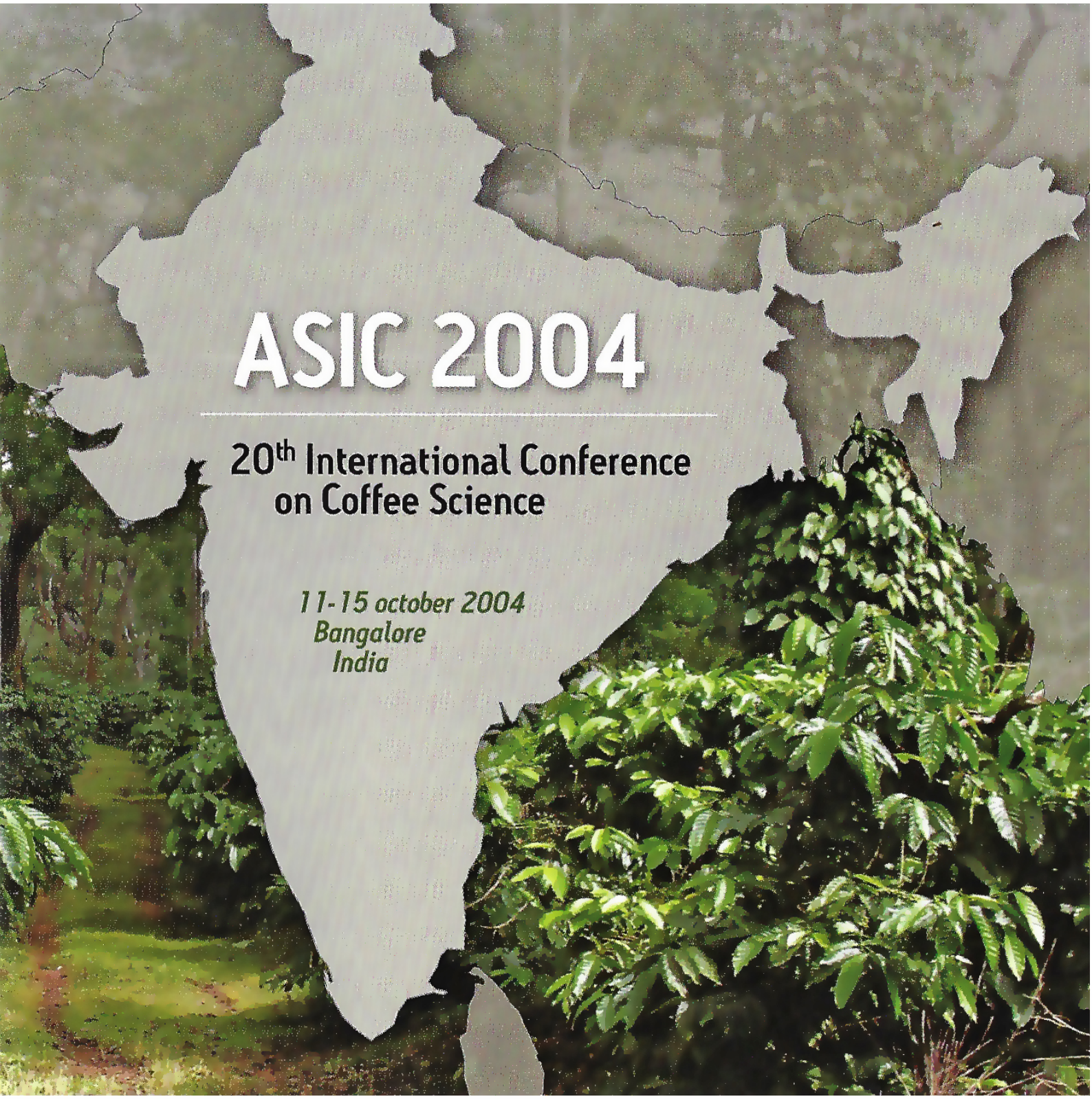
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**VOLUME 1**

**20th International Conference on Coffee Science**
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**20ème Colloque scientifique international sur le café**
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# An Overview of the Coffee Industry in India

**LAKSHMI VENKATACHALAM**

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Structurally, my presentation traverses six key areas. I propose to start with the origins of coffee in India followed by a brief on the Coffee Board, its evolution and current activities. Next I will present the relevant facts of the Indian coffee industry and the current status of our coffee research. I would then like to dwell on the global coffee crisis, the challenges it has thrown up for producing origins like India and concluded with our response to these challenges.

## THE ORIGINS

Like most coffee origins, India has its share of "coffees mythological chestnuts" as Stewart Lee Allen puts in his book "The Devil's Cup". This is the story of Bababudan who is supposed to have lived in the holy cave in the Chandragiri Hills in Chikmagalur, around 1600 AD. On a return from pilgrimage from the holy city of Mecca, he had told his followers that he had brought from the Holy Land, as a gift for them, seven seeds of a wondrous plant which would serve as "food and drink". These seeds were planted on the Chandragiri Hills, which since then came to be known as the Baba Budan Hills. Thus the seven seeds from Mecca have passed into the coffee lore of the world, to be recounted time and again by coffee faithfuls. More than a romantic beginning, or I dare say, "a saintly" beginning to the story of Indian coffee as R.K. Narayan, celebrated writer puts it, "the legend of Baba Budan" identifies India as a producing country in a distinctive and memorable way and lends substance to the antiquity of India's coffee growing traditions".

*"The origin of Indian coffee is saintly. It was not an empire-builder or buccaneer who brought coffee to India, but a saint, one who knew what was good for humanity".*
*- R.K. Narayan, celebrated Indian writer*

The beginnings of commercial cultivation were witnessed in the 19$^{th}$ century by the British entrepreneurs. While the Western ghats were progressively opened up, production reached a level of 30,000 tons by 1870. To quote W.W. Mayne from the Planters Chronicle "It seems clear that the first great expansionary period of the Indian coffee industry must be placed in the eighteen sixties as a result partly of the increasing cost and difficulty of making fresh openings in Ceylon and partly of the prospects of a period of political stability of India". By 1868 came *Hemelia vastatrix* – the deadly disease which within 20 years destroyed the coffee plantations in Ceylon. India did not escape the onslaught – "but thanks to the climate and practice of husbandry that co-operated with rather then exploited the environment, the industry survived to enjoy a second expansionary period". The arrival of Dr. Leslie Colemen heralded the era of organized research which had its beginnings in the experimental research station in Balehonnur (now part of Chikmagalur District in Karnataka State). Dr. L.C. Colemen's research efforts were further strengthened by Mr. W.Wilson Mayne, a key figure among Indian coffee research and this led to improved coffee varieties and stabilization of production.

*"A Spirit of adventure is clearly evident in the efforts of the pioneers. Braving the malarian jungle, these hardy men doggedly worked the land, crafting beautiful and productive estates out of pristine forests. It was enterprise in the best sense, laving a legacy for later generations to build on"*
*- Planting Times*

**THE COFFEE BOARD**

The onset of the second world war brought in a fresh set of problems in terms of price fluctuation and exodus of managers as also passing of British owned properties to Indian hands. It was during this time that the Coffee Act was passed in 1942 which created the Coffee Board and mandated the transfer of marketing from private hands to a central authority. The pool marketing system, which was well set by 1950, was in force for almost 50 years and for the most part, ran in tandem with the global quota system till 1989. Yet another tectonic shift came with the winds of liberalization and reforms that swept various arenas in the early 90s. The demands from growers to free themselves from the pool marketing system was acceded to and over a phased transition period, pooling was given up and there was a return to private marketing. Today, coffee growers are free to grow and market their coffee without any constraints but subject to the regulatory provisions imposed by the Coffee Act and other relevant statutes of the State and Central Government.

The Coffee Board which has been the backbone of institutional support to the industry had necessarily to reinvent itself for a new role as a facilitator, supporting industry through several activities such as research, extension, development, external and internal promotion, quality upgradation, market intelligence and labour welfare.

**COFFEE FACTS**

Let me place before you now, the latest and relevant coffee facts of India which may perhaps be well known to most of you. With a total plantation area of 0.34 million hectares, mostly under small holdings which comprise about 98% of total holdings, India produces some 5 million bags of coffee which is around 4.45% of the global production. More than 75% of the coffee produced in India is exported and this accounts for about 5% of the global exports. The export earnings which rose to over US $400 million in the year 1999-2000 have fallen drastically to around US $ 270 million with the historical low coffee prices that have prevailed in the last 4-5 years.

While coffee may not occupy significant position in terms of total value of agricultural output or exports, it accounts for a significant share of crop area at the state and district level. For example 60% of crop areas in Coorg district is under coffee. It is indeed the engine for development in these regions in terms of supporting the entire social and economic development in some of the most backward areas. The fact that it is also grown on the slopes of western ghats which is one of the most ecologically fragile regions has environmental implications in terms of helping to preserve the microclimate of these regions and preventing losses due to environmental degradation that would have otherwise occurred. Indeed the prices were good for coffee, the per capita income for workers in coffee farms was higher compared to other sectors. The fact that a significant number of workers are women may explain why coffee growing districts in Karnataka State (the largest coffee growing state in the country) recorded a higher ranking in terms of both the human development index and the gender development index, when a survey was undertaken some years ago.

Almost all of Indian coffee is grown at altitudes above 1000 mts nurtured by rich soil, mountain streams and filtered sunlight. Eco friendly cultivation techniques are employed and we are proud of the fact that all of our coffees are shade grown, hand picked and sun dried.

There are however challenges which manifest themselves in terms of diverse climatic regimes across our coffee growing regions. Apart from a long drought period of upto 150 days, there are high variations between the maximum and minimum temperatures (during the growing season) compared to other coffee growing regions of the world, where distribution of rainfall is more even and temperature varies within a closer range. Apart from this, growing coffee under the shade which is a technological necessity, has several advantages as well as constraints. The technology requirements for coffee cultivation in India therefore have to be customized to suit agro-climatic conditions across different regions, in the country, and coffee research in India has to deal with several location specific problems.

India with its geographically distinct coffee areas and wide varieties of plant strains developed over the years offers enormous potential for the growth and development of a wide variety of special and specialty coffees. Some regions with high elevation are highly suited for growing Arabica coffee while those with warmer and relatively humid conditions are more suited for robustas. The varieties of arabicas and robustas developed in India have distinct and discernible quality features in the cup when grown under different agro climatic conditions. Bearing these facts in mind the Board has embarked on a major initiative of demarcating coffee growing areas into distinct and identifiable regions. The objective is to project these regions based on geographical distinctiveness and agro-climatic conditions and local cultural traditions which enable the branding of these coffees and encourage the growers within these regions to develop estate brands. 13 regions have been carved out on this basis and their attractive brand identities or logos are displayed for your view. We also have brand identities for our 3 specialty coffees which are well known in gourmet circles.

**COFFEE RESEARCH**

Coffee, as a crop, has a strong research foundation in India with its origins going back to the opening of Mysore Coffee Experiment Station in 1925. This was merged with Coffee Board during 1946 which later expanded as the Central Coffee Research Institute during 1962. The Central Coffee Research Institute has a large body of trained scientific and technical manpower and has rendered yeoman service to the industry for over 75 years. Our strengths are a strong R&D base, adequate genetic resources for exploitation, trained scientific & technical man power and network to address zonal specific problems.

Some of the important research milestones are release of 15 high yielding disease tolerant and widely adaptable strains for cultivation; development of sound agro-techniques for shade grown coffee; formulation of Eco-friendly IPM & IDM strategies and appropriate on farm and off farm processing methods for product quality.

**THE GLOBAL COFFEE CRISIS**

I now turn to the global crisis in coffee about which Mr. Osorio has already given an illuminating exposition. In this contest, I recall an important observation in the recent World Bank Study entitled - "**Coffee Markets – New paradigms in global supply and demand**" which states *"The history of coffee prices can be regarded as a series of shocks that some times introduced a new paradigm shift. The current shifts are among the most substantial ever experienced".*

Coffee producers are enduring a profound economic and humanitarian crisis. The coffee prices have been at historical lows and below production cost in many producing countries. We are aware that the present crisis is due to the problem of structural over production and a systemic shift in cost structure. The crisis has brought forth paradigm shifts in demand and supply. While global supply is getting more concentrated in few origins the technological innovations in roasting has resulted in enormous flexibility for roasters. Apart from competitiveness in growing costs, the logistics efficiencies that are required in the supply chain today have influenced supply patterns where some origins are able to compete to more effectively.

On the demand side, the potential for growth in newly emerging markets and producing countries is a silver lining – we witness also a proliferation of products reflecting changing life styles/regional preferences. Today more than ever, the trends in consumer behaviour is an important factor that even producing origins will have to tract in order to tailor their product mix and highlight their relative strengths to gain competitive advantage. Above all there is a growing market for differentiated coffees and here I refer to organic, eco-friendly and fair trade coffees all of which get grouped under the umbrella term "sustainable coffees". Then there are the gourmet and specialty coffees. Collectively this segment of the market has shown enormous potential for growth with the promise of higher prices than average for the producer. While mainstream coffees still constitute more than 90% of the total coffee market, no producing origin today can afford to ignore the differentiated coffee market.

I would like to also refer to other significant/concurrent developments that are bound to influence demand and supply. These relate to policy and regulatory environment, business environment and consumer environment which will have significant impact on agricultural policy and programmes in developing countries and not in the least, in coffee. The policy and regulatory environment is influenced by the Multilateral/regional trade agreements, agricultural subsidies in developed countries emerging and the government requirements such as the US Bio-terrorism law, EU Standards for contaminants, pesticide residues, Japanese agricultural standards etc. The Business Environment has seen among others increasing firm consolidation and the emergence of new competitive standards. Finally the consumer environment is being influenced by the rising consumer consciousness about the social/ecological dimensions of human consumption patterns, the globalized consumer tastes and the increased focus on health/diet.

What then is the outlook for coffee? While we know that coffee prices started recovering in 2003-04, there are indications that, it may continue to remain within a range that may leave many producers unprofitable. The challenge therefore is to achieve an orderly balance where prices will guarantee a return not only to efficient producers but to the average producers.

So what are the solutions? Broadly these have centered around increasing consumption, increasing and encouraging the growth of differentiated coffees that fetch higher premiums and promoting diversification.

**INDIA'S RESPONSE TO THE CHALLENGES**

In the last part of the presentation, I will outline India's response to these challenges in the global coffee arena. But first, a few words on the impact of the crisis in India. The unremunerative prices over the last 4 years have resulted in growers not able to recover the cost of production, thereby impacting on maintenance of coffee farms. This has been further exacerbated by the weather conditions particularly in the last two years, which has greatly aggravated problems of pests and diseases and further affected coffee production. Apart from

the economic impact, the humanitarian proportions of the crisis have been significant and quite heart rending. While there have been no reported cases of abandonment of coffee farms, labour employment has declined and families have suffered. More importantly, the financial crisis had compelled the government and the banks to take emergent measures to reschedule the debt of coffee growers.

And yet the growers have shown enormous resilience and fortitude. Adversity has been a great education for us in terms of providing an opportunity to look at our strengths and weaknesses and to reorient ourselves to face future challenges. These are predicated on sustainable production technologies, quality upgradation, adding value to exports, expanding the base for domestic consumption and finally, the empowering the coffee grower to be the arbiter of his own destiny.

In terms of sustainable production technologies, the emphasis has been on developing region specific varieties and packages, reviving traditional farming practices like soil conservation, water harvesting, composting, green manuring etc. and maximizing returns through diversification within coffee farms with compatible cash crops & animal enterprises. We are, in a nutshell, striving to live up to the concept of sustainable development propounded by Mahatma Gandhi who said

"*In my opinion, sustainable development is economic development based on ecological principles like environmental harmony, economic efficiency, resource conservation, local self reliance and equity with social justice".*

The R&D efforts are centered on marker assisted breeding especially for leaf rust and drought tolerance, integrated soil health improvement, farming systems, eco-friendly pest and disease management and quality and food safety aspects.

In this context, I would venture to say that it is perhaps the environmental challenge to sustainability in coffee production that assumes enormous importance particularly in the context of global warming. We know now that changes in climate have the potential for influencing affecting distribution and quality of coffee and there is an urgency for commissioning long term studies on changing weather patterns and identifying appropriate measures that will ensure that production of coffee can internalize the shock of erratic weather conditions.

The efforts on quality upgrading are beginning to bear fruit in terms of capacity building. We have a Post Graduate Diploma course for quality management which aims to train graduates on all aspects of coffee quality. Taking note of the potential of the growing specialty coffee market, an annual cupping competition has been introduced which has enthused the growers to improve quality. To encourage differentiated product development, guidelines for organic and shade grown coffee are established and a sound infrastructure has been put in place for monitoring of pesticide residues and also for dissemination of information on good agricultural and good manufacturing practices.

Our market development efforts in external markets have been centered around a strategy which concentrates on creating a unique image for Indian coffee, in ensuring a high impact at international events and making communication and image building an experiential affair. In other words focus on differentiated strategies for different markets and experiential communication and above all, a belief in ourselves has guided our efforts in recent years to persuade the world to wake up to the exciting aroma of Indian coffee.

The dynamics of the internal market are altogether different. Our efforts here are to understand the market better by launching periodic consumption audits which track trends and attitudes across different regions of our vast country. Two comprehensive market surveys have been released in the last four years. Apart from this, capacity building has been given a special thrust through our training programs on coffee blending, roasting, brewing. We have also decided to address the health concerns in coffee by setting up of a task force following a very successful seminar on Coffee and Health that specifically targeted the medical community.

At the core of the industry is the individual coffee grower for whom the challenges and the pitfalls of liberalization have been quite daunting. Our policy and programs have quite naturally been directed to empower small growers. Apart from financial incentives for capital investment in critical areas such as quality upgradation, water augmentation, replantation so as to improve productivity and quality, Self-Help Groups are being propagated as a mechanism for improving the community approach among growers to resolve problems and also to help to develop greater bargaining power. Research and extension programmes are centered on a participatory approach where the farmer, instead of being a mere user of technology, is encouraged to be a provider of technology, and one who can monitor its impact on his own farm from a total systems perspective. We have also taken steps to improve access to market information through provision of infrastructure, and dissemination of market reports. Finally, there the issue of price risk management, which we are addressing by designing and developing appropriate market based tools. The thrust therefore is to make the grower who is at the centre of coffee economy the arbiter of his own destiny rather than one who is overcome by the sheer force of events.

In conclusion our mission is:

- *To transform the coffee grower from a traditional agriculturist to a technologically literate, quality conscious, self reliant, viable and dynamic entrepreneur.*
- *To ensure that, India is recognized world wide as a reliable and distinctive origin of quality coffee that meets a wide variety of consumer preferences at competitive prices.*

# Coffee World Economy

**N. OSORIO**

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Coffee is a remarkable product. Like wine it involves agricultural and industrial processes. It is complex too in the varieties of possible production and processing systems and its chemical composition, including compounds with measurable effects on the human body.

For this reason scientific research on coffee is of the greatest importance and has resulted both in an increased understanding of the effects of coffee consumption and advances in key areas such as plant breeding, cultivation methods and processing technology.

I am delighted to lend the support of the International Coffee Organization to this 20th ASIC Conference. It is an unparalleled opportunity for scientists working in coffee to disseminate their findings and discuss issues with friends and co-workers. The ICO has provided consistent support to ASIC in the past and has encouraged scientific research for many years. In 1980 the ICO Promotion Fund allocated a million dollars to assist in financing research in the area of coffee and health. In 1988 the Fund agreed to finance a Public Relations programme for coffee in cooperation with EUCA, the European Coffee Roaster's Association, which led to the formation of ISIC, which today plays such an important role in funding research. And it is with the support of ISIC that the ICO has been able to embark on the current Positively Coffee Programmes, designed to disseminate information on the positive health effects of coffee drinking to the public and now especially to the health care professions.

Research is normally undertaken in a context, whether it is to develop resistance to disease, improve quality or investigate the pharmacological effects of particular compounds. It is my role however to inform you of the wider context of current conditions affecting the world coffee economy, which ultimately makes the difference between prosperity and hardship for millions of coffee growers and their families worldwide.

As you are aware the main problem for producers over the last five years has been, one of low world prices caused by an excess of supply over demand. To illustrate, the ICO composite indicator price, which had remained under the old ICO quota system at around 180 US cents per lb in the 1980s has been under 70 cents since April 2000 and was around 61 cents early last week (4 October)

A recent study sponsored by the ICO and funded by the European Commission analysed coffee farm profitability in the 2002/03 coffee year in 8 arabica and 4 robusta producing countries using various production systems. The conclusions were that few coffee producers were able to cover their operating costs, let alone total costs. To take India as a case in point all grower categories using traditional production systems showed losses.

This situation has been recognized by the United Nations as posing threats to the achievement of the Millennium development goals and a setback to the HIPC (Highly Indebted Poor Countries) initiative. We at the ICO have documented for many countries alarming increases in poverty and social problems.

In 2003, the total value of exports of all forms of coffee was estimated at US$9.58 billion of which UD$5.58 billion is attributable to exporting countries for total exports of 85.79 million bags and US$4 billion to re-exports of 27.49 million bags by importing countries, showing the effects of value added by processing. Again it should be recalled that exporting country exports were valued at over 10 billion dollars annually in the 1980s.

Essentially the imbalance in the market was caused by excessive new planting of coffee in the mid 1990s following a price rise caused by a frost in Brazilian coffee areas in 1994. Subsequently Brazil has been able to maintain high levels of production with the help of a continued depreciation of the local currency against the dollar while Vietnam's production has also increased substantially, mainly because production costs are very low as a result of high yields arising from intensive care of coffee trees coupled with substantial use of fertilizers.

The recent development of market fundamentals indicates a substantial reduction in world production during the crop year 2003/04 as 100.69 million bags is recorded compared to 121.94 millions in 2002/03. Production for the crop year 2004/05 is estimated at a level ranging between 112 and 114 million bags. For years ahead, I do not intend to engage in any long term forecasts but there are indications that production for 2005/06 may be reduced as a result of the Brazilian biannual cycle which is likely to record a substantial decrease in production.

Nevertheless it is crucial to recognize that coffee producers have limited options and face a highly imperfect market. Not only are growers constrained by their investment in a perennial crop which takes at least three years before significant output is seen but ecological conditions in coffee areas often restrict the possibility of finding alternatives. Where alternative crops are technically feasible growers are often faced with barriers to market access which further impede diversification.

A big concern is the demand side. World global consumption is estimated at 113.08 million bags from calendar year 2003 including domestic consumption of 27.87 million bags and 85.1 million in importing countries. Nevertheless, despite the low level of world coffee prices and a cut in retail prices in some importing countries, demand remains relatively stagnant and consumption in traditional importing countries show signs of having reached saturation point. Indeed, for the past ten yeas consumption per capita in many major importing countries has decreased, noticeably in Austria, Denmark, Finland, France, Germany, The Netherlands, Norway, Sweden, Switzerland, United Kingdom and the USA.

It is noticeable that many of these countries traditionally value mild arabicas in their blends and evidence suggests that such coffees are being substituted in many cases by naturals and robustas.

Since an increase in consumption continues to be one of the ways of helping to restore a healthy balance between supply and demand, I have appealed to exporting countries to implement programmes for promoting domestic consumption and to donor organizations to finance market development projects to stimulate demand. This is very much a market friendly strategy and I must confess to some disappointment with the response to date of my appeals. However the European private sector is helping us in our Positively Coffee programme which is helping to disseminate important scientificevidence of the positive effects of coffee consumption and we have recently commissioned an important study giving practical guidelines to increase consumption in producing countries which we will

disseminate shortly. Here it is worth remembering that domestic consumption not only helps the overall market balance but increases producer awareness of consumer demands, provides an alternative marketing outlet ad stimulates local small and medium enterprises.

So to researchers my message is straightforward: the priority is to concentrate on areas which will encourage the long-term sustainability of coffee by improving quality, analysing positive effects of consumption and reducing costs. In other words, work which will increase value not quantity.

# Coffee and Risk of Type 2 Diabetes

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## SUMMARY

Data on coffee consumption and risk of development type 2 diabetes from diverse countries have recently been published. These novel findings probably highlight the new dimensions of coffee and its complex components other than caffeine.

## INTRODUCTION

Coffee is one of the most consumed beverages in the world and Finns with 11.3 kg per capita have the first place while the average in Europe is 5 kg. Probably it is some sort of antidote to long, dark and cold winter. Most folk knowledge about coffee attributes the effects of coffee to be synonymous with those of caffeine, even though the other, more abundant components of coffee have not been extensively studied. In fact coffee is a complex compound of potential nutriceuticals. Agricultural factors, roasting, blending, and brewing determine coffee's chemical composition. Coffee is the major source of phenolic polymers, chlorogenic acid (CGA) and also caffeine (Clifford, 2000). Phenolic compounds are known as antioxidants in vitro and might reduce the risk of cardiovascular disease and other degenerative diseases as well (Rice-Evans et al., 1996; Olthof et al., 1976). They may also involve in different stage of glucose metabolism process. The most prevalent phenolic compounds in food are hydroxycinnamic acids (Herrmann, 1976; Kuhnau, 1976), and a major component of this class is caffeic acid, which occurs in food mainly as esters called chlorogenic acids (Clifford, 2000) Although compounds with antioxidant properties (mainly CGA) are lost during roasting of coffee beans (Parliament, 2000), the overall antioxidant activities of coffee brews can be maintained or even enhanced, by the development of compounds possessing antioxidant activity. Chlorogenic acid can also transform into quinides that have been shown to enhance insulin action (Shearer et al., 2003).

Type 2 diabetes, formerly known as non-insulin-dependent diabetes (NIDDM), is the most common form of diabetes and a major health problem associated with excess morbidity and mortality and results in substantial health care costs. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004). In Europe their number will increase from approximately 16 million in1994 to 24 million in 2010 (Amos et al., 2001).

Type 2 diabetes is caused by complicated interplay of genes and environment. Genetic factors play an important role in type 2 diabetes, but the pattern is complicated, since both impairment of beta cell function and abnormal response to insulin are involved. Defective genes that regulate a molecule called peroxisome proliferator-activated receptor gamma (PPARγ) may contribute to type-2 diabetes in some patients. It regulates adipocyte differentiation and lipid and glucose metabolism (Auwerx, 1999). First-degree relative with the disease increases risk of developing diabetes. Large Finnish twin cohort study found that the heritability of type 2 diabetes is 79% (Kaprio et al., 1992). Dramatic changes in the

prevalence or incidence of type 2 diabetes have been observed in communities where there have been major changes in the type of diet consumed, from a traditional indigenous diet to a typical western diet, e.g. Pima Indians in Arizona, Micronesians in Nauru and Aborigines in Australia (Bennett, 1999; Lako and Nguyen, 2001; Hetzel and Michael, 1987). Changing disease rates are almost explained by changes in several dietary factors as well as by changes in other lifestyle related factors (obesity and sedentary lifestyle). This may be particularly important in triggering the genetic elements that cause this type of diabetes. Type 2 diabetes is characterized by development of both microvascular and macrovascular complications. These complications, accounts for most of the increased morbidity and mortality associated with type 2 diabetes (Kannel and McGee, 1997; Fuller et al., 1983; Manson et al., 1991; Nathan, 1993; Stamler et al., 1993). The costs of diabetes care are mostly attributed to the long term complications.

## COFFEE CONSUMPTION AND RISK OF TYPE 2 DIABETES

The association of coffee consumption and the risk of type 2 diabetes were evaluated in a population-based cohort of Dutch men and women (Van Dam and Feskens, 2002). They reported that individuals who drank at least seven cups of coffee a day were less likely to develop type 2 diabetes compared with those who drank two cups or fewer a day (Table 1).

**Table 1. Relative risk of type 2 diabetes by volume of coffee consumption among Dutch people.**

| | Daily coffee consumption | | | | P for trend* |
|---|---|---|---|---|---|
| | ≥ 2 cups (n = 62) | 3-4 cups (n = 84) | 5-6 cups (n = 97) | ≤ 7 cups (n = 63) | |
| Case/100 000 person-years | 319 | 237 | 246 | 200 | |
| Relative risk adjusted for age, sex, and town† | 1.00 | 0.71 (0.51-0.99) | 0.73 (0.53-1.01) | 0.60 (0.43-0.86) | 0.013 |
| Further adjustment for BMI and lifestyle‡ | 1.00 | 0.84 (0.60-1.17) | 0.77 (0.55-1.06) | 0.53 (0.37-0.76) | < 0.001 |
| Further adjustment for cardiovascular disease, hypertension, and hypercholesterolaemia§ | 1.00 | 0.79 (0.57-1.10) | 0.73 (0.53-1.01) | 0.50 (0.35-0.72) | < 0.001 |

**Values were obtained by modeling the median value of each category of coffee consumption as a continuous variable.*
*†Doetinchem or Maastricht.*
*‡Also adjusted for education level (junior secondary school or less, secondary education, vocational colleges or university), leisure time physical activity (low or higher), occupational physical activity (low or higher), alcohol consumption (men: no, or ≤ 1, >1 to 3, >3 drinks/day; women: no, or ≤ 1, >1 to < 2, ≥ 2 drinks/day), and cigarette smoking (never, past, current < 10 cigarettes/day, 10-19 cigarettes/day, ≥ 20 cigarettes/day).*
*§Further adjustment for history of cardiovascular disease (myocardial infarction, stroke, survey for cardiovascular disease), known hypertension, and known hypercholesterolaemia.*

Data from large U.S. cohorts of men and women showed an inverse association between coffee intake and type 2 diabetes. Total caffeine intake from coffee and other sources was associated with a statistically significantly lower risk for diabetes in both men and women (Salazar-Martinez et al., 2004).

In a Swedish cohort study they evaluated the long-term incidence of diabetes in relation to coffee consumption by following a random population sample of 1361 women, aged 39-65 years. The same inverse association between coffee consumption and incidence of type 2 diabetes was achieved (Rosengren et al., 2004).

Also a Japanese study found that coffee intake or caffeine intake from coffee was inversely associated with the prevalence of fasting hyperglycaemia (Table 2) (Isogawa et al., 2003).

**Table 2. Risk of having prevalence of fasting hyperglycaemia (fasting plasma glucose ≥ 6.1 mmol/l) according to caffeine intake and source among Japanese people*.**

| | Odds ratio (95% CI) | P value |
|---|---|---|
| Coffee intake (yes/no†) | 0.61 (0.47-0.80) | < 0.001 |
| Caffeine from coffee (100 mg/day increase) | 0.91 (0.86-0.96) | 0.001 |
| Green tea (Japanese tea) intake (yes/no) | 0.83 (0.59-1.18) | 0.277 |
| Caffeine from green tea (100 mg/day increase) | 1.00 (0.91-1.11) | 0.946 |
| Tea (black tea) intake (yes/no) | 0.95 (0.73-1.25) | 0.705 |
| Caffeine from tea (100 mg/day increase) | 0.97 (0.67-1.30) | 0.839 |
| Oolong tea (a Chinese tea) intake (yes/no) | 1.06 (0.83-1.36) | 0.621 |
| Caffeine from oolong tea (100 mg/day increase) | 1.08 (0.94-1.23) | 0.269 |
| Total caffeine intake (100 mg/day increase) | 0.94 (0.89-0.99) | 0.012 |

**Adjusted for age, sex, body mass index, and family history of diabetes.*
*† "no" means less than once a week.*

Recently, we prospectively followed 6974 Finnish men and 7655 women of 35 to 64 years of age (Tuomilehto et al., 2004). During a mean follow-up of 12 years, 381 incident cases of Type 2 diabetes were found and an strong and graded inverse association between coffee consumption and risk of development of type 2 diabetes was evidenced (Table 3).

Coffee consumption was not associated with incidence of type 2 diabetes in Pima Indians (Saremi et al., 2003) and another Finnish cohort studies (Reunanen et al., 2003). In Pima Indian study the highest category of coffee consumption was only three or more cups of coffee per day, also there was no control for possible factors of lifestyle that may have covered an existing association. Indeed high coffee consumption tends to be related to unhealthy lifestyle behavior (e.g smoking, excessive consumption of alcohol, a poorer diet, and a sedentary lifestyle) in U.S. populations (Salazar-Martinez et al., 2004). Changing coffee consumption habits in Finland during long follow-up period, and also higher consumption of boiled instead of filtered coffee may be explained the lack of association in previous Finnish study (Tuomilehto et al., 2004; Reunanen et al., 2003).

The association between coffee drinking and markers of glycemic control (glucose tolerance, fasting glucose and insulin levels) has been not extensively studied, however in a cross sectional analysis we found that coffee has positive effects on several markers of glycemic control. We found that higher coffee consumption was associated with lower values of 2-hour glucose and fasting insulin among non-diabetic subjects. Prevalence of impaired glucose tolerance, impaired glucose regulation and hyperinsulinemia are lower in higher habitual coffee consumers. However the results were different between men and women. Hormonal differences, which may influence glucose metabolism process, could explain discrepancies in results between sexes (data not shown). This study revealed that coffee may affect postload glucose metabolism and it is rather important because in type 2 diabetes an abnormal rise in blood sugar right after a meal (called postprandial hyperglycemia) is believed to be particularly damaging to the body. Also Ärnlöv et al. who, investigated the association

between coffee consumption and both insulin sensitivity and insulin secretion, found that both coffee and tee consumption were related to improved insulin sensitivity (Arnlov et al., 2004).

**Table 3. Relative risk of type 2 diabetes by volume of coffee consumption among Finnish people.**

| | Daily coffee consumption | | | | | P for trend |
|---|---|---|---|---|---|---|
| | ≤ 2 cups | 3-4 cups | 5-6 cups | 7-9 cups | ≥ 10 cups | |
| Men | | | | | | |
| No. of new case | 41 | 48 | 67 | 28 | 19 | |
| Person-years | 14191 | 20054 | 25704 | 11480 | 10426 | |
| Relative risk adjusted for other factors* | 1.00 | 0.74 (0.48-1.14) | 0.71 (0.47-1.10) | 0.67 (0.40-1.14) | 0.45 (0.25-0.81) | 0.121 |
| Women | | | | | | |
| No. of new case | 46 | 68 | 48 | 13 | 3 | |
| Person-years | 15821 | 30367 | 32036 | 10523 | 4980 | |
| Relative risk adjusted for other factors* | 1.00 | 0.73 (0.50-1.08) | 0.40 (0.26-0.63) | 0.42 (0.22-0.79) | 0.21 (0.06-0.70) | < 0.001 |
| Men and women combined† | | | | | | |
| No. of new case | 87 | 116 | 115 | 41 | 22 | |
| Person-years | 30112 | 50421 | 57740 | 22003 | 15406 | |
| Relative risk adjusted for other factors* | 1.00 | 0.77 (0.57-1.03) | 0.56 (0.41-0.75) | 0.56 (0.38-0.83) | 0.39 (0.24-0.65) | <0.001 |

**Adjusted for age, exam year, body mass index, systolic blood pressure, education, occupational and leisure time physical activity, walking or cycling to and from work, cigarette smoking, alcohol consumption, and tea consumption., †Also adjusted for sex*

## SUGGESTED MECHANISMS

Mechanism or mechanisms that explain association between coffee consumption and risk of type 2 diabetes seems to be complex. Increased insulin resistance in peripheral tissues after caffeine exposure has been reported (Keijzers et al., 2002). However, the thermogenic effect of caffeine through upregulation of uncoupling protein family may overcome the energy imbalance accompanied by unfavorable lifestyle and improve glucose homoeostasis (Yoshioka et al., 1990; Kogure et al., 2002; Schrauwen et al., 2001; Bracco et al., 1995). It is also well known that caffeine and theophylline are strong stimulants of pancreatic β-cells (Tuomilehto et al., 1990). In addition to the effects of caffeine, coffee contains many other substances such as chlorogenic acid that may have metabolic effects on glucose metabolism. For instance inhibition or retardation of the action of α-Glucosidase at the intestinal stage is one of the possible mechanisms. α-Glucosidase, a membrane-bound enzyme located at the epithelium of the small intestine catalyzes the cleavage of glucose from disaccharides (Hauri et al., 1982; Matsui et al., 2001). The inhibition of this enzyme is an effective approach to control hyperglycemia (Matsui et al., 2001), and such inhibitors are used at present in the treatment and prophylaxis of type 2 diabetes, in particular for the control the postprandial blood glucose levels (Toeller, 1994). In addition chlorogenic acid may inhibit glucose transporters ($Na^+$-dependent glucose transporter) at the same stage (Kobayashi et al., 2000). Johnston KL et al suggested that chlorogenic acid might have an antagonistic effect on

glucose transport (Johnston et al., 2003). In addition coffee may influence the secretion of gastrointestinal peptides such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), both of them are known for their glucose lowering effects (Nauck et al., 1993; Meier et al., 2001). At the hepatic stage, Glc-6-Phase may be a key control site in the homeostatic regulation of blood glucose concentration (Newgard et al., 1984; Youn et al., 1986), and Glc-6-Phase is widely held to be significant factor in the abnormally high rates of hepatic glucose production observed in the diabetic state (Arion et al., 1997). Reduced Glc-6-Phase hydrolysis or its inhibition by chlorogenic acid may reduce plasma glucose output leading to reduce plasma glucose concentration (Andrade-Cetto and Wiedenfeld, 2001). Absorption and bioavailability of chlorogenic acid in humans has already examined. Olthof et al. showed that 33% of a 2.8-mmol load of chlorogenic acid was absorbed by ileostomy patients. This may suggest that a certain amount of dietary chlorogenic acid will enter into the blood circulation via absorption from the small intestine (Olthof et al., 2001).

## CONCLUSION

Potentially preventable nature of type 2 diabetes has been evidenced by the implementation of lifestyle measures such as weight control and exercise. In many of the borderline cases of type 2 diabetes, the clinical appearance of the disease and consequent complications may be delayed by diet and exercise for many years. It may show the efficacy of the dietary behaviour in preventing the disease. More or less, coffee has been included to the dietary menu of most of the people and it seems to be helpful in overall glucose metabolism. However we believe that these positive effects cannot be solely achieved by coffee drinking without considering the other lifestyle measures. In addition many people stop or lessen coffee drinking by aging, digestive problems or some other reasons and consequently the overall incidental rate have not changed. But until now, the most important conclusion is to introduce coffee as a safe and useful drink in type 2 diabetes area.

Better knowledge of the coffee component, human consumption and bioavailability is needed in order to properly evaluate the true role of coffee in type 2 diabetes. Eventually research in this area should lead to dietary recommendations optimized for specific population groups in risk of type 2 diabetes.

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# Trace Levels of Animal Carcinogens in Coffee: An Evaluation of their Human Cancer Risk in Light of the Health Benefits from Maillard Reaction Products

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## SUMMARY

In 2002, Swedish researchers found acrylamide in many fried and baked foods, especially potato chips and French fries, at levels from 30-2,300 parts per billion. Acrylamide production was attributed to the higher temperatures reached in Maillard non-enzymatic browning reactions required for desirable color, flavor, aroma and texture production. Both unbrewed and brewed coffees have since been shown to contain measurable levels of acrylamide. Acrylamide is a neurotoxicant and rodent carcinogen. Academic, government and industry research groups have initiated major acrylamide research programs to determine intakes in various countries' diets, means to mitigate its formation and whether or not it is a significant public health risk. The current consensus is that currently available information on acrylamide in foods is not sufficient to draw firm conclusions about its cancer risk to humans. International authorities have stated that there is no indication at this time that consumers need to change their eating or drinking habits.

In late 2003, the European Union initiated a major research program (HEATOX) to expand the acrylamide database in foods and seek ways to mitigate its formation. HEATOX is also focussing on other toxicants/carcinogens produced by heating and cooking foods. In addition, the U.S. FDA in May 2004 released preliminary data on the presence of furan (another rodent carcinogen) in a range of heated foods up to 160 ppb, with concentrations in brewed coffee up to about 80 ppb.

An important toxicological consideration that has received little attention to date is that many Maillard Reaction Products (MRPs) have been shown to be beneficial to health, including many antioxidants, anti-mutagens and anti-carcinogens. MRPs have also been identified that induce the formation of carcinogen-detoxifying enzymes, including glutathione transferase, the enzyme known to detoxify acrylamide. In addition, furan has been shown to be a good antioxidant, as have many other volatile heterocyclic flavor compounds as well as the melanoidins, the complex brown Maillard polymers.

The dietary human cancer risk of acrylamide and furan in coffee must be evaluated in light of their metabolic disposition in the human body, the extensive epidemiological database on coffee consumption and cancer and the beneficial health effects known to be produced by the Maillard reaction. The results of ongoing global research efforts on the health effects of heat processing and cooking of foods and beverages, including coffee, must be used to carefully evaluate both the risks and benefits of these heat-induced compounds.

## INTRODUCTION

There have been dramatically increased worries about diet and health during the past 10-15 years with the constant barrage of anxiety-provoking media stories linking individual foods and food chemicals to diseases such as cancer. What we eat (or don't eat) is often linked to an increased risk of cancer, but a great deal of the information is only preliminary in nature, often yielding to a near absence of concern once a larger body of studies has been accumulated over the ensuing years. The information that builds up during the intervening years, however, can lead to a great deal of nutrition nonsense and "food faddism" that ends up producing nations of "avoiders" of specific foods and ingredients such as salt, fat, carbohydrates (more recently), and for several decades now the avoidance of coffee and caffeine because of health concerns.

An important consideration before examining heat-induced food chemical safety in humans in general, and coffee and coffee chemical safety in particular, is why there is such an intense interest in trace level carcinogens when there is little human epidemiological evidence linking individual foods and beverages with disease risk, including cancer. Carcinogens from heated foods have been a health concern since the 1970's. Trace levels of animal carcinogenic and mutagenic polycyclic aromatic hydrocarbons were found in barbecued steaks, coffee and other heated foods, as were N-nitrosamines in fried bacon and beer. Heterocyclic amines were next reported in over-heated meats, and many of these compounds were eventually shown to be fairly potent mutagens and animal carcinogens. The late 1970's also gave us the first reports on heat-induced "Maillard Browning Reaction" products ("MRPs") as mutagens and possible carcinogens. Dr. Takayuki Shibamoto and I (both at the University of California at Davis) were among the first of a small group of researchers to identify the classical sugar-amine browning reaction as a source of mutagens and carcinogens. My work involved the discovery of the mutagenicN-nitroso fructose-amino acids ("N-nitroso Amadori compounds") in heated model systems. In fact, the key acrylamide intermediate has been shown to be the Amadori compound formed from glucose and asparagine.

This paper will specifically focus on the occurrence and toxicology of acrylamide and furan, since both have been found in roasted and brewed coffees. While it is important to evaluate the toxicological risks of such heat-induced chemicals in foods, it is equally important to fully evaluate the safety of whole foods and beverages using modern epidemiological techniques. It is also becoming increasingly important to recognize that health-beneficial food/beverage chemicals occur naturally, including many in coffee, as do other health-protective compounds produced during heat processing and cooking. Thus, it is critical also to evaluate these beneficial health effects of heated foods/beverages and then to undertake a thorough risk-benefit evaluation of the whole food or beverage. This evaluation must also carefully consider how best to interpret animal toxicology results for individual food chemicals, as well as recent information that coffee may actually be cancer protective.

## ACRYLAMIDE'S DISCOVERY IN FOODS AND BEVERAGES

In April 2002, Swedish researchers shocked the food safety world when they presented evidence of acrylamide [$CH_2$=CHCONH$_2$] in fried/baked foods, most notably potato chips and French fries, at levels of 30–2,300 ppb (Tareke et al., 2002). More moderate levels were found in some protein-rich foods. They also reported that foods cooked or processed at temperatures lower than ~ 120°C did not contain acrylamide, and they attributed this to the higher temperatures reached in Maillard nonenzymatic browning reactions required for desirable color, flavor and aroma production. Acrylamide, a well-known neurotoxicant, had never before been reported in foods, but it has surely been present since man first began

cooking. Acrylamide is manufactured synthetically and is used as the monomer for polyacrylamide with many industrial uses.

Acrylamide's critical safety importance is reflected in its classification by several regulatory agencies and health advisory bodies as a "probable human carcinogen" based on high-dose animal cancer bioassays. Swedish adults' acrylamide intake was initially estimated to be about 100 μg/day. Numerous academic, government and industry research groups have initiated major acrylamide research programs on mechanisms of formation and mitigation, methods of analysis, dietary exposure and biomarkers, toxicology and metabolic consequences and risk communication. The Joint Institute for Food Safety and Applied Nutrition (a program of the U.S. Food and Drug Administration and the University of Maryland) has played a major role in coordinating global acrylamide research and education activities, and it also serves as the FAO/WHO "Acrylamide Infonet" (http://www.acrylamide-food.org/). The European Union's acrylamide activities are reviewed and updated at http://europa.eu.int/comm/food/food/chemicalsafety/contaminants/acrylamide_en.htm. In addition, the EU initiated near the end of 2003 a major research program entitled "Project HEATOX**"** ("Heat-induced food toxicants: identification, characterisation, and risk minimization") as a result of the discovery of acrylamide in foods. The most recent scientific review of acrylamide in food was just published by Taeymans et al. (2004).

**Table 1. U.S. FDA Acrylamide Data (2003).**

| **Food** | **Acrylamide (ppb)** | **Food** | **Acrylamide (ppb)** |
|---|---|---|---|
| Baby food | ND - 130 | French fries | 117 – 1325 |
| Breads/bakery products | ND - 364 | Fruits/vegetables (canned) | ND - 83 |
| Cereals | 11 - 1057 | Gravies/seasonings | ND - 151 |
| Chocolate products | ND - 909 | Infant formulas | ND |
| Coffee (roasted) | 37 - 374 | Nuts/nut butters | ND - 457 |
| **Coffee (brewed)** | **5 - 11** | Potato chips | 117 - 2762 |
| Cookies/crackers | 26 - 620 | Snacks (other salty) | 12 - 1243 |
| Dairy drinks | ND - 43 | Vegetables (frozen) | ND |
| Dried foods/mixes | ND - 1184 | [*ND = non-detectable*] | |

## ACRYLAMIDE CONCENTRATIONS AND EXPOSURE ASSESSMENTS

The FDA has released extensive analytical data on acrylamide levels in hundreds of food samples (U.S. Food and Drug Administration, 2003). Besides the occurrence of acrylamide in fried potato products, cereals, breads, and bakery products, FDA reported significant concentrations of acrylamide in some baby foods, snack foods other than potato chips, nuts and nut butters, chocolate products, various dried foods and roasted and brewed coffees (Table 1). The key difference between acrylamide and other historical "cooked food" carcinogens is that each of these earlier contaminants is found in just a very few foods and only at very low ppb levels, whereas acrylamide is found in so many foods at much higher levels, even up to 1,000-4,000 ppb levels in some foods. In fact, U.S. industry research has shown that acrylamide is found in adult foods contributing up to approximately 38% of total caloric intake, as well as significant percentages of consumers' macronutrients (33% of carbohydrates, 36% of fiber, 28% of fat) and micronutrients (20% of calcium, 47% of iron, 15% of vitamin A, 34% of vitamin E and 22-44% of vitamin B, vitamin C and folic acid) (Food and Drug Administration, 2003).

The FDA also reported an extensive exposure assessment of acrylamide in foods and beverages. While French fries and potato chips were shown to be the major contributors to U.S. dietary acrylamide intakes, coffee appeared among the "Top 8" food items contributing to intake, with concentrations ranging from 5-11 ppb.

## CURRENT FACTS ON ACRYLAMIDE RISK

Two rat chronic bioassays in drinking water have been reported, and this has led to the classification of acrylamide as "reasonably anticipated to be a human carcinogen" (National Toxicology Program, 2002).

However, the rat tumors were all benign, non-malignant tumors (except for male scrotal tumors that have been rarely discovered in man). The tumorigenic animal doses were about 1,000 times higher than human intakes. Three human dietary epidemiology studies have been published to date, and two more are apparently in press. None of these studies has shown any increased cancer risk from the consumption of acrylamide-containing foods, and it is noteworthy that some of these studies have even shown a reduced risk of colorectal cancer.

From recent exposure assessments in several countries, human intakes are now estimated to range from about 25-35 μg/day, not the 100 μg/day estimate in the original Swedish study. Current risk assessments predict that cancer risk may start increasing at ~ 70 μg/day, which is about double the current human intake estimate. The information currently available on acrylamide in foods is not sufficient to draw firm conclusions about cancer risk to humans. FDA, the World Health Organization, the European Union and other health/regulatory bodies have stated that there is no indication at this time that consumers need to change their eating habits in response to the acrylamide findings, but instead advise consumers to follow established dietary guidelines and eat a healthful, balanced diet consisting of a wide variety of foods.

**Table 2. Furan Exposure from Adult Food Types (U.S. FDA, 2004).**

| **Food** | **Intake (μg/kg-bw/day)** |
|---|---|
| **Brewed coffee** | **0.15** |
| **Instant coffee** | **0.001** |
| Chili | 0.04 |
| Soups containing meat | 0.01 |
| Pork and beans | 0.004 |
| Canned pasta | 0.004 |
| Canned string beans | 0.004 |
| Spaghetti sauces | 0.001 |
| Juices | 0.001 |
| Canned corn | 0.0003 |
| Canned tuna (water packed) | 0.00008 |
| Broths | 0.000006 |

## FURAN AS A "NEW" HEAT-INDUCED CARCINOGEN AND ANTIOXIDANT

Furan is a simple, well-known flavor compound produced by heating many foods and beverages, and we have known about its presence at trace levels in coffee for decades. In early 2004, FDA surfaced concern about furan's presence in foods (up to about 125 ppb) and its rodent liver carcinogenicity (Food and Drug Administration, 2004). The European Food Safety Authority recently announced that it will also carefully examine furan in foodstuffs.

Based on analytical data collected through April 2004, FDA estimated that mean furan exposure from adults foods was 0.3 μg/kg-bw/day, with the 90$^{th}$ percentile intake estimated at 0.6 μg/kg-bw/day. Thus, a 70-kg adult is estimated to consume ~ 20 μg furan/day on a mean intake basis. Table 2 shows the FDA estimates of furan intake from various furan-containing foods and beverages, with brewed coffee ranking as the highest source of dietary furan among the foods tested. Consequently, it appears that in this preliminary FDA analysis, brewed coffee may be contributing to about 50% of total furan intake in adults.

Like acrylamide, however, we don't yet know if furan is a human carcinogen. But furan and some other MRPs have been shown to be natural antioxidants (reviewed in Manzocco et al., 2001). Although many researchers have focused on the polyphenolic antioxidants typically found in many foods, Shibamoto and his co-workers have pioneered the discovery of various heat-induced heterocyclic compounds as antioxidants, including furan itself. More recent work by Shibamoto's group has focused on assessing the antioxidative activities of furan and other heterocyclic compounds in brewed coffees (Fuster et al., 2000; Yanagimoto et al., 2002; Yanagimoto et al., 2004), noting that furan and its derivatives are sugar degradation products that are the most abundant volatile compounds in roasted coffee. Most recently, Shibamoto's group found furan derivatives among the active antioxidant fractions of various organic and aqueous fractions extracted from brewed coffee (Yanagimoto et al., 2004). The authors concluded:

"The results from the present study indicate that brewed coffee contains many antioxidants including some heterocyclic compounds. Although the activity of each component is not as strong as the known antioxidant BHT, a total activity of numerous compounds in brewed coffee might be comparable to those of known antioxidants. Therefore, consumption of antioxidant-rich brewed coffee may prevent diseases caused by oxidative damage."

**BENEFITS OF THE "MAILLARD BROWNING REACTION"**

One of the challenges for the public health implications of the presence of acrylamide, furan and other cooked-food carcinogens is that the same chemical processes that create these compounds also produce the chemicals that make food palatable and desirable (i.e., the aroma, color, texture, and flavor of food) as sources of nutritive value. Additionally, as noted above, many beneficial substances are created by these heat-driven reactions, including antioxidants, anti-carcinogens, anti-mutagens and other health-protective compounds (reviewed in Lee and Shibamoto, 2002). These authors and other researchers have concluded that such compounds, including the oxygen-heterocyclic compound furan and its derivatives, may help to prevent *in vivo* oxidative damage such as lipid peroxidation, which is associated with many diseases including cancer, diabetes, atherosclerosis, inflammation, arthritis, immune deficiencies, even "aging." Although living organisms are known to be protected from active oxidants by enzymatic systems, natural antioxidants (α-tocopherol, carotenoids and ascorbic acid in fruits and vegetables) are also known to protect cells from oxidation and humans from various diseases. In addition, it is clear that humans consume some quantity of additional antioxidants, including furan, formed during the heat-treatment of foods.

Therefore, this entire complement of mixed antioxidants (the natural vitamins and heat-induced compounds) may play an important role in protecting humans from oxidative damage associated with the diseases described above. Thus, it is critical to consider the beneficial, health-protective effects of these compounds in evaluating the safety of acrylamide, furan and other animal carcinogens formed during Maillard browning.

## CARCINOGENICITY OF INDIVIDUAL FOOD CHEMICALS

An important report (National Research Council, 1996) on the carcinogenicity of individual food chemicals published by the National Research Council in 1996 came to several key conclusions about the cancer potential of food chemicals: (1) "The great majority of individual naturally occurring and synthetic chemicals in the diet appears to be present at levels below which any significant adverse biologic effect is likely, and so low that they are unlikely to pose an appreciable cancer risk;" (2) "The varied and balanced diet needed for good nutrition also provides significant protection from natural toxicants;" (3) "Current evidence suggests that the contribution of excess macronutrients and excess calories to cancer causation in the United States outweighs that of individual food microchemicals, both natural and synthetic;" and (4) "Closing Remarks…Most naturally occurring minor dietary constituents occur at levels so low that any biologic effect, positive or negative, is unlikely."

## WHAT DO WE KNOW ABOUT COFFEE AND CANCER RISK?

Coffee contains over 50 identified animal carcinogens, including acrylamide, furan, caffeic acid, various aldehydes, polycyclic aromatic hydrocarbons, ochratoxin A and many others. But, after three decades of research, most health authorities across the globe now agree that coffee drinking is NOT a cancer risk. In 1991 the International Agency for Research on Cancer (IARC) published a monograph on coffee, tea, caffeine and related substances (International Agency for Research on Cancer, 1991). IARC concluded that caffeine was not carcinogenic and that coffee was only very weakly associated with an increased risk of bladder cancer. This bladder cancer conclusion was later strongly disputed by a careful meta-analysis published by Yale University epidemiologists, who noted that smoking, an established risk factor for bladder cancer, was as a well-known confounder in the coffee consumption studies (Viscoli et al., 1993). IARC also concluded that coffee may be related to reduced risk of colorectal cancer. This latter conclusion has been strongly supported by many recent studies (reviewed in Tavani and La Vecchia, 2004).

The possible health-protective effect of coffee on colorectal cancer risk has been attributed by some researchers to the antioxidant potential of coffee. A recent Norwegian human study (Svilaas et al., 2004) on the intake of antioxidants from various foods and beverages found that total antioxidant intakes were as follows: coffee >> fruits > tea > wine > cereals > vegetables. The researchers concluded: "These data agree with the hypothesis that dietary antioxidants other than the well-known antioxidants contribute to our antioxidant defense. Surprisingly, the single greatest contributor to the total antioxidant intake was coffee."

## DOES ACRYLAMIDE AND FURAN IN COFFEE POSE A RISK TO HUMAN HEALTH?

To date we have insufficient knowledge about the human relevance of the rodent tumors seen in the acrylamide bioassays, acrylamide's bioavailability from foods, human dietary exposure, comparative species metabolism, acrylamide's activation to glycidamide vs. its detoxification by glutathione. A huge research program is currently underway at the U.S. National Toxicology Program, including chronic feeding bioassays of acrylamide in rat and mouse chow diets, as well as complex mechanistic studies. While acrylamide is not a proven human carcinogen, we hope to be able to show that it has a "practical threshold" in key human organs and that it can be safely consumed by people. These so-called "carcinogens" (acrylamide, furan and others) have produced an increase in tumors in high-dose rodent feeding bioassays, but these results may not be relevant to human cancer risk. The human epidemiology database on coffee generally supports the lack of cancer risk and perhaps even a protective effect of

coffee against colorectal cancer. In terms of overall cancer risk, the greater impact of coffee (and other browned foods and beverages) may actually result from the health-protective compounds produced by the Maillard Browning Reaction. Consequently, we must encourage health and regulatory authorities globally to carefully assess the risk of whole foods and beverages, not trace levels of individual chemicals in these products, and to begin to give much greater public health consideration to the potential health benefits of heated foods and beverages, such as coffee.

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# Coffee, Health and Consumption in Brazil: Past and Present

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**SUMMARY**

The objective of this paper is to show how coffee and health research results have helped Brazil increase coffee consumption, how coffee and health findings have been used and how these results are being used to increase coffee consumption in Brazil.

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Marketing is where art and science meet. Marketing is where rational, concrete research findings are blended with the right amount of emotional appeal to make the consumer move in the direction intended.

This is very much the case when we deal with coffee, health and marketing. We want to use research results, like the ones presented here, to induce consumers to drink more coffee, be it to create new consumers, to recover old ones, and/or to coax current consumers to drink more.

The program that used coffee and health issues to promote coffee consumption in Brazil was pioneered by ABIC, the Brazilian Coffee Roasters Association.

In the late 1980's the roasting community was worried about the decline in coffee consumption, total and per capita. Total consumption had fallen to under 6 million bags. Per capita consumption had fallen to an all time low of 2.8 kg person/year from an average of 5 in the past.

In 1987 ABIC hired a market and consumer survey. Health related restrictions to coffee drinking appeared strongly in the survey results. Out of the total of persons interviewed

- 48.3% said that coffee is bad for the health,
- 43.2% said that coffee should not be drunk by children, and
- 52.3% said that coffee makes children nervous.

Ex-consumers of coffee stated that coffee is bad for the health and that doctors told them not to drink coffee.

In spite of the strong impact of health issues in limiting, restraining and curbing coffee consumption in Brazil, purity, and not coffee and health, gained the center stage of the Brazilian coffee promotion campaign that ABIC launched in 1989, when total consumption reached 5.8 million bags/year, the lowest point in many years.

Coffee and health issues affected consumption in an important way but coffee purity and coffee quality were more critical concerns and easier to address at the time. Coffee and health was not forgotten, but it was not clear how to address it then.

The initial results of the campaign were positive: consumption jumped from 5.8 to 6.4 million bags/year.
In its search for how to handle coffee and health issues, the coffee roasting community came across two personalities, Dr. Darcy Lima, a physician, and Dr. Luiz Trugo, a biochemist. They were both professors at the Federal University of Rio de Janeiro (UFRJ) doing work on coffee compounds other than caffeine and the impacts of coffee drinking on behavior and health.

They started work on the important role of chlorogenic acids and Dr. Lima hinted at the potential role of coffee in preventing alcohol and drug addiction and depression. Dr. Lima carried out an epidemiological study with Brazilian children linking coffee intake with concentration and memory.

Brazil was then getting ready to include coffee and health issues in the programs to promote coffee consumption, which reached 10.2 million bags/year at the end of 1995.

Coffee and health gained center stage in the Brazilian coffee consumption program in 1995 /1996.

The first feature was the "Coffee Minute", broadcast in prime time national TV immediately before the 8:00 PM news program. It was a TV ad designed like an editorial and public service announcement. It featured Dr. Lima himself dressed as a doctor. The setting was a research lab, with a microscope and books.

Four ads and different messages were aired over two years, with strong frequency at the beginning and less often later to control costs. The ads addressed the positive aspects of coffee drinking using rational messages with emotional overtones.

Another major action was an one-page paid article in the main weekly magazine in Brazil. It was also designed as a public service message but the content was more technical/scientific than in the TV ads.

At the end of 1996 consumption of coffee had grown to 11.0 million bags / year.

Let's describe briefly the four groundbreaking TV ads and their different messages.

The ads were conceived around four main themes:

- coffee can be good for your health,
- coffee and youth (it was already known that young consumers were being lost to
- other beverages),
- coffee is a good companion ("coffee is a social drink!") and
- coffee is an active drink.

The language used was simple, clear and objective but the setting was scientific in order to build credibility and trust in the message.

Scientific communication in developing and coffee producing countries may not necessarily follow the same strategies used in consuming countries: income, educational levels, aspirations and consumption drivers may be very different. Simple language and concepts must be used for effective communication. Emotional appeal, besides scientific facts, is

required. Otherwise the message may sound arrogant or academic and not reach the majority of the target audience.

One of the goals of effective coffee and health communication is to get people to talk about the issues proposed, i. e., to make the positive effects of coffee the subject of every day conversation. One wants to get free reliable word of mouth communication of the concepts.

Conflict and arguments about coffee and health within the medical profession and with scientists should not be blown to the wide public. Bad news spread much faster than good news.

Overall, the news that coffee has positive impacts on health was a welcome "shock" for coffee drinking Brazilians

A word of caution: one should be careful about accepting that health issues are a major barrier to consumption everywhere in the same way.

Any program to address health barriers or to transform health issues into drivers of coffee consumption must be based on careful surveys of consumers' perceptions to attack the problem adequately. Local culture and traditions, perceptions about coffee drinking and consumer behavior must be taken into account.

We know, for example, that in Brazil coffee and health is today a stronger barrier in low income, less educated population strata. They tend to take medical advice at face value. They do not question or challenge it because they have only limited access to other sources of information.

The Brazilian age group most sensitive to what they believe to be adverse impacts of coffee drinking is 25 to 35 year old, where consumers are being lost. The situation is the reverse in coffee growing areas where low income children drink coffee and mid to high income parents hesitate to give it to their children.

The coffee and health findings mentioned before supported the creation of a coffee and children program in Brazil in 1997/1998. The objectives of the program were to disseminate coffee culture, to show the positive aspects of coffee drinking and to develop the habit of coffee drinking not only among children but also at their homes. The target public was children and teens in grades 1 to 8 with a view to creating tomorrow's consumers.

The perception that coffee improves performance of school children led to the creation of a coffee in school snacks program from 2000 onwards. This program, directed to children at grades 1 to 4 or 1 to 8, enabled the inclusion of coffee in meals supplied by government in public schools. It started at the local level, in coffee producing municipalities, and it was later adopted by the state of Minas Gerais. There is an on-going lobby to make it a program at the federal level that could eventually reach up to 35 million children.

The coffee and children program helped keep coffee and health issues alive during the lean promotion years between 2000 and 2003.

In 2003 the Brazilian Coffee Fund (FUNCAFÉ) commissioned new market and consumer surveys like the ones done every two years in the 1990's. The idea was to seek guidance to relaunch the domestic consumption program that was scaled down after year 2000.

The survey found that health aspects became the major consumer concern about coffee consumption. Health aspects were found to be the strongest obstacle to coffee consumption by 50% of non-consumers and the strongest reason to decrease coffee consumption by 74% of consumers. Medical recommendation not to drink coffee was identified as the strongest health related obstacle, as mentioned by 60% of each group.

Meanwhile consumption continued to grow very slowly or even fall. Some sources claim it dropped slightly, to 13.6 million bags/year, at the end of 2003.

The relaunching of coffee and health programs is taking place in the current year of 2004. It embraces the following activities and components:

- coffee and heart program,
- coffee and health web site,
- survey with medical doctors,
- TV programs directed at health care professions (HCP) and
- direct mailing to doctors with coffee and health research results.

The coffee and health budget for 2004 is US$ 200 thousand coming from the Brazilian Coffee Fund. Coffee and health is part of a promotion program whose objective is to reach consumption of 16 million bags in 2005 and to make Brazil the largest coffee consuming country in the world by 2010.

Let us describe briefly each major component of the coffee and health program.

The coffee and heart program started last May with a research agreement between the coffee sector and the prestigious São Paulo State University Medical School. The participants were the Brazilian Coffee Roasters Association (ABIC), the São Paulo Heart Institute (INCOR), which pioneered heart transplants in Brazil, and the Zerbini Foundation, created by a leading Brazilian heart expert. The Heart Institute is coordinating the program under the advice of Dr. Mario Maranhão and Dr. Darcy Lima. Dr. Maranhão is a former president of the Geneva based World Heart Federation. The initial objectives of the program are the creation of a Coffee Research Unit to evaluate the role of coffee in the prevention of heart disease and a study of 2,000 Brazilians during a period of 4 to 10 years to investigate the relationship between coffee drinking and heart and coronary performance.

The coffee and health web site (www.cafeesaude.com.br) has been revamped, modernized and updated. Its objectives are to inform about new findings and to update the medical profession. The target public is both the general population and health care professions. The language is not highly technical, often light-hearted. It is not a scientific site directed at the medical professions. It is coordinated by Dr. Darcy Lima.

The survey with medical doctors is about to be made using a statistically representative sample of 380 doctors in all relevant Brazilian regions. The survey will be divided in two stages. The first stage will evaluate the current knowledge about coffee and health held by the medical profession, define doctors' opinions about coffee drinking and orient communication with doctors in current and future programs. The second stage will evaluate the impact of the communication programs after they are implemented and measure the change in perception towards coffee of the target group. The study will be carried out by an opinion survey company with 15 years of experience in the coffee sector. It has been working for the coffee community since 1987.

The TV programs, to be broadcast in a network directed at the healthcare professions, will be specially produced to communicate and disseminate the positive effects of coffee to medical doctors. The programs be in number of 12, to be broadcast by the Medical Connection Network, a private TV network via satellite that uses an internet protocol (TVIP). The audited audience is 40 thousand healthcare professions viewers. The content will be suggested by the survey with doctors, one major subject per program, for example: coffee and heart, coffee and liver and coffee and physical exercise, etc. The strategy of communication, to be oriented by the survey, will use the technical language expected by doctors and other healthcare professions. The efficiency of the TV programs and their recall will be evaluated by the second stage of the survey mentioned above.

Finally, the medical letters program will start later this year. Its objective will be to update doctors on recent research results on coffee and health by means of insertions in major medical publications and the intensive use of the internet. Content will be extracted from recent research on coffee and health and based on issues suggested by the survey with doctors.

I hope I have described properly what the Brazilian coffee sector, specially its coffee roasting community, have done or are doing to promote domestic coffee consumption using coffee and health concepts.

# Awareness of Beneficial Effects of Coffee Among Doctors a Survey Report

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## SUMMARY

The much preferred every day drink of coffee has survived many controversies over the years regarding its beneficial/harmful effect on human health. Coffee drinking, in moderate amounts, was found to reduce the incidence of colorectal cancer, cirrhosis of liver and bronchial asthma and also reduce the risk of developing gallstones. The positive effects of coffee is mainly attributed to the presence of compounds like caffeine, polyphenols including CGA, derivative and degradation products such as caffeic acids, phenylindans & melanoidins which have anti-oxidant properties and hence contribute to the antioxidant defence system in the body.

Coffee Board has taken initiative to disseminate the medical findings related to the positive effects of coffee on human health. In this direction, the Coffee Board of India conducted a survey during Annual CME Conference of the Family Physicians Association during, December 2003 among various sectors of population like doctors, school children etc. to judge the current level of awareness about positive effects of coffee.

The present study summarizes the views/opinions obtained from the survey conducted among the doctors, who are major influencers in the dietary habit of people.

## INTRODUCTION

The much preferred every day drink of coffee has survived many controversies over the years regarding its beneficial/harmful effect on human health. There is a general myth in the minds of people that Coffee drinking is harmful to human health and is the cause of various cardio vascular disorders relating to heart. Recently a plethora of research studies across the world has highlighted the beneficial effects of coffee on health.

Coffee drinking, in moderate amounts, was found to reduce the incidence of colorectal cancer, cirrhosis of liver and bronchial asthma and also reduce the risk of developing gallstones. Three to four cups of coffee per day was found to reduce the risk of Parkinson's disease by two to three fold (Lima, 2001).

An inverse relationship is observed between coffee intake and depression/ suicides and also between coffee intake and alcoholism/ cirrhosis (Steffen, 2001). The positive effects of coffee is mainly attributed to the presence of compounds like caffeine, polyphenols including CGA, derivative and degradation products such as caffeic acids, phenylindans & melanoidins which have antioxidant properties and hence contribute to the antioxidant defence system in the body.

Coffee Board has taken initiative to disseminate the medical findings related to the positive effects of coffee on human health. As a measure of this, Coffee Board has planned a series of awareness programmes on positive effects of Coffee on Health and also initiated a series of surveys among various sectors of population like Doctors, school children etc. to judge the current level of awareness about positive effects of coffee. Many doctors advocate their patients to stop drinking coffee without actually knowing the beneficial effects of coffee.

The current survey was conducted to study the level of awareness among doctors, who are major influencers in the dietary habit of people.

## METHODOLOGY

About 115 medical practitioners, mostly family physicians, in Bangalore was provided with questionnaire and surveyed for their views on coffee drinking, their recommendation to the patients and the effect of Coffee on human health. The views/opinion obtained regarding various questions were detailed under results and discussion section.

## RESULTS AND DISCUSSION

**Question No. 1:** Do you drink Coffee? YES/ NO (If Yes, Everyday/Occasionally/Very Rarely).

**Reponses:** Among the doctors surveyed, 95% were coffee consumers while 5 % did not prefer coffee for consumption. Of the coffee drinkers, 7% were high coffee drinkers i.e., consumed more than 6 cups per day, 20% were medium coffee drinkers (3-5 cups per day) and 72% were low coffee drinkers (consumed 1- 3 cups per day). The result is depicted in the following pie chart.

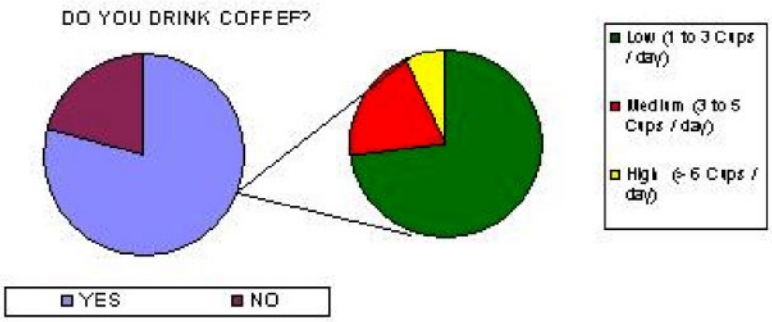


**Figure 1.**

**Question No. 2:** Do you believe coffee drinking in moderation is good for health? YES/ NO

**Responses:** About 77% of the doctors felt that Coffee drinking in moderation is good for Health while 20 % was of the opinion that coffee drinking in moderation is not good for human health. Three percent were neutral on the issue (Pie Chart No. 2).

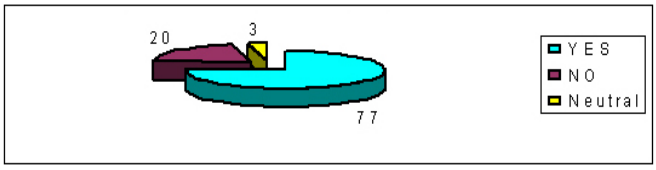


**Figure 2. Coffee drinking moderation is good for Human Health.**

**Question No. 3:** Do you recommend to your patients to stop coffee drinking? YES/ NO

If Yes, What are the reasons for such a recommendation? a) Increases acidity, b) Not good for pregnant women c) Reduces immunity, d) Any other reason (Pl. mention) and e) Increases blood pressure.

**Responses:** About 52% of the doctors surveyed did not recommend their patients to stop drinking coffee. 45% recommended their patients to stop drinking coffee. Among the doctors who recommended their patients to stop drinking coffee, 88% attributed acidity as the reason to recommend their patients to stop drinking coffee while 21% of Doctors suggested increase in Blood pressure as the reason. 17% were of the opinion that Coffee is not good for pregnant women while 2% of the doctors felt that drinking coffee reduced immunity.

Around 17% of the doctors cited other reasons such as incidence of insomnia, anxiety, peptic ulcers, diabetes and cardiac manifestations as the reason for recommending their patients to stop drinking coffee. 3% of the doctors did not advise their patients on coffee consumption and were neutral on the issue.

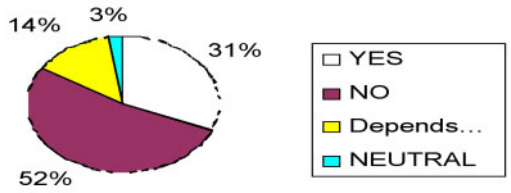


**Figure 3.**

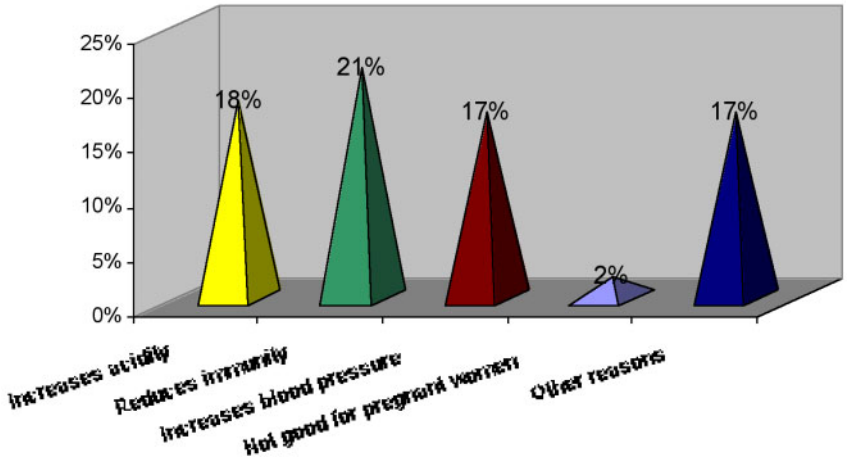


**Figure 4. Reasons for recommending patients to stop drinking coffee.**

**Question No. 4:** Did you know that coffee drinking in moderation (up to 4 cups per day) helps in: a) Increasing alertness, b) Reducing hepatitis disease (Cirrhosis), c) Reducing colorectal cancer, d) Reducing kidney stone, e) Recovery from degenerative brain disorder?

**Responses:** 83% of the doctors were found to be aware of the various positive effects of coffee. Among these majority (97%) were aware of the fact that coffee drinking caused and increase in alertness and 20% of the doctors were also aware of some other positive effects of coffee like reducing colorectal cancer, reducing kidney stones and recovery from degenerative brain disorders. 15% Doctors surveyed were not aware of the positive effects of coffee on

human health when taken in moderate amounts. While 3% of the doctors were of the opinion that drinking coffee in moderate amounts had no positive effects on health.

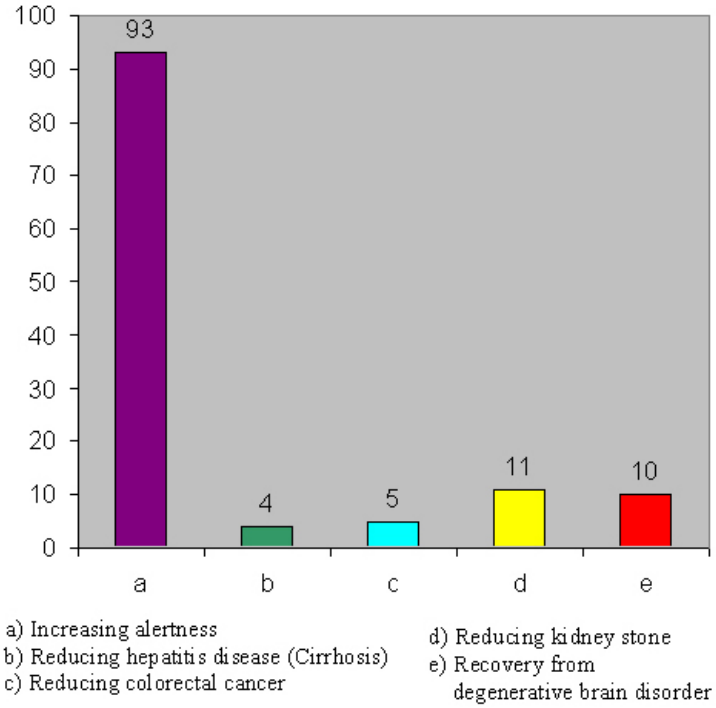


**Figure 5. Awareness on positive health benefits of coffee.**

**Table 1.**

| | | | | | |
|---|---|---|---|---|---|
| | **Pro-forma for Survey of Medical Practitioners about their opinion on Coffee** | | | | |
| 1 | Name of the Respondent | | | | |
| 2 | Address with telephone No. and e- mail | | | | |
| 3 | Do you drink Coffee | 1) YES | 2) NO | | |
| | If Yes | a) Everyday | b) Occasionally | c) Very Rarely | |
| 4 | Do you believe coffee drinking in moderation is good for health? | | | 1) YES | 2) NO |
| 5 | Do you recommend to your patients to stop coffee drinking? | | | 1) YES | 2) NO |
| | If Yes, What are the reasons for such a recommendation? | | | | |
| | | a) Increases acidity | d) Not good for pregnant women | | |
| | | b) Reduces immunity | e) Any other reason (Pl. Mention) | | |
| | | c) Increases blood pressure | | | |
| 6 | Did you know that coffee drinking in moderation (up to 4 cups per day) Helps in: | | | | |
| | | a) Increasing alertness | d) Reducing kidney stone | | |
| | | b) Reducing hepatitis disease (Cirrhosis) | e) Recovery from degenerative brain disorder | | |
| | | c) Reducing colorectal cancer | | | |

**ACKNOWLEDGEMENT**

The authors immensely thank Dr. H. M. Fareed, Secretary, Family Physicians Association who helped in collection of data among the family physicians during the Annual CME

Conference of the Family Physicians Association held on 28/12/2003 held at Bangalore and also thanks to individual doctors who spent their valuable time in responding to the queries.

The above results were presented during the One Day Seminar on Coffee and Health organised by the Coffee Board for the Doctors on 14th February 2004 at Hotel The Grand Ashok, Bangalore.

## CONCLUSION

From the survey conducted with 115 doctors regarding their opinion on coffee consumption and its affect on human health revealed that about 40% of the doctors were aware of the various positive effects of coffee on human health and 38% of the doctors were unaware of the vast positive effects of coffee on human health though they agreed with the fact that coffee drinking caused an increase in alertness. Further, 10% of the doctors were not aware of the positive effects of coffee including increase in alertness while, 11% of the doctors considered coffee consumption to be harmful to human health.

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# Chlorogenic Acids in Coffee – Absorption and Excretion by Human Volunteers

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## SUMMARY

Coffee is a rich source of antioxidant phenolic hydroxycinnamates, particularly the chlorogenic acids, 3-, 4- and 5-caffeoylquinic acids. In a preliminary study to investigate the absorption and excretion of chlorogenic acids, 250 ml of a medium roast filter coffee was consumed by two healthy human volunteers and two subjects with an ileostomy. Ileal fluid and urine collected over a 24 h period were analysed by HPLC with diode array and $MS^n$ detection allowing the identification and quantification of chlorogenic acids and their metabolites. The average recovery of chlorogenic acids and trace amounts of metabolites in ileal fluid was 24% of the amount in the ingested coffee indicating that 76% had been absorbed in the small intestine. The major component derived from the chlorogenic acids to be excreted in urine was a ferulic acid sulphate. In healthy subjects the amount excreted corresponded to ~40 % of the ingested chlorogenic acids while ferulic acid sulphate in the urine of ileal volunteers corresponded to ~28 % of intake. These findings indicate that in healthy subjects the caffeoylquinic acids in the coffee are very efficiently absorbed into the bloodstream from the gastrointestinal tract with 70% of this occurring in the small intestine and the remainder in the large intestine. The chlorogenic acids that pass from the small to the large intestine have antioxidant activity raising the possibility that they may exert protective effects against colon cancer.

## INTRODUCTION

Coffee, one of the most widely consumed beverages in the world (Duthie and Crozier, 2003), is a very rich source of phenolic hydroxycinnamates, particularly the chlorogenic acids: caffeoylquinic (CQA), *p*-coumaroylquinic (*p*CoQA), feruloylquinic (FQA), dicaffeoylquinic (diCQA) and caffeoylferuloylquinic acids (CFQA) (Figure 1) (Clifford, 2000).

Plant-derived phenolics are reported to have wide ranging biological activities. Their potential as antioxidants has led to investigations into protective effects against degenerative diseases such as cardiovascular disease and cancers. However, epidemiological evidence on the protective effects of coffee consumption against coronary heart disease and cancers is, as yet, inconclusive.

Little is known about the absorption, bioavailability and biotransformations of CQAs in the human system, this information is essential in order to evaluate possible health effects. Previous studies have indicated that following coffee ingestion, 5-CQA

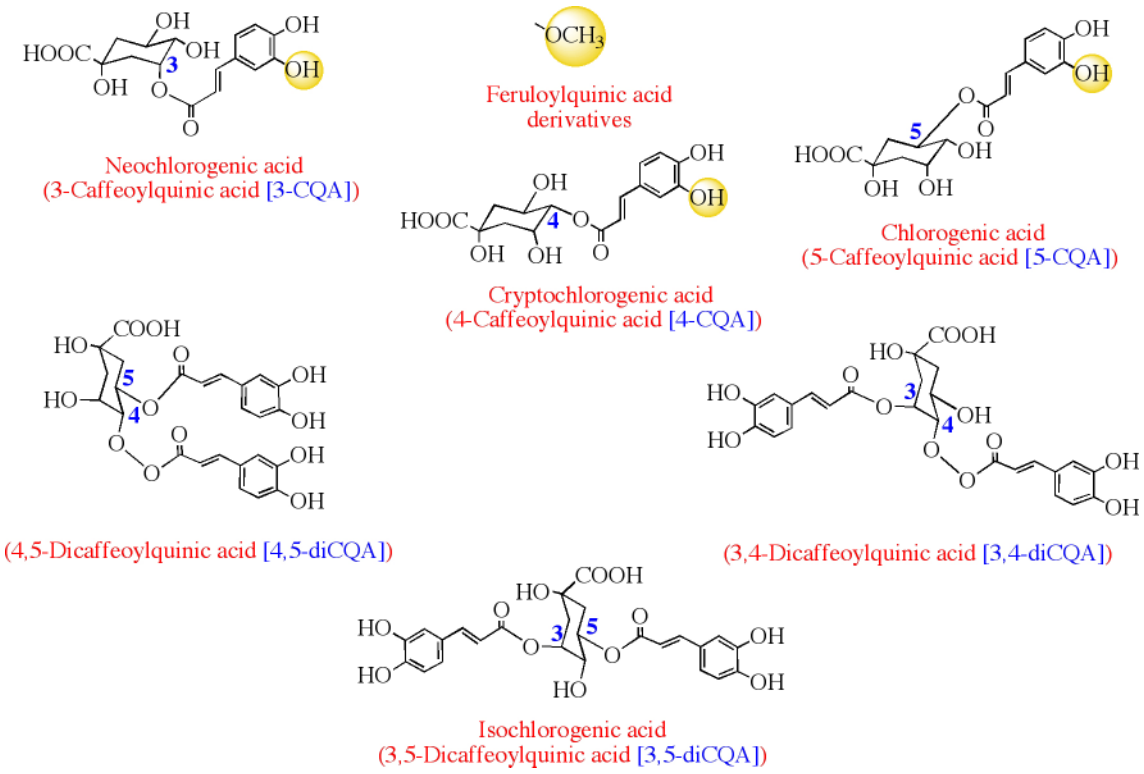


**Figure 1. Structures of the main chlorogenic acids.**

is not absorbed in the small intestine due to the lack of an esterase activity capable of hydrolysing the hydroxycinnamate-quinic acid link (Clifford, 2000). Similarly, absorption studies using rats concluded that 5-CQA is not well absorbed and remains virtually unmodified in the upper digestive tract and that in the large intestine it is catabolised by the colonic microflora yielding phenoxyacetic acids which, to an as yet undetermined extent, pass into the circulatory system and are excreted in urine (Azuma et al., 2000; Gonthier et al., 2003). CQA metabolites have been detected in human plasma and urine following coffee consumption. The plasma metabolites were caffeic acid sulphates and glucuronides (Nardini et al., 2002), while urinary metabolites identified following enzyme and acid hydrolysis include caffeic acid, ferulic acid and hippuric acid (*N*-benzoylglycine) (Olthof et al., 2003).

The use of human volunteers with an ileostomy in studies on the absorption of dietary phenolics provides very useful data as by measuring the levels of phenolics and their metabolites in ileal fluid, compared to what was ingested, it is possible to calculate how much has been absorbed in the small intestine and entered the blood stream. Such data can then be related to the levels of components in urine, which in turn provides information on the metabolic forms in which the phenolics are transported in the circulatory systems and excreted from the body.

In a preliminary study to investigate the absorption and excretion of chlorogenic acids from coffee, two healthy human volunteers and two subjects with an ileostomy were fed coffee and over the next 24 h provided ileal fluid and urine for analysis. The coffee and biological samples collected at intervals over 24 h, were analysed by high performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometric (MS) detection. Data so obtained provides information on what happens to chlorogenic acids in the small intestine, to what extent they are metabolised and/or absorbed and what components pass into the large intestine where, before being expelled in faeces, they may be further modified by the action of colonic bacteria.

## EXPERIMENTAL

*Materials:* Coumaric acid, ferulic acid and caffeic acid were purchased from Sigma (Poole, Dorset, UK). 5-CQA was supplied by AASC Ltd. (Southampton, UK). HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland). [$^{14}$C]Caffeic acid was provided by Dr. R. Hartley, Department of Chemistry, University of Glasgow. Five g of medium roast ground coffee (Sainsbury's Stores, Crow Rd, Glasgow, UK) was used to prepare 250 ml of filtered coffee.

*Feeding study with human volunteers:* Two healthy human subjects and two volunteers with an ileostomy, who were otherwise healthy, were recruited and gave their written informed consent, (non-smokers, one male and one female in each group). The Glasgow Royal Infirmary Ethics Committee approved the study protocol. All subjects were instructed to follow a low phenolic diet for 3 days prior to the study by avoiding foods and beverages containing >15 mg/kg phenolics and minimising the intake of all other fruits and vegetables. On day 4 after an overnight fast, subjects consumed 250 ml of filter coffee. Subjects provided a urine sample prior to coffee consumption and further samples 0-2 h, 2-5 h and 5-24 h after drinking the coffee. Ileostomy subjects provided samples of ileal fluid at the same time points. Urine and ileal fluid were stored at –80°C prior to analysis.

*Sample preparation:* Twenty five ml of methanol, containing $10^6$ dpm [$2^{-14}$C]caffeic acid as an internal standard, was added to 2 g of homogenised ileal fluid and further homogenised using an ultra Turrax mixer for 2 min. Samples were then centrifuged at 2000 *g* for 20 min at 4°C. One ml of the resulting supernatant was collected and reduced to dryness in vacuo before being resuspended in water/methanol/formic acid (89:10:1, v/v) prior to analysis. Urine samples were defrosted and thoroughly mixed prior to analysis.

*Measurement of radioactivity:* Fifty μL aliquots of ileal fluid extracts were added to 5 ml of scintillation cocktail (Optiflow Safe One, Fisons, Loughborough, UK) before determination of radioactivity using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

*HPLC with diode array and mass spectrometric detection:* Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 x 4.6 mm i.d. 4 μm Synergi Polar-RP column (Phenomenex, Macclesfield, UK) eluted with a gradient over 60 min of 5-25% acetonitrile in 0.1% formic acid at a flow rate of 1 mL/min and maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL/min was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface. (Thermo Finnigan) operating in negative ion mode. Analyses were carried out using full scan, data dependant MS-MS scanning from *m/z* 100 to 2000. Capillary temperature was 250°C, sheath gas and auxiliary gas were 60 and 10 units/min respectively, and source voltage was 2kV.

*On-line HPLC-ABTS$^+$ antioxidant detection:* The antioxidant activity of coffee and ileal fluid was determined using the ABTS$^+$ assay based on the method described by Stewart et al. (2005). A 2 mM ABTS$^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated at room temperature in darkness overnight to allow for stabilisation of the radical. ABTS$^+$ reagent was prepared by diluting the stock 8-fold in phosphate buffer at pH 8. Five μl of coffee and 100 μL of ileal fluid extract were injected into a Surveyor HPLC system. Separation was achieved using a 5-25 % gradient of acetonitrile in 0.1% formic acid

as described above but with a 30 min gradient. Following separation and PDA analysis the HPLC eluate was mixed with $ABTS^+$ reagent at a flow rate 0.5 ml/min supplied by a Shimadzu LC-10 AD *vp* pump. Absorbance was then measured at 720 nm.

## RESULTS AND DISCUSSION

*Analysis of coffee:* Freshly prepared coffee was analysed by HPLC-$MS^n$ with chlorogenic acids identified using the hierarchical MS fragmentation scheme of Clifford et al. (2003). This facilitated the detection of 3-, 4-, 5-CQA and a 5-CQA isomer, 3-, 4- and 5-FQA and three undetermined DiCQAs distinct from 3,5-DiCQA. The quantities of these compounds present in the 250 ml of coffee ingested in the feeding study by the human volunteers are presented in Table 1. CQAs were the most abundant chlorogenic acids followed by FQAs and the DiCQAs. Trace amounts of unquantifiable *p*-CoQAs and CFQAs were also detected. In total, the 250 ml of coffee consumed by each of the human volunteers contained 220 mg of chlorogenic acids.

**Table 1. Amounts of chlorogenic acids in 250 ml medium roast coffee consumed by human volunteers[§].**

| Chlorogenic acids | Amount |
|---|---|
| 3-Caffeoylquinic acid | 45 ± 0.2 |
| 4-Caffeoylquinic acid | 47 ± 0.5 |
| 5-Caffeoylquinic acid | 86 ± 1.1 |
| 5-Caffeoylquinic acid isomer | 10 ± 0.1 |
| **Total caffeoylquinic acids** | **188 ± 1** |
| | |
| 3-Feruloylquinic acid | 2.9 ± 0.1 |
| 4-Feruloylquinic acid | 14 ± 0.1 |
| 5-Feruloylquinic acid | 6.7 ± 0.1 |
| **Total feruloylquinic acids** | **24 ± 0.2** |
| | |
| Dicaffeoylquinic acid isomer 1 | 3.4 ± 0.1 |
| Dicaffeoylquinic acid isomer 2 | 1.9 ± 0.1 |
| Dicaffeoylquinic acid isomer 3 | 2.8 ± 0.1 |
| **Total dicaffeoylquinic acids** | **8.1 ± 0.1** |
| | |
| **Total chlorogenic acids** | **220 ± 1.3** |

[§]*Data expressed in mg/250 ml coffee ± standard error (n = 3).*

The antioxidant capacity of the individual chlorogenic acids in coffee was assessed using HPLC with the on-line $ABTS^+$ system (Figure 2A). Antioxidant activity was clearly associated with the presence of the chlorogenic acids with the main contributors being 3-, 4- and 5-CQA. A recent study determined that on a per cup basis coffee had a higher antioxidant capacity than either cocoa, green tea or black tea (Richelle et al., 2001). Furthermore, increased plasma antioxidant activity has been detected after the consumption of coffee (Natella et al., 2002).

*Analysis of ileal fluid:* The average recovery of chlorogenic acids in ileal fluid compared to the quantities consumed by the ileostomy volunteers was 24% (Table 2). Most of the chlorogenic acids were unmodified with only trace amounts of sulphated and glucuronide metabolites being detected. The average recovery of CQAs was 18% and that of FQAs 27%

indicating that in excess of 70% of these compounds were absorbed during passage through the stomach and small intestine. In contrast, the average recovery of DiCQAs was 65% implying a more limited absorption (Table 2).

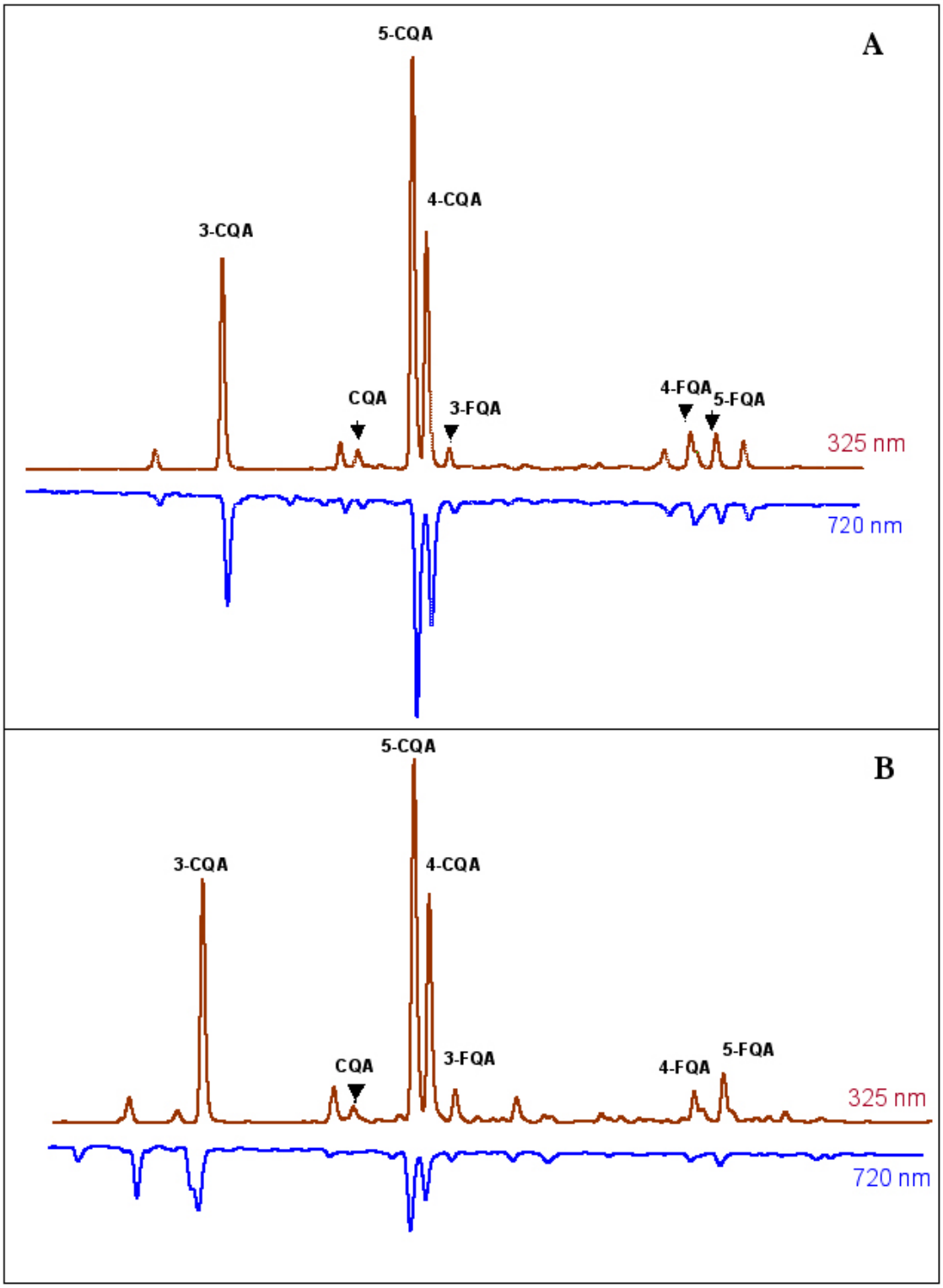


**Figure 2. On-line $ABTS^{+}$ analysis of (A): 5 µL of medium roasted filter coffee and (B) 100 µL of an extract of ileal fluid collected 2-5 h following the consumption of 250 ml of coffee. Samples were injected into a Surveyor HPLC system, separation was achieved on a Synergi Polar-RP column using a 5-25 % gradient of acetonitrile in 0.1 % formic acid over 30 min. Samples were analysed with detection at 325 nm before mixing with $ABTS^{+}$ reagent and monitoring at 720 nm.**

Analysis of ileal fluid using HPLC with the on-line $ABTS^+$ system (Figure 2B) revealed an antioxidant profile very similar to that obtained with the coffee (Figure 2A) although the overall level of antioxidants, in keeping with chlorogenic acid content was only 24% of that in the ingested coffee. None-the-less, this indicates that after drinking coffee the chlorogenic acids that pass from the ileum into the large intestine retain antioxidant activity and, therefore, may exert protective effects within the colon.

**Table 2. Level of chlorogenic acids and their metabolites in the ileal fluid of two volunteers collected 0-24 h after the consumption of 250 ml of medium roasted coffee[§].**

| Chlorogenic acids | Volunteer 1 | Volunteer 2 |
|---|---|---|
| Caffeoylquinic acids | 29.8 ± 1.8 (16%) | 37.8 ± 0.4 (20%) |
| Feruloylquinic acids | 4.1 ± 0.3 (17%) | 5.9 ± 0.3 (24%) |
| 3-Feruloylquinic acid glucuronide* | 0.2 ± 0.0 | 2.4 ± 0.2 |
| Total including metabolite | 4.3 (18%) | 8.3 (35%) |
| Dicaffeoylquinic acids | 5.4 ± 0.3 (67%) | 5.2 ± 0.2 (64%) |
| Ferulic acid sulphate* | 14.1 ± 0.3 | n.d. |
| **Total** | **53.6 (24%)** | **51.3 (23%)** |

[§]*Data expressed in mg ± standard error (n = 3). Italicised figures in parentheses represent the level of the compound expressed as a percentage of the amount ingested. n.d. – not detected; * indicates a metabolite.*

Olthof et al. (2001) fed 5-CQA to human ileostomy volunteers and reported that ca. 30% of the ingested CQA was absorbed in the small intestine, a lower estimate than obtained in the present study following the consumption of coffee. Volunteers were fed a 1 g 5-CQA supplement, which is equivalent to five of the 250 ml cups of coffee used in the present study. This large dose may explain the lower estimate of absorption obtained by Olthof et al. (2001).

*Analysis of urine:* Samples of urine were provided prior to coffee consumption and analysed to ensure that no chlorogenic acids or putative metabolites were present, confirming that the volunteers had adhered to a low phenolic diet prior to the study. Urine samples were then collected from both healthy subjects and ileostomy volunteers over a 24 h period after drinking coffee. Analysis by $HPLC\text{-}MS^n$ revealed that none of the chlorogenic acids originally present in the coffee were excreted in urine. However, trace levels of a number of sulphated, methylated and glucuronide metabolites of the chlorogenic acids were detected but by far the major component excreted in urine was a ferulic acid sulphate (Table 3). This finding is keeping with data obtained in a study by Cremin et al. (2001) in which prunes, which contain caffeic acid, ferulic acid and 5-CQA, were ingested by human volunteers. Caffeic acid and ferulic acid and lower levels of 5-CQA were detected in urine as conjugates with the aglycones being released by incubation with β-glucuronidase and sulphatase.

**Table 3. Excretion of ferulic acid sulphate in urine by healthy and ileostomy volunteers 0-24 h after the consumption of 250 ml of medium roast coffee[§].**

| | Healthy subjects | | Ileostomy subjects | |
|---|---|---|---|---|
| | Volunteer 1 | Volunteer 2 | Volunteer 1 | Volunteer 2 |
| Ferulic acid sulphate | 75 ± 4.8 *(34%)* | 101 ± 6.3 *(45%)* | 67 ± 2.6 *(30%)* | 58 ± 3.0 *(26%)* |

[§]*Data expressed as mg ± standard error (n = 3). Italicised figures in parentheses represent the level of the ferulic acid sulphate expressed as a percentage of the amount of chlorogenic acids ingested.*

The mean level of ferulic acid sulphate excreted, expressed as a percentage of the chlorogenic acids in the ingested coffee was ~40% for the two healthy subjects and ~28% for the ileal volunteers. This implies that around 70% of the absorption of chlorogenic acids into the blood stream from the gastrointestinal tract occurs in the small intestine and 30% in the large intestine. It is also evident that absorption of both CQAs and FQAs from coffee in the gastrointestinal (GI) tract involves hydrolysis, probably by mucosal esterases that occur throughout the GI tract of mammals (Andreasen et al., 2001), releasing caffeic acid and ferulic acid. At some, as yet undetermined, locus prior to excretion, a combination of methylation and sulphation result in the conversion of both caffeic and ferulic acid to ferulic acid sulphate which is excreted in urine in sizable amounts.

Although the amount of ferulic acid sulphate in the urine of the healthy human subjects accounted for ~40% of the ingested coffee chlorogenic acid, the fate of the remaining 60% remains undetermined. Some of the compounds that were absorbed could be sequestered in body tissues while the chlorogenic acids passing into the large intestine that are not absorbed may be catabolised by colonic bacteria to low molecular weight phenoxyacetic acids. Olthof et al. (2003) analysed the urinary excretion of phenoxyacetic acids over a 24 h period following the ingestion of 5-CQA by human volunteers and observed a more than 3-fold increase in the level of hippuric acid. Phenoxyacetic acids were not analysed in the current study. They have a low extinction coefficient and a $\lambda_{max}$ below 250 nm and as a result are not easily detected with an absorbance monitor and, in addition, they do not ionize readily when subjected to MS with an electrospray interface. Although HPLC-MS with an electrospray interface has been used to analyse phenoxyacetic acids the method lacks the sensitivity it exhibits with other phenolics and flavonoids so quantitative analysis has to be based on selected reaction monitoring rather than full scan MS (Gonthier et al., 2003). The method of choice of many investigators when analyzing putative phenoxyacetic acid catabolites is gas chromatography-mass spectrometry with electron impact ionization (Olthof et al., 2003).

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# Coffee, Caffeine and Cognitive Performance

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## SUMMARY

We studied the role of caffeine in action monitoring as expressed by the error-related negativity (ERN), an event-related brain component that reflects anterior cingulate cortex (ACC) activity. In a double-blind, placebo-controlled, within-subjects experiment, two caffeine doses (3 and 5 mg/kg BW) and a placebo were dissolved in a cup of decaffeinated coffee and administered to 15 habitual coffee drinkers. Compared with placebo, both caffeine doses enlarged the ERN. The P2 and P3 amplitudes were not affected by caffeine. Thus, the enlarged ERN after caffeine reflects a specific effect on action monitoring. We conclude that consumption of a few cups of coffee intensifies central information processing, specifically the monitoring of ongoing cognitive processes for signs of erroneous outcomes.

We also studied the effects of caffeine on task switching in alternated task (AABB) blocks and single-task (AAAA) blocks. Participants alternated between two tasks (A and B) in a predictable manner. Reaction time switch costs were reduced compared to placebo. ERPs revealed a negative deflection that developed within the preparatory interval, which was more negative for switch compared to repeat trials. This switch-related difference was increased under caffeine compared to placebo. These results suggest that a few cups of coffee improve task-switching performance by enhancing anticipatory processing such as task-set updating.

## INTRODUCTION

Moderate amounts of caffeine lead to subtle improvements in cognitive operations, the most reported of which are faster reactions and sometimes fewer errors (Lorist et al., 1996; Ruijter et al., 2000). These improvements result from both general caffeine effects on arousal, such as enhanced alertness and wakefulness, and from more specific effects on perceptual, attentional and motor (response preparatory) processes (Lorist & Snel, 1997; Ruijter et al., 2000; Snel et al., 2004). In contrast, sensitivity of central higher-order processes to caffeine has received little attention. Therefore, we tested the assumption that caffeine improves action monitoring, the ability to monitor ongoing processing in the cognitive system for signs of conflict or erroneous outcome.

The psychophysiological index of action monitoring is the error-related negativity ERN, a sharp negative deflection in the event-related brain potential (ERP) with a frontocentral distribution and a peak within 100 ms after an incorrect response. The ERN is said to be generated when a negative reinforcement-learning signal is conveyed from the mesencephalic dopaminergic system to the ACC, which utilizes the signal to modify task performance.

Since caffeine is a psychoactive stimulant we expected larger ERN amplitudes in both caffeine conditions compared to placebo, and larger for a high dose than for a low dose.

The second question we liked to answer was whether caffeine could facilitate switching of tasks. Task-switching paradigms typically require switching back and forth between two choice-reaction time (RT) tasks afforded by the same class of stimuli. In order to succeed, the subject must internally represent and update task-set information about each task, i.e. the appropriate rules that govern the mapping between stimuli and responses. This internal representation and rapid updating of task-set information is critical for enabling a quick reaction to a switch of task.

Changing of task incurs a switch cost: mean RT is longer and the error rate is usually greater than with repetition of a task.

The switch cost is reduced, but not eliminated, by an opportunity for preparation. A "residual" cost remains that is immune to elimination by further lengthening of the preparation interval (Jong, 2000; Rogers & Monsell, 2002). In addition to preparation time, other factors affecting the size of the switch cost include the overlap between stimulus and response attributes (Allport et al., 1994; Meiran, 2000), prestimulus cueing (Meiran, 1996), factors encouraging advance preparation (Nieuwenhuis & Monsell, 2002), and relative task strength or familiarity (Allport et al., 1994; Rubinstein et al., 2001). Although ERP studies are difficult to compare, since the various components are strongly dependent on the specific task manipulations and stimulus materials, they all report a slow-wave negativity within the preparation period, which differentiated between switch and repeat conditions. The authors interpret this component as reflecting anticipatory control processes in task switching.

## TASK AND PROCEDURE OF STUDY 1 AND 2

### Task

Participants had to switch between two simple tasks in a predictable manner (AABB). This paradigm allowed us to test hypotheses about the actions of caffeine on action monitoring (study 1) and on task switching (study 2).

After the task instructions, a grey square with four quadrants (2 $cm^2$ each) was displayed continuously at the centre of a black screen. Stimuli appeared, clockwise, one by one, in the centre of one of these quadrants. The stimuli were red and blue letters, randomly chosen from the set A, E, O, U, G, K, M and R, and printed in an uppercase Arial font (0.5 x 0.8 cm). The participants were instructed to judge whether the colour of the letter was blue or red (colour task), but only if the letter appeared in either of the two upper squares. The other task was to judge whether the letter was a consonant or a vowel (letter identity task) if the letter appeared in the two lower squares, or vice versa. The other half of the participants was instructed to perform the colour task if the letter appeared in either of the two left squares, and the letter identity task when it appeared in the two right squares, or vice versa. Stimuli remained on the screen until participants gave a response or until 2500 ms had elapsed. After a Response-Stimulus Interval (RSI) of 150, 600, or 1500 ms (selected randomly but equiprobably) the next letter appeared on the screen. Stimulus-response mappings were counterbalanced across participants. Speed and accuracy were equally emphasized. The EEG was recorded with a 64-channel tin-electrodes Quikcap (Neuroscan, Inc.) referenced to the left earlobe. Impedance was kept below 5 kΩ. Eye movements were recorded from bipolar tin electrode pairs placed above and below the left eye, and left and right of the outer canthi of both eyes. EEG signals were amplified by SYNAMPS amplifiers (Neuroscan, Inc.) and online filtered with a time constant set to 5 seconds and a low pass of 35 Hz. The data were digitized at 250 Hz.

### Procedure

After a training session the subjects participated in three experimental sessions of 3 h each, starting at 9.30 a.m. The experimental task started 45 minutes after drinking the coffee. A total of 12 blocks were presented with a short break after the sixth block. The task lasted about 90 minutes.

## STUDY 1 ACTION MONITORING: ERRORS

### Subjects

Participants were 15 healthy, non-smoking students (mean age 20.4±2.3 years). They consumed on average 3.6±1.2 cups per day (mean ~374±127mg). The used caffeine doses were 3 and 5mg/kg BW caffeine (low dose), the placebo was 3 mg/kg BW lactose. The doses were dissolved in a cup of decaffeinated coffee was administered. The caffeine abstinence period was 12 hours.

### Statistical analyses

Individual averages for error rates, reaction times, and ERP component amplitudes were analysed with repeated measures analyses of variance (ANOVA). Performance data were analysed with the factors treatment (placebo, low dose, and high dose) and correctness (correct vs. incorrect). Amplitudes were analysed only for error trials. To correct for violations of the sphericity assumption in the ANOVA, degrees of freedom were corrected using Huynh-Feldt method whenever appropriate. Corrected p-values but uncorrected df-values are reported, the latter to facilitate interpretation of the data. Statistically significant main effects of caffeine are followed up by Helmert contrasts analyses, involving two orthogonal contrasts. The first contrast evaluates placebo against the mean of the two caffeine conditions; the second contrast tests the low dose against the high dose condition.

To obtain sufficient numbers of errors, we pooled trials across these conditions, separately for correct and incorrect responses. However, for incorrect trials we selected only those trials that were followed by a 600 or 1500 ms RSI in order to eliminate contaminating effects in the ERP due to processing of the previous response. The mean number of erroneous trials that were averaged into the ERN was 50.60 ± 28.6 (placebo), 43.27 ± 26.1 (3mg caffeine) and 39.33±26.1 (5 mg caffeine). Responses were defined as correct when made with the correct hand between 150 ms and 2500 ms after stimulus onset. Errors were defined as responses made with the wrong hand, regardless of speed.

## RESULTS

*(Only significant results are reported!)*

### Behavioural data

Caffeine dose affected RTs and error rates. Helmert contrasts revealed shorter reaction times and lower error rates with caffeine compared to placebo. Low and high dose conditions only differed on error rates, with slightly fewer errors after a high dose than after a low dose.

Following error trials, participants responded on average 184 ms slower than after a correct trial. This post-error slowing reflecting a strategy change after an incorrect response to prevent future errors was however not affected by caffeine.

**ERN**

Consistent with previous studies, the ERN was largest on frontocentral scalp sites and attained its maximum within 100 msec after the erroneous response (Figure 1).

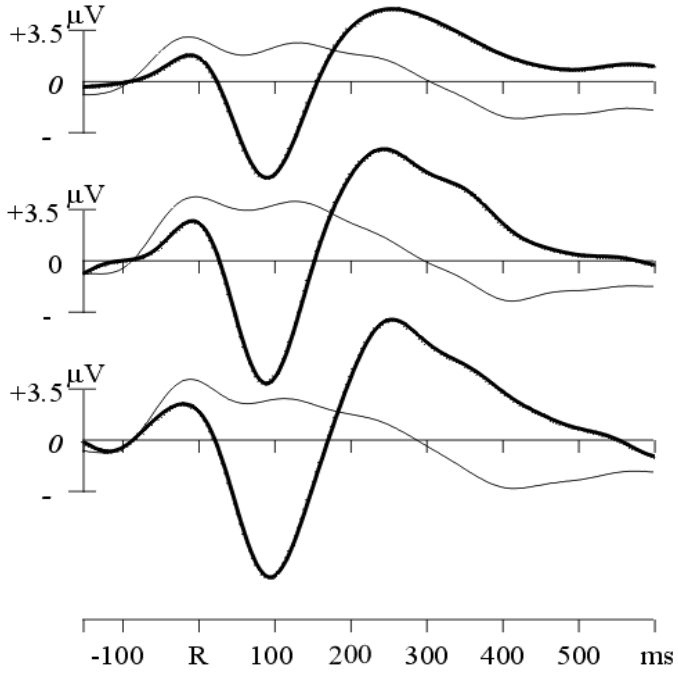


**Figure 1. Response-locked grand-average ERPs recorded from FCz during correct responses (thick line) and errors (thick lines), at placebo, low dose and high dose (top, middle, and bottom, respectively). R denotes the time of the response.**

The ERN amplitude was defined as the negative peak value between 0-150 ms following the erroneous response in the response-locked ERP. This amplitude of the ERN was enlarged by caffeine. Helmert contrasts confirmed a larger ERN for caffeine conditions than for placebo (6.7 µV). Low and high caffeine conditions did not show any difference (9.7 µV and 9.8 µV respectively).

Since caffeine intake induced somewhat lower error rates than placebo the difference in error rate was used as the covariate in two repeated measures ANOVAs in which the low and high dose were each compared to placebo. Both low dose and high dose effects on the ERN remained intact. Therefore, the enlarged ERN amplitude in caffeine conditions was a true effect of caffeine and was not present because of the reduced error rates after caffeine intake. Interesting was that caffeine did affect the ERN but did not affect the P2 and P3. It suggests that the ERN amplitude was selectively enlarged by caffeine and reflects improved action monitoring rather than general arousal changes.

**COMMENTS**

Taken together, the data support our hypothesis that caffeine increases action monitoring as reflected by the ERN. While previous studies have shown that caffeine consistently influences input (perceptual, attentional) and output (motor-related) processes (Snel et al., 2004), this study shows that caffeine also improves central, higher-order control processes, specifically action monitoring.

We also found that the ERN amplitudes were not caffeine dose-dependent. Typically, caffeine effects are found in suboptimal conditions such as during boredom and fatigue (Lorist et al., 1994). In our study the subjects' arousal level was possibly close to optimal during testing since the switch task was complex and demanded high effort. Also the between-subjects variability in reported daily caffeine intake (ranging 154-549mg) could have contributed to performance deterioration. Low users could after a high dose due have reached arousal levels beyond the optimum, while tolerant high users might have benefited from the high dose. Thus, variability in arousal levels after consumption of the high caffeine dose could have caused divergent effects. A related problem is underreporting of absolute caffeine consumption when coffee is used as the only source of caffeine (Wendte et al., 2003). Indeed, when caffeine intake from all caffeine containing beverages was taken as an index for daily caffeine consumption, it almost doubled (range: 154-1063mg; M~568±230). The present findings confirm other studies showing changes in action monitoring after administration of psychoactive substances. At this moment we can only speculate about which brain areas mediate effects of caffeine on the action monitoring system. Animal studies showed that moderate amounts of caffeine selectively enhanced activation in medial prefrontal cortex and basal ganglia (acquas et al., 2002; Nehlig & Boyet, 2000), both of which are part of the circuit for action monitoring. Whether these areas are sensitive to caffeine also in man will be answered in our next study.

To summarize, our results suggest that caffeine intensifies monitoring of ongoing processing in the cognitive system for signs of erroneous outcome. In daily life situations, prevention of errors is crucial; coffee may help.

## STUDY 2 TASK SWITCHING

### Task and procedure (see study 1)

In study 2, 18 students participated. Their age ranged from 18 to 30 years (M = 20.89 ± 3.1 years). Their daily coffee consumption was between 1.21-5.71 cups (M = 3.98 ± 1.3 or 123-583 mg caffeine (M ~406 ± 135). Total caffeine consumption from coffee, tea, and chocolate was between 154-823 mg (M ~406 ±170).

### Data processing

To evaluate ERP effects of preparation in brain activity, response-locked ERPs were obtained aligned to a baseline of -50 to 50 ms around the preceding response. Thus, epochs were averaged separately according to whether the stimulus following the current response required a change in task (switch) or performance of the same task (repeat or single-task). Stimulus-locked waveforms were created by averaging EEG epochs synchronized to stimulus onset, aligned to a baseline from 100-0 ms preceding the stimulus.

### Results

Participants slowed down on switch trials compared to repeat trials, reflecting switch costs. An effect of RSI was observed, indicating faster responses after a 600 ms RSI compared with a 150 ms RSI. The interaction between trial type and RSI was also significant, showing a reduction in switch costs after a 600 ms RSI compared to a 150 ms RSI, which was even further reduced as the RSI lengthened to1500 ms. Error rates were higher for switch trials compared to repeat trials.

**Caffeine**

The main effect of treatment was faster responses in both caffeine conditions compared to placebo. More importantly, the interaction between treatment and trial type was marginally significant [p= .052] indicating a reduction in switch costs in caffeine conditions (154 ms and 144 ms for low and high dose, respectively) compared to placebo [173 ms]. Post-hoc analyses showed that caffeine, averaged over RSI conditions, speeded up responses on repeat trials significantly, but more so on switch trials. The interaction between treatment, trial type, and RSI showed a trend [p = .087]. Helmert contrasts revealed a greater reduction in switch costs after caffeine relative to placebo in the 1500 ms RSI [p = .079] compared to the 600 ms.

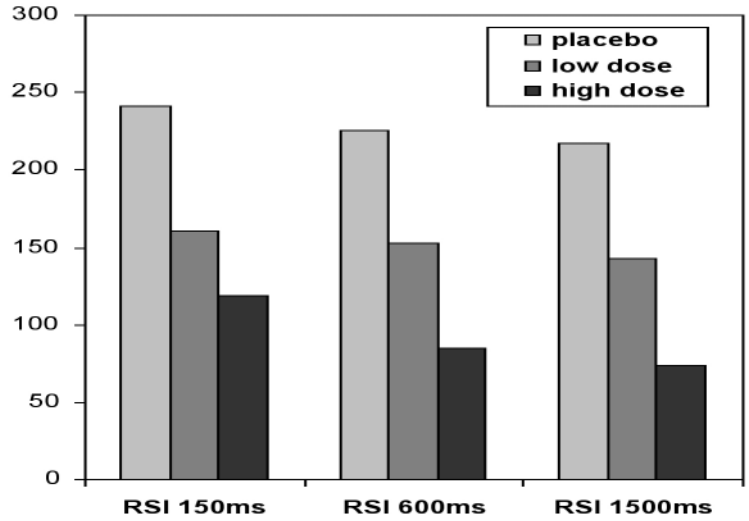


**Figure 2. Mean RT switch costs (ms) as a function of preparation time (response-stimulus interval or RSI) Placebo, low dose, and high dose conditions are represented by light grey, dark grey, and black bars, respectively.**

Thus, participants seemed to benefit most from caffeine if they were given ample time to prepare for the upcoming trial. This was confirmed by post-hoc analyses separately for each RSI, showing that caffeine reduced RT switch costs about 8% in the 150 ms RSI and in the 600 ms RSI, while a reduction of about 33% was observed for the 1500 ms RSI. As for errors, a main effect of treatment was found, that is fewer errors were found in both caffeine conditions compared to placebo. Again, no dose specific effects were found.

**PRESTIMULUS ERPS**

ERP waveforms time-locked to the preceding response were characterized by a build-up of negativity in the interval between the emission of a response and the onset of the next stimulus. The negativity peaked around 400 ms after response onset (early component) and was followed by a slow-wave negativity (late component). These components occurred in all RSI conditions, but were most clearly observed in the 1500 ms RSI due to minimal overlap of the slow wave with poststimulus components. Therefore, we confined analyses of these ERP waveforms to the 1500 ms RSI condition, for the midline electrodes Fz, Cz, and Pz.

**Early negativity: 200-600 ms time window**

In order to examine the early negativity in the prestimulus ERPs, we calculated the area (cumulative amplitude) under the waveform within the time interval 200-600 ms following the preceding response, separately for all treatment and trial type conditions.

A main effect for caffeine occurred in the analysis of single-task and repeat trials. Helmert contrasts showed a trend towards enhanced amplitude of the negativity in caffeine conditions compared to placebo [$p = .059$]. A similar trend was also found in the analysis of repeat and switch trials [$p < .08$].

**Late slow-wave negativity: 800-1200 ms time window**

To examine the slow-wave negativity in the prestimulus ERPs, we calculated the area in the time window 800-1200 ms following the preceding response.

For single-task and repeat trials, we observed a three-way interaction between treatment, electrode, and trial type. Anteriorly, the difference in slow-wave negativity between repeat trials and single-task trials was enhanced in both caffeine conditions compared to placebo, while posteriorly the reverse pattern of a smaller difference between single-task and repeat trials after caffeine occurred, as Helmert contrasts revealed.

For repeat and switch trials, an interaction between treatment, electrode, and trial type was found as well. The enlarged negativity for switch trials compared to repeat trials was increased after caffeine compared to placebo. Separate post-hoc analyses for repeat and switch trials showed that the slow-wave negativity was affected by caffeine on switch trials, but not on repeat trials. In other words, the late negativity associated with anticipatory processing for an upcoming switch was enlarged after caffeine. No dose-specific effects were found.

**POSTSTIMULUS ERPS**

ERP waveforms time-locked to stimulus onset, or poststimulus ERPs, showed P2, N2, and P3 deflections, which were largest at parietal scalp sites. Therefore the analyses were restricted to Pz. A negative shift was superimposed on poststimulus components in the 150 and 600 ms RSI condition.

For single-task and repeat trials, caffeine enlarged the N2 amplitude, but not latency. P2 and P3 amplitudes were not affected by caffeine but their latencies were and shorter compared to placebo.

For repeat and switch trials, again the N2 amplitude was enlarged by caffeine but not its latency. Again, caffeine shortened P2 and P3 peak latencies. In short, caffeine enhanced N2 amplitude and shortened P2 and P3 latencies.

The absence of caffeine effects on P2 and P3 amplitudes suggest that the caffeine-induced changes in the prestimulus slow-wave negativity reflect effects specific to task switching, rather than general changes in arousal or in input or output processes. This notion was confirmed through post-hoc analyses of caffeine effects on the N2, P2, and P3 for the 1500 ms RSI, in which overlap between prestimulus and poststimulus components should be minimal: P2 and P3 amplitudes were still unaffected by caffeine.

## COMMENTS

The ability to reconfigure or update the cognitive system when switching from one task to another was improved by caffeine, as evidenced by reduced RT switch costs after caffeine (and a trend towards reduced error costs). This reduction was largest when participants were given sufficient preparation time. In the ERPs, an early negativity transforming into a slow negativity developed within the preparation interval. The early negativity was smaller for switch than for repeat conditions, and more so posteriorly. Caffeine however did not influence this effect of switching. For switch trials compared to repeat trials the slow negativity was larger, presumably reflecting the greater need for anticipatory control required for an upcoming switch of task. Importantly, this switch-specific modulation of the slow negativity was enhanced after caffeine, indicating strengthened anticipatory control. These caffeine-induced changes in slow negativity are not merely the result of caffeine-induced enhancements in all ERP components, since the early negativity as well as P2 and P3 amplitudes were not affected by caffeine. Rather, this effect of caffeine seems to be specific to preparatory processes involved in task switching.

In sum, anticipatory control processes benefit from caffeine. Specifically, caffeine seems to strengthen processes such as task-set updating, which reduce switch costs. Processes related to task-set maintenance are affected by caffeine to a lesser degree as shown from the non-significant reduction of mixing costs after caffeine.

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# Coffee, Sleep and Wakefulness: Research Trends

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## SUMMARY

The demands of the 24-hour economy may cause a severe disturbance of the wake-sleep cycle. The consequences are increased fatigue and sleepiness and impaired performance and health. Caffeine offers the possibility to stay awake, to ameliorate fatigue and to improve alertness and performance during work and leisure time activities done at irregular times. In long lasting work situations Slow Release Caffeine, bright light or naps form alternative possibilities to cope with the negative consequences of irregular sleep-wake schedules.

## SLEEP

One third of our life we spend asleep, but why we sleep is still unknown. Sleep is a part of the 24-hour endogenous arousal cycle with its peak in the afternoon and its trough shortly after noon and around 03.00AM. The behavioural manifestation of this circadian arousal cycle is expressed as sleep and wakefulness.

A useful method to follow the periodic fluctuations in arousal during sleep is the recording of brain activity (EEG). The recorded sleep structure shows the quality and depth of sleep that runs from stage 1 to stage 4. Stage 1 and stage 2 together form light sleep. Stage 2, the transition from falling asleep to deep sleep is used to objectively measure sleepiness. Stages 3 and 4 together represent deep or Slow Wave Sleep (SWS). When stage 4 is reached, there is a quick return via stages 3, 2 and 1 to a state in which the Rapid Eye Movement-sleep (REM) occurs. In the first half of the night more NREM-sleep (stage 1 to 4), especially more SWS is found; in the second half increasingly more REM- and light sleep. The period of ~1.5 h hour needed to change from NREM to REM is called a sleep-cycle. The 24-hour Sleep-Wake cycle is determined largely by endogenous physiological factors with a free-running length of about 25 hours. Exogenous Zeitgebers force this 25-h period into a 24 hours sleep-wake rhythm. Important Zeitgebers are the light-dark alternation and social factors such as the scheduling of work and leisure time activities. It implies that responding to the demands of the 24-h economy will disturb this regular sleep-wake cycle, which could have serious consequences for task performance and our well being.

## (IR)REGULARITY

To stay awake is of practical concern. Nevertheless, observation learns that in public transport, at home and at work mankind suffers from a serious lack of sleep. Already a shortage of about 1.5 hour sleep for 1 night results in a one third reduction of alertness during daytime. Also 17 to 57% of healthy young adults have sleep onset latencies (SOLs) during daytime about half of the normal SOL and 28% of them sleep regularly less than 6.5 hours each night. Altogether serious sleep loss in at least one third of all adults results in traffic accidents, casualties and an estimated loss of 60 billion dollars in the USA alone. With the 24-hour economy increasingly more people are confronted with several forms of irregular

working schedules: working longer, at inconvenient times, and with changeable working periods. That an irregular lifestyle leads to more intestinal, stomach and sleep-wake problems than a regular lifestyle was found in 210 healthy, 27 year adult men and women (Kikkert et al., 1996). Based on this finding we studied whether working irregularly would have similar but more negative health effects (Snel et al., 2001). The subjects were catering employees at Airport Schiphol who worked for 8-hour periods irregularly between 6.00 AM and 23.00 PM. As for coffee it came out that the more years one worked in this way, the more coffee tended to be consumed and the earlier after a shift one took the last cup. Apparently, coffee was used as a tool to compensate for the fatigue and decreased alertness caused by irregularity of work. Also, the time used to relax after a late duty was positively associated with more cups of coffee. Moreover, coffee drinkers used one hour more to relax after late duty than non- coffee drinkers. As for health, coffee tended to lead to more chronic stomach and intestinal complaints. Since consumption of coffee was significantly positively related to the number of years of irregular work, the stress of it could have caused this worsened health.

Whether coffee may hamper sleep quality was also investigated in 2,202 subjects aged 20-45 years (Janson et al., 1995). In this study information was gathered on having problems with falling asleep, nightmares, nocturnal and early awakenings and the use of psychoactive substances including coffee. Caffeine was not found to be an independent risk factor for difficulties inducing sleep or other sleep disturbances when age, gender, smoking, country or seasonal variation was taken into account. For those who consumed at least 6 cups per day however, there was a negative correlation with nocturnal awakenings.

In sum, caffeine up to 3mg/kgBW hardly influences sleep and even more so when the last coffee is drunk shortly after dinner (Bättig , 1991). Even for high doses up to 6-7mg/kg (~6 to 7 cups per day) the disturbance of sleep in everyday situations with relatively stable sleep-wake cycles seems negligible (Akerstedt & Ficca, 1997). In short, in a *regular* sleep-wake cycle regular caffeine consumption during daytime does not deteriorate sleep quality.

## REGULARITY OF SLEEP-WAKE SCHEDULES

Regularity of sleep is important for a good quality of sleep and should lead to low levels of daytime sleepiness. Hence, regularization of sleep-wake schedules should improve subjective ratings of daytime sleepiness. This assumption was tested prospectively in 39 students (Manber et al., 1996). Subjects in the sleep-only and in the regularity-sleep condition were all given a limited 7.5 hours total sleep time for 4 weeks. Those in the regularity group were explicitly stressed to adhere to a regular sleep schedule. It came out that when nocturnal sleep was not deprived, regularization of sleep-wake schedules led to less sleepiness. In the strict regular schedule condition subjects reported greater and longer lasting improvements in alertness and improved sleep efficiency than the subjects in the sleep only condition. As for coffee consumption there were no differences between the groups.

Irregular patterns of life, voluntarily chosen or imposed, form a risk for health due to excessive sleepiness, disturbed sleep-wake cycles and accidents. To cope with this irregularity one could use bright light, taking naps or a break, improve work scheduling or manipulation of their sleep. Studies on these topics have been done in simulated and real-life situations

## SIMULATED REAL-LIFE SITUATIONS

In a study on simulated driving the influence of 3mg/kg caffeine (~2 cups of coffee) was assessed (Brice & Smith, 2001). The 24 healthy, non-smoking students all regular coffee consumers had on average 4.6 years of driving experience. There was no caffeine abstinence

period before the experiment. In the coffee condition greater alertness and more hits on a repeated digit memory task were found. Also steering variability was significantly less and more stable throughout the 1 hour drive. Reyner and Horne (Reyner and Horne, 2000) sleep deprived 16 students, 23 year old till 24.00PM or for the whole night. After that they had to drive for 2 hours (06.00-08.00AM) on a dull, monotonous roadway. Caffeine (200 mg) improved the driving performance, there were less incidents and less subjective sleepiness. Regular coffee effectively reduced early morning driver sleepiness for about 0.5 hour following total sleep deprivation for one night and for around 2 hours after a 5 hours sleep. The best option to overcome sleepiness is to take caffeine because of its more consistent alerting effects; a break is ineffective. Brice and Smith's study (2001) confirmed that caffeine works beneficially in monotonous tasks, even in non-sleep deprived subjects.

In a simulated shift work situation with 12 participants, 19-36 years old, all none to moderate users (~ 2 cups per day), the influence of 200mg caffeine was studied (Babkoff et al., 2002). Work started at 17.30PM and went on until 10.00AM. During the 1 h rest period from 01.30-02.30AM the participants performed 4 computer tests lasting 90-95 min. Caffeine alone had a beneficial effect on performance during the night.

Modern life may imply the loss of regular sleep schedules with the consequence of partial or complete loss of sleep. To determine the effects of prolonged wakefulness (Kamimori et al., 2000), 50 healthy, non-smoking men (age 18-32 years) were studied. The caffeine doses, 2.1 mg/kg, 4.3 mg/kg and 8.6 mg/kg were given double-blind in sweetened lemon juice, and only once after 49 hours of wakefulness. Sleepiness scores showed dose-related improvements except for the 2.1 mg/kg dose. This latter result was ascribed to possible tolerance effects in these regular coffee consumers. Especially the high dose almost tripled the Sleep Onset Latency (SOL) over the first 4 h post-intake.

The effect of caffeine in simulated jet-lag situations was studied in 5 healthy middle-aged male moderate caffeine consumers (~3 caffeinated beverages) (Moline et al., 1994). The subjects participated in two 16-day sessions in the laboratory. In the first 5 days they followed their habitual sleep-wake rhythm followed by the sixth night from which they were awakened 6 hours before the usual getting up time. This was repeated in the subsequent sleep periods. Throughout the study they got, double-blind 200 mg caffeine or placebo tablets at breakfast. Prior to one of the schedule shifts the caffeine was replaced by placebo. Subjective alertness remained approximately constant following the shift in the drug condition, whereas it declined in the no-caffeine condition ($p < 0.05$). The pre-shift alertness was the same in both conditions. The summarizing conclusion was that a single dose of 200 mg caffeine, taken at breakfast prevented a decline in subjective alertness following a 6 h time advance of the usual waking time.

A comparable study on simulated night work with 2 mg/kg caffeine was done in 15 young adults (Muehlbach and Walsh, 1993). Caffeine improved alertness during three successive night shifts without impairing mood and daytime sleep. Night time performance however, was not significantly improved and sleepiness at the circadian trough remained at weak levels (see also Lee, 1992; Walsh et al., 1995). In a replication study (Muehlbach and Walsh, 1995) the efficacy of caffeine was assessed in maintaining performance and alertness during the circadian trough early in the morning. Ten healthy young adults were totally sleep deprived for 54.5 h. After 41.5 h awake, they got double blind 600 mg caffeine followed by hourly testing. Performance and alertness, assessed every 2 hour were significantly improved by caffeine. Also, caffeine maintained performance and alertness during the early morning hours, when the combined effects of sleep loss and the circadian morning trough of arousal are strongest.

In sum, caffeine is an effective in improving performance and alertness during sleep loss in conditions similar to real-life work situations and notably when performing tasks that are simple, monotonous and not intrinsically interesting enough to keep fatigue away (Snel et al., 2004a).

## REAL-LIFE WORK SITUATIONS

The question whether moderate doses of caffeine (100, 200 or 300 mg) may counter the adverse effects of 72 h sleep deprivation was answered in 68 marine trainees (Lieberman et al., 2002). The subjects were tested 1-8 h after this sleep deprivation period on several cognitive tasks, mood and performance (marksmanship). The sleep deprivation and the stress of the simulated combat situation adversely affected performance and mood. However, caffeine, dose-dependently (200 and 300 mg), improved visual vigilance, reaction time and alertness, but not marksmanship. The largest effect was found 1 hour after intake, but significant effects persisted for 8 hours. The conclusion was that when cognitive performance is critical and must be maintained during severe stress in sleep deprivation conditions, caffeine notably the 200mg dose is effective.

The foregoing studies (simulated and real-life) indicate that caffeine reduces sleepiness and compensates performance decrements due to work during the night. These findings confirm those of studies done by Smith (Smith, 1994). He showed that 1.5 and 3.0 mg/kg doses of caffeine are beneficial for mood and performance during sleep deprivation. An additional advantage is that by its improvement of alertness and improvement problems with safety and loss of performance efficiency are reduced.

Whether coffee could be used in a systematic way in all occupational settings is difficult to say. Caffeine certainly is useful to improve alertness during work, but in general its use is spontaneous and ad hoc. Particularly, the optimal amount and pattern of administration should be elucidated and also its effect combined with other ways to stay awake, among them bright light, naps and more recently the use of Slow Release Caffeine.

## BRIGHT LIGHT

The most important Zeitgeber in entraining the 24 sleep-wake cycle is light. Therefore the 24-h economy, because of its light pollution disturbs the sleep-wake cycle. Paradoxically bright light is also used to regulate disturbed sleep-wake cycles and to keep people awake to sustain good task performance (Wright et al., 1997b; Babkoff et al., 2002). The most effective way to compensate for the increase in fatigue due to sleep deprivation or long working-hours seems to be the combination of 200 mg caffeine and bright light (3,000 lux) followed by caffeine and then by bright light.

## NAPS

Another way to counteract fatigue caused by too less sleep is to take naps. Intuitively, naps and caffeine don't match: naps are meant to induce sleep, while caffeine aims at getting awake. Nevertheless, performance during sleep loss is improved by prophylactic naps and dose-dependently with nap length (Bonnet et al., 1995). In general naps are superior to caffeine in inducing longer and less graded changes in performance and alertness, while caffeine displays a short peak in effectiveness and will loose its effect within 6 hours. The combination of short naps and small repetitive doses of caffeine (150 mg caffeine), however maintains alertness and performance during sleep loss better than no naps or large single

caffeine doses (300 mg and 400 mg). However, beyond 24 hours of sleep loss neither nap nor caffeine is able to preserve function at baseline levels.

Naps in combination with 150mg caffeine were used (Reyner and Horne, 1997) to assess their effect on sleepiness and driving safety on a simulated dull and tedious motor-way. The incident frequency during caffeine+nap and caffeine was respectively 0.09 and 0.34 against 1 for placebo. Taken all results together, the combination caffeine+nap was additive with respect to preventing incidents and sleepiness. During the 2 hours after the caffeine intake and compared with caffeine only, the combination caffeine+naps reduced the number of incidents 3 to 4 fold.

**SHOW-RELEASE CAFFEINE**

Show-Release Caffeine (SRC) is a relatively recent way of caffeine delivery, which could be suitable in long work or shift work schedules that necessitate an elevated and prolonged level of good vigilance and performance. Hence, SRC might be an option to use for workers in health care, transport and certain industries and perhaps for the chronically sleep-deprived general public as well.

A laboratory study on simulated work with SRC was done with 12 young adults (Bonnet & Arand, 1993). The aim was to compare the relatively best effect of either nap for 4 periods of 1 hour each or for one 1-nap period of 4 hours in combination with or without a 200mg SRC dose. Addition and logical reasoning were improved during the night with the combination of the 4-hour nap before the shift and caffeine. Performance after the 1 hour-naps in the beginning of the night was very poor, possibly due to the fact that 60% of the naps ended in SWS against 10% of the prophylactic nap. Apparently, the indicated strategy to stay awake during shift work is to take a nap before starting to work and to use SRC (200 mg) or 2 doses of 200mg caffeine at 01.30 h and 07.30AM (Bonnet and Arand, 1994a, 1994b).

The effect of a short night of 4.5 hours was measured with a driving simulator for 45 min. with or without 300mg SRC (De Valck and Cluydts, 2001). The 12 students with a mean age of 22.5 years, all moderate to normal sleepers had at least 2 years of driving experience. After the normal 7.5 h sleep condition, SRC decreased only lane drifting while after the 4.5 h night the SRC resulted in less lane drifting, smaller speed deviation and accident liability. Subjectively, SCR resulted in less fatigue and more vigour but only in the short 4.5 h sleep condition. Especially when there is no opportunity to take a nap, as is the case in most industrial settings (continuous process and monitoring activities, transport, health care) SRC could be an option to use. An other reason for considering using SRC instead of caffeinated beverages is to prevent the practical problems of transporting and brewing coffee at the work site and the repeated drinking of coffee over long periods.

The effectiveness of a 30-min. nap and 300-mg SRC to counter drivers' sleepiness induced by partial sleep deprivation was evaluated in a second study by De Valck's team (De Valck et al., 2003). The 45-minute driving performance on the simulator was measured at 09.00AM and at 13.00PM as lane drifting, speed deviation, and accident liability. The 30-min. nap opportunity and 300 mg of SRC both were successful in counteracting drivers' sleepiness. The remedial effect of SRC lasted longer than that of the nap, that is, it was also effective in the afternoon session. Apparently, SRC represents a valuable countermeasure that in the case of partial sleep deprivation is preferred to a nap when sleepiness has to be counteracted for longer time.

Beaumont's group (Beaumont et al., 2001) studied the efficacy of a two times 300 mg SRC dose. During 64-h continuous wakefulness sustained attention and cognitive performance were measured. The 16 healthy non-smoking male volunteers, all non-or low caffeine consumers got twice a day at 21.00PM and 09.00AM during the 64-h sleep deprivation period a 300-mg dose SRC or placebo. Measurements were done during the low vigilance periods at 02.00-04.00AM, and at 14.00-16.00PM and during the high vigilance periods at 10.00-12.00PM, and at 22.00-00.00PM. SRC made the subjects significantly less sleepy from the onset to the end of SD. Some cognitive functions were improved till the 33$^{rd}$ hour of SD, others through all the SD period and alertness was better from the 13th hour of SD. In conclusion, a dose of 300-mg SRC given twice daily is able to antagonize the impairment produced on vigilance and cognitive functions by a 64-h SD.

A similar study was done by Patat and co-workers (Patat et al., 2000) with a single 600mg SRC dose during a 36-h SD period. Similar to the Beaumont study (2001) the 600mg SRC induced alerting effects, compensated for performance decrements throughout SD and was able to reverse the deleterious effect of 36-h SD for at least 24 hours with its peak effect 4 hours after intake. To find out which dose SRC is optimal (maximum effect without side effects) 3 doses (150, 300 and 600mg) were compared to placebo in 24 young moderate consumer (~3cups per day) in a 32-h SD experiment (Lagarde et al., 2000). There was a significant effect of the 3 doses of SRC *vs.* placebo on vigilance and performance when subjects became tired. This was found particularly in the number of errors on the 4-letter memory search task and the visual tracking task. Noteworthy was that the effects of SRC lasted 13 hours after treatment. Considering all their results together, the authors concluded that SRC doses of 300 and 600 mg are efficacious but the optimal dose for both men and women is 300mg. SRC (300 mg) seems to be an efficient and safe substance to maintain a good level of vigilance and performance during sleep deprivation, also it permits a long and good quality of wakefulness not only in laboratory settings but also in real-life like in jet-lag and simulated driving conditions.

## STATEMENTS

- Caffeine is used more as an aid to regulate sleep quality and to remain awake in work situations than as a substance that disturbs sleep.
- Caffeine is effective in improving performance and alertness during sleep loss in normal, healthy adults in conditions comparable to those of real-life work situations.
- When wakefulness is critical and must be maintained during severe stress in long-hour sleep deprivation periods, caffeine provides a significant advantage.
- Naps in general are superior to caffeine in showing longer and less graded changes in performance and alertness. The combination of short naps and small repetitive dose of caffeine maintains alertness and performance during sleep loss is superior to no naps or large single caffeine doses
- The most effective way to compensate for fatigue is the combination of caffeine and the exposure to bright light, followed by caffeine and then by bright light.
- Slow Release Caffeine (SRC) is an efficient and safe substance to maintain a good level of vigilance and performance during long-hour sleep deprivation.

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# Antioxidant Components in Roast Coffee

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## INTRODUCTION

With worldwide consumption of about 5 million tons in 2001 (USDA, 2002), coffee is one of the most popular beverages in the world. The habitual consumer highly appreciates coffee beverages for their salubrious, desirable aroma and taste as well as their stimulating properties. Historically, coffee consumption has frequently been related to unhealthy behaviours, such as smoking or a sedentary lifestyle. However, recent knowledge has put coffee into a more positive light, and to date there is growing evidence that moderate coffee consumption has health benefits, among these *in vivo* antioxidant and chemopreventive effects.

On the other hand, the antioxidant coffee components are believed to be a key driver for coffee stability, thus inhibiting lipid peroxidation or the degradation of odor-active thiols. For coffee beverages, the knowledge of the chemical structure and the antioxidant activities of these compounds open the possibility to tailor coffee quality and to extend the shelf-life of coffee products, such as, e.g. ready-to-drink beverages or canned coffee, by slowing down undesirable oxidation reactions.

At present, the strong interest in the antioxidant activity of compounds in coffee products as well as their *in vivo* antioxidant and chemopreventive activity promotes the research on antioxidants in coffee beverages. But as the mechanisms involved in inhibiting lipid peroxidation in the food strongly differ from the biomechanisms involved in antioxidant and chemopreventive activity in the human body, the research on antioxidants need to be carefully defined. The purpose of this paper is to provide a brief overview on the literature status on antioxidants in roast coffee and their activity in food as well as in biological systems.

## ANTIOXIDANT COMPOUNDS IN COFFEE

Multiple investigations were aimed at characterizing the antioxidant capacity of coffee beverages prepared from light, medium, and dark roasted beans obtained from the same blend of raw coffee (Daglia et al., 2000; Del Castillo et al., 2002; Borelli et al., 2002; Piost and Steinhart, 2002; Anese and Nicoli, 2003). But the results published in the literature are very contradictory. This is because rather different *in vitro* assays, which have been developed to assess the antioxidant activity of foods or food fractions, were applied to elucidate the "antioxidant capacity" of coffee; for example, the measurement of radical scavenging activity by measuring the inhibition of ABTS radical cation decolorization (Re et al., 1999), the inhibition of linoleic acid peroxidation (Pryor et al., 1993), inhibition of crocin bleaching (Tubaro et al., 1996), inhibition of ß-carotene/linoleic acid cooxidation (Gazzani et al., 1998), and inhibition of liver microsomial lipids peroxidation (Daglia et al., 2000). Although each of these assays is based on a rather different reaction principle, the results of most of such studies were expressed as "antioxidant capacity" or "antioxidant activity" of coffee. For example, Daglia et al. (2000) measured the inhibitory power of coffees on the coupled

oxidation of ß-carotene and linoleic acid and found that the "antioxidant activity" of coffee decreased from the raw to the medium roast coffee, thereafter increasing again with more severe roasting conditions. Also Steinhart and coworkers (Steinhartet al., 2001) investigated the same objective, but used a ABTS radical decolorization assay (TEAC assay) instead of the ß-carotin/linoleic acid cooxidation assay. In contradiction to the results of Daglia et al. (2000), the authors found that the "antioxidant capacity" increased from raw to the medium roast coffee, thereafter decreasing again towards strongly roasted coffees.

In summary, it can be concluded that the results obtained by such *in vitro* antioxidant measurements are strongly dependent on the assay used, because each assay is addressing a different chemical event contributing to "antioxidant activity", such as, e.g. radical scavenging or inhibition of lipid peroxidation. For future investigations, it will be therefore essential to draw the appropriate conclusions from the results obtained from the individual assays.

In order to elucidate the chemical structure of some antioxidant compounds in coffee, activity-guided separation of coffee beverages by means of solvent fractionation (Daglia et al., Somoza et al., 2003), gel permeation chromatography (Piost and Steinhart, 2002), and multiple-step ultrafiltration (Somoza et al., 2003) pinpointed the chlorogenic acids among the key players for antioxidant activity in coffee beverages. These findings collaborate well with the results of model experiments demonstrated the inhibitory activity of 5-chlorogenic acid on conjugated diene formation from linoleic acid micelles exposed to superoxide (Morishita et al., 1995). But recent quantitative estimations revealed that only a total of 10-15 % of the overall antioxidant capacity of the coffee was found to be due to chlorogenic acids (Somoza et al., 2003), thus indicating that additional compounds are formed during roasting which contribute primarily to the antioxidant capacity of roast coffee beverages.

Aimed at shedding some more light onto roasting-derived antioxidant components, model precursor experiments have been done including chlorogenic acids, carbohydrates, and amino acids (Piost and Steinhart, 2002; Charurin et al., 2002). Measurement of the antioxidant capacity of various roasted model mixtures allowed the conclusion that, with the exception of L-arginine, primary amino acids are able to significantly increase the antioxidant activity of chlorogenic acid/carbohydrate mixtures upon moderate roasting. However, at present no individual antioxidant roast product could be successfully identified in coffee or roast model mixtures.

Besides some low-molecular weight sugars, amino acids, and phenolic components, green coffee beans are rich in heteropolysaccharides and peptidyl glycans which are known to undergo profound molecular changes during the roasting process, thus generating water soluble, brown colored, polymeric melanoidins (Hofmann et al., 1999; Hofmann, 2001; Hofmann et al., 2002; Nunes and Coimbra, 2001). Multiple studies suggest that these macromolecules are responsible for the strong antioxidant properties and metal chelating ability exhibited by roast coffee beverages (Nicoli et al., 1997; Homma and Murata, 1995). As recent studies demonstrated a non-enzymatic, covalent binding of the phenolic compound quercetin to lysine side chains of proteins (Rohn et al., 2004), similar reactions between the lysine side chains or the *N*-terminus of peptidylglycans and chlorogenic acids might give an explanation of how phenol compounds bind via the intermediary ortho-chinone to biopolymers upon coffee roasting, thus giving rise to macromolecular antioxidants as suggested in Figure 1.

In addition, hydrolytic treatment of coffee with sodium hydroxide under reducing conditions revealed a remarkable release of chlorogenic acids and caffeic acid from "bound complexes",

most likely melanoidins (Nardini et al., 2002). This recent finding suggests a simple esterification of the carboxy function of the chlorogenic acid with biopolymers such as, e.g. arabinogalactans, as a potential key reaction channeling the low-molecular weight phenolic compounds into biopolymer-based melanoidins with high antioxidant potential (Figure 1).

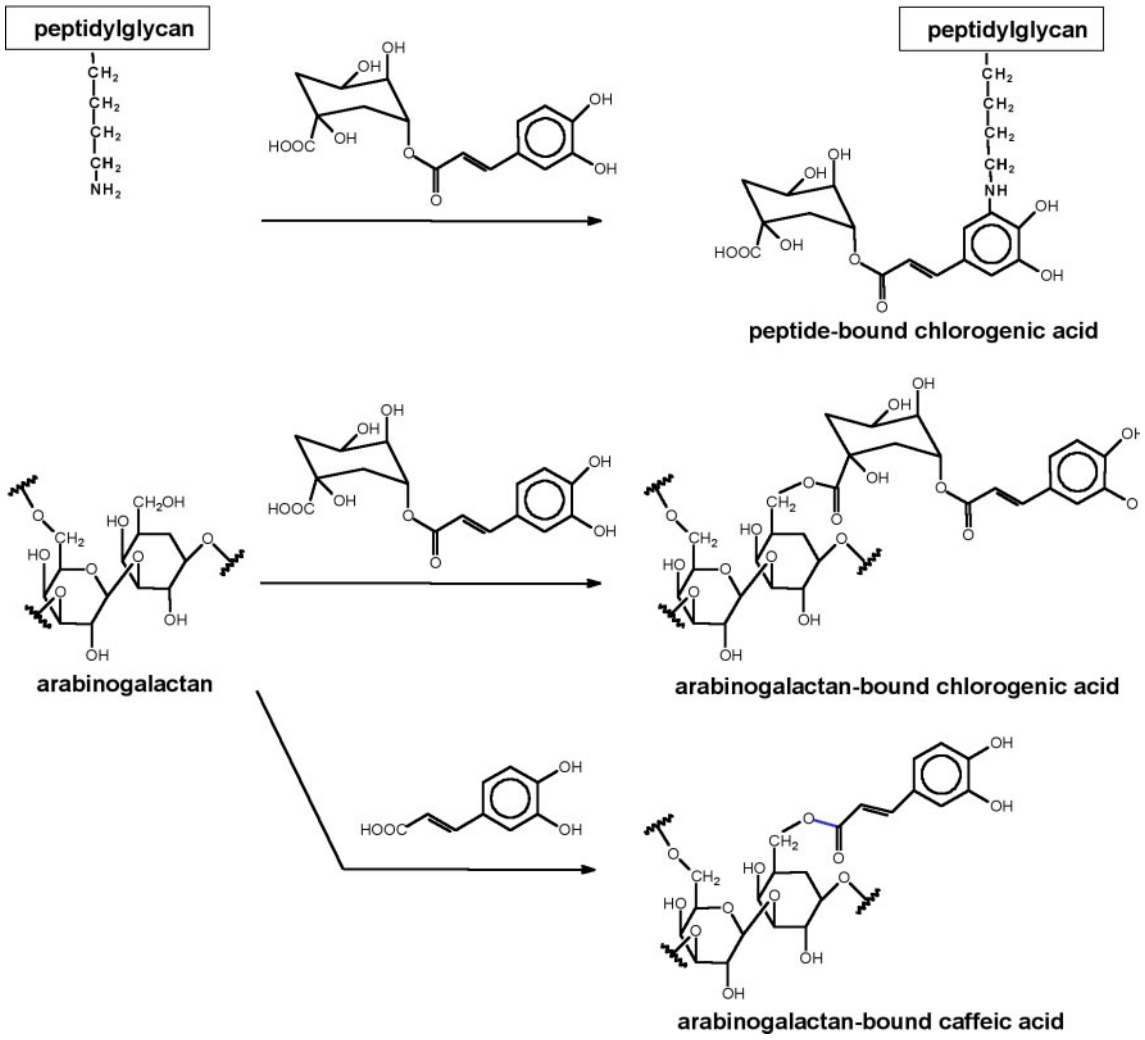


**Figure 1. Hypothetical covalent linking of chlorogenic acid and caffeic acid to polymeric peptidyl glycanes and arabinogalactans.**

Taking all the literature knowledge into consideration, it can be concluded so far that chlorogenic acids are converted into (i) low-molecular weight products exhibiting antioxidant capacity upon the reaction with amino acids as well as (ii) to high-molecular weight, melanoidin-type antioxidants most likely via the esterification with polysaccharides or the oxidative, covalent linkage with peptidyl glycans. However, the knowledge of the exact chemical structures of the antioxidant sites in these melanoidins is still in its infancy; in fact, neither the polymeric backbone, nor the nature and the amount of phenolic compounds incorporated into the brown polymers have been yet elucidated but need to be identified by means of 1D/2D-NMR spectroscopy in future investigations.

## ANTIOXIDANT AND CHEMOPREVENTIVE ACTIVITY OF COFFEE COMPONENTS IN BIOLOGICAL SYSTEMS

Based on the literature knowledge, it is apparent that moderate daily coffee intake is not associated with any adverse effects on cardiovascular outcome or increased incidence of

cancer. In contrast, recent studies show strong evidence that coffee has a significant antioxidant and chemopreventive activity *in vivo*.

To date, most of the physiological effects of coffee beverages have been ascribed to their content in caffeine, the diterpenes kahweol and cafestol (Lam et al., 1987), or phenols such as, e.g. chlorogenic acids (Kitts and Wijewickreme, 1994). As phenolic compounds present in green coffee beans and in roast coffee brews have been demonstrated to exert antioxidative effects in *in vitro* systems (Borelli et al., 2002; Roginsky and Barsukova, 2001), these compounds were hypothesized to primarily increase the total antioxidant capacity in human plasma after drinking of coffee beverages (Natella et al., 2002; Esposito et al., 2003). But to act as an antioxidant *in vitro* does not necessarily mean that a compound can act in the same manner *in vivo*, and vice versa. In fact, the antioxidant capacity and chemopreventive activity of coffee measured *in vitro* is not necessarily consistent with its effect *in vivo*. Many factors such as polyphenol composition, absorption, tissue distribution, biotransformation, and the effect of certain molecules on the endogenous antioxidant defence system have to be taken into consideration.

Aimed at investigating the metabolisation of chlorogenic acid reported as a key antioxidant in coffee, coffee brew was administered to ten healthy subjects in fasting conditions, blood was withdrawn just before (0h), and 1 and 2h after coffee administration, and "free" and "bound" phenolic acids have been quantified by RP-HPLC analysis before and after glucuronidase or alkaline treatment (Nardini et al., 2002). The authors found that the major coffee phenol chlorogenic acid was not detectable at all in human plasma, and caffeic acid was the only phenolic acid detectable in plasma, thus confirming the results of previous studies on rats (Azuma et al., 2000; Camarasa et al., 1988). Quantitation of "*free*" and "*bound*" caffeic acid suggested that plasma caffeic acid is mainly derived from hydrolysis of chlorogenic acid in the gastrointestinal tract, because free caffeic acid was absent in the coffee sample administered. More than 90% of plasma caffeic acid in was found in "bound" form as the corresponding caffeic acid glucuronide (Nardini et al., 2002).

The absence of chlorogenic acid simultaneously with an increase of caffeic acid levels in human plasma after coffee consumption can be explained by the mechanism displayed in Figure 2. Chlorogenic acids from administered coffee is not absorbed as such, but after passing the stomach without any further acidic degradation (Takenaka et al., 2000), it undergoes hydrolysis in the gut mucosa or gut microflora. Cinnamoyl esterases with the capability of chlorogenic acid cleavage are recently reported to be distributed all along the small and large intestine (Andreason et al., 2001). But also bacteria in the mammalian gastrointestinal tract are capable of releasing free phenolic acids from bound complexes (Couteau et al., 2001). Released caffeic acid is then absorbed and enters the vascular system. The glucuronidation is likely to occur in the liver (Scheline, 1978), but also possibly in the small intestine (Spenser, 1999), and colon, where UDP-glucuronyl transferase activity has been reported (Cheng et al., 1999). A previous study reported that, following caffeic acid perfusion, the major products transferred across the rat intestinal epithelium were glucuronides (Spenser, 1999). It seems to be clear that chlorogenic acid is metabolized to give the caffeic acid glucuronide, but is that metabolite still exhibiting antioxidant activity? To give a satisfactory answer on that key question will be an important objective for future investigations.

Based on available published data the chemopreventive effects of coffee seem to involve the antioxidant defence system and to modulate a chemopreventive Phase-II enzyme, the glutathione-S-transferase (GST), on a cellular level. Current data suggest that the balance between the Phase-I carcinogen-activating enzymes and the Phase-II detoxifying enzymes is

critical to determining an individual's risk for cancer. Human deficiencies in Phase-II enzyme activity, specifically GST, have been identified and associated with increased risk for colon cancer (Wilkinson and Clapper, 1997). On the other hand, induction of GST or other Phase-II enzymes, e.g. the UDP-glucuronyl-transferase (UDP-GT), by antioxidants represents a promising strategy for cancer prevention. Although the molecular mechanism by which antioxidants bind to an antioxidant responsive element resulting in the specific, monofunctional induction of Phase-II enzymes has been intensively studied (Prochaska and Talalay, 1988), it is still an open question as to whether a compound showing antioxidant activity *in vitro* may function as a Phase-II inducer in biological systems. Among all of the biological systems suitable for *in vitro* studies, the intestinal Caco-2 cell line is widely used to investigate the effects of dietary compounds on Phase-I/-II enzymes as the colon is clearly one of the most likely sites for the development of different types of dietary induced cancers (Peters and Roelofs, 1989; Schmiedlin-Ren et al., 1997).

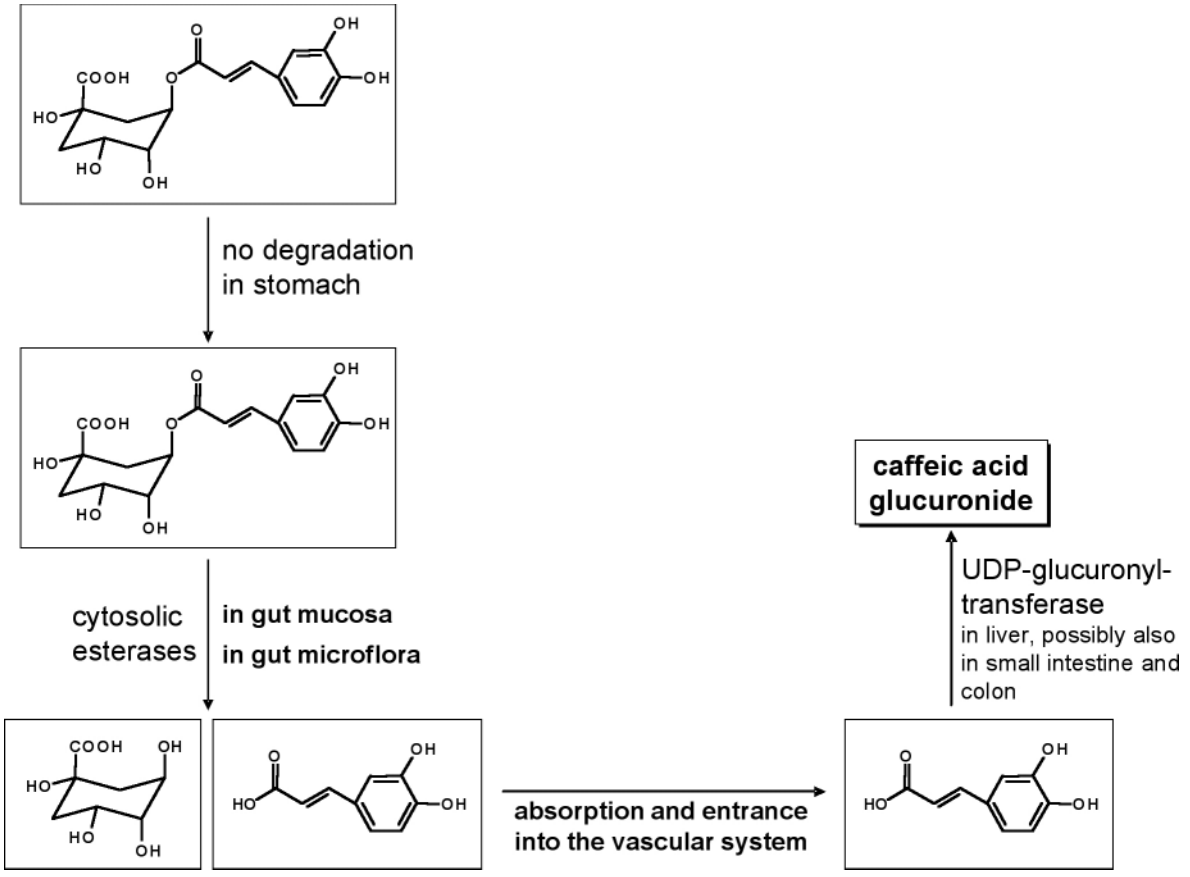


**Figure 2. Metabolisation of chlorogenic acid to caffeic acid glucuronide.**

The induction of Phase-II GST and UDP-GT enzymes *in vivo* has been demonstrated in animal feeding experiments for green coffee beans, coffee brew and for the diterpenes kahweol and cafestol (Lam et al., 1987; Kitts and Wijewickreme, 1994; Lam et al., 1982; Huber et al., 2002). Moreover, regular coffee brew in comparison with de-caffeinated coffee brew was reported to likewise induce the GST activity in mice, indicating that this chemopreventive effect is not related to caffeine (Somoza et al., 2003; Abraham and Singh, 1999). Thus, all of the animal experiments reported so far demonstrate a significant increase in chemopreventive enzyme activities after coffee consumption, but no non-phenolic key compound to which these *in vivo* effects can be fully ascribed has been identified.

Recent human trials showed strong evidence that moderate consumption of coffee induces an increase of the antioxidant capacity in the plasma (Natella et al., 2002) as well as an increase

on the plasma concentrations of glutathione (Esposito et al., 2003). Glutathione is an important antioxidant and cofactor of the detoxifying metabolism and the increase of glutathione concentration induced by food-born molecules is believed to contribute to chemoprevention against environmental carcinogens (Abraham and Singh, 1999). Recently, the coffee diterpens cafestol and kahweol were reported to increase the glutathione levels in the plasma of mice fed non-filtered coffee (Scharf et al., 2001). Despite of these data, there is increasing evidence deriving from nutritional and epidemiological studies that not all these physiological effects detectable upon coffee consumption can be ascribed to caffeine or to bioactive diterpens, and that other unknown compounds might have chemopreventive effects through stimulation of the endogenous antioxidant defence system (Borelli et al., 2002).

This prompted us in a recent study to apply an activity-guided screening procedure to coffee brew, to identify key antioxidant and chemopreventive compounds by means of *in vitro* assays, and to prove the *in vivo* activity of that compound by an animal feeding experiment (Somoza et al., 2003). After solvent fractionation and multiple-step ultrafiltration, the fractions isolated from a coffee beverage were studied in their efficiency in inhibiting the peroxidation of linoleic acid using an *in vitro* antioxidant assay (Lindenmeier et al., 2002) as well as in modulating Phase-I CCR and Phase-II GST activity in human intestinal Caco-2 cells (Somoza et al., 2003). On the basis of these data, it was concluded that, in particular, the hydrophilic, low molecular weight compounds are responsible for the antioxidant potential of the freshly brewed coffee beverage as well as for the chemopreventive activity of coffee. To locate the key player responsible for the PhaseI/II-modulating activity of the low-molecular weight coffee constituents, the LMW fraction was subfractionated by semipreparative RP-HPLC, followed by HILIC-HPLC and each fraction isolated was evaluated in their antioxidant activity by measuring their inhibitory power on linoleic acid peroxidation as well as to determine their Phase-I / -II modulating activity in Caco-2 cells. The *in vitro* antioxidant assay revealed the highest antioxidant activity for the chlorogenic acid, thus collaborating well with the data reported above. In contrast, the highly polar *N*-methylpyridinium ion (NMPY), the structure of which is given in Figure 3, was detected for the first time as a powerful coffee-derived inductor of glutathione-S-transferase in Caco-2-cells (Somoza et al., 2003). This compound could be recently observed to be generated upon decarboxylation of trigonelline during coffee roasting (Stadler et al., 2002).

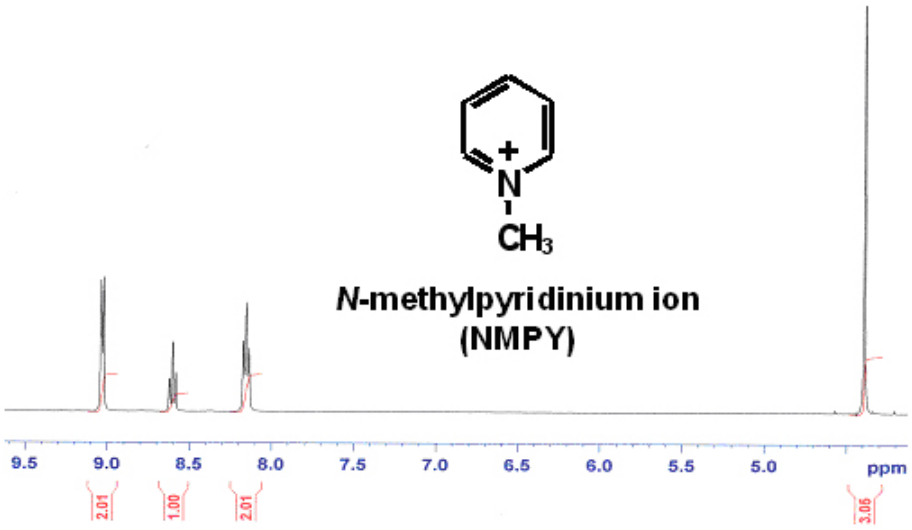


**Figure 3. $^{1}$H NMR spectrum and structure of *N*-methylpyridinium ion (NMPY) (Somoza et al., 2003).**

To test the activity of NMPY in modulating biotransformation enzymes *in vitro*, Caco-2 cells were exposed to NMPY for 72 h. Compared with non-exposed control cells, exposure of either 0.025 or 0.05 mg NMPY per 100 mL cell culture medium resulted in an increased GST activity by +22.4 ± 3.79% ($p<0.05$ vs control cells) or +8.11 ± 6.79 %, respectively (Somoza et al., 2003). In contrast, corresponding results for the CCR activity demonstrated an inhibitory effect of 24.2 ± 6.67% ($p < 0.05$ vs control cells) and –17.9 ± 9.99 % after exposure to 0.025 or 0.05 mg NMPY. Taken together, these results demonstrate that NMPY is the major compound present in coffee beverage that induces the chemopreventive GST activity *in vitro.*

In order to verify the observed effects of NMPY for an *in vivo* system, lyophilized coffee beverage and NMPY were administered to male Wistar rats for 15 days (Somoza et al., 2003). The daily dose for NMPY was calculated on the basis of an average body weight of 351 ± 19 g and a daily intake of 23.4 ± 2.47 g diet, giving 22 mg NMPY per kg body weight. In the liver, feeding of 4.5 % coffee beverage resulted in an increase of Phase-II GST and UDP-GT activity by 24 % and 40 % compared to animals fed the control diet, respectively. Liver samples isolated from animals on the NMPY diet even showed an elevated UDP-GT activity of 65 % ($p = 0.08$) (Somoza et al., 2003). Plasma total antioxidant capacity, calculated on the basis of trolox equivalents (TE values), and plasma tocopherol equivalents were elevated in animals fed the coffee beverage and the NMPY containing diet as given in Figure 4 (Somoza et al., 2003). For the coffee beverage, the results demonstrating a strong antioxidant activity *in vitro* were confirmed by the feeding study.

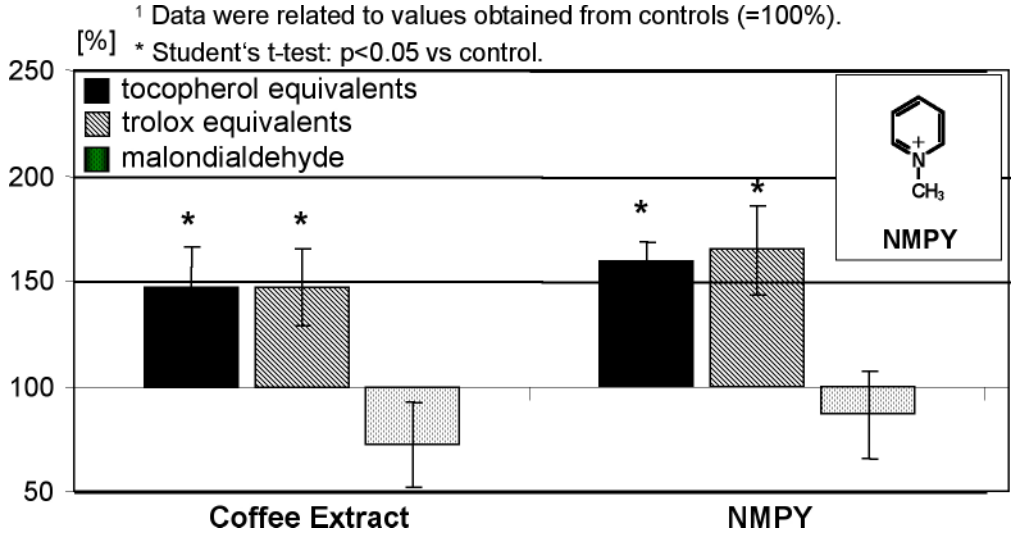


**Figure 4. Percent change in tocopherol equivalents, antioxidant capacity (trolox equ.), and malondialdehyde in plasma of rats fed coffee extract or NMPY for 15 Days (Somoza et al., 2003).**

Suprisingly, feeding of NMPY diet also did result in an increased total antioxidant capacity in the plasma, although NMPY did not show any antioxidant activity *in vitro*. These data clearly indicate for the first time that the mode of action demonstrated for NMPY in biological systems is different from that in foods. It is likely that NMPY is metabolized either by intestinal microorganisms or endogenously by enzymatic activity, resulting in the formation of biologically active compounds. The difference in urine color observed as well as the slightly increased Phase-II GST and UDP-GT activities in rat liver and the increased total antioxidant capacity in rat plasma support the hypothesis that NMPY present in coffee beverage are biologically active.

Although it is still an open question of whether NMPY or its metabolite(s) induce chemopreventive enzyme activities *in vivo*, these recent breaking results strongly support the induction of chemopreventive Phase-II enzymes by dietary coffee beverages containing *N*-methylpyridinium ions. The molecular mechanism for this induction is hypothesized to be based on kinase-mediated reactions rather than binding to the arylhydrocarbon receptor (AhR) which is the main receptor involved in the Phase-I/-II enzyme induction (Iba et al., 2002). N-methylpyridinium, in particular, has been recently demonstrated to induce MAP-kinase activation in two different cell lines, intestinal Caco-2 and renal HEK-293 cells (Zill et al., 2003). Further studies will be necessary to investigate the biochemical pathways by which either N-methylpyridinium or its metabolites induce chemopreventive enzymes *in vivo*.

Taking all these data into consideration, it can be concluded that coffee products display dual antioxidant function. First, coffee *per se* exhibits antioxidant compounds, such as chlorogenic acids, secondly, coffee contains chemicals like NMPY that turn on the endogeneous antioxidant defence system through induction of the phase II biotransformation enzymes.

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# Galactomannans and Arabinogalactan-proteins in the Coffee Bean Cell Wall: Heterogeneity and Localisation

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## SUMMARY

Methods were developed for the isolation and fractionation of the two major groups of non-cellulosic polysaccharides in the coffee bean cell wall: the (galacto)mannans and the arabinogalactans. Chemical analysis demonstrated that both groups were chemically polydispersed with regard to their degree of backbone substitution. The galactose:arabinose ratio in the arabinogalactans varied between 1.9 and 3.3 and the arabinogalactans were shown to exist in the form of arabinogalactan-proteins. The (galacto)mannans, which were studied in coffee beans at several stages of development from 11-37 weeks after flowering, showed mannose:galactose ratios as high as 2. Evidence was obtained that their final degree of galactosylation was affected by the action of an α-galactosidase during the later stages of bean development. A cytochemical and immunological study showed that the different types of polysaccharide were heterogeneously distributed across the cell wall and that during roasting the arabinogalactans were preferentially degraded from the inner zones of the bean.

## RÉSUMÉ

Des méthodes ont été développées pour l'isolation et le fractionnement de deux groupes de polysaccharides non-cellulosiques de la paroi cellulaire du café: les (galacto)mannanes et les arabinogalactanes. L'analyse chimique a montré que les deux espèces sont polydisperses par rapport à leur degré de branchement. Le rapport galactose:arabinose dans les arabinogalactanes varie entre 1.9 et 3.3 et elles se trouvent sous la forme d'arabinogalactanes-protéines. Les (galacto)mannanes qui ont été étudiées à différents stades de développement entre 11 et 37 semaines après la floraison présentent des rapports mannose:galactose allant jusqu'à 1.3. Il a été démontré que le degré de galactosylation final dépend de l'action d'une α-galactosidase durant les dernières phases de développement. Une étude cytochimique et immunologique a démontré que les différents polysaccharides étaient distribuées de façon hétérogène à travers la paroi cellulaire et que durant la torréfaction, les arabinogalactanes des zones internes du grain étaient dégradés préférentiellement.

## INTRODUCTION

As the competition among soluble coffee manufacturers intensifies greater demands are being made on understanding the detailed chemistry and morphology of the coffee bean, so that processes for the preparation of soluble coffee can be improved and refined. Since the cell wall polysaccharides make up ~50% of the dry weight of the bean it is not surprising that this area in particular has been the focus of much research. This has resulted in several publications, which present data on the primary structure of the main non-cellulosic polysaccharides of the coffee bean cell wall, the (galacto)mannans and the arabinogalactans or

as recently established, arabinogalactan-proteins (AGPs) (Redgwell et al., 2002; Fischer et al., 2001; Bradbury and Halliday, 1990).

The physical intractability of the cell wall of the mature coffee bean is largely attributable to the fibrous polysaccharides cellulose and (galacto)mannan, which can associate by hydrogen bonding to form insoluble fibrils. An additional group of polysaccharides, the arabinogalactan-proteins (AGPs), although water-soluble in their pure form, are rendered largely insoluble in the coffee cell wall, either because of entanglement with the fibrous polymers, or by some covalent association which is unknown at the present time. The monosaccharide composition and gross structural features of the galactomannans and AGPs have been documented. However, much remains to be revealed about the extent of their chemical heterogeneity, especially with regard to their degree of substitution and spatial arrangement of the component polysaccharides within the wall. This study presents results on the heterogeneity of the non-cellulosic polysaccharides of the coffee bean cell wall as determined by a combination of chemical analysis and microscopic examination. The latter technique used cytochemical and immunological probes which were specific for the different types of polysaccharide and certain polysaccharide epitopes.

## MATERIALS AND METHODS

Sources of plant material, enzymes, preparation and fractionation of cell wall materials (CWMs), and methods of microscopy and chemical analysis have been detailed previously (Redgwell et al., 2002; Redgwell et al., 2003; Sutherland et al., 2004). For the roasting studies Arabica (var. *Kenya*) were roasted to CTN 182, prior to immunolabelling.

## RESULTS

### Chemical heterogeneity of arabinogalactan-proteins (AGPs)

In order to extract the total AGP content of green arabica beans a series of increasingly severe chemical extractions were performed followed by an enzymatic treatment with a mixture of cellulase and mannanase (Redgwell et al., 2002). Five fractions (AG 1-AG 5) were isolated, purified and compositional analysis performed (Table 1). There was a gradual decrease in the arabinose content of the AGPs as they became more difficult to solubilise from the CWM. Thus the gal:ara ratio varied between 1.9 in the water soluble AGP to 3.3 in the AGP solubilised from the CWM by enzymatic treatment.

**Table 1. Major monosaccharide components of AGP fractions in coffee bean CWM.**

| Fraction | Monosaccharide Composition (mole %) | | | | | Gal:Ara ratio |
|---|---|---|---|---|---|---|
| | | | | | | |
| | rha | fuc | Ara | gal | uronic acid | |
| AG1 | 1.9 | 1.0 | **30.5** | **58.1** | 7.8 | **1.9** |
| AG2 | 1.3 | 1.1 | **24.3** | **62.7** | 9.5 | **2.6** |
| AG3 | 1.4 | 1.1 | **25.2** | **61.0** | 10.1 | **2.4** |
| AG4 | 0.4 | 1.1 | **23.1** | **64.8** | 10.6 | **2.8** |
| AG5 | 0.4 | 1.2 | **21.0** | **69.4** | 7.69 | **3.3** |

The AG fractions 1-5 were either all AGPs or contained some AGPs. They were assayed with the β-glucosyl Yariv reagent which gives a highly specific and characteristic red precipitate with AGPs but no reaction at all with arabinogalactans (Van Holst and Clarke, 1985). Thus, larch arabinogalactan which is not an AGP gives no reaction (Figure 1) .

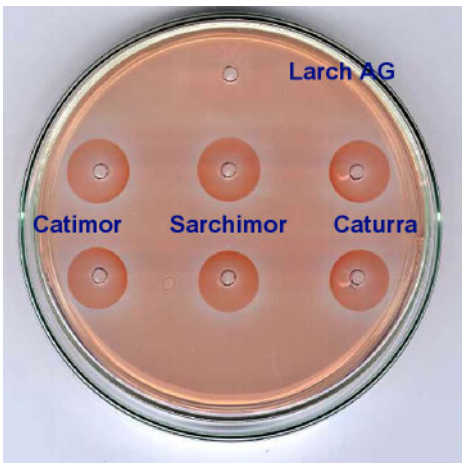


**Figure 1. β-Glucosyl Yariv assay for AGPs from Arabica coffee bean CWM.**

All the AG fractions from coffee bean CWM gave a positive Yariv assay. This fact plus the continued association of protein and carbohydrate during several physical and chemical stages of purification and the identification of hydroxyproline in the protein fraction suggested that all the arabinogalactans in the coffee bean cell wall were AGPs.

**Degree of galactosylation of galactomannans**

The insolubility of the coffee grain is often attributed to the fact that the mannans in coffee behave very much like cellulose and associate by hydrogen bonding into insoluble fibrils. This is more likely to happen when the mannans are unsubstituted and lack side chain sugars. There has therefore been considerable interest in establishing the degree of galactosylation of the coffee mannans. The literature reports several values from 130:1 (Bradbury and Halliday 1990) 30:1 (Fischer et al., 2001) and 40:1 (Oosterveld et al., 2003). With the advent of molecular techniques the idea has been proposed that manipulation of the galactose content of the mannans could be accomplished with coffee plants by down regulating the enzyme α-galactosidase. This would only be effective if α-galactosidase played a role in determining the final galactose content of the galactomannans of the mature bean.

We therefore isolated the galactomannan from the endosperm of developing beans at several stages of their growth, between 11 and 37 weeks after flowering and determined their chemical features by linkage analysis (Redgwell et al., 2003).

**Table 2. Linkage analysis of mannose/galactose content of galactomannans from coffee beans at several stage of development.**

| Sugar | Linkage | Weeks after flowering | | | | | |
|---|---|---|---|---|---|---|---|
| | | 11 | 15 | 21 | 26 | 31 | 37 |
| Man*p* | Terminal | 0.7 | 0.6 | 1.9 | 0.7 | 0.8 | 0.5 |
| | 4- | **27.0** | **30.4** | **32.0** | **19.8** | **22.9** | **19.9** |
| | 4,6- | **13.1** | **15.9** | **16.0** | **7.7** | **3.9** | **2.8** |
| | | | | | | | |
| Gal*p* | Terminal | 17.5 | 19.6 | 20.6 | 10.3 | 7.0 | 6.5 |

Between 21-31 weeks after flowering the ratio of 4-mannosyl residues to 4,6-mannosyl residues increased from 2:1 to 6:1 indicating that there was a gradual loss of galactose from the mannan backbone. Thus the degree of substitution of the coffee galactomannans is

developmentally regulated and may result in part from the modification of a primary synthetic product by the action of an α-galactosidase.

**Cytochemical and immunolabelling of cell wall polysaccharides**

***AGPs***

AGPs were localised by labelling with the AGP-specific β-glucosyl Yariv reagent (Figure 2A) and the monoclonal antibody LM2 (Sutherland et al., 2004) that recognises a carbohydrate epitope containing glucuronic acid. Glucuronic acid has been shown to occupy terminal positions on some of the side chains of coffee AGPs. Both forms of labelling showed a widespread distribution of the AGP across the cell wall. However, there was more intense staining with the Yariv reagent in the region adjacent to the cell lumen.

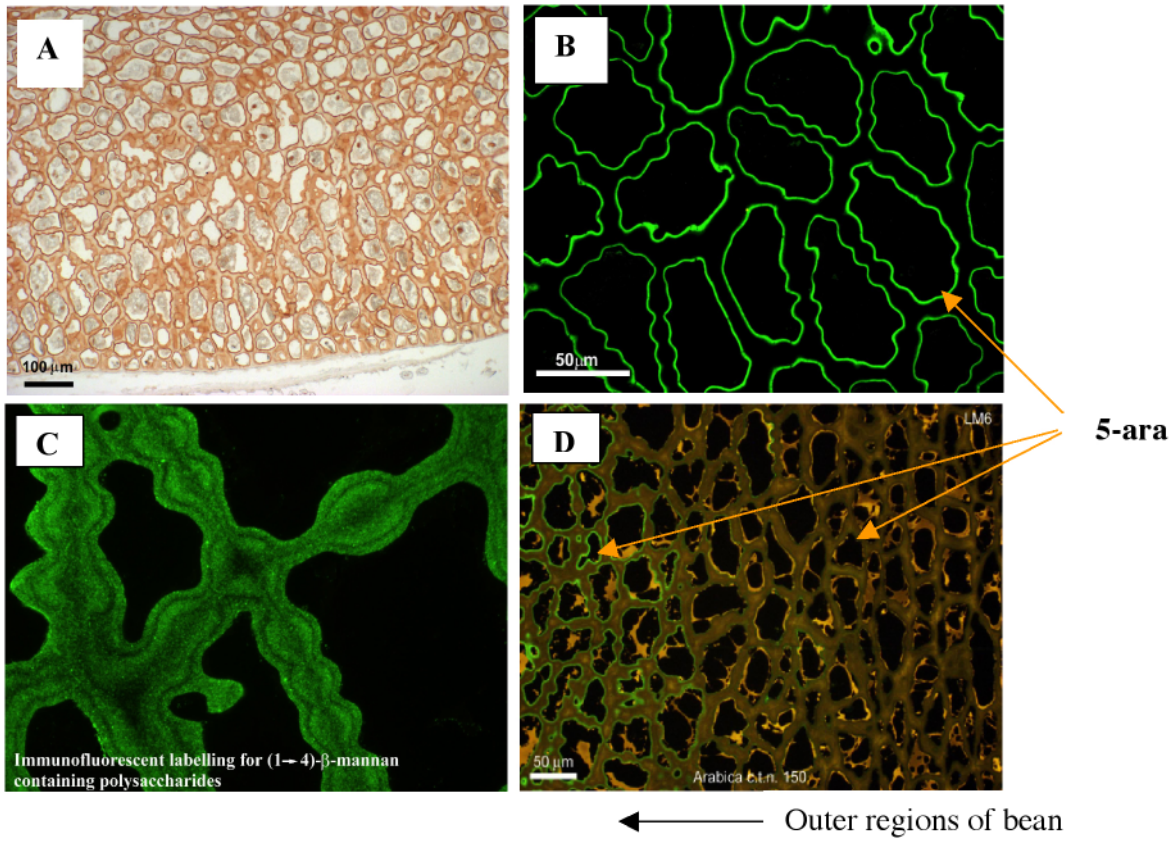


**Figure 2. Cytochemical and immunolabelling of coffee cell wall polysaccharides. A: AGPs labelled with β-glucosyl Yariv reagent. B: 5-arabinan labelled with. LM 6 a monoclonal antibody. C: β-1,4-mannan labelled with monoclonal antibody BGM C6. D: 5-arabinan labelled with LM 6 in roasted bean showing preferential degradation of the arabinan in the inner zones of the bean.**

***Arabinans***

The monoclonal antibody LM6 is specific for linear α-5-arabinans. It labelled the whole cell wall of the single layer of epidermal cells (Sutherland et al., 2004) but in the endosperm itself it labelled only in a compact band adjacent to the cell lumen with no label in the main body of the cell wall (Figure 2B). As this coincided with the intense labelling of the AGPs with the Yariv reagent it suggested that there existed in this region AGPs which contained significant amounts of α-1,5-arabinans in their side chains.

### *Mannans*

Mannans were labelled with the β-1,4-mannan-specific monoclonal antibody BGM C6. The antibody labelled across the entire wall (Figure 2C). However, there was a variation in intensity of the labelling across the wall with more intense staining adjacent to the lumen of the cell and the middle lamella. These two zones were separated by a region of only moderately intense staining (Sutherland et al., 2004).

### *Arabinan breakdown during roasting*

The labelling of a cross section of the cell wall from a roasted bean (CTN 182) with the LM6 antibody showed that there was a preferential breakdown of the 5-arabinan in the inner regions of the cell wall (Figure 2D) We speculate that this occurred because water would be retained longer in these regions during heating converting the inner regions of the bean into what is effectively a hydrolytic cell.

## DISCUSSION

The results of these studies have extended the knowledge of the chemistry of the two major types of non-cellulosic polysaccharides in the coffee bean cell wall, the mannans and the AGPs. Evidence has been presented which points to the degree of galactosylation of the mannans being developmentally regulated. The arabinogalactans have been shown to exist as AGPs and to contain up to 10% of their structure as glucuronic acid. Both types of polysaccharide are extremely heterogeneous, not only with regard to their chemical features but also their distribution across the cell wall. Mannans and AGPs showed a unique distribution pattern. Within each type of polysaccharide there were differences in the location of variant structural forms of the parent polymer across the coffee cell wall.

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# Impact of Wet and Dry Process on Green Coffee Composition and Sensory Characteristics

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## SUMMARY

Two main post-harvest processes are essentially applied to coffee cherries, namely dry process and wet process. The objective of this work was to assess the chemical composition and the sensory profiles of *Robusta* coffees treated by wet and dry processes. As methodology, six *Robusta* coffees grown in an experimental farm (Indonesia) were treated by standard dry and wet processes under well controlled conditions. Extensive chemical and sensory evaluations were carried out. As results, dry and wet processed coffees were significantly different with regard to composition and sensory characteristics. As concerns green bean composition, applying wet process as opposed to dry process resulted in a reduction in free carbohydrate (i.e. fructose, glucose), organic acids (i.e. quinic, oxalic), mineral compounds (e.g. $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$), trigonelline and an increase in chlorogenic acids (e.g. CQA) cell-wall polysaccharides (e.g. arabinogalactans, mannans) and lipids. As concerns sensory characteristics, wet processed coffee as opposed to dry processed ones were perceived as less bitter, burnt, rubbery, woody and slightly more fruity and acid. In conclusion, post-harvest process had a clear impact on the low molecular weight components present in green coffee. Dry process appears to favour degradation mechanisms and wet process the removal of low molecular weight compounds, which contribute to the strong *Robusta* character. The present data do not appear to reflect the occurrence of marked physiological changes during wet processing.

## RÉSUMÉ

Après récolte les cerises de café peuvent être traitées par la voie sèche ou la voie humide. L'objectif de ce travail a été de comparer la composition chimique et les caractéristiques sensorielles de six cafés *Robusta* récoltés dans une ferme expérimentale (Indonésie) et traités selon les deux méthodes. Une analyse détaillée des compositions et les profils sensoriels ont été établis. En ce qui concerne la composition des grains verts, les cafés traités par voie humide présentaient des teneurs plus faibles en glucides libres, acides organiques, composés minéraux et trigonelline d'une part et des teneur plus élevées en acides chlorogéniques, glucides pariétaux et lipides d'autre part. Pour ce qui est des caractéristiques sensorielles, les cafés traités par voie humide étaient moins amers, brûlés, caoutchouc, bois mais plus fruités et acides. Le traitement post-récolte influence l'équilibre en précurseurs d'arômes. Le traitement par voir sèche favorise les mécanismes de dégradation et celui par voie humide le lessivage de composés de petite taille responsables du caractère *Robusta*. Cette étude n'a pas permis de confirmer l'induction de changements physiologiques importants par le traitement humide.

## BACKGROUND

Essentially two main post-harvest processes are applied to coffee cherries, namely the dry process and the wet process. Dry processing consists of drying the whole cherry and then mechanically removing the dried outer parts, whereas wet processing consists of removing the fresh parts by pulping, fermentation and subsequent washing steps before drying. Because of the different conditions prevailing during these treatments, biochemical and physiological changes occurring during post-harvest treatment could differ resulting in different cup quality.

Recently Bytof et al. (2000, 2001) suggested the potent role of germination inhibitors present in the fresh parts to explain the quality differences between wet and dry processed coffees. In the absence of these inhibitors such as in wet process, bean germination could be initiated resulting in the mobilisation of reserves to synthesize low-molecular weight substances. Among these substances described as potential key aroma precursors only free amino acids were quantified.

The difficulties of such studies rely in obtaining the same coffee processed under both post-harvest processes. Six *Robusta* coffees grown on an experimental farm in Indonesia were prepared for that purpose. An extensive chemical characterisation of the green beans and a sensory profiling of the roasted coffees were carried out. This paper compares the chemical and sensory characteristics of dry and wet processed coffees.

## MATERIAL & METHODS

### Green Coffees

Six *Robusta* clones (# 42, 358, 409, 961, 203, 121) selected for their genetic diversity were grown on a Nestlé farm in Indonesia. After harvest, fresh cherries were divided in two parts for well-controlled standard dry and wet processes.

### Chemical Analyses

Initial green coffee humidity was determined on a coffee grinding (d'~1mm) dried at 102°C for 4 hours. All green coffees were further finely ground in a Freezer Mill 6800 to obtain a final average particle size d' below 100 μm. These ground materials were used for extensive chemical analyses.

- ***Lipids*** - Lipids were extracted in a soxhlet with petroleum ether at 70°C. The solvent was evaporated and residual fat measured gravimetrically.
- ***Minerals*** - Ash content was evaluated after total combustion of organic matter at 550°C. The residual ash was determined gravimetrically. In addition, an extensive element profiling was obtained by ICP-MS or ICP-AES.
- ***Carbohydrates*** - Total and free carbohydrate profiles were established by anion exchange chromatography (AEC) equipped with an electrochemical detector (PED). The preparation and separation are summarised in Table 1. Carbohydrates were converted into anhydrosugars and gathered into key polysaccharide families.
- ***Proteins*** - Total nitrogen was evaluated using a LECO FP-200 protein/nitrogen analyser. Total and free amino acid profiles were established by reverse phase chromatography (RP) with fluorimetric and UV detections. The preparation and separation are summarised in Table 2. Amino acids levels were further converted into total protein values, as well as clustered in amino acids classes.
- ***Chlorogenic acids & Caffeine*** - Chlorogenic acids and caffeine were extracted at 40°C in 70% aqueous methanol. Chromatographic separation was carried out on Spherisorb

ODS1 (250 mm x 4) using a water/acetonitrile/phosphate gradient. UV detection was performed at λ 320nm for chlorogenic acids and λ 274 nm for caffeine.

- ***Trigonelline*** - Trigonelline was extracted in HCl 0.1N at 100°C for 5 min. Trigonelline was quantified by HPLC using Nucleosil 50-5 column and UV detection (λ 274 nm).
- ***Organic acids*** - Organic acids were extracted in distilled water at 70°C. Separation of organic acids was carried out by ionic exchange chromatography using a Dionex column AS11 eluted with a NaOH gradient (0.5-25 mM / 35 min) and conductimetry detection.
- ***pH & Acidity*** - Ground green coffee (10 g) was extracted for 5 minutes in boiling water (500 mL). Initial pH was measured. The titratable acidity was evaluated at pH 6.6 and 8 using NaOH 0.1 M and expressed in meq/kg dry green coffee.

**Table 1. Preparation and analysis of free and total carbohydrates.**

| | **Free carbohydrates** | **Total carbohydrates** |
|---|---|---|
| | Mannit, Ara, Gal, Glu, Xyl, Man, Fru, Suc | Mannit, Rha, Ara, Gal, Glu, Xyl, Man, Fru, Agal, AGlu |
| Pre-hydrolysis ($H_2SO_4$ 26N, 20°C/ 2h) | No | Yes |
| Hydrolysis ($H_2SO_4$ 2N, 100°C/ 3h) | No | Yes |
| Neutralisation with NaOH | No | Yes |
| AEC Dionex / separation column | PA100 column | PA1 column |
| Separation medium | Water | Water/NaOH/Na acetate |
| PED with NaOH 0.5N | Yes | Yes |

**Table 2. Preparation and analysis of free and total amino acids.**

| | **Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val** | **Met, Cys** | **Trp** |
|---|---|---|---|
| Oxidation with formic acid | No | Yes | No |
| Hydrolysis with HCl (6N, 110°C/24h) | Yes | Yes | No |
| Saponification with NaOH | No | No | Yes |
| Derivatisation with AccQ-Fluor ™ | Yes | Yes | No |
| HPLC Waters / AccQ ?Tag $C_{18}$ column | Yes | Yes | Yes |
| Fluorimetric detection | Yes | Yes | UV |

**Sensory Analysis**

- ***Roasting & Grinding*** – Batches of 300g were roasted at 240°C in a Neotec Roaster. Roasting duration was adapted to reach the same colour i.e. CTN 90. Roasted coffees were ground in a Ditting® grinder to a particle diameter of 0.6-0.7 mm.
- ***Brew Preparation*** – Coffees were brewed using an electric coffee maker [Technivorm®] and Mélitta® paper filters, at a concentration of 50 g/L. “Vittel Bonne source” plus one part of deionised water was used for the preparation. The prepared coffees were cooled down to 70°C and poured into thermos flasks just before evaluation.
- ***Sensory Evaluation*** – A well-trained panel of 11 internal assessors profiled the samples using a glossary of 22 attributes covering coffee aroma, flavour and texture (Table 3). Each panellist evaluated twice each sample in a monadic way. Each attribute was rated on an unstructured linear scale, anchored at the extremities with “not at all” and “very”. For further analysis, scores were converted into continuous numeric values ranging between 0 and 10.

**Table 3. Sensory attributes describing the aroma, flavour and texture of Robusta brews.**

| Attributes | Aroma | Flavour | From not to very |
|---|---|---|---|
| Citrus | x | x | Intensity of the citrus Aroma or Flavour (bergamot) |
| Fruity | x | x | Intensity of the fruity Aroma or Flavour (red fruit, apricot) |
| Winey | x | x | Intensity of green fruity Aroma or Flavour (cherry, blueberry, prune) |
| Green | x | x | Intensity of green vegetable Aroma or Flavour |
| Hay | x | x | Intensity of dried hay Aroma or Flavour |
| Caramel | x | x | Intensity of caramel Aroma or Flavour |
| Rubbery | x | x | Intensity of green rubbery Aroma or Flavour (burnt tyre) |
| Burnt | x | x | Intensity of burnt Aroma or Flavour (over cooked, smoke) |
| Woody | x | x | Intensity of woody Aroma or Flavour (reminiscent of wood stick chewing) |
| Acid | | x | Intensity of acid taste |
| Bitter | | x | Intensity of bitter taste |
| Astringent | | x | Intensity of astringence |
| Body | | x | Apparent viscosity of the coffee from thin to thick |

## Data Analysis

Mean data of dry and wet processed coffees were compared using a paired t-test (95% confidence level). Multivariate analysis was carried out in order to simultaneously visualise the outcome of many univariate tests. Maps were obtained by discriminant analysis (DA) to visualise the impact of post-harvest process.

## RESULTS AND DISCUSSION

### Chemical Composition

Compared to previous studies (Selmar et al., 2001; Arnold, 1944; Balyaya and Clifford, 1995) this work proposes an extensive comparison of the chemical changes occurring during post-harvest treatment. Tables 4 and 5 compare the chemical compositions of the six *Robusta* clones processed in parallel by dry and wet processes. As suggested by Bytof et al., post-harvest process affects the balance in low molecular weight compounds, key precursors of coffee aroma and flavours.

Applying wet process on *Robusta* clones resulted in lower levels in:

- Free carbohydrates namely free glucose, fructose and galactose;
- Organic acids namely quinic, phosphoric and oxalic acids;
- Mineral compounds namely $K^{+}$, $Ca^{2+}$, $Mg^{2+}$ and $Cu^{2+}$;
- Trigonelline

Applying wet process on *Robusta* clones resulted in higher levels in:

- Chlorogenic acids namely all CQA isomers and 4,5diCQA;
- Lipids
- Cell-wall polysaccharides (i.e. arabinogalactans) and storage polysaccharides (i.e. mannans).

Applying either wet or dry post-harvest processes had almost no influence levels in caffeine, sucrose, malic and citric acids, total and free amino acids.

**Table 4. Chemical composition and mean differences of dry (D) and wet (W) processed *Robusta*. Statistical significance at 95%, the process resulting in higher level indicated in the column.**

| | | 42D | 358D | 409D | 961D | 203D | 121D | Average | 42W | 358W | 409W | 961W | 203W | 121W | Average | Dry-Wet | (SD) | Significant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H2O** | [%] | 13.47 | 14.06 | 12.72 | 12.64 | 13.74 | 13.58 | **13.37** | 12.15 | 11.39 | 10.85 | 10.96 | 11.39 | 11.20 | **11.32** | 2.05 | 0.50 | **Dry** |
| **Lipids** | [%] | 11.44 | 11.87 | 11.60 | 11.80 | 10.56 | 9.80 | **11.18** | 11.63 | 12.04 | 12.44 | 12.84 | 11.44 | 9.56 | **11.66** | -0.48 | 0.51 | **Wet** |
| **Caffeine** | [%] | 2.46 | 2.22 | 2.30 | 2.75 | 2.60 | 3.01 | **2.56** | 2.50 | 2.27 | 2.34 | 2.45 | 2.70 | 3.02 | **2.55** | 0.01 | 0.14 | **-** |
| **Trigonelline** | [%] | 0.62 | 0.67 | 0.66 | 0.64 | 0.60 | 0.58 | **0.63** | 0.60 | 0.68 | 0.65 | 0.63 | 0.54 | 0.53 | **0.61** | 0.03 | 0.02 | **Dry** |
| **Proteins** | [%] | 11.66 | 12.02 | 13.17 | 13.92 | 10.23 | 10.84 | **11.98** | 12.50 | 11.74 | 13.47 | 12.50 | 10.42 | 10.79 | **11.90** | 0.07 | 0.76 | **-** |
| NH4 | [%] | 0.32 | 0.33 | 0.39 | 0.40 | 0.29 | 0.32 | **0.34** | 0.34 | 0.34 | 0.40 | 0.36 | 0.30 | 0.32 | **0.34** | 0.00 | 0.02 | **-** |
| Non polar aliphatic | [%] | 3.65 | 3.76 | 3.91 | 4.20 | 3.06 | 3.21 | **3.63** | 3.95 | 3.60 | 4.00 | 3.72 | 3.13 | 3.24 | **3.61** | 0.02 | 0.26 | **-** |
| Aromatic | [%] | 1.35 | 1.40 | 1.58 | 1.72 | 1.38 | 1.38 | **1.47** | 1.50 | 1.44 | 1.59 | 1.59 | 1.35 | 1.36 | **1.47** | 0.00 | 0.09 | **-** |
| Polar uncharged | [%] | 0.93 | 0.95 | 1.06 | 1.08 | 0.83 | 0.88 | **0.96** | 0.98 | 0.93 | 1.05 | 0.99 | 0.86 | 0.83 | **0.94** | 0.02 | 0.05 | **-** |
| Sulfured | [%] | 0.46 | 0.45 | 0.53 | 0.49 | 0.44 | 0.43 | **0.47** | 0.47 | 0.44 | 0.52 | 0.49 | 0.41 | 0.42 | **0.46** | 0.01 | 0.01 | **-** |
| Carboxylic | [%] | 3.26 | 3.40 | 3.77 | 3.96 | 2.75 | 3.04 | **3.36** | 3.49 | 3.33 | 3.93 | 3.56 | 2.91 | 3.07 | **3.38** | -0.02 | 0.23 | **-** |
| Amino | [%] | 1.68 | 1.73 | 1.95 | 2.06 | 1.48 | 1.58 | **1.74** | 1.78 | 1.67 | 1.97 | 1.79 | 1.45 | 1.56 | **1.70** | 0.04 | 0.12 | **-** |
| Free amino acids | [%] | 0.38 | 0.40 | 0.33 | 0.36 | 0.29 | 0.24 | **0.33** | 0.41 | 0.29 | 0.35 | 0.25 | 0.28 | 0.26 | **0.31** | 0.03 | 0.06 | **-** |
| **Carbohydrates** | [%] | 47.11 | 41.24 | 43.29 | 49.17 | 52.95 | 49.85 | **47.27** | 47.23 | 44.50 | 42.73 | 51.42 | 53.46 | 52.34 | **48.61** | -1.34 | 1.52 | **-** |
| Sucrose | [%] | 4.19 | 4.40 | 4.11 | 3.80 | 2.91 | 3.78 | **3.87** | 4.11 | 4.33 | 3.32 | 3.79 | 4.03 | 3.64 | **3.87** | -0.01 | 0.62 | **-** |
| Arabinans | [%] | 3.70 | 3.22 | 3.54 | 4.02 | 4.30 | 4.21 | **3.83** | 3.77 | 3.49 | 3.54 | 4.20 | 4.59 | 4.48 | **4.01** | -0.18 | 0.12 | **Wet** |
| Galactans | [%] | 11.13 | 9.77 | 10.35 | 12.23 | 13.07 | 12.55 | **11.52** | 11.33 | 10.68 | 10.35 | 12.68 | 13.31 | 13.47 | **11.97** | -0.45 | 0.39 | **Wet** |
| Cellulose | [%] | 6.31 | 5.62 | 6.24 | 6.98 | 8.29 | 7.05 | **6.75** | 6.07 | 5.99 | 6.64 | 7.57 | 7.36 | 7.10 | **6.79** | -0.04 | 0.56 | **-** |
| Mannans | [%] | 19.57 | 16.36 | 17.06 | 19.66 | 21.86 | 20.12 | **19.11** | 19.83 | 18.29 | 17.06 | 20.96 | 22.04 | 21.57 | **19.96** | -0.85 | 0.80 | **Wet** |
| Pectins | [%] | 1.63 | 1.36 | 1.33 | 1.81 | 1.94 | 1.60 | **1.61** | 1.64 | 1.30 | 1.33 | 1.74 | 1.72 | 1.64 | **1.56** | 0.05 | 0.09 | **-** |
| Xylans | [%] | 0.18 | 0.15 | 0.20 | 0.24 | 0.16 | 0.19 | **0.19** | 0.16 | 0.14 | 0.20 | 0.18 | 0.14 | 0.17 | **0.17** | 0.02 | 0.02 | **Dry** |
| Free monosaccharides | [%] | 0.54 | 0.55 | 0.39 | 0.35 | 0.46 | 0.49 | **0.46** | 0.42 | 0.30 | 0.25 | 0.17 | 0.14 | 0.22 | **0.25** | 0.24 | 0.13 | **Dry** |
| **Chlorogenic acids** | [%] | 9.55 | 9.04 | 11.23 | 10.24 | 8.83 | 10.02 | **9.82** | 10.23 | 10.35 | 11.37 | 10.87 | 9.84 | 10.88 | **10.59** | -0.77 | 0.40 | **Wet** |
| 3CQA | [%] | 0.56 | 0.61 | 0.55 | 0.62 | 0.69 | 0.83 | **0.64** | 0.60 | 0.68 | 0.65 | 0.72 | 0.80 | 1.03 | **0.75** | -0.10 | 0.05 | **Wet** |
| 4CQA | [%] | 0.84 | 0.76 | 0.87 | 0.95 | 0.91 | 1.08 | **0.90** | 0.93 | 0.82 | 1.07 | 1.11 | 1.13 | 1.33 | **1.06** | -0.16 | 0.08 | **Wet** |
| 5CQA | [%] | 5.24 | 4.67 | 6.16 | 5.70 | 4.35 | 4.55 | **5.11** | 5.59 | 5.42 | 6.11 | 5.89 | 4.69 | 4.85 | **5.43** | -0.31 | 0.26 | **Wet** |
| 4,5diCQA | [%] | 0.81 | 0.85 | 0.93 | 0.79 | 0.68 | 0.88 | **0.83** | 0.92 | 1.00 | 0.92 | 0.87 | 0.80 | 0.98 | **0.91** | -0.09 | 0.06 | **Wet** |
| 3,5diCQA | [%] | 0.61 | 0.60 | 0.82 | 0.67 | 0.75 | 0.90 | **0.73** | 0.74 | 0.74 | 0.79 | 0.65 | 0.72 | 0.95 | **0.76** | -0.04 | 0.08 | **-** |
| 3,4diCQA | [%] | 0.63 | 0.66 | 0.74 | 0.59 | 0.62 | 0.92 | **0.69** | 0.65 | 0.73 | 0.76 | 0.72 | 0.80 | 0.87 | **0.76** | -0.06 | 0.08 | **-** |
| 4FQA | [%] | 0.00 | 0.00 | 0.13 | 0.00 | 0.00 | 0.00 | **0.02** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | **0.00** | 0.02 | 0.05 | **-** |
| 5FQA | [%] | 0.85 | 0.88 | 1.03 | 0.93 | 0.84 | 0.85 | **0.90** | 0.80 | 0.96 | 1.06 | 0.91 | 0.91 | 0.87 | **0.92** | -0.02 | 0.05 | **-** |
| **Organic acids** | [%] | 1.52 | 1.72 | 1.56 | 2.06 | 2.03 | 2.15 | **1.84** | 1.47 | 1.59 | 1.44 | 1.85 | 1.84 | 1.97 | **1.69** | 0.13 | 0.05 | **Dry** |
| Citric | [%] | 1.02 | 1.22 | 1.01 | 1.20 | 1.13 | 1.19 | **1.13** | 1.06 | 1.18 | 1.02 | 1.15 | 1.07 | 1.19 | **1.11** | 0.02 | 0.04 | **-** |
| Malic | [%] | 0.08 | 0.07 | 0.07 | 0.35 | 0.33 | 0.36 | **0.21** | 0.07 | 0.06 | 0.06 | 0.31 | 0.30 | 0.27 | **0.18** | 0.03 | 0.03 | **-** |
| Quinic | [%] | 0.29 | 0.29 | 0.26 | 0.28 | 0.35 | 0.37 | **0.31** | 0.22 | 0.22 | 0.22 | 0.26 | 0.31 | 0.36 | **0.27** | 0.04 | 0.02 | **Dry** |
| Oxalic | [%] | 0.08 | 0.09 | 0.14 | 0.13 | 0.13 | 0.13 | **0.12** | 0.08 | 0.08 | 0.07 | 0.05 | 0.09 | 0.08 | **0.07** | 0.04 | 0.03 | **Dry** |
| Phosphoric | [%] | 0.05 | 0.06 | 0.08 | 0.10 | 0.09 | 0.09 | **0.08** | 0.05 | 0.05 | 0.06 | 0.07 | 0.08 | 0.08 | **0.07** | 0.01 | 0.01 | **Dry** |
| **Ash** | [%] | 4.46 | 4.70 | 4.65 | 4.70 | 4.54 | 4.84 | **4.65** | 4.31 | 4.51 | 4.27 | 4.38 | 4.36 | 4.54 | **4.40** | 0.25 | 0.09 | **Dry** |
| K+ | [%] | 2.24 | 2.32 | 2.15 | 2.24 | 2.07 | 2.32 | **2.22** | 2.12 | 2.17 | 1.97 | 2.04 | 2.06 | 2.17 | **2.09** | 0.14 | 0.07 | **Dry** |
| Mg2+ | [%] | 0.19 | 0.20 | 0.19 | 0.21 | 0.20 | 0.18 | **0.19** | 0.17 | 0.18 | 0.19 | 0.20 | 0.19 | 0.16 | **0.18** | 0.01 | 0.01 | **Dry** |
| Ca2+ | [%] | 0.12 | 0.15 | 0.15 | 0.14 | 0.13 | 0.15 | **0.14** | 0.11 | 0.13 | 0.14 | 0.14 | 0.13 | 0.13 | **0.13** | 0.01 | 0.01 | **Dry** |
| Fe2+ | [ppm] | 33.5 | 32.6 | 34.4 | 37.8 | 32.5 | 34.7 | **34.2** | 30.7 | 31.6 | 38.1 | 38.2 | 32.7 | 32.7 | **34.0** | 0.2 | 2.3 | **-** |
| Mn2+ | [ppm] | 15.0 | 20.9 | 18.3 | 19.5 | 15.1 | 16.2 | **17.5** | 14.8 | 19.2 | 17.9 | 20.2 | 16.9 | 13.5 | **17.1** | 0.4 | 1.6 | **-** |
| Cu2+ | [ppm] | 20.8 | 17.5 | 20.6 | 21.7 | 20.9 | 16.2 | **19.6** | 14.8 | 16.9 | 16.8 | 18.0 | 19.2 | 14.6 | **16.7** | 2.9 | 2.0 | **Dry** |
| Al3+ | [ppm] | 1.7 | 2.8 | 3.0 | 1.8 | 2.1 | 5.3 | **2.8** | 1.7 | 2.9 | 2.1 | 1.9 | 2.7 | 8.9 | **3.4** | -0.6 | 1.5 | **-** |
| pH | | 6.05 | 6.10 | 6.04 | 5.99 | 6.06 | 6.04 | **6.05** | 6.08 | 5.81 | 5.92 | 5.89 | 5.99 | 5.86 | **5.93** | 0.12 | 0.11 | **Dry** |
| Acidity from 6 to 8 | (meq/kg) | 85.6 | 87.6 | 95.3 | 97.0 | 86.1 | 91.9 | **90.6** | 93.4 | 94.3 | 97.6 | 96.1 | 89.9 | 93.3 | **94.10** | -3.5 | 3.3 | **Wet** |

**Table 5. Amino acids and mean differences of dry (D) and wet (W) processed *Robusta*. Statistical significance at 95%, the process resulting in higher level indicated in the column.**

| | | 42D | 358D | 409D | 961D | 203D | 121D | Average | 42W | 358W | 409W | 961W | 203W | 121W | Average | Dry-Wet | (SD) | Significant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Free** | **[%]** | 0.384 | 0.399 | 0.335 | 0.358 | 0.294 | 0.240 | **0.335** | 0.412 | 0.295 | 0.352 | 0.250 | 0.280 | 0.259 | **0.308** | 0.027 | 0.063 | **-** |
| NH4 | [%] | 0.000 | 0.013 | 0.000 | 0.000 | 0.000 | 0.000 | **0.002** | 0.007 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.001** | 0.001 | 0.006 | **-** |
| Ala | [%] | 0.044 | 0.031 | 0.030 | 0.041 | 0.032 | 0.030 | **0.035** | 0.056 | 0.038 | 0.037 | 0.033 | 0.044 | 0.033 | **0.040** | -0.005 | 0.008 | **-** |
| Gly | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| Leu | [%] | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.002** | 0.015 | 0.016 | 0.015 | 0.013 | 0.012 | 0.000 | **0.012** | -0.010 | 0.007 | **Wet** |
| Ile | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| Val | [%] | 0.014 | 0.014 | 0.011 | 0.013 | 0.012 | 0.000 | **0.011** | 0.016 | 0.012 | 0.012 | 0.000 | 0.000 | 0.000 | **0.007** | 0.004 | 0.007 | **-** |
| Pro | [%] | 0.015 | 0.030 | 0.022 | 0.030 | 0.019 | 0.000 | **0.019** | 0.024 | 0.000 | 0.016 | 0.000 | 0.015 | 0.000 | **0.009** | 0.010 | 0.016 | **-** |
| Trp | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| Tyr | [%] | 0.015 | 0.016 | 0.015 | 0.015 | 0.013 | 0.000 | **0.012** | 0.013 | 0.012 | 0.015 | 0.015 | 0.000 | 0.000 | **0.009** | 0.003 | 0.005 | **-** |
| Phe | [%] | 0.027 | 0.023 | 0.024 | 0.021 | 0.032 | 0.024 | **0.025** | 0.026 | 0.024 | 0.024 | 0.022 | 0.025 | 0.024 | **0.024** | 0.001 | 0.003 | **-** |
| Ser | [%] | 0.024 | 0.035 | 0.024 | 0.025 | 0.023 | 0.021 | **0.025** | 0.026 | 0.028 | 0.019 | 0.017 | 0.015 | 0.017 | **0.020** | 0.005 | 0.004 | **Dry** |
| Thr | [%] | 0.075 | 0.074 | 0.071 | 0.084 | 0.046 | 0.057 | **0.068** | 0.073 | 0.056 | 0.068 | 0.044 | 0.036 | 0.050 | **0.055** | 0.013 | 0.014 | **-** |
| Cys | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| Met | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| Asp | [%] | 0.044 | 0.049 | 0.036 | 0.034 | 0.038 | 0.035 | **0.039** | 0.036 | 0.028 | 0.021 | 0.017 | 0.025 | 0.021 | **0.025** | 0.014 | 0.004 | **Dry** |
| Glu | [%] | 0.082 | 0.072 | 0.069 | 0.066 | 0.061 | 0.059 | **0.068** | 0.075 | 0.038 | 0.085 | 0.057 | 0.079 | 0.102 | **0.073** | -0.005 | 0.027 | **-** |
| Arg | [%] | 0.015 | 0.028 | 0.022 | 0.030 | 0.017 | 0.014 | **0.021** | 0.026 | 0.025 | 0.025 | 0.019 | 0.016 | 0.012 | **0.020** | 0.000 | 0.007 | **-** |
| Lys | [%] | 0.015 | 0.013 | 0.011 | 0.000 | 0.000 | 0.000 | **0.007** | 0.019 | 0.016 | 0.016 | 0.013 | 0.014 | 0.000 | **0.013** | -0.006 | 0.006 | **Wet** |
| His | [%] | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | **0.000** | 0.000 | 0.000 | **-** |
| **Total** | **[%]** | 11.66 | 12.02 | 13.17 | 13.92 | 10.23 | 10.84 | **11.98** | 12.50 | 11.74 | 13.47 | 12.50 | 10.42 | 10.79 | **11.90** | 0.07 | 0.76 | **-** |
| NH4 | [%] | 0.32 | 0.33 | 0.39 | 0.40 | 0.29 | 0.32 | **0.34** | 0.34 | 0.34 | 0.40 | 0.36 | 0.30 | 0.32 | **0.34** | 0.00 | 0.02 | **-** |
| Ala | [%] | 0.45 | 0.45 | 0.50 | 0.69 | 0.41 | 0.42 | **0.49** | 0.50 | 0.46 | 0.52 | 0.48 | 0.43 | 0.42 | **0.47** | 0.02 | 0.09 | **-** |
| Gly | [%] | 0.63 | 0.63 | 0.69 | 0.72 | 0.58 | 0.60 | **0.64** | 0.67 | 0.65 | 0.70 | 0.69 | 0.57 | 0.60 | **0.65** | -0.01 | 0.02 | **-** |
| Leu | [%] | 0.89 | 0.92 | 1.04 | 1.09 | 0.77 | 0.85 | **0.93** | 0.98 | 0.93 | 1.09 | 1.01 | 0.81 | 0.87 | **0.95** | -0.02 | 0.06 | **-** |
| Ile | [%] | 0.44 | 0.45 | 0.50 | 0.51 | 0.40 | 0.40 | **0.45** | 0.46 | 0.44 | 0.51 | 0.46 | 0.38 | 0.40 | **0.44** | 0.01 | 0.03 | **-** |
| Val | [%] | 0.61 | 0.61 | 0.68 | 0.69 | 0.52 | 0.56 | **0.61** | 0.64 | 0.60 | 0.71 | 0.63 | 0.54 | 0.55 | **0.61** | 0.00 | 0.03 | **-** |
| Pro | [%] | 0.64 | 0.69 | 0.49 | 0.50 | 0.39 | 0.39 | **0.52** | 0.69 | 0.52 | 0.47 | 0.46 | 0.40 | 0.41 | **0.49** | 0.02 | 0.08 | **-** |
| Trp | [%] | 0.36 | 0.35 | 0.40 | 0.46 | 0.44 | 0.37 | **0.40** | 0.42 | 0.40 | 0.39 | 0.43 | 0.41 | 0.37 | **0.40** | -0.01 | 0.04 | **-** |
| Tyr | [%] | 0.40 | 0.43 | 0.46 | 0.50 | 0.39 | 0.42 | **0.43** | 0.41 | 0.41 | 0.46 | 0.47 | 0.38 | 0.40 | **0.42** | 0.01 | 0.02 | **-** |
| Phe | [%] | 0.60 | 0.63 | 0.71 | 0.77 | 0.55 | 0.60 | **0.64** | 0.66 | 0.64 | 0.74 | 0.69 | 0.56 | 0.59 | **0.64** | 0.00 | 0.05 | **-** |
| Ser | [%] | 0.50 | 0.51 | 0.57 | 0.59 | 0.45 | 0.47 | **0.51** | 0.53 | 0.50 | 0.56 | 0.54 | 0.46 | 0.45 | **0.51** | 0.01 | 0.03 | **-** |
| Thr | [%] | 0.43 | 0.44 | 0.49 | 0.50 | 0.38 | 0.40 | **0.44** | 0.45 | 0.43 | 0.48 | 0.45 | 0.40 | 0.38 | **0.43** | 0.01 | 0.02 | **-** |
| Cys | [%] | 0.29 | 0.28 | 0.33 | 0.32 | 0.27 | 0.26 | **0.29** | 0.28 | 0.27 | 0.33 | 0.30 | 0.26 | 0.26 | **0.28** | 0.01 | 0.00 | **-** |
| Met | [%] | 0.17 | 0.17 | 0.19 | 0.18 | 0.17 | 0.16 | **0.18** | 0.19 | 0.17 | 0.19 | 0.19 | 0.15 | 0.16 | **0.17** | 0.00 | 0.01 | **-** |
| Asp | [%] | 1.13 | 1.13 | 1.23 | 1.32 | 0.94 | 1.03 | **1.13** | 1.18 | 1.10 | 1.19 | 1.15 | 0.96 | 0.92 | **1.09** | 0.05 | 0.08 | **-** |
| Glu | [%] | 2.13 | 2.26 | 2.54 | 2.64 | 1.81 | 2.01 | **2.23** | 2.31 | 2.23 | 2.74 | 2.41 | 1.95 | 2.14 | **2.30** | -0.07 | 0.17 | **-** |
| Arg | [%] | 0.68 | 0.72 | 0.84 | 0.89 | 0.59 | 0.65 | **0.73** | 0.74 | 0.71 | 0.85 | 0.76 | 0.60 | 0.65 | **0.72** | 0.01 | 0.06 | **-** |
| Lys | [%] | 0.69 | 0.72 | 0.77 | 0.82 | 0.61 | 0.65 | **0.71** | 0.72 | 0.67 | 0.78 | 0.71 | 0.58 | 0.62 | **0.68** | 0.03 | 0.05 | **-** |
| His | [%] | 0.30 | 0.29 | 0.34 | 0.34 | 0.28 | 0.28 | **0.30** | 0.31 | 0.28 | 0.33 | 0.32 | 0.27 | 0.29 | **0.30** | 0.00 | 0.01 | **-** |

**Sensory Profile**

All *Robusta* clones were evaluated by sensory analysis. Rating is presented for all attributes in Table 6. Significant differences in the sensory perception were observed between dry and wet processed *Robusta* as shown in Figure 1a. As result:

- Dry processed *Robusta* were perceived as more rubbery, burnt, bitter and woody. They presented typical attributes of *Robusta* coffees.
- Wet processed *Robusta* were perceived as more acid, fruity, winey and green. Wet process significantly decreased the strong *Robusta* character and slightly enhanced sensory attributes typical of Arabica coffees.

**Discussion**

Globally, dry processed coffees were richer in metabolites from degradation mechanisms (e.g. free glucose and fructose resulting from polysaccharide hydrolysis, quinic acid from chlorogenic acid splitting, phosphoric acid from phospholipids). Degradation reactions could be due to the overall slower drying kinetics of cherry coffee (i.e. dry process) compared to parchment coffee (i.e. wet process). Degradation phenomena could also explain the lower chlorogenic acid level of dry processed coffees, which was already reported by Balyaya et al..

Another interesting chemical difference was the level in minerals (e.g. $K^{+}$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$) that was higher in dry processed coffees compared to wet process. Dry process could favour the transfer of minerals from the fresh parts to the bean core upon drying, whereas wet process could favour the removal of minerals during the washing step.

Finally, cell-wall polysaccharides (i.e. arabinogalactans) and storage compounds (i.e. mannans, lipids) were found in higher contents in wet processed coffees compared to dry processed coffees. No clear mobilisation of reserves to synthesize low-molecular weight substances could be observed when applying wet processed coffees as suggested by Bytof et al.. Additionally, two of the key metabolites involved in germination process (i.e. sucrose, free amino acids) were found in similar amounts for both coffee process.

Although these results are to some extent different from already published data, a clear change in the balance of key aroma precursors is nevertheless observed depending on post-harvest treatment. In the early stage of roasting three key chemical families enter the complex Maillard reaction pathway (1) free amino acids (2) free carbohydrates (3) chlorogenic acids. Different stoechiometric ratio of (1)/(2)/(3) are obtained for dry process i.e. 1/1/11 and wet process i.e. 1/0.6/13, which could explain the different aroma and flavour profiles established.

Figure 1b shows the map of the chemical and sensory data evaluated by discriminant analysis. It clearly demonstrates that cup quality depends on both genetic material (axis 1) and post harvest treatment (axis 2). Post harvest treatment is differentiating typical *Robusta* characters (i.e. burnt, rubbery, bitter, woody. astringent) from more typical *Arabica* characters (i.e. fruity, acid). In the present study, the key chemical parameter differentiating dry from wet processed coffees is the free carbohydrate level.

In conclusion, post-harvest process has a clear impact on the low molecular weight components present in green coffee. Dry process appears to favour degradation mechanisms responsible for a change in the balance of the key aroma precursors. Wet process could favour the removal of precursors, which contribute to the strong *Robusta* character. However, from the present data wet process does not seem to initiate strong physiological changes.

**Table 6. Sensory profile and mean differences of dry (D) and wet (W) processed *Robusta*.**

| | 42D | 358D | 409D | 961D | 203D | 121D | Average | 42W | 358W | 409W | 961W | 203W | 121W | Average | Dry-Wet | (SD) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| citrus | 0.33 | 0.59 | 0.68 | 0.80 | 0.80 | 0.30 | **0.58** | 0.11 | 0.64 | 0.07 | 0.83 | 0.37 | nd | **0.40** | 0.24 | 0.29 |
| fruity | 1.46 | 1.50 | 1.38 | 1.63 | 1.68 | 0.96 | **1.43** | 1.05 | 1.87 | 1.55 | 2.11 | 1.98 | nd | **1.71** | -0.18 | 0.35 |
| winey | 0.29 | 0.20 | 0.19 | 1.36 | 0.91 | 0.41 | **0.56** | 1.24 | 3.15 | 2.42 | 3.46 | 2.64 | nd | **2.58** | -1.99 | 0.73 |
| green | 1.90 | 1.51 | 1.86 | 1.90 | 1.38 | 2.08 | **1.77** | 2.47 | 1.19 | 1.88 | 2.18 | 1.25 | nd | **1.79** | -0.08 | 0.35 |
| hay | 0.35 | 0.50 | 0.76 | 0.78 | 0.41 | 1.21 | **0.67** | 0.86 | 0.67 | 0.49 | 0.26 | 0.72 | nd | **0.60** | -0.04 | 0.43 |
| caramel | 1.99 | 2.01 | 1.68 | 1.25 | 2.26 | 1.91 | **1.85** | 1.73 | 1.42 | 1.53 | 1.60 | 1.83 | nd | **1.62** | 0.22 | 0.36 |
| rubbery | 1.09 | 1.53 | 2.38 | 2.37 | 1.51 | 2.03 | **1.82** | 1.21 | 0.71 | 0.59 | 0.44 | 0.29 | nd | **0.64** | 1.13 | 0.83 |
| woody | 1.31 | 1.40 | 2.00 | 1.38 | 1.60 | 1.62 | **1.55** | 1.79 | 1.06 | 1.21 | 0.82 | 0.96 | nd | **1.17** | 0.37 | 0.50 |
| burnt | 1.70 | 2.18 | 2.08 | 2.37 | 1.90 | 2.17 | **2.06** | 1.64 | 1.18 | 1.22 | 0.79 | 1.00 | nd | **1.17** | 0.88 | 0.54 |
| acid | 2.33 | 2.15 | 1.96 | 2.16 | 2.62 | 2.08 | **2.22** | 2.22 | 2.93 | 1.61 | 2.35 | 3.03 | nd | **2.43** | -0.18 | 0.44 |
| bitter | 3.98 | 4.11 | 3.98 | 5.01 | 2.95 | 4.99 | **4.17** | 3.77 | 2.81 | 3.66 | 3.35 | 2.72 | nd | **3.26** | 0.74 | 0.68 |
| astringent | 2.51 | 2.07 | 2.12 | 2.94 | 2.22 | 2.57 | **2.41** | 2.75 | 1.98 | 1.60 | 2.84 | 2.01 | nd | **2.23** | 0.14 | 0.27 |
| body | 3.38 | 4.05 | 3.39 | 3.62 | 3.70 | 3.41 | **3.59** | 3.75 | 3.12 | 3.93 | 3.96 | 3.39 | nd | **3.63** | 0.00 | 0.61 |

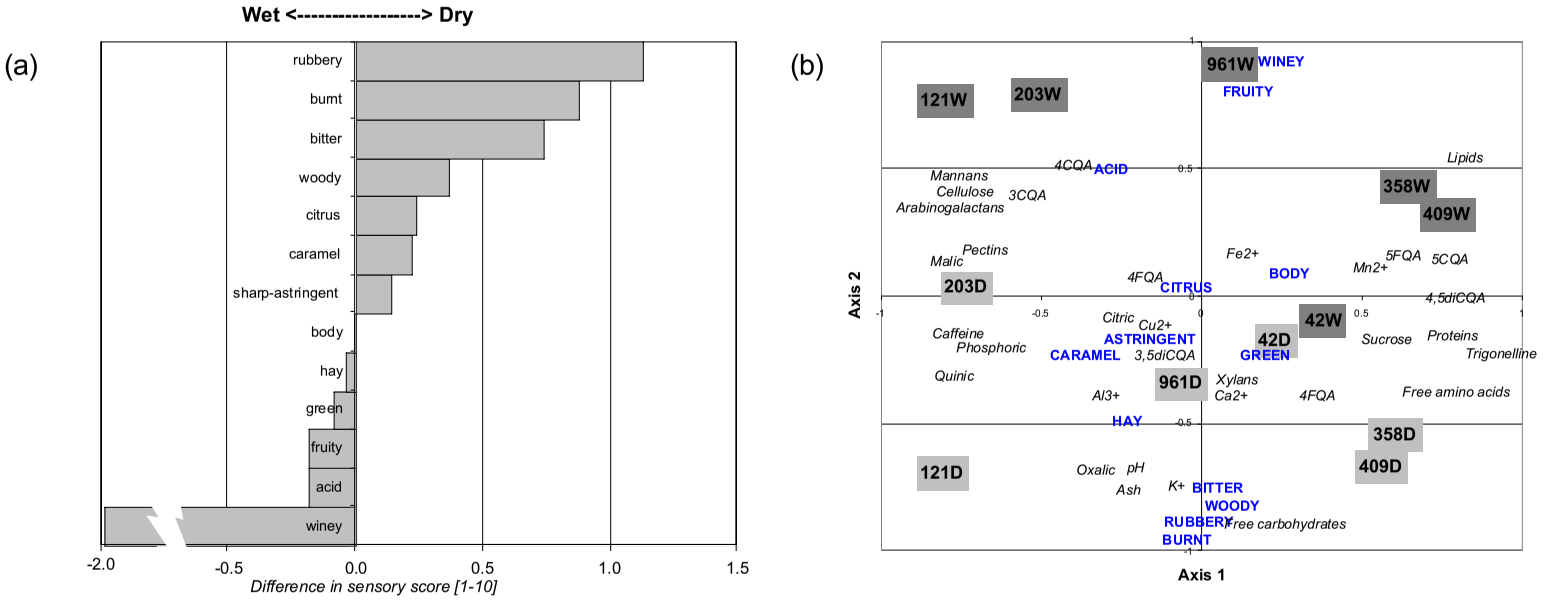


**Figure 1. Sensory characterisation of dry and wet processed *Robusta* (a) Average difference of sensory attributes (b) discriminant analysis of chemical and sensory data.**

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# Green Coffee Chemistry and Its Possible Application for Group Discrimination and Correlation with Cupping Quality

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## SUMMARY

In a preliminary study, forty-three samples of *Coffea arabica* were harvested over two years. Samples from several locations in Hawaii and of several different cultivars were chosen. In addition, two drying methods (forced-air and sun) were examined. The green coffee samples were analyzed for organic acids, simple sugars and chlorogenic acids. A novel HPLC method was developed for simultaneous determination of simple sugars and organic acids. Using this method, typical recoveries of individual acids and sugars used to fortify green coffee samples were: malic acid 100.7%, quinic acid 86.7%, citric acid 112.8%, lactic acid 100.0%, sucrose 93.6%, fructose 109.3%, glucose 110.2%. Chlorogenic acids were determined using HPLC, based upon previously described methods with some modifications. All samples were cupped by a trained panel. The study attempted to discover a correlation between green coffee chemistry and cupping characteristics and the possibility of using the chemistry for sample discrimination. The data were analyzed using multivariate statistical techniques. With the data from the compounds analyzed, no correlation between green coffee chemistry and cupping quality could be elucidated. However, principal component analysis (PCA) showed a separation of coffee samples by drying method. The principal separating factors were lactic acid content and acidity in the cup. Discriminate analysis (DA) supported this result with classification rates greater than 85%. The PCA also showed some groupings of samples by location and cultivar. These results were also confirmed with DA. The corresponding DA suggests that use of these chemical and/or cupping characteristics may be able to accurately discriminate arabica coffee by varieties and locations. Limitations of this study included a low sample: variable ratio and too few samples. Further studies will be undertaken to expand sample size and and the number of components analyzed.

## INTRODUCTION

The sensory aspects of coffee quality, also called cup quality, are determined by a panel of trained individuals. These individuals sample an infusion made from roasted coffee and rate it accordingly. Ratings typically include aspects such as acidity, flavor, aroma and body, all of which are determined by the chemical composition of the coffee, the processing methods, the degree of roast and method of brewing.

While this method of quality measurement has been the industry standard, it is a semi-objective measure. A quantitative method of quality determination would save time and resources, eliminate human subjectivity and offer the means to identify components and their concentrations in green bean, which could be manipulated to influence quality. Green coffee is ideal for such investigations as it eliminates some factors that impact quality. There are up to 850 different compounds in roasted coffee compared with approximately 300 in green coffee (Flament, 2002). Many of these compounds are the result of chemical conversion of precursor compounds found in green bean (Clifford et al., 1985).

Green coffee is known to contain many unique compounds (Clarke and Macrae, 1985), some of which have already been implicated in the taste characteristics of brewed coffee. For instance, research by Ohiokpehai et al. (1982) suggests that the ratio of the monochlorogenic acids/dichlorogenic acids influences coffee quality. In addition, Rivera (Rivera, 1999) has reported that simple organic acids have discrete, concentration dependent sensory effects. A number of authors have demonstrated that roasting sucrose, either under acidic conditions or in the presence of chlorogenic acids, results in the production of simple organic acids (Crean, 1996; Lukesch, 1956; Nakabayashi, 1978). Nakabayashi (1978) further noted that the resulting organic acids were identical to those found in roasted coffee infusions. The involvement of reducing sugars in Maillard reactions and the consequent formation of highly volatile aromatic compounds suggests an additional role for sugars in coffee quality (Flament, 2002). The contribution of acids to pH of the coffee brew will also affect sensorial properties due to the dissociation of various organic acid salts and the liberation of highly volatile free acids (Balzer, 2001).

In Hawaii there are a number of different commercially grown cultivars of arabica coffee. These include, Guatemalan typica, Red catuai, Yellow catuai, and Mokka. In addition there are a wide variety of environments in which coffee is grown, ranging from near sea level to greater than 700 meters above sea level. Some coffees within Hawaii command a relatively high price compared with other coffees in the region. It is therefore desirable to develop chemical methods for objectively discriminating between these coffees and other coffees around the world. This will not only aid in verification, but will also provide useful data for coffee breeding programs.

## MATERIALS

Organic acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sugars were purchased from Mallinckrodt, Inc. (Pons, KY, USA) All solvents were HPLC grade and were obtained from Burdick and Jackson (Muskegon, MI, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ripe *Arabica* coffee cherries were harvested by hand over two consecutive harvests from State variety trial plantings undertaken in 1988. These trials were replicated on Hawaii, Maui, Oahu and Kauai. Replicate samples were therefore harvested on all of these islands. Varieties harvested across all replicates included typica, red catuai, and yellow caturra. In addition, other varieties were harvested from the Hawaii Agriculture Research Center's coffee breeding program located in Kunia, Oahu. These varieties included catimor, mokka, mokka hybrids, and various other typica cultivars.

## SAMPLE PREPARATION AND ANALYSIS

Immediately following harvest, cherries were pulped and the mucilage removed by soaking in water overnight at room temperature. In the first year the parchment coffee was dried at 52°C in a blower oven to a final moisture content of approximately 12% of dry weight, hulled and subsequently stored at -20°C prior to analysis. In the second year, pulped coffee was dried by placing on drying screens and sun drying for up to 14 days to a final 12% moisture content. Samples were then hulled and frozen at –20°C prior to analysis. Immediately prior to analysis and extraction, the green coffee was lyophilized to complete dryness and then ground to a fine powder using an IKA Universalmühle M20 blade grinder (IKA-Labortechnik, West Germany). Larger particles were removed by passage through a 0.6 mm screen.

For organic acid and sugar extraction and analysis, one gram of round green coffee was placed in a 125 ml glass Erhlenmyer flask and sonicated for 15 mins. with 10 ml of 8 mM sulfuric acid containing 50% (v/v) methanol. All samples were then filtered through a

Whatman GF/A glass fiber filter and 5mls of filtrate was evaporated to a volume of less than 2.5 mls using a rotary evaporator (Tokyo Rikakikai Co., LTD, Japan). Distilled water was then added to a final volume of 5mls. Two mls of the extract were passed through a 1ml bed volume Sep-Pak® C18 SPE cartridge (Waters), preconditioned with 1ml of MeOH and 5ml water. Residual unbound extract was eluted by addition of 1ml of distilled water. The eluants were then filtered through a .45 micron nylon filter (Millipore), prior to high performance liquid chromatography (HPLC).

HPLC analysis of sugars and organic acids was undertaken using a Shimadzu SCL-10A controller and LC-10AS pump (Shimadzu Corporation, Japan). Chromatographic separations were achieved using a Rezex ROA Organic acid ion exchange column (300 x 7.5 mm) with a Carbo-H+ guard column (4mm x 3mm), (Phenomenex USA, Torrance, CA). The mobile phase consisted of 6.5 mM sulfuric acid in distilled water at a flow rate of 0.4 ml/min. Twenty ul of sample was injected. The run time was 24 minutes followed by a column wash step, using a mobile phase of distilled water at a flow rate of 0.52ml/min.for 10 mins. The column was re-equilibriated using the original analytical mobile phase at a flow rate of 0.52 mls/min for a further twenty-seven minutes. Organic acids were detected using a Shimadzu SPD-10A UV-VIS detector, measuring absorbance at 220 nm (0 .1 AUF). Sugars were detected from the same chromatography run using a Waters 410 differential refractometer (Waters Associates, Inc, USA), connected in series. Plots and peak integrations were performed using a Shimadzu CR501 chromatopac integrator. Extraction efficiency (data not shown) was determined by fortifying 1 gram of dried, ground sample with known amounts of organic acids, sucrose, fructose, and glucose, and extracting and analyzing as described above. Negative controls, consisting of sample without fortification were also extracted and analyzed. Quantitation was based upon a linear regression equation derived from a standard curve constructed from known concentrations of each analyte. Appropriate standards were chromatographed with each sample set to be analyzed.

Chlorogenic acids were extracted based on method described by Balyaya and Clifford, 1995 (Balyaya and Clifford, 1995) with some modifications. Briefly, 0.5 grams of ground green coffee was refluxed with 100 mls of 70% methanol, 30% water (v/v) for one hour. After cooling, samples were filtered and the filtrate clarified with Carrez reagent. The clarified supernatant was filtered through a 0.45 um nylon filter and twenty microliters were injected an HPLC system (described above) with a YMC Basic S-5 column (4.6 x 250 mm, Waters Corp., Millford, MA, USA). A binary gradient of 0.06% TFA in water to 45% acetonitrile in 0.06% TFA in water over 40 minutes at a flow rate of 1 ml/min was used to elute the acids. The acids were detected by absorption at 315 nm. Seven chlorogenic acids were identified from their relative retention times and molar absorbencies (Balyaya and Clifford, 1995; Clifford, 1985). Quantification was based upon a linear regression equation derived from a standard curve of known concentrations of 5-CQA.

Cupping was undertaken at the University of Hawaii at Manoa. Coffee was roasted the day before the cupping. Seventy grams of green bean was roasted in a Probat PRE-1 lab roaster at 200∞C to an L level of approximately 19 on the LAB scale. The coffee was ground to an "express" size. Each sample contained 7.25g coffee and 150 ml of water at a temperature just below boiling. Coffees were cupped by 7 panelists, in triplicate, in individual tasting booths. Coffees were grouped by variety for the tastings.

Coffees were evaluated on five pre-determined attributes. All ratings were on a 0 (not present) - 5 (intense) scale. Prior to mixing the water and coffee, dry coffee aroma was evaluated. Two minutes after brewing began, wet coffee aroma was assessed. Five minutes after the addition of water, acidity, flavor and body were evaluated. Panelists were allowed to enter terms for defects and other descriptors and rate them accordingly.

Data was statistically analyzed using a standard statistical analysis package (SAS). Principal component analysis (PCA) and discriminate analysis (DA) were performed on all data variables derived from the chemistry and cupping data. PCA biplots and DA classifications were generated and assessed.

## RESULTS

A total of 43 samples were analyzed by means of chemical profiling and cupping. The simultaneous HPLC method developed here for the determination of simple organic acids and sugars showed that adequate recoveries could be obtained for all of the analytes. Table 1 shows the recovery data obtained for each analyte.

**Table 1.**

| Compound | Average % Recovery (n=3) | S.D+/- (%) | Retention time (min) |
|---|---|---|---|
| Malic acid | 100.7 | 8.1 | 15.7 |
| Quinic acid | 86.7 | 3.1 | 16.2 |
| Citric acid | 112.8 | 5.3 | 13.5 |
| Lactic acid | 100 | 10.6 | 19.2 |
| Sucrose | 93.6 | 7.2 | 12.9 |
| Glucose | 110.2 | 6.2 | 15.3 |
| Fructose | 109.3 | 4.6 | 16.1 |

It can be seen from the table above that recoveries of individual analytes from fortified samples were at or near 100%, suggesting that the extraction procedures used were adequate for accurate quantitation of compounds in green coffee samples.

Typical HPLC profiles of sugars and organic acids using the newly developed methodology are shown in Figures 1 and 2. The HPLC profiles from both detection systems revealed easily separable and identifiable compounds. Nearly linear standard curves for both groups of compounds were obtained and are shown in Figures 3 and 4.

Chlorogenic acids analysis also showed that recoveries of the seven identified isomers all exceeded 80% (data not shown).

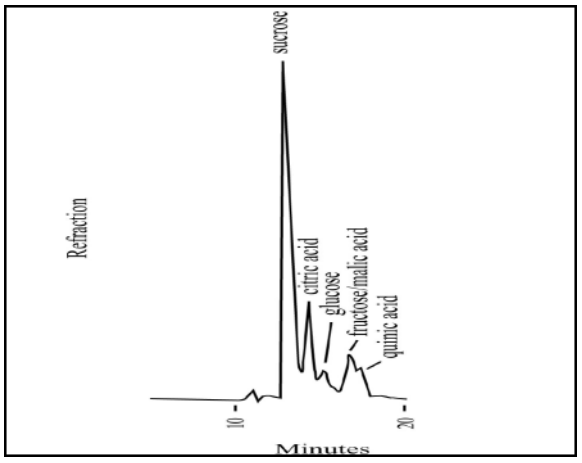


**Figure 1. Sugars and some Organic acids. RI.**

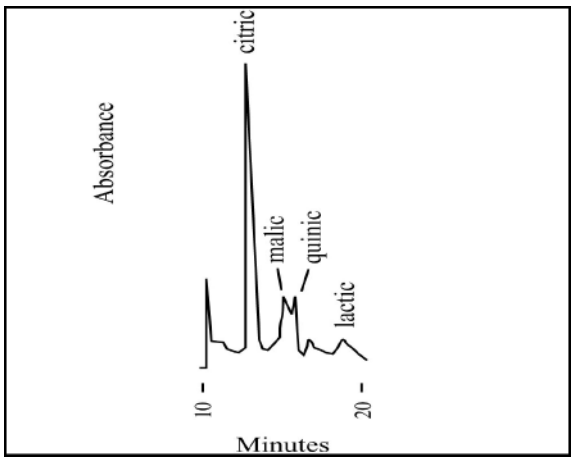


**Figure 2. Organic Acids. UV**

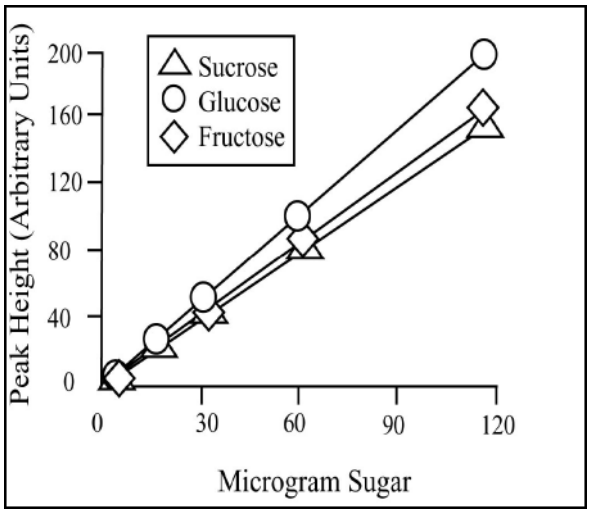


**Figure 3. Std. Curve Sugars. $R^2 > 0.99$.**

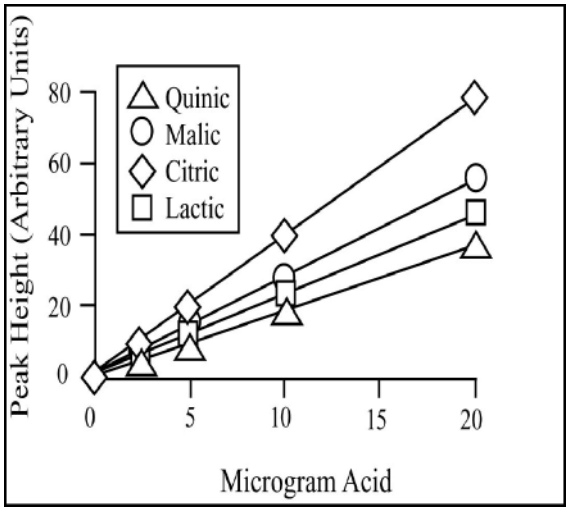


**Figure 4. Std. Curve Organic Acids R2 > 0.99.**

PCA biplots were constructed from chemistry and cupping data (cupping data not shown). The PCA biplots for selected cupping determinants are shown in Figures 5 and 6 below:

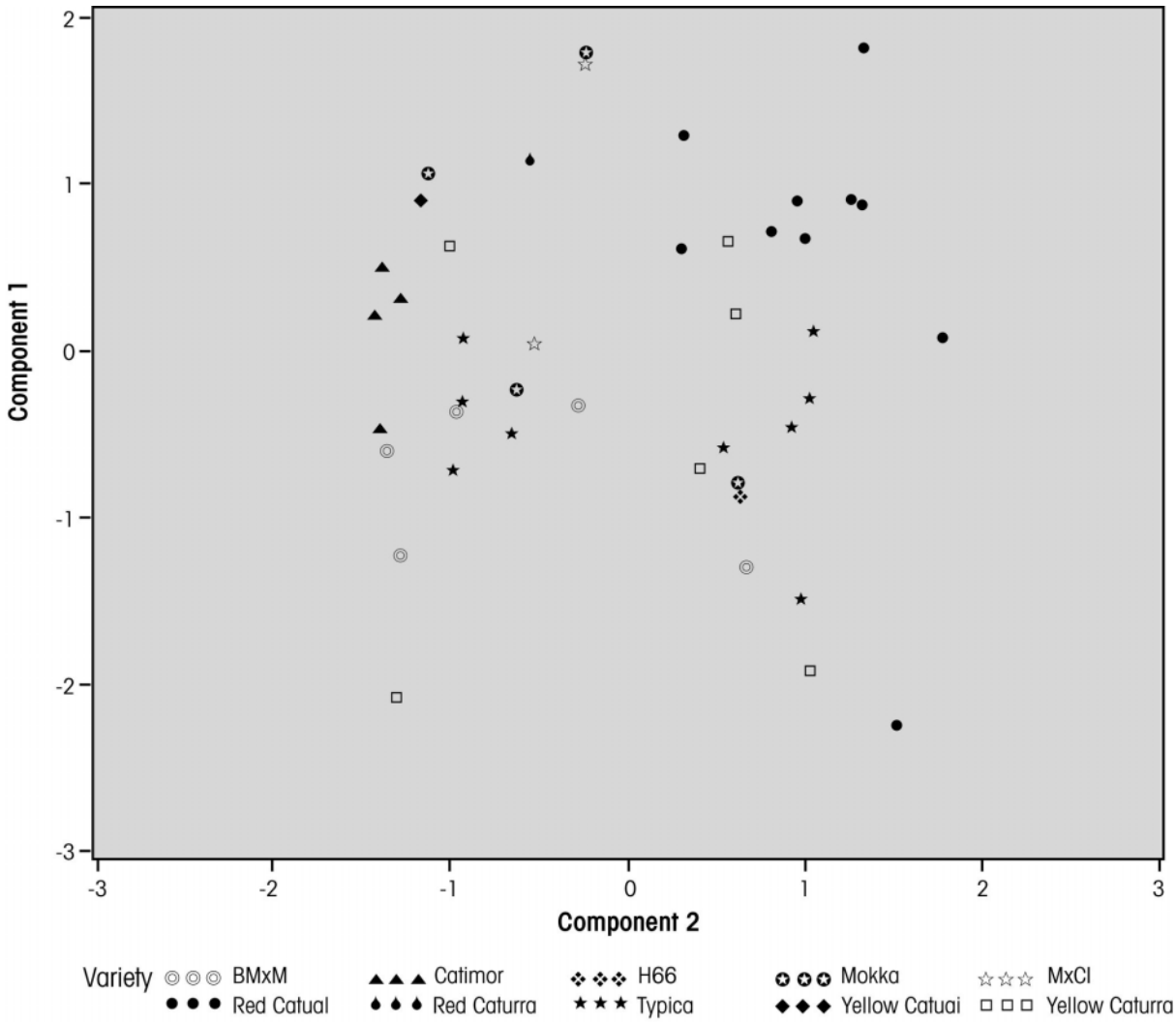


**Figure 5. PCA Biplot of grouped cupping characteristics, identifying data points by variety**

Component 1 represents cupping characteristics related to flavor, dry aroma, and wet aroma. Component 2 represents cupping characteristics related to acidity and body. Individual data points are identified in terms of variety. Distinct data clouds can be seen for Catimor (▲), red catuai (●), and typica (□). This suggests that cupping alone is useful for distinguishing between varieties. In addition to cupping however, chemistry was also found to be useful in distinguishing varieties. For example, chlorogenic acid concentrations alone allowed a distinction between mokka, mokka hybrids and yellow caturra. Malic, citric, and total simple organic acid concentrations allowed distinction of catimor and Blue mountain/mokka hybrids. 3,5-di-CQA concentration and the molar ratio of mono- and di-CQAs facilitated distinction of mokka from other varieties.

Using cupping characteristics as individual components in PCA and analyzing all samples, a striking biplot was generated when individual data points were identified in terms of drying procedure. This is shown in Figure 6 overleaf. Component 1 represents flavor, dry aroma, and wet aroma. Component 2 represents acidity and body. Two distinct data clouds can be seen, corresponding to sun dried samples (□) and oven-dried samples (□). It is interesting to note that a nearly complete split was seen between the drying methods, with the exception of one group, which were determined to be all red catuai samples. The reason for this is not known.

In addition to the data shown in Figure 6, PCA aided in distinguishing other features. For example the Kona location of origin could be determined using chlorogenic acids and 3,5-di-CQA concentrations, and molar ratio of mono- to di-CQAs.

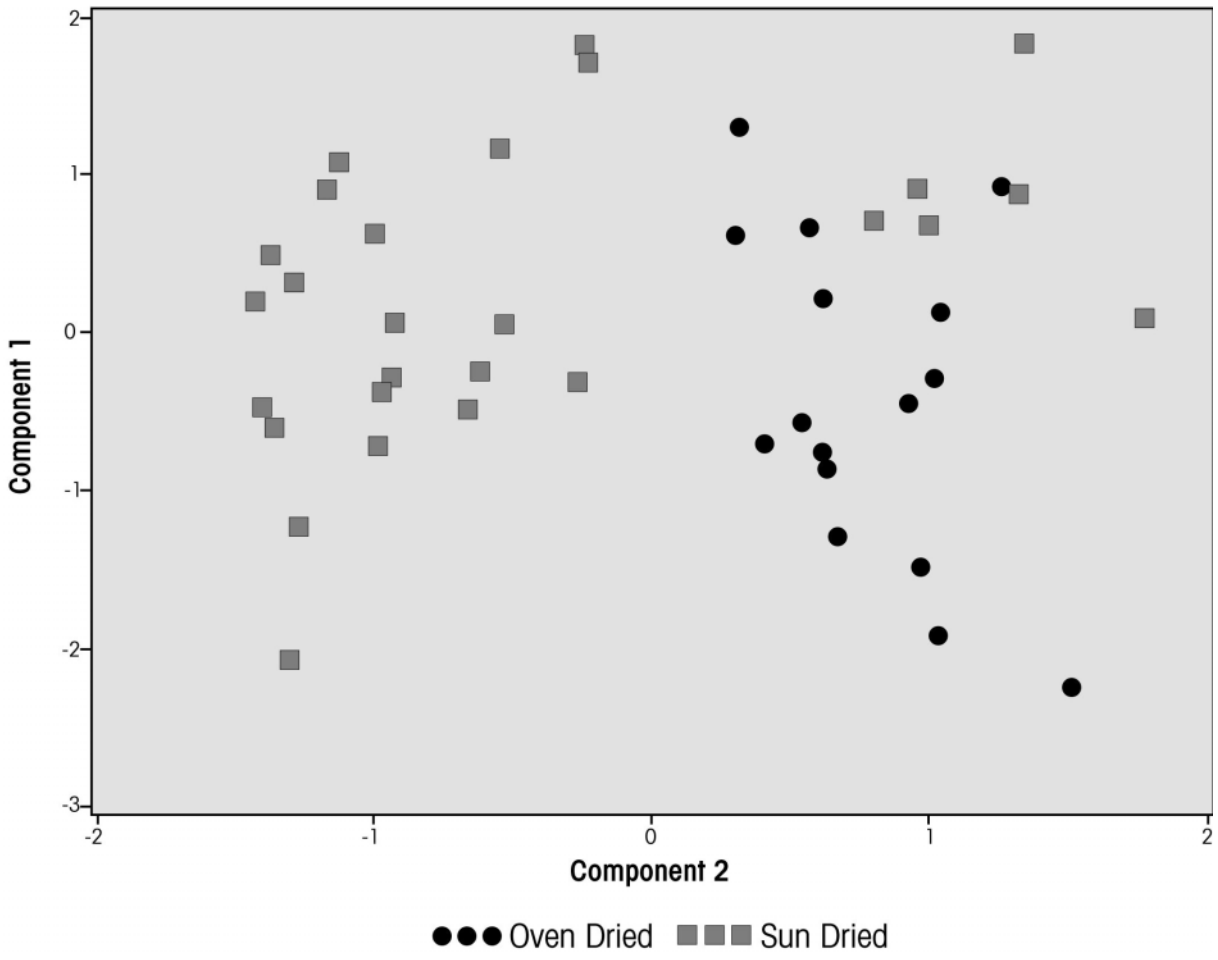


**Figure 6. PCA Biplot of grouped cupping characteristics, identifying data points by drying process.**

**Table 2. Discrimination Between Sun Dried and Oven Dried Samples.**

| Classification Variable | % Correct Classification | % Correct Classification by Jackknife Procedure |
|---|---|---|
| Lactic | 92.5 | 86 |
| Lactic, 3-CQA | 94.5 | 94.5 |
| Dry/wet aromas, acidity, flavor, body | 100 | 97 |
| Acidity | 85 | 85 |
| body | 98 | 95 |

Discriminatory analysis was used to see whether individual group membership could be correctly determined, given the statistical associations determined using PCA. Table 2 shows that there were a number of chemical and cupping parameters that could be used to predict whether samples had been sun dried or oven dried. When one of the samples is removed from the initial classification and then re-inserted after, in a statistical manipulation known as a jackknife procedure, the robustness of the model can be tested. It can be seen that in all cases,

the model is valid, with high percentage classification retained even after this procedure. When samples were split, based on drying procedure and subjected again to discriminatory analysis to determine the location of origin, the results showed that while initial classification of samples based upon the variables studied showed a high percentage of initial classifications, this was not the case after undertaking the jackknife procedure, with one exception. The statistical model using quinic acid, 3,4 di-CQA, 3,5 di-CQA, 5-FQA and the mono-/di- CQA molar ratios allowed for a 100% initial classification and a 94% classification after the jackknife procedure, only when the samples had been oven dried.

Similarly, when samples were split, based on drying procedure and subjected again to discriminatory analysis to determine the variety, sucrose and total chlorogenic acid concentration combined were successful in the initial classification of samples (85%) and again when the jackknife procedure was employed (85%). It is very interesting to note that in no cases could chemistry be used to predict cup rating and vice versa.

## CONCLUSIONS

The aim of this study was to determine if the chemical composition of green coffee could be used to define different varieties of coffee grown in the same location and the same varieties of coffee grown in different locations. The method that was developed to simultaneously measure sugar and organic acid concentrations appeared to be valid, with high recovery and easy identification of individual analytes. Various methods have been described for the detection of sugars and organic acids in green coffee (Rogers et al., 1999; Silwar and Lüllmann, 1988; Van der Stegen and Duijn, 1987), however, no reported methods exist for the rapid detection of both chemical groups simultaneously. PCA analysis showed that cupping and chemistry could be used to some degree to differentiate between varieties and locations, but certainly not all. In contrast, drying procedure could easily be identified based upon cupping or chemistry alone. This will be important in determining drying procedures in commercial coffees, particularly high value specialty coffees.

It was not possible in this study to predict individual cupping discriminator rating or overall cup quality from the chemistry. This may be because the wrong chemical compounds were picked that would facilitate this. However, it seems more likely that this may be because there were too few samples for the statistical analysis. Ideally, PCA requires a much higher sample to variable ratio to give statistically meaningful results. With a much higher sample number and geographically wider locations of origin it may become possible to predict the sensorial properties of brewed coffee by quantitation of chlorogenic acids, organic acids, and sugars in green bean. The elucidation of such a hypothesis has far-reaching implications for the coffee industry. A greater understanding of the importance of flavor precursors in green coffee might create possibilities for flavor manipulation. The ability to quickly and easily determine green coffee chemical profiles suggests research avenues in coffee breeding and genetic transformation that would permit rapid development of varieties with improved cup quality.

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# Biochemical Insights into Coffee Processing: Quality and Nature of Green Coffees are Interconnected with an Active Seed Metabolism

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## SUMMARY

Aroma potential and thereby coffee qualities are strongly influenced by the mode of processing. In the past, we have postulated that these processing-related quality variations are due to specific differences in biochemical reactions occurring within the viable coffee beans during processing. In this paper, unequivocal evidence is presented that various biochemical processes occur within the coffee seeds and that the coffee seeds are able to respond to changes in their environment. Also, evidence is presented that germination is initiated and proceeds during coffee processing. The germination-related metabolism was monitored by both the expression of germination-specific enzymes (i.e. the isocitrate lyase) and the resumption of cell cycle activity / cell division (estimated by ß-tubulin accumulation). The extent and time course of these germination processes depend on the mode of processing. Apparently, the removal of the pulp provides the stimulus for activating the preliminary, hardly perceptible steps of the germination process. Surprisingly, also in dry process seeds, germination-related metabolism occurs, probably due to endogenous triggering. However, here the germination processes reveal a time course different from that of wet process coffees.

Apart from germination, other metabolic processes have been found to occur in coffee beans. The most significant phenomenon is the accumulation of γ-aminobutyric acid (GABA), a typical response to drought stress. However, GABA is exclusively accumulated in dry process, and not in wet process, green coffees. The reason for these differences is the different time frame for the stress-related metabolism. In dry processed beans the stress-related synthesis of GABA is likely to take place over several days, leading to a large accumulation of this non-protein amino acid. In contrast, the induced stress metabolism in wet process beans is terminated before there is a significant accumulation of GABA, as the drying procedure is much faster in wet than in dry process beans. Only in the case of a prolonged drying period, wet process coffees also reveal high levels of GABA.

The data presented in this paper demonstrate the high relevance of the fact that green coffee seeds represent vital and living organisms, in which various metabolic reactions occur during processing, e.g. a germination-related metabolism and a stress metabolism. The extent of these processes depends on the conditions during processing and thereby influences coffee quality. Consequently, specific alterations of these conditions should influence these metabolic processes and affect coffee quality.

Moreover, the new insights can be used to explain potential quality differences of coffees resulting from classical wet and new progressive processing methods with mechanical mucilage removal, e.g. the aquapulping-methods. We deliberately introduced modifications of these progressive methods by adding intermediate storage phases in which the metabolic processes associated with the fermentation stage in wet processing could proceed. Sensory analyses suggested that the introduced modifications led to perceptible changes in the flavour profile.

## INTRODUCTION

The question why wet process coffees reveal cup qualities different from those of dry process coffees may be as old as the introduction of the wet processing itself. However, a conclusive answer is still lacking. Wet process coffees are characterized by pronounced and distinctive aromas and a fine and pleasant 'acidity'. In contrast, dry processed coffees are characterized by their fuller body. In the past, these differences had been attributed exclusively to the facts that wet processing required much more accuracy and diligence, and that only fully ripe cherries were used. In order to monitor the intrinsic effects of processing, standard processings with identical starting material have to be performed in order to exclude any effect other than the mode of processing.

From plant physiological considerations it is obvious that more complex biological aspects should also be involved in this characteristic differentiation. In this context we have to consider that coffee beans – even after processing – are viable seeds, which is evident in the ability of fresh green seeds to germinate and grow. This means that processed coffee beans are able to respond metabolically to changes in their environment. Hence, we have postulated that internal metabolic factors – in addition to external factors – should be responsible for the observed differences in wet and dry process green coffees (Bytof et al., 2000; Selmar et al., 2002).

To elucidate the metabolic differences between wet and dry process coffees, we have performed numerous processing trials under carefully controlled conditions. In order to exclude any other effect than the mode of processing, it was necessary that absolutely identical starting material was used for the experimental processings.

## EXPERIMENTAL

### Model processings

Most of the experimental processings were performed in Brazil, Tanzania, Mexico and Colombia. In all cases – due to a very careful manual sorting – only fully ripe red cherries (*Coffea arabica* L.) were used as "identical starting material" for both, dry and wet processing trials in parallel. The resulting green coffees were shipped by air to the Institute for Plant Biology, TU Braunschweig and were analyzed both sensorially and biochemically. In addition, experimental laboratory processing trials were conducted. In all cases, fresh and sound red coffee cherries were used for both types of processing.

### Sensoric assessment

The sensoric assessments were performed by professional sensory boards of the industrial partners, mainly from Tchibo and Kraft Foods.

**Quantification of free amino acids**

Coffee beans were 'shock' frozen and ground in liquid nitrogen. Amino acids were extracted with sulphosalicylic acid (4% w/v). After derivatization with *o*-phthaldialdehyde / 2-mercapthoethanol (OPA/MCE), they were separated by HPLC on a C18 column and detected fluorometrically ($\lambda_{ex}$ = 334 nm; $\lambda_{em}$ = 425 nm). For quantification, norvaline was added as internal standard.

**Quantification of soluble sugars**

The composition of low molecular carbohydrates was analyzed by a HPAEC system on a DIONEX® PA20 column with a sodium hydroxide gradient. For detection, a pulsed amperometric detector (PAD) was used.

**Expression studies of isocitrate lyase using RT-PCR**

Based on the alignments of known isocitrate lyase (ICL) sequences of various plants, redundant primers were created to generate a homologous probe for the isocitrate lyase of *Coffea arabica*. A corresponding 480 bp-fragment was cloned into a bacterial vector (TOPO TA). The modified plasmid was transformed into *E. coli* (Strain DH 5α), amplified and used for both, as probe for the detection of ICL-messengers in Northern blots and for sequencing. Based on the elucidated sequence, specific isocitrate lyase primers were created, and RT-PCR analysis was performed using RNA isolated from fresh and differently processed coffee seeds (for details see Selmar et al., 2004).

**ß-tubulin as marker for the resumption of cell cycle activity and cell division**

Isolated coffee embryos were homogenized with Modil-buffer (pH 6.8) containing a protease-inhibitor cocktail according to De Castro et al. (1998). The protein extracts were separated by SDS-gel electrophoresis. After blotting on a nitrocellulose membrane, the proteins were detected immunochemically using specific ß-tubulin antibodies (TUB 2.1, Sigma) in a dilution of $1{:}10^{-6}$. Secondary antibody was an anti mouse IgG, conjugated with alkaline phosphatase. Detection was performed using BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitroblue tetrazolium).

**RESULTS AND DISCUSSION**

**Sensorial differences of wet and dry process coffees**

The sensorial assessments confirmed the corresponding results which have already been published in part (Selmar et al., 2002): the known differences in the cup quality of dry and wet process coffees are also evident when identical starting material is used for both types of processing. Thus, we have proved unequivocally that these quality differences are – at least partly – intrinsic to the mode of processing itself. In other words: there are substantial differences in the differentially processed coffee beans, which are principally generated in the course of postharvest treatment. The question arises as to whether these differences could be correlated with definite differences in the metabolic status of the differentially processed coffee seeds.

**Chemical differences of wet and dry process coffees**

In order to determine such metabolic differences, we chose the composition of free amino acids and the content of soluble carbohydrates as related markers. Both represent important precursors for coffee aroma compounds. Extensive and detailed data on the composition of free amino acids have been published by Bytof et al., (2004) and Selmar et al. (2002). In this paper, just the overall results are summarized: in all model and experimental processing trials with identical starting material, the total concentration of free protein amino acids in wet processed seeds is markedly higher than in dry processed seeds (Table 1).

**Table 1. Total content of free protein amino acids in dry and wet processed green coffees.**

| model- and experimental green coffee production by traditional wet and dry processing in parallel | free protein amino acids [ mg / kg f.w. ] | | |
|---|---|---|---|
| | dry processing | wet processing | difference (%) |
| plantation (Brazil, A) | 3,570 | 4,310 | 20,9 |
| plantation (Brazil, B) | 4,030 | 4,360 | 8,2 |
| plantation (Colombia) | 2,760 | 2,850 | 3,3 |
| laboratory (Brazil - Germany) | 5,050 | 5,400 | 6,9 |
| laboratory (Peru - Germany) | 3,710 | 3,940 | 6,2 |
| laboratory ( Kenia - Germany) | 3,250 | 3,570 | 9,8 |

The variations between single trials, even between processed samples from the same plantation but using different batches, were extremely high. These variations offer an explanation for the failure to detect differences in former studies. It is absolutely necessary to use the same plant material for both processing trials. Only an arduous manual sorting, which allows the use of completely identical plant material for both processings, enables the detection of quantitative differences of substantial factors, which *per se* are highly variable. Moreover, it is also necessary that for laboratory trials realistic processing conditions are applied. Unfortunately, in the work of Arnold and Ludwig (1996) the conditions chosen for the dry process did not match the real dry method but rather resembled the “semi-washed” process.

The major sugar present in coffee beans is sucrose, where, between samples, the variation in its content is nearly as high as that of the amino acids. We detected distinctive and reliable differences in the contents of other sugars that correlated with the mode of processing applied. In Figure 1, the glucose and fructose contents in wet and dry process coffees are compared. The contents of glucose and fructose are about five to ten times higher in dry processed than in wet processed beans.

The comparison of the glucose and fructose levels in fresh and processed coffee seeds reveals that fresh and dry processed beans have nearly the same content. In contrast, in wet processed coffees the corresponding levels are low, indicating that during wet processing the content of these sugars decreases. We suggest that this loss originates from an enhanced glucose turnover due to anaerobic fermentation in the endosperm tissue; however, also a leaching cannot be excluded. The data on the differences in the contents of free protein amino acids

and soluble sugar identify for the first time definite differences between wet and dry processed coffee beans, and thereby indicate the occurrence of metabolic changes.

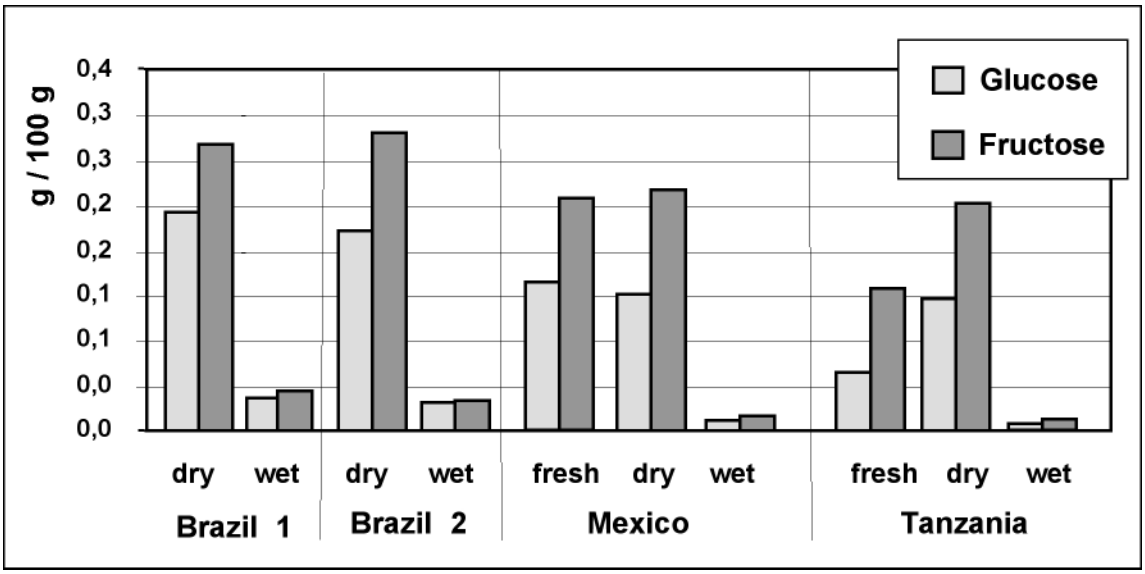


**Figure 1. Glucose and fructose contents in wet and dry process green coffees from experimental processing trials using exclusively fully ripe coffee cherries.**

**Biochemical causales for the differences of wet and dry process coffees: germination**

What might be the biochemical causales of the observed metabolic differences? As outlined earlier (Selmar et al., 2002), the recalcitrant coffee seeds, in principle, are able to germinate within the fruit. However, germination is inhibited by active principles located in pulp (phytohormones, high osmotic potential, germination inhibitors). Metabolism is unlocked, as soon as the pulp is removed such as in wet processing. This induction should alter the composition of substances present in the seeds and thus influence the coffee quality. In contrast with dry processing the pulp remains around the seeds and the metabolism should not be activated until endogenous unlocking occurs.

The exact point of time when germination starts cannot easily be determined, especially in recalcitrant seeds. We chose the expression of putative germination-specific enzymes and the resumption of cell cycle activity and cell division as reliable germination markers. One of the first enzymes expressed in germinating seeds is the isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle, which is responsible for the conversion of stored lipids into carbohydrates. We investigated the expression of ICL during green coffee processing. Unfortunately, related Northern blot analyses revealed that the number of transcripts, even in young seedlings, is very low. Consequently, the related expression studies were performed using the more sensitive RT-PCR technique.

In RT-PCR experiments it could be clearly shown that in fresh coffee seeds ICL is not expressed (Figure 2). In contrast, in wet processed green coffees, a positive signal of the ICL was detected. Surprisingly, also in dry processed coffee seeds, transcripts for ICL were found (Selmar et al., 2004). However, the time course of its expression is different.

In Figure 2, the experimental data of RT-PCR gels from about ten independent experiments are summarized. Whereas in wet processed seeds maximum expression of isocitrate lyase was

detected about one day after the start of the processing, the corresponding maximum in dry processed beans was achieved several days later.

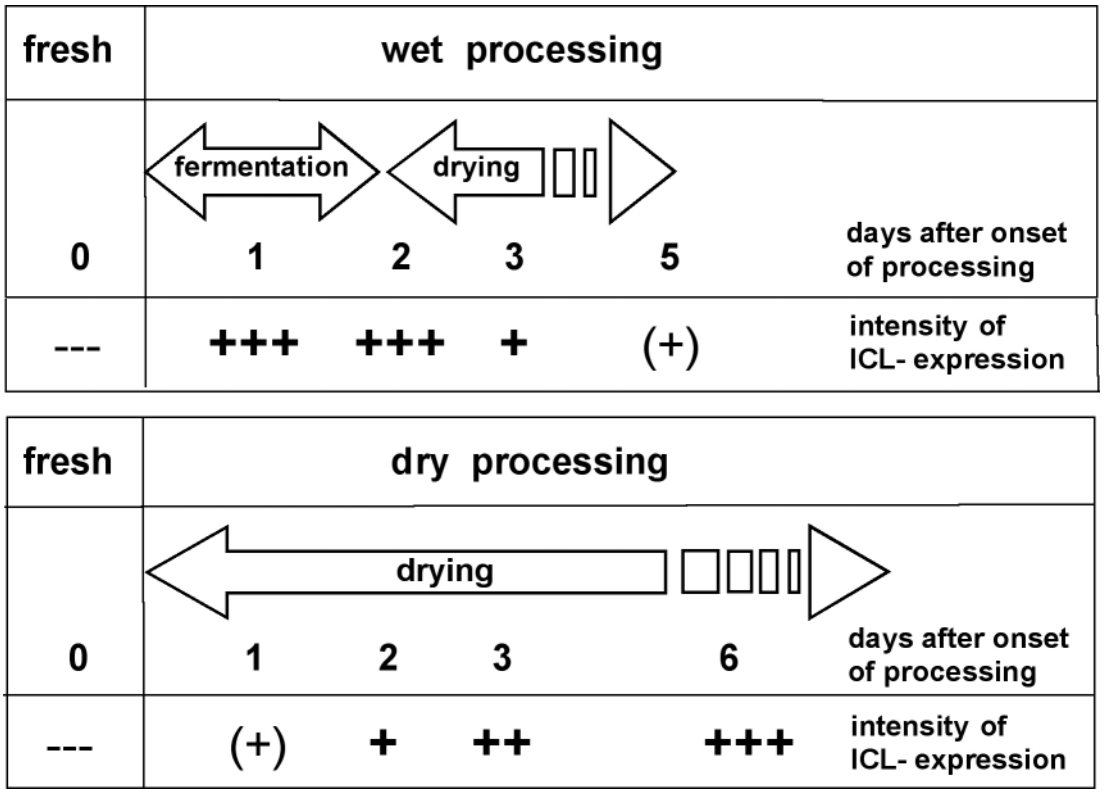


**Figure 2. Expression pattern of ICL in fresh and processed green coffees.**

A further suitable marker for germination is the resumption of cell cycle activity and cell division. To monitor these events, we analyzed the occurrence of ß-tubulin, the major constituent of the microtubules, which are necessary for cell division. The occurrence of this small protein is clear evidence for cell cycle activity and is considered to be a reliable marker for the onset of germination in seeds.

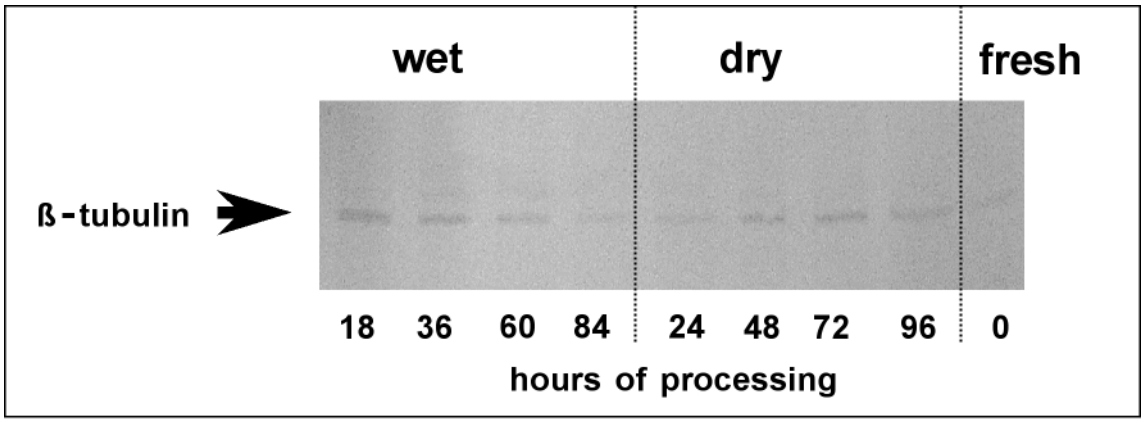


**Figure 3. Western blot analysis of ß-tubulin in fresh and wet and dry processed green coffees. Equal amounts of plant material in each lane.**

As the embryo is the organ where these processes occur first, we prepared embryo protein extracts, separated them electrophoretically, and, after subsequent blotting on a nitrocellulose membrane, analyzed the proteins by using anti-ß-tubulin antibodies.

In embryos prepared from fresh coffee seeds, no ß-tubulin was detectable. In contrast, in wet processed seeds, significant amounts of ß-tubulin were present. ß-Tubulin was determined also in dry processed seeds; however, the time courses of accumulation were quite different. In wet processed coffee seeds, maximum ß-tubulin accumulation was achieved within the first day of processing, shortly after pulp removal, whereas in dry processed seeds, maximum ß-tubulin accumulation was found not earlier than several days after the onset of processing.

The data of the ß-tubulin accumulation fully confirmed the corresponding data from the isocitrate lyase expression studies. Thus, these experiments prove without any doubt that in coffee seeds, during the course of processing, germination-associated processes are initiated and that the extent of the related changes depends on the mode of processing.

**Biochemical causales for the differences between wet and dry process coffees: stress metabolism**

In addition to the germination processes mentioned, further metabolic reactions take place in the coffee seeds during postharvest treatment. In dry process coffee, high amounts of γ-aminobutyric acid (GABA) are accumulated, whereas only small amounts of this non-protein amino acid are present in wet process beans. Untreated fresh seeds contain only traces of GABA. In plants, GABA is considered to be a stress metabolite, typically accumulated in tissues that are exposed to drought stress.

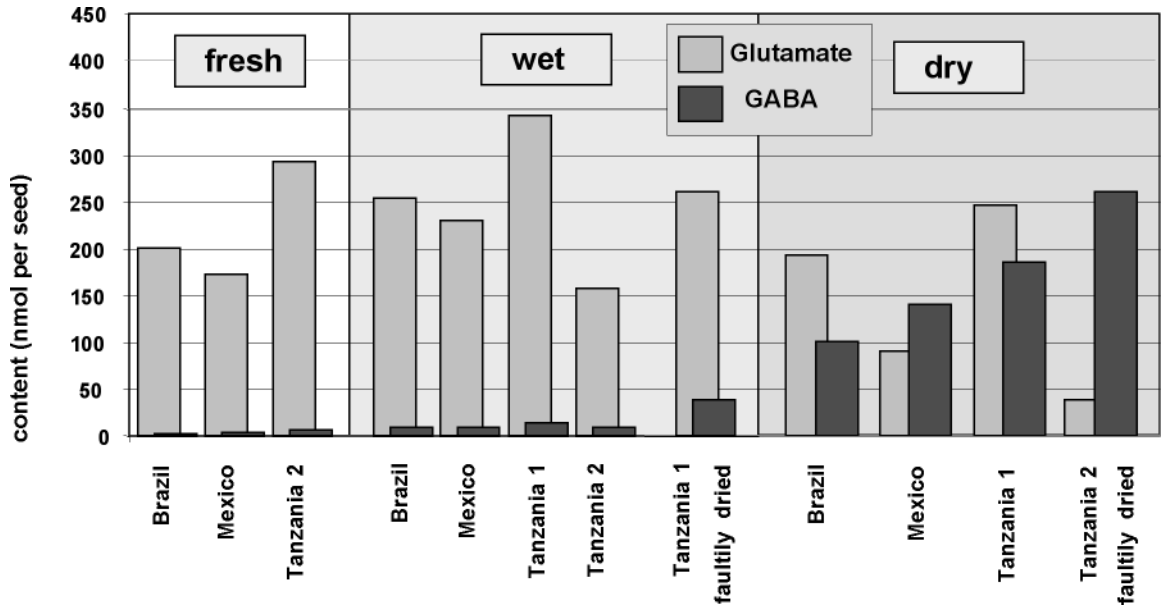


**Figure 4. Occurrence of GABA in fresh and differentially processed green coffees.**

It is apparent that the living and viable coffee seeds, which are dried from an initial moisture content of 50% down to about 12%, suffer massive drought stress and express a related metabolism. Yet, it still has to be explained why a stress related GABA accumulation occurs only in dry process seeds even though wet process seeds are also dried down to the same final moisture content (12%). In this context, the time frames for the drying become relevant. If it is assumed that stress reactions are elicited at about 45% and that all metabolic reactions cease at about 25% moisture content, then in the case of dry processing, an average time span of 4 to 8 days results, in which an active stress metabolism can take place. In wet processing, the drying procedure requires only 2 to 4 days to reduce the moisture content from 50% down to 12%. Consequently, the time window for an elicited stress metabolism corresponds only 1 to 2 days. Assuming that the synthesis and accumulation of GABA starts about 1 to 2 days after

stress induction, it becomes plausible that GABA can be accumulated in response of the drying step only during the dry and not during the wet process.

If indeed the different time frames of the stress-related metabolism are responsible for the observed differences in GABA accumulation, then changes in the time course of the drying procedure should alter the extent of GABA accumulation.

In Tanzania, we performed a field experiment in which the drying period of wet process beans was extended by artificial moistening. In this case of faulty drying, the amount of GABA in wet processed beans was significantly higher than in the control sample (Figure 4). This effect can be much more impressively demonstrated by artificially extending the whole dry process by moistening. In this way, the accumulation of GABA in the dry processed beans is tremendously enhanced. These data confirm the assumption that indeed the GABA accumulated in processed coffees is the result of an active stress metabolism, and that the metabolic processes in green coffees can be modulated by changing the processing conditions.

In plants, GABA is produced by decarboxylation of glutamic acid by the action of a glutamate decarboxylase. Consequently, GABA accumulation should be associated with a decline in the glutamate content. The comparison of the glutamic acid levels in fresh and processed seeds (wet and dry) derived from identical starting material revealed that the stress-related increase in the GABA concentration was accompanied by a corresponding decrease of glutamic acid. This negative correlation is exhibited clearly when the drying time in dry processing is artificially prolonged (faultily dried).

These data suggest that the GABA: glutamate ratio represents a reliable marker for the distinction of differentially processed green coffees. GABA is not accumulated in conventionally wet processed seeds; enhanced ratios point to drying failures. High levels of GABA are indicative of dry processing.

The data presented in this paper prove that green coffee seeds represent vital and living organisms, in which various metabolic reactions occur during processing, i.e. a germination-related metabolism and a stress metabolism. Furthermore, the extent of these processes depends on the conditions during processing and affects coffee quality. Consequently, specific alterations of processing conditions should influence these metabolic processes and thereby offer a promising tool to influence and control coffee quality.

**Practical applications**

In order to illustrate the benefit of these insights, one feasible practical application should be mentioned. At the present, due to aggravated environmental directives, especially for wastewater management, and due to increasing cost pressure, rising amounts of green coffee are being produced by new progressive methods, which utilize mechanical removal of the mucilage. It has been reported that the quality of the coffees produced by such progressive methods is different from that of coffees produced by classical wet processing (Puerta-Quintero, 1999).

There is no doubt that also in the case of these progressive methods, germination and stress-related metabolism will be activated; however the substantial differences originate – again – from different time frames for the corresponding metabolism. In the case of classical wet fermentation, the entire time span from the initiation of the germination-related processes until their termination takes about 2 to 4 days (about 1 to 2 days for washing, sorting and

fermentation and about 1 to 2 days of drying time until the critical water content of 25% is reached – a stage at which metabolic processes virtually cease). In contrast, the time window for the related reactions within seeds which are produced using mechanical mucilage removal is only about 1 to 2 days because the 1 to 2 days allocated for the washing and fermentation steps are omitted.

According to our understanding of an active seed metabolism and its influence on coffee quality, we assume that the extension of the time period of the active metabolism should influence quality. Consequently, in a field experiment in Colombia, we introduced an interim storage phase under moist conditions. The sensorial analyses indeed displayed some positive flavour differences in the samples from the extended process as compared to the control samples. However, when the storage phase time was further extended (more than 24 h), negative effects on flavour were perceived. Present investigations are emphasizing the metabolic differences with regard to germination and stress metabolism in green coffees that result from such modified progressive processing methods.

## ACKNOWLEDGEMENTS

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# Coffee Quality Assurance: Current Tools and Perspectives

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## SUMMARY

The quality of a good cup of coffee, as experienced daily by millions of consumers, is not a matter of chance. It is the result of a quality assurance programme implemented by all the key players of the coffee production-to-consumer chain.

What does it mean at the level of a soluble coffee manufacturer? Clearly, quality assurance starts in the coffee field, where good agricultural and transport practices are essential to develop and preserved the natural quality of the green beans up to the coffee processor's door. This paper focuses on the main controls carried out at the factory level to check the quality and safety of raw materials (green coffee, water used for the extraction process, packaging materials) and finished products, and discusses the rationale behind them. The corresponding analytical procedures, in particular available official standards (ISO, AOAC, DIN, etc), are presented and compared. Current technologies for process monitoring are also extensively reviewed and perspectives for using alternative (e.g. more rapid, simple or efficient) methods are debated. In addition to pure analytical controls, quality systems and procedures are an integral part of the quality assurance programme. The HACCP approach to guarantee food safety, good manufacturing practises to define what is and what is not acceptable in production, and good laboratory practises to ensure the reliability of analytical results have to be implemented too. These systems are also briefly discussed.

## INTRODUCTION

Quality is defined by ISO as "the ability of a set of inherent characteristics of a product, system or process to fulfil requirement of customers and other interested parties" (ISO, 2000). A more practical definition is "the ability of a product to satisfy consumer expectations".

The expectations of the consumers regarding coffee quality are rather high. They mainly include:

- Good sensory characteristics (e.g. aroma, flavour, body, acidity)
- Absence of off-flavours (e.g. mouldy, earthy, fermented, chemical)
- Safety / absence of contaminants (e.g. pesticides, mycotoxins, residual solvents)
- Authenticity (e.g. free of adulterants, correct labelling of the geographic and/or botanic origin, organic product)
- Convenience (e.g. opening of packaging, storage of product, re-use of packs)
- Environmental aspects (e.g. organic product)
- Nutritional value (e.g. dietary fiber, nicotinic acid, potassium)

All these quality characteristics are not a matter of chance. They are the result of planned and systematic activities, preventive measures and precautions taken to ensure that the quality of coffee is attained and maintained day after day. This is the meaning of quality assurance.

The quality of coffee is predetermined by the genotype, the climatic conditions and the soil characteristics of the area in which it is grown. Therefore, quality assurance starts in the coffee field then at the farm, where good farming practices and adequate post-harvest treament are essential to develop the natural quality of the green beans. Then, optimum storage and transport conditions will ensure that this quality is preserved up to the coffee processor's door. At this point, the mastering of the roasting process is key for final cup quality. However, grinding, extraction, and the drying of the extract are additional operations which could alter the quality of coffee and require special precautions. The coffee processor must also systematically control the packing of his product to ensure efficient protection against oxygen, water, light and mechanical shocks. Furthermore, he is responsible for providing all necessary preparation and storage instructions on the product label, in order to guarantee the final cup quality and consumer satisfaction. Once out of the factory, distributors, wholesalers and retailers will all have to take the necessary measures to guarantee the integrity and freshness of the product delivered to the consumer. Therefore, a quality assurance programme has to be implemented by all the key players of the coffee production-to-consumer chain to achieve the common goal: quality and, as a consequence, consumer satisfaction.

The following chapters describe what quality assurance means at the level of a soluble coffee manufacturer, focusing on the main controls carried out from the reception of the raw materials up to the release of the finished packed products.

## GREEN COFFEE

The first principle in the manufacturing of good quality products is to start with good quality raw materials. Within the industry, it is good practice to define the quality of a given raw material in a specification, which is a written document agreed by both the supplier and the manufacturer.

In this connection, the International Organisation for Standardisation (ISO) issued in 1992 guidelines to be used to describe green coffee for sale and purchase (ISO, 1992a). The ISO 9116 standard requires the following information all related to green coffee quality:

- The geographic origin (country, region, state, plantation)
- The botanic origin (species, variety)
- The crop year
- The moisture content or loss in mass
- The total defects and foreign matter
- The content of insect-damaged beans
- The bulk density
- The bean size

The soluble coffee manufacturer buys green coffee according to those or similar guidelines and specifies for each point exactly what he wants. Obviously, he can add other points to the specification (e.g. sensory characteristics, colour, caffeine). When the green coffee arrives at the factory door, he first controls the compliance with the specification using ad'hoc analytical procedures to ensure that he has received the quality ordered.

### Geographic and botanic origin

It is obviously very important to know the geographic and botanic origin of coffee for the purposes of fair international trade (Prodolliet, 1996). On the other hand, the origins used

either alone or in blends impart to the finished product its unique sensory characteristics. Furthermore, premium price is paid for certain origins, which are also often stated on the label of coffee products. Therefore, the purpose of checking the geographic and/or botanic origin of coffee is to support claims and to prevent any deliberate or accidental substitution of a product of reputed origin by a cheaper coffee originating from another country or region.

However, no methodologies have been officially issued. For the geographic origin, the approach using sensory evaluation alone is very limited because of the large number of producing countries and intracountry variability. Objective methods were evaluated (Prodolliet, 2001). Although they all fail to unambiguously determine the geographic origin of green coffee, near infrared reflectance (NIR) spectroscopy (Nzabonimpa, 2001) looks the more promising technique (Figure 1).

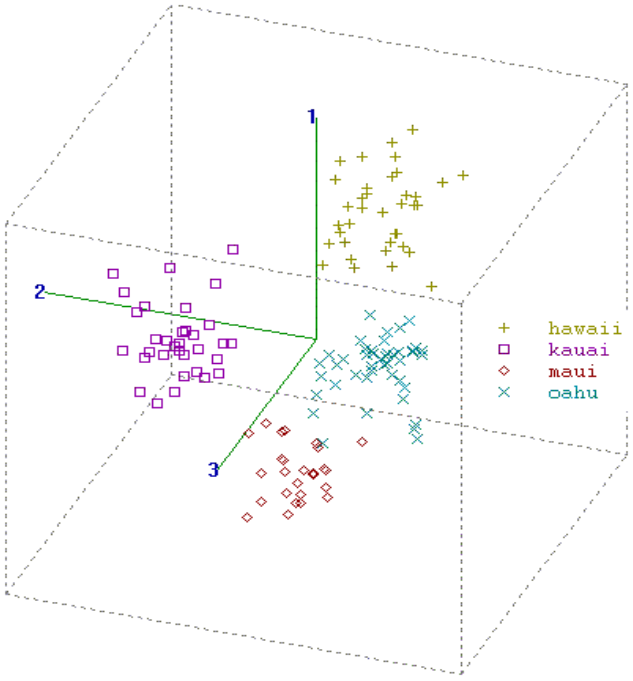


**Figure 1. Discrimination of Hawaiian Arabica coffees by NIR.**

A serious organoleptic examination of a green coffee bag is usually sufficient to assess the botanic origin of coffee as the sensory characteristics and the physical appearance of the two main species, Arabica and Robusta, are usually very different. However, the sensory properties are not only influenced by the coffee species but also, within the same species, by the coffee variety. It may therefore well happen that a specification for Arabica coffee would include restriction on the variety (e.g. Typica, Catuai). The check of origin would then require DNA-based techniques (Meyer, 2000; Rovelli, 2000).

**Sensory evaluation**

The coffee manufacturer's main concern is certainly to deliver to the consumer a product with high quality and regular in-cup taste and aroma. Therefore, the purpose of checking the sensory profile of green coffee (at reception, after roasting) is to ensure the consistent quality of the finished product.

Interestingly, the evaluation of the sensory characteristics of green coffee is not mentioned in ISO 9116 (ISO, 1992a), although this is certainly the most reliable way to assess the quality of the raw material. The basic elements of the sensory evaluation of green coffee consist of:

- A spacious room, equipped with adequate illumination, a sample roaster, a grinder, a cupping table, cups and spoons
- A methodology, describing precisely the roasting conditions, the particle size after grinding, the dosage of coffee in the brew, the type and temperature of the water used to prepare the brew, the way to taste (aroma assessment, removal of floating particles, sucking, use of reference sample, etc), the type of test (discriminative, descriptive, quality control), the number of cups tasted, etc.
- A vocabulary, defining all the sensory attributes to be evaluated
- A well-trained panel

ISO issued guidelines for the preparation of samples (ISO, 1991a). The ISO 6668 standard specifies the sampling, the roasting and grinding conditions and the preparation of the brew. However, it does not make any reference to the vocabulary to be used by the panelists. In this connection, ISO has recently decided to create a new working group to elaborate a vocabulary for the sensory evaluation of coffee products (ISO, 2003b). It will certainly take into account existing and published glossaries (ICO, 2004; ITC, 2002, Lingle, 1986). Hopefully, this will lead to an official ISO standard that should greatly improve and harmonise the communication between the different business partners when talking green coffee quality.

Anyway, sensory evaluation will remain a tedious and complex procedure. Nevertheless very few attempts have been made to develop alternative instrumental methods. The most promising technique, proposed for the evaluation of some key sensory attributes (Feria-Morales, 1991) and for a pass/fail screening (Petracco, 2003), involves the direct analysis of the whole green beans by NIR. Taste sensors have also been developed, but they appeared to be limited to the prediction of only a few attributes, such as acidity and bitterness (Toko, 1995), and cannot assess coffee aroma. On the other hand, gas sensors (or E-nose) were evaluated for the prediction of the aroma pattern (Mitrovics, 1997), but they cannot be used for the assessment of taste attributes.

Colour is also often evaluated as an indirect indicator of the age of the coffee (usually the paler the older) or of the post-harvest treatment. Colour can be assessed either visually or instrumentally using a colorimeter with a large aperture (Brimelow, 2001).

**Moisture content or loss in mass**

Green coffee behaves very differently at high or low moisture content. Although, there is no exact standard defining the ideal moisture content, it is generally recognised that it should range between 8.0-12.5% (Clarke, 2003a; ECC, 2002; ICO, 2002; ITC, 2002; Puerta-Quintero, 2001; Trugo, 2003). The consequences of under-drying and over-drying are as follows:

- Under-drying increases the water activity of the bean and may result in undesirable microbial growth, which may in turn alter the sensory quality of the finished product and/or eventually lead to the formation of mycotoxins
- Buyers want to buy coffee and not water. Consequently, they are more interested to go for low moisture coffee. Conversely, over-drying implies loss of weight and, therefore, loss of money for the sellers
- The moisture content influences the way coffee roasts. Green coffees with a low moisture content tend to roast faster than those with a high moisture content
- Over-dried beans are brittle and tend to break up during handling, generating small particles which may burn too quickly when roasted and affect cup quality

- When the moisture content drops too low, some sensory attributes of the finished product (e.g. aroma, colour, acidity) can be affected

Therefore, the purpose of controlling the moisture content of green coffee is to prevent degradation during transport and storage, to allow the use of constant roasting conditions, to guarantee the sensory quality of the finished product, and for economical reasons.
ISO issued two official standards for the determination of moisture in green coffee:

- ISO 1446 is the basic reference method for the determination of the moisture content of green coffee. After pre-drying and grinding, the coffee is dried to constant mass over $P_2O_5$ at 48°C and under reduced pressure (ISO, 2001).
- ISO 6673, is the "practical" reference method for the determination of the loss in mass of green coffee. The whole beans are dried in an oven with forced ventilation at 105°C for 16 hours (ISO, 2003a).

It is noteworthy that most moisture results within the green coffee trade refer to ISO 6673. The results are insensitive to whether the oven is equipped with a forced ventilation or not (Figure 2). However, it is clearly stated in ISO 6673 that the oven method gives results, which are lower, by about 1.0%, than those obtained with ISO 1446. The reasons for that were recently published (Reh, 2004). In this connection, ISO has decided to create a new working group to review the strategies for moisture determination (ISO, 2003b).

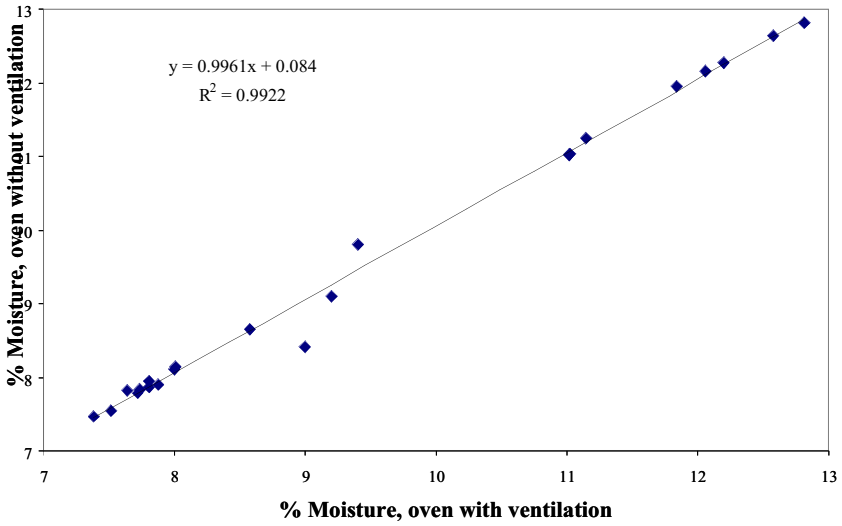


**Figure 2. Moisture in green coffee - ISO 6673 with and without forced ventilation.**

Many benchtop and portable moisture analysers have been developed for rapid determination of moisture in grains. Given a proper calibration they can also be used for whole bean green coffee. The analysis times are very fast, ranging from 5-30 seconds.

Capacitance-based analysers are the most widespread and the cheapest (e.g. Sinar (Figure 3), Dickey-John, Kett, Seedburo). To obtain the best accuracy they should have temperature compensation, density compensation, calibration possibility and bias adjustment options. Proper calibration and validation are obviously key steps to assess the performance and the robustness of the instrument. In this connection, ISO is currently working on guidelines for the calibration of this type of analysers (ISO, 2003b). Capacitance-based analysers can usually operate in a hot and humid environment and are ideal for field applications. Typical performance, as measured by the SD(d) value (standard deviation of the differences between

the rapid and the reference methods), is 0.2-0.3%. Portable moisture analysers were also developed for the analysis of green coffee in-bag and bulk storage (e.g. Sinar).

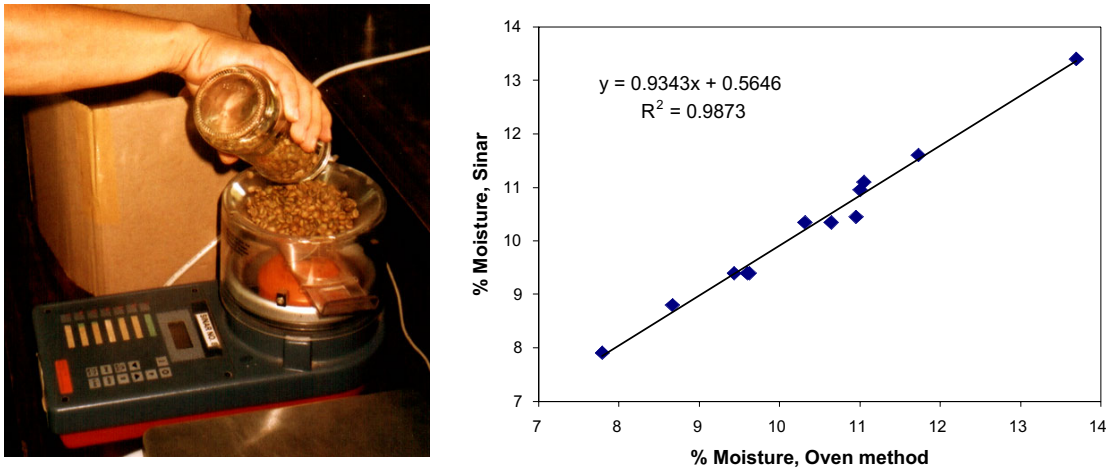


**Figure 3. Determination of moisture in green coffee using Sinar analyser.**

NIR-based analysers are also available for green coffee analysis (e.g. Foss, Kett, Zeltex) and were already successfully applied (Guyot, 1993). They are much more expensive than the capacitance-based analysers, and require more effort to calibrate. However, the performance achieved with NIR analysers is much better, with SD(d) values of the order of 0.1 %. In addition, they can also be used for the determination of other parameters (e.g. caffeine, geographic origin, sensory attributes).

**Total defects, foreign matter and insect-damaged beans**

The ISO 10470 standard has been recently revised and defines defects as "anything divergent from the regular, naked sound green coffee beans expected in a coffee lot" and classify them into five categories (ISO, 2004):

- Foreign materials of non-coffee origin, such as stones, sticks, or metallic matter
- Foreign materials of non-bean origin, such as pieces of parchment or husks
- Beans that are abnormal in shape regularity/integrity, such as broken beans or ear
- Beans that are abnormal in visual appearance, such as black beans (Figure 4)
- Beans that are abnormal in taste of the cup after proper roasting and brewing, such as beans giving off-flavours (stinker, fermented, musty, Rio, phenolic, chemical)

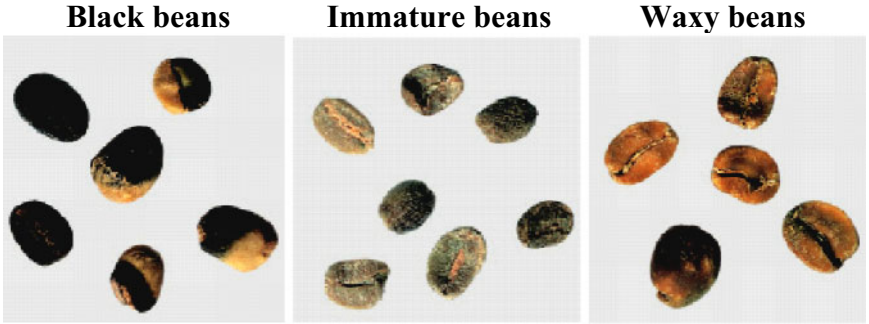


**Figure 4. Example of beans with abnormal appearance.**

It is clear from the above that defects are one of the most important criterion of the evaluation of green coffee, as their presence can greatly alter the final cup quality by generating off-

flavours. They can also lead to material loss due to the presence of foreign matter. Indeed, stones and wood sticks cannot be sold as coffee and must be removed at the factory prior to any further processing. Finally, the presence of defects can affect the roasting and grinding operations. Small bean fragments may burn in the roaster and stones may damage the grinder (Illy, 1995). Not surprisingly, the number of defects is taken into account in most classification or grading systems applied around the world for trade purpose (Clarke, 2003a). Therefore, the purpose of checking the defects in green coffee is to check its grade, to prevent damage of the grinder, to guarantee the sensory quality of the finished product, and for economical reasons.

ISO issued several standards for the determination of defects in green coffee.

- ISO 4149 describes the analytical methodology (ISO, 1980). This standard is under revision (ISO, 2003b). In the new version, defects are manually separated according to their type as defined in ISO 10470. The defects of the same type are weighed together and their percentage compared to the mass of the test portion is calculated. The counting of the defect is no longer mentioned. Each percentage is then multiplied by a coefficient (0, 0.5 or 1) which depends on the influence of the defect on the final cup quality and on the material loss, repectively. The results obtained for each defect are then summed up to yield so-called Quality Impact Units (QIU) for both sensorial concern and material loss (see example below).

**Table 1.**

| **Defect** | **Weight** | | **Coefficient SC** | **Sensorial concern** | **Coefficient ML** | **Material loss** |
|---|---|---|---|---|---|---|
| | **g** | **%** | | | | |
| Stones | 1.2 | 0.4 | 0 | 0 | 1 | 0.4 |
| Bean in parchment | 3.0 | 1.0 | 0 | 0 | 0.5 | 0.5 |
| Black bean | 3.0 | 1.0 | 1 | 1.0 | 0 | 0 |
| Immature bean | 10.5 | 3.5 | 0.5 | 1.75 | 0 | 0 |
| Spongy bean | 9.0 | 3.0 | 0.5 | 1.5 | 1 | 3.0 |
| Brown bean | 7.5 | 2.5 | 1 | 2.5 | 0 | 0 |
| Sound bean | 265.8 | 88.6 | | | | |
| Total | 300.0 | 100.0 | | **6.75 QIU** | | **3.9 QIU** |

*SC: Sensorial concern ; ML : Material loss*

- ISO 10470 provides a comprehensive listing and pictures of the main defects, including clear definition and characteristics, their cause and their influence on the final cup quality and on the material loss (ISO, 2004).
- ISO 6667 describes a microscopic method for the identification and the determination of the proportion of beans damaged by insects (ISO, 1985), mainly by *H. hampei* (coffee bean borer) and *A. fasciculatus* (coffee bean weevil).

The new version of ISO 4149 provides the basis of an harmonised and internationally used system for the determination of defects. Each country could apply ISO 4149 for defect determination, and still keep or develop its own criteria (e.g. maximum number or range of QIU) and wording for defining the final grade of green coffee.

**Density**

Natural causes such as drought, stress or picking of immature cherries may produce beans, which are less dense than normal beans. Their presence may strongly alter the sensory profile (ITC, 2002). Shells, ears and broken beans are also less dense and may lead to uneven roast or charring during roasting (ISO 10470). Therefore, the purpose of controlling the density is to ensure an even roast and to guarantee the sensory quality of the finished product.

The free-flow bulk density can be determined according to ISO 6669, by measuring the ratio of the mass of whole coffee bean to the volume it occupies (ISO, 1995).

**Bean size**

Roasting should ideally be carried out with beans of the same size. When unevenly sized beans are roasted, the smallest tend to burn and the largest tend to be under roasted, affecting the visual appearance of the beans and, more importantly, the cup quality (Barel, 1994). On the other hands, larger beans tend to be more expensive. Consequently, the bean size is taken into account in the classification systems applied around the world for trade purpose (Clarke, 2003a). Therefore, the purpose of checking the size of green coffee is to determine its grade and to guarantee the sensory quality of the finished product.

Bean size can be determined either by manual sieving according to ISO 4150 (ISO, 1991b), or by machine sieving according to ISO AWI 24116, a new ISO standard currently under preparation (ISO, 2003b).

There are obviously other parameters that can be checked on green coffee that are not mentioned in ISO 9116. Most relate to regulatory and safety concerns. The list below is not exhaustive but addresses the main issues.

**Caffeine**

The major active component in coffee is caffeine, a central nervous system mild stimulant. Some consumers who like coffee do not always want the mild stimulatory effect. This is the reason why decaffeinated coffee products were developed. The latter are produced from decaffeinated green coffee. In Europe the absolute caffeine content may not exceed 0.1% in decaffeinated roasted coffee and 0.3% in decaffeinated soluble coffee, but no limit is given for green coffee (Clarke, 1988; EU, 1999). The USA consider coffee to be decaffeinated when 97% of the caffeine originally present in the bean is removed (Clarke, 2003b). Therefore, the purpose of checking the caffeine content in decaffeinated green coffee is to verify the efficiency of the decaffeination operation, to support claims and to guarantee the compliance of the finished product with the legislation.

ISO issued two official standards for the determination of caffeine in decaffeinated green coffee:

- ISO 4052 is the reference chromatographic method (ISO, 1983a). Residual caffeine as low as 0.02% can be detected. However, as stated in the ISO standard itself, the method is particularly sensitive to variations in its application.
- ISO 10095 describes a high-performance liquid chromatography (HPLC) method (ISO, 1992b). The accuracy is very good and the detection limit is 0.01% caffeine.

The HPLC method is now well accepted worldwide but remains a lengthy procedure. In this respect, NIR spectroscopy has already been successfully applied for the determination of

caffeine in regular green coffee (Guyot, 1993). However, the caffeine content in decaffeinated green coffee is much lower than in regular coffee. Despite this, NIR calibrations with acceptable accuracy can be obtained (Figure 5).

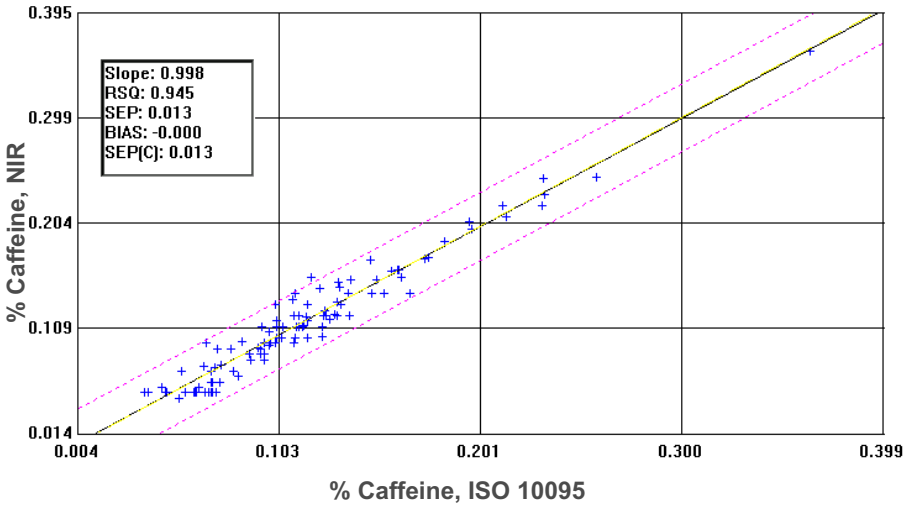


**Figure 5. Determination of caffeine in whole bean decaffeinated green coffee by NIR.**

**Decaffeination residual solvents**

Decaffeination is almost exclusively carried out at the green coffee level, where caffeine is removed with organic solvents (dichloromethane, ethyl acetate), supercritical carbon dioxide or water (Clarke, 2003b). When organic solvents are used, residues are always left in the coffee beans but at very low level. In this connection, the European Union has recently proposed maximum residue limits for extraction solvents in roasted coffee (EU, 2003). Therefore, the purpose of measuring the level of residual solvents is to check the efficiency of the solvent removal operation after decaffeination, to comply with national or international legislation, and to guarantee the safety of the finished product.

Although no methodologies have been officially issued, residual solvents can be determined using azeotropic distillation coupled with capillary gas chromatography (GC). The technique is selective and detection limits at the sub-ppm level can be achieved (Gal, 1971). The method can be simplified using headspace chromatography (Russo, 1989), eventually coupled with solid-phase microextraction.

**Ochratoxin A (OTA)**

Ochratoxin A is a toxic mycotoxin, which has been shown to cause kidney damage and tumours in test animals. It is produced by several mould species, particularly in cereals and cereal products. OTA is also found in green coffee, when mould growth is allowed to occur due to uncontrolled moisture level during drying, storage and transportation (ITC, 2002; Walker, 1997). A Code of Practice was recently issued by the European Coffee Co-operation, which gives good agricultural, transport, storage and manufacturing guidelines intended to prevent OTA contamination and formation (ECC, 2002). In October 2004, the European Union agreed on maximum limits for the toxin in roasted and R&G (5.0 μg/kg) and soluble (10.0 μg/kg) coffee (EU, 2004). Therefore, the purpose of measuring the level of OTA is to comply with national or international legislation and to guarantee the safety of the finished product.

A collaborative study on a procedure using immunoaffinity column cleanup coupled with HPLC for the determination of ochratoxin A in green coffee was recently organized (Vargas, 2004). The method has acceptable accuracy and a very low detection limit (~0.1 μg/kg). A rapid and low-cost alternative method using thin-layer chromatography was also developed (Pittet, 2002). The detection limit is higher (~10 μg/kg), but the method is more appropriate for screening purposes. It can be expected that quicker and cheaper, but very sensitive techniques, using antibodies or protein receptors immobilized on sensor chip surface, will be developed in a near future (Hauck, 1998; Patel, 2000; Shriver-Lake, 2004).

**Pesticide residues**

Pesticides are a broad class of chemical products mainly used to fight against insects, diseases and weeds on crops. They include insecticides, fungicides and herbicides. Other types of pesticides, such as fumigants and rodenticides, are also used to protect green coffee during storage and transport. When pesticides are applied, residues may possibly be left in the coffee beans. Their usage is carefully regulated by national food and drug legislations and the Codex Alimentarius (FAOSTAT, 1999; NCA, 2000). However, the level of pesticide residues in green coffee is generally very low (FDA, 2000; FDA, 2002). It was also shown that, if present, pesticides are substantially reduced during storage, roasting and brewing (McCarthy, 1991). Therefore, the purpose of measuring the level of pesticide residues is to comply with the national or international legislation, and to guarantee the safety of the finished product.

Although no methodologies specific for green coffee have been officially issued, pesticide residues can be determined by using multiresidue methods (CEN, 1998). The technique is selective and detection limits at the sub-ppm level can be achieved. Pesticide residues not included in multiresidue screening can be analysed with single residue methods, e.g. inorganic bromide by capillary GC (CEN, 2000).

**Mineral oil in bags**

For years, jute and sisal fibers were softened with mineral oils before spinning and weaving. In the early nineties, hydrocarbon residues containing high proportion of polycyclic aromatic hydrocarbons (PAH) were found in commodities transported in jute and sisal bags (Grob, 1991a; Grob, 1991b). It was established that the contamination occurred from the use of crude low-grade mineral oils. Hydrocarbon residues were detected (Grob, 1991b) in green (230 ppm) and roasted coffees (10-150 ppm) and, to a very less extent, in soluble coffee (2 ppm). Since then, the International Jute Organization has issued specifications for the manufacturing of jute bags, which limit the amount of unsaponifiable matter to a maximum of 1250 mg/kg (IJO, 1998). Some manufacturers have also developed a hydrocarbon-free lubricant, based on vegetable oil, to soften the fibers. Therefore, the purpose of measuring the presence of mineral oils is to comply with the national or international legislation, and to guarantee the safety of the finished product.

Mineral oils can be checked at two different levels. Firstly, the amount of unsaponifiable matter is determined in the jute or sisal bags. This is performed gravimetrically after saponification, extraction, and solvent evaporation (BSI, 1990; IUPAC, 1987). Secondly, the hydrocarbon residues are measured in the green coffee beans. This can be performed by capillary GC or by using a coupled HPLC-GC system (Grob, 1991b). Detection limits around 50 ppb could be achieved. Alternatively, a mass detector can be used for confirmatory analysis and improve sensitivity.

The green coffee once at the factory had its quality checked using the different techniques presented above. After a formal release (approval) it will go through the different processing steps, which will transform the beans into packed soluble coffee. The main processing steps include roasting, grinding, extraction, evaporation, drying and packing. After each of these unit operations the product should reach specific characteristics (e.g. colour, particle size, total solids, moisture). Analyses are therefore required to ensure the efficiency and the consistency of the process, and, ultimately, that those characteristics are indeed within their corresponding pre-defined tolerance limits. The following chapters review the main analytical controls carried out during the manufacture of soluble coffee.

## ROASTED COFFEE / ROASTED AND GROUND COFFEE

Green coffee must be roasted in order to give the final beverage its unique sensory characteristics. The most important parameters to keep under control are discussed below.

### Degree of roast

Coffee can be roasted to various degrees, from very light to very dark. The degree of roast has a direct impact on the sensory profile of the coffee cup, which is a matter of consumer preference. It has also a great influence on the particle size distribution after grinding and, consequently, on the extractability of coffee. Therefore, the purpose of monitoring this parameter is to control the roasting process and to guarantee the consistent sensory quality of the finished product.

Here is an analytical challenge. As already explained, the ultimate aim of roasting is to achieve a specific and well defined sensory profile. Sensory evaluation should therefore be used to control the process. However, this is a very lengthy and combersome procedure, which is not at all adapted for near-line or on-line monitoring. Consequently, alternative techniques must be applied.

The colour of coffee is correlated to the final roasting temperature and to the bitterness and acidity balance of the cup (Illy, 1995). It is therefore used as an indicator of the end of the roasting process. Experienced roasters do it by visual comparison of the colour of the roasted beans with that of reference ones. The technique is obviously very subjective. The Specialty Coffee Association of America has developed together with Agtron a series of eight colour standard disks ranging from very light to very dark. The procedure simply consists of visually comparing the colour of roasted coffee with the standard disks. It is simple, cheap and more objective than the previous method, but lacks sensitivity as it allows discrete measurements only. Simple colour meters (e.g. Colortest II from Neuhaus Neotec, Colorette 3a from Probat) are now widespread within the coffee industry. They are robust equipment that give the result in arbitrary colour units. Good correlations exist between these colour units with roast weight loss and some sensory charactristics (Figure 6). Interestingly, these colour meters operate in the near infrared region of the spectra. Consequently, they do not measure colour, but rather some still unknown physical parameters. In order to obtain meaningful correlation and reproducible results, it is of utmost importance to properly calibrate the instrument and to grind the samples before measurement. Indeed, in the case of rapid roasting the interior of the bean is less roasted (this means less dark) than the outside. Based on the same principle as the near-line colour meters, on-line colour sensors are also available (e.g. Colour measuring system 2 from Probat), which allow the continuous monitoring of the colour change of the beans during roasting. In this case, the measurement can only be carried out on the whole beans.

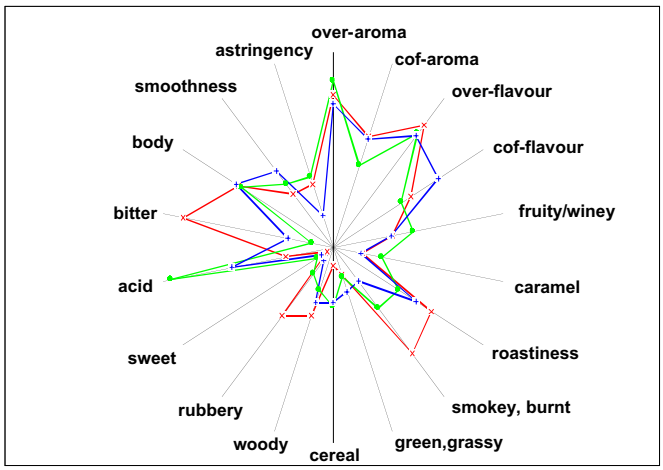


**Figure 6. Correlation between Neuhaus CTn colour unit and sensory attributes of the same Arabica coffee submitted to different roasting conditions.**

### Sensory evaluation

Roasting determines the sensory attributes of the coffee brew. However, instrumental techniques control the roasting process (see above) and the correlation with the sensory quality may vary with the quality of the green coffee and/or the roasting conditions. It is essential that this correlation be verified with a sound reference sensory analysis either to release the product if sold as whole beans or roasted and ground (R&G) form, or to let it enter into the next processing step if soluble coffee is manufactured. Therefore, the purpose of taste testing the roasted coffee is to verify the results obtained with the instrumental techniques or to check the sensory quality of the finished product.

The same techniques described above for the control of the green coffee apply for the sensory evaluation of the roasted coffee.

### Moisture

The moisture level, in combination with the headspace oxygen content, is critical for the shelf life stability of whole beans and R&G coffee (Clarke, 2003c; Walker, 1987). In addition, a number of countries have fixed limits for the maximum water content in roasted coffee (Clarke, 1988). Therefore, the purpose of determining the moisture in roasted coffee to comply with the legislation and to ensure the keeping quality of the finished product.

ISO issued two official standards for the determination of moisture in R&G coffee, that can also be applied to whole bean roasted coffee after grinding:

- ISO 11817 is the reference method for the determination of the moisture content. After extraction with dried methanol at 65°C, water is titrated with the Karl Fischer reagent (ISO, 1994a).
- ISO 11294, is the routine method for the determination of the loss in mass. The sample is dried in an oven at 103°C for 2 hours (ISO, 1994b).

The two methods give very similar results. However, the oven method may lead to over-estimated values if applied to freshly roasted and ground coffee, as it would partly include aroma volatiles, volatile acids and residual carbon dioxide (Walker, 1987).

The same capacitance- and NIR-based analysers used for the rapid determination of moisture in green coffee can be used for roasted coffee. In the case of whole beans, grinding the coffee prior to analysis increases the surface of product reflecting the light and, hence, the sensitivity of NIR techniques. Ground coffee is also much more homogeneous than whole bean coffee, which leads to improved accuracy. A large number of NIR instruments equipped with a measuring probe for powdered products are now available (e.g. Process Sensors MCT600). Thermogravimetric methods using halogen (e.g. Mettler HR73, Sartorius MA50) and infrared (e.g. Sartorius MA45) balances are also available for the near-line analysis of ground coffee. The balances are much cheaper than NIR instruments, but analysis time are longer, typically 10 minutes.

**Caffeine**

In the majority of European countries the absolute caffeine content may not exceed 0.1% in decaffeinated roasted coffee (Clarke, 1988). The USA considers coffee to be decaffeinated when 97% of the caffeine originally present in the bean is removed (Clarke, 2003b). Therefore, the purpose of measuring the caffeine content in decaffeinated roasted coffee is to check the compliance with the legislations.

ISO 4052 and ISO 10095 standards issued for the determination of caffeine in decaffeinated green coffee also apply to decaffeinated roasted coffee. NIR spectroscopy can also be used as an alternative rapid technique either at the whole bean level, or on ground coffee for better sensitivity and accuracy (see above).

**Particle size of R&G coffee**

The particle size distribution after grinding has a considerable influence on the extractability of coffee and, in turn, on the cup quality. This is the reason why the different brewing technique each require specific grinds, whether for home use or for subsequent large-scale extraction. Particle size also has an influence on the shelf life stability of R&G coffee. The smaller the size the larger the oxygen and moisture uptake and the faster the deterioration of the organoleptic quality. Therefore, the purpose of monitoring particle size is to control the grinding process and to guarantee the consistent sensory quality of the finished product.

Particle size distribution analysis is complicated by the fact that the oil present on the surface may aggregate small coffee particles and thus distort the results. The efficiency of the dispersing system, whether mechanical shock, air flow or solvent, is therefore critical for the accuracy of the results.

Manual or machine sieving is the simplest and cheapest method for determining particle size. Although only applicable for green beans, ISO 4150 (ISO, 1991b) and ISO AWI 24116 (ISO, 2003b) standards give useful general guidelines. Methods based on laser diffraction are much quicker and more reproducible than sieving. They are also far more costly. They measure the whole distribution of the particles from a few microns to a few millimeters size (Figure 7). R&G coffee is analysed either in powder form dispersed in air (e.g Sympatec Helos) or dispersed in an inert solvent (e.g. Malvern Mastersizer 2000).

The above-mentioned methods are not all well adapted for process control and require rather standard laboratory environment. In this respect, more robust and compact equipment was developed (e.g. Probat Granutester), which can be used closer to the line. For larger operations, on-line particle sizers are now available that allow continuous measurement of the particle size distribution and hence a better control of the grinder. They are based either on laser diffraction (e.g. Malvern Insitec, Sympatec Twister), light reflectance (e.g. Lasentec,

Messtechnik Schwartz), or camera imaging (e.g. Hydro PartAn). The latter technique also measures the shape of the particles.

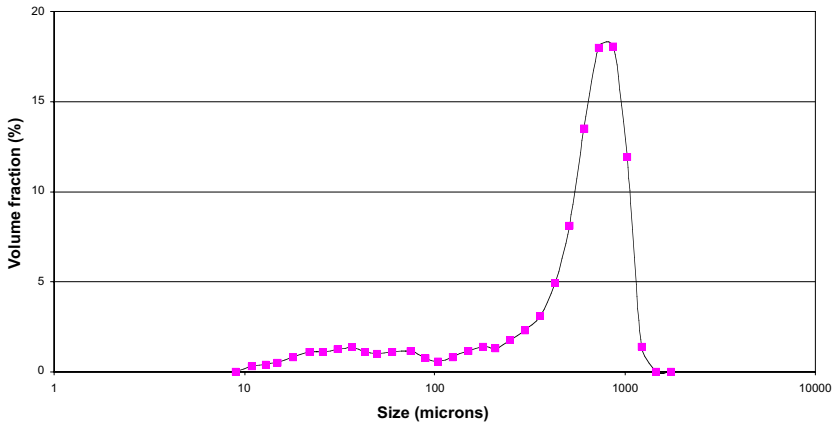


**Figure 7. Particle size distribution of an R&G coffee measured on a Sympatec analyser.**

## SOLUBLE COFFEE

In the manufacturing of soluble coffee, ground coffee is extracted with hot water using percolation batteries, and dried. At the end of the extraction process the "light" extract contains 10-20% solids. However, drying is more economic if done with a concentrated extract. Therefore, the extract coming from the percolation batteries, or light extract, is fed to an evaporator to yield a so-called heavy extract, containing about 50% total solids (Clarke, 2001, Clarke, 2003d). Water removal from the extract is achieved by either spray drying or freeze-drying (Clarke, 2001; Clarke, 2003d). The spray-dried powder can be subsequently agglomerated to match the appearance of freeze-dried granules and to improve its instantination properties (Clarke, 2003d). Re-incorporation of coffee aroma volatiles either prior or after (aromatisation) drying is also usual practice. The conditions under which drying is performed greatly affects the physical (e.g. density, particle size, colour) and sensory characteristics of the coffee powder. The following chapters review the main analytical controls carried out for the check of the coffee extract and of soluble coffee as a finished product.

### Total solids of the coffee extract

The concentration of solids in the light extract reflects the efficiency of the extraction step and is directly linked to the amount of soluble coffee powder that will be produced. The concentration of solids in the heavy extract reflects the efficiency of the evaporation step and is related to the energy needed for drying. It also influences the physical characteristics of the powder (e.g. density, particle size) obtained after drying. Therefore, the purpose of analysing total solids (TS) is to have the economical control of the whole process, to control the evaporator and to guarantee the consistent physical quality of the finished product.

TS of coffee extract is measured using a reference oven method. The extract is previously mixed with sand to avoid splashing and then dried in an oven at 70°C for 16 hours under reduced pressure (LMBG, 1981). This method is obviously not suitable for process control. Alternative methods are available for this purpose. Portable refractometers (e.g. Atago PR series, Figure 8) are certainly the most simple and cheap instruments for near-line analyses.

| | |
|---|---|
| Measurement range | Brix 0.0 to 60.0 % |
| Resolution | Brix 0.1 % |
| Measurement accuracy | Brix ± 0.1 % |
| Measurement temperature | 10 to 40°C (compensation) |
| Measurement time | 3 seconds |
| Power supply | 2 x AAA Batteries |
| Dimensions and weight | 17 x 9x 4cm, 300g |

**Figure 8. Performance of the Atago PR-201α refractometer.**

A large number of devices have been developed for the continuous monitoring of TS in liquid materials, and could well be applied for coffee. They are based on refractometry (e.g. K-Patents, Figure 9), microwave (e.g. Harrer Kassen), ultrasound (e.g. Rhosonics), Coriolis forces (e.g. Krohne, MicroMotion), resonance (e.g. Metra), or NIR.

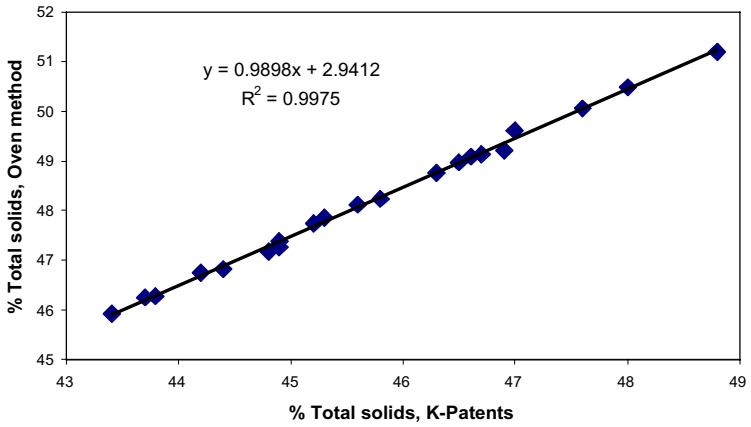


**Figure 9. On-line monitoring of total solids using K-Patents refractometer.**

**Cleanliness of the coffee extract**

It is well known that insoluble substances are also formed during extraction (Bradbury, 1997; Clarke, 2001). These substances may eventually be carried over to the soluble coffee powder leading to an "unclear" coffee cup after reconstitution with hot water. Therefore, the purpose of measuring the cleanliness of the extract is to control the extraction and evaporation steps and to ensure a clean coffee cup.

Insoluble substances can be determined by filtration of the extract and gravimetric evaluation of the amount of residues left on the filter according to the official German method (LMBG, 1990). Turbidimetry, gravimetry after centrifugation, or filtration of the extract followed by visual evaluation of the residues on the filter can be also applied.

**Moisture**

The amount of water left in the powder has a great influence on the shelf life stability of the soluble coffee powder. The higher the moisture level, the higher the risk of caking and degradation of the product, in particular in hot and humid climate. On the other hand, overdrying of coffee leads to losses of aroma coffee volatiles and is economically not justified. In this connection, the European Union has fixed a minimum of 95% (w/w) dry

matter content in soluble coffee (EU, 1999). Therefore, the purpose of analysing moisture is to control the drying process and to comply with national or international legislation.

Moisture can be determined according to ISO 3726 (ISO, 1983b). The method actually measures the loss in mass after the product has been dried in a vacuum oven at 70°C for 16 hours under reduced pressure. The Karl Fischer method is also officially used in Germany (LMBG, 1986). It is as accurate as ISO 3726 and much more rapid. In this connection, ISO has recently decided to create a new working group with the objective of issuing the Karl Fischer method as an international standard (ISO, 2003b).

Reference methods are not suitable for process control. Thermogravimetry-based (Figure 10) or NIR-based analysers (see chapter on roasted coffee) can be used for this.

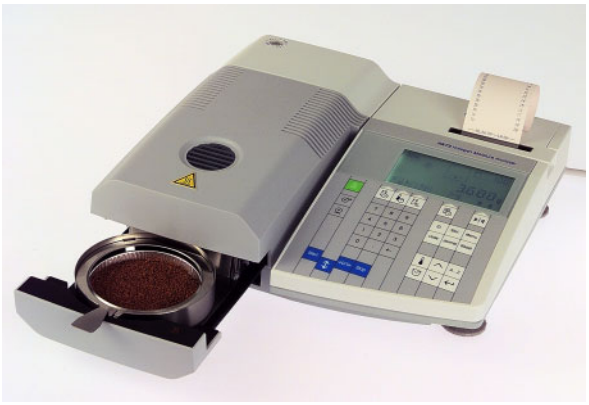

**Figure 10. Near-line monitoring of moisture in soluble coffee using a halogen analyser.**

NIR-based sensors are also available for on-line moisture analysis of powdered food materials and, therefore, allow a much better control of the drying step (e.g. NDC MM710, AIS IRPC). The performance achieved with on-line NIR instruments are usually very good, with SD(d) values of the order of 0.05% (Figure 11).

**Figure 11. On-line monitoring of moisture in powdered products using NDC MM710.**

**Sensory evaluation**

All the processing steps to convert roasted coffe to soluble coffee certainly modify the original sensory profile. The manufacturer wants to make sure that it corresponds well to the target profile and to the consumer's expectations. Therefore, the purpose of taste testing the soluble coffee is to check the aromatisation process and the sensory quality of the finished product as delivered to the consumer.

The same principles described above for the control of the green coffee apply for the sensory evaluation of the soluble coffee. The sensory attributes considered may differ slightly, focusing more on process dependent characteristics (e.g. aroma, flavour, acidity, bitternesss, body). At the same time, the colour of the powder and cleanliness of the cup (see chapter on coffee extract) are also evaluated

**Caffeine**

In Europe the absolute caffeine content may not exceed 0.3% in decaffeinated soluble coffee (EU, 1999). The legislation differs however in other non-European countries (Clarke, 1988). Therefore, the purpose of measuring the caffeine content in decaffeinated soluble coffee is to support claims and to check compliance with the legislations.

ISO 4052 and ISO 10095 standards issued for the determination of caffeine in decaffeinated green and roasted coffee also apply to decaffeinated soluble coffee. NIR spectroscopy can also be used as an alternative rapid technique.

**Bulk density and Hardness**

For a given weight, low density powders occupy a larger volume which could be superior to that of the container itself. In turn, high density powders occupy a smaller volume which may leave an unacceptable large empty volume (head space) in the container and lead to consumer dissatisfaction. Furthermore, soluble coffee is friable and can be subjected to irreversible granule/agglomerate breakdown into finer particles during handling (e.g. packing, transport). This phenomenom can lead to an increase of density of the powder, and to a corresponding increase of the head space and amount of fines in the container. The manufacturer can predict and prevent such effects by carefully controlling the bulk density of the powder. Therefore, the purpose of measuring the bulk density and the hardness of soluble coffee is to guarantee the efficiency and correctness of the filling operation and to ensure the physical integrity of the finished product during distribution.

ISO 8460 describes analytical methods for the determination of bulk densities of soluble coffee (ISO, 1987). The free-flow bulk density is measured by pouring the sample through a funnel into a receptacle of known volume and weighing the content of the receptacle. The compacted bulk density is obtained by measuring the volume of a given mass of coffee after it was subjected to a fixed number of taps in a tapping volumeter. The latter device is commercially available (e.g. Probat 55 LS 250). The difference between the measurements is indicative of the hardness of the granules/agglomerates.

**Solubility, Wettability**

It is important for the consumer when preparing the cup that the coffee powder rapidly dissolves in hot water without forming lumps. The dissolution properties (solubility, wettability) of soluble coffee are indeed influenced by many parameters, including the porosity and the particle size of the powder, shaped during the drying and agglomeration steps. Therefore, the purpose of determining the solubility and wettability is to check the dissolution properties of the finished product.

Solubility and wettability are usually assessed during the sensory evaluation sessions of the finished product, by visually observing the behaviour of the powder upon reconstitution.

**Coffee oil**

As described earlier, coffee aroma volatiles can be re-incorporated into the product after drying. The convenient vehicle for the aroma is coffee oil, which is sprayed or plated on to the coffee powder/granules during the packing operation. The process is known as aromatisation and contributes to the overall sensory quality of the finished product. Therefore, the purpose of analyzing the oil content is to control the aromatisation process.

Coffee oil can be determined gravimetrically by extraction with petroleum benzine in a Twisselmann apparatus and subsequent evaporation of the solvent (SFM, 1973). The use of gas sensors was also reported for the control of the in-jar aroma concentration (Gretsch, 2001).

**Net weight**

The amount of coffee in the container in which it is sold, or the net weight, is always declared on the label and is strictly regulated in all countries. Therefore, the purpose of controlling the net weight is to check compliance with the legislations.

Net weight can be controlled manually by taking samples from the line at a defined frequency and weighing them. The operation is very simple but resource demanding. Nowadays, checkweighers are available for the automated control of net weight (e.g. Mettler Toledo Garvens series). The system checks every container without exception and allows the rejection of non-conforming products.

**Tightness of packaging**

Like any foods, soluble coffee must be protected by an ad'hoc packaging material to minimise water and oxygen uptake and resulting spoilage of the organoleptic and physical properties. Some products, in particular aromatised coffee, additionally require filling under an inert gas atmosphere (Clarke, 2003d). Protection is only efficient if the package is tightly closed. Therefore, the purpose of the different checks on the packed product is to ensure the tightness and integrity of the container.

Procedures for the assessment of tightness depend on the type of packaging and vary from one manufacturer to another. For heat sealable packages, a simple test can be applied, during which a dye solution is injected by means of a syringe into the package and any leak is detected visually. Commercial equipments working on the differential pressure principle are also available (e.g. Uson Qualipak series). The package is placed in a tightly closed measuring chamber under reduced pressure and any pressure increase in the chamber is detected and indicative of a leak. For cans, compressed air can be introduced into the package placed in a water bath and any leak is detected visually by the presence of outside air bubble. More elaborated helium leak testers have also been developed that allow on-line tightness control (e.g. Pfeiffer Vacuum Qualitest).

Analysis of residual oxygen after filling the product under an inert gas atmosphere can be carried out using commercially available oxygen analysers working on different principles. The air in the head space is pumped out by means of a syringe, transferred into a measuring cell, where oxygen can be determined by paramagnetic susceptibility (e.g. Servomex 574 or 1450) or luminescence quenching (e.g. PreSens Microx TX series). Oxygen levels down to 0.1% or even below can be measured.

## WATER

Water is the second basic raw material involved in the manufacture of soluble coffee. The quality of water used during the quenching, extraction and agglomeration process has obviously a direct impact on that of the finished product and, also, on the maintenance of the equipment. The "Guidelines for drinking-water quality" defined by the World Health Organisation also apply to water used in soluble coffee manufacturing (WHO, 2003), whatever its origin (ground, surface, or community water). The following parameters are more relevant for water monitoring in the coffee industry:

- **Pathogens**
  The easiest way to control the absence of pathogenic agents in water is to monitor the presence of residual biocides (e.g. active chlorine, see below).
- **Chlorine**
  Sodium hypochloride is the most widely used disinfection agent. Residual levels of active chlorine of 0.2-0.3 mg/l are usually required to ensure water safety. However, too high level of active chlorine could confer the cup a peculiar unpleasant taste.
- **Hardness**
  On heating, calcium and magnesium cations produce insoluble salts leading to possible scaling and corrosion of equipments. Their concentration, or water hardness, should be kept at a minimum level, typically <1 mg/l expressed as $CaCO_3$.
- **pH**
  Water treated with sodium hypochloride should have a final pH between 5.0 and 7.0. A too low or a to high pH could influence the formation of sediments and also lead to corrosion of the equipment.
- **Turbidity**
  Undissolved particles, if present in water at a too high level, would unavoidably be found in the soluble coffee powder and eventually lead to an unclear cup.
- **Off-tastes/off-odours**
  Water must be free of any off-taste and off-odour, which could alter the final cup quality and be hasardous to the consumer.

Reference analytical methods to ensure the quality and safety of water can be found, for instance, in the WHO guidelines (WHO, 2003) or in specific handbooks (APHA, 1998). Numerous kits are also commercially available (Hach, 1997; Merck, 2004).

## PACKAGING RAW MATERIALS

Soluble coffee is sold in very different types of containers (e.g. cans, glass or plastic jars, soft packs, paper sachets) whose quality, as for green coffee, should be checked at reception. In this case, it is essential to develop good partnership and trust with the packaging supplier, in order to limit as much as possible the number of analyses. However, the following key parameters are usually checked on a well defined proportion of the incoming materials:

- **Appearance**
  The material must be clean, without defect. Cans must not be blown or present any trace of rust, glass jars must be free from cracks and glass splits.
- **Print/Colour**
  The text, illustration and colours on the package must match the reference. Deviations have a direct negative impact on the consumer.

- **Length/Width/Diameter/Thickness**
  Deviations from the specification may greatly influence the machinability of the material and lead to problems during packing operations and/or to untight packages.
- **Grammage**
  The ratio weight over surface, or grammage, is an important parameter for flexible materials. It is an indirect indicator of thickness and is measured gravimetrically.
- **Off-odour**
  Olfactory checks, or sniff tests, are carried out to detect any off-odours released from paper, cardboard and plastic packaging materials. Off-odours include residual solvents, inks, adhesives, monomers, additives, phenols and derivatives. Electronic noses are a possible alternative for the rapid measurements (Zhang, 2003).
- **Lacquer**
  Cans and bottoms are covered by a protective lacquer. The porosity of the lacquer layer should be checked by treating the can with a specific reagent (e.g. $CuSO_4$), or by using an enamel rater measuring the conductivity of the lacquer.

## GOOD MANUFACTURING PRACTICE (GMP)

GMP is a collection of generally recognized rules, procedures and practices that together provide guidelines stating what is and what is not acceptable in the food industry. It is a prerequisite for ensuring the manufacture of safe products of consistent quality. The following points are of particular importance in a soluble coffee factory:

- **Zoning**
  All facilities must separate process areas according to the different hygiene levels required. Typically, internal areas should be divided into medium hygiene zones (warehouse, green coffee cleaning, roasting, extraction) and high hygiene zones, (drying, filling, packaging) depending on the risks of contamination of the product and on the type of cleaning. The authorised movement of people, equipment, raw materials and products between the zones should be strictly defined.
- **Cleaning-procedures**
  Each establishment must have a cleaning plan, including specific instructions for cleaning of each piece of equipment, installation, zone, etc. A soluble coffee factory should exclusively rely on dry cleaning using mainly vacuum cleaners. Wet cleaning should be required for the coffee extraction area only. As coffee powder is sticky, controlled wet cleaning can be occasionally accepted in specific areas (e.g. filling). In this case, the minimal amount of water should be used, followed by rapid drying.
- **HACCP**
  Hazard Analysis at Critical Control Point (HACCP) must be carried out to prevent problems that could affect consumer safety (see corresponding chapter).
- **Foreign-materials**
  Foreign materials, such as hard and sharp metal, glass or wood fragments may cause injury to the consumer and must be completely avoided. The risks associated with whole bean and R&G coffee are very limited (except for “Greek” coffee”), as the brew preparation system acts as a filter of foreign materials. The risks associated with ready-to-drink beverages such as soluble coffee are obviously higher. The main issue is certainly when soluble coffee is packed in glass jars. In this case, X-ray sensors (e.g. suppliers names) should be installed to detect and remove glass splinters. The same devices can also detect the presence of metal pieces.
- **Pest-management**
  All efforts must be made to prevent products from being contaminated by pests or their excrements. Creating efficient physical barriers (proofing of the buildings), keeping the

facilities clean, well in order and in good condition, as well as strict adherence to GMP are certainly the best preventive measures against pests. Despite this, it is nevertheless possible that pests find their way into the processing areas. Therefore, control measures must be implemented in order to detect and eliminate them. Physical control measures including rodent baits, glue traps, insectocutors, or capture (birds, cats) must be privileged. Chemical control measures include insecticides, rodenticides (placed inside baits), pheromone traps, or bird repellents. Clearly, any pesticide treatment should be done by exception. In this situation, all food materials, packaging, and surfaces in contact with food must be protected from contamination. There must be no pests, but no uncontrolled use of pesticides.

- **Storage**
  All precautions must be taken to preserve the quality of green coffee during storage. Official guidelines on good storage practice were published (ECC, 2002; ITC 2002). ISO, in particular, has issued a guide specific for coffee in bags (ISO, 1986). The two main points are preventing re-wetting, which could lead to mould formation and possible occurence of mould toxins, and cross-contamination with any type of foreign materials and chemicals. Only coffee with a maximum moisture content of 12.5% can be safely stored. The beans and the bags should not present any sign of pest infestation or other contamination. In addition, the bags should be clean, free of off-odour and without hole. They must be tightly closed and placed on pallets to protect the coffee against contamination and water damage. The storage area must be leakproof, clean and well-ventilated. The inside temperature and relative humidity should be as low and constant as possible to prevent re-wetting the coffee.

## HAZARD ANALYSIS AND CRITICAL CONTROL POINTS (HACCP)

Ideally, HACCP should be implemented throughout the supply chain. A HACCP plan has already been proposed from the coffee field to the storage and transport of green coffee, mainly in the perspective of the occurrence of OTA (Frank, 1997 & 1999). Several CCP's were identified including the drying rate of coffee, the sorting of green coffee and the elimination of defects, and the moisture of green coffee. Fortunately, the HACCP analysis by the soluble coffee manufacturer is facilitated as the roasting process eliminates all biological hazards (e.g. pathogenic microorganisms). Therefore, he only needs to evaluate the significance of the chemical and physical potential hazards reviewed in this paper.

## GOOD LABORATORY PRACTICE (GLP)

The accuracy and reliability of analytical results is only achieved by the implementation of good laboratory practice. GLP principles are presented in many official documents (CITAC, 2002; Garfield, 2000; ILAC, 2001; ISO 1999). The key points are:

- The analytical methods used must be properly validated (EURACHEM, 1998). The validation process aims at establishing the performance characteristics of a method (e.g. working range, trueness, precision) and proving its fitness for purpose.
- The performance of the equipment and analytical methods must be monitored by applying an internal control plan (ICP). ICP aims at ensuring in the long run the functionning of the equipments according to their specification and the performance of the methods during their routine use. Alternative rapid methods must be properly adjusted, calibrated and validated against reference methods. The sample sets used for the calibration and the validation must be independent with a sufficient number of samples, well representative of the products to be analysed.

- The laboratory should participate in proficiency tests (EURACHEM, 2000). These tests aim at measuring the performance of a laboratory. The Food Analysis Performance Assessment Scheme in UK organises regular tests for the analysis of caffeine, OTA and acrylamide in green and roasted coffee (www.fapas.com).

## CONCLUSION

Keeping consumer's trust is essential. This is achieved by delivering day after day a product with consistent quality. However, quality is not a matter of chance. It is made by people and is the result of deliberate actions. It is a joint effort by all the key players of the coffee production-to-consumer chain. It is certainly worth implementing and running quality assurance programmes, as quality is, at the end, a competitive advantage.

Analytical methodologies are one of the main tools of quality assurance. However, the controls must not be blindly organized. The type of parameter analysed and the frequency of analysis must be selected on a risk evaluation basis. They can range from a simple annual monitoring of the green coffee in order to ensure that everything is under control, to one analysis per batch of finished product to guarantee compliance with regulations, up to an on-line continuous monitoring during production to ensure product consistency.

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# Evaluation of Chemical Composition and Processability of Defective Beans

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## SUMMARY

An ICO coffee quality improvement program is currently revisiting the minimum standard for export quality with a view to propose harmonized and robust assessment method of coffee defects. In the context of coffee manufacture, the occurrence of defects in coffee has a known impact on cup quality and processability. The objectives of this work were therefore to evaluate the global industrial quality of single defects through an in-depth evaluation of their composition and processability behavior. As methodology, a bulk *Robusta* coffee was sorted into perfect and six different single defects. Extensive chemical and processability evaluations were carried out. As results, defective coffee beans could be grouped into three classes on the basis of their composition (1) coffees minor chemical alterations compared of perfect beans (i.e. Infested, Broken) (2) coffees with major chemical alterations compared to perfect beans (i.e. Brown, Half Black, Black) (3) coffees with non coffee bean compounds (i.e. Cherry). As concerns roasting behaviour and extraction level, coffees from Class (1) presented similar performance to that of perfect beans. On the contrary Class (2) and Class (3) were more difficult to roast and exhibited lower extractability. From these results a redefinition of defect classes in term of similar impact on quality and processability of coffee could be envisaged, broken beans and infested beans having lower impact on the global quality of coffee compared to black, brown or cherry beans.

## RÉSUMÉ

Un programme de l'ICO visant à redéfinir objectivement et de manière unique les standards de qualité du café ou encore les défauts a été initié. Dans l'industrie du café, l'impact des défauts du café sur la qualité à la tasse et les performances industrielles est bien connu. L'objectif de ce travail était d'évaluer la qualité globale des différents type de défauts en relation avec leur composition chimique.

Un lot de Robusta a été trié et réparti en fractions correspondant respectivement aux grains sans défauts et six différents défauts simples. Sur la base de la composition chimique des grains sans défauts, trois classes ont pu être identifiées (1) ceux de composition proche (i.e. grains infestés, cassés) (2) ceux de composition altérée (i.e. grains bruns, noirs) (3) ceux contenant du matériel exogène (i.e. grain avec cerise). Pour ce qui est du comportement de torréfaction et du niveau d'extractibilité les cafés de Classe (1) étaient comparables aux grains sans défauts, alors que les Classes (2) et (3) avaient des comportements altérés. Une redéfinition des classes de défauts sur la base de qualité industrielle globale pourrait être envisagée.

## BACKGROUND

In order to ensure and improve green coffee procurement for all parties, quality standards have been defined nationally for local use and worldwide for export. These standards essentially based on moisture level, defect types and counts vary from one country to another and may differ whether it concerns local or export quality. An ICO coffee quality improvement program is currently revisiting the minimum standard for export quality with a view to proposing harmonized and robust assessment methods. Among the proposals is the reduction in defect classes, the weighing of defects as replacement of defect count. In the context of coffee manufacture, the occurrence of defects in coffee has a known impact on in-cup quality and possibly on processability. Some previous studies characterized the chemical specificities of defective beans (Mazzafera, 1999; Franca et al., 2005) but none considered the impact of defect in the global scope of industrial quality of green coffee. The objective of this work was therefore to investigate the impact of single defects on the chemical composition, as wells as the processability.

## MATERIAL & METHODS

### Green Coffees

A bulk of dry processed *Robusta* coffee from Indonesia was sorted for specific defects. The bulk and seven qualities issued from the sorting were obtained for further evaluation (Figure 1).

Bulk Coffee | Perfect Beans | Broken Beans | Infested Beans | Brown Beans

- Bulk beans
- Perfect beans
- Infested beans
- Broken beans
- Half black beans
- Black beans
- Brown beans
- Cherry beans

**Figure 1. Coffee samples obtained by sorting of a bulk Indonesian Robusta coffee. Pictures of a selection of the coffees analysed in this study.**

### Chemical Analyses

Initial green coffee humidity was determined on a coffee grinding (d'~1mm) dried at 102°C for 4 hours. All green coffees were further finely ground in a Freezer Mill 6800 to obtain a final average particle size d' below 100 µm. These ground materials were used for extensive chemical analyses.

- ***Lipids*** – Lipids were extracted in a soxhlet with petroleum ether at 70°C. The solvent was evaporated and residual fat measured gravimetrically.
- ***Minerals*** – Ash content was evaluated after total combustion of organic matter at 550°C. The residual ash was determined gravimetrically. In addition, an extensive element profiling was obtained by ICP-MS or ICP-AES.
- ***Carbohydrates*** – Total and free carbohydrate profiles were established by anion exchange chromatography (AEC) equipped with an electrochemical detector (PED). The

preparation and separation are summarised in Table 1. Carbohydrates were converted into anhydrosugars and clustered into key polysaccharide families.

- ***Proteins*** - Total nitrogen was evaluated using a LECO FP-200 protein/nitrogen analyser. Total and free amino acid profiles were established by reverse phase chromatography (RP) with fluorimetric and UV detections. The preparation and separation are summarised in Table 2. Amino acids levels were further converted into total protein values, as well as clustered in amino acids classes.
- ***Chlorogenic acids & Caffeine*** - Chlorogenic acids and caffeine were extracted at 40°C in 70% aqueous methanol. Chromatographic separation was carried out on Spherisorb ODS1 (250mmx4) using a water/acetonitrile/ phosphate gradient. UV detection was performed at λ 320nm for chlorogenic acids and λ 274 nm for caffeine.
- ***Trigonelline*** - Trigonelline was extracted in HCl 0.1N at 100°C for 5min. Trigonelline was quantified by HPLC using Nucleosil 50-5 column and UV detection (λ 274 nm).
- ***Organic acids*** - Organic acids were extracted in distilled water at 70°C. Separation of organic acids was carried out by ionic exchange chromatography using a Dionex column AS11 eluted with a NaOH gradient (0.5-25 mM / 35 min) and conductimetry detection.
- ***pH & Acidity*** - Ground green coffee (10 g) was extracted for 5 minutes in boiling water (500 mL). Initial pH was measured. The titratable acidity was evaluated at pH 6.6 and 8 using NaOH 0.1 M and expressed in meq/kg dry green coffee.

**Table 1. Preparation and analysis of free and total carbohydrates.**

| | **Free carbohydrates**<br>Mannit, Ara, Gal, Glu, Xyl, Man, Fru, Suc | **Total carbohydrates**<br>Mannit, Rha, Ara, Gal, Glu, Xyl, Man, Fru, Agal, AGlu |
|---|---|---|
| Pre-hydrolysis ($H_2SO_4$ 26N, 20°C/ 2h) | No | Yes |
| Hydrolysis ($H_2SO_4$ 2N, 100°C/ 3h) | No | Yes |
| Neutralisation with NaOH | No | Yes |
| AEC Dionex / separation column | PA100 column | PA1 column |
| Separation medium | Water | Water/NaOH/Na acetate |
| PED with NaOH 0.5N | Yes | Yes |

**Table 2. Preparation and analysis of free and total amino acids.**

| | **Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val** | **Met, Cys** | **Trp** |
|---|---|---|---|
| Oxidation with formic acid | No | Yes | No |
| Hydrolysis with HCl (6N, 110°C/24h) | Yes | Yes | No |
| Saponification with NaOH | No | No | Yes |
| Derivatisation with AccQ-Fluor ™ | Yes | Yes | No |
| HPLC Waters / AccQ ?Tag $C_{18}$ column | Yes | Yes | Yes |
| Fluorimetric detection | Yes | Yes | UV |

**Processability Evaluation**

Processability of coffee was evaluated for roasting behaviour and extraction behaviour.

- ***Roasting*** – 200 g of green coffee ($M_{GC}$) were roasted in a Neotec roaster. Roasting temperature was kept constant (i.e. 240°C) and roasting duration adapted to reach CTN

85. Organic losses (OL) were calculated from final coffee weight ($M_{R\&G}$) and initial ($H_2O_{GC}$) and final ($H_2O_{R\&G}$) humidity of the coffee.

$$OL(\%d.b.) = \frac{M_{GC}x(100 - H_2O_{GC}) - M_{R\&G}x(100 - H_2O_{R\&G})}{M_{GC}x(100 - H_2O_{GC})}x100$$

- ***Extractability*** – 10 g of roasted coffee ($m_{R\&G}$) were suspended in 200 mL of distilled water (V (mL)). The suspension was heated up to boiling and maintained as such for 5 minutes. The suspension was cooled down and passed through a paper filter. The extractability on roasted coffee ($Y_{R\&G}$) was calculated from the concentration (C (%)) of the filtrate. The extractability was calculated on green coffee basis ($Y_{GC}$) taking into account the organic losses.

$$Y_{R\&G}(\%d.b.) = \frac{VxC}{m_{R\&G}x(100 - H_2O_{R\&G})}x100$$

$$Y_{GC}(\%d.b.) = Y_{R\&G}x\frac{OL}{100}$$

## RESULTS & DISCUSSION

### Chemical Composition

This work presents an extensive comparison of the chemical composition of perfect and defective *Robusta* beans (Table 3 to 5). Clear chemical differences were observed between perfect and defective beans and all chemical classes were affected.

***Broken Beans*** are close in composition to perfect beans, although they contain slightly more compounds of low molecular weight. Free carbohydrates (i.e. free glucose, free fructose), free amino acids (e.g. Asp, Glu, Thr), chlorogenic acids (i.e. 5CQA, 5FQA, diCQA), quinic acid and some minerals (i.e. $Fe^{2+}$, $Al^{3+}$) are found in higher amounts.

***Infested Beans*** remain close in composition to perfect beans although they are characterised by lower levels in storage compounds such as lipids and carbohydrates. In particular cell-wall polysaccharides (e.g. arabinogalactans, mannans) and sucrose have decreased most likely metabolised by the infesting organisms. In parallel the content in low molecular weight compounds increases. Free carbohydrates (i.e. free glucose, free fructose), free amino acids (e.g. Asp, Glu, Thr), chlorogenic acids (i.e. diCQA), quinic acid and some minerals (i.e. $K^+$, $Na^+$, $Fe^{2+}$, $Al^{3+}$) are found in higher amounts.

***Brown Beans*** and ***Half Black Beans*** are very close in composition and very far from perfect beans. They are characterised by significant decreases in lipids and in low molecular weight compounds such as sucrose, chlorogenic acids, organic acids (citric, malic) and trigonelline. In parallel increases in proteins (i.e. aliphatic, carboxylic amino acids), in minerals (i.e. $K^+$, $Na^+$, $Fe^{2+}$, $Al^{3+}$) as well as in metabolites resulting from degradation reactions (i.e. free glucose, quinic acid, oxalic acid, phosphoric acid) are observed. Titratable acidity is slightly lowered.

***Black Beans*** present emphasised trends of half black beans. Almost complete degradation of all low molecular weight compounds i.e. sucrose, free amino acids, chlorogenic acids, organic acids (citric, malic) is observed. Trigonelline and caffeine levels are drastically decreased. In

parallel increases in proteins (i.e. aliphatic, carboxylic amino acids), in minerals (i.e. $K^+$, $Na^+$, $Fe^{2+}$, $Al^{3+}$) as well as in metabolites resulting from degradation reactions (i.e. quinic acid, oxalic acid, phosphoric acid) are observed. Titratable acidity is significantly lowered.

**Table 3. Chemical composition of a bulk Robusta coffee and sorted coffee including perfect and defective beans.**

| | | Bulk | Perfect | Broken | Infested | Half Black | Black | Brown | Cherry |
|---|---|---|---|---|---|---|---|---|---|
| | | Composition (% on dry raw material) | | | | | | | |
| H2O | [%] | 11.92 | 11.26 | 9.84 | 9.11 | 9.79 | 11.29 | 10.95 | 11.65 |
| Lipids | [%] | 10.14 | 10.87 | 10.45 | 9.72 | 8.91 | 10.55 | 9.87 | 6.72 |
| Caffeine | [%] | 1.73 | 2.15 | 2.32 | 2.34 | 2.24 | 1.59 | 2.18 | 1.56 |
| Trigonelline | [%] | 0.68 | 0.71 | 0.71 | 0.72 | 0.57 | 0.26 | 0.54 | 0.53 |
| Proteins | [%] | 10.26 | 10.12 | 9.99 | 10.55 | 10.67 | 10.58 | 10.50 | 8.27 |
| *NH4* | [%] | 0.01 | 0.04 | 0.01 | 0.02 | 0.02 | 0.02 | 0.03 | 0.00 |
| *Non polar apliphatic* | [%] | *3.33* | *3.27* | *2.94* | *3.36* | *3.53* | *3.57* | *3.47* | *2.78* |
| *Aromatic* | [%] | *1.25* | *1.24* | *1.26* | *1.29* | *1.29* | *1.22* | *1.24* | *1.01* |
| *Polar uncharged* | [%] | *0.87* | *0.85* | *0.88* | *0.90* | *0.92* | *0.91* | *0.88* | *0.73* |
| *Sulfured* | [%] | *0.39* | *0.39* | *0.39* | *0.41* | *0.41* | *0.42* | *0.40* | *0.32* |
| *Carboxylic* | [%] | *2.97* | *2.90* | *3.03* | *3.09* | *3.08* | *3.11* | *3.09* | *2.23* |
| *Amino* | [%] | *1.44* | *1.44* | *1.48* | *1.49* | *1.40* | *1.33* | *1.39* | *1.18* |
| *Free amino acids* | [%] | *0.49* | *0.31* | *0.44* | *0.40* | *0.30* | *0.04* | *0.27* | *0.07* |
| Carbohydrates | [%] | 49.42 | 52.11 | 52.04 | 46.56 | 49.55 | 50.10 | 50.10 | 43.18 |
| *Arabinogalactans* | [%] | *16.86* | *17.40* | *17.20* | *15.96* | *18.08* | *18.44* | *18.76* | *12.17* |
| *Cellulose* | [%] | *7.50* | *7.59* | *7.65* | *6.76* | *7.48* | *8.02* | *8.80* | *10.96** |
| *Mannans* | [%] | *19.53* | *20.75* | *20.57* | *18.84* | *20.54* | *21.50* | *19.60* | *12.31* |
| *Pectins* | [%] | *1.24* | *1.38* | *1.77* | *1.13* | *1.56* | *1.27* | *1.55* | *1.95* |
| *Xylans* | [%] | *0.21* | *0.22* | *0.15* | *0.17* | *0.20* | *0.20* | *0.27* | *3.31** |
| *Sucrose* | [%] | *3.62* | *4.38* | *4.24* | *3.19* | *1.29* | *0.37* | *0.71* | *1.89* |
| *Free carbohydrates* | [%] | *0.51* | *0.39* | *0.49* | *0.58* | *0.38* | *0.19* | *0.41* | *0.91* |
| Chlorogenic acids | [%] | 6.17 | 9.55 | 10.13 | 9.77 | 7.30 | 1.95 | 6.38 | 5.77 |
| *3CQA* | [%] | *0.52* | *0.80* | *0.78* | *0.75* | *0.59* | *0.25* | *0.58* | *0.45* |
| *4CQA* | [%] | *0.56* | *1.13* | *1.10* | *1.04* | *0.81* | *0.35* | *0.78* | *0.64* |
| *5CQA* | [%] | *2.83* | *4.28* | *4.54* | *4.40* | *2.96* | *0.48* | *2.52* | *2.50* |
| *4,5diCQA* | [%] | *0.55* | *0.84* | *0.88* | *0.85* | *0.67* | *0.47* | *0.63* | *0.54* |
| *3,5diCQA* | [%] | *0.48* | *0.74* | *0.83* | *0.84* | *0.71* | *0.13* | *0.64* | *0.53* |
| *3,4diCQA* | [%] | *0.61* | *0.82* | *0.96* | *0.96* | *0.84* | *0.13* | *0.69* | *0.62* |
| *4FQA* | [%] | *0.12* | *0.17* | *0.17* | *0.15* | *0.13* | *0.00* | *0.07* | *0.00* |
| *5FQA* | [%] | *0.51* | *0.77* | *0.88* | *0.77* | *0.60* | *0.15* | *0.46* | *0.49* |
| Organic acids | [%] | 1.96 | 1.95 | 2.01 | 1.99 | 1.91 | 1.73 | 1.91 | 1.81 |
| *Quinic* | [%] | *0.43* | *0.33* | *0.41* | *0.42* | *0.53* | *0.75* | *0.66* | *0.49* |
| *Formic* | [%] | *0.03* | *0.03* | *0.03* | *0.03* | *0.04* | *0.08* | *0.04* | *0.04* |
| *Malic* | [%] | *0.26* | *0.30* | *0.29* | *0.28* | *0.22* | *0.13* | *0.16* | *0.21* |
| *Oxalic* | [%] | *0.14* | *0.09* | *0.12* | *0.12* | *0.18* | *0.23* | *0.22* | *0.34* |
| *Citric* | [%] | *0.93* | *1.05* | *0.99* | *0.98* | *0.74* | *0.41* | *0.65* | *0.62* |
| *Phosphoric* | [%] | *0.16* | *0.14* | *0.17* | *0.17* | *0.20* | *0.13* | *0.19* | *0.12* |
| Ash | [%] | 4.46 | 4.28 | 4.37 | 4.60 | 4.99 | 4.34 | 4.65 | 5.07 |
| *K+* | [%] | *2.14* | *2.00* | *2.08* | *2.13* | *2.32* | *2.20* | *2.18* | *2.25* |
| *Mg2+* | [%] | *0.20* | *0.20* | *0.19* | *0.18* | *0.19* | *0.17* | *0.19* | *0.17* |
| *Ca2+* | [%] | *0.15* | *0.14* | *0.13* | *0.12* | *0.12* | *0.13* | *0.15* | *0.32* |
| *Na+* | [ppm] | *22* | *19* | *19* | *25* | *57* | *42* | *37* | *29* |
| *Fe2+* | [ppm] | *51* | *43* | *60* | *48* | *68* | *59* | *49* | *83* |
| *Mn2+* | [ppm] | *17* | *16* | *17* | *15* | *17* | *16* | *18* | *25* |
| *Cu2+* | [ppm] | *20* | *21* | *19* | *20* | *22* | *23* | *22* | *20* |
| *Al3+* | [ppm] | *47* | *33* | *71* | *50* | *98* | *61* | *56* | *171* |
| pH | | 6.10 | 6.08 | 6.17 | 6.22 | 6.47 | 6.88 | 6.31 | 6.06 |
| Acidity pH 8(meq/kg) | | 123 | 122 | 118 | 122 | 99 | 44 | 103 | 88 |

***Cherry Beans*** are clearly different in composition. They are characterised by quantitatively and qualitatively different carbohydrate composition. Total carbohydrate content is lower. Levels of coffee bean carbohydrates (i.e. arabinogalactans, mannans) are lowered whereas those of coffee cherry (i.e. xyloglucans, pectins) increases. $Ca^{2+}$ associated to pectins also increases. Lower contents in all the other coffee bean components (e.g. lipids, proteins, chlorogenic acids, citric acid, malic acid, trigonelline, caffeine) are observed. In parallel increase in free carbohydrates (i.e. free glucose, free fructose), in minerals (i.e. $K^+$, $Na^+$, $Fe^{2+}$, $Al^{3+}$) as well as in metabolites resulting from degradation reactions (i.e. quinic acid, oxalic acid) is observed.

***Bulk Beans*** present an intermediate composition between perfect beans and defective beans.

**Table 4. Detailed carbohydrate composition of a bulk *Robusta* coffee and sorted coffee including perfect and defective beans. Total carbohydrates expressed in anhydrosugars.**

| | | Bulk | Perfect | Broken | Infested | Half Black | Black | Brown | Cherry |
|---|---|---|---|---|---|---|---|---|---|
| | | Composition (% on dry raw material) | | | | | | | |
| Total carbohydrates (anhydro-) | [%] | 49.42 | 52.11 | 52.04 | 46.56 | 49.55 | 50.10 | 50.10 | 43.18 |
| *Mannit* | [%] | *0.21* | *0.21* | *0.23* | *0.21* | *0.22* | *0.23* | *0.21* | *0.13* |
| *Rhamnose* | [%] | *0.36* | *0.37* | *0.36* | *0.33* | *0.42* | *0.37* | *0.44* | *0.39* |
| *Arabinose* | [%] | *4.02* | *4.23* | *4.24* | *3.75* | *4.44* | *4.21* | *4.63* | *3.62* |
| *Galactose* | [%] | *12.84* | *13.18* | *12.96* | *12.21* | *13.64* | *14.22* | *14.14* | *8.55* |
| *Glucose* | [%] | *7.50* | *7.59* | *7.65* | *6.76* | *7.48* | *8.02* | *8.80* | *10.96* |
| *Xylose* | [%] | *0.21* | *0.22* | *0.15* | *0.17* | *0.20* | *0.20* | *0.27* | *3.31* |
| *Mannose* | [%] | *19.53* | *20.75* | *20.57* | *18.84* | *20.54* | *21.50* | *19.60* | *12.31* |
| *Fractose* | [%] | *0.27* | *0.19* | *0.23* | *0.30* | *0.18* | *0.08* | *0.18* | *0.47* |
| *Galacturonic acid* | [%] | *0.88* | *1.01* | *1.12* | *0.81* | *1.14* | *0.90* | *1.10* | *1.55* |
| *Glucuronic acid* | [%] | *0.00* | *0.00* | *0.28* | *0.00* | *0.00* | *0.00* | *0.01* | *0.00* |
| *Sucrose* | [%] | *3.62* | *4.38* | *4.24* | *3.19* | *1.29* | *0.37* | *0.71* | *1.89* |
| Free carbohydrates | [%] | 0.514 | 0.387 | 0.493 | 0.575 | 0.383 | 0.190 | 0.405 | 0.910 |
| *Mannit* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Ara* | [%] | *0.003* | *0.002* | *0.003* | *0.002* | *0.004* | *0.002* | *0.005* | *0.007* |
| *Gal* | [%] | *0.053* | *0.073* | *0.066* | *0.039* | *0.023* | *0.016* | *0.051* | *0.059* |
| *Glu* | [%] | *0.175* | *0.106* | *0.181* | *0.220* | *0.163* | *0.085* | *0.155* | *0.371* |
| *Xyl* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Man* | [%] | *0.016* | *0.014* | *0.016* | *0.014* | *0.014* | *0.006* | *0.012* | *0.008* |
| *Fru* | [%] | *0.268* | *0.192* | *0.228* | *0.299* | *0.179* | *0.080* | *0.183* | *0.465* |

**Table 5. Detailed protein composition of a bulk *Robusta* coffee and sorted coffee including perfect and defective beans. Total amino acids expressed in protein equivalent.**

| | | Bulk | Perfect | Broken | Infested | Half Black | Black | Brown | Cherry |
|---|---|---|---|---|---|---|---|---|---|
| | | Composition (% on dry raw material) | | | | | | | |
| Proteins | [%] | 10.26 | 10.12 | 9.99 | 10.55 | 10.67 | 10.58 | 10.50 | 8.27 |
| *NH4* | [%] | *0.01* | *0.04* | *0.01* | *0.02* | *0.02* | *0.02* | *0.03* | *0.00* |
| *Ala* | [%] | *0.45* | *0.44* | *0.44* | *0.46* | *0.50* | *0.46* | *0.46* | *0.38* |
| *Arg* | [%] | *0.60* | *0.59* | *0.61* | *0.62* | *0.59* | *0.58* | *0.60* | *0.47* |
| *Asp* | [%] | *1.01* | *1.01* | *1.05* | *1.09* | *1.09* | *1.00* | *1.01* | *0.82* |
| *Cys* | [%] | *0.25* | *0.25* | *0.24* | *0.26* | *0.26* | *0.26* | *0.25* | *0.20* |
| *Glu* | [%] | *1.95* | *1.89* | *1.98* | *2.00* | *2.00* | *2.10* | *2.08* | *1.41* |
| *Gly* | [%] | *0.56* | *0.55* | *0.57* | *0.58* | *0.60* | *0.59* | *0.55* | *0.47* |
| *His* | [%] | *0.25* | *0.25* | *0.26* | *0.26* | *0.25* | *0.25* | *0.24* | *0.23* |
| *Ile* | [%] | *0.38* | *0.38* | *0.39* | *0.39* | *0.41* | *0.43* | *0.42* | *0.34* |
| *Leu* | [%] | *0.85* | *0.82* | *0.85* | *0.86* | *0.88* | *0.92* | *0.90* | *0.67* |
| *Lys* | [%] | *0.59* | *0.60* | *0.61* | *0.61* | *0.56* | *0.51* | *0.55* | *0.48* |
| *Met* | [%] | *0.15* | *0.14* | *0.15* | *0.15* | *0.16* | *0.16* | *0.15* | *0.13* |
| *Phe* | [%] | *0.56* | *0.54* | *0.56* | *0.57* | *0.57* | *0.59* | *0.58* | *0.45* |
| *Pro* | [%] | *0.55* | *0.54* | *0.55* | *0.55* | *0.55* | *0.59* | *0.57* | *0.46* |
| *Ser* | [%] | *0.49* | *0.48* | *0.50* | *0.51* | *0.51* | *0.51* | *0.50* | *0.42* |
| *Thr* | [%] | *0.38* | *0.37* | *0.38* | *0.39* | *0.41* | *0.40* | *0.38* | *0.32* |
| *Trp* | [%] | *0.33* | *0.35* | *0.34* | *0.35* | *0.33* | *0.27* | *0.30* | *0.26* |
| *Tyr* | [%] | *0.36* | *0.36* | *0.37* | *0.37* | *0.38* | *0.37* | *0.36* | *0.31* |
| *Val* | [%] | *0.55* | *0.53* | *0.15* | *0.52* | *0.58* | *0.59* | *0.58* | *0.46* |
| Free amino acids | [%] | 0.486 | 0.307 | 0.440 | 0.399 | 0.297 | 0.036 | 0.273 | 0.074 |
| *NH4* | [%] | *0.012* | *0.042* | *0.013* | *0.017* | *0.022* | *0.018* | *0.027* | *0.000* |
| *Ala* | [%] | *0.047* | *0.027* | *0.038* | *0.034* | *0.028* | *0.000* | *0.024* | *0.000* |
| *Arg* | [%] | *0.030* | *0.019* | *0.026* | *0.026* | *0.017* | *0.000* | *0.020* | *0.000* |
| *Asp* | [%] | *0.051* | *0.026* | *0.052* | *0.054* | *0.053* | *0.000* | *0.022* | *0.014* |
| *Cys* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Glu* | [%] | *0.076* | *0.047* | *0.075* | *0.067* | *0.065* | *0.018* | *0.036* | *0.012* |
| *Gly* | [%] | *0.014* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *His* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Ile* | [%] | *0.012* | *0.000* | *0.011* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Leu* | [%] | *0.019* | *0.014* | *0.017* | *0.012* | *0.000* | *0.000* | *0.020* | *0.000* |
| *Lys* | [%] | *0.018* | *0.012* | *0.016* | *0.012* | *0.000* | *0.000* | *0.013* | *0.000* |
| *Met* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Phe* | [%] | *0.034* | *0.019* | *0.032* | *0.029* | *0.019* | *0.000* | *0.017* | *0.000* |
| *Pro* | [%] | *0.028* | *0.018* | *0.024* | *0.020* | *0.014* | *0.000* | *0.017* | *0.035* |
| *Ser* | [%] | *0.028* | *0.016* | *0.023* | *0.022* | *0.014* | *0.000* | *0.013* | *0.000* |
| *Thr* | [%] | *0.079* | *0.039* | *0.081* | *0.081* | *0.053* | *0.000* | *0.033* | *0.012* |
| *Trp* | [%] | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* | *0.000* |
| *Tyr* | [%] | *0.019* | *0.015* | *0.017* | *0.013* | *0.011* | *0.000* | *0.015* | *0.000* |
| *Val* | [%] | *0.017* | *0.012* | *0.016* | *0.012* | *0.000* | *0.000* | *0.016* | *0.000* |

In conclusion, based on the composition three classes of defects can be identified. A discriminant analysis of chemical data is given in Figure 2.

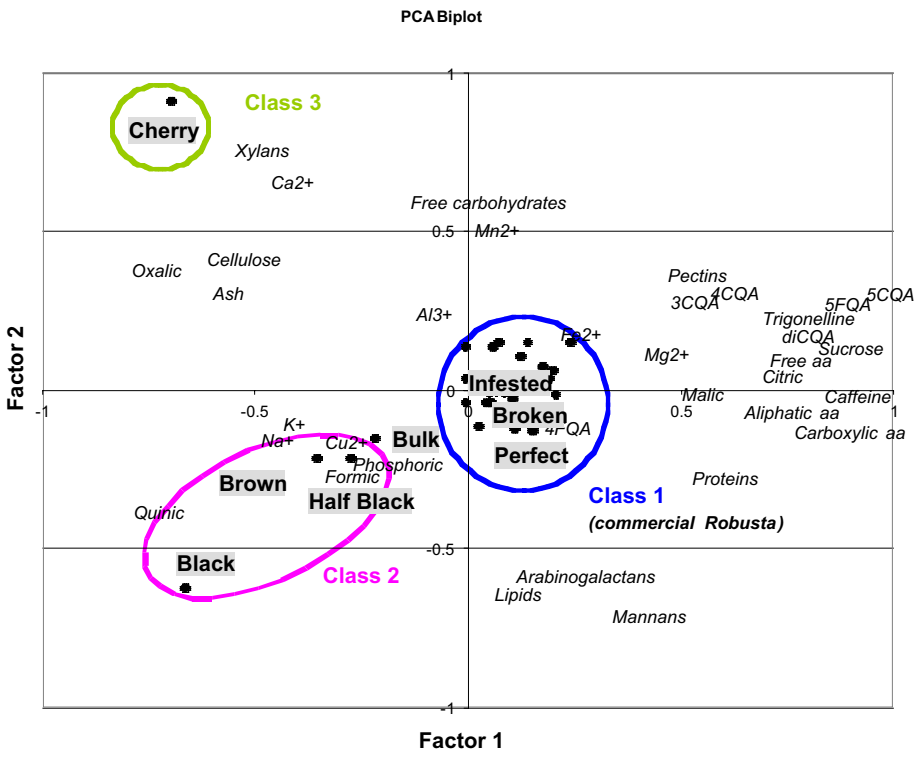


**Figure 2. Discriminant analysis of chemical data obtained for perfect and defective Indonesian *Robusta* and 17 commercial Robusta representative of its chemical diversity.**

1. Coffees, which present limited chemical differences, compared to perfect beans. These coffees are broken beans and infested beans. They also present close composition to that of a representative range of 17 commercial *Robusta* coffees. Based on the comparable chemical composition, these defects should have limited impact on cup quality.
2. Coffees, which present major chemical changes such as reduction in almost all low molecular weight compounds (i.e. sucrose, free amino acids, chlorogenic acids, malic & citric acids) and the generation of metabolites resulting from degradation reactions (i.e. free glucose & fructose, quinic & oxalic acid). These coffees are brown beans, half black beans and black beans. Based on the chemical composition, these coffees are lacking in all key aroma and flavour precursors (Feldman et al., 1969). They will not develop typical coffee aroma (De Maria et al., 1994) and will lack acidity (Ginz et al., 2000).
3. Coffees, which have different chemical composition indicating the presence of non coffee bean compounds (i.e. xyloglucans). These coffees are cherry beans. As class 2, based on their composition these coffees will not develop the typical coffee aroma and flavour.

**Processability Evaluation**

Table 6 compares the processability characteristics of perfect and defective coffees. Clear differences in roasting behaviour and extractability could be observed depending on the type of defect.

***Roasting duration*** to reach CTN 85 was 310s for perfect beans. Half black beans, brown beans and cherry beans presented a slower colour development (i.e. > 370s) compared to all other coffees. The lengthening in roasting duration can be explained by the reduction sucrose, which was shown as being the source of colour during roasting (Charurin et al., 2002).

***Organic losses*** were 7.1% for perfect beans. Half black beans and cherry beans presented very low organic losses (i.e. < 3.7%) whereas broken beans exhibited very high organic losses (i.e. 10.3%).

***Extractability*** of perfect beans was about 29% on R&G. Better extractabilities (+1 pt) were measured for broken and infested beans. These defects modify the cell-wall integrity, further improving the extraction of high molecular weight compounds (e.g. carbohydrates). Lower extractabilities were observed for all other coffees: half black beans (–2 pt), brown beans (–3 pt), black beans (–5 pts) and cherry beans (–7 pts). This loss in extractability performance mainly results from the decrease in low molecular weight compounds. Similar trends can be observed for extractability based on green coffee.

**Table 6. Processability of bulk coffee and sorted coffees including perfect and defective beans evaluated at lab-scale.**

| | | Bulk | Perfect | Broken | Infested | Half Black | Black | Brown | Cherry |
|---|---|---|---|---|---|---|---|---|---|
| | | Composition (% on dry raw material) | | | | | | | |
| **Roasting** | | | | | | | | | |
| *Roasting duration (s)* | [s] | 355 | 310 | 315 | 340 | 370 | 305 | 420 | 370 |
| *Colour (CTN)* | [CTN] | 84 | 84 | 85 | 86 | 83 | 87 | 85 | 83 |
| *H2O $_{R\&G}$ (%)* | [%] | 0.8 | 1.1 | 0.9 | 0.8 | 0.8 | 0.6 | 0.5 | 0.6 |
| *Organic losses (%)* | [%] | 7.3 | 7.1 | 10.3 | 6.6 | 3.7 | 5.6 | 6.6 | 3.3 |
| **Extraction** | | | | | | | | | |
| $Y_{R\&G}$ (% d.b.) | [%] | 26.7 | 28.9 | 29.8 | 29.5 | 27.4 | 22.3 | 25.9 | 21.6 |
| $Y_{GC}$ (% d.b.) | [%] | 24.8 | 26.8 | 26.7 | 27.6 | 26.4 | 21.1 | 24.2 | 20.9 |

## CONCLUSIONS

This work has established clear differences in chemical composition and processability behaviour of defective coffee beans compared to perfect beans. The redefinition of defect classes in term of similar impact on the global quality of coffee could be suggested. In this context, the presence of broken beans and infested beans will have lower impact on the global quality of coffee compared to black, brown or cherry beans.

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# Caffeine Content Distribution among *Mascarocoffea* Species in Madagascar

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## SUMMARY

The wild coffee species of Madagascar and neighbouring islands in the *Mascarocoffea* section has been believed to be caffeine free during almost a century with the exception of *Coffea mauritiana* which present a very low content (0.07%) (Bertrand, 1905; Rossi, 1933; Chevalier, 1938; Chassevent et al., 1974). Unequivocal evidence for the presence of caffeine was published for the first time by Clifford et al. in 1991.

The investigation of caffeine content was extended to 90 populations within 47 species distributed in the 6 botanical subsections of *Mascarocoffea*. No caffeine or only trace was found in 58 populations represented by 30 species distributed in the 6 subsections. 28 populations of 17 species distributed in the 6 subsections also contained very low amount (0.01-0.20% dry weight). While only the *Verae* subsection with all populations of *C. kianjavatensis* and *C. lancifolia* presented caffeine content (0.20-0.80%) as high as some wild east African species such as *C. eugenioides* and *C. sessiliflora*. Nevertheless, no caffeine was found in all the 18 leaf samples of 10 species including *C. kianjavatensis* and *C. lancifolia*.

These results completed the distribution of caffeine content in *Mascarocoffea* previously reported by Rakotomalala et al. (1992). Other particular traits variation in *Mascarocoffea* species will be discussed in relation with caffeine content distribution.

## INTRODUCTION

The section *Mascarocoffea* groups the wild coffees of Madagascar and the neighbouring islands (Chevalier, 1938). It belongs to the subgenus *Coffea* (Bridson, 1987) and contains about 60 taxa divided into 7, then 6 botanical subsections (Leroy, 1967; Charrier, 1978).

The beans of *Mascarocoffea* species have been believed to be caffeine free during almost a century with the exception of a very low content (0.07%) reported in the beans of *Coffea mauritiana* (Bertrand, 1905; Rossi, 1933; Chevalier, 1938; Chassevent et al., 1974). Unequivocal evidence for the presence of 0.55-0.81% caffeine in the beans of population A213 of *C. kianjavatensis* was published for the first time by Clifford et al. in 1991. Rakotomalala et al. (1992) established the presence of caffeine and theobromine in beans from populations of *C. lancifolia* (A320, A405) and from *C. kianjavatensis* (A213, A602). Nevertheless these authors did not find caffeine in the beans of *C. homollei* which belong to the same *Verae* subsection. The investigation of caffeine content has been extended by Rakotomalala (1993) on 56 populations of *Mascarocoffea* distributed in the 6 subsections. No caffeine or only trace amounts were found in few of them with the exception of the two above-mentioned species.

In order to complete the *Mascarocoffea* caffeine content information, 72 populations were analysed at the R&D Center of UCC Ueshima Coffee Co, Japan; using other sample extraction and analysis methods. We now present the synthesis of results from 90 populations obtained by Rakotomalala and by UCC R&D Center.

## MATERIALS AND METHODS

### Plant Material

The collection of *Mascarocoffea* at FOFIFA's Experimental Station of Kianjavato (Madagascar) contains about 3000 trees of 117 populations belonging to 56 species. Some accessions are not described yet. The coffee trees are maintained under a converted natural forest with duplication in the field without shade.

The 90 samples analysed are distributed in the six botanical subsections (Table 1).

**Table 1. Repartition of the samples analysed into the different subsections.**

| | Subsections | | | | | |
|---|---|---|---|---|---|---|
| | *Garcinioides* | *Millotii Complex* | *Multiflorae* | *Subterminales* | *Verae* | *Mauritianae/ Humblotianae* |
| Nb samples | 13 | 18 | 32 | 18 | 8 | 1 |
| Nb species | 4 | 11 | 11 | 8 | 3 | |
| Undescribed taxa | 1 | 1 | 7 | 5 | 1 | |

### Rakotomalala Method

Grinding and $H_2O$ extraction of beans and GC analysis for caffeine were as described in Vitzthum et al. (1974). Preparation and analytical reverse-phase HPLC was performed as described in Rakotomalala et al. (1992).

## R & D CENTER UCC METHOD

### Sample preparation

1g of wet processed green bean was extracted with 50 ml of boiling deionized water. The solution was filtered through filter paper, which was washed 2 times with deionized water into a 50 ml volumetric flask and made up to volume after cooling to room temperature.

### HPLC analysis

A Cosmosil 5C-18 analytical column (6 x 150 mm, 5 µm packing, Waters) protected by a guard column packed with C-18 (4.6 x 10 mm, 5µm, Waters) was used for the separation. Standard of caffeine was prepared in methanol. The mobile phase was 0.2M perchloric acid buffer/methanol (2:8 v/v) with a flow rate of 1 ml/min at room temperature. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 270 nm. The compounds were identified by their retention times, chromatographic comparisons were based on the external standard method. The method for the determination of moisture content is as defined in ISO6673 [1].

[1] ISO6673- 1983 (E). Green coffee determination of loss in mass at 105°C

## RESULTS

Caffeine content for all the 90 populations ranged from 0 (not detected) to 0.80% dw [2]. The frequency distribution is very dissymmetric (Figure 1).

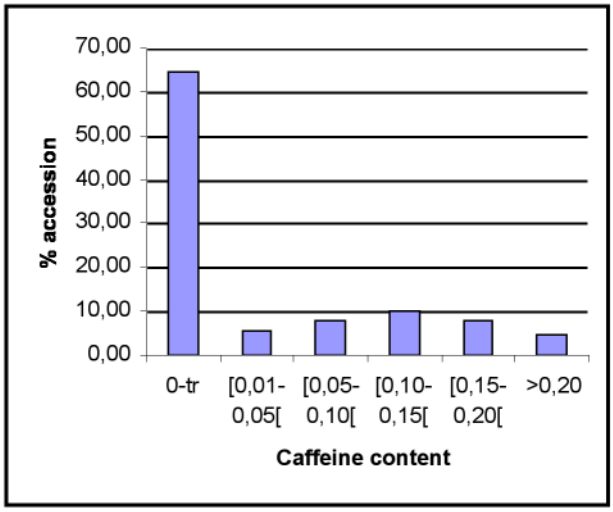



**Figure 1. Caffeine content distribution of 90 populations of *Mascarocoffea.***

No caffeine or only trace was detected in the beans of 58 populations represented by 30 species distributed in the 6 botanical subsections.

28 populations of 17 species distributed in the 6 subsections contained very low amount (0.01-0.20% dry weight).

Only 4 populations presented slightly high caffeine content (0.20-0.80%).

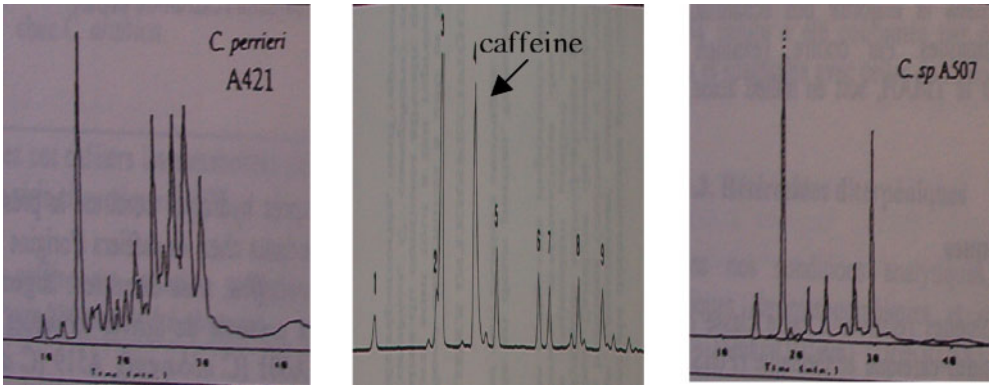
When looking at each subsection, the results show that:

i. all species from the *Garcinioides* subsection (*C. dubardi*, *C. tetragona*, *C. hemii*, *C. mogeneti*) are caffeine free excepted the undescribed taxa A969 which is probably a natural hybrid;
ii. for the *Millotii complex*, 15 populations out of 18 are caffeine free. The remaining three (*C. farafanganensis*, *C. millotii* A222, *C. ambodirianensis* A572) contain very low amounts (0.03-0.05%);
iii. 18 out 32 populations represented by 6 species (*C. perrieri*, *C. resinosa*, *C. vianneyi*, *C. sakarahae*) and some undescribed taxa such as A525, A818, A820, belonging to the *Multiflorae* subsection contain low but perceptible amounts (0.02-0.24); while the other 14 are caffeine free;
iv. for the *Subterminales* group, 14 populations out of 18 are caffeine free. The remaining four (*C. boiviniana*, *C. jumellei*, *C. sakarahae*, *C. pervilleana*) range from 0.01 to 0.21;
v. for the *Verae* subsection, all populations of *C. kianjavatensis* and *C. lancifolia* presented caffeine content (0.20-0.80%) as high as some wild east African species such as *C. eugenioides* and *C. sessiliflora*; while the three populations of *C. homollei* (A574, A743, SZ108) are caffeine free;
vi. *C. mauritiana* A1025 was the only one sample analyzed within the *Mauritiana-Humblotiana* group. The 0.04% caffeine amount detected is similar to the previous reported result.

[2] dw = dry weight

## DISCUSSION

The absence of caffeine observed may sometimes rise from the use of inadequate extraction and detection methods. Rakotomalala (1993) used alcohol/water following by chloroform separation for his investigation. The simplified extraction method applied at the UCC R&D Center could have limited yield loss and allowed to detect caffeine at very low content (<0.01%). Thus, some UCC R&D Center results differed from especially those of Rakotomalala for very low amounts. However, the chromatographic identification of the caffeine by the retention time only, applied by R & D, is not sufficient for many *Mascarocoffea* species, because of the complexity of their chromatograms (Figure 2). Other compounds having the same retention time (depending on the chromatographic conditions) could make interference with the caffeine and could give wrong evaluation. At least checking the spectrum of the pick to be analyzed is needed all the time. Nevertheless, for the majority of the samples analyzed by either method, the main difference was that R & D obtained low caffeine amounts when Rakotomalala detected traces only.




*C. perrieri* *C. canephora* *C. sp. A507*

**Figure 2. HPLC chromatograms of two *Mascarocoffea* species and *C. canephora.***

The synthesis of our results indicates that:

- Within each botanical group, most of taxa are caffeine free, excepted for the *Mauritianae – Humblonianae* subsection where only one species was available; variable amounts of caffeine content are also detected for each group. All these groups present a very high morphological and physiological diversity with the exception of the *Garcinioides* (Charrier, 1978).
- For the *Garcinioides* subsection, the single population with caffeine appears to be a hybrid between C. *dubardi* and another unknown *Mascarocoffea* species. In fact, natural cross pollination between different *Mascarocoffea* species is a common event (Charrier, 1978). Previous study (Rakotomalala 1993) also showed that the taxa from the *Garcinioides* group are highly homogenous for chlorogenic acids isomers and do not contain any rare chemical compounds.
- The *Millotii complex* species present a high morphological diversity. Peculiar chemical compounds such as glycosidic diterpene, *p*-coumarate and sinapate derivates are distributed with variable rate among *Millotii*'s populations; however it appears that the caffeine content distribution is slightly homogenous.
- High morphological and physiological diversity between populations was recorded for the *Multiflorae* subsection. The taxa of this group contain various peculiar chemical compounds such as glycosidic diterpene, *p*-coumarate, sinapate, mono-, di- and tri-methoxycinnamate derivates. The repartition (presence/absence) and the amount of these compounds are unequally distributed within the species of the group. Caffeine content appears also to be unequally distributed in this group.

- The highest morphological and physiological diversity between populations was recorded for the *Subterminales* subsection. Taxa from this group contain also many unequally distributed peculiar chemical compounds such as melilotoside, *p*-coumarate, sinapate, di- and tri-methoxycinnamate derivates. However the group's caffeine variation is slightly low.
- For the *Verae* subsection, which seems slightly homogenous in morphological and physiological point of view, two distinct groups appear: (i) caffeine free group formed by populations of *C. homollei*; (ii) slightly high caffeine content group formed by taxa of *C. lancifolia* and *C. kianjavatensis*. Furthermore, the first group contains *p*-coumarate and tri-methoxycinnamate derivates, while the second contains high theobromine and high sinapate derivates content.
- Finally, for the *Mauritianae-Humblotianae* group, our observation confirms only previous studies.

So, while the complexity of chemical compounds seems correlated to the morphological complexity for the *Mascarocoffea* section, it appears that the caffeine content variation is not correlated to these two traits. Variation of caffeine amounts happen within all each botanical group. Even, sometimes, populations belonging to the same species record different amounts: (i) 0-0.24% for 6 taxa of *C. resinosa*; (ii) 0.13 for the population A602 of *C. kianjavatensis*, while 0.81 for the other population A213.

However, our result confirms the positive correlation between chlorogenic acid (5CQA) and caffeine amounts previously reported (Chassevent et al., 1974; Anthony et al., 1993). The 5CQA values are less than 1.50% for all *Mascarocoffea* taxa with the exception of *C. lancifolia* and *C. kianjavatensis* (3.80-7.50%), which are the only high caffeine content of this group.

Ashihara and Crozier (2001) studying the metabolism of caffeine in various species reported that the low caffeine accumulation in *C eugenioides* is a consequence of a slow rate of caffeine biosynthesis followed by a rapid degradation in leaves. Nevertheless, no caffeine was found in all the 18 leaf samples of 10 species including *C. kianjavatensis* and *C. lancifolia*. Thus, the metabolism of caffeine remains to be an enigma for the *Mascarocoffea* species. The study of this topic is needed as well as the investigation of the metabolism of the coffee peculiar chemical compounds, especially those that affect the beverage quality such as glycosidic diterpenes, theobromine, and those that happen at the end of important metabolism pathways such as theacrine, for the caffeine pathway; melilotoside, mono-, di- and tri-methoxycinnamate, for the chlorogenic acids pathway. Thus, part of the *Coffea* speciation mechanism could be understood and by the way can help for better exploitation and management of coffee germplasm.

Finally, since the detection of perceptible amount of caffeine in the beans of *C. kianjavatensis* (Clifford et al., 1991) the Chevalier's description of *Mascarocoffea* as caffeine free was no longer tenable. Furthermore, when an African species, *C. pseudozanguebariae* was also known as caffeine free (Hamon et al., 1984), it appears that the classification of the subgenus *Coffea* should be revised. The consideration of the chemical compounds diversity including caffeine distribution could be of a help.

## CONCLUSION

To summarize, we can say that the caffeine content determination of *Mascarocoffea* taxa is now more or less complete. A high diversity within and among the botanical subsections defined by Chevalier (1938) is recorded for this trait.

Our synthesis with previous results on (i) morphological classification (Charrier, 1978; Bridson, 1982); (ii) chemotaxonomy (Clifford et al 1991; Rakotomalala, 1993; Rakotomalala et al., 1993 a & b); molecular biology (Cros et al., 1995; Lasherms et al., 1997; Noirot et al., 2003), (iii) particular chemical compounds found in Coffea beans (Rakotomalala, 1993c), and recent knowledge about new African caffeine free species (*C. pseudozanguebariae*, *C. bakossi*), suggest that the review of the subgenus *Coffea* classification, especially those of the *Mascarocoffea*, is needed.

These results are also of help for the exploitation and the management of the *Mascarocoffea* germplasm.

**ACKNOWLEDGMENTS**

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# Applicability of Metal Oxide Sensors for Long Term Measurement of Aroma Concentration and Quality above Coffee Powders

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## SUMMARY

Gas sensors have been widely tested since the nineties to predict the quality of unknown products after a training period with acceptable and not acceptable samples. Most of reported applications screened only a limited number of samples in a relatively short period of time. This study was aimed at screening numerous instant coffee powders characterised by in-jar aromas varying in concentration and quality during a period of 16 months (100 products). Instrumental results (gas sensors and standard gas chromatography) were compared with sensory data.

A good agreement was established between the results from the metal oxide sensors and the results measured by gas chromatography. The FOX 3000 from Alpha M.O.S. was already found to be a reliable and reproducible instrument already in a previous study. Nevertheless, important sensor drifts were observed during the study (base line and sensitivity). However, these drifts could be compensated using built-in calibration procedures. The system could recognise blind samples with a success rate higher than 72%.

Our results showed that the FOX 3000 could be used roughly to predict the concentration of total headspace aroma, and simultaneously the concentration of sulphur compounds in the headspace. This study indicated that the sensors were mainly responding to overall headspace concentration. The information delivered by the sensors on differences in aroma composition was very limited. No correlation with sensory data could be derived from such instrumental analyses up to now. This is not surprising as most key compounds of coffee aroma are present in very low concentrations in the headspace and do not contribute to the signal measured.

## RÉSUMÉ

Les capteurs à gaz ou “nez électroniques” ont été souvent utilisés depuis les années 90 pour prédire la qualité d’échantillons inconnus après avoir entraîné l’instrument à distinguer des échantillons acceptables et inacceptables. Néanmoins, la plupart des applications publiées se réfèrent à un nombre limité d’échantillons analysés et, de plus, sur une courte durée. L’objectif de cette étude était d’analyser un grand nombre de cafés instantanés caractérisés par différentes concentrations, compositions et qualités d’arôme de bocal (une centaine de produits), le tout sur une période de 16 mois.

Les résultats obtenus avec le FOX 3000 se sont révélés en bon accord avec les résultats obtenus par chromatographie en phase gazeuse. Cette étude a confirmé que l’instrument développé par Alpha M.O.S. est fiable dans son fonctionnement et que la reproductibilité des

analyses est satisfaisante. Une dérive importante des capteurs a néanmoins été observée au cours de cette étude mais a pu être corrigée de façon satisfaisante avec les procédures d'étalonnage fournies avec l'instrument. Des échantillons analysés à l'aveugle après 16 mois ont été reconnus avec un taux de succès d'au moins 72%.

Nos résultats ont montré que le FOX 3000 peut être utilisé pour prédire la concentration totale de l'arôme dans l'espace de tête du bocal de façon semi-quantitative, et pour donner simultanément une idée de la concentration des composés soufrés dans l'espace de tête. Cette étude a également montré que les capteurs répondent principalement à la concentration globale de l'arôme et que les informations sur des différences de composition entre les échantillons sont limitées. Aucune corrélation n'a pu être établie à ce jour entre les descripteurs sensoriels de l'arôme et la mesure de l'arôme dans l'espace de tête, que ce soit par GC ou avec les capteurs. Ceci n'est pas surprenant au vu de la faible concentration de la majeur partie des composés odorants-clés de l'arome de café.

## INTRODUCTION

Quality assurance at production floor is the only way of insuring a constant quality of the products delivered to the consumers. Beside physical and chemical controls, sensory procedures play a major role in the final liberation of coffee products, either for roast and ground coffee, or for instant powders. The overall aroma balance of coffee is very difficult to predict based on the chemical composition of the aroma, due to the low concentration of many key aroma compounds and the masking effects between potent odorants of coffee (Vitzthum et al., 1990; Grosch and Blank, 1992; Grosch, 1998; Grosch et al., 1999; Gretsch et al., 1999). Moreover, the techniques used to quantify the aroma fingerprint of a product are by far too complex and time consuming to be applied on a routine basis. However for specific applications, the knowledge of global aroma concentration together with some indications about the content of some tracers of quality would be of great interest for the manufacturer.

"In-jar" aromatisation of soluble coffee is used to deliver a fresh coffee-like aroma to the consumer at first openings of the product. The concentration and the composition of the aroma in the powder headspace are dependent on the aroma source and manufacturing conditions. Therefore, a rapid and simple method to control the global concentration of the aroma in the finished product would be useful at production floor to assure a constant quality of the product.

Gas sensors have been widely tested since the nineties to predict the quality of unknown products after a training period with acceptable and not acceptable samples. However, most of reported applications screened only a limited number of samples over relatively short period of time (Gardini et al., 2000; Bazzo et al., 1998; Schnitzler et al., 2000; Abass et al., 1999; Braggins et al., 1999; Newman et al., 1999; Luzuriaga and Balaban, 1999; Gardner et al., 2000; Ramalho, 2000). This study aimed at screening numerous instant coffee powders with in-jar aroma varying in concentration, composition and quality during a period of 16 months. Moreover, instrumental results (gas sensors and standard gas chromatography) were compared with sensory data.

Since several years, electronic sensors have been tested in our laboratory for their ability to characterise the aroma fraction released from instant coffee aromatised for in-jar aroma (first opening of the package). A first study conducted in 1996 allowed to evidence that conducting polymers were not suited for analysing coffee aroma in finished products. The reason was the high sensitivity of these sensors towards moisture and carbon dioxide, both components always present in much higher concentration than coffee aroma in the powder headspace

(Gretsch et al., 1997). More positive results were obtained with metal oxide sensors. An in depth investigation of the commercial instrument FOX 3000 supplied by Alpha M.O.S. Ltd (Toulouse, France) in 1997 revealed that the apparatus was reliable and could be used to conduct a long term study (Gretsch et al., 1998).

## EXPERIMENTAL

Around 100 instant coffees conditioned in sealed glass jars were analysed for headspace aroma over a period of 16 months. Two product categories were considered (P, R) and 3 variants per category (P1-P3, R1-R3, each variant being further submitted to storage test). These products differed in headspace aroma concentrations and aroma quality already at production. The ca. 100 products were analysed in triplicate taking each time a different sealed jar with the FOX 3000 apparatus equipped with 12 sensors. The samples were also analysed in duplicate from different sealed jars using a reference method based on gas chromatography. Major features of both analytical methods are presented in the Figure 1. The samples were also described by sensory profiling using a trained panel.

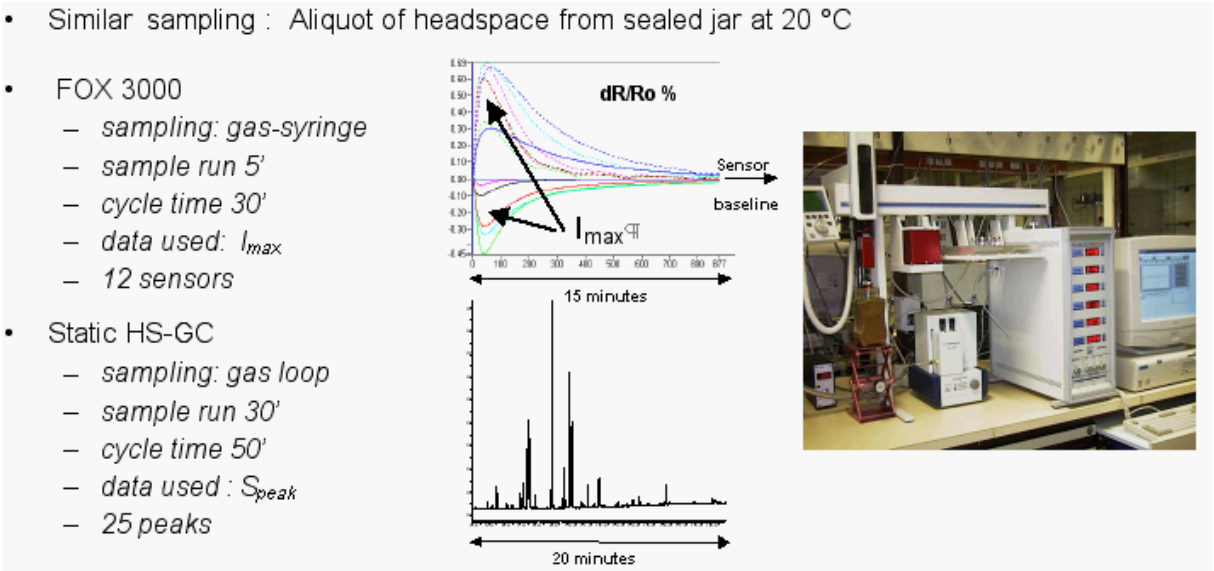


**Figure 1. Comparison of aroma sampling systems, signal measured and data retained for further treatment when using the FOX 3000 or standard gas chromatography**

The calibration of the response over time was performed with external gaseous standards for the analyses performed by gas chromatography. For the metal oxides, we tested two calibration procedures with the supplier. The first one proposed by default in the software used external standards. Pure chemical compounds were dissolved in MCT oil and a set of vials was prepared (one chemical per solution), stored at –20°C and analysed during the study prior to the coffee samples. The second method of calibration used the intrinsic characteristics of the sensors determined simultaneously to the analysis of coffee samples. This approach gave better results and all recognition results presented in this work were obtained with the second method. Both calibration procedures are based on protected software and the user has only access to the raw data.

## RESULTS

The sensor drifts with time and calibration procedures are discussed first. Then the ability of metal oxides to measure aroma concentration is presented, following by the potential of the

system to recognise blind samples. Finally, the discrimination of the samples based on their quality is discussed.

**Sensor drifts and calibration procedures**

The base lines of the sensors exhibited significant drifts during the 18 months of use (Figure 2). The magnitude of the drift depended on sensor. The observed shifts were positive for most of the sensors, except for SY/AA, SY/G and SY/Gh. Sensors presenting highest drifts were SY/gCT*, SY/LG and SY/AA for chamber 1, and PA2*, T30/1* and T70/2* for chamber 2. Four of these sensors (*) were strongly influenced by the change of laboratory premises.

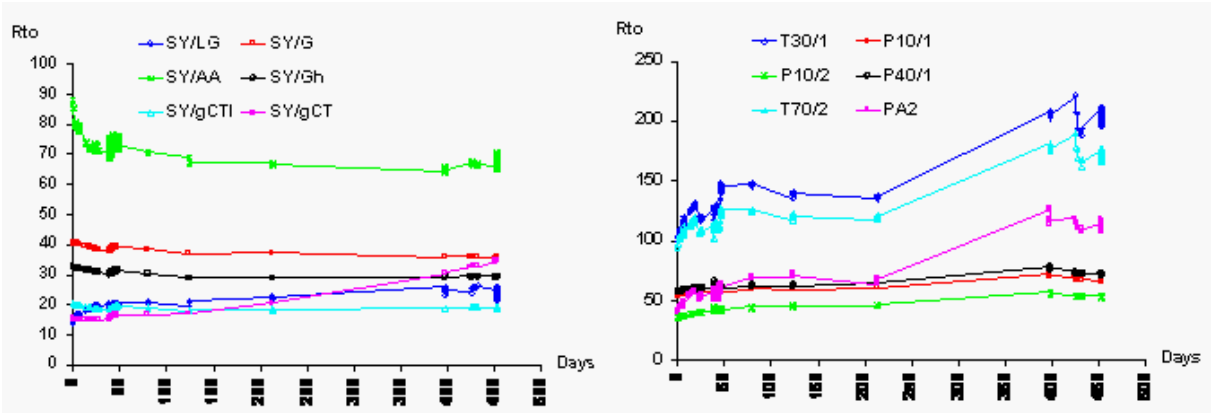


**Figure 2. Evolution of sensor baseline between September 1998 and February 2000. Left: 6 sensors of chamber 1, Right: 6 sensors of chamber 2. Data points represent period of analyses with the instrument. Notice that the laboratory changed premises between days 220 and days 400**

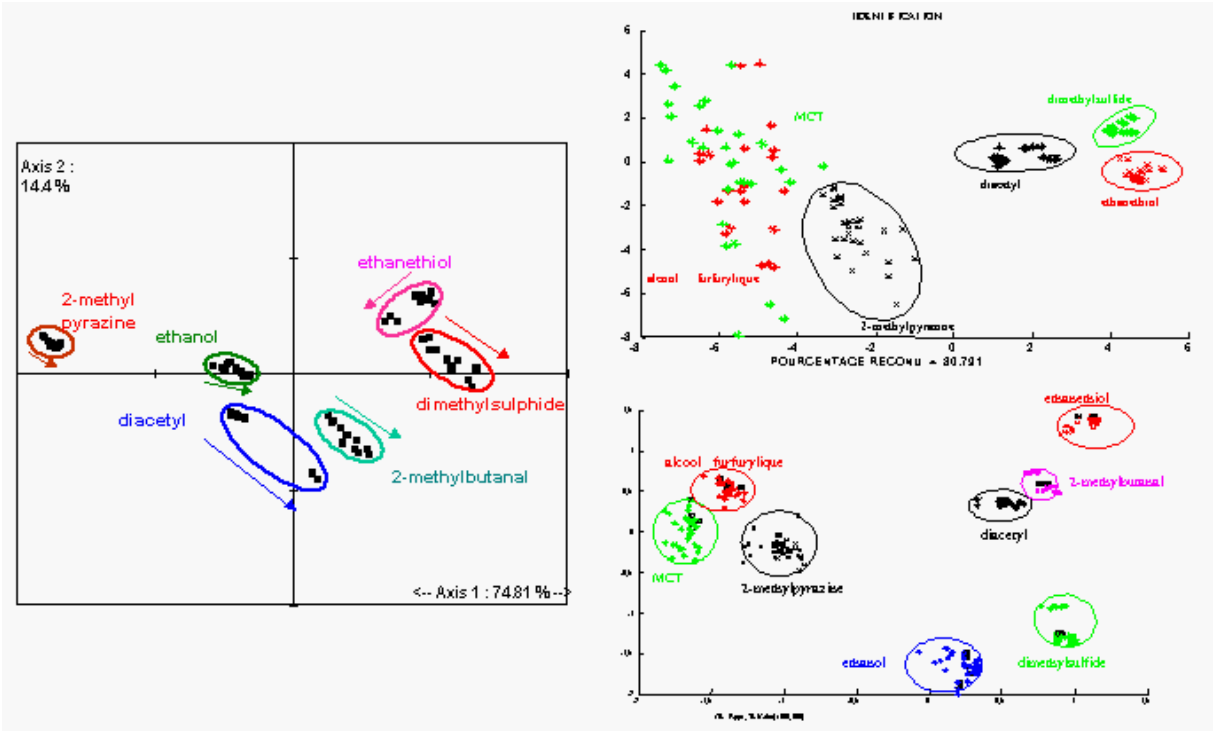


**Figure 3. Comparison of raw data (left) with calibrated data (right) for standard solutions in MCT oil (top right: calibration using 2-methylbutanal and ethanol, bottom right: calibration using intrinsic parameters of sensors)**

These base line drifts were accompanied by changes in sensor response during the study. Moreover the magnitudes of changes were different depending on the standard measured, as shown in Figure 3. Using the built-in calibration procedures, the first two days of analyses were taken as learning days (the system was told about the link between results and chemical compound, and a model was built using a Discriminant Factor Analysis). Samples belonging to the remaining 9 days of analyses were considered as unknown and projected in the model built with the learning period. The success rate for classifying unknown samples passed from 78% on raw data to 80-89% using the external calibration and to 100% using the internal calibration. The internal calibration was indeed the most efficient. It has not only the advantage of liberating the user from additional analyses using chemical standards, but also of providing a better correction for sensor drifts (see also Tan et al., 1998 for external calibration example).

**Assessment of aroma concentration in the headspace of the coffee powders**

To establish better links between global headspace intensity and the response of the sensors, a few coffee powders were coated at pilot scale with an increasing amount of aromatised oil, resulting in an increasing concentration of in-jar aroma. The sensor responses to concentration were either linear (e.g. SY/G, SY/Gh) or followed a power function of concentration (P10/2, P40/1), as shown in Figure 4 (left graph). Similar relations were searched in the long-term study, knowing that the in-jar aroma concentration of the 6 products varied between 100 ppm and 400 ppm. A discriminant analysis was performed to see which sensor would better predict the global concentration in the jar using the data from the whole study. The result evidenced that P10/2 and SY/LG responses presented the best fit with respectively, global aroma and global sulphur concentration in the jar. The relations between sensor response and ppm HS are represented in the Figure 4 (right graphs). The dispersion of the points could be explained by the jar-to-jar variation for one quality (GC and FOX data are obtained from different sealed jars, see also Table 1), and to a lesser extent by the differences in relative aroma composition between products. These results indicate that the FOX could be used to provide a rough quantification of in-jar aroma concentration.

**Recognition of unknown samples**

The coffees analysed were issued from two categories of products (P, R) and 3 variants per category were analysed (P1-P3, R1-R3). Using the internal calibration based on the intrinsic characteristics of the sensors measured during the analyses of the coffees, various models were built by Alpha M.O.S. depending on the level of discrimination desired. Results from the first 50% of analyses were used to build the models; the latter were then validated with remaining results (Figure 4). This study showed that the FOX could discriminate between different products, as long as the built model was restrained to the subclass considered. However, when considering the whole set of results, the discrimination was reduced somehow due to the relatively high dispersion of data, in regard to the distances between the groups.

These models were used to recognise blind samples reanalysed at the end of the evaluation period. The samples reanalysed belong to variants P1, P2 and P3 (model B in Figure 4). When considering first P or R categories (model A), the blind samples were attributed to P products with 72% success. The product variants were then calculated for both types of products categories P and R, using respectively models B or C. Although a better score was found for the P variants (Table 1), the score obtained for R variants evidenced significant overlapping. Interestingly, the product variant inside a category was more easily solved than the product category. This confirms the high correlation of sensor response with headspace global concentration as shown in the table below.

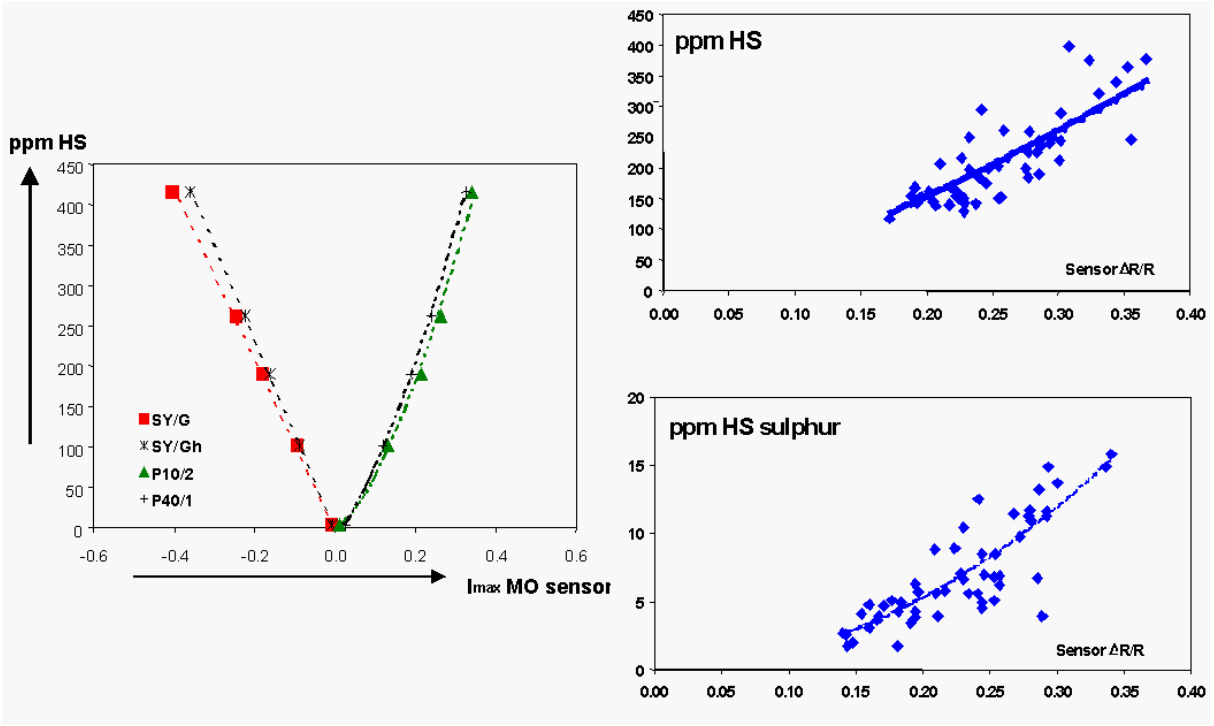


**Figure 4. Response of metal oxide sensors to increasing concentration of in-jar aroma as measured in ppm by GC. Left: Samples produced at pilot scale. Correlation between in jar aroma concentration and sensor response for top right: P10/2sensor and global carbon response (FID detector). bottom right: SY/LG sensor and global sulphur response (FPD detector)**

**Discrimination of samples based on their quality**

When looking at overall evolution of samples during storage, a good agreement was observed between GC and FOX data (Figure 5). However, this evolution was very different from the one drawn with sensory data. P3 variant was more different from the other two variants when considering instrumental data, but P1 variant was more different from the two others when looking at sensory data. Moreover based on FOX and GC data, the magnitude of change during storage was larger for P3, although sensory results exhibited a larger change for P1.

**Table 1. Successful rate of unknown samples towards product categories and variants. Global headspace concentration as measured by gas chromatography, considering all analyses during 17 months**

| | Products P (model B) | | | Products R (model C) | | |
|---|---|---|---|---|---|---|
| **Product** ppm aroma | **P1** 192+/-46 ppm | **P2** 234+/-15 ppm | **P3** 341+/-62 ppm | **R1** 198+/-62 ppm | **R2** 152+/-20 ppm | **R3** 205+/-59 ppm |
| **P1 (blind)** | **75%** | 25% | 0% | 34% | **58%** | 8% |
| **P2 (blind)** | 6% | **94%** | 0% | **79%** | 21% | 0% |
| **P3 (blind)** | 0% | 15% | **85%** | 38% | 8% | **54%** |

When combining sensory and FOX or GC data, no correlation between quality and sensor response could be established up to now. Using a simple principal component analysis, we could see that sensory descriptors were orthogonal to the sensor or GC variables, meaning no correlation at all (Figure 6). This confirms that compounds in high concentration in the headspace above the powder are not reflecting aroma intensity and quality in the case of coffee.

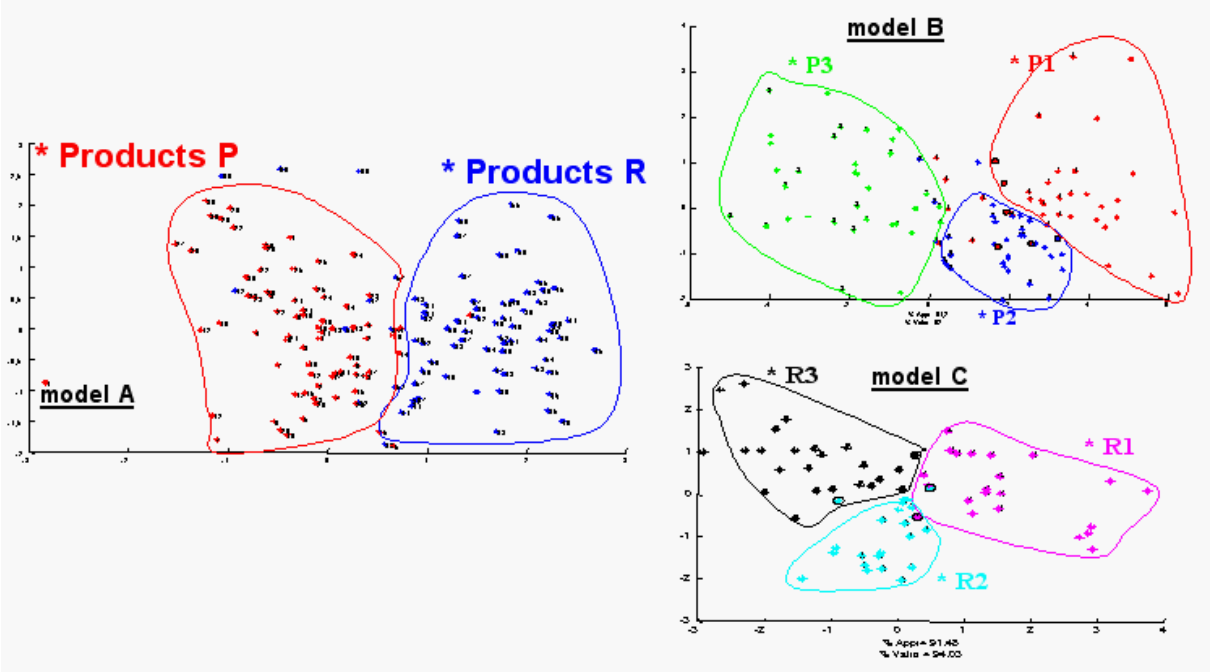


**Figure 4. Models built to discriminate between samples: left: products P and R (learning score 97.4%, validation score 94.5%); top right: variants of products P (learning score 91.2%, validation score 92%); bottom right: variants of products R (learning score 91.5%, validation score 94%)**

## CONCLUSION

The FOX 3000 from Alpha M.O.S is a reliable instrument when operated in a controlled environment (air quality and regulation of the moisture level in the carrier gas). The reproducibility between analyses run within a day lies in the range of a few percents. A good agreement was established between the results obtained with the metal oxide sensors and the results measured by gas chromatography (overall concentration).

Significant drifts of sensor baseline and response were observed over a 16-month period of use, stressing the need for a good procedure of calibration. The recent method developed by the supplier and based on internal calibration using the physical characteristics of the sensors gave better results than the first one available, based on external chemical standards. The system could recognise blind samples with a success rate higher than 72%.

This extensive study revealed that the sensors were mainly responding to overall headspace concentration, when measuring in-jar aroma above coffee powders differing in concentration, composition and aroma quality. The information delivered by the sensors on differences in aroma composition was very limited. No correlation with sensory data could be evidenced, confirming that compounds in high concentration in the headspace above the powder are not reflecting aroma intensity and quality in the case of coffee. However our results indicate that the FOX could be used to provide a rough quantification of in-jar aroma concentration.

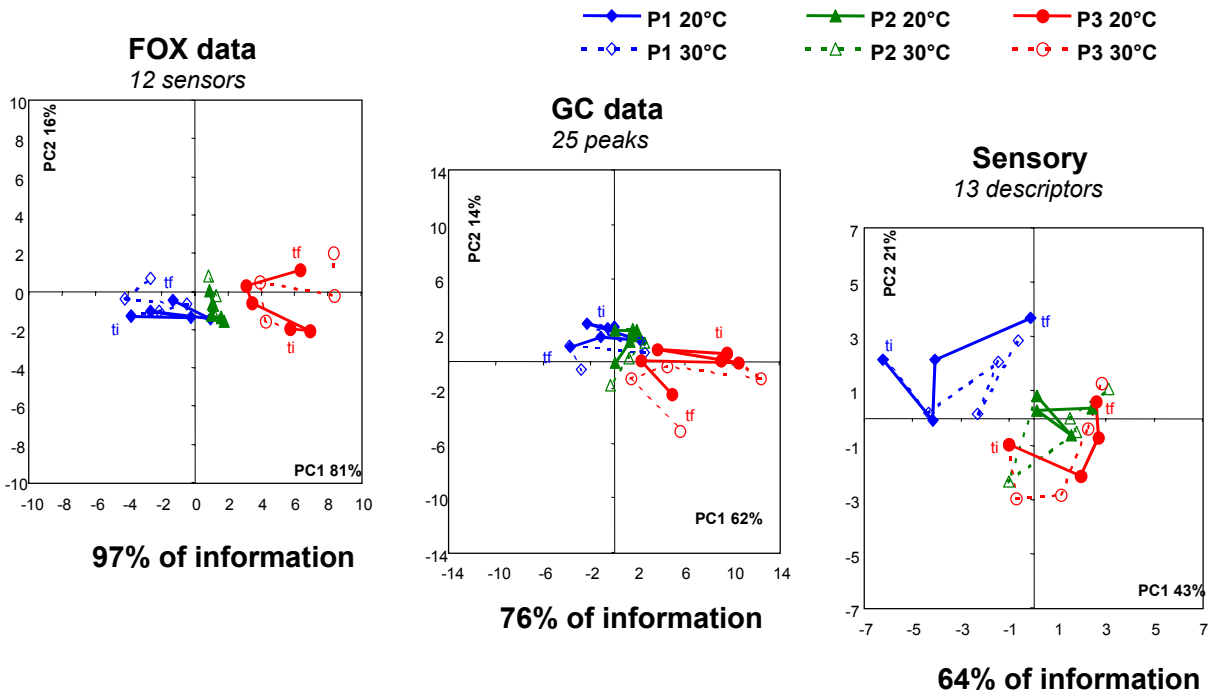


**Figure 5. Evolution of P samples during storage. Comparison of Principal Component Analysis obtained from instrumental (GC, FOX) and sensory analyses**

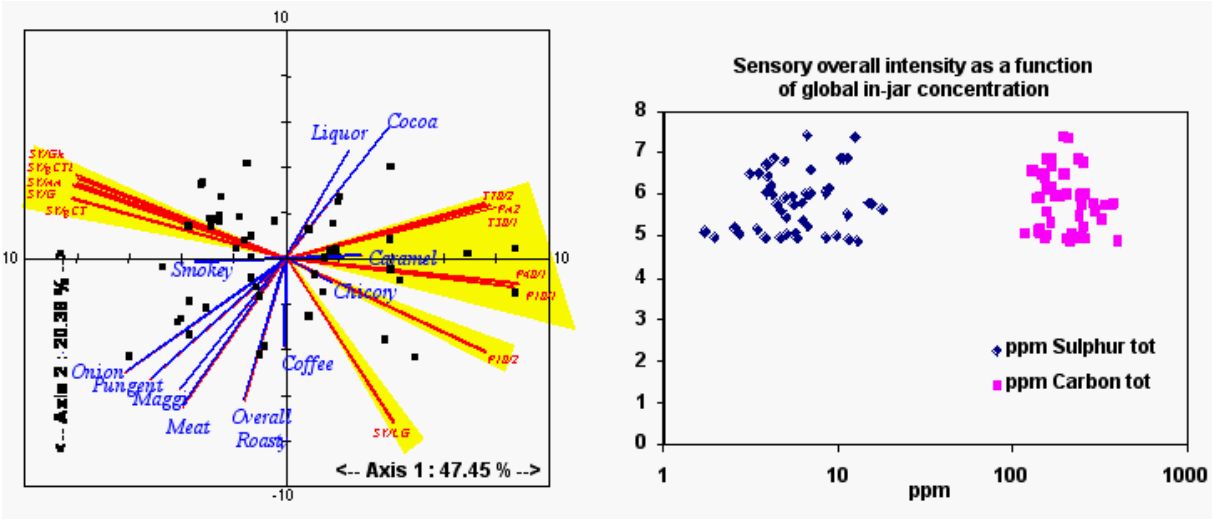


**Figure 6. Principal Component Analysis obtained on FOX and sensory data (left). Plot of sensory overall intensity towards headspace concentration**

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# Raman Spectroscopy as a New and Rapid Method for Green and Roasted Coffee Traceability

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## SUMMARY

Coffee authenticity has always been a challenging problem for the analysts, who have to discover adequate procedures to detect different forms of adulteration. The price paid for coffee depends on coffee varieties(arabica/robusta), the processing conditions (dry/wet) and the altitude where coffee is grown. High Grown arabica is considered of better quality and is the most expensive. It is sold at 2-10 times the price of robusta (according to their geographical origin). Consumers are increasingly willing to pay a higher price for coffee produced in socially and environmentally responsible ways, and in reputable appellations or high altitude. They want to know the origin and the conditions where coffee is grown and to be in position to make informed choices about their coffee. A continuing demand exists for rapid, inexpensive and effective techniques for authenticity of coffee. Raman spectrometry combined with chemometric techniques have been studied for this purpose and can be an alternative rapid analytical method for quality control of coffee.

## INTRODUCTION

In actual coffee market crisis, quality is the best guarantee for negotiating a better price. Botanical origin, geographical origin and altitude where coffee is grown are the important criteria of coffee quality. The two species of economic significance in global coffee trade are *Coffea arabica* (arabica) and *Coffea canephora* (robusta). Arabica are considered better and command a higher price. Altitude at which coffee is grown is increasingly used as a quality qualification for coffee. High grown coffee exhibits superior sensory properties, there is a growing market and has a price differential. It is therefore necessary that proper analytical methods are available to distinguish high grown arabica coffee from others.

## MATERIALS AND METHODS

Two kinds of samples are used in this study: collection from worldwide samples sampled in Belgium from 2 green coffee importers (EFICO and SUPREMO) and from Belgian Coffee Roaster's Association. This collection is constituted by 300 hundred of coffee samples from 40 different countries in which 220 arabica, 78 robusta and 2 liberica. The second collection is constituted by fifteen samples of green coffee (arabica fully washed) from different regions of Rwanda. Small by size but pedologically and climatically diverse, Rwanda offers interesting possibilities for studying the influence of environmental conditions on quality of coffee. Climatic point of view, Rwanda can be subdivided into 2 great regions: The mountainous area in the west and centre of Rwanda with altitude ranging from 1700 to 2500m above sea level, an average temperature of 17°C and annual rainfall between 1300 and 2200 mm. The eastern region is characterized by an altitude ranges from 1300 to 1500m, an average temperature of 21°C and annual rainfall varies between 850 and 1000 mm. This

poster deals with the ability of FT-Raman spectroscopy in discrimination of coffee from different regions of Rwanda (Figure 1 and Table 1).The altitude measurement data of the various points of sampling were collected by means of GPS receiver (Garmin). The Raman spectra of coffee oil were acquired on Perkin-Elmer NIR-FT-Raman spectrophotometer 2000R equipped with a Nd-YAG laser (1064 nm) and an InGaAs detector commanded by Spectrum for Windows software. The spectral data were obtained at a wavenumber resolution of 4 cm-1 and at nominal laser power of 800 mW. Principal component analysis (PCA) was used for classification of robusta in different samples of coffee. Chemometric analysis was made with Unscrambler software 7.6 (CAMO, Norway).

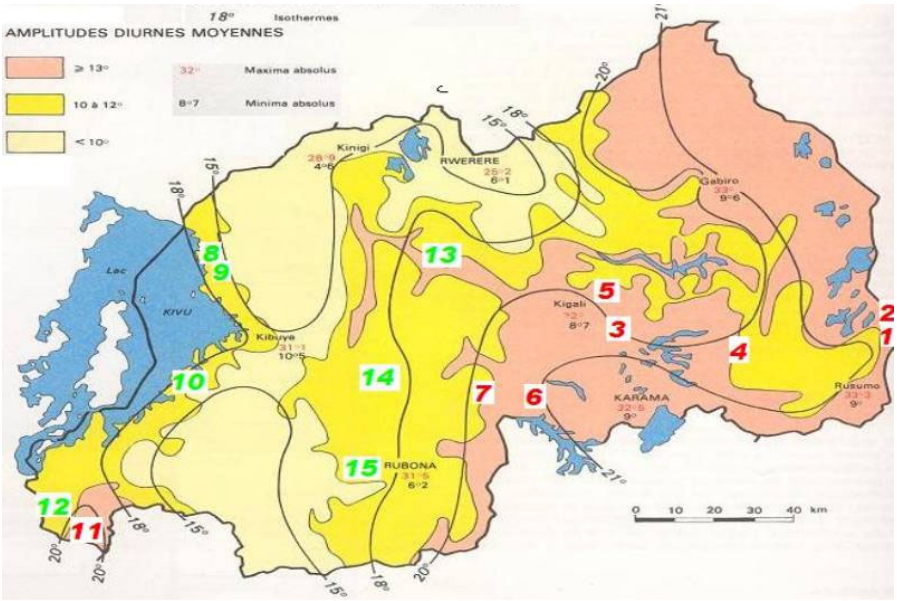


**Figure 1. Rwanda map and distribution of sampling regions.**

**Table1 . Characterization of Samples analyzed.**

| N° | PROVINCE-DISTRICT | REGION | ALTITUDE (m) | RAINFALL (mm) | CODE |
|---|---|---|---|---|---|
| 1 | KIBUNGO-NYARUBUYE | EAST | 1312 | 1-901** | E1(1300) |
| 2 | KIBUNGO-NYARUBUYE | EAST | 1312 | 1-901 | E1(1300A) |
| 3 | KIGALI-NGALI-BICUMBI | EAST | 1503 | 1-901 | E2(1500) |
| 4 | KIBUNGO-KIGARAMA | EAST | 1612 | 902-1073 | E2(1650) |
| 5 | KIGALI-NGALI-GASABO | EAST | 1750 | 902-1073 | E3(1750) |
| 6 | KIGALI-NGALI-NGENDA | EAST | 1470 | 1-901 | E4(1470) |
| 7 | GITARAMA-NTONGWE | EAST | 1500 | 902-1073 | E5(1500) |
| 8 | GISENYI-KAYOVE | WEST | 1792 | 1239-1375 | W1(1800) |
| 9 | GISENYI-KAYOVE | WEST | 1920 | 1239-1375 | W1(1920) |
| 10 | KIBUYE-RUSENYI | WEST | 1637 | 1239-1375 | W2(1640) |
| 11 | CYANGUGU-BUGARAMA | WEST | 1100 | 1375-1545 | W3(1100) |
| 12 | CYANGUGU-GASHONGA | WEST | 1755 | 1375-1545 | W4(1750) |
| 13 | RUHENGERI-NYARUTOVU | WEST | 1820 | 1239-1375 | W5(1825) |
| 14 | GITARAMA-MUHANGA | WEST | 1840 | 1239-1375 | W6(1840) |
| 15 | BUTARE-MARABA | WEST | 1800 | 1239-1375 | W7(1800) |

***Sprinkled and artificial irrigation coffee plantation (NKUBIRI Farm)*

## RESULTS & DISCUSSION

Raman spectrum of arabica samples presents 2 specific peaks at 1567 and 1478 $cm^{-1}$, which are absent in other species (robusta, liberica). Molecule responsible of these differences is isolated in unsaponifiable fraction of coffee oil and characterized. The Raman spectrum of

standard of kahweol (sigma) was compared to coffee oil spectra. These scattering signals are assigned to C = C vibrations in kahweol molecule.

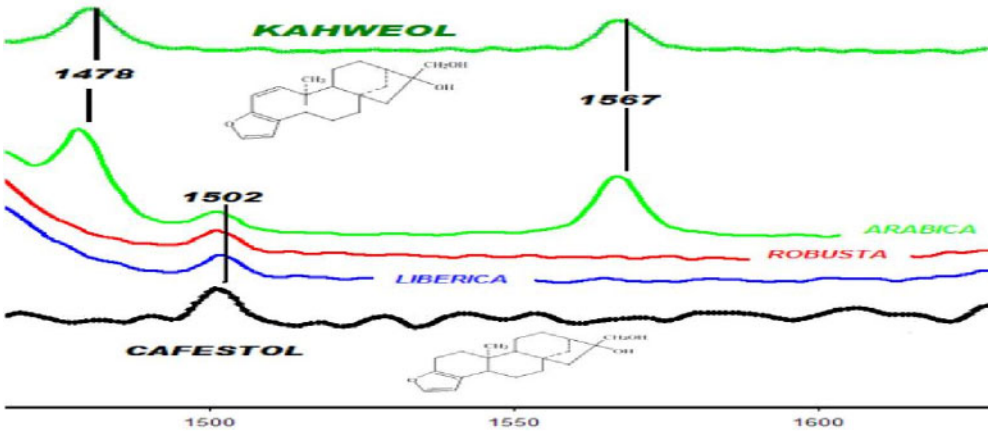


**Figure 2. Raman spectra of coffee lipid fraction, Kahweol and Cafestol.**

On the basis of Raman spectra of 300 samples of green and roasted coffee, a principal component analysis (PCA) was performed and the results allow a total distinction between arabica and the two low coffee quality species: robusta and liberica. Coffee from Australia and Yemen show the lowest intensities of Kahweol peaks and are situated on left side of the Figure 3 while coffee from Kenya and Jamaïca BM have the highest intensities.

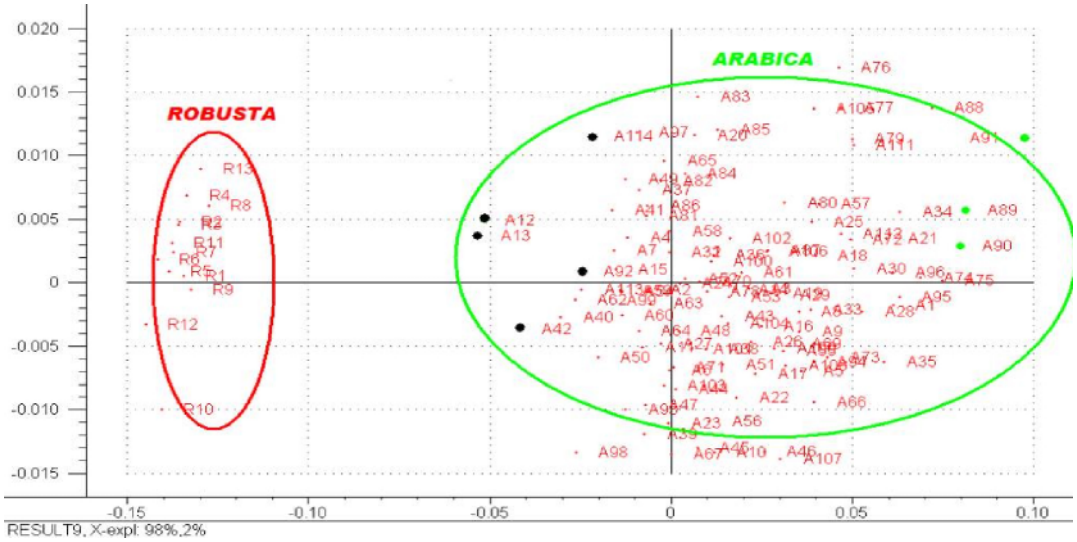


**Figure 3. PCA scores plot of analyzed samples.**

Using coffee samples from different regions of Rwanda, satisfactory classification of coffee samples in two groups was obtained using PCA (Figure 4). The first group is constituted by coffee from West of Rwanda (highlands regions) and the second by coffee from East (lowlands regions). For the same altitude, the content of kahweol is positively correlated to water disponibility (rainfall) (Samples 1, 2, 6 and 11). There is a good correlation between the height of Kahweol peaks and microclimate annual mean temperature where coffee is grown ($R^2$ = .883). These results are in accordance with the observations of Levinsohn et al. (1993), Mc Gavey and Croteau (1995) and Munné-Bosch et al. (2000, 2001) in other plant species.

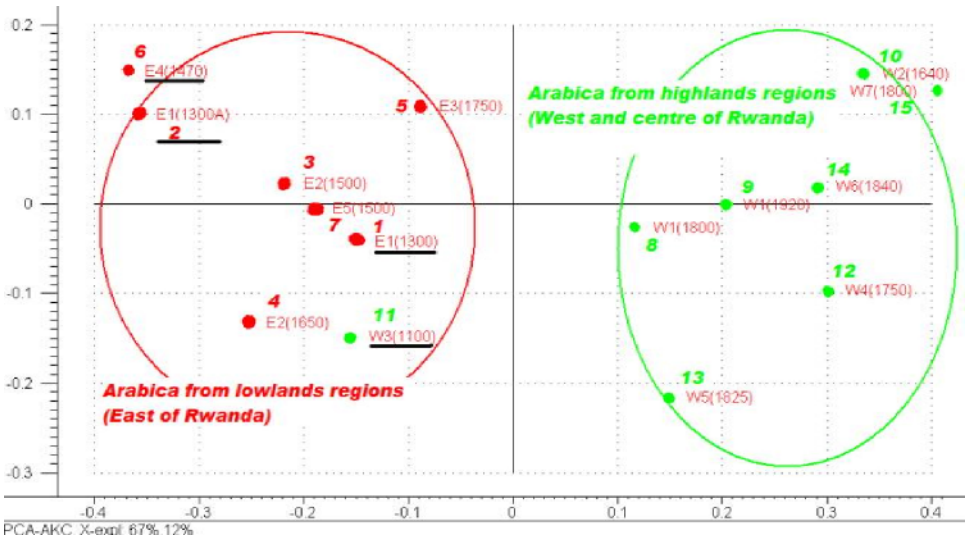


**Figure 4. Rwandan coffee classification according regions.**

Contrary to literature, the proportion of kahweol is not affected by green coffee processing, decaffeination and roasting conditions. This technique can be used in botanical origin authentication of regular or decaffeinated green or roasted coffee.

## CONCLUSION

Raman Spectroscopy can be a useful method in coffee quality control especially in the area of authentication. Kahweol content in arabica coffee is greatly dependent on the climatic conditions where coffee is grown. It can bee used to in authentication of strictly high grown coffee (SHG), a growing market which has a price differential. For Rwanda coffee sector, it is important to regionalize coffee quality management and to set up marketing strategies in order to export coffee from some regions at a premium price in speciality coffee market.

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# Assessment of the Contribution of New Aroma Compounds Found in Coffee to the Aroma of Coffee

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## SUMMARY

Using gc/olfactometry and gc/ms analysis of coffee aroma fractions that were isolated by different mild isolation techniques (e.g. nitrogen stripping, SPME) 6 new sulfur compounds could be detected, 4 of which were positively identified as 3-mercapto-2-butanone, 3-mercapto-2-pentanone, 2-mercapto-3-pentanone and 4-methoxy-2-methylbutan-2-thiol for the first time in coffee. Quantitative analysis of these 4 sulfur compounds in various coffee brews revealed for 3-mercapto-2-pentanone, 2-mercapto-3-pentanone and 4-methoxy-2-methylbutan-2-thiol contents that are significantly above their thresholds (in water). Several mixtures that were formulated with the 3 thiols 3-mercapto-2-butanone, 3-mercapto-2-pentanone, and 4-methoxy-2-methylbutan-2-thiol were added to a coffee beverage. Sensory evaluations of the spiked coffee beverage against a reference (w/o additions) showed a positive impact of these thiols on the sensory profile of the coffee.

## INTRODUCTION

During the last 10 years, coffee aroma has been extensively studied (e.g. reviews by Vitzthum, 1999; Grosch, 2001). The analytical evaluation of the aroma of roast and ground coffee as well as of coffee brews resulted in the identification of 28 character impact compounds (Grosch, 1999). A series of reconstitution experiments were performed that were based on addition of 23 key compounds to a buffered aqueous solution (coffee brew model) or a water/oil mixture (20/1, v/v; roast and ground coffee model) in concentrations that were determined in coffee brew or roast and ground coffee. The direct sensory comparison (orthonasal evaluations) of the aroma models with the respective coffee products revealed that the former exhibited good coffee aroma (Mayer et al., 2000; Grosch et al., 2000). Similarity of odour profiles of models against the references was rated between 2.0 and 2.6 using a 3-point category scale (0: no similarity to 3: identical to coffee product). This shows that a complete match of the full coffee aroma could not yet be achieved. Audouin (2002) performed similarity ratings with two coffee brew models (containing 26 and 43 key aroma compounds in deodorised coffee base) against coffee brews prepared from several commercial roast and ground coffees as well as soluble coffees. 11 trained panelists rated the 'coffeeness' of these products on a similarity scale of 0 (not coffee) to 9 (similar to real coffee). ANOVA showed that the models received lower scores of 3.9 to 4.4 than the reference coffee brews (scores of 5.33 to 7.11).

The results of the above mentioned reconstitution experiments suggest that not all key aroma compounds have been taken into consideration. Therefore, we investigated the identification of new/additional sulfur compounds in coffee based on the assumption that a few reactive compounds have eluded identification. The assessment of the contribution of four newly identified sulfur compounds to the overall aroma of coffee is reported.

## MATERIALS AND METHODS

### Identification of new sulfur compounds

Sulfur compounds were isolated from roasted and ground coffee (100% Arabica; up to 6 kg) by (A) stripping with nitrogen with subsequent cryo-condensation of volatiles and dilution of condensate in dichloromethane (1/10, v/v); and (B) SPME extraction (30 min, 60°C) from the headspace above freshly ground coffee. The aroma isolates A and B were analysed by means of gc/olfactometry on three different columns. Three assessors were asked to evaluate odour quality and intensity of the aroma active compounds. The most abundant sulfur compounds (detected by all assessors on at least 2 columns) were isolated and purified according to Tominaga et al. (1998). Subsequent positive identification of these compounds was based on retention indices on two to three columns of different polarity, co-chromatography of references, odour quality at the sniffing port and mass spectrometry.

### Quantification of sulphur compounds in coffee brew

Coffee brew (5 l, use of 60g coffee/l; from various roasted and ground Arabica and Robusta coffees of CTn 90 and 60) was spiked with the labelled isotope d3-3-mercapto-2-pentanone (internal standard), distilled under high vacuum and extracted with dichloromethane. For 3-mercapto-2-butanone, 2-mercapto-3-pentanone, and 4-methoxy-2-methyl-2-butanethiol recovery factors relative to the internal standard were determined. Isolation and derivatisation of thiols with p-hydroxymercuri benzoate was carried out in accordance to Tominaga et al. (1998). Aroma isolated were analysed by gc/gc/ms. The concentration of 4-mercapto-4-methyl-2-pentanone was below the limit of detection of the analytical method.

### Capillary gas chromatography/olfactometry (HRGC/O)

HRGC was performed on a Fisons gas chromatograph (Type HRGC MEGA SERIES) using three different fused silica thin-film capillaries (DB-Wax, DB-5 and DB-1701, each 30 m x 0.25 mm; film thickness, 0.25 μm; J and W. Scientific, Brechbühler, Switzerland). Samples were applied by cold on-column injection technique at 40°C. After 2 min, the temperature of the oven was raised by 6°C/min to 240°C and held for 10 min. For olfactometry, the gc was connected to a Sniffer 9000 System (Brechbühler, Switzerland) The Kovats retention indices were calculated by co-chromatography of n-alkanes.

### Two-dimensional HRGC/MS (HRGC/GC/MS)

For two-dimensional gas chromatography, two gas chromatographs (GC1+2) of the same type (HRGC MEGA SERIES, Fisons Instruments, Brechbühler, Zürich, Swiss) were inter-connected via a MCSS (moving capillary stream switching) system. In both gas chromatographs capillaries with different stationary phases were installed (DB-Wax and DB-1701). The time windows for cutting of the eluate at GC1 were determined by means of reference substances. Mass spectra in the electron impact mode (MS/EI) were generated at 70 eV on an MD800 (Fisons Instruments, Brechbühler, Zürich, Swiss).

### Sensory evaluations

A soluble coffee (100% Arabica, CTn 90) was spiked with three thiol mixtures, containing super-threshold (in coffee) amounts of 3-mercapto-2-butanone, 3-mercapto-2-pentanone and/or 4-methoxy-2-methylbutan-2-thiol (see Table 4). Stock solutions of the thiols were prepared in ethanol and diluted in water to such an extend that final concentration of ethanol

in the coffee sample did not exceed 50 mg/l water (thus having no impact on the sensory profile). The soluble coffee was freshly prepared before each sensory session as follows: per 1000 ml of soluble coffee beverage, 15 g of soluble coffee were used. Soluble coffee powder was spiked with mixtures I, II or III and reconstituted with hot water (65°C). Solutions were equilibrated for 2 min. while stirring and then served to 8 trained panellists. Panellists were given a reference (non-spiked soluble coffee) and two coded samples per session and were asked to describe the differences in above-cup sensory profiles.

## RESULTS

Gc/olfactometry (gc/o) analysis of coffee aroma isolates obtained by stripping under nitrogen as well as by HS-SPME resulted in the detection of 11 sulfur compounds, 6 of which were unknown (Table 1). Using the enrichment method described by Tominaga et al. (1998) (see also Figure 1) that allows the isolation and analysis of thiols in the ppb to ppt range, 4 of the 6 unknown compounds were positively identified as 3-mercapto-2-butanone, 3-mercapto-2-pentanone, 2-mercapto-3-pentanone and 4-methoxy-2-methylbutan-2-thiol. These thiols are characterised for the first time in coffee. In addition, 3-mercapto-3-methylbutan-2-one and 4-mercapto-4-methyl-pentan-2-one were tentatively identified (based on all criteria mentioned in Table 1 except mass spectra, due to too weak signals). The structure of the 6 new sulfur compounds is shown in Figure 2.

**Table 1. Sulphur compounds identified in aroma isolated from roasted and ground coffee.**

| no. | sulfur compound | odour quality | formerly | RI-value | | |
|---|---|---|---|---|---|---|
| | | | identified in coffee | DB-5 | DB-1701 | DB-Wax |
| 1. | **3-mercapto-2-butanone**[a] | sulfury, cabbage | - | 820 | --- | 1269 |
| 2. | 3-methyl-2-buten-1-thiol | fermented, sulfury | + | 832 | 876 | 1098 |
| 3. | 2-methyl-3-furanthiol | meaty | + | 880 | 936 | 1317 |
| 4. | **3-mercapto-3-methyl butan-2-one**[b] | sulfury | - | 857 | 966 | --- |
| 5. | **3-mercapto-2-pentanone**[a] | sulfury, catty | - | --- | 1015 | 1355 |
| 6. | **2-mercapto-3-pentanone**[a] | sulfury, catty | - | --- | 1011 | 1348 |
| 7. | 2-furfurylthiol | roasty | + | 918 | 996 | 1436 |
| 8. | **4-methoxy-2-methylbutan-2-thiol**[a] | blackcurrant-like | - | 926 | 989 | 1221 |
| 9. | **4-mercapto-4-methyl pentan-2-one**[b] | sulfury, fruity | - | 959 | 1069 | 1400 |
| 10. | 3-mercapto-3-methyl-1-butanol | sulfury | + | 994 | 1120 | --- |
| 11. | 3-mercapto-3-methylbutyl formate | Sulfury, catty | + | 1029 | 1140 | 1538 |

[a] *Positive identification based on odour quality, RI indices on 2-3 columns of different polarity, co-chromatography of references and mass spectra.*
[b] *All criteria mentioned under a except mass spectra.*

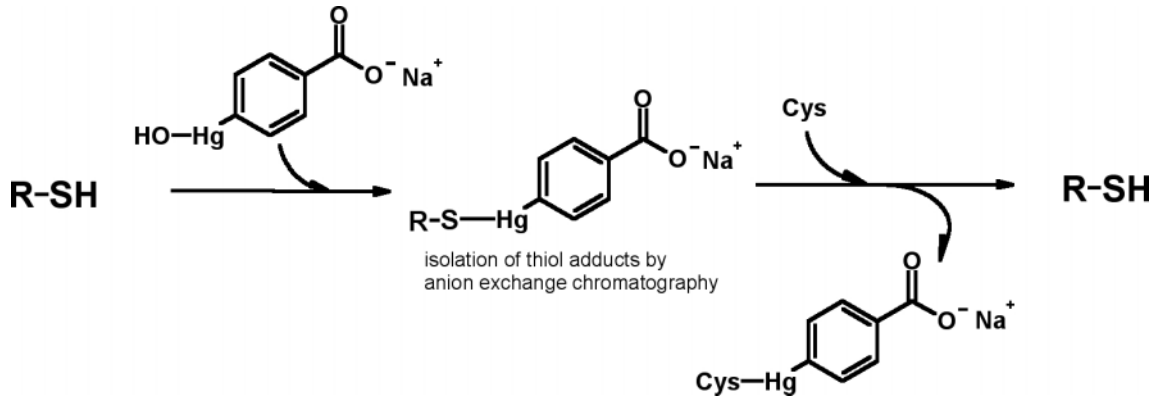


**Figure 1. Reversible addition of the thiols to *p*-hydroxymercuribenzoate and release with cysteine.**

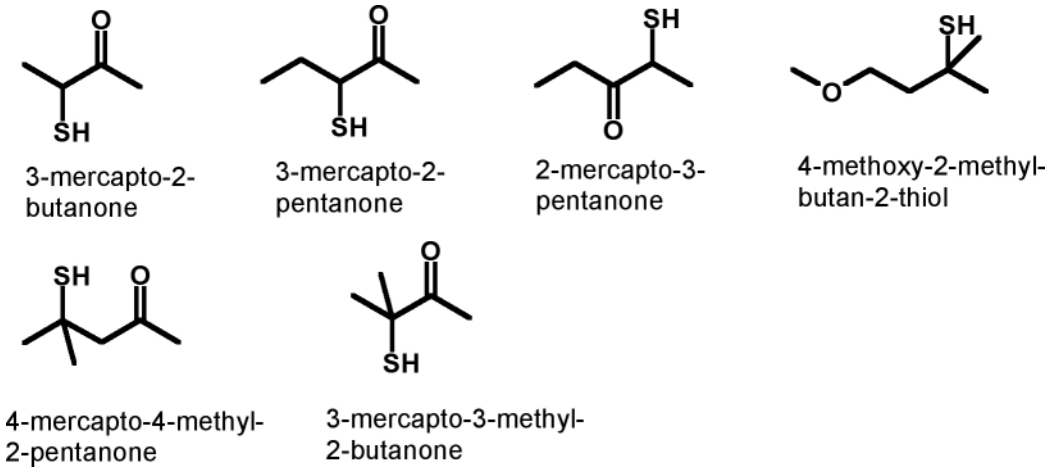


**Figure 2. Structure of newly identified sulfur compounds in coffee.**

The absolute contents of the sulphur compounds 3-mercapto-2-butanone, 3-mercapto-2-pentanone, 2-mercapto-3-pentanone and 4-methoxy-2-methylbutan-2-thiol (MMBT) were determined in various coffee brews (ex Robusta Vietnam, CTn 60; Arabica Central US/Santos, CTn 60; Arabica Mexico/Ethiopia, CTn 90) by means of stable isotope dilution analysis. Concentrations of three mercaptoalkanones ranged between 1 and 5.5 ppb, whereas the content of 4-methoxy-2-methylbutan-2-thiol was found to be around 0.015 ppb (Table 2). Both roast degree and coffee species/variety had a negligible or minor impact on the contents of the thiols. Calculation of the odour activity values (based on the odour thresholds of the compounds in water, that are shown in Table 3) revealed high values for MMBT (OAV of 683-788), followed by the two mercaptopentanones (OAV of 3-8). These thiols (a.o. MMBT) should have a high impact on the aroma of the coffee brews. For 3-mercapto-2-butanone, OAV's of ≤1 were obtained, which indicated that its contribution to the coffee aroma is lower than the three other thiols.

Odour thresholds were additionally determined in a soluble coffee using Triangular tests with spiked and non-spiked coffee beverages. Thresholds were found to be by a factor of 29 (3-mercapto-2-butanone), 276 (3-mercapto-2-pentanone) and 4250 (MMBT) higher than in water. This result underlines the importance of the sensory evaluation of reconstituted aroma models or spiking experiments (using real coffee samples) to confirm the contribution of these sulfur compounds to the coffee aroma.

**Table 2. Concentrations (ppb) of new sulfur compounds in brews from various roasted coffees.**

| aroma compound | concentration [ppb] in coffee brew from R&G | | | odour activity values (OAV)[a] | | |
|---|---|---|---|---|---|---|
| | Rob Vietn. CTN 60 | Ara, Centr. US/Sant. CTN 60 | Ara Mex/Eth. CTN 90 | Rob Vietn. CTN 60 | Ara Centr. US/Sant. CTN 60 | Ara Mex/Eth. CTN 90 |
| 4-methoxy-2-methyl butan-2-thiol | 0.014 | 0.014 | 0.016 | 701 | 683 | 788 |
| 3-mercapto-2-butanone | 1.0 | 1.0 | 4.1 | < 1 | < 1 | 1 |
| 2-mercapto-3-pentanone | 2.4 | 5.4 | 4.0 | 3 | 8 | 6 |
| 3-mercapto-2-pentanone | 2.5 | 5.5 | 4.1 | 4 | 8 | 6 |

[a]*OAV based on odour thresholds in water (see Table 3).*

**Table 3. Odour thresholds of thiols in water and soluble coffee.**

| **aroma compound** | **odour threshold [ppb] in water[a]** | **odour threshold [ppb] in coffee[b]** | **ratio** |
|---|---|---|---|
| **4-methoxy-2-methyl butan-2-thiol** | 0.00002 | 0.085 | 4250 |
| **3-mercapto-2-butanone** | 3.6 | 87 | 29 |
| **2-mercapto-3-pentanone** | 0.7 | n.d. | - |
| **3-mercapto-2-pentanone** | 0.7 | 193 | 276 |

[a]*Orthonasal thresholds according to:*
[b]*Own results; orthonasal thresholds determined in soluble coffee (100% Arabica) using Triangular tests.*
[c]*Ratio of thresholds in coffee/in water.*

However, our primary interest was to formulate building blocks with the three new sulfur compounds 3-mercapto-2-butanone, 3-mercapto-2-pentanone, and 4-methoxy-2-methylbutan-2-thiol that can be used in coffee aroma formulations, rather than confirming their contribution to coffee brew. To this end, three mixtures with 1-3 thiols were formulated and added (in super-threshold (coffee) amounts) to a coffee beverage. Sensory profiling of the spiked coffee beverages against a reference (w/o additions) showed that all three mixtures had a positive impact on the sensory profile, i.e. they reinforced the overall aroma, sulfury, roasty and fresh coffee character (Table 4).

**Table 4. Sensory evaluation of a coffee beverage spiked with mixtures of 3 newly identified sulfur compounds against a reference coffee (w/o additives).**

| **Mixture[a]** | **addition of** | *sensory description[b]* |
|---|---|---|
| I | 2-mercapto-3-butanone, 3-mercapto-2-pentanone | more overall aroma, more sulfury, more fresh, more roasty, more full/complex |
| II | 4-methoxy-2-methylbutan-2-thiol | more overall aroma, more sulfury, more full/complex |
| III | 2-mercapto-3-butanone, 3-mercapto-2-pentanone, 4-methoxy-2-methylbutan-2-thiol | more overall aroma, more sulfury, more fruity |

[a]*Amounts relate to super-threshold levels (each compound in mixtures I and II as well as MMBT in mixture III)*
[b]*Panel described above-cup aroma of samples relative to reference (soluble coffee).*

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# Coffee Aroma Quality: Investigation of the Reaction Pathways Involved in the Degradation of Thiols

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## SUMMARY

A mixture of volatile thiols covering a large range of physico-chemical properties was monitored over time in the presence of a coffee brew. The analysis was conducted by SPME-GC-MS. Additives inhibiting specific reaction pathways were preincubated with the brew prior to thiol addition. Degradation kinetics of the volatile thiols were characterised by their rate constants k(obs). The results suggest that thiols might disappear *via* two different major mechanisms depending on their structure. Aliphatic thiols like ethanethiol react by irreversible nucleophilic addition to matrix components generated by oxidation. Benzylic thiols like 2-furfurylthiol react partially following this same pathway but degrade also via radical cascade reactions.

## RÉSUMÉ

L'évolution d'un mélange de thiols volatils représentant un large domaine de propriétés physico-chimiques est suivi au cours du temps en présence d'un café filtre. L'analyse est conduite par MEPS-CG-SM. Des additifs inhibant certains chemins réactionnels sont préincubés avec le café avant l'addition des thiols. Les cinétiques de dégradation sont ensuite mesurées et décrites par la constante de vitesse apparente de réaction k(obs). Les résultats indiquent que les thiols se comportent différemment en fonction de leur structure chimique. Les thiols aliphatiques tels que l'éthanethiol réagissent par addition nucléophile sur des composés de la matrice générés par oxydation. Les thiols benzyliques comme le 2-furfurylthiol peuvent subir en plus des réactions de dégradation radicalaires.

## INTRODUCTION

Fresh coffee aroma has a very elusive composition both in ground coffee beans and in coffee brew. It has been shown that this is mainly due to the quick decrease of volatile sulfur aroma components responsible for the roasty, fresh and burnt notes (Hofmann and Schieberle, 2002). Isolated coffee aroma on the other hand is more stable although its thiols also decrease but over a longer timescale. It has therefore been postulated that coffee thiols disappear by chemical interaction (or reaction) with coffee matrix components leading to the irreversible formation of new covalent bonds (Hofmann and Schieberle, 2002; Charles-Bernard et al., 2003). Several reaction partners from the coffee matrix have been proposed and various chemical mechanisms can be imagined to explain this chemical degradation ranging from radical cascade reactions to nucleophilic addition (see Figure 1) (Hofmann and Schieberle, 2002; Blank et al., 2002; Pascual et al., 2002). The purpose of this study was to gain some information on the relative contribution of the suspected mechanistic pathways to the overall degradation.

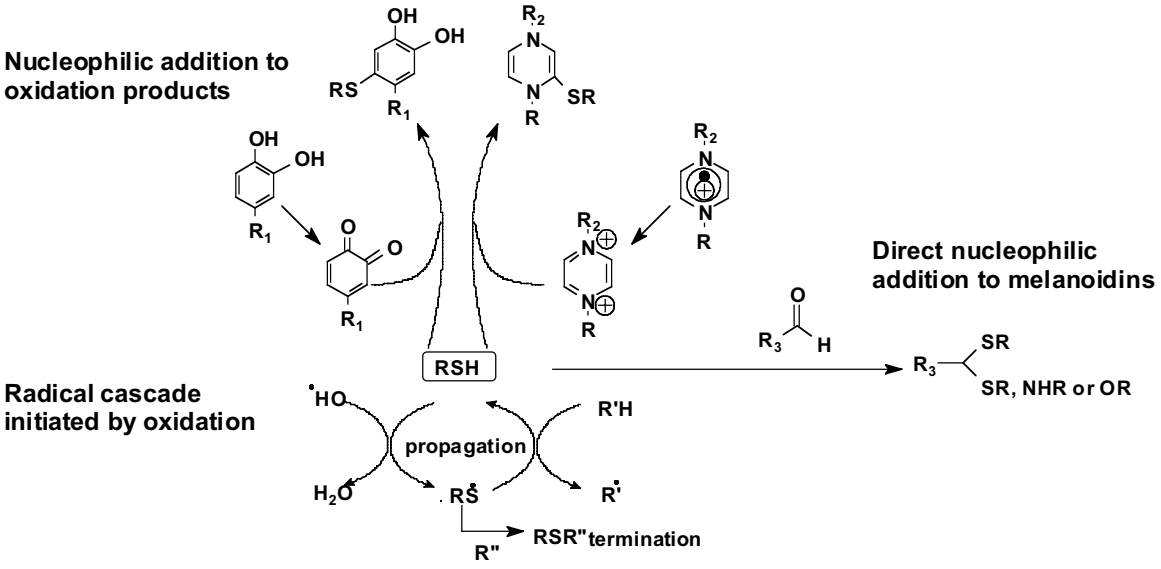


**Figure 1. Possible mechanistic pathways for the reaction of volatile coffee thiols with non-volatile coffee matrix.**

## MATERIALS AND METHODS

### Coffee Sample

Coffee brew models were produced from a blend comprising Santos (Arabica) 50%, Colombia (Arabica) 30%, Ivory Coast (Robusta), medium roasting level (CTN = 85), by countercurrent extraction 3 x 12 min at 120°C. The volatile aroma was vapour stripped and the extract spray-dried without pre-concentration, (extraction yield 22.7%). The resulting powder was considered as a "dearomatised dry coffee brew". Coffee stock solutions were prepared at 2.5% t.s. in acetate buffer 0.01 M at pH 5.2.

### Volatile Compounds

The thiol stock solution was prepared at twice the final concentrations listed below. When prepared in a glove-box these solutions could be stored for 4 weeks at –20°C. At room temperature (RT) the mixture containing the 9 thiols was shown to be stable over 24 hours in the working buffer (0.01 M acetic acid, pH 5.2). The final concentrations [µmol/L] were: 2-methyl-2-propanethiol (2M2P), 3.7; 3-mercapto-3-methylbutylformate (MMBF), 4.9; 2-butanethiol (2BT), 3.7; ethanethiol (EtSH), 5.4; propanethiol (PropSH), 4.4; butanethiol (BuSH), 3.7; pentanethiol (PentSH), 3.2; 2-furfurylthiol (FFT), 3.9; benzylthiol (BnSH), 3.4; thiophenol (PhSH), 9.7.

### Additives

All chemicals were purchased from Fluka/Aldrich/Sigma (Buchs, Switzerland) and used without further purification. Stock solutions of the additives were prepared in the working buffer at ten times the final concentrations listed below. Final concentrations [mmol/L] were gradually increased: hydroxylamine hydrochloride, 0.9-220; L-ascorbic acid sodium salt, 1-50; sodium thiosulfate pentahydrate, 1-5; sodium sulfite anhydrous, 1-10; diethylenetriaminepentaacetic acid (DTPA), 1-5; $H_3BO_3$, 50; and catalase from bovine liver, 10-15 mg/L. With sodium sulfite, sodium ascorbate, hydroxylamine hydrochloride, boric acid and DTPA the additive stock solution in acetate buffer was adjusted to pH 5.2 with HCl and NaOH respectively.

**Measurement of the Interactions**

*Reference aroma sample with additive **A***: aroma stock solution, additive stock solution and working buffer were mixed in a ratio 5:1:4. The mixture was stirred 15 min. 800 µL were transferred into a 2 mL amber silane-treated glass vial and equilibrated one hour on the autosampler before headspace analysis. *Coffee sample with aroma and additive **B***: the coffee stock solution (2.5% t.s.) was diluted 4:1 with the additive stock solution. The mixture was stirred for one hour at RT and diluted 1:1 with the aroma mixture. After 15 min stirring, an aliquot (800 µL) was filled into a 2 mL vial and equilibrated one hour on the autosampler before headspace analysis. For kinetic studies, vials containing reference samples **A** and coffee samples **B** were prepared at time zero and put alternately on the autosampler so that the headspace was sampled every two to four hours in different vials. Headspace concentrations of vials **A** and **B** were measured after the same time. The ratio of the two integration surfaces was plotted as a function of additive concentration and/or time for each additive. $Na_2SO_3$ and $Na_2S_2O_3$ both decreased the headspace concentration of the buffered blank thiol mixture even upon short time equilibration (1 h). The results with these additives are therefore expressed relative to a blank flavoured buffer solution without additive. Trials under anaerobic conditions were entirely prepared in a glove-box (Easy Box EB 80-1 spez., MecaPlex, Switzerland). *SPME-GC-MS analysis.* The headspace of the vials was sampled using a Varian CP-820 autosampler. A SPME fiber (PDMS/DVB; 65 µm, Supelco, Buchs, Switzerland) was inserted into the headspace and allowed to equilibrate for 1 min. Aroma compounds were desorbed in the injector port of the GC for 5 min at 240°C. During the first 3 min the purge was off. GC separation was performed on a Hewlett Packard 5973 gas chromatograph equipped with a DB-Wax column (J&W Scientific, 30 m, 0.25 mm ID, 0.25 µm film, 0.9 mL/min, constant flow). The oven temperature was held at 35°C for 3 min then programmed to 170°C at 4°C/min, then to 220°C at 20°C/min and held at 220°C for 10 min. Mass spectra were acquired in scan mode from 29 to 300 amu. *Data treatment.* The SPME-GC-MS peak area, *i.e.* concentration, of volatile compound in presence of coffee brew and additive was expressed in percentage relative to SPME-GC-MS peak area of the volatile compound in a reference aroma sample in working buffer or reference aroma sample in working buffer + additive. Each datapoint was measured in duplicate and deviations from average were plotted as error bars. Kinetic data were treated assuming pseudo first order rate law. For each volatile compound the Ln of concentration was expressed as a function of time [s]. The slope of the curve gave –k(obs) the observed rate constant. The individual k(obs) for each volatile compound were then expressed as k(rel) relative to the corresponding k(obs, no add), rate constant in the coffee sample without additive: krel = k obs,no add / k obs, add.

**RESULTS AND DISCUSSION**

This study focused on volatile thiols since they were previously determined to be the most reactive components of coffee aroma when exposed to coffee brew (Hofmann and Schieberle, 2002; Charles-Bernard et al., 2002). Thiols of this study were chosen to give mechanistic information on the interactions between thiols and coffee matrix components. They were not necessarily “real coffee thiols” but cover certain physico-chemical properties that represent the main coffee thiols. The concentrations were set to be in the linear range of the SPME fiber, at a ppm level and as close as possible to the real concentrations determined in a coffee brew. The additives were chosen in order to slow down or inhibit specific reaction pathways as shown in Table 1.

**Table 1. Role of additives used in this study.**

| Additives | Expected action | Additive final concentration [mmol/g dry coffee] |
|---|---|---|
| Hydroxylamine x HCl | Strong competitor nucleophile | 0.09-22 |
| Ascorbic acid | Radical scavenger | 0.1-5 |
| DTPA | Transition metal chelating agent / Fenton inhibitor | 0.1-0.5 |
| $Na_2S_2O_3.5H_2O$ | Weak reducing agent (peroxides) | 0.1-5 |
| $Na_2SO_3$ | Reducing agent and oxygen scavenger | 0.1-1 |
| $H_3BO_3$ | Phenol complexing agent (catechol) | 5 |
| Catalase | $H_2O_2$ reducing agent | 1-5 [mg/g dry coffee] |

A 1 h incubation time of the coffee brew with the various additives was decided arbitrarily. For certain additives such as $NH_2OH$ incubation times of 1 h and 24 h of coffee brew with the additive led to very similar results (data not shown). Sampling and analysis are depicted in Figure 2. Only results for primary thiol ethanethiol (EtSH), tertiary thiol, 2-methyl-2-propanethiol (2M2P), and the benzylic thiol 2-furfurylthiol (FFT) are shown in Figures 3-6. The other thiols follow similar trends.

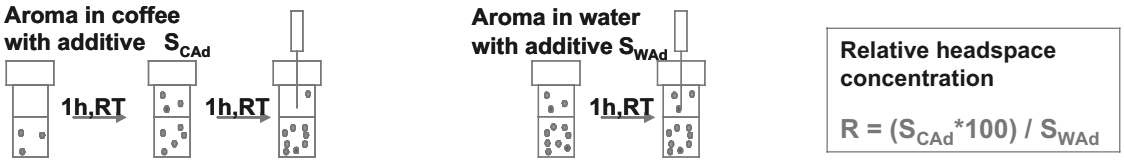


**Figure 2. Stability of thiols in the presence of various additives, sampling and analysis.**

**Nucleophilic addition**

The competitor nucleophile hydroxylamine blocks the electrophilic reactive sites in the matrix and therefore prevents thiols from reacting *via* this pathway. The aliphatic thiols (e.g. ethanethiol) were quantitatively recovered when 8-10 mmol of hydroxylamine/g dry coffee were added 1 hour prior to the contact between thiols and matrix (Figure 3). FFT still degraded even in the presence of 22 mmol hydroxylamine/g dry coffee, suggesting that a parallel reaction mechanism occurs. At this point of the study we concluded that the reaction between aliphatic thiols (e.g. ethanethiol) and the coffee matrix is mainly due to nucleophilic additions. The addition might occur on electrophiles present as such in the matrix or formed by oxidation (see Figure 1).

**Oxygen and radical mediated pathways**

For linear aliphatic thiols the absence of oxygen allows to recover 80% of the initial concentration after 24 hours (data not shown). This stabilisation suggests that: either thiols add to oxidation products that are generated in the coffee matrix only in the presence of oxygen, or oxygen is needed to create a reactive environment (radical species) involved in thiol degradation. The preincubation of coffee brew with sodium sulfite ($Na_2SO_3$) led to a remarkable stabilisation of EtSH and FFT (Figure 3). In the coffee beverage (pH 5.2) sodium sulfite is a weak reducing agent and oxygen scavenger. It may also react with matrix electrophilic counterparts (e.g. aldehydes). The observed stabilisation is therefore due to a cumulative effect on both mechanistic pathways.

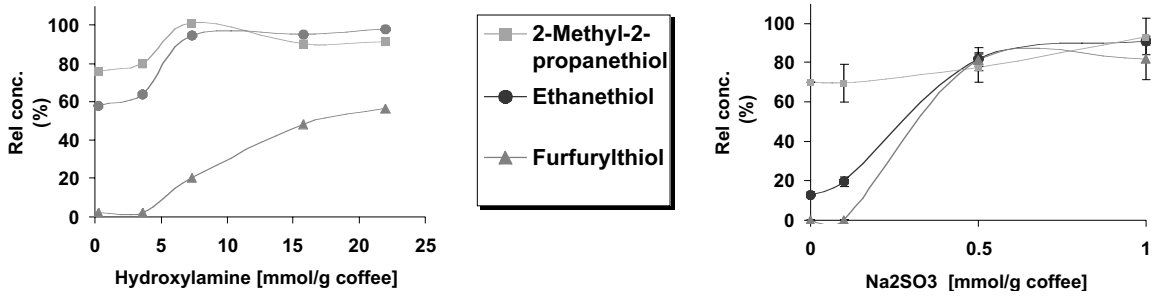


**Figure 3. Degradation kinetics of ethanethiol, 2-methyl-2-propanethiol and 2-furfurylthiol in the presence of coffee brew preincubated for 1 h with competitor nucleophile $NH_2OH$ or reducing agent $Na_2SO_3$, (t.s. 1%, pH 5.2).**

In the presence of a weak reducing agent like sodium thiosulfate ($Na_2S_2O_3$) the thiol mixture was slightly destabilised in a concentration dependent way (Figure 4). Similarly the addition of catalase to reduce $H_2O_2$ into $H_2O$ resulted in no significant effect on the 3 thiols (Figure 4). Therefore the trapping of reactive oxygen species (ROS) such as hydroperoxides and peroxide radicals is not sufficient to prevent degradation of the thiols under investigation. Many other radical species are present in the beverage to maintain the radical cascade even under these conditions (Pascual et al., 2002).

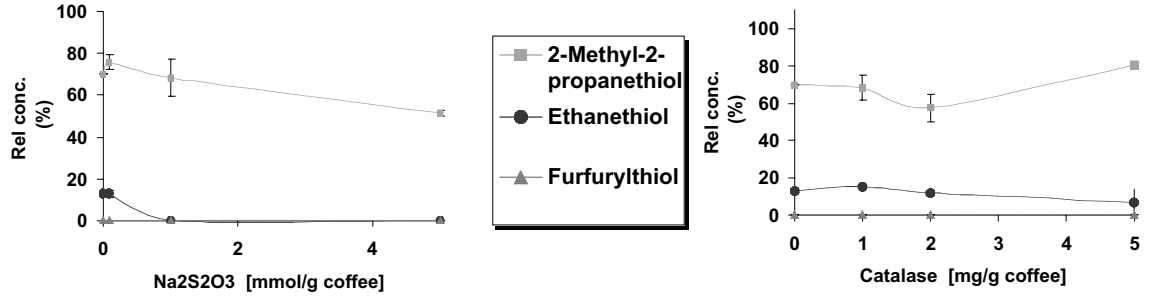


**Figure 4. Degradation kinetics of ethanethiol, 2-methyl-2-propanethiol and 2-furfurylthiol in the presence of coffee brew preincubated for 1 h with reducing agent $Na_2S_2O_3$ or catalase (t.s. 1%, pH 5.2).**

The addition of the radical scavenger ascorbic acid resulted in a stabilisation of all 3 thiols studied (Figure 5). It is however weaker than in the absence of air. This suggests that the radical environment does not play the major role in the degradation of aliphatic thiols (e.g. EtSH) and the nucleophilic addition to oxidation products in the matrix becomes more plausible. Confirming this hypothesis, the addition of a transition metal chelator (DTPA) expected to slow down radical generation via the Fenton reaction shows only a very weak stabilisation effect of linear thiol EtSH. FFT was undetectable after 1 hour in the presence of this additive (Figure 5).

Finally the synergic or cumulative effect of competitor nucleophile and inert atmosphere was explored (Figure 6). For both thiols an additional stabilisation was observed in the absence of air. The degradation rate of FFT however is still faster than for EtSH. This suggests that FFT degrades in parallel following another mechanistic pathway (e.g. radical cascade reaction and addition to the matrix).

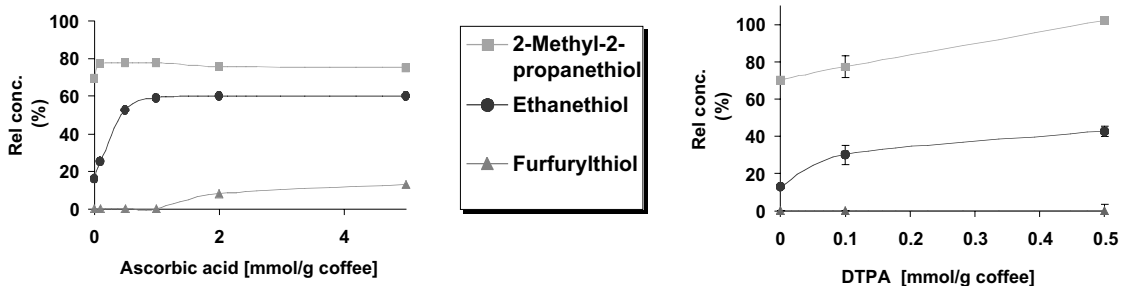


**Figure 5. Degradation kinetics of ethanethiol, 2-methyl-2-propanethiol and 2-furfurylthiol in the presence of coffee brew preincubated for 1 h with radical scavenger ascorbic acid or chelating agent DTPA (t.s. 1%, pH 5.2).**

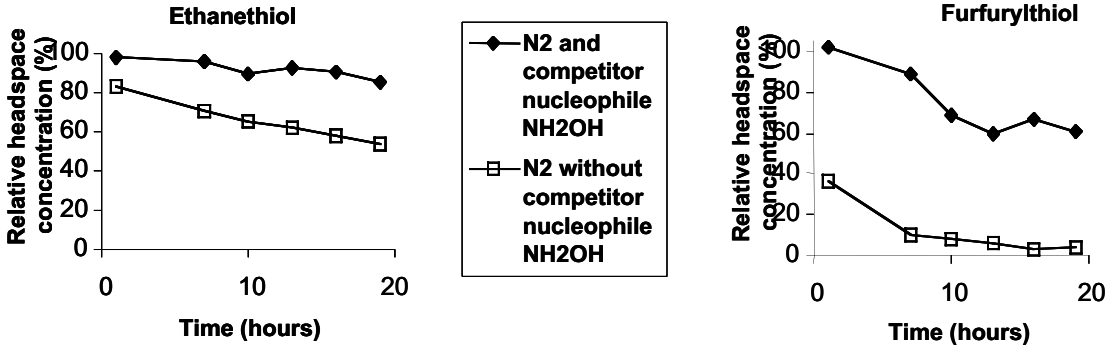


**Figure 6. Degradation kinetics of ethanethiol and 2-furfurylthiol in the presence of coffee brew preincubated for 1 h with competitor nucleophile $NH_2OH$ with and without air (t.s. 1%, pH 5.2).**

Relative rate constants of thiol decay in coffee brew with and without additive give a semi-quantitative mechanistic information (see Table 2). For EtSH and FFT, the strongest attenuation of the degradation is seen under the combined effect of a competitor nucleophile and inert atmosphere. From independent experiments we conclude that for the aliphatic thiol, EtSH, this effect is mainly due to the competitor nucleophile and for the benzylic thiol FFT, mainly to the absence of oxygen. Sodium sulfite as reducing agent and nucleophile had a strong stabilising effect on both thiols. Additives quenching reactive oxygen species or radicals had a weaker effect except for ascorbic acid, which had a significant stabilising effect on FFT.

**Table 2. Relative rate constants (rate attenuation) of thiol degradation in the presence of reconstituted coffee brew with additive as compared to coffee brew without additive, t.s. 1%, pH 5.2, krel = $k_{obs\ no\ add}$ / $k_{obs,add}$.**

| Additive | amount (mmol/g) | Ethanethiol | Furfurylthiol |
|---|---|---|---|
| **Aerobic, coffee no additive** | | 1.0 | 1.0 |
| **Anaerobic** | | 17.9 | 45.1 |
| **Hydroxylamine** | 10.0 | 10.5 | 4.0 |
| **Anaerobic + hydroxylamine** | 21.0 | 64.8 | 92.6 |
| **$Na_2SO_3$** | 2.0 | 51.5 | 67.9 |
| **Ascorbic acid** | 1.0 | 9.0 | 23.6 |
| **DTPA** | 0.5 | 0.5 | 1.0 |

These results suggest that thiols disappear *via* one or two major mechanisms depending on their structure. Aliphatic thiols like ethanethiol and benzylic thiols like 2-furfurylthiol (FFT), both react by irreversible nucleophilic addition to the matrix. The reaction partner very likely is an oxidation product generated in the presence of air. These experiments do not determine whether the reactive species is of the "quinone" or "Crosspy" (Hofmann et al., 2002) type. Benzylic thiols like FFT degrade in parallel via radical cascade reactions that are partially inhibited by ascorbic acid.

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# Aroma Extract Dilution Analysis of Espresso Coffee

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## SUMMARY

Different extraction techniques for aroma isolation of espresso coffee have been compared by gas chromatography mass spectrometry. Key aroma compounds have been identified by aroma extract dilution analysis. Unknown aroma compounds have been isolated by preparative gas chromatography and characterised by nuclear magnetic resonance. Differences between the two phases, "crema" and liquid of an espresso beverage have been semi-quantitatively compared by static headspace especially for aroma active sulphur compounds.

## ZUSAMMENFASSUNG

Verschiedene Extraktionstechniken zur Aromaisolierung aus Espresso Getraenk wurden mittels Gaschromatographie Massenspektrometrie verglichen. Schluesselaromastoffe wurden ueber Aroma Extrakt Verduennungsanalyse identifiziert. Unbekannte Verbindungen wurden mittels praeparativer gas chromatography isoliert und mittels Kernresonanzspektroskopie aufgeklärt. Unterschiede in Schwefelkomponenten der „crema" zum Getraenk wurden semi-quantitative mittels statischer headspace Analyse verglichen.

## INTRODUCTION

Espresso coffee is the "Italian way" of consuming coffee, and gets well exported to the whole world with the Italian way of life. It is a small cup, only half filled, with a very dense coffee beverage, obtained by pressurised extraction (percolation) of the coffee cake, prepared on the consumers order. Visually it is covered by the "crema", a thick reddish-brown foam of tiny bubbles, above a cup of rich flavoured, aromatic beverage. One of the most important organoleptic characteristics is its aroma, besides the mouthfeel and the somewhat correlated amount and stability of the foam. Despite the increasing daily consumption of about 50 million cups of espresso over the world, only few works dealing with the characterisation of its aroma can be found in literature. Recently, the headspace (HS) (Ishikawa et al., 2004) of Espresso was evaluated by gas chromatography mass spectrometry GC-MS and the results were correlated to sensory data (Maeztu et al., 2001). Together with other groups (Andueza et al., 2003; 2002; Bicchi et al., 2002) Maeztu et al. (2001) mainly focussed on statistical discrimination between different beverages or coffees or dealt with instant beverages (Pollien et al., 1997; Sanz et al., 2002). Sarrazin et al. (2000) and Ramos et al. (1998) further investigated the representativeness of different extraction techniques prior to the aroma analysis. Buffo and Cardelli-Freire (2004) reviewed the knowledge about aroma of coffee in general. In the present study we used different extraction techniques to separate the aroma from the coffee matrix and evaluated the chromatographically separated substances via mass spectrometry and sniffing. To simplify the complex mixture, column chromatography and preparative gas chromatography was used to isolate sub-fractions and single compounds were analysed by nuclear magnetic resonance spectroscopy (NMR).

## MATERIALS

### Coffee

Commercial Arabica-blends and Arabicas of single origins were used for espresso preparation, whereas commercial blends or the same coffees as for espresso preparation were used in case of drip.

### Beverages

Espresso preparation was achieved by a home use machine (Gaggia, Italy), extracting 13 g of coffee with water of 90°C under a pressure of 10 bar, and stopping the percolation after 60 ml had passed in approximately 30 seconds. Drip was prepared using a Cafe City (Tefal, Germany) automatic drip machine using 50 g of coffee for 1 litre of water.

### Extraction

SDE: 1 litre of the freshly prepared beverage was extracted for two hours by simultaneous distillation and extraction (SDE, Normschliff Geraetebau, Wertheim, Germany) using 50 ml mixture of pentane/diethylether, followed by concentration.

HSSE: A head-space vial was filled with 10 ml of the freshly prepared beverage. A twister (length 1 cm, film thickness 0.5 mm) was placed in the glass insert (Gerstel, Muehlheim, Germany), the vial was closed with a PTFE coated septa and conditioned for 30 minutes at 60°C.

SAFE: The entire beverage (60 ml) was placed in the funnel of the distillation apparatus without the addition of any solvent. After evaporation of nearly all liquid, the condensed aqueous aroma isolate was melted and 10 ml were extracted by twister for 20 minutes at room temperature.

Static Headspace: An HP 7694 headspace sampler coupled via silco-steal transfer line to a DB5 wide bore column was used to sample 3 ml of Headspace volume after 30 minutes of equilibration at 60°C of vials filled with 10 ml of liquid. Detection was done for sulfur compounds by pulsed flame photometric detector (PFPD, OI-Analytical, USA) in parallel with FID.

### Analysis

Analyses were performed on a 5973N GC-MS system (Agilent, Waldbronn, Germany) in electron impact mode. A Carbowax column (60 m x 0.25 mm) was ramped from 35 to 240°C under constant flow of helium. Liquid extracts were injected in split mode (1:5). The Twister was desorbed in a TDSA (thermal desorption unit and autosampler, TDS2, Gerstel) in splitless mode and trapped in the programmable temperature vaporizer (PTV). PTV was in solvent venting mode at 50 ml helium flow during desorption, rapidly ramped from –120 to 250°C.

Preparative GC and GC-sniffing was performed on Carbowax or DB5 wide bore column using a coupled to a preparative fraction collector (PFC, Gerstel) or an olfactory detection port (ODP2, Gerstel) heated to 200°C. The deactivated traps were cooled using liquid nitrogen.

NMR: NMR experiments were performed on a Varian Inova spectrometer equipped with an 11.75 Tesla super-conducting magnet (500 MHz for $^{1}H$). Single compounds obtained by preparative GC were recovered by washing the trap with deuterated solvents.

## RESULTS AND DISCUSSION

Changing the extraction technique significantly alters the composition of the obtained aroma extract. Whereas typical headspace sampling describes the composition above a cup of coffee, we here focussed on the differences inside the cup, as studies on in vivo aroma release showed differences between the HS and aroma perceived ortho-nasal on the one hand, and the retro-nasal aroma perceived during swallowing (Weel et al., 2004) on the other hand. In addition, HS is not capable of extracting high boiling compounds. Therefore we first compared the standard extraction technique (simultaneous distillation and extraction, SDE), to solid phase techniques and SAFE (solvent assisted flavour evaporation) followed by twister extraction of the aqueous distillate. Extracts were then analysed via GC-MS and GC-sniffing. For visualization of the chemical differences, labelled total ion chromatograms are presented in Figures 1, 2, and 3.

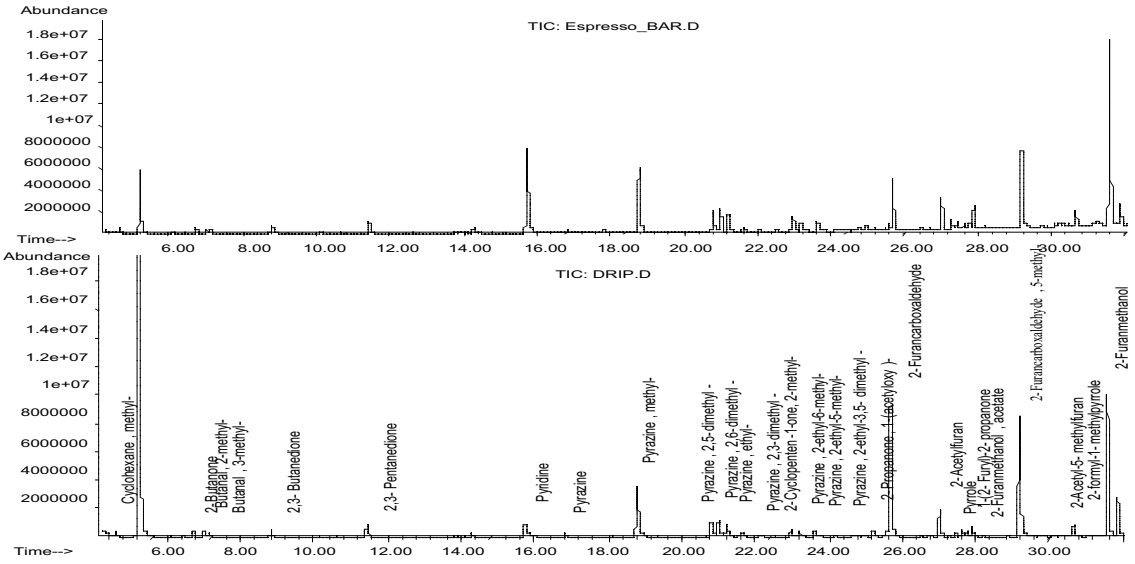


**Figure 1. GC-MS analysis (TIC) of Espresso and Drip beverage after SDE.**

To gain information about the sensory importance of the differences between the various extraction techniques and the coffee preparations, the effluent of a GC was evaluated by a trained four person panel. Hereby information from literature about character impact compounds from coffee brews and coffee powder (Holscher et al., 1990; Blank et al., 1992; Semmelroch and Grosch, 1995) as well as sniffing of reference compounds and standard coffee extract was used. Aroma extract dilution analysis (AEDA) was performed diluting the liquid extracts consecutively 1:2. The results are assembled in Table 1.

Comparing the results to drip coffee, higher dilution factors for nearly all compounds were present in espresso beverage. One reason is the higher amount of coffee used for preparation of espresso beverage compared to drip, 220 g/l in case of espresso, between 25–50 g/l for drip. Noteworthy are the higher flavour dilution values for the buttery compounds 2,3-butandione and 2,3-pentandione compared to the results in the literature for drip. Here several effects are superposed. One is the different type of coffee used, as in drip preparations Robusta is almost always part of the blend. From own studies and literature (Sanz et al., 2002) Robusta is known to produce less 2,3-butandione, but more 2,3-pentandione upon roasting.

Furthermore the roasting degree is normally darker in Italian type coffee, resulting in less of this early products from caramelisation. In our opinion the dominating effect is the pressure applied during extraction and the two phase system in the cup. Even analysing a drip beverage of the same roast and ground these differences could by confirmed (Figure 2). For isolation of unknown compounds preparative GC was used. So far only the first compound could be characterised by heteronuclear multi bond correlation (HMBC) as dihydro-2-methyl-3(2H)-furanone. It is was first identified already 1964 by Gianturco et al. (1964) and is reported to posses a caramely, sweet odour impression at 30 ppm, so the flavour dilution factor was below 32.

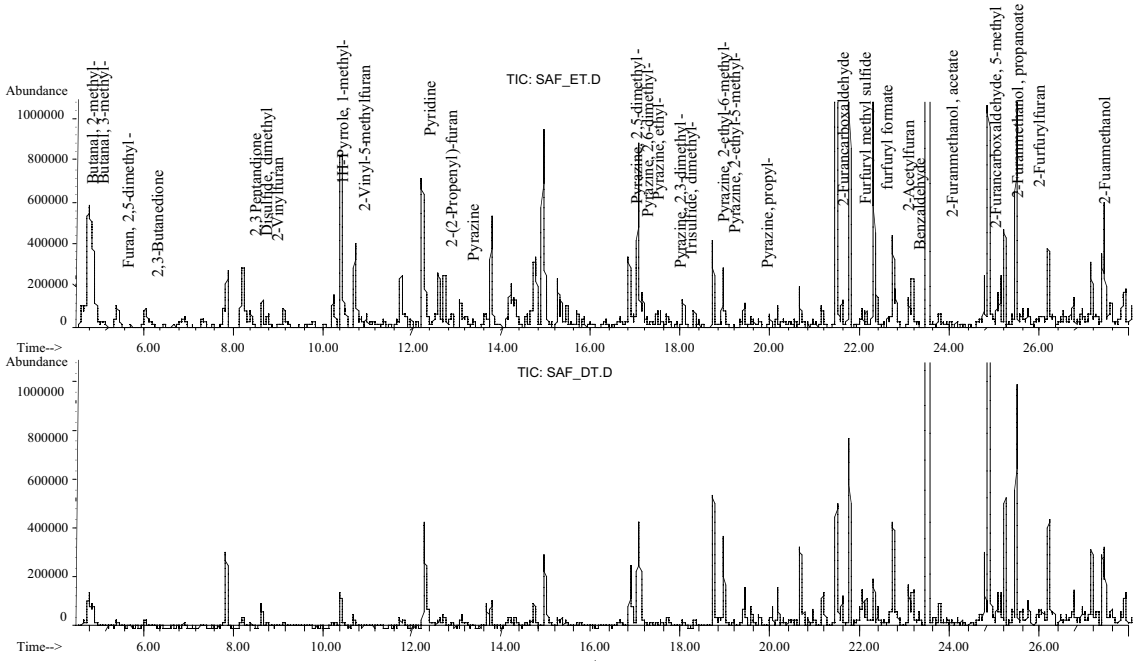


**Figure 2. GC-MS analysis (TIC) comparing Espresso and Drip beverage using SAFE followed by SBSE.**

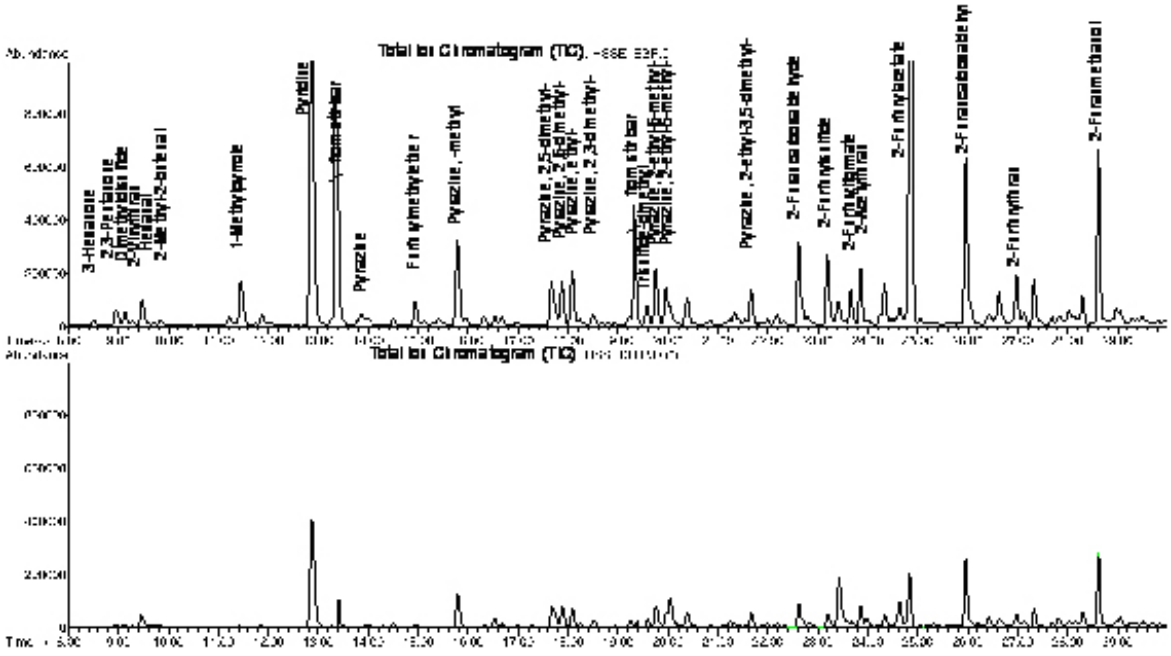

**Figure 3. GC-MS analysis (TIC) of HSSE (60°C, 60 min) of Espresso vs. Drip beverage.**

**Table 1. aroma impact compounds in Espresso brew (SDE).**

| No. | RI (CW) | Compound | Odor impression | FD |
|---|---|---|---|---|
| 1 | < 500 | Unknown | putrid, sulfury | 128 |
| 2 | 921 | 2-methylbutanal | pungent, fermented | 128 |
| 3 | 935 | 3-methylbutanal | pungent, cocoa | 256 |
| 4 | 982 | 2,3-butandione | Buttery | 512 |
| 5 | 1024 | Thiophene | spicy, garlic, rubber | 256 |
| 6 | 1060 | 2,3-pentanedione | butter, rancid | 512 |
| 7 | 1077 | 3-methyl-2-buten-1-thiol | rotten, skunky | > 2048 |
| 8 | 1098 | Unknown | asparagus-like | 64 |
| 9 | 1200 | Pyridine | typical, pyridine | 32 |
| 10 | 1238 | 4-(Z)-heptanal | Fishy | 128 |
| 11 | 1290 | 1-octen-3-one | mushroom-like | 512 |
| 11 | 1292 | 2-methyl-3-furanthiol | roasted meat-like | 1024 |
| 12 | 1380 | *Mercaptomethylpentanone* | sweaty, catty | 256 |
| 13 | 1412 | 2,3,5-Trimethylpyrazine | nutty, musty | 128 |
| 14 | 1414 | 2-Furfurylthiol | roasty, coffee like | > 2048 |
| 15 | 1422 | 2-Isopropyl-3-methoxy-pyrazine | peasy | 2048 |
| 16 | 1455 | Acetic acid | vinegar-like | 512 |
| 17 | 1469 | Methional | cooked potato-like | 1024 |
| 18 | 1475 | 2-Ethyl-3,5-dimethyl-pyrazine | roasty, musty | 256 |
| 19 | 1485 | *2-vinyl-5-methyl-pyrazine* | roasty, musty | 256 |
| 20 | 1514 | 2-Isobutyl-3-methoxy-pyrazine | paprika-like | 2048 |
| 21 | 1527 | 3-Mercapto-3-methyl-butylformate | catty, roasted coffee-like | > 2048 |
| 22 | 1544 | Linalool | fruity | 512 |
| 23 | 1623 | 2-Phenylacetaldehyde | Honey | 128 |
| 24 | 1645 | 3-Mercapto-3-methylbutanol | soup-like | 512 |
| 25 | 1665 | 2/3-Methylbutanoic acid | sweaty, pungent | > 2048 |
| 26 | 1825 | (*E*)-β-Damascenone | honey-like, fruity | > 2048 |
| 27 | 1863 | Guaiacol | burnt, phenolic | > 2048 |
| 28 | 2035 | *4-Hydroxy-2,5-dimethyl-3(2H)-furanone* | Caramel | 2048 |
| 29 | 2037 | 4-Ethylguaiacol | clove like | 1024 |
| 30 | 2205 | 4-Vinylguaiacol | clove like | 1024 |

*Peaks sorted by retention time on DB-Wax column. Reported are compounds with FD ≥ 32. In italics tentatively identified compounds.*

Once the key aroma compounds in Arabica Espresso coffee are identified, a detailed analysis of the effects of roasting, blending and extraction is possible using quantitative analysis by GC-MS for the identified character impact compounds. Application of fast simplified solid phase extraction techniques (SPME, SBSE) a routine control of processing parameters like degree of roast or extraction technique are possible. Influence of the “crema” on the beverage is hereby very important and characteristic for the espresso type beverage. To gain a first information about distribution of the key aroma compounds between the two layers, a HS analysis of the separated liquids was performed. Of special interest were the potent sulphur containing aroma compounds, analysed by semi-quantitative static headspace GC pulsed flame photometric detection (PFPD). In Table 2 the area counts for the same amount of liquid of the upper and lower layer are reported. It seems that especially methanthiol is effectively protected by the lipophilic layer above the cup, being present in higher amounts in the liquid phase.

**Table 2. semiquantitative data of sulphur compounds above "crema" and coffee beverage.**

| Area counts | H2S | Methanethiol | DMS | CS2 | Thiofuran |
|---|---|---|---|---|---|
| "Crema" | 748 | 453 | 293 | 1498 | 195 |
| liquid | 158 | 5971 | 765 | 175 | 304 |

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# The Body of the *Espresso* Coffee: the Elusive Importance

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## SUMMARY

The 'body' of the *espresso* coffee is the sensory attribute, which "is easier to recognise than to define". This statement, originally adopted to underline the complexity in defining the gels, seems to be very appropriate for an attribute, which, in addition to visual aspect and flavour, greatly contributes to the enjoyment of drinking *espresso* coffee.

The 'body' of *espresso* coffee is often described as the tactile sensation due to the interaction between the beverage and the oral cavity (mouthfeel). However, 'body' may include also olfactive perceptions connected to the intensity of aroma and is described by resorting to terms like "rich", "lingering", or "long lasting".

The classification of mouthfeel characteristics of *espresso* coffee and the subsequent development of a proper vocabulary, permitted to individuate a set of physico-chemical properties, which may contribute to *espresso* coffee mouthfeel perception.

The present study reports on the physico-chemical properties of *espresso* coffee, which may be relevant in the mouthfeel perception. In particular, total solids (concentration), density, rheological and interfacial properties have been determined as a function of cup volume and temperature. The correlation between physico-chemical properties and sensory properties have been described in the framework of oral processing problems.

In order to remove possible ambiguity, in addition to the term 'body', the use of the Italian term *corpo* to describe pure tactile sensation elicited by physico-chemical properties is proposed.

## INTRODUCTION

The 'body' of the *espresso* coffee is the sensory attribute, which "is easier to recognise than to define". This statement, originally adopted by D. Jordan Lloyd (1926) to underline the complexity in defining the gels, seems to be very appropriate for an attribute which in addition to visual aspect and flavour (integration between taste and olfactive sensations (Civille and Lyon, 1996)) greatly contributes to the enjoyment of drinking *espresso* coffee.

In spite of the fact that in Food Science texture is recognised as the third fundamental element to food quality in addition to appearance and chemical senses, its description with an appropriate vocabulary is adopted mostly for solid and semi-solid foods. When espresso coffee texture has to be described, coffee tasters generally use a very limited vocabulary, and in many cases they use the term 'body', only. Unfortunately, the same textural term 'body' can be used indiscriminately in sensory evaluation of dilute coffee infusions and/or of very concentrated coffee beverages like *ristretto* (20 mL or less *espresso* coffee), which strongly differ not only in terms of overall sensory properties but also in their physico-chemical properties.

Several definitions have been proposed for the term 'body' (ASTM E253-78a, ISO 5492:1992, Jowitt 1974, Lingle 2001) which are not univocal, on the contrary they tend to contradict one another, or even themselves. For instance the one proposed by the International Coffee Organization (ICO, 1991) define the term 'body' as an "attribute used to describe the physical properties of the beverage" includes the following statement: "A strong but pleasant full mouthfeel characteristic as opposed to being thin". In this statement 'strong' is intensity related (high score on a graduated scale) but does not describe the sensation; 'pleasant' is a hedonic judgement and 'thin' is a descriptor commonly used in texture analysis (Szczesniak, 1979; 2002) as opposed to thick.

*Espresso* coffee tasters in Italy use the term '*corpo*' to describe the perceptions other than appearance and flavour. This term, used in Italy to describe red wine organoleptic characteristics at least since the middle of the 18$^{th}$ Century (Trinci, 1738) can be translated literally into the word 'body'. However, as far as *espresso* coffee is concerned, the term '*corpo*' can be better related to another well known texture-related concept, the 'mouthfeel', linked to tactile sensations (ISO Standard 5492:1992, Jowitt 1974) and defined as "the mingled experience deriving from the sensations of the skin in the mouth during and/or after ingestion of a food or beverage. It relates to density, viscosity, surface tension and other physical properties of the material being sampled" (quoted in Bourne 2002).

In a recent study (Navarini et al., in press) the texture terms used to describe *espresso* coffee have been classified resorting to a methodology introduced by Alina S. Szczesniak to investigate the mouthfeel characteristics of beverages (Szczesniak, 1979). The terms used by Italian professional *espresso* coffee tasters to describe the *corpo* of *espresso* coffee resulted to fall into six of the eleven categories of sensory mouthfeel terms of beverages and liquid foods individuated by Szczesniak (1979).

These categories are:

- Viscosity-related terms, like thick and viscous;
- Feel on soft tissue surfaces, which includes creamy, smooth, velvety;
- Substance (body)-related terms, like rich/heavy, full-mouthed, consistency;
- Coating of oral cavity, with terms like mouthcoating, clinging, tongue-coating, oral cavity-coating;
- Resistance to tongue movement, as resistance to tongue-palate movements, round, syrupy;
- Afterfeel-mouth terms, like lingering, intense flavour after swallowing, long lasting, prolonged flavour intensity.

*Corpo* of *espresso* coffee, just like 'mouthfeel' is a multi-parameter attribute (Szczesniak, 2002) and then not just a sensory descriptor. Moreover, the term *corpo* may be a synonym of mouthfeel in the case of coffee beverages prepared by a method which permits to modulate the physical properties of the beverage, like *espresso* brewing, whereas the term 'body' may be properly used to describe the mouthfeel of coffee beverages in which flavour, but not physical properties, plays a relevant role.

A few studies have been devoted to correlate the 'body' of *espresso* coffee to measurable variables. Petracco (1989), in a detailed physico-chemical study on *espresso* coffee brew, found viscosity and the presence of particulate matter remarkably relevant in discriminating two pure Arabica blend *espresso* coffees differing in perceived 'body'. The small particles (<10 μm) suspended in the beverage as revealed by optical microscopy, have been recognised as cellular wall fragments of roasted coffee, lipids droplets and gas bubbles, being lipids

droplets particularly abundant, smaller and with narrower size distribution in the sample having the high score for 'body'. In the same study, a comparison between pure *Arabica* and pure *Robusta espresso* coffees showed that the latter was characterized by a higher score for 'body' only when tasted just after preparation (about 2 s), suggesting that in spite of the lower content of lipids droplets the higher foaminess of *espresso* brewed from pure *Robusta* coffee is involved in the 'body' perception. The contribution given by the dispersed phases (lipids droplets and/or bubbles) to the beverage viscosity has been suggested to play a major role in the 'body' perception and to vary depending on botanical variety.

A more recent study (Maeztu et al., 2001), aimed at comparing *espresso* coffees from a physico-chemical and a sensory point of view, confirmed the higher foaminess induced by the presence of high *Robusta* content in the roasted coffee blend (80%) but no statistically significant differences in 'body' have been reported between pure *Arabica* and 80% *Robusta* blend *espresso* coffees. In this study, a highly significant ($p < 0.001$) correlation between total solids on filtrate and 'body' has been found.

Petracco (1989) and Maeztu et al. (2001), focussed their attention on the physico-chemical properties of *espresso* coffee prepared under well defined fixed conditions. However, it is well known that by changing even only one of the several elements, which compose the set of ingredients and technical conditions necessary to prepare an *espresso* coffee, the physico-chemical properties of the beverage can be affected. Recently, for instance, the influence of water pressure (Andueza et al., 2002) and extraction temperature (Andueza et al., 2003) have been reported. Moreover, it has been observed that the *espresso* coffee mouthfeel increases as total extraction volume ('cup volume' for the sake of brevity) decreases (Petracco, 1989), and it is relatively easy to recognize that a *ristretto* (20 mL or less *espresso* coffee) has a stronger sensory impact, including mouthfeel, compared to a *lungo* (40/50 mL or more *espresso* coffee).

These findings stimulated the present investigation aimed at studying the influence of cup volume on both physical and sensory properties of *espresso* coffee. The scope is to offer an overview of the beverage physico-chemical properties which may contribute to the *espresso* coffee mouthfeel perception and thus to attempt to render less elusive a sensory descriptor like 'body' (*corpo*) which differentiate *espresso* coffee from other coffee-based beverages.

## EXPERIMENTAL

### Materials

Medium roasted *Coffea arabica* blend has been ground at an appropriate particle size distribution for *espresso* brewing technique by using a professional grinder (Mazzer, Italy). *Espresso* coffee beverages have been prepared by using two different professional *espresso* coffee machines ("La Marzocco" and "Cimbali", Italy) according to *espresso* coffee preparation standard (Petracco, 2001) except percolation time which has been varied to obtain a wide range of cup volumes. After percolation, the beverages have been subjected immediately to the characterisation or as previously described (Navarini et al., 2004).

### Methods

*Espresso* coffee concentration has been determined as previously described (Maeztu et al., 2001). Density has been determined by means of an Anton Paar densimeter, viscosity has been measured by means of a FVM-80A process viscometer (CBC Europe, Italy) and rheological behaviour has been characterised by using a rate controlled and a stress controlled

Haake rotational rheometers equipped with different geometries and operating in the shear rate range $1–10^3$ $s^{-1}$. Surface tension measurements have been performed in the short-medium time range (0.001–50 s) by means of a Dynamic Maximum Bubble Pressure tensiometer (Gammalab, Germany).

Unless otherwise specified, measurements were performed at 20°C.

As far as sensory evaluation is concerned, eight trained assessors, recruited on the basis of their experience in *espresso* coffee tasting, evaluated differences in perception of *corpo* of *espresso* coffee, brewed at different cup volume, from 5 mL to 35 mL, according to forced choice method. For every beverage exactly 5 mL were presented (in order to avoid inducing correct answer on the basis of volume perception).

In order to eliminate possible recognition due to differences in flavour profiles or appearance, orthonasal *viae* were closed by nose clips, and panellists were blindfolded. They were presented with 2 cups and they had to choose that with a higher perceived *corpo*.

Samples were randomized according to an unbalanced design, with more tests on small differences (diff = 5 mL: 38 tests; diff = 10 mL: 23 tests; diff = 15 mL: 8 tests, diff = 20 mL: 4 tests; diff = 25 mL: 2 tests).

Having two samples, the probability of correct answer, $P_c$, by chance is 0.5. The probability of correct answer is related to the perception probability, $P_d$, by the formula

$$P_c = P_d+1/2(1- P_d)=0.5\ P_d +0.5$$

Since sensory perception threshold is usually defined as the intensity of the stimulus at which $P_d = 0.5$, then $P_c = 0.75$. Therefore we consider not discriminable any recognition under 75%.

## RESULTS AND DISCUSSION

### *Espresso* coffee physico-chemical properties: influence of cup volume

The typical *espresso* coffee overall concentration (associated with the total solids content of the drink) has been reported to be 52.5 g/L (Petracco, 2001). This is the typical result of an extraction process characterised by well-defined conditions, in particular: 6.5 ± 1.5 g for ground coffee portion, 90 ± 5°C for water temperature, 9 ± 2 bar for inlet water pressure and 30 ± 5 s for percolation time. This process permits obtaining a total extraction volume of about 25-30 mL giving a cup of the beverage generally called *espresso* coffee or regular *espresso*. However, if the percolation time is only varied, it is possible to obtain lower or higher extraction volume beverages, well known in Italy as *ristretto* or *lungo espresso* coffees, respectively.

In Figure 1, the *espresso* coffee overall concentration is reported as a function of cup volume. A cup volume of 15 mL (like that used in Southern Italy) corresponds to a concentration of 80-90 g/L whereas concentration lower than 50 g/L (but higher than the typical concentration of coffee beverages prepared with no-pressure methods (Petracco, 2001)) can be obtained at extraction volumes higher than 50 mL. The experimental data follow a power law with an exponent equal to –0.69.

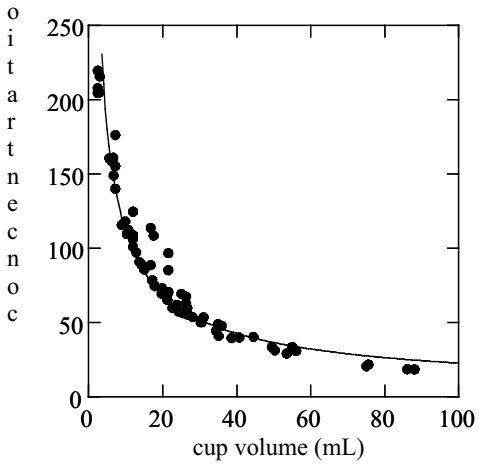


**Figure 1. Concentration as a function of cup volume.**

The wide range of beverage concentrations which can be obtained by the *espresso* brewing technique is expected to affect the beverage physical properties and in particular density, rheological and interfacial properties.

The *espresso* coffee density, as shown in Figure 2, is clearly affected by cup volume, however, the influence is very modest (power law with an exponent equal to –0.015). Density values are in full agreement with literature data.

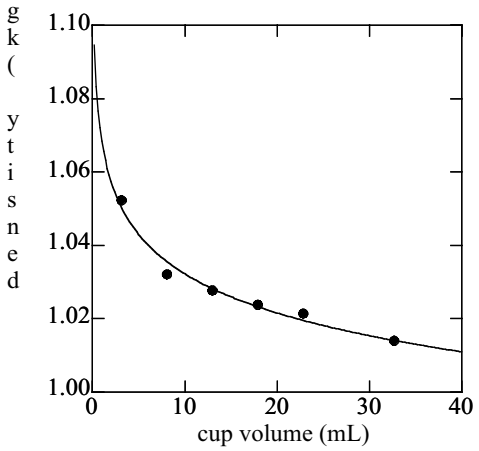


**Figure 2. Density as a function of cup volume.**

A remarkable cup volume influence has been observed for *espresso* coffee viscosity (relative to water at the same temperature), as reported in Figure 3. Between 20 and 40 mL the relative viscosity has been found to range from about 2 to 1.4, respectively, in agreement with previous data (Petracco 1989; Maeztu et al, 2001). The relative viscosity increased up to about 3 passing from 20 mL to 10 mL. Below 10 mL relative viscosity higher than 9 has been measured. As for overall concentration, also in this case the experimental data have been successfully fitted with a power law characterised by an exponent close to –0.66.

The rheological behaviour has been found to be Newtonian without evidence of time-dependent behaviour. Shear-thinning behaviour has been observed at very low (< 5 mL) cup volume only. As expected for low viscosity Newtonian fluids, *espresso* coffee exhibited turbulence at high rates of shear. The shear rate at which the transition from laminar to

turbulent flow occurs, linearly increased by decreasing the cup volume (from 20 s-1 at about 30 mL to 80 s-1 at about 4 mL).

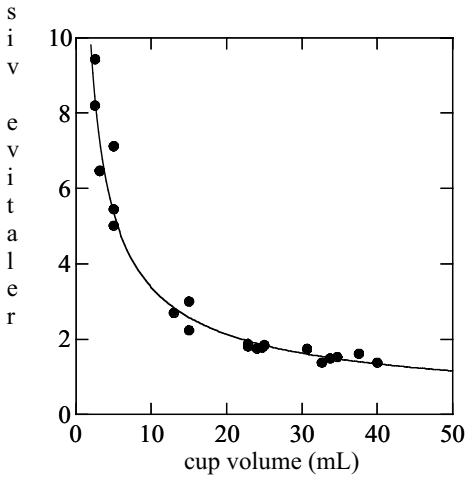


**Figure 3. Relative viscosity as a function of cup volume.**

*Espresso* coffee exhibits a remarkable decrease of the surface tension with time with a behaviour consistent with a system having good wetting properties for oral cavity (Navarini et al., 2004). Figure 4 shows the cup volume dependence of the surface tension (at 10 s) of air – *espresso* coffee interface. The surface tension absolute values are considerably lower than that of pure water at the same temperature confirming a remarkable adsorption phenomenon at air/beverage interface; the tendency to approach high surface tension values by increasing the cup volume is clearly evident. The experimental data resort to a power law with an exponent close to 0.042.

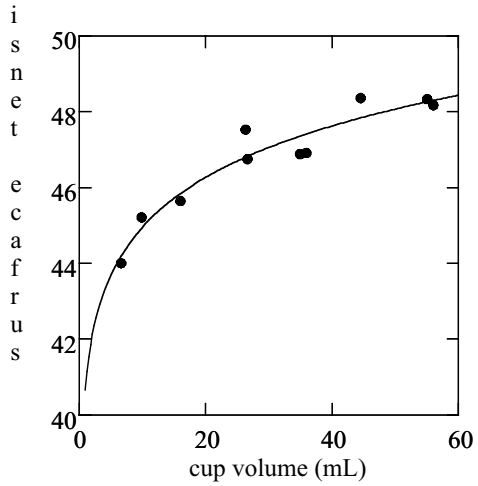


**Figure 4. Surface tension as a function of cup volume.**

**Influence of Temperature**

It is well known that temperature affects the physical properties of liquid foods and beverages (Bourne, 2002). If the temperature range is relatively narrow, a relatively small effect on the correlation between experimentally determined physical properties (e.g. viscosity) and sensory evaluation has been reported (van Vliet, 2002). *Espresso* coffee is prepared at high temperature and it is therefore subjected to a strong temperature gradient between preparation and “in-mouth” temperature when it is consumed. The physical properties are expected to be

remarkably influenced by such a gradient, and this influence has been found to be concentration dependent in other liquid food systems (Bourne, 2002).

Figure 5 shows the temperature dependence of regular *espresso* coffee viscosity. By reducing the cup volume, a more pronounced decrease in viscosity as a function of temperature can be observed (data not shown).

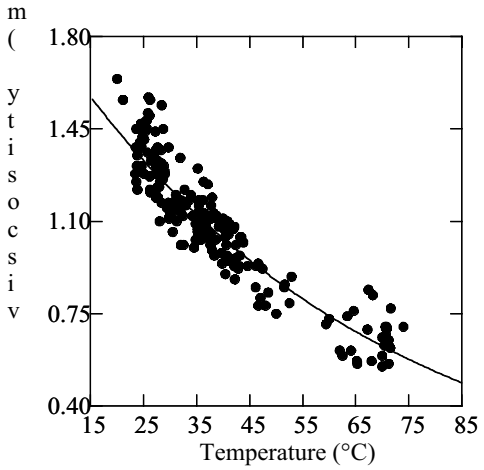


**Figure 5. Viscosity as a function of temperature.**

It is conceivable that the temperature gradient influences the rheological behaviour (e.g. laminar to turbulent flow transition), as well, leading to a very complex rheological process, the set of events beginning with the ingestion of the beverages through to swallowing.

The increase of the temperature from 20 to 37°C has been shown to decrease surface tension values of regular *espresso* coffee (Navarini et al., 2004). Interfacial properties, including contact angles (very important quantities involved in many phenomenological processes like wetting, adhesion and lubrication), have been shown to be strongly influenced by temperature for low cup *espresso* coffee volume (Ferrari, 2004).

**Oral processing**

Bolus formation and swallowing of liquids involves a rapid sequence of events: a volume of fluid is propelled from the oral cavity, down and across the pharyngeal surface of the tongue and enters the hypopharynx and subsequently the oesophagus (quoted in Malone et al., 2003). The interplay between beverage physical properties and oral cavity (saliva and oral surfaces) during this complex set of events is crucial in texture perception.

It has been postulated by Szczesniak (1979), that the viscosity of saliva at rest is taken by the human being as the norm for viscosity judgements. “Thus, beverages called ‘thin’ should, when mixed with saliva in the mouth, exhibit a viscosity lower than saliva while those called ‘thick’ should have a viscosity greater than saliva” (Szczesniak, 1979). Although conceivable, this view represents an oversimplification, being the human saliva a very complex system. In all cases, the statement suggests that the (viscosity) oral perception should be elicited by a physical stimulus.

In healthy individuals, whole human saliva (WHS) is mainly composed of several secretions from various salivary glands, which have their own specific biochemical properties, making WHS a strongly varying mixture of different secretion fluids (van der Rejiden et al, 1993)

with strong interindividual variation of flow rate (Sreebny, 2000). Unstimulated WHS viscosity at 37°C has been reported to be equal to 1.60 ± 0.37 mPas at 1 $s^{-1}$ shear rate and equal to 1.48 ± 0.30 at 300 $s^{-1}$ shear rate, suggesting a non-Newtonian behaviour in the shear rate range occurring in the oral cavity (van der Rejiden et al, 1993). In term of relative viscosity, unstimulated WHS is characterized by values range 2.94 – 1.76. This range of relative viscosity approximately falls within the cup volume range 12 – 27 mL for *espresso* coffee viscosity. By stimulation (mastication or alimentary functions), human whole saliva changes its glandular (and thus chemical) composition and a strong enrichment of (low viscosity) parotid saliva occurs. Stimulated WHS has been reported to be characterized by relative viscosity values ranging from 1.24 ± 0.10 to 2.01 ± 0.47 (Ortega Pantaleon et al, 1998) corresponding approximately to *espresso* coffee cup volume higher than 16 mL.

During assessment of fluid consistency (appraisal of the ease with which the beverage flows between the upper surface of the tongue and the roof of the palate), the shear rate which operates in the mouth has been reported for low viscosity fluids (1 – 10 mPas) to be $10^3 – 10^2$ $s^{-1}$ (Parkinson & Sherman, 1971). This means that during oral processing an *espresso* coffee can be perceived under laminar or turbulent flow, depending on viscosity and then on cup volume.

The *espresso* coffee long-lasting after-taste, a sensation perceived for a while (up to 15 min) after having swallowed and emptied the mouth, has been related to the beverage surface properties (Petracco 2001). Navarini et al. (2004) found the dynamic tensiometric properties of regular *espresso* coffee consistent with those of an efficient 'wetting system' for the oral cavity. In facts, human saliva-coated tooth surface and saliva-conditioned oral mucosa are completely wet by a liquid having its surface tension within the range 35-38 mN/m and 25-27 mN/m, respectively (Christersson, 1991). Regular *espresso* coffee at 37°C (*Arabica*) has been reported to achieve surface tension close to 36 mN/m (Navarini et al., 2004). Stimulated WHS is characterized by surface tension equal to 56.2 mN/m at 30 s (Christersson et al., 2000) at 37°C. By comparing this value with those reported in Figure 4 (obtained at 20°C and at 10 s), it is evident that *espresso* coffee shows a higher surface activity, in agreement with previous studies.

**Sensory evaluation**

In order to assess a possible correlation between instrumentally determined beverage physical properties and texture perception, eight trained assessors evaluated differences in perception of *corpo* of *espresso* coffee in the cup volume range 5-35 mL. The cup tasters were asked to evaluated *corpo* and not 'body', since the lack of flavour profile (noseclips) and visual aspect (blindness) reduce testing to an evaluation of the overall texture (mouthfeel) and not just of a single sensory descriptor.

Figure 6 shows the results of the forced choice test, with the percentage of discriminated samples as a function of the beverage cup volume. The three curves refer to volume differences of 5, 10 and 15 mL between the tested samples.

For a volume difference of 5 mL, the threshold of discrimination for samples is between 10 and 15 mL, that is when a sample has 15 mL and another 20 mL volume, discrimination percentage in terms of corpo is below 75%.

For a volume difference of 10 mL, the threshold of discrimination for samples is between 20 and 25 mL, that is when a sample has 25 mL and another 35 mL volume, discrimination

percentage falls below 75%, but for lower volumes (e.g. 20-30) the panel can discriminate between samples in terms of corpo.

For a volume difference of 15 mL, samples are discriminated (volumes being 20 mL vs. 35 mL); for higher volumes, discrimination percentage drops.

These findings strongly suggest that in the lack of any interference by flavour and appearance, different cup volume *espresso* coffee beverages can be discriminated in term of *corpo*. The threshold of discrimination is remarkably close to the cup volume range corresponding to beverages characterised by relative viscosity similar to that of whole human saliva. In agreement with previous studies (Navarini et al., in press), the sensory perception of 'viscosity' appears to be the most conscious important single mouthfeel sensation of *espresso* coffee.

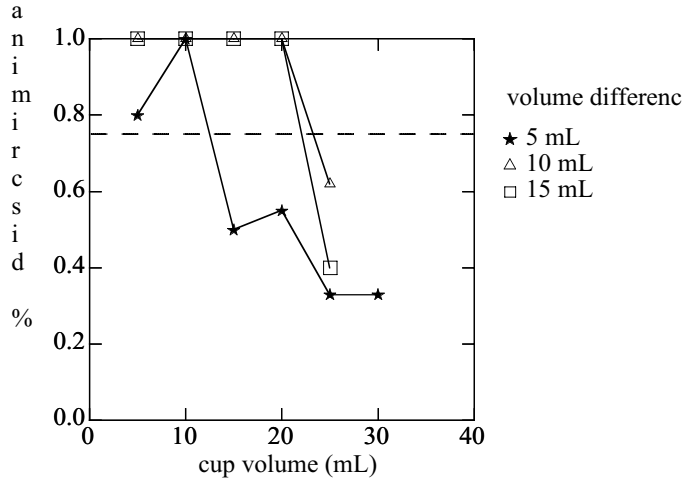


**Figure 6. Discrimination percentage as a function cup volume for different volume differences.**

## CONCLUSIONS

*Espresso* coffee cup volume remarkably affects the physical properties generally mentioned in literature as important in mouthfeel perception. Viscosity (both measured and perceived) seems to play a major role, particularly if beverage-oral cavity interactions are considered.

It cannot be excluded a priori a relevant role also played by *espresso* foam and particulate matter in mouthfeel perception, being particles in food as small as 6 μm detected by a sensory panel (quoted in Bourne 2002).

By eliminating flavour and appearance, diluted beverages have not been discriminated on the basis of oral tactile perceptions suggesting that a proper vocabulary should be adopted in order to describe the texture of coffee beverages. The term *corpo*, used by Italian *espresso* cup tasters, seems to be more appropriate to describe oral sensations, other than flavour and visual aspects, elicited by physical properties whereas the term 'body' may be properly used to describe the mouthfeel of coffee beverages in which flavour, but not physical properties, plays a relevant role.

Taking into the account the difficulties in studying the interaction of texture with taste, smell and flavour, which has only begun to be investigated (Delwiche, 2004) and the interindividual variability in terms of human oral cavity, the adjective "elusive" in discussing the texture of *espresso* coffee may be well justified.

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# Identification of Carotenoids and Carotenoid Degradation Products in Coffee

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## SUMMARY

Carotenoid compounds as well as degradation products from carotenoids have been identified in green coffee origins. The xanthophylls lutein and zeaxanthin were identified by means of HPLC with detection in the visible wavelength region following extraction with acetone. In addition, glycosidically bound ionols were identified as carotenoid degradation products known to be formed by the oxidative degradation of lutein and zeaxanthin. These glycosides were analyzed by adsorption on XAD-1180 resin followed by enzymatic hydrolysis of the eluted extract with ß-glucosidase and GC-MS analysis of the released aglycones.

To investigate the impact of carotenoids on aroma formation a model roasting system was used. For this purpose, "depleted" green coffee was enriched with ß-carotene, a structurally similar carotenoid, since the contents of the naturally occurring lutein and zeaxanthin are small. ß-Carotene was degraded under coffee roasting conditions to yield ß-ionone, its known major degradation product. Furthermore, the importance of 3-oxo-α-ionol as an aroma precursor was also investigated in the model system. 3-Oxo-α-ionol is a precursor for isomeric megastigmatrienones which were also identified in green coffee after aroma isolation by simultaneous-distillation-extraction (SDE). It is suggested that the content of megastigmatrienones is influenced by the sample preparation procedure.

## INTRODUCTION

Carotenoids are a class of natural compounds, which has been extensively studied due to their nutritional importance. Furthermore carotenoids are valued as pigments for use in food products. The role of carotenoids as flavor precursors deserves further attention. A range of powerful aroma compounds is carotenoid-derived. It is mainly foods like tea, wine and fruits in which the composition of carotenoid-derived aroma compounds was thoroughly investigated (Winterhalter and Rouseff, 2002). A scheme, which depicts the potential cleavage of carotenoids, is shown in Figure 1. Carotenoids yield a variety of degradation products with a range of aroma relevance. Predominant carotenoid-derived aroma compounds are $C_9$-$C_{13}$ norisoprenoids.

ß-Damascenone, a $C_{13}$ norisoprenoid, is among the very few carotenoid-derived aroma compounds described for coffee (Holscher and Steinhart, 1994). ß-Damascenone exhibits a very low flavor threshold and was found to be part of the list of potent odorants for the powder and brew of Arabica coffee (Blank et al., 1992).

The occurrence of carotenoids in green coffee is stated in the literature (Holscher and Steinhart, 1994; Yeretzian et al., 2002) and their precursor relevance for ß-damascenone is also mentioned. However, a screening of the literature reveals that no clear identification and quantification of carotenoids in green coffee has been reported so far. Therefore we attempted a quantitative analysis of carotenoids and also conducted model studies to evaluate their

relevance as flavor precursors for coffee. In addition, this study on glycosidically bound flavor precursor compounds will add to the knowledge on glycoconjugated progenitors in coffee. Previously, linalool disaccharides, sugar esters of 3-methylbutyric acids as well as diterpenoid glycosides (Weckerle et al., 2002, 2003; Bradbury and Balzer, 1999) were identified in coffee.

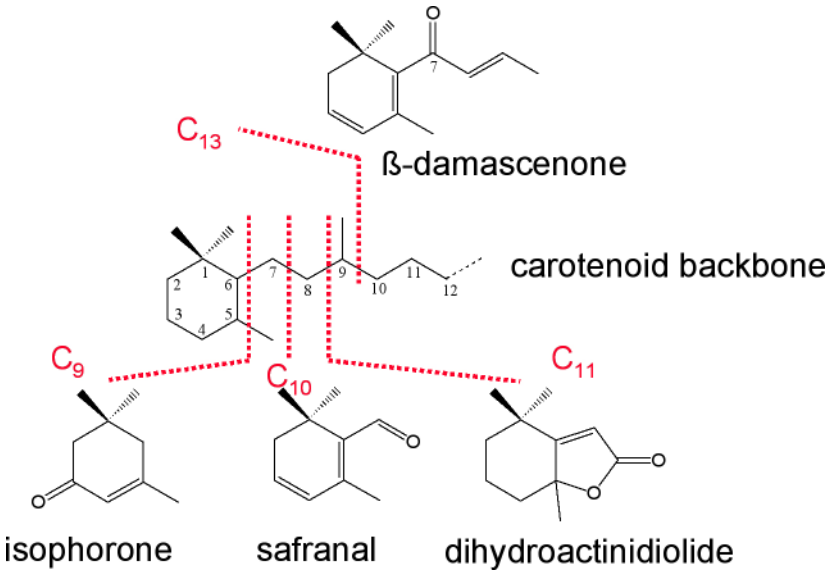


**Figure 1. Scheme for cleavage of carotenoids (adapted from Winterhalter and Rouseff, 2002).**

## MATERIAL AND METHODS

### Analysis of carotenoids

Carotenoids: Ground green coffee (50 g) was extracted with 2 × 200 mL of acetone for 30 min each. The extract was filtered and evaporated nearly to dryness. The pigments were extracted into diethylether. The extract was saponified with 10 mL of methanolic KOH (30 g KOH/l methanol) at room temperature for 10 min under constant shaking. The solution was washed with water (10% NaCl was added for better phase separation) until pH 7 was reached. The ether was removed, the residue taken up in acetone and made up to 5 mL. The solution was filtrated over 0.45 µm hydrophobic filters and injected into the HPLC (Luna RP18, 5 µm, 250×4.6 mm; MeCN/$H_2O$ (solvent A, 9/1, v/v), ethyl acetate (solvent B), linear gradient: 0-60% B in 16 min, 60-100%B in 16-36 min, flow rate 1 mL/min, detection at 440 nm. HPLC equipment: Agilent 1100 Series with diode array detector.

### Analysis of carotenoid degradation products

Ca. 200 g of green coffee was deactivated with 100 mL of ethanol and 120 mL of water for 24 h at room temperature. After evaporation, drying and grinding, 100 g was defatted with 300 mL of n-hexane. The beans were then extracted with methanol (300 mL and 2 × 250 mL at room temperature). The methanol was evaporated, the residue taken up in 150 mL water and cleaned up on a small XAD-1180 column (filled with 40 g of the resin). The column was washed with water (500 mL) and eluted with methanol (ca. 500 mL). The methanolic eluate from that column was evaporated to dryness (yield ca. 800 mg). 400 mg were dissolved in 50 mL of buffer solution pH 5.0 and extracted three times with 40 mL of pentane/ether (1/1, v/v) to remove free volatile aroma compounds. After evaporation of residual solvent, the solution was hydrolysed by emulsin (75 mg, ca. 7 units, ß-glucosidase from almonds, at 37°C for 72

h). The liberated aglycones were extracted with 3 × 40 mL pentane/ether (1/1, v/v, after addition of tridecane as internal standard), washed with 2 × 40 mL of 0.1N HCl, concentrated on a small Kuderna-Danish column to ca. 0.5 µl and analysed by GC-FID or GC-MS (60 m DB-WAX, 0.32×0.25 µm, 40-240 °C, 5°C/min).

**Synthesis of reference compounds**

- 3-Oxo-α-ionol was synthesized according to Aasen et al., 1973.
- The isomeric megastigmatrienones were prepared by dehydration of 3-oxo-α-ionol with $KHSO_4$ according to Aasen et al., 1972.

**Model roasting trials**

Depleted Robusta beans were produced by extraction with water at 80°C. The extraction was considered as complete if roasting of extracted and dried beans and subsequent tasting showed an almost complete loss of the typical coffee flavor and coffee tasted cereal and popcorn-like. These "depleted" beans were then used for infusion of ß-carotene and 3-oxo-α-ionol, respectively. 10 mg of the compounds were dissolved in a few mL of ethanol, diluted with 100 mL of water and infused into 150 g of the depleted beans at 80°C for 2h, dried and roasted (Neuhaus Neotec Signum, 260°C, 180 sec.).

**Isolation of aroma compounds from model roasting trials**

The R&G coffee was extracted using a conventional Likens-Nickerson simultaneous-distillation-extraction (SDE) apparatus. 100 g of coffee was extracted for 2h using a pentane-ether mixture (1/1, v/v). The resulting extract was concentrated on a Kuderna-Danish column and dried over $Na_2SO_4$. The analysis by GC-MS was performed on a 60 m DB-WAX (0.32 × 0.25 µm) with the following temperature gradient: 40-240 °C at a rate of 5°C/min.

**RESULTS AND DISCUSSION**

**Identification of carotenoids in green coffee**

The xanthophylls lutein and zeaxanthin were identified in green coffee by HPLC-DAD analysis and detection in the visible wavelength region. The structures are displayed in Figure 2.

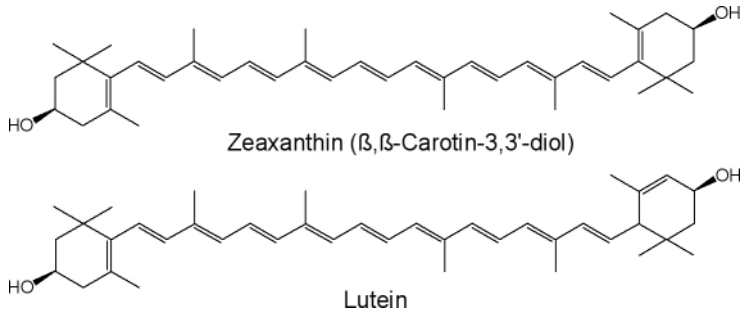


**Figure 2. Structures of carotenoids identified in green coffee.**

A representative HPLC chromatogram of an acetone extract of green coffee is shown in Figure 3. Besides the carotenoids with the characteristic UV-VIS spectra with three maxima, a range of chlorophyll derivatives can be also traced in the chromatographic run.

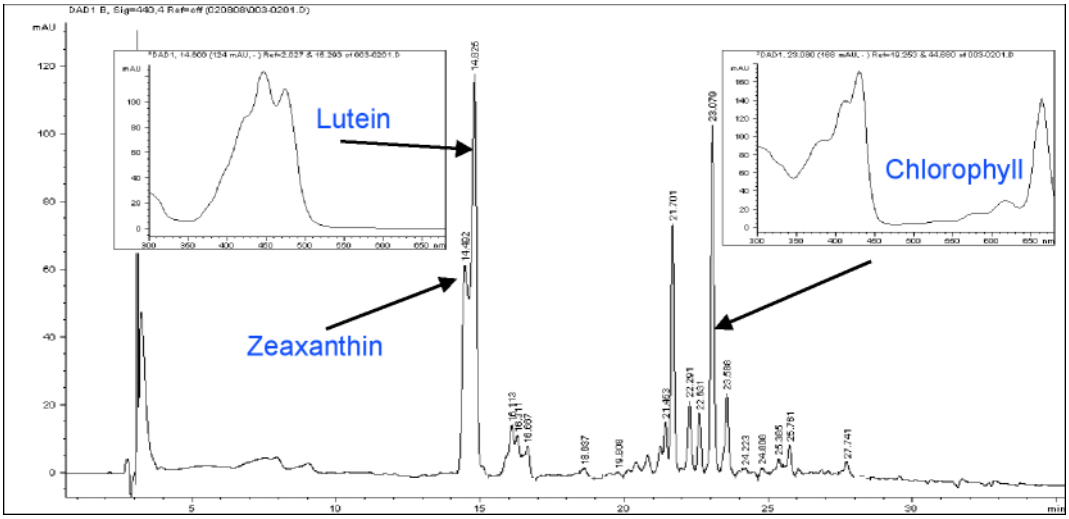


**Figure 3. HPLC chromatogram of green extract with inserts showing UV-VIS DAD spectra.**

A method for the quantitative determination of carotenoids in green coffee involving alkaline saponification was developed and results are presented below. It was found that general levels are low and that Robusta contains a higher concentration of carotenoids than Arabica. Kenya, Brazil and Colombia were analyzed for carotenoids and 0.2-0.7 mg/kg carotenoids (calculated as sum of lutein and zeaxanthin) were found in the green coffees, whereas contents up to 1.5 mg/kg were found for Robusta.

**Identification of carotenoid degradation products**

Carotenoids are labile compounds. By oxidative degradation, primary cleavage products are formed which undergo subsequent enzymatic modification (Knapp et al., 2002). These are usually glycosidically bound, non-volatile compounds and can be cleaved using ß-glucosidase enzyme preparations to release the respective aglycones. The aglycones are the potent aroma compounds and can then be analyzed by GC-MS (Winterhalter and Rouseff, 2002). By using this approach, two glycosidically bound ionols (3-oxo-α-ionol, ***1*** and 3-oxo-7,8-dihydro-α-ionol, ***2***) were identified in green coffee (cf. Figure 4 for structures) after enzymatic liberation from the isolated glycosidic mixture (Strauss et al., 1987).

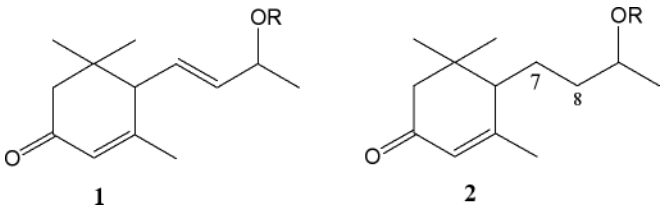


**Figure 4. Structures of glycosides found in green coffee: 3-oxo-α-ionol (*1*, R=sugar moiety); 3-oxo-7,8-dihydro-α-ionol (*2*, R=sugar moiety).**

The presence of 3-oxo-α-ionol (***1***) was verified with a synthesized standard reference compound, whereas 3-oxo-7,8-dihydro-α-ionol (***2***) was only tentatively identified based on its retention index (RI) in conjunction with the MS spectrum.

These two ionols are known lutein- and zeaxanthin-derived aroma precursors and have been identified in many foods (Enzell, 1985; Williams et al., 1992). So far, the data on the presence

of glycosidically bound carotenoid-degradation products in green coffee is scarce. The presented data will help to increase the knowledge of flavor precursors in coffee and elucidate further generation pathways of aroma compounds. In the light of the present investigation, the search for ß-damascenone precursors in the form of glycoconjugates, such as previously identified in other plants (Roberts et al., 1994; Straubinger et al., 1997) might be successful.

**Model roasting trials**

In order to elucidate the significance of carotenoids for the flavor generation potential in coffee, model roasting trials with added amounts of carotenoids were performed. For this purpose, green coffee beans were depleted, i.e. extracted with hot water to remove all flavor precursor compounds. These "empty" beans were then used as a reaction chamber for the added compounds. In this model system, ß-carotene, which does not naturally occur in green coffee, was added and the green beans roasted. The aroma generated from the carotene was determined following simultaneous-distillation-extraction (SDE) with a Likens-Nickerson-type apparatus. As a result, the known carotenoid degradation product ß-ionone could be identified by GC-MS with the help of an authentic reference compound. This finding confirms that the degradation reaction of carotenoids occurs during coffee roasting and that carotenoids are a source of potential aroma for roasted coffee.

In further model roasting trials, synthesized 3-oxo-α-ionol ***1*** (Aasen et al., 1973) was infused into the depleted beans and the bean matrix roasted. By analysis of the roasted coffee by SDE and GC-MS, four newly formed compounds were detected. By comparison with literature MS spectra (Aasen et al., 1972), they were identified as isomeric megastigmatrienones (Figure 5). A generation pathway for the megastigmatrienones via 3-oxo-α-ionol (***1***) has been proposed by Wahlberg, 2002.

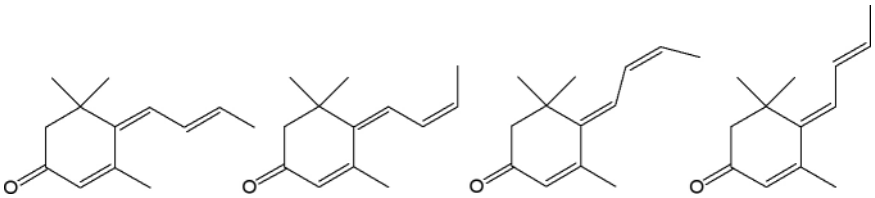

**Figure 5. Isomeric megastigma-4,6,8-trien-3-ones identified in roasted coffee model system with added 3-oxo-α-ionol.**

The megastigmatrienones were described in the literature as character impact compounds of Burley tobacco (Enzell, 1985). This prompted us to search for their occurrence in R&G coffees. However, in R&G coffee no megastigmatrienones were detected, whereas they are present in green coffee. Therefore, megastigmatrienones are potentially newly identified green coffee constituents. It has to be taken into account that the sample preparation consisted of SDE which involves boiling of green coffee at acidic pH levels over prolonged time periods. Since glycoconjugates are labile products which undergo cleavage and rearrangement reactions in their free forms, it must be considered that the content of megastigmatrienones is manipulated by the sample preparation step (Winterhalter and Schreier, 1988). Their aroma relevance as such and as precursor compounds for other aroma compounds remain to be established and requires further model reactions as well as GC-olfactometry studies.

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# Novel Instant Coffee Aromatizing Compositions

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## SUMMARY

Instant coffee products typically lack the intense aroma that characterizes roast coffee. Consequently, preparing an instant coffee beverage deprives the consumer of the full sensory experience that brewing roast coffee affords. A new controlled-release delivery technology has been developed that can significantly increase peak aroma intensity during instant coffee preparation. Key to this technology is the use of unconventional, buoyant, water-immiscible, volatile carrier liquids to create novel aromatizing compositions with improved functionality. A model aroma comprised of physically and chemically diverse volatile liquids was compounded into a variety of carrier liquids and encapsulated in instant coffee granules designed for addition to coffee products. Reconstituting granules containing aromatized unconventional carriers in hot water more effectively directed the aromatizing composition onto the beverage surface where rapid evaporation imparted intense preparation aroma without adversely affecting flavor or appearance. The effect of carrier physical properties on functionality and aroma release in a model system will be presented.

## MATERIALS

2-ethylfuran, 2-methylpropanal, diacetyl, isobutyl acetate, 4-ethylguaiacol, eugenol, and ethyl acetate were obtained from Sigma-Aldrich. Ethanol was obtained from Aaper and d-limonene from Firmenich. Soybean oil obtained from ADM was used as a reference to generally depict the performance of predominantly triglyceride coffee oil.

## METHODS

### Encapsulation

The following process was used to separately encapsulate aromatized d-limonene, 2-ethylfuran, ethyl acetate, ethanol, and soybean oil in coffee granules. Each carrier was aromatized by combining 70 volumes with five volumes each of 2-ethylfuran, 2-methylpropanal, diacetyl, isobutyl acetate, 4-ethylguaiacol, and eugenol. A 50% coffee solution, prepared by dissolving 21g freeze-dried Kenco® coffee in 21 g water, was chilled to 3°C and aerated for one minute at 10,000 rpm with an immersion mixer. 7 g aromatized carrier was added and the aeration process repeated to emulsify or disperse the aromatized carrier in the coffee solution. The solution was dripped into liquid nitrogen using a syringe with 24-guage needle to form frozen particles which were then dropped onto a bed of finely milled coffee powder and allowed to desiccate for two days in a closed jar. The powder was sieved between U.S. no. 10 and 12 mesh screens to collect the dry granules containing encapsulated aromatized carriers. The granules had density < 1.0 g/cc and floated on the surface of water.

**Analysis**

The following process was used to quantify the release of aroma components from coffee granules into the air above a simulated hot beverage. 0.1g granules were dropped onto the surface of 200 mL 85°C water within a closed 250 mL jar while sweeping the headspace with nitrogen gas at a rate of 300 mL/minute. Immediately upon granule addition, the nitrogen stream was diverted into a series of time-sequenced Tenax traps. Traps containing aroma released during 0-10, 10-20, 20-30, 30-60, 60-90, and 90-120 second time segments were later desorbed and analyzed using GC-MS to quantify aroma component counts released during each segment.

**RESULTS**

Aroma release data are summarized in Tables 1-10. Carrier and aroma component physical properties are outlined in Tables 11-12. Aroma components were totaled, with 30-second segments normalized to average 10-second increments, and plotted versus midpoints to illustrate aroma release rate for each encapsulated carrier (Figure 1) and carrier performance relative to soybean oil (Figure 2). All data presented are averages of duplicate experiments and aroma totals do not include 2-ethylfuran counts since this aroma component was also used as a carrier.

**Table 1. Aroma released in 0-10 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 5.6 | 7.8 | 61.3 | 15.8 | 27.5 |
| diacetyl | 3.0 | 1.3 | 18.5 | 5.1 | 20.2 |
| 2-ethylfuran | 10.1 | 26.5 | --- | 38.0 | 59.6 |
| isobutyl acetate | 7.9 | 33.7 | 119 | 27.9 | 63.3 |
| 4-ethylguaiacol | 2.3 | 9.6 | 16.9 | 4.2 | 6.0 |
| eugenol | 2.4 | 8.4 | 24.4 | 3.8 | 4.0 |
| Total Aroma | 21.2 | 60.8 | 240.1 | 56.8 | 121.0 |
| Versus Oil | 1.00x | 2.87x | 11.33x | 2.68x | 5.72x |

**Table 2. Aroma released in 10-20 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 52.4 | 33.8 | 185 | 149 | 152 |
| diacetyl | 18.5 | 6.2 | 81.0 | 46.6 | 89.8 |
| 2-ethylfuran | 153 | 127 | --- | 377 | 332 |
| isobutyl acetate | 114 | 163 | 464 | 377 | 411 |
| 4-ethylguaiacol | 4.8 | 9.4 | 36.2 | 20.9 | 23.7 |
| eugenol | 3.2 | 8.3 | 18.7 | 18.4 | 18.0 |
| Total Aroma | 192.9 | 220.7 | 784.9 | 611.9 | 694.5 |
| Versus Oil | 1.00x | 1.14x | 4.07x | 3.17x | 3.60x |

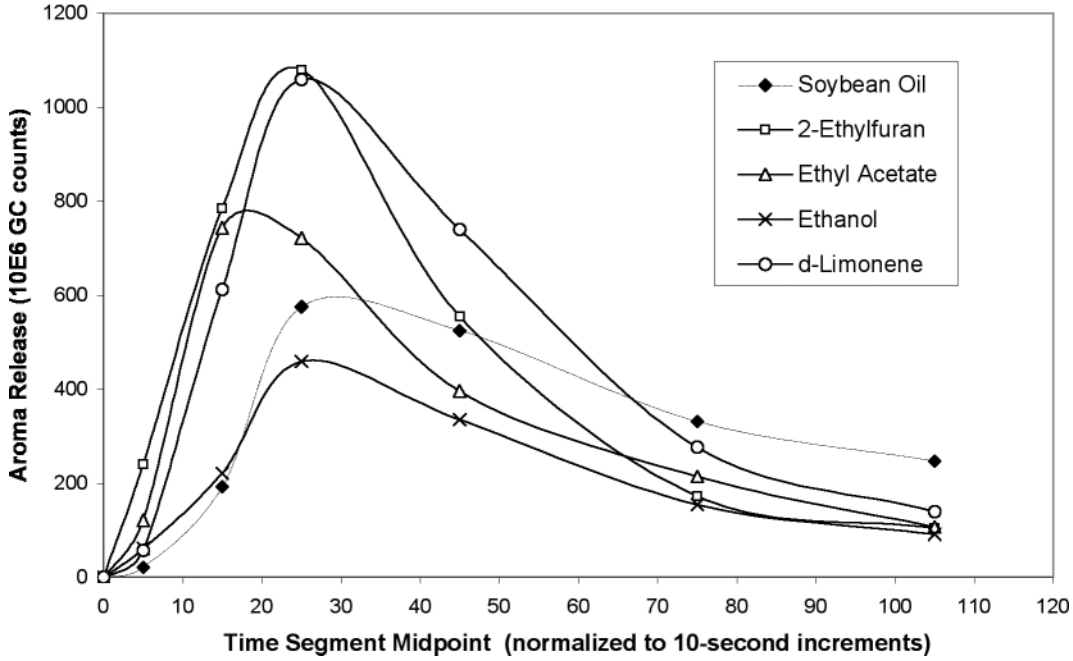


**Figure 1. Aroma release from encapsulated carriers.**

**Table 3. Aroma released in 20-30 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 126 | 76.8 | 158 | 247 | 131 |
| diacetyl | 46.3 | 18.4 | 88.7 | 77.7 | 84.3 |
| 2-ethylfuran | 358 | 231 | --- | 582 | 319 |
| isobutyl acetate | 337 | 317 | 695 | 666 | 417 |
| 4-ethylguaiacol | 56.6 | 21.7 | 64.0 | 38.1 | 23.3 |
| eugenol | 9.7 | 24.9 | 74.0 | 30.1 | 16.8 |
| Total Aroma | 575.6 | 458.8 | 1,079.7 | 1,058.9 | 672.4 |
| Versus Oil | 1.00x | (0.80x) | 1.88x | 1.84x | 1.17x |

**Table 4. Cumulative aroma released in 0-30 seconds.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 184.0 | 118.4 | 404.3 | 411.8 | 310.5 |
| diacetyl | 67.8 | 25.9 | 188.2 | 129.4 | 194.3 |
| 2-ethylfuran | 521.1 | 384.5 | --- | 997.0 | 710.6 |
| isobutyl acetate | 458.9 | 513.7 | 1,278 | 1,070.9 | 891.3 |
| 4-ethylguaiacol | 63.7 | 40.7 | 117.1 | 63.2 | 53.0 |
| eugenol | 15.3 | 41.6 | 117.1 | 52.3 | 38.8 |
| Total Aroma | 789.7 | 740.3 | 2,104.7 | 1,727.6 | 1,487.9 |
| Versus Oil | 1.00x | (0.94x) | 2.67x | 2.19x | 1.88x |

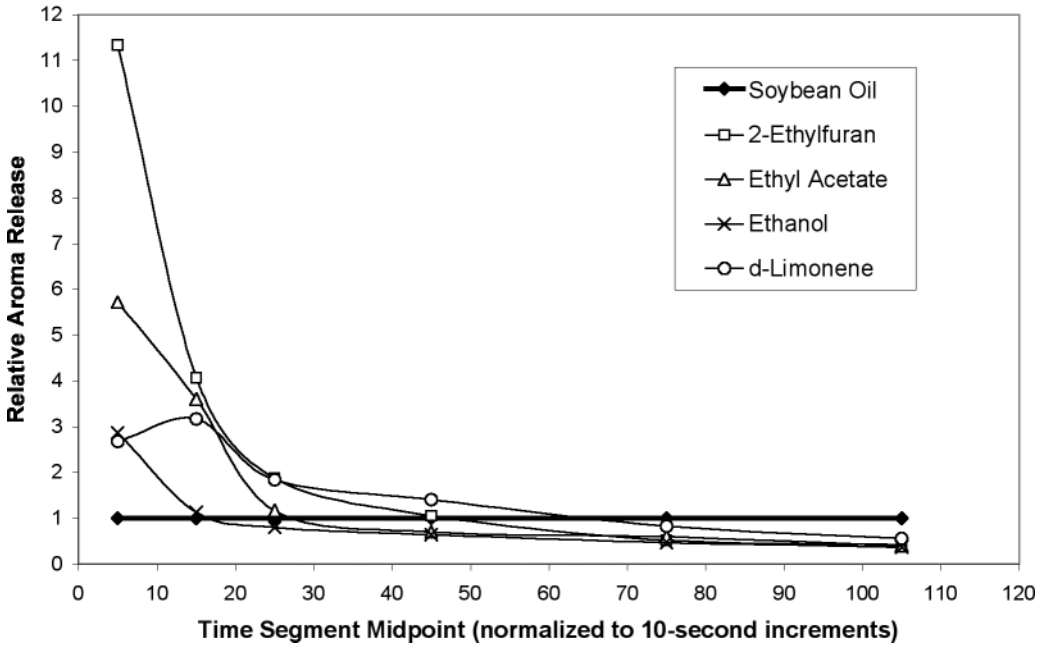


**Figura 2. Aroma release relative to Soybean oil.**

**Table 5. Aroma released in 30-60 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 312 | 160 | 254 | 485 | 165 |
| diacetyl | 119 | 53.4 | 158 | 135 | 143 |
| 2-ethylfuran | 853 | 441 | --- | 1,017 | 502 |
| isobutyl acetate | 993 | 641 | 916 | 1,379 | 673 |
| 4-ethylguaiacol | 91.6 | 71.2 | 145 | 103 | 66.4 |
| eugenol | 59.8 | 80.8 | 190 | 117 | 62.3 |
| Total Aroma | 1,575.4 | 1,006.4 | 1,663.0 | 2,219 | 1,109.7 |
| Versus Oil | --- | (0.64x) | 1.06x | 1.41x | (0.70x) |

**Table 6. Aroma released in 60-90 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 151 | 56.8 | 47.4 | 100 | 45.6 |
| diacetyl | 82.1 | 30.0 | 45.8 | 57.6 | 54.1 |
| 2-ethylfuran | 539 | 199 | --- | 331 | 276 |
| isobutyl acetate | 610 | 264 | 188 | 436 | 357 |
| 4-ethylguaiacol | 88.9 | 58.5 | 109 | 113 | 77.7 |
| eugenol | 62.8 | 54.4 | 128 | 123 | 66.0 |
| Total Aroma | 994.8 | 463.7 | 518.2 | 829.6 | 600.4 |
| Versus Oil | --- | (0.47x) | (0.52x) | (0.83x) | (0.60x) |

**Table 7. Aroma released in 90-120 second segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 105 | 23.6 | 19.2 | 41.7 | 23.5 |
| diacetyl | 78.1 | 20.3 | 28.8 | 35.6 | 34.7 |
| 2-ethylfuran | 378 | 71.8 | --- | 107 | 135 |
| isobutyl acetate | 382 | 97.2 | 75.2 | 162 | 145 |
| 4-ethylguaiacol | 102 | 74.4 | 88.2 | 102 | 54.8 |
| eugenol | 73.9 | 57.1 | 104 | 77.0 | 40.4 |
| Total Aroma | 741.0 | 272.6 | 315.4 | 418.3 | 298.4 |
| Versus Oil | 1.00x | (0.37x) | (0.43x) | (0.56x) | (0.40x) |

**Table 8. Total aroma released (0-120 seconds).**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Aroma Component | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 2-methylpropanal | 752.0 | 358.8 | 724.9 | 1,038.5 | 544.6 |
| diacetyl | 347.0 | 129.6 | 420.8 | 357.6 | 426.1 |
| 2-ethylfuran | 2,291.1 | 1,096.3 | --- | 2,452.0 | 1,623.6 |
| isobutyl acetate | 2,443.9 | 1,515.9 | 2,457.2 | 3,047.9 | 2,066.3 |
| 4-ethylguaiacol | 346.2 | 244.8 | 459.3 | 381.2 | 251.9 |
| eugenol | 211.8 | 233.9 | 539.1 | 369.3 | 207.5 |
| Total Aroma | 4,100.9 | 2,483.0 | 4,601.3 | 5,194.5 | 3,496.4 |
| Versus Oil | 1.00x | (0.61x) | 1.12x | 1.27x | (0.85x) |

**Table 9. Aroma release versus time segment.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Time Segment | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 0-10 sec | 21.2 | 60.8 | 240.1 | 56.8 | 121.0 |
| 10-20 sec | 192.9 | 220.7 | 784.9 | 611.9 | 742.7 |
| 20-30 sec | 575.6 | 458.8 | 1,079.7 | 1,058.9 | 721.3 |
| 0-30 sec | 789.7 | 740.3 | 2,104.7 | 1,727.6 | 1,585.0 |
| 30-60 sec | 1,575.4 | 1,006.4 | 1,663.0 | 2,219.0 | 1,187.4 |
| 60-90 sec | 994.8 | 463.7 | 518.2 | 829.6 | 642.7 |
| 90-120 sec | 741.0 | 272.6 | 315.4 | 418.3 | 320.4 |
| 0-120 sec | 4,100.9 | 2,483.0 | 4,601.3 | 5,194.5 | 3,735.5 |
| Versus Oil | 1.00x | (0.61x) | 1.12x | 1.27x | (0.91x) |

**Table 10. Aroma release normalized to soybean oil.**

| | GC Counts (1x10E6) | | | | |
|---|---|---|---|---|---|
| Time Segment | Soybean Oil | Ethanol | 2-Ethylfuran | d-Limonene | Ethyl Acetate |
| 0-10 sec | 1.00x | 2.87x | 11.33x | 2.68x | 5.72x |
| 10-20 sec | 1.00x | 1.14x | 4.07x | 3.17x | 3.60x |
| 20-30 sec | 1.00x | (0.80x) | 1.88x | 1.84x | 1.17x |
| 0-30 sec | 1.00x | (0.94x) | 2.67x | 2.19x | 1.88x |
| 30-60 sec | 1.00x | (0.64x) | 1.06x | 1.41x | (0.70x) |
| 60-90 sec | 1.00x | (0.47x) | (0.52x) | (0.83x) | (0.60x) |
| 90-120 sec | 1.00x | (0.37x) | (0.43x) | (0.56x) | (0.40x) |
| 0-120 sec | 1.00x | (0.61x) | 1.12x | 1.27x | (0.85x) |

**Table 11. Physical properties of carrier liquids.**

| Carrier Liquid | Density (g/cc) | Boiling Point (ºC) | 25ºC Vapor Pressure (mm Hg) | Water Solubility |
|---|---|---|---|---|
| 2-Ethylfuran | 0.91 | 92 | ~ 50 | Insoluble |
| d-Limonene | 0.84 | 175 | 2 | Insoluble |
| Ethyl Acetate | 0.90 | 77 | 94 | Slight (8%) |
| Ethanol | 0.79 | 78 | 59 | Miscible |
| Soybean Oil | 0.92 | none | 0 | Insoluble |

**Table 12. Properties of model aroma components.**

| Aroma Component | Chemical Classification | Density (g/cc) | Boiling Point (ºC) | Water Solubility |
|---|---|---|---|---|
| 2-methylpropanal | Aldehyde | 0.91 | 64 | Low (10%) |
| diacetyl | Ketone | 0.84 | 88 | Moderate (20%) |
| 2-ethylfuran | Heterocyclic | 0.91 | 92 | Insoluble |
| isobutyl acetate | Ester | 0.90 | 118 | Very Low |
| 4-ethylguaiacol | Aromatic | 0.79 | 235 | Very Low |
| eugenol | Terpene | 0.92 | 255 | Insoluble |

## DISCUSSION

2-ethylfuran had the most desirable combination of physical properties and produced the most rapid aroma release and highest peak intensity. Soybean oil had the least desirable physical properties and produced the lowest peak intensity. The other novel carriers, d-limonene and ethyl acetate, also performed well. The latter provided very high peak aroma intensity with only somewhat reduced total aroma release attributed to slight solubility in water. The relatively poor performance of ethanol was attributed to rapid miscibility with water. Each novel carrier released significantly more aroma than soybean oil and ethanol in the critical first 30 seconds after granule addition to water. Only aromatized soybean oil produced an unsightly residual oil slick of the type common to use of triglyceride-based carriers like coffee oil.

## CONCLUSIONS

The unique combination of volatility, water immiscibility, and buoyancy possessed by these novel carriers can significantly increase aroma release rate, peak intensity, and recovery from encapsulates used in hot beverage applications.

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# Forecasting Coffee Quality by Near Infrared Transflectance Spectra: a Virtual Cup-Tester Panel

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## SUMMARY

Espresso coffee value stems mainly from its sensory quality, as traditionally determined by cup-testers' panels. Since this approach is time-consuming and expensive, the number of tested samples per day is often inadequate to meet with industrial needs.

An instrumental screening procedure, able to discard *a priori* undesirable samples likely to be rejected by the panel, would be therefore of help if rapid, non destructive, and reliable.

We developed a procedure for checking the reliability of predictions obtained examining a large number of Near Infra Red Transflectance spectra with the related sensory Merit data, using a data base regression technique know as LOCAL in the WinISI software package.

From a total of 3438 raw coffee samples from Brazil, collected over a seven year period, 1753 samples were chosen to build a library. We studied predictions based on global calibrations and by local data base regression, selecting appropriate neighbours to an individual calibration for each sample.

Results show that LOCAL data base regression technique performs better than global calibration by partial least squares regression, and is precise enough for industrial purposes.

## INTRODUCTION

### Coffee quality

Coffee, often referred to as the second most globally traded good after petroleum, is not needed for nutritional purposes but is much appreciated for its taste appeal, along with its physiological effects on alertness and performance. The assessment of quality is of paramount importance to both of these aspects, in order to supply the customers with a pleasant and wholesome product. This applies especially to espresso coffee (Illy and Viani, 1995), a way to enjoy a cup of coffee that is gaining large popularity throughout the world because of the greater sensory impact it has on the consumer, when compared with coffee brews prepared by other methods (Petracco, 2001).

Because of the high complexity of the raw seed matrix, and even more so when dealing with the roasted finished product, proximate analysis methods able to dig into the structure of ill-known families of compounds are required. The chemical approach to the sensory sphere benefits nowadays of the development of increasingly sophisticated analytical methods, where

the parts per billion of volatile aromas are not the final frontier of detection limits (Vitzthum, 1999).

**Sensory analysis**

Anyway, no matter the progress of instrumental techniques, the good old cup-testing approach still remains the ultimate quality assessment tool. After all, the reason why coffee has become so popular, achieving the position of the second most largely consumed beverage after water, is its flavour or, even better, its overall impact on our senses. Sensory evaluation, which used to be considered the magic because "taste is a matter of taste", is nowadays earning the status of a highly respected analytical tool, able to produce key information with good reliability (Meilgaard et al., 1999).

In industrial coffee routine, some form of objective evaluation is needed to ascertain product overall quality, along with the constancy of that quality on time and on varying process conditions.

The "tool" commonly put to use is a panel of assessors, who may be either coffee experts (professional cup-testers) or naïve consumers after a basic training (I.C.O., 1991). The reason for employing more than one people is obvious: by averaging responses, the risk of incorrect judgement due to a possible bad shape or minor illness of one person is minimised. Another panel potential is the synergy that can be gained by debating coffee characteristics among the assessors during open sessions: this procedure may extract more information, since individual sensitivities and perception thresholds may be different.

Unfortunately, implementing such a practice is neither simple nor inexpensive. Cup-testing sessions cannot be too long or frequent during the day, because some fatigue develops after the first dozen of cups or so. This is particularly true for espresso tasting, due to the presence of tiny coffee oil droplets in emulsion (Petracco, 1989), which stick on the tongue and on mouth membranes imparting a lingering after-taste. As a consequence, industry strives to take advantage of sensory data collections, using them as raw experimental data to calibrate instrumental screening methodologies.

**Instrumental testing**

Near-infrared spectroscopy is a good instance of such a rapid, non-destructive fingerprinting technique. It is based on a absorption measurements of scanned monochromatic near-infrared light, whose energy is dissipated in rotational and vibrational movements of the molecular bonds of the material under examination, and ultimately transformed into heat (Murray and Williams, 1987). Energy absorption patterns contain a lot of implicit information about molecular response to specific wavelengths and to their combinations. The procedure generally employed to analyse solid materials, opaque to visible light, is known under the name of diffuse reflectance. Since infrared radiation is able to penetrate some millimetres under the surface of solid materials, the diction Near Infra Red Transflectance (NIRT) may apply to better describe the technique used in the present work.

NIRT has been shown suitable for supplying simultaneous forecasts of many chemical characteristics of the sample examined, provided that a good calibration has been previously obtained by statistical correlation with traditional, time-consuming analytical methods. This secondary method has widely been used with agricultural products (Shenk, 1992) and sometimes in the specific domain of coffee (Davrieux et al., 2001; Downey and Boussion, 1996; Guyot et al., 1993), also for modelling sensory data (Feria-Morales, 1991).

**Objective of this work**

In the present work, we have arranged NIRT data to predict a proprietary overall sensory quality variable, called Merit, in order to allow the screening of large quantities of unground raw coffee samples as a preliminary to the orthodox cup-testing evaluation for acceptance.

Our purpose is not to replace the panel by an instrument, but rather to develop an automatic shielding procedure that, while taking into account the risk of discarding good coffees (which, in particular years, are a rare commodity), eliminates those samples that at any rate would have been rejected by the panel.

**EXPERIMENTAL**

**Materials**

Several thousand raw coffee samples from different origins have been examined during seven years in a NIRSystems 6500 analyser (Foss Tecator), equipped with a translational sample presentation module. This module allows to average multiple NIRT scans of approximately 100 g of whole coffee beans, which happens to be the correct sample size used to roasting and brewing a cup for panel testing.

Each of those samples has been further processed and prepared for espresso cup-testing, according to a standardised protocol described elsewhere (Petracco, 2000). The evaluation of several organoleptic variables of the beverage, including the overall Merit, was collected.

**Methods**

Spectra, consisting in 1050 log 1/R data points from 400 to 2498 nm in 2 nm steps, have been processed by the software WinISI II version 1.50 (InfraSoft International). A product library has been built with 1753 spectra of Brazilian raw coffees selected from a data set of 3438 samples, retained after the elimination of some evident spectral outliers and the streamlining of the population in the denser spectral zones (see Figure 1).

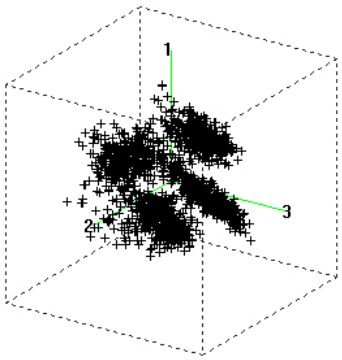


**Figure 1. xyz plot of the first three principal components of spectral data point in the library.**

To predict Merit, we evaluated several options for calibration, namely for establishing a correlation between the response of the instrument and the feature values assumed by the samples in a given product library. We correlated the NIRT spectra with the Merit scores assigned by the cup-testing panel, applying multivariate calibration tecniques based on the

algorithm PLS (Partial Least Squares), an improved version of the powerful dimension reduction tool Principal Components Analysis (PCA) (Beebe et al., 1998).

For the sake of comparison, we implemented first the conventional method called Global Calibration (GLOBAL), which establishes a single regression equation by using all the samples in the product library, allowing 11 terms of a modified PLS algorithm with cross-validation in 4 groups.

Then we tested a recent method called Local Calibration (LOCAL), whose goal is to select from a product library a certain number of nearest-neighbours, namely those samples whose spectra are most similar to the target sample on the basis of their Mahalanobis distance (Mahalanobis, 1936), and to use them for producing a dedicated calibration equation for each sample to be predicted.

In both trials we predicted a test-set of 1447 samples by restricting the spectral wavelegth range to a proprietary selection of only 84 data points in four intervals, suggested by previous experiments.

## RESULTS AND DISCUSSION

Local calibration had been developed to evaluate large databases of spectra and reference values, using the single sample prediction concept with the aim of performing predictions in real time (Shenk, 2000). It has been shown to give, with PLS, results as good as conventional calibration techniques (Barton et al., 2000).

### Validation statistics

Statistics of our test-set LOCAL predictions, compared to GLOBAL ones, are in Table 1.

**Table 1. Statistics of predictions obtained by the two methods.**

| | Standard Error of Prediction (SEP) | Regression Slope | R-squared |
|---|---|---|---|
| LOCAL | 0.910 | 0.587 | 0.400 |
| GLOBAL | 0.949 | 0.436 | 0.308 |

The R-squared data may appear not exciting, if compared to those usually obtained with chemical constituents; however, this is not surprising when acknowledging that spectra of raw coffee samples are here being used to predict quality of a coffee beverage prepared through a series of steps, namely roasting, grinding, brewing and cup-testing. Moreover, one should bear in mind that they refer to sensory variables, prone to intra-panel, inter-session and inter-panel noise. Cues on how NIR chemometrics may help filtering some noise out from laboratory data can be found in the literature (Coates, 2002; Lu and McClure, 1998).

### Prediction evaluation

The performance of our predictions has been evaluated splitting the 1447 test-set samples in two classes according to their predicted Merit values: the ones below a given cutoff threshold (parameter) represent those that in industrial practice would be discarded *a priori* without being submitted to the panel's assessment. Assuming that a cup-tested sample would be accepted as "good" by the panel if exceeding a fixed Merit rating, we computed the percentage of discarded samples in the test-set that would have scored "good", and defined it

as "missed good coffees". Conversely, the number of samples that would have scored "no good", divided by the number of all discarded ones, was defined as "bypassed bad coffees".

**Decision rule**

To study the optimal cutoff for a splitting decision, we built a Naive Bayes Classifier (Michell, 1997) on our product library used as a Training Set. The envelopes of the frequency histograms, reported in Figure 2, intersecate at value 4.4, which apparently is the best choice. Nevertheless, reasons to choose a different cutoff may raise from strategical considerations, as for instance raw material shortage.

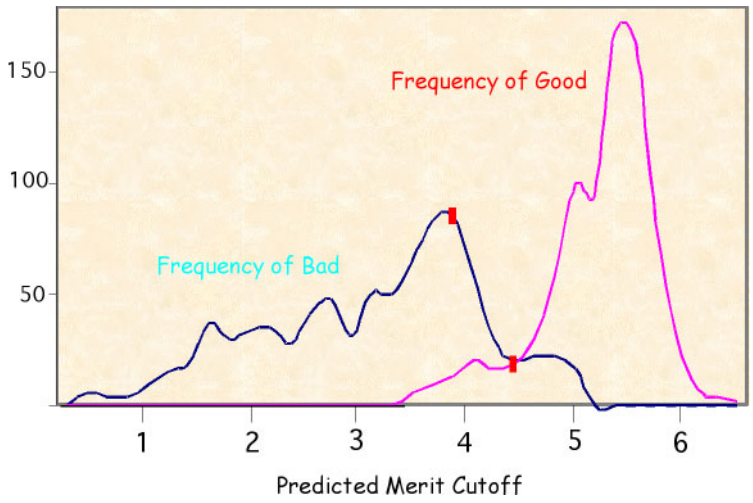


**Figure 2. Frequency hystograms' envelopes.**

**Performance forecasting**

The plot of "missed good coffees" and of "bypassed bad coffees" against the parameter "Merit Cutoff" is reported in Figure 3, which can be used as an operating curve where one can forecast LOCAL prediction performance, in terms of panel effort savings and related quality giveaway, by entering a cutoff Merit threshold value.

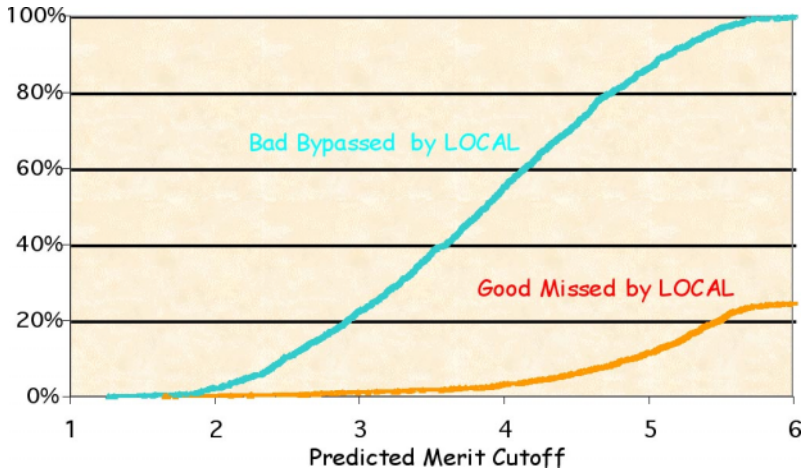


**Figure 3. Operating curve to forecast prediction performance comparing calibration algorithms.**

A similar exercise has been done with GLOBAL predictions, resulting in Figure 4, where the operating curves of both prediction algorithms are compared: LOCAL prediction consents

larger savings in the range of interest (around cutoff 3), with comparable good samples' losses. These aggregated results show that LOCAL data base regression technique performs better than GLOBAL calibration, and is suitable for coffee industrial purposes.

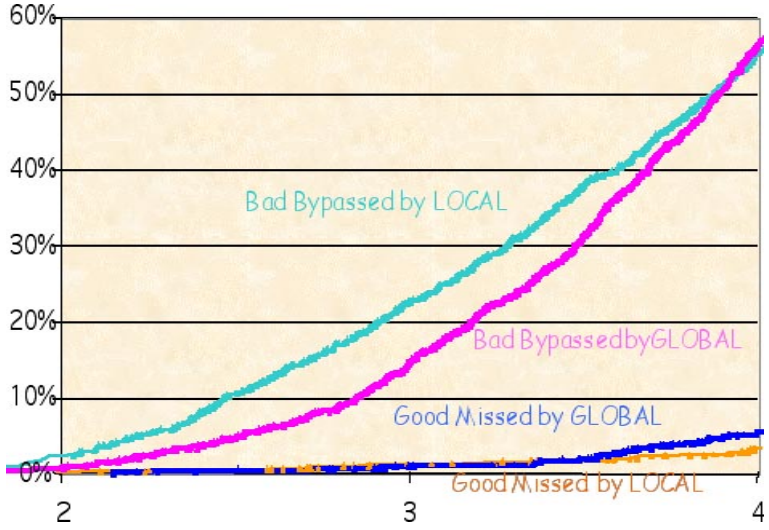


**Figure 4. Operating curves from LOCAL and GLOBAL predictions, compared in the key industrial range.**

It is worth noticing that both savings and losses grow monotonically when the rejection cutoff is raised, as shown in Figure 5 obtained combining the two curves of Figure 2 by elimination of the Merit cutoff parameter. This means that there is no such a thing as an optimum cutoff threshold, which must be chosen by compromise to other factors, like available resources limits or acceptable losses.

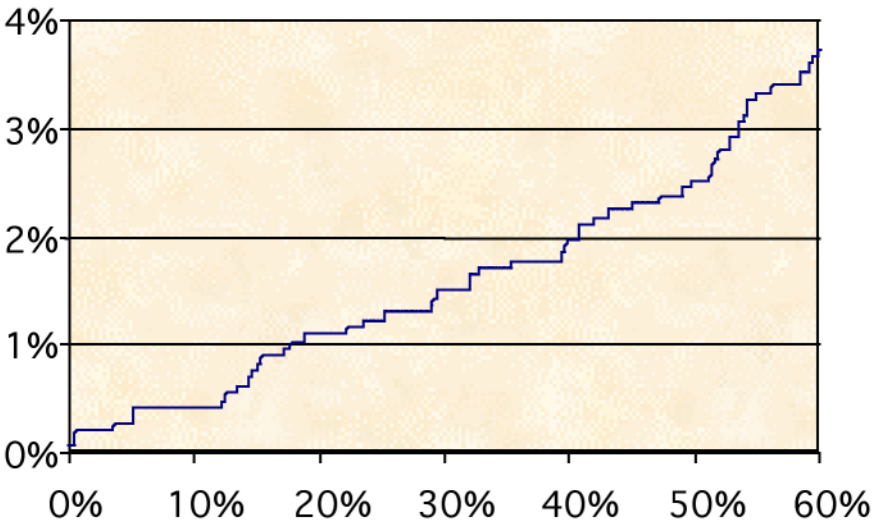


**Figure 5. Prediction performance diagram, showing the percent of missed good samples (y) as a function of the percent of bypassed bad samples (x).**

## CONCLUSIONS

Few everyday experiences can compete with a good cup of coffee, as long as sheer sensory pleasure is considered. It is clear that most of the quality of such a beverage is determined by

its overall sensory impact. In this context, espresso is the brewing method that offers the consumer the most powerful experience, even if a high quality cup is not easy to obtain.

The relevant conventional quality assurance tool, cup-testers' panel, is expensive and time consuming; this often makes the number of tested samples per day inadequate to meet with industrial needs.

A screening procedure, able to discard *a priori* those undesirable samples that are likely to be rejected by the panel, would be therefore of help if rapid, non destructive and worthy of being depended on. Near Infra Red Transflectance spectra acquisition fulfils the two former conditions: the last one must be checked by setting up an evaluation procedure.

In this paper we showed how such a procedure can be designed and applied to appraise the prediction of espresso beverage quality from spectral data of raw coffee samples.

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# Quality Control of Taste Intensity of Blended Coffee

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## SUMMARY

A quality control method of blended coffee products applying the gust value estimated from physico-chemical properties was suggested. Using this method together with the current quality control method judged by sensory testing, the quantitative nature, the reproducibility, and the objectivity of the quality control of coffee tastes was improved.

Trade varieties including the genotype and the grade, the seasonal changes in a producing country, and roast degree influenced the intensity of acidity, and roast degree influenced the intensity of bitterness.

By means of controlling titratable acidity and luminosity, variations in the taste intensities of blends could be reduced.

## RÉSUMÉ

Une méthode de contrôle de qualité pour les cafés mélangés qui applique la valeur “gust”, estimée à partir de propriétés physico-chimiques, a été suggérée.

Cette méthode, utilisée conjointement avec la méthode de contrôle de qualité du test sensoriel a amélioré la nature quantitative, la reproductibilité et l’objectivité du contrôle de qualité des saveurs de café.

Des caractéristiques telles que le génotype et la qualité, les changements climatiques saisonniers du pays d’origine, et le degré de grillage ont influencé l’intensité de l’acidité, et le degré de grillage a influencé l’intensité de l’amertume.

Les variations dans les intensités de saveur de cafés mélangés pouvaient être réduites en contrôlant l’acidité titrable et la luminosité.

## INTRODUCTION

When consumers have selected a blended coffee product and purchased it continuously, the balance of the taste intensities, the intensities of acidity and bitterness, is one of the important criteria for its perceived quality. Therefore, it is necessary for coffee processors to make and supply blended coffee products that fit for the consumer’s preference or the processor’s recommendation in the perceived acidity and bitterness. And, coffee processors have to control the taste intensities of blended coffee products so they are made as intended.

However, the taste intensities of blended coffee products depend on a large number of factors. Some of these derived from varieties of the green coffee related to the genotype, the grade, and the seasonal changes in a producing country, while others are variables of the roasting process.

Additionally, as the taste intensities of blended coffee products have been judged by sensory testing, objective methods to evaluate the taste intensities of coffee become necessary from the viewpoint of the quantification and the reproducibility.

Moreover, coffee processors have been looking for objective methods to assess the variability and the interchangeability of coffee from different origins for reasons of the control of the taste intensities.

At the last conference, we presented a quantitative evaluation method (Aino and Motoyoshi, 2001) to estimate the taste intensities of coffee, called the gust values (Lewis, 1948; Beebe Center and Waddel, 1948; Beebe Center, 1949), from its physico-chemical properties. The purpose of this study was to apply this method to the quality control of the taste intensities of blended coffee products.

In this paper, an objective quality control method for reducing the variation in the taste intensities of blends is shown.

## MATERIALS AND METHODS

### Coffee samples

For the experiment involving trade varieties and their seasonal changes, the coffee samples coming from 5 different countries (Brazil, Colombia, Guatemala, Zimbabwe, Indonesia) were used. For the experiment of grade, the samples from Brazil, Colombia, and Guatemala were tested. These coffee samples were roasted with Probat RZ-4000 to light roast.

For the experiment involving roast degree, 5 trade varieties with 4 roast degrees were examined.

### Coffee brew preparation

Twenty grams of roast and ground coffee was extracted by 250 milliliters of boiling water using Kalita computer coffee machine HG-115. After filtration, the brew was cooled in a cold-water bath to about 20°C.

The concentration of coffee brew was adjusted to brix 1.50% by adding distilled water.

### Measurements of physico-chemical properties

Luminosity in Lab chromaticity diagram (L-value) of coffee was measured with Nippon Denshoku Kogyo color meter Model ZE-2000. Brix of coffee brew was determined by Atago RX-3000 digital refractometer. Titratable acidity was measured by neutralizing 100 ml of coffee brew with N/10-NaOH using Hiranuma auto titrater COMTITE-980.

### Estimation of the gust values from physico-chemical properties

A scale of taste intensity based on a ratio judgment, called the gust scale, was used to evaluate the taste intensities of coffee. The unit in this scale is defined as the subjective strength of a one percent solution of sucrose. Using the gust scale, the taste intensities in coffees were evaluated as quantitative values with an equi-ratio property.

By the following experimental equations (Beebe Center, 1949), the gust values of coffee were estimated.

- For acidity; log ψa = 0.154TA – 0.949
- For bitterness; log ψb = (-0.038LV+1.838)*Pa + (-0.031LV+1.737)*Pr

Where the different terms are :
ψa : gust value for acidity; ψb : gust value for bitterness,
TA : titratable acidity; LV : L-value of roast and ground coffee,
Pa : percentage of arabica; Pr : percentage of robusta

## RESULTS AND DISCUSSION

### Influenced of some factors on taste intensity

The taste intensities of blended coffee products depend on a large number of factors, such as trade varieties, grade, seasonal changes in a producing country, and roast degree. These factors were investigated individually.

### Influence of trade variety on taste intensity

The taste intensities for various trade varieties were shown in Figure 1.

The intensities of acidity and bitterness were plotted as logarithms of the gust value. On the horizontal axis, the intensity of acidity was plotted and in the vertical axis, the intensity of bitterness was plotted.

As shown in Figure 1, the intensities of acidity were different, especially in relation to the genotype, arabica and robusta. The intensities of bitterness were similar for the same roast degree, regardless of trade variety. However, because coffee that had weak acidity tended to have a strong bitterness value, the bitterness of robusta coffee was slightly stronger than that of arabica coffee.

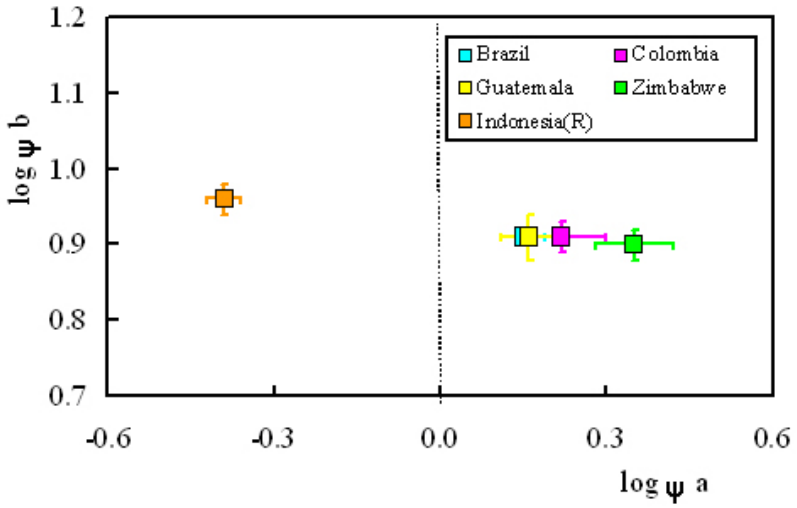


**Figure 1. taste intensities for various trade varieties. Log ψa: logatithm of gust value for acidity; log ψb: logatithm of gust value for bitterness.**

**Influence of green coffee grade on taste intensity**

Figure 2 shows the taste intensities of some green coffee grades.

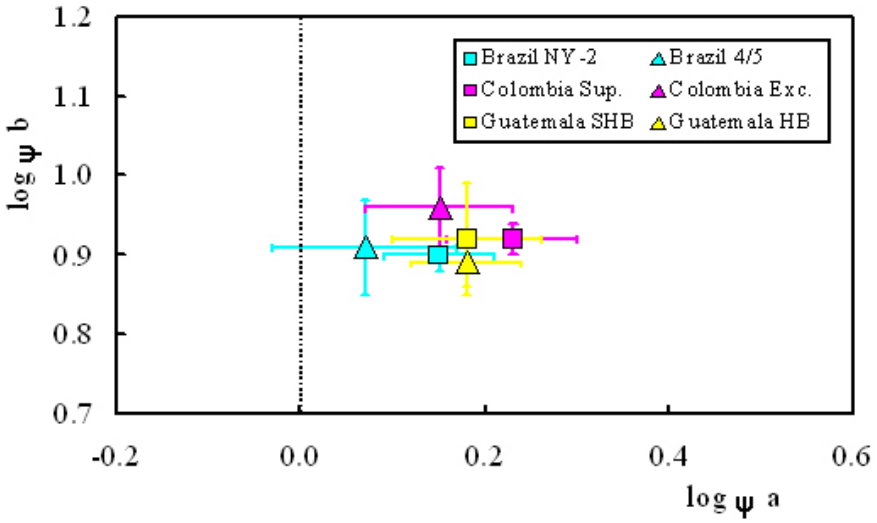


**Figure 2. Taste intensities of some greeen coffee grades.**

The acidities of high-grade coffees, such as Brazil NY-2, were stronger than those of corresponding low-grade coffees. The intensities of bitterness were similar for the same roast degree, regardless of grade.

**Seasonal changes of intensity of acidity**

The seasonal changes of the intensity of acidity are shown in Figure 3.

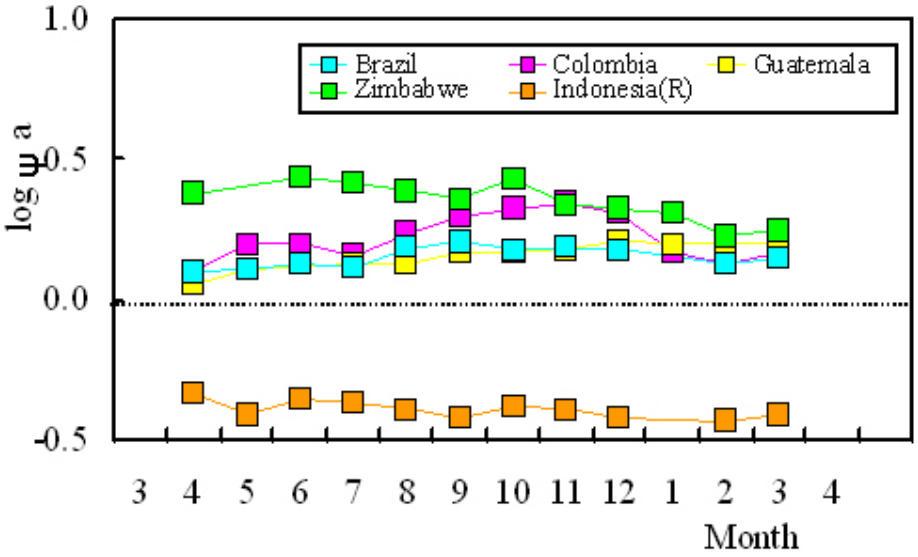


**Figure 3. Seasonal changes of the intensity of acidity.**

For Brazil coffee, the intensity of acidity showed relatively large values from August to December. The main harvesting season in Brazil is from May to September, and it takes two or three months by sea to reach Japan. Thus, the so-called new crops of Brazil coffee were

used from August to December in Japan. This is the reason for the seasonal changes of the intensity of acidity in Brazil coffee.

The seasonal changes of acidity in other trade varieties were explained similarly.

**Influence of roast degree on taste intensity**

Figure 4 shows the taste intensities in different roast degrees.

With the progress in roast degree, the intensities of acidity were decreased, and the intensities of bitterness were increased.

The influences of roast degree were relatively large for the intensity of acidity and bitterness. As mentioned before, for the same roast degree, the intensities of acidity were influenced by trade varieties, the grade, and the seasonal changes. However, the influences of these factors were small concerning bitterness intensities.

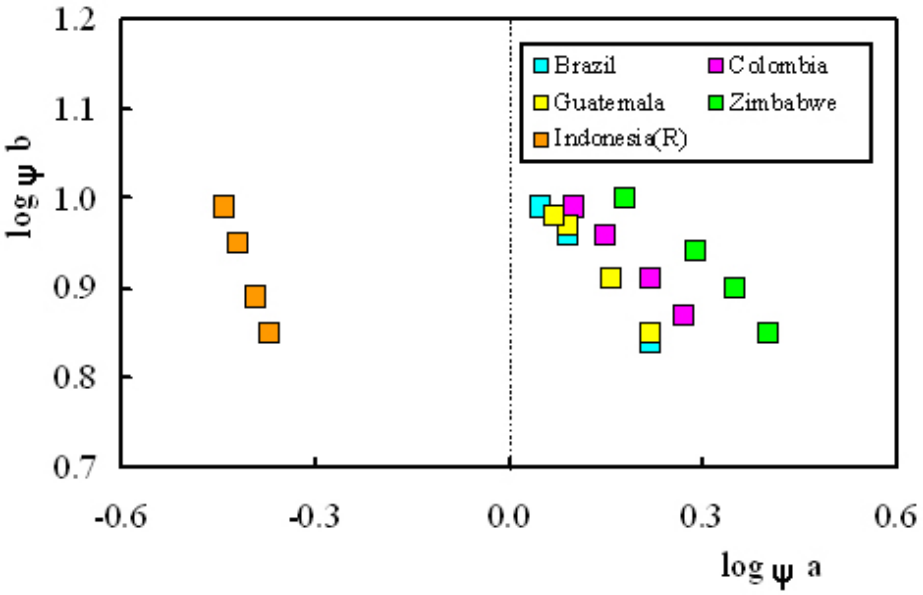


**Figure 4. Taste intensities in different roast degree.**

**Factors influencing taste intensity**

The factors influencing taste intensities are summarized as follows.

Trade varieties including the genotype and the grade, the seasonal changes in a producing country, and roast degree influenced the intensity of acidity. Roast degree influenced the intensity of bitterness.

From these results, we considered that controlling titratable acidity and luminosity could reduce these influences on the taste intensities of blended coffee.

**Taste intensity specifications**

The taste intensity specifications for blended coffee were illustrated in Figure 5.

The indistinguishable range of the taste intensity by sensory testing was about 0.02 in variations of logarithm of the gust value. This value was corresponded to 0.13 ml in titratable

acidity, and 0.5 in luminosity (L-value). From the indistinguishable range of the taste intensity, an example of the specifications was decided and described in this figure.

By keeping the taste intensities of blended coffee within the specifications, the quantitative nature, and the objectivity in quality control of coffee tastes were improved compared with the current quality control method judged by sensory testing.

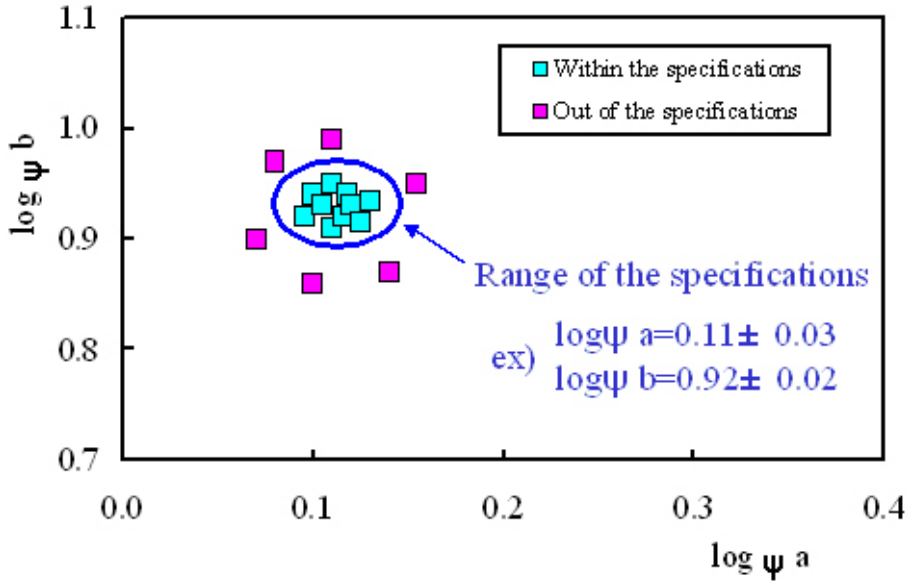


**Figure 5. Taste intensity specifications.**

**Adjustment of blend**

Some examples for the adjustment of blend are simulated in Table 1.

**Table 1. Adjustment of blend.**

| Trade Variety | TA (ml) | Blend ratio(%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Std | A | B | C | D | E |
| Colombia -1 | 9 | | 25 | | 25 | | 10 |
| Colombia -2 | 7 | 25 | | | | 20 | 10 |
| Colombia -3 | 5 | | | 25 | | 5 | 10 |
| Guatemala -1 | 8 | 30 | 30 | 30 | | 30 | 25 |
| Guatemala -2 | 6 | | | | 30 | | |
| Brazil | 7 | 30 | 30 | 30 | 30 | 30 | 35 |
| Indonesia(R) | 4 | 15 | 15 | 15 | 15 | 15 | 10 |
| TA (ml) | | 6.85 | 7.35 | 6.35 | 6.75 | 6.75 | 6.95 |
| $\log_{\psi}$ a | | 0.11 | 0.18 | 0.04 | 0.09 | 0.09 | 0.12 |
| $\Delta\log_{\psi}$ a | | 0.00 | 0.07 | 0.07 | 0.02 | 0.02 | 0.01 |

** Specification log ø a = 0.11 ± 0.03*
*Ä log ø a > 0.03; Out of the specification, Ä log ø a? 0.03; Within the specification*

We supposed L-values are 25, and the intensities of bitterness are within the specifications in these examples.

Compared with the standard case, the case A and B have a same blend ratio, but the intensities of acidities are clearly distinguishable. These are classified out-of-specification products.

To control the taste intensities within the specifications, the case A and B are changed to the case C or D, keeping the blend ratio.

If we can change the blend ratio, it is possible to use the case E that has almost same taste intensities with the standard case.

By a detailed adjustment of blend, variations in the taste intensities of blended coffees could be reduced.

**Quality control method for taste**

Quality control methods for coffee taste are summarized in Figure 6.

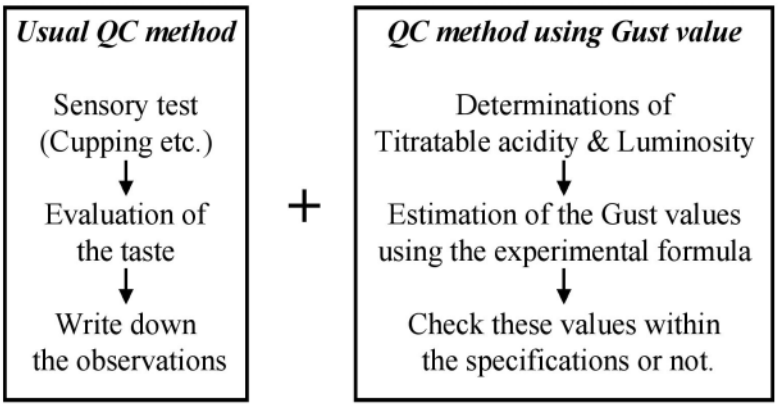


**Figure 6. Quality control method for taste.**

In addition to the usual quality control method, the method using the gust value is carried out. After the determinations of titratable acidity and luminosity, the gust values are estimated using the experimental formula. We then check that the gust values are within the specifications.

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# Analytical Method and Model for Measuring Mannan Oligomer Size Distribution in High Yield Soluble Coffees

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## SUMMARY

The depolymerization of poly mannan to its soluble oligomers is central to achieving high yields in soluble coffee processing. Measurement of mannan oligomers and their distribution is, therefore a potentially excellent indicator of progress of high yield processes.

Until now good measurement of high yield processes has been hampered by the lack of analytical method specific to mannan oligomers, especially in whole coffee extract and by the lack of a theoretical model for expected oligomer distribution as a function of the degree of reaction.

This paper outlines an analytical method and a model for prediction of DP distributions. The method is based on capillary electrophoresis of derivatise mannan oligomers, which is specific and reliable, and can be applied to whole coffee extracts.

The model is based on a statistical analysis of bond scission and has been used to explore the kinetics of high yield coffee extraction.

## RÉSUMÉ

La dépolymérisation des polymères de mannanes en oligomères solubles est une étape clé pour atteindre un haut rendement dans le procédé de préparation du café soluble. La mesure de la taille des oligomères de mannanes et de leur distribution est donc potentiellement un excellent indicateur de progression dans la mise au point de procédés à haut rendement.

Jusqu'à présent la caractérisation de procédés à haut rendement, en particulier pour des applications dans les extraits de café finis, était limitée par le fait qu'une méthode analytique adaptée aux oligomères de mannanes n'était pas disponible; un modèle théorique prédisant la distribution des oligomères en fonction du degré de polymérisation (DP) manquait également. Cette étude présente une méthode analytique et un modèle de prédiction des distributions de degré de polymérisation des oligomères de mannanes. La méthode s'appuie sur l'application de l'électrophorèse capillaire à des oligomères de mannanes dérivés, ce qui la rend spécifique et précise et étend son application aux extraits de café finis. Le modèle est basé sur une analyse statistique des ruptures de liaisons; il est ici appliqué à l'exploration de la cinétique d'extraction à haut rendement du café.

## BACKGROUND AND INTRODUCTION

In the manufacture of instant coffee, the achievement of commercial yields depends on the solubilisation of three key classes of material. The first class consists of the salts, alkaloids, acids, flavours, simple sugars, melanoidins and a small proportion of the two main carbohydrates, arabinogalactan and mannan, which are freely soluble in hot (< 100°C) water.

The second class of materials consists of the majority of the arabinogalactan and melanoidins and proteinaceous material, which seem to be associated with it, which are solubilised at temperatures between 130 and 180°C (Bradbury, 2001). The third class, the mannans, requires higher temperatures to make them and their accompanying melanoidins and proteinaceous material soluble. Most commercial soluble coffee processes use a staged process to maximise quality, so that each class of material is only exposed to the conditions needed to release them (Vitzthum, 1995; Gerhardt-Rieben, 1996; Scoville, 1992; Pigman; Heeb and Mandralis, 1998).

The arabinogalactans are inherently soluble due to their highly branched helical structure (Bradbury, 2001). The elevated temperature needed to release them is evidence of binding to or entrapment in the cellular matrix. The mannans, by contrast, are based on linear polymers of degree of polymerisation (DP) about 20-40 (Bradbury, 2001) with a 2-fold axis ß1-4 linked mannose unit backbone. The polymers and larger oligomers have low solubility. It is known that there are varying levels of galactose side units present in the mannan, hence the use of the nomenclature 'galactomannans' in some texts. It has been shown that the more highly substituted polymers are extracted early in the extraction process due to their higher solubility.

In order to extract the mannan the native chains of DP 20-40 must undergo a controlled de-polymerisation to DP 7 or less, where the oligomers are freely soluble. This de-polymerisation is typically achieved by hydrolysis at temperatures from 150-220°C in a conventional percolation battery or in some from of batch or plug flow reactor system (Scoville, 1992; Stahl, 1993).

Control of this de-polymerisation is essential. Excessive hydrolysis leads to too much of the mannan polymer being converted to mannose, which is both sweet and labile. The sweet taste can be a flavour issue itself. More importantly the degradation pathway for simple sugars at the reaction temperatures required for de-polymerisation leads to generation of aliphatic acids which can have a negative effect on flavour.

Under-reaction can be a problem for manufacture since it leads to a preponderance of marginally soluble oligomers, especially DP8-12. These are soluble at the extraction temperatures, but then precipitate in cooler down-stream storage and heat-transfer equipment leading to excessive fouling.

## MEASUREMENT OF DP DISTRIBUTION

Measurement of the degree of reaction (DoR) for the hydrolysis process is achieved by analysing the resultant oligomers size or DP distribution, usually using gel permeation chromatography (GPC) (Gerhardt-Rieben, 1996; Leloup et al., 1997) or ion exchange chromatography (IEC). In practice these are both limited to the measurement of DP distributions in isolated hydrolysates. Even then the measurements require considerable analytic and interpretive skill.

Figure 1 shows a typical ion exchange chromatograph of a high yield faction.

Each peak is complicated by one or more leading or following peaks or shoulders, which make peak allocation and integration problematic. In any case, only DP1-6 can be reliably measured above the base-line error.

In whole coffees, this measurement is even more problematic because of the interference and overlay of other moieties from the whole coffee matrix.

Figure 2 shows a typical ion exchange chromatogram for a whole soluble coffee.

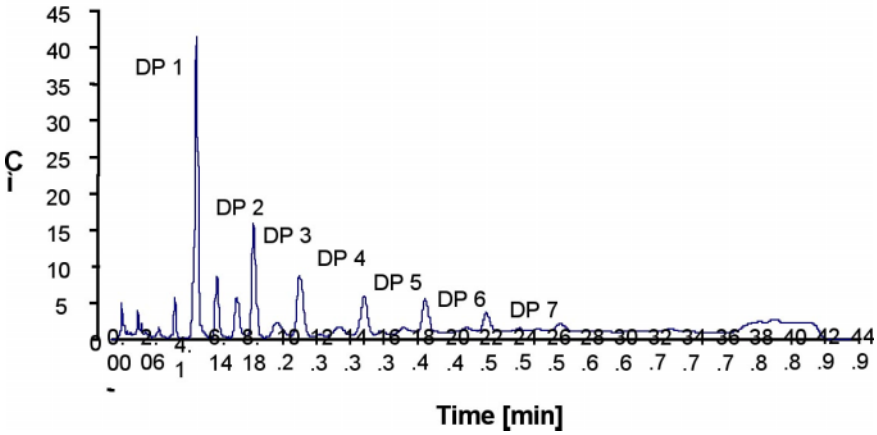



**Figure 1. Ion exchange chromatograph: high yield fraction.**

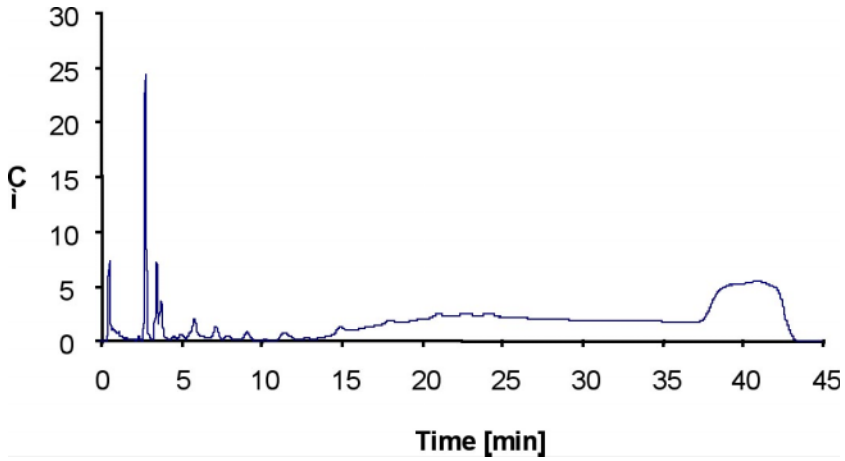



**Figure 2. Ion exchange chromatograph: Kenco soluble coffee.**

Those familiar with interpreting DP distributions will know that, as described earlier, high DoR material has a preponderance of low DP's and low DoR material has more large DP's. However, there was a lack of mathematical formula for the expected shape of the distributions and the whole distribution must be specified to characterise a particular DoR. This leads to problems in interpreting measurements in relation to the process and also means there is no way of cross checking that a measured distribution is free from analytical error. In summary then;

1. The analytical techniques required highly skilled interpretation.
2. The analytical methods can only be reliably used on isolated high yield fraction.
3. The expected DP distribution is unknown so the shape of the distribution must be specified in full: it cannot be characterised by a simple number or index.

The first of these two problems have been resolved by a new analytical method. The third by the development of a relatively simple model for the shape of the DP distribution.

## NEW ANALYTICAL METHODOLOGY

The preferred analytical technique is capillary electrophoresis (CE) with UV detection. Since oligo-mannans possess neither an appropriate charge or a specific UV absorbance it is necessary to derivatise them with a charged, UV specific moiety.

This has been satisfactorily achieved by incubating a 10% w/w analyte solution with an excess of 7-aminonapthaline-1,3-disulphonic acid and sodium cyanoborate at 30°C for 4 hours at pH 2.5.

The derivatised sample is then analysed by a proprietary capillary electrophoresis instrument fitted with a UV detector under the following conditions.

| | |
|---|---|
| Capillary: | 48.5cm x 50 μm ID (extended light path) |
| Voltage: | –20 kV |
| Injection: | 25 mbar * 4 sec |
| Wavelength: | 232nm |
| Temperature: | 25°C |
| | Buffer: 120 mM Tris (hydroxymethyl) – amino-methane adjusted to pH 2.0 with phosphoric acid |
| Conditioning: | Flush 2.0 min with 0.1M Phosphoric acid, subsequently 3.0 min with buffer before analysis |

Linearity was confirmed by analysing samples of pure DP1 (mannose) to DP6 (mannohexose) over a range of concentrations from 0.021 to 0,336 mM. The results are shown in Figure 3.

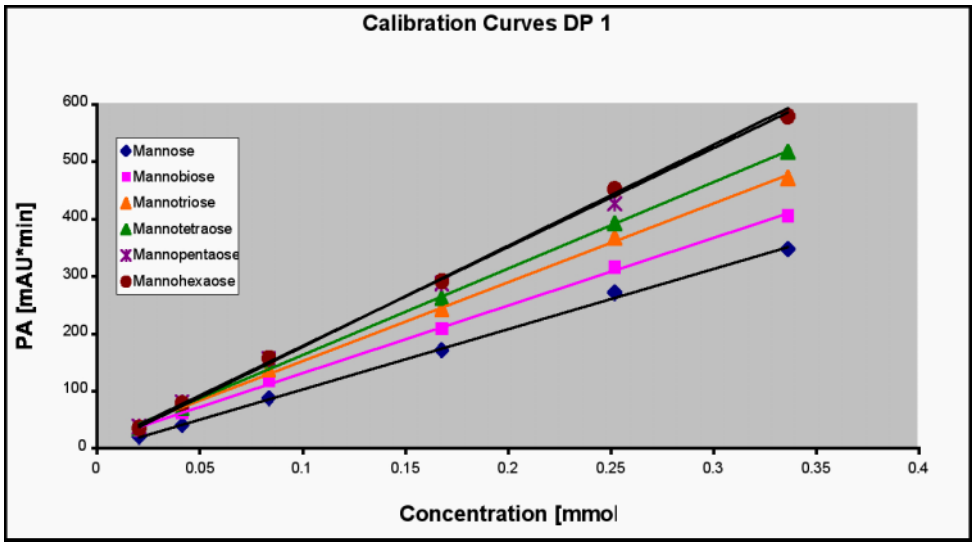



**Figure 3. Calibration graphs of DPs 1-6 as determined by 6-point calibration.**

Due to the high cost of pure oligomers, response factors are calculated so that a pure mannose solution can be used as a reference standard for day-to-day calibration.

Typical migration times and response factors (relative to mannose) are given in Table 1.

Spiking experiments lead to recoveries over 95% so the derivatisation can be considered quantative.

Typical eletropherograms for an isolated high yield fraction and a typical instant coffee are shown in Figures 4 and 5.

**Table 1. CE migration times and response factors DP's 1-9.**

| DP | Migration time [min] | Relative response factor | DP | Migration time [min] | Relative response factor |
|---|---|---|---|---|---|
| 1 | 7.7 | 1 | 6 | 14.6 | 1.65 |
| 2 | 9.2 | 1.1 | 7 | 16.0 | 1.65 |
| 3 | 10.6 | 1.32 | 8 | 17.4 | 1.65 |
| 4 | 11.8 | 1.47 | 9 | 18.8 | 1.65 |
| 5 | 13.2 | 1.67 | | | |

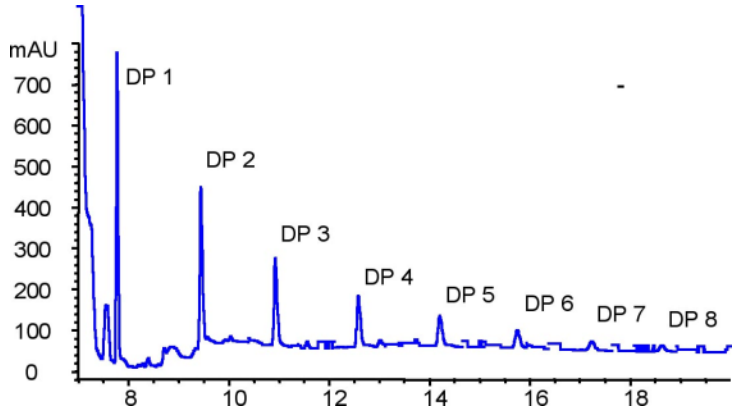



**Figure 4. Capillary electropherograph: high yield fraction.**

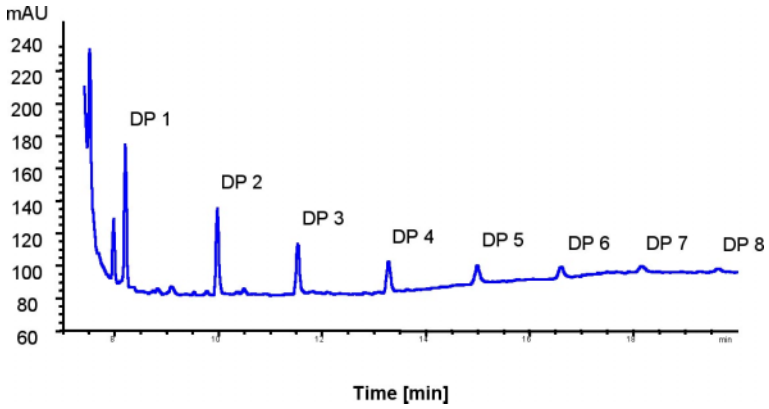



**Figure 5. Capillary electropherograph: Kenco soluble coffee.**

## PROBABILITISIC MODEL FOR DE-POLYMERISATION

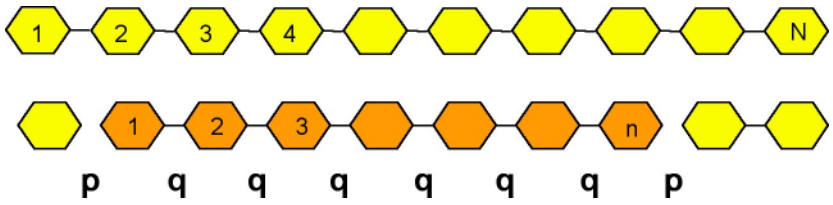
The third issue is that of developing a model for calculating the DP distribution based only on the original substrate DP and the overall Degree of Reaction (DoR).

Previous work (Nattrop Anders, 1999) has been done in this field. Nattorp's calculations, for example, require complex solution to $N^{th}$ order differential equations (where N in its original DP) and have many parameters. Whilst the chemistry and mathematics of Nattorp's approach is thorough, the multi-dimensionality makes it difficult to apply to simple predictive modelling.

It was found, in this present study, that a very simple probabilistic model based on the assumption that all glycosidic links in the chain have an equal likelihood of being hydrolyzed, is surprisingly effective.

Assuming that the breakage of bonds in a chain during hydrolytic de-polymerisation is random, it is easy to see intuitively how the distribution of oligomer size can be calculated. Consider, for example the fate of the following DP N chain, when an oligomer of DP n is formed.

Let p = the probability of any one bond breaking,
So q = 1-p = the probability that a bond remains intact.




The probability of creating this particular DP n oligomer is

$$pq^{n-1}p = p^2q^{n-1}$$

By considering all the ways that DP n oligomers can be formed from a DP N polymer we can generalise and show

$$Pr(n,N) = (2p+(N-n-1)\ p^2).\ q^{n-1}$$
Except when n=N, then
$$Pr(N,N) = q^{N-1}$$

This same expression can be derived more rigorously (Clifford, 1998) by considering the probability that a particular monomer ends up as part of a DP n.

This more rigorous analysis also shows that the distribution is only a very weak function of its original DP in the range considered (20-40) and that it is a function only of the mean original DP. That is, the distribution of the original polymer length does not affect distribution of the de-polymerised oligomers.

In practice we have found it satisfactory to assume that N = 20.

To create a mass distribution for comparison to CE analytical data these probability distributions need to be corrected for molecular length and for the water added at each hydrolysis point and then normalised. This can readily be achieved in a spreadsheet.

By standardising the original DP, N at N = 20 (as above) we now have a simple single parameter model to describe any distribution. This parameter, p, the probability of any one bond breaking during hydrolysis is, post hoc, also the fraction of bonds which have broken. This is termed the degree of reaction, DoR, as before, and

$$DoR = p$$

Typical shapes for its DP distribution as a function of DoR are shown in Figure 6.

By fitting the model to an observed oligomer distribution measured by, for example, CE it is possible to calculate the DoR. This is usually done by least square fitting in a spreadsheet.

Figure 7 shows typical charts where the model has been fitted to actual CE data to determine a DoR

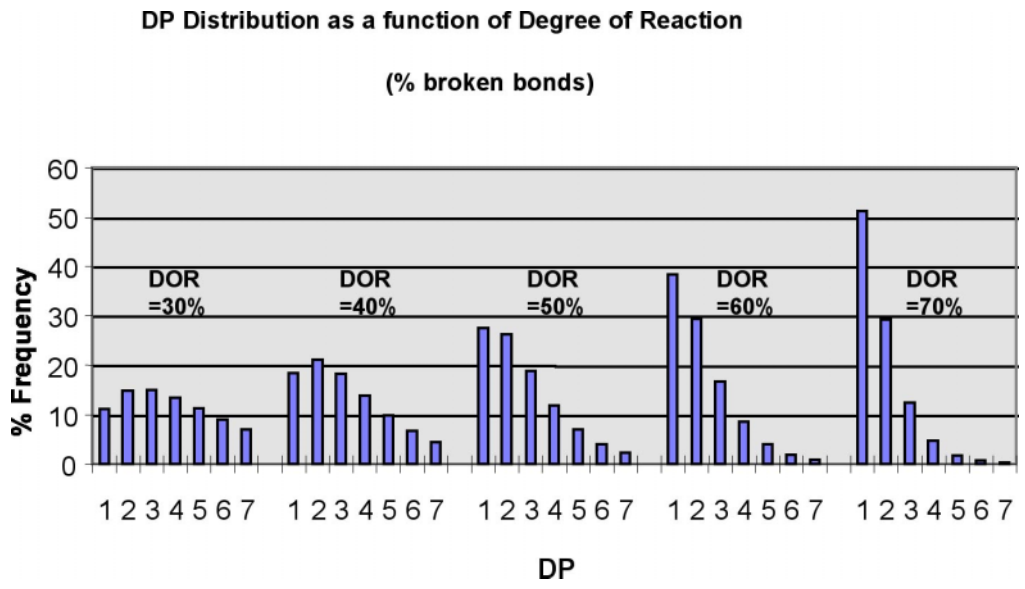



**Figure 6.**

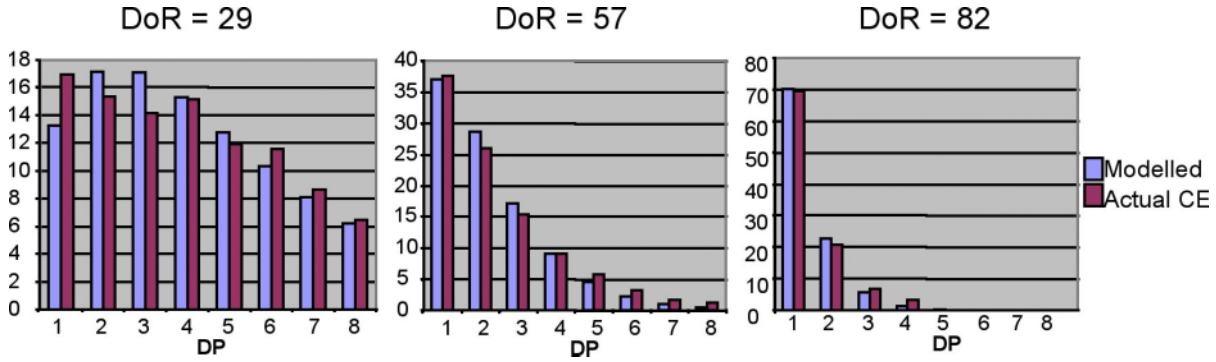



**Figure 7. Modelled and actual data.**

The residual square error is a useful measure of the goodness of fit and can be used to indicate either an analytical error, an out-of-control situation in the process or that the hydrolysis is not random, as would be the case in enzymatic hydrolysis.

## KINETIC MODELLING

The final strand which gives confidence in the validity of this simple model is its robustness in relating observed DP distributions to known hydrolysis conditions via an equally simple kinetic model. The approach taken was to consider the hydrolysis reactions as first order in bonds themselves rather than consider the fate of the individual molecules generated during hydrolysis. In this way the extent of the reaction, the DoR, can be modelled in time as

$$DoR = 1\text{-}e^{-kt}$$

Where t = time (sec)
k = rate constant ($sec^{-1}$)

A classic Arrhenius function was used to model k as a function of temperature.

$$k = A\, e^{\frac{-G}{RT}}$$

Where G = activation energy ($kJ\ mol^{-1}$)
R = gas constant ($kJ\ mol^{-1}\ K^{-1}$)
T = absolute temperature (K)
A = frequency factor ($sec^{-1}$)

This two parameter model in A and G was fitted to the DoR outcomes of 38 experiments in which partially extracted grounds of varying origins and primary yield were hydrolysed in a pilot scale batch reactor at temperatures ranging from 180 to 230°C for times ranging from 120 to 720 seconds.

Figure 8 shows a parity plot of the modelled versus observed values. As can be seen the correlation of the least square fitted model to the observed DoR's falls almost exactly on the parity line and the correlation co-efficient is very high. The values for activation energy, G found by the best fit model was 122 kJ/mol, very close to published data for hydrolysis of the corresponding linkage in cellobiose (Hassid and Ballou, 1957).

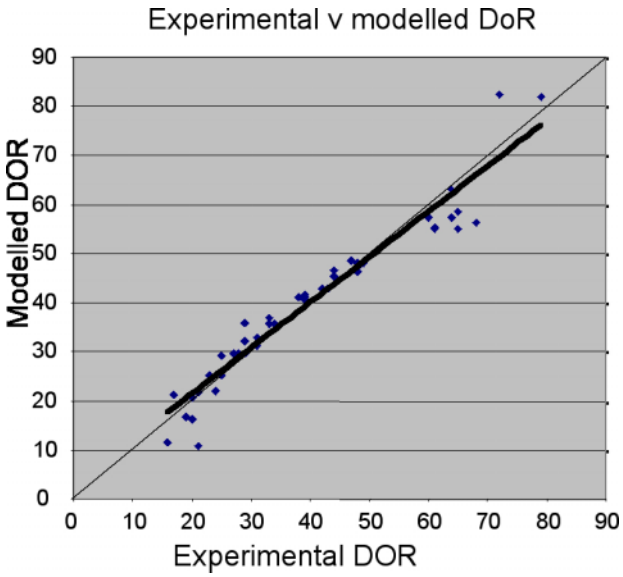



**Figure 8.**

Furthermore it was not possible to materially improve this fit by considering the effects of the primary yield, the origin of the coffee or more sophisticated kinetics.

The parsimony of this model in conjunction with its explanatory power is a good indication of the robustness and predictive quality of the approach.

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# Antimicrobial Effect of Coffee Extract on *Streptococcus Mutans*

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## SUMMARY

The objective of this study was to investigate the *in vitro* antimicrobial activity of coffee on *Streptococcus mutans*, which is the major causative agent of dental caries in humans. The influence of the extraction/brewing process, brand, and coffee chemical compounds in the presence or absence of the aqueous extract was evaluated. The method of diffusion in agar was used to investigate the antimicrobial activity. Sterile disks were soaked with 20 μL samples, added to Mueller Hinton agar previously seeded with $10^8$ cfu 24 h *S. mutans* and incubated at 37ºC/48 h, in the presence of 5% $CO_2$. The diameters of the inhibition zones were measured and submitted to statistical analysis. Every extract tested showed antimicrobial activity. Significant difference was observed on the pH values of the extracts and also on the pH and color characteristics of aqueous extracts from coffees of different brands. However, no significant difference was observed on the antimicrobial activity among ethanolic, aqueous (100ºC/3 min), and espresso extracts and also among brands analyzed. No significant difference was observed on the antimicrobial activity among tested substances (2 mg/mL) by themselves; however, when associated with coffee aqueous extract, there was higher antimicrobial activity, except for chlorogenic acid.

## RESUMO

Este trabalho teve como objetivo investigar a atividade antimicrobiana *in vitro* do café sobre o *Streptococcus mutans*, que é o principal agente causador de caries dentárias no ser humano. A influência do método de extração, da marca e de compostos químicos do café na presença ou não de extrato aquoso foi pesquisada. O método de difusão em agar foi usado para investigar a atividade antimicrobiana. Discos estéreis foram adicionados de 20 μL de amostras e adicionados ao agar Mueller Hinton previamente inoculado com $10^8$ UFC de *S. mutans* 24 h e incubados a 37ºC/48 h, na presença de 5% de $CO_2$. Os diametros das zonas de inibição foram medidos e submetidos à análise estatística. Todos os extratos analisados apresentaram atividade antimicrobiana. Diferença significativa foi observada nos valores de pH dos extratos e também nos valores de pH e características de cor das marcas analisadas. Entretanto, não foi detectada diferença significativa entre os extratos etanólico, aquoso (100ºC/3 min) e espresso, e entre as marcas com relação ao diâmetro da zona de inibição. Não foi observada diferença significativa na atividade antimicrobiana dos compostos testados (2 mg/mL) por si só. Entretanto, quando associados ao extrato aquoso do café foi observado um aumento da atividade antimicrobiana, exceto para o ácido clorogênico.

## INTRODUCTION

The use of natural products has been one of the strategies of greatest success for the discovery of new drugs (Harvey, 2000). From 1983 to 1994, 78% of the new antibiotics approved by the

US Food and Drug Administration were natural products or their derivatives (Cragg et al., 1997). The role of dietary factors as anti-infective agents has also been recently investigated, with particular emphasis on their role in inhibiting bacterial adhesion to host tissues, which is the first step of the infectious process.

*Streptococcus mutans* has been identified as capable of producing dental caries in experimental animals and in humans (Hamada et al., 1984). The potential for *S. mutans* to form dental caries is determined by its ability to adhere to the tooth and to produce metabolic acids (Hamada et al., 1984; Tao & Tanzer, 2002). Adherence to the tooth surface involves two stages: an initial reversible interaction between the organism and the saliva-coated tooth surface and an irreversible stage which is mainly mediated by water insoluble glucans synthesized from sucrose by enzymatic action of glucosyltransferase (Hamada et al., 1984).

Studies have been carried out in order to investigate the influence of natural compounds on the reduction of the incidence of dental caries and of gum illnesses. Cranberry was able to modify the gum microflora, resulting in an efficient control of the gum and of periodontal diseases (Weiss et al., 1998). The inhibitory effects of oolong tea extract and of reconstituted dried milk on caries inducing properties of mutans streptococci were also determined (Matsumoto et al., 1999; Almeida et al., 2004). Topical application of propolis twice daily, or its inclusion in water available to rats *ad libitum*, reduced the incidence of dental caries. Among food constituents, polyphenols in tea were observed to reduce the rate of acid production and to diminish bacteria's growth rate. Polyphenols identified in propolis were also responsible for the reduction of dental caries in rats (Koo et al., 2003).

Coffee contains significant amounts of polyphenols (Furahata et al., 2002); therefore, its potential use as a natural antibacterial agent should be evaluated. Daglia et al. (1994) reported the antibacterial activity of coffee against Gram-positive (*Staphylococcus aureus, Streptococcus pyogenes, S. faecalis, Bacillus subtilis*) and Gram-negative bacteria (*Proteus vulgaris, Salmonella typhimurium, Escherichia coli, Enterobacter cloacae*). According to their studies, the brewing process did not affect the antibacterial activity. However, *Coffea robusta* had higher antibacterial activity compared to *Coffea arabica.* Furthermore, coffee with higher degree of roasting had superior antibacterial activity compared to average and low roast, suggesting that products of the Maillard reaction formed during roasting could also be involved in the antibacterial activity. According to Daglia et al. (2002), coffee had anti-adhesive properties on *Streptococcus mutans* which were due to naturally occurring compounds (trigonelline, nicotinic acid and chlorogenic acid) and to roasting induced low molecular weight melanoidins.

Brewed coffee also showed antibacterial activity against a strain of *Legionella pneumophila*, bacteria involved in respiratory infections. The acids protocatechuic (3,4-dihydroxy benzoic acid), chlorogenic, and caffeic exhibited marked bactericidal action although the response was not fast (Dogasaki et al., 2002; Furuhata et al., 2002).

The objective of this study was to investigate the antimicrobial activity of coffee extracts and of coffee chemical compounds on *Streptococcus mutans*. The following parameters were investigated: (i) extraction solvents and brewing; (ii) coffee brands; (iii) some coffee chemical compounds and (iv) aqueous extract with coffee chemical compounds.

## MATERIAL AND METHODS

### Material

Four different brands of coffee (*Coffea arabica*) were purchased in Belo Horizonte, state of Minas Gerais, Brazil. Chemical constituents of coffee, among them, protocatechuic acid (Sigma P5630), caffeic acid (Sigma C0625-26), trigonelline (Sigma T5509) and chlorogenic acid (Aldrich C44206) were purchased from Sigma Chemical Co (St Louis, MO, USA) and from Aldrich (Milwaukee, WI, USA). The microorganism used was *Streptococcus mutans* from the collection of the Universidade Federal do Rio de Janeiro (Rio de Janeiro, RJ, Brazil). The Muller Hinton medium used was obtained from Dialab Diagnósticos (Montes Claros, MG, Brazil). Sterile disks (6 mm diameter) and disks containing 30 μg of the antibiotic amoxilin clavulanic acid were purchased from Cecon (São Paulo, SP, Brazil).

### Sample preparation

The coffee grains were ground by means of a domestic mill (Braun, USA) and sieved through a 20 mesh sieve (Tyler series). Ethanolic extracts were obtained by mixing 2 g of coffee with 10 mL ethanol in a ultrasonic bath at 24 ± 1°C for 15 min. The aqueous extracts were obtained by adding 8 g of coffee to 40 mL boiling water. The mixture was agitated for 3 minutes in boiling water and filtered in qualitative filter paper under vacuum (Vitorino et al., 2002). The espresso coffee was prepared by weighing 8 g of sample and extracting with 40 mL of water in an espresso coffee maker (Cremissimo Ariete, Fashion Coffee, Italy). Solutions of 2 mg/mL of each of the coffee chemical compounds (protocatechuic acid, caffeic acid, trigonelline and chlorogenic acid) were prepared in distilled water and in aqueous coffee extract in order to have solutions in the absence and presence of aqueous coffee extract, respectively.

The coffee grains were analyzed for CIE color characteristics. The extracts were submitted to pH determination and to the *in vitro* antibacterial activity against *S. mutans*. The antibacterial activity of solutions of 2 mg/mL protocatechuic acid, caffeic acid, trigonelline and chlorogenic acid in the presence and absence of aqueous coffee extract on *S. mutans* was also investigated

### Methods of analysis

#### *In vitro susceptibility test*

*In vitro* antimicrobial activity on *S. mutans* was performed according to the disk diffusion test (Collins et al., 1995). A 0.2 mL aliquot of a 24 hr culture of *S. mutans* incubated in nutritive agar at 37 ºC, in the presence of 5% $CO_2$, corresponding to 0.5 turbidity on the Mc Farland scale ($10^8$ cfu/mL) was spread over the surface of a Petri dish (150 mm diameter) containing 50 mL of Muller Hinton agar. Sterile disks were soaked in 20 μL of the tested products and placed onto the inoculated surface. After 48 hr incubation at 37ºC in presence of 5% $CO_2$, the diameters of the inhibition zones were measured using a caliper. Positive and negative controls of the disks containing 30 μg of the antibiotic amoxilin clavulanic acid, ethanol 98° GL and distilled water were used.

### *CIE L*a*b**

The color characteristics of ground coffee were determined using the ColorTec colorimeter PCM (Accuracy Microsensor, Pittsford, USA). The chroma ($c = (a^2 + b^2)^{1/2}$) and the hue (h = arctg a/b) were obtained using the colorpro software (Colorpro, 2004).

### *pH*

The pH values of the extracts were measured using a Digimed pHmeter model DM20 (Digimed, Santo Amaro, SP, Brazil).

### *Statistical analysis*

The diameters of the inhibition zones obtained were submitted to analysis of variance and the means were compared by the Tukey test at 5% probability.

## RESULTS AND DISCUSSION

### Influence of extraction solvent

According to Figure 1A, there was a significant effect of the type of solvent and brewing process on the pH values obtained for the coffee extracts, with higher values observed for the ethanolic extract (7.17), followed by the espresso (6.07) and by the aqueous extract (5.29).

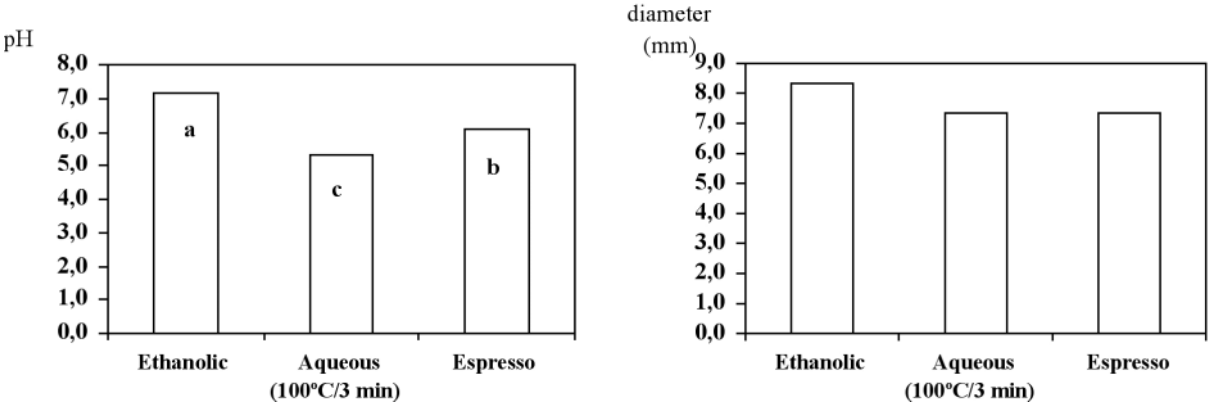


**Figure 1. pH values (A) and diameter of *in vitro* antimicrobial activities (B) of ethanolic, aqueous and espresso coffee extracts. Mean values (n = 5) with different letters are statistically different (Tukey test, 5% probability).**

Every extract showed *in vitro* antimicrobial activity on *S. mutans* (Figure 1 B) however, no significant difference was observed among the types of extracts used. Daglia et al. (1994) and Furuhata et al. (2002) also found no significant effect of the brewing process on the antimicrobial activity of several Gram-positive and Gram-negative bacteria and of *Legionella pneumophila*, respectively.

### Influence of coffee brand

The CIE L*a*b* color characteristics of ground coffee from the different brands are indicated in Table 1. There was no significant difference on a* values and hue angles. However, luminosity (L*), b value and chroma varied significantly. According to Borges et al. (2002), L values correlate significantly with the degree of roasting. Based on this information, the

brands differed with respect to the roasting degree. Samples A and B would be the ones with a higher degree of roasting.

**Table 1. Characteristics of different coffee brands: CIE L*a*b*, chroma and hue angle of ground coffee and pH and *in vitro* antimicrobial activity of aqueous coffee extracts.**

| Parameter | Brand[1] | | | |
|---|---|---|---|---|
| | A | B | C | D |
| CIE color | | | | |
| L* | 20.92 ± 1.18 b | 21.18 ± 1.99 b | 29.44 ± 0.37 a | 26.39 ± 1.08 a |
| a* | 5.98 ± 2.99 | 5.89 ± 0.29 | 10.28 ± 1.36 | 8.53 ± 2.16 |
| b* | 11.70 ± 2.08 b | 12.03 ± 3.15 b | 26.01 ± 0.77 a | 20.78 ± 2.17 a |
| chroma | 13.34 ± 2.26 c | 13.47 ± 2.64 c | 28.00 ± 0.24 a | 22.57 ± 1.37 b |
| hue ° | 63.35 ± 12.87 | 62.84 ± 8.03 | 68.42 ± 3.16 | 67.49 ± 7.07 |
| pH | 5.29 ± 0.03 a | 5.23 ± 0.05 a | 4.88 ± 0.03 c | 5.02 ± 0.01 b |
| Diameter[2] (mm) | 7.33 ± 1.26 | 6.92 ± 0.88 | 7.00 ± 1.00 | 7.00 ± 1.00 |

[1]*Means with different letters (a,b,c) in the same row are significantly different (Tukey test, $p \leq 0.05$).*
[2]*Diameter of the inhibition zones of in vitro antimicrobial activity to S. mutans.*

The pH values of the aqueous extracts obtained from the different brands are indicated in Table 1. Significant difference was observed among samples, with higher values observed for samples A and B.

Every brand of coffee analyzed showed an inhibition zone (Table 1) indicating antimicrobial activity to *S. mutans*. However, even though there was significant difference among samples based on color and pH values, no statistical difference was observed for the antimicrobial activity. These results suggest that there might be other factors, besides the ones investigated in this study, that can affect antimicrobial activity.

**Table 2. Diameters of inhibition zones of the *in vitro* antimicrobial activity of *Streptococcus mutans* exposed to coffee chemical compounds individually and incorporated into the aqueous extract.**

| Chemical compound | Diameter of inhibition zones (mm) of coffee chemical compounds | |
|---|---|---|
| | Individually | In aqueous extract |
| Chlorogenic acid | 7.58 ± 0.38 a | 8.25 ± 0.66 a |
| Caffeic acid | 7.33 ± 0.29 b | 8.67 ± 0.29 a |
| Protocatechuic acid | 7.50 ± 0.50 b | 8.50 ± 0.50 a |
| Trigonelline | 7.33 ± 0.29 b | 8.33 ± 0.58 a |

*Mean values (± standard deviation) with different letters (a,b) in the same row are significantly different (Tukey test, p≤0,05).*

**Influence of the coffee compounds and its incorporation into coffee extract**

The chemical compounds at the concentration of 2 mg/mL, when evaluated individually, provided similar diameter of the inhibition zones to *S. mutans* (Table 2). This suggests that they affected antimicrobial activity in a similar manner and extent. However, when the coffee extract was incorporated to the chemical compounds, there was a significant increase in the

antimicrobial activity of most of the compounds, except for chlorogenic acid. Based on these results, there seems to be a synergistic effect among different coffee compounds.

The results obtained in this study indicate a possible antimicrobial effect of coffee to *S. mutans* and, therefore, a potential to prevent caries. However, further studies are needed in order to evaluate the effect of coffee chemical concentrations on the antimicrobial activity and also synergistic effects. It is anticipated that in the future, by determining the concentration of effective compounds, the antimicrobial activity of a coffee could be predicted.

## CONCLUSIONS

Coffee extracts showed antimicrobial activity against *Streptococcus mutans.* The extraction, brewing process and the brand of coffee used did not affect antimicrobial activity. The chemical compounds chlorogenic acid, caffeic acid, protocatechuic acid and trigonelline at 2 mg/mL, showed similar antimicrobial activity to *Streptococcus mutans.* The addition of caffeic acid, protocatechuic acid and trigonelline to coffee aqueous extracts improved the antimicrobial activity.

## ACKNOWLEDGMENTS

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# Enzymatic Synthesis of Caffeic Acid Esters from Chlorogenic Acid by Transesterification and Condensation Reactions

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## SUMMARY

Immature green coffee beans contain considerable amounts (4.8-5.8g $100g^{-1}$) of 5-chlorogenic acid derivatives. The enzymatic conversion of their 5-chlorogenic acid derivatives into value-added products was investigated. A chlorogenate hydrolase (EC 3.1.1.42) was found to synthesize from 5-chlorogenic acid and 2-phenylethyl alcohol into 2-phenylethyl caffeate (by transesterification) and from caffeic acid and 2-phenylethyl alcohol into 2-phenylethyl caffeate (by condensation) as well as hydrolysis of 5-chlorogenic acid. Some reaction conditions including pH, temperature, substrate and solvent concentrates, and reaction time were optimized for the production of 2-phenylethyl caffeate. A maximal molar yield of 53% was achieved by transesterification and 13% by condensation. Among the parameters studied for optimization, the pH of the buffer solution and concentration of solvent affected the production of 2-phenylethyl caffeate. The optimum pH for the hydrolysis reaction was within the neutral range (pH 6.5), whereas the residual two reactions were only catalyzed within the acidic range (pH 3.0-4.0). The optimum concentration of 2-phenylethyl alcohol for two reactions were 5-70 vol.% and no 2-phenylethyl caffeate was produced in the anhydrous solvents.

## INTRODUCTION

Low quality green coffee beans are not marketed in coffee drinks or utilized effectively as resources. However, these beans contain considerable amounts (4.8-5.8 g 100 $g^{-1}$) of 5-chlorogenic acid (5-CQA) derivatives. We are investigating the enzymatic conversion of their 5-CQA to value-added products.

Chlorogenate hydrolase (CQA hydrolase) can be purified from a wheat bran medium of *Aspergillus japonicus* and a culture medium of *Aspergillus niger* only reported on the catalytic hydrolysis reaction of 5-CQA, caffeoyl and *p*-coumaroyl tartarates, and ethyl and benzyl cinnamates.

In this report, we describe the enzymatic synthesis of ethyl caffeate (CAET) from 5-CQA and ethyl alcohol, and 2-CAPE from 5-CQA or caffeic acid (CA) and 2-phenethyl alcohol (2-PA) using CQA hydrolase.

## MATERIALS AND METHODS

### Chemicals

CQA hydrolase was obtained from Kikkoman Co., (Chiba, Japan). Other chemicals of analytical grade were obtained from Tokyo Kasei Kogyo Co., Ltd., Wako Pure Chemicals Ltd., Sigma-Aldrich Co. (St. Louis, MO), and Nacarai Tesque Inc. (Kyoto, Japan).

**Enzymatic synthesis**

These reactions were carried out in two-phase system (total volume of 20 ml) consisting of 5-CQA or CA in a buffer solution and 2-PA. A typical reaction was carried out with shaking in a reaction mixture consisting 2-PA (1.0-8.0 ml at 8.3-65 mmol) and 50-200 mM citrate buffer (pH 3.0 or 4.0) containing 5-CQA or CA (90-355 mg at 0.25-1.97 mmol) and enzyme (4.0 ml with 0.96 mg of protein).

**Optiminization of enzymatic reaction conditions**

To optimize the enzymatic reaction, the following factors were examined: the concentrations of 5-CQA (0-1.5 mmol), 2-PA (0-160 mmol), CA (0-1.97 mmol), reaction time (1-24 h), concentration (50-1000 mM) and pH (1-8) of the buffer, and temperature (25-70°C).

**RESULTS AND DISCUSSION**

**Ester synthesis from 5-CQA and ethanol and 2-PA by using CQA hydrolase**

CQA hydrolase catalyzed hydrolytic activity even in 20-30 vol.% lower alcohol, acetone, and dimethylsulfoxide aqueous solutions as well as buffer solution. We found that the hydrolytic activity of the enzyme from *A. japonicus* fell to half in a 20 vol.% ethanol-buffer solution, but a new product was detectable on a spot (Rf 0.71) and a peak (retention time 15.2 min). The new product was isolated from the reaction mixture using the following procedure: vacuum concentration, adsorption to HP-20 resin (Nippon Rensui Co. Tokyo, Japan) and elution with an ethanol gradient (10 to 50%), and silica gel column chromatography with a solvent system of chloroform: methanol/100:5 (v/v). The purified substances were analyzed by LC-MS (HP1100 HPLC, Hewlett Packard, USA, and JEOL The MS station JMS-700 TKM, JEOL Ltd, Tokyo, Japan), $^{1}$NMR (JEOL EX-270, JEOL Ltd.) and $^{13}$C NMR (JEOL EX-270): LC-MS, *m/z* 208 ([M-H]$^{-}$, 207), $^{1}$H NMR ($CD_3OD$) δ 1.30 (t, 3H, 7.26Hz), 4.21 (q, 2H, 7.26Hz), 4.85 (br, 2H, OH), 6.24 (d, 1H, 16.17Hz), 6.78 (d, 1H, 8.24Hz), 6.94 (dd, 1H, 8.24 and 1.98Hz), 7.04 (d, 1 H, 1.98 Hz), 7.53 (d, 1H, 15.83Hz). $^{13}$C NMR ($CD_3OD$) δ 14.65 ($CH_3$), 61.35 ($CH_2$), 115.09 (C2'), 115.25 (C2), 116.46 (C5), 122.84 (C6), 127.71 (C1), 146.72 (C1'), 146.79 (C4), 149.52 (C3), 169.29 (C = O). These results confirmed the formation of the transfer product as ethyl caffeate.

A new peak on HPLC (retention time of 16.2min) and a spot on TLC (Rf 0.55) analyses were also detected for the reaction mixtures that contained 5-CQA and 2-PA as substrates. All products were identified as 2-CAPE from the mass and NMR spectra: LC-MS, m/z 284 ([M-H]$^{-}$, 283), $^{1}$H NMR ($CD_3OD$) δ 2.98 (q, 2 H, 6.93 Hz, H at $CH_2$), 4.36 (q, 2 H, 6.93 Hz), 4.88 (br, 2H, OH), 6.25 (d, 1H, 16.17Hz), 6.78, (dd, 1 H, 8.24 Hz), 6.94 (d, 1H, 1.98Hz), 7.01 (d, 1 H, 1.98 Hz), 7.21 (d, 2 H, 1.65 Hz), 7.29 (d, 2 H, 0.99 Hz), 7.54 (d, 1 H, 15.83 Hz). $^{13}$C NMR ($CD_3OD$) δ 33.23 ($CH_2$), 61.35 ($CH_2$), 115.09 (C2'), 115.25 (C2), 116.46 (C5), 122.84 (C6), 125.93 (C4, Benzene), 127.71 (C1), 128.31 (C2, C6, benzene), 129.74 (C3, C5, benzene), 146.72 (C1'), 146.79 (C4), 149.52 (C3), 169.29 (C = O).

CAET and 2-CAPE were also detected for the reaction mixtures from ethanol and CA, and from 2-PA and CA, respectively. These results indicate that CQA hydrolase is able to catalyze the trans- esterification and condensation reactions as well as hydrolysis reaction of 5-CQA (Figure 1). CQA hydrolase was only reported on the catalytic hydrolysis reaction of 5-CQA, caffeoyl and *p*-coumaroyl tartarates, and ethyl and benzyl cinnamates, but no trans-esterification and condensation reactions were reported. This is the first report on the transesterification reaction by CQA hydrolase.

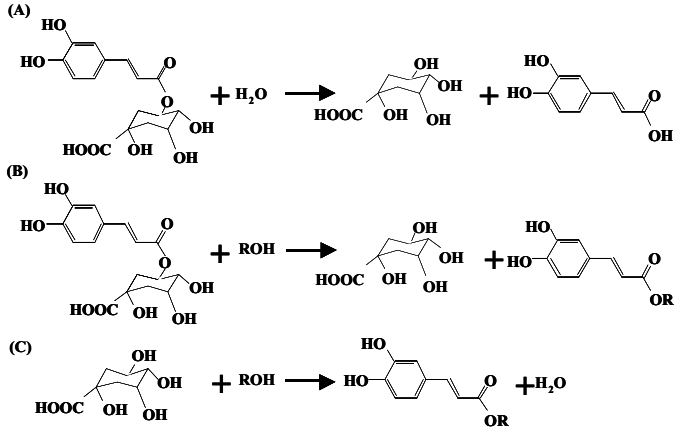


**Figure 1. Hydrolysis of 5-CQA and synthesis of CA esters catalyzed by CQA hydrolase. A, hydrolysis; B, transesterification; C, condensation.**

**Optiminization of enzymatic reaction conditions**

Ethanol was the preferred alcohol for the production of CA esters among alcohols tested. For repeated use of the enzyme and reactions at a higher concentration of alcohol, the enzyme was immobilized. CAET synthesis in ethanol solution was optimized. In the presence of 20 mM 5-CQA-30 vol.% ethanol solution (total volume 20 ml) and 0.3 g of immobilized CQA hydrolase (11.5 mg protein/g of wet resin), 13 mM CAET (65% yield) could be obtained within 24 h at 40°C.

The optimum pH of the hydrolysis reaction of 5-CQA was within the neutral range (pH 6.0-7.0), whereas the optimum pH of the transesterification and condensation reactions into 2-CAPE shifted to the acidic pH range (pH 3.0-4.0) (Figure 2). The pH of the reaction system is responsible for selecting the type of enzymatic reaction. At acidic pH, 2-CAPE as well as CA was detected in the reaction mixture, but CA was only detected at neutral pH. The modifications of the enzyme and substrate charge states due to the reduced pH may enable the catalyzation from 5-CQA to 2-CAPE at acidic pH.

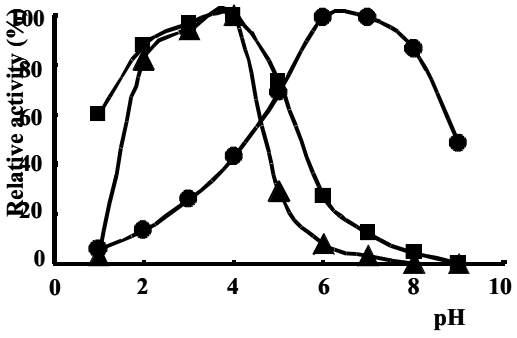


**Figure 2. Effect of pH on the activity of CQA hydrolase. pH 1-2, HCl-KOH buffer; pH 2-3, glycine buffer; pH 3-6, citrate buffer; pH 6-8, phosphate buffer; pH 7-9, tris-HCl buffer. ●, hydrolysis; ▲, condensation; ■, transesterification.**

For the transesterification between 5-CQA and 2-PA, the greatest amount of 2-CAPE (74 mg at 0.26 mmol) and CA by hydrolysis accumulated at 45°C for 4h in a two-phase system consisting of 5-CQA (184 mg, 0.52 mmol)- 50 mM citrate buffer solution (pH 4.0, 15 ml), 2-PA (1.0 ml, 8.3 mmol) and free CQA hydrolase (4.0 ml with 0.96 mg of protein), with a 50%

molar conversion yield. Furthermore, for the condensation reaction between CA and 2-PA, the enzyme synthesized 2-CAPE (18 mg at 65 μmol) with 13% molar conversion yields under the following optimum conditions: the enzyme was incubated at 45°C for 24 h in a reaction mixture consisting CA (90 mg at 0.50 mmol)-200 mM citrate buffer (pH 4.0, 8.0 ml), 2-PA (8.0 ml, 65 mmol) and the enzyme (4 ml with 0.96mg of protein). The transesterification catalyzed by CQA hydrolase was especially well-suited for the production of 2-CAPE because of the high molar conversion yield (50%) and short reaction time (4h). Although CA was also detected in the reaction mixture, the enzyme synthesized from 5-CQA and 2-PA into 2-CAPE by transesterification and not by condensation with CA formed from the hydrolysis of 5-CQA, because 2-CAPE were detected along with CA and a higher molar conversion yield (50%) was achieved for the transesterification than that of the condensation (13%). The products accumulated in 2-PA solution because of their sparingly solubility in water, and they were detected in the solvents.

**Table 1. Substrate specificity of the condensation reaction catalyzed by CQA hydrolase.**

| | Methanol | 2-PA |
|---|---|---|
| *p*-Hydroxy benzoic acid | N.D. | N.D. |
| 3,4-Dihydroxy benzoic acid | N.D. | N.D. |
| Cinnamic acid | 3.7 | 1.1 |
| 3-Phenyl propionic acid | 8.1 | N.D. |
| 4-phenyl but-3-enoic acid | N.D. | N.D. |
| 5-Phenylpenta-2,4-dienoic acid | N.D. | N.D. |
| Caffeic acid | 19 | 13 |
| 3-(3,4-Dihydroxyphenyl)-propionic acid | 46 | 28 |
| *o*-Coumaric acid | N.D. | N.D. |
| *m*-Coumaric acid | 1.1 | 0.84 |
| *p*-Coumaric acid | 22 | 10 |
| 6-Hydroxynaphthalene-2-carboxylic acid | 2.7 | 0.38 |
| Ferulic acid | 0.52 | N.D. |
| 4-Nitro cinnamic acid | N.D. | N.D. |
| L-Tyrosine | N.D. | N.D. |
| L-Phenylalanine | N.D. | N.D. |

*Numeric data, mole conversion rate %; N.D., not detected.*

**Substrate specificity**

Hydroxyphenylpropionic acid derivatives were active as the substrates of the condensation reactions with 2-PA, while the following aromatic compounds were inert as substrates: hydroxybenzoic acids, phenyl alkanoic acids with a $C_4$ or $C_5$ alkyl side chain length, *o*-coumaric acid, 4-nitrocinnamic acid, and aromatic amino acids (Table 1). Among the substrates tested, 3-(3,4-dihydroxyphenyl)- propionic acid gave the highest conversion yield for the condensation reaction with 2-PA or methanol (Table 1). These results suggest that the distance between the binding site with hydroxyphenyl moiety of the substrate and the catalytic site of the enzyme corresponds to the length of the $C_2$-$C_3$ alkyl chain.

In contrast, the addition of a hydroxyl group at the *para* position of the phenyl group and not the *ortho* and *meta* positions increased the yield of products (Table 1), which suggests that the hydroxyl group at the *para* position can bind with an amino acid residue in the active center of the enzyme by hydrogen bonding. Such bonds may increase the stabilization of the

enzyme-substrate complex. On the other hand, the spaces around the *ortho* and *meta* positions of the phenyl group in the active center may be too narrow to form an enzyme-substrate complex, because small quantities of products produced from *m*-coumaric acid and feluric acid compared with *p*-coumaric acid, and no product was produced from *o*-coumaric acid.

# Accumulation of the Stress Metabolite γ-aminobutyric Acid (GABA) in Coffee Seeds (*Coffea arabica* L.) During Post Harvest Processing

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## SUMMARY

In dry process coffee high amounts of γ-aminobutyric acid (GABA) are accumulated, whereas only small amounts of this non-protein amino acid are present in wet process beans. Untreated fresh seeds contain GABA only in traces. In plants, GABA is considered as stress metabolite, which especially is accumulated in tissues that are exposed to drought stress. It is apparent that the living and vital coffee seeds, which are dried from an initial water content of 50% down to about 12% suffer massive drought stress and express a related metabolism. The cause, why the stress related GABA accumulation occurs only in dry process seeds, although also the wet process seeds are dried down to a final water content of 12% is due to different time frames for the drying. Whereas in dry processed seeds, the time span for an induced, active stress metabolism is about 4 to 8 days, it is only 1 to 2 days in the case of drying the wet processed parchment beans.

GABA accumulation is enhanced when the time span for drying is elongated, e.g. due to artificial moistening. GABA accumulation is accompanied by a corresponding decrease in glutamate content, confirming that GABA is produced by decarboxylation of glutamic acid.

## INTRODUCTION

Green coffee is produced *via* two different methods of post harvest treatment, both of which are aimed to remove the outer layers of the fruit, and to reduce the water content of the coffee seeds (*"beans"*) from originally 50% to 12%. However, after roasting, the corresponding green coffees produce beverages of different qualities. As the coffee seed remains viable during processing, we postulated that physiological responses of the coffee seed to the different treatments were responsible for the differences observed (Bytof et al., 2000; Selmar et al., 2002).

In viable tissues, a massive decrease in water potential during drying should initiate stress reactions. In order to investigate whether or not green coffee seeds suffer drought stress in the course of drying, we performed processing experiments, and analyzed the occurrence of the stress metabolites.

## MATERIAL AND METHODS

*Coffea arabica* L. fruits of various cultivars (*Caturaì, Acaiá,* and *KP345*) were submitted to processing experiments, which were conducted in various coffee growing countries (Brazil, Tanzania and Mexico). Moreover, realistic model-processing experiments were performed in our laboratory, using freshly imported fruits from the named countries. The free amino acids were extracted with 4-sulphosalicylic acid (4% w/v) and analyzed on HPLC with the OPA/MCE method (for experimental details, see Selmar et al., 2002).

## RESULTS AND DISCUSSION

The effect of different post harvest treatments on the free amino acids in green coffees were already subject of an earlier work (Arnold & Ludwig, 1996). However, the authors could not find any specific impacts of the treatments on the amino acid compositions. This was mainly due to the fact, that the method used for amino acid analyses (FMOC-Cl), was apparently not suitable for a reliable GABA-detection (Arnold, 1995). Moreover, the way of dry model processing applied did not match the real conditions of a dry processing at all, but rather those of a "semi-washed" post harvest treatment.

Our data show, that coffee beans submitted to the dry processing contain extraordinarily high amounts of the non-protein amino acid γ-amino-butyric acid: GABA (Figure 1 and Figure 2). These increased amounts are accompanied by decreased contents of glutamic acid. The reciprocal correlation between the contents of glutamic acid and GABA in commercial coffee samples was already annotated in the work of Trautwein (1987), however, an explanation for this phenomenon was not given.

In plant tissues, GABA is known to be accumulated as a response to various stress situations: e.g. water stress, osmotic stress, hypoxia etc. (Shelp and Bown, 1999). GABA is rapidly formed by the enzymatic α-decarboxylation of glutamate. Hence, we conclude that the GABA-accumulation in dry process coffee beans is a consequence of a water stress situation occurring during the drying phase of post harvest treatment. Remarkably, proline is not affected by the treatment (data not shown).

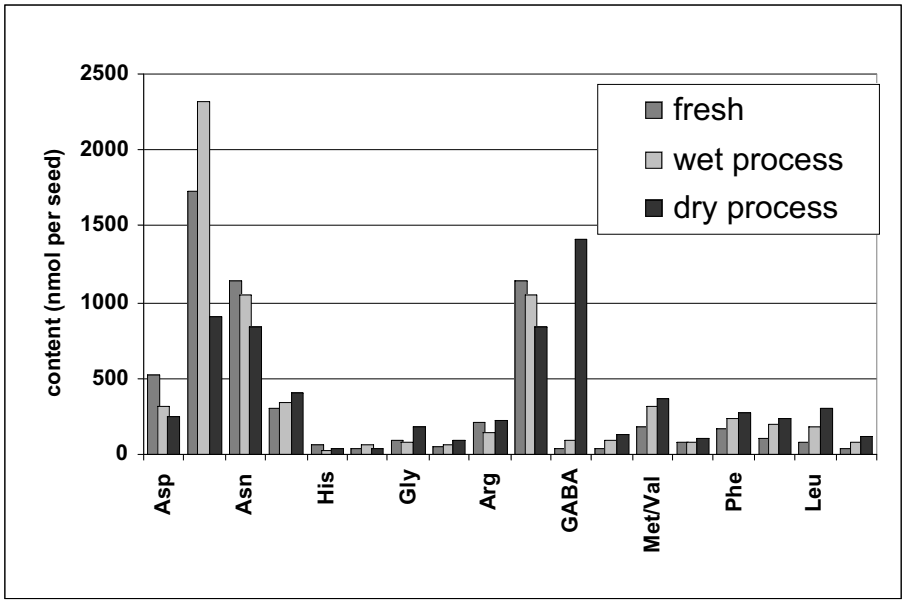


**Figure 1. Free amino acids in fresh and differently processed Arabica-coffees.**

Although the drying of the coffee beans is part of both kinds of post harvest treatments, GABA is not accumulated during the wet process. Apparently the time frame for a similar response of the beans is too narrow: if we assume that stress reactions are elicited at about 45% and all metabolic reactions are shut down at about 25%, in the case of dry processing, an average time span of 4 to 8 days results, in which an active stress metabolism can take place. In wet processing, the drying procedure from 50% down to 12% takes only 2 to 3 days. Consequently, the time window for an elicited stress metabolism is just about 1 to 2 days. Assuming that the synthesis and accumulation of GABA starts about 1 to 2 days after stress

induction, it becomes plausible that GABA can be accumulated as response of the drying only within dry process coffee beans and not in wet processed ones. However, if the drying of the washed parchment beans is prolonged (e.g. as a consequence of artificial moistening or insufficient drying capacity during the peak of harvest), also here, a relative accumulation of GABA can be observed.

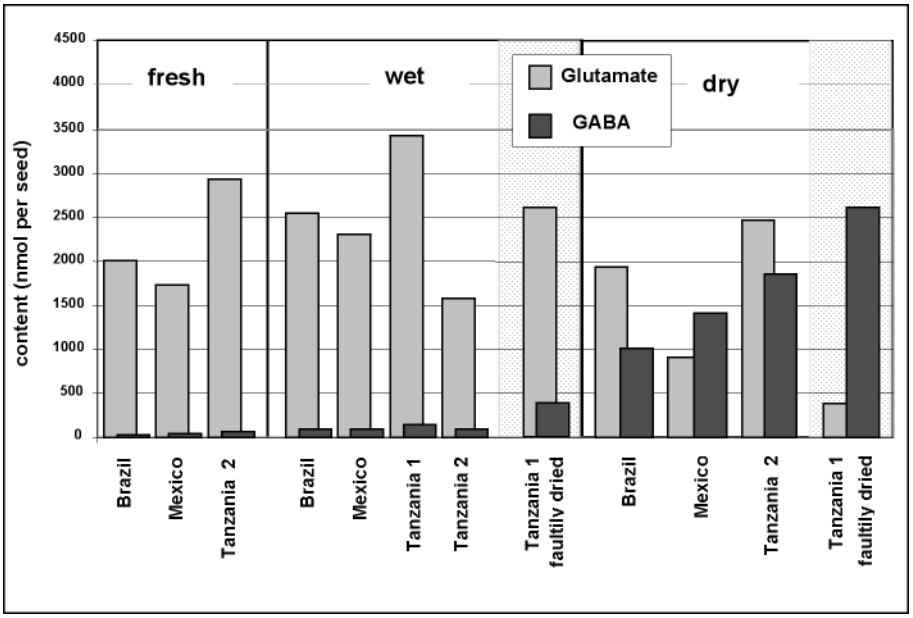


**Figure 2. GABA and glutamic acid contents in fresh and differently processed Arabica-coffees.**

The processing-specific changes in the amino acid composition of coffee beans should be due to the GABA-shunt acting in the coffee endosperm. These metabolic alterations of the composition of essential flavour precursors demonstrate the high relevance of coffee seed physiology for coffee quality.

## ACKNOWLEDGEMENTS

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# Expression of Isocitrate Lyase and the Abundance of ß-tubulin: Feasible Markers for the Estimation of Germination Processes in Differently Processed Green Coffees

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## SUMMARY

In order to monitor germination processes in coffee beans during post harvest treatments, two approaches have been pursued: 1) expression studies with germination-specific enzymes i.e. that of isocitrate lyase (ICL) and 2) analyses of cell cycle activity and cell division. ICL was quantified by RT-PCR, using specific primers deduced from a homologous probe. As reliable marker for cell division, the occurrence of ß-tubulin was determined. This protein assembles the microtubules and thus is an indispensable constituent of cell division. The abundance of ß-tubulin was visualized in Western blots using specific antibodies.

Whereas in fresh coffee seeds neither ICL transcripts nor ß-tubulin molecules were abundant, both components are found in significant amounts in processed ones. In the case of wet process coffees the highest rate of ICL expression and the highest amount of ß-tubulin were detectable already at the first day after the onset of processing. In contrast, in dry process seeds, peak levels were found after about four to six days after the start of post harvest treatment.

These results unequivocally prove that germination plays a role in coffee processing and that the extent of this metabolism differs between wet and dry processed coffees. These new insights establish a basis to explain the quality differences of differently processed green coffees and underline the so far underestimated relevance of coffee seed physiology for questions of coffee quality.

## INTRODUCTION

In living organs and organisms, the chemical composition strongly is influenced by the metabolic status of the related tissue. We postulated that the well-known variations in the chemical composition of coffee seeds processed under different conditions are largely caused by germination (Bytof et al., 2000; Selmar et al., 2002). According to Bewley & Black (1994) for *orthodox* seeds there is a clear definition for beginning (water uptake) and ending of germination (protrusion of the radicle). In contrast, for the *recalcitrant* coffee seeds only the ending is clearly marked: the coffee seed, freshly prepared from a red ripe fruit reveals a water content of nearly 50% and does not really take up water for germination. Therefore, the onset and progress of germination must be characterized biochemically. We chose the expression of the isocitrate lyase (ICL) – a key enzyme of the glyoxylate cycle – and the resumption of cell division, monitored by the abundance of ß-tubulin, as tools to specify the onset and progression of germination.

## EXPERIMENTAL

### Model processings

Most of the experimental processings were performed in our laboratory in Braunschweig. In all cases – due to a very careful manual sorting – only fully ripe red cherries from Brazil, Tanzania, or Mexico were used as "identical starting material" for both, dry and wet processings in parallel.

### Expression studies of isocitrate lyase using RT-PCR

Based on the alignments of known isocitrate lyase (ICL) sequences of various plants, redundant primers were created to generate a homologous probe for the isocitrate lyase of *Coffea arabica*. A corresponding 480 bp-fragment was cloned into a bacterial vector, (TOPO TA). The modified plasmid was transformed into *E. coli* (Cell DH 5α), amplified and used for both, as probe for the detection of ICL- messengers in Northern blots and for sequencing. Based on the elucidated sequence, specific isocitrate lyase primers were created: ICL 380 (gggattgggacctgcctagaacc) and ICL 520 (gccagtgtgtcaacctcatgg) resulting in a 433 bp-PCR product. RT-PCR analysis was performed using RNA, isolated from fresh and differently processed coffee seeds.

### ß-tubulin as marker for the resumption of cell cycle activity and cell division

Isolated coffee embryos were homogenized with Modil-buffer (pH 6.8) containing a protease-inhibitor cocktail according to De Castro et al. (1998). The protein extracts were separated by SDS-gel electrophoresis. After blotting on a nitro-cellulose membrane, the proteins were detected immunochemically using specific ß-tubulin antibodies (TUB 2.1, Sigma) in a dilution of $1{:}10^{-6}$. The secondary antibody was an anti mouse IgG, conjugated with alkaline phosphatase. Detection was performed using BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitroblue tetrazolium).

## RESULTS AND DISCUSSION

One of the first enzymes expressed in seeds that germinate is the isocitrate lyase, the key enzyme of the glyoxylate cycle, which is responsible for the conversion of stored lipids into carbohydrates. We investigated the expression of isocitrate lyase during coffee processing. Unfortunately, related Northern blot analyses revealed that the number of transcripts, even in young seedlings, is very low. Consequently, the related expression studies had to be performed using the more sensitive RT-PCR technique.

The corresponding RT-PCR experiments clearly show that in fresh coffee seeds no isocitrate lyase is expressed (Figure 1). In contrast, in wet process green coffees, a clear signal of the isocitrate lyase is found. Surprisingly, also in dry processed coffee seeds, transcripts for isocitrate lyase are detectable. However, the time course of expression is different. On the basis of about 10 independent RT-PCR-experiments, an average time course for the expression of isocitrate lyase was established. In wet process seeds, already one day after the start of the processing, maximum expression of ICL can be observed. In contrast, the corresponding maximum in dry process beans appears several days later.

A further reliable marker for germination is the resumption of cell cycle activity and cell division in the embryo. To monitor these events, we analyzed the occurrence of ß-tubulin, the major constituent of the microtubules, which are necessary for cell division. The occurrence

of this small protein is a clear evidence for cell cycle activity, and in the embryo, for the onset of germination.

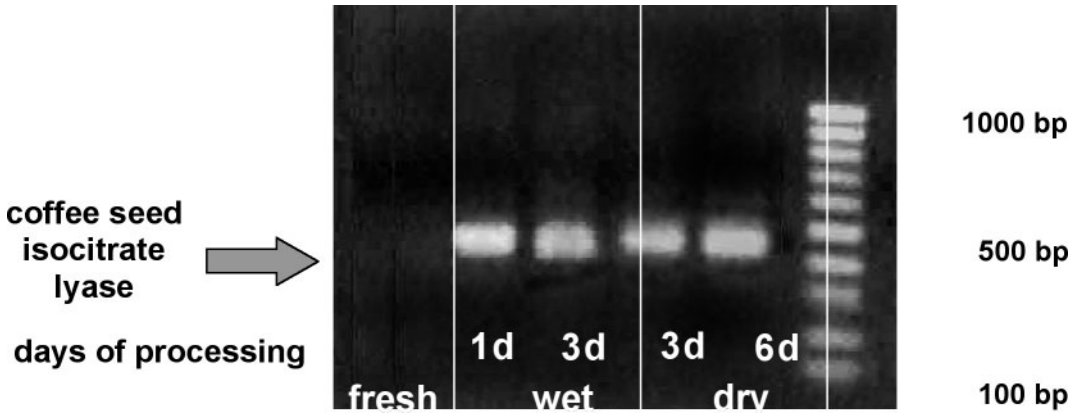


**Figure 1. Expression of ICL in fresh and differently processed green coffees.**

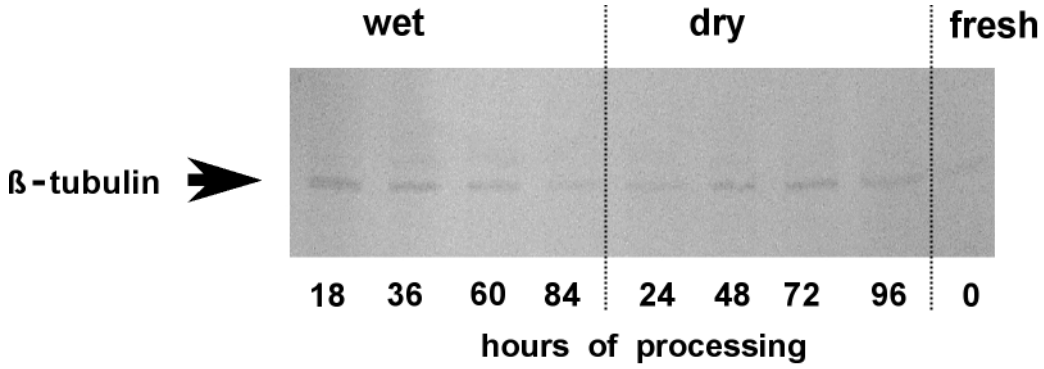


**Figure 2. Western blot analysis of ß-tubulin in fresh and differently processed green coffees. Equal amounts of plant material in each lane.**

In fresh coffee seeds, ß-tubulin is not detectable; in contrast, in wet process seeds, significant amounts of ß-tubulin are present. Yet, also in dry process seeds ß-tubulin can be determined, however, again, the time courses of ß-tubulin accumulation are quite different. In wet process coffee seeds, maximum ß-tubulin accumulation is observed within the first day of processing, shortly after pulp removal. In contrast, in dry process seeds maximum ß-tubulin accumulation is found several days after the onset of processing.

The data of the ß-tubulin accumulation fully confirmed the corresponding data from the isocitrate lyase expression studies. Thus, these experiments prove without any doubt that in the course of processing, germination is initiated in the coffee beans, and that the extent of the related metabolism depends on the mode of post harvest treatment.

## ACKNOWLEDGEMENTS

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# SPME Technology and its Application in the Coffee Industry

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## SUMMARY

SPME has been commercially available for only eight years and new applications for the food industry are rapidly being developed and published. The aim of this study is to confirm application of SPME (Solid Phase Micro Extraction) technology for the soluble coffee industry. The separation of 50 volatile compounds on soluble coffee has been accomplished with a reproducibility of 5% RSD (Relative Standard Deviation) for most analytes. Significantly, 16 pyrazine-containing compounds, which are known to be major contributors to pleasant nutty/roasty notes, were successfully detected in soluble coffee. In conclusion, SPME methods require no solvent, are rapid, simple and inexpensive and can be used to concentrate volatile components from liquid and soluble coffee. SPME is an excellent tool for the analysis of key pyrazine compounds in soluble coffee.

## INTRODUCTION

Sample preparation techniques based on adsorption have been widely used for trace analysis to preconcentrate trace compounds and/or separate analytes from sample matrix. Recently, a new variation of adsorption technique called solid-phase microextraction (SPME) has been developed by Pawliszyn and co-workers (Arthur and Pawliszyn, 1990) and SPME devices are now commercially available. The key component of a SPME device is a piece of fused silica fiber (typically 1 cm long and 0.11 mm outer diameter), coated with an absorbent polymer. When the SPME fiber is immersed in an liquid sample, a partitioning of the compounds in the sample between the aqueous phase and fiber surface occurs. The adsorbed compounds can then be thermally desorbed in a GC injection port. This technique has been studied mainly for analysis of pollutants in environmental water samples (Arthur et al., 1992a) Headspace sampling using SPME has also been reported (Zhang and Pawliszyn, 1993). SPME is also a potentially useful techniques for flavor analysis. Hawthrone et al. (1992) successfully applied SPME with an uncoated fused silica fiber for determination of caffeine in beverages. Virtually all samples that can be analyzed by conventional static headspace can be determined with SPME. But, no one individual method is suitable for all purpose because of the complexity of food samples, the wide variation of flavor composition, and different analytical objectives. Each of the preconcentration techniques has its own advantages and disadvantages, and these methods are generally complementary to each other. SPME may work best by headspace or by liquid sampling depending on the nature of the matrix and the volatility of the flavor components. The major objective of this study was to investigate the application of SPME technology in the coffee industry.

## MATERIALS AND METHODS

### Sample preparations

14.3 g of soluble coffee were weighed into a 100 ml headspace vial. Distilled water was then added to make the solution up to mark 100 ml. Dissolve the soluble coffee powders with

distilled water into sonicator. Transfer 3.5 ml of coffee solution with pipette into 10ml headspace vial and add 1.0 g of NaCl into the headspace vial. A 5ml of internal standard 2ppm concentration, ethyl crotonate (Aldrich Chemical Co., Inc.), and magnetic stirrer were then added. The headspace vial was sealed with a septum and then secured by a cap, and then SPME protective sheath pierces the septum the plunger is lowered to expose it to the sample headspace. The headspace vial was stirred and heated at 40°C for 30 minutes in a water bath. After a pre-determined absorption time, the fiber coated with 100 μm polydimethylsiloxane (PDMS /Carboxen) is withdrawn back into the protective sheath, then the sheath is pulled out of the sampling vial. The sheath is immediately inserted in the GC injector and the plunger is again lowered to expose the fiber. This time, the fiber is exposed to a high temperature in the injector liner (GC) at 230°C where the concentrated analytes are thermally desorbed on 3 minutes and, consequently refocused onto the GC column and finally detected flame ionization detector (FID). The results are expressed as the peak concentration of the 50 volatile components calibrated by the internal standard.

**Gas Chromatography(GC) and Mass Spectrometry (MS)**

An Hewlett Packard 6890A gas chromatograph with FID was used. The column used was a J & W DB-1 (30 m x 0.52 mm, film thickness, 1.5 μm). Helium was used as the carrier gas at a flow rate of 35 ml/min. The temperature program was : 50°C for 2 min, then raised 4 /min until 150°C was reached. These conditions were held for 5 minutes. GC-MS was performed on a HP 5972 mass spectrometer, coupled to a 5890 Series II gas chromatograph and a fused silica capillary column (J & W DB-1, 30 m x 0.52 mm, film thickness, 1.5 μm). The mass spectrometer operated in electron impact mode with an electron energy of 70 eV and an emission current of 50°C. The mass spectrometer scanned from m/z 29 to 400 at 1.9 scan $s^{-1}$. The components of interest were tentatively identified by comparing their mass spectra with those contained in NIST/WILEY Mass Spectral Database and by comparison of LRI.

**RESULTS AND DISCUSSION**

**Optimization of Experimental Conditions**

***"Salting-Out" Effect***

Generally, the presence of electrolyte in an adsorption system can influence the adsorption in two ways : changing the properties of the phase boundary and decreasing the solubility of the hydrophobic compounds in the aqueous phase. The latter is more often observed in analytical chemistry, being referred to as "salting out". The presence of electrolyte in aqueous solution can lower water activity, and, consequently, decrease the solubility of hydrophobic compounds in aqueous phase. This "salting out" effect can also be used to increase the adsorption on SPME fibers. For practical reasons, 1.0 g of amount of salt was used for this study to enhance the sensitivity of analysis.

***Sample Amount and Headspace Volume***

The nature of the sample matrix and the SPME fiber under given experimental conditions determines the distribution coefficient of an analyte between sample and SPME fiber. Moreover, the amount of analyte adsorbed on an SPME fiber also depends on the amount of sample and its headspace volume. If the concentration of the analyte in the sample after adsorption is significantly lower, the amount of adsorbed on the SPME fiber can be by increased by increasing the sample amount, while the distribution coefficient remains constant. With increasing sample amount, the amount adsorbed also increases, at first, but

then remains practically constant thereafter. For a solid or liquid sample, analytes distribute among the condensed phase, the adsorption phase and the gas phase. Because the total amount of analytes in the three phase is constant at equilibrium, lowering the headspace volume will increase the amount of the analytes in the other two phases, and therefore, increase the sensitivity of SPME-based methods. The effect of headspace volume is often most pronounced for volatile trace flavor components. For this study, 14.3 g of soluble coffee were weighed into a 100 ml headspace vial. Distilled water was then added to make the solution up to mark 100 ml. Dissolve the soluble coffee powders with distilled water into sonicator. Transfer 3.5 ml of coffee solution with pipette into 10ml headspace vial.

***Adsorption and Desorption Rate***

For liquid sampling, the adsorption rate of SPME is mainly determined by mass transfer and diffusion process, and is thus accelerated by agitation (Liu et al., 1997; Louch et al., 1992). The time needed to reach the plateau is typically around 10 minutes (Coleman, 1996). For headspace sampling, the SPME process involves evaporation of the analytes into gas phase, and diffusion to the surface of the fiber. Adsorption from gas phase onto the fiber surface is fast in comparison with that from a liquid phase because the diffusion rate is much greater in former case. However, evaporation rates can be very different for various analytes. For compounds with lower vapor pressure, evaporation seems to be rate determining step. Continuing to sample until the adsorption equilibrium has been reached can achieve maximal sensitivity, as well as minimal variation, for an SPME-based method. If the sampling time and conditions are accurately controlled, acceptable reproducibility can be achieved. If the temperature of the GC injection port used for desorption was set at 200°C, carryover was observed in our lab. We generally used sampling time for 30 minutes in a water bath at 40°C with magnetic stirrer, and 3 minutes for desorption time in GC injector at 230°C for this study.

**Quantitative Analysis**

A high recovery is generally required for an accurate and precise quantitative analysis using external calibration. Because SPME as a preconcentration procedure is a single batch method, quantitative recovery in most cases is very difficult, if impossible, to accomplish. In practice, to compensate for substantially incomplete analyte recoveries, an internal standard that possesses similar physical and chemical properties is often used. However, choosing an appropriate compound as an internal standard is difficult due to the high selectivity of SPME fibers. The quantitative analysis used for this study is an internal standard method. A 5 ml of internal standard 2ppm concentration, ethyl crotonate (Aldrich Chemical Co., Inc.) is used.

**Determination of the detected analytes**

Figure 1 illustrates a chromatogram resulting from the SPME extraction of soluble coffee. The peaks were mainly aldehydes, ketones, hetrocyclic compounds, esters and pyrazine compounds. These components were identified by library search and/or through matching for retention times and of the mass spectra with those of the authentic specimens and also Kovats Index were used.

**Repeatability of the analytical procedure**

The day–to-day precision of the method was determined by running Korean soluble coffees in triplicate, one analysis per day. 50 Volatile compounds were identified in Table 1. Quantitative analysis for peak area between individual component and ISTD were measured. The mean and the standard deviation of each analyte for Korean soluble coffee samples are summarized

in Table 1. In general, the range of relative standard deviations (RSD) were 0.5 ~ 5.0% except for pyrazine (6.9%) The repeatability of the procedure was confirmed periodically by reanalyzing a previous sample.

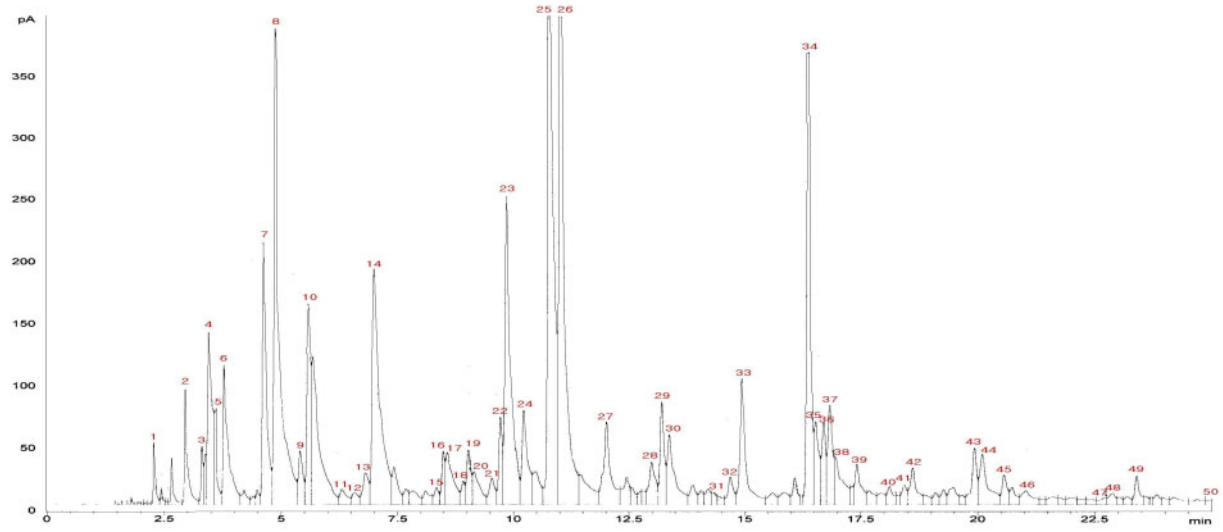


**Figure 1. Chromatogram of soluble coffee by SPME headspace sampling.**

**SPME application in the coffee industry**

Pyrazines are nitrogen-containing heterocyclics that are potent characterisitics flavorants found a wide range of raw and processed foods (Maga, 1982). In coffee, 79 members of this family were found (Flament, 1991). Pyrazines are usually associated with the generation of roasted, nutty and burnt flavor notes. These unique and desirable sensory properties make pyrazines essential to the soluble coffee industry. From this study, A total of 50 volatile compounds on soluble coffee were detected using a SPME method and Significantly, 16 pyrazine-containing compounds, which are known to be major contributors to pleasant nutty/roasty notes, were successfully identified. The SPME method can be used for getting the correlation between pyrazines compounds and nutty/roasty note in the soluble coffee industry.

## CONCLUSIONS

The separation of target compounds from the sample matrix is a challenge to many analytical chemists. The extraction process is generally the step at which most analyte loss occurs; therefore, efficient methods of extraction are continually being sought. Solid-phase microextraction is a relatively new technique whereby analytes of interest partition from the sample matrix into a polymeric liquid coating. The application of the headspace SPME of flavor volatile compounds of soluble coffee was investigated. 50 different common flavor volatile organics from soluble coffee were examined and accomplished with a reproducibility of 5% RSD (Relative Standard Deviation) for most analytes. On the basis of this study, it is concluded that the SPME analysis technique described herein is technically feasible for the extraction of the trace volatile compounds and the reliable characterization of soluble coffee.

**Table 1. Relative Standard Deviation (RSD(%[a])) for volatile compounds identified in the soluble coffee.**

| Peak No. | Compound Name | RSD (%) | Peak No. | Compound Name | RSD (%) |
|---|---|---|---|---|---|
| 1 | Acetone | 4.5 | 26 | Furfuryl alcohol | 0.2 |
| 2 | 2-Methyl propanal | 4.1 | 27 | Dihydro-2-furanone | 2.7 |
| 3 | 2,3-Butanedione | 5.0 | 28 | 1-(2-furanyl) ethane | 1.4 |
| 4 | Methyl ethyl ketone | 3.9 | 29 | 2,5-Dimethyl pyrazine | 2.9 |
| 5 | Methyl formate | 4.1 | 30 | Ethyl pyrazine | 2.3 |
| 6 | 2-Methyl furan | 3.7 | 31 | 2,3-Dimethyl pyrazine | 3.4 |
| 7 | 3-Methyl butanal | 4.6 | 32 | Benzaldehyde | 0.7 |
| 8 | 2-Methyl butanal | 4.2 | 33 | 5-Methyl furfural | 0.3 |
| 9 | 2-Pentanone | 2.9 | 34 | Furfuryl acetate | 1.2 |
| 10 | 2,3-Pentanedione | 3.9 | 35 | 2-Ethyl-6-methyl pyrazine | 3.4 |
| 11 | 2,5-Dimethyl furan | 4.3 | 36 | 2-Ethyl-5-methyl pyrazine | 3.3 |
| 12 | Pyrazine | 6.9 | 37 | 2,3,5-Trimethyl pyrazine | 1.9 |
| 13 | Methyl pyrrole | 3.7 | 38 | 2-Ethyl-3-methyl pyrazine | 2.3 |
| 14 | Pyridine | 3.1 | 39 | Benzacetaldehyde | 4.3 |
| 15 | 3-Methyl-2-buren-ol | 4.5 | 40 | Methylphenol | 3.5 |
| 16 | 2,4-Dimethyl-3-pentanone | 4.1 | 41 | 2-Cyclopenten-1-one | 1.2 |
| 17 | 3-Hexanon | 3.2 | 42 | 2-Acethyl pyrrole | 0.5 |
| 18 | 3-Furanone | 3.7 | 43 | 3-Eethyl-2,5-dimethyl pyrazine | 2.1 |
| 19 | 2,2-Dimethyl-3-pentanone | 4.3 | 44 | Furfuryl propionate | 3.3 |
| 20 | Hexanal | 3.7 | 45 | 3-Methyl-1,4-heptadione | 2.7 |
| 21 | 4-Methyl thiazole | 4.1 | 46 | Furfuryl formate | 3.6 |
| 22 | 2-Methyl pyrazine | 1.2 | 47 | 2,3-Diethyl-5-methyl pyrazine | 4.7 |
| 23 | Furfural | 3.9 | 48 | 3,5-Diethyl-2-methyl pyrazine | 3.6 |
| 24 | 2-Methoxy methylfuran | 5.0 | 49 | N-Furfuryl pyrrole | 2.9 |
| 25 | Ethyl crotonate (Internal Standard) | 2.3 | 50 | 2-Isobutyl-3-methoxy pyrazine | 0.9 |

[a]*Calculated from a minimum of three replicates*
[b]*Components are presented in order of elution*

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# Levels and Profiles of Bioactive Amines in Coffee as Affected by Roasting

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## SUMMARY

The present study aimed at an evaluation of the effect of roasting on the levels of amines in coffee. Also, since coffee quality is associated with the presence of defective beans, an evaluation of amine levels for each specific defect (black, brown, immature) in comparison to healthy beans was performed. Samples were roasted to three different levels. HPLC analysis was carried out for the detection and quantification of the following amines: putrescine, spermidine, spermine, agmatine, cadaverine, serotonin, histamine, tyramine, tryptamine and phenylethylamine. Putrescine, agmatine and spermidine were detected in all samples prior to roasting. Putrescine was the prevailing amine, followed by agmatine, spermine and spermidine. This distribution was observed for all samples except for black beans, for which agmatine was the prevailing amine. Histamine was only detected in the defective coffee beans. There was a significant decrease in total amine content after roasting and only small amounts of serotonin, agmatine and spermidine remained. No significant differences were detected in amine levels among defective and non-defective coffee beans after roasting.

## INTRODUCTION

Amines are organic bases formed during metabolic processes in living organisms, being encountered in most food products. They are classified as (i) natural, formed during *de novo* polyamine biosynthesis, or (ii) biogenic, formed by action of decarboxylase-positive microorganisms. Thus, biogenic amines have been used as a criterion for quality evaluation of food products (Bardócz, 1995).

A few studies have discussed the presence of amines in coffee (Amorim et al., 1977; Cirilo et al., 2003; Casal et al., 2004; Oliveira et al., 2005). Amorim et al. (1977) investigated the presence of polyamines in coffee, prior to and after roasting at 240$^{o}$C for 12 min. Putrescine, spermine and spermidine were detected before roasting. Only putrescine was detected after 10 min of roasting and no amines were detected after 12 min. Cirilo et al. (2003) investigated the presence of both natural and biogenic amines in coffee submitted to two levels of roasting (300$^{o}$C for 6 and 12 min, respectively). These authors encountered serotonin, putrescine, spermine and spermidine in crude coffee and, even though the total amine content decreased after roasting, agmatine was detected after 12 min roasting. Furthermore, amine levels were quite different from those reported by Amorim et al. (1977). Casal et al. (2004) evaluated the levels of biogenic amines (putrescine, cadaverine, serotonin, tyramine, spermidine, and spermine) in robusta and arabica coffees. Their results indicated that putrescine, the main biogenic amine present in the green beans, could be used in the discrimination of the referred species. These authors also mentioned that amines could be used for discrimination between green coffees subjected to different postharvest processes. Oliveira et al. (2005) evaluated the effect of roasting on the levels of amines in high (soft) and low (rio) quality coffees.

Putrescine levels were significantly higher for the low quality sample. Also, both histamine and tryptamine were only present in the low quality sample. Their results indicate that both amine levels and profiles could be associated with the presence of defective beans.

Thus, in view of the fact that the content of amines is known to be related to quality of food products in general, an investigation of the profiles of amines in healthy and defective coffees, as well as the effects of processing on those compounds is of relevance. Therefore, the present study aimed at an evaluation of the effect of roasting on the levels of amines in both healthy and defective coffee beans.

## METHODOLOGY

Arabica green (crude) coffee samples (2002/2003 crop) were obtained from Santo Antonio Estate Coffee (Minas Gerais, Brazil). The coffee beans were subjected to selection in an electronic sorter. The beans rejected by the sorting machine were used in the present study. This mixture of coffee beans consisted of 7.5% black, 18.0% immature, 34.0% sour and 40.5% non-defective beans in weight, and will be herein designated as PVA mixture. This notation (PVA) stands for "Preto, Verde e Ardido", the Brazilian denominations for black, immature and sour beans, respectively. Black, sour, immature and non-defective beans were manually separated from the PVA mixture. Samples of randomly selected 100 beans were separated from each lot (black, sour, immature, non-defective and PVA mixture) and roasted in a convective oven at 200$^{o}$C for 30 min (light roast), 1h (medium roast) and 2h (dark roast).

HPLC analysis was carried out for detection and quantification of the following amines: putrescine, spermidine, spermine, agmatine, cadaverine, serotonin, histamine, tyramine, tryptamine and phenylethylamine, according to the methodology described by Cirilo et al. (2003).

Experiments were performed in triplicate. The obtained data were submitted to analysis of variance and the means were compared by the Duncan test at 5% probability.

## RESULTS AND DISCUSSION

Results obtained for amine levels in crude coffee in comparison to the ones available in the literature are presented in Table 1. Total amine levels ranged from 3.0 mg/100g (black beans) to 13.8mg/100g (non-defective beans). No significant differences were found in total amine levels among non-defective, immature and sour beans. However, black beans presented much lower amine levels compared to the others. Average values were in the same range reported by Oliveira et al. (2005), but higher than those found by Cirilo et al. (2003).

Seven of the ten evaluated amines were detected in crude coffee: putrescine, histamine, serotonin, agmatine, spermidine, spermine and tryptamine (Table 2). Among those, only putrescine, agmatine and spermidine were detected in all samples above trace levels. Putrescine was the prevailing amine, being responsible for approximately 50% of the total amine content, followed by agmatine, spermine and spermidine. This distribution was observed for all samples but black, for which agmatine was the prevailing amine. The presence of putrescine, spermidine and spermine was expected, since these amines are usually present in most plants (Flores et al., 1989; Cirilo et al., 2003) and were detected in previous studies for coffee (Amorim et al., 1977; Cirilo et al., 2003; Casal et al., 2004; Oliveira et al., 2005). Putrescine is a precursor of spermidine and spermine, which could explain its high levels. Histamine was only present in the defective coffee beans. This is in agreement with the results presented by Oliveira et al. (2005), who reported that histamine was detected only for

low quality coffee. Serotonin was detected in some of the samples. Traces of cadaverine were found in the black beans.

**Table 1. Total levels of bioactive amines in green coffee (mg/100 g).**

| Value | This study | | | | | Oliveira et al. (2005) | Cirilo et al. (2003) |
|---|---|---|---|---|---|---|---|
| | Defective | | | | Non-defective | | |
| | Black | Immature | Sour | PVA | | | |
| Minimum | 2.86 | 11.24 | 9.37 | 9.63 | 10.97 | 8.48 | 3.03 |
| Maximum | 3.14 | 12.99 | 11.67 | 16.93 | 16.8 | 13.65 | 4.44 |
| Mean | 3.00 | 11.95 | 10.40 | 13.09 | 13.79 | 10.9 | 3.21 |

**Table 2. Contribution of bioactive amines to total levels in green coffee (%).**

| | Defective | | | | Non-defective |
|---|---|---|---|---|---|
| | Black | Immature | Sour | PVA mixture | |
| Putrescine | 29.3 | 45.1 | 40.5 | 49.3 | 54.9 |
| Histamine | 14.7 | 5.8 | 9.8 | 4.9 | 0 |
| Serotonin | 13.0* | 3.5* | 0 | 0 | 3.5* |
| Agmatine | 47.0 | 16.0 | 21.1 | 17.7 | 12.2 |
| Spermidine | 5.8* | 13.3 | 11.9 | 13.6 | 14.4 |
| Spermine | 0 | 15.9 | 16.7 | 13.4 | 16.4 |
| Tryptamine | 0 | 4.8 | 0 | 3.4 | 2.8 |

**Amine was detected in only one sample of triplicate analysis.*

There was a significant decrease in total amine content after roasting. According to previous studies, both putrescine and spermine should be completely consumed even for mild roasting conditions, regardless of coffee quality (Oliveira et al., 2005). Only serotonin, agmatine and spermidine were detected, in most samples in amounts below the quantification limit (0.37 mg/100 g green coffee). For a light roast (~12% weight loss), traces of serotonin were detected in all samples, traces of agmatine were present in the black and sour samples, and traces of spermidine were detected for immature, sour and non-defective beans. After a medium roast (~14.5% weight loss), traces of serotonin still remained in all samples. Agmatine was detected only in the black beans and traces of spermidine still remained in the immature and PVA samples. After more intensive roasting (~17% weight loss), traces of serotonin were still present in the black, sour and PVA samples. Traces of agmatine were still found in the sour samples, whereas a slight increase in agmatine concentration was observed for the black beans (0.39 mg/100 g). It is noteworthy to point out that these results confirm that serotonin is quite resistant to the effects of roasting, as discussed by Casal et al. (2004).

## CONCLUSIONS

The effect of roasting on the profile and levels of bioactive amines in coffee was evaluated. Putrescine, agmatine and spermidine were detected in all samples prior to roasting. Putrescine and agmatine were the prevailing amines, followed by spermine and spermidine. Histamine was present only in the defective coffee samples. Total amine content decreased considerably after roasting, and only small amounts of serotonin, agmatine and spermidine remained. The results obtained in this study show that roasting promotes drastic changes in the amines profiles and also indicate that detection of histamine in green coffee could be associated to the presence of defective coffee beans.

**ACKNOWLEDGEMENTS**

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# Two New Diterpenes in Roasted Coffee

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## SUMMARY

In commercial roasted coffees, two new diterpenes were discovered. They were elucidated by means of EI- and CI-high-resolution mass spectrometry and several NMR-spectroscopic methods. They were named isokahweol and dehydroisokahweol.

The pentacyclic diterpenes of *Coffea arabica* and *Coffea canephora var. Robusta* are well known: Arabica coffee beans contain the diterpenes cafestol and kahweol, whereas Robusta coffee beans contain cafestol, small amounts of kahweol, and in addition, 16-O-methylcafestol and 16-O-methylkahweol (Speer and Kölling-Speer, 2001; Kölling-Speer and Speer, 2001).

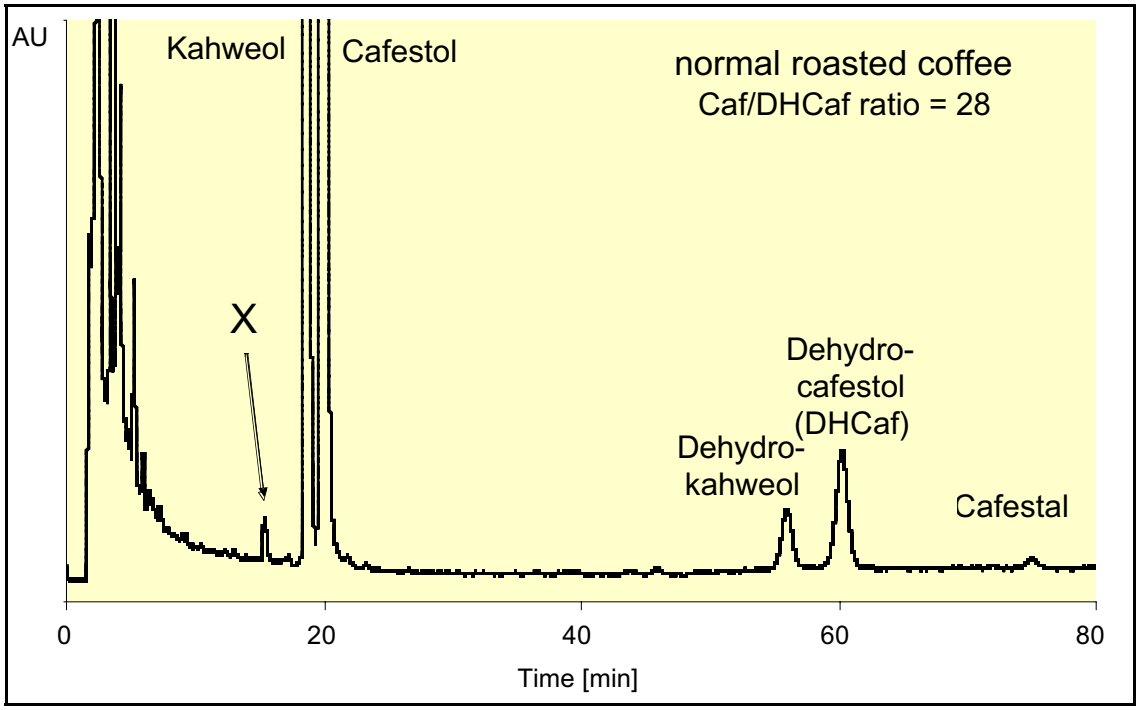
During the roasting process, cafestol is decomposed to dehydrocafestol and cafestal as well as kahweol to dehydrokahweol and kahweal. All of these degradation products were identified by our work group for the first time a few years ago (Tewis et al., 1993; Speer et al., 2000).

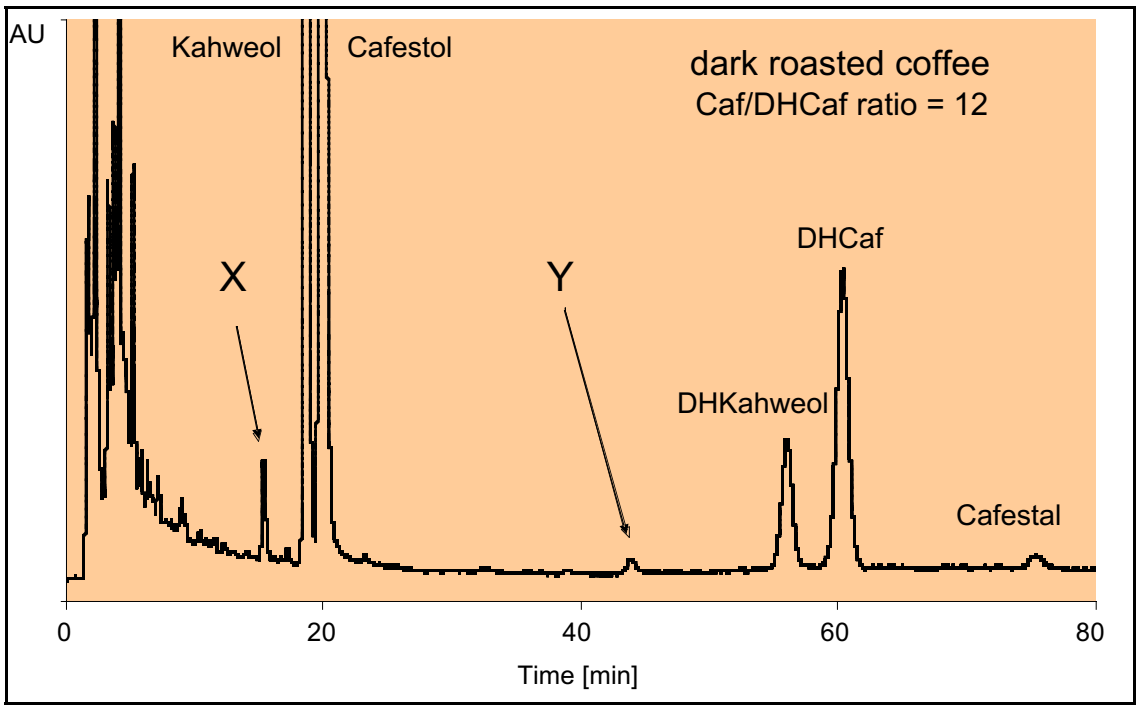
Recently, while analysing various commercial roasted coffees on containing Robusta parts by using the DIN method No. 10779 (DIN 10779, 1999), two small additional peaks were discovered in the HPLC chromatograms (X and Y in Figure 1, 2). They increased at a higher roasting degree determined via the cafestol-dehydrocafestol ratio (Kölling-Speer and Speer, 1997).

Now, both peaks were elucidated by means of EI- and CI-high-resolution mass spectrometry and several NMR-spectroscopic methods ($^1$H-NMR, COESY, NOESY). The EI-mass spectra are presented below in Figure 3 and 4, the appropriate $^1$H-NMR spectra and the structural formulas of Peak X and Peak Y in Figure 5 and 6. The UPAC name of peak X is 7-(hydroxyl) -10b-methyl-5,6,7,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta[5,6]naphto[2,1-*b*]furan-7-ol, the UPAC name of peak Y is (10b-methyl-5,6,9,10,10a,10b,11,12-octahydro-5a,8-methanocyclohepta[5,6]naphto[2,1-*b*]furan-7-yl)methanol.

Both compounds were unknown until now.

Due to their similarity to kahweol and dehydrokahweol we have named the new diterpenes isokahweol and dehydroisokahweol.




**Figures 1, 2. HPLC chromatograms of two commercial coffees with different roasting degrees.**

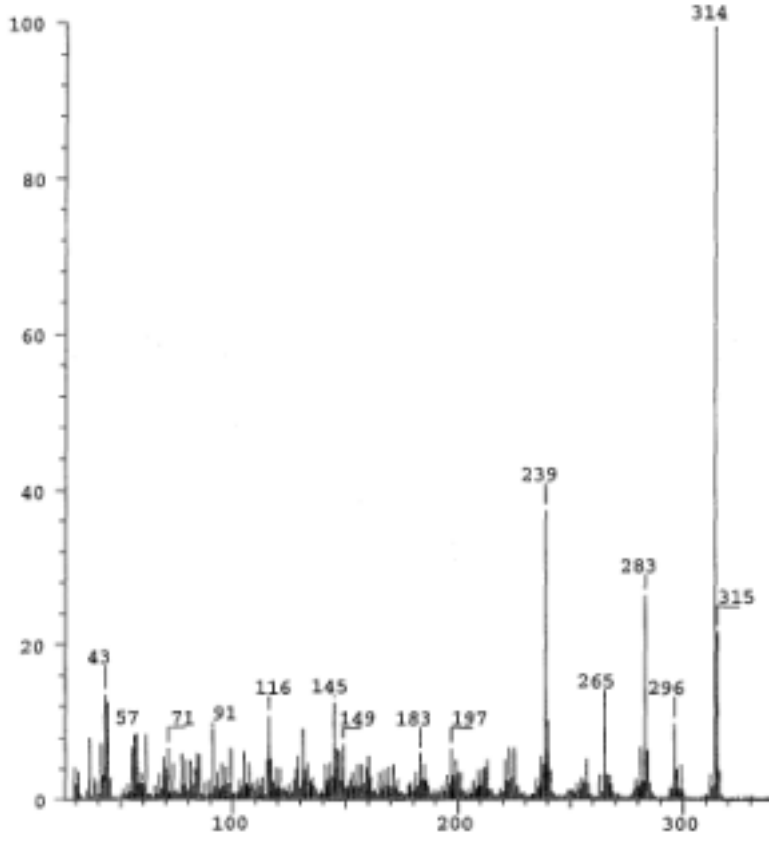



**Figure 3. EI-mass spectrum of isokahweol.**

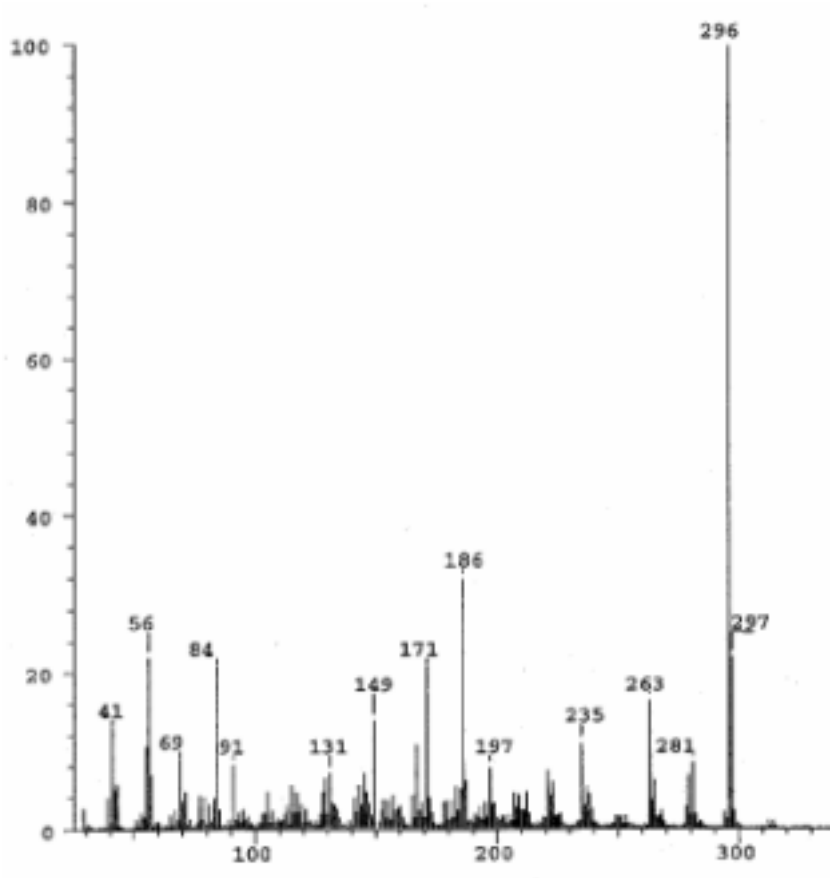



**Figure 4. EI-mass spectrum of dehydro (Peak X) isokahweol (Peak Y).**

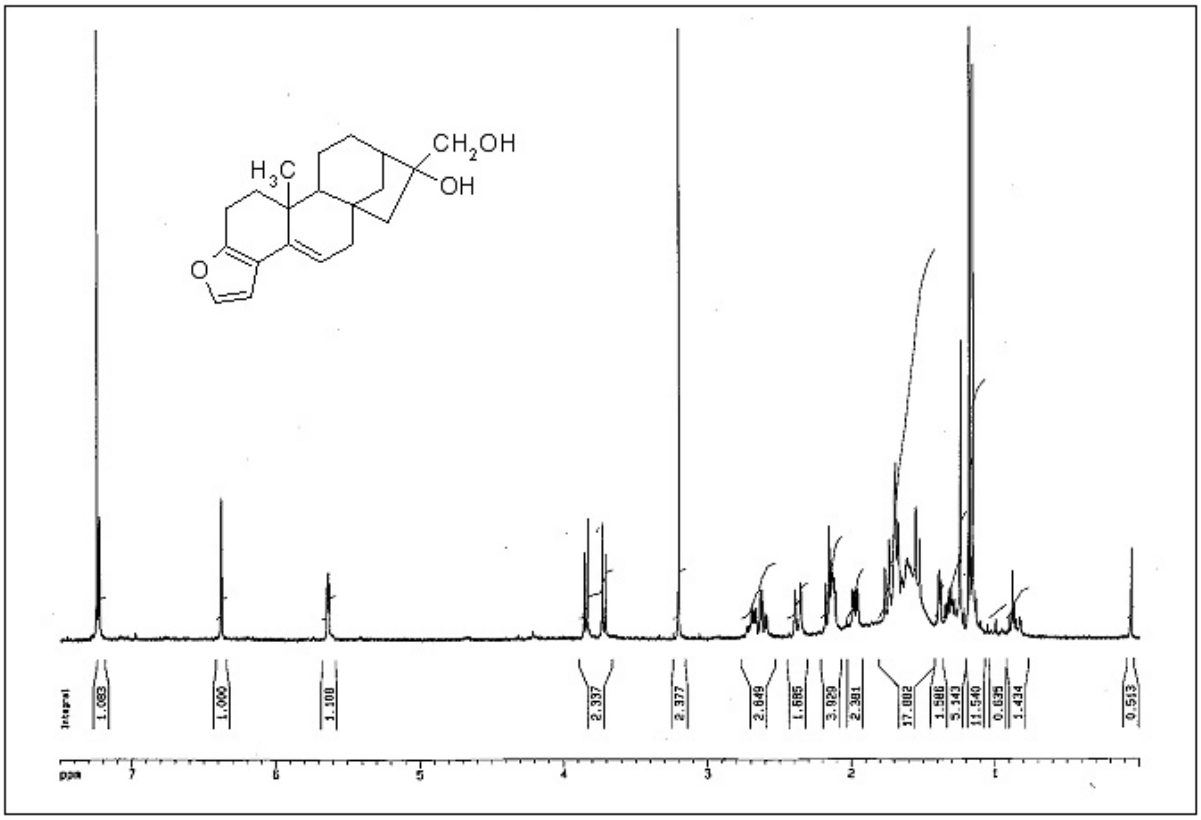



**Figure 5. $^{1}$H-NMR spectrum of isokahweol.**

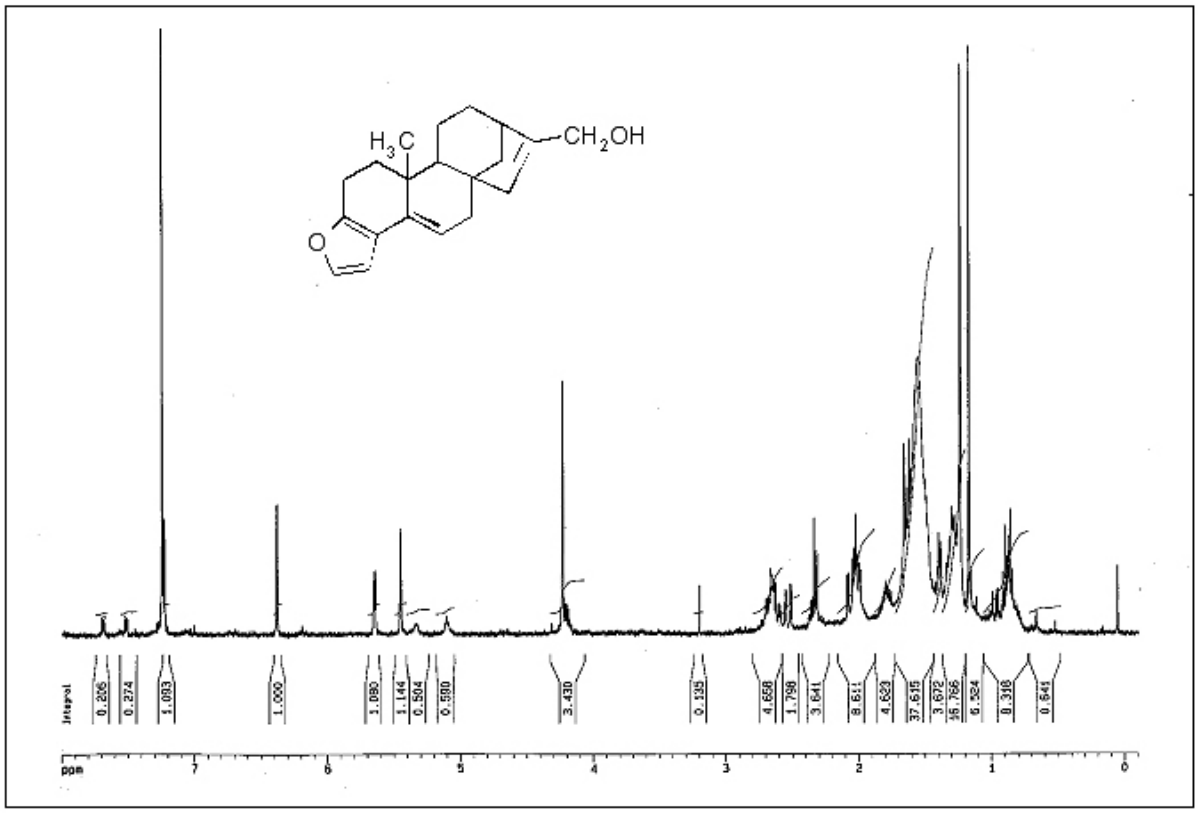



**Figure 6. $^{1}$H-NMR spectrum of dehydroisokahweol.**

**ACKNOWLEDGEMENTS**

We would like to thank Dr. Gruner and Mrs Shapoval for acquiring the NMR data and the high-resolution mass spectra.

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# Moisture Content Gain of Raw Coffee Beans at Constant and Alternated Temperatures and Different Equilibrium Relative Humidities

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## SUMMARY

The aim of this work was to study the moisture percentage gain (wet basis) of raw coffee beans during storage and transportation. Coffee beans were submitted to different conditions of equilibrium relative humidity (ERH, 80, 87, 95%) at a constant temperature of 25°C and alternated temperatures of 14 (night time) and 25°C (day time). The results suggest that the moisture gain is faster at the alternated temperatures than at constant temperature. The moisture gain percentile of 80, 87 and 95% at the alternated temperatures was 16, 18 and 23% higher than at constant temperature, respectively. The moisture gain of constant and alternated conditions was described by linear and second order polinomium equations, respectively. Moreover, the difference between one or another condition is statistically significant according to Tukey test at 5%. This difference is mainly due to condensation, which takes place during alternate temperature changes.

## INTRODUCTION

The moisture content (MC) measurement of coffee beans, expressed in wet weight (w.w.), is very important for the production and sale of coffee. The moisture content affects the quality during drying, storage and transport as well as the cost and properties of beans for processing. Significant changes in moisture content may occur when there are differences in the temperature in different regions with the same lot of coffee beans. In this situation condensation may appear. This condensation causes an increase in the moisture content of beans, which are close to a wall in the storage area. The temperature oscillation also favours an increase in relative humidity in coffee container headspace during transportation. Similarly, condensation can occur in grain storage silos and barns, especially when an adequate ventilation system is not available. The excess in moisture content may influence coffee whiting, fungal development on stored grain and cause physiological changes. Regitano et al. (1964) recommend a limit of maximum moisture content of 11% for coffee. The aim of the present work was to study the moisture percentage gain (w. w.) of raw coffee beans, at constant and alternated temperatures and different equilibrium relative humidities (ERH).

## MATERIAL AND METHODS

### Coffee

Raw arabic coffee beans from São Paulo State with an initial moisture content of 11% (w.w.) were used.

**Saturated solutions**

The following saturated solutions were prepared in desiccators to obtain different ambients of equilibrium relative humidities (ERH): a) ammonium sulphate ($(NH_4)_2SO_4$); b) sodium and potassium tartrate ($NaKCH_4O_{6)}$ and c) lead nitrate ($Pb\ (NO_3)_2$ , which gave 80, 87 and 95% ERH, respectively.

**Moisture gain tests**

Flasks with 25 grams of coffee were placed in the desiccators. A total of 18 desiccators were used, 6 for each salt solution. The desiccators were incubated at 25ºC.

Nine previously identified desiccators were alternated every 12 hours from 14ºC ± 2ºC to 25ºC. The other 9 were maintained at 25ºC. The experiment lasted 39 days.

**Determination of moisture gain**

Determination of moisture gain was carried out by sample weight increase. The weight control was carried out weekly, using an analytical calibrated balance (Metler Toledo – Switzerland).

**Statistical analysis**

The data of average coffee moisture gain at constant and alternated temperatures at the three different ERH were compared by the Tukey test at the level of 5% of probability.

**RESULTS AND DISCUSSION**

The average variation of storage moisture percentage at a constant temperature of 25ºC, and alternated temperatures of 14 and 25ºC are presented in Figures 1 and 2.

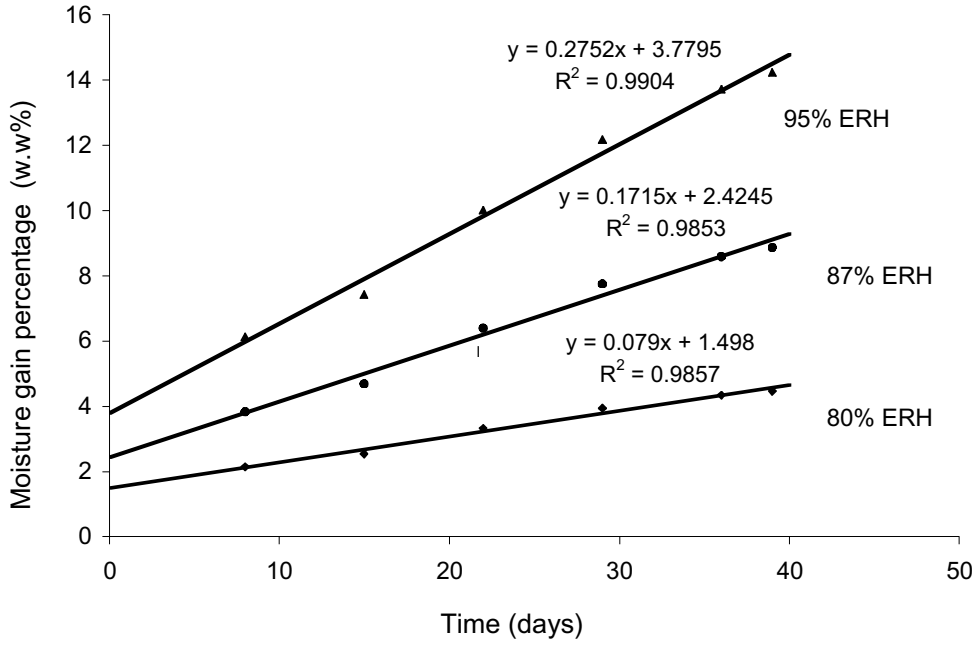


**Figure 1. Moisture gain of coffee beans at constant temperature of 25ºC and ERH of 80, 87 and 95%.**

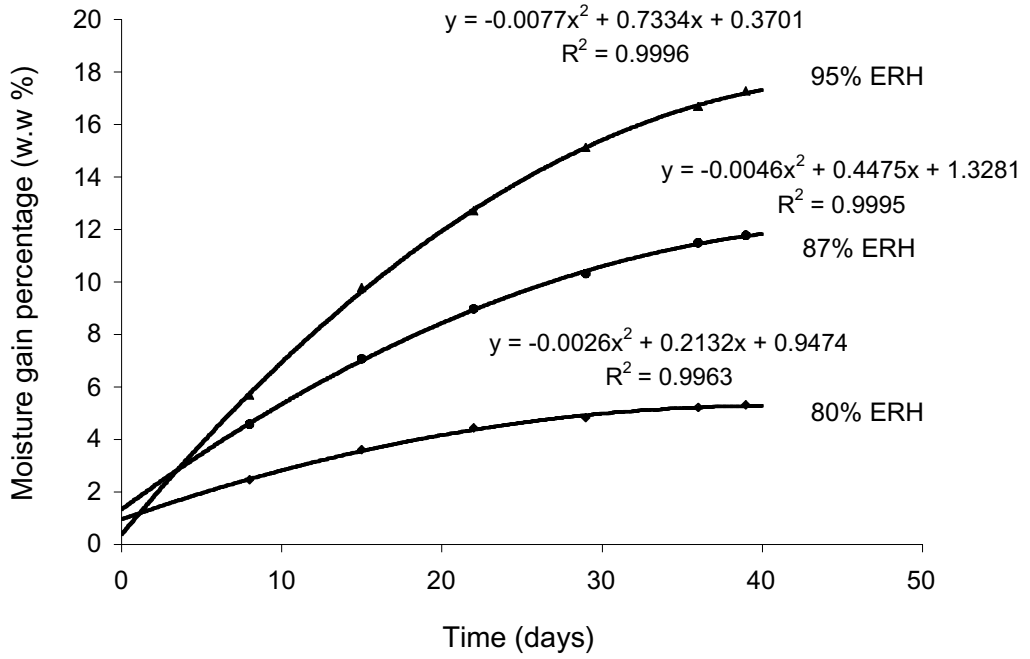


**Figure 2. Moisture gain of coffee beans at alternated temperatures of 14ºC and 25ºC and ERH of 80, 87 and 95%.**

The moisture gain of coffee at different ERH at constant temperature was linear according to the equations presented in Figure 1. The moisture gain at alternated temperatures of 25 and 14ºC was shown by the polinomium equations of the second order (Figure 2).

The predictive equations obtained from this assay are limited to the test conditions. However it can be useful in non-ventilated storage and transportation systems. These data simulate possible conditions of transitory moisture content of coffee beans, especially at the top or side during storage in barns, and later in containers, when fluctuation in temperatures can cause condensation. In well-sealed isothermal condition, the gain of moisture depends basically on the relative humidity of headspace. On the other hand, in condition of non or partially sealed, the moisture gain will also depend on the external relative humidity.

Table 1 presents the analysis of variance and comparison of the averages (Tukey test) obtained between the coffee moisture content gain at alternated and constant temperatures at three different ERH (80, 87 e 95%) for 39 days.

Table 1 shows that after 15 days of storage, the differences of coffee moisture gain at alternate and constant temperatures were statistically significant. Formation of condensation on the desiccator stored at alternated temperatures, was observed. This may explain the significant differences presented in these two conditions, since at constant temperature, this condensation was not observed.

Any type of innovation which has an objective of reducing temperature gradient that occurs between day and night, or winter and summer, will contribute for the decrease in moisture content gain in stored grains in a closed system, without ventilation or partially hermetic. Further research is necessary to keep the initial moisture content of grains at the safety margin.

**Table 1. Moisture content gain of coffee beans at alternated temperatures (AT) of 14 and 25ºC and constant temperature (CT) of 25°C, on different days and at equilibrium relative humidities (ERH).**

| Days | ERH | AT | CT | M.s.d. |
|---|---|---|---|---|
| **8** | 80% | 2.45 ± 0.10 a | 2.14 ± 0.29 a | 0.486 |
| | 87% | 4.57 ± 0.27 a | 3.82 ± 0.15 b | 0.490 |
| | 95% | 5.67 ± 0.59 a | 6.12 ± 0.20 a | 0.993 |
| **15** | 80% | 3.60 ± 0.06 a | 2.54 ± 0.32 b | 0.523 |
| | 87% | 7.07 ± 0.29 a | 4.68 ± 0.19 b | 0.562 |
| | 95% | 9.77 ± 1.06 a | 7.42 ± 0.21 b | 1.738 |
| **22** | 80% | 4.44 ± 0.07 a | 3.32 ± 0.35 b | 0.570 |
| | 87% | 8.97 ± 0.33 a | 6.39 ± 0.26 b | 0.673 |
| | 95% | 12.70 ± 1.16 a | 10.01 ± 0.16 b | 1.880 |
| **29** | 80% | 4.81 ± 0.16 a | 3.94 ± 0.33 b | 0.591 |
| | 87% | 10.08 ± 0.41 a | 7.75 ± 0.19 b | 0.730 |
| | 95% | 15.10 ± 0.46 a | 12.17 ± 0.33 b | 0.898 |
| **36** | 80% | 5.20 ± 0.26 a | 4.34 ± 0.29 b | 0.631 |
| | 87% | 11.21 ± 0.47 a | 8.59 ± 0.11 b | 0.770 |
| | 95% | 16.67 ± 0.59 a | 13.71 ± 0.38 b | 1.125 |
| **39** | 80% | 5.31 ± 0.27 a | 4.46 ± 0.26 b | 0.604 |
| | 87% | 11.47 ± 0.50 a | 8.87 ± 0.12 b | 0.829 |
| | 95% | 17.26 ± 0.53 a | 14.23 ± 0.41 b | 1.071 |

*M.s.d.: minimal significant difference of Tukey test at error level of 5% of probability. The samples (average and ± standard deviation) followed by the same letter do not differ at the 5% level.*

## CONCLUSION

From this work, the following conclusions can be made:

- The moisture gain of raw coffee beans at constant and alternated temperatures can be depicted according to linear and quadratic equations respectively.
- The significant difference between both situations was possibly caused by the condensation found at alternated temperatures.

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# Comparison of Different Methodologies to Determine Raw Coffee Bean Moisture Content

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## SUMMARY

The aim of this work was to compare methods to determine the moisture content on a wet weight basis (w.w.) of raw coffee beans. Six methodologies were evaluated in the moisture range from 9 to 17%: a) oven with forced ventilation (ISO method); b) oven with forced ventilation (Brazilian Agriculture Ministry method); c) Vacuum oven at 98ºC for 24 h; d) Vacuum oven at 70ºC for 24 h; e) Capacitance method using the Gehaka G 600 equipment; f) calorimetric method, using the infrared "AND" (130ºC for 15 min). The ISO 1447 is the official method for raw coffee and was used as a standard for comparison with the other methods. All results were analysed by the Dunnet test at a level of 1 to 5% probability. The results indicated that the methods tested presented lower moisture values than the ISO 1447 method, except for Gehaka in the range from 11.8 to 14.2%. The Brazilian Agriculture Ministry method was the closest to ISO 1447 with approximately 1% of difference. The vacuum oven at 70ºC for 24h was the method that differed most with a difference of approximately 4%. The infrared results were similar to the oven method at 98ºC for 24h. Both differed about 2% from ISO 1447. The results from Gehaka suggested that the equipment over-estimates the moisture value in the range from 9 to 11% w.w. On the other hand, in the range from 12.4 to 16.6% it is closer to ISO 1447. Most of the methods tested presented a statistically significant difference when compared to ISO 1447.

## INTRODUCTION

The moisture content of raw coffee beans is very important from two aspects: economic, since the grain is commercialised on a mass basis and quality, since the moisture content is an indicator of storage quality. If the grains are stored at high moisture content, fungal growth may occur. For this reason, knowledge of the moisture content is useful to prevent or reduce post harvest losses, mainly during storage and transport.

It is recommended that coffee beans be stored and transported at moisture contents lower than 12% (w.w.). Changes in colour, flavour and texture are observed when the moisture content is above 13% w.w. (Godinho et al., 2000; Vilela et al., 2000; Lee 1999). Rigitano et al. (1964) and Wilbaux & Hahn (1966) suggested a maximum moisture content of 11% w.w. as a safety limit during coffee storage. There are several methods for coffee moisture content determination. The most used method in Brazil is that of the Brazilian Agriculture Ministry (1992), which uses an oven at 105°C ± 3°C for 24 h or up to constant weight (Godinho et al., 2000; Corrêa et al., 2000) with or without forced air circulation. Rapid electronic methods such as capacitance have been widely used at the field level and by coffee traders. This method uses electronic equipment, previously calibrated with the standard oven method, and produces the moisture content data in a few seconds. Some coffee companies and cooperatives use the infrared (IR) method to determine moisture, standardizing the potency

and time of exposition of the product under an IR lamp. The moisture content data obtained are corrected according to the standard oven method.

The methodology for determining raw coffee bean moisture using a vacuum oven at 70°C for 24 h is a non-destructive method and only removes the available free water. Considering the importance of the moisture content determination in raw coffee bean, the objective of the present study was to compare six methodologies used in Brazil and overseas, using the standard ISO 1447 method (1978) as the reference method.

## MATERIAL AND METHODS

### Coffee sample preparation

Raw arabica coffee beans were prepared with moisture contents of: a) 9%; b) 11%; c) 12.5%; d) 14% and e) 16.5%.

### Moisture content determination

The following six methodologies for the determination of moisture content were compared:

a. Oven with forced ventilation (ISO 1447 method, 1978): 130ºC for 6h plus 4h.
b. Oven with forced ventilation (Brazilian Agriculture Ministry method, 1992): 105ºC ± 3 °C for 24 h or to constant weight.
c. Vacuum oven: 98ºC for 24h;
d. Vacuum oven: 70ºC for 24h;
e. Capacitance method using the Gehaka G 600 equipment;
f. Calorimetric method using the infrared "AND" (130ºC for 15 min).

### Statistical analysis

The experimental design was completely random. ISO 1447 was used as the standard for comparison with the other methods. All results were analysed using the Dunnet test at a level of 1 to 5% of probability.

## RESULTS AND DISCUSSION

The data obtained for the moisture content of coffee beans on a wet weight basis are presented in Table 1. The 5 different methodologies were compared to the ISO 1447 method. According to Table 1 there were significant differences between the ISO 1447 method and the other methodologies.

Table 2 presents the linear regression equations and coefficients of correlation found in this study.

The angular coefficients of the linear regression curve varied from 0.96 to 1.41. The linear coefficients varied from 0.89 to 5.94. The equation and the coefficients of correlation obtained suggest that an adequate correlation for the determination of moisture content in raw coffee beans could be obtained in the range from 9.05 to 16.62%, whichever method was used.

In the Code of Practice prepared by the European Union and International Coffee Organization (ICO), the ISO 6673 (1983) method was recommended. This method uses an

oven at 105°C for 16h, which is similar to the Brazilian Agriculture Ministry method. The only difference is the drying time, which is 24h instead of 16h. In future studies comparing moisture content methodologies, the ISO 6673 method should also be included.

**Table 1. Moisture content (% w.w.) of raw coffee beans, comparing ISO 1447 with 5 other methods.**

| Methodologies | Moisture content (% w.w.) | | | | |
|---|---|---|---|---|---|
| Oven: Brazilian Agriculture Ministry 105ºC/24h | 7.98 ± 0.02 b B | 9.95 ± 0.01 b B | 11.41 ± 0.04 b B | 13.12 ± 0.02 b B | 15.62 ± 0.04 b B |
| Electronic: Capacitance | 10.90 ± 0.10 b B | 11.80 ± 0.00 b B | 12.8 ± 0.06 a A | 14.20 ± 0.06 a A | 16.10 ± 0.10 b B |
| Calorimetric Infrared | 7.00 ± 0.40 b B | 9.20 ± 0.26 b B | 10.50 ± 0.9 b B | 11.87 ± 0.32 b B | 13.8 ± 0.10 b B |
| Vacuum oven: 70°C/24 h | 5.02 ± 0.09 b B | 6.99 ± 0.03 b B | 8.42 ± 0.16 b B | 10.04 ± 0.12 b B | 12.96 ± 0.05 b B |
| Vacuum oven: 98°C/24 h | 7.33 ± 0.04 b B | 9.10 ± 0.12 b B | 10.62 ± 0.09 b B | 12.26 ± 0.11 b B | 14.87 ± 0.16 b B |
| Oven: ISO method ref. 1447 | 9.05 ± 0.02 a A | 10.97 ± 0.05 a A | 12.45 ± 0.09 aA | 14.18 ± 0.07 a A | 16.62 ± 0.06 a A |
| Msd level 1% | 0.545 | 0.374 | 0.457 | 0.474 | 0.292 |
| Msd level 5% | 0.412 | 0.285 | 0.348 | 0.361 | 0.222 |

*M.s.d. = minimum significant difference by the Dunnet test at error level of 1 to 5% of probability. Samples (average ± standard deviation) followed by the same small letters in the same column do not differ at the 1% level; samples with capital letters do not differ at the 5% level.*

**Table 2. Linear regression equations and coefficients of correlation between the moisture content (Y) determined by ISO 1447 method and moisture content (x) determined by the other methodologies.**

| | Moisture content determination (w.w.) by different methodologies* | | | | |
|---|---|---|---|---|---|
| **Statistic** | A | B | C | D | E |
| Regression equation | Y=0.994x+1.1076 | Y=1.413 x –5.9414 | Y=1.1228 x+0.8942 | Y=0.9631 x+4.2888 | Y=1.0036 x+1.7788 |
| Coefficient of correlation | 1.00 | 0.99 | 0.99 | 1.00 | 1.00 |

*A) Oven method of the Brazilian Agriculture Ministry; B) Electronic method (capacitance); C) Calorimetric method (Infrared); D) Vacuum oven method at 70°C/24 h; E) Vacuum oven method at 98°C/24 h.*

## CONCLUSIONS

Significant differences were observed when five methodologies were compared with the standard ISO 1447 method. The electronic method of capacitance (Gehaka G 600) showed no significant differences in the moisture content range from 12.45 to 14.18%. This suggests that in this range the equipment gives equivalent values for moisture content to those obtained by the ISO method. The calibration curve and the respective equations and coefficients of correlation obtained in this study suggest that the moisture content of raw coffee beans can always be determined by conventional methodologies if a correlation factor based on a standard method is considered.

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# Identification of Chlorogenic Acids in Coffee Beans and Their Physiological Activities

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## SUMMARY

Seven hydroxycinnamic acid derivatives were isolated from Robusta green coffee beans and identified as 3-, 4-, 5-CQAs, 5-FQA, and 3,4-, 3,5-, 4,5-diCQAs by MS and HPLC analysis. CQAs and diCQAs showed potent scavenging activities of DPPH free radicals and superoxide anion radicals, and tyrosinase inhibitory activities. The potency order of these activities was diCQAs > CQAs > 3,4-diOH HCA, which suggests that the number of caffeoyl groups in the compound and the double bond at the C7 position of the cinnamoyl group contributes to the expression of these activities rather than the linkage positions of the caffeoyl group to quinic core. DiCQAs also showed potent antiproliferative activity on adherent cancer cell lines tested, especially on KB cells.

## INTRODUCTION

Coffee beans contain considerable amounts of hydroxycinnamic acid derivatives, 4.3-7.2% CQAs, 0.3-1.2% FQAs, and 0.8-2.5% diCQAs (Clifford, 1985). The ratio of CQAs in total chlorogenic acids (containing CQAs, FQAs, and diCQAs) of green coffee beans increases with maturity while the ratio of diCQAs decreases (De Menezes, 1994). Contamination of low quality beans negatively affects the coffee beverage flavor because of their high ratio of diCQAs to CQAs. These low quality beans are noted among unused agricultural resources and we are currently investing the effective utilization of these coffee beans. It is well known that the hydroxycinnamic acid compounds have various physiological activities. In this study, we isolated seven kinds of hydroxycinnamic acid derivatives from green coffee beans. We also compared their following physiological activities of these compounds under the same conditions; DPPH free radical and superoxide anion radical scavenging activities, tyrosinase inhibitory activities, and antiproliferation activities.

## MATERIALS AND METHODS

### Sample preparation

Robusta green coffee beans were grounded and extracted with 70% (v/v) aqueous methanol under reflux. The extract were concentrated and stored to form a yellow precipitate of hydroxycinnamic acid derivatives-K-caffeine complex (Uritani and Muramatsu, 1952; Martin et al., 1994). The precipitate was dissolved in tartaric acid solution, and the resulting potassium tartarate crystal was removed. Then, the solution was extracted with $CHCl_3$ to remove caffeine. The aqueous layer was subjected to a Sephadex LH-20 column and scanned for absorbance 326 nm. Fractions absorbed were collected by a linear gradient of methanol (0-70%) in 0.2% (v/v) acetic acid aqueous solution. Each peak concentrated was subjected to a reversed-phase column in a

preparative HPLC. The collected fractions were once again, subjected to a Sephadex LH-20 column and eluted with methanol. The purified fractions were freeze-dried.

**DPPH radical and superoxide anion radical scavenging activity**

The DPPH radical and superoxide anion radical scavenging activity of the samples were analyzed according to a slightly modified procedure of Yoshikawa et al. (1994) and Matsushige et al. (1996) respectively. These activities were expressed as the sample concentration necessary to give a 50% reduction in the sample absorbance ($IC_{50}$).

**Tyrosinase Inhibitory Activity**

The activity of mushroom tyrosinase was determined using L-tyrosine and L-DOPA as substrates by a slightly modified method of Tada et al. (2002). The activity was expressed as the sample concentration that gave a 50% inhibition in the enzyme activity ($IC_{50}$).

**Antiproliferation activity**

Three culture cells containing human histiocytic lymphoma U937, normal human diploid lung fibroblast WI38 and SV40 virally transformed WI38 (WI38VA) were obtained from the Health Service Research Resources Bank, Osaka, Japan. Human breast carcinoma MCF-7 (ATCC #HTB22) and human oral carcinoma KB cells were obtained from ATCC (Rockville, USA) and Tottori University (Yonago, Japan), respectively. The antiproliferation activities of the samples were examined in four human cancer cell lines (U937, KB, MCF-7 and WI38VA) and one normal cell line (WI38), and viable cells were enumerated after incubation with the isolated hydroxycinnamic acid derivatives by WST-1 assay (Shinmoto et al., 1996).

**RESULTS AND DISCUSSION**

**Isolation of hydroxycinnamic acid derivatives from green coffee beans**

The purification combined with Sephadex LH-20 column chromatography and preparative HPLC was effective in isolating of seven kinds of compounds at 326 nm from the extracts. HPLC retention time, UV absorption maximum, FAB-MS and MS-MS data of the isolated seven compounds from green beans are listed in Table 1.

**Table 1. Identification of Hydroxycinnamic acid derivatives from Green Coffee Beans Using Their Spectral Characteristics in HPLC and Positive and Negative Ions in FAB-MS and MS-MS.**

| | | | positive ions | | negative ions | | | |
|---|---|---|---|---|---|---|---|---|
| compd.no. | HPLC retention time (min) | absorption (nm) | $MS[M+1]^+$ | MS-MS (m/z) | $MS[M-1]^-$ | MS-MS (m/z) | estimated MW | identification |
| 1 | 14.5 | 325 | 355 | 163 | 353 | 191 | 354 | 3-CQA |
| 2 | 17.7 | 325 | 355 | 163 | 353 | 191 | 354 | 4-CQA |
| 3 | 23.6 | 325 | 369 | 177 | 367 | 191 | 368 | 5-FQA |
| 4 | 14.8 | 325 | 355 | 163 | 353 | 191 | 354 | 5-CQA |
| 5 | 38.6 | 325 | 517 | 163 | 515 | 179,353 | 516 | 3,5diCQA |
| 6 | 35.8 | 325 | 517 | 163 | 515 | 179,353 | 516 | 3,4diCQA |
| 7 | 44.0 | 325 | 517 | 163 | 515 | 179,353 | 516 | 4,5diCQA |

**Antioxidative activity of hydroxycinnamic acid derivatives**

DPPH radical and superoxide anion radical scavenging activities ($IC_{50}$) of the seven hydroxycinnamic acid derivatives ranged in the concentrations from 5.6 to 10 and 4.3 to 36 μM, respectively. DiCQAs had a greater DPPH radical scavenging activity than α-tocopherol and ascorbic acid. The potency of radical scavenging effects by 3- and 4-CQAs and 5-FQA was also higher than that of α-tocopherol ($p < 0.01$). DiCQAs showed a higher superoxide anion radical scavenging activity compared with CQAs, 5-FQA and caffeic acid ($p < 0.01$). The activity of CQAs was similar to caffeic acid, but 5-FQA showed very weak activity. Furthermore, quinic acid and 3,4-diOH HCA had no activity. DiCQAs containing two caffeoyl groups showed potent antioxidative activity compared to CQAs containing a caffeoyl group and caffeic acid, whereas 3,4-diOH HCA which are saturated at the C7 double bond position of caffeic acid had no activity. A double bond at C7 position in caffeoyl group might be essential for the expression of antioxidative activity. Two hydroxyl groups in caffeoyl group might also play an important role in their expression of activity, because methoxylation at the C3 position of the caffeoyl group (5FQA) lowered the scavenging activity of superoxide anion radical radicals to half of that of 5CQA.

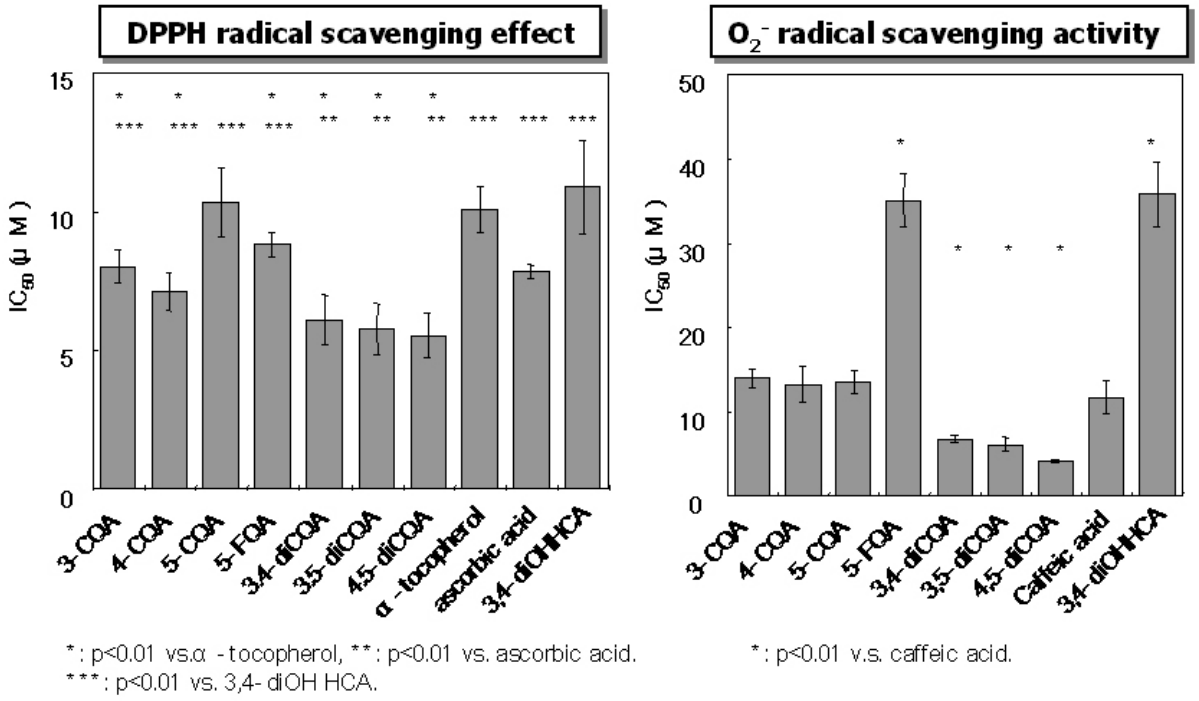


**Figure 1. DPPH radical and superoxide anion radical scavenging activities of chlologenic acid isomers.**

**Tyrosinase inhibitory activity of isolated hydroxycinnamic acid derivatives**

The activity of tyrosinase was evaluated by measuring the amount of dopachrome produced from L-tyrosine or L-DOPA by tyrosinase. DiCQAs most significantly inhibited the formation of dopachrome from L-tyrosine and from L-DOPA among the samples tested ($p < 0.01$). No inhibitory activity was detected in 3,4-diOH HCA, which suggests that double bond at the C7 position in caffeoyl group might be also essential for the expression of their tyrosinase inhibitory activity other than antioxidative activity. However, 5-FQA also showed strong activity. In the case of tyrosinase inhibitory activity, the number of caffeoyl group might not be the only factor in determining the potency of inhibitory activity. The double bond at the C7 position of caffeoyl group was considered

group was considered to be required to form a stable Schiff base, which should show the inhibitory activity, with a primary amino group in the enzyme.

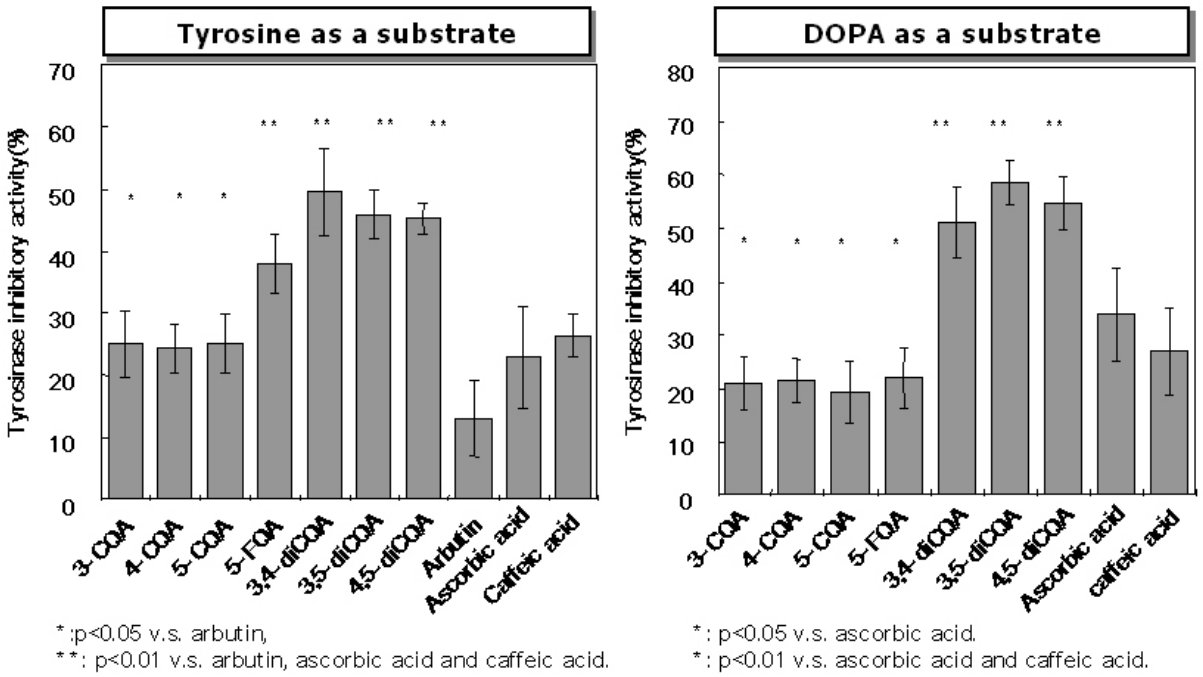


**Figure 2. Tyrosinase inhibitory activity of chlologenic acid isomers using L-tyrosine and L-DOPA as a substrate.**

**Table 2. Antiproliferation Ativity of Hydroxycinnamic Acid Derivatives of Chlorogenic Acid Isomers on Several Cancer Cell Lines ($IC_{50}$, mM).**

| | cancer cell lines | | | | |
|---|---|---|---|---|---|
| compound | U937 | KB | MCF-7 | WI38 | WI38VA |
| 3-CQA | 1.20 ± 0.31 | 0.53 ± 0.02 | 0.89 ± 0.44 | 5.87 ± 2.47 | 4.93 ± 0.84 |
| 4-CQA | 0.74 ± 0.03 | 0.16 ± 0.03 | 0.92 ± 0.48 | 3.56 ± 0.09 | 5.99 ± 2.08 |
| 5-CQA | 1.54 ± 0.27 | 0.14 ± 0.02 | 0.74 ± 0.38 | 5.87 ± 2.47 | 8.18 ± 3.42 |
| 5-FQA | 3.07 ± 0.55 | 0.56 ± 0.03 | 1.62 ± 0.81 | 3.46 ± 0.41 | 4.65 ± 0.48 |
| 3,4-diCQA | 0.57 ± 0.01 | 0.16 ± 0.02 * | 0.31 ± 0.15 * | 0.50 ± 0.03 * | 0.75 ± 0.05 * |
| 3,5-diCQA | 0.56 ± 0.05 | 0.18 ± 0.01 * | 0.32 ± 0.16 * | 0.52 ± 0.04 * | 0.47 ± 0.03 * |
| 4,5-diCQA | 0.76 ± 0.06 | 0.10 ± 0.01 * | 0.37 ± 0.19 * | 0.62 ± 0.03 * | 0.58 ± 0.02 * |

*$p < 0.01$ vs CQAs(3-CQA, 4-CQA, 5-CQA) and 5-FQA .*

**Antiproliferative activity of hydroxycinnamic acid derivatives isolated from green coffee beans**

Among the culture cells tested, KB cells were most sensitive to hydroxycinnamic acid derivatives ($p < 0.01$), CQAs, 5FQA and diCQAs. DiCQAs showed antiproliferative activities at lower $IC_{50}$ against WI38, WI38VA and MCF-7 ($p < 0.01$) compared to CQAs and 5FQA. These results suggest that the number of caffeoyl groups might be a dominant factor in determining the potency of inhibitory activity on the growth of adherent cells. WI38 and WI38VA cells had lower sensitivity against CQAs and FQA ($p < 0.01$). However, the $IC_{50}$ values of CQAs and diCQAs on normal cell line WI38 were almost the same as those on the WI38VA cancer cell line, which suggest that those compounds have some cytotoxicity for normal cells. 5-FQA was not effective for the antiproliferation of

for the antiproliferation of U937, KB and MCF-7 cancer cell lines compared to CQAs and diCQAs ($p < 0.01$). Methoxylation at the C3 position of the caffeoyl group might be required to express the antiproliferative activity of cancer cell lines other than the scavenging activity of superoxide anion radicals (Figure 1).

## CONCLUSIONS

We isolated seven kinds of hydroxycinnamic acid derivatives, three diCQAs, three CQAs, and one FQA from green coffee beans. DiCQAs exhibit more potent antioxidative, tyrisinase inhibitory, and antiproliferation activities compared to FQA and CQAs. Our findings suggest that hydroxycinnamic acid derivatives, especially diCQAs, which are unused agricultural resources, might be useful as protective agents against active oxygen species, as skin-whitening agents in cosmetics, and as chemopreventive agents.

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# Caffeine Distribution in Coffee Beans

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## SUMMARY

The mathematical equation model for the decaffeination process of green coffee bean is useful for the optimization procedure. One of the most important data for the model formulation is the knowledge of the substrata distribution in the solid matrix, in this case caffeine in green coffee beans. This is the main objective of this work and therefore, the paper shows an experimental macroscopic approach exclusively based on the dissection of green coffee beans.

## RÉSUMÉ

L'utilisation d'un modèle mathématique est très important pour l'optimisation du processus de décaféination du café vert. Une des données importantes qui entre en jeu dans la formulation de l'équation mathématique est la connaissance de la distribution du substrat dans la matrice solide. Dans ce cas, la caféine est le substrat et le grain de café vert représente la matrice solide. Cette étude de distribution de la caféine est l'objet de cette recherche, basée sur une analyse macroscopique exclusivement pointée sur la dissection du grain de café vert.

## INTRODUCTION

This paper concerns the caffeine distribution in green coffee beans, and shows an experimental approach exclusively based on the dissection of the grain where it is possible to individualize two different sides: an external layer "shell" and an internal part "stone". This distribution can be studied by employing a macroscopic method where the caffeine content in both part is evaluated by using the Levine method.

The evaluation of the caffeine distribution in the green coffee beans is presented and discussed.

## EXPERIMENTS

The materials used for this analysis are: green Arabica coffee beans "Santos Brazil", green Robusta coffee "Vietnam" purchased from Demus S.p.A (Trieste – Italy), and another materials like ammonia, sodium hydroxide, sulphuric acid, ether, chloroform, celite and glass wool cap used to determine the caffeine concentration with Levine method. In this experimental phase, the apparatus like grinder, dryer, chromatographic columns and UV – spectrophotometer were used.

The figures below shows clearly the two parts after the dissection phase. These parts are dried by setting the dryer temperature point in order to reduce the moisture and to obtain the same

coffee beans humidity. After this operation, it is then possible the determination of the caffeine concentration of each part by using the Levine method.

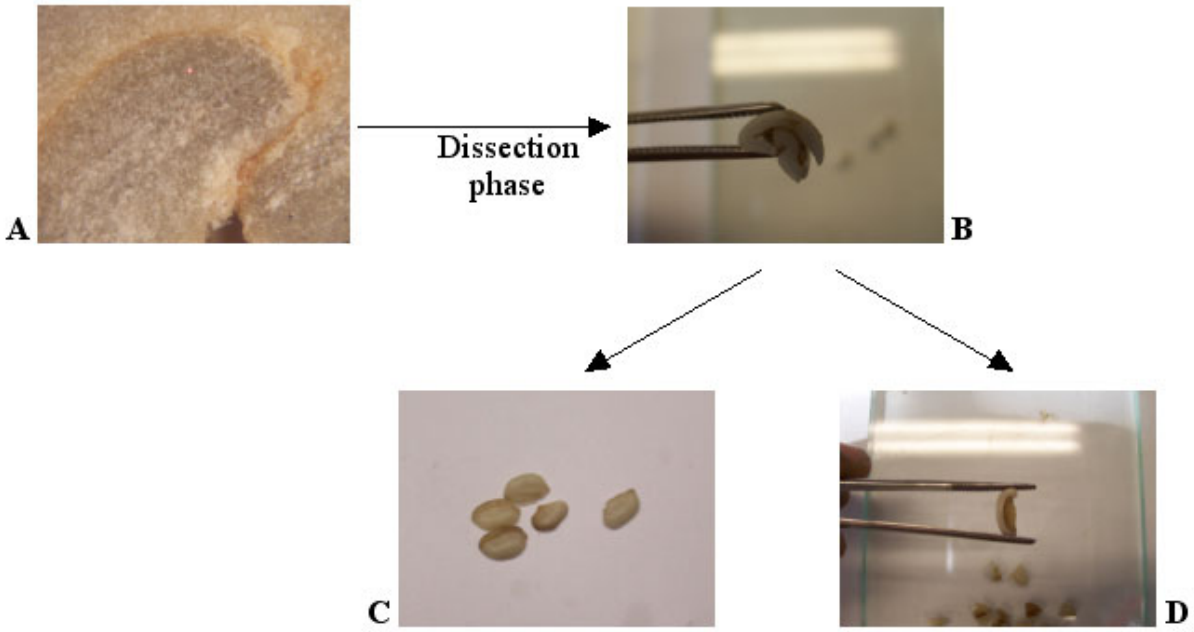


**Figure 1. A) Section of green coffee bean. C) Internal part of green coffee bean "stone". D) External part of green coffee bean "shell".**

For each part, ten analysis were done and then, the table below of analysis results presents the average data of caffeine concentration.

**Table 1. Average values of caffeine content (wt/wt) determined with Levine method.**

| Green coffee beans | Average values of caffeine content in the "stone" | Average values of caffeine content in the "shell" | Difference between "shell" and "stone" |
|---|---|---|---|
| Arabica: "Santos Brazil" | 0,97% | 1,16% | 16,4% |
| Robusta: "Vietnam" | 1,82% | 2,24% | 18,7% |

## RESULTS AND DISCUSSIONS

- The results of the analysis (see table) evidence for both types of coffee, a difference of more or less 17% between the internal part, "stone", and the external layer, "shell", with a greater concentration value in the "shell" side. Therefore the caffeine could be considered by weight percentage present in a higher amount in the external layer, and then it is not homogeneously distributed in the whole coffee bean.
- The extraction processes are essentially based on mass transfer mechanisms and diffusion phenomena which can vary from system to system. In this case, regarding the results obtained, it is very important to consider in the mathematical model formulation, the mass transfer resistance in the fluid phase, and the resistance located in the solid phase. (see Figure 2 and 3)

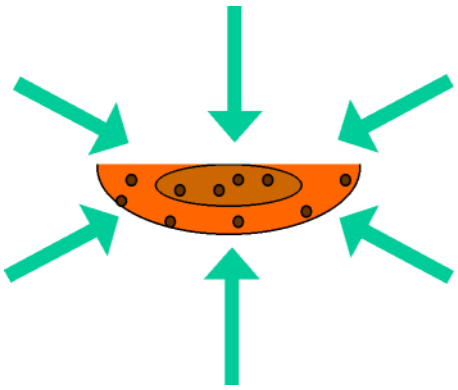

**Figure 2. Coffee bean aspect before the contact with extraction solvent.**

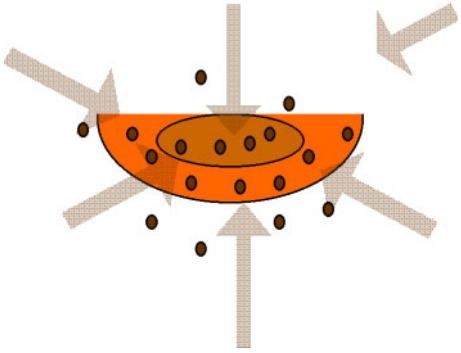

**Figure 3. Coffee bean after the contact with solvent: fluid phase and solid phase resistance.**

## CONCLUSION

In this work, the results obtained show that the caffeine must not be considered homogeneously distributed in the whole coffee bean. In fact, a difference of more or less 17% between "shell" and "stone" has been obtained with a greater concentration value in the "shell" side. This results is very important for a correct formulation of the mathematical model. Another method for the knowledge of this distribution using an electronic microscope is under investigation.

## ACKNOWLEDGEMENT

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# Chemosystematic Study of Diterpenoids in Green Coffee Beans

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## SUMMARY

The diterpenic fraction of Variedad Colombia and nine other related genotypes of green coffee beans were studied by gas chromatography mass spectrometry. Eleven diterpenic compounds were identified and quantified. Cafestol , kahweol, 16-*O*-methylcafestol, 15,16-dehydrocafestol, 13,16-dehydrocafestol, 15,16-dehydrokahweol, 13,16-dehydrokahweol, 16-*O*-methylkahweol and the new diterpene 11,12-dehydrokahweol. The 11,12-dehydrokahweol was found in typical variety but not in Caturra variety, allowing to differentiate these two varieties of C. *arabica.* The 16-*O*-methylcafestol and 16-*O*-methylkahweol were found only in the genotypes of *C. canephora* allowing to discriminate these coffees of the rest of materials.

## INTRODUCTION

The most studies of diterpenes in coffee have been related to thepresence of cafestol, kahweol and/or 16-*O*-methylcafestol. The cafestol was found in both *C. arabica* and *C. canephora* species (Nackunstz and Maier, 1987). The kahweol was detected in *C. arabica* at concentrations 100 times higher than in *C. canephora* (Lercker, 1995), while the 16-*O*-methylcafestol has been found more concentrated in *canephora* coffees than in *arabica* coffees (Speer and Montag, 1989), these data have been used as quality control for making possible to identify mixtures of these coffees (Speer et al., 1991). Diterpen glycosides also has been reported in *Coffea* but in low concentrations and its presence in some cases has been associated with the origin (Ducroix et al., 1975; Poisson, 1977; Prewo et al., 1990).

Following with the study of composition from Variedad Colombia and other related genotypes of coffee, this paper describes the isolation and analysis of diterpenes composition, in order to establish the principal differences among these coffee genotypes that allow evaluation of hybrid material. Special attention was given to the new diterpenoids tentatively identified by gas chromatography-mass spectrometry (GC-MS).

## MATERIALS AND METHODS

*Material.* The study was made with green coffee beans of ten genotypes: Variedad Colombia (advanced generation of Caturra x Híbrido de Timor crossing), cultivar resistant to rust (*Hemileia vastatrix*), the first generation of the crossing of Caturra x Híbrido de Timor (F1), two varieties of *C. arabica*, Caturra and Típica; three introductions of Híbrido de Timor (1343, 832 and 2252) and three introductions of *C. canephora* (BP4, BP46 and Centro 1). These materials were planted in Cenicafé germplasm collection in Chinchiná, Caldas, Colombia. The fruits were processed under identical conditions and the seeds were sun dried and grounded in presence of liquid nitrogen.

*Diterpenic fraction extraction.*The diterpenic fractions of each genotype were extracted from 2 g of sample as describes Speer (Speer and Lebensm, 1989), but using terbutyl methylether as solvent and five hours soxhlet extraction. The eluate was concentrated under nitrogen current for subsequent with GC analysis.

*Identification.* The diterpenic fractions were analyzed with a Hewlett Packard 6890 GC-MS equipped with a HP-5MS column (30 m x 0.250 mm i.d., 0.25 μm film thickness). The temperature program was 150ºC for 2 min followed by arise to 250ºC at a rate of 20ºC-$min^{-1}$. After 30 min the temperature was raised to 280ºC at a rate of 1ºC-$min^{-1}$ and held for 3 min. Helium was used as carrier gas, split ratio 20:1; saver flow 15.0 $mL.min^{-1}$. The injector temperature was 280ºC and 1μL of each sample was injected. Mass spectra were taken at 70 eV. The relative retention times, were calculated with relation to the cafestol and the retention indexes were estimated according to the Kovats method, based on *n*-hydrocarbons. The most of diterpenic compounds were identified by comparison with mass spectra of standard compounds and/or mass spectra previously reported (Pettitt, 1987; Speer et al., 1989; Tewis et al., 1993; de Roos et al., 1997). Other compounds were tentatively identified by comparison of mass spectra with those of standard or structurally similar compounds.

The quantification of each compound was performed using the external standard method, integrating the area of each of the chromatographic peaks and relating them to cafestol acetate (1 $mg.mL^{-1}$).

## RESULTS AND DISCUSSION

A total of 11 compounds were identified and quantified in diterpenic fractions analyzed by GC and GC-MS. Table 1 shows these results together with relative retention times to cafestol and Kovats indices. These compounds presented the same basic structure but with differences among them in the position, number of double bonds and/or in the radical on C-16. Cafestol, 16-*O*-methycafestol and kahweol were identified in *C. canephora* and *C. arabica*. The isomers of the dehydrocafestol: 15,16-dehydrocafestol and 13,16-dehydrocafestol and the isomers of the dehydrokahweol: 15,16-dehydrokahweol and 13,16-dehydrokahweol, were tentatively identified in this study for first time in green coffee beans. These 4 compounds had been found in low amounts in roasted coffee and were reported as dehydration products of cafestol and kahweol during the roasting process (Tewis et al., 1993). The 16-*O*-methylkahweol was previously reported in green beans of *C. stenophylla* (de Roos et al., 1997) but it is the first report in *C canephora*. The three remaining compounds, 11,12-dehydrokahweol, 16-*O*-isobutylcafestol and 16-*O*-isobutylkahweol were found for the first time in this work, their spectra are shown in the Figure 1.

The main differences among genotypes were: the presence of kahweol, 13,16-dehydrokahweol and 15,16-dehydrokahweol in *C. arabica*, (Caturra and Tipica varieties), but not in *C. canephora* accessions; the presence of 16-*O*-methylcafestol and 16-*O*-methylkahweol in *C. canephora* but not in *C. arabica.* It was also found significant difference within the varieties of *C. arabica* studied. The main compound of Tipica variety, 11,12-dehydrokahweol was absent in Caturra, while kahweol, one of the highest peaks of Caturra variety, was detected in low concentration in Tipica. Both compounds would differentiate between the two varieties of *C. arabica*. These results correspond well with earlier studies on discrimination of these two varieties (Guerrero et al., 2001) and all this information would contribute in the selection to obtain hybrid materials from them. Additionally the presence of the 11,12-dehydrokahweol in the Variedad Colombia and F1,

could be attributed to the inheritance of the Híbrido de Timor, which might come from the natural crossing of *C. arabica* x *C. canephora* (Moreno, 1982).

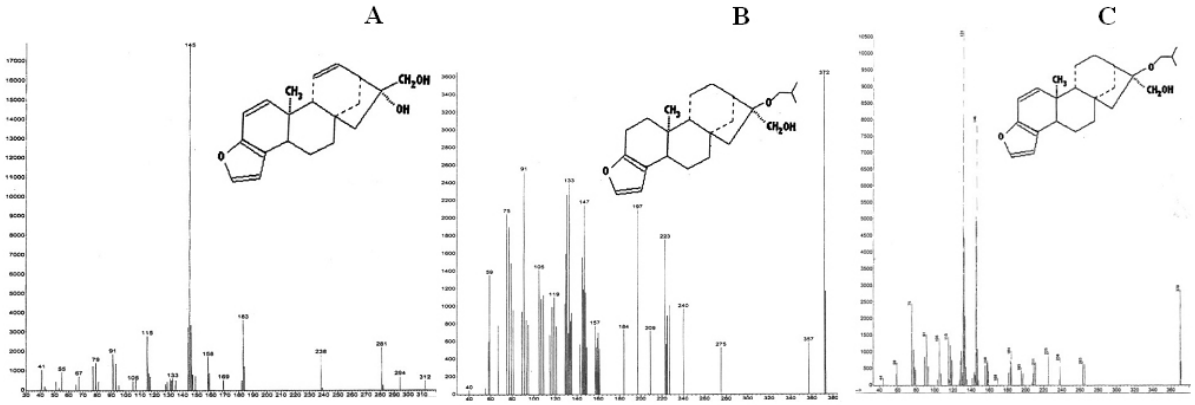


**Figure 1. Mass spectrums of new diterpenes found in green coffee beans (A) 11,12-dehydrokahweol, (B) 16-*O*- isobutylcafestol and (C) 16-*O*-isobutylkahweol.**

**Table 1. Diterpenes identified by GC-MS in genotypes of green coffee beans comparing Retention Times Relatives to cafestol (tR') and Kovats indices (KI). Variety (V.), Hibrido de Timor (HT), Canephora (CAN), and First generation of Caturra x Híbrido de Timor (F1).**

| COMPOUND | $t_R$ | KI | CATURRA | TIPICA | V. COLOMBIA | F1 | HT1343 | HT2252 | HT832 | CANCEN1 | CANBP4 | CANBP46 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13,16 - dehydrokahweol | 0.75 | 1573.89 | + | ++ | ++ | +++ | ++ | ++ | + | — | — | — |
| 15,16 - dehydrokahweol | 0.77 | 1591.45 | + | ++ | ++ | +++ | + | + | + | — | — | — |
| 13,16 - dehydrocafestol * | 0.78 | 1600.82 | ++ | ++ | + | ++ | + | +++ | ++ | ++ | ++ | + |
| 15,16 - dehydrocafestol | 0.80 | 1620.73 | + | + | ++ | ++ | — | — | + | + | ++ | + |
| 11,12 - dehydrokahweol | 0.81 | 1626.58 | — | +++ | ++ | ++ | — | — | ++ | — | — | — |
| 16-O-isobutilkahweol | 0.88 | 1658.13 | + | ++ | + | + | — | — | — | + | — | — |
| 16-O-isobutilcafestol | 0.93 | 1726.11 | — | + | — | — | — | — | — | ++ | + | + |
| 16-O-methylkahweol | 0.95 | 1743.68 | — | — | — | — | — | — | — | + | + | + |
| Kahweol | 0.96 | 1765.23 | +++ | + | — | + | +++ | + | +++ | — | — | — |
| Cafestol | 1.00 | 1798.01 | ++++ | +++ | — | — | ++ | ++++ | — | | | |
| 16-O-methylcafestol ** | 1.00 | 1798.01 | — | — | — | — | — | — | — | ++++ | +++ | ++++ |

*< 10 mg / g of green coffee oil (+); 10-30 mg / g (++) ; 30-70 mg / g (+++) ; 70-110 mg / g (++++); --- = Undetected; * Overlapped with ptalate in HT 2252 coffee; ** 16-O-methylcafestol is overlapped with cafestol in C. canephora coffees.*

The composition of Variedad Colombia and the F1 were similar. In both, the most important diterpenes were the dehydrokahweol and dehydrocafestol, which might indicate that the selection made by agronomic characters, did not affect the composition of this fraction, confirming the results previosly reported (Guerrero et al., 2001). Furthermore, differences were found among Híbrido de Timor accessions, the introduction 2252 exhibited high concentration of cafestol and low concentration of kahweol while in 1343 and 832 introductions the cafestol was not presented or it was found in low concentrations and the kahweol was the major compound and there were not found important differences between *C. canephora* accessions, the three presented the same compounds with the 16-*O*-methylcafestol overlapped with the cafestol.

On the other hand the 16-*O*-isobutylcafestol was detected only in low concentrations in Típica variety and *C. canephora* accessions while the 16-*O*-isobutylkahweol was not found in Híbrido de Timor accessions and in *C. canephora* introductions BP4 and BP46 but it was detected in the other genotypes.

*Mass spectral analysis.* The 11,12-dehydrokahweol was the main compound of Típica variety, its spectra (Figure 1), showed $[M]^{+\bullet}$ 312, $[M\text{-}H_2O]^{+\bullet}$, $[M\text{-}CH_2OH]^{+}$, common fragmentation of this kind compounds and one important fragment (*m/z* 238), resulted from the loss of 74 amu, which allows to establish the presence of a double bound in 11-12 position. This was confirmed by spectral comparison with a compound of similar structure and double bound in the same position (Herz, 1982). The 16-*O*-methylkawheol presented in its spectra $[M]^{+\bullet}$ 328, $[M\text{-}CH_2OH]^{+}$, $[M\text{-}CH_3OH]^{+\bullet}$, $[M\text{-}CH_2OH\text{-}\ CH_3OH]^{+\bullet}$ and as main fragments *m/z* 131, *m/z* 145 and *m/z* 146 which were contained in kawheol spectra indicating C1-C2 unsatured. The fragment *m/z* 59 confirms *O*-sustitution in C16 as in 16-*O*-methylcafestol spectra.

The compounds 16-*O*-isobutylkahweol ($M^{+\bullet}$ 370) and the 16-*O*-isobutylcafestol ($M^{+\bullet}$ 372) had mass spectra a little different to other diterpenes. These compounds did not show neither of two fragments $[M\text{-}H_2O]^{+\bullet}$ nor $[M\text{-}CH_2OH]^{+}$ which may indicate *O*-sustitution in C-16. Additionally the fragment *m/z* 59 confirms this substitution. In the 16-*O*-isobutylkahweol spectrum was found main ionic fragments presented in the kahweol spectrum and the 16-*O*-isobutylcafestol presented *m/z* 133 and *m/z* 147 fragments as in the cafestol spectrum.

In conclusion, the results of this study allowed to differentiate between *C. arabica* Caturra and Típica varieties, and also among all Hibrido de Timor accessions and differentiate *C. canephora* accesssions from the other genotypes. There were not found important differences in the composition between First generation of the crossing of Caturra x Híbrido de Timor and Variedad Colombia. Furthermore it was possible to identify tentatively three new furanekaurenic diterpenes, which are also discriminating compounds. This information would be used for evaluation of hybrid material, very important in plant breeding programs.

## ACKNOWLEDGEMENTS

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# Moisture Determination in Green Coffee – A Method Comparison

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## SUMMARY

Green coffee behaves very differently at high and low moisture content with a number of unwanted consequences like microbial growth, mycotoxin formation, altered sensorial quality of end product, instable production conditions and unclear trade issues. Generally a moisture content ranging between 8.0-12.5% is considered to be adequate to avoid the mentioned issues. ISO has therefore issued a number of standards for reference, routine and rapid methods. Nevertheless, on-going discussions on how effective the methods are capable to principally determine the moisture content lead to modifications of the official approach. This work was therefore focused on clarifying the specificity and accuracy of several available methods. We could demonstrate that only ISO 1446 (2001) exclusively measures water but leaves some residual water content difficult to extract from the dried coffee matrix. For all drying oven based methods we observe degradation of the product contributing to the overall weight loss. We used near infrared spectroscopy to establish the degree of degradation and the completeness of the drying process. Repeatability was found excellent for all methods despite degradation and incomplete drying which should negatively affect accuracy.

## INTRODUCTION

Moisture content is certainly the most critical quality parameter of green coffee, as it governs fermentation and mould growth during storage and transport, which could lead either to the development of off-flavors at the cup level or to the formation of mycotoxins. It is also important for economical reasons as coffee is paid by weight and buyers are obviously more interested to buy solid coffee materials than water. In this connection, ISO had issued three official standards. ISO 1446 is the basic $P_2O_5$ reference method (slow drying at 48°C) for the determination of the moisture content in green coffee. ISO 1447 (rapid drying at 130°C) is the routine oven method. ISO 1446 and 1447 (1978) are supposed to give identical results. The third procedure, ISO 6673 (medium drying at 105°C) (1983a), is the practical method for the determination of the loss of mass, in which coffee is dried in an oven with forced ventilation. It is clearly stated in ISO 6673 that the method gives results, which are about 1.0% lower than ISO 1446. ISO recently decided to withdraw standard ISO 1447, which was the method used up to now to calibrate all moisture analyzers based on indirect methodologies such as capacity, conductivity or near infrared spectroscopy. Consequently, the withdrawal imposes a recalibration of all analysers. The purpose of this study was to precisely assess the performance of ISO 6673 and to further test the robustness of the method.

## MATERIALS AND METHODS

The present work is based on the comparison of the following methods and practices. Near infrared spectroscopy was used to support conclusions on the specificity and accuracy of the moisture content determination methods.

**Drying method ISO 1446 (2001):** ISO 1446 describes the basic reference method for the determination of the moisture content in green coffee. It is based on the desiccation of about 3

to 4 g of ground green coffee under phosphorus pentoxide ($P_2O_5$) at 48°C ± 2.0°C up to weight constancy after about 2 weeks.

**Drying method ISO 1447 (1978):** ISO 1446 describes the routine method for the determination of the moisture content in green coffee. Approximately 5 g of whole green coffee beans were dried for 6 h ± 15 min at 130.0°C ± 2.0°C using a T6060 oven from Hereaus. The sample was then removed from the oven and weighed after it has cooled down to ambient conditions. In a second drying step the sample was put under identical drying condition for 4 h ± 15 min. After an identical weighing procedure the moisture content is calculated as sum of the weight loss after the first drying step and half the weight loss of the second drying step.

**Drying method ISO 6673 (1983a):** ISO 6673 measures the mass loss of complete green coffee beans. ISO 6673 is using an electrically heated drying oven with forced air ventilation controlled at 105.0°C ± 1.0°C. Approximately 10 g of whole green coffee beans are dried for 16 h ± 0.5 h. We used model Salvis Thermocenter (Renggli AG, Switzerland), which was checked identically to the standard ovens. The analysis was performed in duplicate and the results averaged. Additionally, we carried out the same analysis but using an oven without forced air ventilation (T6060, Hereaus). In order to investigate the influence of the laboratory conditions some samples the oven was placed in a climatic chamber at 32 ± 1°C and 63 ± 2% RH.

**Near infrared spectroscopy:** All the samples were scanned using a scanning near infrared spectroscopy (NIRS) spectrometer model Infralyzer 500 from Bran&Luebbe, Germany. The measurements were done using small ring cups. Samples were scanned over the whole range of wavelength (400 to 2500 nm) with a resolution of 2 nm. However, an averaged spectrum was used for further work. For the measurement all samples were rapidly ground. The spectra were normalized at the wavelengths of 1680 and 2232 nm and analyzed at the 1940 nm waterband.

**Samples:** Arabica and Robusta green coffees from different geographic origin were selected in order to cover a wide range of moisture content. Humidification of samples to obtain higher moisture levels was not necessary. After reception the green coffee beans were placed into cans and allowed to thermo-dynamically equilibrate for minimum one month before analysis.

## RESULTS AND DISCUSSION

For the studied sample set ISO 1446 shows the lowest results of all tested methods. The new ISO 6673 gives results, which are approximately 0.3 weight % higher than ISO 1446. ISO 1447 gives results, which are about 1.3 weight % higher than ISO 1446 and about 1 weight % higher than ISO 6673. We almost did not find any differences when we exposed the drying ovens operated according ISO 6673 to tropical conditions.

We have chosen one Robusta sample to assess the effects of the grinding and the drying processes in more detail. Enough beans were ground and rapidly sieved for obtaining about 100 g of particles ranging between 1 mm and 5 mm. Table 1 and Figures 1 and 2 summarize the results. For the sample dried according ISO 1446 we measured 12.24 weight % as extracted water and we found 0.53 weight % residual water content (according to a NIR calibration). Assuming no weight loss due to degradation we can calculate the “true” water content of 12.77 weight %. For ISO 6673, extracted water was 12.53 weight %. Based on the assumption that the near infrared absorption at 1940 nm is in linear correlation to the residual water content (ranged between 0 to 1.5%) we could determine the residual water content as

0.63 weight %. The sum of both extracted and residual water by-pass the true water content determined as 12.77 by 0.39 weight %. We attribute this weight loss to degradation reactions during the drying process.

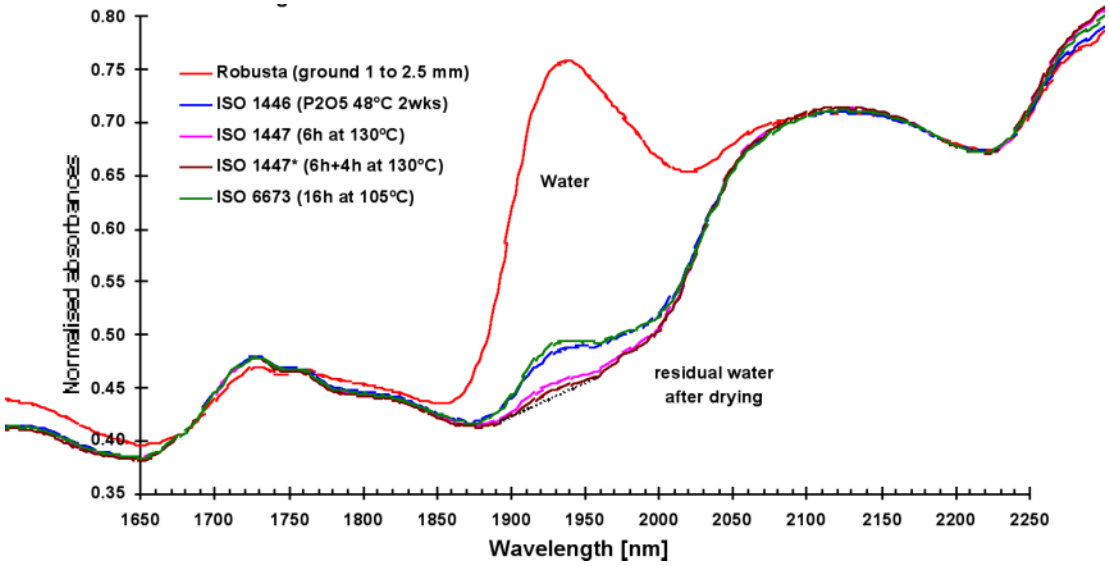


**Figure 1. Green coffee wet and dried under different conditions.**

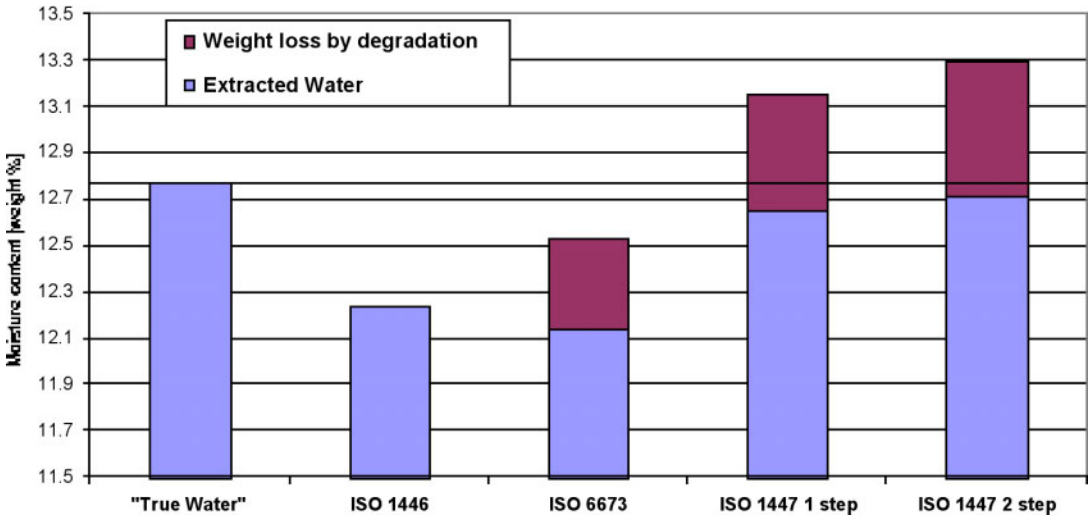


**Figure 2. Comparison of results for Robusta sample.**

Accordingly we also performed the calculation for ISO 1447. After the first drying step we determined a residual water content of 0.12 weight % and extracted water content of 13.16 weight %. We calculated therefore a weight loss by degradation of 0.51 weight %. The second drying step leads to an overall weight loss of 13.30 weight % and ISO 1447 would report 13.23 weight % as final result. The additional weight loss of 0.14 weight % is at the lower end of what we have observed during our study and all differences given for this sample are not statistically representative for the whole population. Nevertheless they allow understanding the occurring phenomena during the drying process. Of this additional weight loss 0.06 weight % were attributed to water loss based on the near infrared spectra. The additional weight loss for degradation in the second drying step would be in this example 0.08 weight %. Compared to the overall weight loss related to degradation of 0.59 weight % this amount stays quite small, meaning that the most significant weight loss related to degradation occurs in the first drying step. This also supports the theory that water is required for some of the

degradation reactions. Globally we can conclude that ISO 1447 almost completely removes the water but also includes significant degradation of the sample. Here, the degradation at 130°C in the ground particles was less extensive than in the whole beans due to the more efficient drying kinetics. Overall the weight loss related to degradation is larger for ISO 1447 than for ISO 6673. This would be an argument supporting the change of method versus ISO 6673.

**Table 1. Repartition of Residual Water, Extracted Water and Weight Loss by Degradation for a Robusta Sample.**

| Robusta 513032 | | | NIR Absorbtion [abs] at 1940 nm | Residual Water [weight %] | Extracted Water [weight %] | Overall weight loss [weight %] | Weight Loss by Degradation [weight %] |
|---|---|---|---|---|---|---|---|
| Method | Temperature | Time | | | | | |
| ISO 1446 | 48 °C | 316 h | 0.489 | 0.53 | 12.24 | 12.24 | 0.00 |
| ISO 6673 | 105 °C | 16 h | 0.495 | 0.63 | 12.14 | 12.53 | 0.39 |
| ISO 1447 | 130 °C | 6 h | 0.460 | 0.12 | 12.65 | 13.16 | 0.51 |
| | | 6 h + 4 h | 0.453 | 0.06 | 12.71 | 13.30 | 0.59 |
| | | Result | | | | 13.23 | |

The second part of the study was focused on the expected change from using ISO 1447 to ISO 6673. Different samples covering 7% to 13% moisture content were analyzed by both methods. The regression analysis between ISO 6673 and ISO 1447 shows a very good correlation with $R^2$ of 0.999 and a standard error of differences of 0.078 weight %. Based on the 18 samples we found a significant bias of 1.05 weight %. This bias is constant over the moisture range and independent of the origin of the green coffee.

## CONCLUSIONS

The repeatability standard deviation is very good and similar for all the tested methods: about 0.06 weight %. The reference method ISO 1446 has proved to let residual water content at the end of the drying (e.g. 2 weeks). This residual value was very sensitive to the particle size, due to the very low water diffusion in dehydrated green coffee material. Results obtained with ISO 6673 present a significant bias of 0.3 weight % compared to ISO 1446. The bias is constant over the whole moisture range. All methods are highly correlated with each other with $R^2$ values above 0.990. This will allow the upgrade of the calibrations of the moisture analyzers by a simple bias adjustment procedure. ISO 1447 method surprisingly gave not results similar to ISO 1446 method, but higher values (average difference: 1.38%). However the usual average difference of 1.05% was measured between ISO 1447 and ISO 6673. Further trials showed that the results obtained with ISO 6673 were independent of the climatic conditions and the presence of forced ventilation. This would allow the use of cheaper standard ovens in laboratories where climatic conditions are not necessarily well controlled. ISO 6673 requires the least input of labor as no grinding is required and only one drying step is applied. It is therefore well suited for routine purposes.

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# 16-O-Methylcafestol in the Quality Control of Instant Coffees

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## SUMMARY

16-O-methylcafestol is the indicator substance for the proof of Robusta in roasted Arabica coffee mixtures. The analysis is performed using the DIN method No 10779. After some modification this method is also suitable for determining 16-OMC in instant coffees. Data of numerous commercial instant coffees are presented.

Alongside roasted coffee, instant coffee, with nearly 30% of the world market, has reached high economic significance. The products are often verified by the trade designations “Pure Highland Coffee” or “100% Arabica Coffee”. How can this be proved?

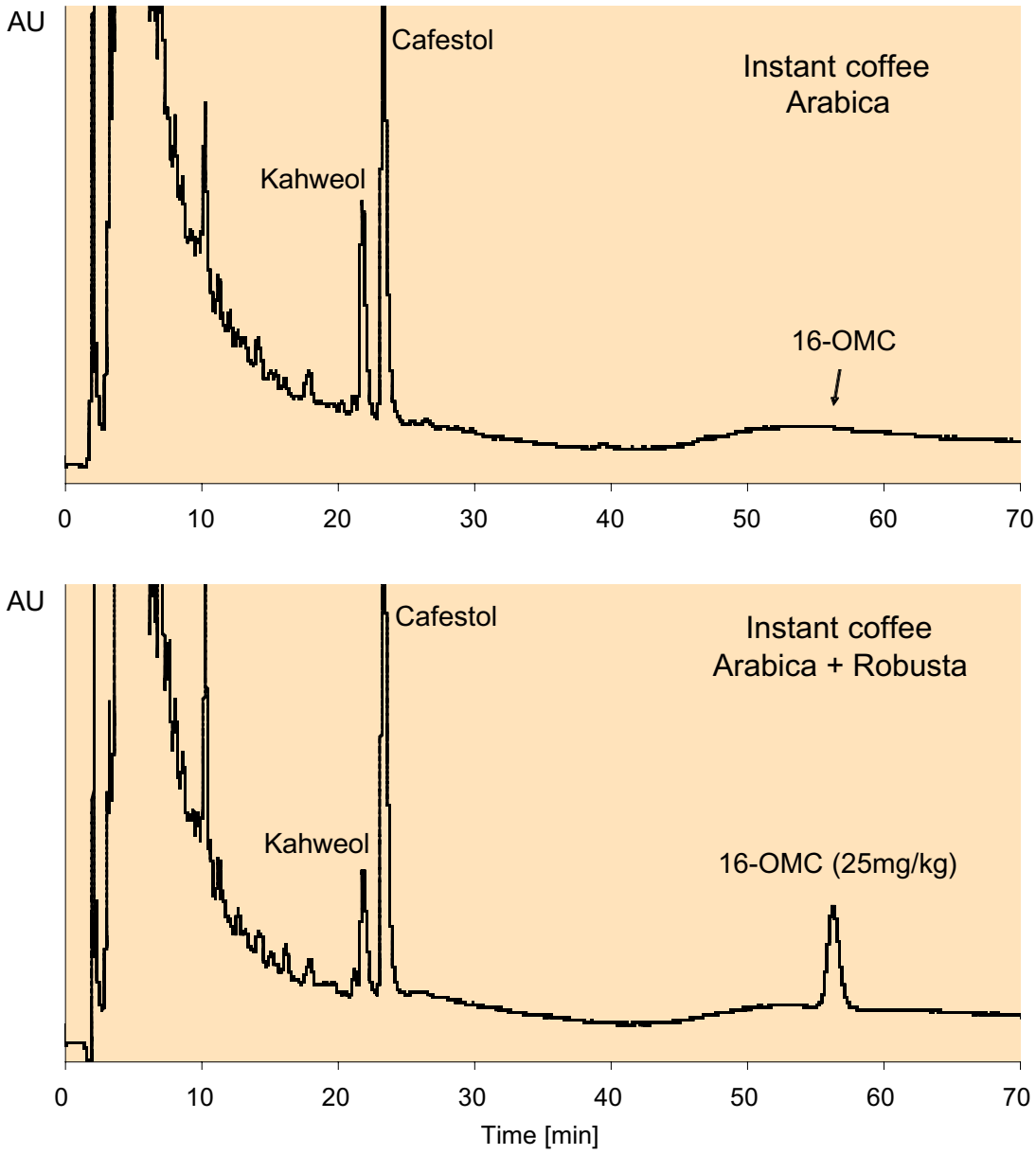
In case of roasted commercial coffees, such designations can be proved by analysing the content of 16-O-methylcafestol (16-OMC) as an indicator for Robusta coffee (Speer et al., 1991). Using the DIN-method 10779 (1999), coffee oil is extracted and saponified. The diterpene 16-OMC is then determined in the unsaponifiable matter by means of HPLC.

Instant coffee contains very little coffee oil due to production methods, where only water may be used for extraction under optimisation of the pressure and the temperature. However, on the basis of investigations by Sehat et al. (1993), and then later also by Urgert et al. (1995) and Gros et al. (1997), it could be shown that coffee lipids merge with the brew.

Via the detection of 16-OMC it should, therefore, be possible to detect whether an instant coffee was produced from pure Arabica coffee or whether Robusta properties were used additionally. Nevertheless, due to the small amounts of 16-OMC to be identified, DIN 10779 could not be applied to instant coffee. Rather, it was necessary to directly saponify the instant powder, and beyond that it became possible only after relinquishing the dilution steps to reliably analyse the 16-OMC content with the HPLC by using a diode array detector (Figure 1, 2).

The modified method was validated for dosaging pure Arabica instant coffee with 16-OMC directly prior to saponification. The results of fourfold-classifications for the three concentrations are summarised in Table 1.

Numerous commercial instant coffees from the European coffee market were then analysed (Figure 3). Aside from coffees in which 16-OMC could not be detected, there was a group of coffees that contained only small amounts of 16-OMC. Furthermore, there was another group found to have higher 16-OMC contents.




**Figures 1, 2. HPLC chromatograms of commercial instant coffees.**

**Table 1. Recovery of 16-OMC additions to Arabica instant coffee.**

| **Addition of 16-OMC [mg/kg]** | **5** | **25** | **50** |
|---|---|---|---|
| **Recovery [%]** | 85 | 85 | 85 |
| | 83 | 82 | 87 |
| | 85 | 84 | 87 |
| | 83 | 82 | 88 |
| **Mean Recovery [%]** | **84** | **83** | **87** |

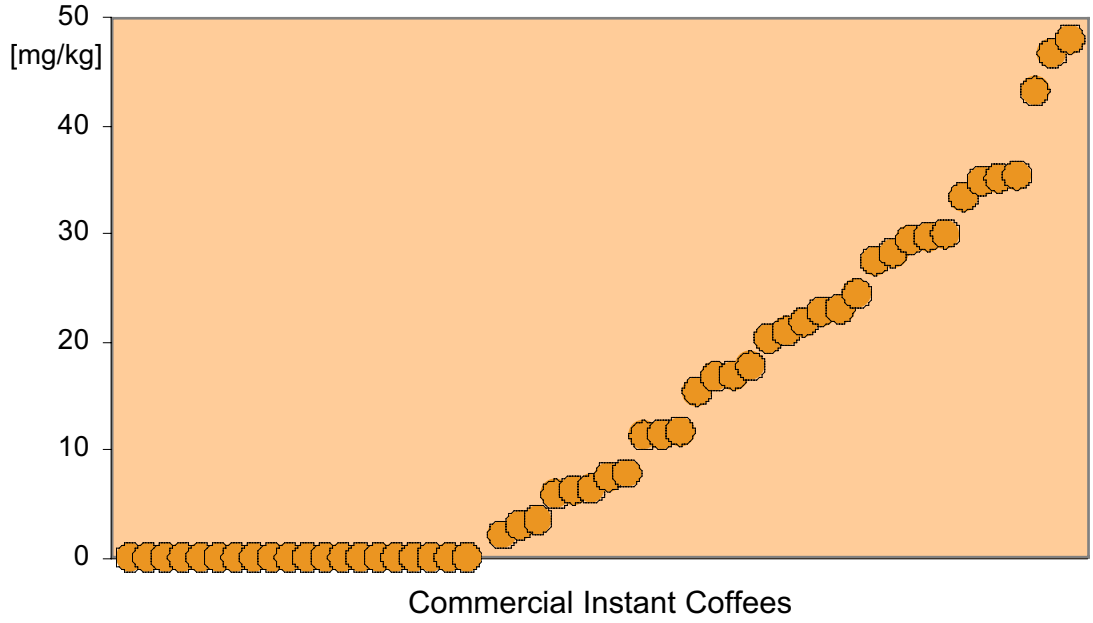



**Figure 3. Contents of 16-OMC in commercial instant coffees.**

## CONCLUSIONS

16-OMC is the indicator substance for the proof of Robusta, also in instant coffees. However, statements in regard to mixing ratios are not possible because the low 16-OMC contents detected very strongly depend on the extraction conditions used.

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# Chromatography of Carbonic Acid-5-Hydroxytryptamides

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## SUMMARY

In coffee wax, six carbonic acid-5-hydroxytryptamides are well known. Now, by means of LC/MS two new C5HTs were elucidated: the 5-hydroxtryptamides with the odd-numbered fatty acids henicosanoic acid and tricosanoic acid.

## INTRODUCTION

The surface of green coffee beans is covered by a thin waxy layer with a proportion of 0.2 to 0.3 per cent. In first investigations concerning the wax composition of green Arabica coffee beans, Wurziger and his co-workers (Dickhaut, 1966; Harms, 1968) isolated and identified the carbonic acid-5-hydroxytryptamides (C5HT), namely arachidic acid-, behenic acid-, and lignoceric acid-5-hydroxytryptamide.

Furthermore, Folstar et al. reported the presence of stearic acid-5-hydroxytryptamide and, later, the presence of ω-hydroxyarachidic acid- and ω-hydroxybehenic acid-5-hydroxytryptamide (Folstar et al., 1980) (Figure 1). All these components were confirmed by König and Sturm (König & Sturm, 1982).

With a modified chromatographic method we are able to demonstrate that coffee contains more fatty acid-5-hydroxytryptamides than described until now. Resulting LC/MS data are presented for the first time.

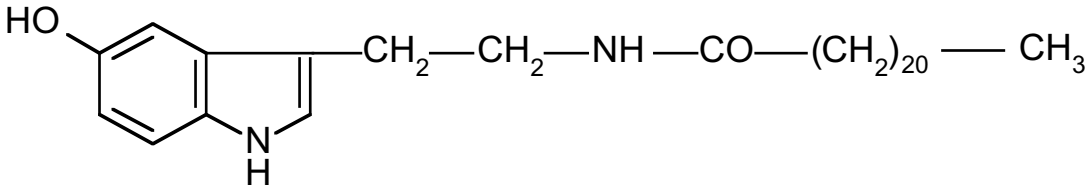


**Figure 1. Structural formula of 5HT-behenate.**

## LC/MS

Instruments: HPLC-Series 1100, Agilent, equipped with mass spectrometer API 4000 Q Trap, Applied Biosystems.

Positive ionisation, Atmospheric Pressure Chemical Ionization (APCI).

## RESULTS

Wax from the industrial decaffeination process of coffee was dissolved in dichloromethane. After several clean-up steps, the C5HTs were analysed by HPLC combined with fluorescence detection (Figure 2) and mass spectrometry.

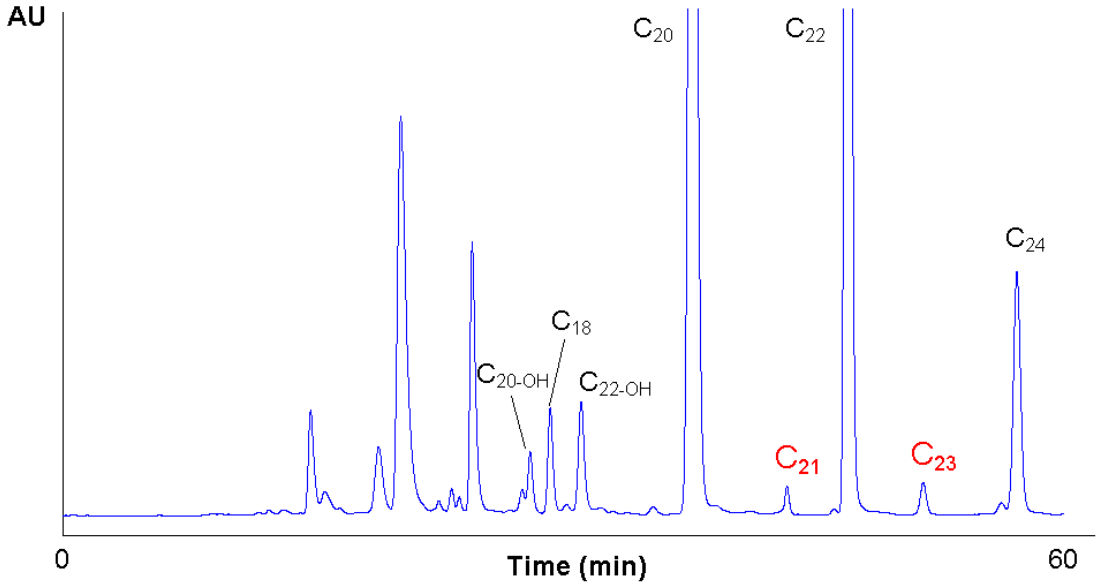


**Figure 2. HPLC chromatogram of C5HTs.**

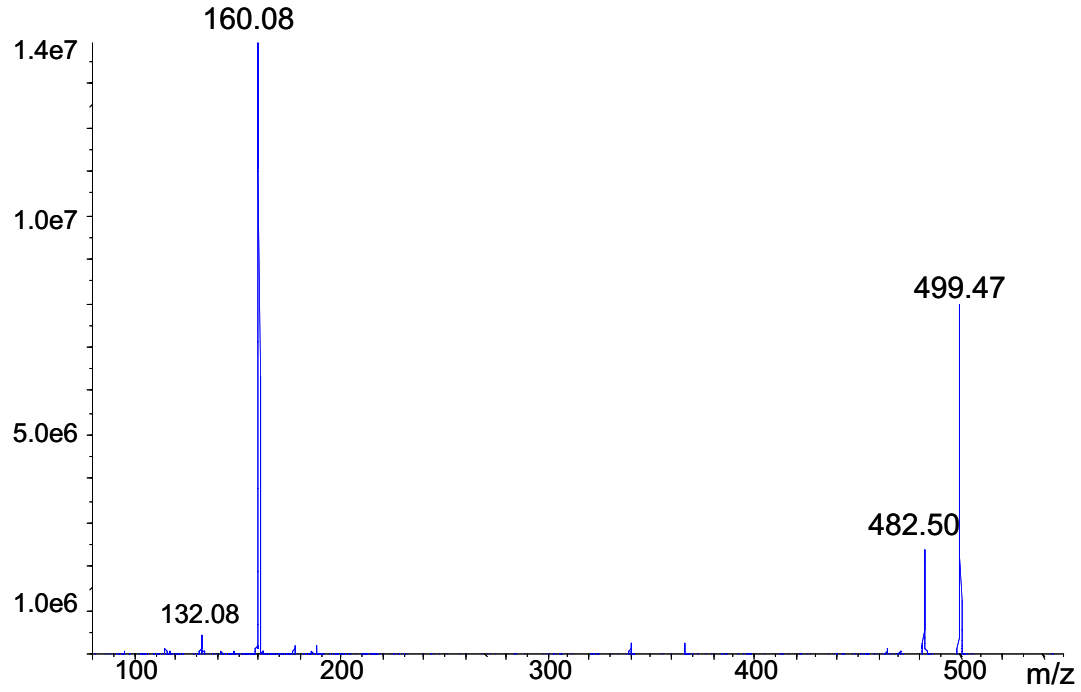


**Figure 3. LC mass spectrum of 5HT-behenate.**

In order to confirm the main peaks as the known carbonic acid-5-hydroxy tryptamides (namely arachidic acid-, behenic acid-, and lignoceric acid-5-hydroytryptamide) behenic acid-5-HT (Figure 1) was synthesized by Hinkel (2004), who modified the method by Hubert et al. (1979), and analysed with LC/MS.

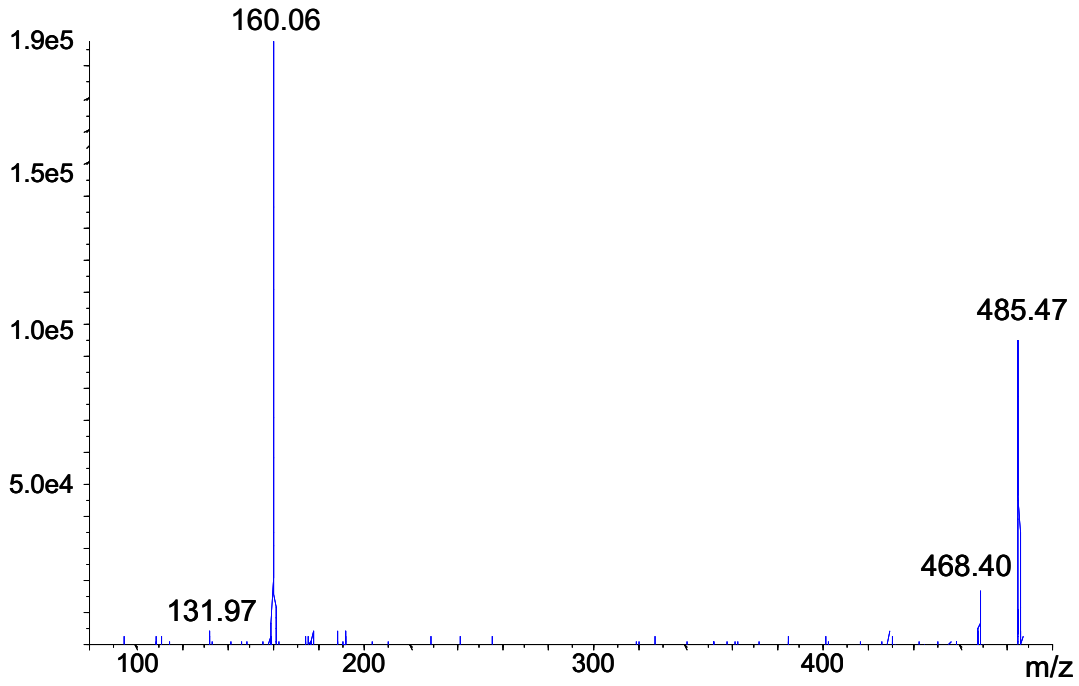


**Figure 4. LC mass spectrum of 5HT-henicosanate.**

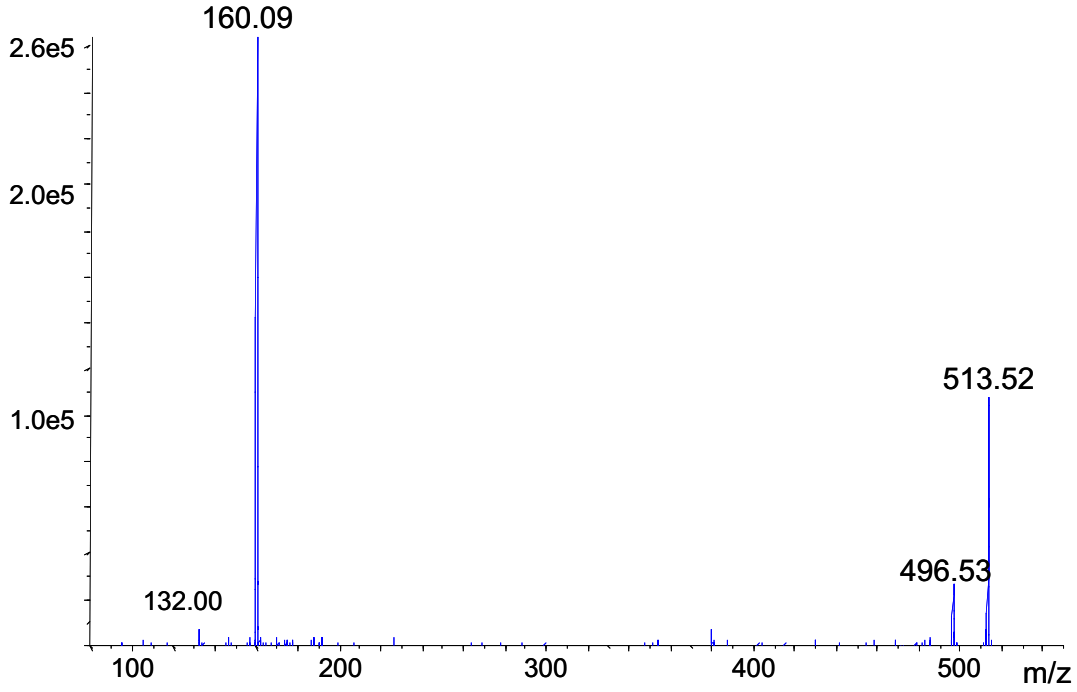


**Figure 5. LC mass spectrum of 5HT-tricosanate.**

In Figure 3, the LC/MS-spectrum presents the peak $[M+H]^+$, a fragment ion $[(M+H)-17]^+$ and above all, a base peak at m/z 160. Now, due to their LC/MS spectra, the known hydroxytryptamides with $C_{18}$, $C_{20}$, $C_{22}$, and $C_{24}$ as well as the hydroxytryptamides with ω-hydroxyarachidic acid and ω-hydroxybehenic acid can be assigned (Figure 2).

Apart from these known substances a number of additional peaks were detected. Two of them – the peaks marked red – were elucidated as the hydroxytryptamides with the odd-numbered fatty acids $C_{21}$ and $C_{23}$. The mass-spectra are given in Figure 4 and 5.

The occurrence of these odd-numbered fatty acids was not unexpected because they were already detected in triacylglycerides and diterpene esters (Speer and Kölling-Speer, 2001).

Concerning the other peaks, we have only obtained some hints at the moment. To ascertain these, we are isolating these components for further identification and characterization with common spectroscopic methods.

**ACKNOWLEDGEMENT**

We would like to thank Dr. Schreiber (Applied Biosystems, Darmstadt, Germany) for the LC/MS data acquisition.

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# Influence of KCl Fertilization and Roasting on the Levels of Bioactive Amines in Coffee

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## SUMMARY

*Coffea arabica* L. catuaí was cultivated in São Sebastião do Paraíso, Minas Gerais, Brazil. It was fertilized with KCl at levels of 0, 100, 200 and 400 kg of K/ha. The green coffee was roasted at 300°C for 6 and 12 min (American and French). The samples were analyzed for bioactive amines and the roasted ones were also analyzed for moisture, water activity and color characteristics. Total amines in green coffee ranged from 1.80 to 4.38 mg/100 g. The predominant amine was putrescine (34%), followed by serotonin (30%), spermine (19%) and spermidine (17%). An increase in the levels of the KCl caused a decrease in putrescine in the grain. The different types of roasting did not affect moisture and water activity. However, roasting time affected significantly color characteristics: American roasted samples produced lighter grains with higher intensities of a* and b*. During roasting, there was a significant decrease in total amines levels with total loss of putrescine and spermine and formation of agmatine.

## RESUMO

*Coffea arabica* L. catuaí cultivada em São Sebastião do Paraíso, Minas Gerais, Brasil, foi adubada com KCl em níveis de 0, 100, 200 e 400 kg de K/ha. O café verde foi torrado a 300°C for 6 e 12 min (Americana e Francesa). As amostras foram analisadas quanto a aminas bioativas e as torradas foram também analisadas quanto a umidade, atividade de água e características de cor. O teor total de aminas no café verde variou de 1,80 a 4,38 mg/100 g. A amina predominante foi a putrescina (34%), seguida da serotonina (30%), espermina (19%) e espermidina (17%). Quanto maior a adubação com KCl, menor o teor de putrescina nos grãos. A torrefação não afetou a umidade e a atividade de água, entretanto, afetou as características de cor: a torrefação americana produziu grãos mais claros com maior intensidade de a* e b*. Durante a torrefação houve diminuição dos teores totais de aminas, com perda total de putrescina e espermina e formação de agmatina.

## INTRODUCTION

Polyamines such as spermidine and spermine have been found in all higher plants. They are considered to be of importance in growth processes and in membrane stabilization. The precursors for polyamine synthesis in plant cells are ornithine and arginine, with putrescine as an obligate intermediate (Flores et al., 1989). It has been known that nitrogenous compounds can accumulate in plants as a result of drastic changes in the environment. Various mineral deficiencies can also increase soluble N. Potassium deficiency in plants leads to disappearance of protein and increase in amine and amide N. The build up of putrescine in the leaves of potassium deficient plants has been observed in a wide range of plants (Basso &

Smith, 1974) and appears to be universal. Putrescine increases under deficiency of potassium, phosphorus, calcium, magnesium, iron, manganese, sulfur and boron, but to a much larger extent in potassium and phosphorus deficient plants. The response to ionic stress involves a rise in putrescine biosynthesis and a block in polyamine synthesis (Flores et al., 1989). Putrescine content may be a useful guide to the mineral status especially if accumulation of this amine may be detected before deficiency symptoms appear.

It is the goal to increase productivity, improve coffee quality and optimize costs by use of adequate production and processing techniques. Therefore, the objective of this work was to investigate the influence of potassium fertilization and roasting on the levels of bioactive amines in coffee.

## MATERIALS AND METHODS

### Materials

*Coffea arabica* L. red catuai MG-99 was cultivated in purple latosoil in the farm of EPAMIG in São Sebastião do Paraíso, MG, Brazil. Potassium fertilization was performed with KCl at 0, 100, 200 and 400 kg of K/ha. Four samples were obtained under each condition. Coffee was harvested 10 months after flowering, sun dried, and roasted at 300°C (Probat roaster, Germany) for 6 min – American and 12 min – French roasting. The experiment was performed in triplicate. Green and roasted samples were analyzed for bioactive amines and roasted samples were also analyzed for moisture, water activity and color characteristics. Analyses were performed in duplicate.

### Methods of analysis

***Determination of bioactive amines.*** Amines were extracted with 5% trichloroacetic acid (Cirilo et al., 2003), separated by ion-pair RP HPLC and quantified fluorimetrically after post-column derivatization with *o*-phthalaldehyde (Vale & Glória, 1987). **Moisture content** was determined by Karl Fisher (IAL, 1985). **Water activity** was determined in a TESTO 650 (Lenzkirch, Germany). **CIE L*a*b* color characteristics** were determined in a Colortec PCM (Clinton, USA). **Statistical analysis.** The data were submitted to Anova and the means compared by the Duncan test at 5% of probability.

## RESULTS AND DISCUSSION

### Types and levels of bioactive amines in green coffee

Putrescine, spermidine, spermine and serotonin were detected in green coffee. The presence of the first three amines was expected but serotonin had not been reported in the literature. Total amine levels ranged from 1.80 to 4.38 mg/100 g. Putrescine was the predominant amine (34%), followed by serotonin (30%), spermine (19%) and spermidine (17%).

### Influence of potassium fertilization on amine levels

The dose of potassium fertilization (Table 1), affected significantly the levels of the majority of the amines detected. However, it did not affect total amine levels. An increase in KCl doses caused a significant reduction on putrescine levels to 82% with 100 kg/ha, and to ~58% with 200 and 400 kg/ha. Therefore, the addition of KCl to the soil at doses of 200 kg of K/ha, caused a significant decrease in the accumulation of putrescine in the coffee grain. With

respect to the levels of serotonin, significantly higher levels were found in samples fertilized with 100 and 200 Kg of K/ha.

**Table 1. Bioactive amines in green coffee fertilized with different doses of KCl.**

| KCl | Amine levels (mg/100 g)* | | | | |
|---|---|---|---|---|---|
| (kg K/ha) | Putrescine | Spermidine | Spermine | Serotonin | Total |
| 0 | 1.65 a | 0.71 b | 0.77 a | 0.83 c | 3.96 a |
| 100 | 1.35 b | 0.46 c | 0.51 b | 2.06 a | 4.38 a |
| 200 | 0.96 c | 0.91 a | 0.55 b | 1.31 b | 3.73 a |
| 400 | 0.97 c | 0.52 c | 0.69 ab | 0.64 c | 2.82 a |

**Mean values with similar letters in the same column do not differ significantly by Duncan test at 5% de probability.*

**Influence of roasting on amine levels**

The different types of roasting did not affect moisture content and water activity (Table 2). However, it affected significantly color characteristics. American roasted samples produced lighter grains with higher a* and b*. During roasting, there was a significant decrease in total amines levels with total loss of putrescine and spermine and formation of agmatine. Roasted coffee was characterized by the presence of 3 amines, with predominance of serotonin followed by spermidine and agmatine. There was significant correlation between the levels of the fertilizer KCl and the levels of spermidine and of spermidine in samples submitted to American roasting.

**Table 2. Water content, water activity, color and amines levels of coffee submitted to roasting at 300°C for 6 and 12 min.**

| Parameter | Roasting * | |
|---|---|---|
| | American (6 min) | French (12 min) |
| Water content (g/100 g) | 3.70 | 3.76 |
| Water activity | 0.40 | 0.37 |
| Color characteristics | | |
| L* | 31.58 a | 20.30 b |
| a* | 12.63 a | 7.03 b |
| b* | 18.20 a | 6.04 b |
| Amines | | |
| spermidine | 0.12 b | 0.20 a |
| serotonin | 0.16 b | 0.29 a |
| agmatine | nd | 0.12 |
| total | 0.28 b | 0.61 a |

**Mean values with similar letters in the same line do not differ significantly by Duncan test at 5% de probability.*

**CONCLUSIONS**

Putrescine, serotonin, spermine and spermidine were detected in green coffee. KCl fertilization affected putrescine and serotonin accumulation in coffee grains. Roasting affected amines levels and profile. There was a decrease in amines levels with total loss of putrescine and spermine and formation of agmatine.

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# Antiproliferation and Anti-Influenza Viral Activities of Caffeic Acid Phenethyl Esters Synthesized Enzymatically from Chlorogenic acid and Phenethyl Alcohol

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## SUMMARY

Phenylethyl caffeates synthesized enzymatically from 5-chlorogenic acid and alcohols absorbed a part of long-wavelength ultraviolet rays because of there absorption maximum over 320 nm. 1,1-Diphenyl-2-pycrylhydrazyl radical and superoxide anion scavenging activities ($IC_{50}$) of 1- and 2-phenylethyl caffeates ranged from 11 to 12 and 5 to 6 μM, respectively, and those activities were 1.8 times higher than activity of ascorbic acid which is commonly used as antioxidants. Both phenylethyl caffeates inhibited the growth of *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* at 0.22-0.44, 0.44-0.88, 0.44-0.88 mM, respectively, by 96-well microbioassay system used a liquid broth culture system in a dose response format. 2-Phenylethyl caffeate activated the growth of normal human diploid lung fibroblast WI38 cells at 35 μM, whereas SV40 virally transformed WI38 cells (WI38VA) were sensitive to 2-phenylethyl caffeate ($IC_{50}$ 60 μM). Human histiocytic lymphoma U937 and human oral carcinoma KB cells were also sensitive to 1- and 2-phenylethyl caffeates (26-48% inhibition at 35 μM). Influenza viruses A and B were inhibited 95 and 92% of their replication by 8.8 μM 1-phenylethyl caffeate, respectively, and virus A was inhibited 99% by 35 μM 2-phenylethyl caffeate. These phenylethyl caffeates might inhibit the virus replication in infectious cells.

## INTRODUCTION

Low quality green coffee beans are not marketed in coffee drinks or utilized effectively as resources. However, these beans contain considerable amounts (4.8-5.8 g 100 $g^{-1}$) of 5-chlorogenic acid (5-CQA) derivatives. We are investigating the enzymatic conversion of their 5-CQA to value-added products, and synthesized caffeic acid esters by using CQA hydrolase as reported at PC351 of 20$^{th}$ international conference on Coffee Science at Bangalore.

Trace amounts of caffeic acid esters are widely distributed in plants and propolis from honeybee hives. Methyl caffeate is contained in the leaves of *Melissa officinalis* L. (lemon balm) (Tagashira and Ohtake, 1998) and *Lonicera japonica* (Chang and Hsu, 1992), ethyl caffeate (CAET) is contained in the seeds of *Ipomoea muricata* (Ysrael and Nonato, 1999), and 2-phenylethyl caffeate (2-CAPE) is an active ingredient derived from propolis (Na et al., 2000). These esters have a broad spectrum of biological activities including antioxidant properties, antifungal action, inhibitory activity against platelet activation, anti-viral actions, and anticancer effects (Tagashira and Ohtake, 1998; Chang and Hsu, 1992; Ysrael and Nonato, 1999; Na et al., 2000).

In this report, we describe the biological activities of 1- and 2-phenylethyl caffeate (1- and 2-CAPE) synthesized from 5-CQA and 1- or 2-phenethyl alcohol (1-, or 2-PA) using CQA hydrolase, respectively.

## MATERIALS AND METHODS

### Chemicals

1- and 2-CAPEs were synthesized from 5-CQA and 1- or 2-PA by using CQA hydrolase (Okamura and Watanabe, 1982). Other chemicals of analytical grade were obtained from Tokyo Kasei Kogyo Co., Ltd., Wako Pure Chemicals Ltd., Sigma-Aldrich Co. (St. Louis, MO), and Nacarai Tesque Inc. (Kyoto, Japan).

### Diphenyl-2-pycrylhydrazyl (DPPH) radical and superoxide anion scavenging activity

The DPPH radical and superoxide anion scavenging activities of the samples were analyzed according to a procedure of Iwai et al. (2004).

### Antimicrobial activity

Antimicrobial activities of the samples were tested against four bacteria and two fungi by an agar dilution method and a 96-well microbioassay system used a liquid broth culture system in a dose response format.

### Antiproliferation activity

Antiproliferation activities of the samples were examined in three human cancer cell line (U937, KB, and WI38VA) and one normal cell line (WI38) according to a procedure of Iwai et al. (2004).

### Antiviral activity

Antiviral activities of the samples were determined by a plaque assay method using Influenza virus A, B, and Madin Darby Canine Kidney (MDCK) cells. The production of interferon by 2-CAPE was assayed by the addition of samples to the MDCK cells before influenza virus infection and incubated at 37°C for 24 h. Polyinosinic polycytidilic acid (Poly I:C, Sigma-Aldorich Co., 25 μg/ml) was used as inducer of interferon (Tsumura et al., 1989). The inhibition of virus infection by 2-CAPE was assayed by the contact the virus with samples before virus infection.

## RESULTS AND DISCUSSION

### UV absorption spectra

2-CAPE in 50% ethanol solution exhibited absorption maximum at 329 nm (absorbance 0.95 at 48 μM), whereas CA in 50% ethanol showed absorption maximum at 317 nm (absorbance 0.73 at 48 μM), which suggesting that 2-CAPE may be able to absorb a part of long wavelength UV ray (320-400 nm) as well as the middle wavelength UV ray (280-320 nm).

### Antioxidative activity of CAPEs

DPPH radical and superoxide anion scavenging activities ($IC_{50}$) of the CAPEs ranged from 11 to 12 and 5 to 6 μM, respectively. DPPH radical scavenging activity of CAPEs exhibited 1.8 fold higher than ascorbic acid, and similar to those of alfa tocopherol, 5-CQA, and CA.

**Antimicrobial activity of CAPEs**

CAPEs inhibited the growth of *S. aureus* NBRC 12732, *B. subtilis* NBRC 3009, *P. aeruginosa* NBRC 3080 and *C. albicans* NBRC 1594 at 0.22-0.44, 0.44-0.88, 0.44-0.88, and 0.88-1.8 mM, respectively, by 96-well microbioassay system used a liquid broth culture system in a dose response format, whereas no growth inhibition against *E. coli* NBRC 3301 and *A. niger* NBRC 4414 was detected at 1.8 mM. Those antimicrobial activities were higher than those of CA and 5-CQA (Table 1).

**Table 1. Antibacterial activity of CA esters by micro liquid dilution method.**

| | MIC (mM) | | | | | |
|---|---|---|---|---|---|---|
| Sample | *S. aureus* | *B. subtilis* | *E. coli* | *P. aeruginosa* | *C. albicans* | *A. niger* |
| CA | >2.8 | >2.8 | >5.6 | >2.8 | >0.69 | >5.6 |
| CQA | 1.4 | 0.71 | >2.8 | 1.4 | 2.8 | >2.8 |
| 1-CAPE | 0.22 | 0.44 | >1.8 | 0.44 | 0.88 | >1.8 |
| 2-CAPE | 0.44 | 0.88 | >1.8 | 0.88 | 1.8 | >1.8 |

**Antiprolifearation activity**

2-CAPE activated the growth of normal human diploid lung fibloblast WI38 cells at 35 μM, whereas the cancerated WI38VA cells were sensitive to 2-CAPE (IC50, 60 μM). Morphological characteristics of WI38 cells cultured with 35 μM 2-CAPE were the same as those of cells without 2-CAPE, whereas WI38VA cells cultured with 35 μM 2-CAPE were peeled off from the bottom face of plate and the morphology of cells changed from fibloblastic to histiocytic form. Human histocytic lymphoma U937 and human oral carcinoma KB cells were also sensitive to 1- and 2-CPAEs (26-48% inhibition at 35 μM).

**Antiviral activity**

Influenza viruses A and B were inhibited 95 and 92% of their replication by 8.8 μM 1-CAPE, respectively, and virus A was inhibited 99% by 35μM 2-CAPE. The virus A caused cytopathic effects against the MDCK cells (Figure 1-B), but the addition of 2-CAPE lowered the relative frequency of cytopathic effect at 35 μM (Figure 1-C). The cell lysate prepared from MDCK cells infected by virus formed a number of plaque on MDCK cells, but the number of plaque can be greatly diminished by the incubation of cells with 1- and 2-CAPEs after virus infection.

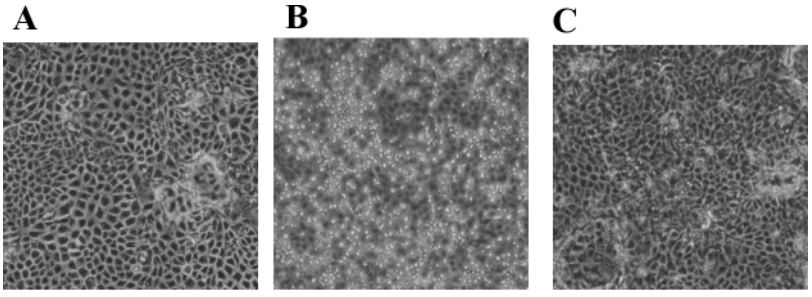


**Figure 1. Anti-influenza viral activity of 2-CAPE. A, MDCK cells grown without virus infection; B, Cytopathic effect appeared on the MDCK cells infected by influenza virus; C, Repression of cytopathic effect by addition of 2-CAPE after virus infection.**

On the other hand, inhibition ratio of 2-CAPE against virus A replication fell to about half (51%), when MDCK cells were incubated in the culture liquid containing 35 μM 2-CAPE before virus infection. However, the incubation with polyinosine polycytidic acid (25 μg/ml) as interferon inducer instead of 2-CAPE inhibited completely the replication of virus. Furthermore, the incubation of influenza virus with 2-CAPE before virus infection exhibited no inhibition of virus replication. These results suggest that 2-CAPE inhibited the virus replication without production of interferon and without inactivation of virus before infection. CAPEs might inhibit the virus replication in infectious cells.

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# The Influence of Fungi on the Flavour of Coffee Beverages

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## SUMMARY

To investigate the influence of fungi on the flavour of coffee beverages, samples of raw coffee beans of varying quality were collected from different regions of Brazil. Samples were surface disinfected with 0.4% chlorine solution for 1 min, then 50 beans from each sample were plated directly (10 particles per plate) onto Dichloran 18% Glycerol agar, and incubated at 25ºC for 5 to 7 days. After incubation, fungi were isolated and identified. To study beverage quality, raw coffee beans from each sample were roasted and ground. The samples were evaluated by three degustation tests: infusion, diluted espresso and espresso. Sensory analyses were carried out, evaluating the quality of the beverage in respect to body, aroma, acidity, bitterness, astringency and sweetness. Besides that, the presence of positive flavours and aromas such as caramel, chocolate or floral and negative characters such as immature, fermented, stinker, woody, rancid, mouldy, rioy and smoky were also evaluated. The most common fungi isolated from raw coffee beans, which still produced a good, clean beverage were *Cladosporium* spp, *Alternaria* spp, *Fusarium* spp, *Penicillium* spp., black moulds and yeasts. The high occurrence of *Penicillium* spp. in the Sorocabana region gave a positive sensory evaluation On the other hand, the most common fungi isolated from raw coffee beans which had a wood and rioy taste were *Aspergillus niger, A. ochraceus, Eurotium* spp., *Fusarium* spp. and dematiaceous fungi. Prepared coffee which had a fermented taste was infected mostly with *Aspergillus* and *Eurotium* species, while coffee with a wood smoke taste was infected mostly with *Aspergillus niger, A. tamarii, A. flavus, Eurotium* spp, and dematiaceous fungi.

## INTRODUCTION

Coffee flavour depends on a number of factors including coffee species and variety, climatic conditions, harvesting, drying techniques, and technological conditions used for the roasting and grinding of coffee beans. However, it is also known that the growth of microorganisms on coffee fruit, either in the plantation or during drying and storage, can be important in the definition of final beverage quality. This contamination is very diversified, with the predominance of one or another group dependent on the processing stage and environmental conditions (Silva et al., 2000).

Several volatile metabolites such as 2,4,6-trichloroanisole (TCA), geosmin and terpenes have been isolated from coffee beverages (Curtis et al., 1974; Gee & Peel, 1974; Spadone et al., 1990). On the other hand, some species of *Penicillium* and *Aspergillus* are able to produce volatile metabolites which can affect the flavour and aroma of food and beverages (Börjesson et al., 1992; Schnürer et al., 1999).

The objective of this work was to investigate the fungal microbiota on coffee and the influence of these fungi on the flavour of coffee beverages.

## MATERIAL AND METHODS

### Coffee samples

Samples of arabica raw coffee beans of varying quality were collected from two regions in Brazil: 1) Sorocabana in south western São Paulo State, a relatively cold, rainy region with an average of 17.7ºC and 64.9 mm/month rainfall, and where the samples collected were at an altitude of 800 to 1000 m (Farms 1 and 2); and 2) Minas Gerais State, a temperate, dry region with an average of 19ºC and 14.8 mm/month rainfall, and with a high altitude above 1100 m (Farms 3 to 8). A total of 40 samples were collected.

### Mycological methods

Samples were surface disinfected with 0.4% chlorine solution for 1 min. Fifty beans from each sample, subdivided into 5 plates of 10, were plated directly onto Dichloran 18% Glycerol agar (DG18), and incubated at 25ºC for 5 to 7 days (Pitt & Hocking, 1997). After incubation, the plates were examined and the percentages of different fungal genera estimated. Representatives of all fungal types were isolated onto malt extract agar plates, then identified after growth under standard conditions, with the help of standard texts (Pitt & Hocking, 1997; Klich & Pitt, 1988; Pitt, 2000). The presence of each common fungus was then calculated as a percentage of beans in each sample.

### Coffee beverage quality

To study drinking quality, raw coffee beans from each sample were roasted and ground.

The samples were evaluated by three different degustation tests, i.e. infusion, diluted espresso and espresso.

Sensory analyses were also carried out, evaluating the quality of the beverage in respect to body, aroma, acidity, bitterness, astringency and sweetness. In addition, the presence of positive flavours and aromas such as caramel, chocolate and floral, and negative character including immature, fermented, stinker, woody, rancid, mouldy, rioy and smoky were also evaluated.

## RESULTS AND DISCUSSION

Samples with more significant results are presented in Tables 1 and 2, which show the percentage of fungi in coffee and its possible influence on sensory evaluation, in the two regions studied.

In the Sorocabana region coffee beans were highly infected with fungi, especially with *Penicillium* species. The high occurrence of *Penicillium* spp. was found together with a positive sensory evaluation. Most of the beverages had a low to regular body, discrete aroma, caramel taste and were slightly astringent. On the other hand, when *Aspergillus niger* was present in a percentage higher then 20%, the sensorial evaluation was negative and the sensorial characters associated with this species were woody, fermented, mouldy and bitter.

**Table 1. Percentages of coffee beans from Sorocabana region infected by particular species of fungi and their influence on sensory evaluation.**

| Sample number | Processing stage | Total fungal infection (%) | Infection by the most common species (%) | Sensory evaluation | Overall sensory evaluation |
|---|---|---|---|---|---|
| 1 (Farm 1) | Pulped natural coffee from storage | 96 | *Aspergillus ochraceus* (2)<br>*Fusarium stilboides* (4)<br>*Mucor* spp. (6)<br>*Penicillium brevicompactum* (50)<br>*Penicillium palitans* (16)<br>*Penicillium* spp. (2)<br>Yeasts (4) | Regular body<br>Discrete aroma<br>Caramel<br>Slightly astringent | Positive |
| 2 (Farm 1) | Pulped natural coffee from storage | 96 | *Aspergillus niger* (2)<br>*Eurotium rubrum* (6)<br>*Fusarium stilboides* (4)<br>*Fusarium* spp. (4)<br>*Penicillium brevicompactum* (52)<br>*Penicillium citrinum* (2)<br>*Penicillium palitans* (2)<br>*Penicillium* spp. (22)<br>Dematiaceous fungi (2) | Discrete aroma<br>Caramel<br>Little body<br>Slightly astringent | Positive |
| 3 (Farm 2) | Dried immature coffee from storage | 68 | *Aspergillus* spp. (2)<br>*Eurotium herbariorum* (4)<br>*Eurotium repens* (2)<br>*Eurotium rubrum* (38)<br>*Eurotium* spp. (6)<br>*Nigrospora sphaerica* (2)<br>*Penicillium brevicompactum* (6)<br>*Penicillium citrinum* (4)<br>*Penicillium* spp. (2)<br>Dematiaceous fungi (2) | Floral aroma<br>Caramel<br>Little body | Positive |
| 4 (Farm 2) | Pulped natural coffee from storage | 72 | *Alternaria* (2)<br>*Aspergillus niger* (22)<br>*Aspergillus ochraceus* (4)<br>*Eurotium rubrum* (18)<br>*Eurotium* spp. (16)<br>*Penicillium brevicompactum* (8)<br>Dematiaceous fungi (2) | Woody taste<br>Fermented<br>Strong immature taste (green defect) | Negative |
| 5 (Farm 2) | Dried floaters from storage | 88 | *Alternaria* sp. (4)<br>*Aspergillus niger* (34)<br>*Aspergillus* sp. (2)<br>*Cladosporium* sp. (2)<br>*Eurotium rubrum* (12)<br>*Eurotium* spp. (14)<br>*Fusarium semitectum* (2)<br>*Fusarium stilboides* (4)<br>*Fusarium* spp. (10)<br>*Nigrospora oryzae* (2)<br>*Penicillium brevicompactum* (20)<br>Dematiaceous fungi (4) | Fermented<br>Mouldy<br>Bitter | Negative |

In Minas Gerais, samples with fungal infection lower than 20% showed positive results from sensory evaluations. The most common fungi isolated, which still presented had good body and aroma, clean beverage, and good acidity and bitterness were *Cladosporium* spp, *Alternaria* spp, *Fusarium* spp. and several yeast species.

**Table 2. Percentages of coffee beans from Minas Gerais infected by particular species of fungi and their influence on sensory evaluation.**

| Sample number | Processing stage | Total fungal infection (%) | Infection by the most common species (%) | Sensory evaluation | Overall sensory evaluation |
|---|---|---|---|---|---|
| 6 (Farm 3) | Dried coffee from storage | 20 | *Alternaria* sp. (2)<br>*Cladosporium* sp (2)<br>*Fusarium* sp. (2)<br>Yeasts (6)<br>Dematiaceous fungi (8) | Clean beverage<br>Good body<br>Good aroma<br>Good acidity and bitterness | Positive |
| 7 (Farm 4) | Dried coffee from storage | 12 | *Aspergillus ochraceus* (2)<br>*Cladosporium* sp. (2)<br>*Penicillium brevicompactum* (2)<br>Yests (4)<br>Dematiaceous fungi (2) | Clean beverage<br>Good body<br>Good aroma<br>Good acidity and bitterness | Positive |
| 8 (Farm 5) | Dried coffee from storage | 2 | *Fusarium* sp. (2) | Clean beverage<br>Good body<br>Good aroma<br>Good acidity and bitterness | Positive |
| 9 (Farm 6) | Dried coffee from storage | 4 | *Fusarium* sp. (2)<br>*Penicillium brevicompactum* (2) | Clean beverage<br>Good body<br>Good aroma<br>Good acidity and bitterness | Positive |
| 10 (Farm 7) | Dried coffee from storage | 90 | *Aspergillus niger (24)*<br>*Aspergillus ochraceus* (20*)*<br>*Aspergillus tamarii* (2)<br>*Chaetomium* sp. (2)<br>*Cladosporium* sp. (2)<br>*Fusarium* sp. (10)<br>*Penicillium brevicompactum* (2)<br>*Phoma* sp. (2)<br>*Rhizopus* sp. (2)<br>Yeasts (2)<br>Dematiaceous fungi (22) | Woody taste<br>“Rioy taste” | Negative |
| 11 (Farm 8) | Dried coffee from storage | 94 | *Aspergillus niger* (50)<br>*Aspergillus flavus* (14)<br>*Aspergillus tamarii* (6)<br>*Aspergillus* sp. (2)<br>*Eurotium* sp. (18)<br>*Mucor* sp. (2)<br>Dematiaceous fungi (2) | Woody taste<br>Smoky | Negative |

In the Minas Gerais samples, as in those from Sorocabana, samples with *A niger* infections higher than 20% showed negative sensory evaluations. The negative characters associated with this species were woody, rioy taste and smoky.

Besides *A niger*, some other fungal species produced a woody and rioy taste. These included *Aspergillus ochraceus, A. flavus, Eurotium* spp. and dematiaceous fungi.

In the present work, some correlation was found between the presence of certain species of fungi and their effect on both good and bad flavours in coffee beverages. However, more studies should be carried out in order to evaluate the influence of fungal metabolites on coffee beverages more thoroughly.

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# Incidence of Elevation on Chemical Composition and Beverage Quality of Coffee in Central America

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## SUMMARY

Chemical and beverage quality of green coffee samples from eight farms with different elevation in Poas region in Costa Rica (Trial 1) and from ten experimental plots with different elevations in El Salvador, Honduras and Costa Rica (Trial 2) were evaluated in 2003-2004 harvest. Coffee arabica trees in Trial 1 were at two different production years in a three-year rotational pruning cycle; while in Trial 2, trees were two and three year old. Bean size, dry matter weight and beverage quality were measured for Trial 1 samples, while biochemical content were estimated for both trials. Bean size and dry weight increased as elevation was higher and as shoot age was younger. Significant differences in chemical composition were observed in function of elevation in both trials and for beverage quality in Trial 1. Caffeine, chlorogenic acid and fat contents increased with elevation while trigonelline and sucrose decreased. Significant negative correlation between sucrose and fat content was observed. For beverage quality, tendency was that at higher elevation, better was classified the coffee with exception of one farm at intermediate elevation that was well classified. This farm was located in a very fertile area with outstanding plant nutrition and durable good cultural management. The study confirm that elevation is a very important factor for produce high quality coffee, but also that quality can be get by adequate cultural management in intermediate elevation.

## INTRODUCTION

In Central America, in general coffee buyers and producers know that best quality coffees are produce in the highlands. Guyot et al. (1996) in a study made in Guatemala confirmed that coffee from higher elevation showed a better quality than those from lower elevations. Unfortunately, the lack of an accurately statistical design of the experiments did not permit clearly to differentiate the effects attribute to elevation of those attributes to use of shade, plant age or cultural management. The effects of tree physiological conditions on coffee quality are thought to be important, but have been rarely investigated. Coffee tree experiences a strong alternating production pattern, where after a series of high yields, the tree becomes exhausted. In intensive coffee cultivation, the coppicing practice takes place every five years or less. Bertrand et al. (2004) found that the year of production and canopy region influence the bean characteristics and beverage quality of the coffee. The purpose of this study was to examine the relationships between elevations and shoot age on bean size and coffee beverage quality.

## MATERIALS AND METHODS

### Trial 1

The experiment was carried out during 2002/2003 harvest in commercial high density plots without shade (7000 plants.ha$^{-1}$) of 'Caturra and Catuaí' cultivars in eight farms with different elevation, located in a linear transect in the south slopes of Poas volcano, Alajuela province, Costa Rica (Table 1). Plants were intensively managed receiving 1000 kg.ha$^{-1}$ of 18N-3P-10K-8Mg-0.5B fertilizer annually split equally into two applications in May and August, 250 kg.ha$^{-1}$ of N in November and two foliar applications of copper hydroxide (1,5 kg.ha$^{-1}$) to prevent leaf and fruit diseases such as coffee leaf rust (*Hemileia vastatrix*) and leaf and fruit brown eye spot (*Cercospora coffeicola*). In all farms, trees were managed on a three-year rotational pruning cycle, where one every three rows is stump 40-100 cm above ground level depending of each farm management (only two and three year old shoot are productive). Soils from this coffee region have a common volcanic origin, generally with high organic matter content (8-15%) and moderately acid pH (4,5 to 5,5), major cations calcium and magnesium are generally deficient, as availably phosphorus too. Soil amendment is a strongly recommended practice done every year before rain season begins (May to November). In order to study the effects of elevation and shoot age on bean chemical content and beverage quality, three replicates of ten trees randomly selected in the same row on second year after pruning (Y1) and ten from third year (Y2) were harvested. The statistical design used was a split-plot, were the farm is considered as main plot and the year of the production as sub-plot.

### Trial 2

Samples from ten farms located in different Central America coffee regions in elevations between 700 to 1600 m were evaluated (See Table 1). Trial 2 samples represent the differences found in commercial coffee farms between soils, climatic conditions and agronomy practices used in each country. Because coppicing practices differ in each country, samples came from three year old trees of "Caturra, Catuaí, Pacas, Bourbon and Pacamara" cultivars randomly selected from shade and without-shade plots with 5000 plants.ha$^{-1}$ each. Caturra, Catuaí and Pacas are dwarf cultivars while Bourbon is a tall cultivar, Pacamara derived from (Pacas x Maragogype) and Maragogype is a mutant from Bourbon that produces low yield and very large fruit. In Costa Rica, plants received same inputs described before. In El Salvador and Honduras, plants received 800 kg/ha/year of N-P-K (20-10-10) in May and September and 150 kg/ha/year of N in November, along with 1 foliar application of copper hydroxide. Elevations were compared by ANOVA for the following groups of elevations (namely [700-899], [900-1099], [1100-1199], [1200-1399] and [1400-1600]).

### Harvest, processing and chemical analysis

Full ripe fruits were harvested and were prepared by wet processing method (wet de-pulping, 12 hours of anaerobic fermentation, sun-drying until reach 12% of water content and mechanical de-husking of parchment). For Trial 1, green coffee bean size percentage (diameter > 6,75 mm) was calculated. Dry weight of 200 beans was also recorded. For both trials, 50 g of dry green coffee sample from each farm was analyzed for caffeine, trigonelline, fat and sucrose content following Guyot et al. (1988) protocol. The analyses were performed by near infrared spectrometry by reflectance after grinding the green coffee to <0,5 mm. A NIR spectrometer system (Model 6500, NIRSystem, Inc. 1201 Tech Road Silver Spring, 20904, MD, USA) driven by NIRS2 (4.0) software (Intrasoft Int. LLC, Rd. 109, Sellers Lane, Port Matilda) was used for biochemical determination.

**Table 1. Sample origin description: farm, location, elevation (m), presence of shade and variety of Trails 1 (Costa Rica) and 2 (Central America).**

| | Farm | Location | Elevation | Shade | Cultivar |
|---|---|---|---|---|---|
| Trial 1 | La Trinidad | Alajuela, CR | 900 | No | Catuaí |
| | La Esperanza | | 1000 | No | Catuaí |
| | Bariloche | | 1100 | No | Caturra |
| | La Fortuna | | 1200 | No | Catuaí |
| | La Emilia | | 1300 | No | Catuaí |
| | La Luisa | | 1350 | No | Catuaí |
| | Alsacia | | 1400 | No | Caturra |
| | Loma Bonita | | 1450 | No | Catuaí |
| --- | | | | | |
| Trial 2 | ICAFE | Pérez Zeledón, CR* | 680 | No | Caturra, Catuaí |
| | San José | Usulatán, ES* | 850 | Yes | Caturra |
| | Hda. San Rafael | Naranjo, CR | 930 | Yes | Caturra |
| | San Jorge | Santa Ana, ES | 1120 | Yes | Pacas, Bourbon |
| | CICAFE | Heredia, CR | 1120 | No | Caturra, Catuaí |
| | Pirineos | Usulatán, ES | 1250 | Yes | Catuaí, Bourbon |
| | El Milenio | Auchapán, ES | 1350 | Yes | Bourbon, Pacamara |
| | Las Lagunas | Marcala, Hon* | 1440 | Yes | Caturra, Catuaí |
| | Doka | Alajuela, CR | 1400 | No | Catuaí, Pacamara |
| | Solís | Dota, CR | 1580 | No | Caturra, Catuaí |

*(*) CR = Costa Rica, ES = El Salvador, Hon = Honduras*

**Table 2. Elevation effect on beverage quality of samples from Trial 1 (Costa Rica).**

| | Elevation | Flavor | Body | Acidity | Bitterness | Preference |
|---|---|---|---|---|---|---|
| | 900 | 3,30 ab | 2,76 ab | 2,53 dc | 1,93 a | 2,53 c |
| | 1000 | 3,45 a | 2,90 ab | 3,32 ab | 1,58 ab | 3,12 ab |
| | 1100 | 3,30 ab | 2,56 b | 2,40 d | 1,99 a | 2,53 c |
| | 1200 | 3,0 b | 2,58 b | 3,32 ab | 1,22 b | 2,41 c |
| | 1300 | 3,53 a | 2,85 ab | 2,85 bc | 1,53 ab | 2,75 ab |
| | 1350 | 3,48 a | 2,96 ab | 3,37 a | 1,55 ab | 3,16 a |
| | 1400 | 3,51 a | 2,83 ab | 3,16 ab | 1,51 ab | 3,25 a |
| | 1450 | 3,61 a | 3,19 a | 3,41 a | 1,35 b | 3,38 a |
| F probability | 0,01 | 3,61 a | 3,19 a | 3,41 a | 1,35 b | 3,38 a |

*(*) Means within a column separated for Duncan test, P=0,05*

**Beverage quality assessment**

A sample of 150 g of large bean size green coffee from each treatment from trial 1 was roasted for 10-11 minutes a 230°C. Cup quality tests were carried out on an infusion prepared with 12 g of ground coffee in which 120 ml of boiling water was added, when temperature descend to 50-60°C. A panel of nine professional judges tasted three cups of each sample. The main beverage attributes (aroma, body, acidity) were estimated using a scale ranging from 0 to 5, where 0 = nil, 1 = very light, 2 = light, 3 = regular, 4 = strong and 5 = very

strong. A preference score was used ranging from 0-5 where, 0 = not good for drinking, 1 = very bad, 2 = bad, 3 = regular, 4 = good and 5 = very good. The tests were repeated two times. Values presented are means of the two tasting sessions.

**Data analysis**

Statistica© software (Statsoft, Inc. 1993) was used to perform all statistical analyses. The mean values of relevant factors were compared by the Duncan test at $P \leq 0.05$.

## RESULTS

**Effect of elevation on coffee bean size (Trial 1) and chemical composition (Trials 1 and 2)**

In Trial 1 coffee bean size was significantly affected by the elevation (Figure 1). The higher is the elevation, higher is proportion of large size beans. This relation is observed until 1400 m. At 1450 m, bean size decreased significantly. Dry weight increased as well with the elevation. Elevation had significant effect on chemical composition in Trials 1 and 2; trigonelline, caffeine, fat and ACG contents were dependent of elevation.

**Effect of elevation on beverage quality (Trial 1)**

Samples from 1300 m and higher elevations were preferred for cuppers than those elevations below, with exception from samples from 1000 m farm (Table 2). Higher elevation samples showed more acidity and aroma, and less bitterness than those from lower elevations. Samples from 1100 m and below presented more bitterness and less acidity.

**Effect of shoot age on bean size and dry weight (Trial 1)**

Shoot age had significant effect over dry weight of 200 beans sample (P = 0,03). Dry weight from beans produced on two year old shoot (Y1) was slightly higher than three year old (Y2) [33,45 and 33,01 respectively] (Data no shown).

## DISCUSSION

Ripening problems due to extremely cloudy and cold temperatures can explain why the size, dry weight and fat content of the green bean decrease at 1450 m of elevation in Trial 1, these data suggest that in extremely high growth coffee areas, in years with rainfall excess (when lack of dry season avoid to concentrate flowering induction) and when prolonged low temperatures periods set up especially during coffee ripening, a yield lost can be hide for the producer.

Elevation influence on cup quality and chemical content is very well known, but this is the first report of a work done commercial conditions in the same area, with equal tree conditions (Trial 1) and where same cultivar trees with equal age and physiological condition but growth in different elevations (Trial 2) are compare for different chemical content variables.

Results demonstrates that good quality coffee can be produce in intermediated elevation if soil conditions and agronomy management are efficiently conducted, and that this quality also comes together with high levels of fat content, caffeine and chlorogenic acids.

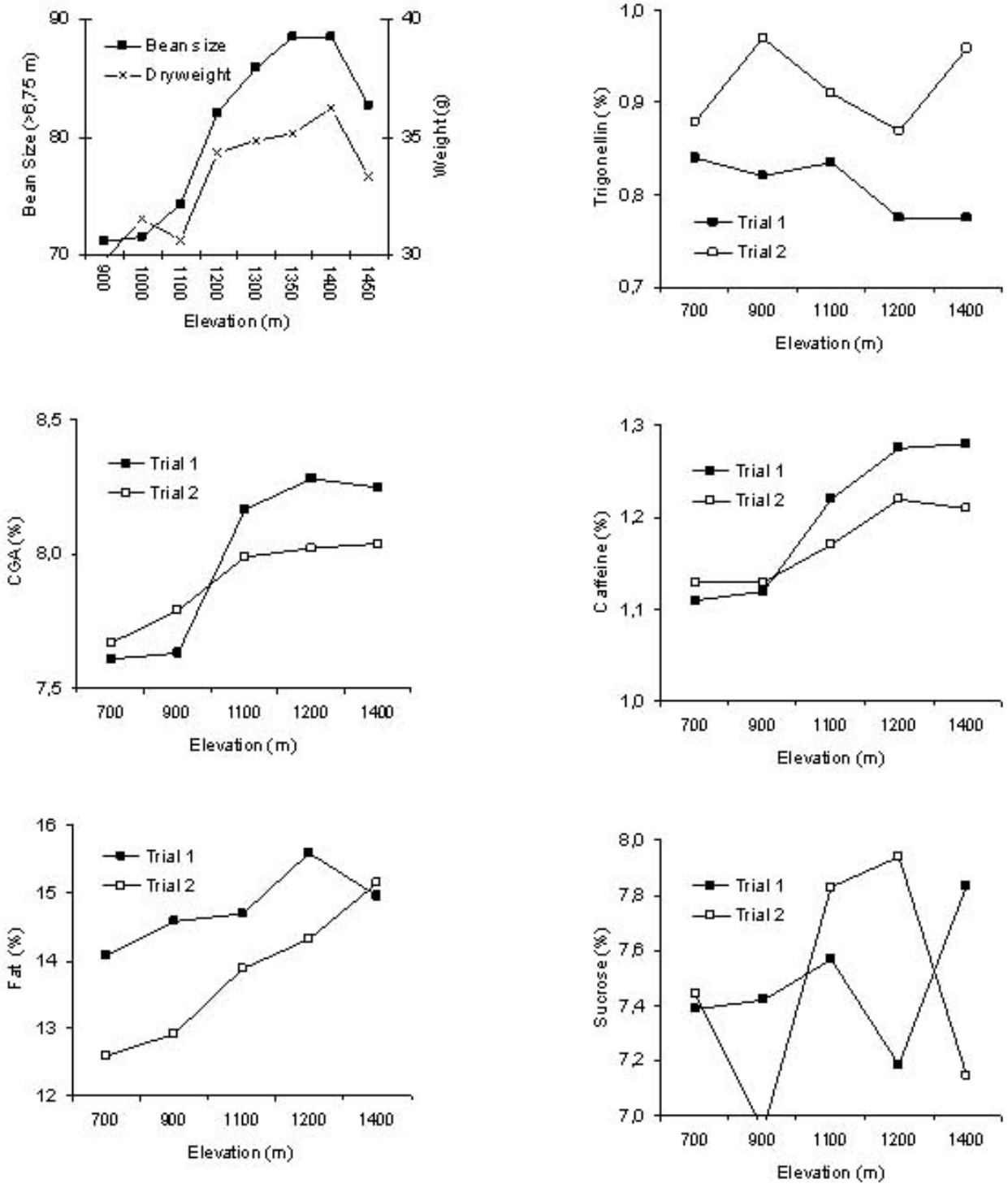


**Figure 1. Elevation effect on bean size for Trial 1, and chemical composition for Trials 1 and 2**.

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# Evaluation of Chinese Made Moisture Meters

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## SUMMARY

Coffee drying is a systematic operation, which contributes greatly to the visual and intrinsic attributes of the bean. Improperly dried coffee results in various **"Off-Flavours"** in the cup. Under-dried coffee samples turns **"Mouldy"** and imparts **"Mouldy"** taste in cup and over-dried coffee causes **"Woody"** taste in cup. In India, different types of moisture meters viz., Kappa moisture meter, Sinar moisture analyzer, Digital coffee moisture analyzer and Digital moisture meter are being used for rapid moisture measurement for the routine quality control purpose.

In the present study, two different types of Chinese made moisture meters viz., a) QCS- 3Z model moisture meter and b) LDS- ID Moisture Analyzer were evaluated for their suitability and widespread field use by operators in the coffee chain. These moisture meters were evaluated at two levels viz., pre- calibration and post– calibration. Initially, these moisture meters were checked for their accuracy in moisture readings as such (without calibration) and then calibrated using green coffee samples of known moisture content. The results of this study revealed that:

1. In case of QCS-3Z model Chinese moisture meter, "Channel-2" is found suitable for measuring the coffee bean moisture while, "Channel-1" is found suitable in respect of LDS- ID Moisture Analyzer.
2. Both the types of Chinese made moisture meters viz, "QCS-3Z model Chinese moisture meters" and LDS-ID Moisture Analyzer are highly reliable for moisture determination of green coffee samples having a wider range of moisture from 8.0 to 15%.

## INTRODUCTION

Drying is an important post harvest operation in coffee processing intended to reduce the moisture content of the harvested cherries/wet parchment to a level, which will not be detrimental during storage of coffee. The harvested cherries in most cases have a moisture range of 60-65% which is brought down to a safer moisture level of 10% in the case of parchment and 10.5% and 11% in the case of arabica and robusta cherry respectively. These safer moisture levels, apart from providing storage stability and easy removal of husk/parchment, also helps in keeping the enzymatic activity and mold growth at minimum.

In India, at estate level, the most commonly practiced method of moisture determination is **"Test Weight".** The test weight is expressed in-terms of kg/forlit (40 liter). It is followed that when a forlit of parchment (both arabica and robusta) records 15.5 kg, arabica cherry 16kg and robusta cherry 18kg, the moisture content deemed to have reached 10% in the case of parchment (both arabica and robusta) and 10.5% and 11% in respect to arabica and robusta

cherry. This test weight method always does not ensure the desired moisture level in the bean, as the size of bean is not uniform (Coffee Guide, 2000).

To-date, different types of moisture meters viz., Kappa moisture meter, Sinar moisture analyzer, Digital coffee moisture analyzer, Digital moisture meter and Farmex MT-3 grain moisture testers are being used for measuring moisture content in green coffee samples (Gopinandhan, 2003; Gopinandhan et al., 2001).

A study was undertaken by Coffee Board of India during the year 2003 to evaluate the Chinese made moisture meters for their suitability and widespread field use by operators in the coffee chain. Under this study, two types of moisture meters viz. a) QCS- 3Z model moisture meter and b) LDS- ID Moisture Analyzer received from FAO, Rome during April 2003 were evaluated at the Analytical Laboratory, Bangalore, Coffee Board of India. The results of this study are discussed in this paper.

## MATERIALS AND METHODS

### A) Preparation of Standard Coffee Samples

25nos. of green coffee samples were collected randomly and their moisture content was determined following the procedure set out in the ISO 6673–1983(E) Green Coffee-Determination of Loss in Mass at 105°C (5). Out of 25 nos. of samples tested, 15 nos. of coffee samples having a moisture range from 7.6 to 14.75% were selected for further work.

### B) Pre-Calibration Evaluation of Chinese Moisture Meters

As a first step, all the four Chinese moisture meters were checked for their accuracy in moisture readings without calibration by using green coffee samples of known moisture content. In the case of QCS- 3Z model Chinese moisture meters, moisture readings were recorded in all the four channels while, Channel –1 (P-1) was used for recording moisture content in case of LDS- ID Chinese Moisture Analyzer. Moisture readings of the same samples were also recorded using SINAR Moisture Analyzer (SINAR-AP 6660-Moisture Analyzer Model) for comparison purpose. The data on moisture percent (%) was analyzed for Standard Deviation (Sd) as well as Coefficient of Correlation (r), to see which of the Chinese made moisture meters or which channel of the given Chinese model moisture meter is showing nearest readings. The statistical analysis of the above data is given in Table 1.

### C) Calibration of Chinese Model Moisture Meters

All the four Chinese moisture meters were calibrated following the guidelines given in the operating manual supplied along with the instrument and also based on the experience gained in the calibration of various moisture meters at Analytical Laboratory, Coffee Board of India. Moisture meters were calibrated using green coffee samples of known moisture content ranging from 8.6 to 14.7%.

### D) Post - Calibration Evaluation of Chinese Model Moisture Meters

After calibrating channel “2” of the three “QCS- 3Z Moisture Meters” and Channel “P-1” of the “LDS- ID Moisture Analyzer”, green coffee samples of un known moisture collected randomly were used for recording their moisture content using all the four Chinese moisture meters. Soon after recording moisture readings from different meters, the samples were checked for their moisture by oven dry method.

**Table 1. Pre-calibration evaluation of Chinese model moisture meters.**

| | | MOISTURE PERCENTAGE OF GREEN COFFEE SAMPLES * | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample No. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | r-value |
| Oven Dry Method (ISO Method NO. 6673) | | 7.6 | 8.4 | 8.4 | 8.5 | 8.6 | 8.6 | 9.2 | 9.3 | 9.5 | 9.7 | 10.5 | 12.9 | 13.2 | 14.2 | 14.75 | |
| Sinar Moisture Analyzer *** | | 7.8 | 8.5 | 8.2 | 8.5 | 8.9 | 8.6 | 9.7 | 9.9 | 9.9 | 10.3 | 10.9 | 13.5 | 14.1 | 15.8 | 15.9 | 0.99** |
| LDS-ID Moisture Analyzer | | 9.9 | 10.8 | 10.6 | 10.6 | 10.8 | 11 | 11.8 | 11.7 | 11.8 | 11.8 | 12.8 | 15.3 | 16.5 | 16.9 | 18.2 | 0.99** |
| QCS-3Z (A) | Ch-1 | 13.3 | 15 | 14.2 | 14.9 | 15.5 | 15.2 | 18.2 | 18.8 | 18.2 | 19.3 | 20.5 | 24.8 | 23.1 | 24.2 | 24.8 | 0.95** |
| | Ch-2 | 9.0 | 10.2 | 9.6 | 10.1 | 10.5 | 10.3 | 12.4 | 12.8 | 12.3 | 13.1 | 13.9 | 16.8 | 15.7 | 16.4 | 16.9 | 0.95** |
| | Ch-3 | 6.2 | 7.0 | 6.6 | 7.0 | 7.3 | 7.2 | 8.5 | 8.8 | 8.4 | 9.0 | 9.6 | 11.7 | 10.8 | 11.4 | 11.7 | 0.95** |
| | Ch-4 | 10.7 | 12.1 | 11.4 | 12 | 12.5 | 12.3 | 14.7 | 15.2 | 14.6 | 15.5 | 16.5 | 20.2 | 18.6 | 19.5 | 20 | 0.95** |
| QCS-3Z (B) | Ch-1 | 9.0 | 9.7 | 9.4 | 9.5 | 10.3 | 10.1 | 12.2 | 12.5 | 12.1 | 12.7 | 13.7 | 16.7 | 15.7 | 16.8 | 17.4 | 0.96** |
| | Ch-2 | 7.5 | 8.0 | 7.8 | 7.9 | 8.5 | 8.3 | 10.1 | 10.4 | 9.9 | 10.5 | 11.3 | 13.8 | 13.0 | 13.9 | 14.4 | 0.96** |
| | Ch-3 | 4.4 | 4.8 | 4.7 | 4.7 | 5.1 | 5.0 | 6.0 | 6.2 | 5.9 | 6.3 | 6.8 | 8.2 | 7.8 | 8.3 | 8.6 | 0.96** |
| | Ch-4 | 15.4 | 16.6 | 16.1 | 16.2 | 17.5 | 17.4 | 20.8 | 21.3 | 20.5 | 21.7 | 23.3 | 28.4 | 26.8 | 28.5 | 29.7 | 0.96** |
| QCS-3Z (C) | Ch-1 | 13.2 | 14.3 | 14.5 | 14.6 | 15.7 | 15 | 18.4 | 17.7 | 17.9 | 19.1 | 19.7 | 24.6 | 22.6 | 23.9 | 24.5 | 0.95** |
| | Ch-2 | 8.7 | 9.56 | 9.6 | 9.7 | 10.5 | 9.9 | 12.2 | 11.8 | 12 | 12.8 | 13.2 | 16.4 | 15.1 | 16 | 16.4 | 0.95** |
| | Ch-3 | 6 | 6.6 | 6.6 | 6.7 | 7.2 | 6.9 | 8.4 | 8.1 | 8.2 | 8.8 | 9.1 | 11.4 | 10.4 | 11 | 11.3 | 0.95** |
| | Ch-4 | 10.2 | 11.1 | 11.1 | 11.3 | 12.1 | 11.6 | 14.2 | 13.6 | 13.8 | 14.7 | 15.2 | 19 | 17.5 | 18.5 | 19 | 0.96** |
| Average | | 9.26 | 10.18 | 9.92 | 10.15 | 10.73 | 10.49 | 12.45 | 12.54 | 12.33 | 13.02 | 13.80 | 16.91 | 16.06 | 17.02 | 17.57 | |
| Co-Variance (%) | | 8.33 | 9.97 | 9.40 | 9.77 | 11.16 | 10.76 | 16.27 | 16.53 | 15.57 | 18.61 | 19.66 | 29.48 | 24.45 | 26.90 | 28.84 | |
| Standard Deviation (S.D) | | 2.99 | 3.27 | 3.17 | 3.24 | 3.46 | 3.40 | 4.18 | 4.21 | 4.09 | 4.36 | 4.59 | 5.62 | 5.12 | 5.37 | 5.56 | |

** - Average of Two Replications: r-value - Correlation of Co-efficient; **- Highly Significant; ***- Sinar Moisture Analyzer was calibrated during February 2003 using coffee samples of known moisture content ranging from 8% to 13%.*

**Table 2. Evaluation of Chinese model moisture meters (after calibration).**

| | Moisture Percent (Average of three readings) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | r-value |
| Oven Dry Method | 8.6 | 9.2 | 10.6 | 13 | 13.3 | 14.1 | 14.7 | |
| Type of Meters | | | | | | | | |
| Sinar Moisture Analyzer*** | 8.4 | 9.3 | 10.2 | 13.6 | 13.1 | 14.9 | 15.6 | 0.99** |
| QCS-3Z (A) | 8.5 | 9.9 | 10.9 | 12.8 | 12.9 | 13.9 | 14.3 | 0.99** |
| QCS-3Z (B) | 8.7 | 10 | 11.1 | 12.8 | 13.3 | 14 | 14.4 | 0.99** |
| QCS-3Z(C) | 8.7 | 10.2 | 11 | 13.1 | 13.3 | 13.9 | 14.4 | 0.99** |
| LDS- ID Moisture Meter | 8.6 | 9.2 | 10.5 | 12.4 | 13.3 | 14 | 15.5 | 0.98** |
| Average | 8.5833 | 9.6333 | 10.7166 | 12.9500 | 13.2000 | 14.1333 | 14.8167 | |
| C.V. (%) | 0.0114 | 0.1689 | 0.0980 | 0.1325 | 0.0233 | 0.1222 | 0.2847 | |
| SD | 0.1169 | 0.45019 | 0.3430 | 0.3987 | 0.16733 | 0.3827 | 0.5842 | |

*r-value - Correlation of Coefficient; **- Highly Significant; ***- Sinar Moisture Analyzer was calibrated during February 2003 using coffee samples of known moisture content ranging from 8% to 13%.*

The moisture data obtained after calibration of the four Chinese moisture meters was again subjected to statistical analysis to find out Standard Deviation, (sd) Co-Variance (cv) and Correlation Coefficient (r) between oven readings and Chinese moisture meters and the same is given in Table 2.

## RESULTS AND DISCUSSION

The Chinese made moisture meters were evaluated at two levels viz., pre- calibration and post-calibration. The results on the pre-calibration performance of these Chinese model moisture meter were given in Table 1.

As could be seen from the table 1 that in case of QCS- 3Z model Chinese moisture meters, comparison of meter readings of the four channels, Channel-2 (left to right) of all the three QCS- 3Z model Chinese moisture meters recorded least deviation from the oven readings. Hence, Channel-2 of QCS-3Z model Chinese moisture meter was selected for calibration and further evaluation. While in case of LDS- ID Moisture Analyzer Channel P-1 was calibrated and further evaluated.

The observations on the post-calibration performance of the Chinese model moisture meters were given in table-2 which indicated that the differences between the moisture readings of Chinese moisture meters and oven readings are found to be least and highly significant. Statistical analysis of these data indicated high degree of correlation between Chinese moisture meters and oven readings.

## CONCLUSION

In conclusion, the results of the present study revealed that:

1) Both the types of Chinese made moisture meters viz, "QCS- 3Z model Chinese moisture meters" and LDS- ID Moisture Analyzer are highly reliable for moisture determinations of green coffee samples having a wider range of moisture from 8.6 to 15%.
2) In case of QCS- 3Z model Chinese moisture meters, Channel "2" should be used for measuring the coffee bean moisture, while in LDS- ID Moisture Analyzer Channel "1" can be used for measuring the coffee bean moisture after appropriate calibration against a standard method.

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ISO 6673–1983 (E) Green Coffee- Determination of Loss in Mass at 105°C.

# Survey of Indian Coffee Samples for Ochratoxin-A (OTA) Contamination

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## SUMMARY

Ochratoxin-A (OT-A) is a naturally occurring mycotoxin produced by moulds belongs to the genera of *Penicillium* and *Apsergillus*. The presence of OT-A contamination in coffee is currently much discussed in the international circle as, it is reported to be nephrotoxic and possibly carcinogenic.

Under FAO/CFC/ICO sponsored multi-country global project on **"Enhancement of Coffee Quality through Prevention of Mould Formation"** an analytical laboratory was established at the Coffee Board of India. A total of 113 green bean samples from commercial trade have been analysed for OT-A content as per the internationally accepted norms. Prior to the establishment of the Analytical laboratory, the Board had a collaborative project with National Institute of Nutrition, Indian Council of Medical Research Institute (ICMR), Hyderabad under which a total of 158 green coffee samples were analysed for OT-A content. Out of the 271 samples analyzed, 257 samples had OT-A levels below 5 parts per billion (ppb) including those samples with below detection limits. Only 14 samples had OT-A levels more than 5 ppb. The highest level of OT-A recorded was 11.7 ppb in a robusta cherry sample. The results of this screening exercise indicated that majority of the green coffee samples analyzed had insignificant level of OT-A contamination. Indian green coffee samples will be able to meet maximum permissible limits prescribed for green coffee by various importing countries and also will be able to meet the proposed norms of the European Union (EU).

## INTRODUCTION

In recent time, reports on the occurrence of Ochratoxin-A (OT-A) in coffee are causing much concern in the trade. OT-A is a mycotoxin produced by three main species of fungi, *Aspergillus ochraecus, A. carbonarius* and *Penicillum verrcucosum* with a minor contribution from *A. niger* (WHO Food Additives Series 47, 2001). OT-A is a potent nephrotoxic agent (Hald, 1991) and has been classified by the International Agency for Research on cancer into the group 2B as possibly carcinogenic to humans (Solfrozzo et al., 1998).

In coffee, the occurrence of OT-A has attracted worldwide attention ever since the Hungarian Health Authorities first rejected a coffee consignment during 1994 citing excess levels of OT-A. Coffee seeds are liable to mould and OT-A contamination especially, if they are not dried

to properly or if re-hydrated during any stage of drying, storage and transportation (Bucheli et al., 2000).

Determination of OT-A levels in export and commercial coffees have been extensively carried out since the first report of OT-A contamination in coffee by Levi et al. (1974). Around the globe, several investigators have demonstrated the natural occurrence of OT-A in green coffee (Micco et al., 1989; Studer-Rohr et.al., 1993; Nakajima et. al., 1997) and commercial coffee (Patel et al., 19979; van der Stegen et al., 1997; Jorgensen, 1998; Wolff, 2000). Extensive sampling of green coffee from various origins has shown that OT-A contamination may be more frequent in some areas but no producing country is entirely free from OT-A contamination.

The Coffee Board of India has initiated a series of Research Studies in 1994-95 to find the causative factors for mould and OT-A formation in coffee and to identify the critical stages during production and processing of coffee with an aim to formulate preventive/control strategies. An Analytical laboratory was established at the Coffee Board of India during 2001 with capacity to analyse OT-A as per the internationally accepted norms. A total of 113 green bean samples from commercial trade have been analysed for OT-A content. Prior to the establishment of the Analytical laboratory, the Board had a collaborative project with National Institute of Nutrition, Hyderabad under which about 158 green coffee samples were analysed for OT-A content.

The present paper summarizes the over all results generated so far on the extent of Ochratoxin-A (OT-A) levels in various types of coffee samples of Indian origin.

## MATERIALS AND METHODS

### Coffee Samples

Raw and Green coffee samples of various types (Parchment/Cherry) of different varieties (Arabica/Robusta) and also Monsooned and Organic coffees were collected from various loci (Curer, Trader and Auction Platforms). The coffee samples upon collection were oven-dried at 100°C for overnight and then milled finely using Cyclotec Sample Mill. The powdered coffee samples transferred into a double-layered polythene bags, labeled properly and stored in refrigerator until further analysis.

Extraction and purification of OT-A from samples were carried out according to the method of Patel et al. (1997) with modifications as follow:

### Extraction of OT-A from Samples

Ten grams of finely powdered green coffee sample was taken into a 250 ml capacity amber colored conical flask. The OT-A from the samples was extracted with 50 ml of dichloromethane containing 5 ml 0.1 M Orthophosphoric acid. The sample flasks were placed on a orbital shaker set at 200 rpm for an hour time. The crude extract was filtered through Whatman No.1 filter paper and collected in a 250 ml capacity amber colored RB flask. The crude filtrate was evaporated using a rotary evaporator set at 40°C. The residue was dissolved in 5 ml of dichloromethane.

**Purification of Crude Extract**

Clean up of the crude extract was performed by taking 10 gram of activated silica gel and slurry was prepared in dichloromethane and packed in a glass column (1.5 dia x 30 cm L). After silica gel slurry settled, sodium sulfate anhydrous was added. The residue was loaded on to silica gel column and drained by gravity. The column was washed with 100 ml dichloromethane: methanol (97:3) followed by diethyl ether: hexane (75:25) and both the washate were discarded. OT-A was eluted with 100 ml of toluene- acetic acid (90:10). The washate is collected in a 250 ml capacity amber colored RB flask and evaporated using a rotary evaporator at 40°C. The residue was again re-dissolved in 5 ml toluene-acetic acid (90:10) and transferred to 10 ml capacity amber colored pear shaped flaks and evaporated using a rotary evaporator set at 40°C. The final residue was dissolved in a small volume of HPLC mobile phase, filtered through nylon membrane (0.2 micron and 13 mm dia) and used for analysis in HPLC.

**Analysis of OT-A using HPLC Instrument**

HPLC analysis was performed using Acetonitrile (55%) Water (45%) and Acetic Acid (2%) as the mobile phase with a flow rate of 1ml/min. The excitation maximum was 330nm and emission at 460nm. OT-A was detected and quantitated by comparison of peak areas of samples and standard OT-A.

**RESULTS AND DISCUSSIONS**

The levels of OT-A found in the green coffee samples analyzed were shown in Table 1 and 2.

**Table 1. Extent of OT-A levels in different types of Indian coffee samples [Results of the study carried out at National Institute of Nutrition (NIN), Hyderabad under a sponsored programme for 3 years from October 1997 to 2000].**

| Type of Coffee Samples | Number of Samples Analyzed | Ochratoxin-A Level (ppb) | | | | | |
|---|---|---|---|---|---|---|---|
| | | No. of Samples and Range of Contamination | | | | | |
| | | BDL | 0.5-1.0 | 1.1 - < 5 | 5.1 - < 10.0 | 10.0-20 | 20-30 |
| Arabica Parchment | 74 | 39 | 21 | 11 | 01 | 02 | NIL |
| Arabica Cherry | 07 | 02 | 01 | 03 | Nil | 01 | |
| Robusta Parchment | 21 | 13 | 06 | 02 | Nil | Nil | |
| Robusta Cherry | 20 | 06 | 06 | 05 | Nil | 03 | |
| Whole Cherry | 16 | 00 | 07 | 08 | Nil | 01 | |
| Monsooned Coffee | 20 | 03 | 06 | 08 | 01 | 02 | |
| Total | 158 | 63 | 47 | 37 | 02 | 09 | |

In general, cherry samples were recorded higher levels of OT-A contamination than parchment samples. Examination of analytical results given in table 1 and 2 revealed that out of 271 samples analysed, a total of 257 (95%) coffee samples had OT-A contamination below 5 ppb including those samples with below detection limits. Only 14 samples had OT-A levels more than 5 ppb, concentration ranging from 5.1 to 11.77 ppb. The highest level of OT-A recorded was 11.7 ppb in a robusta cherry sample.

**Table 2. Extent of OT-A levels in different types of Indian coffee samples [Results of the study carried out at Analytical Laboratory, Coffee Board, India under the on–going FAO/CFC/ICO Multi-Country Global Project on "Enhancement of Coffee Quality through Prevention of Mould Formation"].**

| Type of Coffee Samples | No. of Samples Analyzed | Ochratoxin-A Level (ppb) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of Samples and Range of Contamination | | | | | | | |
| | | BDL | 0.5-1.0 | 1.1-2.0 | 2.1-3.0 | 3.1-4.0 | 4.1-5.0 | 5.1-15 | 15-30 |
| Arabica Parchment | 45 | 09 | 33 | 2 | 1 | Nil | Nil | Nil | NIL |
| Arabica Cherry | 14 | 01 | 11 | 1 | 1 | Nil | Nil | Nil | |
| Robusta Parchment | 27 | 08 | 17 | 2 | Nil | Nil | Nil | Nil | |
| Robusta Cherry | 14 | 00 | 5 | 1 | 3 | 1 | 1 | 3 | |
| Monsooned Coffee | 09 | 00 | 8 | 1 | Nil | Nil | Nil | Nil | |
| Organic Coffee | 04 | 00 | 04 | Nil | Nil | Nil | Nil | Nil | |
| Total | 113 | 18 | 78 | 07 | 05 | 01 | 01 | 03 | |

*BDL- Below Detection Limit*

## CONCLUSION

In conclusion, the OT-A levels detected in the present study indicated that green coffee samples from India will be able to meet the maximum limits prescribed for green coffee by various importing countries (ranging from 5 to 10 ppb) and also will be able to meet the proposed maximum permissible limits of 3-4 ppb and 6-10 ppb prescribed for roasted coffee and soluble coffee respectively by the European Commission (EC).

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# Isolation of the Diterpene 16-O-Methylcafestol from *Coffea Canephora* var. *Robusta* by High Speed Countercurrent Chromatography (HSCCC)

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## SUMMARY

High speed countercurrent chromatography was successfully used for the isolation and purification of 16-O-methylcafestol (16-OMC), a diterpene from *Coffea canephora* var. *robusta*. 16-OMC is a useful analytical parameter for the discrimination of the two coffee species *arabica* and *canephora* var. *robusta*.

After saponification of extracted coffee lipids, the unsaponifiable matter was separated by HSCCC. The solvent system consisted of hexane / ethyl acetate / ethanol / water 5/2/5/2 (v/v/v/v). The identity and purity (>95%) of the isolated compound was verified by means of thin layer chromatography, high-performance liquid chromatography / mass spectrometry as well as NMR spectroscopy.

## ZUSAMMENFASSUNG

Das Diterpen 16-O-Methylcafestol (16-OMC) konnte erfolgreich aus Robusta-Röstkaffeebohnen mittels der High-Speed Countercurrent Chromatography (HSCCC) isoliert werden. Die Bestimmung des Gehaltes an 16-OMC ist eine wichtige Methode zur Differenzierung der beiden Kaffeevarietäten *Coffea arabica* und *Coffea canephora* var. *robusta.*

Nach Verseifung des Kaffeeöls wurde der unverseifbare Anteil mittels HSCCC aufgetrennt. Die Trennung erfolgte mit einem Gemisch aus Hexan / Ethylacetat / Ethanol / Wasser in einem Verhältnis von 5/2/5/2 (v/v/v/v). Die Identität und die Reinheit (> 95%) der isolierten Verbindung wurde mit Hilfe von Dünnschicht-chromatographie, RP-HPLC, LC-MS sowie NMR-Spektroskopie bestimmt.

## INTRODUCTION

Coffee is the most popular beverage in Germany. Each German drinks round about 160 litre of coffee-beverages in one year (second place: beer 120 litre) (Deutscher Kaffeeverband, 2003).

Of major importance for the world coffee production are the two species *coffea arabica* and *coffea canephora* var. *robusta*. The two economical important species differ especially in flavour and, because of that, in price, too. A unit of high quality arabica costs nearly twice as much as robusta coffee (Deutscher Kaffeeverband, 2003). Often the high-quality arabicas, described as “Pure arabica blend” or “Highland coffee”, are mixed with less expensive

robustas. Therefore it is very important to have a possibility for controlling the quality of the products with appropriate methods for the discrimination of the two species.

Robusta beans are smaller in size and rounder in shape than arabica beans. Hence, a differentiation by comparison of the bean-size before roasting and grinding is possible. Also quantities over 15% of robusta in arabica blends can be detected by sensory testing (Speer et al., 1991). But the most important method for discrimination is an analysis of the ingredients. Latest research has described the discrimination between the two species on the basis of their amino acid enantiomers (Casal et al., 2003).

Another very useful analytical parameter is the content of the diterpene 16-O-methylcafestol (16-OMC), which is only present in robusta coffee (Speer et al., 1991). Therefore a DIN-method for the determination of 16-OMC in roasted coffee has been developed employing reversed phase high-performance liquid chromatography (DIN 10779, 1999). This method allows detection of up to 2% of robusta in arabica blends (Speer et al., 1991).

When we started our investigation, there was no 16-OMC commercially available. The lack of an authentic reference compound prompted us to develop an efficient method for the preparative isolation of 16-OMC using the all-liquid chromatography technique of HSCCC.

## COUNTERCURRENT CHROMATOGRAPHY (CCC)

Countercurrent Chromatography is an automated version of liquid-liquid extraction, comparable to the repeated partitioning of an analyst between two immiscible phases by vigorous mixing in a separatory funnel. Modern CCC started in 1970 with the development of the so-called Droplet Countercurrent Chromatography (DCCC).

The real breakthrough of CCC came with the invention of the Coil Planet Centrifuge that was introduced by Ito in 1981. Today this technique is known as High-speed Countercurrent Chromatography (HSCCC).

HSCCC separation takes place in a so-called “multi-layer coil” that is made by wrapping inert Teflon tubing around a holder in multiple layers. Consequently the technique is also known as Multilayer Coil Countercurrent Chromatography (MLCCC). The tubing usually has an inner diameter between 1.6 and 2.6 mm and the length can reach 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and the sample capacity. During separation the coil is rotated in a planetary fashion; it rotates at 800 to 1000 rpm around its own ‘planetary’ axis and simultaneously around a parallel ‘solar’ axis. This planetary rotation has two effects:

1. The rotation creates a fluctuating acceleration field, which enables vigorous mixing of the two phases followed by settling within the coil. In areas of the coil, which are close to, the centre of rotation the force field is weak. As a consequence the phases are mixed. At a further point of their orbit, when they are far away from the centre of rotation, the force field becomes stronger and the two phases are separated. Alternate mixing and settling is repeated with each rotation and in this way up to 50000 partitioning steps per hour can be achieved.
2. Rotation of the coil also enables retention of stationary phase. During rotation of a coil filled with two immiscible liquids it can be observed that the two phases move towards opposite ends of the coil, known as **head** and **tail**. Generally the less dense phase displaces the heavier phase towards the tail, but the orientation is also to be influenced by viscosity and interfacial tension. This phenomenon, known as hydrodynamic equilibrium,

requires choosing the elution mode carefully but it also gives the analyst the freedom to select either the lighter or the heavier as mobile or stationary phase. When the heavier phase is selected as the mobile phase, the proper elution mode is **head to tail** and the mobile phase is introduced from the head of the system. By choosing the lighter phase as the mobile phase the elution order of the compounds is reversed, the correct elution mode is **tail to head** and the mobile phase is pumped into the tail of the system.

CCC as an all liquid chromatographic technique operates under gentle conditions and allows non-destructive isolation even of labile natural compounds. Due to the absence of any solid stationary phase, adsorption losses are minimised and hence a 100% sample recovery is guaranteed.

## ISOLATION

For the isolation of 16-OMC, roasted robusta coffee beans (India) were ground and extracted with *tert*-butyl methyl ether (TBME). The coffee lipids were saponified under reflux with ethanolic potassium hydroxide and the unsaponifiable matter was separated by liquid-liquid extraction using TBME (Casal et al., 2003). After removal of the solvent, the residue was separated by HSCCC.

Solvent system used for the separation of 16-OMC consisted of hexane / ethyl acetate / ethanol / water 5/2/5/2 (v/v/v/v, the less dense layer as the stationary phase, flow rate: 4.0 ml/min). 1g of the unsaponifiable matter could be applied for each HSCCC separation in one run (Figure 1).

## IDENTIFICATION

First control of identity and purity of the received HSCCC-fractions was made with TLC on silica gel (mobile phase diethyl ether-chloroform 1/1). 16-OMC shows a characteristic red coloration after spraying with a solution of vanillin/sulphuric acid (Speer and Mischnick, 1989).

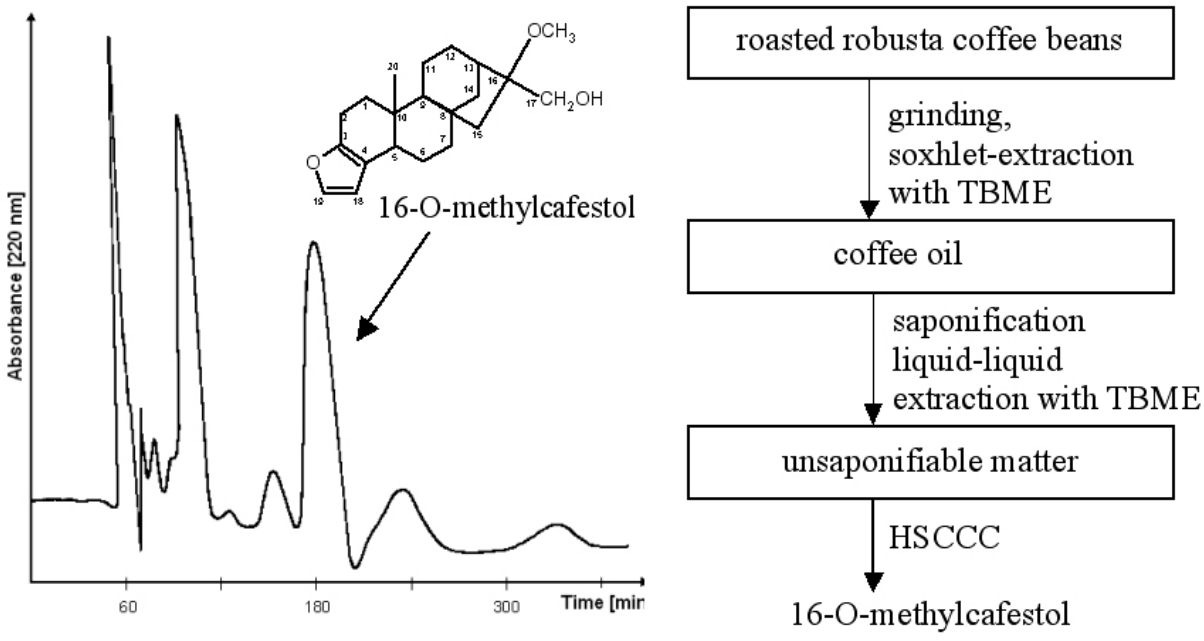


**Figure 1. HSCCC-separation and work-up protocol for the isolation of 16-OMC.**

Moreover the identity and purity (> 95%) of the isolated compound was verified by means of high-performance liquid chromatography / mass spectrometry as well as NMR spectroscopy.

## YIELD

140 mg of pure 16-OMC was obtained in a single run.

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# Flavour Perception of White Coffee Beverages – Oral Breath Sampling and Sensory Analysis of the Influence of Milk Proteins

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## SUMMARY

Enriched milk proteins and different recombined 'milks' were added to a coffee beverage. Sensory analysis in particular intensity tests was performed. In addition to the sensory analysis, apparative analysis was performed too. The retronasal perception was examined by 'Oral Breath Sampling (OBS)' and GC/MS analysis. From the results of the intensity tests, flavour profiles were derived. The investigations showed that all additives affect the flavour release from the coffee beverage in both the orthonasal and the retronasal way.

## INTRODUCTION

In the presence of proteins, the flavour release is reduced because of the proteins' capability to form covalent and reversible hydrogen bonds with the flavour substances (Leland, 1997). Kinsella (1990) indicated that proteins form different bonds with aldehydes and ketones due to their different size, structure and surface. Investigations of Buecking and Steinhart (2002) showed that different milk additives have a retarding effect on the flavour release of roasted coffee. An objective of this study was the investigation of the influence of defined manufactured milk products and constituents on the orthonasal and retronasal perception of the coffee flavour. For the investigation of the retronasal flavour, a method based on studies by Roozen and Legger-Huysman (1994) was developed which makes it possible to examine the flavour in the oral cavity directly after the consumption of coffee.

## MATERIAL AND METHODS

### Coffee

Arabica coffee beans from Kenya were dark roasted (roast degree: 70 scale division; roasting temperature: 266°C, supplied by Tchibo, Hamburg, Germany) and the coffee brew was prepared in a household coffeemaker with 42 g coffee powder and 800 mL water.

### Milk products

Different protein quantities (casein and whey proteins) were always suspended in 30 g water to avoid dilution effects. Additionally, 'milk' recombinants ('milk' with 3.5% fat and 2.8% [recombinant A], 4.2% [recombinant B] or 5.6% [recombinant C] casein) were supplied. The addition to the coffee amounted to 100% (1:1, w:w).

### Sensory Analysis

The coffee beverage was placed in paper cups closed with a cap. The flavour was tested by a panel of up to 16 assessors (from a pool of 24). All panellists had previously received a special training in intensity sensory evaluations of coffee drinks. The assessors performed an intensity test (DIN 10966, 1997) for smell and taste of the coffee beverage. The descriptors used were the same as in a previous study (Buecking and Steinhart, 2002) and the intensities of these attributes were assessed using a discrete six-point scale from 0 (no smell / taste) to 5 (predominant smell / taste).

### Oral Breath Sampling

A 'Gas Sampler' (Gerstel, Germany) equipped with a mouthpiece was used as 'Oral Breath Sampler (OBS)'. An assessor took a quantity of 20 mL fresh brewed coffee for a defined period (25 sec) into the mouth area and rinsed the oral cavity with it. After the swallowing of the beverage, the lips enclosed the mouthpiece. While the assessor breathed by the nose, the gas area of the oral cavity was sucked off by the 'Oral Breath Sampler'. With a flow of 0.3 L/min and a volume of 1.0 L the duration amounted to approx. 3.3 min; i.e. at a number of three assessors the duration of the sampling is approx. 10 min. In this period the temperature of the coffee beverage sank from about 55°C to 45°C. The volatiles were collected on Tenax TA and then examined via thermal desorption, cryo focusing and GC-MS analysis (GC: HP 5890 GC series II; MSD: HP 5971; column: OV-1701).

## RESULTS AND DISCUSSION

### Sensory results

On investigating single components, the influence of the milk proteins was obvious in the sensory analysis, particularly with the coffee descriptors. As shown for some tracers (selected aroma compounds) in the GC/MS-analysis (see Figure 3), the whey protein additive exhibited a saturation effect with higher amounts (data not shown). In contrast to the OBS tests, the whey protein showed a smaller influence than the casein. With the casein additions, the influence on the milk descriptors rose evenly with rising amounts of casein (Figure 1). At first, the coffee descriptors showed a small influence in the taste range, which increased strongly. For the odour descriptors, a clear influence was immediately recognizable which flattened with higher amounts of added casein. An acid-binding effect of the casein is prominent in the taste range.

This phenomenon was also observed by Fischer and Widder (1997) while examining the flavour release from aqueous casein solutions. With the casein-enriched recombined milks, the smaller perception of the roasty odour in the flavour profile (Figure 2) may be due to protein - flavour interactions. It has to be noted that the effects of the casein are stronger here than with the addition of the pure casein, because first of all the absolute casein amount in the recombinants exceeded the highest added amount in the investigations with the single components and secondly synergistic and antagonistic effects with other milk constituents (e.g. milk fat) are conceivable.

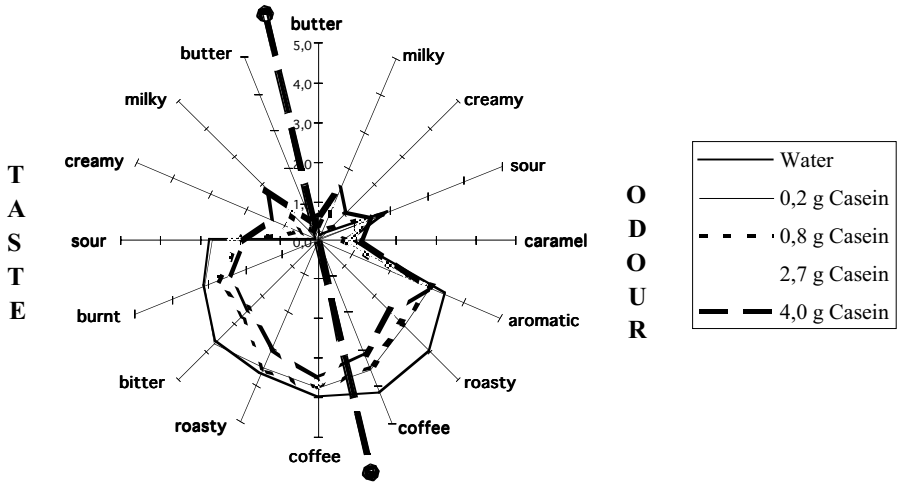


**Figure 1. Intensity Test – odour and taste profiles of black coffee and of coffee with different amounts of casein additive.**

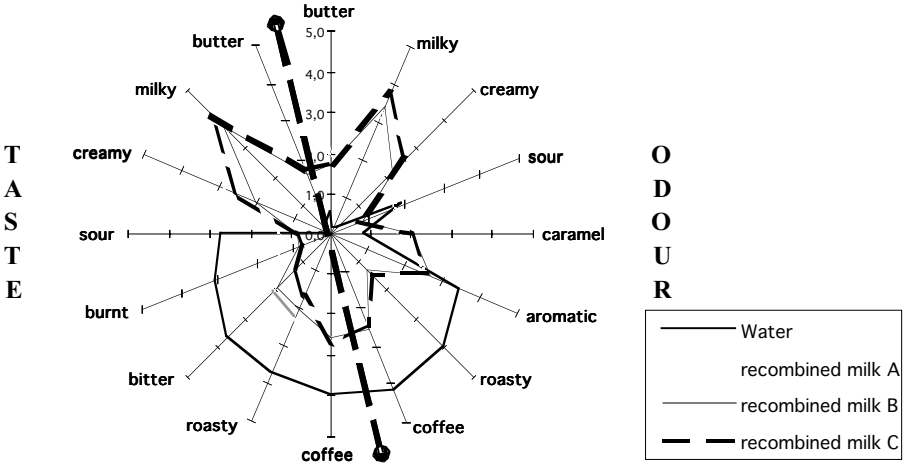


**Figure 2. Intensity test – odour and taste profiles of black coffee and of coffee with milk additives (increasing casein content).**

## Instrumental results

Additional information's are obtained by the instrumental analysis. However the results are not directly comparable with the results of the sensory analysis, because the tracers cover only a small range in the total flavour. With the addition of whey protein, an obvious decrease in the release of all tracers to partly only 50% (2-methoxyphenol) with increasing whey protein addition was observed (Figure 3).

The retardation of 2,3-pentanedione, cis-4-heptenal, phenylacedaldehyde, 2-ethyl-3,5-dimethylpyrazine and 2-methoxyphenol (guaiacol) could not be increased by adding more whey protein to the coffee beverage. Though a similar effect was observable with the addition of casein, the retardation was weaker than with the whey protein. For cis-4-heptenal, phenylacetaldehyde and 2-ethyl-3,5-dimethylpyrazine even a slight increase in the flavour release was recognizable before it decreased with higher amounts of the casein additive. With the addition of the recombined milks, a reduction of the flavour release was observable for most of the tracers, whereby the recombinant A displayed the strongest effect (Figure 4). An exception was the 2,3-butanedione, here the flavour release even increased. An increased

flavour release appeared also with cis-4-heptenal, phenylacetaldehyde and 2-ethyl-3,5-dimethylpyrazine at the addition of recombinant B, which comprised a casein content of 4.2 g. With a further increase of the casein amount (to 5.6 g), the release decreased.

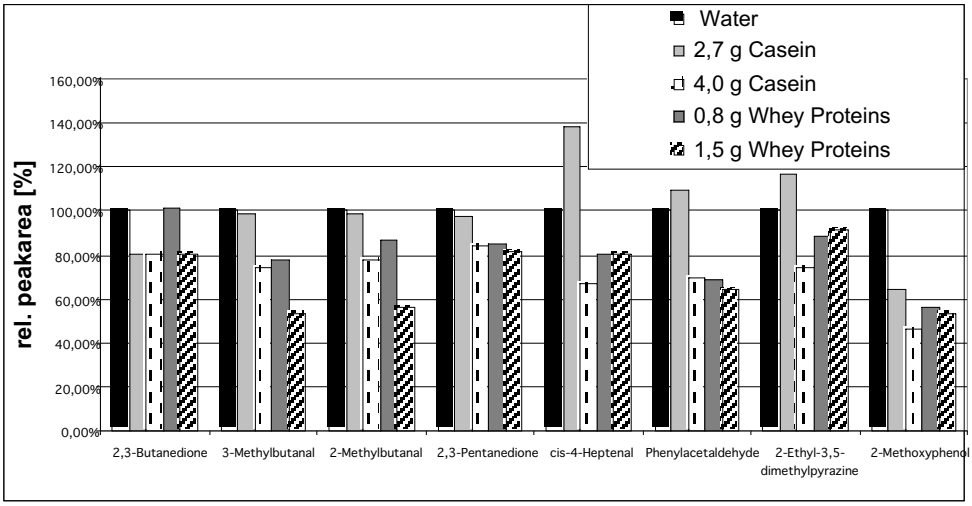


**Figure 3. Oral Breath Sampling - Effect of milk proteins on flavour perception: GC/MSD peak areas (average of three panellists) of some tracers shown as percentage related to black coffee (only water additive).**

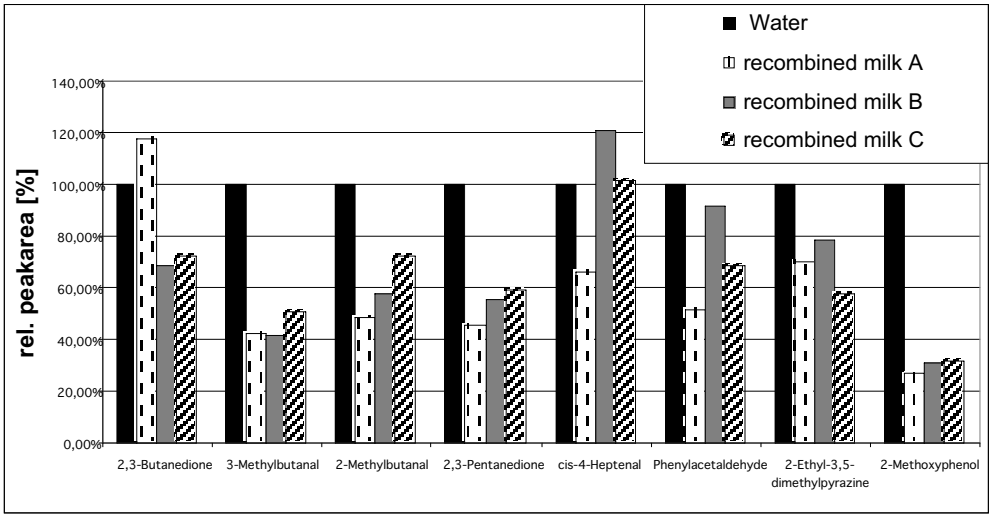


**Figure 5. Oral Breath Sampling – Influence of different casein-content in milk: GC/MSD peak areas (average of three panellists) of some tracers shown as percentage related to black coffee (only water additive).**

In Addition to the OBS, external headspace analysis (orthonasal perception) was performed (data not shown). In the case of the retronasal perception, only a small reduction of the release was observable. A possible explanation may be that the retarding properties of the added proteins compete with the releasing properties of the saliva, which results in a lower effect upon the flavour release.

## ACKNOWLEDGEMENTS

The technical assistance of Geertje Drichelt and Jana Paucke is gratefully acknowledged. Thanks apply to our sensory panel and to the Tchibo-Frisch-Röst-Kaffee GmbH for supplying the coffee beans.

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# Analysis of Enantiomeric Linalool Ratio in Green and Roasted Coffee

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## SUMMARY

Enantiomeric distribution of the monoterpene alcohol linalool (3,7-dimethylocta-1,6-dien-3-ol) was determined by multi dimensional gas chromatography (MDGC) and dual column switching (DCS) GC-MS for the first time in green and roasted coffee after extraction by simultaneous distillation extraction (SDE) and stir bar sorptive extraction (SBSE). Sensory impact of the two enantiomers was evaluated in water and by addition experiments to espresso beverage. Only a slight excess of the (S)-isomer in green and roasted coffee was found. Depending on post harvest treatment higher enantiomeric excess was found in dry processed samples.

## ZUSAMMENFASSUNG

Die Enantiomerenverteilung des Monoterpenalkohols Linalool (3,7-Dimethylocta-1,6-dien-3-ol) wurde mittels multi-dimensionaler Gaschromatographie (MDGC) und Säulenschaltung (DCS) in Kombination mit GC-MS das erste Mal in grünem und geröstetem Kaffee bestimmt. Als Extraktionstechniken wurden die simultane Destillation-Extraction (SDE) und die mikro Festphasenextraktion mittels beschichtetem Rührfisch (SBSE, Twister) verwendet. Die Erkennungsschwellen in Wasser und Espresso wurden bestimmt und sensorisch bewertet. In Kaffee konnte ein kleiner Überschuss des (S)-Isomers festgestellt werden, der von der Erntenachbehandlung des Kaffees abhing. Trocken aufgearbeiteter Kaffee zeigte einen höheren Anteil an (S)-Linalool.

## INTRODUCTION

Linalool (3,7-dimetyl-1,6-octadien-3-ol) is an important flavour and fragrance compound. It contributes to the characteristic aroma of a vast number of natural products like fruits and spices, as well as tea and chocolate (Bernreuther and Schreier, 1991; Wang et al., 1994; Jiang and Kubota, 2004; Bazemore et al., 2003). Two optical isomers with different odour profiles and thresholds are described: 3S-(+)-linalool is perceived as sweat, floral, pertigrain-like (odour threshold 7.4 ppb) and the 3R-form as more woody and lavender-like (0.8 ppb) (Ohloff and Klein, 1962; Padrayuttawat et al., 1997).

Plants mostly produce only one linalool isomer, so that the enantiomeric excess can be used as an indicator for authenticity. The R-form e.g. prevails in basil oil (*O. basilicum* L.) and Japanese pepper (*X. piperitum* DC.), whereas the 3S-(+)-linalool dominates in orange oil, strawberries and cocoa products (Preis, 1997). Plants with a balanced distribution of both enantiomers are also known (e.g. pineapple). Recently changes in the enantiomeric

composition during beer production has been monitored using stable isotope dilution analysis (Steinhaus et al., 2004).

In coffee, enantiomeric separation of chiral compounds has been reported only for short chain strecker products (Stalcup et al., 1993; Maier et al., 1999), aliphatic alcohols as well as limonene and α-terpineol (Stalcup et al., 1993).

In the case of coffee authors already speculated about the influence of processing and beverage preparation on the chirality of small molecules (Stalcup et al., 1993).

## MATERIALS AND METHODS

### Coffee

Coffee beans (*Coffea arabica*) originating from Dominic Republic, Brazil and Guatemala were ground after liquid nitrogen cooling. Roast coffee was freshly prepared in a lab scale prototype roaster (Research & Technical Development, Illycaffè). Colour was characterised by a prototype lab scale leucometer to a colour equivalent to Italian roast (equivalent 38, LKB, Dr. Lange, Berlin, Germany). Grinding of roast coffee was performed by a blade mill (Super Jolly, Mazzer Luigi, Venezia, Italy). Preparation of Espresso beverage was achieved by Gaggia home-user espresso machine using standard conditions (Illy and Viani, 1995).

### Extraction techniques

The green or roast coffee volatiles were extracted by simultaneous distillation extraction (SDE, Normschliff Geraetebau, Wertheim, Germany) or by stir bar sorptive extraction (SBSE, Twister, Gerstel, Muehlheim, Germany) from a liquid infusion or a cup of espresso coffee, respectively. A comparison between SDE (Illy and Viani, 1995) and SPME, a technique similar but less sensitive than SBSE, has been recently published (Garcia-Esteban et al., 2004).

### Instrumental analysis

The MDGC system consisted of two Fisons Mega 8000 gas chromatographs coupled by a Moving Column Stream Switching (Garcia-Esteban et al., 2004) (MCSS, Carlo Erba, Germany) device. The pre-column was a Carbowax (30 m x 0.25 mm, 0.25 df), ramped from 35°C at 4°C/min to 240°C. Split injection was applied, with a constant head pressure of 235 kPa of Helium. Pressure in the dome was set to 125 kPa for the Lipodex (Heptakis (2,3,6-tri-O-methyl)-beta-Cyclodextrine/Polysiloxan, M&N, Germany) main column. Both detectors were FIDs.

The MDGC-MS was built in a 5973N GC-MS system (Agilent, Waldbronn, Germany). A Carbowax pre-column (60 m x 0.25 mm) was coupled via dual column switching (DCS, Gerstel) device to the same main column as in MDGC. Liquid extracts were injected in split mode (1:5). A Twister (length 1 cm, film thickness 0.5 mm) was desorbed in TDSA (thermal desorption unit and autosampler, TDS2, Gerstel) in splitless mode and trapped in the programmable temperature vaporizer (PTV). PTV was in solvent venting mode at 50 ml helium flow during desorption, rapidly ramped from –120 to 250°C and starting run at constant pressure of 195 kPa. Pressure in the cross piece was kept at 46 kPa during a ramp from 70 to 110°C at 5°C per minute on pre- and main column, followed by an isothermal period at 90°C and heat out. Control flow was switched off to transfer the linalool peak from 34.8 to 35.5 minutes to the chiral column, analysing by SIM in electron impact for ions m/z 71,93,121 and 55 (100ms dwell time each).

## References

Samples of 3R- and 3S-linalool were a gift by Dr. C. Margot, Firmenich, 3R- and racemic linalool was purchased from Aldrich (Milano, Italy).

## RESULTS AND DISCUSSION

In multi dimensional gas chromatography (MDGC) a switching device is used to transfer the target compounds after pre-separation on a first column onto a second column with different separation characteristics. In the present case, a first separation of the SDE extract of green coffee was performed on a polar Carbowax column. As second column a permethylated cyclodextrine phase was used in order to achieve separation of the enantiomers of linalool. By using only FID detection one has to be aware that even with a very small cut window, more compounds than only the linalool peak can be transferred from the complex mixture of coffee volatiles using the MCSS system. For clear and undoubted identification of the isomers eluting from the chiral column, mass spectrometry must be applied, even though the MDGC system already suggested enantiomeric excess of S-(+)-linalool. Crucial is the MS identification especially in roasted coffee, as the number of known volatile compounds rises from 300 for green coffee, to about 1000 in roasted coffee. A schematic outline of the system used for the analysis is shown in Figure 1, the resulting chromatogram for a liquid extract from Guatemala roast coffee is presented in Figure 2.

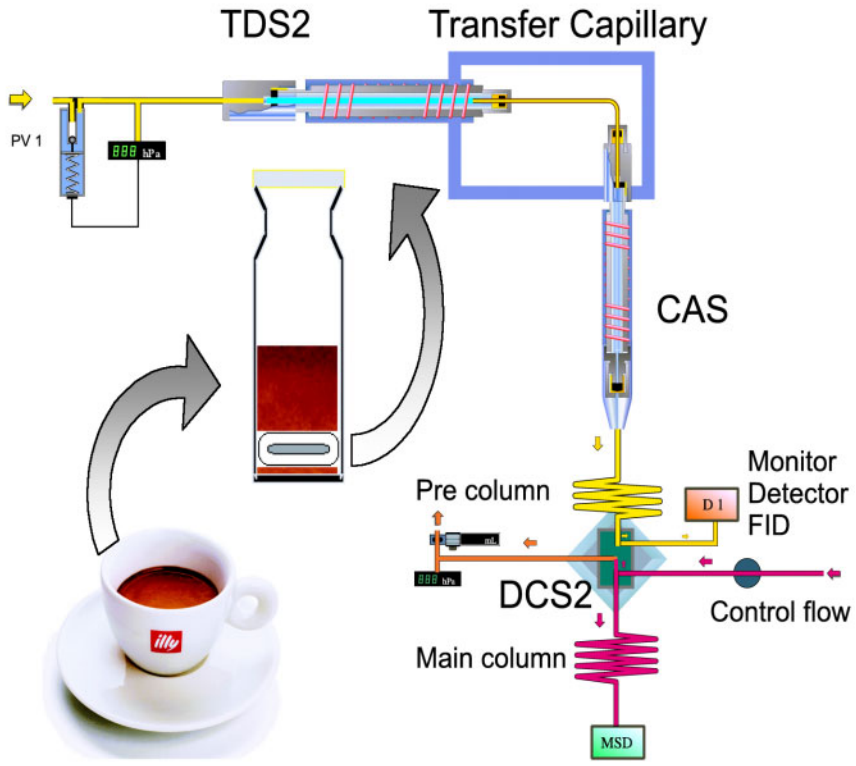


**Figure 1. TDS-MDGC-MS in control flow on, coffee volatiles get registered at monitor detector and then vented off (control flow supplies carrier for main column and MSD).**

The analysis confirmed a small excess of S-enantiomer of linalool (ee 13.2% S). Stir bar sorptive extraction (SBSE) was used to extract the monoterpene alcohol linalool from coffee preparations in a more gentle manner, as racemisation of linalool under acidic extraction conditions is known (Sulzbach, Patent DE 4017909) to occur. However, no differences in the enantiomeric ratio were observed between the two sample preparation procedures.

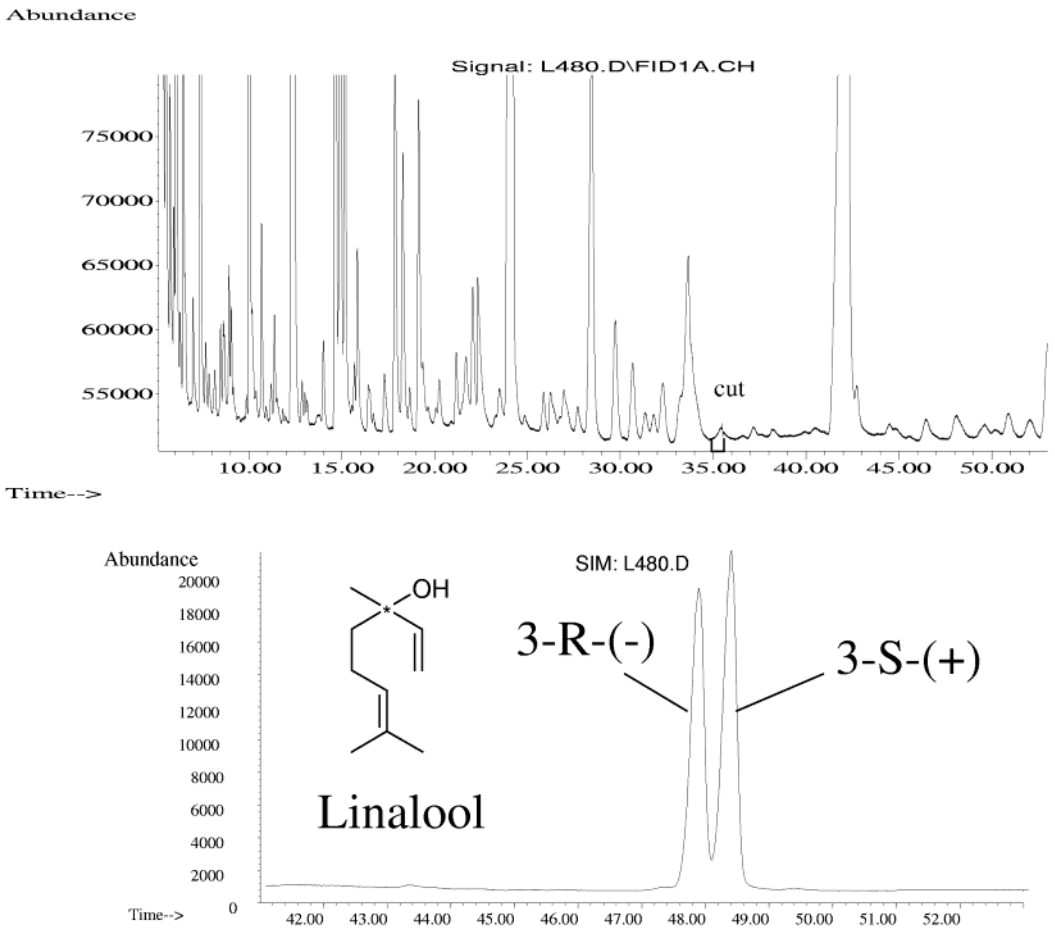


**Figure 2. MDGC-MS Separation of roast coffee from Guatemala upper chromatogramm carbowax (60 x 0.25 mm, FID) lower permethylated cyclodextrine phase (25 x 0.25 mm, SIM).**

Still, the SBSE seems to be more favorable, since the total time for the analysis could be dramatically reduced as extraction took place during only 20 min at room temperature.

Analyses of coffees from different origins revealed massive differences in the enantiomeric ratios of linalool, thus contradicting the theory of authenticity.

**Table 1. Enantiomeric excess in arabica coffees from different origins and post harvest treatments**

| Origin (G/RG) | Post harvest | % ee (S)-Linalool (± SD, n=6) |
|---|---|---|
| Brazile RG | Dry | 22.8 ± 1.7 |
| Guatemala | Washed | 8.9 ± 1.6 |
| Santa Domingo | Dry | 45.0 |
| Santa Domingo | Washed | 11.7 |

After examination of the sample history and analysis of an identical coffee processed by the dry and the wet procedure, the observed differences could be correlated to the post harvest method (see Table 1). A possible explanation for the change of enantiomeric excess in favour of S-(+)-linalool for dry processed coffee could be due to the aroma precursors present in green coffee. Schreier et al. (2003) found only 3(S)-linalyl-glycosides, but not the

correspondent R-forms. Selmar and Bytof (2001) proposed that during dry processing coffee seeds are more active in metabolism, because of the slower drying the seed germination is initiated. To gain information about sensorial influences of the enantiomeric distribution we determined flavour recognition thresholds of the two stereoisomers in water and in espresso coffee. For water we found approximately ten times the detection threshold published by Padrayuttawat (1997), resulting in a recognition threshold of 95 ppb in water for S-linalool and 22 ppb for R-linalool. The best estimate threshold (BET) (Meilgaard et al., 1999) for each assessor was determined using the 3 alternative forced choice.

In espresso coffee the corresponding levels were 6.84 mg/l for S and 0.93 mg/l for R-linalool, respectively. Sensory description for the R-isomer in high concentrations was honey-like, fruity, caramel whereas the S-isomer elicited a flowery, slightly woody note.

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# Antioxidant Activity of Green Coffee Conserves

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## SUMMARY

Antioxidant-rich fractions were extracted from green coffee using methanol plus water mixtures. In arabica and robusta, methanol to water ratio of 60:40 gave highest yield of conserves followed by 70:30 and 80:20. The extracts were screened for their potential as antioxidants using in vitro models, such as ß-carotene-linoleate and 1,1-diphenyl-2-picryl hydrazyl (DPPH) model systems. The methanol to water ratio of 60:40 robusta coffee conserve showed 70 and 73% antioxidant activity at 200 ppm using the ß-carotene-linoleate and DPPH model systems, respectively. Similarly, the methanol to water ratio of 70:30 extracts of arabica coffee conserve showed 65 and 70% antioxidant activity at 200 ppm using the same model systems, respectively. The results showed that the ferulic and *p*-coumaric acids, made a greater contribution to total antioxidant activity than chlorogenic acids. The conserves exhibited maximum antioxidant activity than individual chlorogenic acids. An HPLC method has been developed to determine chlorogenic acids in green coffee extracts and this method has repeatability, precision, and rapidity.

## INTRODUCTION

Coffee is one of the important agricultural commodities is cultivated on 11.6 million ha and its production is 63 lakh tons (Indian Coffee 2003). As an agro based rural enterprise in India, this industry is a source of direct employment for about 4,00,000 people in the area of cultivation apart from providing indirect employment to many in processing and trade sections. Green coffee contains a large amount of polyphenols exemplified by chlorogenic acid, caffeic acid, ferulic acid ad p-coumaric acid (Myriam et al., 2001). Chlorogenic acid in green coffee has six sub groups namely, caffeoyl quinic acids (CQA), 2. p-coumaroylquinic acids (PCQA), feruloylquinic acids (FQA), dicaffeoyl quinic acids (diCQA), caffeoylferuloyl quinic acids (CFQA) and feruloylcaffeoyl quinic acids (FCQA) (Clifford 1985). In addition to CQA, ß-coffeoyl, l-tryptopha, ß-coffeoyl, l-tyrosine and traces of unesterified *e*-caffeic acid, ferulic acid and 3-*e*-*p*-coumaric acid have been reported (Clifford et al., 1989). Degradation of chlorogenic acids during roasting of green coffee was reviewed (Clifford, 1985). Chlorogenic acid largely undergoes hydrolysis to caffeic acid and quinic acid during roasting. Since quinic acid occurs at about 0.5% in roast coffee, the hydrolysed acids undergo further chemical changes. The antioxidant activity of this diverse group of compounds on the individual structuring number of hydroxyl groups present in coffee are compared with tea and wine (Myriam Richelle et al., 2001).

The chlorogenic acids is the group of compounds where the individual compounds showing its own function in the aspect of bioactivity. In the common beverage coffee, the phenolic acids such as caffeic acid ferulic acid and p-coumaric acid are in the bound form which (release their bonds after) on alkaline hydrolysis form in free acids. Of these, caffeic acid is found in high level in human blood plasma and most of the caffeic acid present in plasma is in bound form mainly in the glucuranate /sulphate forms (Nardini et al., 2002) due to absence of

free caffeic acid in coffee. Plasma caffeic acid is likely to be derived from hydrolysis of chlorogenic acid (CGA) in the gastrointestinal tract.

The food industry today is finding that the traditional commercial synthetic antioxidants such as BHA and BHT are becoming less/not acceptable, and a new range of natural antioxidants are to be developed to protect processed foodstuffs from rancidity and off flavors (Amito et al., 1997). Roasting markedly influences the composition of coffee; chlorogenic acids (CGA) credited with antioxidant property are lost during roasting of coffee beans (Clifford, 1989). The overall antioxidant property of coffee brews can be maintained, or even enhanced, by the development of compounds possessing antioxidant activity, including Maillard reaction products during roasting (Daglia et al., 2000). Investigations (Nicoli et al., 1997., Daglia et al., 2000, Richelle et al., 2001) demonstrate that antioxidant property of roasted coffee may be attributed to Maillard reaction products formed during roasting in addition to certain phenolic compounds that are present in green coffee including CGA, caffeic acid, ferulic acid and p-coumaric acid. The contribution of volatile heterocyclic compounds to the antioxidant activity of brewed coffee has also been reported (Fuster et al., 2000). Recently, effect of roasting on the antioxidant activity of coffee brews has been reported (Maria Dolores Del Castillo et al., 2002; Pojjana Charurin et al., 2002, Nidhi Singh and Madhava Naidu 2004).

An attempt has been made in this investigation to explore the potential of green coffee as a source of antioxidants. The aim of this work was to study the anti-oxidative activity of the conserves of green coffee beans prepared by employing methanol and water and to produce conserves exhibiting maximum antioxidant activity. The antioxidant activity of the extracts was determined using β-carotene-linoleic acid and DPPH model systems. Further, an attempt was made to characterize the coffee conserves/ extracts by HPLC.

## MATERIALS & METHODS

### Materials

Coffee seeds viz., Arabica parchment and Robusta parchment were procured from the local market. Caffeic acid, chlorogenic acid, 3,5 - dimethoxy hydroxy cinnamic acid, *o*-coumaric acid, p- coumaric acid, ferulic acid, β - carotene, 1,1-diphenyl-2-picryl hydrazyl (DPPH), linoleic acid, butylated hydroxy anisole (BHA), and Tween-80 were procured from Sigma-Aldrich, Chemical Co. (St.Louis,MO) USA. All solvents/chemicals used were of analytical grade and obtained from SD Fine Chemicals, Mumbai. UV-visible spectral measurements were taken using a GBC Cintra-10, Australia. Coffee conserves were de-solventized using Rotavapor (Buchi, USA). Coffee conserves were characterized using high performance liquid chromatography (Waters 2487, USA).

### Extraction

The green coffee beans were ground to a fine powder using Kamas hammer mill (Falling No. AB, S-12611 Stockholm, Sweden, Type 120) and then passed through 30 mm sieve. Ground coffee sample (20 g each in duplicate) was transferred to a glass column (2.5 X 18 cm) and extracted individually with a mixture of solvents, methanol and water ratio. Material to solvent ratio of 1:12.5 was used for extraction in each case. The solvent was added in 10 installments of 25 ml each allowing 30 minutes contact time after each addition. The extracts were drained and pooled. The pooled extracts were distilled on hot water bath under reduced pressure and the solvent-free extract was further distilled to remove water using the rotavapor and stored at $4^{o}$C till further use. The yields of extracts/conserves are presented in Table 1.

**HPLC Analysis**

Coffee bean extracts/conserves were dissolved in HPLC grade water and was injected on to a C18 column (25 cm x 4.6 mm id particle size 5 microns, pore size 100 A). The mobile phase used was a gradient of acetonitrite (containing 3.5% acetic acid) and water containing 3.5% acetic acid. The analysis was done with respect to assay of caffeic acid, chlorogenic acid, 3,5-dimethoxy hydroxy cinnamic acid, *o*-coumaric acid, p-coumaric acid, ferulic acid against respective reference standards (Figure 1 to 3). The percentage of respective acids was expressed as absolute assay as well as in terms of its chromatographic purity based on peak area normalization method. The detection was done using a UV detector set at 325 nm.

| | |
|---|---|
| Column | : 25 cm x 4.6 mm id particle size 5 microns, pore size 100 $A^o$ |
| Mobile Phase | : A : 3.5% acetic acid acetonitrile |
| | B : 3.5% acetic acid acetonitrile |
| Gradient form | : 10% at A to 30% of A over 10 minutes and back to10% of A after 15 minutes |
| Flow rate | : 0.7 ml/min. |
| Detector | : UV set at 325 nm. |
| Standard conc.: | 400 µg/ml prepared in water |
| Sample conc. : | 500 µg/ml prepared in water. |
| Sample size : | 20 µl |

**PROCEDURE**

Preparation of mobile phase

**Solvent A**

A measured quantity of HPLC grade acetonitrile (400 ml), and glacial acetic acid (14 ml) was mixed, stirred through 0.22 µ membrane filter at the same time degassing the solvent applying vacuum.

**Solvent B**

A measured quantity of HPLC grade water (400 ml) and glacial acetic acid (14 ml) was mixed, stirred well and filter through 0.22 µ membrane filter at the same time degas the solvent applying vacuum.

**Preparation of solutions**

*a) Preparation of standard solution*

Chlorogenic acid standard (4 ng) was transferred it into a 10 ml volumetric flask, dissolved in 2 ml of HPLC grade water and diluted up to the mark using HPLC grade water. The standard solution (400 µg/ml) was filtered through a 0.22 µ membrane filter using a syringe-based system.

*b) Preparation of sample solution*

Coffee conserve (25 mg) was transferred into a 50 ml volumetric flask, dissolved in 30 ml of HPLC grade water and diluted up to the mark using HPLC grade water. The sample solution had a concentration of 500 μg/ml.

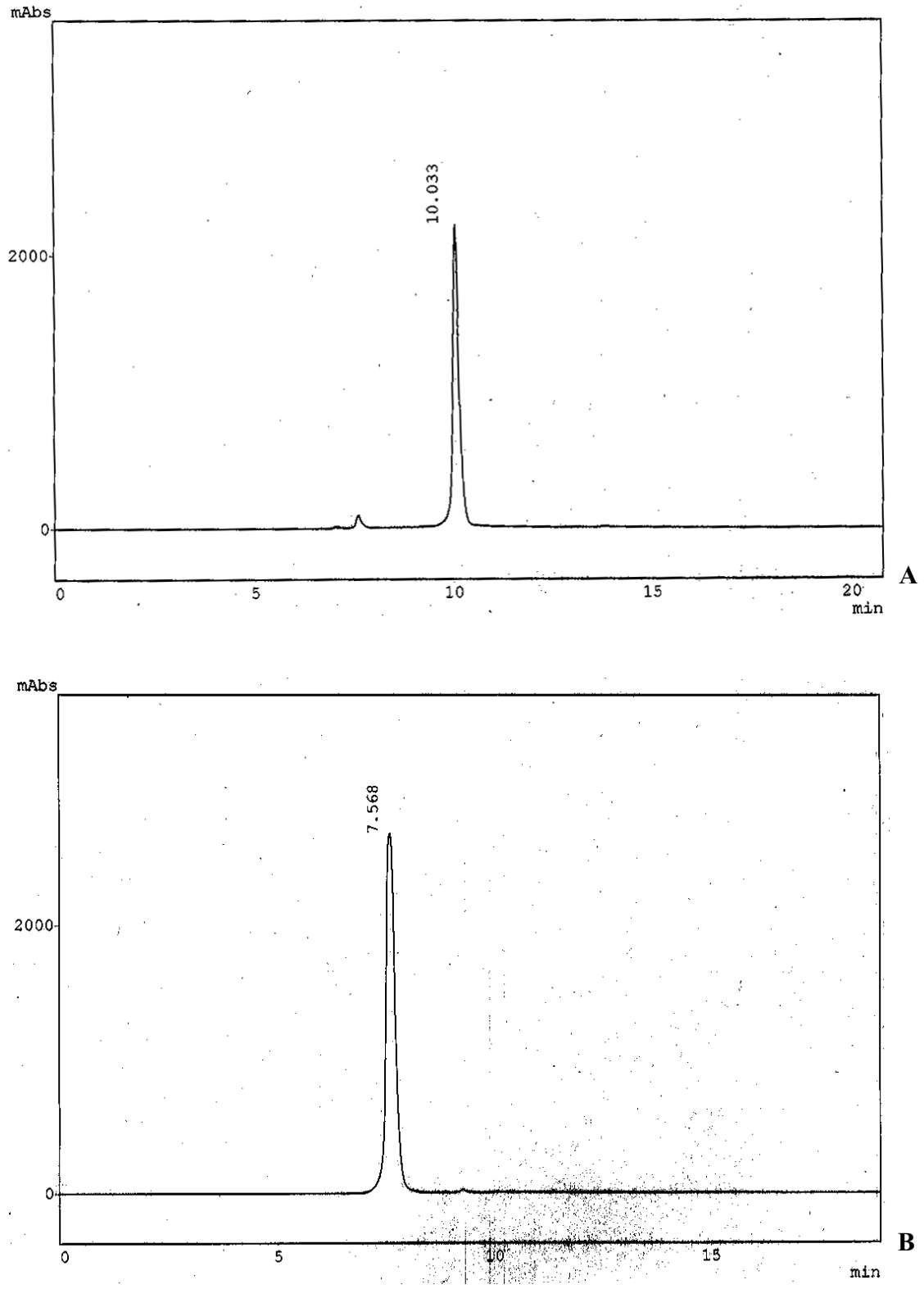


**Figure 1. A) HPLC chromatogram of standard *o*-coumaric acid; B) HPLC chromatogram of standard caffeic acid.**

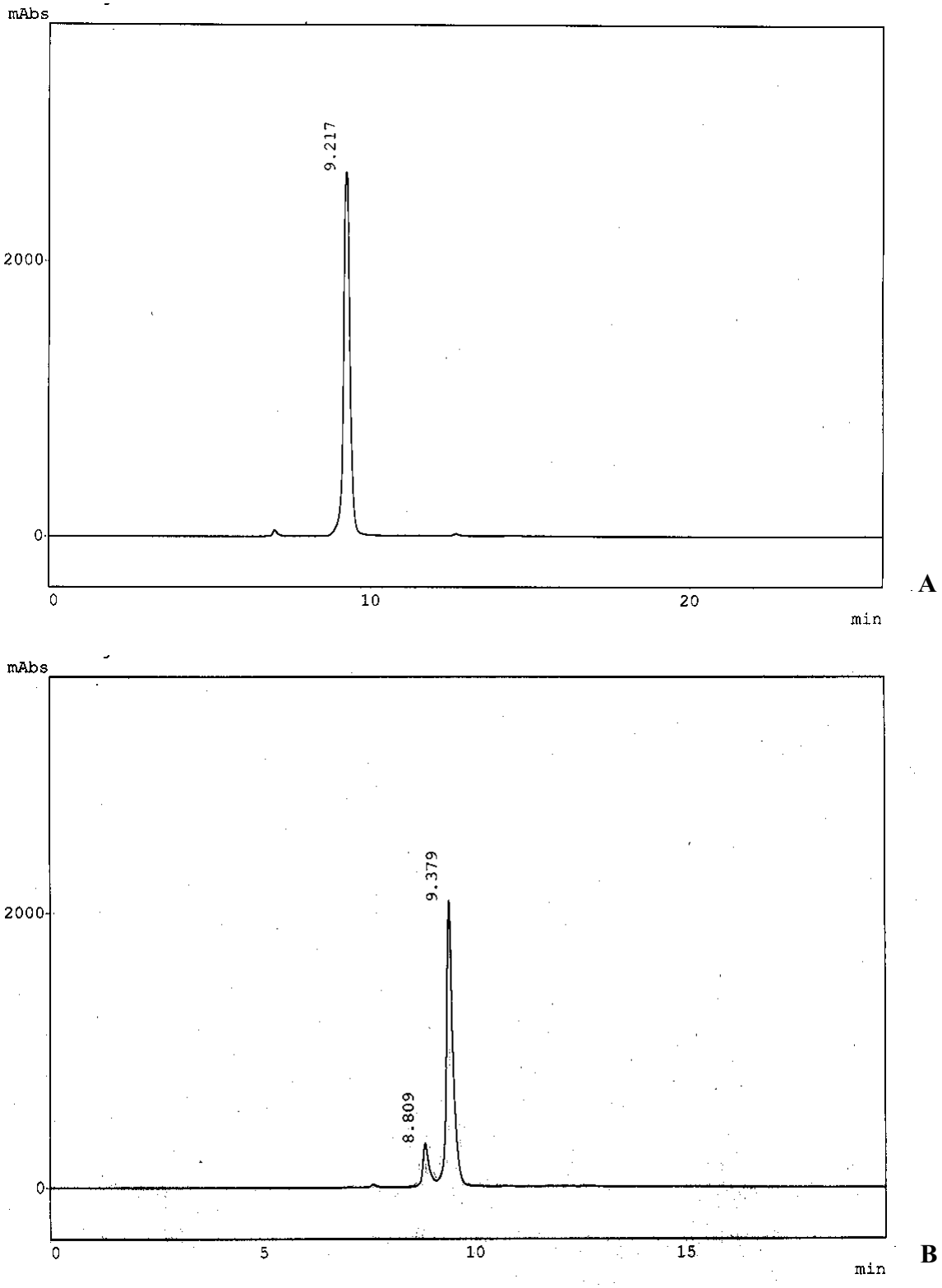


**Figure 2. HPLC chromatogram of standard ferulic acid; B) HPLC chromatogram of standard 3,5-dimethoxy-4-hydroxy-cinnamic acid.**

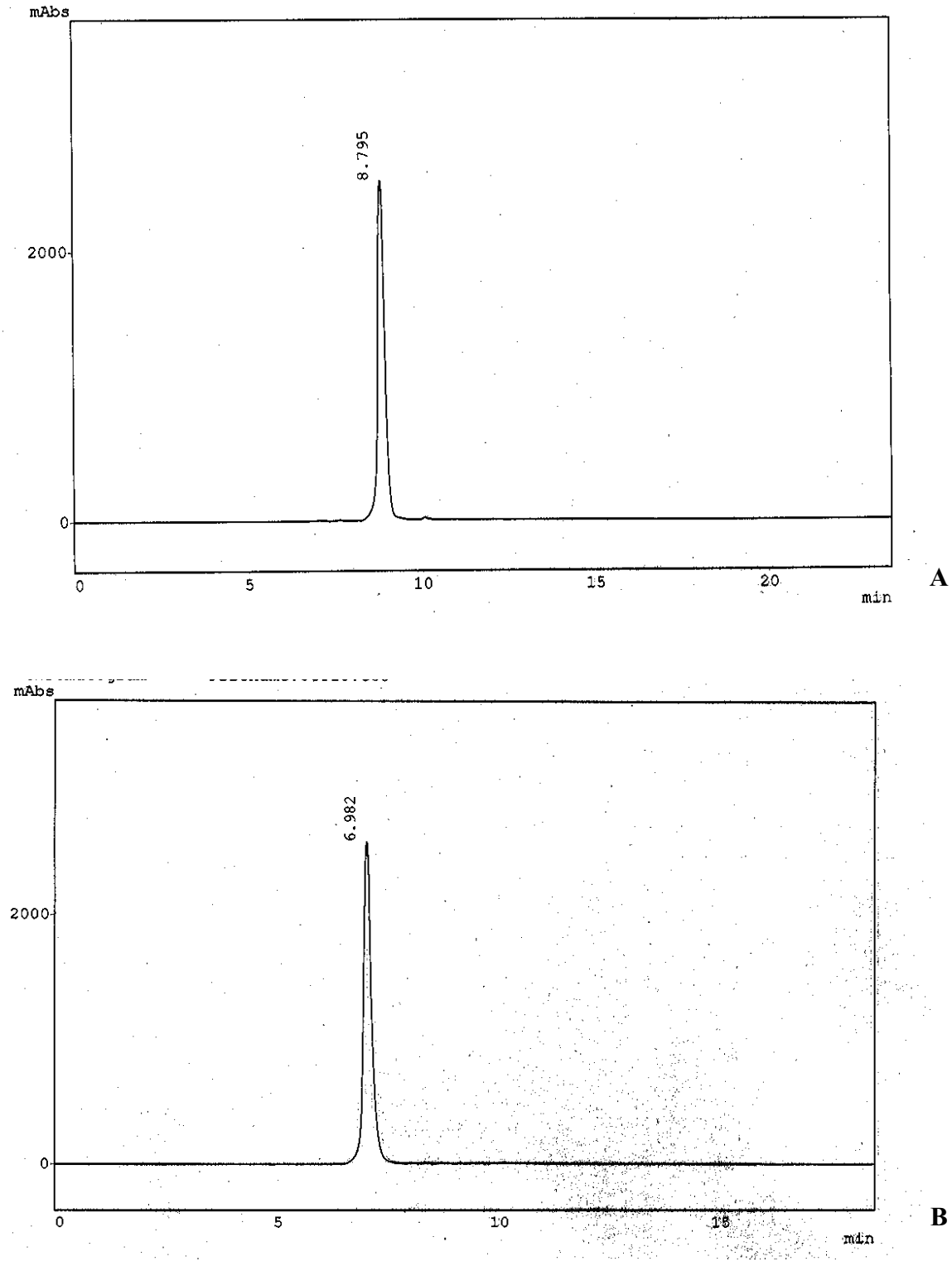


**Figure 3. A) HPLC chromatogram of standard *p*-coumaric acid; B) HPLC chromatogram of standard chlorogenic acid.**

**Operating condition of HPLC**

Adjusted the detector at 325 nm

Gradient table on the instrument as given below

| Gradient | A | | B |
|---|---|---|---|
| | 10 | : | 90 |

| Time | % A | Flow |
|---|---|---|
| 0.00 | 10.0 | 0.7 |
| 5.00 | 30.0 | 0.7 |
| 10.00 | 30.0 | 0.7 |
| 15.00 | 10.0 | 0.7 |
| 20.00 | 10.0 | 0.7 |
| 25.00 | 10.0 | 0.7 |

The column was flushed with HPLC grade water at a flow rate of 1 ml/ min for a period of 30 min followed by HPLC grade methanol for 30 min. The gradient was run as per the conditions as given in the above table. A blank gradient was also run and the signals noted. The standard solution was injected into the system and noted the retention time and peak area. This was repeated 5 times and the average area of 5 injections determined.

Inject the sample solution into the system and recorded the retention time for different standards (chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid and o-coumaric acid) from the sample. Repeated the injection triplicate and perform the system suitability test for the sample. Calculate the mean area for different standards from the sample.

**Calculation**

The compound was assayed and the purity determined using the following expression

$$\text{Assay purity of compound from sample} = \frac{\text{Weight of std X Area of Sa X Dilution of Sa X Purity X 100}}{\text{Weight of sa. Area of Std. Dilution of Std}}$$

Weight of the Standard taken : Wt.std
Weight of the sample take : Wt.sa
Area corresponding to : Asa
Dilution of standard : Dstd
Dilution of Sample : Dsa
Purity of standard : P

Chromatographic purity of different standards (chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid and o-coumaric acid) were calculated based pn peak area.

$$\text{Percentage chromatographic purity} = \frac{\text{Purity of compound X 100}}{\text{Pt}}$$

This total peak area is taken as Pt.

**Antioxidant assay using β carotene – linoleic acid method**

The procedure of Adegoke et al., (1998) was followed with minor modifications for determining the antioxidant activity. Eight milligrams of crystalline β-carotene, 80 mg of linoleic acid and 800 mg of Tween-80 (polyoxyethylene sorbitan manopalmitate) were dissolved in 2 ml of chloroform in a 250 ml R.B flask. The added chloroform was removed at 45$^{o}$C under vacuum using a rotary evaporator. The resulting mixture was immediately diluted with 30 ml of distilled water and the emulsified concentrate was equilibrated in dark for 1.5 h. Triple distilled oxygenated water was added to this emulsion (120 ml) and stirred using magnetic stirrer and used for analysis (A). Oxidation reactions were performed in test tubes fitted with standard tapered glass joints.

A stock solution of coffee conserve prepared in absolute ethanol (1 mg/ml). A 500 µl of this solution and 500 µl of alcohol were added to 4 ml of the dilute oxygenated emulsion (A). This represents a dosage of 100 ppm on emulsion basis. The tubes were immediately placed in a water bath maintained at 50$\pm$ 1$^{0}$C. At 30 minutes intervals up to 180 minutes, 4 ml aliquots from each tube were pipetted directly into the calorimetric cuvette and absorbance was recorded against 95% ethanol blank at 470 nm in a UV-Spectrophotometer. The antioxidant activity (AA) of each extract was evaluated in terms of bleaching of the β- carotene using the following equation:

$$\textbf{Percentage of AA} = 100 \left[1 - \frac{A_0 - A_t}{A^0_0 - A^0_t}\right]$$

AA = Antioxidant activity
$A_0$ = Initial absorbance of the sample
$A_t$ = Absorbance of the sample after time 't'
$A^0_0$ = Initial Absorbance of the blank
$A^0_t$ = Absorbance of the blank after time 't'

The efficacy of the conserves at 100 and 200 ppm levels were similarly evaluated. Set of control-determinations of antioxidant activity was carried out using butylated hydroxy anisole (BHA) at 50, 100 and 200 ppm level. Relative antioxidant activity of the green coffee conserves was compared as a percentage of BHA activity at corresponding concentration (Figure 5).

**Radical scavenging activity by DPPH method**

Different concentrations (50, 100 and 200 ppm) of coffee conserves and BHA were taken in different test tubes. The volume was adjusted to 100 µL by adding MeOH. Five ml of a 0.1 mM methanol solution of DPPH was added to these test tubes and shaken vigorously. The tubes were allowed to stand at 27$^{0}$C for 20 minutes. The control was prepared as above without any conserve, and MeOH was used for the baseline correction. Changes in the absorbance of the sample were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Radical scavenging activity (%)= (Control OD - Sample OD/Control OD) x 100.

On the basis of the results (Figure 6) of the above two experiments, the conserves of coffee, which showed significant activity by both methods, was selected for further studies.

**Results and Discussion**

The yield of extracts obtained from arabica and robusta coffee using various solvent mixtures are shown in Table 1. In both arabica as well as robusta, solvent mixture ratio of 60:40 (methanol: water) gave highest yield followed by 70:30 and 80:20. With increase in the water content in the solvent mixture, yield of conserves increased in both varieties. In case of robusta coffee conserve the higher yield (29.51%) was obtained from 60:40 solvent mixture than 70:30 (26.93%) and 80:20 (26.55%) methanol: water, respectively. The lowest yield (24.78%) obtained from in arabica coffee extracts in solvent ratio of 80:20. The total chlorogenic acids content in the extracts are estimated spectrophotometrically and results were presented in Table 1. The highest chlorogenic acids (36.8%) were observed in robusta coffee conserves. The lowest chlorogenic acids (24.8%) was observed in arabica coffee extracts. With increasing the water content in the solvent mixture chlorogenic acids content was decreased in both varieties.

**Table 1. Yields of extracts and estimation of chlorogenic acids in green coffee conserves.**

| SL NO | SAMPLE | RATIO OF SOLVENTS | PERCENTAGE YIELD OF EXTRACTS | CHLOROGENIC ACIDS % |
|---|---|---|---|---|
| 1 | COFFEE ARABICA | 80:20<br>70:30<br>60:40 | 24.55 ± 0.29<br>26.67 ± 0.12<br>28.30 ± 0.32 | 47.90 ± 0.21<br>51.78 ± 0.59<br>41.9 0 ± 0.90 |
| 2 | COFFEE ROBUSTA | 80:20<br>70:30<br>60:40 | 26.48 ± 0.13<br>26.56 ± 0.37<br>29.88 ± 0.32 | 48.67 ± 0.19<br>38.71 ± 0.15<br>45.41 ± 0.24 |

**Table 2. Characterization of green coffee extracts through HPLC.**

| SL NO | SAMPLE | ARABICA | | | ROBUSTA | | |
|---|---|---|---|---|---|---|---|
| | RATIO OF SOLVENT EXTRACTS | 80:20 | 70:30 | 60:40 | 80:20 | 70:30 | 60:40 |
| 1 | CAFFEYOLQUINIC ACID | 26.16 ± 0.25 | 26.18 ± 0.18 | 19.67 ± 0.16 | 23.63 ± 0.02 | 21.69 ± 1.13 | 21.38 ± 0.53 |
| 2 | FERULIC ACID | 2.76 ± 0.25 | 17.60 ±0.17 | 2.20 ± 0.26 | 6.26 ± 2.00 | 4.04 ± 0.04 | 6.22 ± 0.23 |
| 3 | CAFFEIC ACID | 0.64 ± 0.02 | 0.67 ± 0.02 | 8.52 ± 0.03 | 4.26 ± 0.20 | 3.78 ± 0.10 | 3.83 ± 0.22 |
| 4 | *o*-COUMARIC ACID | 1.84 ± 0.02 | 0.64 ± 0.02 | 2.72 ± 0.02 | 12.08 ± 0.07 | 8.22 ± 0.26 | 2.68 ± 0.71 |
| 5 | *p*-COUMARIC ACID | 2.44 ± 0.02 | 23.02 ± 0.19 | 8.74 ± 0.02 | 2.68 ± 0.27 | 2.43 ± 0.39 | 12.33 ± 0.14 |

The total chlorogenic acids content of the methanol plus water extract was maximum; the chlorogenic acids content was low in the ratio of 60:40 extracts, despite high extract yield than the chlorogenic acids content of 70:30 and 80:20 extracts. The antioxidant activity may be directly correlated to the chlorogenic acid content of various green coffee extracts; thus, the methanol: water (60:40) extract of robusta coffee conserve showed higher activity as compared to the other extracts.

The HPLC pattern of the methanol plus water extracts (selected) of green coffee (robusta and arabica) is shown in Figure 4 and 4a. The percentage of chlorogenic acid is shown to be the major component in the HPLC patterns of the six extracts are comparable. Six major (peaks) compounds (Figure 1-3) were identified in the extracts as presented in the Table 2. Based on the results, it can be concluded that chlorogenic acids, ferulic acid, caffeic acid, 3,5-dimethoxy 4 hydroxycinnamic acid, o-coumaric acid and p-coumaric acids. The HPLC analysis showed that the ferulic acid and p-coumaric acids made a greater contribution to total antioxidant activity than chlorogenic acids (Figure 7).

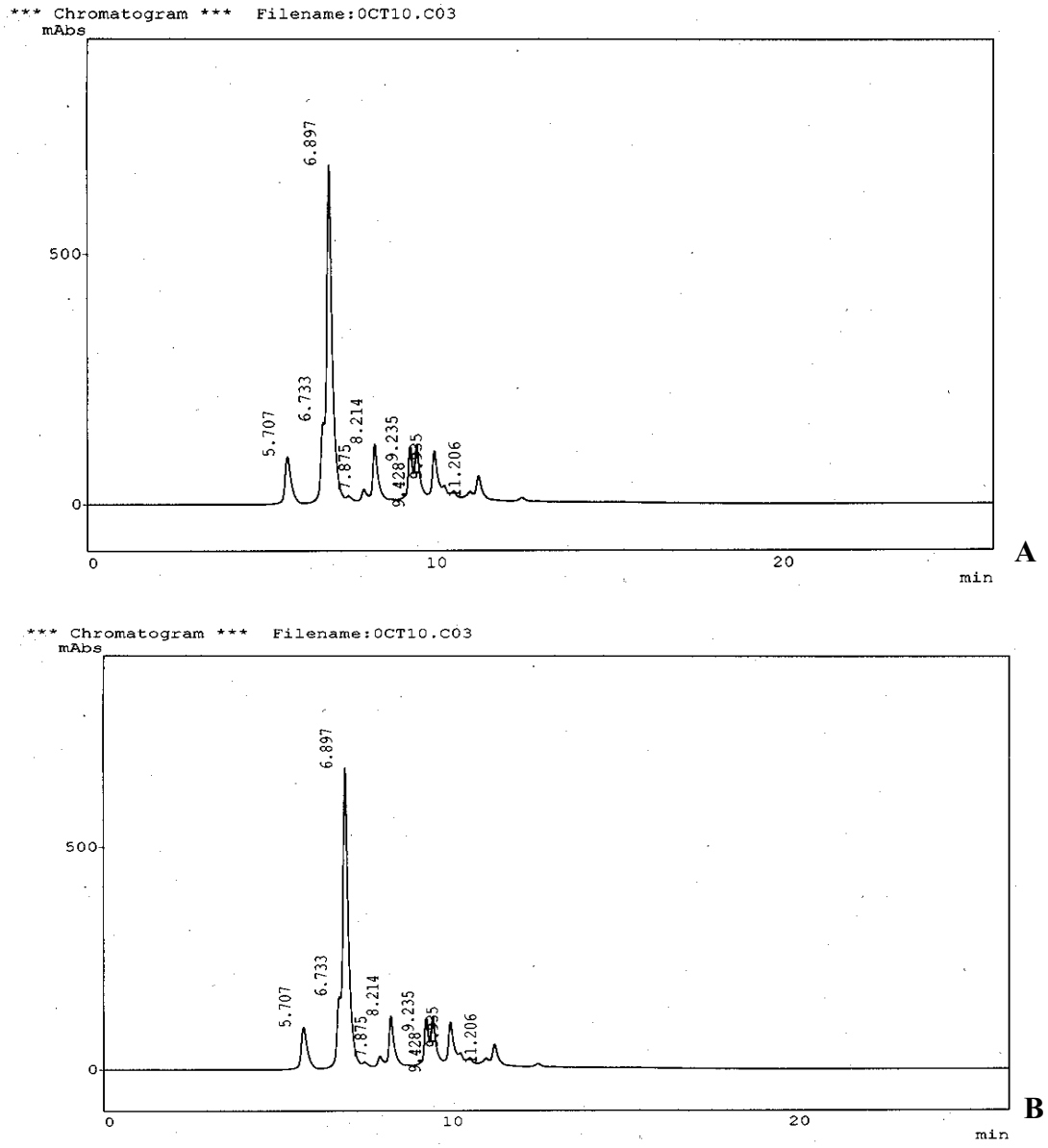


**Figure 4. A) HPLC chromatogram of *Coffea arabica*; B) HPLC chromatogram of *Coffea robusta.***

The antioxidant activity of green coffee extracts as measured by the bleaching of ß-carotene is presented in Figure 5. It can be seen that arabica and robusta coffee extracts preferred by different solvent mixtures exhibited various degrees of antioxidant activity. At 200 ppm concentration, arabica coffee extracts of methanol: water 60:40, 70:30 and 80:20 exhibited 58, 70 and 65% antioxidant activity, respectively. At 200 ppm concentrations, extracts of robusta coffee (60:40, 70:30 and 80:20) exhibit 70, 48 and 45% antioxidant activity respectively. The mechanism of bleaching of ß-carotene is a free radical mediated phenomenon resulting from

the hydro peroxides formed from lenoleic acid. In this model system, ß-carotene undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated ß-carotene molecules. As ß-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically. The presence of different extracts can hinder the extent of ß-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

Free radical scavenging potentials of green coffee extracts at different concentrations were tested by the DPPH method, and the results are shown in Figure 6. Antioxidant reacts with DPPH, which is a stable free radical, and converts it to 1,1-diphenyl-2-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. At 200 ppm, 60:40, 70:30 and 80:20 extracts of robusta coffee exhibit 73, 65 and 62% free radical scavenging activity, respectively. At 200 ppm, methanolic: water extracts (60:40, 70:30 and 80:20) of arabica coffee exhibit 70, 65 and 64% free radical scavenging activity, respectively.

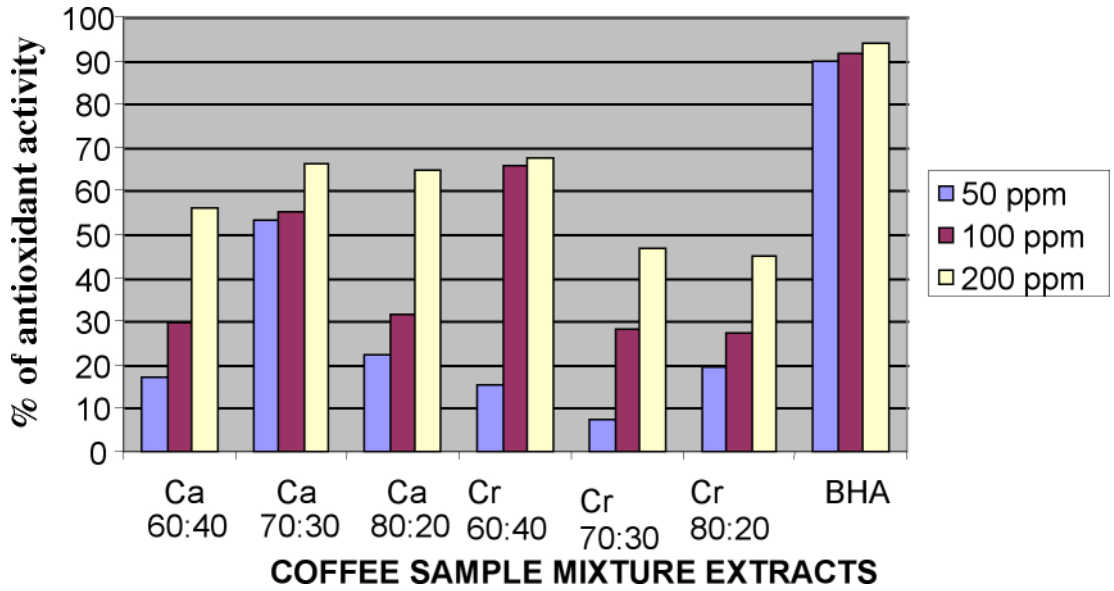


**Figure 5. Antioxidant activity of green coffee extracts.**

The activity of the extracts is attributed to their hydrogen donating ability (Shimada et al., 1992). It is well-known that free radicals cause autoxidation of unsaturated lipids in food (Kaur and Perkins, 1991). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of the lipid (Sherwin 1978). The data obtained reveal that the extracts are free radical inhibitors and primary antioxidants that react with free radicals.

The results shown above indicate that the extraction with methanol not only gives high yield of the extract but also gives high antioxidant activity, which was confirmed by ß-carotene linoleic acid and DPPH methods used for the antioxidant assay. Ferulic acid and p-coumaric acids made a greater contribution than chlorogenic acids (Figure 7) The conserves exhibited maximum antioxidant activity than individual compounds. Thus, the results of the present work indicate that the selective extraction of antioxidant from natural sources by appropriate solvents, and variety / cultivar used for extraction is very important in obtaining fractions with high antioxidant activity.

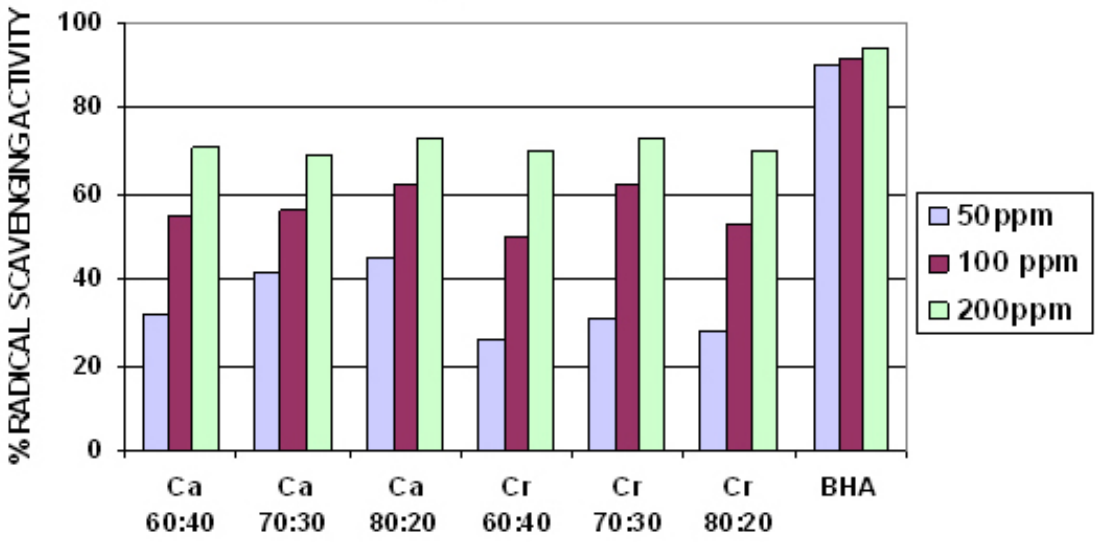


**Figure 6. Radical scavenging activity of green coffee extracts and BHA by DPPH method at different concentrations (ppm).**

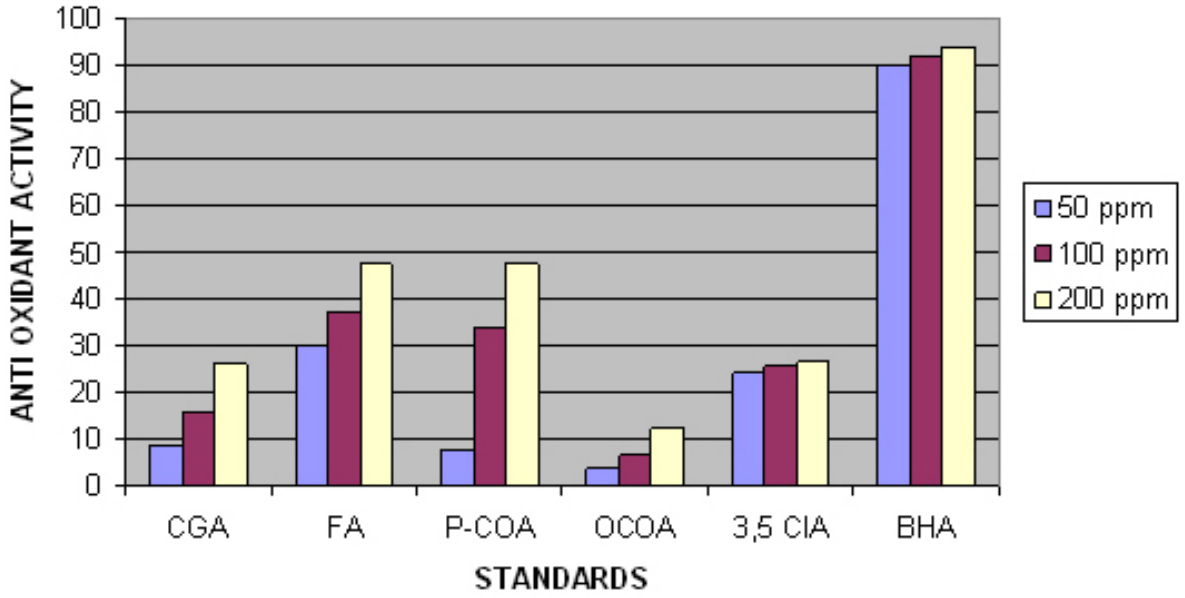


**Figure 7. Antioxidant activity of standard compounds.**

The results of the present work indicate the presence of compounds possessing antioxidant activity from green coffee as an enriched source of the antioxidants exhibiting higher activity as compared to roasted coffee extracts (Nidhi Singh and Madhava Naidu, 2004). The difference in the antioxidant activity of the arabica and robusta may be ascribed to their different phenolic compositions. This is the first report on the description of antioxidants from green coffee, and further studies are needed on the isolation and characterization of individual phenolic compounds to elucidate their antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

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# Investigations of On-farm Processing and Drying of Coffee in India to Develop a GAP and GMP Plan for Prevention of Mould Formation

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## SUMMARY

Experiments were carried out at CCRI and CRSS, Chettalli on various aspects of processing (Good Agricultural Practices and Good Manufacturing Practices) both in arabica and robusta parchment and cherry. The experiments comprised, layer thickness, stirring frequencies, stage of fruit maturity, pre washing fruits before pulping, fruit injury, pulp contamination, heaping of fruits, re wetting of coffee, different surfaces for drying coffee and use of test weight and moisture meter for measuring bean moisture on incidence of mould. From the trials drying of coffee on bare soil was found to invite higher mould incidence compared to other surfaces. It was also found that drying surfaces had no major influence on the incidence of Ochre moulds in the final product, if the fruits were already infested with Ochre group moulds. Drying of parchment and cherry at 4 cm and 8 cm with minimum 4 rakings per day was found to minimise the ochre mould infection. Mixing of tree dried and gleaning during processing of coffee was found to increase the risk of toxigenic mould in the final product. Pre washing and removal of floats before processing was found to bring down the mould contamination considerably. Injury to fruits and pulp/skin contamination in cherry and parchment coffee respectively increased the risk of ochre incidence. Heaping of fruits before pulping even for one day was found to invite fungal contamination. Rewetting of cherry during drying (especially towards the end of drying) was found to significantly increase the risk of OTA producing moulds. Parchment coffee was found over dried and cherry to under dried by test weight method.

## INTRODUCTION

The ICO-CFC-FAO Global Mould Project entitled “Enhancement of Coffee Quality through the Prevention of Mould Formation” is being implemented in India by Central Coffee Research Institute, Coffee Board of India since 2001. Under this project, various field studies related to mould prevention during on farm processing were taken up at Central Coffee Research Institute (CCRI), Balehonnur; Coffee Research Sub-station (CRSS), Chettalli during 2001-2004 with the objective of understanding the toxigenic (*Aspergillus ochraceus*) and other mould association in coffee during on-farm processing.

## MATERIAL AND METHODS

### Effect of different drying surfaces on mould incidence in coffee

Trials on drying surface both in arabica and robusta parchment and cherry coffee were conducted using tiles, concrete, soil, cow dung, coloured polythene sheets, tarpaulin and parabolic dryer at both the stations. In all the trials, the parchment and cherry coffee were dried at 4-5cm and 7-8cm thickness respectively with 6-8 stirrings per day.

### Effect of layer thickness on mould incidence

The experiment was carried out at CRSS, Chettalli on both robusta and arabica parchment and cherry during the year 2001-03 to understand relationship between the thicknesses of drying on mould infection. Parchment coffee was dried at single bean, 2 m, 4 m and 8cm layer thickness and cherry was dried at single fruit, 4 cm, 8 cm and 15 cm layer thickness. The lots were raked 6-8 times per day and parchment was covered during night.

### Effect of stirring frequency on mould incidence

The trial was conducted at CRSS, Chettalli on both robusta and arabica parchment and cherry during the year 2001-03 to understand relationship between the stirring frequency and mould infection. Coffee lots were stirred 2, 4 and 8 times per day and un-stirred lots served as control.

### Effect of fruit ripeness on mould incidence

The experiment was taken up during 2001-02 on arabica in CCRI and on robusta coffee at CRSS, Chettalli to understand the association of toxigenic mould in various fruit categories viz., greens, half-ripe, ripe, over-ripe, tree dried and gleanings.

### Effect of Pre washing of fruits on mould incidence

This experiment was carried out at CCRI during 2001-02 both in arabica and robusta coffee, to understand the effect of pre washing of fruits on mould reduction in cherry coffee. Freshly harvested fruits were soaked for pre-wash and to separate the floats before spreading them for drying. Un washed fruits were maintained as control.

### Impact of fruit injury on mould incidence

The trial was conducted at CRSS, Chettalli during 2001-03 both in robusta cherry and arabica cherry to find out the impact of fruit injury during processing on mould incidence. Injuries to fruits were made by sterilized hands to the level of 1%, 2%, 5% and 10%. Un-injured fruit lot served as control.

### Effect of pulp contamination on mould incidence

The trial was conducted at CRSS, Chettalli during 2001-03 both in robusta parchment arabica parchment to find out the impact of pulp/fruit skin contamination on mould incidence. 1, 2, 5 and 10% of pulp was mixed with wet parchment. Un-contaminated lot served as control.

**Effect of heaping of fruits on mould incidence**

This experiment was conducted at both the stations in arabica and robusta with an objective to understand the effect of heaping fruits on mould contamination. Freshly harvested fruits were heaped for 1, **2**, 3 and 4 days and then sun dried without covering during night. The fruits spread on the same day and covered during night were maintained as control.

**Effect of re-wetting during on-farm drying on mould incidence**

At both the stations arabica and robusta was used to find out the effect of re-wetting of drying coffee on mould infection. Rewetting was done on $4^{th}$, $8^{th}$, and $15^{th}$ days after drying using different quantities of water.

**Revalidation of on farm moisture measurement of coffee**

Experiment was conducted at CRSS, Chettalli to understand the differences in bean moisture content of arabica parchment, cherry and floats when measured by test weight, oven and moisture meter method.

The drying pattern and days, initial and final mould incidence and OTA contamination were tested in all the trials.

**Table 1. Effect of drying surfaces on mould incidence in arabica (2001-02).**

| Drying Surfaces | Total infection % | | Niger infection % | | Ochre infection % | | Drying Days | |
|---|---|---|---|---|---|---|---|---|
| | CCRI | CRSS | CCRI | CRSS | CCRI | CRSS | CCRI | CRSS |
| **Parchment** | | | | | | | | |
| Tiles | 99.3 | 86.4 | 44.9 | 50.2 | 4.40 | 0.00 | 5 | 6 |
| Concrete | 99.6 | 82.1 | 50.7 | 28.5 | 7.60 | 0.00 | 5 | 6 |
| B.Sheet on soil | 100.0 | 97.3 | 48.9 | 62.8 | 4.20 | 0.00 | 5 | 7 |
| W. Sheet on soil | - | 95.2 | - | 55.3 | - | 0.00 | - | 6 |
| Bl. Sheet on soil | - | 97.6 | - | 64.6 | - | 0.00 | - | 8 |
| G. Sheet on soil | 98.7 | - | 30.0 | - | 2.80 | - | 5 | - |
| Cow dung | 99.5 | - | 44.7 | - | 2.60 | - | 7 | - |
| **Cherry** | | | | | | | | |
| Tiles | 96.3 | 86.0 | 26.6 | 86.0 | 21.6 | 4.70 | 7 | 11 |
| Concrete | 100.0 | 80.9 | 33.8 | 71.4 | 19.7 | 4.70 | 7 | 11 |
| B.Sheet on soil | 100.0 | 94.6 | 42.8 | 93.2 | 16.6 | 6.60 | 7 | 12 |
| W. Sheet on soil | - | 88.8 | - | 84.2 | - | 5.50 | - | 12 |
| Bl. Sheet on soil | - | 98.3 | - | 96.8 | - | 5.88 | - | 13 |
| G. Sheet on soil | 100.0 | - | 38.8 | - | 9.20 | - | 7 | - |
| Road | - | 100.0 | - | 100.0 | - | 7.23 | - | 11 |
| Soil | 100.0 | 100.0 | 24.3 | 72.6 | 7.30 | 9.00 | 11 | 14 |
| Cow dung | 100.0 | - | 41.6 | - | 10.0 | - | 11 | - |

*B.Sheet-Black polythene sheet, W.Sheet-White polythene sheet Bl.Sheet-Blue polythene sheet, G.Sheet- Green polythene sheet, "-" not included as treatment*

## RESULTS AND DISCUSSION

### Effect of different drying surfaces on mould incidence in coffee

At both the centers ochre group aspergilli were found in the fruits of arabica before drying. Ochre incidence was found in final product of parchment at CCRI but not at CRSS (Table 1) at the same time, higher incidence of Ochre mould was recorded in CCRI compared to CRSS in cherry coffee. In both parchment and cherry drying, number of days taken to reach the desired moisture level was same in cement, tiles and polythene surfaces. This clearly indicated that, the carry over of ochre fungi to final beans from initial load in the fruits might not be controlled by use of different drying surfaces. In arabica experiment (2003-04), at CRSS, Chettalli (Table 3), Ochre incidence was found to high on coffee dried in parabolic dryer (3%) compared to other surfaces. In cherry drying soil surface recorded higher ochre incidence (7.6%).

**Table 2. Effect of drying surfaces on mould incidence in robusta coffee (2003-04).**

| Drying Surfaces | Total infection % | | Niger infection % | | Ochre infection % | | OTA (ppb) | | Drying Days | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CCRI | CRSS | CCRI | CRSS | CCRI | CRSS | CCRI | CRSS | CCRI | CRSS |
| **Parch (Run-1)** | | | | | | | | | | |
| Cement | 66.0 | 95.3 | 1.38 | 81.6 | 1.36 | 2.30 | BDL-2.35 | BDL | 5 | 8 |
| Tarpaulin | 70.0 | 97.0 | 0.00 | 84.0 | 2.72 | 3.30 | BDL-1.40 | 0.99-1.58 | 5 | 8 |
| Parabolic | 67.0 | 95.3 | 4.08 | 77.0 | 5.10 | 4.30 | BDL-1.12 | 0.42-0.65 | 5 | 10 |
| **Parch (Run-2)** | | | | | | | | | | |
| Cement | 69.0 | 98.6 | 18.0 | 86.6 | 0.71 | 3.60 | 0.22-0.44 | 0.18-1.37 | 5 | 7 |
| Tarpaulin | 67.0 | 98.6 | 16.0 | 87.0 | 0.00 | 4.00 | BDL-1.73 | 0.16-0.39 | 5 | 8 |
| Parabolic | 49.0 | 98.0 | 10.0 | 91.3 | 0.71 | 5.00 | 0.18-0.44 | 0.24-0.56 | 4.5 | 9 |
| **Cherry (Run-1)** | | | | | | | | | | |
| Tarpaulin | 53.0 | 98.3 | 0.00 | 85.0 | 6.46 | 2.60 | BDL-0.60 | 1.20-1.37 | 7 | 17 |
| Soil | 47.0 | 100.0 | 0.00 | 88.3 | 2.72 | 4.30 | BDL-3.07 | 2.02-2.53 | 8 | 21 |
| Cow dung | 63.0 | 98.6 | 0.00 | 72.3 | 8.16 | 2.00 | 0.15-0.47 | 1.57-2.64 | 8 | 20 |
| **Cherry (Run-2)** | | | | | | | | | | |
| Tarpaulin | 81.0 | 100.0 | 47.0 | 92.0 | 0.95 | 4.00 | 0.33-1.57 | 0.80-1.18 | 6 | 14 |
| Soil | 99.0 | 100.0 | 19.0 | 94.3 | 1.18 | 4.60 | 0.52-0.97 | 2.34-3.03 | 7 | 20 |
| Cow dung | 57.0 | 100.0 | 34.0 | 80.0 | 0.71 | 1.30 | BDL-2.72 | 1.79-2.76 | 7 | 19 |

In robusta experiment, both at CCRI and CRSS, Chettalli, higher Ochre incidence was recorded on coffee dried on parabolic dryer compared to other surfaces. Ochre incidence was 5.1% & 0.71% and 4.3% & 5% in parchment coffee under run-1 and run 2 trials at CCRI and CRSS respectively (Table 2). While in robusta cherry, ochre incidence was more in coffee dried on soil compared to other surfaces. Ochre incidence was 2.72% & 4.30% and 1.18% & 4.6% in robusta cherry under run-1 and run 2 trials respectively at CCRI and CRSS.

**Table 3. Effect of drying surfaces on mould incidence in arabica coffee (2003-04).**

| Drying surfaces | Total infection % | Niger infection% | Ochre infection% | OTA (ppb) | Drying days |
|---|---|---|---|---|---|
| **Parchment** | | | | | |
| Tarpaulin | 97.0 | 82.6 | 2.60 | BDL-0.399 | 10 |
| Cement | 93.6 | 83.6 | 1.30 | 0.22-1.06 | 11 |
| Parabolic | 98.0 | 95.0 | 3.00 | BDL-1.11 | 10 |
| **Cherry** | | | | | |
| Tarpaulin | 98.0 | 82.3 | 3.00 | 0.30-0.58 | 14 |
| Soil | 100.0 | 94.0 | 7.60 | 0.45-1.87 | 15 |
| Cow dung | 99.3 | 77.3 | 1.00 | BDL-0.22 | 16 |

**Effect of layer thickness on mould incidence**

Both arabica and robusta cherry dried at 15 cm depth resulted in 100% bean infection and 2.59% and 9% ochre contamination respectively in the final product. In case of arabica and robusta parchment dried at 8 cm thickness, mould infection was very high to an extent of 92.8% (arabica) and an incidence of 1.08% and 4% ochre respectively (Table 4). The cup quality was also found affected when the drying thickness was more than 8 cm in respect of cherry and 4 cm in case of parchment. Thus spreading at 8cm and 4 cm thickness was found to be optimum for drying the cherry and parchment coffee respectively.

**Table 4. Effect of layer thickness on mould incidence.**

| | Ara. Parchment (cm) | | | | Ara. Cherry (cm) | | | | Rob. Parchment (cm) | | | | Rob. Cherry (cm) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **2** | **4** | **8** | **S** | **4** | **8** | **15** | **S** | **2** | **4** | **8** | **S** | **4** | **8** | **15** |
| T % | 82.4 | 83 | 84.6 | 92.8 | 82.1 | 92.8 | 95.4 | 100 | 61.9 | 52.3 | 80.9 | 90.4 | 57.1 | 66.6 | 71.4 | 100 |
| N% | 78.5 | 86 | 78.4 | 84.6 | 83.2 | 88.4 | 89.3 | 100 | 56.2 | 42.1 | 53.5 | 31.0 | 92.0 | 73.0 | 83.0 | 85 |
| O% | 0.00 | 0.0 | 0.00 | 1.08 | 0.00 | 0.00 | 0.00 | 2.59 | 0.00 | 0.00 | 0.00 | 4.00 | 0.00 | 0.00 | 0.00 | 9.0 |
| Dd | 5 | 6 | 7 | 8 | 9 | 11 | 12 | 14 | 4 | 5 | 6 | 8 | 10 | 12 | 13 | 16 |

*T- Total infection, N-Niger infection, O-Ochre infection, Dd-Drying days, S-Single layer*

**Effect of stirring frequency on mould incidence**

Initially *A.niger* was the dominant flora in both cherry and parchment coffee before initiation of the drying process. *A.ochraceus* incidence was 1.29 and 7.14% in arabica parchment and cherry dried without stirring respectively. The same 1 and 4% respectively robusta (Table 5). The final coffee samples from these lots had also contained OTA. The cup quality was found to improve with the increase in number of stirrings, in both the cases.

**Effect of fruit ripeness on mould incidence**

A study on mould association in beans of fruit ripeness process indicated that beans from gleanings (28.6%), tree dried fruits (19.5%) and bulk/unsorted cherry (6.3%) of arabica had the highest incidence of Ochre compared to robusta. whereas the beans from greens and just ripe fruits were free of Ochre (Table 6). From this study it is evident that gleanings and tree dried fruits would be one of the source for toxigenic mould in dry processed coffee. Harvesting of fruits at correct stage of ripeness without allowing the fruits to over dry on the

tree is most important for reducing the mould incidence, especially the Ochre incidence in coffee.

**Table 5. Effect of Stirring frequency on mould incidence.**

| | Ara. Parchment (No. of stirrings) | | | | Ara. Cherry (No. of stirrings) | | | | Rob. Parchment (No. of stirrings) | | | | Rob. Cherry (No. of stirrings) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **C** | **2** | **4** | **8** | **C** | **2** | **4** | **8** | **C** | **2** | **4** | **8** | **C** | **2** | **4** | **8** |
| **T %** | 100 | 92.8 | 84.3 | 78.5 | 100 | 100 | 88.7 | 82.0 | 100 | 100 | 85.7 | 76.1 | 100 | 95.2 | 57 | 52.3 |
| **N%** | 100 | 100 | 85.7 | 64.2 | 100 | 100 | 88.9 | 85.4 | 70 | 56 | 47 | 52 | 95 | 90 | 91 | 100 |
| **O%** | 1.29 | 0.0 | 0.0 | 0.0 | 7.14 | 4.76 | 1.89 | 1.22 | 1.0 | 0.0 | 0.0 | 0.0 | 4.0 | 0.0 | 0.0 | 0.0 |
| **Dd** | 8 | 7 | 7 | 6 | 14 | 14 | 12 | 11 | 8 | 7 | 5 | 5 | 15 | 15 | 12 | 11 |

*T-Total infection,N-Niger infection,O-Ochre infection,Dd-Drying days,S-Single layer,C-Control*

**Table 6. Effect of stages of fruit maturity on mould incidence.**

| | Arabica | | | | | Robusta | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | G | R | TD | Gl | B | G | HR | R | OR | TD |
| **T %** | 87.0 | 91.0 | 100 | 100 | 96.0 | 71.4 | 76.1 | 76.1 | 100 | 100 |
| **N%** | 38.0 | 47.0 | 50.5 | 62.0 | 60.0 | 33.0 | 42.0 | 74.0 | 95.0 | 80.0 |
| **O%** | 0.0 | 0.0 | 19.5 | 28.6 | 6.30 | 0.0 | 0.0 | 0.0 | 4.00 | 9.00 |
| **Dd** | 15 | 16 | 11 | 0 | 14 | 13 | 13 | 12 | 12 | 10 |

*G-Greens, HR-Half-ripe, R-Ripe, OR-Over-ripe, TD-Tree dried, B-Bulk, Gl. Gleaning, T-Total infection, N-Niger infection, O-Ochre infection, Dd-Drying days*

**Effect of Pre washing of fruits on mould incidence**

The total fungal infection rate in the beans from unwashed fruits was more than those from the pre-washed (floats separated) both in arabica and robusta **(**Figure 1). The final product of unwashed fruits had higher incidence of niger group aspergilli when compared to pre-washed fruits. The ochre group incidence was noticed only in unwashed robusta on the initial day of drying, but was absent in the final product. Separation of floats and over dried fruits using water floatation method was found to be effective and efficient in bringing down the total mould incidence.

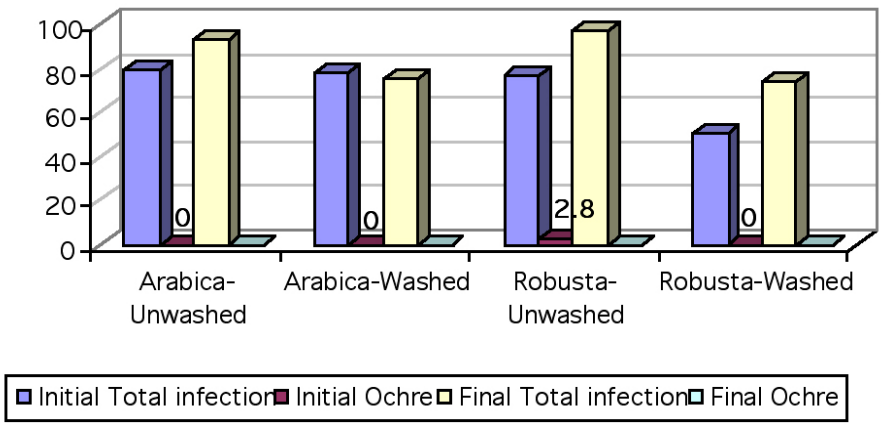


**Figure 1. Effect of pre-washing fruits on mould infection.**

**Impact of fruit injury on mould incidence**

Fruit injury higher than 1% in cherry lot found to increase the total mould as well as ochre group in the final beans (Table 7). The incidence of Ochre mould was found to be more on robusta compared to arabica when fruits were damaged.

**Table 7. Effect of fruit injury on mould incidence.**

| | Arabica | | | | | Robusta | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C | 1% | 2% | 5% | 10% | C | 1% | 2% | 5% | 10% |
| **T %** | 73.4 | 89.3 | 97.1 | 100 | 100 | 85.7 | 95.2 | 100 | 100 | 100 |
| **N%** | 72.4 | 85.6 | 92.1 | 100 | 100 | 80.0 | 87.0 | 88.0 | 89.0 | 91.0 |
| **O%** | 0.0 | 1.78 | 2.85 | 5.71 | 14.2 | 0.0 | 0.0 | 4.00 | 13.0 | 13.0 |

*T- Total infection, N-Niger infection, O-Ochre infection, C-Control*

**Effect of pulp contamination on mould incidence**

The study indicated the higher ochre incidence in the final beans when the pulp contamination was high. Arabica showed higher incidence of Ochre mould compared to robusta when contaminated with pulp (Table 8). This could be possibly due to higher mucilage content and moisture content in arabica pulp.

**Table 8. Effect of pulp contamination in wet parchment on mould incidence.**

| | Arabica | | | | | Robusta | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C | 1% | 2% | 5% | 10% | C | 1% | 2% | 5% | 10% |
| **T %** | 78.6 | 94.8 | 98.6 | 100 | 100 | 95.0 | 100 | 100 | 100 | 100 |
| **N%** | 79.4 | 84.0 | 88.4 | 100 | 100 | 83.0 | 90.0 | 95.0 | 87.0 | 84.0 |
| **O%** | 0.0 | 2.85 | 3.57 | 8.57 | 14.2 | 0.0 | 4.00 | 6.00 | 8.00 | 12.0 |

*T- Total infection, N-Niger infection, O-Ochre infection, C-Control*

**Effect of heaping of fruits on mould incidence**

Even thought yeast was predominant species on fresh fruits, there was a reduction in yeast population and slight increase in niger population as the heaping progressed. The samples subjected for heaping upto 2 days had higher incidence of niger where as the sample subjected for heaping for 3 days had higher incidence of *Aspergillus sp* in CCRI trials. But at CRSS, the incidence of Ochre increased as the heaping days increased (Table 9). Hence heaping of fruits should be avoided to prevent mould infection.

**Table 9. Effect of heaping of fruits on mould incidence in final product.**

| | CCRI (Robusta) | | | | CRSS, Chettalli (Arabica) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C | 1d | 2d | 3d | C | 1d | 2d | 3d | 4d |
| **T %** | 88.5 | 100 | 100 | 100 | 79.4 | 92.6 | 100 | 100 | 100 |
| **N%** | 31.4 | 37.1 | 54.2 | 40.0 | 72.6 | 92.6 | 100 | 100 | 100 |
| **O%** | 0.0 | 0.0 | 0.0 | 0.0 | 2.85 | 5.61 | 8.16 | 10.2 | 14.2 |
| **Dd** | nd | nd | nd | nd | 13 | 12 | 11 | 10 | 10 |

*T- Total infection, N-Niger infection, O-Ochre infection, Dd-Drying days, C-Control, nd-Not determined*

**Effect of re-wetting during on-farm drying on mould incidence**

Upon re wetting, the population of niger group aspergilli and ochre group aspergilli was increased by 1.3-1.7 and 2.5 to 6 times respectively as compared to control (without rewetting). The results clearly indicate that rewetting of cherry lots during drying (especially towards end of drying process) will significantly increase the risk of OTA producing moulds both in arabica and robusta (Table 10).

**Table 10. Effect of re-wetting fruits on mould incidence in final product.**

| | CCRI (Robusta) Coffee re-wetted on 9th d of drying | | | CRSS, Chettalli (Arabica) | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | 25ml/kg | 50ml/kg | C | 4th d | 8th d | 15th d | 8-15th d |
| **T %** | 85.3 | 100 | 100 | 82.4 | 87.1 | 92.1 | 100 | 100 |
| **N%** | - | - | - | 62.4 | 92.6 | 94.4 | 100 | 98.6 |
| **O%** | 0.60 | 2.80 | 7.10 | 2.85 | 18.3 | 23.0 | 28.3 | 16.0 |
| **Dd** | - | - | - | 14 | 17 | 16 | 16 | 20 |

*T- Total infection, N-Niger infection, O-Ochre infection, Dd-Drying days, C-Control*

**Revalidation of on farm moisture measurement of coffee**

The data on moisture measurement using test weight and oven method is presented in Figure 2. Parchment reaches desired moisture of 10% in 12-13 days by oven method, where as 15-17 days were required to get 15.5kg/forlit test weight. Hence coffee gets over dried for 3-5 days as per the test weights standards. In case of cherry, the desired moisture level of 11% is reached by 15-17 days by test weight method, where as it takes 18-19 days for getting desired moisture by oven method. Hence cherry is under dried. In case of floats, desired moisture is achieved by 15th day itself by oven method, which is 3-4 days earlier than cherry

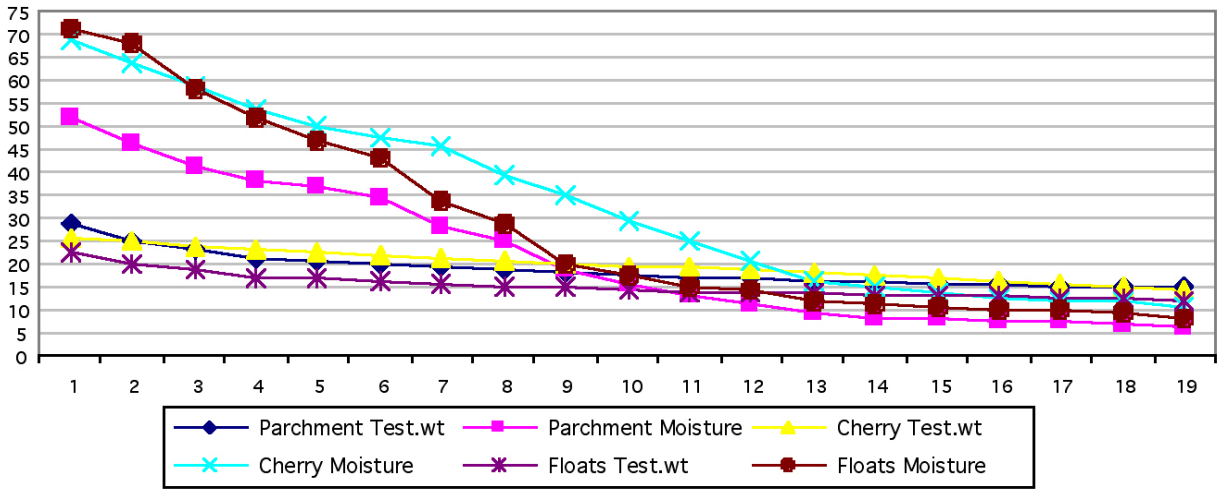


**Figure 2. Comparison of test weight and oven method for moisture measurement.**

From these studies, it is evident that Good Agricultural and manufacturing practices during coffee production reduces the incidence of toxigenic as well as total mould to a greater extent.

# Application of Splitting of Coffee Cherries Prior to Sun Drying as an Alternative to Enhance Drying Performance

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## SUMMARY

Drying is the most intensive and critical step in coffee processing. In order to enhance the process, splitting of coffee cherries has been applied by many farmers since decades a go. Previously the system has been applied in big plantations, in order to enhance their production capacity. The coffee product under the system has been known as "OIB" (*Oost Indische Bereiding*), which separated from the natural process ("GB" = *Gewone bereiding*). Currently, the system is applied by farmers in some coffee areas. The splitting is conducted by using a various coffee splitter from a wooden to metal type models. Drying performance of split coffees was evaluated and compared to natural and de-pulped coffees at 3 different locations. The moisture content and $a_w$, were followed daily and physical quality and organoleptic, as well as ochratoxin A (OTA) status of the coffee products were analyzed. Application of splitting prior to sun drying was apparently enhanced drying performance. To reach 12% of moisture, natural drying took between 13-15 days while split coffee took 10-13 days, which comparable to the drying of parchment coffees at the same load of 30 kg of cherries or parchments per $m^2$. Splitting also improved quality (cup taste) of coffee product, and did not promote OTA contamination. Advantages and disadvantages of the splitting system are also presented.

## INTRODUCTION

Coffee is one of agriculture products that established since 3 centuries ago in Indonesia. Coincided with the development of big plantations, technology of coffee processing also developed. The most critical step in primary coffee processing is drying, due to the long, costly and very sensitive to deterioration. For big plantations handling of tons of coffee cherries per day would be a serious problem particularly for the drying process. It is amenable if attempts pursued, one of the most effective solution is the application of the wet method by the development of pulping machine at the end of 18s in Java. The coffee produced by the wet process at that time known as *West Indisch Bereiding (WIB)*, which also used currently by certain producers and buyers. Although big plantations have applied the wet process for red-sound cherries, but they still apply the dry process for the inferior (float, green, yellow, and dry) cherries.

Parallel to the wet processing technique the dry process also developed by the invention of coffee cherries splitter in the same era. The famous splitter was "Vis Kneuzer" developed by N.V. Stoomwerkplaats Smeroe, Malang-East Java end of 19$^{th}$ century. The dry process with splitting is known as *Oost Indisch Bereiding (OIB)*, which separated from the common natural dry process *(Gewone Bereiding)*.

Due to the better drying performance, the method has been developed and applied by farmers mostly in East Java and many in Lampung. Traditionally, coffee cherries of various maturities are split then sun dried on tarpaulin sheet with a thickness 1-3 cm for 4-9 days. Farmers split their coffee by using various model of splitter which currently available from a simple wooden type to metal type splitter with the variants. In farmers community there are available mobile type splitters for rent. The cost of splitting in East Java is about IDR 10000 (USD 1.1) per 100 kg coffee.

The splitting system has a potential to be applied in other coffee producing areas. Before being recommended for adoption widely, the split method needs to be verified, particularly for it reliability and safety to mycotoxins contamination.

Complement to our previous report( Ismayadi et al., 2001), this article presents results of drying trials of split coffee compared to whole cherries and coffee parchment with mucilage (*descascado*). It includes development of moisture contents and $a_w$, along drying period, and the status of moulds infestation, OTA contents, defect numbers, and cup quality of the bean products.

## TECHNICAL FEATURES OF COFFEE SPLITTER

The old model coffee splitter was developed by N.V. Stoomwerkplaats Smeroe, Malang, known as Vis Kneuzer. Beside the old model, currently various models of coffee splitter are available developed locally. Splitting of coffee cherries is based on the squeezing or crushing coffee cherries in a gap between a rotating drum and a breast plate, similarly to the de-pulping process of a pulping machine. By the action of mounds on the rotating drum the cherries are squeezed against the breast plate. The main different the machine to a pulper is there is no a separating plate in the splitter, both coffee and pulp mixed altogether at the outlet. The drum rotation of coffee splitter is opposite to a pulping machine; if the drum of a pulper moves toward the outlet, while the splitter moves to backward.

Many type of mounds on the drum available, the common ones are made by welding a steel bar (1–2 cm) on the surface of the rotating drum in a zig-zag mode. The second type is made by scrapping the rotary drum to get a saw toothed shape horizontally or in a curve way. Since the mounds are made of a hard metal, it can be used to split both mature and immature cherries, with no water required. A wooden framed splitter uses an aluminium drum with mounds made of aluminium nipples is also available. Size of rotating drums varies 10–20 cm in diameter and 10–30 cm in length. The rotation is about 300 rpm or higher. It is generated by an electric motor (5.5 HP) or a diesel engine (7.5 HP). A smaller gasoline engine is also used for a wooden type splitter.

The common coffee undergoes dry process in Indonesia is consisted of a mixture of various maturity coffee from immature ones to overripe and dry cherries. By such coffee, the output of a splitter is a mixture of whole cherries (normally consist of small-green ones or dry cherries), split cherries, parchments, and pulp. Splitting “degree” is altered by adjusting the gap between breast plate and the rotary drum. The degree also can be altered by adjusting the drum speed (engine speed). The adjustment for each type of coffee is required to get a good splitting with minimum broken. Normal capacity of a splitter is about 1 ton/h.

## TRIALS ON DRYING OF SPLIT COFFEE CHERRIES

### Materials and Method

In order to evaluate the effectiveness of splitting in enhancement of coffee drying, a series of trial was conducted in 3 locations with different climatic condition, at Silo (East Java), Kaliwining Experimental Station (East Java), and Liwa (West Lampung), the last has more humid condition. Coffee materials were taken from a plantation in East Java and farmer groups in West Lampung. It consisted of Robusta coffee cherries with ±98% of reds. Coffee splitting was conducted by using a metal type coffee splitter in East Java and wooden type splitter in West Lampung, both are available in farmer communities in those areas. Beside split coffees, normal cherries and coffee parchment with mucilage ("descascado") were also included.

Coffee drying was conducted on a tarpaulin sheet with the load each 30 kg/m$^2$ (6 m$^2$ per unit and wooden framed with 6 cm height). Each type of coffees (treatments) replicated 4 times and each unit of the treatment was placed randomly at each location. The coffee mass was turned 5 times per day. Moisture content of materials was measured gravimetrically every day by using an oven at 105°C for 18 hours, together with $a_w$ (measured with Novasina ms-1) and weight of constant volume (19 l). Coffee bean products were analysed further for its OTA contents, moulds, defect numbers and cup quality. OTA analysis based on the immuno-affinity column clean up and HPLC procedure of Pittet et al. (1996) with minor modification (injection volume 50 µl instead of 75 µl). Defect numbers calculated according Indonesian standard SNI 01-2907-1999. Mould infestation analysed by direct plating of 98 beans on DG-18 media; and cup taste evaluation was conducted by 4 trained panellists and reported only for flavour with score 0 (the worst) – 5 (the best) and defect/taint with score 0 (none) – 3 (max).

The drying rate of each type of coffee was calculated as follow:

$$R = \frac{Xo - Xt}{d}$$

$R$ = **Drying rate**; $Xo$ = **Moisture content at day 0**; $Xt$ = **moisture content at day t**; $d$ = **number of day to get moisture** $Xt$ **around 10%.**

$R$ and other parameters were analysed statistically according to randomised block design.

Weather conditions along the drying trial at each area was also noted especially the evaporation potential.

### Results and Discussion

#### *Drying performance*

Drying performance of split coffee was compared with normal cherries and parchment coffee with mucilage attached. The drying performance is followed by measuring the moisture content until about 10% or less (Figure 1) and $\mathbf{a_w}$ (Figure 2). From statistical analysis of the drying rate $R$, it is evidence that splitting caused faster drying compared to normal/whole cherries and the rates were in between normal cherries and parchments (Table 1). To some extent the drying rate of split cherries similar to parchment at the same coffee mass load. However, since the parchment yields more coffee beans, the total bean yield of split coffee much less (about half) of parchment coffee. Better drying rate of the split coffee compared to normal cherries was due to the wider surface of coffee mass and better exposure of coffee for evaporation.

Drying rate depended on weather condition at each location. The trial at Liwa was a bit hampered by rains at early stage of drying. During first 2 days practically the coffee was covered, while in between the drying period there were rainy parts of the days. Such low evaporation potential made the drying also slow. In Silo, there were also rains at the first 3 days but only in the night; while in Kaliwining there where no rain during the trial. Long or slow drying of coffee in moist area is risky for mould growth, which might promote mycotoxins contamination and worse quality of the coffee products. The drying could have been improved by reducing the load of coffee mass (less thickness) and by applying more frequent turnings.

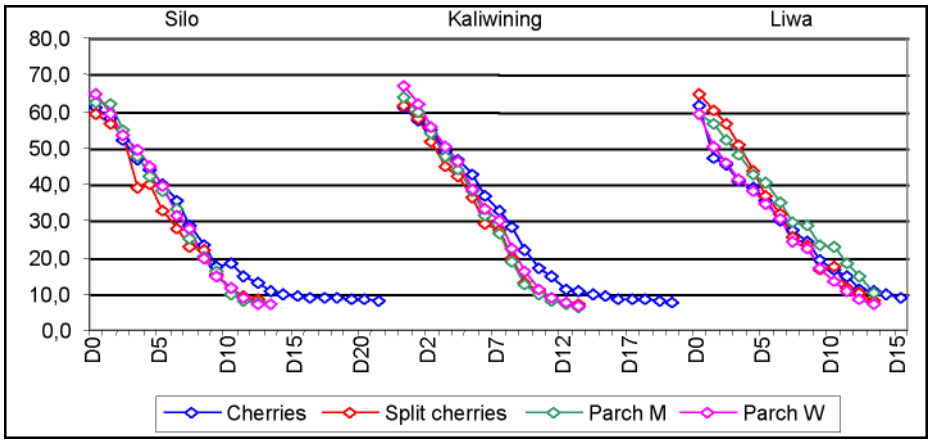


**Figure 1. Reduction of moisture during the drying courses for 4 types of coffee in 3 locations.**

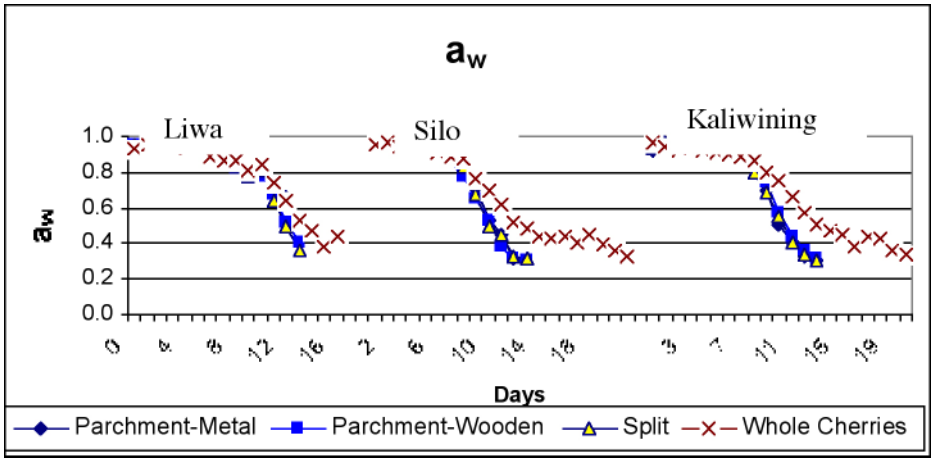


**Figure 2. Reduction of aw during the drying courses for 4 types of coffee in 3 locations.**

Reduction of $a_w$ values during the drying of coffees were similar to the reduction of moisture contents. All the coffee types had similar $a_w$ reduction rate at early drying period up to about 0.8 followed by different rate after, where the split coffee had similar faster rate to the descascado coffee and the normal cherries had the slowest reduction rate (Figure 2). Since many moulds grow only in such condition with $a_w$ higher than 0.7, it can be expected that split coffee is saver for mould growth compared to the normal cherries.

**Table 1. Means of Drying rates (*R*) for coffee types at each location.**

| Coffee type - location | Drying rate (%/day) | Duncan's test ($\alpha = 0.05$) |
|---|---|---|
| Parchment M- Kaliwining | 5.440 | A |
| Parchment W- Kaliwining | 5.413 | A |
| Parchment M- Silo | 5.253 | AB |
| Parchment W- Silo | 5.065 | B |
| Split - Kaliwining | 4.970 | B |
| Split - Silo | 4.548 | C |
| Split - Liwa | 4.510 | C |
| Parchment W - Liwa | 4.245 | C |
| Parchment M - Liwa | 3.935 | D |
| Cherries - Kaliwining | 3.773 | D |
| Cherries - Liwa | 3.693 | DE |
| Cherries - Silo | 3.440 | E |

***OTA contents***

There is no evidence that splitting causes OTA accumulation in the beans product. Very low content of OTA were detected to some samples (Table 2). Only one sample of coffee from split cherries at Liwa was detected contained OTA 0.12 ppb; while two sample of normal cherries from the same location were detected contained OTA at 0.14 ppb and 0.24 ppb (the highest value), respectively. Three coffee samples from parchment were also detected contain OTA with very low concentration (0.09 ppb and 0.03 ppb). The OTA might developed during the drying especially during the first 6-8 days when $a_w$ still higher than 0.85. Drying of coffee cherries is susceptible for mould growth and OTA contamination particularly for the (over) ripe ones as already reported by Bucheli et al. (2000). It was reported that OTA mostly accumulated in the husk and only about 1% present in the beans derived from natural processed cherries. Since we used red cherries, it is probable that the coffee might be contaminated with OTA especially for a long drying of whole/normal cherries.

**Table 2. Means of OTA content in coffee beans.**

| Coffee types | Silo | Kaliwining | Liwa |
|---|---|---|---|
| Normal cherries | 0 B | 0.015 B | 0.095 A |
| Split cherries | 0 B | 0 B | 0.03 AB |
| Parchment Metal | 0 B | 0 B | 0 B |
| Parchment Wooden | 0 B | 0.023 B | 0.03 AB |

*Note: Numbers followed by the same letter are not significantly different (Duncan's Multiple Test $\alpha$=0.05)*

***Moulds infestation***

Mould species infested the beans during the drying course predominantly were *Aspergillus*, only very few were *Penicillium* and other species. The most dominant ones were black *Aspergillus*, which the higher rates were found in the coffee beans from split cherries (Figure 3). The second dominant group was flavi, which mostly present in Silo and Kaliwining, while very few found in Liwa. Split cherries tended to be contaminated with flavi at a higher rate compared to other types. *A. ochraceus* only found in 5 samples with very low (2-8%) infestation rate.

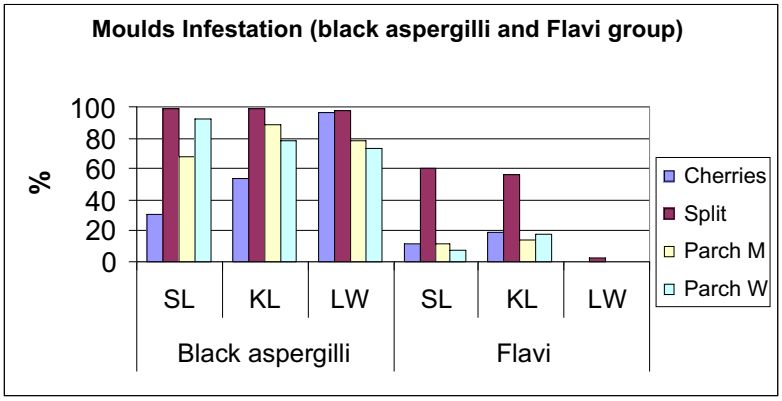


**Figure 3. Infestation rate of black aspergilli and flavi group.**

***Defects***

Defect numbers of coffee beans might be originated from the coffee cherries or might be developed during the drying period. There is no significant different of defect numbers of each type of coffee tried in Silo and Kaliwining (Table 3). This is proved that the pre-treatment of coffee prior to drying did not change the defect number. In case of Liwa, the defect number was higher compared with other 2 locations. This is might come from the coffee material used that already worst. Dominant defect components were black beans, partly black beans, brown beans, immature beans, and holed beans. Statistically, splitting caused the defect number higher compared with the 2 types of coffee in Liwa. It is need to reconfirm the higher defect come whether due to the processing or from the coffee source.

**Table 3. Means of defect number of coffee beans.**

| Coffee types | Silo | Kaliwining | Liwa |
|---|---|---|---|
| Cherries | 48.3 C | 49.8 C | 112.0 B |
| Split cherries | 56.4 C | 43.4 C | 191.1 A |
| Parchment Metal | 36.7 C | 53.8 C | 109.1 B |
| Parchment Wooden | 49.9 C | 51.7 C | 123.0 B |

*Note: Numbers followed by the same letter are not significantly different (Duncan's Multiple Test $\alpha$=0.05)*

***Cup taste***

Coffee samples from trials in Liwa in general had a better flavour score although some of them had a low level defect taints of fermented and mouldy, which encountered by only fewer of the panellists. Development of the defect might due to the wet condition on the first days of drying. Fermented taint also found in some sample of normal cherries from Kaliwining and Silo, while not present in split cherries and parchments. Our experience before also proved that splitting can reduce the fermented taint of coffee beans.

From the above data showed that the splitting is favourable at a dry and even condition, while if the weather is uneven likely splitting might worsen the coffee. To avoid that, the thickness of the coffee can be reduced to 3 cm or less and applying more frequent turnings. In practice, drying of split coffee is done on a plastic sheet with the thickness only 1–3 cm, which different to this trial (7.5 cm). At that thickness normally the drying can be finished in about 6–8 days (in a good weather).

**Table 4. Means of flavour value and taints degree of coffee product.**

| Location | Coffee type | Flavor* | Fermented# | Mouldy# |
|---|---|---|---|---|
| Liwa | Cherries | 3.47 bc | 0.38 d | 0.25 abc |
| | Split cherries | 3.34 c | 0.63 c | 0.08 c |
| | Parchment Metal | 3.72 ab | - | 0.125 bc |
| | Parchment Wooden | 3.85 a | - | 0.5 abc |
| Silo | Cherries | 3.13 c | 1.0 b | 0.75 ab |
| | Split cherries | 3.19 c | - | 0.79 a |
| | Parchment Metal | 3.38 c | - | 0.125 bc |
| | Parchment Wooden | 3.44 bc | - | - |
| Kaliwining | Cherries | 1.94 d | 2.1 a | - |
| | Split cherries | 3.38 c | - | - |
| | Parchment Metal | 3.44 bc | - | - |
| | Parchment Wooden | 3.38 c | - | - |

*Note: * = Flavour score 0 – 5 (best); #= taint score max 3.*
*Numbers followed by the same letter are not significantly different (Duncan's Multiple RangeTest $\alpha = 0.05$)*

Farmers never store the dried split coffee. Instead the coffee de-hulled immediately, since the split coffee is too "bulky" for storage. Farmers in Lampung applying the system only for cash money. While for a long time storage they dry the whole coffee normally, and store the dried cherries. Coffee maturity also influenced moulds infestation and taints, which the most susceptible ones are the ripe and overripe cherries (Ismayadi et al., 2001; Bucheli et al., 2000). By the splitting and drying at a thinner layer the development of moulds and taints like fermented and mouldy can be avoided due to the faster drying.

## CONCLUSION

- Splitting improved drying performance of coffee cherries.
- Splitting was not promoting OTA contamination and defects of coffee beans and could reduced such taint defect like fermented that frequently occur in normal red cherries.
- Application of splitting method in an uneven weather area can be done with precaution and by applying less thickness and more frequent turnings on drying.

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# Amélioration de la Qualité du Café par la Prévention contre les Moisissures

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**RÉSUMÉ**

La présence de l'ochratoxine A (OTA), mycotoxine provenant du métabolisme de certaines moisissures particulièrement du genre Aspergillus, affecte la qualité sanitaire et commerciale du café. Les nouvelles mesures réglementaires au niveau du commerce international fixe des seuils d'OTA de 8 à 10 µg/kg dans le café vert et de 3 à 4 µg/kg dans le café torréfié.

Afin de garantir les revenus des opérateurs de la filière, des activités de recherche portant sur, les techniques de séchage, les délais de traitement après récolte, l'influence de la maturité des cerises sur la qualité sanitaire du café, la charge des séchoirs, la microbiologie, les défauts… sont conduites simultanément en Côte d'Ivoire en vue de dégager des mesures de préventions (bonnes pratiques agricoles) qui, adoptées, contribueront à la production d'un café marchand de bonne qualité sanitaire.

Les résultats obtenus ont montré que le séchage des cerises de café est d'autant plus rapide que les cerises sont mures (rouge), les charges de 30 kg et de 40 kg/$m^2$ de séchoir sont les charges optimum respectives pour le traitement par voie sèche et par voie humide. Le séchage sur le séchoir basculant mis au point au CNRA offre un rapide et meilleur séchage quelque soit le mode de traitement (voie humide ou voie sèche).

Des moisissures du genre Aspergillus, des levures et des bactéries constituent la flore externe (communauté externe) et la flore interne (communauté interne) de la cerise de café.

Les analyses relatives à la détermination du taux d'ochratoxine A aux différents points critiques sont en cours au CIRAD à Montpellier.

**SUMMARY**

Ochratoxin A (OTA), a mycotoxin derived from the metabolism of some moulds, particulary Aspergillus, affect the commercial and sanitation of coffee quality.

The new international trade regulations set the OTA limits for green coffee from 8 to 10 µg/kg and for roasted coffee from 3 to 4 µg/kg.

To secure the incomes in the coffee channel production, some research activities were conducted. These activities concern drying technique, delay of post harvest treatment, influence of coffee cherries maturity on coffee sanitation quality, dryer capacity, microbilogy aspect and defaults. These research activities are being conducted in Côte d'Ivoire in order to take some measures to improve good agricultural practices. These measures if adopted can contribute in producing good, healthy and marketable coffee quality.

Results show that drying of coffee cherries is faster when the cherries are mature (red) and that the dryer optimum capacity should be30 kg and 40 kg/$m^2$ respectively for dry and wet processing. Drying which CNRA rocking dryer offer fast and better drying regardless of the treatment used. Moulds of Aspergillus genus, yeast and bacteria give the external and internal flora of coffee cherries. The analysis of OTA determination on different critical points are being conducted at CIRAD in Montpellier (France).

## INTRODUCTION

Après avoir maîtrisé les problèmes de santé liés aux intoxications alimentaires provoquées par les pathogènes vrais comme les salmonelles, les listeria et certains microorganismes comme les Staphylocoques, sont apparues dans les années 60, des pathologies chroniques provoquées par des microorganismes responsables de la production de mycotoxines, produits du métabolisme de. On note entre autres moisissures, *Aspergillus flavus,* responsable de la production de l'aflatoxine, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus sulphureus*, *Asprgillus citricus*, certaines espèces de *Penicillium* comme *Penicillium verrucosum*, responsables de la production des ochratoxines, les *Fusarium* qui produisent la fumonisine. Ces mycotoxines sont responsables de diverses pathologies humaines appelées mycotoxicoses. Christensen et Lopez (1963), Christensen (1957, 1972), Creppy et al. (1995) et Studer-Rohr (1995) ont montré que ces mycotoxines perturbent également le métabolisme glucidique et la coagulation sanguine. Ces pathologies étaient à l'origine peu connues. En effet, des grains de céréales consommés après conservation prolongée dans le sol ont provoqué des mortalités massives par millier en Russie et en Mandchourie (situation de guerre). Il a été prouvé par la suite que ces mortalités étaient dues à l'Ergotoxine, issue de l'ergot du seigle caractérisée par une atteinte du système nerveux. Une maladie décima en 1960 tous les dindons de la Grande Bretagne. Ces derniers présentaient des tâches sur le foie. Analysés, il a été mis en évidence une mycotoxine appelée aflatoxine.

De façon globale, ces mycotoxines ont des propriétés carcinogènes (cancer du foie), néphrotoxiques, immunodépressives, génotoxiques, tératogènes neurotoxiques et se développent dans de nombreux produits agricoles, principalement dans les céréales. A côté des céréales, figurent le café et le cacao qui représentent les principales sources de revenu des pays producteurs. Le café et le cacao sont principalement contaminés par l'ochratoxine A (OTA. La présence de l'OTA dans le café vert a été signalée dès 1974 par Levi et al., dans le café-boisson par Tsubouchi en 1988 et par Studer-Rohr en 1995.

Le comité scientifique de l'alimentation humaine, dans son rapport du 17 septembre 1998, a estimé nécessaire, compte tenu des effets néfastes des mycotoxines sur la santé humaine, de veiller sur les teneurs en ces toxines et en particulier la teneur de l'OTA dans le café et dans certains produits alimentaires. Selon ce rapport, il serait prudent de réduire autant que possible la teneur en OTA dans le café à 5 ng/kg p.c. /jour.

Cette norme se pose à toutes les variétés de café de toute origine quel que soit le mode de traitement (voie sèche ou voie humide) pratiqué. Par conséquent, tous les pays producteurs de café sont concernés par le problème de l'ochratoxine A.

## PROBLÉMATIQUE

Les produits d'exportation des pays africains sont pour la plupart dominés par le café et le cacao. Ces échanges pourraient être négativement bouleversés compte tenu des projets de normes en voie d'adoption par l'Union Européenne et fixant le taux maximum de l'OTA entre 8 et 10 µg/kg dans le café vert et 3 à 4 µg dans le café torréfié. Notons que dans le même

temps, certains lots de café en provenance des différents pays producteurs présentent des taux d'OTA supérieurs à 10 ppb.

En effet, des échantillons de café prélevés en Europe et en provenance d'Afrique de l'Est et de l'Ouest (OIC, 1996), ont révélé des taux de contamination respectivement de 9% et de 18% qui représenteraient d'importantes pertes de devises. Pour certains pays de ces mêmes régions, le taux de contamination s'élèverait à 25%. Dans le cas de la Côte d'Ivoire, l'adoption de ces normes se traduirait par le refus 54.000 tonnes de café vert, soit une perte de plus de 20 milliards de francs CFA. Des rejets de café en provenance de la Côte d'Ivoire ont déjà été enregistrés en Italie et en Espagne.

Selon les informations fournies par l'Union Européenne, une limite de 2 µg/kg dans les fèves et poudre de cacao et 1 µg/kg dans le chocolat serait proposée par une minorité des états membres de l'Union Européenne. L'application de ces normes engendrerait une perte estimée à 142 milliards de francs CFA par an soit 2,3% du PIB et 5,7%des exportations totales de la Côte d'Ivoire.

Face à cette situation qui se traduit par la disparition des entraves traditionnelles (barrières tarifaires) au commerce international des produits agricoles au profit de nouvelles exigences de qualité sanitaire, il conviendrait que la Côte d'Ivoire, qui est un des principaux pays producteurs de café et de cacao dans le monde, réagisse pour limiter la contamination du café par l'OTA et cela afin de préserver ses intérêts tout en garantissant les revenus des 670.000 producteurs que compte le pays.

Pour atteindre cet objectif, le Centre National de Recherche Agronomique (CNRA), qui a en charge la recherche agronomique en Côte d'Ivoire conduit plusieurs activités de recherche en vue de maîtriser la prévention des moisissures responsables de la production de l'ochratoxine A dans le café et dans le cacao et participe dans le même temps aux activités du comité national chargé du problème de la qualité des produits agricoles de façon générale en Côte d'Ivoire.

Les objectifs globaux poursuivis à travers ces différentes activités ont porté sur la défense des intérêts des produits agricoles, en particulier le café et le cacao.

De façon spécifique, ces activités doivent garantir à terme, l'accès du café et du cacao ivoirien au marché international tout en respectant les nouvelles exigences de qualité sanitaire, par l'adoption de bonnes pratiques agricoles devant permettre la production de café et de cacao de bonne qualité marchande.

## ACTIVITÉS

Les activités conduites au CNRA en vue de l'amélioration de la qualité du café et du cacao se repartissent en activités de recherche et en activité de sensibilisation et de formation des producteurs aux bonnes pratiques agricoles.

### Activités de recherche

Malgré la situation socio-politique la Côte d'Ivoire, à travers le CNRA, participe au projet intitulé ''Amélioration de la qualité du café par la prévention contre les moisissures.'' Six autres pays producteurs de café (Brésil, Colombie, Inde, Indonésie, Ouganda et Kenya) participent également à ce projet financé à hauteur de USD 6.242.000 par le Fonds Commun

des produits de base (CFC), l'industrie européenne du café, le Gouvernement Allemand et une contribution des 7 pays impliqués dans ledit projet. Le projet est administré par la FAO.

L'objectif principal de ce projet est de rechercher les moyens pouvant contribuer à l'amélioration de la qualité du café afin de faire des recommandations de bonnes pratiques agricoles et hygiéniques aux intervenants de la filière.

Pour ce faire, des essais de recherche portant sur :

- la distribution des moisissures productrices de l'OTA dans l'écosystème des plantations en début et en fin de saison,
- l'impact du délai de traitement des cerises après la récolte tant par la voie sèche que par la voie humide sur le développement des microorganismes producteurs d'OTA,
- l'impact des cerises tombées et associées aux récoltes sur la contamination des cerises de café,
- des études mycologiques devant permettre de comprendre la relation moisissures – environnement et son influence sur la contamination du café et du cacao,
- le séchage en fonction de la maturité des cerises afin de déterminer l'impact de la qualité de la récolte sur la contamination et la production de l'OTA dans le café,
- la distribution (localisation) des moisissures productrices de l'OTA dans les cerises de café (communauté fongique externe et interne),
- la dynamique et l'adaptation des moisissures productrices de l'OTA dans les cerises de café au cours des étapes de récolte, de séchage, de stockage et de transport,
- le stockage afin de déterminer les conditions optimales de conduite d'un bon stockage,
- la détermination des points critiques de contamination des cerises.
- et enfin, la fiabilité d'humidimètres d'utilisation simple par les producteurs et les industriels impliqués le long de la chaîne de production du café et du cacao,

Les zones d'études sont les grandes régions de production du café et du cacao avec pour sites expérimentaux, les stations de recherche sur le café et le cacao du CNRA et les plantations privées.

## RÉSULTATS PARTIELS DES ACTIVITÉS

### Activités de sensibilisation et de formation

Acquis:

**1) au niveau du CNRA**
- Conduite d'activités de recherche portant sur l'amélioration de la qualité du café et du cacao,
- Mise sur pied d'une équipe chargée de la diffusion des bonnes pratiques agricoles,
- Existence de modules de formation des opérateurs économiques de la filière.

**2) au niveau de l'Agence Nationale d'Appui au développement Rural (ANADER)**
- Existence d'une équipe de vulgarisateurs de bonnes pratiques agricoles dans les zones de production du café et du cacao,
- Existence et diffusion de modules de formation sur les bonnes pratiques agricoles

**3) au niveau du Ministère de l'Industrie et du développement du secteur privé (MIDSP)**
- Existence d'une équipe chargée de la diffusion du management de la qualité dans les secteurs du café et du cacao,

- Existence de modules de formation en management de la qualité dans les secteurs du café et du cacao.

**4) au niveau national**

- Existence au niveau national d'un comité LOBBYING et d'un comité QUALITE
- Existence de résultats d'une étude commanditée, financée par l'Union Européenne et exécutée par le Bureau d'Etudes Landell Mills.
- Montage et équipement d'un laboratoire complet d'analyses d'OTA au LANADA,
- Enquête sur le niveau réel de contamination du café et du cacao par l'OTA en cours d'exécution par le LANADA,
- Etude de l'incidence des traitements industriels appliqués au café vert et au cacao sur la réduction de la teneur en OTA dans les produits finis en cours d'exécution à l'UFR de pharmacie de l'Université de Cocody,
- Renforcement des capacités analytiques du CNRA, du LANADA, du laboratoire de toxicologie de l'UFR de pharmacie.

L'ensemble de ces acquis doit contribuer à la maîtrise de la prévention de la production de l'OTA dans le café et dans le cacao.

**SENSIBILISATION**

L'agriculture en Côte d'Ivoire représente plus de 70% du PIB national et constitue la base de l'économie dont le café et le cacao sont les principaux soutiens. Ces deux cultures assurent le revenu de plus de 670.000 producteurs.

Des journées portes ouvertes organisées sur les stations de recherche sur le café et le cacao du CNRA participent à l'information et à la sensibilisation des producteurs sur les conséquences du rejet du café et du cacao sur le marché international :

- Perte d'une importante part du marché international avec pour conséquence première, la diminution des rentrées de devises au niveau national,
- Baisse des revenus des 670. 000 producteurs de Côte d'Ivoire.

**FORMATION**

Le CNRA dispose de trois formateurs de formateurs aux bonnes techniques agricoles et hygiéniques. Ils ont à charge la formation des vulgarisateurs des bonnes pratiques agricoles et hygiéniques auprès des opérateurs économiques de la filière café-cacao.

Sont en cours de préparation :

- Un séminaire de formation aux bonnes pratiques agricoles et hygiéniques post récolte des opérateurs économiques de la filière café des pays francophones membres de l'Organisation Internationale du Café (OIC)
- Un séminaire de formation aux bonnes pratiques agricoles et hygiéniques post récolte des opérateurs économiques de la filière café en Côte d'Ivoire.

Ces deux séminaires, financés par le projet ''Amélioration de la qualité du café par la prévention contre les moisissures'' auront lieu au cours de cette année 2004.

**ACTIVITÉS DU COMITÉ LOBBYING**

Plusieurs voyages auprès de la CAOBISCO, de l'ECA de la DG SANCO

## Partenaires

a) Au niveau local :
- LANADA
- ANADER
- UFR Pharmacie
- Ministères techniques

b) Au niveau international
- Union Européenne
- CFC et Landell Mills

# Checking Drying

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## INTRODUCTION

Drying, be it natural or artificial, is by far the key stage in coffee post-harvest processing. However, although natural or sun drying is the most common method and the simplest to use, it calls for particular care if it is to be done properly. Complete drying before storage ensures safe conditions and is a guarantee against risks of toxigenic mould growth and OTA production as long as storage facilities and practices are appropriate.

Experiments carried out through the "Enhancement of coffee quality through the prevention of mould formation" project have shown that appropriate drying equipment is required, such as plastic tarpaulins or cemented areas. In addition, appropriate and scientifically tested practices have to be applied, such as turning frequency which optimum seems to be 4 stirrings per day and covering during night and when it rains.

Dryer loading is also a parameter which must controlled as well as possible, or else drying course is lengthened and homogenization of coffee is more difficult.

## HOW TO DETERMINE THE SIZE OF A DRYING YARD

Sizing the drying area is the first obstacle to be overcome for good drying management. The cost of investment, or lack of space, are often blamed for under-sized drying areas, but it is also necessary to consider the lack of technical knowledge among farmers and extension officers, who do not possess a simple way of assessing drying requirements.

The minimum area required for proper coffee drying can easily be calculated with a simple formula:

$$S = \frac{T \times W_d}{L}$$

where

- S ($m^2$) is the surface of cemented drying yard, table or tarpaulin
- $W_d$ (kg) is the daily weight of cherries which have been collected or parchment which has been processed,
- T (days) is the average length of drying in a given location and
- L ($kg/m^2$) is the weight of fresh cherries or wet parchment per $m^2$ of yard

All these variables can be quantified using information obtained from interviews with farmers, and data drawn from experimental work.

**Daily weight of coffee**

The first thing needed is as accurate as possible an estimation of the amount of cherry or parchment coffee that will have to be dried by the end of one day's harvest or one day's wet processing. This information can be obtained from farmers or through calculations based on farm production and harvest peak intensity.

This weight of cherries depends on their degree of ripeness and, of course, on the labour available and processing capacity for the wet process.

In many coffee producing countries, the harvest period is spread over 3 or 4 months with a peak at the mid season which lasts for 3 to 4 weeks. On average, it is estimated that 80% of total production is picked at the harvest peak or 3 to 4% of total production per day. For example, a farm producing 10 tonnes of cherries per year will collect 300 to 400 kg of cherries per day on average.

In countries where there are two picking seasons, this estimation must, of course, be done for the most important harvest peak.

**Length of drying**

This variable is linked with weather conditions, practices and load of dryer. As there is a relation between two variables, it is considered in general that an acceptable length of drying should not exceed 2,5 weeks for parchment and 3,5 weeks with on average respectively 2 and 3 weeks. An optimal length of drying means that quality is not altered by fermentation and moulds at the end of drying.

These recommendations are confirmed by the experiments which have been carried out in the project.

The length of drying corresponds to the time during which the dryer will be occupied by coffee and will therefore not be available for the following days' harvests.

**2.3. Load of dryer**

Research experiments have made it possible to determine the optimum load of a drying area, in $kg/m^2$. This load is used to determine the optimum drying time in accordance with perfectly defined practices, such as the coffee turning frequency, and with local climatic conditions.

Graph 1 clearly shows differences in curves according to loads. With the highest load, drying starts effectively on day D3 whereas it starts on day D1 at a load of 10 $kg/m^2$ and length of drying is much longer to complete drying.

Under the conditions of the experiment the load of 10 $kg/m^2$ should be recommended but the investment is high and probably not profitable. The choice is therefore between 30 $kg/m^2$ and 50 $kg/m^2$ loads. In this case, risks of unacceptable contamination which might occur when the water activity remains above 0,80 for a too long time. Thus, in this experimental case, it is preferable to choose a load of 30 $kg/m^2$. This critical water activity limit is achieved after 10 days of drying instead 12 days for 50 $kg/m^2$ load. The shape of drying curve for 50 $kg/m^2$ load might favour mould growth the first 6 days even if water activity is more suitable for yeasts.

Appropriate drying equipment and practices also require that the moisture content be controlled throughout the drying process, especially towards the end. Several ways of determining moisture content can be used: direct and indirect methods.

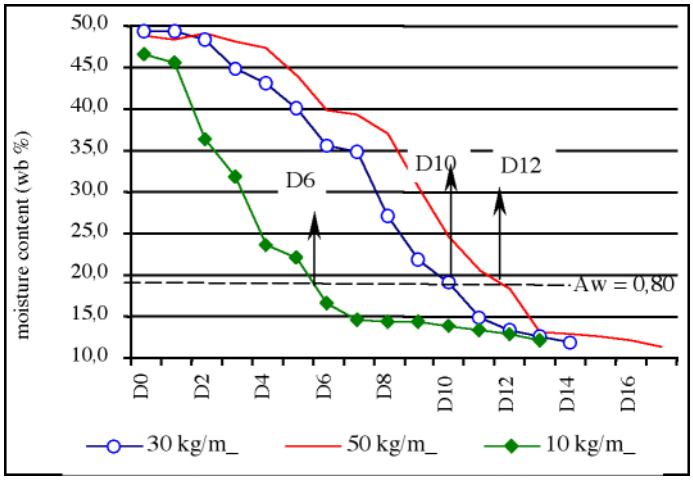


**Figure 1. Drying curves according to load of dryers (Sources: Data from Côte d'Ivoire, 2003; project GCP/INT/743/CFC).**

**Table 1. Estimation of drying surfaces needed using results in Figure 1.**

| Load (kg/m$^2$) | 10 | | 30 | | 50 | |
|---|---|---|---|---|---|---|
| Length of drying (days) for mc=12% | 12 | | 13 | | 15 | |
| Daily harvest (kg/day) | 300 | 400 | 300 | 400 | 300 | 400 |
| Surface (m$^2$) | 360 | 480 | 130 | 173 | 90 | 120 |

## CONTROL OF MOISTURE CONTENT THROUGHOUT DRYING COURSE

Different methods can be used to estimate moisture content during drying and they can be grouped in three categories:

- empirical methods
- direct methods
- indirect methods

### Empirical methods

In practice, many farmers and middlemen use trial and error to estimate the moisture content of their coffee. They are based on hardness of coffee beans, which is assessed by biting, or tinkling of cherries or screeching of parchment but the observation cannot be quantified and evidences of correlation between moisture content and the state of coffee have never been established.

These methods have no scientific basis and can in no way be correlated to moisture measurements taken in an oven. They should therefore be done away with.

### Direct method

The direct method involves oven-drying and has been standardized for coffee beans (ISO standards). However, it can be applied to cherry and parchment coffee for applications in the field and for research. The method implies to dispose of laboratory equipments such as oven

and analytical balance and takes at least 12 hours to determine moisture content, so that direct method cannot be used on routine basis. It is precise and has to be applied to calibrate the equipment used in the indirect methods. Moisture content is generally given on wet basis but for peculiar work it can be expressed on dry basis (some relations between water activity and moisture content are defined by using mc on dry basis).

$$mc_{wb}\ \% = 100 \times \frac{W_w - W_d}{W_w} \qquad mc_{db}\ \% = 100 \times \frac{W_w - W_d}{W_d}$$

where

- $W_w$ is the wet weight of the sample
- $W_d$ is the dry weight of the sample

**Indirect methods**

The advantage of indirect methods is that they give results rapidly, subject to appropriate calibration of the measuring instruments. They can therefore be used on a routine basis. Different indirect methods have been tested:

- moisture meters
- weight of a constant volume
- relative humidity of the air between coffee cherries in an enclosed chamber

***Moisture meters***

Moisture meters are the instruments most frequently used, but they need to be carefully calibrated. Their principle lies in measuring the passage of an electrical current through the product, which is then converted into a moisture %. These instruments are designed to give a linear result and usually they have been calibrated for cereals or other small seeds by the manufacturer. As calibration depends on types of seeds, a new calibration must be done for coffee beans, cherries and parchment. It therefore has to be checked first of all that the values displayed by the instrument perfectly tally with the moisture contents of control samples or in other words in curves drawn from standards and readings corresponding to the standards are parallel. In the example shown on graph 2, it is clear that instrument 1 (Figure 2a) provides plausible indications whereas instrument 2 (Figure 2b) is not satisfactory. It can be checked statistically that the curves in one case are parallel, but not in the other. Instrument 2 cannot be calibrated and must resent to the manufacturer.

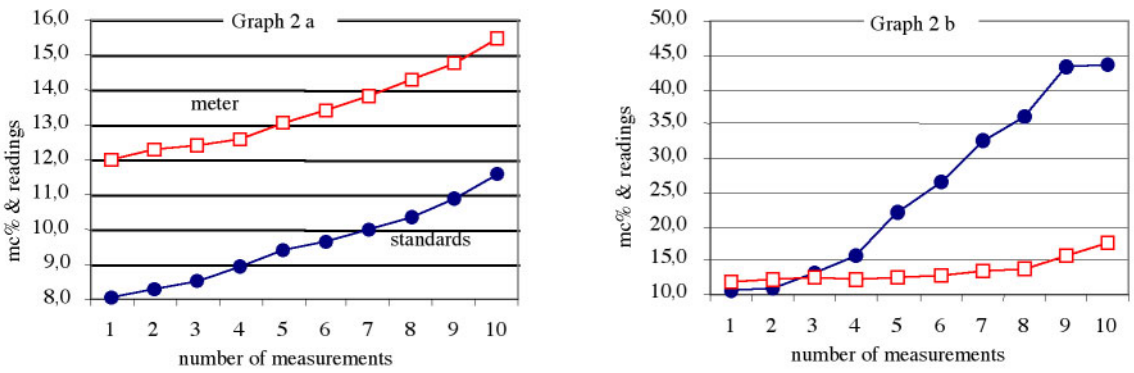


**Figure 2. Responses of two different meters (Sources: Data from Indonesia, 2004; project GCP/INT/743/CFC).**

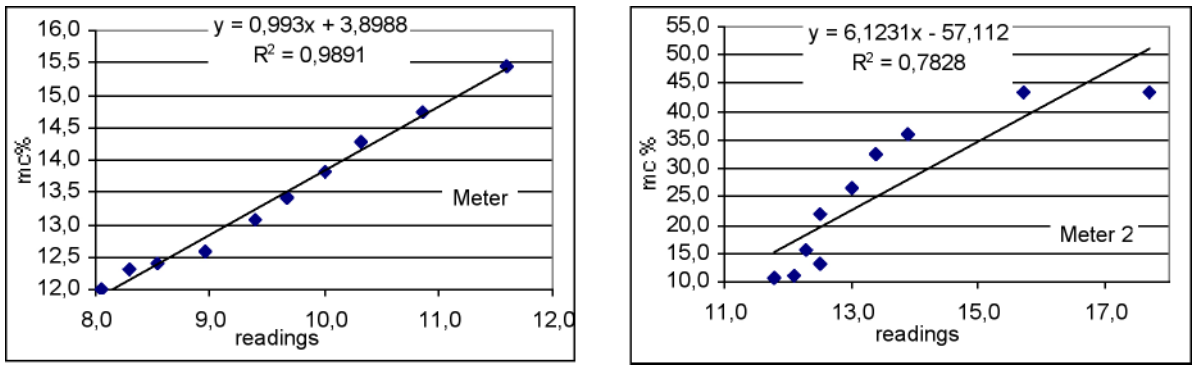


**Figure 3. Calibration of moisture meter.**

The second stage consists in establishing the relation between the values given by the instrument, and the moisture contents of the control samples, hence by superposing the two curves obtained for instrument 1. It is clear that a translation downwards of the curve plotted from readings of the meter will more or less overlapped with the curve plotted from standards. The regression equation y = a X + b in which y is the exact moisture content value of an unknown sample and x is the value given by the instrument is computed. Coefficient of regression is near 1 and the coefficient a ≈ 1 (graph a). The adjustment is excellent. In graph b, coefficients confirms that calibration cannot be done. From the regression equation a calibration chart is established which will be used.

Another point should be taken into account: moisture meters are conceived and built to work within a given range which is mentioned in the manufacturer's manual. In general, this range is between 10 and 25% mc for sophisticated meters. The example in graph 3b shows that there is a high deviation with standards above 30% mc. A calculation was done with standards below 30% and the regression still remains bad.

A third stage consists in checking that the same control always gives the same value. The precision of the instrument is then determined by statistical analysis.

***Weight of a constant volume***

A second indirect way of assessing coffee moisture content is to measure the weight of a constant volume of coffee. Although tempting, this method is not satisfactory as the correlations are very poor between weight and constant volume. Figure 4 shows that regressions are different according to locations, thus it is not possible to determine a unique calibration chart.

In fact, this amounts to assessing the bulk density of the coffee depending on its moisture content. Yet, that density is closely linked to agricultural practices and the environment, hence to locations. Moreover, there is substantial scatter of the measurements for the same location. As it stands, the method is probably not reliable. However, it should be possible to adapt it, not by monitoring changes in density, but by using the fact that this weight is more or less constant when the coffee is dry. It would then be enough to assess the water content of coffees when weight varies by more or less 100 to 200 g on 3 to 4 consecutive days.

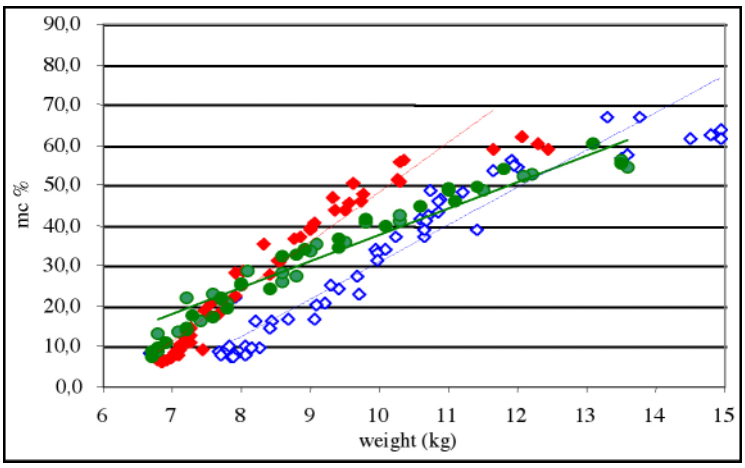


**Figure 4. Weight of a constant volume in 3 different locations (Sources: Data from Indonesia, 2004; project GCP/INT/743/CFC).**

***Relative humidity***

A third method, reserved for research purposes, is based on the relative humidity of the air between coffee cherries in an enclosed chamber. In this case the water activity of the product is measured. This method therefore means establishing the relation between Aw and the coffee moisture content. These are sorption/desorption curves.

Different models have been published and under the project the model, which has been used, is adapted from published models. The general equation, which has been used, is:

$$Aw = 1 - \alpha e^{a \times mc^2 + b \times mc + c}$$

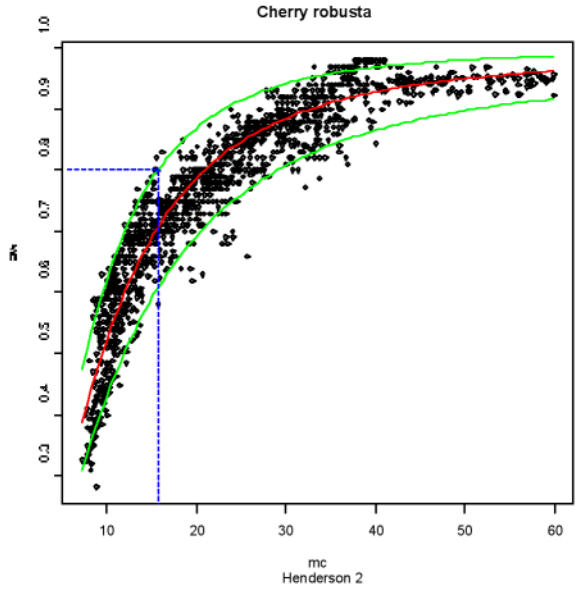


**Figure 5. Desorption study fro robusta cherries (Sources: Data from project GCP/INT/743/CFC).**

The advantage of the method is that it can be used to assess coffee moisture content based on the biological criteria of mould growth. Precise measurements call for special equipment, but

measurements taken in the field provide an idea of coffee moisture content when Aw reaches the critical value of 0.80. With a confidence interval of 1%, cherries are considered free of contamination risks once their moisture content has reached 12.5%. For parchment coffee, the value would be 13.8%. These values also depend on the mathematical models used.

Graph 5 gives an idea of the distribution of points (Aw, mc) for robusta cherries. All measurements from each trial have been pooled to estimate the relation.

**Table 3. Moisture content for an Aw = 0,80.**

| % mc for Aw = 0,80 | Interval of confidence | | |
|---|---|---|---|
| | ± 5 % | ± 1 % | ± 0,1 % |
| Robusta cherries | 15,7 | 14,1 | 12,5 |
| Parchment arabica | 14,9 | 13,8 | 12,6 |

Table 4 gives the results of desoprtion studies for robusta cherries and arabica parchment. Practically, moisture content must be below 14% mc at an interval of confidence of 1% before coffee is safe and thus can be stored in order to minimize risks of mould growth and OTA production during storage. Is this level sufficiently low to ensure safe conditions for storage and transportation or should mc at an interval of confidence of 0,1% be taken into account? Further investigations are needed to answer this question.

## CONCLUSION

To conclude, effective management of coffee moisture content during drying relies on the application of good technological practices, the availability of adequate drying areas, and the availability of reliable ways of measuring that moisture. Moisture meters are the most useful instruments for that purpose. Granted, some of them are expensive, but cheaper models that are sufficient for farmers do exist. The method based on coffee density at the end of drying should also be looked into more closely, to check whether or not it is applicable.

# Development and Assessment of Various Models of Solar Dryer for Drying of Coffee Cherries

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## SUMMARY

Despite remarkable increase of the domestic coffee production, several problems related to the coffee bean quality remain unsolved. Recently Indonesia produces approximately 600.000 tons and smallholders dominate 90% of this. Referring to the defect-based quality system, the quality of smallholders' coffee beans is considered very poor. Some survey reported that the drying was the key operation in the smallholders' coffee processing practice and was assumed as the major factor affecting the final coffee quality. Various models of solar dryer had therefore been developed and tested for the drying of coffee at the farmers' field using both solar and furnace as heating sources. The model varied from a small scale drying capacity of 500 kg coffee cherries till a large-scale solar dryer up to 5000 kg one. The small scale dryer essentially consisted of a flat plate air heating collector, a tunnel-like drying chamber and a small fan of 0.125 kW to provide the required airflow over the coffee cherries layer. Both the solar collector and the drying chamber were covered with a UV stabilized transparent plastic sheet. The average time required for drying fresh coffee cherries to the final moisture content of 12% was 76 to 80 hours, whilst at the same time, the moisture content of comparable samples dried in the traditional sun drying were only 35% (wb). The average daily solar radiation during the tests was about 2.50-3 kWh per $m^2$ resulting an average drying chamber temperature of 38-40$^oC$. Firewood consumption for the additional heating during nighttime was 0.5 kg per kg-dried coffee. The medium scale dryer utilized a solar greenhouse model. A UV stabilized plastic sheet was used to cover the roof and sidewalls. The height of the house was 2.80 m. The available floor under the greenhouse was 6 m long and 3 m wide. A flat bed type dryer of 3 m x 2 m x 1 m was installed in the middle part of the floor. The dryer was equipped with a 0.4 kW exhaust blower. Test results with several deeps of coffee bed had indicated that the highest drying efficiency was attained at 57% when the drying chamber was loaded with 1100 kg. This amount was equal to a 0.30 m deep bed of wet coffee cherries. The specific energy was 5.20 MJ per kg water evaporated. The average daily solar radiation was 3.50 kWh per $m^2$ producing an average drying chamber temperature of 38-40$^oC$, and rH of 45%. The total drying time was 72 hours when the final moisture reached 11.20%. The large scale of solar coffee dryer was provided with a 144 $m^2$ solar collector incorporated on the roof of the drying building. A flat bed type dryer of 6 m x 3 m x 1 m was installed under the roof and the capacity was approximately 5000 kg of coffee cherries. The airflow of the hot air through the collector was 150-250 $m^3$ per min and was generated by a 1.25 kW multiple-operated axial fans. The collector was able to heat the drying air up to 60°C and rH of 24% when the average solar radiation was 3.50 kWh per $m^2$. Burning of 55 kg wood per hr in the furnace during nighttime could maintain the drying air at a relatively stable temperature of 55-60°C and rH 25-30%. The total drying time was 48 hours when the final moisture reached 12% at a 0.30 m deep bed. The heat output of the furnace ranged from 55 to 70 kW at the combustion efficiency of 50 to 60%. The entire trials showed that the models of solar dryer performed sufficiently even during cloudy and rainy days. The mean quality of the coffee dried in the solar dryer was physically better than that of the sun drying samples. The

improvement of drying rate was able to lower the moisture content of the coffee cherries at the right time. As a result, the risk of spoilage and microbial infestation to the dried coffee beans could be reduced. The proper protection of the coffee cherries during the entire drying process could also avoid the contamination of the product against soil, insects and other undesirable materials. These combined factors contributed a significant improvement of the coffee bean quality as well as its uniformity and consistency.

## INTRODUCTION

Annual production of the coffee in Indonesia is approximately 600.000 tons and smallholders (small family farms) produce 90% from this amount. The average land holding of an individual farmer ranges from 0.2 ha to 1 ha and 90% of them cultivate Robusta coffee plant. Due to limiting harvesting amount and lack of capital for purchasing a proper drying facility, farmers prefer to dry the coffee cherries by traditional sun drying.

Several surveys reported that sporadic rains are common during the harvest season of coffee, which makes it difficult to consistently sun-dried coffee and thus lowers significantly the quality of sun-dried coffee beans. The traditional sun drying methods often yield poor quality, since the produce is not protected against dust, insects, and animals while drying. Soiling, contamination with microorganisms, formation of mycotoxins, and infection with disease-causing germs are the result. The report also concluded that the drying is the key operation in the smallholders' coffee processing practice and is assumed as the major factor affecting the final coffee quality.

The Coffee and Cocoa Research Institute have conducted some efforts to design and implement solar dryers to improve the coffee production in Indonesia. Solar drying can be considered as an elaboration of sun drying and a solution of all the drawbacks of natural drying. Even with a very simple mean, solar dryer is provided with devices to supply; more sufficient heat to draw out moisture, dry air to absorb the released moisture, and adequate airflow to carry off the moisture out from the dryer. Thus, solar drying can be used for the entire coffee drying process or for supplementing artificial drying systems to reduce the total amount of fuel energy required.

For the purpose of classifying the cases studied, categories have been established according to the intended use of each type of system. Various models of solar dryer have therefore been developed and tested for the drying of coffee at the farmers' field using both solar and furnace as heating sources. The model varies from a small scale drying capacity of 500 kg coffee cherries till a large-scale solar dryer up to 5000 kg one.

This work presents the performance of several individual, medium and large-scale coffee drying systems. Demonstrated achievements through practical applications will also be described, emphasizing on the technology aspect.

## THE DRYER SYSTEM INVESTIGATED

### Small-Scale Solar Dryer for Individual Family Unit

Individual family units can be described as those systems designed to dry small quantities of coffee cherries ranging from 200 to 500 kg. The drier essentially consisted of a flat plate air heating collector, a tunnel-like drying unit and a small fan to provide the required airflow over the product to be dried. These were connected in series as shown in (Figure. 1).

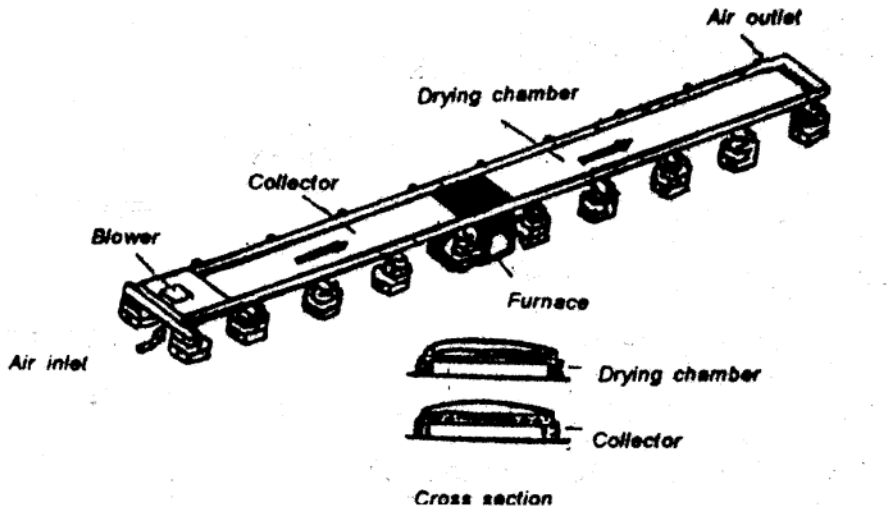


**Figure 1. A small-scale solar tunnel drier.**

Both the collector and the drying unit were covered with a transparent plastic sheet. Galvanized steel plates painted black were used as an absorber in the collector. Coconut fiber was used as insulation material under the collector plate and drying chamber surface to reduce the heat loss. The whole system was placed horizontally on a raised platform. Ambient air was forced through the collector by a small fan of 0.125 kW. The coffee cherries to be dried were spread evenly in a thin layer on the floor of the drying chamber. The loading capacity of the drying floor ranged from 12 to 15 kg coffee cherries per $m^2$. The drying was started usually at 7.0 am and ended at 5.0 pm. At the nighttime, the drying was continued by an additional heat source from the furnace. Continuous drying regime was necessity particularly when the coffee was still wet (the moisture content was above 45%) and sporadic rains were coming. To compare the performance of the tunnel drier with that of the sun drying, control samples of coffee cherries were placed on trays in a single layer on a raised platform beside the drier. Both experimental and control samples were dried simultaneously under the same weather condition.

Solar radiation passed through the transparent cover of the collector and heated the absorber. Heat was then transferred from absorber to the air, which was flowing above the collector surface. As a result, the drying air temperature increased from ambient condition to 38 to 40°C (Figure 2). During a sunny day, the maximum temperature of drying air could reach 45$^o$C when the solar radiation was at 800 kW per $m^2$. Solar radiation also passed directly through the transparent cover of the drying chamber and reheated the drying air to produce higher temperature at the end of the drying chamber. While passing over the products, the hot air absorbed moisture from the cherries and brought it away out from the drying chamber. The biomass furnace was urgently required as an additional heat when heavy rain occurred during the daytime. Burning of 5 to 10 kg of air-dried wood in the small furnace during nighttime could maintain the drying air at a relatively stable temperature of between 35–38$^o$C and rH 40-45%.

Comparison of the moisture contents of coffee cherries in the solar tunnel drier with those obtained, by the traditional sun drying is shown in Figure 3. The moisture content of coffee cherries in the solar tunnel drier gradually reduced from 65% to 11.50% within 78 hours of drying while at the same time, the moisture content of similar amount of samples in the sun-drying floor just went down to approximately 35%. This is due to the fact that the coffee cherries dried in the tunnel drier received energy both from the collector and from incident solar radiation, whilst the control samples received energy only from incident radiation. Moreover, the constant airflow within the dryer was able to remove the evaporated water from

the cherries out from the drying chamber keeping the relative humidity lower than that of in the environment. Turning of the cherries during the entire drying process was required to obtain a uniform moisture content of the product. Turning was carried out 2 to 3 times a day and was done by moving the cherries layers up side down and forward-backward direction throughout the length of drying chamber.

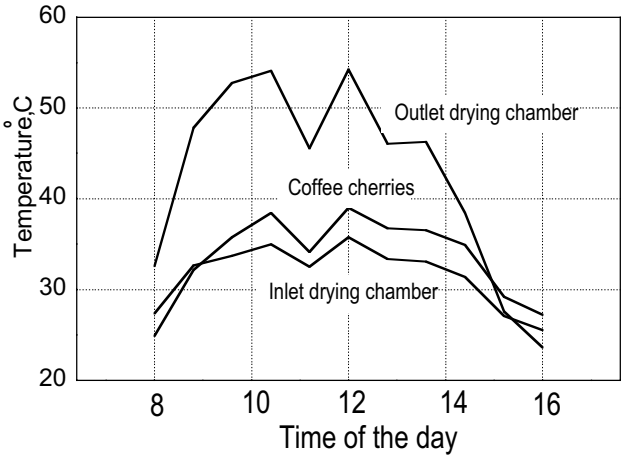


**Figure 2. Temperature profile of air and coffee mass within the drying chamber.**

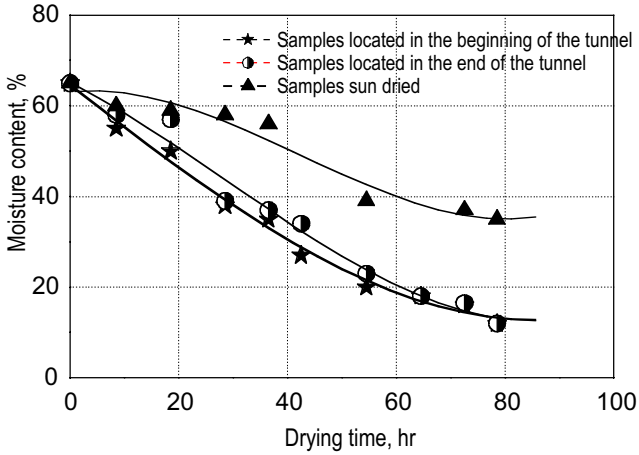


**Figure 3. Variations of moisture content with time of coffee cherries inside the solar tunnel dryer and the sun-drying floor.**

The operation cost of the drying process was relatively cheap which was approximately Rp 35 per kg coffee cherries. The cost was mainly to provide the electricity, whereas the labor cost was almost neglected as the farmers did the same for sun drying operation. The investment cost of the dryer depends of the size and the construction of the dryer. It might be cheaper as the materials for construction are locally available and the construction can be done by village workers. For the one in the Figure 1, the price was less Rp 1 million (1 US$ = Rp 9150,-). Enlarging the size, increases the prices also, however the investment cost can be reduced according to the number of units produced.

**Medium Scale Solar Dryer**

Medium scale systems meet the need of individual and of groups, cooperatives, or farmers associations to supply a greater quantity of product under constant conditions, thus empowering them to reach more markets. A greenhouse (GH) model was considered to fulfill

the criteria for both individual [for rich farmer] and a group of farmers consisted of 10-20 memberships (Figure 4). The GH type solar dryer was made of UV stabilized plastic sheet, 1.5 mm thickness for covering the roof and sidewalls. The sheet was fixed on the steel angle frame supported by 1.24 cm x 1.24 cm mesh wire net with 0.8 mm in diameter. To gain an optimum incident solar radiation, the side walls were inclined to shape trapezoidal cross section connected to triangular roof top. The height of the top of triangular roof from the floor was 2.80 m. The available floor area under the greenhouse was 6 m long and 3 m wide. A flat bed type dryer of 3 m x 2 m x 1 m was installed in the middle part of the floor. The dryer was equipped with a 0.4 kW exhaust fan.

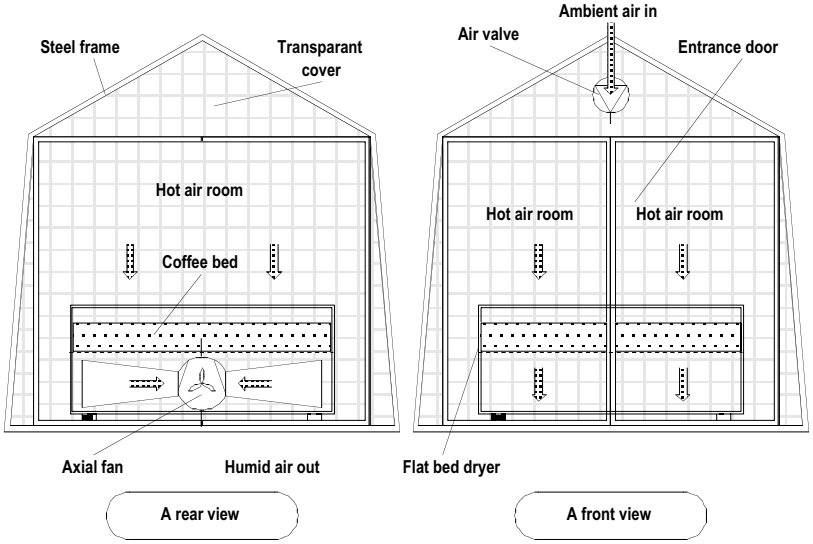


**Figure 4. A green house type dryer.**

The drying test results with several bed depths had indicated that the highest drying efficiency was attained at 57.70% when the dryer was loaded with 1.1 tons or equal to approximately 0.3 m in deep bed of wet coffee cherries. The total drying time was 72 hours when the final moisture reached 11.20%, as shown in Figure 5.

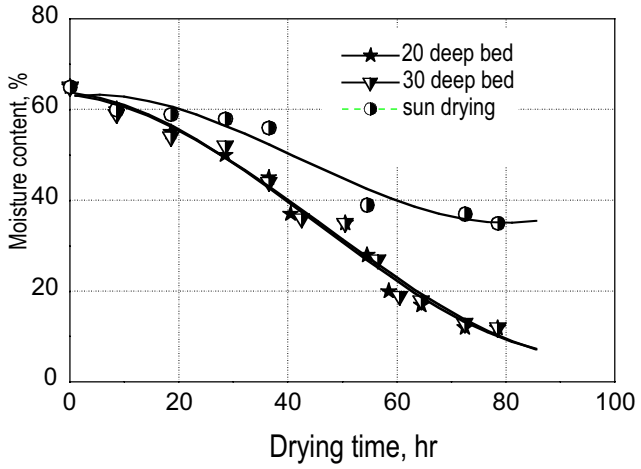


**Figure 5. Variations of moisture content with time of coffee cherries inside the green house dryer and the sun drying floor.**

Turning of the cherries during the entire drying process was required to obtain a uniform moisture content of the product. Turning was carried out regularly 2 to 3 times a day and was done by mixing the cherries mass up side down within the box of drying chamber. The

specific energy in terms the total input energy was calculated approximately 5.2 MJ per kg water evaporated. During the entire tests, the average solar radiation was 525 W per $m^2$, the drying air temperature within the green house ranged from 38 to 40°C, and rH of 45%. The maximum attainable temperature within the structure was between 42 to 43°C, whereas the air flow rate was varied between 0.57 to 0.68 kg per sec.

The operation cost of the drying process using this system was much higher than that of operating the tunnel dryer one. The operating cost was approximately Rp 60 per kg coffee cherries which was mainly to pay a higher electricity consumption and labor cost for loading coffee cherries, stirring during drying and unloading the product. Due to more complex construction and more materials used to produce a green house dryer, for the one in the Figure 4, the investment cost was approximately Rp 7 to 9 million (1 US$ = Rp 9150,-). Enlarging the size, increases the prices also, however the investment cost can be reduced according to the number of units produced.

**Large Scale Commercial Applications**

Large-scale commercial applications require greater capitalization, and are designed to dry very large quantities of coffee with better control of temperature and hygienic conditions. These systems are appropriate for farmers associations or village cooperatives consisted of 50-100 memberships as well as for large commercial coffee estates. A drying building system is considered to fulfill the criteria for large scale operation. Several commercial uses of the system have been implemented, as shown in Figure 6.

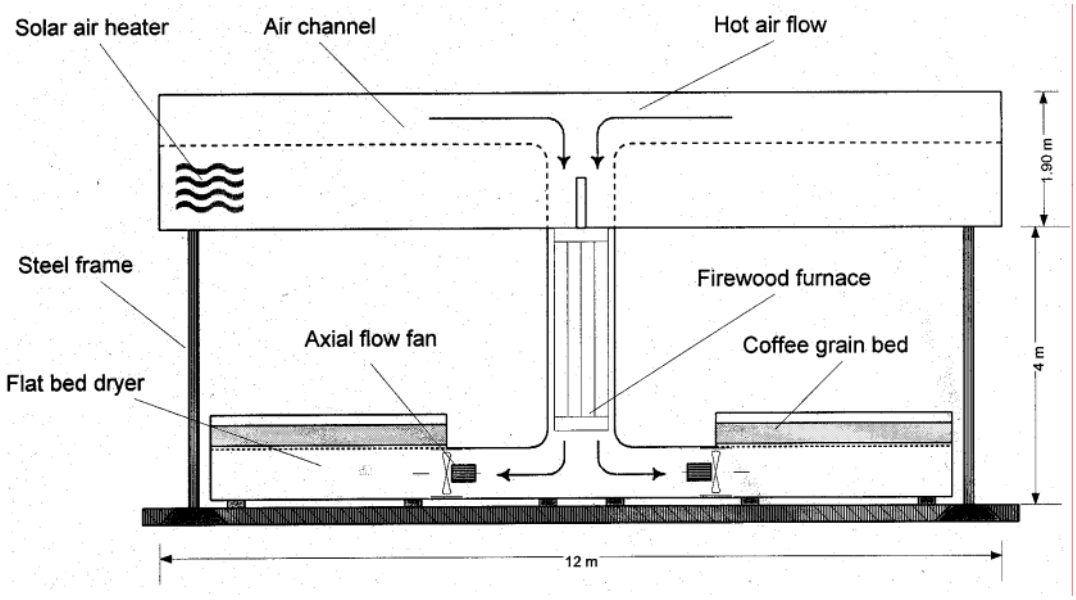


**Figure 6. A side view of the drying building, an array of solar modules installed on the roof.**

The drying building was erected on the middle part of a 14 x 14 $m^2$ concrete platform. The collector was designed as a modular system using conventional building materials, such as wood beams, C steel profiles, plywood, sheet metal and fiberglass. Each module was formed by attaching the black-painted galvanized iron sheets as an absorber on a 0.75 m wide and 6 m long wood frame. The total number of the solar collector module was 30 pieces to construct the entire roof surface areas of 144 $m^2$. Transparent corrugated polycarbonate sheets were finally installed to cover the whole surface of the solar collector modules. A flat bed type dryer was used as a drying facility. The box has dimension 3 m wide, 7 m long and 1 m high.

The box consists of 6 plenums in parallel. Each plenum is equipped with an individual air valve and an axial blower driven by an electric motor of 0.25 HP, 1500 rpm and 220 V. Drying floor is made up from perforated aluminum sheets over it the coffee being dried is spread out evenly. The maximum loading capacity of each drying floor was about 5 tons or equivalent to approximately 0.40 m layer of coffee bed.

A downdraft type wood furnace was used an additional heat source at the nighttime drying operation. The furnace was provided with a tube heat exchanger to transfer the smoke-free heat into the drying air. The outer tube heated up the ambient air flowing in a direction parallel to the exhaust gas stream within the tube. A centrifugal blower was used to suck the combustion air through the wood bed inside the furnace and to force the combustion gases through a chimney. The blower was driven by an electric motor of 220 V, 1 HP and 2980 rpm.

Figure 7 presents the drying air temperature at the drying system during a relatively sunny day operation. The temperature of the drying air varied linearly with the amount of solar radiation available during the day. The drying air was heated up gradually from low temperature in the morning to the higher one in the following hours corresponding with the increase of the solar radiation. The attainable maximum temperature at the outlet of solar collector was 65-70°C, at a peak solar radiation of about 900 W per $m^2$. The profile of the drying air temperature within the plenum was slightly lower than that of the outlet collector due to the heat losses occurred to the airflow inside the entire air ducting system.

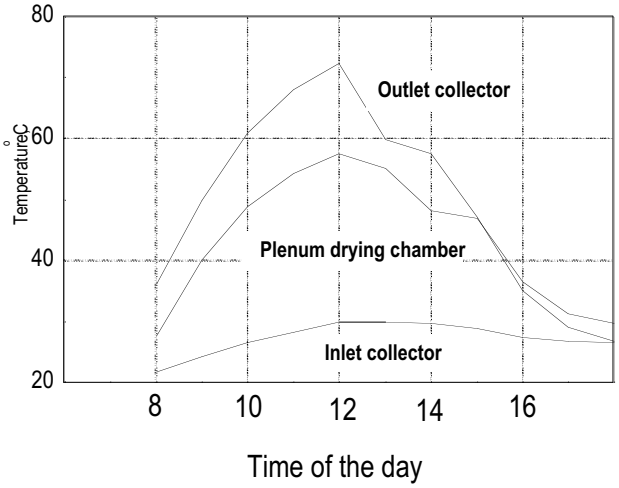


**Figure 7. Temperature profile of drying air at the entire system.**

During nighttime, the hot drying air was produced by the furnace. The ambient air was heated up by the exhaust gas flowing in the inner tubes of heat exchanger. The drying air temperature increased gradually from low temperature during the initial step of wood combustion to attain a relatively stable temperature at 50-55°C, which corresponds to 20 to 25% relative humidity (Figure 8).

At the burning rate of firewood at 55 kg per hr, the furnace could produce the heat output approximately 65-70 kW and the heat efficiency of 50 to 60%. An irregularity of the firewood combustion slightly occurred resulting unstable drying air temperature, but it was still at tolerable and acceptable level for coffee drying. However, the combustion high moisture content of firewood should be avoided as the temperature of drying air fluctuated extremely and the firewood consumption increased considerably. The use of air-dried wood with 20-22% moisture content is recommended to produce an optimum combustion condition within the furnace. As a result, the wood consumption throughout the drying process could also be reduced to

approximately 2-3 $m^3$ per ton dried coffee. Conventional coffee dryers as used in large coffee estates consume large amounts of wood and electricity to dry the coffee. In fact, they utilize a firewood furnace as a single heat source, which increases significantly the firewood consumption to approximately 7 to 9 $m^3$ per ton, dried coffee.

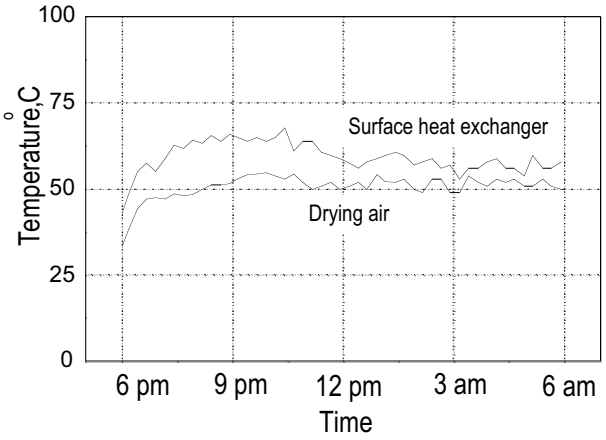


**Figure 8. The temperature profile of drying air and the outer surface of heat exchanger.**

The layers of coffee bed resisted considerably the airflow causing a pressure built-up under the coffee mass and consequently increasing the electricity consumption to drive the blower. To reduce the electrical energy consumption, the newly designed solar dryer was therefore equipped with low consuming energy blowers. The electrical energy consumption was about 100 kWh per ton dry coffee, which was comparatively lower than that of the conventional coffee dryer, which reached 200-250 kWh per ton dry coffee. Figure 9 shows the static pressure development by the blowers varied significantly depending on the depth of the coffee bed within the drying chamber. The pressure decreased along with the running of the drying process, while the airflow increased steadily when the coffee beans dried up.

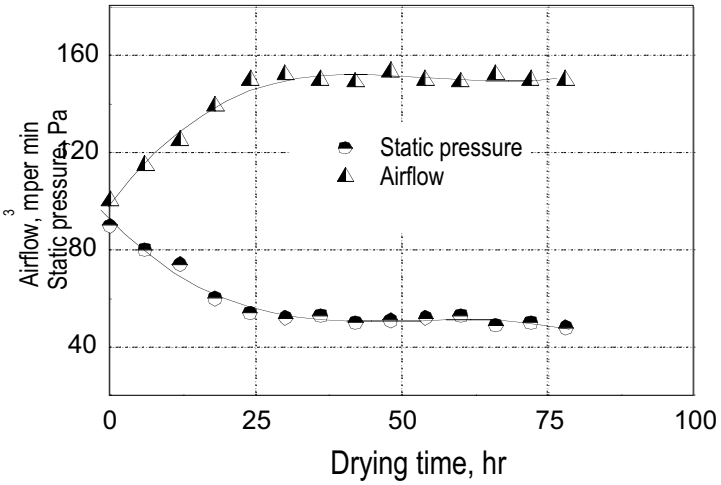


**Figure 9. The profile of static pressure and airflow inside plenum during drying.**

The effect of coffee moisture content on resistance to airflow was more pronounced in the initial stage of drying. When the moisture content of coffee was 60% (w.b), the static pressure developed inside the plenum was indicated at the maximum value of 95 Pa. As the physical nature of coffee cherries shrank gradually due to water evaporation during the drying process, the pressure decreased steadily toward the minimum value when the moisture content of coffee dropped below 30%. The pressure reduction corresponded to the decreasing of the bulk

density of coffee as indicated by the declining of the coffee bed approximately 25% from the initial depth. Meanwhile, the airflow through the coffee bed increased remarkably from 100 to 150 $m^3$ per min.

The drying curves of coffee cherries at a constant deep bed of 0.40 m is presented in Figure 10. A regular procedure of drying carried out the farmers was initiated during daytime at 13-14 am. After all harvested cherries had been loaded completely into the drying chamber, the drying was started immediately. At first, the solar collector at a relatively low level was used as a heat source, thus the moisture was removed slowly from the coffee cherries. The drying was then continued during nighttime by using the hot drying air generated from the furnace. The drying temperature was easily maintained at relatively stable of 50-55°C by adjusting a proper combustion device. As a result, the drying rate became significantly faster as the furnace was able to give sufficient energy to remove more moisture from the coffee. A continuous drying operation like that gave a significant reduction on the drying time. The total drying time was only 48 hours when the final moisture of coffee reached 12%, whilst an intermittent drying regime by using a single solar collector as a heat source was only able to produce the same final moisture level of coffee for approximately 76 hours.

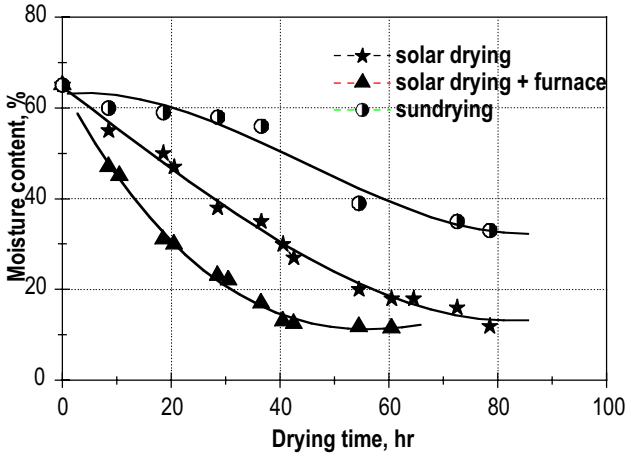


**Figure 10. Moisture content development of coffee cherries during drying process at different drying regimes.**

Medium and large scale commercial systems have the advantage of allowing the user (a group of framers) to increase the commercial value of the product as they can produce more uniform and consistent coffee at a reasonable amount to sell directly to the big trader. The products are also eligible for exportation to areas where they may receive a higher price for their goods.

Large scale solar dryer needs high capital cost. However, it can be reduced by using an existing farm building or adding a solar air heater to an existing conventional coffee dryer. Moreover, large scale commercial systems can be utilized as multi-crop dryers. In fact, users mostly prefer to have a dryer, which is applicable to one or two crops or to a specific kind of crop, such as cocoa, pepper, maize, paddy etc. Sustainable coffee production can however be achieved when the quality of the coffee is such that it demands market premiums. While providing the processing facilities, farmers should be educated continuously enable them to produce good quality coffee.

## STORAGE

Recent research work on the use of solar collector installed on the roof cocoa store house has been carried out intensively, Figure 11.

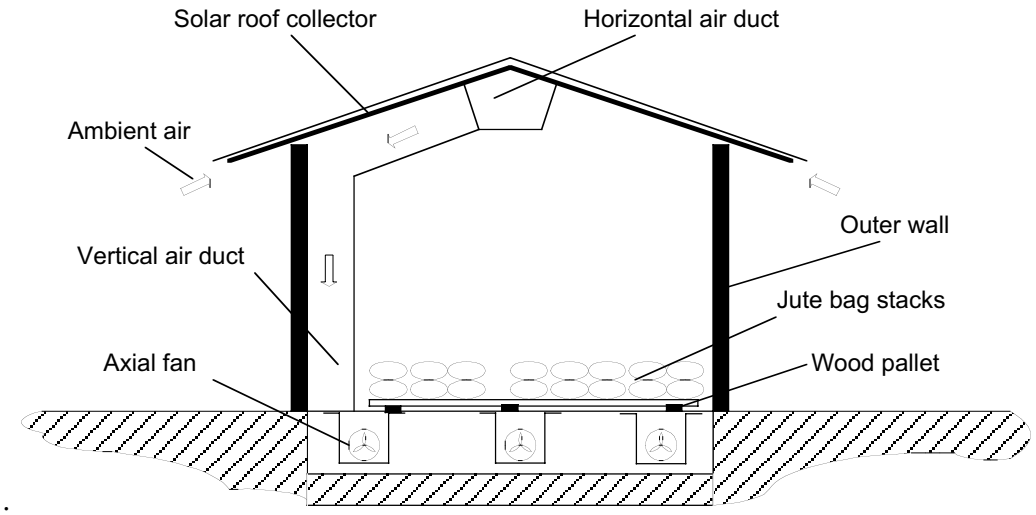


**Figure 11. A front view of the solar assisted coffee store house.**

The hot air from the solar roof collector was injected continously during the daytime through underground channels within the store house. As the moisture content of the dried coffee has been at the equilibrium state, the available heat supplied by the collector was not used to evaporate the moisture anymore but it was stored by the dried coffee within the jute bags. The stored heat was then deliberated during the nighttime and heated up the ambient air within the store house as consequently reduced relative humidity of the ambient air within the storehouse from 95% to nearly 75% as indicated in Figure 12. Due to the fact that the ambient relative humidity of the store house was constantly controlled, the moisture of dried coffee remains at a relatively stable level of 12%, which was considered to be a safe storage level at the tropical region.

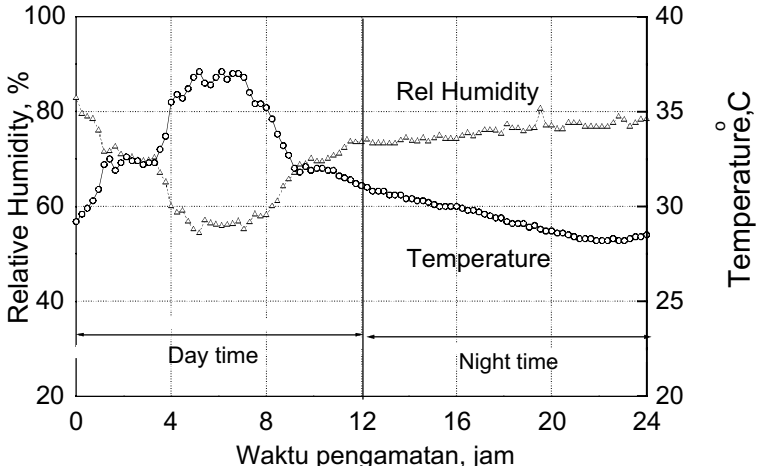


**Figure 12. The profile of temperature and relative humidity of air within atmosphere controlled store house.**

**Potential application**

The most important feature of any dryer is the quality of the dried product. It was evident that the mean quality of the coffee dried in the solar dryer was virtually better than that of the sun drying one. Some constraints for using sun drying were that this technique needed relatively a long period of sunny weather and the drying rate was slow. Thus, there was a danger for the

coffee beans to getting moldy and consequently developed off flavor or even mycotoxins contamination.

The advantage of using solar dryer system was able to generate a relatively high temperature and low humidity of drying air. Both could significantly improve the moisture removal from the coffee cherries and reduce the risk of spoilage and microbial infestation to the coffee beans during the drying process and in subsequent storage. Moreover, the product being dried was protected against dust, soil, insects and other undesirable materials. All of these factors contributed significantly to improve and more consistent product quality.

The land requirement as well as its cost, was becoming a decisive factor in constructing the sun-drying floor in some years to come. Since the throughput per unit area of the solar dryer was larger, i.e., 15-20 kg fresh coffee cherries per $m^2$, the land requirement for the drying process could be reduced. For large scale drying capacity, sun drying is a labor intensive operation mainly for stirring the coffee on the drying floor and heaping the coffee during rainy days or ending the sun drying process. Oppositely, stirring is less intensive in the drying chamber due to sufficient airflow distribution so that the workers could do some other productive works on the farm.

To reduce the electrical energy consumption, the dryer was equipped with low consuming energy blowers. The electrical energy consumption was about 100 kWh per ton dry coffee, which was comparatively lower than that of the conventional dryer, i.e., 200-250 kWh.

## DISSEMINATION AND COMMERCIALISATION

The early reports showed that solar dryers have not been able to make noticeable impacts on the users in the agricultural sector. The constraint of solar dryers application might be more of social and educational aspects. It requires information program which shows that solar drying is able to provide benefit in economic sense.

The Government sponsoring demonstration programs of the use of solar drying units in the production scale are urgently required. Providing performance data on the successful use of solar dryers is an important role to promote encouragement to the private sector (Figure 13). The economic incentive in term of quality which is convertible to cash should be established and will be appreciated by the user particularly farmers' community.

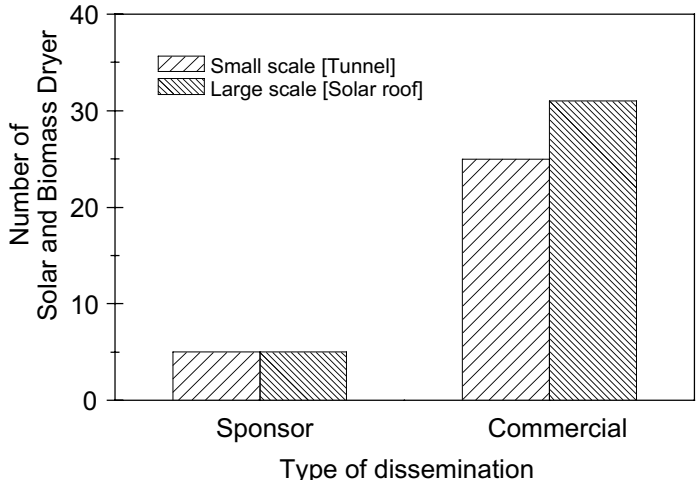


**Figure 13. Dissemination and commercialization chart of the solar and biomass dryer within 2002–2003.**

The demonstration facility will also promote village workshops ability to fabricate solar dryers with the help of locally available materials, labors and tools. It may be recommended that subsidy on solar dryers manufactory and application should be considered either in the form of taxation or credit-policy.

A well coordinated research-development-demonstration programs for creating awareness is required for proper implementation of a solar drying technology. National and international relevant research organizations should be identified and entrusted with the responsibility of evaluating techno-economic aspect of different designs and validating the models through extensive field testing.

## CONCLUSION

The solar coffee drying process has several technical and product-quality advantages,

- A moderate-temperature process, conducted right after harvest, relies on consistent heat, gently circulated across the coffee can optimally preserve the quality of the beans.
- A solar drying system can be used by wide range business scale from an individual farmer, small group of farmers association to a large scale coffee business.
- A solar drying system can be proved to be most useful device from energy conservation point of view. It is not only save energy but also save lot of time, occupying less area, protects environment as well.

The constraint of solar dryers application might be more of social and educational aspects. It requires information program which shows that solar drying is able to provide benefit in economic sense.
The demonstration facility will also promote village workshops ability to fabricate solar dryers with the help of locally available materials, labors and tools. It may be recommended that subsidy on solar dryers manufactory and application should be considered either in the form of taxation or credit-policy.

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# Coffee Cherry Drying: A Two Layer Model

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## SUMMARY

Drying of *Coffea arabica* cherry was carried out in thin layer dryer at air temperatures of 40, 50 and 60$^0$C at bed depths of 50,62.5 and 75 mm with airflow rates 1.5 and 2 m$^3$/m$^2$/min. Since the coffee cherry consists of skin and bean, a two layer-drying model was developed to characterize the overall drying process for relative humidity range of 46 to 83 per cent. The diffusion coefficient was determined using drying and equilibrium data and expressed as a Arrhenius type function. The study revealed that quality of the coffee was unaltered over the temperature range studied.

## INTRODUCTION

A coffee cherry has relatively thick skin, which encloses the parchment. It can be considered to consist of two distinct layers; namely, the outer skin and parchment separated by an air space.

During drying moisture from the centre of the cherry has to move through the parchment, the intervening air space and the outer skin before it leave the cherry. This moisture migration is a result of a concentration gradient established between the outer surface of the skin and the interior of the parchment. Resistance encountered in the parchment and the skin, provided that the corrective resistance at the boundary is small thus limit moisture transfer rates.

## REVIEW OF LITERATURE

A widely accepted mechanism for moisture transfer during drying of cereal grains and similar products is liquid and /r vapour diffusion. The basic diffusion model employed in the drying literature is Fick's second law of diffusion

$$\partial M/ \partial t = \nabla.(D \nabla M) \quad (1)$$

Where

- M- Average moisture content (fraction dry basis)
- D- Effective diffusion coefficient (mm2/h)
- T-time,(h)

With a suitable set of initial boundary conditions, the analytical solution tom the equation is usually expressed in the form of series of terms. If it is assumed that: 1.Cherry as a spherical in shape, 2. the diffusion coefficient is independent of moisture content at a given temperature ( Steffe and Singh 1980); 3. the equilibrium moisture content is constant with time; and 4. the grain temperature is constant during drying (Becker and Sallans 1955), the solution to Fick's law is given by

$$\frac{M-M_e}{M_0-M_e} = \frac{8}{\pi 2}\left[\left[\exp\frac{-\pi^2 D t}{4a^2}\right] + \frac{1}{9}\left[\exp\frac{-\pi^2 D t}{4a^2}\right] + \frac{1}{25}\left[\exp\frac{-25\pi^2 D t}{4a^2}\right]\right] \quad (2)$$

where

- M- Moisture content (fraction d.b) at any time
- Me- equilibrium moisture content (fraction d.b)
- Mo- initial moisture content (fraction d.b)
- half thickness of the cherry (mm)

The coefficient is an effective coefficient because the individual diffusivities of the skin and parchment are lumped into single value.

Robertson (1962) presented an analytical procedure in which a model is conceived as being formed several phases or layers. Once the number and size of the layers have been determined, the problem reduces to the determination of rate constant of the interacting layers. Following this recommendation, Sharma et al. (1982) used two and three term models for drying of rough rice with the model layers representing the hull, bran and endosperm. St John and Otten (1989) used the same approach to describe the thin layer microwave drying characteristics of unshelled peanuts.

It is reasonable, therefore, to propose the following two layer model to describe the drying of coffee cherry.

$$M-M_e / M_0-M_e = A_1 e^{(-k1t)} + A_2 e^{(-k2 t)} \quad (3)$$

Where,

- $A_1$,$A_2$- characteristics constant( dimensionless)
- $k_1$,$k_2$- drying constants for the two compartments of cherry ($h^{-1}$)

**PROCEDURE**

Eighteen experiments were run to include three levels of drying temperatures and three levels of thickness and two levels of air flow rate. Relative humidity and temperatures were monitored every 2 to 3 hours. The drying temperatures were measured using copper-constantan thermocouple having ± 1°C. The variables considered were drying temperatures, relative humidity, initial moisture content of cherry, final moisture content of cherry, drying time, bed depth and air flow rate. The moisture contents of cherry were determined by AOAC (1971) method.

The effective diffusion coefficient of cherry was obtained by comparing the experimental data with the data obtained from equation 2 for various assumed values of the diffusion coefficient. For each drying temperature, the diffusion coefficient was allowed to vary until the sum of squared deviations between the experimental and predicted values was a minimum. The quality of the dried cherry namely strength, acidity and off taste were determined by means of cup test at Coffee Board, Govt of India, Bangalore.

## RESULTS AND DISCUSSION

The drying results were analysed with equation 3 and the R2 values obtained was less than 0.8. A power term 'n' was introduced for the time 't'; the predicted value and experimental value were in agreement over the whole drying period as the r2 value was above 0.998 in all cases and hence the following model is proposed for the coffee cherry drying. The values of drying constants for different experiments conducted are given in Table1.

**Table 1. Effect of temperature, bed depth on drying constant.**

| Bed depth, mm | Temp, °C | $A_1$ | $A_2$ | $k_1$ | $k_2$ | n |
|---|---|---|---|---|---|---|
| | 40 | 0.6123 | 0.4129 | 0.1236 | 0.1102 | 0.952 |
| 50 | 50 | 0.5924 | 0.4245 | 0.1176 | 0.1209 | 0.947 |
| | 60 | 0.5856 | 0.4339 | 0.1015 | 0.1339 | 0.936 |
| | 40 | 0.5928 | 0.4312 | 0.132 | 0.1485 | 0.935 |
| 62.5 | 50 | 0.5811 | 0.4209 | 0.1198 | 0.1576 | 0.928 |
| | 60 | 0.5702 | 0.4130 | 0.1008 | 0.1653 | 0.919 |
| | 40 | 0.5768 | 0.4521 | 0.1446 | 0.1528 | 0.915 |
| 75 | 50 | 0.5631 | 0.4405 | 0.1302 | 0.1648 | 0.908 |
| | 60 | 0.5520 | 0.4325 | 0.1208 | 0.1781 | 0.898 |

The drying constant k1 for the skin linearly increase with the depth but decrease with the temperature, whereas the $k_2$ (parchment) linearly increases for both. The air velocity does not significantly affect the drying rate of a thin layer cherry at nominal velocities ≤ 0.25m/s

The first term of the developed equation results from the skin of the cherry whereas the second term represents the parchment. The above hypothesis was arrived at by the determination of moisture migration from the two layers as given in Table 2. The developed model is similar to the model proposed by Sharma et al. (1982) for rough rice but for power term 'n' for drying time 't'.

**Table 2. Drying rate of skin and parchment.**

| Drying time, h | Skin layer kg of water/ka of dry matter | Parchment kg of water/kg of dry matter |
|---|---|---|
| 0 | - | - |
| 2 | 0.0539 | 0.0107 |
| 4 | 0.0435 | 0.0108 |
| 6 | 0.0336 | 0.0112 |
| 8 | 0.0250 | 0.0125 |
| 10 | 0.0140 | 0.0140 |

The rate of moisture removal in layer 1 (skin) is about 5 times faster than that of layer 2 (parchment) in the initial stage; it falls gradually as drying proceeds. At the start of drying moisture is removed at significantly higher rate from the skin than from the parchment; with time the moisture removal rate from the skin decrease because moisture loss from the skin equals the moisture gain from the parchment.

The effective moisture diffusion coefficients of coffee cherry at the three drying temperatures are shown in table 3. The magnitude of the diffusivities are about four times reported by

Suarez et al. (1980) for sorghum and 1.25 times those reported by Ezeike and Otten 1991) for melon seeds.

**Table 3. Diffusion coefficient of cherry at indicated temperatures.**

| Temperature, °C | Diffusivity, $m^2/h$ | $R^2$ |
|---|---|---|
| 40 | 3.15 x 10-6 | 0.985 |
| 50 | 3.30 x 10 -6 | 0.980 |
| 60 | 3.60 x 10 -6 | 0.992 |

Assuming an Arrhenius-type function of diffusion coefficient on temperature (Aguerre *et al.* 1982, the following result was obtained.

$$D = 3.315 \exp ( -0.9466 \text{ X } 10^6 / ( T+273)) \qquad R^2 = 0.978$$

Where, T- temperature (°C)
The quality parameters of the coffee cherry like strength, acidity and off taste were not changing for the range of temperatures tested and the same is presented in Table 4.

**Table 4.Cup quality evaluation of coffee cherry.**

| Bed depth, mm | Air temp. $^0$C | Strength/Body | Acidity | Off taste | Cup quality rating |
|---|---|---|---|---|---|
| | 40 | Fair to good | Fair plus | - | Average |
| 50 | 50 | Fair | Light | - | Average |
| | 60 | Fair | Fair | - | Average |
| | 40 | Light to fair | Fair plus | - | Average |
| 62.5 | 50 | Fair | Fair | - | Average |
| | 60 | Fair to good | Light | - | Average |
| | 40 | Fair to good | Fair | - | Average |
| 75 | 50 | Fair | Light | - | Average |
| | 60 | Fair | Fair plus | - | Average |

## CONCLUSIONS

The drying characteristics of coffee cherry were modeled using a two layer expression. The drying rate of the skin was faster than that of parchment. The diffusion coefficient of the coffee cherry was determined and found to follow the Arrhenius expression for the range of temperatures studied. The quality of the coffee did not change over the range of temperature tested.

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# Influence of Coffee Processing and Defects on the Incidence and Occurrence of Ochratoxin A

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## SUMMARY

762 coffee samples (1 kg) – most of dried processed arabica coffee from several stages of pre and post harvest – were collected from different regions of Brazil according to a sample history questionnaire. Among them 60 samples (16 kg) were classified and sorted by defects, according to Brazilian Classification, in 13-17 types of defects: black, sour, insect damage beans, malformed, shell, immature, bean with fox silver skin, pulp nipped bean, broken, parchment, sticks, among others. All 762 samples and fractions of defects (446 subsamples) in the coffee samples were analysed for OTA and the influence and impact of coffee processing and the presence of defects in the OTA contamination were determined.

## INTRODUCTION

Ochratoxin A (OTA) a naturally occurring mycotoxin in coffee and other products is produced by the genera *Aspergillus* and *Penicillium*. It is considered a renal carcinogenic to animals and possibly to humans (IARC, 1993) and its presence in food has been a concern in many countries which have been laid down regulation and guidelines (FAO, 1997; CE, 2002a; CE, 2002b). During cultivation, processing and hulling, coffee is subject to a hazard i.e. ochratoxin A contamination. The implementation of good agricultural practice may minimize contamination; therefore, the identification of critical points is fundamental for the implementation of the HACCP system. Many factors have been reported in literature as critical for the production of a good quality and safe coffee such as cultivation practices, harvest, wagging, separation or washing, drying: terrace management and type of terrace, storage, hulling and transportation (PAS, 2004). The presence of defects in coffee is an indicative of its quality given by a type number (Brasil, 2003) and will determine the price and its acceptability in the market (Mattielo, 1991). In this study, we have characterized some aspects of coffee quality (defects), cultivation and processing that contribute to the occurrence of ochratoxin A in coffee, specially those related to dry processing such as the harvest and post harvest reflected herein as floats, natural cherry; husked cherry; mixture of unripe/natural cherry; mixture of unripe/ripe and floating (*"café da roça*") and sweeping coffee beans.

## EXPERIMENTAL

### Part 1

762 coffee samples (1 kg), most of dried processed arabica coffee, were collected from different regions of Brazil according to a sample history questionnaire what allowed the characterization of the farm infrastructure and facilities, as well as the characterization of approximately 12 aspects of the agricultural practices involved in the pre-harvest and pos-harvest of the sample such as shading, fertilization, irrigation, coffee variety, degree of maturation, type of harvest (mechanical and manual), wagging, contact with soil, separation and washing, drying process (terrace, dryers). The effect (independent) of each aspect of the agricultural practice in the occurrence of OTA contamination was determined using the Qui-square ($\chi^2$) and the exact Fisher test. For the evaluation of the difference between the types of coffee the t student test was applied. The influence of the occurrence of OTA in coffee was done using logistic regression based on categorical data with probability of significance below 25% selected through the univariate analysis. All results were considered significant for a probability smaller than 5% ($p < 0.05$), having, therefore, at least 95% confidence level at the conclusions presented.

### Part 2

Sixty (60) green coffee samples were collected according to a sampling protocol designed for green coffee (Vargas et al., 2004a, 2004b). The 16 kg sample were homogenised and divided in 03 samples (~5 kg). One of the 5 kg samples was split in 02 samples of 1 kg and 4 kg sample named original and composed sample, respectively. The 04 kg samples were decomposed (hand picked) in defect free (picked-up) and defect subsamples. The defects were sorted out and classified according to the Brazilian Classification System and ISO 10470 in 13-17 types of defects taking in consideration the most severe defect giving a total of 446 defect subsamples: As an example a sour/insect damaged bean was classified as a sour bean. The contribution of the mass of the defect and of the picked-up sample, as well as their contamination to the overall OTA contamination of the composed sample (4 kg) was determined by using the following equations:

$$\mathrm{OTA}_{\mathrm{Composed}} = \frac{\sum_{i=1}^{n} Mass_i \times OTA_i}{\sum_{i=1}^{n} Mass_i}$$

where: $Mass_i$ → Mass of the defect sample i
$OTA_i$ → level of contamination of the defect i

$$\%\mathrm{OTA}_i = \frac{Mass_i \times OTA_i}{\sum_{i=1}^{n} Mass_i \times OTA_i}$$

where: $Mass_i$ → Mass of the defect sample i
$OTA_i$ → level of contamination of the defect i

The **t** Student test was used to evaluate the difference between the OTA contamination levels in the original and composed sample, and in defect free subsamples and defects. The relationship between OTA contamination, quantity, and mass of defects was determined using Pearson's correlation (r). The frequency of a given defect in composed samples with

contamination> 5 ng/g was determined by using Fisher test. All results were considered significant for a probability of $p < 0.05$. The test non-parametric "Kappa" was used to estimate the degree of concordance between the number (%) of samples with contamination i.e. ≤5 ng/g and >5 ng/g due to mass (1 kg and 4 kg) and presence of defects.

All samples and defects (part 1 and part 2) were prepared by grinding down the coffee beans through 0.5-1.0 mm screens and thoroughly homogenised. Samples were analysed by immunoaffinity column with liquid chromatography (Vargas et al., 2003).

## RESULTS AND DISCUSSION

### Part 1

The distribution of the 762 samples classified according to type of coffee was husked cherry (28.9%), mixture (20.5%), sweeping coffee (varrição) (20.1%), float (15.9%), unripe/natural cherry (8.0%), and natural cherry (6.7%). The **a**nalysis of OTA in the 762 samples revealed a frequency of 83.6% of samples with contamination up to 5ng/g with 48.4% of the sample free of OTA (nd = 0.06 ng/g, LD = 0.12 ng/g), and 16.4% of samples with OTA contamination ≥ 5ng/g (Figure 1).

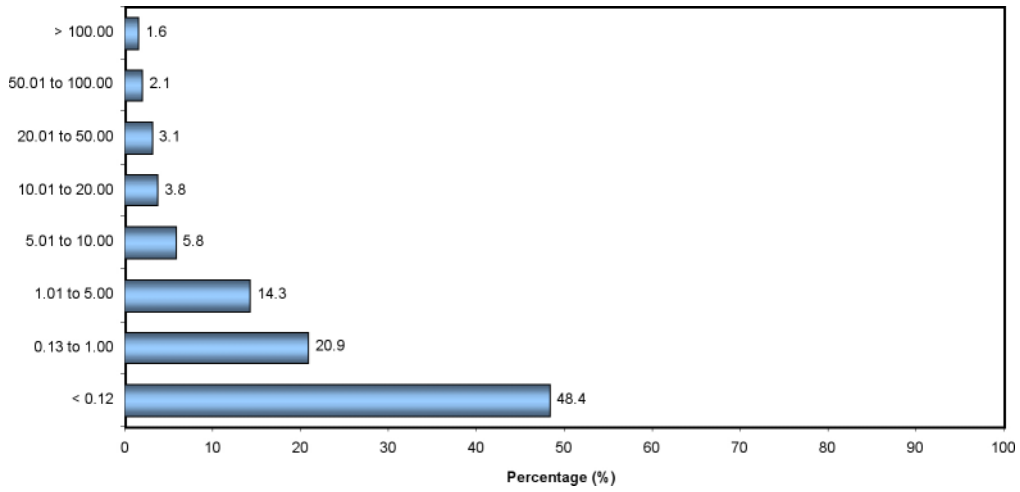


**Figure 1. Distribution of samples per range of OTA contamination.**

There were significant differences between the contamination of sweeping coffee (*varrição*) and other types of coffee. It was observed that coffee from sweeping was highly likely to increase the contamination of the whole samples significantly, in the extent of 41.8% of the samples with OTA contents > 5 ng/g (16.8%) (Table 1 and 2). Standing as second major contributor towards the presence of OTA, float type occurred in 20.6% of the samples with OTA >5 ng/g. The two types (sweeping and float) were originated in the plantation site, occasioned by the drying of the fruit on the trees and from the ones fallen down on the soil. It is remarkable to notice that even coffees from sweeping show a certain percentage of samples free of OTA, indicating the existence of pre-conditions for the occurrence of OTA. In this respect the hypothesis is that the degree of humidity of the soil, the time of contact with the soil and the extent of damage suffered by this fallen-down coffee when its permanence on the tree, are pre-conditions for major or minor occurrence of OTA. The occurrence of coffee berry borer on the coffee, in its various stages of development and the occurrence of damages, both physical and phyto-pathological to the fruit when still on the tree, thus causing ruptures for precocious contamination by fungi and production of OTA. Still on the Table 1 and 2 one can observe the development of OTA in the mixture green/natural cherry (17.6%), which was

attributable to improper handling of these coffees in the drying terrace. The complete mixture (T5) of unripe and ripe cherry, raisin/dry (floats) on the tree, as it is customarily done in most of Brazilian harvest has presented a sampling contribution of 14.5% > 5 ng/g. Such contamination is expected to have been partly originated from raisin/dry coffees from the tree, already with a high fungus invasion, and partly has been developed at the drying terrace, due to either deficiency or inadequacy of the drying process. Lastly, it was observed that the presence of OTA in husked cherry coffee was practically not existing (1.1%). That stands for the absence of toxin in cherry coffee, i. e., in the mature and healthy form, as the coffee should be harvested, as well as the absence of contamination of the product in the post-harvest process, indicating that the drying process has been well conducted.

**Table 1. Descriptive analysis OTA contamination per group of coffee: sweeping coffee x others**

| **Types of coffee** | **Descriptive Analysis** | | | | | |
|---|---|---|---|---|---|---|
| | **Minimum** | **Maximum** | **Median** | **Mean** | **s.d** | **p** |
| Sweeping coffee | 0.06 | 773.73 | 3.12 | 21.12 | 75.14 | **< 0.001** |
| Others | 0.05 | 295.76 | 0.06 | 4.45 | 20.10 | V > O |

*V → sweeping coffee  O → Others: unripe, natural cherry, raisin/float, husked cherry*
*p < 0.001 – t student test*

**Table 2. Comparison of OTA contamination between different types of coffee.**

| | **Type of coffee** | | | | | |
|---|---|---|---|---|---|---|
| **OTA** | ***T1*** | $T_2$ | $T_3$ | $T_4$ | $T_5$ | $T_6$ |
| ≤ 5ng/g | 79.4 | 90.7 | 98.9 | 82.4 | 85.5 | 58.1 |
| > 5 ng/g | 20.6 | 9.3 | 1.1 | 17.6 | 14.5 | 41.9 |
| **Total** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** |

*$T_1$ →Float  $T_2$ → Natural Cherry  $T_3$ → Husked cherry  $T_4$ →Unripe/Natural Cherry*
*$T_5$ → Mixture  $T_6$ → Sweeping (Varrição) (p < 0.001 - $\chi^2$ Qui square test)*

Regarding the type of harvest (mechanical and manual) it was observed a strong tendency ($p < 0.052$, $\chi^2$ Qui square test) of having higher OTA contamination in the samples manually harvested. Upon the occurrence of higher content of OTA on coffees, which have been collected mechanically, it is not believed to be due to the harvesting itself, but to disturbances caused along the flow of drying, due to the dimensioning of drying terraces and dryers.

The manual or mechanical stripping directly on the soil has shown highly harmful to coffee increasing significantly ($p < 0.001$, $\chi^2$ Qui square test) the levels of OTA contamination when compared to those stripped on a cloth, on a wide basket or on a sieve. That is a practise of harvesting that has been abandoned in Brazil, and replaced by a harvest on cloth or plastic, and even by a mechanical process. The practice of it is characterised by collection of all the fruits of the tree and the lifting of these fruits together with the coffee beans already fallen down on the soil longer before contributing in this way to OTA contamination.

No difference in OTA contamination was observed due to use of dryers in combination or not with terrace ($p < 0.541$, $\chi^2$ Qui-square) as expected from any of these processes, since well conducted.

However, the type of terrace influences the levels of OTA contamination as can be observed in Table 3. Asphalt (mud) terraces contributed most to the production of OTA, followed by earth terrace and cement terrace. The asphalt (mud) terrace has been adopted by small coffee growers in substitution to earth, rather because of their low cost. So, this paradox of improvement of quality of terraces attributed to the asphalt (mud) and the present data finds explanation in the lack of conservation on the surface of the terrace, to deficient handling practices exercised by small coffee planters still not so well conscious of the necessary good practices. The asphalt terrace has some problems related to conservation of the surface, and along a few years of use, it turns to present frequently cracks and holes not allowing a proper cleaning of the surface thus facilitating the fungi growth (hot spots) and OTA contamination. Earth surface terraces are of difficult handling during rain periods, and this can be the explanation for the elevated number of contaminated samples. Regarding the cement terraces, which predominate in the coffee activity in Brazil, no other explanation for such level of contamination but improper handling, cracks on the surface (poor conservation), the occurrence of rain falls or even the origin of the coffee (from sweeping and floats) already contaminated in the plantation site. At suspended and brick terraces no OTA formation was observed at levels above 5 ng/g. These latest two types of terraces are rather rare in Brazil. The suspended terrace, have nowadays been adopted by growers who search for a better quality for their products and correspond preferentially to cherry coffees and/or husked cherry coffees, therefore, less subjected to the occurrence of OTA. Hence, the zeal at handling, associated with the characteristics of being suspended can explain this good performance. The brick terraces, which have been preferred, as compared to cement terraces, have shown a good result. This diversity of factors turns the explanation of the influence of type of drying terrace on the occurrence of OTA a rather complex issue.

**Table 3. Influence of the Type of Terrace in the OTA contamination.**

| | **Terrace** | | | | | |
|---|---|---|---|---|---|---|
| **OTA** | **Earth** | **Cement** | **Asphalt (mud)** | **Suspended** | **Brick** | |
| ≤ 5ng/g | 82.7 | 86.8 | 62.0 | 100.0 | 100.0 | |
| > 5 ng/g | 17.3 | 13.2 | **38.0** | 0.0 | 0.0 | |
| *Total* | 100.,0 | 100.0 | 100.0 | 100.0 | 100.0 | |

*$p < 0.001$ (p refer to Fisher test)  Suspended = Brick < (Cement) < Earth < Asphalt*

Another factors were evaluated concerning the influence in the OTA contamination. Among them altitude, irrigation, fungicides, harvest time, shading, wagging, topography, rainfall. It was notice a trend of smaller incidence of OTA as the altitude increased ($p < 0.01$, Fisher test). Coffee plantation in lower altitudes, with higher temperatures provide a more precocious ripening of the coffee, and under these conditions, at full sunlight, one can observe more incidence and sunray effects on the top of the tree increasing the percentage of dry/raisin coffee on the tree, that associated to a poor post-harvest management is likely to promote a higher content of OTA. Concerning the influence of the type of irrigation system in the OTA contamination, it was observed no significant difference in the OTA contamination of samples due to ordinary irrigation systems. However, on the "central pivot" system, a significant reduction of OTA occurrence ($p < 0.01$, Fisher t) was noticed which was not interpreted as the cause for the low levels of OTA. Plantations in the areas with such system "central pivot" are considered highly technical and conducted according to good agricultural practices (good harvest, post-harvest, drying and storage management). All this, associated to low relative air humidity conditions and absence of rainfalls, which are prevailing in the harvest period in these regions, provides the production of coffees practically free from OTA, being, most of

them, husked cherries (36.2%). No influence of fungicides, harvest time, shading, wagging, topography, and rainfall) on the OTA contamination was determined.

**Part 2**

The highest occurrence of defects were: sour (98.3%), immature (85.0%), black (80.0%), insect-damaged (85.0%), broken (75.0%), shell and its pulp (88.3%), green (85.0%), black/immature (60.0%), insect-damaged - bluish (53.3%), malformed (41.7%), fox silver skin (25.0%), bean in parchment (22.3%), dried cherry-pod (15.0%), sticks/stalks (8.3%), husk (5.0%), pulp-nipped (3.3%), stones (1.7%). Concerning the variety of defects in the samples, it was observed that 66.7% of samples showed at least 9 types of defects.

The defect-free coffee sample (clean) contributed with around 80.0% of the composed sample (4 kg) overall mass and with 45.9% of the OTA contamination. Although the picked-up sample has contributed with 45.9% of OTA contamination the mean (2.97 ng/g) and median (1.77 ng/g) were relatively low. On the other hand, the sour defect contributes with 6.9% of the sample overall mass and with 25.3% of OTA contamination. The OTA contamination level for this defect showed a median of 4.8 ng/g and a mean of 79.8 ng/g. Following, the insect damaged - bluish defect, which contributes with 1.5% of the overall mass of the 4 kg sample and with 7.4% of OTA contamination with a median of 0.1 ng/g and a mean of 27.8 ng/g. The insect damaged and black defects contribute with 4.9% and 3.1% of OTA contamination, respectively.

From this result the overall sample contamination (composed sample) was re-calculated, by eliminating the main defects (sour, insect damaged - bluish defect, insect damaged and black), which was denominated as composed without defects.

Although the picked-up sample, sour, insect damaged - bluish, insect damaged, black, malformed, broken contribute the most to the presence of OTA contamination in coffee, the defects “black, stick/stalk, bean in parchment and insect damage-bluish” were more present in samples with contamination $> 5$ ng/g with 95% confidence ($p < 0.05$, Fisher test). The presence of these defect in coffee may be consider as indicative of OTA contamination in coffee.

Comparing the OTA contamination determined in the original sample (1 kg) and in the composed sample (4kg), a significant difference was not identified, although a reduction in the value of contamination was observed (Figure 2). On the other hand, significant differences (t student test, $p < 0.05$) were observed, when comparing the OTA contamination (mean) between the clean (picked-up) x defect, clean (picked-up) x original, clean (picked-up) x composed and clean (picked-up) x composed defect-free, showing that the presence of defects increase the levels of OTA contamination in coffee (Figure 2). From the comparison of OTA contamination in the composed sample and in the composed sample without the main defects it was observed that in fact the presence of the following defects: sour, insect damaged and insect damaged-bluish and black beans significantly increase OTA contamination (t student, $p < 0.05$).

It was possible to determine the degree of concordance between the size (1 kg or 4 kg) and type of sample (picked-up, original and composed) on the number of samples with contamination $\leq 5$ ng/g and $> 5$ ng/g (Kappa test). Considering the cut off of 5 ng/g, it was observed that the percentage (78.3%) of the picked-up samples with contamination $\leq 5$ ng/g when compared with the original samples (65%) showing that the absence of defects in fact contribute to reduce the contamination in coffee (Kappa = 0,44, $p < 0.001$).

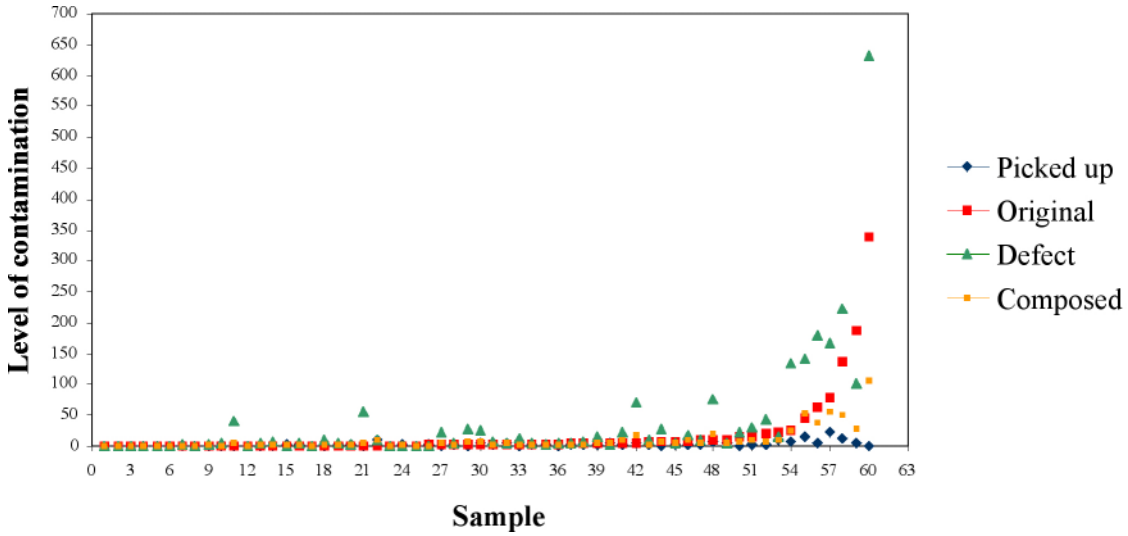


**Figure 2. OTA contamination level in the original, clean, composed samples and in the defect.**

Less impact was observed in the number of samples with OTA contamination ≤ 5 ng/g due to sample sizes of 1 kg (65%) and 4 kg (60%) (Kappa = 0.61, $p < 0.001$). The elimination of the main defects (sour, insect damaged and insect damaged-bluish and black beans) from the 4 kg samples increased to some extent the number of samples with OTA contamination ≤ 5 ng/g when compared to the 1 kg sample (Kappa = 0,53, $p < 0.001$).

There was no correlation of the quantity of defects and the percentage of mass with defect with the original and composed contamination (Pearson correlation).

## CONCLUSION

From the univariate analysis it was possible to identify independent aspects (effects) of pre and post harvest that concur to the incidence and levels of OTA contamination in coffee. The multivarite analysis (logistic regression) indicates that besides the significant independent factors determined in the univariate analysis, the presence of OTA contamination with levels > 5 ng/g in coffee samples was (Central Pivot) influenced by the type of irrigation, the harvest method i.e. the contact of coffee with soil during harvest and the type of terrace. As discussed before the "Central Pivot" represents here the highly technical processing system conducted according to good agricultural practices showing the coffee processed under good management system are less susceptible to OTA contamination.

The presence of defects impacted significantly and negatively the incidence and levels of OTA in coffee. Among the defects the sour, insect damaged - bluish, insect damaged, black, malformed and broken were the ones that most contribute to the incidence and levels of OTA contamination in coffee. The defects "black, stick/stalk, bean in parchment and insect damage-bluish" were more present in samples with contamination > 5 ng/g.

No correlation between the quantity of defects and the percentage of mass with defect with the OTA contamination in both original and composed sample was determined.

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# Presence of Fungi in Natural Coffee and the Beverage Quality

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## SUMMARY

A reduction in the quality of natural coffee for "espresso" beverage has been noticed in the last few years in Brazil. The present study was carried out with the aim of investigating every processing stage. Six farms were studied in different regions of Cerrado in Minas Gerais state, Brazil. Fungal presence was also analyzed to find out if it would interfere in the quality of the end product. The harvest was carried out by stripping when the majority of the fruit was dry on the trees. All the fruits were processed naturally (whole dried). The coffee sample, which had the best quality, was from Area 2. It presented a good aspect and was well dried, with good taste and without any fermentation problems. In the other coffee sample areas, it was observed some beans with an excessive mucilage of brown color in its ventral grooves and some of them were fermented. Some fermented immature beans were also found. The results show that the inferior quality found before the drying at the patio stage was probably because of plant stress caused by the high temperature and water deficiency from May to July. Some immature fruits and abnormal fruits with only one bean developed were found probably due to low levels of potassium application. A great percentage of fungal infection was found on ripe cherries put to dry on the patio. On the other hand, when the farmer follows all the correct processing steps with an adequate fertilization, if the fruits are harvested when the majority is at the dry stage on the trees (in the Cerrado region), then it is possible to produce good natural coffee for "espresso".

## INTRODUCTION

Coffee fruits can be processed by three different methods: a) the dry process, where the whole fruits are dried on the patio or in mechanical dryers, after been harvested; this process gives natural coffee, characterized on cupping by a full body and a mild aroma; b) wet process (washed coffee), where the coffee is pulped by fermentation for 12 to 36 h; this process produces a highly aromatic cup, with a fine body and a high acidity; c) wet process (pulped natural coffee), where the coffee is pulped usually mechanically; this process is intermediate between the natural and washed processes.

Brazil is the largest coffee producer in the world and there is a great interest regarding natural Brazilian coffee. Natural coffee develops the highest solid concentration in the cup because the whole fruit sugars are transferred to the bean, providing more body. This characteristic is very desired when making espresso coffee. Unfortunately, a reduction in natural coffee quality has been noticed in the last few years. Therefore the objective of the present work was to perform a technical control over the processing of natural coffee in order to study its behavior and investigate the real causes for the fall in its quality, including studies of fungal infection.

## MATERIAL AND METHODS

### Coffee

From harvest to storage, all the processing stages were submitted to a technical control.

### Variety and fertilization

All data regarding variety and fertilization of N, P and K were carried out according to each grower's criteria, and were compared.

### Climatic

Climatic variations of temperature and rainfall of previous years and those during the current study were collected from the farms.

### Localization of the farms

Six farms located in the best region for natural coffee, in Brazil, were chosen as follows:

- area 1: Patos de Minas – 6ha. and 24,480 coffee trees.
- area 2: Estrela do Sul – 18 ha and 59,760 coffee trees.
- area 3: Indianópolis – 18 ha. and 61,524 coffee trees.
- area 4: Serra d o Salitre – 6 ha. and 30,000 coffee trees.
- area 5: Rio Paranaíba – 8 ha. and 28,560 coffee trees.
- area 6: Coromandel – 20 ha. and 50,000 coffee trees.

### Agricultural practice

#### *Harvesting*

The harvest had to be carried out when the majority of fruits were dried on the tree, i.e. during August and September because the maturation stage is considered ideal for producing good natural coffee. All the fruits had to be processed naturally (whole dried) on the tree. Only one sample of ripe cherry coffee from area 1 (called 1-a) was harvested to be compared with the others. All the dried cherries were mechanically harvested.

#### *Drying*

The dried cherries were immediately transported to the patio. Coffee, with around 15% of moisture content, was spread into thin layers on the patio and turned over continuously the whole day. Coffee was piled up late in the evening and covered with waterproof material. The drying was finished in approximately 4 days.

#### *Storage*

Coffee was stored with about 11% of moisture content for approximately 30 days.

#### *Hulling, grading and sorting*

The coffee lots were hulled, graded and sorted using mechanical and electronic machines.

***Physical and sensorial analysis***

The raw coffee beans from each sample were analysed physically (moisture content) and sensorial (beverage quality).

The beverage was analyzed after the visible defects of the beans were excluded.

Each sample were roasted and ground. The samples were evaluated by three different degustation tests, i.e. infusion, diluted espresso and espresso.

Sensory analyses were also carried out, evaluating the quality of the beverage in respect to body, aroma, acidity, bitterness, astringency and sweetness. In addition, the presence of positive flavours and aromas such as caramel, chocolate and floral, and negative character including immature, fermented, stinker, woody, rancid, mouldy, rioy and smoky were also evaluated.

**Mycological methods**

Samples were surface disinfected with 0.4% chlorine solution for 1 min. Fifty beans from each sample, subdivided into 5 plates of 10, were plated directly onto Dichloran 18% Glycerol agar (DG18), and incubated at 25ºC for 5 to 7 days (Pitt & Hocking, 1997). After incubation, the plates were examined and the percentages of different fungal genera estimated. Representatives of all fungal types were isolated onto malt extract agar plates, then identified after growth under standard conditions, with the help of standard texts (Pitt & Hocking, 1997; Klich & Pitt, 1988; Pitt, 2000). The presence of each common fungus was then calculated as a percentage of beans in each sample.

**Water activity determination**

The water activity was measured using an Aqualab model 3TE (Decagon, USA) with three repetitions.

**RESULTS**

Table 1 shows the data concerning the coffee variety and the soil fertilization of each farm in different regions. Table 2 shows the percentage of coffee ripeness at harvest time in the farms from different areas.

The climatic conditions (maximum average temperature and rainfall) for the year 2001-2003 from May to July are shown in Table 3. The average of maximum temperature in the year 2003 was 3ºC higher than the previous years. In the same way it can be observed that the pluviometric index was very low in 2003.

The physical (moisture content) and sensorial analyses of coffee are shown in Table 4.

**Fungal Presence in Dry Beans**

The percentage of fungi in coffee beans and its water activity are presented in Table 5.

**Table 1. Coffee variety and soil fertilization of the farms.**

| Area | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Region | Patos de Minas | Estrela do Sul | Indianópolis | Serra do Salitre | Rio Paranaíba | Coromandel |
| Variety | Mundo Novo | Mundo Novo | Yellow Catuai | Mundo Novo | Red Catuai | Red Catuai |
| N (kg/ha) | 379 | 320 | 445 | 330 | 249 | 204 |
| P (kg/ha) | 96 | 70 | 137 | 100 | 66 | 0 |
| K (kg/ha) | 379 | 224 | 445 | 400 | 66 | 325 |

**Table 2. Percentage of coffee ripeness at harvest time.**

| Area | Dry cherries (%) | Ripe cherries (%) | Immature (%) |
|---|---|---|---|
| 1 | 70 | 30 | - |
| 2 | 95 | 2 | 3 |
| 3 | 96 | 4 | - |
| 4 | 98 | 2 | - |
| 5 | 86 | 10 | 4 |
| 6 | 98 | 2 | - |

**Table 3. Maximum average temperature and rainfall.**

| Period | Maximum average temperature (ºC) | Rainfalls (mm) |
|---|---|---|
| 2001 | 26.0 | 47.00 |
| 2002 | 27.0 | 50.00 |
| 2003 | 29.3 | 05.00 |

**Table 4. Physical and sensorial analysis of coffee.**

| Area | Moisture content (%) | | Analysis of beans with visible defects | Final sensorial evaluation |
|---|---|---|---|---|
| | Initial | Final | | |
| 1 | 15.1 | 11.2 | Some immature fermented beans | positive |
| 1a (ripe cherries) | 65.0 | 11.1 | Many fermented beans | negative |
| 2 | 15.0 | 11.1 | Many immature beans | positive |
| 3 | 14.9 | 11.5 | Many immature fermented beans | negative |
| 4 | 15.0 | 12.0 | Some immature fermented beans | positive |
| 5 | 14.7 | 11.5 | Many immature fermented beans | negative |
| 6 | 15.7 | 11.1 | Some immature fermented beans | positive |

**Table 5. Percentage of coffee beans from different area infected by particular species of fungi.**

| Area | Total fungal presence (%) | Identified fungus (%) | Water activity |
|---|---|---|---|
| 1 | 12 | 6% *Fusarium* spp.<br>2% *Eurotium* sp.<br>2% *Penicillium* sp. | 0.524 |
| 1a (ripe cherries) | 40 | 22% *Aspergillus niger*<br>8% *Aspergillus ochraceus*<br>6% *Eurotium* sp.<br>2% *Aspergillus* sp.<br>2% *Fusarium* sp. | 0.502 |
| 2 | 8 | 4% *Aspergillus niger*<br>2% *Aspergillus ochraceus*<br>2% *Mucor* sp. | 0.532 |
| 3 | 20 | 18% *Fusarium* sp.<br>2% *Aspergillus ochraceus* | 0.553 |
| 4 | 2 | 2% *Fusarium* sp. | 0.575 |
| 5 | 20 | 10% *Aspergillus ochraceus*<br>8% *Fusarium* sp.<br>2% *Penicillium* sp. | 0.553 |
| 6 | 6 | 2% *Aspergillus niger*<br>2% *Eurotium* sp.<br>2% *Fusarium* sp. | 0.538 |

## DISCUSSION

The drying process of natural coffee does not need sophisticated installations; a clean patio, with good sunshine and adequate dimensions are enough. This fact explains that, if the coffee fruits are harvested when the fruits are at the raisin stage or already dried on the tree, the greater part of drying occurred on the tree. This coffee called “boia” when arrives in the drying area, will have approximately 15% of moisture content. In the case of ripe cherries, the moisture content is approximately 65%; to decrease from 65% to 11%, it would be necessary to remove 54% of water. If over 50% is removed on the tree, in the drying area it will be necessary to remove only 4% to reach 11% of moisture content. Therefore, 93% of drying will occur on the tree, and 7% in the drying area. This process takes approximately only 3 to 4 days of work in the Cerrado region where the climatic conditions are appropriate. During this period, 3 to 4 days, it is more difficult for the coffee quality to be affected if 100% of fruits are raisin and dried. However, it was observed in this experiment in some samples that the presence of some ripe cherries, immature and badly formed fruits with only one seed, influenced negatively on the drying process and consequently the good quality of coffee beverage.

The coffee, which achieved the best performance in the drying aspect, was from area 2. The beverage was positive, and no fermented immature beans or fermented beans were observed. The drying was very homogeneous.

The sample of ripe cherries put to dry on the drying patio presented a beverage of bad quality and a great percentage of fungal infection.

Three factors might be responsible for this: a) excessive amount of water within the ripe cherries; b) large quantity of sugar in the mucilage and; c) high temperature during the drying process.

A delay in maturation and some abnormal fruits with only one bean were observed in area 5. This may be due to the low levels of potassium in the soil.

Samples of dried cherries from areas 3 and 5 presented a beverage of bad quality with a high fungal presence.

This might be due to an unbalanced nutritious state of the trees plus unfavorable climatic conditions.

Samples of dried cherries from areas 1, 2, 4 and 6, which gave a beverage of good quality might be due to a well-run coffee plantation, a balanced fertilization and good preparation process, even under unfavorable climatic conditions.

## CONCLUSIONS

Some conclusions can be taken from the present work: it is possible to make good natural coffee in the Cerrado region, free of fungal infection, which can affect the quality. For this, it is necessary to use the best fruits at the raisin and dried stages, normally obtained in the August or September, to use good agricultural practice, to use balanced fertilization and to follow good processing techniques, even with unfavorable climatic conditions.

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# Green Coffee transport

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## SUMMARY

As already reported during the previous ASIC Colloquium (Blanc et al., 2001), conditions allowing mould to grow could exist during green coffee transport. A few previous trials already allowed us to identify some episodes at risk. New trials have been carried out to complete these previous investigations and assess the influence of some specific arrangements in the containers. Relative humidity and temperature are recorded for the entire journey and the results are the key element to draw our conclusions. Some recommendations are made in this paper on how to make transport safer.

## EQUIPMENT AND METHODS

The recording equipment for temperature (T) and relative humidity (HR) together with the calibration procedure were described in the previous paper (Blanc et al., 2001).

## REMINDER OF PREVIOUS TRIALS

The results of three different transport tests have been previously reported. These results have already allowed to identify two main transport phases at risk for mould development. The first one is the storage of a container in a north European harbour, mainly during winter season. Figure 1 illustrates this risk when the relative humidity reaching 100%, condensation takes place and water droplets fall onto the coffee, locally increasing the risk of mould development.

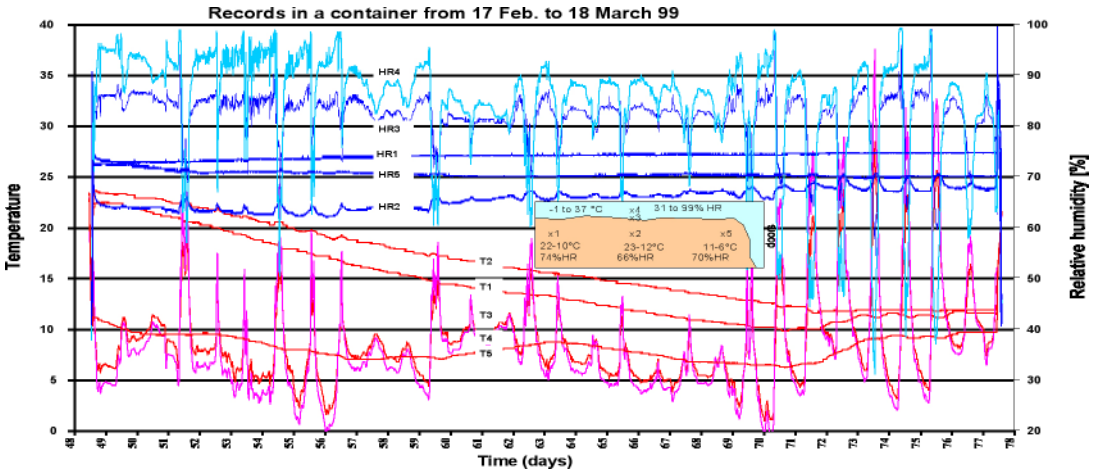


**Figure 1. Storage in container.**

A second period at risk during green coffee transport is the terrestrial transport in the producing countries. The Figure 2 is an illustration of what can happen.

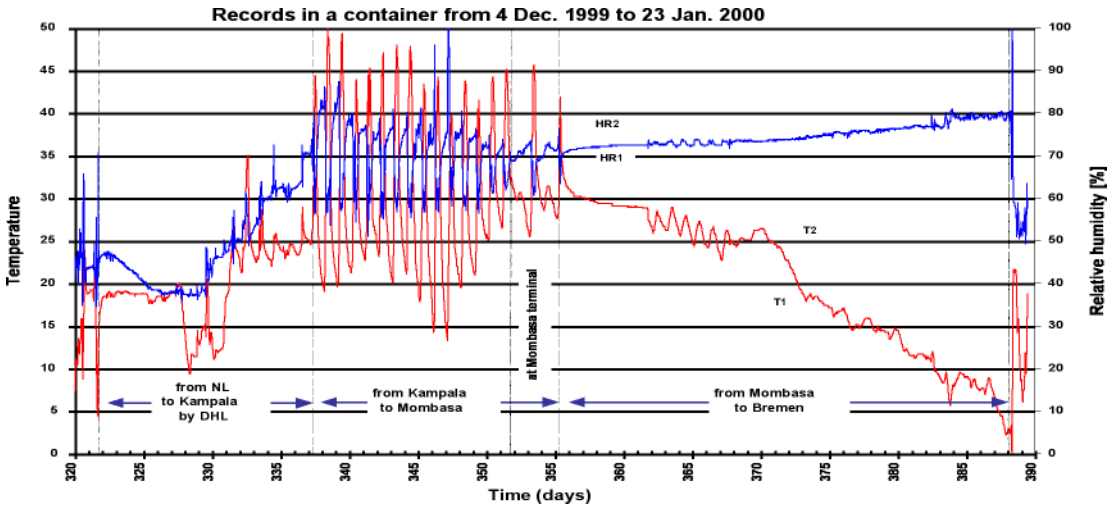


**Figure 2. Evolution during the different transport phases.**

On the other hand, according to these first results, the maritime phase of transport did not indicate condensation risks at all. The influence of external conditions, mainly the fluctuation of the temperature, is preponderant and can induce condensation. The upper layer of coffee is mainly affected by the risk of condensation. The rest of the coffee, perhaps with the exception of what is in contact with the door of the container, is not really affected. In this part, which represents the majority of the volume, the relative humidity and the temperature evolve very slowly, in particular for bulk rather than bags, indicating already a benefit of bulk transportation. What is important is that the moisture of the coffee loaded in the container is low enough to avoid mould development at least in this part. Based on other investigations a moisture lower than 12.5% is highly advised. But even after the completion of these trials several questions are still pending mainly to avoid the negative consequences of condensation on the surface of the coffee.

## RECENT TRANSPORT TESTS

The main pending questions concerned the following points:

- Despite the safety aspect of maritime transport is he coffee affected differently depending on the position of the container in the vessel?
- In case of a call in a harbour in a tropical zone during transport, is there a risk of condensation or to the opposite a too high temperature in the container?
- Do different specific arrangements inside the container help to protect green coffee against the risks of condensation?
- Can dry bags absorb enough a significant amount of water to justify their addition at different positions in the container?

A new set of tests have been carried out to try to answer these questions. The influence of the position of the containers in the vessel has been investigated by a Brazilian team (Palacios-Cabrera et al., 2004).

Other trials have been carried out between a South-East Asian producing country and Europe. Taking into account the benefits already observed for bulk transport this trial focused on this mode of transport. Several containers were transported in parallel to investigate the effect of:

- Wrapping green coffee with a woven fabric liner
- Adding cardboard at the roof and the sides

- Adding dry bags in various numbers and positions
- Storing the containers upon arrival in Europe

Cardboard had been installed to ensure certain thermal protection and also protection against water dripping. Woven fabric liner wraps the green coffee avoiding direct contact with the walls (Figure 3).

**Figure 3. Installation of cardboard and liner.**

Dry bags are also added in different locations in the containers, either directly in contact with the coffee or in between the liner and the cardboard or in both locations (Figures 4 and 5).

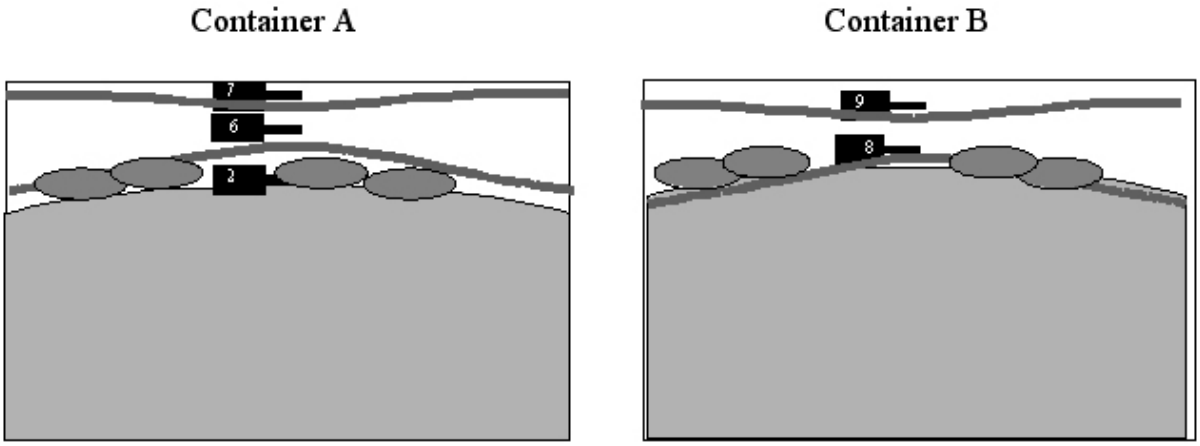


**Figure 4. Example of dry bags locations.**


**Figure 5. Pictures of dry bags.**

We will discuss the results obtained in container A and B with the arrangements in the previous pictures. The outcome in other containers is similar and enough conclusions and guidance can be drawn from what is observed in these two containers.

Figure 6 shows the evolution of the relative humidity and the temperature throughout transport.

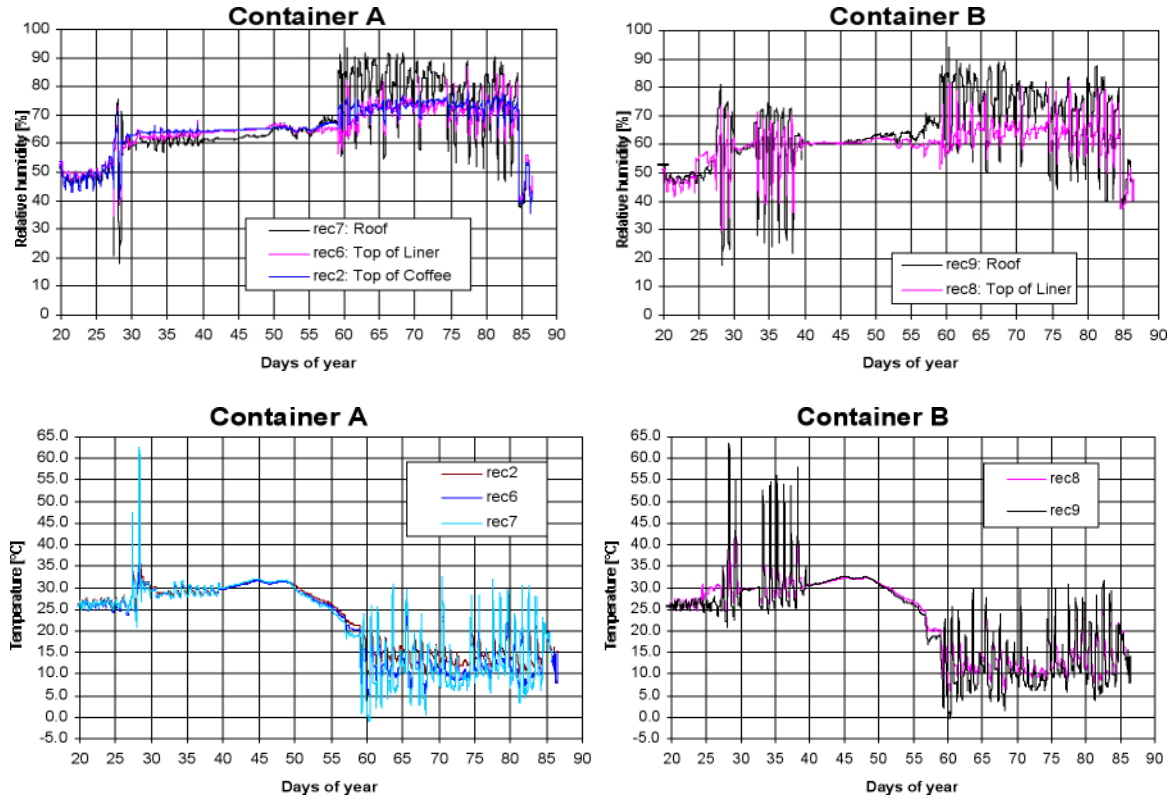


**Figure 6. Evolution of relative humidity and temperature.**

The first general observations can already be indicated:

- Nothing particular happened during the maritime transport phases (days 31 to 34 and days 40 to 60). The slow progressive decrease of the temperature does not results in a large increase of relative humidity.
- The two containers show a different reaction during the call in an south-eastern Asian harbour. Figure 7 shows the data recorded during these days (day 34 to 40)

Container B has been stored in sunlight, which explains that temperatures as high as 58°C were reached near the roof. Container A was covered by other containers and does not show any variation for both temperature and relative humidity.

- The three weeks of storage upon arrival in Europe confirmed previous observations. As opposed to the call in Asia external night temperatures dropped below 2°C. Figure 8 shows detailed records for this period. Frequent periods with HR above 90% were reported at the roofs. But for the other records, no risk of condensation was observed either between the liner and the cardboard or below the liner. The lowest HR recorded in container B in comparison to container A cannot be explained by the presence of dry bags in the headspace between the liner and the cardboard. The efficiency of dry bags are commented on below.

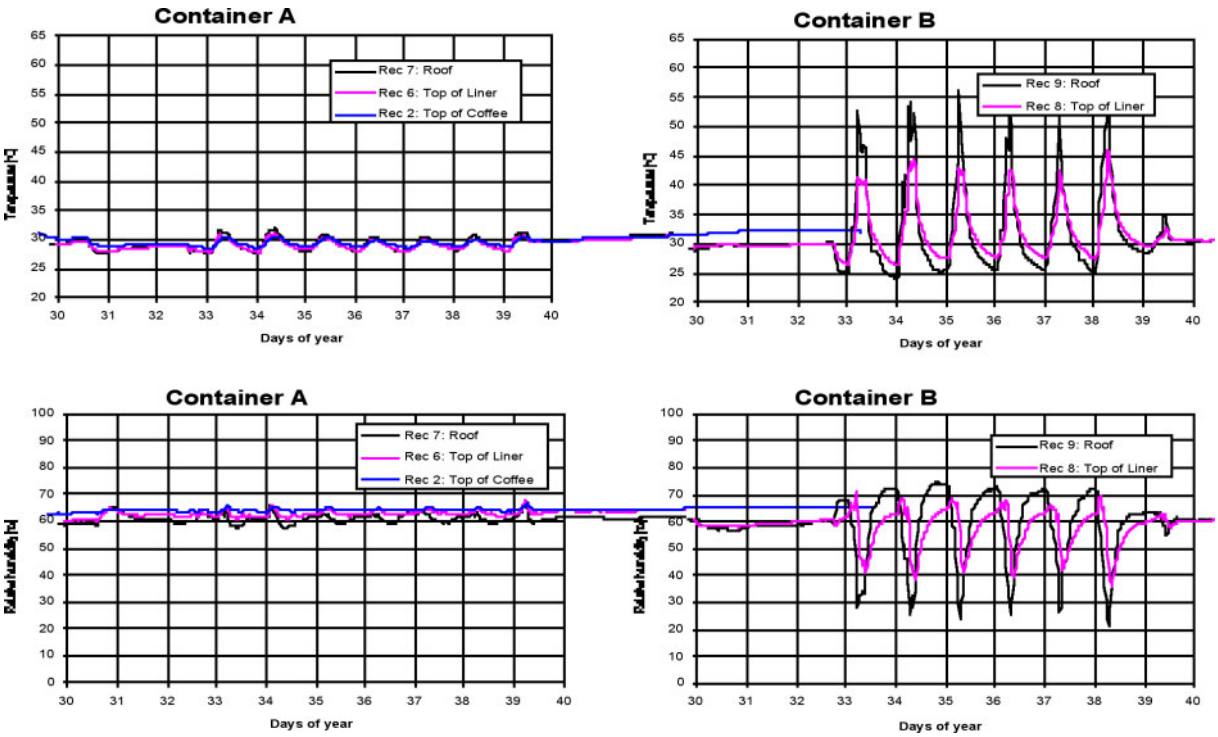


**Figure 7. Call in a southeastern Asian harbour.**

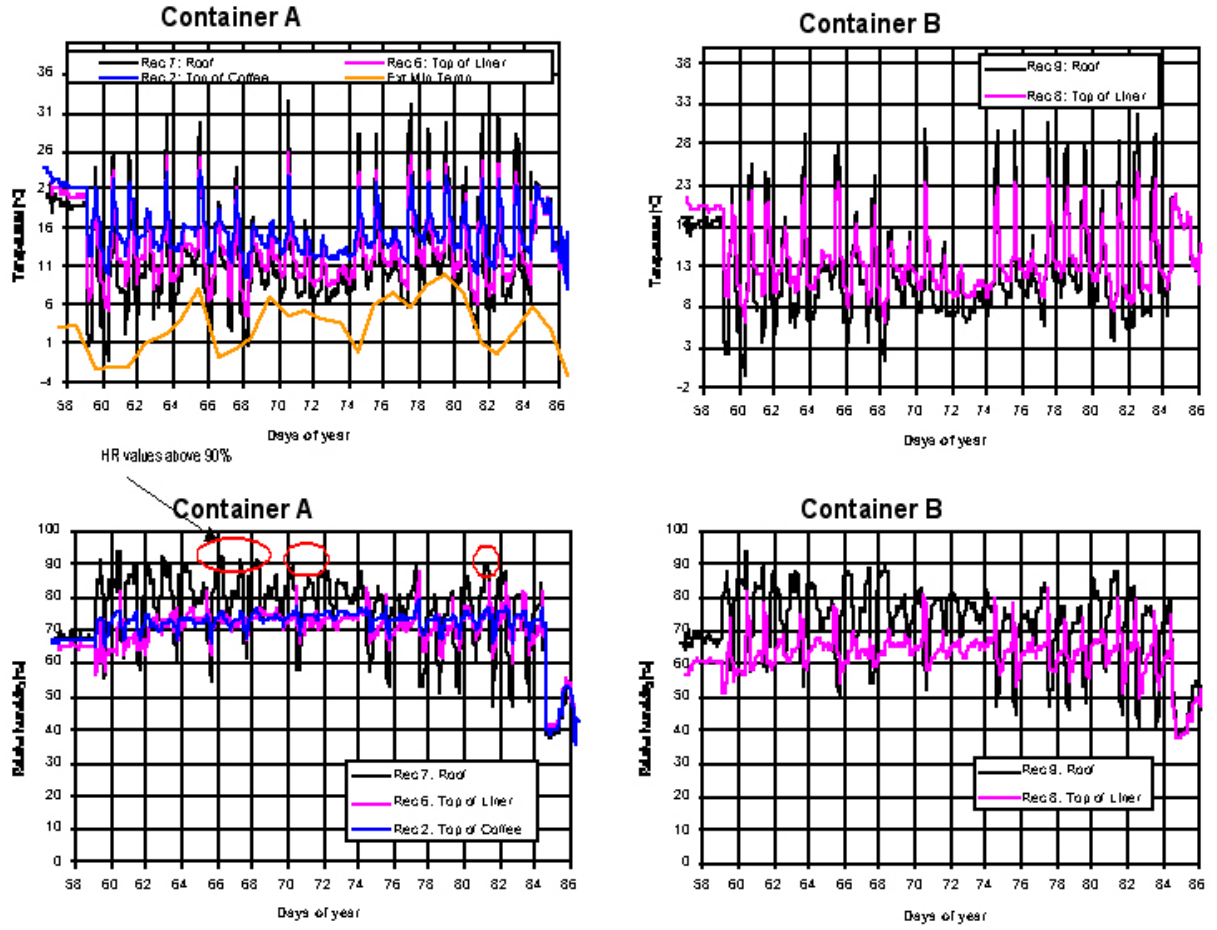


**Figure 8. Storage period at arrival in Europe.**

Despite all the precautions taken in the experimental design, the temperature variations differed in the containers. These differences do not allow direct comparison of their HR

values and made the evaluation of the impact of the dry bags difficult. The following Table 1 indicates the typical water uptake recorded of a few dry bags.

**Table 1.**

| | Dry bag 1 | Dry bag 2 | Dry bag 3 |
|---|---|---|---|
| Weight before drying (g) | 2649.6 | 2555.5 | 2661.2 |
| Weight after drying (g) | 2542.9 | 2466.3 | 2561.2 |
| Difference (g) | 106.7 | 89.2 | 100 |
| Difference (%) | 4.0 | 3.5 | 3.8 |

From these results it is evident that the dry bags do not absorb enough water to have an important role. However, it is possible that they absorb enough water to act as a dynamic HR regulator system by trapping the excess humidity and releasing it afterwards. Further trials with more dry bags and different absorbents could give more information.

## CONCLUSIONS

These recent green coffee transport tests confirmed previous observations. The main phases at risk during transport are the terrestrial phases and in particular the possible storage period in a European harbour during winter. However, in this series of tests the different protection installed in the containers showed some efficiency by isolating the green coffee from the influence of external conditions. A call to a harbour in a tropical zone showed the possibility of very high temperature in the containers, depending on whether they are positioned on top of a pile or not. Dry bags absorption capacity is not important enough to really have a significant influence.

Further transport tests would be important to confirm these different observations. Nevertheless, it is already advisable that green coffee transport safety would be improved when combining different conditions such as a moisture content of green coffee lower than 12.5%, bulk transport when feasible, equipping the container with a cardboard liner, adding a cardboard coverage on the top of the coffee and wrapping the coffee in a liner.

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# Trial Shipment of Coffee from Santos (Brazil) to Livorno (Italy) Monitoring Relative Humidity, Temperature, Grain Moisture Content and Condensation in Non-ventilated Commercial Containers

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## SUMMARY

Six containers (three commercial and three prototype) were monitored during a shipment from Brazil to Italy. The containers were stowed in three different places (hold, below deck and on deck) on the ship. Each prototype was located next to a conventional container. Temperatures, relative humidity, condensation and changes in moisture content, were monitored. The coffee container located below deck was less affected by variations (0.7%) in relation to the moisture content than those in the hold and deck, especially close to the upper layer of the cargo. Coffee located in the hold showed the highest variation in moisture content (3%) and in addition, the container showed visible condensation. Coffee transported on the deck had an intermediary moisture variation (2%) and there was no visible condensation. The coffee moisture content variation of the prototype containers was similar to that of the commercial ones, mainly in the upper layer of the cargo (2 to 3%). The moisture content gain in the upper layer of the cargo was 1 to 3% while the increase in water activity was small (0.70). This suggests that the diffusion of moisture occurs very slowly inside the cargo. In this situation there may be sufficient time and conditions for fungal growth. The regions of the container near the wall and ceiling are susceptible to condensation since they are close to the headspace with its high relative humidity. More details of the data collected during the trip will be presented.

## INTRODUCTION

The gain or loss of moisture inside dynamic, non-hermetic systems such as the containers used for coffee transportation, is a common occurrence on maritime routes between regions of tropical climate such as Brazil and temperate climate such as the Northern hemisphere (Sharp & Greve, 1984; Pfeiff, 1998; Lee, 1999).

Changes in temperature during grain transportation can result in humidity transfer. Differences of 2 to 3ºC favour migration, while differences of 5 to 10ºC from one region to another favour faster humidity transfer (Lazzari, 1997). The warmer region tends to lose water, which is absorbed by the colder region (Pixton, 1982). The monitoring of the grain intrinsic factors such as: moisture content and water activity, as well as the extrinsic factors:

temperature and relative humidity, are useful parameters to evaluate some inherent grain quality characteristics (Sauer, 1988).

Little research has been carried out on coffee transportation so the objectives of the present work were: a) to supply data on relative humidity and temperature in different locations inside the vessel (hold, below deck and deck), as well as in different regions inside the container (top, middle, bottom and headspace); b) to compare and validate the data obtained from real and prototype containers; and c) to evaluate ochratoxin A production in coffee during transportation.

## MATERIAL AND METHODS

### Coffee

Sixty kg of export type raw arabica coffee beans were put in conventional containers while 2 kg bags were used for the prototype.

### Conventional and Prototype Containers

Three conventional containers with dimensions of: 5.88m x 2.32m x 2.19m
Three prototype containers with dimensions of: 1.86m x 0.73m x 0.73m
In order to validate the prototype data as compared to that of the conventional container, polystyrene was placed on three lateral walls of the prototype. The top and one wall did not have polystyrene. That would simulate one segment of a real container. The prototypes were placed in a vertical position close to the end of the conventional ones, as shown in Figure 1.

**Figure 1. Prototype container next to conventional container on the deck of the vessel.**

### Coffee sampling

From the conventional containers, nine bags (3 per container) were selected at random and identified. A sub-sample of 1kg was collected from each bag. The sub-samples were mixed and the following analyses were carried out: moisture content, water activity and ochratoxin A. This first sampling corresponded to the initial determination of the parameters that were monitored. From the same identified bags, sub-samples of 1kg were collected in Trieste (Italy), for the final determination.

For the prototypes, sampling was carried out before the coffee was filled into the mini bags. Six kg from 30 coffee bags were collected at random. During the sea voyage, two samplings were performed. The prototype had 9 holes sealed with screws, which allowed for samples to be taken using a special bag sample collector. Samples were taken from the bottom, middle and top of the containers for moisture content measurement. In the final sampling in Livorno (Italy), 1kg of each region was collected for: moisture content, water activity and ochratoxin A analysis.

**Moisture content analysis**

The moisture content was determined using electric capacitance equipment Gehaka 600, previously calibrated by the oven method at 105ºC ± 2ºC for 24 h. The Gehaka 600 measured the moisture content *in situ* during the voyage. A temperature correction was also considered in the final results.

**Water activity determination**

The water activity was measured using an Aqualab model 3TE (Decagon, USA) with three repetitions. The water activity was measured at the beginning and end of the voyage.

**Analysis of ochratoxin A (OTA) in the coffee**

The coffee samples were analysed for OTA according to Pittet *et al.* (1996). The samples were extracted with a solution of methanol: 3% sodium bicarbonate (50:50). The extracts were filtered and diluted with phosphate buffered saline and applied to an immunoaffinity column (Vicam, Watertown, Mass.) containing a monoclonal antibody specific for OTA. After washing, the OTA was eluted with HPLC grade methanol and quantified by reverse-phase HPLC using a fluorescence detector. The mobile phase used was acetronitrile/4 mM sodium acetate with 0.5% acetic acid solution (42:58). The flow rate was 1mL/min. The equipment used was a Shimadzu LC-10VP system (Shimadzu Corporation, Japan) set at 330 nm excitation and 470 nm emission. The HPLC was fitted with an ODS Hypersil (5 μm, 25 mm X 4.6 mm) pre-column and Supelcosil$^{TM}$ LC-18 (5 μm, 250 mm X 4.6 mm) column (Supelco, USA). The detection limit of the method was 0.2 μg/kg OTA.

**Extrinsic factors monitored during the voyage: temperature and relative humidity**

Temperature and relative humidity were monitored using Onset (HO8-032-08) calibrated sensors. Four sensors were placed inside the conventional containers at the bottom, middle, top and headspace. In all, 12 sensors were used for the conventional containers. The sensors were protected inside a stainless steel cage. In the prototype the same location criteria was used.

**Coffee transportation trajectory from Leme (Brazil) to Santos**

The coffee was first brought from the farms to a storage place in Leme. It was classified and selected by the importer. In Leme the chosen coffee beans were returned to the 60 kg bags. The sensors were placed in the bags and mini-bags at this time. The coffee bags remained in the storage place for three days until the road transportation authorization was obtained. They were then loaded onto the truck and covered by a canvas top. The total road trajectory from Leme to Santos was 274 km and the journey took approximately 8 hours.

**Container filling in Santos**

The coffee bags were filled into the container in Santos. The identified bags containing the sensors were placed at the centre of the conventional containers. The coffee bags were covered with kraft paper to protect them against humidity. The containers were closed and only opened at the final destination in Italy. In a similar way, the prototypes were also filled in Santos. The sensors were put into place at that time. The containers were stored at a terminal called Tecondi in Santos for 12 days. This was the time needed to get the authorization for maritime transportation and for the arrival of the vessel.

**Coffee embarkation onto the vessel**

The conventional and prototype containers were placed on the vessel in three positions: deck (Deck 7), below deck (Deck 3) and hold (Deck 1). Deck 7 is located at the top where the containers are drastically affected by the climatic changes. Deck 3 is on the first floor of the vessel and is above sea level and the place is ventilated. Deck 1 is in the hold of the vessel, 5m below sea level where air circulation is greatly reduced as compared to Deck 3.

**Maritime transportation**

The sea voyage took 14 days. Outside temperature and relative humidity measurements were carried out every morning and afternoon, as well as airflow measurements using appropriate equipment.

In Livorno (Italy) the sensors from the 3 prototype containers were removed and samples for OTA, moisture content and water activity were collected. The prototype containers returned to Brazil on the same ship.

**Railway transportation from Livorno to Trieste**

The 3 conventional containers were transported by train from Livorno to Trieste. This trip took 10 days.

**Coffee unload in Trieste**

In Trieste the conventional containers were opened. The coffee bags were unloaded and taken to a storage place until the coffee was roasted. The sensors and samples from the location of the sensors were taken for later analyses, as also from the prototypes. It was verified if the container ceiling showed condensation. Some bags were wet and were separated from the others. Samples from these wet bags were also collected.

## RESULTS AND DISCUSSION

Table 1 presents the moisture contents and water activities of the coffee beans monitored during transportation from start (Leme, Brazil) to finish (Trieste, Italy). The locations of the containers in the vessel correspond to: Deck 7 (deck); Deck 3 (below deck, first floor) and Deck 1 (Hold) and the samples from the three regions of the container: bottom, middle and top.

**Table 1. Initial and final moisture contents on wet and dry weight bases (%) and water activity ($a_w$) of coffee beans during transportation from Leme to Trieste.**

| Moisture content ( wwb/dwb %) and water activity ($a_w$) of coffee beans | | | | | |
|---|---|---|---|---|---|
| Location in the vessel | Container region | $a_w$ (initial) | wwb/dwb % (initial) | $a_w$ (final) | wwb/dwb% (final) |
| *Deck 7* On deck | Bottom | 0.59 | 10.87/12.20 | 0,59 | 10.87/12.20 |
| | Middle | 0.59 | 10.85/12.17 | 0.58 | 12.10/13.69 |
| | Top | 0.59 | 10.83/12.20 | 0.64 | 12.43/14.19 |
| *Deck 3* Below deck | Bottom | 0.53 | 10.03/11.14 | 0.53 | 10.03/11.14 |
| | Middle | 0.58 | 11.01/12.38 | 0.54 | 10.17/11.32 |
| | Top | 0.58 | 11.01/12.38 | 0.60 | 11.58/13.10 |
| *Deck 1* Hold | Bottom | 0.56 | 11.01/12.38 | 0.57 | 10.87/12.20 |
| | Middle | 0.61 | 11.30/12.74 | 0.58 | 10.87/12.20 |
| | Top | 0.61 | 11.44/12.92 | 0.63 | 12.00/13.64 |
| | Wet bags | -* | - | 0.700 | 14.00/16.83 |

**Not determined.*

In general there was a decrease in moisture content at the end of the voyage in the three decks studied, with the exception of the top and middle regions of the container on deck 7, and the top region on deck 1.

Table 2 shows the moisture content and water activity data of the three prototypes located next to the conventional containers, taken at the start (Leme), during the journey and finish (Livorno). The prototype stopped at Livorno and returned to Brazil in the same vessel; they did not accompany the conventional containers to Trieste. In general the prototype containers showed similar results to those of the conventional containers.

**Table 2. Moisture contents on wet weight basis (% MC) and water activities ($a_w$) of prototype containers.**

| Moisture content (wwb %) and water activity ($a_w$) of coffee beans | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sampling | | | | | |
| Location in the vessel | Container region | Initial | | 29/01 | 3/02 | Final | |
| | | MC (%wwb) | Aw | | | MC (%wwb) | Aw |
| Deck 1 Ital #2 | Bottom | 10.73 | 0.57 | 9.60 | 10.45 | 12.15 | 0.57 |
| | Middle | 10.73 | 0.57 | 9.32 | 9.88 | 11.86 | 0.58 |
| | Top | 10.73 | 0.57 | 9.60 | 10.31 | 12.85 | 0.60 |
| Deck 3 Ital #3 | Bottom | 10.73 | 0.57 | 10.43 | 10.31 | 11.86 | 0.58 |
| | Middle | 10.73 | 0.57 | 10.01 | 9.88 | 11.86 | 0.59 |
| | Top | 10.73 | 0.57 | 10.01 | 10.17 | 12.29 | 0.59 |
| Deck 7 Ital # 1 | Bottom | 10.73 | 0.57 | 10.45 | -* | 11.86 | 0.60 |
| | Middle | 10.73 | 0.57 | 10.45 | - | 11.86 | 0.59 |
| | Top | 10.73 | 0.57 | 10.45 | - | 12.43 | 0.63 |

**Not determined.*

While unloading the coffee bags from the conventional containers in Trieste, some of those in the container located in the hold (deck 1) showed signs of wetness. These bags were at the top

of the container and were wet due to condensation dripping from the container ceiling. These bags were separated from the others. The coffee beans inside these bags had a whitish colour and some were lumped together. Coffee beans from these bags showed high moisture contents (14%) and water activities (0.70) and an OTA content of 13 μg/kg. This condensation was not observed in the containers located on decks 7 and 3, possibly because the high humidity in the headspace of the containers was lost to the environment as vapour and part was transferred to other cargo. The air conditions on deck 7 might have contributed to the drying and evaporation of condensation since there were 4 ventilation holes in the container sides. Deck 3 had limited ventilation and on deck 1 the ventilation was very poor.

With the container located on deck 7, the water retention capacity was reduced at low temperatures and the bags in the top region gained moisture, as presented in Table 1. In the other two containers, the temperature decreased slowly, and consequently the water retention capacity was higher; the tendency being for the water in the environment to reach the relative humidity equilibrium. This water, in vapour form, rises by convection, gets cold and forms condensation, due to the high temperature gradient between the headspace and the cargo and between the headspace and the external environment. It was observed that the container located on deck 3 had little ceiling condensation as compared to that on deck 1, while that on deck 7 showed no condensation.

Figure 2 shows the temperature and relative humidity changes in the conventional containers located on the decks of the vessel during storage at Santos terminal, the sea voyage and rail transportation. It can be observed that the temperatures inside and outside the containers started to decrease at the end of the sea voyage. During rail transportation in Italy, the temperature decreased abruptly and the relative humidity in the headspace increased proportionally.

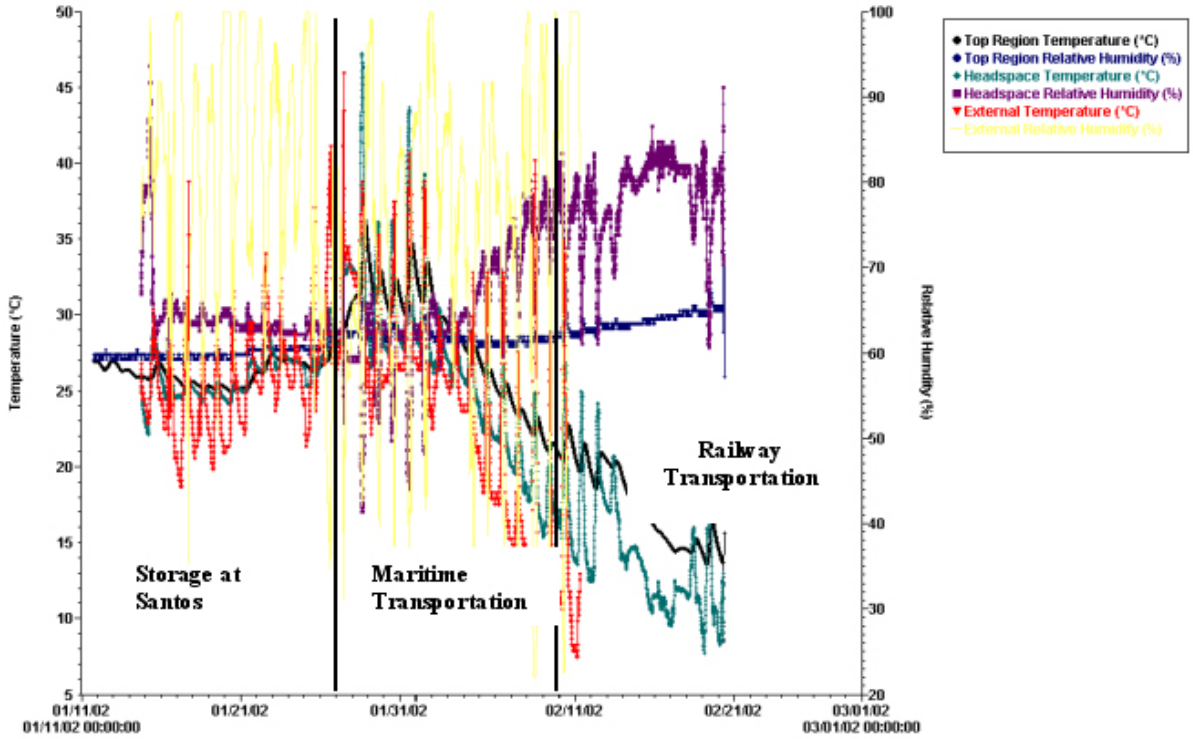


**Figure 2. Temperature (ºC) and relative humidity (%) of the conventional container located on the deck of the vessel.**

Tables 3 and 4 present the OTA concentrations of the coffee in the conventional and prototype containers respectively, at the beginning and end of the voyage. According to Tables 3 and 4 there was a slight increase in OTA production during transportation, especially at the top of the container located on deck 7, close to the headspace. In this location the temperature oscillation was more drastic and consequently the humidity diffusion inside the container was more dynamic than on the other decks, mainly during the sea voyage. A similar situation occurred at the top of the prototype containers, which presented higher OTA production. From the results presented, deck 1 would be the most appropriate location for container transportation, since temperature oscillation is less drastic, and the inter-granular microenvironment presented less adequate conditions for fungal growth and toxin production. Since the temperature changes drastically affected container condensation, it is recommended that the unloading of coffee in the importer countries be quick, since this condensation will increase and if the spores of toxigenic fungi are present in the coffee beans, they will develop and produce toxin.

**Table 3. Initial and final ochratoxin A (OTA) concentrations (μg/kg) in coffee from the conventional containers.**

| OTA concentration in coffee μg/kg | | | |
|---|---|---|---|
| Location in the vessel | Container region | OTA concentration (μg/kg) | |
| | | Initial | Final |
| *Deck 7* On deck | Bottom | ND* | 0,33 |
| | Middle | ND | 0,1 |
| | Top | ND | 7.91 |
| *Deck 3* Below deck | Bottom | 1.65 ± 0.003 | 0.16 |
| | Middle | 0.19 ± 0.018 | <0.1 |
| | Top | 1.03 ± 0.50 | 0.74 |
| *Deck 1* Hold | Bottom | 1.54 ± 1.33 | 0.17 |
| | Middle | 0.30 ± 0.18 | 0.26 |
| | Top | ND | 0.28 |
| | Wet bags | -** | 13.13 |

* *Not Detected;* ** *Not Determined*

**Table 4. Initial and final ochratoxin A (OTA) concentrations (μg/kg) in coffee beans transported in prototype containers.**

| Location in the vessel | Container region | OTA concentration (μg/kg) | |
|---|---|---|---|
| | | Initial | Final |
| *Deck 7* On deck | Bottom | 0.20 | 2.85 |
| | Middle | | 1.01 |
| | Top | | 2.38 |
| *Deck 3* Below deck | Bottom | 0.20 | 1.22 |
| | Middle | | 2.06 |
| | Top | | 0.45 |
| *Deck 1* Hold | Bottom | 0.20 | 0.20 |
| | Middle | | 1.29 |
| | Top | | 0.66 |

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# Infective Fungi Profile in a Wet Coffee Processing Chain in Kenya

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## SUMMARY

Coffee was picked from a block within a large coffee estate in Kenya and subjected to wet processing method (with fermentation) up to the fully dry stage. Samples were taken throughout the chain and fungi isolated from inside the coffee beans by plating on DG18 medium after surface sterilization with 1% sodium hypochlorite. Emphasis was laid on the lowest and highest grades after separation by water. It was observed that total fungal infection increased with decrease in density and sorting the dry coffee (by colour and insect damage) gave coffee of different microbial loads. The dominant genera were *Penicillium* spp, *Fusarium* spp, *Cladosporium* spp and *Aspergillus* spp. The occurrence of these genera with potential mycotoxin production among other adverse quality attributes is of concern.

## INTRODUCTION

Moulds are a group of fungi that form woolly or furry growth on their substrates. They are universal in occurrence and agricultural produce such as coffee is in contact with them throughout the process of production up to consumption. When the ambient conditions especially moisture and temperature are favourable, the moulds grow on and into the coffee, causing large economic losses due to discolouration, off-flavours and taste, and production of metabolites that may be toxic to consumers (mycotoxins). One mycotoxin that is of great concern recently is Ochratoxin A (OTA) produced by *Aspergillus* and *Penicillium* species. International maximum limits of this mycotoxin may be set in coffee trade, which may have major consequences to developing economies (Studer-Rohr et al., 1993, 1995; Duris, 2002). In Kenya, Masaba (1991) isolated many fungal genera from coffee canopies including *Alternaria, Penicillium, Aspergillus, Fusarium, Cladosporium, Colletotrichum* and *Phoma* which may affect coffee quality in one or combination of the effects mentioned above. It is therefore of importance to study fungi that occur in Kenyan coffee after harvest during subsequent processing stages. Kenya produces mainly Arabica coffee (more than 98**%),** that is wet processed basically as shown in Figure 1. The objective of the work was to assess mould dynamics in the processing chain on 'as is' basis.

## MATERIALS AND METHODS

### Sampling

During 2001 early crop harvest (April-August), it was arranged with the farm management of Jacaranda estate, at Coffee Research Station in Ruiru, Kenya, that one day's harvest of a certain block would be used for the studies and thus treated without mixing with other coffee during the whole study. The coffee was subjected to recommended processing procedures at routine commercial standards but the different grades were situated in the same location and treated similarly to avoid differences due to location or in operations like efficiency of covering in case of rains.

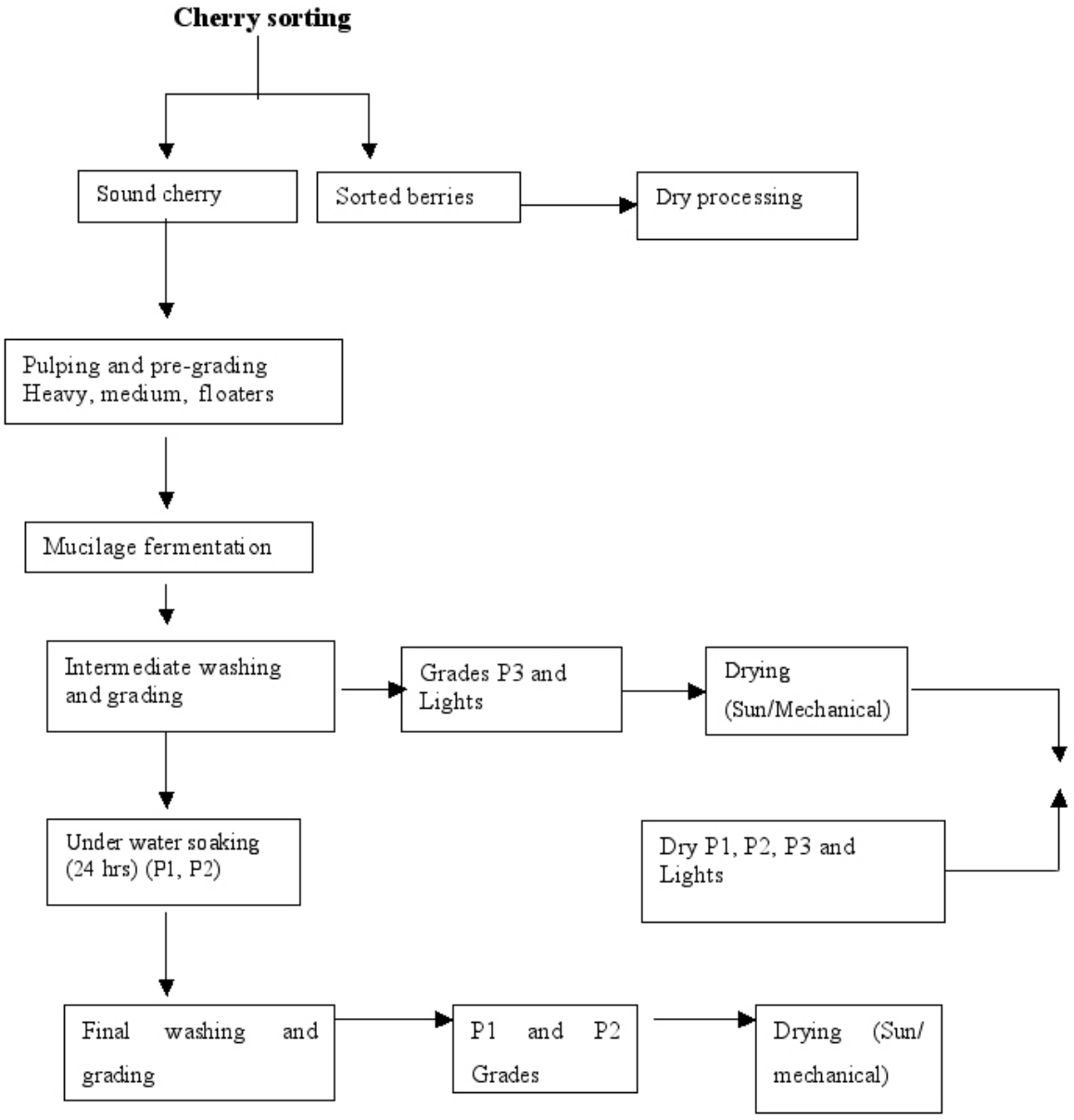


**Figure 1. The principal steps in primary wet processing of coffee in Kenya. Key: P1- Parchment 1, P2- Parchment 2, P3- Parchment 3.**

While picking was in progress, the technical staff sampled from trees cherries of two categories i.e. best cherry by visual assessment and those dried on the trees. When the coffee was delivered to the pulping station in the evening (on farm), samples was done after sorting as the pickers poured the coffee into the pulping hopper by accumulating small handfuls from their containers to obtain about 10kg. This was thoroughly mixed and 0.5 kg taken for analysis.

After pulping (which involves density grading by floatation in water) and fermentation (natural) of the coffee overnight (16 hours), the coffee was sampled after being washed (which involves further grading again by density in water). There was no intermediate washing and this was the final washing. Two categories were followed i.e. Parchment 1 (P1, the heaviest) and Parchment Light (PL, the lightest). After washing and grading, PL gave rise to two grades and it was the lighter of them PL (L) that was further followed. This grade is usually milled on the farm together with dry processed sortings (Buni) because it is uneconomical to deliver it for commercial milling. Both categories were sampled at start of drying and end of drying while sampling of PL(L) was extended to cover after storage and

milling stages. Before the coffee was milled the waste disposal blowout pipe (a temporary fitting) was ridded of particles and sampling delayed until about a third of the designated coffee (2 bags) was milled. This was to reduce the risk of contamination with previous coffee. Sampling was always by small increments followed by thorough mixing and about 0.5 kg taken for laboratory analysis.

**Microbial analysis**

Infective fungi were isolated from inside the coffee beans after removing the parchment and surface sterilizing the beans using 1% sodium hypochlorite for 10 minutes. For fresh cherry, the beans were squeezed out (one per berry) and sun-dried for 2-3 hours to reduce sliminess of the mucilage and the parchment separated out. Seven beans were plated per 9 cm diameter petri-dishes containing DG18 medium (selective fungal medium). Two sub-samples were analyzed with seven plates per sub sample and incubated at room temperature (22-24$^{o}$C). Fugal colonies that grew were recorded after 12 days.

**RESULTS AND DISCUSSIONS**

Various fungi were isolated from the coffee analyzed in the study with heavily infected beans having more than one fungal type (Figure 2). One consistent observation in this study was that sorted defective coffee had more mould infections than both general population and "best" sorted coffee (Table 1). In the farm, the visually faultless coffee cherries had lower percentage of infection than the cherry population that went into the pulping hopper. The coffee that had dried on the trees could have even been remnants of earlier seasons or infected by Coffee Berry Disease. This coffee had high mould infection hence a risky category to pick. Selective picking is thus very useful in reducing mould population in the processing chain. The practiced sorting was not very thorough as done during the picking of "best" cherry in the farm and high degree of thoroughness may not be economically feasible due to labour requirement.

After pulping, fermentation and final washing, which is the last grading by density in water and after which drying starts; the lightest coffee PL (L) had the highest mould infections. After drying the coffee, the general mould infections increased in all the coffee categories. Considering the length of time taken (15 days) and the ambient conditions (Table 2) it was possible for mould infections to take place either as new or those superficial ones to grow deeper. The sortings from the unsorted coffee had more infections. The sorting was based on discolouration and insect damage. After this stage P1 coffee was delivered to commercial mill and was not followed further. The observed increase in infection during drying need further investigation and if need be, the drying conditions can be modified by artificial means. This is subject to favourable economic analysis.

After storage (69 days), PL (L) was milled. The random sample from the bag after milling had less infection than the sample from the mill hopper. This shows a cleaning process. This could be due to unsound beans being broken and blown away together with light beans and the husks. This was tested by sampling the broken and light beans that are blown into the waste piping. These fall just below the outlet of the pipe. They had a much higher infection percentage than the rest of the samples.

As a general conclusion, density grading reduces the rate of infection of dense coffee and visual sorting has a similar effect. These attributes can be improved.

**Table 1. Fungi isolated from the inside of coffee beans at different stages along the processing chain.**

| Micro flora genera | Initial samples | | | After Fermentation | | | After Drying | | | | | Milling (PL (L)) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | P1 | | PL(L) | | | | | | |
| | Best cherry | Dry on tree | Hopper | P1 | PL (H) | PL (L) | Unsorted | Sortings | Unsorted | CBB | Black | Hopper | Bag | Best | Waste end |
| *Aspergillus* | | 0.08 | | | | 0.47 | | 0.03 | | | | 0.67 | 0.6 | 0.5 | 0.02 |
| *Penicillium* | | 0.4 | 0.14 | | | 0.24 | 0.18 | 0.11 | 0.04 | 0.55 | 0.15 | | | 0.25 | 0.04 |
| *Fusarium* | | 0.23 | 0.14 | | 0.5 | 0.12 | 0.55 | 0.42 | 0.87 | 0.18 | 0.54 | | | | 0.7 |
| *Cladosporium* | | 0.25 | | | | 0.12 | 0.18 | 0.08 | 0.08 | | 0.24 | | 0.04 | | 0.11 |
| *Phoma* | | | | | | | | 0.08 | | | | | | | |
| Yeasts | 0.67 | 0.05 | 0.43 | 1.0 | | 0.06 | | 0.29 | | 0.15 | 0.02 | 0.33 | 0.16 | | |
| *Colletotrichum* | | | | | 0.5 | | | | | | | | | | |
| *Alternaria* | 0.33 | | 0.14 | | | | 0.09 | | 0.04 | 0.06 | 0.02 | | | | |
| *Epicoccum* | | | | | | | | | | | | | 0.16 | 0.17 | |
| Unidentified | | | 0.14 | | | | | | | | 0.02 | | 0.04 | 0.08 | 0.13 |
| Total colonies | 3 | 40 | 7 | 1 | 2 | 17 | 11 | 38 | 24 | 33 | 41 | 36 | 25 | 12 | 53 |
| **% infection** | **6.12** | **73.43** | **12.24** | **2.04** | **4.08** | **34.69** | **20.41** | **69.39** | **46.94** | **65.71** | **81.63** | **65.31** | **44.9** | **24.29** | **81.63** |

**Table 2. Weather parameters at the study site (Coffee Research Station) in the year 2001.**

| Parameters | | Month | | | |
|---|---|---|---|---|---|
| | | June | July | August | September |
| | Date | | | | |
| Rainfall | 1-10 | 12.5 | 0.8 | 5.7 | 21.3 |
| | 11-20 | 0 | 0 | 2.6 | 0 |
| | 21-end | 1.5 | 0.3 | 0 | 0 |
| **Monthly total** | | **14** | **1.1** | **8.3** | **21.3** |
| Maximum temperature | 1-10 | 23.5 | 22.0 | 22.5 | 25.4 |
| | 11-20 | 23.1 | 23.1 | 26.7 | 27.3 |
| | 21-end | 23.5 | 21.6 | 25.8 | 28.5 |
| **Mean** | | **23.4** | **22.2** | **24.7** | **27.1** |
| Minimum temperature | 1-10 | 11.2 | 10.3 | 9.1 | 11.2 |
| | 11-20 | 10.5 | 11.1 | 10.9 | 11.1 |
| | 21-end | 10.8 | 10.7 | 8.4 | 10.6 |
| **Mean** | | **10.8** | **10.7** | **9.5** | **11.0** |
| RH0900hrs | 1-10 | 88 | 89 | 89 | 91 |
| | 11-20 | 89 | 89 | 90 | 87 |
| | 21-end | 90 | 91 | 87 | 88 |
| **Mean** | | **89** | **90** | **89** | **89** |
| RH1500hrs | 1-10 | 88 | 86 | 89 | 94 |
| | 11-20 | 85 | 91 | 90 | 93 |
| | 21-end | 88 | 89 | 89 | 95 |
| **Mean** | | **87** | **89** | **89** | **94** |

The most common mould genera observed were *Aspergillus, Penicillium, Fusarium, Cladosporium* and yeasts. This infection is likely to lead to presence of various mycotoxins, off-flavours and taste. However the mould complex can be explored further to know if the

safe fungi can be utilized to control the dangerous ones (biological control). Other research needs include investigations into the influence of agro-ecological zones and cropping patterns and systems on the occurrence of the moulds.

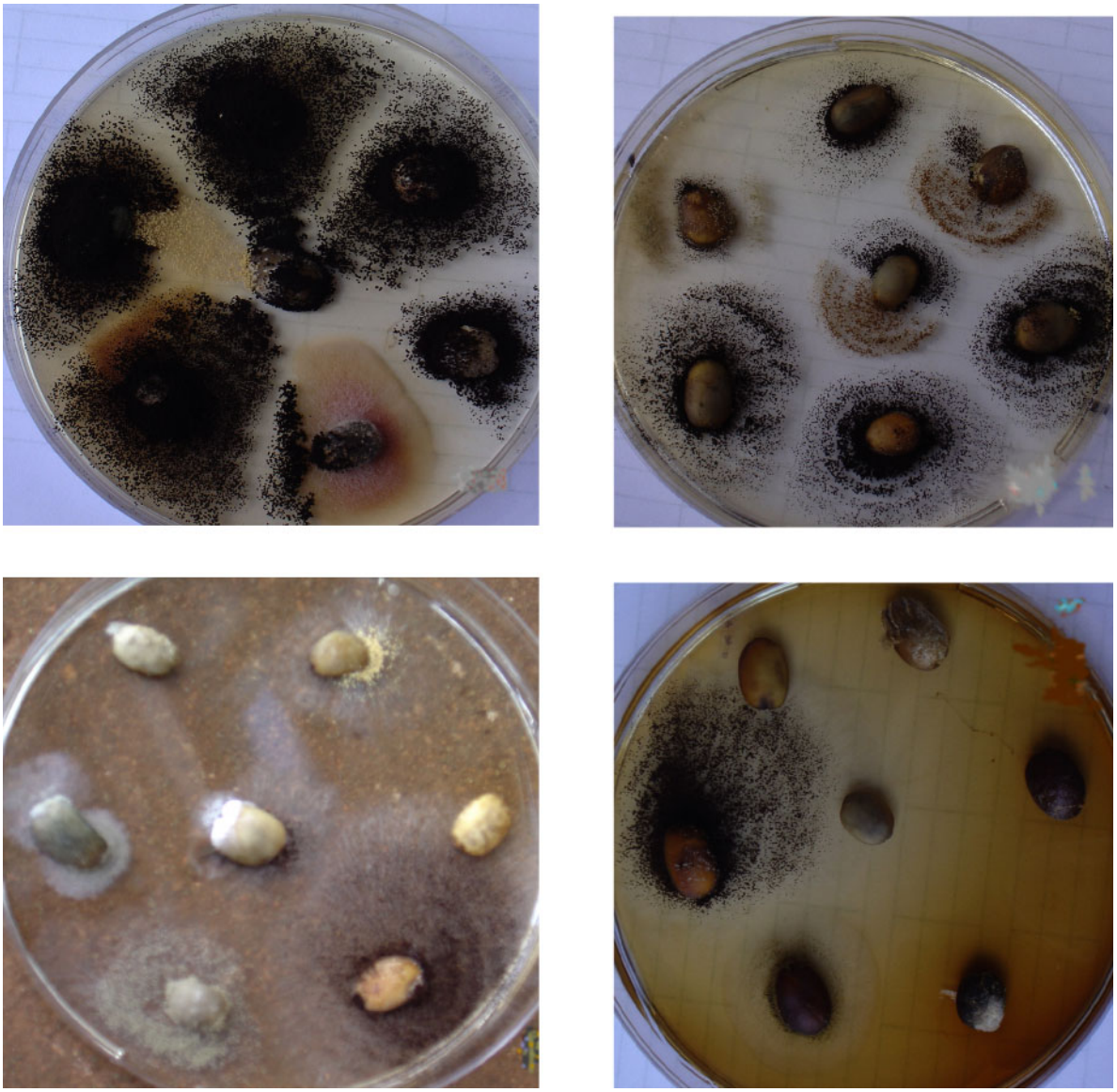

**Figure 2. Different moulds isolated from inside the coffee beans on DG18 after 12 days of during the study.**


## ACKNOWLEDGEMENTS

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# Evaluation of Coffee Blends Made from Semi-dry Processed (Via Peeled Cherry) Robusta and Dry Processed Arabica Coffees Using a Sensory Consumer Test

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## SUMMARY

The objective of this study was to evaluate coffee blends made from semi-dry processed (via peeled cherry) Robusta and dry processed Arabica coffees in the proportions of 10, 20, 30, 40 & 50% Robusta plus a 100% Arabica sample, using an affective sensory test – the acceptance test – with habitual coffee consumers. Fifty-four consumers (university students, lecturers and employees) were consulted, selected as a function of their coffee consumption frequency, requiring a minimum consumption of 1 cup per day. The consumers first evaluated the aroma of the ground coffee samples and subsequently the overall appreciation, aroma and flavour of the respective beverages, using a 9-point hedonic scale (9 = liked extremely; 5 = neither liked nor disliked; 1 = disliked extremely). The results were evaluated by way of distribution histograms, ANOVA, Turkey's test ($p \leq 0.05$) and preference maps. The results showed no significant difference, at the 5% level, in the appreciation of the different blends. More than 62% of the consumers gave scores above 6 (liked slightly) for the overall appreciation of all the sample beverages, the majority of the scores being 7 (liked moderately). Despite the relatively small scale of the test, the results suggest that the use of good quality Robusta coffee in blends with Arabica coffee could occur to a greater or lesser degree depending on the targeted public and the interests of the roasting company, without prejudice to the final beverage quality as expected by the consumer.

## RÉSUMÉ

L'objectif de cette recherche a été d'évaluer des mélanges de Café Arabica et de Café Robusta, ce dernier étant préparé post-cueillette par via cerise déjà épluchée (procédé demi-sec), en différentes proportions de 10, 20, 30, 40, et 50% Robusta et de 100% Arabica. L'évaluation a été faite par test sensoriel affectif – Test d'acceptabilité – avec cinquante cinq consommateurs (étudiants universitaires, professeurs et employeurs) habitués au café (minimum une tasse par jour). Les consommateurs ont d'abord évalué l'arôme du café moulu de chaque échantillon et ensuite l'appréciation générale, l'arôme et la saveur des échantillons en utilisant une échelle hédonique de 9 points (9 = apprécié extrêmement ; 5 = neutre ; 1 = pas du tout apprécié). Les réponses ont été analysées par des graphiques de distribution, ANOVA, test de Turquie et cartes de préférence. Les résultats ont montré qu'il n'y avait pas de différence significative ($p \leq 0,05$) pour l'appréciation générale des échanttillons. Plus de 62% des consommateurs ont donné des notes supérieures à 6 (apprécié légèrement) pour l'appréciation générale de tous les mélanges, avec la majorité des notes étant de 7 (apprécié modérément). Malgré que ce test soit à petite échelle, le résultat suggère que le pourcentage de Café Robusta de qualité utilisé en mélanges avec du Café Arabica dépendra du publique visé et de l'intérêt des industriels, sans compromettre la qualité final de la boisson désirée par les consommateurs.

## INTRODUCTION

In recent years, Brazil, the largest World coffee exporter and third largest consumer, has registered increasing efforts by the coffee producing sector to improve the quality of their product by way of genetic improvement, new planting and harvesting techniques etc.

In this context, the development of a new post-harvest method of preparation, known as the semi-dry process or via peeled cherry, deserves mention. This consists of the existing wet process without the fermentation step. With this process one can obtain coffee with colour and body characteristics similar to those of dry processed coffee but with increased chances of obtaining a high standard beverage since the unripe fruits are removed, processing only the mature ones. In addition to the improved sensory quality of the final product, another advantage of the semi-dry process over dry processing is an economy of energy in the drying process, since the bean plus the parchment is about 40% smaller than the whole fruit. This advantage extends throughout storage since the dry bean plus parchment is smaller than the whole dry cherries. In comparison with the wet process, the semi-dry process, in addition to costing less, has the additional advantage of being less detrimental to the environment, consuming less water and discharging less organic material into the effluent.

Arabica coffee prepared in this way is annually awarded various international prizes. On the other hand, Robusta coffee, despite recent expansions in its participation on both internal and external markets, requires even greater efforts to widen its acceptance, since its use is traditionally limited to the soluble coffee industry and to low quality ground roasted blends, aiming at reducing their price. Currently, Robusta coffee producers, concerned to improve the quality of their product, have started using the post-harvest semi-dry process, obtaining most satisfactory results. The use of this coffee in blends with Arabica coffee has thus become, depending on the proportion, an alternative to reduce costs without decreasing final product quality.

## METHODOLOGY

### Raw materials

Dry processed, type 5 Arabica coffee (A), classified as hard beverage, 10% moisture content, obtained from Coopercitrus, Catanduva, SP, Brazil. Semi-dry processed, type 5/6 Robusta (Conilon) coffee (R), classified as having light/medium Robusta taste, 11.1% moisture content, obtained from Coogabriel, São Gabriel da Palha, ES, Brazil.

### Experimental design to evaluate the blends

**Table 1: Percent (%) mass concentrations of Arabica (A) and Robusta (R) coffees in the blend formulations for ground, roasted coffee.**

| | % mass | | | | | |
|---|---|---|---|---|---|---|
| **A** | 100 | 90 | 80 | 70 | 60 | 50 |
| **R** | 0 | 10 | 20 | 30 | 40 | 50 |

The Arabica and Robusta coffees were roasted separately (laboratory scale PROBAT-WERKE type PRE 1 rotary electric drum roaster) with a weight loss during roasting of close to 15.0%. The Arabica coffee was roasted at 210°C for approximately 10 min. and the Robusta coffee at 210°C for approximately 13 min.

The beverages were prepared using Melitta® type paper filters, using 80g ground coffee per litre filtered water at approximately 98°C, and stored for a maximum of 30 min. in thermos flasks before tasting.

**Sensory analysis**

Acceptance tests (McFie & Thomson, 1994; Meilgaard et al., 1987; Stone & Sidel, 1985) were carried out using 54 consumers selected as a function of their coffee consumption frequency, a minimum consumption of 1 cup per day being accepted. The consumers evaluated the samples as follows: first they evaluated the aroma of the ground, roasted coffee and then they evaluated the respective beverages for overall appreciation, aroma and flavour using a 9-point hedonic scale (9 = liked extremely; 5 = neither liked nor disliked; 1 = disliked extremely). The samples were presented at random in a monadic way, in two sessions, serving in white plastic cups codified with 3 digits, 3 samples being evaluated per session. The consumers sweetened their own samples with either sugar or sweetener, following instructions as to how to sweeten in a standard manner. The results were evaluated by way of distribution histograms, ANOVA, Turkey's test ($p \leq 0.05$) and preference maps.

**RESULTS & DISCUSSION**

The following histograms (Figures 1, 2, 3 and 4) show the distributions of the results obtained for each attribute evaluated.

The score distributions for the aroma of the coffee powders (Figure 1) were similar for all the samples. About 90% of the scores were positive, that is, equal or greater than 6 (liked slightly), samples 20%R and 10%R obtaining the greatest number of scores of 8 (liked a lot) and 9 (liked extremely), 72.22% and 66.67% respectively.

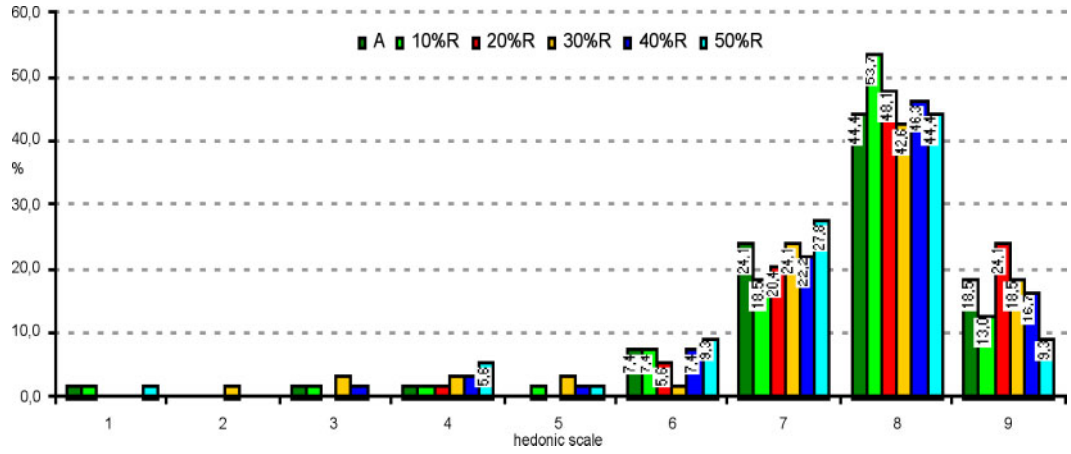


**Figure 1. Histograms of the % distribution of the scores obtained on the hedonic scale (9 = liked extremely; 5 = neither liked nor disliked; 1 = disliked extremely) for Aroma of the ground roasted coffee (powder) samples in the sensory consumer test.**

With respect to overall appreciation (Figure 2), 62% of the scores for all the samples were equal or greater than 6, with the majority concentrated in 7 (liked moderately). The samples with the greatest number of scores equal or greater than 7 were 10%R, 20%R and 40%R, whilst 50%R obtained the greatest percentage of scores indicating consumer disapproval (below 5): 31.48%.

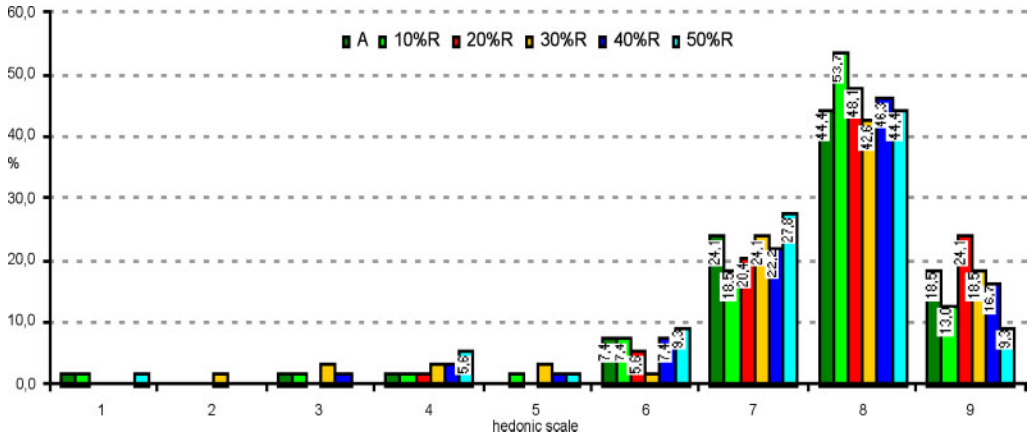


**Figure 2. Histograms of the % distribution of the scores obtained on the hedonic scale (9 = liked extremely; 5 = neither liked nor disliked; 1 = disliked extremely) for Overall Appreciation of beverages prepared from the ground roasted coffee samples using paper filters in the sensory consumer test.**

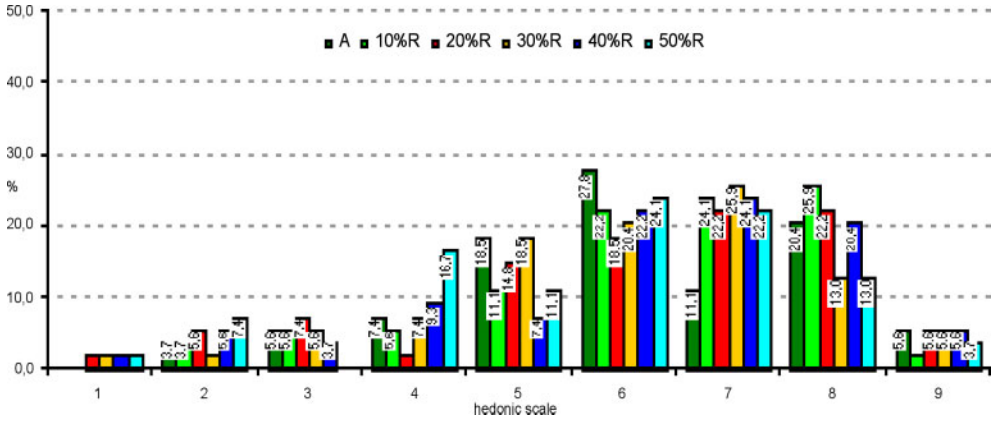


**Figure 3. Histograms of the % distribution of the scores obtained on the hedonic scale (1 = disliked extremely; 5 = neither liked nor disliked; 9 = liked extremely) for the Aroma of beverages prepared from the ground, roasted samples using paper filters in the sensory consumer test.**

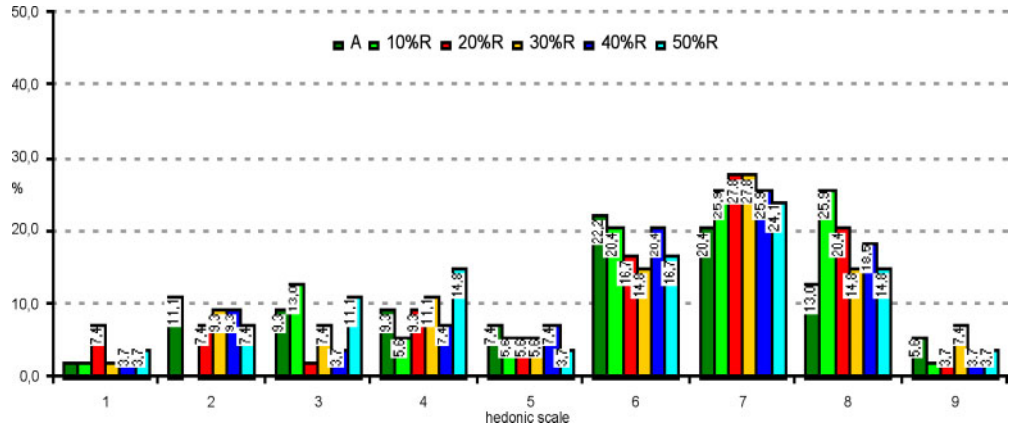


**Figure 4. Histograms of the distribution of the scores obtained on the hedonic scale (9 = liked extremely; 5 = neither liked nor disliked; 1 = disliked extremely) for the Flavour of beverages prepared from the ground, roasted coffee samples using paper filters in the sensory consumer test. a) Arabica 100% and blends with: b) 10% Robusta, c) 20% Robusta, d) 30% Robusta, e) 40% Robusta and f) 50% Robusta).**

For all the samples, 62% of the scores for beverage aroma were equal or greater than 6, samples 10%R and 20%R receiving the largest number of scores 8 and 9 (27.78%). Once again, sample 50%R received the largest number of scores below 5 (25.93%). For beverage flavour (Figure 4), 60% of the scores were equal or greater than 6 and similar to the results for aroma, samples 10%R and 20%R showed the largest number of scores 8 and 9: 27.78 and 24.04% respectively. As always, sample 50%R received the greatest number of scores below 5 (37.03%).

Although the results of the histograms cannot be considered conclusive, a clear tendency for a greater acceptance of samples 10%R and 20%R can be noted. Table 2 shows the results of the ANOVA test with respect to the scores:

**Table 2: Means of the scores obtained in the consumer sensory analysis for the ground, roasted coffee samples, prepared using paper filters.**

| Samples | *Aroma** | *Overall* | *Aroma*** | *Flavour* |
|---|---|---|---|---|
| 100%A | 7.50 [ab] | 5.80 [a] | 6.04 [a] | 5.56 [a] |
| 10%R | 7.44 [ab] | 6.20 [a] | 6.28 [a] | 6.19 [a] |
| 20%R | 7.85 [a] | 5.74 [a] | 6.11 [a] | 5.83 [a] |
| 30%R | 7.35 [ab] | 5.87 [a] | 6.02 [a] | 5.83 [a] |
| 40%R | 7.50 [ab] | 5.93 [a] | 6.13 [a] | 5.85 [a] |
| 50%R | 7.22 [b] | 5.76 [a] | 5.76 [a] | 5.50 [a] |

*- Means with the same letter in the same column indicate no significant difference between the samples at the 5% level. In each column, the highest score is in red and the lowest in blue. *aroma of powder; **aroma of beverage.*

By Turkey's test, the only significant difference was between 20%R and 50%R for the aroma of the ground, roasted coffee powder. For the other samples and attributes, there was no significant difference in appreciation according to the consumers consulted.

The lack of difference in appreciation of the samples may be due to the great variability in scores, a frequent occurrence in this kind of test. In order to confirm and illustrate the ANOVA results, preference maps were constructed for overall appreciation of the beverages and for the aroma and flavour scores (Figure 5). The maps place the samples and the consumers in a geometric space, as also the analysis of the principal components. Samples in the same vicinity showed greater similarities with respect to the attribute in question, and consumers were placed preferentially in the region of the sample to which they gave the highest score (Moskowitz, 1994). The maps shown in Figure 5 explained 56.8% of the variation occurring in the appreciation of the samples and demonstrated that the consumers consulted were distributed in a homogenous way amongst the samples, without the formation of preferential groups, that is, there was no general tendency to greater appreciation of a particular sample or group of samples. The same occurred for the evaluation of beverage aroma and flavour, where the maps explained, respectively, 53.2% and 49.0% of the variation found for these attributes.

## CONCLUSIONS

In general, although the sensory analysis responses represented by the histograms indicated that the blends 10%R and 20%R received the highest percentage of high scores (8 and 9) on the hedonic scale, whilst 50%R received the lowest scores, the statistical analyses of the

results showed no significant differences between the samples according to the consumers consulted.

Thus these results demonstrated the viability of using Robusta coffee in the ground, roasted coffee sector contrary to the belief existing amongst some coffee producers, roasters and experts, who consider it impossible to ally Robusta coffee with a quality beverage. Another good example of the acceptance of Robusta coffee by consumers can be seen in a multi-laboratory study carried out in various European countries (McEWAN, 1998). In this study a washed Robusta coffee (processed by the wet method) was highly appreciated by consumers consulted in Poland and the United Kingdom. It is important to insist on the importance of consumers, since their opinion of what quality is, sometimes conflicts with the concept rooted in the ideas of the producer (Stone et al., 1991). The addition or otherwise of Robusta coffee, and the amount to be added, must be chosen as a function of the targeted public, that is, as a function of what the consumer expects in the way of quality. It is important to emphasise that only if good quality raw coffees are used will there be no loss in sensory quality.

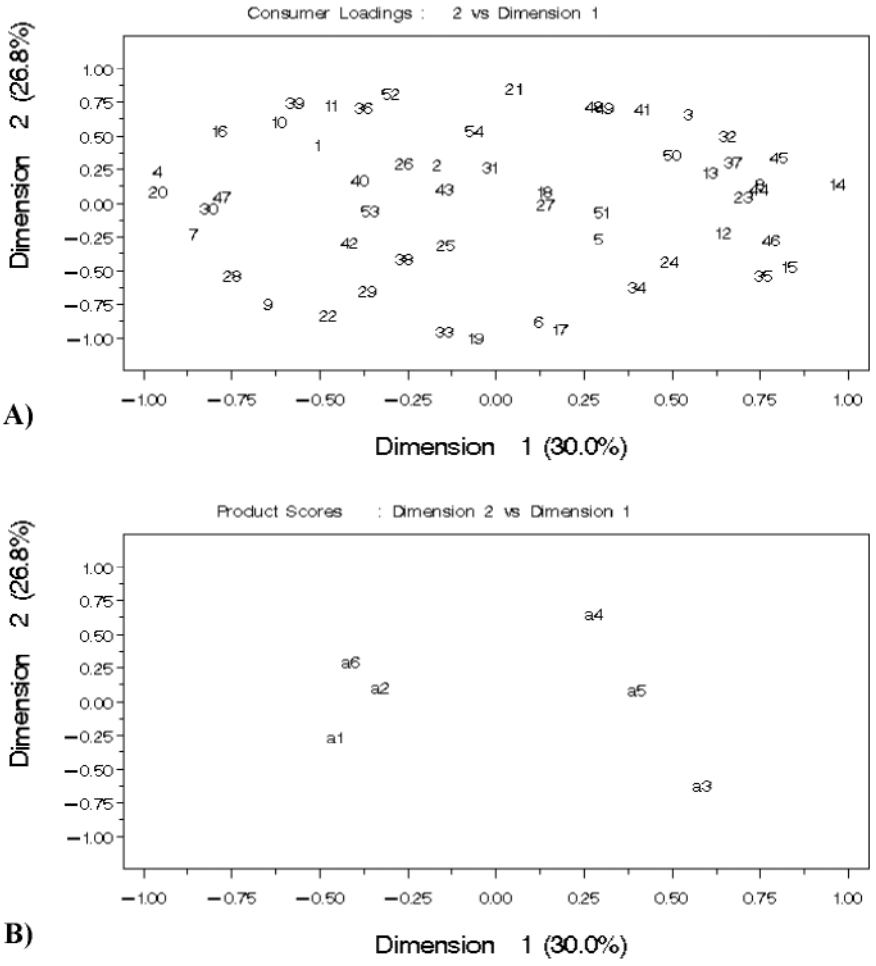


**Figure 5. Internal preference maps for the evaluation of overall appreciation a) in relation to the samples and b) in relation to the consumers. (a1 = 100%A, a2 = 10%R, a3 = 20%R, a4 = 30%R, a5 = 40%R, a6 = 50%R).**

## ACKNOWLEDGMENTS

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# Recent Developments in Coffee Roasting Technology

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## SUMMARY

Roasting of coffee beans leads to considerable changes in microstructure and structure resulting in a remarkable bean volume expansion. The extent of the volume expansion influences mass transfer during storage and has to be considered as important for bean quality changes during storage.

Therefore, a concept for the mechanism of volume expansion was developed. During roasting, structural cell wall polymers such as mannan, arabinogalactan and cellulose are changing to a rubbery state allowing limited changes in volume, but preventing the beans from disintegration. From DMTA data, a state diagram of cell wall polymers and coffee beans was developed and the conditions for an elastic behaviour allowing volume expansion were identified.

Besides structure resistance forces, driving forces are required in order to achieve a volume expansion. During roasting, a considerable quantity of moisture and dry mass is evolved. The quantity of carbon dioxide and moisture evolved during High-Temperature-Short-Time (260°C 170 s) and Low-Temperature-Long-Time (228°C 720 s) roasting process was determined online in the exhaust roasting air using Near-Infrared-Absorption technique (NIR). In both processes a maximal moisture evaporation rate caused by the evaporation of initially present moisture was observed before the roasting process was stopped. Under HTST conditions, carbon dioxide evolution rate increased exponentially at temperatures above 180-200°C, whereas under LTLT conditions, carbon dioxide evolution rate was rather constant above 180-200°C until the end of the roasting process. Finally, the weight ratio of carbon dioxide and moisture in the total roast loss were calculated and a mass balance for the roasting process was developed.

## INTRODUCTION

Roasting presents an important process step. The influence of roasting on coffee bean and cup quality was put in research focus only since several years. In our work, we were focussing on the influence of the roasting process on changes of microstructure and on the mechanisms involved in volume expansion. In the work of Geiger (2004), the influence of structure resistance forces and driving forces on volume expansion was studied in detail.

## STRUCTURE RESISTANCE

Dynamic mechanical thermal analysis DMTA was applied for the identification of transition phenomena of polymeric cell wall compounds. With DMTA texture modifications such as

softening and hardening of materials can be identified and related to the changes of the physical state of materials.

For the analysis coffee beans of different moisture content were cross-sectioned to slices of 3mm thickness and mounted on a DMTA Solids Analyzer RSA II (Rheometrics, Piscataway USA) with plate-plate configuration. Then, the specimen were heated linearly from 30°C to 250°C with a heating rate of 5°C/min.

A DMTA thermogramme of a coffee bean slice is presented in Figure 1. Therein, the storage modulus G' is shown. The fast drop of storage modulus G' between 130 and 170°C represents a transition of polymers from the glassy to the rubbery state, the coffee bean texture is softening. The storage modulus G' increase between 200 and 230°C represents the reversion of the transition, interrupted by a melting phenomenon of a compound between 212 and 217°C. The coffee bean texture is hardening again.

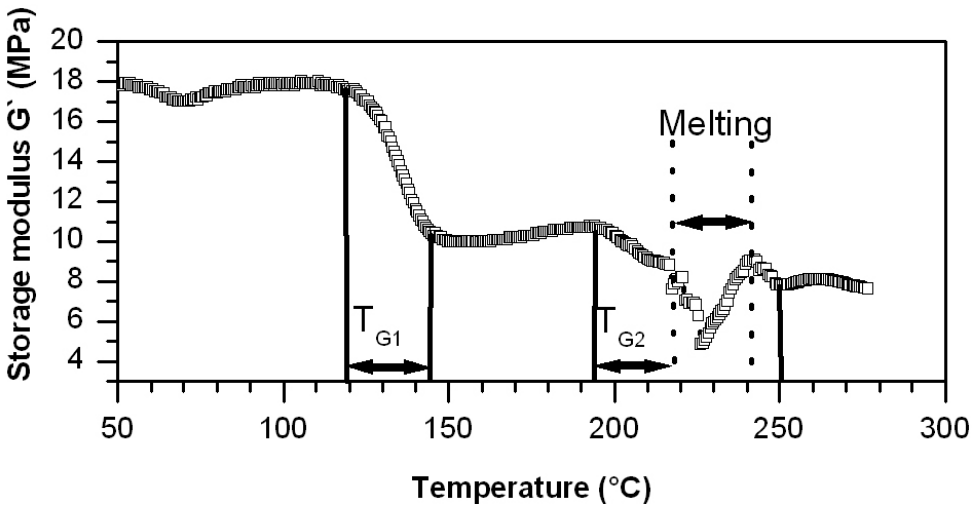


**Figure 1. DMTA-thermogramm of a coffee bean.**

There is a strong relationship between initial moisture content and the temperature range of the glass transition. Because DMTA analysis was performed under non-moisture-controlled conditions, the moisture content as determined immediately at the beginning of the transition was taken into account.

By variation of the initial moisture content a state diagram for coffee beans was developed (Figure 2). Because no sharp and pronounced transition was found in DMTA, the on- and offset of the transition was used to describe the state transition.

In order to identify the compounds involved in the state modifications in green coffee beans an adapted DMTA method was applied to analyse the behaviour of pure amorphous or semi-crystalline polymers prevailing in coffee bean cell walls, namely cellulose, arabinogalactan and mannan. The observed state diagram of coffee beans correlates to cellulose and mannan, whereas arabinogalactan melted completely above 210°C. Probably, the melting phenomenon observed in DMTA analysis was caused by arabinogalactan.

Finally, a mixture of cell wall compounds representing the approximative composition of coffee beans was analysed (Figure 3). In fact, the changes of storage modulus G' were comparable to structured coffee beans in quality, which allows to conclude that the overall state changes in coffee beans are composed by the state changes of individual cell wall polymers.

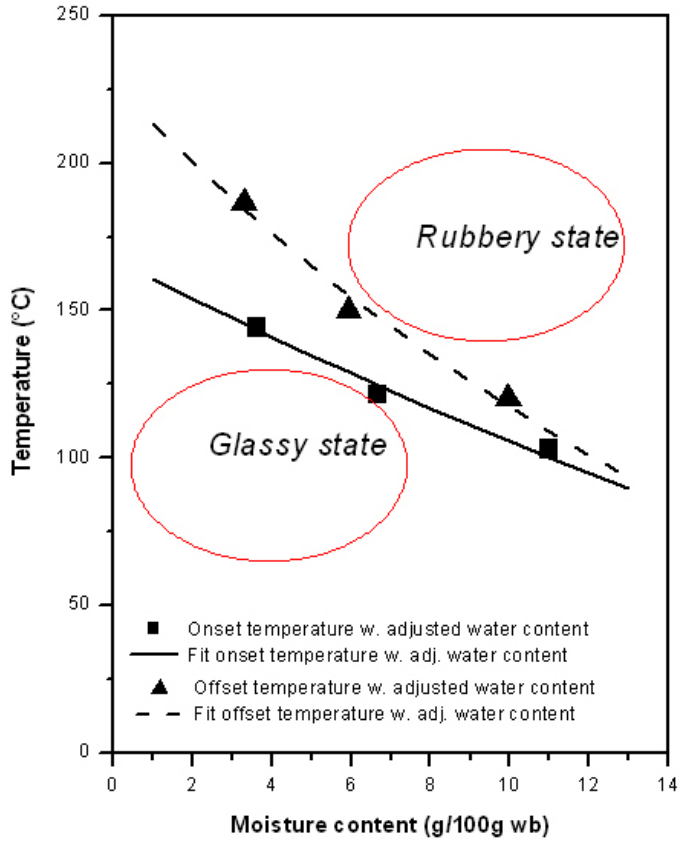


**Figure 2. State diagram for coffee beans.**

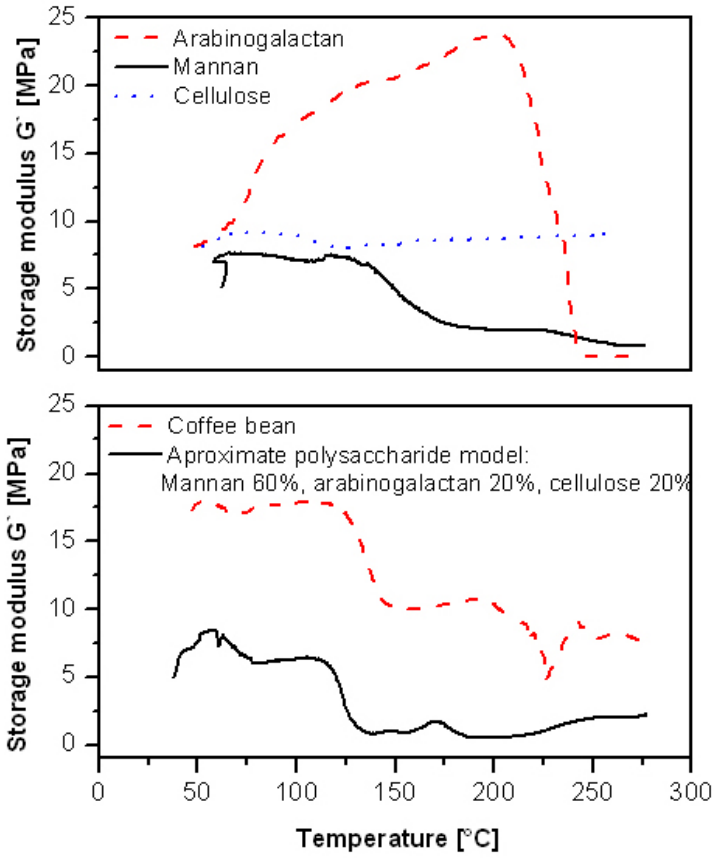


**Figure 3. Thermograms of coffee cell wall polymers, a mixture of cell wall polymers and coffee beans.**

## DRIVING FORCES

During roasting coffee beans are exposed to hot temperatures, where flavor compounds are formed during non-enzymatic browning and pyrolytic reactions. Moisture as present in the raw beans is evaporated, and reaction products from non-enzymatic and pyrolytic reactions evolve.

Geiger et al. (2004) studied driving forces during coffee roasting in detail, where they applied a high-temperature-short-time (HTST) and a low-temperature-long-time (LTLT) process with air temperatures of 260°C for 170 s (HTST) and 228°C for 720 s (LTLT). Roasting was carried out on a laboratory fluidized bed roaster as described in detail by Schenker (2000) and Schenker et al. (2000) to an equal bean color, which corresponded to a L*-value of 22-23. Sample size was 100 g coffee beans, the initial moisture of the beans was 8.3 g/100g wb. In addition coffee beans were roasted which had been pre-dried in an air-dryer at 85 °C for 6 days to a residual moisture content of 1.1 g/100 g wb.

In order to identify driving forces the evolution of gases such as carbon dioxide and moisture vapour were analysed during roasting by linking a LI 6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, Nebraska) to the exhaust air tube of laboratory roasting machine. An aliquote of the air (< 0.2%) was lead from the exhaust air tube to the detector. In order to compensate for potential variations of air velocity over the exhaust tube cross section (r = 0.05 m) the sampling tube (r = 0.0025 m) was positioned in the exhaust tube.

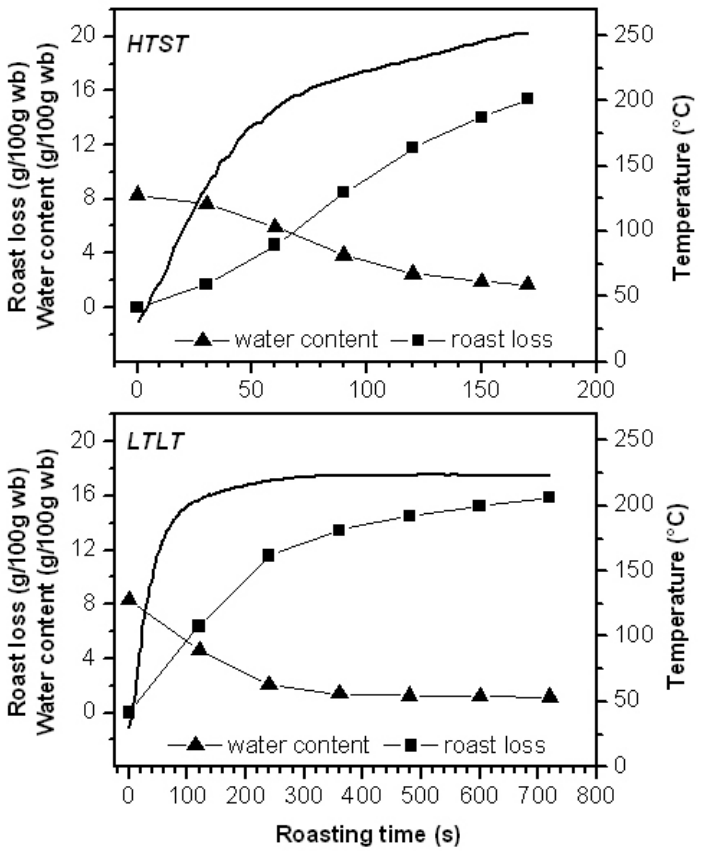


**Figure 4. Bean center temperature, moisture content and roast loss during HTST and LTLT roasting.**

Figure 4 shows the development of coffee bean core temperature, roast loss and moisture content of beans during the isothermal high-temperature-short time (HTST) and low-

temperature-long time (LTLT) roasting process to equal bean color and roast loss. The increase of roast loss and the decrease of moisture content were almost linear at HTST conditions. Only towards the end, the curve for moisture content started to level off. In contrast, exponential curves over the roasting time were observed at LTLT conditions for both roast loss and moisture content. As a consequence, the roasting temperature is the most decisive parameter in controlling overall changes in coffee beans.

The evolution of carbon dioxide is presented in Figure 5. The carbon dioxide concentration increased sharply in the end phase of HTST roasting while the concentration in LTLT roasting stayed much lower and levelled off in the end phase. One could imagine that HTST roasting moved towards pyrolytic conditions at the end of the process. The cumulative value show that more carbon dioxide evolves in the LTLT process than in the HTST process due to the much longer roasting time.

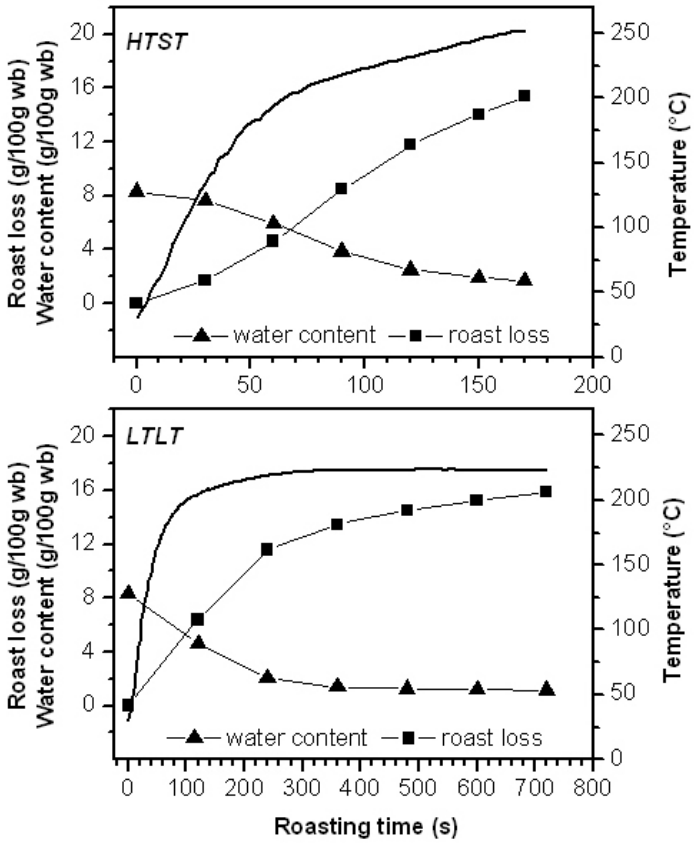


**Figure 5. Evolution rate and cumulative evolution of carbon dioxide during HTST and LTLT roasting.**

The cumulative values in Figure 5 do not reflect the quantity of carbon dioxide formed completely. A substantial part of this quantity is trapped in the coffee beans and is released only during storage. Therefore, cumulative values of evolved carbon dioxide during roasting and storage were combined in Figure 6. Roast loss and storage time, respectively, were chosen as independent variables for the two steps. The lower gas evolution during HTST roasting is more than compensated during storage by a much higher cumulative gas release. Under the given roasting conditions, HTST and LTLT roasting result in an almost equal total formation and evolution of carbon dioxide. Total carbon dioxide formation and evolution seems to be in particular dependent from roast degree and to a reduced extent from roasting temperature.

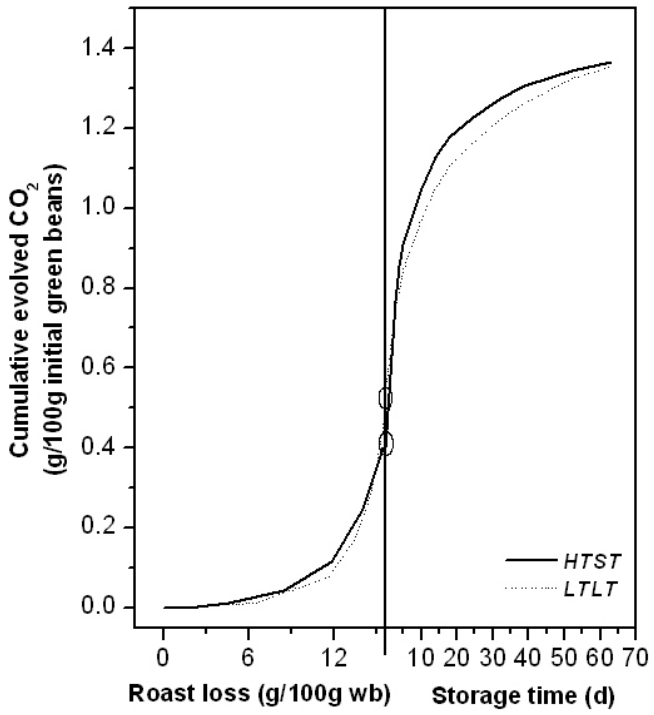


**Figure 6. Cumulative evolution of carbon dioxide during LTLT and HTST roasting and during subsequent storage.**

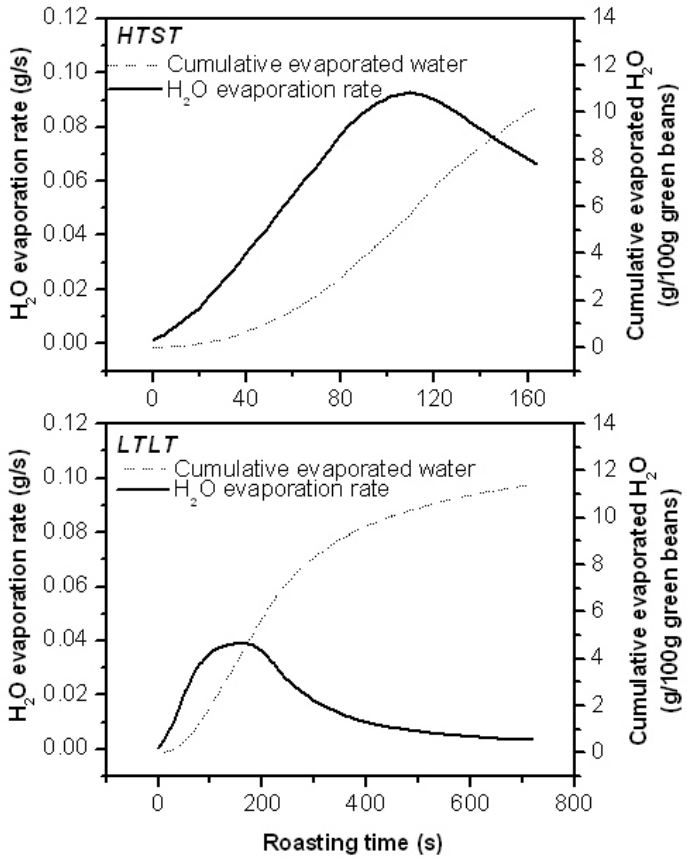


**Figure 7. Moisture evaporation rate and cumulative moisture evaporation during LTLT and HTST roasting.**

Figure 7 presents the averaged data for water evaporation during roasting. Both roasting processes lead to a peak evaporation rate and a subsequent decrease. The HTST and LTLT

process differ primarily in the extent of the peak rate. Moisture evaporation rate depends on the roasting temperature.

The cumulative evaporated quantity of water is composed of water, which evaporates due to dehydration of initial moisture of coffee beans, and of water which is generated by chemical reactions. To evaluate the formation of moisture in chemical reactions, HTST and LTLT roasting trials with pre-dried beans (1.1 g/100 g wb) were carried out. It was assumed that in this case the detected moisture in the exhaust air was exclusively formed by chemical reactions due to a negligible initial water content of the green coffee. In Figure 8 the chemical reaction water and the initial water evaporation rates are shown. The evaporation rate of initial water was calculated from the difference between the evaporation rate of total moisture during roasting of non pre-dried coffee (water content: 8.30 g/100 g wb) and the evaporation rate of total moisture during roasting of pre-dried coffee.

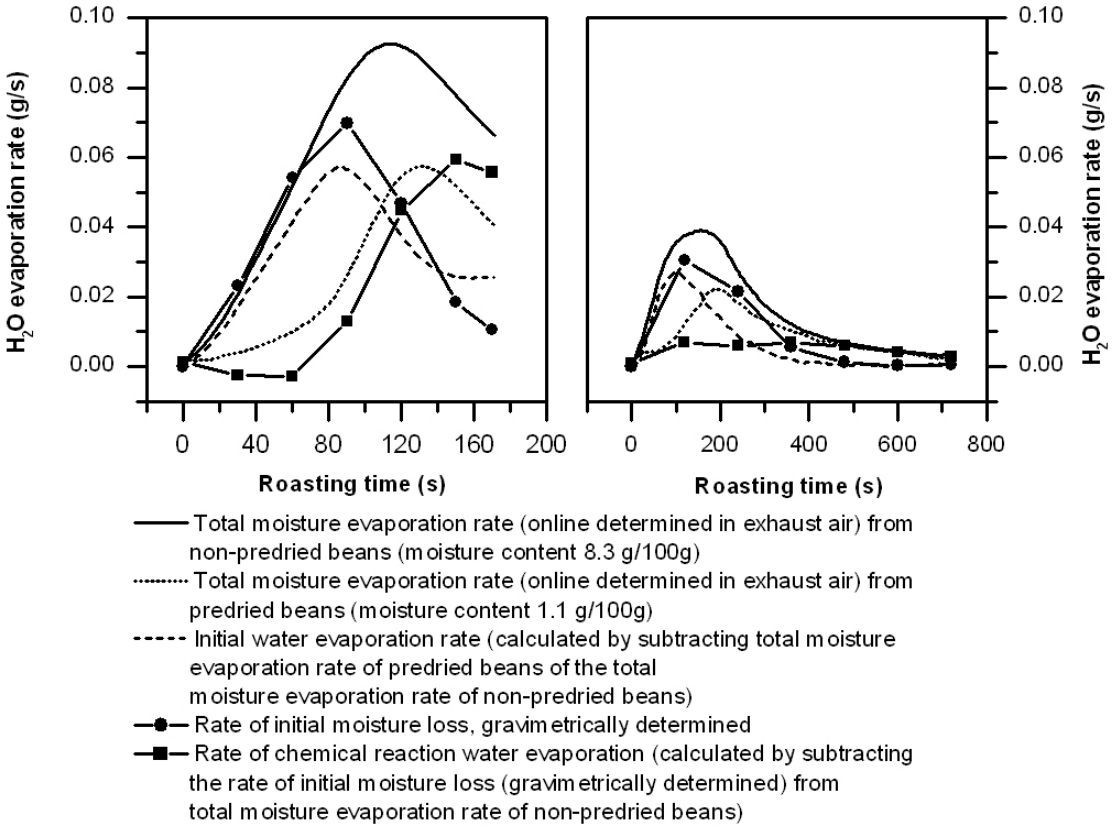


**Figure 8. Evaporation rate of total moisture, initial moisture and chemical reaction water during HTST and LTLT roasting.**

Water evaporation is temperature dependent. The peak rate for HTST roasting was higher than the peak rate under LTLT roasting conditions, whereas more moisture evaporates during LTLT roasting due to the much longer roasting time. In an early roasting phase of both roasting processes only initial water has been released from the beans. With increasing roasting time, in particular to see for LTLT roasting, total moisture evaporation mainly consists of evaporating chemical reaction water. Initial water evaporation became almost negligible after approximately 300 s and the formation and evaporation of chemical reaction water were equaled. The peak rate of total moisture evaporation is due to the overlapping effect of initial and chemical reaction water. The decrease of chemical reaction water evaporation could be the consequence of running out of the substrate or formed chemical reaction water will be used in the cells for other chemical reactions and will therefore not

occur in the exhaust air. It seems in Figure 8 that chemical reaction water was already produced from the beginning of the process. In fact, most of the residual moisture of 1.1 g/100g wb in the pre-dried green beans had to be removed first before the effective chemical reaction water could be detected. The evaporated moisture from the pre-dried coffee, considered as chemical reaction water, amounted for 41% (HTST) and 36% (LTLT) respectively of total moisture evaporation from the non pre-dried green coffee.

In Table 1, a mass balance over roasting and storage is presented on the basis of evolved carbon dioxide, evaporated water and losses of solids in the form of silver chaffs. An estimation of the loss of gases during the cooling step was done by linear extension of the cumulative carbon dioxide and moisture evaporation values for another 20 s after the end of roasting. A standard deviation of the evolved carbon dioxide and moisture was calculated from the deviation of air velocity and gas concentration measurements.

**Table 1. Mass balance for the roasting and storage of coffee beans and comparison with overall roast loss.**

| Step | Weight [g] | |
|---|---|---|
| | HTST | LTLT |
| Initial beans | | |
| Total solids | 91.7 | 91.7 |
| Moisture | 8.3 ± 0.2 | 8.3 ± 0.2 |
| Sum | 100.0 | 100.0 |
| Roasting loss (on-line) | | |
| Carbon dioxide | 0.4 ± 0.0 | 0.5 ± 0.3 |
| Total water | 10.2 ± 1.2 | 11.4 ± 1.4 |
| Silver chaff | 1.0 | 1.0 |
| Sum | 11.6 ± 1.2 | 12.9 ± 1.7 |
| Cooling loss (calculated) | | |
| Carbon dioxide | 0.1 ± 0.0 | 0.0 ± 0.0 |
| Water | 1.6 ± 0.2 | 0.1 ± 0.0 |
| Sum | 1.7 ± 0.2 | 0.1 ± 0.0 |
| Total weight loss (on-line/calculated) | 13.3 ± 1.4 | 13.0 ± 1.7 |
| Roast loss (gravimetric) | 15.38 ± 0.05 | 15.86 ± 0.02 |
| Storage loss | | |
| Carbon dioxide | 0.99 ± 0.02 | 0.83 ± 0.01 |

Taking the variations of the data in Table 1 into account, approximately 93% (LTLT) and 96% (HTST) of the gravimetrically determined roast loss could be explained by measuring gas evolution and determination of silver chaff. It must be pointed out that the balance values in Table 1 still do not account for all of the roast loss. Material from abrasion, e.g. tippings, and evolving gases other than $CO_2$ and water vapor also contribute to the total roast loss. The remaining difference for both roasting processes could be explained by inaccuracies of air velocity and gas concentration measurements as well as by inaccuracies of the raw material.

In Figure 9 the volume expansion rate is plotted in combination with total gas evolution rate and moisture evaporation rate. It becomes obvious that the evaporation of moisture presents a very strong driving force for volume expansion. It is worth mentioning, that the highest volume expansion rate is reached even before moisture evaporation has peaked. Carbon dioxide evolution is of minor importance only, but in case of HTST roasting contributes to an

increase in volume expansion rate at the end of the roasting process because it restrengthens the total gas evolution.

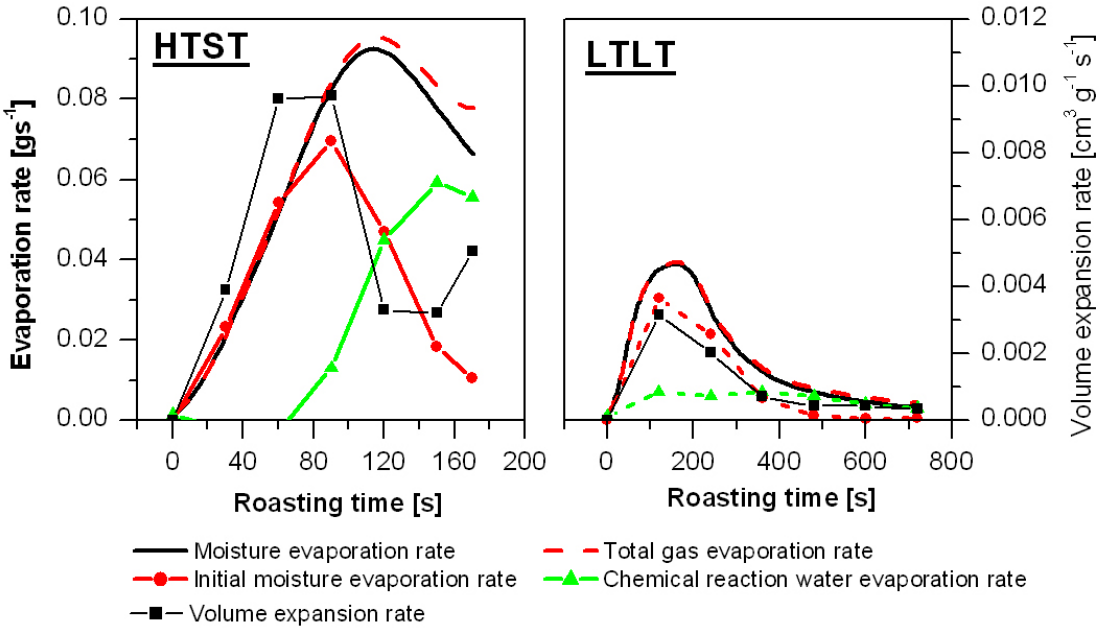


**Figure 9. Characterisation of the driving forces during HTST and LTLT roasting.**

## PROCESS OPTIMIZATION

The observed volume expansion is of major concern for storage stability of roasted coffee beans. The degradation of cellular and subcellular microstructure represents a sink for mass transfer from the environment. Furthermore, the increase in porous volume and porosity accelerates mass transfer. Both are a consequence of volume expansion and allow oxidation to start. Furthermore, carbon dioxide released from the coffee beans after roasting may lead to an aroma stripping. Therefore, it will be crucial to achieve an optimal balance of volume expansion allowing optimal extractability and minimal mass transfer during storage.

As a next step in the investigations on coffee roasting processes we will investigate the influence of evolution of carbon dioxide and water vapor during roasting on the development of coffee been structure, the aroma retention and the aroma release during roasting and storage.

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# Quality and Efficiency with New Emission Controls on the Basis of Proforte

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## SUMMARY

Emission control has come a long way. The obligations are much different throughout the world. What is appropriate in one country may not be necessary or not enough in another. But the tendency to stricter pollution control does not seem to be negotiable. Yet, not everything what is technically possible may be viable in practice.

The presentation highlights an insight into a possible future state-of-the-art emission control by means of an FRTO (flameless regenerative thermal oxidation).

Advantages and disadvantages are discussed on the basis of preliminary operating experience. Quality and efficiency is compared side by side to existing technologies. Results of in depth studies are presented versus feasibility from different perspectives.

## RÉSUMÉ

Le contrôle d´émission a eu une longue voie de développement. Les régulations par ce contrôle sont très différentes dans tout le monde. Les uns sont appropriés dans un pays et dans un autre pays certaines régulations ne sont pas nécessaires ou sont même pas suffisamment. Mais la tendance envers un contrôle de pollution plus stricte n´est pas négociable. Cependant, tout qui est possible du point de vue technique n´est pas réalisable dans la pratique.

La présentation montre un aperçu sur un contrôle d´émission moderne par le biais d´un FRTO (flameless regenerative thermal oxidation = oxydation sans flammes, régénératrice et thermique)

Les avantages et désavantages sont discutés sur la base des premières expériences avec les opérations. La qualité et l´efficacité sont comparées avec les technologies existantes. Les résultats des études profondes sont présentés et comparés avec la faisabilité des perspectives différentes.

## GENERALITIES

The combustion of harmful and/or bothering gaseous organic impurities contained in the exhaust gases, which mainly are carbon hydrides, or odoriphores can convert them to generally harmless substances. The exothermal reaction of a complete combustion makes carbon hydride convert to pure $CO_2$ and water vapour. The carbon monoxide, which might be contained in the process gas, is oxidated to $CO_2$ along with the carbon monoxide produced in the intermediate step.

The oxidative exhaust gas cleaning units available on the market are operated with or without catalyzers or use of heat.

## HISTORICAL BACKGROUND

The regenerative thermal afterburning has been on the market for more than 20 years now and a variety of designs is offered by several companies. The oxidation bed is heated and its temperature is kept at a constant level by a support heating with separate burner.

Proforte does not need any burner. This process involves a flameless combustion. The process has been developed and patented by ADTEC AB from Göteborg / Sweden. In 1994, ADTEC AB was taken over by MEGTEC SYSTEMS. The original designation of "Combu-Changer" (ADTEC AB) was changed into "VOCSIDIZER" by MEGTEC. More than 750 units have been supplied in the past 16 years.

Searching for alternative solutions towards the exhaust gas cleaning, our R&D Department has taken a close look to the VOCSIDIZER. The flameless combustion producing no additional combustion products and the excellent thermal efficiency degree of as much as 98 % have been convincing.

In 1998, the first industrial trials with the VOCSIDIZER were effected. Although the results have been promising, this process has not been pursued further at that moment. In 2001, R&D brought the conversation round to the regenerative thermal oxidation again.

In May 2001, the first trials with "FRTO" were started. Almost 200 trials with different settings and after several modifications have been run in Emmerich.

The long-term behaviour has been tested at a Belgian company. For that purpose, the trial unit operated by R&D has been sold to this company. The "FRTO" unit has been connected to drum roaster R 1500/1 without recirculation of the roasting gas. From September 2003 up to now (July 2004) a great many discoveries have been gained.

The headword "Proforte" is composed of the first three letters of the name of our company and the Latin word "forte" which means "strong, powerful". Besides, "forte" involves the first letters of the most important features:

"f" for flameless
"o" for oxidation
"r" for regenerative
"t" for thermal

Some essential process parameters in conjunction with hardware modifications have been adjusted during research and development work. The regenerative thermal exhaust gas cleaning equipment available on the market - which are usually designated "RTO" - does not work for the described cases.

## PROFORTE - DISTINCTIVE FEATURES

Our exhaust gas cleaning plant "Proforte" has some distinctive features - particularly in comparison with the catalytic exhaust gas cleaning, - which are described hereinafter.

1) low energy costs
2) low service costs
3) no separate burner necessary
4) high cleaning efficiency

5) robust construction
6) many years of experience
7) low installation costs
8) frequency-controlled fan drive
9) no production of NOx caused by flame oxidation
10) requires much space
11) high self-weight
12) visible steam trail

**FUNCTIONAL DESCRIPTION OF PROFORTE**

The heart of the plant is a highly effectively insulated equipment bin filled with ceramic bodies.

The ceramic pillow serves as oxidation bed and heat exchanger. In the middle of the bed there is a temperature of approx. 900°C to 1000°C. The hydrocarbon and carbon monoxide compounds contained in the exhaust gas are oxidated when flowing through this bed.

The flow direction of the exhaust gases is reversed cyclically in order to ensure that the oxidation zone remains in the middle of the bed. This also allows a maximum use of energy inside the regenerative heat exchanger.

In the first halve of the ceramic bed, the exhaust gas to be cleaned is heated up and in the second halve - which means after the oxidation - the exhaust gas releases the energy again. For that purpose, the valve positions are preset at certain cyclic intervals by a programmed control.

The exhaust peak with non-purified air generated during the reverse interval is routed into a buffer tank. After the end of the reverse procedure and until the beginning of the next reverse, the non-purified air is emptied out of the buffer tank via a duct and cleaned air is filled into the buffer tank. The non-cleaned air buffered during the reverse procedure is pressed into the Proforte unit by means of a process air fan along with the crude gas to be cleaned.

Because of the optimum use of heat, there only is a slight increase of the outlet temperature of the cleaned exhaust air.

During a production stop, e.g. at night and/or weekends, the Proforte unit is turned to "stand-by mode". If a certain temperature in the middle of the oxidation bed is not reached anymore, the temperature of the unit will automatically be kept by adding small amounts of fuel gas with a highly throttled air quantity.

At the beginning of production, the exhaust gas loaded with contaminants is routed through the ceramic bed. The gaseous organic compounds contained in the exhaust gas are oxidated to water and carbon monoxide and the carbon monoxide oxidates to carbon dioxide. Additional combustion gas is only added if required, i.e. if the concentration of contaminants is too small.

The energy released during this combustion serves to maintain the temperature inside the ceramic bed. If the concentration of contaminants is high, the Proforte system can even work auto-thermal, this means without additional heat supply (fuel gas).

The processing procedures are not influenced by the Proforte system.

**REMARKS**

All mentioned figures are reference values which merely are intended to serve as a comparison. Any guarantee, warranty or similar cannot be given for these and similar values.

The calculation is based on the following energy prices:

- natural gas 38 Eurocent / 10 kWh
- electrical energy 10 Eurocent / kWh

The heat energy released during exhaust gas cleaning by the oxidation of the contaminants have not been taken into consideration for the energetic examination.

The costs applicable to the depreciation of the catalyzer elements depend on the prices applicable for precious metal on the world market, the yearly interest rate, the general cost development and, in the end, from the service life of the catalyzers. The costs mentioned here for the depreciation of catalyzers refer to

- price situation of 1st July 2004
- a yearly interest rate of 6 %
- a yearly cost increase of 3.5 % and
- a service life of 24,000 hours or 6 years (twice as much as the guaranteed period)

Three variants have been taken into consideration for the production conditions. These variants correspond to some extent to a one-shift operation, two-shift operation and three-shift operation. As a consequence, a daily production time of 7 hours, 15 hours and 24 hours has been taken as basis with 5 working days per week.

The specific energy values calculated for the roasting and the roasting exhaust gas cleaning refer to the so-called nominal processing capacity, i.e.

- a certain green coffee moisture (11.5%)
- a certain green coffee temperature (approx. 20°C)
- a certain batch weight
- a certain roasting time
- a certain roasting degree (120 scale grad. - Colorette 3)
- a certain quantity of pre-cooling water and
- a certain roasting process.

**OPERATING COSTS**

The thermal process (Proforte) and the catalytic process (catalyzer 420°C) are compared with each other.

With a production time of 7 hours per day, the energy costs for catalytic exhaust gas cleaning related to room temperature are four times as high as those incurred for Proforte. With an operation time of 24 hours, the energy costs are seven times higher.

The difference related to the cleaning of coffee roasting exhaust gas is less high due to the higher outlet temperature of 180°C. With a production time of 7 hours per day, the catalytic afterburning has a factor of 2.7, which is increased to factor 4.5 for a 24-hour-operation.

In addition, the costs incurred for depreciation of the catalyzers have to be borne in mind for the catalytic exhaust gas cleaning. These costs currently are as follows per 1000 $m^3n$:

- 24 hours/day approx. 0.70 Euro
- 15 hours/day approx. 0.90 Euro
- 7 hours/day approx. 1.90 Euro

**POSSIBILITIES OF USING PROFORTE**

The regenerative thermal afterburning system Proforte can in principle be used for the cleaning of exhaust gases from coffee, cocoa and cereal roasting plants.

Proforte can also be used for the cleaning of odorous exhaust air from diffuse sources provided that sufficient oxygen is existing and that the concentration is limited, which, as experience has proven, is no problem for the diffuse sources related to the processing of coffee.

In most cases, these exhaust air currents consist of exhaust air enriched with flavours. On the one hand, this involves little concentrations but on the other hand it also results in very intensive odours. The afterburning unit has the almost exclusive task of reducing the odours.

The combined cleaning of coffee roasting exhaust gases and the cleaning of exhaust gas from diffuse sources may be an ideal and reasonable solution. Depending on how the exhaust air of the sources is composed, particularly in respect to the oxygen portion, this exhaust air quantity can substitute the fresh air required for the roasting process either partially or completely.

In different words, this means that when cleaning coffee roasting exhaust gases, the Proforte system offers possibilities allowing for the additional treatment of odorous, oxygenous exhaust air.

**INFLUENCE OF PROFORTE ON THE QUALITY OF THE FINAL PRODUCT**

In the course of the many individual trials, special attention has been paid to the influence of Proforte on the quality of the roasted coffee. For that purpose, numerous coffee samples have been tested and compared in both a sensory and analytical way, i.e. by means of a fluid chromatography (HPLC). The results clearly show that Proforte does not have an influence on the quality of the final roasted product.

The roasted process is not influenced in any way by the Proforte system installed downstream. A special regulation ensures that the Proforte process is automatically adjusted to the roasting process. The roasting process is independent from the roasting exhaust gas cleaning.

**INVESTMENT COSTS / AMORTIZATION**

Generally speaking, the investment costs incurred for the Proforte system are higher than those for the catalytic exhaust gas cleaning. As sales prices have not yet been fixed we can only set out ideas on the amortization, aroused by lower energy costs, which point the way ahead.

From a mere economical point of view, the Proforte roasting exhaust gas cleaning only represents an alternative to the catalytic roasting exhaust gas cleaning for roasting plants with high processing capacity (size). The following data provides the calculated amortization periods in an exemplary fashion for roasters types 1500 and 4000 taking into consideration different production times.

| roaster type | production time h/day | amortization period with puff-cleaner years |
|---|---|---|
| R1500/1 R-LT | 7 | 50 |
| R1500/1 R-LT | 15 | 11 |
| R1500/1 R-LT | 24 | 6 |
| RZ 4000/1 | 7 | 9.5 |
| RZ 4000/1 | 15 | 4 |
| RZ 4000/1 | 24 | 2.5 |

Nevertheless, the Proforte system is an interesting solution for roasting plants working with high roasting supply air temperatures and an inevitably low roasting air volume such as the drum roaster.

The running costs incurred for roaster R 1500/1 of simple design without recirculation and equipped with our Proforte system are more reasonable than those of a roaster with recirculation and catalytic roasting exhaust gas cleaning.

When cleaning the exhaust gas of diffuse sources for example, the Proforte system is more reasonable than the catalytic roasting exhaust gas cleaning due to the lower inlet temperature which generally is equal to the room temperature.

As for an exhaust gas volume to be cleaned of 3000 $m^3n/h$ and a Proforte of construction size 4.5, the Proforte system is amortized within a period of 1.2 to 3.8 years depending on the production time.

| exhaust gas volume $m^3n/h$ | production load h/day | amortization period years with puff-cleaner |
|---|---|---|
| 3000 | 7 | 3.8 |
| 3000 | 15 | 1.9 |
| 3000 | 24 | 1.2 |

**CONCLUSION**

Given its high thermal efficiency, its high and constant cleaning degree and the safe design requiring little service, the Proforte system is an afterburning unit which can be used in many processes.

The low nitrogen monoxide and nitrogen dioxide concentrations in the cleaned exhaust air are remarkable for the cleaning of roasting exhaust gases. The NOx concentrations in the crude gas are very low, particularly when compared to the catalytic exhaust gas cleaning.

The Proforte system with puff-cleaner ensures clean gas values, which are far below the limit values mentioned in the recently passed TA Luft. Even the mentioned nitrogen oxide concentrations to be aspired as target value can be adhered to when using our Proforte system with puff-cleaner.

Moreover, the Proforte system can remain under the limit value for the total carbon concentration in the crude gas indicated in the VDI directive 3892 so that one can do without a proof of single substances in the exhaust gas such as acetic acid and formaldehyde.

# Semi-Fluidizing Batch Roaster for Roasting of Coffee

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## INTRODUCTION

Roasting of coffee beans represents a standard technology. It's only recently that roasting technology of coffee was put in the focus of research groups. Schenker (2000) identified technological key factors for optimal quality coffee roasting:

- Process duration 6-15 min at moderate temperatures for optimal flavor generation
- Reduction of energy supply at the end of the roasting process in order to maintain a high concentration of flavor compounds in the bean
- Optimal Air-To-Bean Ratio (ABR) for optimal roasting atmosphere
- High speed cooling in order to immediately stop the roasting process

Derived from further research work a uniform treatment at low mechanical energy input and increasing supplementation of hot gases with fresh air towards the end of the process is of importance as well.

## ROASTER DESIGN

In order to meet these stated requirements a semi-fluidizing batch roaster CR was developed (Figure 1). The roasting system CR was constructed with a roasting zone and a cooling zone, both based on a round chamber. Hot air is distributed into the product via bended arms and through vertical nozzles (Figure 2). The nozzles dive into the product and end up 20 mm above the solid bottom. Thus after leaving the nozzle, the air is rejected, creating a vertical air stream through the product. This effect generates a semi-fluidizing bed. As the arms are rotating, a smooth radial mixing action is brought to the product. In order to cool the roasted coffee as fast as possible the water cooling system HWP was developed. Thereby, water is injected in the hot air flow at 180 bars creating an aerosol of a huge surface to bind energy fast.

## RESULTS

Derived from industrial trials it was found that roasting in the semi-fluidizing batch roaster CR resulted in an even, uniform and repeatable treatment, whereas a wide variation of process conditions are applicable (Figure 3). High Water Pressure Cooling HWP in the roasting zone allowed to immediately stop the roasting process and to reduce the product temperature below 150°C within 1 min. Due to the cooling step in the roasting zone, the starting conditions for different batches were indeed comparable, allowing a high process repeatability. Thanks to these positive results the semi-fluidizing roasting system CR was commercialized, and industrial systems are successfully in duty since more than three years (Figure 4).

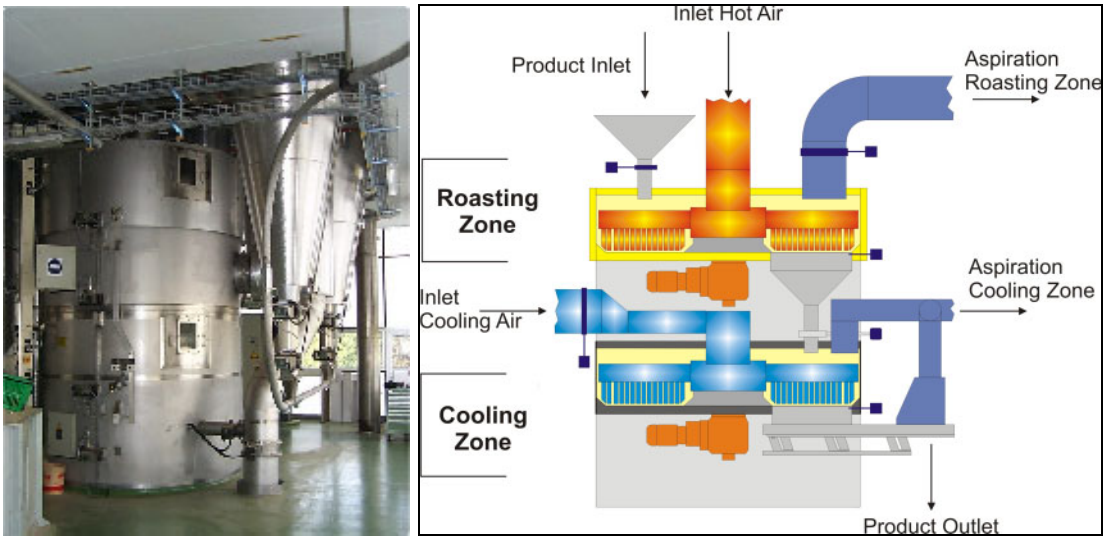


**Figure 1. View and scheme of the semi-fluidizing batch roaster.**

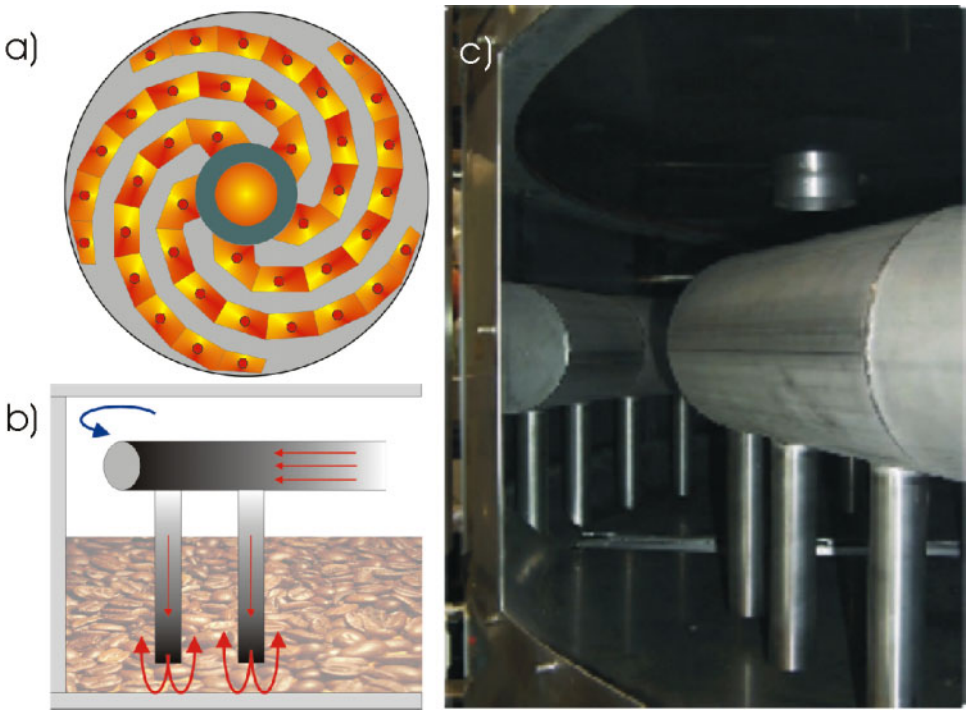


**Figure 2. a.) Top view cross-section on roaster: The hot air enters the system in the center and is evenly distributed over the product area with rotating bended arms. The air nozzles attached to the arms are coextensively arranged and supply the air equally into the coffee bean bed. b.) and c.) Side view of roasting chamber: The hot or cold air distributed by the rotating arms enters the coffee bean bed through vertical air nozzles. At the nozzle outlet, the air flow is reversed, leading to a partial fluidisation of the bed.**

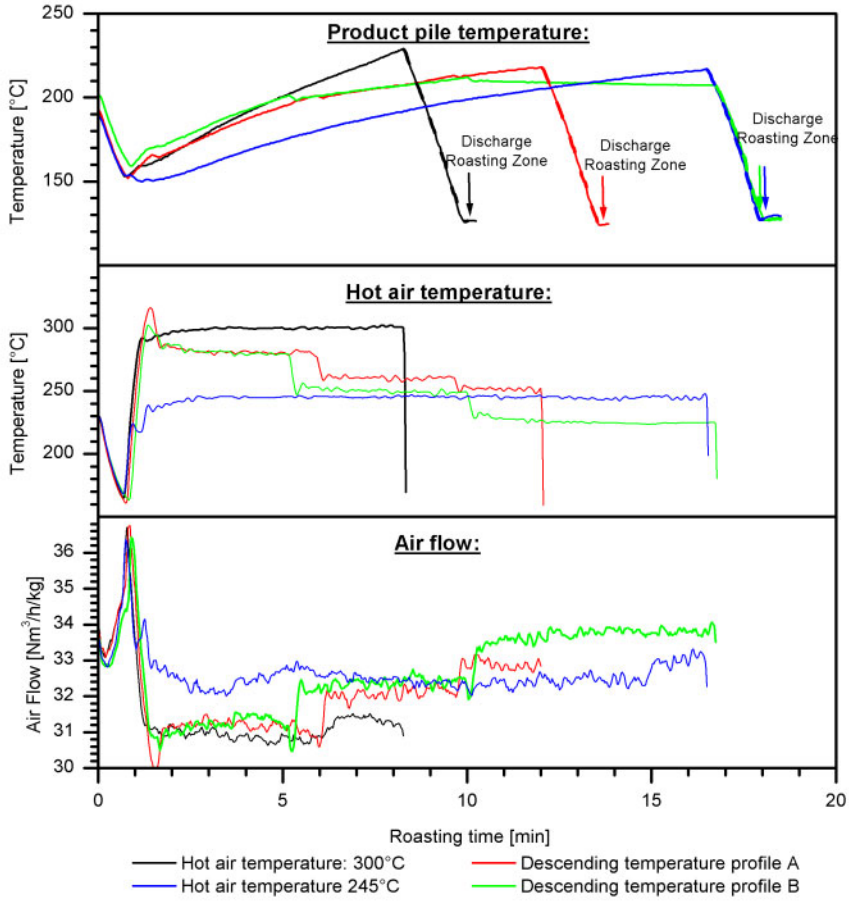


**Figure 3. Presentation of different roasting procedures in the CR roasting system. All beans were roasted to a roasting degree lightness L*-value 19-20 (CIELAB).**

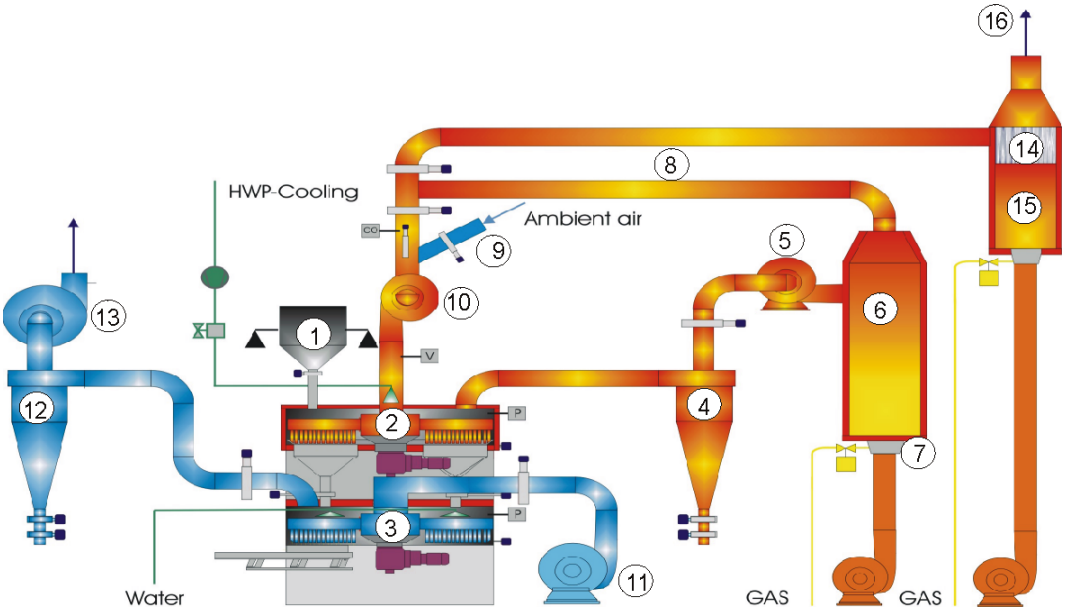


**Figure 4. Layout of an industrial roasting system CR with different roasting and cooling areas incl. heat recovery and off-air cleaning (1: weighing hopper; 2: roasting area, 3: cooling area; 4: roasting cylone; 5:roasting fan; 6: burner chamber; 7: industrial burner; 8: hot air pipework; 9: ambient air inlet; 10: main fan; 11: cooling fan; 12: cooling cyclone; 13: aspiration fan; 14: catalyst; 15: burner chamber; 16: cleaned off-air).**

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# Temperature Field during Roasting and Cooling of Coffee Beans

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## SUMMARY

Aim of this work is to describe the heat and mass transfer phenomena and to record and calculate the changing parameters as the transient temperature distribution inside coffee beans during roasting and cooling. Therefore a number of changing material properties that influence the temperature profile inside a coffee bean have been determined by on-line monitoring these processes, analysing differently roasted and quenched coffee beans and coffee batches, as well as analysing coffee beans and coffee grounds by thermal analyses. Thus a basis for a mathematical model is provided with which, apart from temperature, various changing material properties during variably performed roasting and cooling processes can be estimated, aiding an optimisation of these processes with regard to evenly roasted coffee beans.

## INTRODUCTION

The technical challenge of roasting and quenching coffee is to control the temperatures and stop the roasting process when the aroma has fully developed, the colour of the coffee is homogeneous throughout the whole bean and its physico-chemical properties have changed in a way that subsequent grinding and extraction processes are facilitated.

The difficulties in controlling the process arise from the changes in nearly all the parameters related to the process: the temperatures, the material properties, and the geometry of the bean. When heat energy is supplied to the surface of a coffee bean the temperature of the bean increases as a result of heat conduction into the porous material. A front of endothermic evaporation of the bean moisture starts moving towards the centre, followed by exothermic roast reactions at higher temperatures that yield mainly water vapour and carbon dioxide. Consequences are a pressure build-up leading to mass transfer outwards, volume increase and "cracking" of the bean. Moreover the kinetics of the temperature rise, the compo-sition, and the caloric and physical properties of the beans dramatically change. During quenching the cooling rate, and amongst others the composition and the fracture behaviour of the coffee beans are highly influenced by the cooling medium, its temperature and mass flow. During water cooling the intense evaporation at the surface leads to high heat transfer rates and a mass transfer of water and water vapour inwards, thus altering the abovementioned material properties. Several interacting heat and mass transfer phenomena are illustrated in Figure 1.

## EXPERIMENTAL

The experimental setup for roasting and quenching single coffee beans allows for roasting the coffee bean in still or circulating roast gas at temperatures up to 280°C inside a view cell. The changes in mass and volume are recorded using an analytical balance and a CCD video system respectively. The temperatures inside the bean and at its surface are recorded

simultaneously. The temperatures at different positions inside the bean are measured by thermocouples of 0.25 mm in diameter that do not influence the roasting process. The surface temperature is measured contactless by an infrared pyrometer. The off-line determination of further thermophysical properties such as caloric data, density and water content is enabled by terminating the roasting process at specific times. This quenching is performed by cooling with air or by water spray of different conditions.

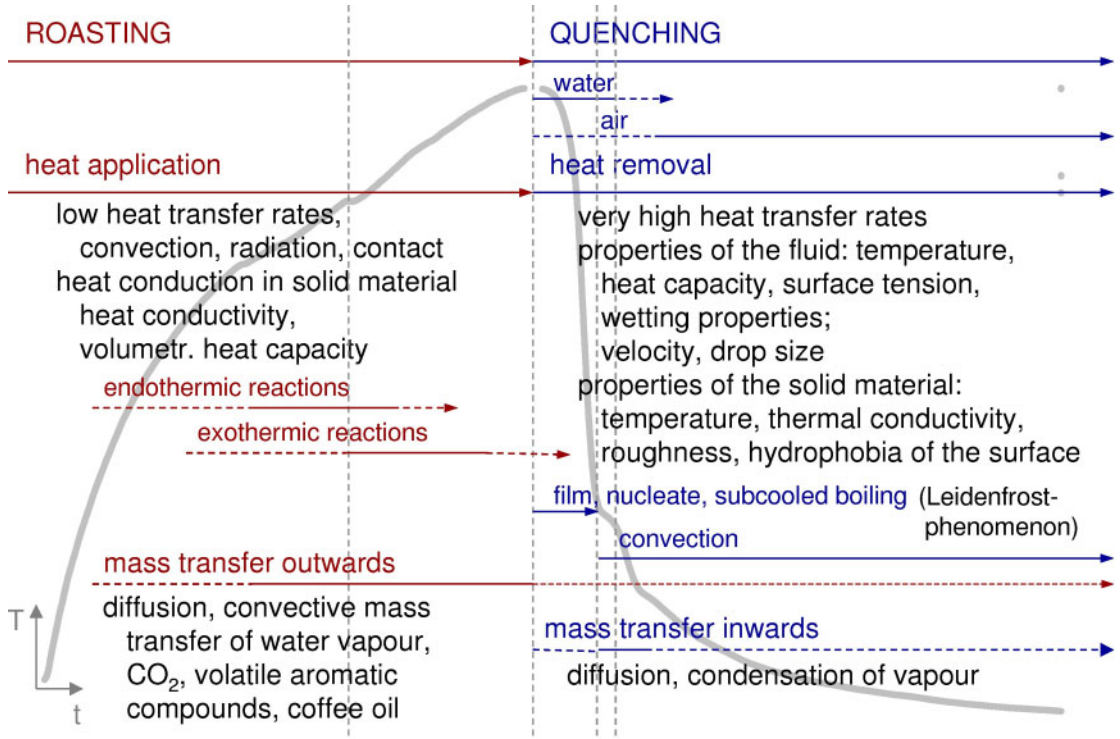


**Figure 1. Heat and mass transfer phenomena and qualitative temperature profile inside a coffee bean.**

The principal temperature profile and the changes in thermo-physical material properties during roasting are depicted in Figure 2. The mass loss of about 15% is composed of evaporated water and dry matter loss due to roast reactions. The volume is increased by at maximum 90% resulting in a porous body with half the apparent density of a green coffee bean. Swelling and drying lead to a strong decrease in heat conductivity.

Starting at ambient conditions the temperatures increase until a nearly constant temperature has been reached. A small temperature drop occurs at about 220°C because of an endothermic flash. Some water remaining in the bean evaporates spontaneously once the cracking of the bean entails a pressure release.

Latent reaction enthalpies during roasting have been analysed applying both conventional plate DSC of green, ground coffee at a heating rate of 10 K/min and reaction calorimetry of whole coffee beans at 1 K/min (Figure 3). The latter method reveals that the matrix of unbroken coffee beans considerably influences the kinetics of roast reactions due to mass transfer resistances. The quantity of the endothermic peak and the DSC results are alike, 184 kJ/kg and about 150 kJ/kg respectively, whereas the peak maximum shifts from about 90°C to 140°C when analysing coffee beans instead of coffee grounds. The transition to exothermic roast reactions also occurs at higher temperatures. However, the quantification of the latter, being in the same order of magnitude as the endothermic reaction enthalpy, strongly

depends on the abortion temperature of the roasting process, since during thermal analyses the roast reactions gradually pass into highly exothermic reactions of decomposition.

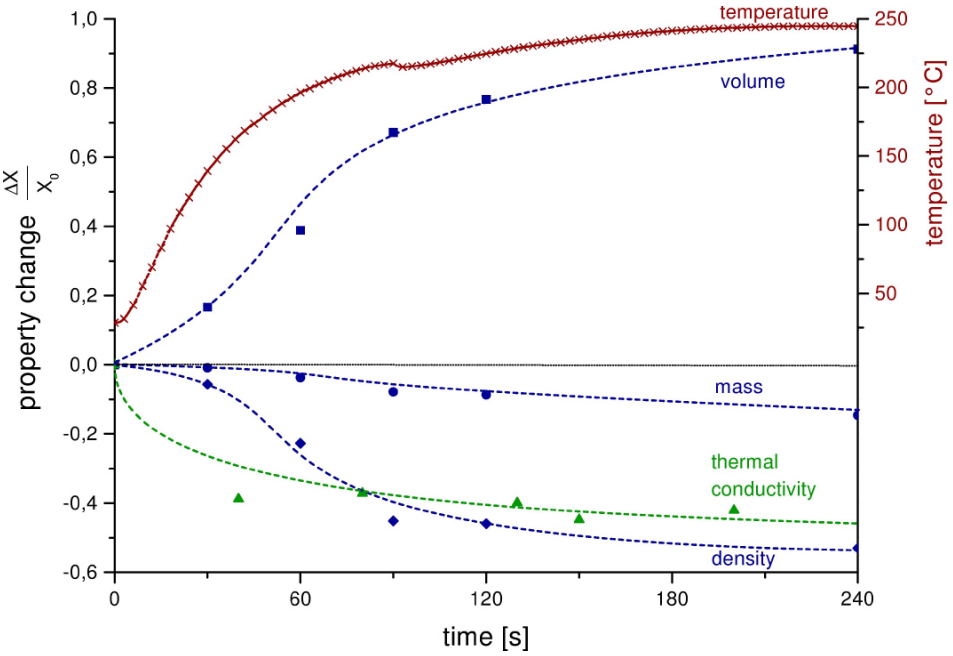


**Figure 2. Changes in material properties during roasting.**

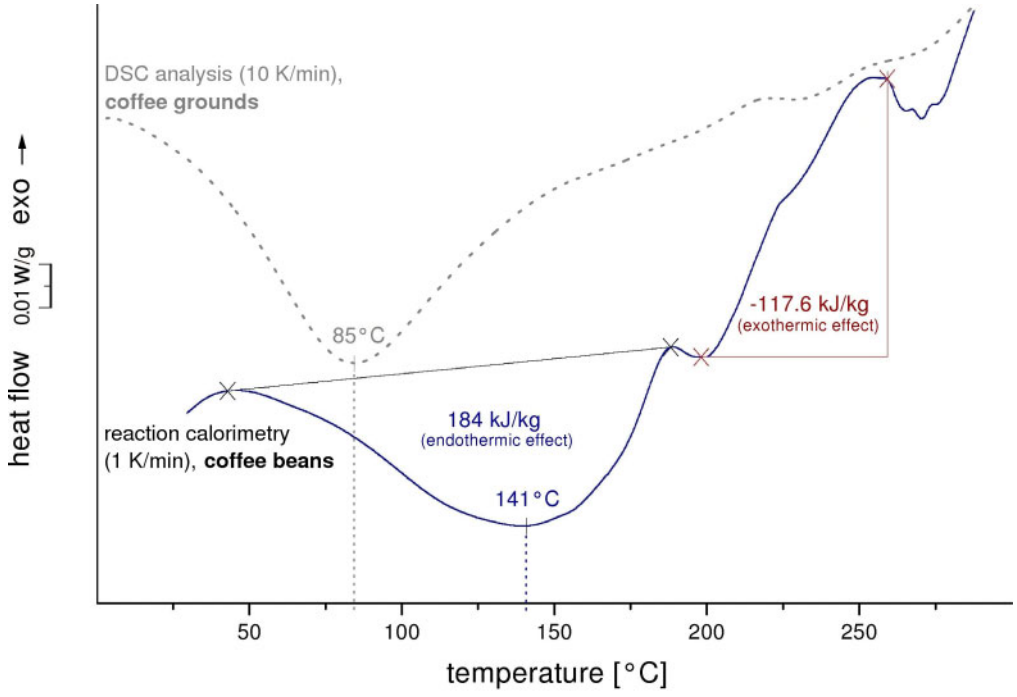


**Figure 3. Thermal analyses of coffee grounds and whole coffee beans.**

To terminate the roasting process the coffee is cooled down rapidly by water quenching. Intense evaporation and condensation effects at the surface and in the pores account for rapid cooling, a decreased cooling rate below 100°C, and a higher humidity of water quenched coffee. Both re-moistening and equalisation of the humidity are enhanced considerably by raising the temperature of the applied water. Whereas quenching with air results in a uniformly decreasing cooling rate (Figure 4) and a final humidity of about 1-2%.

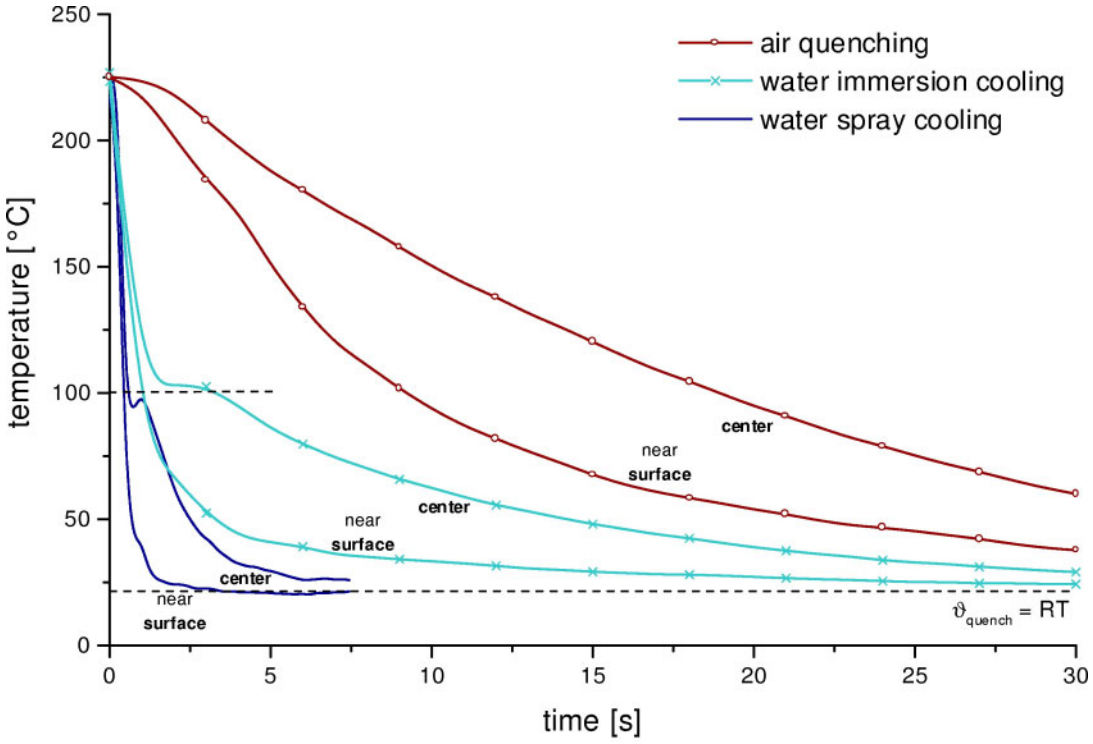


**Figure 4. Temperature profiles during quenching.**

## MATHEMATICAL MODELLING

The transient temperature distribution inside the coffee bean is calculated by solving the partial differential equation of heat conduction including temperature dependent material properties and superimposed heat sources and sinks for the equivalent sphere:

$$\rho c_{p(T)} \cdot \frac{\partial T}{\partial t} = \operatorname{div}\left[\lambda_{(T)} \operatorname{grad} T\right] + \dot{W}_{(T,x,t)}, \qquad \text{boundary condition } -\lambda_{(T)} \cdot \left.\frac{\partial T}{\partial x}\right|_{x=R} = \alpha \cdot (T_R - T_\infty).$$

The heat transfer coefficient $\alpha$ in the afore mentioned boundary condition is depicted as dimensionless Nusselt number against the relevant range of roast gas velocities displayed as Reynolds number in Figure 5. In industrial roasting processes the coffee batch is usually fluidised so that the bulk properties, characterised by the Archimedes number, play a decisive role with regard to the heat transfer. In a fluidised bed the heat transfer, passing a slight maximum, cannot be enhanced significantly with increasing the relative velocity. With increasing roasting degree both Archimedes and Nusselt numbers as well as heat conductivity and heat transfer coefficient are on the decrease.

Boundary conditions and the gained material property data are implemented in a physically founded model specifying the heat transfer phenomena. A coupled model of mass transfer accounts for evaporation, condensation, and diffusion of the bean moisture. Herein the diffusion resistance is related to the changing porosity of the bean. Along with the analysed adsorption behaviour it provides for the calculation of the mass flow rate of vapour and the latent energy density. It even includes the endothermic flash due to sudden evaporation. Results of thermal analyses are incorporated in order to consider exothermic effects.

The model allows for approximately calculating the transient temperature distribution inside a coffee bean during roasting and cooling (Figure 6). It is valid for both a single bean in still or circulating gas and coffee beans in a fluidised coffee batch in industrial processing.

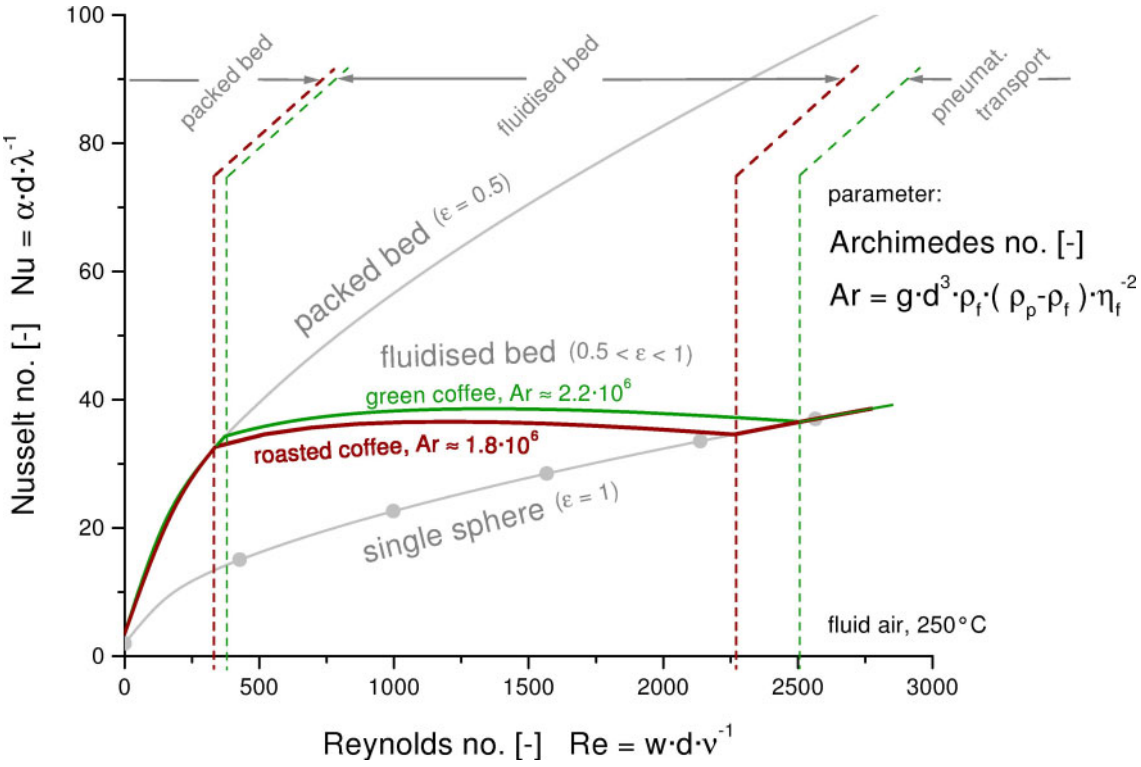


**Figure 5. Heat transfer to sphere equivalent coffee beans.**

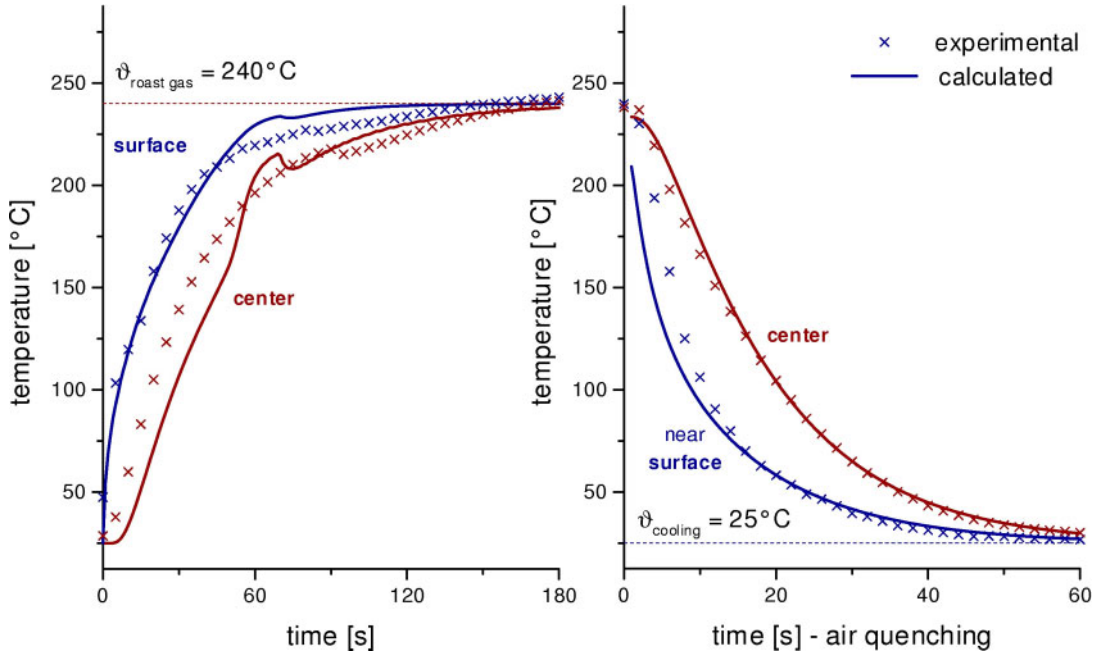


**Figure 6. Comparison of measured and calculated temperature profiles.**

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# Influence of Coffee Processing, Water Activity, Temperature and pH on Mould Growth and Ochratoxin A Production in Coffee

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## SUMMARY

Ochratoxin A (OTA) is the main mycotoxic contamination known in coffee. OTA occurrence in coffee beans can be due to both environmental and processing conditions. The incidence of tree post-harvest process (traditional wet processing, ecological wet processing and dry natural processing) on mould grown and OTA production by strains of *A. ochraceus* and *A. niger* was evaluated and the effects of water activity (*Aw)*, temperature and pH and their interactions on (a) growth and ochratoxin A production on a green coffee extract medium, (b) ochratoxin A production on raw coffee by *A. ochraceus* were investigated. The method used to produce green coffee does not have any effect on *Aspergillus ochraceus* and *A. niger* growth ability and toxigenesis. However, temperature and especially $A_w$ are very important factors that need to be controlled during drying and storage

## INTRODUCTION

Three OTA producing moulds have mainly been found in association with coffee: *A. niger, A. carbonarius* and *A. ochraceus* (Frank, 2001; Joosten et al., 2001). OTA production can occur throughout post-harvest processing, drying, storage and transport of coffee. Understanding the conditions required for OTA production could help in reducing contamination incidence and in controlling the processes. Moisture, temperature and substrate type are the main factors that play a major role in the development of OTA producing strains and in toxigenesis (Le-Bars, 1988; Northolt et al., 1979). Work was carried out on the conditions for *A. ochraceus* and *A. carbonarius* growth and OTA production on green coffee, notably the effect of water activity at 25°C (Taniwaki et al., 2001), and on OTA production by *A. ochraceus* depending on $A_w$, alternating 2 storage temperatures (Cabrera et al., 2001). *A. ochraceus* growth and OTA production during coffee storage was reported for $A_w$ values between 0.94 and 0.80 (Frank, 1999). However, the influence of the incubation temperature and of $A_w$ in the medium and in coffee beans has not been totally explored. It also remains to be checked whether the type of post-harvest processing method used affects the capacity of green coffee to be reinfected by toxigenic strains during storage, thereby enabling toxigenesis, by affecting the intrinsic quality of the beans (linked to their physico-chemical properties, particularly the pH).

## MATERIAL AND METHODS

### Strains

We used strains isolated from parchment coffee: *A. ochraceus* (MULC 44639) and *A. niger* (MULC 44640).

**Tested coffee processing**

The batches of green coffee (*Coffea arabica)* came from the Coatepec region, Mexico (2001-2002 harvest). Coffee was processed by the "dry", "wet" and "ecological wet" methods

**Culture media**

CMEA: 30 g of ground green coffee and 15 g of agar/l. $A_w$ (0.70, 0.75, 0.80, 0.85, 0.90, 0.95) was adjusted with glycerol. The pH values were obtained with 0.1 M sodium citrate buffer solutions.

**Inoculation, incubation and growth rate measurement**

Spores obtained on PDA medium were deposited in the centre of a Petri dish containing CMEA. Each day, the diameter of the colonies was measured and the growth rate (mm/day) was calculated.

**OTA production by *A. ochraceus* on green coffee beans for different $A_w$**

Batches of 120 g of sterile green coffee were placed in desiccators under ERH values of 75, 80, 85, 90, 95 and 99%. Once equilibrium was reached, the desiccators were placed at 28°C.

**OTA quantification**

From the culture medium, the modified Bragulat, *et al* method (Bragulat et al., 2001) was used. From coffee beans, the coffee was dried at 70°C, frozen at –80°C then ground. Samples were analysed according to Nakajima (Nakajima et al., 1990).

**Statistical processing of the results**

A D-optimal experimental design was generated so as to study the main effects and double interactions. It was analysed by the Henderson 3 model of the general linear model (Searle, 1987).

**RESULTS AND DISCUSSION**

**Study on a model medium**

***Growth rate***

Analysis of variance showed that of the 4 factors studied (processing method, temperature, $A_w$ and pH), only the temperature, $A_w$ and temperature-$A_w$ interaction were significant for both strains. The minimum and optimum $A_w$ values were 0.80 and 0.95 respectively for *A. ochraceus* and 0.85 and 0.99 respectively for *A. niger*.

***OTA production***

$A_w$ conditions for OTA production by *A. ochraceus* were more limited than those necessary for its development: the $A_w$ limit was 0.90 and the optimum 0.95. This was not the case for temperature, as toxin production began right from 10°C with a compatible $A_w$. The main factor limiting OTA production was $A_w$. For *A. niger*, $A_w$ and temperature were also

important factors, but OTA production (7 μg $kg^{-1}$) was only obtained for an $A_w$ of 0.99 at 35°C.

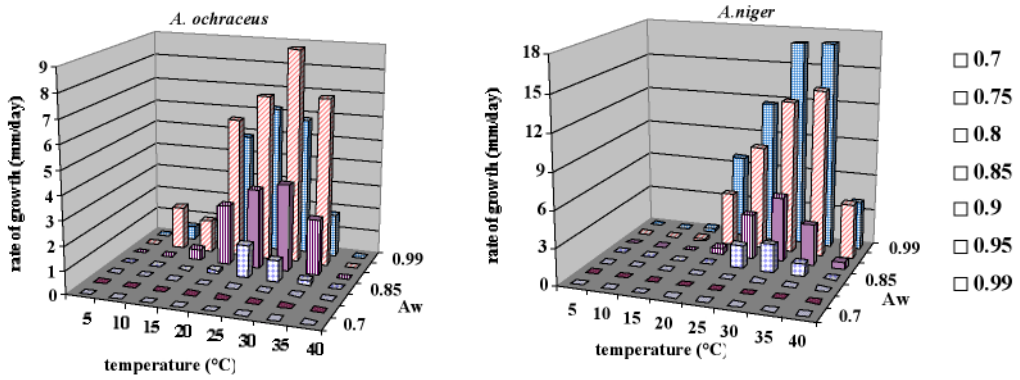


**Figure 1. Interaction of $A_w$ and temperature on growth rate (mm $day^{-1}$) of *A. ochraceus* and *A. niger* on CMEA.**

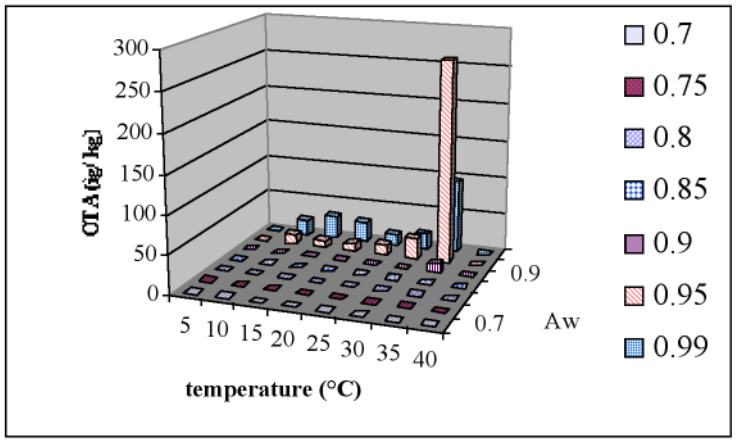


**Figure 2. Interaction of $A_w$ and temperature on ochratoxin A production by *A. ochraceus* on CMEA.**

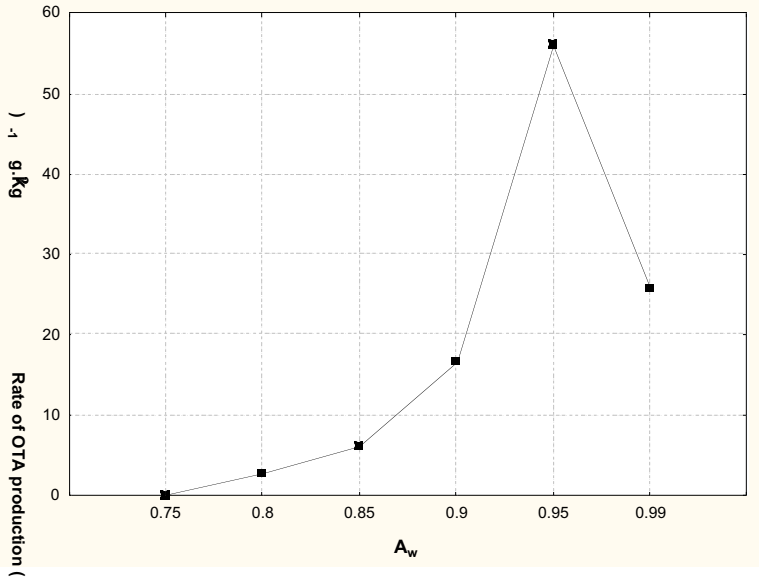


**Figure 3. Rate of Ochratoxin A production by *A. ochraceus* on green coffee under various $A_w$.**

**Study on coffee**

After 25 days, growth was only visible with the naked eye from an $A_w$ of 0.80. Optimum production was obtained at 0.95, confirming the results obtained on CMEA.

## CONCLUSION

The method used to produce green coffee does not have any effect on *Aspergillus ochraceus* and *A. niger* growth ability and toxigenesis. However, temperature and especially $A_w$ are very important factors that need to be controlled during drying and storage.

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# Evaluation of the Hygienic Quality of Green and Roasted Coffee Beans and Ground Roasted Coffee from Different Brazilian States

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## SUMMARY

This study aimed to evaluate the hygienic level of commercial coffee brands from various Brazilian states. Raw materials were evaluated for external and internal insect infestation and roasted samples (espresso and ground coffee) for heavy (sand, soil, metallic particles etc.) and light (larvae, insects, mites, insect fragments, rodent hairs etc.) filth. Raw samples showed infestation by crop related insects (*Hypothenemus hampei*) and storage insects (mite, psocoptera, lepidoptera and other coleoptera), indicating raw material external filth control as one critical point and damaged beans (borer exit/entry holes; bean tunnelling) as another, since the latter can house whole insects, fragments and cast-off skins, leading to contaminated final products. 92.2% of the roasted bean samples (205 espresso and 375 roast, ground) showed from 1 to > 300 insect fragments and only 7.8% conformed to current Brazilian legislation (absence of filth, parasites and larvae). It is impossible to grow, harvest and process raw products free of naturally occurring defects, as required by the current legal limit, but the implementation of good manufacturing and storage practices throughout the coffee production chain could reduce the levels. A survey of hygiene levels is useful in quality control to identify critical points. The recognition and significance of this insect material could lead to a review of current legislation, establishing reasonable limits for natural defects presenting no health hazards, in foods for human use.

## INTRODUCTION

Microscopic analysis can identify external and internal insect infestation and light filth, determining food product hygiene levels and indicating critical contamination points during processes where control practices should be emphasised. Such information could also be used to establish maximum limits for filth.

Extraneous material consists of undesirable elements associated with inadequate conditions or practices during cultivation, harvest, transport and storage of the raw material, handling, industrialisation, storage and distribution of the final product, including filth, decomposed matter and mixtures of materials such as sand, soil, glass, metallic fragments and other extraneous material, excluding bacteria (Barbieri, 1994; Ziobro, 2000).

Filth is classified as heavy or light according to the density of its elements as compared to the floatation medium used for separation (Dent, 1978). Heavy filth, such as soil, clumps of earth, stones, metallic fragments and other filth resulting from inadequate cleaning (US FOOD …., 1998), is separated from the coffee by sedimentation. Light filth (lypophyllic) is separated by floatation in an oil/water mixture, e.g. acarids, larvae, insects and their fragments, rodent hairs, barbules etc. (Ziobro, 2000).

The main coffee pest is the fruit borer (*Hypothenemus hampei* (Ferrari)), attacking fruits at any maturation stage. After mating, adult females perforate fruits and construct galleries, disaggregating skin particles. They take about 7h to reach the parchment, and once inside the seed, widen the gallery before laying their eggs. After hatching, the larvae feed on small particles from this chamber until reaching adulthood. Thus larvae, pupae, excrement and larval and pupal skin changes can be found in the final product. Another common pest causing bean damage is the woodworm *Araecerus fasciculatus* (DeGeer), attacking dried cherries, parchment coffee and hulled coffee. Coffee aged for more than 3 years shows greater infestation than new coffee (Gallo et al., 1988; US Food …, 1998).

The item "Microscopic Characteristics" of the Brazilian technical norm for ground, roasted coffee requires the absence of filth, parasites and larvae (Brasil, 1978), being incompatible with reality, since it is impossible to produce, harvest and process foods free of natural defects by conventional open field agriculture. Such requirements can lead to increased use of pest-controlling chemicals, exposing the consumer to toxic residues instead of aesthetically unacceptable but innocuous pests (FDA, 1995). Nevertheless the implementation of good manufacturing and storage practices throughout the coffee production chain would reduce contamination levels.

Aiming to protect the consumer against this practice and inadequate manufacturing conditions of hygiene, the USA and Canada have established directives to evaluate the level of extraneous matter in foods involving multiple stages: the specific foods and their defects (filth) are identified, analytical methods developed and adequate sampling plans designed to guarantee industrial safety. The samples are collected, analysed and the levels adjusted, pertinent legislation being implanted, establishing tolerance limits for extraneous material, with periodic revisions (FDA, 1995).

The objective of this study was to survey hygiene levels, identifying the critical points for filth contamination and providing subsidies for a revision of legal standards for innocuous and inevitable filth, reflecting both reality and product quality.

## MATERIAL AND METHODS

- 681 samples of raw coffee beans were examined (200 for espresso and 481 for ground, roast coffee), plus 205 samples of roast coffee for espresso and 375 for ground, roasted coffee, acquired during 2000 and 2001 from 40 commercial brands produced in the States of Roraima, Mato Grosso, Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, Santa Catarina, Paraná and Rio Grande do Sul.
- The samples of raw beans from the batches used to produce the roast coffees destined for the espresso and ground, roasted coffees, were evaluated for:

a) External infestation – 250 g raw coffee beans, sieved through the n° 8 (2.38 mm) sieve, were examined microscopically for external filth and insects. External filth (sand, earth, twigs and others) was quantified and the insect origins identified (GOHRAM, 1991; PACHECO et al., 1995).
b) Internal infestation – 100 raw beans were microscopically examined for the numbers of damaged beans (borer exit/entry holes) and healthy beans. The healthy and damaged beans were broken separately in a coffee grinder and internal infestation determined by method n°988.16 – item (b) of A.O.A.C. (ZIOBRO, 2000) and the insect origins identified.

- The samples of roast beans for espresso (previously ground) and ground, roasted coffees were evaluated for:

a) Heavy filth/100g by method n° 988.16 – item (a) of A.O.A.C. (ZIOBRO, 2000).
b) Light filth/25g by method n° 988.16 – (b) of A.O.A.C. (ZIOBRO, 2000).

**RESULTS & DISCUSSION**

Of the 681 (200 espresso [E] & 481 ground, roasted [GRC]) raw bean samples evaluated, 100 (14.7%) (3 E & 97 GRC) presented quantifiable (0.1-0.5g) external filth (soil, sand, husk and insects). Of the remaining 581, the presence of insects (1-11) and acarids (1-93) was observed in 19 of the EC samples and 91 of the GRC samples. The insects were identified as being crop related, eg fruit borer (*Hypothenemus hampei)* or storage pests, eg psocoptera (*Lyposcelides sp.*), lepidoptera (*Ephestia sp.*) and other coleopteras (*Araecerus fasciculatus, Tribolium castaneum, Oryzaephilus surinamensis* (L.) and *Cryptolestes spp*). For internal infestation, of the 481 raw GRC samples, 419 (87.1%) presented damaged beans (1-30 beans with holes) and 62 (12.9%) showed only healthy beans. Of the 419 samples with damaged beans, 159 (38.0%) showed no internal insect infestation and 260 (62.0%) showed 1 to 40 insects. With respect to the healthy beans, of the total of 62 samples, only 5 (8.0%) presented internal infestation (1-3 insects). Of the 200 samples of raw E beans, 76 (38.0%) showed only healthy beans and 124 (62.0%) presented damaged beans (1-8 beans with holes). Of the damaged beans, 32 (25.8%) showed internal infestation (1-4 insects). Of the healthy beans, 5 (6.6%) of the 76 samples showed internal infestation (1-2 insects). The raw material data allows for the indication of external filth control and internal infestation of damaged beans as critical control points.

The determination of heavy filth showed 62.6% of the roast coffees (E and GRC) with 0.04 to 32.96 mg/10g. Considering that the raw material can be contaminated by soil and/or sand in the field and during patio drying, similar to spices used for infusion (teas) for which the tolerance limit is 150mg/10g (Brasil, 1998), these values were relatively low.

Of the 205 roast samples for espresso (RE) and 375 roast samples for ground, roasted coffee (RGRC), 535 samples (92.2%) showed light filth (1- >300 insect fragments) and 45 (7.8%) were completely free of filth, conforming to current legislation. 6.7% also presented larvae (1-10), 0.7% whole insects (1), 0.7% acarids (1), 0.2% larvae + whole insect (1+1) and 0.2% acarid + rodent hair (1+1), in addition to the insect fragments. Contamination of RE with insect fragments was generally lower (45 free and 160 from 1 to 28) than RGRC (375 from 1 to uncountable >300), emphasizing that raw material quality and the use of good manufacturing practices are important to reduce insect fragments in the final product.

According to the *Canadian Health Protection Branch* Guidelines for Extraneous Material in Food (HPB, 1984), 73.5% of the samples presenting filth (535) were within the tolerance limit of 60 insect fragments per 25g, whereas according to Brazilian legislation, only 7.8% conformed, showing the need to establish a new tolerance limit in Brazil. However, the presence of samples with more than 60 fragments/25g, up to uncountable (>300), indicates the need for implementing good manufacturing practices.

**CONCLUSIONS**

- This study indicates the following as critical contamination points: external filth adhering to the bean and internal infestation, especially of damaged beans (with borer exit hole), which could contain larvae, whole insects, fragments and skin change residues (exuvae).

- A tolerance limit of 60 insect fragments/25 g sample appears to be a reasonable suggestion for a revision of current legislation. The implementation of good manufacturing practices in the coffee production chain is also necessary.

**ACKNOWLEDGMENTS**

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# The Application of Desiccant Dehumidification to Coffee Processing

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## SUMMARY

Desiccants are hydrophilic substances, which have traditionally been used to remove moisture from the atmosphere, thereby reducing relative humidity. The technology has been widely used in controlling humidity of coffee in storage. On observation of a Bry-Air compact industrial desiccant dehumidifier, the concept of applying the technology to the coffee drying process was derived.

Drying conditions, primarily temperature can have critical impact on the volatile component of coffee and by extension, the quality of the beans It is generally agreed that the lower the drying temperature the better the quality of the green beans. The dehumidifier operates at an average temperature of 36$^{o}$C and depending on the size can remove moisture/water at a rate of several tons per hour.

Dried air at a temperature of 36$^{o}$C, generated by the desiccant dehumidifier was passed through coffee in a metal box, stacked with trays of wet coffee to dry the coffee. Three samples of parchment coffee were dried from approximately 50% moisture to 12% moisture in this manner and the drying time, weight, appearance and cup quality compared with samples of the same batch of coffee that was dried mechanically and by sun. The results showed that:

- Desiccant drying is faster.
- Desiccant dried coffee has more weight than mechanically dried coffee at the same percentage moisture.
- Desiccant dried coffee has better colour and cup quality.

The results of this research are preliminary but very encouraging and could bring great benefits to the cost and quality of coffee processing.

## INTRODUCTION

The initial stages in the processing of most agricultural commodities involves the removal of moisture. The case of coffee is no exception. The pericarp comprising mainly of pectin and the mucileagenous mesocarp are first mechanically removed. The wet parchment, which is derived from that process, has a moisture content of approximately 50%. This water is to be found in substantial amounts of hydrophilic colloidal macromolecular substances such as proteins and polysaccharides.

The amount of moisture present in coffee beans, plays a very important part in determining storage stability against deterioration. At high moisture content the rate of degradation of

quality is very rapid. Greatest stability of the coffee beans occurs at a moisture content of between 11%-13%.

The process of moisture removal (drying) is a critical set in the processing of coffee. The usual method of drying is either sun drying, mechanical drying or a combination of both. In both these methods moisture removal is achieved through elevated temperatures. Temperatures as high as 80$^{o}$C are sometimes used.

The aroma of coffee is one of its most important attributes. Coffee quality is largely accessed on the basis of its aroma, flavour and physical appearance of the beans. The characteristic aroma/flavour of coffee is derived from the volatile components of the roasted beans. These volatiles are generated during roasting by complex pyrolitic chemical reactions, involving the non-volatile chemical components such as sugars, amino acids and phenolic compounds of the raw beans. The final aroma/flavour is determined by the non-volatile precursors present in the bean before roasting.

Drying conditions, primarily temperature will have a significant impact on the non-volatile components of the green beans. It is generally agreed that the lower the drying temperature, the better will be the quality of the green beans. Theoretically, desiccant drying of coffee (which operates at a maximum temperature of 36°C) should enhance the quality of coffee processed using this technology.

**OBJECTIVES**

This research sets out to evaluate the potential for the application of desiccant dehumidifier technology to coffee processing and determine its impact on the final quality of the green bean and the brew.

**MATERIALS AND METHOD**

- One Bry-Air compact industrial dehumidifier.
- Coffee samples for drying (100 lbs each)
- Moisture meter
- Drying box
- Drying Trays

The samples of wet parchment coffee (100 lbs) were selected and one sample dried using the dehumidifier and the other sun dried to approximately 12% moisture. When the batch from which the samples were taken had completed mechanical drying, a sample (10 lbs) was taken as the mechanically dried sample.

After resting for about eight weeks, the samples were hulled, polished, physically examined and cup tasted by a panel. Blind testing was done by the CIB's panel of four tasters.

**DISCUSSION**

The drying of coffee by Desiccant Dehumidification has been shown to be feasible, and appears to have several advantages. From the results it was observed that:

- The dehumidifier operates at a constant temperature of 36$^{o}$C.
- The dehumidifier dries coffee in 20% less time
- Coffee dried with the dehumidifier had a more blue/green colour

- Coffee dried with the dehumidifier had about 2%-4% more weight.
- Coffee dried with the dehumidifier had better cup quality.
- The impact of desiccant dehumidifier drying technology on coffee quality is the same for first or second quality.

**Table 1.**

RESULTS – Experiment #1

| Parameter | Dehumidifier | Mechanical | Sun |
|---|---|---|---|
| Wet Weight | 100 lbs | | 100 lbs |
| Initial % Moisture | 40.6 | 38.5 | 40.6 |
| Drying Time | 16.0 hrs | 20.0 hrs . | 56.5 |
| Dried Weight | 50.5 | | 48.0 |
| Final % Moisture | 12.5 | 11.9 | 12.7 |
| Resting Time | 8 weeks | 8 weeks | 8 weeks |
| Bean Appearance | 1.0 | 3.0 | 1.5 |
| Cup Quality | 1.0 | 3.0 | 2.0 |

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**Table 2.**

RESULTS – Experiment #2

| Parameter | Dehumidifier | Mechanical | Sun |
|---|---|---|---|
| Wet Weight | 100.0 lbs | 100.0 lbs | 100.0 lbs |
| Initial % Moisture | 52.3 | 48.5 | 52.3 |
| Drying Time | 17.0 | 24.0 | 59.0 |
| Dried Weight | 49.5 | | 47.0 |
| Final % Moisture | 12.3 | 11.6 | 12.2 |
| Resting Time | 7.5 | 7.5 | 7.5 |
| Bean Appearance | 1.5 | 3.0 | 1 5 |
| Cup Quality | 1.5 | 2.5 | 2.0 |

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**Table 3.**

RESULTS – Experiment #3

| Parameter | Dehumidifier | Mechanical | Sun |
|---|---|---|---|
| Wet Weight | 100.0 lbs | | 100.0 lbs |
| Initial % Moisture | 50.2 | 46.4 | 50.2 |
| Drying Time | 17.5 | 22.5 | 76.0 |
| Dried Weight | 47.4 | | 46.2 |
| Final % Moisture | 12.1 | 12.4 | 12.5 |
| Resting Time | 7 weeks | 7 weeks | 7 weeks |
| Bean Appearance | 2.0 | 3.5 | 2.5 |
| Cup Quality | 2.5 | 3.0 | 3.0 |

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## CONCLUSION

It has been demonstrated that the desiccant dehumidifier can be feasible applied to the drying of coffee and the technology has the potential to improve quality, increase out-turn and reduce drying cost. However further work is necessary to obtain more verify these results and derive more data on the commercial application

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# Variations in the Occurrence of Fungi in Coffee during Processing within and between Factories in Kenya

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## SUMMARY

Coffee samples (*Coffea arabica* L.) were collected from nine factories (three in each coffee agro-ecological zone) in Thika District, Kenya. The samples included fresh cherry and parchment grades before and after drying. Surface fungi were determined by wash and dilution method while internal fungi were determined by direct plating of the beans on Malt Extract Agar (HI-Media) after surface sterilization with 1% sodium hypochlorite for 10 minutes. Fungal growth was recorded from 4 to14 days of incubation at 25$^{o}$C. Yeasts were the dominant fungi on the external and in the internal surfaces of the cherry while *Fusarium* spp. occurred as the dominant moulds. Drying of the parchment was found to reduce the levels of the internal fungal infections.

## INTRODUCTION

Mould spores are widespread in nature and coffee being an agricultural commodity is in contact with them in all stages of production and processing. Under certain conditions these moulds may affect the quality of the product (Devonshire, 1956; Wrigley, 1988). Some moulds in their growth may also produce toxic waste products which may diffuse into the surrounding substrate beyond the area visibly affected by mould (Engenlhaardt et al., 1999). Ochratoxin A (OTA) a nephrotoxic and nephrocarcinogenic mycotoxin (Joosten et al., 2001) is an important toxic metabolite of some species of *Penicillium* and *Aspergillus* (Davis and Diener, 1987). In this study samples of coffee were assessed in terms of fungal infection of material during processing.

## MATERIALS AND METHODS

### Determination of external fungi on fresh cherry

Fifty (50) cherries were washed in 100 mls of 0.1% peptone water in sterilized screw-top jars. Serial dilutions of the washings were made up to $10^{-5}$ and 0.1 ml of each of the last three dilutions ($10^{-5}$, $10^{-4}$, $10^{-3)}$ spread on MEA (HI-media) containing chloramphenical and glycerol. Each dilution was done in duplicate. The various fungal colony-forming units were counted and recorded from day 4 to 14 days of incubation at 25$^{o}$C.

### Determination of internal fungi in parchment coffee

Coffee in parchment was de-husked manually and surface sterilized in 1% sodium hypochlorite (NaOCl) for 10 minutes and rinsed three times in sterilized distilled water The beans were blotted off excess water by use of sterile filter paper. Seven beans per plate were pressed gently onto MEA with the longitudinal slit facing downwards using sterile forceps.

The plates were incubated at 25°C. The total fungi were recorded and the specific types were expressed as proportions of the total of fungal population in the case of fresh cherry. The uninfected coffee beans out of the 49 plated per sample were recorded and the percentage fungal infections computed to give the overall bean infection.

## RESULTS AND DISCUSSIONS

### Mycological examination of the external and internal surfaces of fresh cherry

The results of the levels of yeasts and moulds obtained on the external and the internal surfaces of cherry from different factories in the three coffee agro-ecological zones are given in Table 1 and Table 2 respectively. The total fungal colony forming units on the external surfaces ranged from $10^5$-$10^6$, $10^6$-$10^7$, $10^6$-$10^8$ in the higher altitude (HA), Medium altitude (MA) and Lower altitude (LA) zones respectively. Yeasts were observed on the surfaces of cherry from all factories in the three zones. The most dominant moulds occurring on the external surfaces of cherry were *Fusarium* spp. and *Cladosporium,* spp. For the wet processed coffee most of the surface microflora are eliminated together with the pulp while some are washed off in the pulping water.

**Table 1. Levels of yeasts and moulds obtained on the external surfaces of cherry [colony forming units (cfu)/ cherry].**

| | HA | | | MA | | | LA | | |
|---|---|---|---|---|---|---|---|---|---|
| **Type of fungi** | **F1** | **F2** | **F3** | **F1** | **F2** | **F3** | **F1** | **F2** | **F3** |
| Yeasts | 9.20 x106 | $1.22x10^6$ | 8.20 $x10^5$ | 1.36 $x10^7$ | $8.20x10^7$ | 1.08 $x10^6$ | $6.5 x10^6$ | 1.28 $x10^8$ | 4.30 $x10^5$ |
| *Fusarium* spp | – | – | $1.00x10^4$ | – | – | $2.20x10^5$ | $1.00x10^6$ | $3.00x10^5$ | $4.00x10^4$ |
| *Aspergillus* spp | $2.0X10^4$ | – | $1.0x10^4$ | – | – | – | – | – | – |
| *Cladosporium* spp | – | $7.00x10^4$ | $4.00x10^4$ | – | – | _2.6 $x10^5$ | – | $1.00x10^6$ | $8.00x10^4$ |
| *Penicillium* spp. | $3.24x10^4$ | – | $3.24x10^4$ | – | – | – | – | – | – |
| *Colletotrichum* spp | – | – | $3.24x10^4$ | – | – | – | – | – | – |
| Others | $1.0X10^4$ | $3.30x10^5$ | $3.24x10^4$ | | | $3.2x10^4$ | $1.0x10^4$ | $2.0x10^6$ | $7.0x10^4$ |
| Total cfu /cherry. | 9.26x106 | 1.62 x 106 | 9.77x105 | 1.36x107 | 8.202x107 | 1.59 x 106 | 7.51 x 106 | 1.31 x 108 | 6.2.0 x $10^5$ |

**KEY**

- Others: filamentous moulds, which were not identified.
- (-) Type not recovered.
- HA (Higher Altitude 1700-1820M), MA (Medium altitude zone 1580-1760M), LA (Low altitude zone 1520-1580M).
- F1, F2 and F3 are factories in each of the agro- ecological zones where the samples of cherry assessed were obtained.

The internal surfaces of cherries from the medium altitude were less infected by fungi than those from the higher altitude and low altitude (LA) coffee zone. Fresh cherry samples from the LA coffee zone were observed to have higher proportions of *Fusarium* spp. and *Colletotrichum* spp.

**Table 2. Types of fungi in the internal surfaces of fresh cherry expressed as proportions of the total fungal population.**

| | HA | | | MA | | | LA | | |
|---|---|---|---|---|---|---|---|---|---|
| **Type of fungi** | F1 | F2 | F3 | F1 | F2 | F3 | F1 | F2 | F3 |
| Yeasts | 0.87 | 0.25 | - | - | 0.83 | 0.41 | 0.34 | 0.76 | - |
| *Cladosporium* spp | 0.02 | 0.15 | 0.60 | 0.50 | - | - | 0.04 | - | 0.02 |
| *Aspergillus* spp. | 0.02 | 0.05 | - | - | - | - | - | - | - |
| *Alternaria* spp | 0.02 | - | - | 0.25 | - | - | - | - | - |
| *Fusarium* spp. | 0.03 | - | 0.40 | | - | 0.14 | 0.57 | 0.12 | 0.45 |
| *Colletotrichum* spp | - | 0.25 | - | - | - | 0.40 | 0.04 | 0.13 | 0.55 |
| Others | 0.06 | 0.29 | - | 0.25 | 0.17 | 0.07 | - | - | - |
| Percentage fungal infection | 95.90 | 36.73 | 12.24 | 12.24 | 16.33 | 14.29 | 69.39 | 79.59 | 97.70 |

**KEY**

- Others: filamentous moulds, which were not identified.
- (-) Type not recovered.
- HA (Higher Altitude 1700-1820M), MA (Medium altitude zone 1580-1760M), LA (Low altitude zone 1520-1580M).
- F1, F2 and F3 are factories in each of the agro- ecological zones where the samples of cherry assessed were obtained.

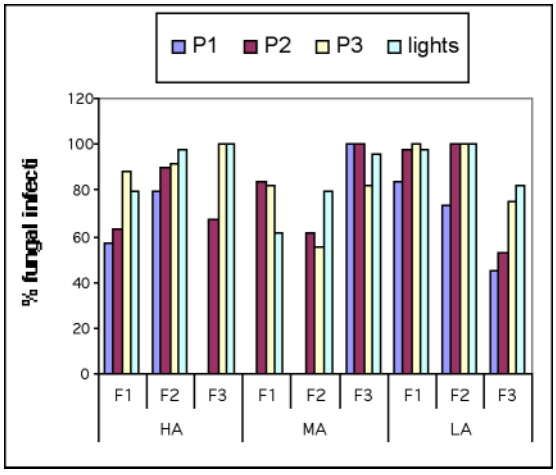


**Figure 1. Fungal infection in 'wet' parchment coffee.**

**FUNGAL INFECTIONS IN PARCHMENT GRADES**

At the processing units, cherry is pulped and the resulting parchment put in 'fermentation' tanks. Fermentation process is a natural process aimed at degrading the mucilaginous layer, which covers the pulped parchment (done by inherent and microbial enzymes), so that it can be easily washed off with water. Wet parchment is a term used to describe samples taken before drying commenced. The levels of the percentage fungal infections in parchment grades

before and after drying are shown in Figures 1 and 2 respectively. Parchment one (P1) showed lower levels of fungal infections in all the factories where this grade was available except in factory three in the main coffee zone. P1 is the heaviest and the best parchment grade. No discernable trend was observed in the other grades. The levels of fungal infections in the parchment grades declined on drying of the coffee.

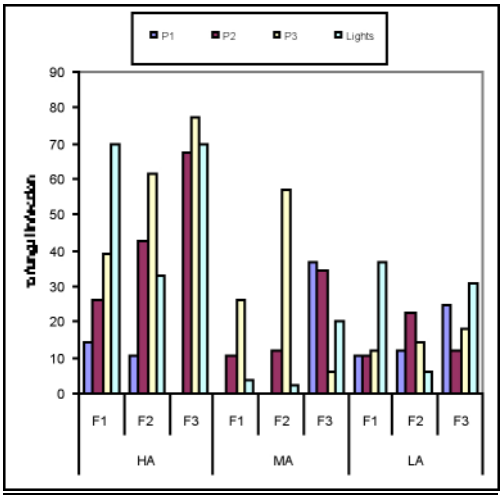


**Figure 2. Fungal infection in dry parchment.**

**KEY**

- Bars represent the percentage fungal infections in Parchment 1, Parchment 2 Parchment 3 and lights.
- HA (Higher Altitude 1700-1820M), MA (Medium altitude zone 1580-1760M), LA (Low altitude zone 1520-1580M).
- F1, F2 and F3 are factories in each of the agro- ecological zones where the samples of parchment coffee grades assessed were obtained.

**CONCLUSION**

The internal surfaces of fresh cherries from the medium altitude were less infected by fungi than those from the higher altitude and low altitude coffee zone. Yeasts occurred as the dominant fungi on the external and in the internal surfaces of the cherry while *Fusarium* spp. occurred as the dominant mould. Drying of the parchment grades reduced the levels of fungal infections.

**ACKNOWLEDGEMENTS**

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# Screening, Isolation and Identification of Brazilian Coffee Bean Microorganisms and the Impact of the Roasting Processes on the Bean Microbiota

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## SUMMARY

The aim of this work was to isolate microorganisms from beans of different Brazilian coffees that might be used for coffee industrialization. Samples were collected from lots of *Coffea arabica* (arabica) and *Coffea canephora* (conilon) of different producing areas. As a general evaluation of the potentially toxin producing flora, this study will provide indications about the methodologies and probable substances to look for in the samples. The effect of roasting process on the bean microbiota was investigated.

L'objective de ce travail était d'isoler des micro-organismes des grains des différents cafés brésiliens qui pourraient être employés pour l'industrialisation de café. Des échantillons ont été rassemblés de lots de *Coffea arabica* (arabe) et de *Coffea canephora* (conilon) de différentes régions productrices. Comme évaluation générale potentiellement de la toxine produisant la flore, cette étude fournira des indications au sujet des méthodologies et des substances probables de rechercher dans les échantillons. L'effet de torréfie sur le microbiota de grain a été étudié.

## INTRODUCTION

Technological improvements and discoveries in the study of mold toxins used to affect the public safety policy at all levels, from the anonymous consumers and retailers to the government agencies in charge of food trade or public welfare. Nevertheless, it is not advisable to remain only over powerful but expensive analytical devices to determine very small amounts of a given substance. It is also necessary to have a general evaluation about probable substances to look for in the samples. The purpose of this work was to evaluate the toxin producing microorganisms in coffee beans of different Brazilian coffees that might be used for coffee industrialization. The effect of roasting process on the bean microbiota was investigated.

## MATERIAL & METHODS

Microbiological analyses were performed for molds, yeast and bacteria screening, isolation and identification. The sampling was performed at Iguaçu green coffee storage warehouse. The samples were collected from lots of *Coffea arabica* (arabica) of different Brazilian producing regions (South of Minas Gerais; Parana) and *Coffea canephora* (conilon) from Rondônia, a State at northwestern Amazonian region. Samples of beans rejected during further cleaning from conilon and arabica blends were also analyzed. Samples of microorganisms presented at environmental air were also collected.

## ANALYTICAL METHODS

The analyses were carried out according to the methodology of the American Public Health Association[1,2]. Another analytical methodology employed to identify yeasts used classification kits (Kit API 20 E) purchased from BIOMÉRIEUX BRASIL S.A (Rio de Janeiro) and the bacteria were also identified with Kits API 50 CH from Biomerieux. The molds strains were identified to the specie level using optical microscopy observation of samples structures stained with lactophenol blue. The identification procedures had been based on the TUITE recommendation[3]. According to their morphologic characteristics, molds strains had been compared and identified with pertinent literature[4,5,6].

## RESULTS & DISCUSSION

A total of 474 bacteria counts were identified and the results of the sampling at the raw material warehouse are presented at Table 1. All the coffee beans samples that were analyzed for internal infection presented a positive result (data not shown). The fungi strains isolated from these samples (338 counts) were further identified to the species levels as shown at Tables 2 & 3.

**Table 1. General classification of bacteria from warehouse samples: PCA, total spore mesophilic aerobics; TGE, spore mesophilic aerobics; DTA, spore thermophilic aerobics; PE-2/35, spore mesophilic anaerobics – 35°C; PE-2/55 spore thermophilic anaerobics – 55°C.**

| Samples | Bacteria (%) | | | | |
|---|---|---|---|---|---|
| | Total | Spores forming | | | |
| | PCA | TGE | DTA | PE-2/35 | PE-2/55 |
| Arabica Beans Silo | 7.14 | 6.25 | 6.20 | 0.00 | 0.00 |
| Conilon Beans Silo | 5.10 | 6.25 | 6.20 | 0.00 | 0.00 |
| Swab Arabica Beans Silo | 3.06 | 6.25 | 6.20 | ND | ND |
| Swab Conilon Beans Silo | 7.14 | 6.25 | 6.20 | ND | ND |
| Warehouse Environment Air | ND | ND | ND | 0.00 | 0.00 |
| Arabica South MG Bags | 29.08 | 31.25 | 29.46 | 41.67 | 50.00 |
| Arabica Parana Bags | 8.16 | 6.25 | 6.20 | 8.33 | 0.00 |
| Arabica Blend | 5.61 | 6.25 | 6.20 | 8.33 | 12.50 |
| Arabica Residues | 8.16 | 6.25 | 6.20 | 8.33 | 0.00 |
| Conilon Rondônia Bags | 10.20 | 12.50 | 12.40 | 16.66 | 25.00 |
| Conilon Rondônia Blend | 6,12 | 6,25 | 8,53 | 8,33 | 0,00 |
| Conilon Residues | 10,20 | 6,25 | 6,20 | 8,33 | 12,50 |

The aerobic thermophilic spore forming count (DTA) includes species of facultatives or strict thermophlic Bacillus, among them the spoilage type "flat-sour" (*B. coagulans* e *B. stearothermophilus*), acid producers but not gas producing during growth. The anaerobic spore forming thermophilic (PE-2/55°C) include sacarolitic clostridium $H_2S$ non-producing, which species type is *Clostridium thermosaccharolyticum* (gas producing) and *Desulfotomaculum nigrificans*, $H_2S$ producing during growth. The aerobic mesophilic spore forming counts (TGE) include species of aerobic and facultative anaerobic Bacillus such as *B.macerans*, *B.polymyxa* and *B.licheniformis*, among others. The anaerobic mesophilic spore forming counts (PE-2/35°C) include species of Clostridium, mainly proteolitics (putrefatives) such as *C. sporogenes*, *C. bifermentans*, *C. putrefasciens*, *C. hystolyticum* and *C. botulinum*,

*C. butyricum* and *C. pasteurianum*. The culture medium also permits the growth of mesophilic facultative anaerobic Bacillus, making it difficult to differentiate contaminated samples with these microorganisms.

**Table 2. Fungi strains occurrence on green coffee storage samples.**

| Samples | Isolated Microorganisms (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Aspergillus* | | | *Fusar.* | *Penicil* | *Cladosp* | others |
| | *niger* | *flavus* | *ochra.* | | | | |
| Arabic Silo | 22.22 | 33.34 | 11.11 | 18.52 | 0.00 | 3.70 | 11.11 |
| Conilon Silo | 50.00 | 14.28 | 0.00 | 14.28 | 0.00 | 7.14 | 14.28 |
| Swab Silo | 30.00 | 50.00 | 0.00 | 0.00 | 0.00 | 20.00 | 0.00 |
| Environment air | 43.34 | 10.00 | 11.67 | 3.33 | 11.67 | 11.67 | 8.33 |
| Arabic South MG | 50.79 | 9.52 | 14.29 | 9.52 | 1.59 | 9.52 | 4.77 |
| Conilon | 50.00 | 11.11 | 11.11 | 11.11 | 0.00 | 11.11 | 0.00 |
| Residue Conilon | 32.00 | 20.00 | 4.00 | 16.00 | 0.00 | 4.00 | 24.00 |
| Residue Arabic | 25.00 | 15.62 | 3.12 | 40.62 | 6.25 | 9.38 | 0.00 |
| Parana Arabic | 26.67 | 13.33 | 13.33 | 0.00 | 13.33 | 20.00 | 13.33 |
| Arabic blend | 60.00 | 10.00 | 20.00 | 0.00 | 10.00 | 0.00 | 00.00 |
| Conilon blend | 61.54 | 15.38 | 7.69 | 0.00 | 0.00 | 0.00 | 15.39 |

**Table 3. Incidence of different yeast species on the group and the total of isolated counts identified to the specie level.**

| **Yeast species** | **Isolated Counts** | **Group %** | **Total %** |
|---|---|---|---|
| *Candida guillermondii* | 15 | 68.18 | 4.82 |
| *Rhodotorula mucilaginosa* | 04 | 18.18 | 1.29 |
| *Crytococcus albidus* | 02 | 9.09 | 0.64 |
| *Crytococcus humicolus* | 01 | 4.55 | 0.32 |
| Yeast Total Isolated | 22 | 100 | 7.07 |
| Fungi Total Counts | 311 | | 100 |

## CONCLUSIONS

1. The majority of the molds isolated from samples correspond to the *Aspergillus* genus with the species were *A.niger* (61.98%), *A.flavus* (23.96%) and *A.ochraceus* (14.06%).
2. Beside *Aspergillus*, *Fusarium* was the second more common isolated genera.
3. Although all the coffee beans samples presented toxigenic species the moulds and yeasts were quite common and there is nothing like pathogenic microorganisms.
4. Molds isolated from the low quality rejected Arabic beans belong mainly to the genera *Fusarium* (40.62%). *Aspergillus* responds for other 43.74% counts distributed as by the *A.niger* (57%) and the toxigenic *A.ochraceus* (7%) and *A.flavus* (36%).
5. In Conilon rejected beans the Aspergillus molds respond for 56% of the isolated counts with the same proportion observed in arabica residues for *A.niger*, *A.ochraceus* and *A.flavus* but a 2.5 lower level of *Fusarium*. This is an indication that the overall selective and cleaning process does contribute to reduce the contamination levels.
6. Besides other minor frequent molds genera, it is worth to mention *Penicillium* and *Cladosporium.* Parana Arabica and Rondônia Conilon coffees, did not present *Fusarium*.
7. The yeasts strains isolated from coffee beans belong to the genera *Candida* and *Rhodotorula* (only 6.2% of total counts), being the species *Candida Guillermondii* and

*Rhodotorula mucilaginosa* more often isolated, the latter was isolated only from arabic beans;

8. The more abundant aerobic spore forming bacteria were those developed under mesophilic conditions; it was not detected anaerobic hydrogen sulphide ($H_2S$) producing thermophilic spore forming bacteria but a positive detection for non-producing ones. Sulphite reducing microrganisms were also detected (*Clostridium*, total aerobic mesophilic, molds and yeasts).
9. After roasting, no count was detectable in beans and also in the extracts obtained with low temperature water (data not shown). The roasting process assures the elimination of microorganisms and these data lead to an approach based upon the scanning for possible levels of the toxin derived only from the toxigenic species found in this initial screening.
10. Despite the presence of toxigenic organisms, samples detailed monitoring for known toxins, as those of importance to the coffee beans like the Ochratoxin A (OTA), has shown very reduced values from the different Brazilian producing origins (data not shown). The averages of OTA have been in most of the cases below to 6 ppb.

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# Small Coffee Processing Unit Using Renewable Energy for Village Development

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## SUMMARY

As an attempt to trigger industrialization in rural Indonesia, a Small Coffee Processing Unit (SCPU) with input capacity of 2-3 tons wet coffee berries was installed in 1999 at Batudulang village, in the Island of Sumbawa. The unit uses a combined Greenhouse Effect (GHE) solar dryer and biomass stove as one of its main processing facility. Beside the dryer the unit also was provided with a 746 W mechanical husker, a 373 W roaster, a 373 W milling unit and simple packing tools to produce pure and blended ground coffee ready for the market. A local cooperative was assigned to operate the facility after a proper training by our Research Center and was capable to produce good quality products. In order to study the viability of the project as an effective means to improve the quality of life of the people in the village and to eradicative poverty, the cooperative has been provided with small grant acquired from ISESCO.

The fund now has been used as working capital for the cooperative so that there will available budget to purchase and process raw materials, and finally sell the product to the market. In this way more than 171 small holders living in the village and its surroundings can get benefit with better price of their products by processing their coffee in the SCPU since the quality of the products can be improved with more local contents of their products. It is expected therefore, more time can be made available for farmer to work in other income generating activities tons and ultimately can solve the multitude problems related to backwardness, poverty, accessibility to better education and health facilities. The project is now has been adopted and under trial as a CDM pilot project by the South-South North (SSN)-Pelangi, since most of the SCPU energy is supplied from the locally available renewable energy sources.

**Keywords:** processing unit, solar dryer, ground coffee, village development, micro-credit.

## INTRODUCTION

The Millennium Development Goals (MDG) as well as the results of the WSSD conducted in Johannesburg, South Africa, in 2002 have confirmed the world's commitment to work closely in various plans of action to alleviate poverty all over the world. This commitment is also in line with Kyoto Protocol where coercive effort between the developed and developing countries is encouraged to reduce GHG emissions while at the same time initiate sustainable development. To this end, renewable energy, which is already available in the rural areas, can serve both the purpose of triggering rural economic activities but avoiding additional GHG emission. In this respect, therefore, it is highly recommended that developing countries, should take the advantage of the clean and environmental friendly renewable energy

technology as a legitimate means to achieve sustainable and equitable development from the grass roots and consequently to the national level.

In 1999 the Government of Japan had granted a grassroots project to CREATA -IPB for the purpose of community empowerment through productive uses of renewable energy to process potential agro and marine products, in selected rural areas in East Java, Bali and West Nusatenggara. The use of the available grant was strictly limited to the fabrication and installation Small Processing Unit (SPU) facilities in the above mentioned project sites. Since the grant did not provide the needed working capital to operate the facilities, efforts have are continuously being made to find source of revolving fund schemes from several sources, both from in country as well as from overseas. Fortunately, in 2003, the ISESCO/UNESCO had showed interest in providing assistance to make the project sustainable.
This paper describes some of the results of how the provided working capital had improved significantly, the performance and the viability of the established SPUs. As an example, the case of SPU for coffee processing will be presented here.

## THE SPU FOR COFFEE AT BATUDULANG VILLAGE

The SPU at Batudulang is managed by local cooperative, the Kelompok Serba Usaha Samalewa ( Cooperative of General Merchandise), and has head office in Sumbawa Besar. The SPU at Batudulang is managed by the village chief, who owned 3-4 hectares of cash crops such as tumerics, ginger, anona, candle nuts, and coffee.

In order to conduct the current SPU project using ISESCO and UNESCO support, the following job description has been agreed upon in the contract agreement signed with CREATA-IPB.

- CREATA IPB is responsible for the general policy for project execution
- Head of KSUS cooperative is responsible for managing and coordinating activities of the SPU and provide regular report on the progress of the activity but also help in marketing the products on Sumbawa Island, within Sumbawa city and as far as Dompu and Bima regency.
- The Chief of Batudulang village, which is also member of the KSUS cooperative is responsible for the operation of the SPU and produce marketable products of the SPU such as ground coffee, tumeric, ginger, anona fruits, shelled candle nuts , etc.
- One student from Faculty of Economic of Mataram University, was assigned for marketing of products on Lombok island

### a. Standard operating and processing procedures

The SPU in Batudulang follows the following manufacturing procedures, shown in Figure 1 below.

### b. Production and sales data records

Batudulang village produces a variety of agricultural products of high values and available almost all year round. The following Table 1 and Figure 2. show coffee production potential in Sumbawa regency. It shows that Batudulang area is only number four in terms of the quantity of coffee production. However, its location is accessible within 45 minutes by car from the city of Sumbawa Besar, the capital city of Sumbawa regency. Table 2 shows the availability of products to be processed using the SPU for coffee at Batudulang village. Except for candle nut most of the products are sold in the form of ground materials, blended

with coffee or used as medicinal product. Besides producing commodity shown in Table 1, Batudulang, is also well known as the main producer of honey. Mr. Junaidi is one of the main supplier of honey produce in Batudulang village.

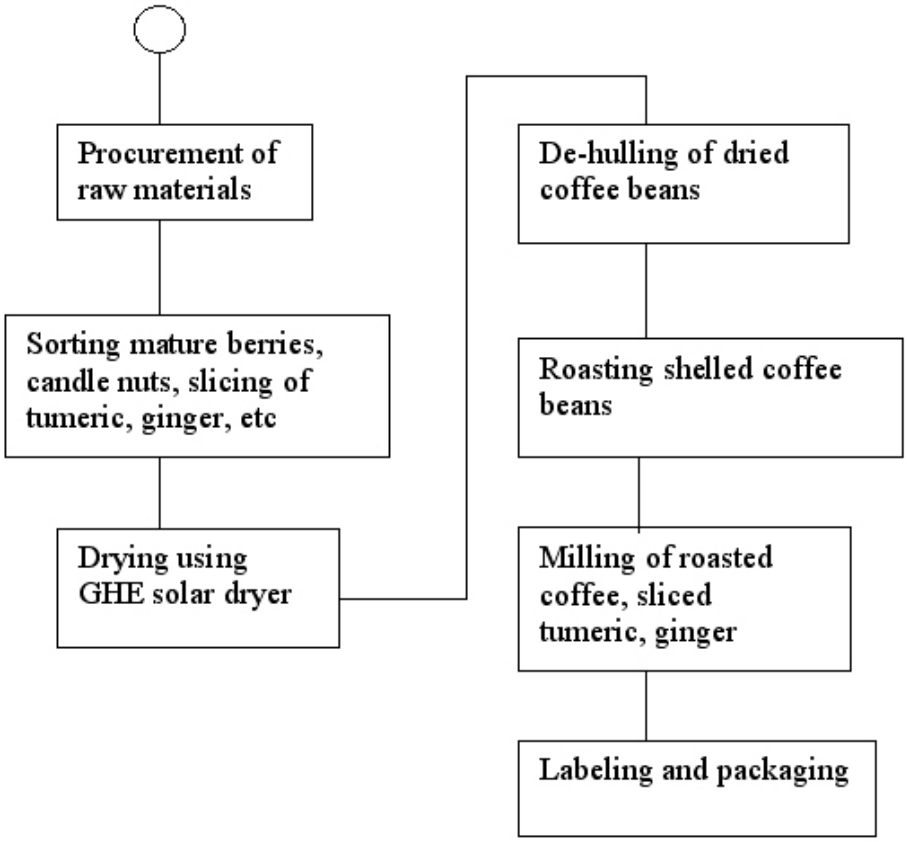


**Figure 1. Operating process of SPU at Batudulang village.**

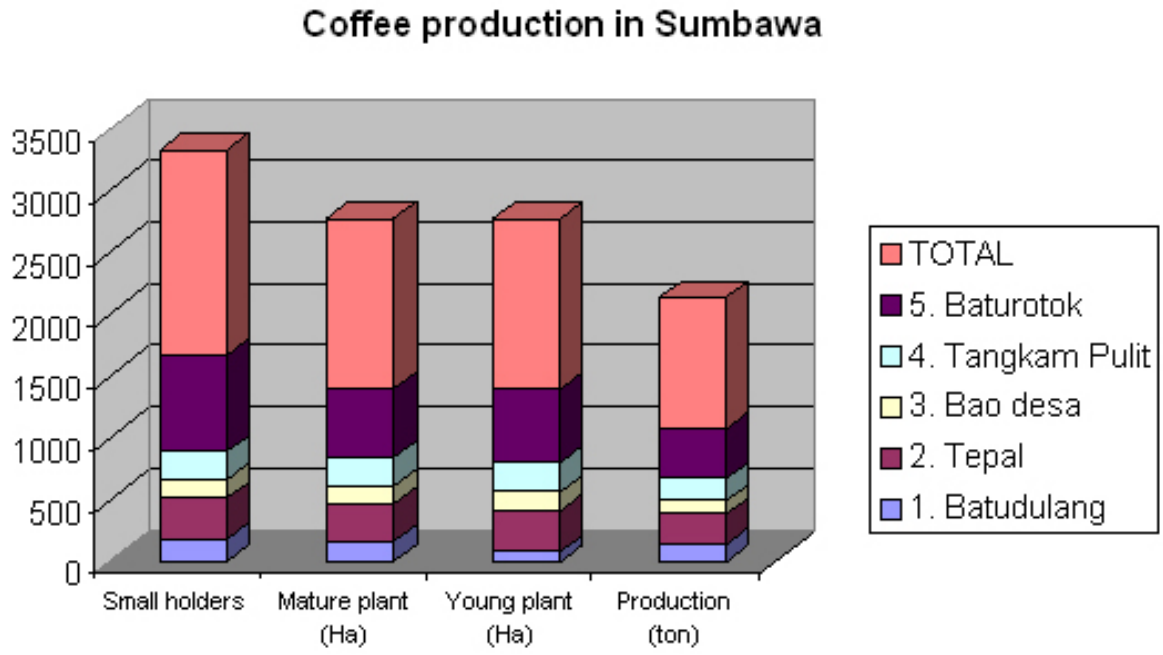


**Figure 2. Coffee production in Sumbawa regency ( Junaidi, 2003).**

**Table 1. Coffee production in Sumbawa (Junaidi, 2003).**

| Location | Small holders | Mature plant (Ha) | Young plant (Ha) | Production (ton) |
|---|---|---|---|---|
| 1. Batudulang | 171 | 152.5 | 75.4 | 142.9 |
| 2. Tepal | 334 | 310.5 | 331.5 | 238.5 |
| 3. Bao desa | 150 | 142.5 | 152.5 | 106.9 |
| 4. Tangkam Pulit | 228 | 238.3 | 239.3 | 177.5 |
| 5. Baturotok | 783 | 541.3 | 589.3 | 401.1 |
| TOTAL | 1666 | 1385.1 | 1388 | 1066.9 |

**Table 2. Availability of raw materials to be processed using the SPU.**

| Product/Month | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Candle nut | v | v | | | | | | | v | v | v | v |
| 2. Tumeric | v | v | v | v | v | v | v | v | v | v | v | v |
| 3. Coffee:<br>• Arabica<br>• Robusta | | | | v | v | v | v | v | | | | |
| 4. Ginger | | | | | | v | v | v | | | | |

Since the month of April as the harvest began to take place, the processing of coffee from drying to milling and packaging was started and the amount of process products increased as the harvest time approaching the peak harvest between June through August. The sales also began to increase accordingly, particularly, after the Ministry of Trade and Industry had approved on the used of product label under the name of the KSUS cooperative. Figure 3 shows facilities at the SPU and its main product in the form of strings of sachets.

**Figure 3. General overview of SPU for coffee processing at Batudulang village, Sumbawa (left) and its main product of ground coffee (right).**

## FINANCING SCHEME

One of the main purpose of providing working capital to the SPU was to enable the manager to purchase raw materials, hire laborers to operate different equipment in the SPU, make improvements of the facility whenever necessary, marketing the processed products, etc. By making use of the facility effectively it can bring benefit not only to the cooperative it self but also to the people living the village. To study the viability of the SPU key parameters for analysis such as technical and production cost data as well as amount of sales had been collected from the SPU for coffee processing at Batudulang village.

Table 3, shows the results of calculation using technical data obtained from the three months operation of the SPU. The designed loading capacity of the GHE solar dryer was 3 tons. Calculation results indicated that with the drying time of 3 days consecutively, and loading of only 1600 kg, the rate of return on investment (RoR) was higher that the current bank rate of 12%. This condition was achieved with regular payment of principal and interest rate for the period of 5 years. It should be noted that although coffee harvesting is only conducted within the three months period, the SPU can be operated all year round buy purchasing dried coffee stored by the small holders in the village, not to mention the income generated by processing other cash crops such as ginger, tumeric, candle nuts, anona fruits grown and owned by the SPU manager.

Another calculation using the break even method also indicated that providing initial working capital for operating the SPU in the first year, the income generated in the first year could be used as working capital in the 2nd year, if the payment of loan can be started at the 2nd or 3rd year after the load is provided. In case that the SPU could only sell 50% of the processed products, the delay in loan repayment may not exceed 3 or 5 years of operating time. Figure 4 shows how the RoR will change as the loading capacity is increased. Marketing survey indicated that better sales can be expected by improving further the processing technique and quality of packaging.

**Table 3. Calculation example for coffee processing at Batudulang village, Sumbawa.**

| *Commodity:* | *Coffee berries* | | |
|---|---|---|---|
| Drying times (days.) | 3 | | |
| Recovery factor (-) | 0.27 | | |
| Price of ground coffee(US$/kg) | 1.61 | | |
| Price of raw coffee (US$)/kg | 0.08 | | |
| | | | |
| *Investment* | | *Variable cost* | *US$/kg/yr* |
| 1. Price of the SPU system | 1.12E+04 | 1. Biomass fuel (US$/kg coffee) | 0.004 |
| 2. Economic life (yr) | 10 | 2. Electricity US$./kg coffee | 0.013 |
| 3. Salary-(US$/yr)- 3 employees | 1200 | 3. Labor US$./kg coffee | 0.004 |
| 4. Procurement of raw (US$) | 4000 | 4. Wear and tear US$./kg coffee | 0.002 |
| 5. Administration (US$./yr) | 1.12E+03 | 5. Drying frequency (batch/yr) | 30.000 |
| 6. Interest rates (%/y) | 4.00E-02 | 6. Load per batch (kg) | 1600.000 |
| 7. Electricity US$/yr) | 1.75E+01 | 7. Total drying (kg/yr) | 48000.000 |
| 8. Total (US$) | 1.76E+04 | 8. Variable cost (US$/kg/yr) | 0.278 |

**) 1 US$ =Rp.9000*

| | |
|---|---|
| Investment (US$) | 1.76E+04 |
| Drying load (kg/y) | 48000 |
| Income from sales (US$) | 20880 |
| Annual Cost*) (US$) | 1.76E+04 |
| Beneit-Cost (US$) | 3.33E+03 |
| PWFS (n=10, i=?_) | 5.28E+00 |
| RoR (%) | 1.51E-01 |

**). Include payment of principal and interest rates.*

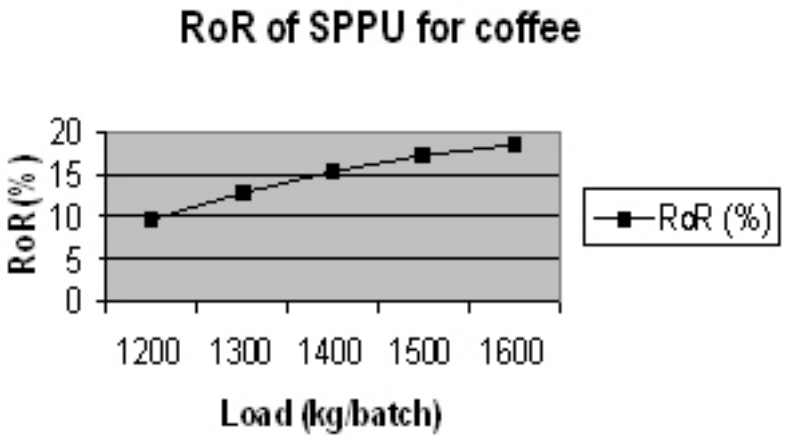


**Figure 4. Change in RoR value under different loading capacity of the GHE solar dryer.**

## CONCLUSIONS

1. GHE solar dryer can help to generate income by means of Small Processing Unit for coffee in Batudulang village, Sumbawa, provided that working capital can be provided.
2. The technology provided in the form of SPU is manageable by local entrepreneur.
3. Several improvements on the processing technique for the blend coffee produced and its packaging are still required in order to enhance sales and expand market share.

## ACKNOWLEDGEMENT

The authors extend their gratitude to ISESCO and UNESCO for providing the assistance so that the installed SPU is now beginning to operate accordingly. Also thanks are due to the ASIC committee for providing support to present this paper during the 20th International Conference on Coffee Science (ASIC 2004) in Bangalore, India.

# Determination of a Coffee Factory Capacity

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## SUMMARY

Coffee factories, which commonly deliver high grade coffee, can occasionally experience a sudden drop in quality of the processed coffee. In most cases, the cause of such a constraint can be traced back to a sudden peak cherry input (due to sudden ripening of the coffee cherry in the farms), which the factory could not cope with. There being no doubt that much earnings can be lost in this way, the health and safety of the consumers can also be seriously compromised.

There is a strong tendency to construct more factories in order to "release" the existing ones from congestion in order to surmount any likely constraints from these and other unforeseen factors. However, since coffee is a seasonal crop, the coffee factory will be in use for only a short time of the year. Further more, the factory nevertheless consumes capital in the form of for instance depreciations even during the time it is not in use. As such, the more factories there will be the higher the total costs thus leaving the farmer with a lower payout. Therefore, although in some cases the construction of new factories can be considered, in general the existing processing capacity in all coffee growing areas suffices greatly. For this purpose, ways and means on how to handle sudden peak crops in an economic way without loss of coffee quality and quantity are demonstrated.

## INTRODUCTION

If a primary coffee processing factory is not well designed to cope with the expected cherry intake at any time, congestion can be experienced at the peak of the season. This can occasionally lead to a sudden drop in coffee quality in some consignments from coffee factories, which do commonly deliver high grade coffee. This can occur whenever there is a sudden peak crop due to sudden ripening of the coffee cherry in the farms. Lack of adequate knowledge of determining the optimal processing capacity can also induce such constraints.

Although in some cases the construction of new factories might be considered, in general the existing processing capacity in all coffee growing areas suffices greatly (Anon., 1990). The following procedure for determining the processing capacity in a primary coffee factory can therefore be very useful in enhancing coffee quality.

## DETERMINATION OF A COFFEE PROCESSING FACTORY CAPACITY

The following expressions are essential in calculating the various capacities of a coffee factory (Wrigley and Gordon, 1988; Anon., 1990).

- Ripening trend ≅ 20% in 2 weeks or, 15% in 1 week at the peak of the season.
- Annual harvest ≅ 40% and 60% from the minor and main seasons respectively.
- 1000 kg heavy cherry ≡ 200 kg dry parchment or 160 kg clean coffee.
- 1000 kg light cherry ≡ 100 kg clean coffee.

- Pulping rate per disc = 1000 kg cherry per hour.
- Pre-grading rate with = 300kg coffee/hr.
- 1000 kg cherry ≡ 0.5 $m^3$ parchment and needs 0.6 $m^3$ fermentation.
- 1000 kg cherry needs 40 $m^2$ in a 2.5 cm coffee layer on the drying tables,
- 1000 kg cherry need 20 $m^2$ in a 2.5 cm coffee layer on the drying tables, ∴ only 10 $m^2$ are required when the drying layer of coffee is 5 cm,
- 1 bag of heavy parchment ≅ 50 kg,
- 200 kg dry parchment requires about 0.5$m^2$ bins or store space,
- Bag of parchment stacked 8 bags high needs 0.1$m^2$ store space,
- One (1) tonne clean coffee (green coffee) = 16.5 gags gross.

**CHERRY INTAKE CAPACITY**

Annual production = p kg
Main season production = 0.6p kg
Peak production: 20%/2weeks = 0.12p kg
15%/week = 0.09p kg

**PULPER SELECTION**

**Table 1.**

| **Cherry production, Kg** | **No. of discs (n)** |
|---|---|
| <50,000 | Mini pulper (Hand/Motorized) |
| 50,000 - 150,000 | 1 |
| 150,000 – 350,000 | 2 |
| 350,000 – 800,000 | 3 |
| 800,000 – 1,200,000 | 4 |

**PULPING**

Since 1 disc can process 1000 kg cherry per hour,
Pulping capacity = 1000dtn kg cherry per week
Where d processing days per week
t processing hours per day
n No. of discs/pulper; n = 1-4

However, always make sure the pulper and grading devices well adjusted and maintained such that no breakdown occurs during the peak season.

**FERMENTATION**

**Assumptions**

- Coffee cherry can be graded into 1 and 2 and both grades pulped separately.
- If the lights of cherry No. 1 and 2 are fermented in the same tank, then at least 5 fermentation tanks are required per day of processing.
- The heavy parchment coffee from cherry No. 2 can be mixed with the seconds from cherry no. 1 to reduce the tanks required from 5 to 4.

$\therefore$ Pulping capacity per day $= \frac{1000dtn \text{ kg}}{d} = 1000tn \text{ kg}$

Required fermentation space $= \frac{1000tn \times 0.6 \text{ kg}}{1000} = 0.6tn \text{ m}^3$

If fermentation and soaking will take y days, then, the total requirements are 0.6nty $m^3$ of fermentation space or 5y tanks. It is advisable, however, to have 2-3 more tanks, so as to be able to switch from one tank to the other depending on the ratio between "firsts" "Seconds" and "lights. Other methods of increasing capacity at this stage include: Soaking the "firsts" only after final grading and washing; re-circulating the pulping water, pectic enzymes or covering the fermentation tanks with suitable materials to speed up the fermentation and; considering not to put any attention to the "lights" any more but instead dump these in the skin pit as long as the performance and adjustment of the pre-grading machinery has been checked to be sure that no "heavies" are in between the "lights".

**SKIN DRYING**

At this stage, an uncontrolled fermentation can take place when drying is done too slowly (Anon., 1991) resulting in low quality and poor colours.

Skin drying area $= \frac{1000tn \times 40}{1000} = 40tn \text{ m}^2$ per day

No of tables $= \frac{40tn}{lw}$

Where l Table length (m)
w Table width (m)

During adverse weather, drying tents of plastic or PVC can be used. Alternatively, a suitable cover above the coffee or stacking trays of wet parchment on racks under a corrugated iron shed will render useful services. The same shed can be used for emergency bins. Always release the drying area for wet parchment without delay besides putting not more than 2.54 cm wet parchment on the wet parchment tables.

**DRYING**

Drying creates the biggest problems besides being highly labour consuming where it is conducted only in the sun, while commonly a large area is also required. Nevertheless, it should not be all that difficult with good management.

**Assumptions**

- The coffee layer on the drying tables will be 2.5 cm during "white stage" and 5 cm during "hard black" and "fully dry" stages. Hence, the average coffee layer thickness will be [(2.5+5)/2= 3.75 cm throughout the drying.
- The drying time is also 14 days. Hence, the drying area has to hold the parchment produced in 2 weeks

Cherry intake in two weeks = 2 x 1000dtn kg
= 2000dtn kg

Equivalent parchment volume = $\frac{2000\text{dtn} \times 0.5}{1000}$ $m^3$
= dtn $m^3$

∴ Required area = $\frac{\text{dtn} \times 100}{3.75}$

No of tables = $\frac{\text{dtn} \times 100}{3.75 \text{xlw}}$

To enhance coffee drying: Open the drying tables as early in the morning as possible and close as late as possible in the evening during the peak time; Make sure all tables are in good order and easily accessible; Stir the coffee quickly and frequently; Transport the dry parchment to the store immediately and consider the construction of drying tents or covers over the drying tables. However even with dull weather and occasional rain spells it is easily possible to dry parchment, in 14 days or even less. The hard black parchment may also be transported to the emergency bins and kept there until more tables become available again.

**STORAGE**

Adequate storage space and empty bags are necessary to releases the drying tables for further drying. Only one season's produce has to be stored for some time in the factory

Annual production of dry parchment = $\frac{0.5p}{1000}$ kg

∴ Storage requirements = $\frac{0.5p \times 0.6}{1000}$ kg
= $\frac{0.5p \times 0.6}{1000 \times 50}$ bags
= $\frac{0.5p \text{ x}0.6 \times 0.1}{1000 \times 50 \times 8}$ $m^2$ floor space
= $7.5p \times 10^{-8}$ $m^2$ floor space

**LABOUR**

The lack of labour often presents difficulties some solutions to which lie in: dividing the work force in groups, appointing a group leader and assigning to each group particular duties; making sure that all workers get sufficient time off and avoiding long overtime since this will decreases the interest and productivity of the workers. During peak time, it is worthwhile to divide the labour force into early and late shifts or try to find ways and means of making the work easier. In case communal labour by the member is available, use it for sorting on the wet parchment tables and transport of parchment to the store. In case it is also used for the drying, well defined instructions have to be given. The factory manager must also make a work program for each day so that he can plan a proper labour utilization.

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# The Current Recommendations for the Processing of High Quality and Safe Coffee in Kenya

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## SUMMARY

In Kenya, the predominantly produced arabica variety is processed by the wet method.

The relatively little coffee from other varieties like Robusta, as well as the coffee, which is rejected from the wet method at the sorting stage is subjected to dry processing. For proper processing and handling of coffee, a high level of management regime is recommended towards the enhancement of coffee quality via good agricultural and post harvesting practices. However, since the need to produce high quality and safe food products is increasingly becoming not only sensitive but of great concern to the whole world, it is important that all the available information towards this end does not just remain in theory but in actual practice.

For this reason, the critical stages, prevailing mainly during the post harvesting processing, are reviewed. The role played by every technical recommendation at every stage is also identified as well as the respective impact with respect to quality improvement. The constraints facing the implementations of any of the recommendations are also identified, discussed and a possible solution presented.

## INTRODUCTION

Processing is a very important activity in coffee production and prays a crucial role in quality determination (Mburu, 1999). However, good field coffee management and post harvest processing enhances coffee quality as well. Subsequently coffee is either processed by the wet or dry methods, which vary in complexity and expected quality of the coffee. The former whose stages between sorting and drying are carried out with the assistance of water is predominantly for arabica coffee. The dry method simply entails drying the cherry into Buni coffee, which is hulled at the end of the season. This involves just drying of the cherry on suitable surfaces or even mechanically. Once dry, the coffee is temporarily stored to ensure that all the coffee harvested at different times of the season is ready prior to delivered to the miller for further processing. In some farms, the dry processed coffee and dry light parchment from the wet method are hulled on the farm and delivered to the market via the miller as estate cured coffee. This is mainly meant to improve the value of such coffee before submitting it to the market.

To ensure that coffee quality is improved during processing, each stage must be undertaken in the right manner and with facilities, which are in good order (Anon., 1990, 1991,1997). For this purpose, the critical requirements for each stage of processing are highlighted as follows.

## HARVESTING

It is important to ensure timely and selective picking of just ripe coffee cherry judged by their deep red colour; Mixed stages of maturity and ripeness of cherries cause pulping and fermentation problems which lower the coffee quality. Farmers should therefore avoid the overripe and under ripe coffee cherry. Protect the harvest from getting hot by avoiding direct sunlight through shading with coffee bushes or tree crops in the farm; Prevent cherry from falling to the ground during harvesting. Pick the diseased and insect pest damaged cherry, before actual cherry picking day. Use hygienic harvesting equipment.

## SORTING

Avoiding contact of cherry with soil during sorting by use of clean material like canvas; carefully sort the cherry by removing the immature, green, under-ripe, overripe, dry, and diseased and insect damaged cherries as well as other plant leaves, twigs and any other foreign materials from the good cherry; Small cherry should also be sorted before pulping starts. Process the sorted out poor coffee by the alternative dry method. In some cases, the red ripe cherries can be processed separately from the slightly under and overripe cherries.

## PULPING AND PRE-GRADING

Pulping is the mechanical removal of the pulp from the cherry to have parchment coffee. Prior to the seasons pulping, the pulping machine should be adjusted on the basis of the size of the majority of the berries to ensure that there is no berry damage or passage of many unpulped cherries during pulping which would result into poor quality coffee. Keep the pulper and the pre-grader clean and in good mechanical. The pulping unit must be repaired and tested before coffee is taken to the factory; Spares parts, fuel and any other thing that may be needed in emergency cases should be in store to avoid congestion and unnecessary delay during processing. Coffee picked must be delivered to be pulped on the same day to avoid commencement of fermentation before pulping. Pre-grade the coffee precisely into firsts, seconds and floaters or lights; use clean river water which is free from chemicals, undesirable tastes and odours for processing; practise water re-circulation to enhance the subsequent fermentation stage; flash the system with clean water immediately after pulping; dispose re-circulated water immediately after pulping; prevent the coffee from contacting grease, oils or exhaust fumes from diesel engines.

## FERMENTATION PROCESS

Fermentation breaks down the mucilage on the parchment into simple non-sticky substances, which are easily washed off. The removal of mucilage is important because it is sticky, inhibits drying, attracts dust, makes handling difficult and is a good media for spoilage microorganisms to thrive on. A gritty feel with the hand determines completion of fermentation after washing a bit of the parchment with clean water; ferment the different grades of parchment separate in fermentation tanks; never leave the parchment for a longer time than absolutely necessary in the fermentation tanks; observe the correct fermenting coffee not more than 1 m depth. Intermediate washing is practised (to hasten the fermentation rate) by gently stirring the parchment in water every night and the water drained off. Finally never use the fermentation tanks as a temporary store for wet parchment.

## FINAL WASHING, GRADING CHANNEL AN SOAKING

Always use clean water, paddles and dikes during washing and grading; wash the completely fermented parchment thoroughly to ensure complete removal of mucilage; no perfumes or

drugs like cigarettes are permitted for they are likely to impart undesirable tastes (taint) to the coffee; ensure that grading is efficiently done for complete separation of the different parchment grades. Clean facilities with no cracks or rough surfaces are required.

The parchment may be soaked in absolutely clean water and equipment for between 16-24 hours to improve the quality. Parchment coffee can be soaked for 7 days without deterioration of quality as long as parchment is dried carefully to prevent cracking; the soaking water should be changed every morning; use of dirty water may bring in bad colour, taste and even undesirable chemicals, which lower the quality of the bean. Salty water commonly found in boreholes is not suitable for soaking coffee; clean running water from streams is better.

**PARCHMENT DRYING**

Execute the skin drying process within the shortest time possible. Skin drying can proceed even in dull weather as long as it does not rain. Parchment should not be left overnight on the skin drying tables; when skin drying, defective beans can be distinguished and sorted out easily. Use suitable drying and water proof covering materials while allowing ventilation from beneath; Maintain the overall tables in a clean condition and absolutely flat for even drying; Practise slow drying during the white stage for 2-4 days to avoid cracking. Defective beans are also conspicuous at this stage and should be sorted out; The drying parchment should be stirred regularly and the layer controlled not exceed 2.5 cm thick; At the soft black stage, which lasts at least 2 days of actual sunshine, parchment coffee may be transferred to ventilated conditioning facilities or the drying depth increased to 5.0 cm on the tables to avail drying space to the wettest and most vulnerable parchment; Avoid re-wetting or pro-longed static moisture balance at any stage of drying; The drying parchment should not fall on the grass or bare ground to avoid contracting the grassy or earthy flavour; The final moisture content for parchment should be a maximum of 10%; Avoid over drying parchment to safeguard against fading and moisture re-absorption (to prevent mould growth) in storage.

**BUNI DRYING**

Start drying immediately on the day of harvesting by spreading the coffee cherry on a clean and well drained surface. Avoid mixing cherry harvested on different days (i.e. of different moisture contents); never heap but instead control the thickness of the drying layer and complement this with regular stirring; avoid soil contact by preferably using drying tables or other suitable drying surfaces, which must be clean; cover from adverse weather conditions to avoid rewetting while still keeping the coffee well ventilated at the same time; damp rooms are unsuitable for cherry drying; the moisture content of dry cherry is a maximum of 12%.

**STORAGE AND CONDITIONING**

Never store parchment and clean coffee together with Buni in the store; store only under controlled but adequate ventilation; Provide water and dust proof floors, walls, and roofs; the roof must also provide adequate insulation in order to minimise heat transfer; storage temperatures at not more than 22°C and relative humidity 50-70% with minimal fluctuations are ideal; minimise storage at humid/warm conditions implying no storage in a harbour unless in an air conditioned warehouse; allow no contact with concrete wall by placing the coffee bags on wooden pallets 15 cm away from either wall or floor surface.

## COFFEE FACTORY HYGIENE

All the factory operators, equipment and materials must be very clean. The recommended maintenance procedures e.g. painting should be strictly adhered t0; all the surfaces should be of the correct specifications e.g. smooth and; disposal of the wastes should be such as not to pollute the environment.

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# Coffee Processing Waste Management

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## INTRODUCTION

The key steps in the wet coffee process are: removal of the outer skin from the coffee bean by pulping; pre-grading the coffee beans; release of the mucilage covering the coffee bean by fermentation; washing and grading the mucilage free coffee bean according to its density in water; sun drying the coffee bean over 10-14 days and temporary storage of dry coffee. This system is characterised with: the excessive consumption of water (at each stage of the process), which generally varied greatly from 59-105$m^3$ (Mburu et al., 1994) and 24-114$m^3$ (Finney, 1990) of water per tonne of dry parchment; the contamination of water due to inappropriate design of waste treatment pits; the generation of large amounts of waste pulp and seepage of contaminating leachate from pulp heaps into the environment. Surveys in the early 1990s showed that some rivers in the coffee growing areas had BOD values ranging between 14 and 55 mg/litre (Mwaura, 1995) instead of a maximum of 6mg/litre (WHO guideline for drinking water).

To overcome the problems related to the disposal of the wet coffee processing wastes, a research project was conducted in Kenya between 1993-1997 (Wood et al., 2000). Its specific research objectives were to: conduct an environmental impact assessment (EAI) of the coffee industry; identify procedures for reducing water usage and effluent discharges in process operations; evaluate current effluent treatment systems, identify the wastewater components that contribute to the largest negative impact and develop improved treatment technology and; develop a practical protocol for water conservation and reduction in water pollution in accordance with the requirements of the water Act of Kenya 1972.

## THE PRELIMINARY ENVIRONMENTAL IMPACT ASSESSMENT (EIA)

Its objectives were to identify:

### The institutional and legislative context for water management for the coffee industry in Kenya

The strategy for sustainable development in Kenya, developed in response to Agenda 21, was being promoted through the implementation of Kenya's National Environmental Action Plan (NEAP) in conjunction with the ministry of land Reclamation, Regional and water Development (MLRWD); the national Irrigation Board (under MLRWD).

The ministry of Agriculture; The Coffee Research foundation; the Inter-ministerial Committee on the environment; based on the existing Legal framework for water resource and environmental management in Kenya.

### Sources of industrial coffee pollution

Drainage and seepage of raw effluents from the seepage pits and decomposing pulp heaps into the natural watercourse.

**Priority Areas For Improvement**

***Current practices of coffee processing and effluent treatment***

The EIA identified the actions, which would improve the control of the coffee process and its product to include:

- Management improvements for immediate implementation,
- Carrying out regular preventive maintenance;
- Ensuring that skin towers, operate effectively;
- Limiting water usage to the permissible levels of 22.5$m^3$ per tonne of dry parchment, Regular (off season) cleaning of seepage pits,
- Use of cut off ditches to Prevent rainwater run-off from entering the waste pits,
- Discharging the process effluent from tanks immediately after pulping,
- Flushing the system through with clean water after pulping,
- Installation of small tanks where large re-circulation tanks prohibit the use of a re-circulation system early and late in the season,
- Preventing water leaking from pumps, valves and pipe work and,
- Prevent diesel/oil leaks from engines.

***Areas for research to reduce the output and improve the treatment of coffee effluent***

Process modification by research – The EIA recommended research towards:

- Controlling the release of effluent run off from heaps of coffee skin and pulp;
- Reducing water utilisation to meet limits set by the Kenya water authorities;
- Improving the management of wastewater from the coffee process.

**RESEARCH FINDINGS**

**Efficiency Improvements in the Traditional Process**

***Controlling the release of effluent runoff from heaps of skin pulp***

The simplest form of effluent management is to improve the controlled channelling of skin tower effluents into a suitably designed percolation and decomposition trench. However, improved measures to manage the release of liquor from pulp heaps and its discharge into appropriate pits, as well as the management of the pulp heaps themselves and their removal from the site, are therefore to be encouraged. Pulp heaps attracts insects, can produce bad odours and have high pollution potential.

**Pressing Option**

The options available for coffee producers are screw press or piston press.

***The potential benefits of conducting pressing***

- Controlled management of the pulp while the liquor fraction can be piped to treatment trenches.
- Press cake is easy to handle for use as mulch, livestock feed or any other purpose;
- Potential value added to the pulp if the cake is sold or replaces other bought in materials;
- Reduced costs of transportation per tonne of pulp produced and;

- The concentrated, warm press liquor may enhance the fermentation process.

**Potential for Reducing Water Utilisation to Meet Limits Set by the Kenya Water Authorities**

These include: re-circulation of soaking and final grading water for pulping and mucilage removal, and washing the fermented beans through a pump, which can remove 65% of the total mucilage solids by rapid agitation in water for 10 seconds, and 85% after two passes through the centrifugal pump. After picking up soluble and insoluble solids at the washing stage, the water is directed towards the waste pit. By so doing, the volume of water designated for re-circulation is equivalent to 4.5 $m^3$ per tonne of cherry.

The process line must be fitted at appropriate points with totalising water flow meters, and that water volumes are measured and installed. The alternative would be to use batch volumes of water from tanks marked with the desired quantities.

**Potential for Mucilage Sedimentation from Effluent Streams**

This will reduce the potential for pit clogging; improve its action as a percolating filter or digester of organic compounds. The research findings in this area are in Table 1

**Table 1. Performance of cement in mucilage sedimentation.**

| Consideration | Results |
|---|---|
| The most successful flocculants | Portland cement |
| Application rate | 1..5 g/litre (1.5kg/$m^3$) of wastewater |
| Settling rate | By gravity within 5 minutes |
| Concentration sediment | 20% of the original column of wastewater |
| Volume of sediment | Not changes even after 3 days |
| Other requirements | Settling tank, a pump, pipes and valves |
| Effluent composition | 50% soluble and 50% suspended solids |
| Soluble sugars and organic acids | Treated by percolating trenches or other systems |

**Alternative wet coffee processing Technologies**

Existence of technologies developed for minimisation of processing water requirements by dry pulping, mechanical removal of mucilage and use of endless Archimedean screws to transport beans and waste was noted. However, these technologies omit the fermentation, soaking and grading steps. Therefore, they need to be tested in Kenya at pilot scale level to obtain their precise effect on coffee quality.

**Opportunities for Waste Treatment**

Since the availability of land for effluent control is often limited or expensive, investigation were on technologies which: would not increase land requirements beyond those used by current practices; were less capital intensive, require unsophisticated operating and monitoring regimes and necessitate a low level of infrastructure support.

***Technologies, which were evaluated***

- The Kenyan seepage pit, with modified design and improved operation;
- The Leeds design of seepage trench, correctly engineered and operated;

- Anaerobic stabilisation ponds and
- An upward flow anaerobic sludge blanket (UASB) reactors.

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It can be noted from table 2 that as treatment system engineering becomes complex, its performance improves while its cost increases. On that basis the Kenyan seepage pit technology, with modified design (7m circumference, 1.5m deep and waste inlet channel 1m wide by 0.5m deep) and improved operation was found the most viable.

**Table 2. Cost of COD Removal in Various Treatment Systems.**

| Option | Capital Cost/year (Ksh) | O & M Cost/year (Ksh) | COD Removal (%) | Kg COD Removed year (Ksh) | Cost/kg COD Removed (Ksh) |
|---|---|---|---|---|---|
| No Change | 3,600.00 | 0 | 5-15 | 851.00-16,171.30 | 4.23-10.22 |
| Improved Pit | 3,591.02 | 58,200.00 | 30-80 | 5,106.73-13,617.94 | 4.54-12.10 |
| Trench | 19,772.93 | 54,000.00 | 40-85 | 6,808.97-14,469.06 | 5.10-10.84 |
| 2d Pond | 38,675.90 | 153,022.40 | 55 | 9,362.33 | 20.48 |
| 4d Pond | 72,263.50 | 153,022.40 | 70 | 11,915.69 | 18.91 |
| 8d Pond | 139,438.30 | 153,022.40 | 80 | 13,617.94 | 21.48 |
| UASB | 100,639.40 | 150,294.00 | 65 | 11,064.57 | 22.68 |

## CONCLUSION

According to the results, improvements in river water quality will have a direct impact on improving the quality of coffee processed by the wet method. The findings also indicate that the low cost simple technologies available to the wet coffee industry can have a rapid impact on levels of water abstraction, effluent management and discharge. Improving the design, operational and maintenance of wastewater pits is an essential step, which the industry must take.

The findings also supported the project's objectives. The options for improved process and waste control must be critically appraised and where proven to be effective, adopted. There is also some evidence that pesticides are finding their way into watercourses as a result of coffee processing. Information is sparse and inconclusive, but there are sufficient data to indicate the need for environmental research by the coffee industry on this problem

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# Fate of Artificially Inoculated *Aspergillus ochraceus* and OTA Production during Wet Processing of Coffee

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## SUMMARY

In an experiment on artificial inoculation of *Aspergillus ochraceus* during wet processing of coffee, fruits, pulped beans and washed parchment were inoculated in different lots with *Aspergillus ochraceus* spore suspension at 20ml/kg of fruits ($10^7$ spores/ml). After infection, samples were drawn at different stages of processing for assessing the carry over of Ochre spores in the beans. The results showed that the percent reduction of inoculated *A.ochraceus* spore from fruit to dried beans, pulped beans to dried beans and washed parchment to dried beans was 84.5%, 71.1% and 26.0% respectively. The OTA contamination in beans from inoculated fruits, pulped beans and washed parchment was 0.40, 0.48 and 1.25 ppb respectively.

## INTRODUCTION

The contamination of coffee with *Aspergillus ochraceus* and subsequent OTA production in the coffee beans was found to cause health hazards after consumption (Boorman, 1989). There are no comprehensive studies of exact stage of infection and OTA production of level in coffee during processing. Hence a study was taken up at Central Coffee Research Institute during the year 2001 to under stand the most vulnerable stage in wet processing of coffee for ochre infection and OTA production in final beans.

## MATERIALS AND METHODS

Arabica fruits (30 Kgs) collected from Central Coffee Research Institute were equally divided into three parts of 10 Kg each. The divided fruit lots were pulped separately, fermented, washed and dried as per the normal processing method using hand pulper and manual washing. The first lot of 10 Kg fruits was surface sterilized with 1.0% sodium hypo chlorite solution for 10 minutes and then washed three times in sterile water and air dried in laminar airflow chamber. The air-dried fruits were artificially inoculated with *A.ochraceus* spore suspension at 20 ml per Kg fruits ($10^7$ spores/ ml). The infected fruits were incubated for 16 hours and then processed as per the normal processing methods. The second lot of 10 Kg fruits was pulped and infected with *A. ochraceus* similar to earlier method. In the third lot of fruits washed parchment were obtained and then infected with *A.ochraceus* after surface sterilization. Fruits without inoculation were maintained as control. Samples were drawn at different stages of processing for assessing the carry over of *A.ochraceus* in beans.

**Analysis of Internal bean infection**

One hundred beans samples were collected at different stage of processing and surface sterilized with 1.0% Sodium hypochlorite solution for 8-10 minutes and rinsed thrice with sterile water. The sterilized beans were plated on DG 18 medium (Hocking and Pitt, 1980) @ 10 beans per plate. All the inoculated plates were incubated at room temperature for 5-7 days for mycological examination.

**Analysis of Ochratoxin –A (OTA)**

After drying, the dried samples were analyzed for OTA contamination. Ochratoxin analysis was carried out by HPLC method. Extraction and purification of samples were carried out according to the method of Patel Et al., 1997 with the following modifications. Ten gram of powdered samples was extracted with 50 ml CHCl3 and 5 ml 0.1M orthophosphoric acid for half an hour in a mechanical shaker. The extract was filtered using Whatman No.1 filter paper. The filtrate was evaporated under vacuum in rotary evaporator at below 400C. Clean up was performed on silica gel (60-120 mesh) column. Five gram of silica gel was slurred in chloroform and packed a glass column 1.5 X 30 cm with stopper. After silica gel settled, one gram of anhydrous sodium sulfate was added.

The evaporated residue was dissolved in 2 ml CHCl3 (x2) and loaded on to silica gel column and drained by gravity. The column was washed with 50 ml CHCl3- MeOH (97:3) and washes discarded. Ochratoxin –A was eluted with 50 ml of toluene –acetic acid (9:1) and evaporated under vacuum at 400C, transferred to 5ml test tube with toluene –acetic acid (9:1) and evaporated under nitrogen. The residue was dissolved in the HPLC solvent and used for HPLC analysis.

HPLC analysis was performed on a 15 cm Bandaclone RPC-18 Spherisorb column using acetonitrile (55%) water (45%) and acetic acid (2%) as mobile phase with a flow rate of 1ml/min.the excitation maxima was 330 nm and emission at 460 nm. The OTA was detected and quantified by comparison of peak areas of samples and standard OTA. Presence of OTA was confirmed with Boron trifluride reaction with samples extracts (AOAC, 1995). 50 ml of BF3 was added to dried extract, reacted at 600C for seconds, evaporated, dissolved in mobile phase and injected into HPLC column. The OTA peaks were confirmed by changes in the retention time of OTA in samples and standards.

**RESULTS AND DISCUSSION**

The results on carry over of *A.ochraceus* spores from fruits to dried beans are presented in Figure 1.

From the data it was found that the initial ochre load in fruits came down from 74.0% to 11.5% in dried beans after passing through fermentation, washing and drying during wet processing. The total reduction of ochre was found to be 84.5%. This clearly indicated that even if coffee gets infected with ochre during initial stages, its load could be brought down through wet processing.

The data on inoculated ochre incidence in pulped beans and its carry over to dried beans is given in the Figure 2.

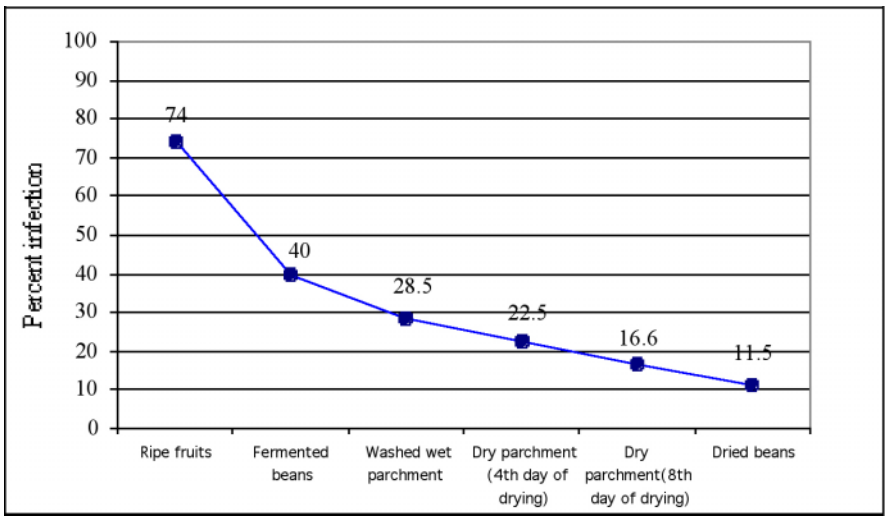


**Figure 1. Survival of inoculated *A. ochraceus* in fruits to dried beans.**

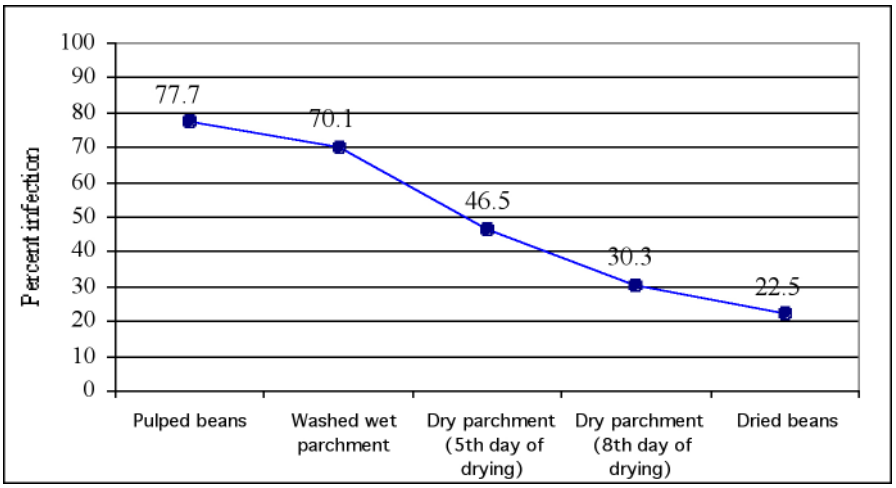


**Figure 2. Survival of inoculated *A. ochraceus* pulped beans to dried beans.**

From the data it was found that the initial ochre load in pulped beans came down from 77.7% to 22.5% in dried beans passing washing and drying process. The total reduction of ochre from pulped beans to dried beans was found to be 71.1%. This indicated that even if ochre enters the coffee during pulped bean stage, its carry over to dried bean could be reduced to minimum level when compared to fruit stage.

The data revealed that initial ochre load in washed parchment came down from 75% to 55.5% in dried beans after drying. The total reduction of ochre from washed parchment to dried beans was found to be 26.0%. This indicated that when ochre enters the coffee during wet parchment stage, its carry over to dried bean could not be reduced considerably **(**Figure 3**)** when compared to infection of ochre at fruit and pulped beans stages.

The OTA contamination in beans from inoculated fruits, pulped beans and washed parchment is given in Table 1. OTA contamination was found in proportion with ochre infection at different stages of wet processing.

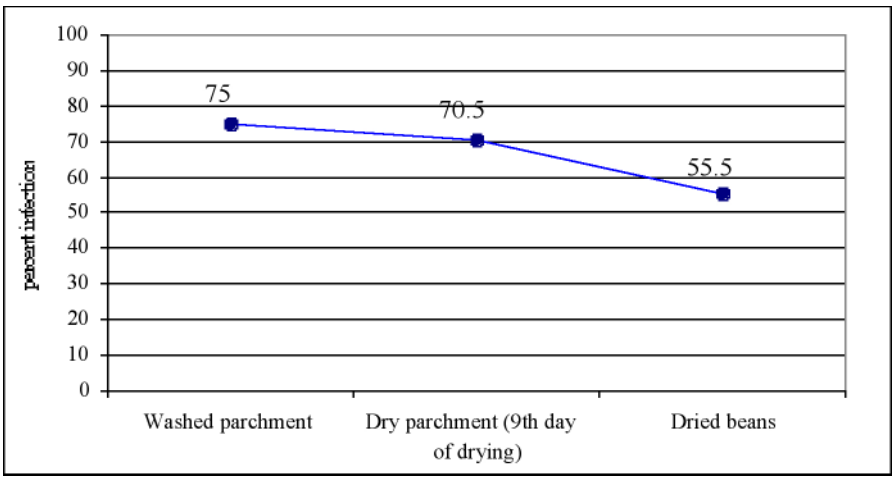


**Figure 3. Survival of inoculated *A. ochraceus* in washed parchment to dried beans.**

**Table 1. Ochratoxin –A contamination in artificially infected coffee samples.**

| Sample details | OTA (ppb) |
|---|---|
| Artificial infection in Fruits | 0.40 |
| Artificial infection in pulped beans | 0.48 |
| Artificial infection in washed parchment | 1.25 |
| Control (No artificial infection) | Not detected |

From this study, it was found that washed parchment is highly susceptible for mould infection as compared to fruit and pulped beans during wet processing of coffee.

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# Quality of "Espresso" Coffee: a Study Performed through Italian Coffee Shops

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## SUMMARY

A study on "espresso" coffee quality was carried out through a large area of the centre and north of Italy. A total of 148 coffee bar were visited during four years (2000-2003) and the "quality in cup" was evaluated by some characteristics such as pH, total solid content, volume, powder of coffee utilised, consistency of cream, rate of extraction. Moreover samples of roasted coffee, used to obtain the brews, were analysed for colour (lightness and red index), moisture, water activity and density of beans in order to verify the homogeneity of the samples after the roasting process, coming from the same roasting company. The homogeneity of roasted samples was proved; moreover tested coffee brew samples resulted quite standardized on the basis of some most important characteristics of espresso quality, despite the known different habit to prepare the "espresso" in different areas of Italy.

## INTRODUCTION

"Espresso" extraction is the most common brewing method in Italy. The Italian "espresso" coffee brew had peculiar chemical, physical and sensorial characteristics that increase its popularity not only in Italy, but also in other countries. The peculiar features of this Italian beverage were influenced by many factors: roasting process, extraction procedure, processing conditions such as water/coffee ratio, coffee grind degree, temperature, pressure and time, together with quality of raw material (Sivetz & Desrosier, 1979; Clarke & Macrae, 1985; Illy & Viani, 1985). The most recent results about the quality of "espresso" coffee concern the influence of water pressure on the coffee quality (Andueza et al., 2002), the characterization of "espresso" obtained by different botanical varieties (Maetzu et al., 2001), the dynamic tensiometric characterization (Navarini et al., 2004) and the preference for "espresso" derived from different blends of coffee beans based on sensory analysis (Cristovam et al., 2000).

The different habits to obtain "espresso" coffee affect chemical, physical and organoleptic characteristics of coffee brew. The preparation of this kind of brew gives good results only if it is carried out under the best and standardised conditions. It is know that the way to prepare the "espresso" may vary, depending on personal preferences and regional traditions. In the present work a study on the quality of Italian "espresso" coffee brew was carried out through a large area of the centre and north of Italy. A total of 148 coffee shops were visited and the "quality in cup" was evaluated by some characteristics of brew. Samples of roasted and ground coffee, collected from every shop, were also analysed for some roasting parameters. On the basis of considered roasting parameters and quality characteristics of tested coffee samples, we try to evaluate the more evident differences between features of "espresso" brews taken in different areas of Italy. The same study had been carried out during previous four years (1996-1999) and results showed a strong difference among beverages based on their features obtained from different areas of Italy (Severini et al., 1997; Severini et al., 1999).

Since then, the trend have been to improve and standardize the quality of "espresso" by training of coffee shop operators.

## MATERIALS AND METHODS

### Raw material

Experiments were carried out on a total of 148 "espresso" coffee beverage and respective beans and ground roasted coffee samples. Samples of brew, roasted and ground coffee were taken in a same number of shops located in different regions of the centre and north of Italy in the course of four years (2000-2003).

### Roasting parameters

To study the uniformity of the roasting process, the following parameters were evaluated on beans and ground roasted coffee samples, coming from the same roasting company, and used to obtain the "espresso" brew:

- *colour change*: using a *tristimulus* colorimeter (Chromameter-2 Reflectance, Minolta, Japan), equipped with a CR 100 measuring head. The standard C.I.E. conditions, with illuminant C (6774K) were used. The instrument was calibrated on a white tile (L* = 95.3, a* = -1.0, b* = 0.8). Colour is expressed in L* (lightness) and red index (a*). The samples were prepared by placing ground coffee or whole beans on suitable supports. Mean values and standard deviations were obtained from five measurements for each sample;
- *density of coffee beans (g/L)*: using a suitable picnometer according to the methodology described by Lerici et al. (1980);
- *moisture content (%)*: determined by mass loss of ground coffee samples, after heating in an oven according to AOAC methods, N° 968.11 (2000). Measurements were made in triplicate;
- *water activity:* using the hygrometer Aqualab CX – 2 (Decagon, Washington, USA).

### Characteristics of espresso coffee

The quality of "espresso" coffee beverage was evaluated by some characteristics such as:

- *total solids* (%): determined by drying the brew to a constant weight in an oven at 105°C;
- *pH*: determined using an electronic pH meter (AMEL 334 – B, Milano, Italy);
- *volume (mL)*: determined by a graduated cylinder;
- *grams of ground coffee used*: by weighing each portion of ground coffee;
- *rate of extraction (mL/s)*: by relating the measured volume of one cup of brew to its extraction time;
- *consistency of cream (s)*: following the methodology described by Severini et al. (1997).

### Statistical Analysis

Principal component analysis (PCA) was used to reduce the number of variables in the data matrix and to select the most discriminating parameters. The statistical package STSG Statistica for Windows, version 6.0 (Statsoft Inc., Tulsa, UK) was used.

## RESULTS AND DISCUSSION

### Roasting parameters

As well recognised, the most important physical changes that occur in coffee beans during the roasting process are colour change and density reduction (Lerici et al., 1987). Each sample of beans and ground-roasted coffee was analysed for the previously mentioned parameters together with the moisture content and water activity; the data are reported in Table 1. Based on these data, the coffee samples roasted at the dark level presented the characteristics suitable to prepare an Italian-style "espresso" coffee brew. The values obtained for these parameters between different samples are within the typical ranges for the defined roasted degrees (Da Porto et al., 1991). As expected this result indicates that the roasting process was uniformly conducted. The highest variability observed for moisture and water activity is probably due to the environmental conditions in which is stored the ground coffee before its extraction. In Table 2 are reported results about the quality characteristics of all analysed "espresso" coffee. On average we can see that the tested brews presented quality attributes nearly all close to those of a well-prepared Italian espresso coffee (pH>5; total solids: 7-8%; powder utilised: 6.5-7 g; rate of extraction: 1ml/s). Nevertheless, rate of extraction and consistency of cream data showed the highest variability; this because of the different way to extract the espresso brew in the different coffee shops. Moreover, it is known (Dalla Rosa et al., 1986) that a multitude of variables, that interact each others at the same time, can affect the foam stability and consistency of coffee brew.

**Table 1. Overall results of coffee samples roasting parameters.**

| | | ***Mean*** | ***Min.*** | ***Max.*** | **± S. D.** | **V.C.%** |
|---|---|---|---|---|---|---|
| **Beans** | Lightness (L*) | 26.26 | 26.25 | 29.14 | 1.44 | 5.47 |
| | Red index (a*) | 3.83 | 3.82 | 4.54 | 0.40 | 10.36 |
| | Density (g/l) | 0.56 | 0.51 | 0.60 | 0.02 | 3.19 |
| **Ground** | Lightness (L*) | 27.81 | 25.00 | 30.78 | 1.33 | 4.79 |
| | Red index (a*) | 7.35 | 5.85 | 9.03 | 1.00 | 13.63 |
| | Water activity ($a_w$) | 0.16 | 0.09 | 0.27 | 0.05 | 29.85 |
| | Moisture (%) | 1.14 | 0.03 | 3.32 | 0.47 | 40.81 |

**Table 2. Overall results of "espresso" coffee brew quality parameters.**

| | | ***Mean*** | ***Min.*** | ***Max.*** | **± S. D.** | **V.C.%** |
|---|---|---|---|---|---|---|
| **Coffee brew** | pH | 5.60 | 5.21 | 6.02 | 0.19 | 3.38 |
| | Total solids (%) | 7.73 | 4.35 | 12.39 | 1.44 | 18.59 |
| | Volume (ml) | 22.04 | 14.00 | 33.00 | 3.92 | 17.80 |
| | Powder utilised (g) | 7.12 | 6.00 | 9.00 | 0.42 | 5.90 |
| | Rate of extraction (ml/s) | 0.95 | 0.40 | 2.73 | 0.30 | 31.99 |
| | Consistency of cream (s) | 2.79 | 0.50 | 10.00 | 2.11 | 75.82 |

To better assess which factors were the most important to describe the quality of espresso coffee and to determine differences among considered brew samples, obtained from coffee bar located in different areas of Italy, a principal component analysis (PCA) was performed. Figure 1 (a and b) shows bidimensional representations of PC1 and PC2 scores for all the variables and espresso samples, respectively. PC1 and PC2 explain the 58.83% of the total variance. The first PC (PC1) explains 38.08% and the second (PC2) explains 20.75% of the total variance. The variables total solid content, volume of one cup of brew and rate of

extraction are the descriptors of highest contribution to PC1; while only consistency of cream to PC2. As seen, the percentage of soluble solid decreased in the brews with high volume and extracted quickly. This important characteristic of "espresso" did not seem to depend on ground coffee portion used. High consistency of foam is related to high quantity of ground coffee portion and to high pH values. We can see, as shown in Figure 1b, that some espresso samples from the centre-east of Italy had higher soluble solid content and a cream more consistent than the coffee brews from the northwest of Italy. Volume and rate of extraction appear at the right side of the plot with some of espresso coffee samples from the north-west of Italy, that present higher values of the mentioned descriptors. Samples from centre-west of Italy were the most grouped in the middle of figure. Generally, both PCs did not separate the tested espresso coffee samples well; actually, most of them are grouped in the middle of score plot. In conclusion, after applying PCA method we cannot easily differentiate espresso coffee beverages according to their most important characteristics. On the basis of selected variables it seems that tested coffee brew samples resulted quite standardized despite the known different habit to prepare the "espresso" in different areas of Italy. Moreover, results show that a suitable training of coffee shop operators could lead to a standardized high quality of "espresso" coffee.

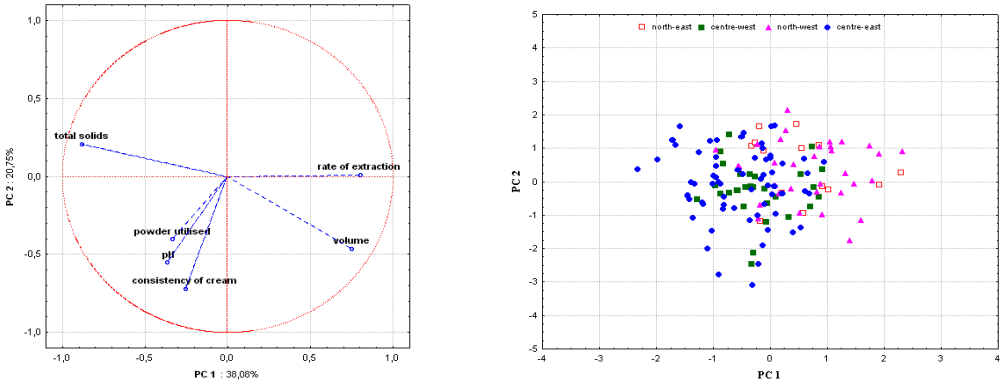


**Figure 1. Principal component projection of espresso coffee samples from different area of Italy. (a) Loading plot for coffee variables (b) Score plot of espresso coffee samples.**

## ACKNOWLEDGEMENTS

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# Effect of Caffeine on Fungal Growth

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## SUMMARY

Studies conducted on coffee beans revealed that the monsooned coffee beans had significantly lower caffeine content than the cherry and parchment coffee in Robusta variety compared to Arabica. Also, monsooned coffee beans had more number of fungal species associated with it than the non-monsooned beans and so the effect of caffeine on fungal growth was studied. Fungi like *Rhizopus stolonifer* and *Absidia heterospora* failed to grow even at a low concentration (5 mg caffeine/ml of MEA) while two species of *Penicillium* showed sparse growth. Fungi of *Aspergillus* group grew fairly well even up to the highest concentration used i.e., 20 mg caffeine/ml of MEA. An evident reduction in growth on solid media was observed on a daily basis as the concentration increased. At each concentration (5, 10, 15 and 20 mg caffeine/ml of MEA), the fungi showed an initial lag phase in their growth as measured by its percent reduction when compared to control but increased exponentially as the days of incubation increased. Microscopic examination revealed that there was a great reduction in the sporulation, formation of pigments in the spores, and branching of phialides as the concentration of caffeine increased. Using a gradient-HPLC system consisting of a phenylhexyl column it was found that one potential species out of the 9 fungal species isolated from coffee beans was capable of using caffeine as the sole nitrogen source and thereby degrading it completely in synthetic liquid media. Results point out the potential use of this fungus in bringing about decaffeination of coffee using fungal enzymes.

## INTRODUCTION

Phytochemical compounds such as caffeine are secondary metabolites secreted by the coffee plant for protection against insect pests and fungal diseases. In spite of high caffeine contents, there are various microorganisms like bacteria and fungi found associated with the coffee plant and coffee beans. Monsooned coffee is a specialty coffee from India whose coffee beans are characterized by higher moisture contents and lesser caffeine than non-monsooned coffee beans. Therefore our work was to isolate fungi from these coffee beans, determine the effect of different concentrations of caffeine on their morphology and growth, and also find the possibility of bringing about decaffeination using these fungi.

## MATERIALS AND METHODS

The fungi studied were *Aspergillus niger, A. ochraceous, A. tamarii, A. flavus, Penicillium* sp., *P. rugulosum, Absidia heterospora, Rhizopus stolonifer* and *Verticillium* sp. which were isolated from the coffee beans using the Standard Blotter Method (Neergaard, 1977). Fungi were inoculated by the point inoculation method on malt extract agar (MEA) plates (solid media) for 7 days (pH 5.5) at varying concentrations of caffeine (0 to 20 mg/ml) and in 50 ml of synthetic Czapex Dox agar (CDA) containing 1mg of caffeine/ml of CDA. The measurements of the control were used to calculate the percentage of inhibition of the fungus due to the influence of caffeine. The level of caffeine in the samples growing on liquid synthetic media (CDA) was measured by HPLC analysis (ISO, 2002). All experiments were

carried out in replicates. The effect of the caffeine on the fungal growth rate was measured by multifactorial analysis using Minitab release 8.0 version.

## RESULTS AND DISCUSSION

Monsooned coffee beans had more number of fungal species associated with it than the non-monsooned beans and fungi such as *Penicillium* sp., *P. rugulosum* and *Absidia heterospora* were found only in the monsooned coffee beans (data not shown). The occurrence of other species of fungi found on the non-monsooned beans was also higher in the monsooned beans which was due to the higher moisture and changed substrate conditions of the specialty coffee called Monsooned coffee (Tharappan, 2003). Also, monsooned coffee beans had significantly lower caffeine content than the cherry and parchment coffee in Robusta variety compared to Arabica and so the effect of caffeine on fungal growth was studied.

*Rhizopus stolonifer*, though by far the fastest growing fungi of the lot (as seen in control), failed to grow even at the lowest concentration of caffeine i.e., 5 mg/ml of MEA (Figure 1). The other Phycomycete, *Absidia heterospora* showed sparse growth at a caffeine concentration of 5 mg/ml of MEA only and this growth happened after five days of inoculation. The percent reduction in its growth was around 96-98% compared to the control. *Verticillium* sp. grew very slowly at concentrations of only 5 and 10 mg/ml of caffeine in MEA and not at higher concentrations. Growth occurred after three to four days of incubation but this growth was faint compared to the control.

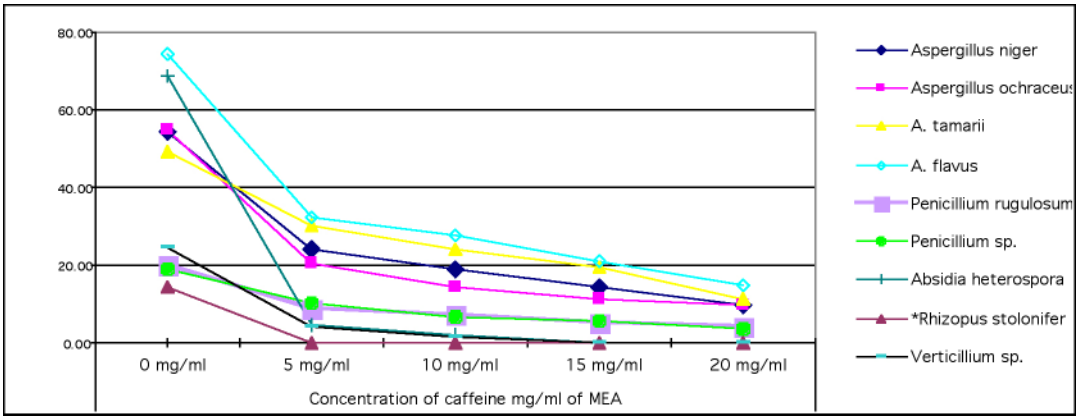


**Figure 1. Growth of fungi at varyng concentrations of caffeine on the seventh day of incubation on malt extract agar (colony diameter in mm).**

Both the *Penicillial* species studied showed similar growth patterns to the different treatment conditions. Both species were slow growing and took time to establish on media. All the *Aspergillus* spp. grew fairly well even up to the highest concentration used i.e., 20 mg/ml of MEA. An evident reduction in growth was observed on a daily basis as the concentration increased i.e., from 5 to 20 mg/ml of MEA. At each concentration the fungi showed an initial lag phase in their growth as measured by its percent reduction when compared to control but increased exponentially as the days of incubation increased (data not shown). On the seventh day of incubation, *A. niger* and *A. ochraceous* showed higher percent reduction in their growth (81.8%) at 20 mg/ml of caffeine in MEA while *A. flavus* showed 79.8% followed by *A. tamarii* at 71.4%.

There was great variation in the morphology of the fungal mycelia and spores with an increase in caffeine concentration (on solid media). Microscopic observation revealed that there was less branching of phialides, a great reduction in sporulation, and the spores were

immature lacking pigmentation and spines indicating that at higher concentrations caffeine was interfering with the physiology of the fungi. Studies point out that some *Aspergillus* sp. were capable of using caffeine as the sole carbon source at low concentrations which may be because of the toxicity being overcome by deliberate induction of detoxification system by the microbe, so as to render the caffeine as a safe substrate but at higher concentrations it proved toxic by interfering in its physiology.

In liquid media *Aspergillus* sp. isolate 1 brought about complete degradation of caffeine when it was given as the sole nitrogen source, as determined by HPLC analysis (Figure 2) and the biomass produced was also substantial when compared to control (Table 1). Isolate 2 was capable of growing in the caffeine supplemented media but only when an external nitrogen source ($NaNO_3$) was furnished and the biomass produced was comparable to the control. When only caffeine was supplied there was no appreciable growth seen. Some workers have reported species of fungi such as *Aspergillus, Penicillium, Trichoderma* and *Humicola* to have caffeine degrading ability out of which five *Asperillus* strains and two *Penicillium* strains could degrade 100% of caffeine in liquid medium (Roussos et al., 1994). Also (Gutiérrez-Sánchez et al., 2003) on their studies on *A. tamarii* reported that caffeine degradation and biomass production were affected with the addition of a simple nitrogen source such as $(NH_4)_2SO_4$ which greatly reduced the lag phase. On solid media clear zones were observed around *Aspergillus* colonies but their occurrence couldn`t be justified. Also unsuccessful extraction of caffeinases from solid culture has been reported (Hakil et al., 1999) perhaps owing to the intracellular nature of the decaffeinases. The breakdown product of caffeine couldn`t be identified however it may be possible to do so by the new HPLC method which enables the identification of caffeine demethylating metabolites present in the supernatant of the fungal culture in liquid medium (Denis et al., 1998). So further work is needed to ascertain the breakdown products of caffeine, find the conditions and mechanism of action of the decaffeinase enzyme, the use of carbon source and other nitrogen sources. Use of microbial enzymes to bring about decaffeination will be a very viable method in terms of feasibility, ease of operation, biosafety and expenses involved.

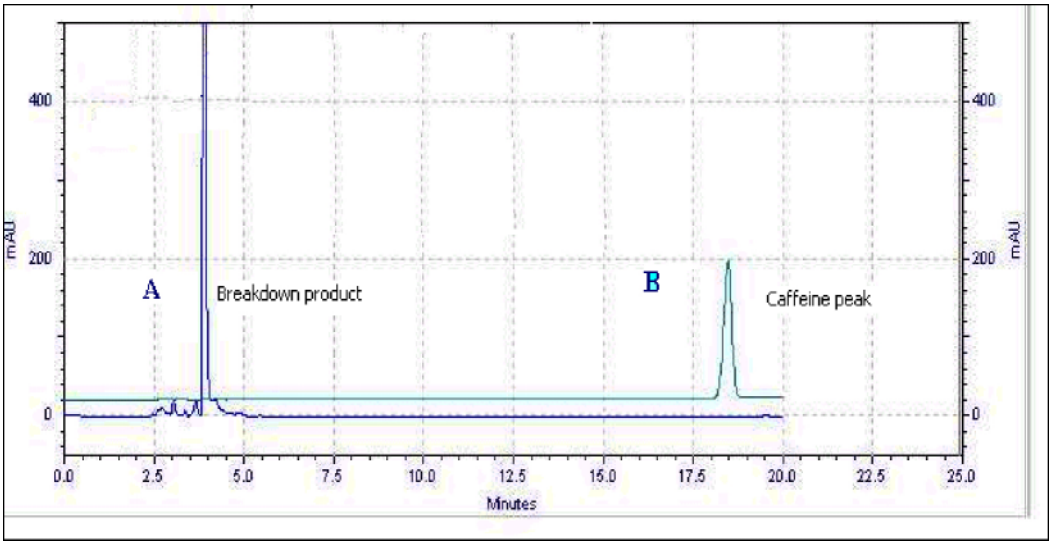


**Figure 2. HPCL chromatograms of samples initially containing 1 mg of caffeine/ml CDA medium. A. Sample of Aspergillus isolate 1 groown on CDA medium containing caffeine; B. Uninoculated CDA medium containing caffeine.**

**Table 1. Effect of caffeine on biomass production and pH of CDA media.**

| | *Aspergillus* sp. Isolate 1 | | | *Aspergillus* sp. isolate 2 | | |
|---|---|---|---|---|---|---|
| | Biomass g/l | PH | | Biomass g/l | pH | |
| Treatment | | initial | final | | initial | final |
| Control (with nitrogen source) | 8.52 ± 1.36 | 7.0 ± 0.0 | 7.0 ± 0.0 | 7.57 ± 0.84 | 7.0 ± 0.0 | 4.7 ± 0.6 |
| Control (without nitrogen source) | 1.35 ± 0.15 | 6.8 ± 0.3 | 6.0 ± 0.0 | 1.37 ± 0.26 | 7.0 ± 0.0 | 5.0 ± 0.0 |
| Only caffeine | 7.13 ± 0.52 | 6.8 ± 0.3 | 4.0 ± 1.0 | 1.31 ± 0.10 | 6.5 ± 0.0 | 4.7 ± 0.3 |
| Caffeine+nitrogen source | 8.08 ± 1.40 | 7.00 ± 0.0 | 5.80 ± 0.3 | 7.89 ± 1.75 | 7.0 ± 0.0 | 3.8 ± 0.8 |

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# Distribution of *Aspergillus ochraceus* in Major Coffee Growing Regions of India

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## SUMMARY

During 2001 and 2002 a total of 65 estates have been surveyed in Karnataka, Kerala and Tamil Nadu to understand the distribution of *Aspergillus ochraceus* in coffee. The occurrence of ochre mould was observed to be more in soil followed by beans and air in arabica when compared to robusta estates. Presence of ochre mould in soil appeared to be a predisposing factor for ochre incidence in the final product. Arabica coffees were less likely to be get infected by ochre mould compared to robusta coffee. Higher incidence of ochre mould was observed in dry processed coffee compared to wet processed. In drying yards, mud surface recorded higher incidence of ochre mould. The farm practices viz., picking method; use of harvest mats, fermentation, layer thickness, stirring frequencies had no definite relationship on the presence of ochre mould in final bean. The incidence of ochre infection had positive relationship with higher altitude and high rainfall. Both in arabica and robusta higher ochre incidence were noticed in estates situated at higher elevations (900-1100 m) and high rainfall (1500-3000 mm). There appears to be a wide variation among different isolates of *Aspergillus ochraceus* in their OTA production capability.

## INTRODUCTION

Coffee occupies a prime position among the plantation crops in India. India grows both arabica and robusta coffee over an extent of 3,40,000 ha, over a wide range of agro-ecological conditions. The coffee quality is influenced by several internal and external factors, among which microbial contamination is known to bring down the quality considerably. In recent years, the occurrence of Ochratoxin-A (OTA) in coffee has gained great concern in coffee trade. OTA is reported to be a nephrotoxic mycotoxin and suspected carcinogen (Boorman, 1989) produced by moulds *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum* in coffee. Levi et al. (1974) first reported the natural occurrence of OTA in coffee. In India the presence and distribution of OTA producing fungi in coffee growing regions has not been studied earlier in detail. In this paper the distribution of OTA producing fungi in traditional coffee growing regions of India is discussed in detail.

## MATERIAL AND METHODS

Under the mould survey programme a total of 65 coffee estates were surveyed in the traditional coffee growing states of Karnataka, Kerala and Tamilnadu during 2001 and 2002 with the participation of Dr. Mick Frank, International Mycologist. These 65 estates include the 16 estates, which were revisited during 2002 for confirming the Ochre incidence in final

product. The estates surveyed included both arabica and robusta processed by wet and dry methods. During the survey, information on climatic conditions, cultivation & processing practices was collected.

Soil, air-surrounding drying yard, drying yard swabs (Note: In case of robusta estates, sampling of drying yard surface could not be done) and final dried product (parchment and cherry) samples were collected for mycological analysis as well as for organoleptic evaluation. The mycological analysis and enumeration of mycoflora was done as per the methods prescribed in "Handbook of Mycological Methods".

## RESULTS AND DISCUSSION

### Mould survey - 2001

The details of estates surveyed during 2001 and 2002 are presented in Table 1.

**Table 1. Details of mould survey during 2001 & 2002.**

| | |
|---|---|
| Total number of estate surveyed | 65 |
| Karnataka<br>Kerala<br>Tamilnadu | 32<br>10<br>23 |
| Total number of Arabica estates | 47 |
| Total number of Robusta estates | 18 |

During the year 2001, thirty-three coffee farms processing both arabica and robusta by wet and dry methods were visited in five zones distributed over three major coffee growing states of the country. Four estates were surveyed from Baba Budan Giris, Koppa and Gonikoppal zones of Karnataka. 10 farms of Wyanad region of Kerala and 11 farms of Pulneys region in Tamil Nadu were surveyed.

In Karnataka, higher incidence of *A. ochraceus* was observed in the bean of only 3 estates (16-24.4%) out of 12 estates surveyed. The occurrence of *A. ochraceus* incidence was high in parchment samples of Koppa zone followed by Giris as compared to Gonikoppal. In Gonikoppal zone, *A. ochraceus* was not observed in soil and air, but was noticed in the final product of cherry in the range of 1.08-4.8%. In which *A. niger* was found to be predominant in cherry samples (69.5%-93.5%). The frequency of *A.ochraceus* was found to be less in Wyanad zone of Kerala region as compared to Karnataka. *A.ochraceus* incidence was observed in cherry samples of only 3 (1.0-7.9%) of 10 estates surveyed. The incidence of ochraceus was not observed in soil and air of nine estates. *A.niger* incidence was found to be predominant in all cherry samples (28.0-92.0%) when compared other filamentous fungi. In Pulneys zone of Tamil Nadu, *A. ochraceus* was observed in the samples (6.2%-11.7%) of 3 estates. In most of the estates, the fungi presence was noticed in soil and air from drying yard, but was not observed in parchment and cherry samples. Yeast association was observed to be dominant in most of the coffee samples from Tamil Nadu region.

### Mould survey - 2002

Based on the results of first survey, the estates with incidence of *Ochre* group in the final produce were identified for taking up follow up studies during Year 2002.

During this period, a total of 35 estates (Re-sampled and newly visited farms) were surveyed for studying the frequency of occurrence of OTA moulds in coffee plantations.

In Karnataka state, a total of 20 estates were surveyed, which included 17 arabica and 3 robusta. The survey was conducted in Koppa (4 estates), Giris (3 estates), Balehonnur (4 estates), Mudigere (7 estates) and Gonikoppal (2 estates) zones. Among the estates surveyed, 11 estates (4 in Balehonnur and 7 in Mudigeri) were surveyed for first time and the remaining estates (3 in Giris, 4 in Koppa and 2 in Gonikoppal) were re-visited to confirm the presence of OTA moulds in the final product.

Out of 11 new estates surveyed, only two estates one each from Balehonnur (2.8%) and Mudigeri zones (4.0%) had incidence of ochre group fungi in the final product. Coffee samples from Balehonnur zone had Niger group aspergilli as predominant sp, while those from Mudigere zone had the yeast. In the re-surveyed estates, all the three estates in Giris zone (arabica) were found to be free from ochre incidence in the final product. On the other hand, two robusta estates out of four in Koppa zone (4%) and both the re-surveyed robusta estates in Gonikoppal region (31-32%) had ochre group aspergilli in the final product. This indicated that out of 9 estates, which were positive for Ochre incidence during the year 1 survey, only 4 proved to be positive for *A.ochraceus* in the second year.

In Kerala, primarily a robusta zone, totally three estates were surveyed in which one was surveyed for first time. Samples from all the three estates had much lower Ochre infection of 4-6% with Niger group aspergilli as predominant sp. In Tamil Nadu, survey was conducted in 12 estates of Pulneys zone. Out of these, six estates were revisited for confirming the presence of *A. ochraceus* in final beans. Among the re-surveyed and newly surveyed estates, five estates from each group had incidence of *A.ochraceus* (2% to 25%).

From the two years survey of 65 farms comprising arabica and robusta, occurrence of ochre group was very high in soil samples (72.3% and 44.4%) in arabica and robusta respectively) followed by occurrence in bean (51.1% and 61.1% respectively in arabica and robusta) and air samples (36.8% and 8.3% respectively in arabica and robusta) (Table 2). Further, ochre distribution was found very high in arabica bean samples when the rainfall ranges from 2500-3500mm and at an elevation of 1100-1650 MSL. Where as the same was vice versa in robusta farms and higher ochre incidence was found when the rainfall ranges from 1500-2500 mm at elevation of 700-1100 MSL (Figure 1 & 2). Distribution of ochre in soil followed the same trend as in case of bean samples both in arabica and robusta (Figure 3 & 4). No definite relation could be established between the Ochre incidence in the final beans and various climatic conditions, cultural practices and processing conditions (Table 3).

There appears to be a wide variation among different isolates of *Aspergillus ochraceus* in their OTA production capability (Table 4), as indicated by the fact that OTA in beans is independent of ochre population on the beans. Besides this, bean samples from certain estates had higher OTA than those from other estates in the same region.

**Table 2. Distribution of ochre group aspergilli in coffee plantations**.

| Sample | Arabica | | Robusta | |
|---|---|---|---|---|
| | Total No.of + ve Estates | % Distribution | Total of No.+ ve Estates | % Distribution |
| Soil | 34/47 | 72.3 | 8/18 | 44.4 |
| Air | 14/47 | 36.8 | 1/18 | 8.3 |
| Bean | 24/47 | 51.0 | 11/18 | 61.1 |

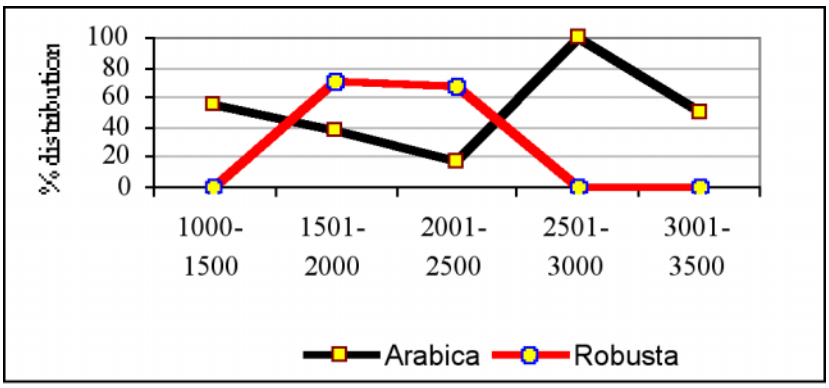


**Figure 1. Distibution of Ochre in final bean samples in relation to rainfall (mm).**

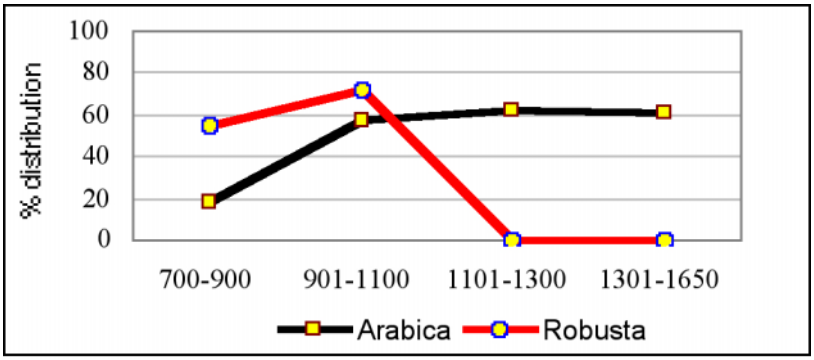


**Figure 2. Distribution of Ochre in final bean samples in relation to elevation (Meters).**

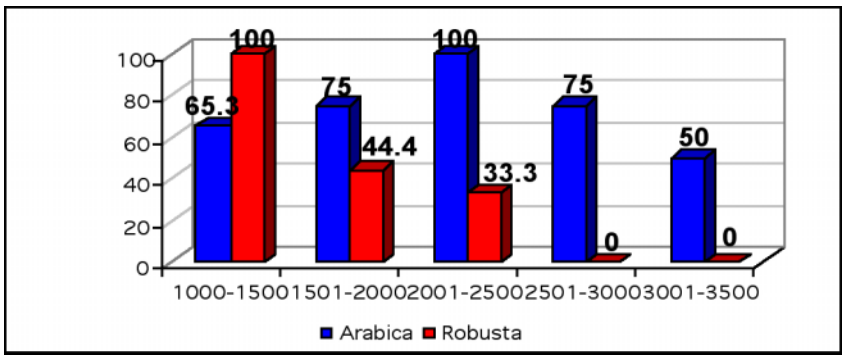


**Figure 3. Distribution of Ochre in soil in relation to rainfall.**

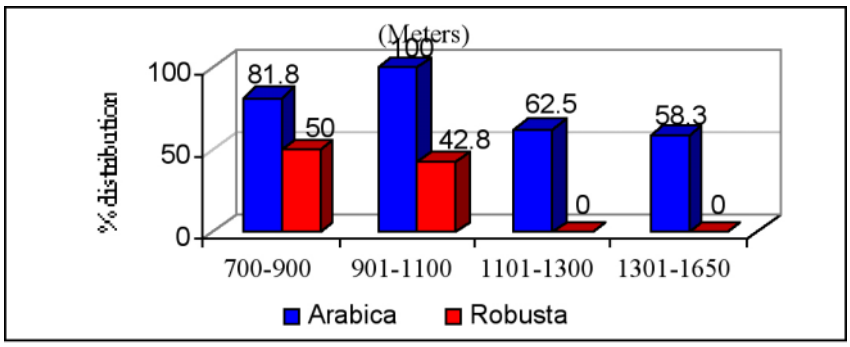


**Figure 4. Distribution of Ochre in soil in relation to elevation.**

**Table 3. Distribution of Ochre group aspergilli in relation to on farm practices.**

| On farm practices | Arabica | | Robusta | |
|---|---|---|---|---|
| | Total No.of + ve Estates | % Distribution in beans | Total of No.+ ve Estates | % Distribution in beans |
| Selective picking | 24/46 | 52.1 | 11/18 | 61.1 |
| Harvesting mats | 24/47 | 51.0 | 11/16 | 68.0 |
| Wet process | 24/46 | 52.1 | - | - |
| Dry process | 0/1 | 0.0 | 11/18 | 61.1 |
| Fermentation | 8/15 | 53.3 | - | - |
| Direct wash | 16/32 | 50.0 | - | - |
| **Drying yard** | | | | |
| a) Cement | 14/24 | 58.3 | 6/7 | 85.7 |
| b) Tiles | 10/19 | 52.6 | 0/1 | 0.0 |
| c) Mud | - | - | 5/8 | 62.5 |
| d) Cow dung | 0/3 | 0.0 | 0/2 | 0.0 |
| **Drying Thickness** | | | | |
| a) 2-3 cm | 15/26 | 57.6 | 11/16 | 68.75 |
| b) 4-5 cm | 8/21 | 38.0 | 0/1 | 0.0 |
| c) 6-7 cm | - | - | 0/1 | 0.0 |
| **No. of Stirring/day** | | | | |
| a) No stirring | - | - | 4/6 | 66.6 |
| b) 2-3 | 2/3 | 66.0 | 1/3 | 33.3 |
| c) 4-5 | 13/25 | 52.0 | 2/5 | 40.0 |
| d) 6-8 | 9/19 | 47.3 | 4/4 | 100.0 |

**Table 4. OTA mould and OTA contamination in different coffee growing regions.**

| Farm code | Coffee | % Ochre in beans | OTA (ppb) |
|---|---|---|---|
| **Tamil Nadu** | | | |
| PCE | Arabica.Parch | 3.00 | 2.64 |
| NCP | Arabica.Parch | 3.00 | 2.40 |
| ARK | Arabica.Parch | 13.0 | 3.51 |
| PNK | Arabica.Parch | 3.00 | 3.67 |
| NOP | Arabica.Parch | 10.0 | 4.00 |
| SJP | Arabica.Parch | 8.00 | 6.16 |
| PJP | Arabica.Parch | 2.00 | 12.7 |
| CDF | Arabica.Parch | 25.0 | 15.5 |
| **Karnataka** | | | |
| KTM | Robusta. Chy | 31.0 | 1.83 |
| SLC | Robusta. Chy | 32.0 | 3.80 |
| **Kerala** | | | |
| RKS | Robusta. Chy | 4.0 | 1.10 |
| NVT | Robusta. Chy | 4.0 | 0.86 |

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# Microbial Biodiversity of Arabica and Robusta Coffee Ecosystem of Coorg Region – Karnataka, India

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## SUMMARY

Samples of rhizosphere, phyllosphere, caulosphere and pomosphere of coffee; rhizosphere of pepper and shade tree were collected for microbial analysis from five arabica and robusta farms in Coorg district, Karnataka. The total CFU's, bacteria, fungi, yeast and actinomycetes and other beneficial microorganisms like Pseudomonas, Azotobacter, Phosphorous solubilizing bacteria and carbohydrate utilizing microbes were enumerated for their quantitative and qualitative nature in coffee ecosystem. Soil samples were also subjected for physico-chemical properties. In general, higher microbial population was recorded in arabica farms compared to robusta farms. Bacterial and yeast population was normal in all the farms, but the population of fungi and actinomycetes was found to be very low. The bacterial population in arabica farms was found to be higher as compared to robusta farms and in the case of fungi it was vice versa. pH of soil had negative relationship with the total microbial population. There was no definite relationship between organic carbon and phosphorous status of soil to the microbial load. Higher coarse sand, fine sand and silt content in soil was found to affect the microbial population, where as higher clay content found to have positive relationship.

## INTRODUCTION

Microorganisms are fundamentally important component of the soil habitat, where they play key roles in ecosystem functioning through controlling nutrient cycling reactions essential for maintaining soil fertility and also contributing to the genesis and maintenance of soil structure.

In coffee, there are no comprehensive studies on microbial association and distribution in coffee ecosystem in the world. In view of the above, preliminary studies have been initiated on the association and distribution of microbial communities in traditional coffee growing regions of India.

## MATERIALS AND METHODS

The soil and plant samples from five arabica and robusta farms were collected in Coorg region, Karnataka during the year 2003-04. Sample were analysed for soil texture and nutrient status as per the standard soil analysis method.

Microbial counts were made on selective media (Nutrient agar for bacteria, Czapek agar for fungi, Kenknight agar for actinomycetes and buffered yeast agar for yeast) after decimal dilution of soil samples ($10^4$) using sterile 0.1% peptone water by pour plate method and the

plates were incubated at optimum temperature in triplicates. The colonies that appeared after incubation period were counted as Colony Forming Units (Cfu)/g of sample. The colony characteristics were observed and representative single colonies were isolated and sub cultured on respective media. Cell morphology was observed microscopically after staining. All the bacterial cultures were identified according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Identification of yeast and fungi were done as per the Manual "Illustrated genera of imperfect fungi" (Barnett, 1960).

## RESULTS AND DISCUSSION

The elevation in arabica farms ranged between 2900-3000 MSL while in robusta farms it was 2560-3036 MSL and the rainfall in arabica farms was 40-53 inches while in robusta it was 40-50 inches/ annum

The physical and chemical properties of the farms selected for the study are presented in Table 1. In general, the pH of both arabica and robusta soils ranged 5.8-6.8, which is ideal for coffee. The organic carbon level in arabica soil was found to be in the range of 2.68-3.35%, while in robusta it was 1.50-3.10%.

**Table 1. Physical and Chemical properties of selected farms.**

| Farms | pH | EC dSm1 | OC % | P (Kg) | K (Kg) | Size separates % | | | | Texture |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | C.S | F.S | Silt | Clay | |
| **Arabica** | | | | | | | | | | |
| Kudaloor-U | 6.5 | 0.009 | 3.35 | 20 | 320 | 19.0 | 38.0 | 26.0 | 20.0 | SCL |
| Salisbury | 6.8 | 0.008 | 3.19 | 38 | 380 | 33.9 | 40.1 | 10.0 | 16.0 | SL |
| Kothagiri | 5.9 | 0.006 | 2.79 | 24 | 290 | 26.8 | 41.2 | 10.0 | 22.0 | SCL |
| Kohinalli-B | 5.8 | 0.008 | 2.68 | 62 | 280 | 25.5 | 40.5 | 14.0 | 20.0 | SC |
| Kudaloor-G | 6.8 | 0.008 | 3.20 | 40 | > 400 | 30.3 | 41.7 | 10.0 | 18.0 | SL |
| **Robusta** | | | | | | | | | | |
| Basavanagudi | 6.8 | 0.013 | 3.10 | 108 | 400 | 32.3 | 39.1 | 12.0 | 16.0 | SL |
| Maldare-K | 6.7 | 0.008 | 2.52 | 64 | 330 | 28.3 | 35.7 | 12.0 | 24.0 | SCL |
| Maldare-G | 6.5 | 0.006 | 2.06 | 04 | 270 | 27.9 | 34.1 | 8.00 | 30.0 | SCL |
| Maldare-N | 6.5 | 0.007 | 1.50 | 53 | 290 | 40.5 | 37.5 | 6.00 | 16.0 | SL |
| Thappa | 6.5 | 0.013 | 2.12 | 22 | 250 | 35.0 | 37.0 | 8.00 | 20.0 | SCL |

In general the coarse sand and clay fraction was more in robusta soil than arabica soils and the fine sand and silt fractions were found to be more in arabica soils. The soil textural classes of both arabica and robusta were found to be Sandy loam to sandy clay loam.

The microbial distribution in rhizosphere of arabica and robusta farms is presented in Figure 1 & 2. From the data it was found that populations of bacteria, actinomycetes and yeast was more in arabica compared to robusta, where as the fungal population was more in robusta rhizosphere. This could be due to non-spraying of Bordeaux mixture and other copper fungicides in robusta and is very common in arabica. The total Cfu/gram of soil was also high in arabica than robusta. The data on microbial distribution in Coffee phyllosphere (leaf surface), Caulosphere (stem surface) and pomosphere (fruit surface) are presented in Figure 3, 4 & 5. In phyllosphere, robusta recorded higher total Cfu than arabica; this is mainly because of larger leaf area and lesser cuticle wax in robusta leaves. In caulosphere, the total Cfu was more in arabica than robusta, with lesser fungal counts than robusta. In pomosphere, arabica recorded higher total Cfu, bacterial and yeast counts but fungal and actinomycetes counts were more on robusta fruits. The higher population on arabica fruits could be attributed to

more sugar content of the fruit and very favorable weather conditions during the ripening period with generally falls during Nov-Dec.

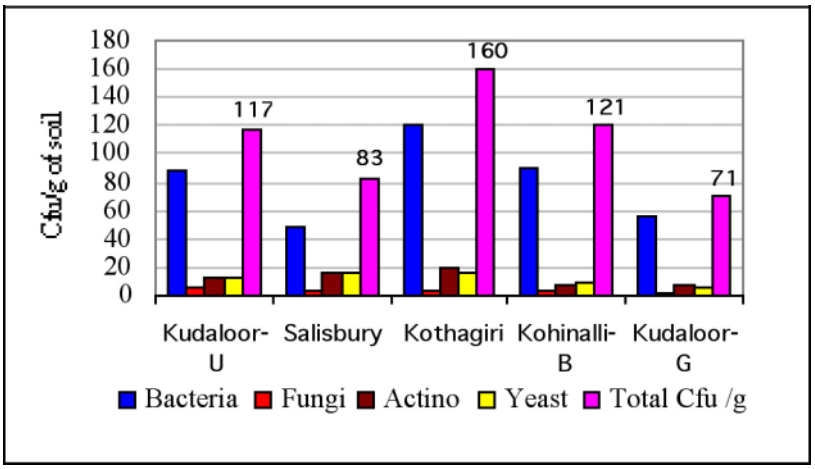


**Figure 1. Soil microbiological properties of arabica farms.**

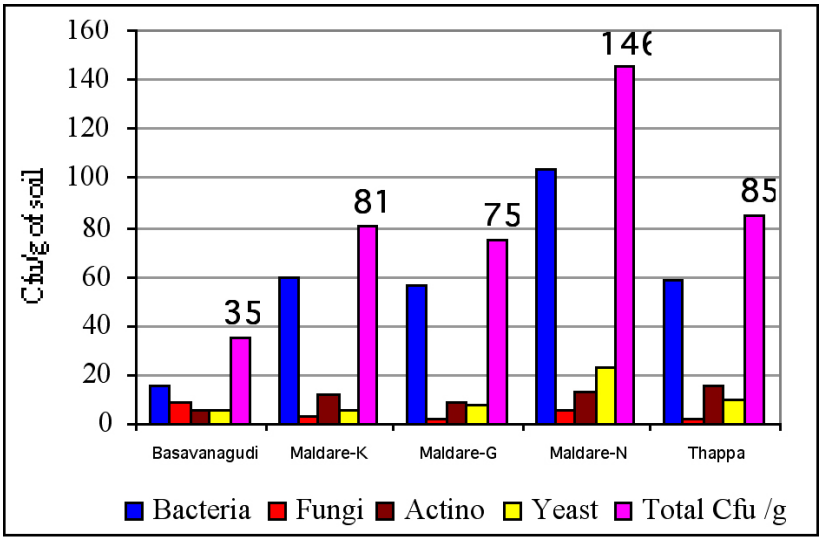


**Figure 2. Soil microbiological proprerties of robusta farms.**

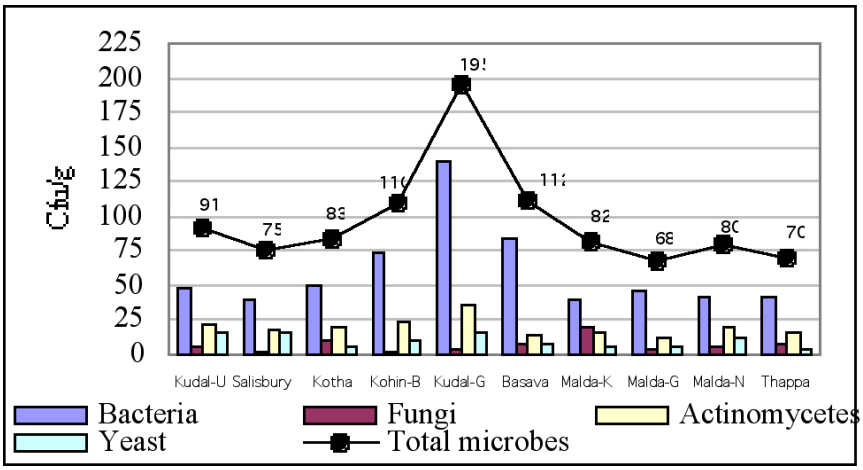


**Figure 3. Microbes associated with Caulosphere of coffee.**

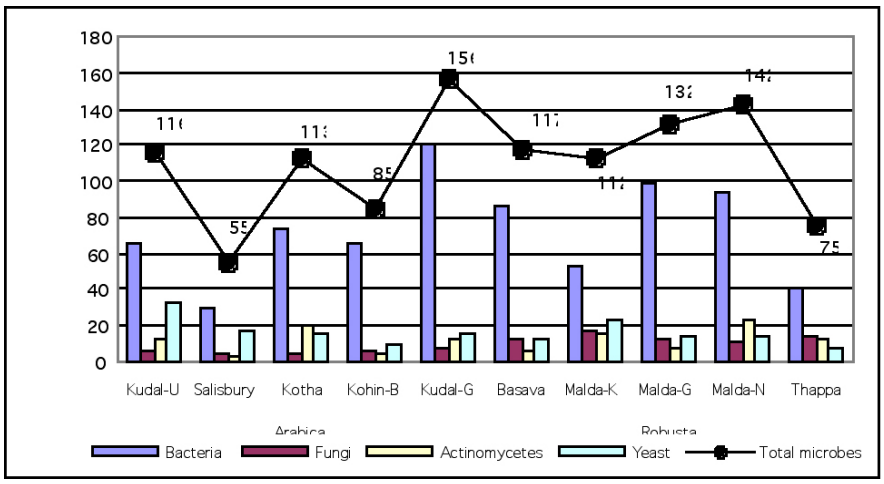


**Figure 4. Microflora associates with coffee phyllosphere.**

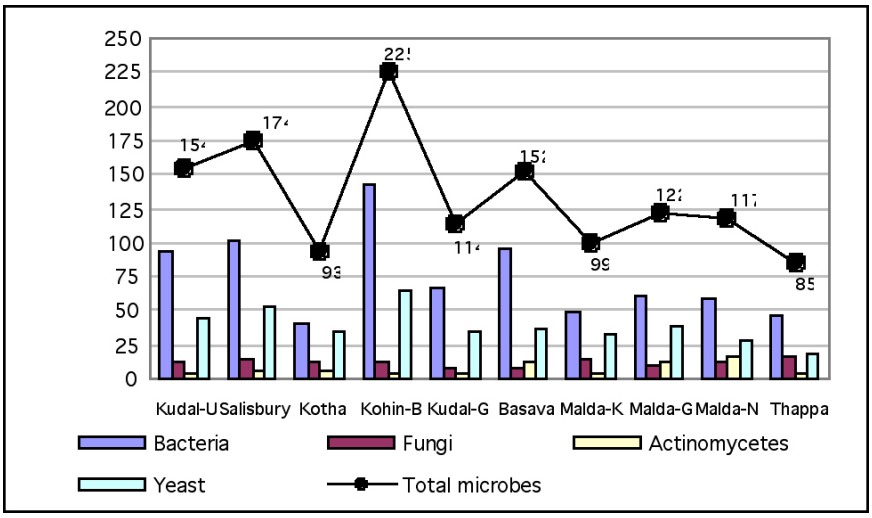


**Figure 5. Microbes associated with coffee pomosphere.**

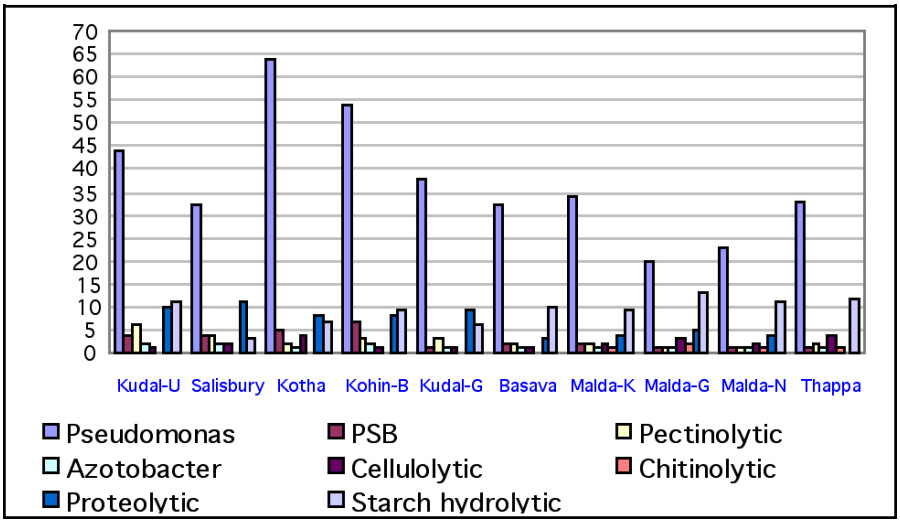


**Figure 6. Beneficial microbes of coffee rhizosphere in select farms.**

The microbial distribution in pepper and shade tree rhizosphere of arabica and robusta farms were presented in Table 2. In pepper rhizosphere, bacterial and actinomycetes population was more on pepper grown in arabica farms than robusta farms, but the population of fungi and

yeast was vice versa. The total microbial population per gram of soil was more in arabica than robusta farms. The same trend was observed in shade tree sample also with respect to arabica and robusta.

Analysis of rhizosphere samples of arabica and robusta for beneficial microbes is presented in Figure 6. From the result it was found that Pseudomonas sp, Proteolytic microbes, Chitinolytic microbes, Pectinolytic microbes, PSB and Azotobacter sp were more in arabica rhizosphere than robusta, higher Starch hydrolytic and Cellulolytic microbes were recorded high in robusta than arabica.

In general higher microbial population in rhizosphere could be due to high organic matter content of the coffee soils and root exudation contributed by coffee and shade tree canopies.

**Table 2. Soil microbiological properties of Pepper and tree rhizosphere.**

| Farms | Pepper rhizosphere | | | | | Shade tree rhizosphere | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | F | A | Y | Total Cfu/g | B | F | A | Y | Total Cfu/g |
| **Arabica** | | | | | | | | | | |
| Kudaloor-U | 48 | 4 | 22 | 11 | 85 | 20 | 8 | 16 | 14 | 58 |
| Salisbury | 64 | 2 | 24 | 9 | 99 | 120 | 2 | 32 | 12 | 166 |
| Kothagiri | 80 | 3 | 12 | 23 | 118 | 120 | 6 | 12 | 12 | 150 |
| Kohinalli-B | 72 | 5 | 28 | 11 | 116 | 74 | 6 | 24 | 18 | 122 |
| Kudaloor-G | 136 | 3 | 15 | 25 | 179 | 80 | 2 | 10 | 7 | 99 |
| **Robusta** | | | | | | | | | | |
| Basavanagudi | 64 | 6 | 16 | 24 | 110 | 110 | 9 | 19 | 28 | 166 |
| Maldare-K | 12 | 4 | 6 | 2 | 24 | 48 | 3 | 9 | 13 | 73 |
| Maldare-G | 112 | 3 | 14 | 24 | 153 | 92 | 4 | 14 | 12 | 122 |
| Maldare-N | 69 | 3 | 14 | 10 | 96 | 48 | 6 | 9 | 24 | 87 |
| Thappa | 44 | 2 | 10 | 6 | 52 | 94 | 4 | 13 | 16 | 127 |

# Coffee Cherry Husk, a Feed Stock for Methane Generation

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## SUMMARY

Cherry husk is a major agro-waste generated by coffee industry. Due to its phytotoxic nature its use is restricted in agriculture but used widely as a low cost fuel. The combustibility of this waste is very low and large quantities of carbon dioxide and monoxides are generated, which are pollutants per-se.

Efforts to utilize the cherry husk as feedstock for the generation of methane. Earlier when cherry husk was used alone gas generation was highly restricted due to its low pH and high acid value. Hence different combinations were tried, such as coffee husk with cow dung and vegetable waste. Fermentation was carried out as solid phase biogas generation system. Results indicated that gas generation started by 20-30 days and continued for 100 days from a single feed.

Gas production was maximum and was at the rate of 19.72 ml/kg/day in control and the least was 9.47 ml/kg/day in coffee husk: cow dung (70:30). Change in total solids, volatile fatty acids, pH, and total anaerobic count were followed at regular intervals. PH changed from 3.5 to 7.15 in 15 days in mixture containing market waste. Maximum amount (19.3 g/l) of volatile fatty acids were produced in 70:30 mixture of coffee husk and cow dung.

## INTRODUCTION

The rapid rice in the price of fossil fuels and their scarcity all over the world has aroused interest to develop newer resource of energy. Bio methanation is preferred over other process as it provides fuel in the form of methane; high value digested slurry as manure (Nand, 1990) However cow dung is an limited resource even in countries like India (Chand, 1989) and to produce larger quantity of biogas, it is inevitable that other resource such as leaf biomass have to be utilized (Molnor and Bartha, 1988; Gosh, 1990). Cherry husk is a major agro waste generated by coffee industry. Due to its phytotoxic matrix its use is restricted in agriculture but used as a low cost fuel. With a low combustibility rate of 2300 kcal/kg, coffee husk generates more carbon dioxide and monoxide, which are pollutants per-se.

In this study efforts were made to use cherry husk as alternate feedstock for the production of Biogas. As it contains high amounts of Polyphenolic materials, which maintain the pH in the range, so 3-6-4.5 our earlier affords to use the husk as such did not yield good results. In this experiment, coffee husk was fortified with various percentages of cow dung and vegetable market waste.

## MATERIALS AND METHODS

### Design of fermentor

The fermentor was made up of PVC pipe of 45cm height and a 12cm wide, which had a total volume of 8.1 liter. Top of the ferment was covered with stainless steel plate sealed with petroleum jelly. Four inch below from the top a hole was made. A single holed rubber cork was tightly fitted along with the glass tube. This was so adjusted to coincide with the headspace. To that glass tube a meter length of flexible pipe was fitted which was fitted wh8ich was connected to the gas-measuring cylinder of 1L capacity. Biogas fementor as well as measuring cylinder was kept in water through out the study. The measuring cylinder was filled with water. The amount of gas collected in the cylinder was measured by water displacement method.

### Feed Stock

Coffee husk with cow dung (60:40 and 70:30) Coffee husk with vegetable waste (70:30) was used as feedstock. Cow dung served as control.

### Estimations

Biogas produced in the ferment or was estimated by water displacement method.

Moisture percentage was measured by using advance Moisture meter. PH was analyzed by Digisuim electronic pH meter. Total acidity was measured using titrimetric method with citric acid as standard. Total solids, volatile and non-volatile solids were estimated gravimetrically (Costa, 1998). Volatile fatty acid was determined by distillation method (Costa, 1998). Total anaerobic population was estimated using anaerobic jar method (Cappucciano and Sherman, 1999).

**Figure 1. Lab scale fermenter used for biogas production.**

## RESULTS AND DISCUSSION

Biogas production is known to occur largely through a two-step reaction system. Polymer in the biomass is first Hydrolyzed and converted predominately to acetic acid and hydrogen (acidogenesis). These intermediates from acidogenesis are converted to biogas by two distinct groups of methanogens (Methanogenesis). It has been reported that many simple constituents of herbaceous biomass feed stocks (such as sugars, pectin’s and hemicelluloses) were rapidly

hydrolyzed and converted to methanogenic intermediates mainly volatile fatty acids (Chanakya et al., 1995; Vishwanath et al., 1992)

In the present investigation bioreactors in control and cow dung: Market waste combination showed gas production within 8 days as against 40 days with the other two samples. (Figure 2). But the gas production continued for more than 100 days in samples with coffee husk. Samples with market waste showed better production of gas for a longer duration of time when compared to that of mixtures with cow dung.

Such changes with various combination of biomass was earlier observed by Srivastava et al. (1994) and Chanakya et al. (1995).

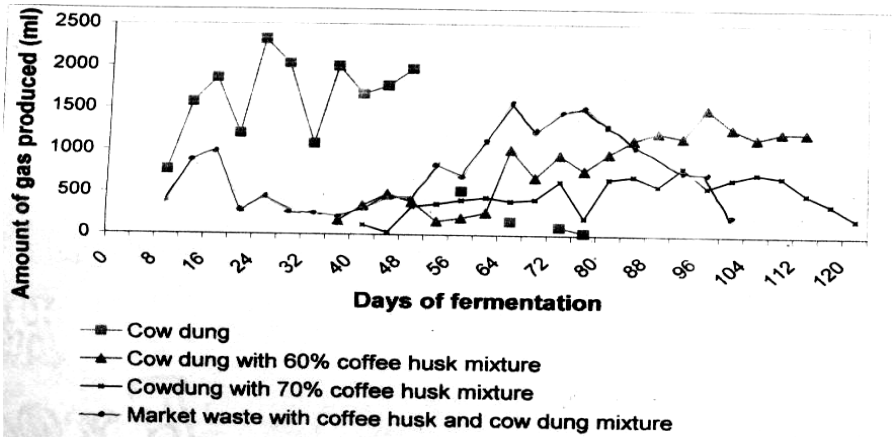


**Figure 2. Total gas production for different fermenting substrates at different time intervals.**

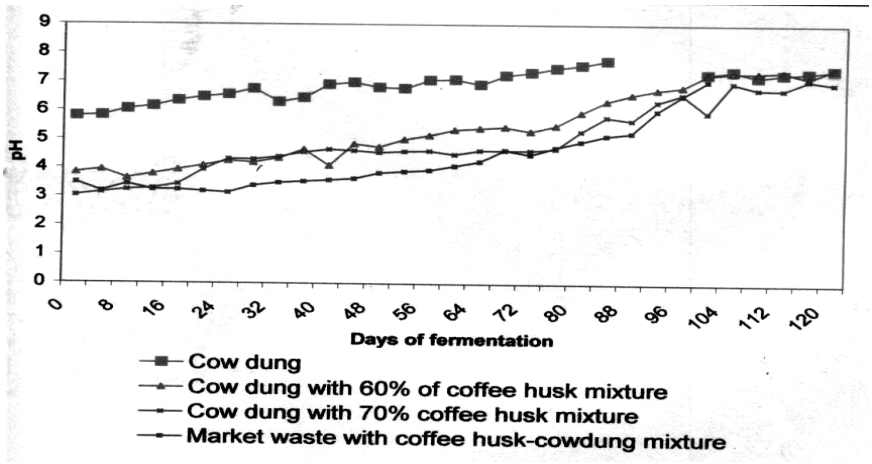


**Figure 3. pH for different fermenting substrates at different time intervals.**

Volatile Fatty acids (VFA) was very high in all feedstock with coffee husk when compared to negligible amounts present in cow dung (Table 1). Such high level of VFA hinders the methanogenesis as the low pH associated with high VFA decreases the conversion of VFA into methane. The acetate levels should fall low once the high gas production was achieved (Chanakya et al., 1995).

The failure of methanogens and interspecies hydrogen transfer in the digester could be deduced by the accumulation of higher as well as branched chains if volatile fatty acids such

as butyrate and valerate above 0.03 mg/Lt.This leads to the lack of complete conversion of acetic acid indicating the less than desired level of performance. (Chanakya et al., 1992).

**Table 1. Comparision between the total solids of cow dung, cow dung with % coffee husk mixture, cow dung with 70% coffee husk mixture and coffee husk, cow dung with market mixture.**

| Sl.No. | No. of days | VFA in Cow dung (mg/L) | VFA in cow dung with 60% coffee husk mixture (mg/L) | VFA in cow dung with 70% coffee husk mixture (mg/L) | VFA in coffee husk, cow dung with market waste mixture (mg/L) |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 16073 | 19340 | 16073 |
| 2 | 4 | 0 | 16000 | 19000 | 15080 |
| 3 | 8 | 765 | 15071 | 21300 | 15750 |
| 4 | 12 | 1574 | 15734 | 18040 | 15050 |
| 5 | 16 | 1860 | 15483 | 19130 | 15349 |
| 6 | 20 | 2200 | 12349 | 17100 | 15349 |
| 7 | 24 | 2320 | 12301 | 17920 | 15250 |
| 8 | 28 | 2730 | 14312 | 14300 | 15200 |
| 9 | 32 | 1580 | 13103 | 16010 | 14900 |
| 10 | 36 | 2000 | 12030 | 16310 | 15110 |
| 11 | 40 | 2670 | 12314 | 17920 | 14800 |
| 12 | 44 | | 11399 | | 14850 |
| 13 | 48 | 1770 | 11000 | 15840 | 13500 |
| 14 | 52 | 1970 | 11349 | 17300 | 12950 |
| 15 | 56 | 1200 | 11430 | 17980 | 11500 |
| 16 | 60 | 525 | 11120 | 15340 | 10350 |
| 17 | 64 | | 10013 | 18140 | 9500 |
| 18 | 68 | 165 | 13013 | 14960 | 9350 |
| 19 | 72 | | 10043 | 12130 | 8350 |
| 20 | 76 | 100 | 9013 | 13330 | 7950 |
| 21 | 80 | 30 | 7045 | 12310 | 8500 |
| 22 | 84 | | 6432 | 15130 | 8300 |
| 23 | 88 | | 7500 | 14000 | 7500 |
| 24 | 92 | | 8031 | 11100 | 6800 |
| 25 | 96 | | 6321 | 13390 | 5400 |
| 26 | 100 | | 6320 | 10900 | 5321 |
| 27 | 104 | | 9432 | 10340 | |
| 28 | 108 | | 6134 | 6340 | |
| 29 | 112 | | 7384 | 9310 | |
| 30 | 116 | | 6400 | 11310 | |
| 31 | 120 | | 5825 | 8900 | |

The low conversion of VFA by methanogenic organisms is highlighted by the slow change of pH to near neutrality (Figure 3). In all the treatments pH change to near neutral occurred in about 120 days. Whereas in control such changes was noticed in about 30 days. Such slow change of pH was earlier by Chanakya et.al. (1997) working with dry biomass.

The total acidity gives an indication of the substrate to resist establishment of alkaline condition (Molnar & Bartha, 1988). The maximum total acidity in control was 680 mg/L at a pH of 5.80 at 0 days which reduced to 346 mg/L by 16 days. Whereas all the samples total acidity was between 1525 mg/L to 1750 mg/L. During the coarse of fermentation there was a

drop in total acidity in 60:40 coffee husk: cowdung sample by 10 days (Figure 4). But this did not lead to high gas production.

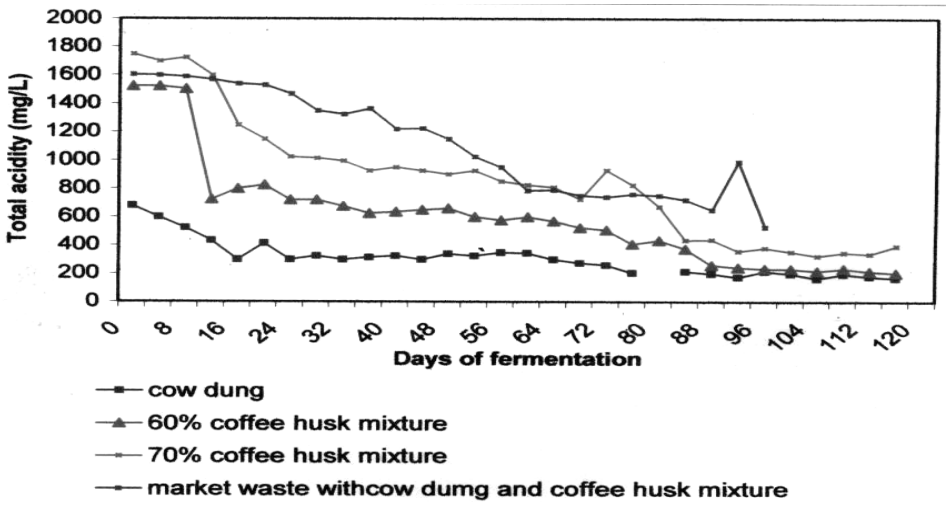


**Figure 4. Total gas production for different fermenting substrates at different time intervals.**

Microbial succession depends on the availability of organic as well as trace elements for their growth. The inhibitor compounds does not permit the colonization of microbes even in the presence of organic matter. (Francese et al., 2000). The succession of microbes thus depends on the total nutrients present along with the inhibitor compounds. The presence of a group of inhibitors may lead to the succession of a very different group of organism. In the present study even though the total count of the anaerobic organism reached almost to the same level as shown in Table 2. The lack of gas production to optimum level indicates the increase of non-methanogenic forms of bacteria in the fermentor.

**Table 2. Anaerobic count at different time intervals for different fermenting substrates.**

| Substrate | Colony in CFU/g of sample | | | |
|---|---|---|---|---|
| | 20th day | 40th day | 60th day | 80th day |
| Cow dung | $28 \times 10^6$ | — | $56 \times 10^8$ | $89 \times 10^{10}$ |
| Cow dung with 60% of coffee husk | $28 \times 10^5$ | $56 \times 10^6$ | $46 \times 10^8$ | $52 \times 10^{10}$ |
| Cow dung with 70% of coffee husk | $30 \times 10^5$ | $38 \times 10^5$ | tntc | $50 \times 10^8$ |
| Cow dung, coffee husk and market waste | $20 \times 10^4$ | $49 \times 10^6$ | tntc | $96 \times 10^7$ |

## CONCLUSION

As the initial VFA was high leading to low pH we presume it has inhibited the colonization of methanogenic bacteria. Further work on neutralization the substrate or increase the nutritional value is to be tried. Field trials on the standardized methods are to be taken up.

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# A Robusta Consensus Genetic Map using RFLP and Microsatellite Markers for the Detection of QTL

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## SUMMARY

A genetic map is an important tool to guide and speed up plant breeding programs. Nestlé has initiated a collaborative action with ICCRI and Cornell University in order to generate a reliable genetic map of the allogamous diploid species *Coffea canephora* var. Robusta.

A segregating population of 93 individuals was created by ICCRI from the cross of two elite clones BP409 and Q121. These clones have a different genetic background, BP409 is a Congolese genotype and Q121 seems to be a hybrid between Guinean and Congolese genetic groups. After propagation by cuttings the resulting progenies were planted in two different locations in Indonesia, in order to assess the environmental effects on quantitative traits such as agronomic performances or cup quality.

The two parents, BP409 and Q121 are highly heterozygous (63% and 55%, respectively). Due to this genetic status, it was possible to establish one backcross genetic maps for each parent and then to elaborate a consensus map of both. Using 453 molecular markers such as RFLP and microsatellites, we have identified eleven linkage groups, covering 1258 cM, which most likely represent the eleven gametic chromosomes of the *Coffea canephora* species.

The construction of the consensus map was made easier thanks to the great level of polymorphism of the microsatellite markers. In comparison to RFLP markers they allow the mapping of a higher percentage of loci (30% instead of 14%) on both parental maps. The microsatellites are therefore suitable not only to create a consensus Robusta genetic map but also to constitute a core set of markers easily transposable for genetic mapping of other Robusta progenies but also for *C. arabica* genetic studies.

The progenies were planted in both locations early 2001 and quantitative data were recorded every year. The QTL identified for the yield of the first crop and vigor are specific of one location, suggesting a strong interaction between genotype and environment, as it might have been expected for the first stages of coffee production.

**RÉSUMÉ**

La cartographie génétique est un outil important pour guider et accélérer les programmes d'amélioration des plantes. En collaboration avec l'ICCRI et l'Université de Cornell, Nestlé a initié un projet d'établissement d'une carte génétique fiable chez l'espèce diploïde allogame *Coffea canephora* var. Robusta.

Une population ségrégeante de 93 individus a été créée par l'ICCRI en croisant deux clones élites indonésiens BP409 et Q121. Les deux clones parentaux ont une origine génétique différente, BP409 est un génotype Congolais et Q121 apparaît comme issu de l'hybridation entre les groupes Guinéen et Congolais. Après multiplication végétative par bouturage les descendants ont été plantés dans deux sites en Indonésie afin de pouvoir étudier l'effet environnemental sur des caractéristiques quantitatives telles que les performances agronomiques et de qualité à la tasse.

Les deux parents BP409 et Q121 présentent des taux d'hétérozygotie élevés de respectivement 63% et 55%. Ainsi grâce à cette caractéristique génétique, il a été possible d'obtenir une carte génétique backcross pour chacun des parents et de réaliser la carte consensus des deux. 453 marqueurs moléculaires de types RFLP et microsatellites ont permis la détection de onze groupes de liaison couvrant 1258 cM, qui représentent certainement les onze chromosomes gamétiques de l'espèce *Coffea canephora*.

La construction de la carte génétique consensus a été facilitée par le haut niveau de polymorphisme des marqueurs microsatellites. Ils ont permis la cartographie d'un plus grand nombre de loci (30%) sur les deux cartes parentales que les marqueurs RFLP (14%). Ainsi, les microsatellites apparaissent comme des outils de choix non seulement pour obtenir la carte génétique consensus de Robusta mais aussi pour obtenir des marqueurs de référence facilement transposables pour la cartographie d'autres descendances de Robusta ou pour des analyses génétiques chez Arabica.

Les descendances ont été plantées en 2001 dans les deux sites et les données quantitatives ont été enregistrées chaque année. Les QTL identifiés la première année pour les caractéristiques de rendement et de vigueur sont spécifiques d'un lieu suggérant une forte interaction génotype x environnement, ce qui était prévisible pour les premiers stades de production du caféier.

## INTRODUCTION

Twenty years ago the first plant molecular genetic linkage map was established on tomato (Tanksley et al., 1992). Over the last 12 years, numerous genetic studies on major crops have been developed with molecular markers with the main ultimate goal of assisting and speeding up the breeding programs. Generally, tropical crops receive poor attention in the development of biotechnology tools and coffee is a good example of these "orphan" crops compared to the main field crops such as corn, rice or tomato.

Coffee production is based on *C. arabica* and *C. canephora* species. Although *C. arabica* represents 70% of world production, *C. canephora* is an important coffee source for soluble coffee. This species is also at the origin of many disease resistance traits used so far in *C. arabica*. *C. canephora* is a strict allogamous species leading to polymorphic populations and strongly heterozygous individuals.

The main objective of this study was to develop a complete linkage map of *C. canephora* using co-dominant markers such as RFLP and microsatellites (SSR) in order to develop a reliable and reproducible tool for further quantitative genetic studies.

## MATERIAL AND METHODS

### Plant material

ICCRI has created a progeny by crossing two *C. canephora* var. Robusta clones named BP409 (Congolese) and Q121 (hybrid). These two clones were selected according to their genetic differences but also to their phenotypic characteristics such as yield, vigor, nematode resistance or bean size and cup quality. A total of 93 individuals were used to establish the genetic consensus map. These segregating individuals were propagated by cuttings and planted in 2001 in two locations at Jember (East Java) and Lampung (South Sumatera) in Indonesia.

A set of 218 *C. canephora* accessions from various origins (including the two parental clones) was fingerprinted with 24 RFLP probes in order to evaluate the genetic diversity of these accessions relative to the two parental clones.

### RFLP and microsatellite markers

DNA from each coffee accession was obtained and purified according to Crouzillat et al., 1996. Genomic *Pst*I probes (coded as CFG or CAG) were generated from a Robusta or Arabica genomic library, respectively. The cDNA probes named cccl, cccp or cccs are respectively from leaf, pericarp or seed Robusta tissues, (Lin et al., 2004). These probes were screened for RFLP using Southern blots of parental DNA (BP409 and Q121) restricted with *Eco*RV, *Hin*dIII, *Dra*I, *Hae*III, *Pvu*II, *Rsa*I and *Sca*I to identify the best combination of probe and endonuclease digest that detected polymorphism.

A genomic library enriched for $(GA)_n$ and $(GT)_n$ microsatellite (SSR) was constructed from Robusta and Arabica according to the procedure of Edwards et al., 1996. Other microsatellite markers were obtained from web databases (Rovelli et al., 2000; Cristancho et al., 2002; Combes et al., 2000) and were recorded as A, AY or ZAP locus names.

### Microsatellite analysis on ABIprism 310

Amplified fluorescent-labeled DNA products were separated on a ABIprism 310 sequencer using capillary electrophoresis. Each run on ABIprism 310 was performed with four different microsatellite markers, each of them with a specific fluorescent labeling (FAM, VIC, NED or PET).

PCR amplification products and experimental data were analyzed by GENSCAN and GENOTYPER (Applied Biosystems) softwares. Every microsatellite allele is recorded according to its size (bp). The genetic status of each progeny is coded according to the segregation type.

### Construction of the genetic linkage maps

The segregation of each molecular marker was tested with a chi-square test for the goodness-of-fit to the expected Mendelian segregation ratios depending on the parental genotype configuration. The different genetic maps (backcross BP409, backcross Q121 and the

consensus map) were established using Joinmap software version 3.0 (Van Ooijen and Voorrips, 2001). A LOD score of 5 was used to identify the linkage groups. The Kosambi mapping function was selected to convert recombination frequencies into map distances (cM) (Kosambi, 1944).

## RESULTS

### Genetic diversity among *Coffea canephora* accessions

218 accessions were analyzed with 24 RFLP probes in order to establish a survey of the genetic diversity of this species. A total of 90 alleles were detected leading to an average of 3.8 alleles per locus. The Principal Component Analysis (PCA) resulting from these data indicates a clear differentiation between the three main genetic groups: Congolese, Guinean and Conilon (Figure 1).

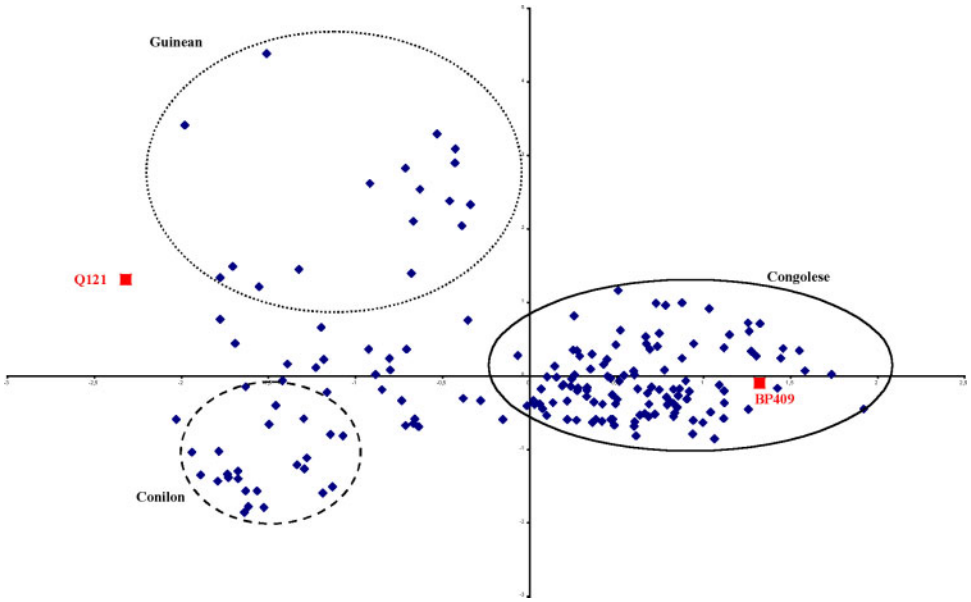


**Figure 1. Principal Component Analysis showing the genetic diversity among 218 *Coffea canephora* accessions. The three main genetic groups are indicated in conjunction with the two parental clones used to obtain the progeny. The two axes explained 26.5% of the variance.**

The two *C. canephora* clones selected for the creation of the mapping progeny are quite distant. The clone BP409 is part of the Congolese group and the clone Q121 is more related to the Guinean cluster.

### Molecular markers for the coffee genetic map

550 RFLP markers from a *Pst*I genomic library (Table 1) were screened for their DNA polymorphism, using *Eco*RV and *Dra*I restriction enzymes. Then they were used for the creation of the genetic map. 118 RFLP markers (21%) have been mapped on the eleven linkage groups.

In the same way, 700 EST's were analyzed using 7 restriction enzymes and 218 (31%) of these EST's were successfully mapped. The better map efficiency of EST's compared to *Pst*I genomic probes is certainly due to the higher number of restriction enzymes used for the EST's screening.

The microsatellite screening was performed on 370 SSR's and 117 (32%) of them were mapped. This map efficiency value is similar to that of the EST markers using 7 restriction enzymes.

Another aspect to be considered is the transposition rate of the mapped genetic markers. These bridge loci are heterozygous on both parents ($F_2$ segregation type) and consequently they are highly important for the creation of the consensus genetic map. A percentage of 30% of the SSR markers against 13% for genomic and 15% for EST loci (Table 1) reveals that the microsatellites are the most efficient markers for this aspect of the mapping procedure.

**Table 1. Comparative mapping efficiency and transposition rate of the genomic, EST and SSR markers.**

| | Genomic *Pst*I | EST | SSR |
|---|---|---|---|
| **Process of analysis** | **RFLP** | | **PCR** |
| **Markers screened** | **550** | **700** | **370** |
| **Markers mapped** | **118** | **218** | **117** |
| **% Mapping efficiency** | **21** | **31** | **32** |
| **$F_2$ markers** | **15** | **32** | **35** |
| **% Transposition** | **13** | **15** | **30** |

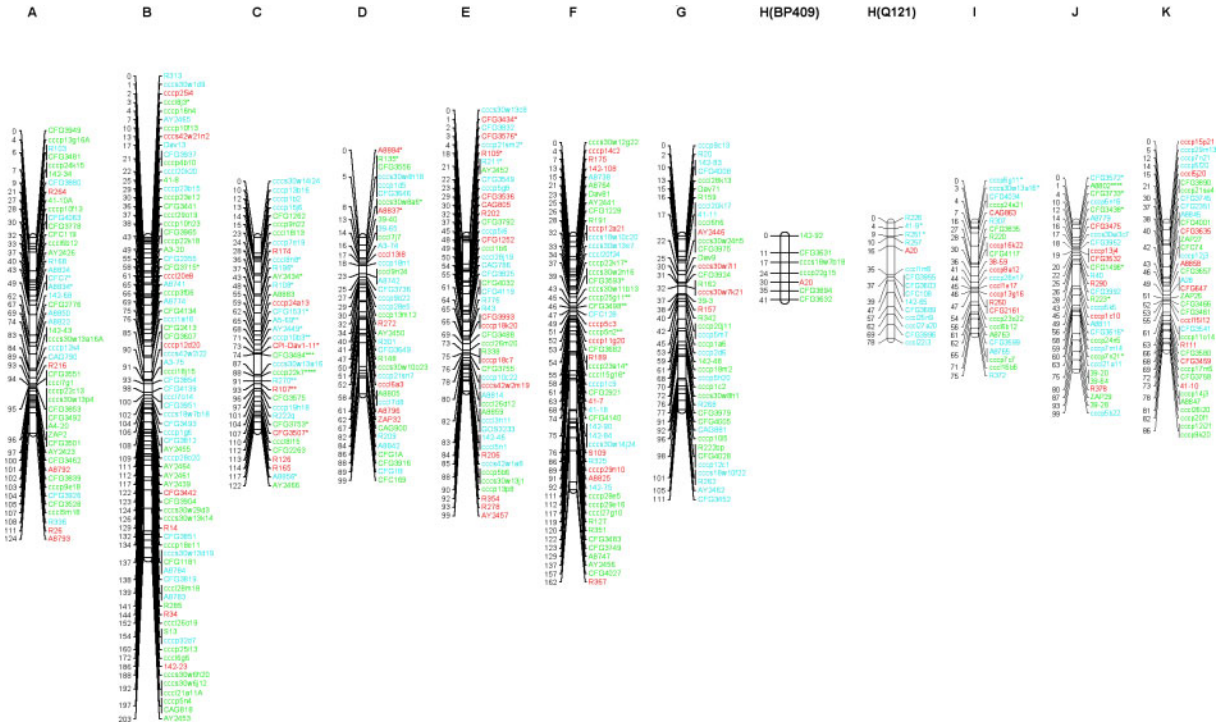


**Figure 2. Genetic consensus linkage map of *Coffea canephora* derived from the cross BP409 X Q121. The 453 loci were distributed to eleven linkage groups (A to K). The numbers to the left of each linkage group represent the cumulative map distance in cM (Kosambi function). Loci identified by the symbols: *, **, ***, **** displayed distorted segregating ratios with P < 0.05, P < 0.01, P < 0.001 and P < 0.0001, respectively. The red loci indicate bridge markers that segregate in both parental gametes; green and blue loci are markers segregating only in BP409 and Q121 gametes, respectively.**

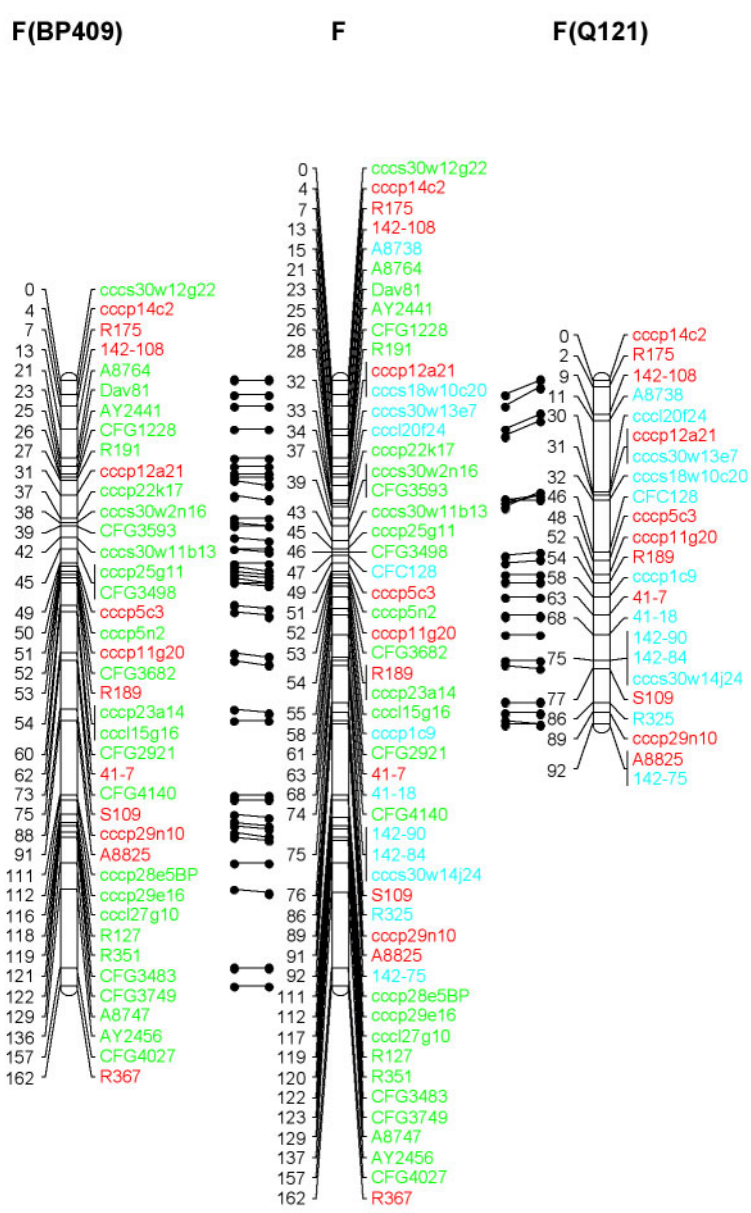


**Figure 3. Linkage group F of the genetic map obtained from the cross between BP409 X Q121. The red loci indicate the 12 bridge markers that segregate in both parental gametes; green and blue loci are markers segregating only in BP409 and Q121 gametes, respectively.The construction of the consensus genetic map.**

Eighty nine percent (89%) of the 453 mapped markers fitted the Mendelian segregation ratio expected from the genotypes of the two parents. Eleven percent (11%) of these markers deviated significantly ($P < 0.05$) from the expected segregating ratio. These markers are not randomly distributed among the 11 linkage groups as shown on linkage groups C and F where these loci were clustered together (Figure 2).

A total of 468 markers were used for the genetic mapping of BP409 and Q121 clones, 453 (97%) of these loci allows the definition of 11 linkage groups, which likely represent the eleven chromosomes of haploid *C. canephora*. These 11 linkage groups covers 1258 cM with an average distance of 2.8 cM between markers. The size of the chromosomes varies from 75 to 203 cM for the linkage groups B and I respectively. All the linkage groups detected, except H, have at least two bridge markers allowing the construction of each consensus linkage group. The H group had only one $F_2$ type marker (A20) and up to now, it is still impossible to

define the chromosome orientation of the two backcross linkage groups (H BP409 and H Q121) and consequently to obtain the consensus map for this linkage group.

Based on the 453 mapped markers 203 (45%) segregates for BP409, 168 (37%) for Q121 and 82 (18%) for both, the latest constituting the bridge loci. Using the mapped markers, the heterozigosity values for the two parents BP409 and Q121 are, respectively, 63% and 55%. The similar level of heterozygosity allowed the construction of each of the two-backcross parental genetic maps (data not shown) and then the creation of the consensus coffee map. Generally, a good co-linearity is observed between the two-backcross genetic maps (Figure 3). As an example, the consensus linkage group F shared 12 bridge markers that segregate in both parental backcross maps. No significant co-linearity bias was observed on these two maps in regards to the consensus map. The only difference is due to the differential covering of this chromosome where no heterozygous markers have been found in the lower part of this linkage group for the parent Q121. Due to this lack of polymorphism in this genome area of the parent Q121, the coverage of the linkage group F is reduced from 162 cM (BP409) to 92 cM (Q121).

## DISCUSSION

The use of co-dominant markers such as RFLP and SSR allowed the creation of the first high-density consensus genetic map of C. *canephora* var Robusta. The reference map described here has a high density marker that could be easily transfer to other mapping progenies of *C. canephora* or other coffee species of commercial value such as *C. arabica*. The mapping of PCR based markers, such as microsatellites, will facilitate this step. Several linkage maps of *C. canephora* were already published (Paillard et al., 1996; Lashermes et al., 2001) but they were not complete and they were mainly based on RAPD or AFLP which are not loci specific and consequently are not easy to transfer across different mapping populations. Due to their co-dominant nature, specific single locus behavior, and their high level of polymorphism, microsatellites are therefore valuable markers for further development of genetic studies in coffee.

This genetic consensus map, based on co-dominant markers, will be easily transferred on other progenies (*C. canephora* or *C. arabica*) and consequently it should allow the comparison of QTL's for agronomic and quality traits.

Coffee EST sequencing (Lin et al., 2004) has also produced a new source of markers such as SSR and SNP for use in future genetic studies.

By checking the matching of the identified QTL's with candidate genes, biochemicals and pathways this map could also be used for the validation of some hypothesis concerning the determinants of key traits of interest. Syntheny studies or even chromosome walking on some these QTL's could also allow to discover some genes and subsequent biochemicals which have never been supposed to be involved in these traits.

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# Toward an Integrated Physical Map of the Coffee Genome

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## SUMMARY

Availability of very large-insert, ordered DNA libraries are crucial for genomics research including physical mapping, map-based cloning of agronomically important genes, and analysis of gene structure and function. The construction of an integrated genetic and physical map of the coffee genome represents therefore an important challenge that has justified a recent initiative. A bacterial artificial chromosome (BAC) library of the allotetraploid species, *Coffea arabica*, was constructed. This large insert DNA library derived from a multi-disease resistance line contains 88 813 clones with an average insert size of 130 kb, and represents approximately 8 *C. arabica* haploid genome equivalents. The undertaken mapping approach combined hybridisation with mapped markers and BAC fingerprinting. We completed hybridisation with both low-copy RFLP markers distributed on the 11 different chromosomes and probes corresponding to disease resistance gene analogues. BAC clones from subgenomes $E^a$ and $C^a$ were assembled into separate contigs. Accuracy of the map was verified using several approaches. These preliminary results represent the first step toward the construction of a physical map of the coffee genome.

## INTRODUCTION

Integrative physical mapping is the central of genomic research. For instance, integrated maps have been proven in human and several plant and animal species the essential infrastructure and tools for structural, functional, and comparative genomics research. Regarding the coffee genome, the integration of physical and genetic mapping information would represent therefore an important challenge that has justified a recent initiative.

A physical map actually consists of continuously overlapping contigs of large-insert genomic DNA clones, in which the distance between two markers is measured in kb. The ideal physical map is integrated with genetic and cytogenetic maps (Figure 1). In this report, we intend to present progress toward the development of a large-insert coffee DNA library as preliminary step for genetic and physical map integration.

## MATERIALS AND METHODS

### BAC library construction

Isolation of high molecular weight DNA, digestion and ligation were done as recently described by Chalhoub et al. (in press). Nuclei were isolated from 25 g of leaf tissue, embedded in low melting point agarose plugs and the high molecular weight DNA was partially digested by the *Hind*III restriction enzyme. Restriction fragments were submitted to triple size selection by pulse field gel-electrophoresis (PFGE). Ligation reactions were carried out using the pIndigo BAC vector, and the four different sizes selected partially digested

DNA samples. Ligated products from the selected ranges 100-150, 150-200, 200-250 and 250-350 kb were noted ligation 1, ligation 2, ligation 3 and ligation 4, respectively. Competent *E. coli* DH10B cells (Invitrogen) were transformed by electroporation and transformant-colonies were picked using a Genetix Q-Bot and stored in 384-well microtiter plates at -80°C.

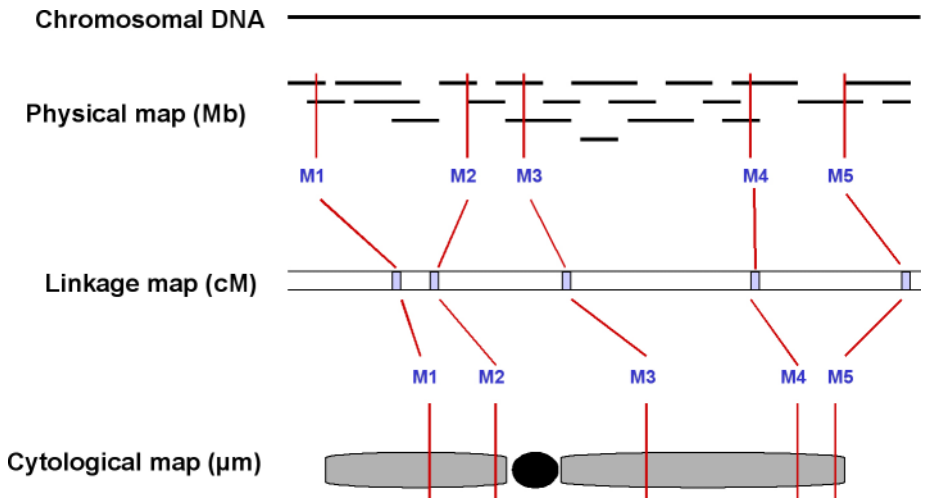


**Figure 1. An ideal physical map of the coffee genome.**

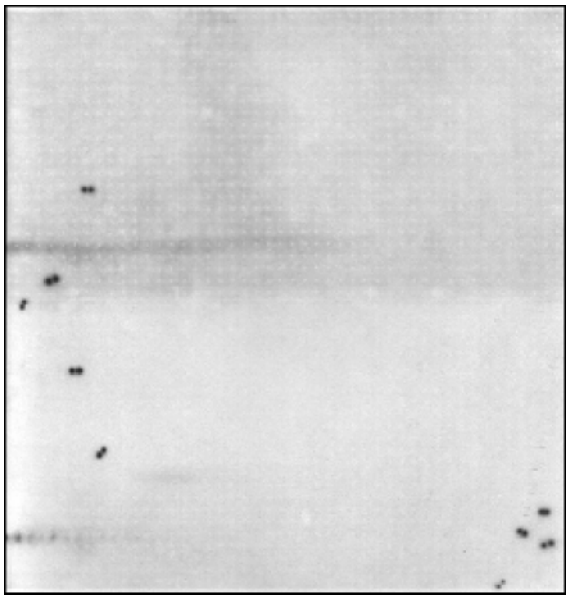

**Figure 2. Colony hybridisation of BAC filters: 27 648 clones were double spotted onto nylon membrane and hybridised with nuclear single-copy probe gA 6.**

***BAC filter and Southern hybridisation***

High-density colony filters were robotically prepared. BAC clones were gridded in double spots onto filters using a 4 x 4 pattern as described by Tomkins et al. (1999). In total, 27 648 clones (i. e. 44 plates of the ligation 4, 27 plates of the ligation 3 and 1 plate of the ligation 2) of the BAC library were spotted onto a 22.5 x 22.5 cm filter. Probes were labelled with [$^{32}$P]-dCTP and Southern hybridisation was carried out as described by Sambrook et al. (1989). The BAC DNA was extracted using an alkaline lysis procedure (Sambrook et al., 1989).

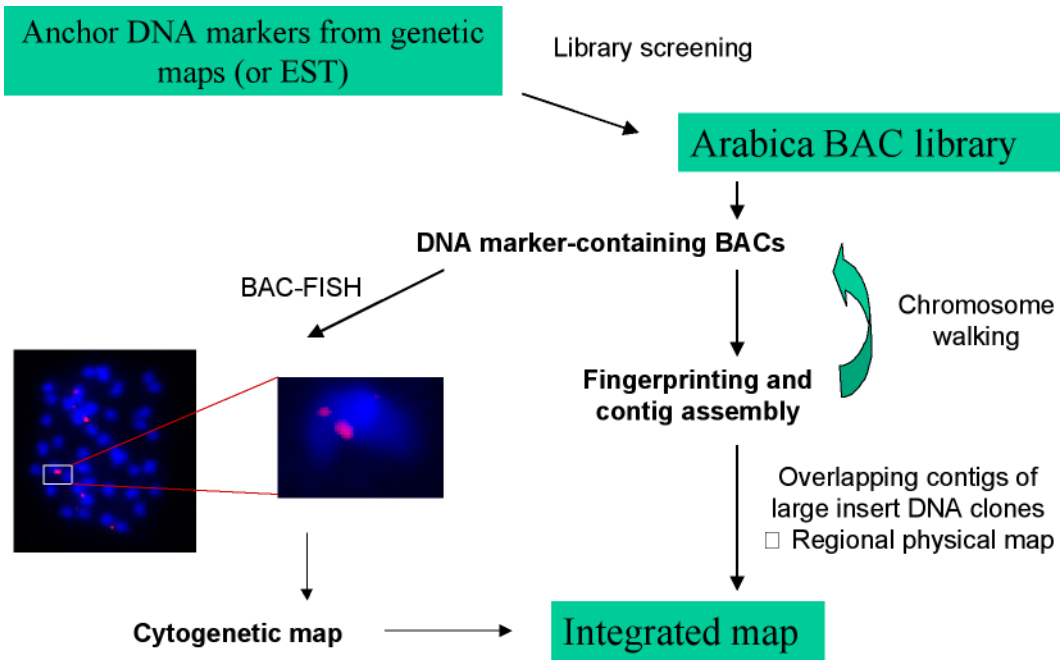


**Figure 3. Strategy used for integrative mapping of the coffee genome.**

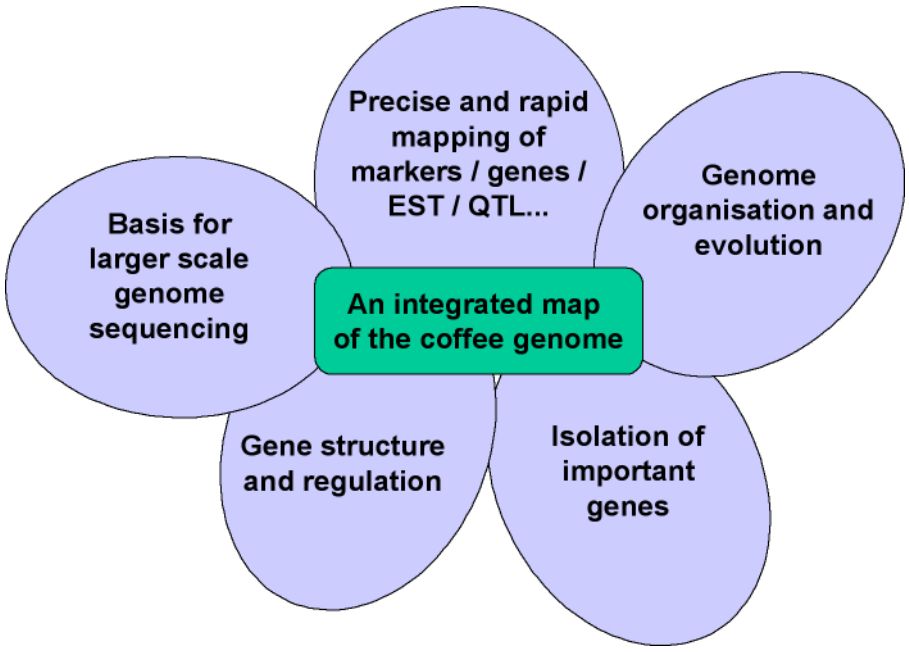


**Figure 4. Applications of an integrated physical and genetic map in coffee genomics research.**

## RESULTS AND DISCUSSION

### BAC Library

An Arabica BAC library using the cultivar IAPAR 59 was successfully constructed (Noir et al., 2004). This introgressed variety, derived from the Timor Hybrid (Lashermes et al., 2000b), is widely distributed in Latin America. The cultivar IAPAR 59 was selected for resistance to leaf rust and root-knot nematodes (Sera, 2001) and it presents the introgressed DNA fragment carrying the *Mex-1* locus that confers the resistance to *Meloidogyne exigua* (Noir et al., 2003). The described Arabica library consists of a total of 88 320 BAC clones

arranged in four sublibraries (corresponding to the different ligation reactions) with 55% of BAC clone inserts presenting a size superior to 200 kb. Although nuclear isolation and purification steps were performed, results of the screening with mitochondrial and chloroplast probes showed that approximately 4.5% of the library sequences were derived from the organellar DNA.

**Table 1. Composition of the Arabica BAC library.**

| Ligation reaction | Size selection range (kb) | Number of clones picked | Mean insert size +/- SD[a] (kb) | Insert size range (kb)[b] | Proportion (%) of the whole library size |
|---|---|---|---|---|---|
| 1 | 100 - 150 | 34 944 | 96 +/- 13 | 70 - 120 | 26 % |
| 2 | 150 - 200 | 23 040 | 130 +/- 9 | 120 - 150 | 25 % |
| 3 | 200 - 250 | 13 824 | 183 +/- 39 | 130 - 240 | 20 % |
| 4 | 250 - 350 | 16 896 | 210 +/- 65 | 80 - 310 | 29 % |

[a] *Standard deviation.*
[b] *Lower and higher observed BAC insert size.*

The haploid genome size of *C. arabica* has been estimated at 1300 Mb (Cros et al., 1995). Thus, considering the average BAC insert size of 130 kb, the entire library corresponds to 10 506 Mb of cloned nuclear DNA and would represent approximately 8 *C. arabica* genome equivalents. The probability (P) of having a particular nuclear sequence represented at least once in this library would be $P = 1\text{-}e^{N[\ln(1\text{-}I/GS)]} = 0.9997$ (Clarke and Carbon, 1976; I being the average insert size, N the total of clones in the library and GS the genome size).

**Genome coverage**

To test the distribution and genome-coverage of the BAC library, fifteen RFLP probes distributed (Lashermes et al., 2001) on the eleven gametic chromosomes of the *Canephora* genome (C genome) were used to screen high-density filters containing 27 648 BAC clones and representing approximately 44% of the overall BAC library size. All these probes distributed throughout the analysed genome hybridised positive BAC clones. The number of positive clones in each case was roughly consistent with the allotetraploid structure and the estimated eight-times genome coverage of the library.

**Subgenomes identification**

RFLP analysis was performed to estimate the contribution of the two diploid constitutive sub-genomes of *C. arabica* ($C^a$ and $E^a$) in the BAC library derived from the homozygous inbred line IAPAR 59. For each probe, restriction enzymes were chosen to reveal duplicate loci in the Arabica accessions and polymorphism among a set of diploid species accessions of *C. canephora* and *C. eugenioides*, corresponding to modern-day progenitor species of *C. arabica* (Lashermes et al., 2000a). By comparison, these duplicate loci were interpreted as two homeologous loci associated with the two homeologous genomes, $C^a$ and $E^a$, expected in the case of the allotetraploid Arabica genome. These observations indicate that the two sub-genomes constitutive of *C. arabica* are represented in the IAPAR 59 BAC library. In addition, the association between BAC clones analysed and attributed $C^a$ or $E^a$ sub-genomes suggested that constitutive genomes of Arabica are represented in the roughly same proportion in the library. Altogether, these evaluations indicated that the Arabica allotetraploid genome is well covered and represented by the BAC library.

## CONCLUSIONS

In conclusion, the Arabica BAC library described here represents the first large-insert DNA library for the genus *Coffea*. It contains large inserts, has sufficient genome coverage (8 X), combines the 2 sub-genomes $C^a$ and $E^a$, and contains a low fraction of clones with mitochondrial or chloroplast DNA. These features indicate that the BAC library is suitable for many applications in classical genome research (i.e. physical mapping, map-based cloning, functional and comparative genomics).

Besides in term of polyploid genome analysis, this BAC library offers the opportunity to reconstruct the phylogeny of homeologous loci in this allotetraploid genome in order to better investigate the mechanisms of speciation of the *C. arabica* species.

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# Anchor Markers for Comparative Mapping within the *Coffea* Genus

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## SUMMARY

In order to unravel the *Coffea* genomic reorganization, we focused on comparative mapping within the *Coffea* genus and between *Coffea* and related families such as Solanaceae. Prerequisites for comparative mapping are a genetic linkage map for each species and a common set of DNA markers that can be used to align the maps. With this objective, we started developing anchor markers corresponding to two classes: 1) anonymous genomic sequences such as SCAR markers derived from AFLPs as well as microsatellite markers, and 2) coding genomic sequences (derived from ESTs: e.g. EST-SSR, COS). For all markers analysed in this study, we obtained good transferability among *Coffea* species even when considering species belonging to different phylogenetic clades. These locus-specific markers will facilitate assembly of a panel of "anchor" PCR-based markers for the study of similarities and differences in the structure and function between *Coffea* genomes. Furthermore, we have defined an initial set of 54 highly conserved, single copy genes—COS markers—which can be used as markers for comparative mapping between the tomato and coffee genomes. These comparative maps will not only shed light on the nature of genome evolution, but will also facilitate comparative mapping studies of qualitative and quantitative traits.

## INTRODUCTION

Coffee trees belong to the *Rubiaceae* family and originated from the intertropical forest of Africa, Madagascar, and Mascarene islands. All species share the same chromosomic number, x = 11, and are diploid, except *C. arabica*, which has 44 chromosomes.

Phylogenetic relationships based on cpDNA (Cros et al., 1998) and rDNA ITS-2 (Lashermes et al., 1997) analyses highlighted a strong geographical differentiation between East African, West and Central African and Malagasy species.

This genetic differentiation is supported by results on the fertility of the F1 interspecific hybrid (Louarn, 1992). Indeed, considering only African species, fertility observed on the basis of pollen viability or seed set is much higher within than between geographical clusters. With intra-cluster combinations, fertility can reach up to 90%, while it is very low in inter-cluster combinations, with complete sterility often occurring.

In all cases, the sterility of interspecific hybrids is correlated with the number of univalents in pollen mother cells and could be due to a lack of chromosome pairing during meiosis.

Moreover, despite a same number of chromosomes for diploid species, the amount of DNA per genome 2C (qDNA) ranges from 0.93 pg (*C. racemosa*) to 1.78 pg (*C. humilis*) (Cros et al., 1994; Noirot et al., 2003). Interestingly, this amount is correlated with the geographical

origin of the species. For African diploid species, the amount of qDNA increases from East to West Africa.

How can we explain differences in chromosome pairing during meiosis and differences in qDNA between species? Two nonexclusive hypotheses could be put forward: i)- gain/loss of species specific noncoding DNA including repetitive DNA and transposable elements; ii)- genomic reorganization following the size increase/decrease of genomes during speciation.
In order to test the genomic reorganization hypothesis, we first focused on comparative mapping within the *Coffea* genus and between *Coffea* and related families such as Solanaceae (Fulton et al., 2002). With this objective, we started developing anchor markers corresponding to two classes: 1)- anonymous genomic sequences such as SCAR markers derived from AFLPs as well as microsatellite markers, and 2) coding genomic sequences (derived from ESTs).

## SEQUENCE-CHARACTERIZED AMPLIFIED REGION (SCAR) MARKERS

Genetic maps based on interspecific BC1 progenies have been previously produced by our research team (Ky et al., 2000; Coulibaly et al., 2003). These maps were constructed with AFLP markers specific to the donor species. We first considered the [(*C. pseudozanguebariae* x *C. liberica* var *dewevrei*) x *C. liberica* var *dewevrei*] cross (Ky et al., 2000) and analyzed specific sequences of the *C. pseudozanguebariae* (PSE) genome relative to *C. liberica* var *dewevrei* (DEW) produced from *Eco*RI and *Mse*I digestion (Poncet et al., 2004).

SCAR markers were derived from these PSE AFLP sequences. Finally, 23 AFLP bands were cloned, successfully sequenced, analyzed and the corresponding primer pairs tested on other species to determine whether they could amplify homologous sequences across *Coffea* species.

We noted two key features when considering the nature of these sequences. First, no similarities between PSE sequences were observed, indicating that none of them corresponded to disperse repetitive DNA. Then comparison of PSE sequences to public database sequences revealed significant similarity with five sequences (BLASTx and BLASTn, E-value less than $e^{-04}$), with two of them being expressed proteins from *Arabidopsis*.

The study of the base composition of the PSE AFLP sequences showed an AT content distribution ranging from 49.2% to 75.2% with two modes (58.6% and 71.4%), while for *Coffea* expressed sequences (EST) the AT content distribution was unimodal with a mean of 55.0%. Interestingly, the two AFLP sequences which showed some similarity with *A. thaliana* proteins, had an AT content of around 55%. An AFLP sequence showing similarity to repetitive DNA, had an AT content of 61.4%.

These results are in line with the hypothesis whereby the main part of AFLP bands produced from *Eco*RI and *Mse*I digestion more likely correspond to noncoding sequences. Indeed, due to i)-the relative importance of noncoding *vs.* coding sequences in eukaryote genomes and ii)-the DNA methylation insensitivity of *Eco*RI, access to noncoding sequences is more frequent.

In the next step, 23 SCAR primers were designed and tested on genomic DNA (gDNA) of nine *Coffea* species representative of the natural distribution of coffee trees, and gDNA of *A. thaliana* (THA). Fifteen primer pairs efficiently amplified at least one *Coffea* species and four of them gave good amplification with the nine *Coffea* species tested. *Arabidopsis thaliana* was only amplified with one SCAR primer (Table 1). The amplification success was found to be independent of the species considered and did not reflect the relatedness between

the species as defined by Lashermes et al. (1997). In general, amplification of a single fragment of the expected size was obtained with the PSE genotype.

However, despite this high transferability potential, we observed a very low level of polymorphism, even between species. Consequently, only two SCAR primers could be mapped on the [(*C. canephora* x *C. pseudozanguebariae*) x *C. canephora*] progeny and one was used for the [(*C. liberica* x *C. canephora*) x *C. canephora*] mapping.

This result is not specific to the *Coffea* genome and it is well known that during SCAR development the loss of the original polymorphism often poses experimental challenges. Indeed, the original polymorphism was found to be mainly located at the restriction sites. It was not always possible to design primers covering the corresponding region. Nevertheless, when SCARs are produced from AFLPs linked to QTLs of interest, the amount of variation they could reveal should be further studied, for example through enzymatic digestion of the PCR products (cleaved amplified polymorph sequences, CAPS).

**Table 1. Amplification with SCAR primers across nine *Coffea* species and *Arabidopsis thaliana* and analyses on mapping progenies.**

| Species[a] | PSE | DEW | CAN | CON | EUG | HET | MIL | MOL | ARA | THA | Mapping potential[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amplification (%) | 10/21 (47.6) | 5/21 (23.8) | 8/21 (30.9) | 9/21 (42.8) | 7/21 (33.3) | 10/21 (47.6) | 7/21 (33.3) | 8/21 (30.9) | 8/21 (30.9) | 1/21 (4.8) | |
| Weak amplification (%) | 2/21 (9.5) | 3/21 (14.3) | 5/21 (23.8) | 3/21 (14.3) | 4/21 (19) | - | 4/21 (19) | 3/21 (14.3) | 3/21 (14.3) | 0 | |
| Total | 12 | 8 | 13 | 12 | 10 | 10 | 10 | 11 | 11 | 1 | 5 |

[a]*: PSE = C. pseudozanguebariae, DEW = C. liberica var dewevrei, CAN = C. canephora, CON = C. congensis, EUG = C. eugnenioides, HET = C. heterocalyx, MIL = C. millotii, MOL = C. sp Moloundou, ARA = C. arabica, THA = Arabidopsis thaliana.*

[b] *: mapping analyses: at least one mapping progeny concerned*

## MICROSATELLITE MARKERS FROM ANONYMOUS GENOMIC SEQUENCES

Microsatellite primer sets were designed on *C. arabica* sequences obtained from Genbank/EMBL databases (Combes et al., 2000; Rovelli et al., 2000) or on *C. canephora* sequences (Dufour et al., 2001). Primers obtained from Baruah et al. (2003) were also evaluated. All of them were tested for amplification and their ability to reveal polymorphism on several *Coffea* species. Indeed, as expected, a previous microsatellite study carried out on *C. canephora* (CAN) and *C. pseudozanguebariae* (PSE) revealed a high level of genetic diversity (measured with PIC* value) within species (Poncet et al., 2004). Furthermore, both PIC distributions were bimodal but there was no correlation between the two PIC sets. This result is important since it indicates that it is not possible to predict the polymorphism level of one given species from the polymorphism observed in another species (Poncet et al., 2004).

Finally, out of 355 primer pairs available, only 53 could be effectively used for CP mapping while 33 have to be tested on the progeny (Table 2).

* ($PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$ , where $P_{ij}$ is the frequency of the jth allele for the ith marker and summed over n alleles)

**Table 2. Results obtained with the three sets of primer pairs tested. Only results related to the ((*C. canephora* x *C. pseudozanguebariae*) x *C. canephora*) (CP) mapping are reported.**

| Primer origin | # primer pairs | No PCR product | analyzed on CP progeny | to test on CP progeny |
|---|---|---|---|---|
| *C. arabica*[1] | 116 | 48 (41.4%) | 25 (21.6%) | - |
| *C. canephora*[2] | 230 | 115 (50%) | 26 (11.3%) | 33 (14.3%) |
| *C. arabica*[3] | 9 | - | 2 | - |

[1]*: Poncet et al. (2004) based on sequences from Combes et al. (2000) and Rovelli et al. (2000)*
[2]*: based on sequences from Dufour et al. (2001)*
[3]*: from Baruah et al. (2003)*

Likewise, some of these primers were tested on another interspecific progeny ((*C. canephora* x *C. heterocalyx*) x *C. canephora*) and 34 couples were easily readable on the BC1 progeny (Coulibaly et al., 2003).

## SSR MARKERS DERIVED FROM *COFFEA* EST SEQUENCES

EST databases provide a valuable resource for the development of SSR-markers, which are associated with transcribed genes. Two *C. canephora* cDNA libraries were produced from two organs, i.e. leaves and fruits and sequencing is ongoing. Currently, two sets of 5814 fruit EST sequences and 3112 leaf EST sequences are available in our Coffee database (Table 3).

In a first step, nonnuclear sequences were eliminated. Then, search of microsatellite motifs was performed on the remaining sequences using a modified version of Tandem Repeat Finder (http://tandem.bu.edu) (C. Tranchant, christine.tranchant@mpl.ird.fr).

**Table 3. Summary of *Coffea canephora* EST libraries (in progress).**

| | Leaf library | Fruit library |
|---|---|---|
| Total sequences | 3112 | 5814 |
| Clean sequences | 2709 (87 %) | 5706 (98 %) |
| Mean size | 575 bp | 599 bp |
| Potential Unigenes (singletons + contig) | 1859 (1520 (56 %) + 339) | 3436 (2552 (45 %) +884) |
| | 4852 (3551 + 1301) | |
| Microsatellites | 446 (~10%) | |

In a second step, sequences including microsatellites were compared to public database sequences using the BLASTx algorithm. The search was conducted in July 2004 using the default parameters. Similarities were considered significant when the E-value was less than $e^{-30}$.

In a third step, the 25 more significant E-values were chosen for primer designing (Primer 3 software). Out of these 25 primer pairs, 23 gave an amplification with at least one of the seven *Coffea* species involved in the mapping populations. Since only diagnostic loci – distinguishing the two species of an interspecific cross – can be considered, intraspecies polymorphism was assessed. Finally, between 8 and 12 primer pairs could be used for mapping purposes i.e. 34.8 to 52.2% of the primers tested (Table 4). These numbers are higher than that obtained from anonymous genomic SSR sequences.

**Table 4. Results concerning the 23 primer pairs giving a PCR product and obtained on the five mapping progenies currently studied.**

| PCR amplification / 25 pairs | Mapping progeny | Useful for mapping / 23 |
|---|---|---|
| PSE: 20 (80%) | CP | 11 (47.8%) |
| HET: 18 (72%) | CH | 11 (47.8%) |
| DEW: 21 (84%) | PD | 12 (52.2%) |
| LIB: 23 (92%) | LC | 9 (39.1%) |
| EUG: 22 (88%) | CE | 8 (34.8%) |
| CAN: 23 (92%) | | |

*CP : (C. canephora x C. pseudozanguebariae) x C. canephora*
*CH : (C. canephora x C. heterocalyx) x C. canephora*
*PD : (C. pseudozanguebariae x C. liberica var dewevrei) x C. liberica var dewevrei*
*LC : (C. liberica x C. canephora) x C. canephora*
*CE : (C. canephora x C. eugenioides) x C. canephora*

## SSR MARKERS DERIVED FROM *LYCOPERSICON* EST SEQUENCES

Coffee belongs to Rubiaceae and is closely related to the Solanaceae family, as these two families are included in the Asterid I class. We could thus take advantage of the sequencing of the tomato genome thanks to the generation of shared markers. Among tomato (*Lycopersicon esculentum*) sequences, EST sequences including microsatellite motifs were identified and some of them mapped on the *L. esculentum* LA925 x *L. pennellii* LA716 linkage map (http://www.sgn.cornell.edu/cgi-bin/mapviewer/mapTop.pl?map_id=1). We selected EST markers which mapped with a LOD score of at least 2 and then looked for similarity with our *Coffea* EST sequences using BLASTn algorithms. Of the 32 *Lycopersicon* EST sequences retained, only one had good similarity (E-value = $e^{-138}$), with a *Coffea* EST sequence similar to an histone H3.2 protein. Thus, except for this sequence, only heterologous primers previously designed and available on the **Solanaceae Genomics Network** website (http://www.sgn.cornell.edu) could be used and tested for amplification on the seven *Coffea* species involved in mapping progenies.

Among the 32 primer pairs tested, 8 did not give any amplification. Between 11 and 21 were not useful for mapping purposes and 12.4 % to 50 % will be analyzed to determine their progeny mapping potential (Table 5). A lower number of primers are useful for mapping purposes compared to the results given in Table 4. These results are not surprising since these SSR primers are derived from heterologous sequences.

**Table 5. Results concerning the 24 primer pairs giving a PCR product and obtained on the five mapping progenies studied.**

| Mapping progeny | Amplification but not useful for mapping | Total # for mapping (%) |
|---|---|---|
| CP | 11 (45.8%) | 12 (49.9) |
| CH | 16 (66.6%) | 8 (38.5) |
| PD | 12 (50%) | 12 (50) |
| LC | 21 (87.5%) | 3 (12.4) |
| CE | 20 (83.3%) | 4 (16.6) |

*CP : (C. canephora x C. pseudozanguebariae) x C. canephora*
*CH : (C. canephora x C. heterocalyx) x C. canephora*
*PD : (C. pseudozanguebariae x C. liberica var dewevrei) x C. liberica var dewevrei*
*LC : (C. liberica x C. canephora) x C. canephora*
*CE : (C. canephora x C. eugenioides) x C. canephora*

## COS MARKERS DERIVED FROM *LYCOPERSICON-ARABIDOPSIS-COFFEA* CONSERVED ORTHOLOG SET SEQUENCES.

To overcome the problem of sequence divergence between *Coffea* and the Solanaceae family and to define a set of shared markers, we screened our EST database for sequences that could correspond to COS (conserved orthologous set) markers. These markers are derived from the identification of unique genes which are highly conserved between plant species – initially tomato – and arabidopsis and their functions have been conserved throughout evolution.

In a first step, we considered the 1025 conserved ortholog set (COS) sequences as defined by Fulton et al. (2002). Out of them, 311 were effectively mapped on the tomato genome with a LODscore ≥ 2. Considering only this set of sequences, their comparison to our *Coffea* EST sequences using BLASTn revealed close similarity with 54 of them (E-value < $e^{-10}$ with a mean value of $e^{-43}$). Moreover, the corresponding 54 markers are distributed all over the 12 tomato chromosomes. Using BLASTx, a putative function was attributed to 42 sequences (e-value <-30). Finally, this set of 54 EST sequences would be a first initial set of COS markers to map on our progenies.

In a second step, multiple alignments were performed to identify conserved and variable regions of the EST sequences. ORFs (open reading frames) were identified.

An analysis is currently under way to take the location of conserved regions and of ORFs into account. The primer pairs will be designed and tested for amplification on the seven *Coffea* species. When possible, they will be used for *Coffea* interspecific mapping projects.

## CONCLUSION

For all markers analysed in this study, we obtained good transferability among *Coffea* species even when considering species belonging to different phylogenetic clades such as CAN, EUG, and PSE. The amplification success was found to be independent of the species considered and did not reflect the between-species relatedness. This is in agreement with the recent speciation scenario noted within the *Coffea* genus.

All these locus-specific markers will facilitate assembly of a panel of "anchor" PCR-based markers for comparative mapping studies in coffee trees and for marker-assisted selection. Moreover, microsatellites associated to ESTs as well as COS markers have the advantage of detecting unique expressed regions of the genome. They will facilitate the study of similarities and differences in the structure and function between *Coffea* genomes.

Furthermore, we have defined an initial set of 54 highly conserved, single copy genes – COS markers – which can be used as markers for comparative mapping between the tomato and coffee genomes.

These comparative maps will not only shed light on the nature of genome evolution, but will also facilitate comparative mapping studies of qualitative and quantitative traits.

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# Characterization of Simple Sequence Length Polymorphisms (SSLP) in a Sample of *Coffea* spp. Germplasm

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## SUMMARY

To assess usefulness of microsatellites to discriminate *Coffea* genotypes, thirty-four fluorescently labeled microsatellite markers were used in a set of 30 *Coffea* accessions from the CENICAFE germplasm bank in Colombia. The plant material included seven east African accessions representing five diploid species (C. canephora, C. liberica, C. eugenioides, C. kapakata, C. congensis), and 23 wild and cultivated tetraploid accessions of *C. arabica* from Africa, Indonesia and South America, using one sample per accession. Similarity coefficients, number of alleles, and PIC values were estimated. The similarity coefficientes between all possible pairs of genotypes, ranged from 0 to 0.84. The average similarity coefficients were 0.18, 0.34 and 0.59 among diploids, wild tetraploids and cultivated tetraploids. These values show that the cultivated tetraploids are the most closely related and that diploids are the most diverse accessions. The allele number ranged from 1 to 8 for all genotypes, with an average of 4 alleles per locus for diploids, 3 for wild tetraploids and 2 for cultivated tetraploids. The PIC value ranged from 0 to 0.86, with 0.60 for diploids, 0.35 for wild tetraploids and 0.22 for cultivated tetraploids.

The microsatelites showed fifty-five percent of the alleles from the wild tetraploids not shared with cultivated tetraploid*s*, suggesting that these wild ancestors from Ethiopia could be used productively as a source of novel genetic variation to expand the gene pool of elite *C. arabica* germplasm. It was concluded that microsatellites are a very useful tool to discriminate genotypes, especially among tetraploids that present lower genetic variability that the diploids and therefore have a great potential to be utilized on breeding of *C. arabica.*

## INTRODUCTION

Coffee trees belong to the subgenus *Coffea* of the large Rubiaceae family. The two main cultivated species, are *Coffea arabica* L. and *Coffea canephora* Pierre. Another species of coffee that is cultivated less extensively is *C. liberica* Bull ex Hiern (Wellman, 1961).

*C. arabica* is native to the south-western highlands of Ethiopia and is the most widespread cultivated species throughout the world. It is considered a segmental allotetraploid (Meyer, 1969), is autogamous and has relatively uniform phenotypic characteristics. As a cultivated species, *C. canephora* is second to *C. arabica*. It is widely grown in Africa and Indonesia and was described by Pierre in 1895 (Wellman, 1961). *C. canephora* originated in the humid lowland forests of tropical Africa, which stretch from Guinea to Uganda and Angola (Charrier and Berthaud, 1985). It is an open pollinated diploid species and is characterized by its great variation in forms and ecotypes including a high level of resistance to coffee leaf rust caused by *Hemileia vastatrix* Berk and Br. (Wellman, 1961).

DNA markers provide powerful and reliable tools for evaluating genetic variation both within and between populations (Qamaruz et al., 1998; Powell et al., 1996). Microsatellites, or

simple sequence repeats (SSRs), are widely used in genetic studies because of their abundance, distribution in the genome and their hypervariability (Powell et al., 1996). SSR's are recognizable as tandem arrays of di, tri, or tetra-nucleotide motifs found in the DNA of most eukaryotic organisms (Weber and May, 1989). SSR primer pairs have been used in a range of different crops including coffee (Combes et al., 2000).

While previous studies using RFLP (Paillard et al., 1993) and RAPD (Lashermes et al., 1993 and 1996) markers in coffee reported very low levels (~10%) of polymorphism within wild and cultivated forms of tetraploid *C. arabica* and within different Arabusta hybrids, higher levels of diversity in *C. arabica* and related *Coffea* species have been detected using SSR markers (Combes et al., 2000). Fluorescent labeling of microsatellite primers in combination with high throughput sequencers and automated detection systems allows data to be recorded automatically for multiple markers (Ziegle et al., 1992, Reed et al., 1994). These systems facilitate data analysis and lay the foundation for large-scale genotyping experiments.

The objective of the present study was to evaluate genetic diversity in a set of 30 *Coffea* spp. genotypes, including both diploids and tetraploids, using 34 SSR fluorescent labelled microsatellite markers and automated allele detection.

## MATERIALS AND METHODS

### Germplasm

A total of 30 genotypes of *Coffea* spp. were used including five diploid species and 23 different wild and cultivated accessions of the tetraploid species *C. arabica* (Table 1).

These accessions form part of the National Center for Coffee Research (CENICAFE) germplasm bank in Colombia. Seven diploid accessions were selected to represent the five most important diploid species in the collection, with two accessions each representing *C. eugenioides* and *C. liberica*. For tetraploids, we sampled eleven wild tetraploids from Ethiopia as well as ten cultivated tetraploids and two accessions of 'Timor hybrid'.

Of the ten cultivated tetraploids, the most widely grown in the 870,000 ha of the Colombian coffee region are the improved *C. arabica* varieties 'Colombia' (27%) and 'Caturra' (43%). The traditional variety, 'Tipica' (30%), is still planted by small farmers (Federacion Nacional de Cafeteros, 1997). 'SL28', 'K7', and 'Rume Sudan' are commercial varieties in Kenya. 'Timor Hybrid' was discovered on a private estate at the end of the 1940's in Timor island, Indonesia (Goncalves and Rodrigues, 1976). It was used in the development of the composite variety 'Colombia', which provides multi-genic resistance to coffee leaf rust *H. vastatrix* (Castillo and Moreno, 1988).

Three of the 11 wild Ethiopian accessions (E), were collected by the FAO mission in 1964/65 (Meyer et al., 1968), and the other eight (Et) are part of the ORSTOM collection (Guillaumet and Halle, 1966) and of the Ethiopian collection at CENICAFE.

### DNA extraction

The extraction of DNA was done using lyophilized tissue of a single plant per genotype. The method used was a modification of McCouch et al. (1988), by addition of 2% CTAB to the extraction buffer, and by adding PVP (Polyvinyl-pyrrolidone) and DIECA (Diethyl-dithiocarbamic acid) to the nitrogen for grinding.

**Table 1. Coffee genotypes used for diversity analysis.**

| Genotype | Ploidy | Species | Degree of Dometication | Origin |
|---|---|---|---|---|
| *C. kapakata* | 2X | *C. kapakata* | Unknown* | Angola |
| *C. liberica Col 24* | 2X | *C. liberica* | Cultivated | Ivory cost |
| *C. liberica Col 25* | 2X | *C. liberica* | Cultivated | Ivory cost |
| *C. congensis* | 2X | *C. congensis* | Unknown* | Congo river basin |
| *C. canephora 1020* | 2X | *C. canephora* | Unknown* | Congo |
| *C. eugenioides I 900* | 2X | *C. eugenioides* | Unknown* | Congo |
| *C. eugenioides 'Ofic'* | 2X | *C. eugenioides* | Unknown* | Congo |
| VAR COLOMBIA | 4X | *C. arabica* | Cultivated | Colombia |
| TIMOR HYBRID 522 | 4X | *C. arabica* | Cultivated** | Timor Island |
| TIMOR HYBRID CV 2 | 4X | *C. arabica* | Cultivated** | Timor Island |
| BORBON AMARILLO | 4X | *C. arabica* | Cultivated | Reunion Island |
| BARBUK SUDAN 1716 | 4X | *C. arabica* | Cultivated | Sudan |
| CATURRA AMARILLO | 4X | *C. arabica* | Cultivated | Brazil |
| CATURRA ROJO | 4X | *C. arabica* | Cultivated | Brazil |
| K 7 | 4X | *C. arabica* | Cultivated | Kenya |
| RUME SUDAN 1222 | 4X | *C. arabica* | Cultivated | Sudan |
| RUME SUDAN 104 | 4X | *C. arabica* | Cultivated | Sudan |
| SL28 | 4X | *C. arabica* | Cultivated | Kenya |
| TIPICA ROJO | 4X | *C. arabica* | Cultivated | Yemen |
| VAR COLOMBIA | 4X | *C. arabica* | Cultivated | Colombia |
| ET38C3 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| ET38C4 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| E 20 | 4X | *C. arabica* | Wild | Ethiopia (Sidamo) |
| ET35CC1 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| ET15 | 4X | *C. arabica* | Wild | Ethiopia (Illubabor) |
| ET56 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| ET42 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| E 456 | 4X | *C. arabica* | Wild | Ethiopia (Illubabor) |
| E 41 | 4X | *C. arabica* | Wild | Ethiopia (Kaffa) |
| ET26 | 4X | *C. arabica* | Wild | Ethiopia (Illubabor) |
| ET18 | 4X | *C. arabica* | Wild | Ethiopia (Illubabor) |

**Microsatellite markers**

Thirty four microsatellites were used for this study. They were extracted from a short insert genomic library made from cv. Caturra based on hybridization based screening with a 26-mer oligonucleotide containing a poly $(GA)_{13}$ motif.

**Detection of polymorphism**

Microsatellite polymorphism was initially evaluated on silver stained polyacrylamide gels using the 30 germplasm accessions described above. Based on the observed molecular weights and the ranges of allele sizes, eight multiplex panels of fluorescently-labeled coffee

microsatellites were designed to survey allelic diversity using an automated DNA sequencer (Applied Biosystems, Model 377).

The forward primers of selected markers were 5' labeled with either hexacloro-6-carboxyfluorescein (HEX), tetrachloro-6-carboxyfluorescein (TET), or 6-carboxyfluorescein (FAM) dye phosphoramidites and synthesized by The Cornell BioResource Center (Cornell University). Each multiplex panel included combinations of markers whose alleles would be separated by at least 20 bp from each other when labeled with the same color dye in order to avoid overlapping.

PCR amplifications were carried out for a single marker at a time, in a total reaction volume of 25ul. The optimum mix of reagents was 2 ul of DNA (10 ng/ul), 0.75 ul of each primer (diluted to 10uM concentration), 2.5 ul of 10X buffer, 0.2 mM each of dNTPs and 1 unit of *Taq* polymerase. The PCR profile, was a initial denaturation of 94°C for 5 min followed by 30 cycles of denaturing at 94°C for 1 min, annealing at the primer specific optimum temperature for 2 min, extension at 72°C for 2 min, and a final incubation at 72°C for 5 min.

The PCR products for each multiplex set were mixed together in a 1:1:2 ratio FAM:TET:HEX and 0.5 ul of the mixed solution was combined with 1 ul of loading buffer (80% formamide in blue dextran), and 0.1 ul of internal-lane size standard, Genescan-500 TAMRA (Applied Biosystems, Foster City, CA). The samples were denatured at 95°C for 5 min, and loaded into a 64-well, 36 cm, 5% denaturing LongRanger® (FMC) gel in 1X TBE buffer. Gels were run for 2.5 hours on an automated DNA sequencer (Applied Biosystems, model 377), using virtual filter C.

**Data analysis**

Molecular weights (bp) for microsatellite bands were estimated by the Local Southern method using GeneScan® software (Elder et al., 1987) v. 3.12 (Applied Biosystems). The individual amplified fragments were assigned as 'alleles' of the appropriate microsatellite loci with Genotyper® software v. 2.5 (Applied Biosystems).

The polymorphism information content (PIC) for each microsatellite marker was calculated according to Weir (1990), based on allele frecuencies of all the varieties analyzed:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where $P_{ij}$ is the frequency of the jth allele for the ith marker and summed over n alleles.

All fragments were used to generate a genetic similarity matrix using the software NTSYS-PC v. 2.0 (Numerical Taxonomy and Multivariate Analysis System, F. James Rohlf, 1997). The Dice coefficient ($r_d$) of association was chosen to measure the similarity between genotypes (Dice 1945).

Relationships among accessions were analyzed using both the Unweighted Pair-Group Method (UPGMA), and Principal Coordinate Analysis (PCOORDA) (Sneath and Sokal, 1973).

## RESULTS

### Polymorphism

The level of polymorphism was evaluated by calculating the percent of markers detecting variation in the 30 genotypes, using all 34 microsatellites. Ninety percent of the markers detected polymorphism among the diploids (n = 7), 70% among the wild tetraploids (n = 10) and 56% among the cultivated tetraploids (n = 10). The allele number ranged from 1 to 8 for all genotypes, with an average of 4 alleles per locus for diploids, 3 for wild tetraploid accessions, and 2 for cultivated tetraploids.

**Table 2. PIC values for 34 microsatellite markers grouped by ploidy and showing differences between cultivated and wild tetraploids.**

| SSLP | Tetraploids | | Diploids | Tetraploids |
|---|---|---|---|---|
| | Cultivated 4x | Wild 4x | | |
| CFGA100 | 0.35 | 0.00 | 0.44 | 0.21 |
| CFGA189 | 0.46 | 0.69 | 0.75 | 0.57 |
| CFGA2 | 0.00 | 0.20 | 0.45 | 0.08 |
| CFGA20 | 0.14 | 0.74 | 0.63 | 0.55 |
| CFGA202 | 0.47 | 0.64 | 0.69 | 0.57 |
| CFGA207 | 0.60 | 0.51 | 0.64 | 0.58 |
| CFGA222 | 0.41 | 0.43 | 0.67 | 0.54 |
| CFGA227 | 0.00 | 0.18 | 0.69 | 0.08 |
| CFGA236 | 0.00 | 0.31 | 0.45 | 0.15 |
| CFGA249 | 0.44 | 0.58 | 0.78 | 0.52 |
| CFGA260 | 0.00 | 0.00 | 0.72 | 0.00 |
| CFGA276 | 0.26 | 0.55 | 0.78 | 0.40 |
| CFGA280 | 0.00 | 0.00 | 0.73 | 0.00 |
| CFGA281 | 0.49 | 0.00 | 0.78 | 0.35 |
| CFGA285 | 0.00 | 0.50 | 0.00 | 0.21 |
| CFGA311 | 0.29 | 0.67 | 0.82 | 0.44 |
| CFGA35 | 0.00 | 0.41 | 0.61 | 0.17 |
| CFGA38 | 0.20 | 0.00 | 0.67 | 0.12 |
| CFGA465 | 0.54 | 0.66 | 0.64 | 0.60 |
| CFGA485 | 0.00 | 0.17 | 0.61 | 0.08 |
| CFGA491 | 0.26 | 0.00 | 0.00 | 0.22 |
| CFGA494 | 0.26 | 0.43 | 0.82 | 0.63 |
| CFGA499 | 0.00 | 0.20 | 0.57 | 0.08 |
| CFGA502 | 0.72 | 0.78 | 0.61 | 0.81 |
| CFGA529 | 0.00 | 0.00 | 0.67 | 0.00 |
| CFGA54 | 0.00 | 0.48 | 0.38 | 0.28 |
| CFGA547a | 0.26 | 0.66 | 0.86 | 0.47 |
| CFGA55 | 0.13 | 0.64 | 0.73 | 0.44 |
| CFGA69 | 0.00 | 0.00 | 0.78 | 0.00 |
| CFGA74 | 0.00 | 0.48 | 0.00 | 0.22 |
| CFGA75 | 0.36 | 0.00 | 0.57 | 0.23 |
| CFGA91 | 0.32 | 0.00 | 0.75 | 0.26 |
| CFGA92 | 0.50 | 0.54 | 0.57 | 0.53 |
| CFGA99 | 0.00 | 0.30 | 0.61 | 0.15 |

The average PIC value (Table 2), was 0.45 overall, with 0.60 for diploids, 0.35 for wild tetraploid accessions, and 0.22 for cultivated tetraploids.

These data provide us with a general picture of the relative levels of diversity among the three groups. Based on the objectives of a specific study, it is possible to select the most informative SSLP markers to efficiently evaluate the material.

**Genetic relationships**

Similarity coefficients between all possible pairs of genotypes ranged from 0 to 0.84. The average similarity coefficients within groups were 0.18 among diploids, 0.34 among wild tetraploids and 0.59 among cultivated tetraploids. These values reflect the proportion of shared alleles among genotypes within each group and are consistent with the data presented above, in that the cultivated tetraploids embody the least genetic diversity (are the most inter-related) and that diploids are the most diverse group of accessions.

Similarity coefficients were used to examine the genetic relationships among the 30 germplasm accessions. A dendrogram based on UPGMA analysis of similarity data is shown in Figure 1.

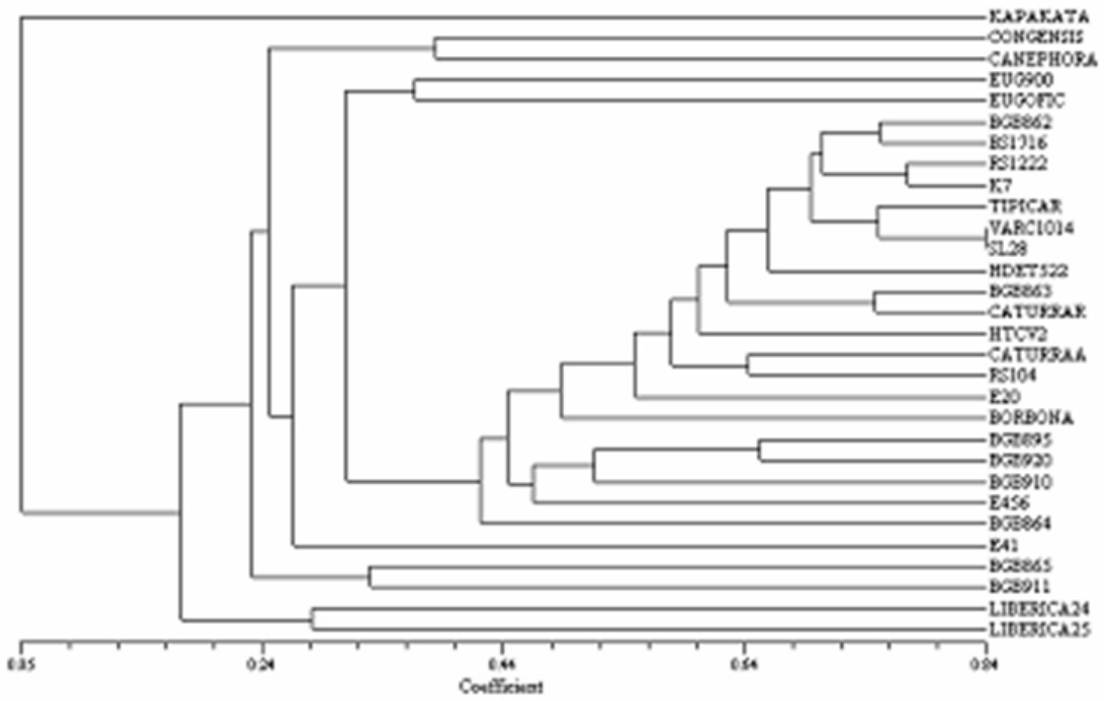


**Figure 1. Dendrogram of relationship among 32 coffee accessions based on microsatellite markers.**

The correlation between the similarity coefficients matrix and the cophenetic matrix derived from the tree matrix produced by UPGMA was 0.928, indicating that the two procedures gave similar groupings.

Results from Principal Coordinate Analysis (PCOORDA) based on similarity data is shown in Figure 2. The accessions form clusters that are generally consistent with both ploidy and cultivation status. As can be seen from these figures, genotypes are clustered in a very similar manner as UPGMA.

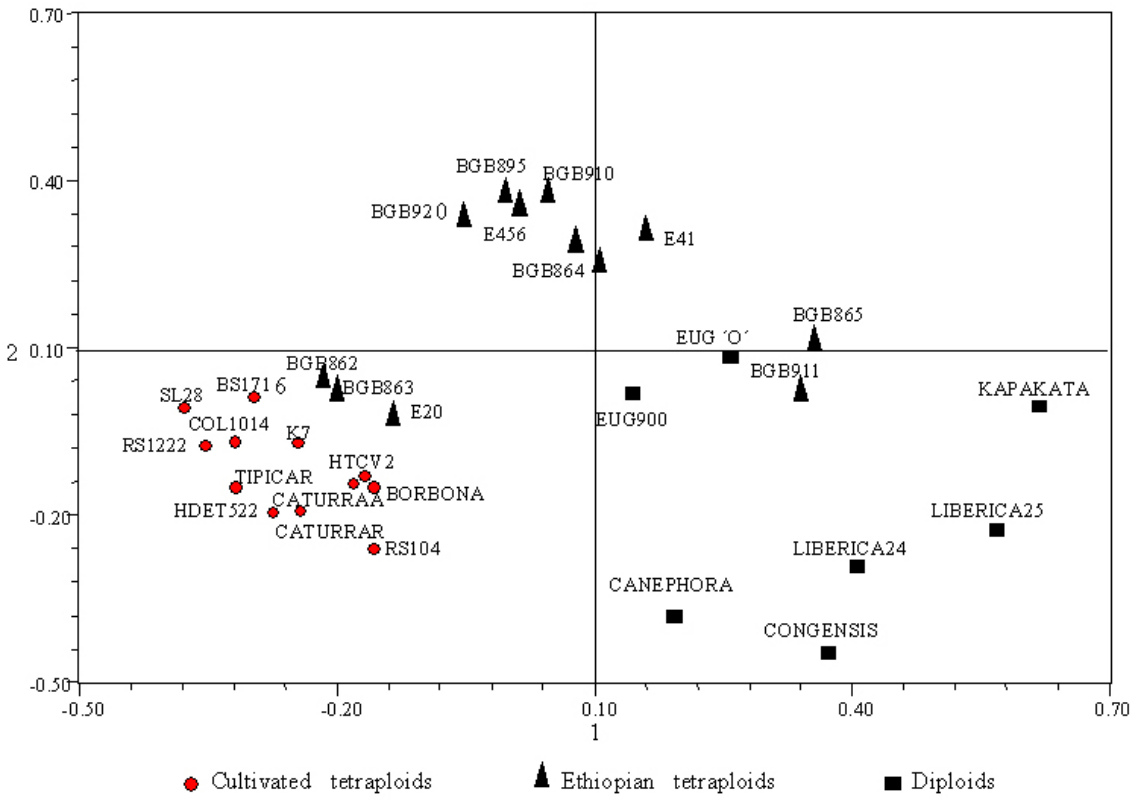


**Figure 2. Spatial distribution of the genotypes based on Principal Coordinate Analysis.**

## Tetraploids

The cultivated tetraploids showed the lowest level of within-group genetic diversity. Although these accessions form an ill-defined group in the dendrogram (Figure 1), their genetic similarity is more apparent in the PCA analysis (Figure 2). The two accessions of Timor Hybrid analyzed here appear to be more closely related to *C. arabica* cultivars than to *C. canephora* in both the dendrogram and the PCA. The similarity coefficient between the Timor Hybrid and *C. arabica* cultivated accessions averaged $r_{d=}0.58$, while with *C. canephora* it was $r_{d=}$ 0.35.

The wild Ethiopian tetraploids embodied a high level of genetic diversity at the SSLP level. They are scattered in 3 of the 4 quadrants in Figure 2 and most were genetically differentiated from the cluster of cultivated accessions. In Figure 1 the diversity of the wild accessions can be more clearly seen in relation to the cultivated tetraploid varieties. Accessions BGB862, BGB863 and E20 are most closely related, with an average similarity coefficient of 0.58. Accessions BGB864, BGB895, BGB920, BGB910 and E456 are intermediate in terms of shared alleles, with a similarity coefficient of 0.44. Accessions 'BGB865', 'BGB911', and 'E41' are the most distinct from the cultivars, with an average similarity coefficient of 0.27.

## Diploids

*C. kapakata* was the diploid species that was most genetically distinct from all other germplasm, with a Dice similarity coefficient of $rd_{=}0.05$. When compared with the other diploids the similarity coefficient was $r_{d=}0.04$, with wild tetraploid *C. arabica* the similarity coefficient was 0.06 and with cultivated *C. arabica*, it was 0.05.

After *C. kapakata*, *C. liberica* was the second most distantly related species to all other germplasm in this study ( $r_{d=}0.18$). Because we included two different accessions of *C.*

*liberica,* we were able to evaluate within-species variation. As can be seen in Figures 1 and 2, *C. liberica* showed high intraspecific variation ($r_d = 0.29$).

Compared to other diploids in this study, *C. congensis* was most closely related to *C. canephora* (Figure 1). In fact, the accessions of *C. canephora* and *C. congensis* shared more microsatellite alleles ($r_d = 0.39$) than did the two accessions of *C. liberica* described above. This is in agreement with studies by Charrier and Berthaud (1985) suggesting that the two species are close relatives

The two different accessions of *C. eugenioides* had similarity coefficients that were similar to those reported for *C. congensis* and *C. canephora* ($r_d = 0.37$) suggesting that *C. eugenoides*, like *C. liberica,* is a diverse species. Because both cultivated and wild tetraploids are believed to have originated from the same two progenitor diploid species, *C. eugenioides* and *C. canephora* (Lashermes et al., 1999), we compared the similarity coefficients between the two different accessions of *C. eugenoides* and the *C arabica* accessions to determine whether one lineage of *C. eugenioides* was genetically more similar to *C. arabica* than the other.

## DISCUSSION

Significantly higher levels of polymorphism were observed among diploid or wild tetraploid species of coffee than within cultivated *C. arabica*. This is in agreement with historical data, indicating that commercial varieties of *C. arabica* have undergone a genetic bottleneck. The cultivated tetraploids embodied approximately one third the amount of SSR diversity as the diploid species, and three fourths that of the wild tetraploids, based on number of alleles, PIC values, and similarity coefficients.

This study illustrates the particular value of microsatellite markers for discriminating among closely related accessions. The two earliest introductions to Latin America, 'Tipica' and 'Borbon', which are known to be very closely related, were distinguishable at 25% of SSR alleles. These results agreed with those of Orozco et al. (1994), where scientists were able to differenciate Tipica and Borbon using RAPDs. Compared to other studies using RFLPs (Paillard et al., 1993) or RAPDs (Lashermes et al., 1996) where only 10% of alleles detected variation among cultivated tetraploids, an average of 18% of SSR alleles showed intra-subspecific polymorphism in the cultivars surveyed here.

The lack of diversity within existing *Coffea* spp. cultivars in Latin America presents coffee breeders and geneticists with a need to look beyond the elite gene pool to identify suitable parents for crossing. Microsatellite markers provide an efficient way to estimate genetic similarity and can be used to efficiently select parental candidates for crossing. The data suggests that crosses between cultivated and wild tetraploids can be reliably used in breeding projects aimed at enhancing the long-term productivity of the coffee industry in South America.

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# Analysis of Expressed Sequence Tags (ESTs) and Microsatellite Sequences in Coffee *(Coffea arabica*)

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## SUMMARY

Despite its global importance, very little information has been gathered from the coffee plant at the genetic level. Here we describe the analysis of a set of Expressed Sequence Tags (ESTs) and microsatellite sequences developed from coffee (*Coffea arabica*).

A non-directional cDNA library was constructed from young coffee leaves tissue. Seven hundred and sixty clones were selected, subjected to single-pass sequencing and identified by sequence similarity searches against sequences in the EST and non-redundant GeneBank databases. Of the 751 sequences, 42% exhibit similarity to known plant genes, 19% to sequences with unidentified function, and 39% represent new gene sequences. Analysis of the identified clones indicated sequence similarity to a broad diversity of genes encoding proteins such as enzymes, structural proteins and regulatory factors.

Additionally, simple sequence repeat (microsatellite) loci were identified in coffee in clones isolated from genomic libraries. Coffee turned out to be among plant species containing a low frequency of microsatellites. Sequence analysis showed that $(AC)_n$ microsatellites in coffee are frequently associated with other microsatellites, mainly $(TC)_n$ and $(AT)_n$ motifs, while $(AG)_n$ microsatellites are not normally associated with other microsatellites. The results also suggest that the coffee genome harbors a low number of poly (AAT) microsatellites, a very common microsatellite motif in other plant genomes. Dot-blot and sequence analysis revealed that a high number of coffee microsatellites are associated to miniature inverted-repeat transposable elements or MITES.

We have developed nearly 400 microsatellite markers and currently are developing a *C. arabica* molecular map with them. These markers have been also used to examine the genetic diversity of CENICAFE germplasm collection.

## INTRODUCTION

Coffee is one of the most important agricultural commodities in the world, providing large resources for the economies of many developing countries. Despite its global importance, very little information has been gathered from this plant at the genetic level. As of October 2001, only 26 gene sequences from coffee had been deposited in the Genebank database.

cDNAs sequence analysis provides several advantages for genome investigations in plants. Isolated and partially characterised cDNA clones have contributed to the construction of linkage and physical maps (Davis et al., 1999), studies of the mechanisms of expression of various isozymes and family genes (Yamamoto and Sasaki 1997) and the development of expressed sequence tags. Expressed Sequence Tags (ESTs) analysis refers to the application of large-scale partial sequencing of anonymous cDNA clones from cDNA libraries and their subsequent identification through homology searches of public databases (Adams et al., 1991).

Simple sequence repeats (SSRs), also known as microsatellites, are DNA sequences composed of tandemly repeated short motifs (Tautz and Renz, 1984). These motifs exhibit extensive site-specific length polymorphism due to differing numbers of repeat units; they are also robust, easily transferable, co-dominant, chromosomally located to single-locus and can be developed as PCR-based markers (Weber and May, 1989; Tautz, 1989).

The genus *Coffea* includes two cultivated species of economic importance, *C. arabica* L. and *C. canephora* Pierre. *C. arabica* (*2n* = 4X = 44) is an amphidiploid formed by a recent event of hybridization between the diploid species *C. eugenioides* and *C. canephora* (Lashermes et al., 1999); all other *Coffea* species are diploid (*2n* = 2X = 22). ESTs and microsatellite markers have not been extensively developed in coffee as in other crops. Only eleven microsatellite markers were obtained by Combes et al. (2000) and they have been used for the study of allele number and heterozygosity level in several diploid and tetraploid coffee species.

The aim of the present work was to establish the frequency of several microsatellite motifs in the coffee genome, the isolation and characterizaton of cDNAs and microsatellite sequences from *C. arabica* and the analysis of ESTs expressed in coffee leaves.

## MATERIALS AND METHODS

### ESTs Development

Total RNA was isolated from *C. arabica* var. Caturra young leaves by using a total RNA kit (Promega). The mRNA was isolated from total RNA using oligo dT cellulose columns. A non-directional cDNA library was constructed from the isolated mRNA in an *EcoRI* digested PCR2.1 vector. Random clones were selected and single-pass sequenced in an ABI377 sequencer. This stage of the project was contracted with the company DNA Technologies, Inc.

### Microsatellite Development

Two types of genomic libraries were used for the isolation of coffee microsatellites. One random genomic library and several genomic libraries enriched for different microsatellite motifs were constructed. The libraries were screened with short di- and tri-repeat oligonucleotides of 10-15 bases, and positive clones were isolated and sequenced.

### Sequence Analysis – *ESTs*

For the annotation of predicted gene families, we followed the annotation tools described at Cold Spring Harbor Laboratory. We used comparative methods in the analysis of EST sequences. The comparative methods primarily involve searches for homology within publicly available databases and multiple sequence analyses generated locally. Non-redundant GeneBank and dbEST databases searching was performed with the programs Gapped BLAST and BLASTX (Altschul et al., 1990) at NCBI.

#### *Microsatellites*

The high quality sequences obtained were analyzed for the presence of microsatellite motifs by using the programs RepeatMasker (University of Washington) or SSRIT (Cornell University). Microsatellite loci were defined as those di-nucleotide motifs having more than 7 repeat units and tri-nucleotide motifs containing more than 5 repeat units. Vector sequences were trimmed with the commercial software SEQUENCHER® (Gene Codes Inc., Ann

Harbor MI) or LASERGENE (DNASTAR, Inc., Madison, WI). Redundant sequences were eliminated with the program Stand-alone BLAST.

## RESULTS

### ESTs Analysis

Seven hundred and fifty one clones that contained inserts were randomly isolated and single-pass sequenced. Vector sequences were trimmed and redundant sequences were identified and eliminated for the construction of a non-redundant EST database for coffee with the remaining sequences. Among the 751 sequenced clones, 445 sequences were unique; this represents a 40% redundancy. This redundancy was expected since the cDNA library constructed was not normalized. However, only 6 sequences had a representation in the set of 10 or more clones, that we consider high.

Some of the homologies of the sequences searched are shown in Table 1. Of the 751 sequences, 42% exhibit similarity to known plant genes, 19% to sequences with unidentified function (UF class), and 39% represent new gene sequences (NSH class). Likewise, 36% of maize ESTs developed from different tissues had homology to genes of known function (Davis, et al., 1999). The larger number of ESTs with homology to genes of known function found in coffee might only represent a larger number of characterized ESTs deposited currently in public databases.

Analysis of the identified clones indicated sequence similarity to a broad diversity of genes encoding proteins such as enzymes, structural proteins and regulatory factors. Most of Arabidopsis genes and presumably most of plant genes belong to gene families. For example, 273 members represent the cytochrome P450 monooxygenase gene family in Arabidopsis (www.arabidopsis.org). In the ESTs coffee set analyzed we identified several gene families. Among these families are the Histone family, the cytochrome P450 protein family and ribosomal RNA gene families.

CAEST74 was the most represented clone in the library and its sequence has a weak homology to a nuclear repair endonuclease from Arabidopsis. The second clone most represented in the coffee ESTs set was CAEST126 that shows some homology with a defense-related gene from tobacco. Most of the coffee sequences have homology to genes from plants of the Solanaceae family.

### Frequency of Microsatellites in Coffee

Coffee turned out to be among plant species that contain a low frequency of the microsatellite motifs tested (Table 2). However, the average distance between two loci, estimated at 127kb for poly (AC), is among the shortest in any plant genome (Table 3) given that coffee has a small genome size of 1158Mb (Arumuganathan and Earle, 1991). In contrast, the distance between two poly (AC) loci, estimated at 769kb, is among the largest in plant genomes.

*Microsatellite Sequence Analysis:* A total of 367 poly (AG) and poly (AC) microsatellite sequences were identified. The number and type of the identified coffee microsatellites is summarized in Table 4. Coffee $(AC)_n$ microsatellites are frequently associated with other microsatellites; fifty four (29%) were associated to $(TC)_n$ microsats, 34 (18%) were associated to $(AT)_n$ sequences, and 7 were associated to different tetranucleotide motifs (4%). On the other hand, coffee (AG)n microsatellites are not normally associated with other microsatellites, hence the number of perfect motifs is higher. Only 7 (4%) microsats were associated to (TG), 7 (4%) to (AT) sequences and 6 (3%) to different tri-nucleotide sequences. The rest of the compound sequences correspond to imperfect poly (AG) motifs.

**Table 1. Distribution of cDNAs in classes of putative function.**
**The class assignment is based on the description of the best match from BLAST similarity searches to the EST database and BLASTX similarity searches to the non-redundant GenBank database (NCBI). The number of EST clones and the percentage of the total from each category in the coffee database are listed. cDNA clones that were sequenced from both 5' and 3' ends were counted only once.**

| CODE | DESCRIPTION | COUNT | PERCENTAGE |
|---|---|---|---|
| AA | Amino acid metabolism | 3 | 0.40 |
| C | Carbohydrate metabolism | 39 | 5.19 |
| CDC | Cell division cycle | 1 | 0.13 |
| CHP | Chaperonin, heat shock, folding | 1 | 0.13 |
| CSK | Cytoskeleton | 3 | 0.40 |
| CW | Cell wall | 0 | 0.00 |
| D | Defense | 43 | 5.73 |
| DEV | Development | 7 | 0.93 |
| DNA | DNA - modifying enzymes | 49 | 6.52 |
| ER | Endoplasmic reticulum proteins | 0 | 0.00 |
| HOR | Hormone biosynthetic enzymes | 0 | 0.00 |
| L | Lipid metabolism | 1 | 0.13 |
| LEA | Lea - Late embryogenesis abundant | 0 | 0.00 |
| MT | Membrane, transporters, receptors | 34 | 4.53 |
| NM | Nitrogen metabolism | 0 | 0.00 |
| NSH | Non-significant homology | 293 | 39.01 |
| NUC | Nucleus | 15 | 2.00 |
| NUM | Nucleotide metabolism | 0 | 0.00 |
| OX | Oxygen-detoxifying enzymes, peroxidases | 41 | 5.46 |
| PA | Proteinases, ubiquitin | 23 | 3.06 |
| PS | Photosynthesis | 5 | 0.67 |
| RB | Ribosome, protein translation | 9 | 1.20 |
| RNA | Acting on RNA | 4 | 0.53 |
| RR | Ribosomal RNA | 21 | 2.80 |
| RS | Respiration | 2 | 0.27 |
| SEM | Secondary metabolism | 1 | 0.13 |
| SM | Sulfur metabolism | 0 | 0.00 |
| SP | Storage protein | 0 | 0.00 |
| STD | Signal transduction (kinases, calmodulin, etc.) | 6 | 0.80 |
| T | Transcription factors | 5 | 0.67 |
| TON | Tonoplast | 0 | 0.00 |
| TRF | Subcellular trafficking | 0 | 0.00 |
| UF | Unidentified function | 145 | 19.31 |
| | TOTAL | 751 | 100.00 |

Sequence analysis of the (AC)n microsatellites identified in coffee revealed the possible association of these repeated elements with miniature inverted-repeat transposable elements or MITES. The coffee sequences contain distinctive characteristics of the MITE family such as short length (up to 400 bp) and terminal inverted repeats of 10-15bp (Zhang et al., 2000). They might represent the first family of transposable elements reported in coffee.

**Table 2. Estimated frequency (percentage) of microsatellite motifs in several plant genomes.**

| | GA | CA | GAA | AAT | Reference |
|---|---|---|---|---|---|
| **Wheat** | 4.55 | 2.84 | N/A | N/A | Roder et al. (1995) |
| ***Pinus taeda*** | 4.6 | 3.8 | N/A | N/A | Echt and May-Marquardt (1997) |
| **Rice** | 4.5 (2.8) | 4.1 (2.7) | 0.1 (0.02) | (0.03) | Panaud et al. (1995) and this study |
| **Peach** | 1.98 | 0.47 | N/A | N/A | Sosinski et al. (2000) |
| **Chickpea** | 0.21 | N/A | 0.16 | 0.37 | Winter et al. (1999). |
| **Arabidopsis** | 0.106 | 0.018 | 0.355 | N/A | Depeiges et al. (1995) |
| **COFFEE** | 0.47 | 0.08 | 0.06 | 0.02 | This study |

*N/A represents data not available.*

**Table 3. Number and type of microsatellites (repeat units > 7) identified in the coffee genome.**

| | TYPE OF MOTIF | |
|---|---|---|
| | **GA** | **CA** |
| **Perfect Repeat** | 122 | 56 |
| **Compound Repeat** | 59 | 130 |
| **Maximum Length of Perfect Repeat** | 38 | 30 |
| **Mean Length of Perfect Repeat** | 14.88 | 13.39 |
| **%GC Content of Flanking Regions** | 37.84 | 40.83 |
| **Total of Unique Sequences** | 181 | 186 |

**Table 4. Amplification of coffee microsatellites in diploid and tetraploid species.**

| | Repeat Motif | PCR primer sequence (5′ –> 3′) | Product size (bp) | Number of Alleles/ Polymorphism | |
|---|---|---|---|---|---|
| **GA Microsats** | | | | Diploids | Tetraploids |
| **CFGA574**[2] | $(AG)_6$ TG $(AG)_8$ | F: GTATGGCTCTGCATTCTGTCA<br>R: GCCAACCTCTCAAATGCTTC | 106 | 3/Yes | 1 /No |
| **CFGA627**[2] | $(AG)_{16}$ | F: GGGAAGGAATTCTTTCAACTCT<br>R: CTTGGAAATTACCATGCAACC | 134 | 2 /Yes | >10/ Yes |
| **CFGA792b**[2] | $(AG)_{12}$ | F: GATCAGAACTTTGAGCTCAGCA<br>R: AATGTGGCACGCTAGAAGTG | 182 | 4 /Yes | 6 /Yes |
| **CFGA1122**[1] | $(AG)_{13}$ | F: AATGCTTCCAGTCACCCGT<br>R: GGCTTTCAAGCATCCAGTCT | 127 | 4 /Yes | 3 /No |
| **CFGA1255**[1] | $(AG)_{11}$ | F: TTGACGTTCTCGTCCGTATG<br>R: AAAGGCTTGAAAACTTGCGG | 147 | 4 /Yes | 2 /No |
| **CFGA1258**[1] | $(AG)_{18}$ $(N)_{20}$ $(CCA)_5$ | F: CAAGACAAATGTCTCTGCTGG<br>R: AATGTATCAAGCGCGCAAC | 132 | 3 /Yes | 1 /No |
| **CA Microsats** | | | | | |
| **CFCA14A**[2] | $(AC)_{12}$ | F: CTGGTAGGAGCTCTTCAAATTG<br>R: TGGCCAAATCTTGTCCGT | 161 | 4 /Yes | 2 /No |
| **CFCA281**[2] | $(AC)_{13}$ | F: GCGTCCACGTGTTAAGTCTT<br>R: TCAAGTGGCAGACATGTCAC | 155 | 2 /No | 10 /Yes |
| **CFCA331**[2] | $(CT)_{17}$ $(AC)_{18}$ $(CT)_4$ AT TT $(AC)_6$ | F: TGATGGACAGGAGTTGATGG<br>R: CACTCATTTTGCCAATCTACC | 150 | 2 /No | 10 /Yes |
| **CFCA334**[2] | $(AC)_5$ AA $(AC)_{13}$ | F: AGCCACCACAGGAAGTTTCA<br>R: GGGAGTGAAAGACATCAGGTG | 154 | 1 /No | 1 /No |
| **CFCA360**[2] | $(AC)_{15}$ | F: TTAAGACATCGGTGCATTCA<br>R: TGTGTACTGGGTTTTTTGATGT | 135 | 2 /Yes | >10 /Yes |
| **CFCA530**[2] | $(ACC)_8$ $(AC)_{11}$ | F: GTGTTTGCTTGTCAATATGCC<br>R: ACTCCTAACCTAACCGGGAA | 122 | 5 /Yes | 4 /No |

[1] *Microsatellite loci isolated from the random genomic library.*
[2] *Microsatellite loci isolated from the enriched libraries.*

## DISCUSSION

### ESTs in Coffee

The ESTs data set analysed in this study provides initial characterisation of a significant number of genes in the coffee genome. This set will be very useful for the study of gene expression patterns in coffee, the development of markers for the construction of a coffee molecular map and the identification of defence related genes expressed in the coffee leave.

It is difficult to estimate what fraction of genes in a genome is specifically expressed in any tissue of the plant, but it seems that the majority of plant genes are expressed in all tissues of the plant with diverse levels of expression. Estimates of plant genes not having strong tissue-specific expression range from 50 to 90% (Girke et al., 2000; White et al., 2000). Consequently, it is expected that the cDNA library from leaves might have a fair representation of the majority of genes encoded in the coffee genome.

All but one of the sequences were new coffee sequences not represented in the Genebank database. This shows the small number of coffee sequences deposited in public databases; only 26 coffee gene sequences were deposited in Genebank as of October 2001. The only coffee sequence identified in this study already reported in Genebank was the gene for the Rubisco-large subunit protein, rbcL. This sequence was identified in two clones. The sequence for the Rubisco-small subunit protein was identified in this set of ESTs, and it was also represented in two clones.

Carson and Botha (2000) found that most of the ESTs identified in sugarcane showed homology to animal but not plant ESTs. At the time of that study it was presumed that it was due to the small number of ESTs from plants deposited in databases. Most coffee ESTs in this study displayed homology to other plant sequences demonstrating that there is a growing number of plant ESTs being deposited in public databases. However, the fraction of plant ESTs in the dbEST database is still small, < 10%, compared to the number of animal ESTs that represents close to 80% of the sequences of this database.

### *Microsatellites in Coffee*

After hybridization experiments using radioactive oligonucleotides, it was determined that the enrichment was successful for the motifs (AC)n and (AG)n but not for the motifs (ATT)n and (AAG)n. One of the reasons for the failure to enrich the libraries for the tri-nucleotides motifs could be the difficulty to optimize the hybridization temperature for these repeated sequences. Another reason could be the very low frequency of these motifs as shown in the screening of the coffee genomic library with the corresponding oligonucleotides. Therefore, for future developments on microsatellite markers search it is suggested to concentrate on those with poly (AG) and poly (AC) motifs, which, because of their frequency, can provide the appropriate saturation for mapping purposes. The common presence of compound microsatellites in coffee is unusual since they are rare in other plant genomes (Cardle et al., 2000). It is interesting that $(AC)_n$ $(CT)_n$, the most common compound microsatellite in coffee, is also a very common compound microsatellite in humans (Bull et al., 1999).

The relatively low GC content of the flanking regions of the coffee microsatellite motifs could be explained by the fact that *Tsp*509I and *Eco*RI, the enzymes used to construct the libraries, recognize AT-rich sites (AATT and GAATTC respectively). Microsatellites in animals are confined mostly to AT-rich sequences (Matula and Kypr, 1999) and this could be also the case for coffee. If it is probed that the majority coffee microsatellites are mostly located in AT-rich regions, this would put microsatellites away from CpG islands, regions associated with the presence of housekeeping genes.

It was remarkable to find a high number of microsatellites in coffee associated to transposon-like sequences. Association of microsatellites with other classes of repetitive DNA has been detected in other organisms. Forty five percent of AT dinucleotide repeats in rice were found to be associated to members of a MITE family (Temnykh et al., 2001). Some barley microsatellites are frequently associated to MITE elements, mostly of the Stoneway type (Cardle et al., 2000) and 41% of 290 clones containing SSRs showed an association with known repetitive elements, especially retrotransposons (Ramsay et al., 1999). Finally, it would be interesting to develop transposon markers for coffee from these sequences, similar to the markers developed in petunia and maize described as the transposon-display technique (Van den Broeck et al., 1998; Casa et al., 2000) in order to pinpoint the location of agronomically important genes in coffee.

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# Transcriptomics of Resistance Response in *Coffea arabica* L.

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## INTRODUCTION

Coffee is one of the most important agricultural export commodities in the world. It represents the he main export resource for some developing countries. Despite the tremendous economic importance of coffee, our understanding of its biology is still very poor and the genetics of *Coffea* is almost non existing. The reasons for such a low research effort are several and they range from the low economic resources allocated to fundamental genetic research to the complexity of its genome.

*Coffea arabica*, is affected by an array of diseases caused by fungi, bacteria, flagellates and viruses. The two most devastating coffee pest and diseases are the leaf rust or orange rust (*Hemileia vastatrix*) and the coffee berry disease or CBD (*Colletotrichum coffeanum*). Leaf rust ruined the economy of Sri Lanka in the last quarter of the 19$^{th}$ century, rendering it bankrupt and changing the social habit of people from coffee drinking to tea. In addition, nematodes are in some areas very damaging to *C. arabica*, especially the so-called root-knot nematodes (*Meloidogyne* spp.*)* and the root-lesion nematodes (*Pratylenchus* spp.*)* (Carvalho, 1988). Pesticides provide effective protection but their applicability can be compromised by adverse environmental effects and by the emergence of resistant pathogen strains. For these reasons, much effort has been invested towards understanding the innate resistance mechanisms in plants (McDowell and Woffenden, 2003). To counteract the pathogenic infection, plants activate a variety of defence responses. A local defence include strengthening of cell walls, activation and/or synthesis of antimicrobial compounds, and expression of many defence-associated proteins, including the pathogenesis-related (PR) proteins. A hypersensitive response, characterised by a genetically programmed suicide of infected cells, frequently develops. The local response can, intern, trigger a long lasting systemic response (systemic acquired resistance, SAR) that primes the plant for resistance against a large spectrum of pathogens (Shirano et al., 2002). Plants maintain constant vigilance against pathogens by expressing large arrays of “R genes” (R, resistance). R genes encode putative receptors that respond to the products of “Avr genes” (Avr, avirulence) expressed by the pathogen during infection. Avr signal recognition initiate downstream signalling that make the infection unsuccessful.

Plant-pathogen interactions and downstream signalling are extremely complex and dynamic. The ongoing interactions between the pathogen and the plant are difficult to monitor with more traditional genetic and biochemical methods. With the advent of the large-scale genomic sequencing, EST (expressed sequence tag) projects and development of DNA microarray technologies, it is now possible to monitor the expression of thousands of genes simultaneously irrespective of different defence-related treatments and timing of the analysis.

Furthermore, SAR-inducing chemical compounds provide a unique opportunity to investigate the induced resistance mechanisms in plants in the absence of biological model systems. In particular, the two components, 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3) thiadiazole-7-carbothionic acid S-methyl ester (BTH) were demonstrated to be the potent inducers, acting independently or downstream of SA in SAR signalling but activating the SAR signal transduction pathway through the same signalling cascade. A biologically induced SAR response in Coffee has not been previously described. Based on the previous studies on SAR responsive system in tobacco, arabidopsis and other dicots, it is likely that the resistance and gene activation in *Coffea arabica* are SAR-like responses induced by BTH. The present study describes SAR response in *Coffea arabica*, induced by BTH, analysing the cDNA microarray expression profile of two different tissue, leaf and root as these tissues are eventually the target of most pathogens.

## EXPERIMENTAL PROCEDURES

The construction of cDNA library from root and leaf was carried out as described by Pallavicini et al. 2001 (19$^{th}$ Colloquium ASIC-2001). The detailed procedures on EST annotation, microarray hybridisation and data analysis are well documented in the web databank, http://www.coffeedna.net/array

## RESULTS AND DISCUSSION

### cDNA microarray analysis of coffee gene expression after BTH treatment

Changes in the abundance of transcripts corresponding to 1506 *C. arabica* ESTs were examined by cDNA microarray analysis after treatment with the SAR inducer BTH. Both leaf and root tissues showed changes in their gene expression level after treatment with acibenzolar-S-methyl. Statistical analysis showed that 55 genes were significantly over-expressed and 16 genes were significantly under-expressed in leaf, while in the case of roots, 37 genes were over-expressed and 42 genes were under-expressed. The false discovery rate (FDR) was calculated using SAM parameter and was found to be 0.7% and 0.95% in leaf and root respectively. Therefore, less than one false positive is expected for each tissue analysis.

### Physiological function of BTH-regulated genes in leaf

The SAR response in leaf has been widely studied in *Arabidopsis* and Tobacco, which provide the possibilities to critically validate the results of coffee SAR response. Chitinase have been proposed as an important factor in the active defence response of plants (Kasperzewska, 2003). In the set of up-regulated genes, we have annotated some antimicrobial peptides such as class III endochitinase (EST accession: Li-04-C10) and chitinase (RM-00-A08). These sequences were classified as pathogenesis-related proteins (PR) as their substrate is chitin, one of the main components of fungal cell wall.

The most common feature of SAR is the activation of the oxidative burst in metabolic pathway. The treated leaf showed increased transcript abundance of lipoxygenase (RMi-01-E05), secretary peroxidase (RM-00-A24) and L-ascorbate peroxidase (RM-00-A20). These enzymes are unique to primary defence response in where it produces reactive oxygen species (ROS) with a direct anti-microbial action. Lipoxygenase has significant role in pathogenic response; it can produce toxic volatile and nonvolatile fatty acid-derived secondary metabolites to directly attack the invading pathogens. It may also cause irreversible membrane damage, which would then lead to the leakage of cellular contents and ultimately result in cell death. Furthermore, lipoxygenase can also generate new signal molecules, which

co-ordinately amplify specific response by activating other metabolic pathways. One of the well known actions of lipoxygenase is the induction of peroxidase activity in plant tissues, which has often been used as an enzymatic marker in early SAR studies (Kogel et al., 1994; Young et al., 1995; Rasmussen et al., 1995). The first reaction to a biotic stress is the induction of the cell-wall synthesis and modification. Pectin acetylesterase (RMi-01-G12) is a pectin-degrading enzymes, involved in softening the primary cell wall, which is up regulated in nematode feeding sites induced by root-knot and cyst nematodes (Vercauteren et al., 2002).

The response to BTH treatment appears to involve SA-mediated signalling pathway, which overlaps with other hormone-dependent, signalling pathways (Schenk et al., 2000; Cardinale, 2002). One of the transcripts (RM-00-H18) that showed increased abundance was similar to a vegetative storage protein (VSP). In addition to their putative role in nitrogen storage, VPS is induced by environmental stress such as wounding, treatment with methyl jasmonate (MeJA), sucrose and coronatine (Utsugi et al., 1998). Moreover, VSP transcript and protein expression were significantly higher in *Erwinia carotovora*-resistant chicory inbred lines compared with susceptible lines, suggesting that involvement of VSP in resistance to pathogens attack (Richard-Molarda et al., 2004). Other genes were related to abiotic stress response, such as pleiotropic drug resistance like protein (Li-01-G08), wound induced protein (RMi-08-C11), cochaperonine DNA-J (L-10-E01), metallothioneine like proteins (RM-00-J16; RM-00-C07), cyclophilin (RMi-01-H12) and dehydration induced proteins (RM-00-E16; RMi-07-F04). Cyclophilin is an immunophilins plays a major role in protein folding besides mRNA processing, protein degradation, and signal transduction. Hence cyclophilin is considered to be crucial in development and stress responsiveness (Romano et al., 2004). Dehydration induced protein RD22, a seed-specific protein, found in *Arabidopsis*, and is induced in vegetative-stage plants during drought, salt stress, or application of ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). The other up regulated genes in coffee are likely to be involved in hormone-dependent response, such as abscissic stress ripening protein (RM-00-J03) and auxin-repressed protein (RMi-04-H05).

Transcripts of some of the genes required for protein synthesis (such as ribosomal proteins, RM-00-C20 and RMi-08-G01) and genes involved in energy metabolism seemed to be expressed at a higher level after treatment. Hexose sensing in the secretory pathway is essential for mediating the activation of defence-related genes as well as repression of photo-synthetic genes (Herbers et al., 1996). Some other up regulated transcripts showed similarity with regulative factors; RNA-binding protein (RM-00-F18), histone H1 (Li-03-H10; L-10-F02), translation factor (RMi-01-D09).

**Physiological function of BTH-regulated genes in root**

Total RNA was isolated from roots of untreated controls and treated plants at 16 h after BTH application with four independent replicate experiments. Analyses of these data revealed that 79 ESTs on the microarray showed significant differential expression in response to the treatment and 80% of these ESTs showed similarity with known proteins. Unlike leaf, root had more down-regulated genes. Some up regulated transcripts belong to pathogenic-related genes such as thaumatine-like protein (RM-00-F17) and major allergen protein/PR10 protein (RM-00-D15). Thaumatin-like proteins are grouped in pathogenesis-related (PR)-5 families, members of this group have been shown to have anti-fungal activity against a broad spectrum of fungal pathogens (Hu and Reddy, 1997). Some defence-related proteins are cross-reacting allergens that activate the plant defence mechanisms (Hanninen, 1999).

In contrast to leaf, there are no up regulated genes with antifungal or scavenging activity, but many transcripts were found to be involved in cell wall fortification. A total of 5 genes were

directly involved in cell wall strengthening such as biosynthesis of cellulose (cellulose synthase, RM-00-B23), biosynthesis of lignin (caffeic acid O-methyltransferase II, RMi-09-F02; phenylalanine ammonia-lyase 1, RM-00-G16) and extensine (RMi-01-A09, RM-00-H19) that code for a major structural cell wall protein. Phenylalanine ammonia-lyase probably participates in root growth reduction in association with cell wall stiffening, related to the formation of cross-linking among cell wall polymers and lignin production (dos Santos et al., 2004). Moreover other 3 up regulated transcripts were putative cell wall proteins.

Many up regulated genes are expressed in abiotic stress response. Some of these genes showed increased abundance also in leaf such as methallothionein-like protein 3 (RM-00-C07), cyclophilin (RMi-01-H12) and dehydration-responsive protein RD22 (RMi-07-F04). Aquaporin was also over expressed in root. It is known that aquaporins were induced during the resistance response even if the actual role of water transport during plant defence response is yet to be determined (Mysore et al., 2003).

As in leaf, transcripts of some genes are required for protein synthesis (5 ribosomal proteins and one amino acid transporter-like protein, L-07-E09) and also the genes involved in energy metabolism seemed to be required at a higher level after treatment. Putative NADPH-dependent mannose phophatase (Li-02-B01) and hexose transporter (RM-00-F23) were involved in sugar metabolism. Very few up regulated transcripts showed similarity with regulative factors such as cyc02 protein (RM-00-I05) and translation elongation factors (L-10-F07).

Transcript abundance of many genes was decreased. One fourth of the 42 down regulated genes showed no similarity with well-annotated proteins. Half of the remaining transcripts were involved in metabolism processes of sugar (alcool dehydrogenase, Li-03-F10; putative quinone oxidoreductase, Li-03-H03) and protein synthesis (ribosomal proteins, O-succinylhomoserine, Li-02-H10). Regulative proteins and transcription factors compose another significant part of under expressed genes such as zinc finger proteins (RM-00-C14), translation factors, MYB transcription factor like (RMi-05-A08), protein kinase and GTP-binding proteins.

As reported by Bostock (1999), salicylic acid reduces jasmonic acid production by switching off genes involved in JA biosynthesis and in fact, we found that the allene oxide cyclase precursor (RMi-02-A03) was down-regulated, which is a protein involved in jasmonate synthesis.

Normally, antioxidant enzymes like glutathione S-transferase (Gst), glutathione peroxidase and L-ascorbate peroxidase are typically over expressed during pathogenic infection to eliminate reactive oxygen species (ROS), produced as a consequence of environmental stress. All these genes are not only missing the up regulated class of transcripts, but strangely showed a decreased transcript abundance.

**Different modulation of SAR response in root and leaf**

The global studies of gene expression of stress in *C. arabica* show a large difference in the defensive response between root and leaf. We have carried out the experiments simultaneously from the same plant and we believe that a comparison between root and leaf expression profile is appropriate.

There are 8 transcripts that show common regulation between the two organs (RM-00-B07, RMi-03-H08, RMi-07-F04, RMi-04-H01, RMi-01-H12, RM-00-P11, RM-00-C07 e RM-00-

H19). Interestingly, most of the clones showed similarity to the sequences involved in biotic stress response in plants in the public database. These clones could be directly used as a putative markers for stress response in *C. arabica.*

It is interesting to note that the identification of some genes that seem to have opposite regulation between root and leaf after the treatment (L-11-H11, Li-02-B12, RM-00-H14, RMi-06-A01). Of these only 2 were identified with putative function. One codes for a superoxide dismutase (SOD). The activation of oxidative burst is considered one of the first response after pathogenic attack. But in root, this activation seems to be repressed. As we have described earlier, Gst is also repressed.

The second one is a root specific gene, whose expression has been reported to be repressed after several kind of treatments (Munoz et al., 1997). In coffee, this gene has the similar behavior in root, but interestingly, in leaf, it is found to be up regulated.

## CONCLUSION

Coffee is not a trivial economic issue in the developing world, while it is the second-largest export earner for developing countries. The countries like Burundi and Uganda, the main source of foreign exchange accounts for more than half of export earnings. The disease susceptibility of Coffee plant directly affects approximately 20 million families that depend on beans as their main source of income. Coffee is very sensitive to pests and diseases. At least six major diseases could possibly affect coffee plantations, of which coffee rust pathogen, *Hemileia vastatrix*, is currently found almost all over the world. It is important to note that coffee-growing plantations affected by this fungus may never be recovered. In the present study, the effect of the treatment with BTH on the subsequent activation of defence related genes in *Coffea arabica* have been investigated. This is the first step towards elucidating the mode of action of BTH, ultimately to establish SAR in this tropical crop.

To analyse the SAR in *C. arabica*, two cDNA libraries were constructed using mRNA isolated from leaves and roots. A total of 1516 non-redundant EST groups were obtained, consisting of 217 contigs and 1299 singletons. The exact number of genes represented by the EST sequences was slightly lower, due to the ambiguous reads and stringent clustering parameters hindered singletons from being clustered. Considering the fact that EST sequencing effort preferentially captures the abundantly transcribed genes and the cDNA microarray is particularly effective for this class of transcripts we assume that the amount of data generated was sufficient for the first gene expression analysis in this crop.

The competitive hybridisation on the *C. arabica* specific cDNA microarray prepared from root and leaf, treated with BTH, revealed over-expressed a set of genes. This expression profile reflects a wide spectrum of defence related response against different pathogens as described in various plant species. As biologically induced SAR response in coffee has not been described earlier, it is difficult to draw a definite conclusion that SAR is induced by BTH in coffee. Indeed, although the basic mechanism of induced SAR seems to be highly conserved, every plant species exhibits a specific response.

To our knowledge, SAR response in plant using cDNA microarray technology has been compared for the first time in the two organs that are exposed to pathogenic attacks aggressively. A substantial difference was observed in the expression profile of these tissues. Interestingly, the leaf tissues exhibit a negative response in the normal metabolic pathway, which can be explained that the cells in the leaves initiate the synthesis of pathogenesis related proteins in response to the pathogenic attack. In contrast, the roots mainly down

regulate the metabolism and increased the biosynthesis of proteins involved in storage processes. A tentative comparative analysis of expression profiling between Coffee and Arabidopsis thaliana (TAIR: Gene Expression Information Resources) provides newer vision that one third of genes over-expressed in Coffee SAR overlap with Arabidopsis gene profile but also that the 20 per cent of the genes have a contrasting expression.

In general, BTH was reported to stimulate resistance in mono and dicotyledonous plants against a wide spectrum of viral, bacterial and fungal parasites. A comparative analysis of the SA-dependent defence pathway in tobacco and tomato show a significantly different defence response and there fore BTH treatment in one plant species cannot be simulated for the other. However, the results presented in this study clearly shows that the resistance response in Coffee at transcriptomic level could possibly be altered by means of a chemical induction. It also discloses new vision on the possibilities to study effectiveness of Coffee SAR in resistance against biotic agents.

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# Identification and Mapping of AFLP Markers Linked to a Leaf Rust Resistance Gene in Coffee – A Step towards Marker Assisted Selection in Coffee

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## SUMMARY

Coffee leaf rust caused by the obligate parasitic fungus *Hemileia vastatrix* is an economically important disease and a major limiting factor for arabica coffee (*Coffea arabica*) production. Occurrence of different physiological races of leaf rust pathogen has been the major constraint for durability of genetic resistance in cultivated strains. Interactions between coffee and rust pathogen are governed according to gene for gene hypothesis and the host resistance appears to be conditioned at least by nine major resistance factors designated as $S_H1$ to $S_H9$, either singly or in combination, while the corresponding virulence have been indicated as $V_1$ to $V_9$. Molecular markers linked to $S_H$ genes would be extremely useful in improving the selection efficiency for durable resistance.

In this direction, we have identified AFLP markers tightly linked to a rust resistance gene $S_H3$, naturally introgressed in to *Coffea arabica* from a related species *C. liberica*. $F_2$ progeny (101 individuals) derived from a cross between an arabica accession, Matari and an introgressed line S.288 was used for analysis. The progeny segregated into two groups for rust resistance, one manifesting resistance to physiologic race II ($V_5$) due to the presence of $S_H3$ gene while the second group showed susceptibility due to the absence of $S_H3$. AFLP profiles generated by using the 15 primer combinations selected after initial screening showed 35 polymorphic fragments associated with alien genome introgression. Among these, 21 markers were found associated with the $S_H3$ resistance gene and one marker co-segregated perfectly with the $S_H3$. Linkage analysis of the introgressed markers resulted in four linkage groups. All the markers linked to $S_H3$ resistance gene were closely associated (6.3 cM) and grouped together. The present findings provide a starting point for further refinement of marker-trait associations. The potential applications of the identified markers for improving the selection efficiency in arabica coffee are discussed.

## INTRODUCTION

Of the several diseases affecting arabica coffee (*Coffea arabica*), leaf rust disease caused by the pathogenic fungus *Hemileia vastatrix* is the most devastating and responsible for substantial crop losses in all arabica producing countries. Considering the economics and also to minimise the chemical inputs for disease management, development and cultivation of tolerant cultivars is the best disease management option being followed. Therefore, breeding for rust resistance has been one of the major objectives of arabica improvement programmes

in many countries including India. But the occurrence of different physiological races and ever increasing racial diversity of the pathogen is often a bottleneck for durability of resistance in commercial strains. To date as many as 40 physiological races are known to be infecting different coffee genotypes in various coffee growing countries (Rodrigues et al., 1993; Sreenivasan et al., 1994).

In view of the low genetic variability in *C. arabica*, transfer of resistance genes from either spontaneous accessions collected in the primary centre of diversity or diploid wild relative *Coffea* species has therefore been a constant priority of coffee breeding programmes (Bettencourt and Rodrigues, 1988). Nine major dominant resistance factors to *H. vastatrix* have been so far inferred according to the gene-for-gene concept. The resistance to leaf rust of coffee plants therefore appears to be conditioned by at least nine resistance genes designated as $S_H1$ to $S_H9$, either singly or in combination, while the corresponding virulence have been indicated as $V_1$ to $V_9$ (Bettencourt and Rodrigues, 1988). Of these resistance factors, $S_H1$, $S_H2$, $S_H4$ and $S_H5$ have been found in *C. arabica*. The other genes $S_H6$, $S_H7$, $S_H8$ and $S_H9$, have been introgressed from the diploid species *C. canephora,* while $S_H3$ probably originates from another diploid species, *C. liberica* (Wagner and Bettencourt, 1965; Vishveshwara, 1974; Bettencourt and Rodrigues, 1988). Interestingly, the resistance genes of diploid origin i.e from *C. liberica* and *C. canephora* have been reported to be more effective in providing durable protection under field conditions compared to the genes of arabica origin (Srinivasan and Narasimhaswamy, 1975; Ramachandran and Srinivasan, 1979; Sreenivasan et al., 1994). Availability of molecular markers linked to $S_H$ genes and other genes conditioning resistance to coffee leaf rust would greatly facilitate the combined use of resistance genes introgressed from diploid coffee species.

The aim of the present study was to identify molecular markers closely linked to the $S_H3$ gene for leaf rust resistance and to gain insight into the mechanism of introgression of $S_H3$ rust resistance gene into *C. arabica* from *C. liberica*.

## MATERIAL AND METHODS

### Plant material

An $F_2$ population of 101 individuals derived from a cross between two inbred lines, Matari and S.288 was used for this study. Matari is a *C. arabica* accession and manifests high susceptibility to rust due to the absence of major rust resistance genes, while S.288 is a *C. arabica* line introgressed with *C. liberica* genome (Prakash et al., 2002). The S.288 is reported to carry the $S_H3$ and $S_H5$ rust resistance genes (Rodrigues et al., 1975). The $F_2$ individuals were raised by self pollination of two $F_1$ trees (H.536/17 and H.536/12) of the cross Matari x S.288, under controlled conditions at Centro de Investigacao das Ferrugens do Cafeeiro (CIFC, Oeiras, Portugal). Besides this $F_2$ population and the two parents, six selected accessions of *C. arabica* representing the different diversity groups and two accessions of *C. liberica* were also included for molecular analysis.

### Evaluation of rust resistance

The entire $F_2$ population was screened for leaf rust resistance at CIFC (Oeiras, Portugal) by artificial inoculations in greenhouse/controlled conditions using selected rust races, as detailed by Bettencourt and Rodrigues (1988). Details on the rust races screened, their virulent gene combinations and rust reaction group are presented in Table 1. The races of *H. vastatrix* were selected to allow a segregation analysis of the $S_H3$ resistance gene.

**Table 1. Screening data of $F_2$ population (101 individuals) derived from a cross between Matari (the susceptible line) and S.288 (line carrying the $S_H3$ and $S_H5$ rust resistance genes) with three different races of *H. vastatrix*.**

| Plant resistance pattern | | Response to different races (Resistance vs Susceptibility) | | | Frequency of $F_2$ individuals |
|---|---|---|---|---|---|
| Group | Resistance Genes [a] | II ($v_5$) | VIII ($v_{2,3,5}$) | XIX ($v_{1,4}$) | (%) |
| A | $S_H3$, $S_H5$ or $S_H3$ | R | S | R | 78 |
| B | $S_H5$ | S | S | R | 19 |
| C | - | S | S | S | 3 |

[a] *As anticipated according to the gene-for-gene concept and assuming a Mendelian segregation*

**DNA isolation and AFLP assay**

DNA was extracted from fresh leaf samples and the AFLP protocol described by Vos et al. (1995) was basically followed with minor modifications to suit coffee DNA as reported by Lashermes et al. (2000).

**Data analysis**

The polymorphic AFLP bands were designated in order of decreasing fragment size and according to the primer combinations used. AFLP markers were manually scored as binary data with presence as '1' and absence as '0'. The segregation pattern of each marker both associated and non-associated with rust resistance, in the $F_2$ population was subjected to $\chi^2$ analysis to test goodness of-fit to the expected Mendelian segregation ratio. Linkage analysis and map construction were performed using the computer program MAPMAKER version 3.0b (Lander et al., 1987). Multipoint analysis was used to designate the most likely order of the markers. Recombination frequencies were converted into map distances or centiMorgan (cM) values by using the Kosambi function (Kosambi, 1944).

**RESULTS AND DISCUSSION**

**Rust resistance pattern in the $F_2$ population**

Rust screening data of $F_2$ individuals carrying the $S_H3$ and $S_H5$ rust resistance genes against three different races of *H. vastatrix* carrying different virulence factors is presented in Table 1. As anticipated from the gene-for-gene concept, three groups of plants presenting differential resistance patterns were identified. These groups were interpreted as representing the presence of the $S_H3$ gene either alone or in association with $S_H5$ gene (group A), the presence of the $S_H5$ gene alone (group B) or the absence of both $S_H3$ and $S_H5$ genes (group C). Among the 101 $F_2$ plants analysed in this study, the $S_H3$ resistant genes appeared to be present in 79 individuals (i.e. 78%) and absent in 22 individuals (i.e. 22%). The segregation fits the 3:1 ratio expected for a single dominant gene ($\chi^2 = 0.558$; $p = 0.455$). Thus the segregation data obtained in the present study confirm the hypothesis of a single dominant gene for the $S_H3$ resistance factor. It was earlier reported that $S_H$ genes are dominant and condition total susceptibility to compatible races and specific resistance to incompatible races (Rodrigues et al., 1975). Accordingly, coffee plants with the $S_H3$ resistance gene are expected to manifest susceptibility to the corresponding virulence factor ($V_3$). The rust screening data of the present population confirmed the simple inheritance of the $S_H3$ gene for rust resistance, as observed in earlier screening studies (Wagner and Bettencourt, 1965).

**AFLP polymorphism**

A total of 80 primer combinations were screened on a subset of DNA samples including both parents (i.e. Matari and S.288), $F_1$ and two $F_2$ individuals as well as six accessions of *C. arabica* representing the different diversity groups and two accessions of *C. liberica*. All the 80 primer combinations used gave good amplification profiles and the clear amplified products per sample ranged from 20 to 50, depending on the genotype and primer combination used for AFLP reaction. In all, a total of 260 polymorphic bands were scored in the sub set of plants analysed. Out of these, 154 bands were found to be originated from S.288 and 81% of these bands were also seen in at least one of the *C. liberica* accessions included in the analysis. None of these markers were seen either in 'Matari' or in other arabica accessions analysed and were therefore considered as markers introgressed from *C. liberica*. The remaining 106 bands originated from Matari. The data indicates that at least 50% of the total polymorphism in the $F_2$ population analysed is the result of introgression of *C. liberica* chromosome fragments.

The AFLP technique appears to be highly efficient in generating DNA markers in the coffee plant material analysed in this study. In accordance with previous reports (Lashermes et al., 2000b; Prakash et al., 2002), a large part of the detected polymorphism is related to the introgression of foreign genetic material in *C. arabica*. The line S.288 carrying the $S_H3$ gene is the selfed offspring of S.26, a natural hybrid between *C. arabica* and *C. liberica* identified and used in rust resistance breeding programmes of in India. Hence, almost all identified marker bands in S.288, appeared to be derived from introgressions of *C. liberica* chromosome fragments.

**Identification of candidate markers associated with $S_H3$ resistance gene**

From among the initially screened 80 primer combinations, 60 primer pairs that gave parental polymorphism were selected for further analysis. These selected primers were used to screen a set of 15 plants including the two parents, $F_1$ and 12 $F_2$ (Matari x S.288) plants (six plants each of group A containing $S_H3$ gene and group B without $S_H3$). Analysis of marker data revealed 21 marker bands derived from 15 different primer combinations as candidate markers associated with the $S_H3$ resistance gene because these markers were found present in 6 plants classified in group A and absent in the 6 plants classified in group B. As probably the consequence of the introgressed origin of the $S_H3$ resistance gene, the AFLP technique proved to be a powerful tool for identifying linked DNA markers. A total of 21 markers tightly linked to the $S_H3$ rust resistance gene were identified. Similarly, Noir et al. (2003) demonstrated the efficiency of AFLP in identifying markers linked to an introgressed major gene (*Mex*-1) conferring resistance to root-knot nematode (*Meloidogyne exigua*) in *C. arabica*.

**Segregation and linkage analysis**

In order to study the segregation and linkage analysis, the whole $F_2$ population of Matari x S.288 (101 plants) was analysed using the 15 primer combinations that generated the candidate markers (Table 2). From the profiles of these 15 combinations, a total of 57 polymorphic bands were scored in the population, of which 35 bands were found to be the introgressed fragments, originated from S.288 while the remaining 22 polymorphic bands derived from Matari, the second parent. All marker bands from S.288, with only one exception, displayed an introgressed origin. The segregation ratios in the $F_2$ population of all the 35 marker bands derived from S.288 corresponded well with the 3:1 inheritance pattern expected for a dominant marker (Table 2). Linkage analysis in the $F_2$ population of those marker bands using an LOD threshold of 5.0 resulted in 3 linkage groups (namely 1, 2 and 3)

of 21, 6 and 7 markers, respectively (Figure 1). Only the marker not associated to introgression remained unlinked. As the LOD score was decreased to 2.5, no modification in linkage association was detected.

**Table 2. Segregation analysis in the $F_2$ (Matari x S288) population (101 individuals) of the 35 identified AFLP markers derived from the parent S288.**

| Code | Marker designation # size (bp) [a] | $\chi^2$ value [b] | Linkage group |
|---|---|---|---|
| M1 | E-AAC/M-CAG (1) # 380 | 0.01 (0.953) | 1 |
| M2 | E-AAC/M-CTA (3) # 60 | 0.06 (0.813) | 1 |
| M3 | E-ACA/M-CAG (1) # 280 | 0.00 (1) | 1 |
| M4 | E-ACA/M-CAG (2) # 70 | 0.03 (0.860) | 1 |
| M5 | E-ACG/M-CTA (1) # 60 | 0.16 (0.684) | 1 |
| M6 | E-ACG/M-CTA (3) # 180 | 0.41 (0.523) | 1 |
| M7 | E-ACG/M-CAA (1) # 200 | 0.00 (1) | 1 |
| M8 | E-AGC/M-CTG (1) # 300 | 0.42 (0.515) | 1 |
| M9 | E-CAA/M-CCT (1) # 350 | 0.03 (0.861) | 1 |
| M10 | E-CAA/M-CCT (5) # 60 | 0.16 (0.684) | 1 |
| M11 | E-CAT/M-CAT (2) # 180 | 0.17 (0.675) | 1 |
| M12 | E-CAC/M-CGT (3) # 150 | 0.01 (0.907) | 1 |
| M13 | E-CTA/M-CTA (1) # 310 | 0.00 (1) | 1 |
| M14 | E-CTA/M-CTA (2) # 100 | 0.01 (0.954) | 1 |
| M15 | E-CAT/M-CTG (1) # 180 | 0.12 (0.726) | 1 |
| M16 | E-CAT/M-CTG (2) # 150 | 0.05 (0.817) | 1 |
| M17 | E-CAT/M-CCA (1) # 200 | 0.01 (0.954) | 1 |
| M18 | E-CTA/M-CGT (1) # 390 | 0.01 (0.954) | 1 |
| M19 | E-CTA/M-CGT (2) # 200 | 0.01 (0.954) | 1 |
| M20 | E-CTA/M-CTT (1) # 300 | 0.13 (0.720) | 1 |
| M21 | E-CTA/M-CCA (1) # 250 | 0.05 (0.817) | 1 |
| M22 | E-AAC/M-CAG (2) | 0.05 (0.817) | 2 |
| M23 | E-AAC/M-CAG (3) | 0.05 (0.817) | 2 |
| M24 | E-AAC/M-CTA (1) | 0.11 (0.740) | 2 |
| M25 | E-CAA/M-CCT (3) | 0.05 (0.817) | 2 |
| M26 | E-CAT/M-CAT (1) | 2.72 (0.098) | 2 |
| M27 | E-CAC/M-CGT (1) | 0.01 (0.907) | 2 |
| M28 | E-AAC/M-CTA (2) | 0.69 (0.404) | 3 |
| M29 | E-ACG/M-CAA (2) | 1.51 (0.218) | 3 |
| M30 | E-AGC/M-CTG (2) | 0.42 (0.515) | 3 |
| M31 | E-CAA/M-CCT (2) | 0.34 (0.559) | 3 |
| M32 | E-CAA/M-CCT (4) | 0.41 (0.523) | 3 |
| M33 | E-CAC/M-CGT (2) | 0.48 (0.488) | 3 |
| M34 | E-CTA/M-CTT (2) | 1.92 (0.165) | 3 |
| M35 | E-ACG/M-CTA (2) | 0.41 (0.523) | Unlinked |

*[a] Marker band number designated in order of decreasing fragment size is indicated in parentheses following the respective primer combination. When estimated, the size of the AFLP fragment is specified in base pairs (bp).*

*[b] Comparison between the observed segregation and the expected values assuming a 3:1 segregation ratio. Probability values are indicated in parenthesis.*

Earlier, based on the analysis of the number of introgressed markers, it was suggested that the introgression in S.288 was restricted to a few chromosome segments (Prakash et al., 2002). In this study, only three distinct introgressed fragments were identified, corresponding to a total length of 52.8 cM. It was interesting to note that all the markers corresponding to the three *C. liberica* introgressed fragments exhibited no segregation distortion and that recombination

events occurred between markers. These findings support the earlier reported view that introgression of genes into *C. arabica* from diploid species like *C. canephora* appears not to be limited by differences in either sequence homology or chromosomal structure (Herrera et al., 2002; Noir et al., 2003).

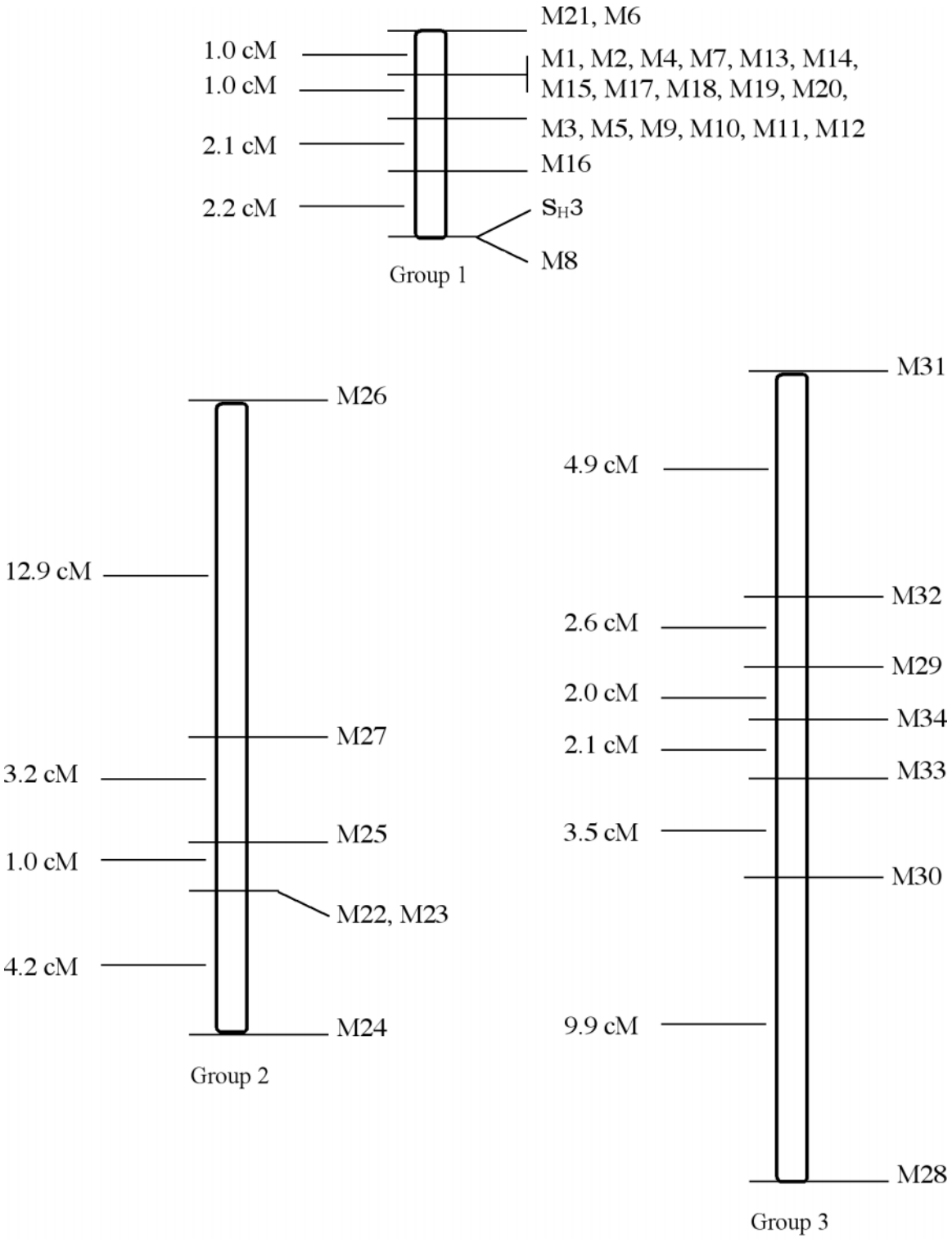


**Figure 1. Genetic linkage map of the three introgressed fragments identified in the S.288 line as deduced from the AFLP analysis of the $F_2$ (Matari x S.288) population. Molecular marker names are indicated on the right side while the estimated map distances in centimorgans are shown on the left. The $S_H3$ gene for resistance to rust in coffee is located in group 1.**

All 21 candidate markers associated with the $S_H3$ resistance gene were grouped together in a single linkage group (i.e. group 1) of 6.3 cM. The two other linkage groups (i.e. groups 2 and 3) covered 21.4 and 25.1 cM, respectively. The linkage between the candidate markers belonging to group 1 and the $S_H3$ resistance gene was confirmed when analysing the whole $F_2$ population (LOD score > 14). The size of the marker bands ranged from 60 to 390 base pairs (Table 2). Among the markers, the marker M8 co-segregated perfectly with the $S_H3$ gene and exactly positioned with the $S_H3$ locus at one end of linkage group while all other markers flanked on the other side (Figure 1). Hence, it could be inferred that the $S_H3$ resistance gene was located close to marker M8.

It is apparent that the usefulness of the identified AFLP markers linked to the $S_H3$ gene in MAS will depend on the distance and orientation of the markers with respect to the gene (Hospital, 2001; Frisch and Melchinger, 2001). In our study, all the markers linked to the $S_H3$ gene were distributed in a distance of 6.3 cM and the recombination within the introgressed region was also evident as six recombinants were identified in the population. However, no recombinant individuals were detected between $S_H3$ and marker M8. In fact this marker was found to co-segregate perfectly with $S_H3$, which determines the high selection efficiency of this marker for resistance genotypes.

Identification of molecular markers linked to rust resistance genes is an important starting point towards improving the selection efficiency in coffee breeding programmes. This is the first report on DNA markers linked to a gene conferring rust resistance in coffee. As the resistance in coffee is known to be governed by the gene-for-gene concept, there exists scope for high similarities among the $S_H$ resistance genes and this molecular information generated on $S_H3$ affords insight and scope to approach other $S_H$ genes. Further, the identified markers may also be used for pyramiding of the different resistance sources (Duvick, 1996; Lui et al., 2000). Also molecular markers helps in quick screening of the resistant plants at seedling stage and thus reduce the time required to identify resistant breeding lines or the segregating lines carrying desirable alleles.

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# Gene Introgression through Interspecific Hybrids: Molecular Analyses and Implications for Coffee Breeding

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## SUMMARY

Transfer of desirable genes from wild relative species to cultivated varieties through wide crosses is one of the proven breeding strategies for crop improvement. However, inherent problems of interspecific introgression such as hybrid instability, infertility, non-Mendelian segregations, and low levels of intergenomic crossing-over can constitute important limitations. In coffee, the transfer of desired characters, in particular disease resistances, from the diploid relative species such as *C. canephora* into *C. arabica* cultivars without affecting quality traits has been the main breeding objective world wide. However, the ploidy difference between *C. arabica* and the donor species, and the lack of information regarding genome recombination in interspecific hybrids and DNA introgression into *C. arabica* constitute major bottlenecks. During the last few years, the behaviour of interspecific hybrids between the diploid species, *C. canephora* and *C. eugenioides,* and *C. arabica* has been investigated. Numerous plant populations resulting from the backcross ($BC_1$) of either triploid or tetraploid interspecific hybrids to *C. arabica* were analysed. Flow cytometric analysis of the nuclear DNA content revealed that most of the BC1 individuals were nearly tetraploid suggesting that among the gametes produced by the interspecific hybrids, those presenting 22 chromosomes are strongly favoured. Furthermore, molecular markers (i.e. RFLP, microsatellite and AFLP) combined with evaluations of morphological characteristics and resistance to leaf rust were applied to verify the occurrence of gene transfer from the donor species into *C. arabica*, and to estimate the amount of introgression present in $BC_1$ individuals. While a high amount of introgression was observed in the progenies derived from the tetraploid interspecific hybrids, the $BC_1$ individuals generated from the triploid interspecific hybrids exhibited contrasted situations. The different results will be discussed in relation to the mechanism of introgression into *C. arabica* and the efficient use of genetic resources in arabica breeding.

## INTRODUCTION

*Coffea arabica* is the only tetraploid species (2n = 4x = 44) in the genus and is self-fertile while other species are diploid (2n = 2x = 22) and generally self-incompatible. Recent investigations established that *C. arabica* is an amphidiploid formed by natural hybridisation between *C. eugenioides* and *C. canephora* (Lashermes et al., 1999). *C. arabica* is characterised by low genetic diversity which has been attributed to the allotetraploid origin, reproductive biology and evolution process of this species. In contrast, considerable variability was reported among diploid coffee species and some of these species form valuable gene reservoirs, for different breeding purposes (Carvalho, 1988).

Transfer of desirable genes from wild relative species to cultivated varieties through wide crosses is one of the proven breeding strategies for crop improvement. Gene exchange is possible due to the meiotic recombination process which allows information from the parental chromosomes to be combined into new genetic entities that are passed to the next generation. However, inherent problems of interspecific crosses such as hybrid instability, infertility, non-Mendelian segregations, and low levels of intergenomic crossing-over can constitute important limitations (Stebbins, 1958).

When *C. arabica* is used as maternal parent, successful crosses have been obtained with a large number of diploid species. The resulting interspecific hybrids are usually triploid and rather vigorous. Triploid hybrids are highly sterile because of its disturbed meiosis. Nevertheless, viable gametes occur occasionally and these hybrids have been successfully backcrossed to *C. arabica* (Orozco, 1976). At the contrary, the hexaploid hybrids (i.e. obtained by duplication of triploid hybrids) and tetraploid hybrids (i.e. resulting from the hybridisation between *C. arabica* and auto-tetraploidised diploid parents), appear reasonably fertile. Interestingly the meiotic behaviour in tetraploid interspecific hybrids seems to differ markedly from that of *C. arabica*. Although bivalents have been reported, the nature of pairing as deduced from the mode of inheritance appears fundamentally different (Lashermes et al., 2000).

Despite a considerable interest for coffee breeding, genetic studies in relation to either interspecific triploid or tetraploid hybrids has been very limited. In particular, development of efficient strategies for selection of hybrid-derived progenies would require a better knowledge of genome interactions and factors affecting genetic exchange in coffee hybrids. Therefore, the purpose of this study was to analyse the mechanisms affecting gene introgression between the diploid related *C. canephora* and *C. eugenioides* species and the cultivated *C. arabica* through interspecific hybrids. We were particularly interested on behaviour of the diploid genomes and its interaction with the *C. arabica* genome in the context of the hybrid genome. Using different molecular markers (i.e. RFLP, microsatellites and AFLP markers), allele segregation and chromosome recombination were studied in several $BC_1$ populations derived from either tetraploid (i.e. arabustas) or triploid hybrids. The results are discussed in relation to the mechanism of introgression into *C. arabica* and the efficient use of genetic resources in Arabica breeding.

## MATERIALS AND METHODS

### Plant material

The plant material surveyed consisted of several $BC_1$ populations resulting from the backcross of either interspecific tetraploid or interspecific triploid $F_1$ plants to *C. arabica*. Backcrossed plants involving tetraploid hybrids were derived from a two different hybrid plants which resulted from a cross between a plant of *C. arabica* used as female parent, and a tetraploid plant of *C. canephora* previously obtained by colchiploidisation of one clone named IF181.

Likewise, backcrossed plants involving triploid hybrids were generated by crossing tetraploid *C. arabica*, with different diploid accessions of either *C. eugenioides* or *C. canephora* species. Triploid hybrids were then backcrossed to *C. arabica* plants. This material has been developed in the framework of the breeding program of the Colombian National Center of Coffee Research (CENICAFE) and was grown under appropriate experimental field conditions in Colombia.

**Molecular marker assay**

Genomic DNA was isolated from lyophilised leaves through a nuclei isolation step as described by Agwanda et al. (1997). Procedures for restriction fragment length polymorphism (RFLP), microsatellite (SSR) and AFLP analysis, were performed as previously reported (Lashermes et al., 2000; Lashermes et al., 1995; Combes et al., 1995). Molecular markers were tested for polymorphism between the accessions of the parental species *C. arabica* and *C. eugenioides* or *C. canephora*. For a given type of $BC_1$ population, only the markers exhibiting clear and reliable polymorphic products (i.e. *C. eugenioides* or *C. canephora* specific alleles) were surveyed in the corresponding $BC_1$ progenies.

**Determination of nuclear DNA content**

The total DNA amount in nuclei of parental species, triploid hybrids and $BC_1$ individuals was assessed by flow cytometry (Dolezel et al., 1989). To ascertain the ploidy level of hybrid and $BC_1$ individuals, the 2C peak values were compared with the values determined (four replicates) for the diploid (*C. canephora* accession) or tetraploid (*C. arabica* var. Caturra) parental species. Statistical analysis were used to compare the mean values of DNA content of the different plant material types.

**Data analysis and interpretation**

Restriction fragments (i.e. RFLP locus) as well as PCR-amplified products (i.e. microsatellite locus) of different sizes were identified and easily interpreted as either *C. canephora* or *C. arabica* specific markers by comparing the parental accessions (Figure 1). When only one *C. canephora* specific marker was identified in the arabusta hybrids at microsatellite loci, allelic interpretation was not undertaken since dosage could not be determined. In contrast for RFLP loci, variations in banding intensity within the same lane were considered to represent differences in allele copy number as previously reported (Lashermes et al., 2000). The alleles of *C. canephora* were therefore designated by the letter C in single or double dose. In all cases, segregation of each marker was tested for goodness of fit to the expected segregation ratio assuming random chromosome segregation by chi-square analysis.

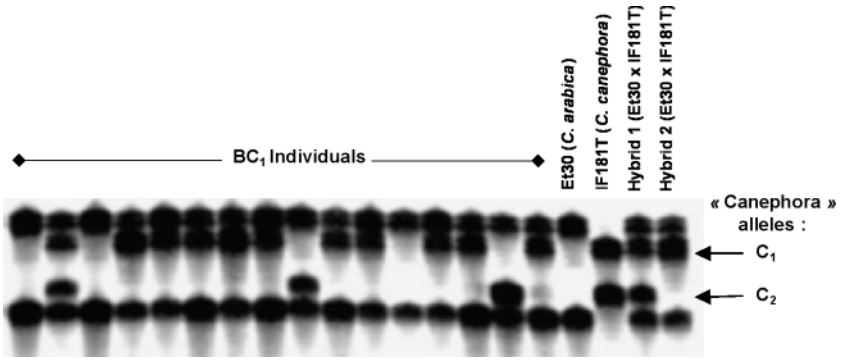


**Figure 1. DNA marker analysis of introgression. Example of microsatellite locus (M166) showing allele segregations among $BC_1$ individuals.**

**RESULTS AND DISCUSSION**

**Intergenomic behaviour in tetraploid hybrids**

For each $BC_1$ plant derived from tetraploid interspecific hybrids, the frequencies of *C. canephora* alleles were determined. The distribution of the 73 individuals for the frequency of

*C. canephora* alleles is presented in Figure 2. The observed distributions were not significantly different from the expected values for a theoretical binomial distribution assuming random chromosome segregation at all loci (Herrera et al., 2002a). A remarkably high frequency of *C. canephora* specific markers was observed in $BC_1$ populations. The segregation of *C. canephora* alleles transmitted by the arabusta hybrids conformed to the expected ratio in the absence of selection at almost all loci analysed. These results suggest an overall random association of chromosomes in the functional gametes. However, involvement at a low frequency of gametes resulting from irregular meiosis cannot be discounted.

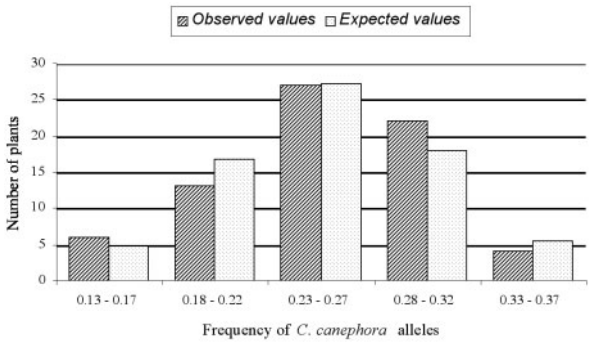


**Figure 2. Histograms of the numbers of $BC_1$ individuals in which particular frequency of *C. canephora* alleles were detected. Expected values for a theoretical binomial distribution are also illustrated.**

Co-segregation studies of specific *C. canephora* markers detected in the tetraploid arabusta hybrids were also carried out based on the analysis of $BC_1$ populations. The observed cosegregation patterns were compared with the expected values for unlinked markers. For 3 of the 6 marker pairs analysed, the observed co-segregations in at least one $BC_1$ population were not significantly different ($P > 0.05$) from those expected for unlinked markers (Table 1). The recombination rate of *C. canephora* chromosome segments estimated in the arabusta hybrids was found to be very similar to the recombination frequencies reported in *C. canephora*. The compared equivalent marker intervals, although widely distributed, do not cover the whole genome and local differences in recombination frequency may exist. However, our results clearly demonstrate that recombination in the tetraploid arabusta hybrid is not significantly affected by the genetic differentiation between chromosomes belonging to the different genomes (Herrera et al., 2002a). Therefore, the four sets of chromosomes present in the arabusta hybrid might not only display preferential pairing but also have no difficulty in recombining.

**Table 1. Comparison for different *C. canephora* chromosome segments of the recombination frequencies estimated in the arabusta hybrid (*C. arabica* x *C. canephora* 4x) and in *C. canephora* (from Herrera et al., 2004).**

| Linkage groups | Marker intervals | Recombination fractions | | $\chi^2$ value [a] |
|---|---|---|---|---|
| | | Arabusta Hybrid | *C. canephora* | |
| **3** | M41 - gA71 | 0.24 | 0.31 | 0.86 (0.355) |
| | gA71 - M157 | 0.37 | 0.32 | 0.36 (0.548) |
| | M157 – M42 | 0.13 | 0.04 | 3.94 (0.047) |
| | M41 – M42 | 0.27 | 0.40 | 1.66 (0.198) |
| **4** | M47 - cR167 | 0.10 | 0.07 | 0.15 (0.700) |
| **7** | gA72 - gA61 | 0.37 | 0.20 | 6.65 (0.010) |
| **9** | gA1 – gA19 | 0.35 | 0.49 | 2.29 (0.129) |

[a] *Probability values are indicated in parenthesis*

***DNA content variation in progenies derived from triploid hybrids***

2C nuclear DNA contents of parental species, triploid hybrids and $BC_1$ individuals were successfully assessed (Figure 3). As expected, all interspecific triploid hybrids exhibited a DNA content intermediate between the two parental species, thus confirming their ploidy level (i.e. 3x) and the possibility of comparing genome sizes by flow cytometry. The DNA content of $BC_1$ plants varied appreciably around the value of the recurrent tetraploid *C. arabica* parent. The flow cytometric analysis revealed that the generated $BC_1$ individuals had a DNA content close to that of *C. arabica*. Although allowing only a crude estimation, this observation indicates that most $BC_1$ plants were nearly tetraploids with about 44 chromosomes. This mining that among the male gametes produced by the interspecific triploid hybrids, only those presenting a high number (i.e. diploid) of chromosomes could successfully generate viable hybrid seeds. Apparently, pollen with about 22 chromosomes would have a selective advantage over gametes presenting a lower number of chromosomes (Herrera et al., 2002b; Herrera et al., 2004).

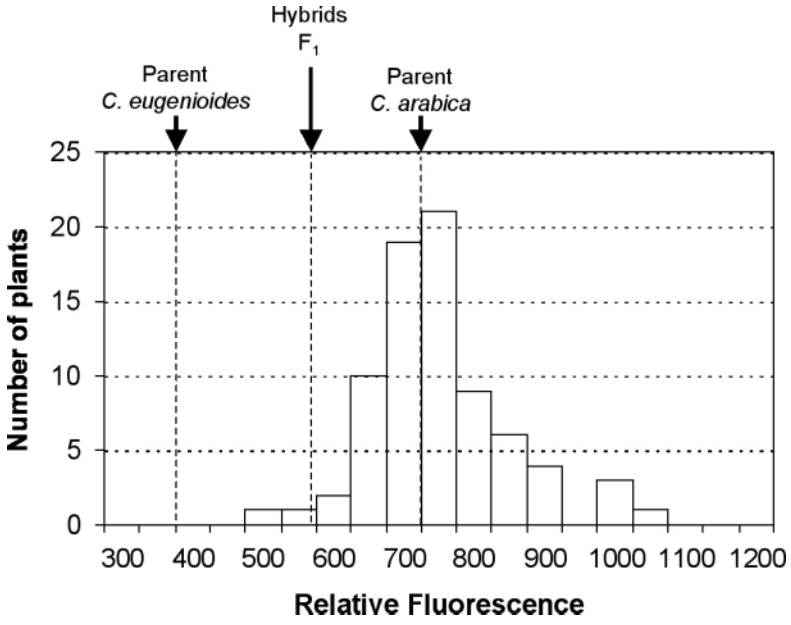


**Figure 3. Distribution of $BC_1$-EUG individuals according to their relative DNA content (expressed as relative fluorescence units) as determined by flow cytometry. For comparison, mean values estimated for the *C. arabica* and *C. eugenioides* parents as well as the triploid interspecific hybrids ($F_1$) are indicated.**

**Alien genome introgression through triploid hybrids**

***a- Introgression in BC1 plants involving triploids from different diploid parents***

As observed in Table 2, introgression analysis of $BC_1$ plants from triploids involving the two diploid species *C. canephora* (i.e. $BC_1$-CAN) and *C. eugenioides* (i.e. $BC_1$-EUG) showed a high level of gene transfer (Herrera et al., 2004). Original data showed an important variation of introgressed markers (between 14 to 71 %) depending on the locus and species considered. Nevertheless, comparison between the two studied types of populations revealed a significant effect of the genetic background. The mean frequency of introgressed plants per loci in $BC_1$-EUG was significantly lower than in $BC_1$-CAN populations. This reduction appeared to concern a large proportion of the studied loci, suggesting an unsystematic process affecting the overall genome in $BC_1$-EUG plants.

**Table 2. Overall analysis of $BC_1$-CAN (21 plants) and $BC_1$-EUG (43 plants) progenies for the presence of introgressed markers originating either from *C. canephora* or *C. eugenioides* at different microsatellite loci (from Herrera et al., 2004).**

| $BC_1$ – CAN progenies | | | $BC_1$ – EUG progenies | | |
|---|---|---|---|---|---|
| **Total studied loci** | **Frequency of introgressed plants [a] (%)** | **$\chi^2$ value [b]** | **Total studied loci** | **Frequency of introgressed plants [a] (%)** | **$\chi^2$ value [b]** |
| 11 | 57.8 | 1.0 | 14 | 39.0 | 11.0 ** |

*[a]Percentage of introgressed plants was calculated as: number of plants exhibiting C. eugenioides or C. canephora specific markers / total of scored plants. [b]Comparison between the observed percentage and the expected value (i.e. 66 %) assuming random chromosome segregation and diploid gamete formation in the triploid hybrids. *, ** indicate significance at P = 0.05 and P = 0.01, respectively.*

***b- Introgression in BC1 plants from reciprocal crosses***

The comparison of reciprocal backcross progenies between *C. arabica* and $F_1$ triploid interspecific hybrids (*C. arabica* x *C. canephora*) used as male or female parent was performed using a common set of nine microsatellite markers (Table 3). The analysis of reciprocal progenies between *C. arabica* and triploid interspecific hybrids (*C. arabica* x *C. canephora*) used as male or female parent revealed a very strong effect of the backcross direction (Herrera et al., 2004). In fact, when triploid hybrids were used as pollen donor, gene introgression appeared drastically reduced. This large reduction was also observed using further AFLP analysis (Herrera et al., 2002b).

**Table 3. Comparison of reciprocal backcross progenies between *C. arabica* and $F_1$ triploid interspecific hybrids (*C. arabica* x *C. canephora*) used as male or female parent (from Herrera et al., 2004).**

| **Backcross direction** | **Number of studied plants** | **Number of scored loci** | **Frequency of *C. canephora* specific markers (%)[a]** | **Number of introgressed plants[b]** | **Mean of introgressed markers in the introgressed plants (range)** |
|---|---|---|---|---|---|
| ♀ x ♂ | | | | | |
| $F_1$ x *C. arabica*[c] | 21 | 189 | 55.0 | 20 | 5.2 (2-9) |
| *C. arabica* x $F_1$[d] | 20 | 180 | 1.7 | 2 | 1.5 (1-2) |

*[a]Percentage of introgression was calculated as: number of detected C. canephora markers / total number of scored loci. [b]A plant was considered as introgressed when at least one C. canephora specific marker was detected. [c]Data from this study. [d]Data from Herrera et al., (2002 b).*

Restricted introgression suggested to be due to mechanisms associated with the formation of diploid gametes since the presence of *C. canephora* alleles in the functional gametes has been reported neither significantly beneficial nor detrimental (Herrera et al., 2002b). The contrasting situation observed in the present study suggests a dissimilar behaviour of male and female meiosis in triploid hybrids. The formation of functional diploid gametes carrying introgressions from *C. canephora* would either be promoted during the female meiosis or prevented during the male meiosis.

## CONCLUSIONS

1 - Our results clearly demonstrate that recombination in the tetraploid arabusta hybrid (*C. arabica* x *C. canephora* 4x) is not significantly affected by the genetic differentiation between chromosomes belonging to the different genomes. The four sets of chromosomes present in the arabusta hybrid might not only display preferential pairing but also have no difficulty in recombining. Further, in the arabusta hybrid context, introgression of desirable genes into *C. arabica* from *C. canephora,* and most probably from other diploid related species, should not be limited by differences either in sequence homology or in the chromosomal structure.

2 - Overall molecular analysis carried out on different BC progenies confirms that interspecific triploid hybrids remains as useful genetic bridges for the transfer of genes from diploid related species to tetraploid cultivated forms. Current evidence indicates that gene flow in interspecific-interploidy crosses is a complex phenomenon involving natural barriers at the pre- and post-zygotic levels. Hence, understanding of introgression through triploid hybrids does provide an opportunity to control introgression of genes and gene complexes between the cultivated and diploid relative species.

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# The Drop of Beverage Quality Caused by *Coffea canephora* Gene Introgression Can be Avoided by Selection

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## SUMMARY

The introgression of *Coffea canephora* genes in *C. arabica* is suspected of causing a drop in beverage quality. Coffee samples from lines introgressed by *C. canephora* via the Timor Hybrid, and well-known for their resistance to coffee leaf rust (*Hemileia vastatrix*) and to the nematode *Meloidogyne exigua* were studied for beverage quality, chemical composition and amount of introgressed genetic material. Chemical analyses (caffeine, chlorogenic acids, fat, trigonelline, sucrose) were carried out. The amount of AFLP markers introgressed from the Timor Hybrid varied from 1 to 37 for the studied lines. In a first study the introgressed cv. CR95, was compared to the traditional non-introgressed cv. Caturra in 13 locations. In a second study, the cv. 'Veranero', was compared to Caturra in 14 contrasted locations. Finally, 15 introgressed pure lines were compared with the tradional cv. Caturra, Villa-Sarchi and Catuai. The cv. CR95 revealed a lower beverage quality to Caturra and a lower content of fat. In the second study, there were significant differences between lines for all the biochemical compounds analysed and for the acidity and the overall standard of the beverage. Two lines (T17927, T17924) were significantly poorer than the controls for sucrose and beverage acidity. T17924 also had more chlorogenic acids and was poorer for the overall standard. However, two highly introgressed lines, T17934 and T17931, (25 and 30 AFLP markers respectively) did not differ from the non-introgressed controls. There were no correlations between the amount of AFLP markers and the chemical contents or beverage attributes. It was concluded that it should be possible to find lines with resistance genes and good beverage quality. Selection can avoid accompanying the introgression of resistance genes with a drop in beverage quality.

## INTRODUCTION

Two types of coffee are consumed worldwide, Robusta (*Coffea canephora* Pierre) and Arabica (*C. arabica* L.). Robusta coffee has been characterized as a neutral coffee, weak-flavoured, and occasionally strong and pronounced bitterness (Charrier and Berthaud, 1985). Arabica fetches a higher price as it makes a milder, fruitier and acidulous beverage. The species *C. arabica* (2n = 4x = 44) is an allotetraploid containing two genomes that originated from two different diploid wild ancestors, *C. canephora* and *C. eugenioides* Moore (Lashermes et al., 1999). The species is characterized by low genetic diversity (Lashermes et al., 1996), which is attributable to its reproductive biology and evolution. Among other things, the low variability is reflected in its susceptibility to most diseases (Bertrand et al., 1999). In contrast, *C. canephora* is a diploid species (2n = 2x = 22) with considerable variability (Charrier and Berthaud, 1985; Lashermes et al., 1999). Since the second half of the 20$^{th}$ century, most breeding programmes implemented throughout the world (Brazil, Colombia, Kenya, Ethiopia, Costa Rica, Honduras) have transferred resistance to rust (*Hemileia vastatrix* Berk. and Br.), root-knot nematodes *(Meloidogyne* sp.) and Coffee Berry Disease (*Colletotrichum kahawae* sensu Hindorf) from the Timor Hybrid to cultivars of *C. arabica*.

The original Timor Hybrid from the island of Timor (Bettencourt, 1973) is derived from a wild interspecific cross between *C . arabica* and *C . canephora.* The Timor Hybrid has been crossed with commercial varieties such as 'Caturra' or 'Villa-Sarchi'. The $F_1$ hybrid has been selfed and a plant breeding programme, based on pedigree selection (Carvalho et al. 1989), has been carried out for five to eight generations. Based on this strategy, several cultivars generally known as 'Catimors' or 'Sarchimors' have been released in Brazil ('IAPAR 59', 'TUPI', 'OBATA'), Colombia ('Colombia') or Central America ('IHCAFE 90', 'Costa Rica 95' or 'T5175'). These varieties are resistant to most known races of rust and therefore produce around 20% more than traditional varieties. It has been estimated that several hundred thousand hectares have been planted with these new varieties. Given this success, it can be expected that breeding of the Arabica species for resistance to pests and diseases will be based for some time on crosses derived from the Timor Hybrid. Lashermes et al. (2000a), using AFLP (Amplified Fragment Length Polymorphism) markers recently estimated that the approximate amounts of introgressed materials in many introgressed Arabica lines ranged from 8% to 27% of the *C. canephora* genome. The amount of alien genetic material is therefore substantial. It thus seems likely that the introgression process has not been restricted to resistance traits but could also involve undesirable genes. For example, Herrington et al. (1983) discovered that introgression can be a source of bitterness in watermelon (*Citrullus lanatus*). As Robusta does not have such a good beverage quality (BQ) as Arabica, it is reasonable to wonder whether introgression might have a negative impact on BQ. In addition, the defence exhibited by plants against pathogens depends to a large extent on chemical compounds (Agrios 1997), which might interfere with end-use quality. Ky et al. (1999) suggested that coffee species like Robusta, producing more chlorogenic acids (12-13% *vs* 7-8% for Arabica), are well protected against many pathogens but of poor BQ. Guerrero et al. (2001) were able to discriminate the two *Coffea* species, using quantitative and qualitative differences of chlorogenics acids. The caffeine content is higher in the *C. canephora* beans (2-4%) than in the *C. arabica* beans (0.8-1.7%), and fat, sucrose and trigonelline are lower (Clifford, 1985). Based on organoleptic evaluation and using scientific procedures, introgressed lines of Arabica were found to produce good BQ, that was similar to the non-introgressed standard (Fazuoli et al*.,* 1977; Castillo, 1990; Moreno et al., 1995; Puerta, 1998; Puerta, 2000; Owuor, 1988). However, most coffee buyers claim that new introgressed varieties have a poorer BQ than the 'Caturra' standard. In this study, by linking the amount of alien genetic materiel as estimated by AFLP analysis in Timor Hybrid-derived lines with beverage quality and the chemical compositions of beans, we attempted to adress this question, which has crucial implications for genetic improvement of the species.

## MATERIALS AND METHODS

### Plant material

#### *Experiment 1*

The cv 'CR95' (see Table 1), was compared to cv 'Caturra'. Samples of the two cultivars coming from 13 contrasted locations were compared.

#### *Experiment 2*

The cv 'Veranero' (see Table 1), was compared to cv 'Caturra'. Samples of the two cultivars coming from 14 contrasted locations were compared. The cv 'Veranero' is an introgressed cultivar derived from the cross between Typica and *C. canephora* (Anthony et al., 2002).

**Table 1. Description of plant material. Numbers of AFLP markers attributable to introgression detected in 22 introgression lines and a non-introgressed cultivar, cv Caturra. Resistance (R) and susceptibility (S) to leaf rust *Hemileia vastatrix* (race II) and to root-knot nematode *Meloidogyne exigua* (population of Costa Rica) are from Bertrand et al. (1997) and Bertrand et al. (2001), respectively.**

| Line | Description | Origin | Introgression markers | Reaction to Leaf rust | Reaction to nematode |
|---|---|---|---|---|---|
| T17924 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 32 | R | R |
| T17925 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 14 | R | R |
| T17926 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 28 | R | R |
| T17927 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 30 | R | R |
| T17928 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 26 | R | S |
| T17929 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 1 | S | S |
| T17930 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 14 | R | R |
| T17931 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 30 | R | R |
| T17933 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 16 | R | R |
| T17934 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 25 | R | R |
| T17935 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 16 | R | R |
| T17936 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 37 | R | R |
| T17937 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 33 | R | R |
| T17938 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 10 | R | R |
| T17940 | Introgressed cultivar « Colombia » (CIFC1343) | Colombia | 19 | R | R |
| CV 'Veranero' | Introgressed cultivar | Costa Rica | ? | S | S |
| cv CR95 | Introgressed cultivar (CIFC 832/1) | Costa Rica | 12 | R | S |
| cv Caturra | Non-introgressed cultivar | Costa Rica | 0 | S | S |
| cv Catuai | Non-introgressed cultivar | Costa Rica | 0 | S | S |
| cv Villa-Sarchi | Non-introgressed cultivar | Costa Rica | 0 | S | S |

***Experiment 3***

15 introgressed Arabica lines (from generation $F_4$ and onwards) derived from different progenies of the Timor Hybrid (i.e. CIFC 832/1, CIFC 832/2, CIFC 1343) and three non-introgressed commercial cultivars, cv Caturra, cv Catuai, cv Villa-Sarchi (Table 1) formed the plant material. The samples came from a trial set up at the ICAFE research centre in Costa Rica in 1998, located in Heredia at 1,200 m above sea level on an andosol type soil. In each of the four replicates of the trial, the plots consisted of 10 trees (i.e. 40 trees per line).

**Samples taken for organoleptic and chemical analysis**

Composite samples were taken from plots, using ripe, healthy cherries harvested from the upper branches of the trees during the harvest peak. Two kg of coffee cherries were subjected

to the wet process (pulping, fermentation and drying) to obtain 1 kg of green coffee beans. The samples of green coffee were screened through a size 17 sieve and the most defective beans were eliminated. In experiment 1 and 2, samples were submitted for organoletpic analysis in 2000. In experiment 3, samples harvested in 1998 (Y1) and 1999 (Y2) were submitted for organoleptic analysis (see below). The samples harvested in Y1 and Y3 (2000) were submitted for chemical analysis (see below). For the Y3 harvest, 15 out of 18 lines were harvested, the three other lines (T17936, T17937 and T17938) did not produce a sufficient quantity for harvest.

**Organoleptic analysis**

After roasting for 6-7 min, BQ tests were carried out on an infusion (120 ml) prepared from 12 g of roasted coffee. In experiment 1 and 2 a panel od 16 evaluators compared the two cultivars for each location (repsctiveley CR95 vs Caturra and Veranero vs Caturra) following a triangular test. In experiment 3, a panel of eight evaluators tasted 120 ml of infusion following a quantitative test. The major taste and flavour attributes, aroma, body (i.e. strength), acidity were scored using scales ranging from 0 to 5 where 0 = nil, 1 = very light, 2 = light, 3 = medium, 4 = strong and 5 = very strong. There was also an overall standard for liquor quality based on the above attributes and ranging from 0 to 5 where 0 = unacceptable, 1 = bad, 2 = regular, 3 = good, 4 = very good, 5 = excellent.

**Chemical analysis**

The analyses were performed by near infrared spectrometry by reflectance (Williams and Norris 1990) of green coffee (50 g) after grinding (ground to < 0.5 mm) using a NIR spectrometer system (model 6500, by NIRSystem, Inc. 1201 Tech Road Silver Spring, Md 20904) driven by NIRS2 (4.0) software (Intrasoft Intl., LLC, RD109, Sellers Lane, Port Matilda, Pa 16870). For the Y1 and Y3 samples from Trial 1 (experiment 2) and for all samples from experiment 1, a NIR spectrum (NIRS) was acquired in reflectance (R) mode in the 1104-2456 nm range (Downey et al., 1994; Downey and Boussion, 1996; Scanlon et al., 1999). Using specific calibrations (Guyot et al., 1988; Guyot, 1993), it is possible to determine the caffeine, trigonelline, fat and sucrose contents. These contents were determined for all samples (experiment 1) and for Y1 samples (experiment 2). For the Y3 samples, a NIR spectrum was acquired in reflectance (R) mode at intervals of 26 nm for a total of 52 data points. The reflectance, expressed as log (1/R) values, gave a characteristic signature for each sample.

**AFLP protocol**

Leaf samples were taken from 20 trees representing 17 introgressed lines and three non-introgressed lines as controls (cv Caturra, cv Catuai, cv Villa Sarchi) (Table 1). The AFLP protocol described by Vos et al. (1995) was basically followed with minor modifications to suit coffee DNA as reported by Lashermes et al. (2000a). For each sample, 500 ng of genomic DNA were digested using two restriction enzymes, EcoRI and MseI. Restricted DNA fragments were ligated with EcoRI and MseI adapters using T4 DNA ligase (Gibco BRL). In pre-selective amplification, the ligation mixture was amplified using primers complementary to the adapters with one additional selective 3'-nucleotide. Two sets of primers with three selective nucleotides were used for amplification. The EcoRI primers were end labelled with $\gamma$ -[$^{33}$p]-ATP using T 4 polynucleotide kinase. PCR amplifications were carried out using a total of 42 AFLP primer combinations as described in Lashermes et al. (2000a). Amplification products were electrophoresed on 6% denaturing polyacrylamide gel. The gels were dried and exposed to Kodak Bio Max X-ray film.

**Data analysis**

Experiment 1 and 2 were tested using a triangular test design. The normal dsitribution was used like an approximation of the binomial distribution to test the significance of the differences (P = 0.05). For the experiment 3, for each introgressed line, the number of introgressed AFLP marker bands was determined as previously described (Lashermes et al., 2000a). Number of introgressed AFLP markers was calculated for each line, and subsequently analysed for possible association, using a Pearson correlation test, with mean values for each line for chemical contents (Y1 samples) and organoleptic scoring (Y1 and Y2 samples). Results obtained in experiment 3 were used to analyse the amount of variation between lines for chemical contents determined on the Y1 samples as well as for the organoleptic scoring performed on the Y1 and Y2 samples. Data were therefore subjected to analyses of variance (ANOVA) using lines as the grouping variable (each lines represented by 3-4 values for the different plots), and followed by a comparison of the means for the different lines using a Newman-Keuls multiple range test. Based on the NIR wavelength data from the Y3 samples, a discriminant function analysis was performed (Statistica 4.3, Statsoft Inc. 1993). The Squared Mahalanobis distances between lines were calculated and significance of the variation among distances was determined by a Wilks' lambda test.

**RESULTS**

**Experiment 1**

The jury was able to discriminate the 2 cultivars in 73% of the tests ($P < 0.001$). Caturra was preferred to CR95 in 65% of the tests ($P < 0.01$).

**Experiment 2**

The jury was able to discriminate the 2 cultivars in 84% of the tests ($P < 0.000$). Caturra was preferred in 75% of the tests ($P < 0.000$).

**Experiment 3**

The mean number of markers introgressed per line was 21.1 among the 22 introgressed Arabica lines, with extreme values ranging from 1 (T17929) to 37 (T17936) (Table 1). The previously determined resistances to race II of coffee rust as well as to *M. exigua* are indicated in Table 1. The variations in chemical compound contents were low (Table 2) and within the range determined for a set of more than 300 Arabica coffee samples of ‘Caturra’ and ‘Catuai’ produced in Central America (Guyot, unpublished data). There were significant differences between lines for all the biochemical compounds analysed. For caffeine, five introgressed lines had significantly higher values than the non-introgressed controls (1.26 to 1.27%). Lowest and highest values were noted in lines T17925 (1.23%) and T17933 (1.45%). For chlorogenic acids, a single line (T17924) had a content (8.34%) that was significantly higher than in the controls (7.43 to 7.66%). However, this extreme content was not beyond the range found for the chlorogenic acid content of Arabica coffee produced in Central America (7.5% to 8.3%; Guyot, unpublished data). Two lines (T17933 and T17937) had a lower chlorogenic acid content than the controls. For the fat content, significant differences existed between the lines. One line (T17928) was characterized by a significantly lower content (13.78%) than the three controls (14.42, 14.46 and 14.50%). Again, this value was within the range for this compound in Central America (13.0% to 15.0%). The trigonelline content varied between 0.95 (T17930) and 1.14% (T17926). Seven lines had higher contents than the controls (1.02 to 1.03%) and two had lower values. Sucrose revealed more marked differences between lines

than the other compounds studied. The non-introgressed controls had the highest values (7.14 to 7.23%). Four lines had significantly lower values than the controls. However, deviations were slight (6.41 to 7.10%) and still within the range for Arabica from this region (6.2% to 9%).

**Table 2. Comparison of chemical components (% of dry weight) of coffee produced by the Timor Hybrid-derived lines and cv Caturra, cv Catuai and cv Villa-Sarchi as non-introgressed controls. Means followed by the same suffix in the same column are not significantly different at $p \leq 0.05$.**

| Line | Caffeine | Chlorogenic acids | Fat | Trigonelline | Sucrose |
|---|---|---|---|---|---|
| T17924 | 1.37 bcd | 8.34 a | 14.47 abc | 1.127 a | 6.41 c |
| T17925 | 1.23 d | 7.75 b | 14.31 abc | 1.125 a | 6.78 abc |
| T17926 | 1.30 cd | 7.77 b | 14.20 abc | 1.143 a | 6.66 bc |
| T17927 | 1.31 bcd | 7.73 b | 14.30 abc | 1.127 a | 6.71 bc |
| T17928 | 1.37 abc | 7.45 bc | 13.87 c | 1.017 d | 7.01 ab |
| T17929 | 1.32 bcd | 7.41 bc | 14.03 bc | 1.022 bc | 6.92 ab |
| T17930 | 1.38 ab | 7.39 bc | 14.43 abc | 0.952 e | 6.83 abc |
| T17931 | 1.30 bcd | 7.28 cd | 14.36 abc | 1.032 bc | 6.88 abc |
| T17933 | 1.45 a | 7.06 d | 14.61 ab | 0.962 e | 7.02 ab |
| T17934 | 1.31 bcd | 7.55 bc | 14.83 a | 1.072 bc | 6.89 abc |
| T17935 | 1.37 bc | 7.75 b | 14.26 abc | 1.097 ab | 6.86 abc |
| T17936 | 1.28 cd | 7.26 cd | 14.29 abc | 1.016 d | 7.10 ab |
| T17937 | 1.28 cd | 7.22 d | 14.21 abc | 1.022 cd | 6.96 ab |
| T17938 | 1.33 bcd | 7.59 bc | 14.21 abc | 1.095 ab | 6.64 c |
| T17940 | 1.37 abc | 7.73 b | 14.16 abc | 1.095 ab | 6.79 abc |
| cv Caturra | 1.26 d | 7.43 bc | 14.42 abc | 1.030 cd | 7.23 a |
| cv Catuai | 1.27 d | 7.54 bc | 14.46 abc | 1.042 cd | 7.16 a |
| cv Villa-Sarchi | 1.26 d | 7.66 bc | 14.50 abc | 1.017 d | 7.14 a |

There were no significant differences between the introgressed lines and the non-introgressed controls for body attributes (Table 3). For the flavour attribute, the differences found in Y1 were barely significant and there were no significant differences between lines in Y2. However, significant differences were found for acidity and the overall standard in Y1 and Y2. For example, it was found that line T17926 was poorer than the non-introgressed controls for the overall standard and for acidity in Y1. Nevertheless, that result was not confirmed in Y2 since T17926 was not significantly different from the controls. Only two lines were significantly poorer than the controls for two years running. They were line T17927, which was poorer than the controls for acidity in Y1 and in Y2, and line T17924, which was significantly poorer than the controls for acidity and for the overall standard in Y1 and in Y2.

**Discrimination between lines based on their NIRS**

Based on the NIR wavelength data from the Y3 samples, squared Mahalanobis distances between lines were calculated (Table 4). It was not possible to distinguish between the non-introgressed controls (probabilities indicated in the upper matrix of Table 4) based on their NIR wavelength. Three introgressed lines could not be distinguished from one or two of the non-introgressed controls. They were T17929, T17935 and T17940. There was significant discrimination between the other lines and the three non-introgressed controls based on their NIRS. The 15 lines are represented on the two principal components in Figure 1.

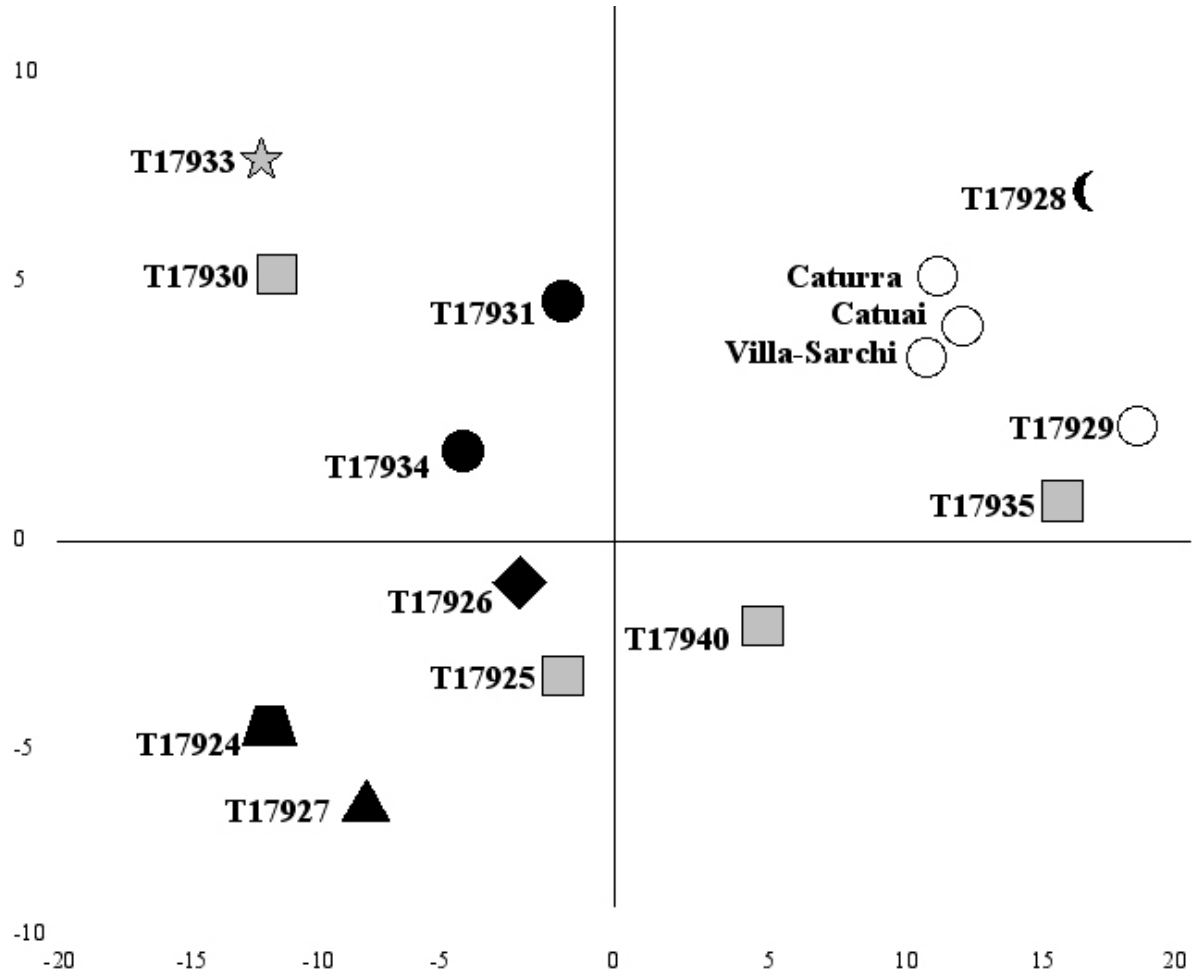


**Figure 1. Representation of 15 *Coffea arabica* accessions based on the Mahalanobis distance calculated on the near infrared spectroscopy spectrum from the Y3 samples. For each accession, the line number (centroid) represents the means for 3-4 samples on the two principal components. Differences, for chemical contents from Y1 samples and the organoleptical attributes from Y1 and Y2, between the introgressed lines and the non-introgressed controls are indicated by a symbol (shape and colour). *Circle*: non-introgressed controls and introgressed lines identical for chemical contents and BQ; *square*: lines significantly higher than the controls, for trigonelline and/or caffeine contents; *moon*: line significantly higher than the controls for caffeine contents and significantly lower for fat contents; *star*: lines significantly lower than the controls for chlorogenic acids and trigonelline contents; *lozenge*: line significantly higher than the controls for trigonelline and lower for sucrose contents; *triangle*: line significantly lower than the non-introgressed controls for sucrose content and for beverage acidity; *trapeze*: line significantly lower than the controls for sucrose and chlorogenic acid contents and for beverage acidity and overall standard. The *black symbols* represented lines with 21-32 introgressed markers; the *grey symbols* represented lines with 5-20 introgressed markers; the *white symbols* represented lines with 0-1 introgressed markers.**

**Relations between introgression levels and line characteristics**

There were no significant correlations between the BQ attributes of the lines or their chemical contents and their amount of introgressed AFLP markers. It can be seen in Figure 1 that line T17929 (1 introgression marker) and two highly introgressed lines (T17934 and T17931, 25 and 30 markers respectively) did not differ from the non-introgressed controls for either the chemical contents or the organoleptic analysis attributes. The other introgressed lines differed

from the non-introgressed controls to varying degrees. Lines T17935, T17940, T17930, and T17925, which displayed fewer than 20 introgression markers, differed from the controls for caffeine or trigonelline content. Line T17928 had a higher caffeine content than the controls, but a lower fat content. Line 17933 (16 markers) had less trigonelline and sucrose than the non-introgressed controls. Line T17926 (26 markers) had significantly more sucrose and trigonelline than the control. Line T17927 (30 introgression markers) differed significantly from the controls for trigonelline and sucrose and the beverage of this line was judged to be less acidic in Y1 and Y2. Lastly, line T17924 (32 introgression markers), differed from the controls for trigonelline, sucrose, chlorogenic acids, beverage acidity and the overall standard in Y1 and Y2.

**Table 3. Beverage characteristics of Timor Hybrid-derived lines and cv Caturra, cv Catuai and cv Villa-Sarchi as non-introgressed controls. Scores for acidity, body, flavour estimated using scales ranging from 0 to 5, 0 = nil, 5 = very strong. Overall standard, 0 to 5, 0 = unacceptable, 5 = excellent. Data obtained from a panel of eight evaluators. Means followed by the same suffix in the same column are not significantly different at $p \leq 0.05$.**

| | Year 1 | | | | Year 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Overall Standard | Acidity | Body | Flavour | Overall Standard | Acidity | Body | Flavour |
| T17924 | 2.18 bc | 2.21 bc | 2.50 a | 2.50 ab | 1.86 b | 1.64 c | 2.57 a | 2.93 a |
| T17925 | 2.39 ab | 2.18 bc | 2.50 a | 2.82 ab | 2.36 ab | 2.36 abc | 2.57 a | 2.93 a |
| T17926 | 1.75 c | 1.75 c | 2.40 a | 2.40 ab | 2.36 ab | 2.64 ab | 3.14 a | 3.50 a |
| T17927 | 2.11 c | 2.00 bc | 2.54 a | 2.75 ab | 2.00 ab | 1.86 c | 2.71 a | 3.14 a |
| T17928 | 2.18 bc | 2.11 bc | 2.46 a | 2.75 ab | 2.64 a | 2.64 ab | 3.07 a | 3.07 a |
| T17929 | 2.47 ab | 2.33 ab | 2.80 a | 2.91 ab | 2.28 ab | 2.14 abc | 2.86 a | 2.71 a |
| T17930 | 2.47 ab | 2.50 ab | 2 .61a | 2.75 ab | 2.14 ab | 2.14 abc | 2.57 a | 3.21 a |
| T17931 | 2.24 abc | 2.14 bc | 2.57 a | 2.76 ab | 2.79 a | 2.57 ab | 2.86 a | 3.35 a |
| T17933 | 2.19 bc | 2.14 bc | 2.47 a | 2.62 ab | 2.69 a | 2.39 ab | 3.15 a | 3.07 a |
| T17934 | 2.76 a | 2.71 ab | 2.86 a | 2.91 ab | 2.36 ab | 2.14 abc | 2.57 a | 2.85 a |
| T17935 | 2.53 ab | 2.57 ab | 2.75 a | 2.82 ab | 2.79 a | 2.86 ab | 3.14 a | 3.14 a |
| T17936 | 2.19 bc | 2.09 bc | 2.71 a | 2.62 ab | 2.36 ab | 2.57 ab | 3.00 a | 3.21 a |
| T17937 | 2.53 ab | 2.46 a | 2.64 a | 2.96 ab | 2.29 ab | 2.07 abc | 2.71 a | 3.50 a |
| T17938 | 2.04 bc | 1.93 c | 2.43 a | 2.29 b | 2.57 ab | 3.07 a | 3.14 a | 3.07 a |
| T17940 | 2.36 ab | 2.25 bc | 2.53 a | 2.57 ab | 2.71 a | 3.07 a | 2.71 a | 3.43 a |
| cv Caturra | 2.79 a | 2.81a | 2.81 a | 3.13 a | 2.33 ab | 2.33 ab | 2.90 a | 3.04 a |
| cv Catuai | 2.73 a | 2.99 a | 2.67 a | 2.60 ab | 2.97 a | 2.87 ab | 2.90 a | 2.84 a |
| cv Villa-Sarchi | 2.60 a | 3.05 a | 2.86 a | 2.36 ab | 3.04 a | 3.05 a | 2.81 a | 2.82 a |

**Table 4. Discriminant analysis performed to classify 20 lines using Mahalanobis distances on the basis of NIRS wavelengths. Each sample was characterized by 52 wavelengths in the 1104-2448 nm interval. Lower matrix, Mahalanobis distance between the lines, upper matrix, probability of Wilks' lambda tests between the lines.**

| Lines | T17924 | T17925 | T17926 | T17927 | T17928 | T17929 | T17930 | T17931 | T17933 | T17934 | T17935 | T17940 | Villa-Sarchi | Catuai | Caturra |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T17924 | | 0.15 | 0.02 | 0.50 | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T17925 | 191.28 | | 0.92 | 0.38 | 0.00 | 0.01 | 0.02 | 0.32 | 0.00 | 0.27 | 0.01 | 0.28 | 0.01 | 0.02 | 0.00 |
| T17926 | 218.49 | 62.40 | | 0.36 | 0.00 | 0.00 | 0.01 | 0.17 | 0.00 | 0.12 | 0.00 | 0.35 | 0.01 | 0.00 | 0.00 |
| T17927 | 109.56 | 194.33 | 149.72 | | 0.00 | 0.00 | 0.04 | 0.07 | 0.01 | 0.05 | 0.00 | 0.07 | 0.00 | 0.00 | 0.00 |
| T17928 | 1512.76 | 785.81 | 755.71 | 1203.07 | | 0.42 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 |
| T17929 | 1270.40 | 561.27 | 537.36 | 1014.76 | 121.71 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.22 | 0.03 | 0.04 | 0.07 | 0.04 |
| T17930 | 209.57 | 337.91 | 280.69 | 276.13 | 1374.67 | 1209.44 | | 0.05 | 0.10 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T17931 | 266.61 | 158.34 | 134.43 | 267.41 | 750.66 | 658.07 | 164.99 | | 0.01 | 0.43 | 0.00 | 0.08 | 0.00 | 0.00 | 0.00 |
| T17933 | 398.59 | 495.76 | 370.52 | 399.12 | 1249.32 | 1139.57 | 107.97 | 228.23 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| T17934 | 234.06 | 150.19 | 126.37 | 261.09 | 820.45 | 671.72 | 197.71 | 74.92 | 334.14 | | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 |
| T17935 | 1157.21 | 652.51 | 599.42 | 952.27 | 278.67 | 245.81 | 1183.39 | 713.04 | 961.59 | 702.92 | | 0.05 | 0.00 | 0.16 | 0.16 |
| T17940 | 392.64 | 167.80 | 100.48 | 271.19 | 503.67 | 360.37 | 412.90 | 173.07 | 353.95 | 204.09 | 306.29 | | 0.00 | 0.10 | 0.03 |
| Villa-Sarchi | 916.01 | 451.88 | 350.58 | 695.34 | 426.96 | 324.15 | 753.59 | 424.20 | 782.57 | 428.56 | 621.28 | 366.63 | | 0.08 | 0.11 |
| Catuai | 737.73 | 347.46 | 295.87 | 597.31 | 303.54 | 236.92 | 617.66 | 300.02 | 481.47 | 343.07 | 183.30 | 134.18 | 194.35 | | 0.18 |
| Caturra | 970.56 | 546.37 | 423.35 | 790.93 | 353.32 | 310.29 | 843.23 | 425.17 | 616.21 | 486.46 | 210.28 | 234.32 | 169.28 | 127.15 | |

## DISCUSSION

The variation in number of markers introgressed per line reflected a level of variability similar to that detected with another set of samples of introgressed lines by Lashermes et al. (2000a). All but two of the lines (T17929, cv 'Veranero') were resistant to leaf rust (race II). That resistance was introgressed from *C. canephora* via the Timor Hybrid (Kushalappa and Eskes, 1989; Gonçalvez and Pereira, 1998). Resistance to *M. exigua*, which also came from *C. canephora* (Bertrand et al., 2001) was found in 13 of the 17 lines. Line T17929, which only had a single AFLP attributable to introgression was susceptible to both parasites. The Timor Hybrid-derived lines therefore had great variability for the number of introgression markers. The presence of large amounts of introgressed genetic materials from *C. canephora* in many introgressed Arabica lines indicates that plant breeding has resulted in contrasting situations between lines. These lines are choice germplasm for studying the effect of introgression on BQ.

For many crops, undesirable effects are often associated with introgressed segments (Grandillo et al., 1999). For Arabica, most work published on the quality of introgressed lines concludes that BQ has not been modified by the introgression of genes from *C. canephora* (Fazuoli, 1977; Moreno et al., 1995; Puerta, 1998). Nevertheless, our results seem to show that these conclusions need to be moderated. For the two cultivars CR95 and 'Veranero' and for some lines in selection there would seem to be a drop in quality attributable to introgression. That was the case with line T17924 which displayed significant differences from the non-introgressed controls for most of the chemical contents (trigonelline, sucrose and chlorogenic acids), and for beverage acidity and the overall standard. However, there were also highly introgressed lines that revealed no difference from the non-introgressed controls. Such was the case with lines T17934 and T17931, which did not differ for either the chemical content or the BQ. As the latter reveal genetic resistances to coffee leaf rust and *M. exigua*, it can be concluded that the presence of resistance genes has no pleiotropic effects on beverage quality. This is an encouraging result for the future of genetic improvement programmes based on the introgression of resistance genes from *C. canephora* via the Timor Hybrid. However, if it is to be more effective and, in particular, if it is to avoid maintaining undesirable introgressed fragments suspected of having a negative effect on BQ, selection could be assisted by specific markers of resistance to pests/diseases (Lashermes et al., 2000b). This programme would be much more efficient if it were possible to detect chemical compounds with variations that are highly correlated to quality defects attributable to introgression. In our study, the lowest sucrose contents and the highest chlorogenic acid contents seemed to be linked to a poor BQ.

Genetic improvement of Arabica, based on the introgression of genes from the species *C. canephora* in order to create varieties resistant to the main parasites of the crop, has resulted in lines that have a variable amount of introgression markers, thereby illustrating the problems involved in reducing introgression to only those genes of agronomic interest via traditional selection. Nevertheless, it would seem that selection can avoid accompanying the introgression of resistance genes with a drop in BQ.

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# Functional Development and Maturation of Coffee (*Coffea arabica*) Fruit and Seeds

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## SUMMARY

Despite the global economic importance of coffee, little is known about the factors that are essential for the establishment of the mature coffee seed and final crop yield. Genomic gene expression studies show great potential and appear essential to unravel the complex molecular mechanisms and regulatory programs that act during coffee fruit and seed development. However, the success of this type of study is largely dependent on the precise identification, timing and positioning of the developmental events under study. The present study provides a functional overview of coffee fruit and seed development, as a pre-requisite to assist in gene expression studies. We have focused on cell and tissue structure (light microscopy), cell cycle activity coupled to tubulin accumulation (western blotting) and microtubular cytoskeleton configurations (immunocytochemistry), in relation to the timing of physiological events during development, pre-maturation and maturation (dry weight, water content, germinability, desiccation tolerance). The present study clarifies the origin of the different tissues, and allows a precise definition of organogenesis and phases of development in relation to seed physiology and anatomy.

## INTRODUCTION

The intrinsic quality of a seed is defined during its development by means of specific physiological events which are interrelated with the accumulation of reserves during maturation. These reserves mainly constitute of proteins, lipids and carbohydrates (Baud et al., 2002; Hills, 2004) and have significant importance (a) as a contribution to the acquisition of germination ability and of desiccation tolerance upon maturation; (b) as support to the initial growth of seedlings upon reserve degradation during imbibition and germination; and (c) as a source of food and nutrition for humans and animals.

Not surprisingly, much is known about seeds as propagules, as well as source of food and nutrition. Yet, the morphological and physiological processes that occur in coffee seeds as propagule or as source of food and nutrition are far from being understood. Recent information about regulatory mechanisms controlling coffee seed development has begun to emerge, as exemplified by studies on the molecular physiology of carbohydrate accumulation during seed development and maturation (Marraccini et al., 2001; and 2004 in this conference). Many aspects have also been unveiled on the molecular physiology of coffee seed germination (Marraccini et al., 2001; Silva et al., 2002). The introduction of plant biotechnology, including all genomic technologies, appears to have great potential in unveiling the complex behavior of seeds (reviewed by Bewley and Black, 1994; Bino et al., 2004; Borevitz and Chory, 2004; Ecker and Cook, 2004; Gallardo et al., 2003; Galau et al.,

1991; Goldberg et al., 1994; Harada, 1997; Hills, 2004; Kermode, 1995; Phizicky et al., 2003; Raghavan, 1997), and will certainly contribute to clarify the complex behavior of coffee seeds. The world wide efforts to set up coffee ESTs databases will be extremely helpful in attaining this goal.

From the molecular and biochemical point of view the study of seed development is difficult, since a population of seeds does not complete the process of maturation in synchrony. In coffee (*Coffea arabica*), the production of seeds typically is characterized by the desuniformity of flowering, long cycle and desuniformity of fruit development, as well as the relative desiccation sensitivity, which characterizes the coffee seed as having an intermediate behavior (Ellis et al., 1990; Estanislau, 2002). Due to the traditional economic importance of coffee in Brazil, much of the efforts has been in the direction of improvement of the crop, in search of superior agronomic characteristics of the coffee plant, mainly aiming at resistance to biotic and abiotic stresses in superior genotypes, and of recognized productive yield and supreme quality of 'beans' for beverage. Consequently, little attention has been dedicated to the improvement of the quality of the seed itself, either as propagule or as product for beverage. The inherent characteristics of coffee fruit and seed development is of such complexity that it generates significant difficulties to production, harvest and storage, as well as to germination and certification of seeds for seedling production (*i.e.* in the same year of harvest). All this together has negative implications to the quality of the seeds, as much as for the plantation and formation of new coffee crops, and, hence, for its use as a beverage. Furthermore, recent studies, as demonstrated in this conference, provide evidence and novel insights of a relation between the quality of the coffee beverage and the induction of germinative events during the wet processing of coffee seeds (Mazzafera & Purcino, 2004; Selmar et al., 2004; in this conference).

The present study aimed at the characterization of the different phases of coffee fruit and seed development, in relation histodifferentiation, organogenesis, germinability, desiccation tolerance and cell cycle events, as a means of establishing a functional overview of coffee fruit and seed development, which could assist studies at biochemical, molecular and genomic levels, towards a better understanding of the mechanisms acting on coffee seed development which could contribute to better processing, conservation, germination and establishment of new coffee crops, as well as improvement of beverage quality.

## MATERIAL AND METHODS

### Plant Material

Coffee fruit and seeds were obtained from the first blooming of a 3 year old *Coffea arabica* L. (cv. Acaiá Cerrado MG-1474) crop cultivated on an experimental field at the Federal University of Lavras, located in the county of Lavras in the southern region of the state of Minas Gerais in Brazil in 2000. Blooming (anthesis) branches were labeled and fruit harvested at different stages of development, from 90 up to 225 days after anthesis (DAA), at intervals of 30 (until 120 DAA) or 15 days (between 120 and 225 DAA). Each group of harvested fruit was screened through serial 16, 20, 24, 28 and 32 mesh metal screens in order to standardize fruit size. Each stage of development was represented by the screen that retained maximum number of fruit, which were then used for analysis as non-dried fresh or as dried fruit for the analysis of seed germination and desiccation sensitivity. After excision, whole seeds, isolated endosperm or isolated embryos were either immediately used or frozen in liquid nitrogen and stored at -80°C. Water content (fresh weight basis) was determined at all stages of development by measuring the weight of 3 replicates of 10 fruit, 20 seeds or 20 embryos before and after drying at 105°C ± 3°C for 24 hours.

### Germinability

Germination capacity was tested by placing 4 replicates of 20 seeds in paper towel rolls moistened with an amount of water equivalent to 2.5 times the weight of the dry paper, and incubated at 30°C with 8h light and 16h darkness. Germination was scored every 2 days for 30 days. A seed was considered germinated when the protruded radicle length was greater than 1 mm. The ability to germinate was tested also on replicates of 20 isolated embryos individually incubated on MS culture medium (Murashige and Skoog, 1962) containing 2% gibberellic acid ($GA_3$) in tubes placed in a growth chamber at 20ºC under 8h light and 16h darkness. An embryo was considered germinated after a final period of 60 days if it showed a greenish coloration of the cotyledons and a minimum radicle length of 1 mm.

### Desiccation Tolerance

Acquisition of desiccation tolerance was tested by drying whole fruit with different drying protocols (rapid drying and slow drying) and testing seed and embryo germinations as described above. Fruit were dried either by placing them on top of a retaining screen inside a hermetic chamber with air circulation ('hygrostat') containing at the bottom (1) a saturated solution of lithium chloride to provide a relative humidity of 13% at 15$^{o}$C (rapid drying); or (2) a saturated sodium chloride solution to provide a relative humidity of 73% (slow drying). Fruit and seed dehydration on both methods was monitored every two days until the water content had dropped to about 13%. Water content was determined as described above.

### Immunochemical Detection of ß-tubulin

Total protein extraction from endosperm or embryo tissues, as well as protein concentration determinations, electrophoresis, western blotting and immuno-chemiluminescence detection of ß-tubulin were conducted as described previously (De Castro et al., 1995, 1998).

### Immunocytochemical Detection of ß-tubulin

Isolated endosperms or embryos were fixed in paraformaldehyde, dehydrated in an ethanol series and embedded in butylmethylmetacrylate, sectioned, and affixed on slides according to Baskin et al. (1992). ß-tubulin immuno-labeling with anti-ß-tubulin (1:200 v/v) as first antibody (Amersham, Buckinhamshire, UK), FITC conjugated goat anti-mouse (1:200 v/v) as second antibody (Amersham, Buckinhamshire, UK), as well as microscopy were conducted according to Xu et al. (1998). Nuclear DNA was counterstained with 1 mg/ml propidium iodide (PI) (Molecular probes, Eugene, OR, USA) in some sections. In all sections, omission of the first antibody and application of pre-immune serum served as controls and showed no fluorescence. From each of the studied stages at least 5 perisperm tissue parts, 5 endosperm tissue parts or 5 embryos were randomly selected. Of each tissue 10-20 sections on the same slide were observed. One of the median sections was selected as representative for the whole population. Independent repetitions, following this protocol, yielded essentially similar results.

## RESULTS AND DISCUSSION

### Flowering and fruit development

The stimuli for floral induction occurred with the first rains of the spring season in September 2000, after the usually long period of drought of the winter months (Figure 1A, B). This is the normal pattern of flowering in arabica coffee (*Coffea arabica*) shrubs in the southern region

of Minas Gerais. The opening of the flowers in arabica coffee can be observed in a single day or during a few days, in one or more flowerings in one same reproductive period (Wormer, 1964). The first flowering generally occurs in the upper two-thirds of the plant, and generally it corresponds to the biggest proportion of flowers amongst all the flowerings. Each flowering lasts only 2 or 3 days and is followed by intense vegetative development. The reconstitution of the plant foliage, lost during the previous harvest and period of drought, is important for a good load of fruit and seeds (Maestri and Barros, 1977).

In the conditions of the present study we did not observe an immediate growth of the fruit from the first flowering, which remained in a latent state until approximately 60 DAA. This period was characterized by a continuation of the dry period, during which a short period of rains occurred that stimulated the first flowering (Figure 1B). This indicates that the fertilized ovaries were capable to maintain themselves in a certain state of 'latency' for a relatively long period under extreme conditions of water deficit. Reactivation of growth occurred as soon as favorable conditions for fruit development were established. The growth of the fruit was only initiated from 60 DAA onwards (not shown), immediately after a second flowering at the lower one-third portion of the plants, followed by an intense vegetative development. This overall response was probably due to the availability of water as the summer rainy season started in November, similar to the observations of Maestri and Barros (1977).

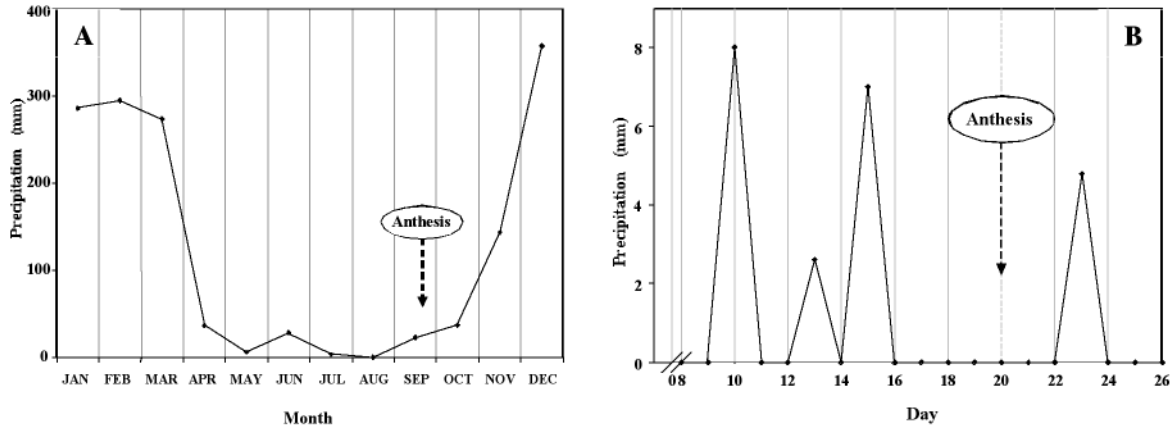


**Figure 1. (A) Monthly precipitation between January and December 2000; and (B) Precipitation during September 2000 showing the period of anthesis in Lavras–MG, Brazil.**

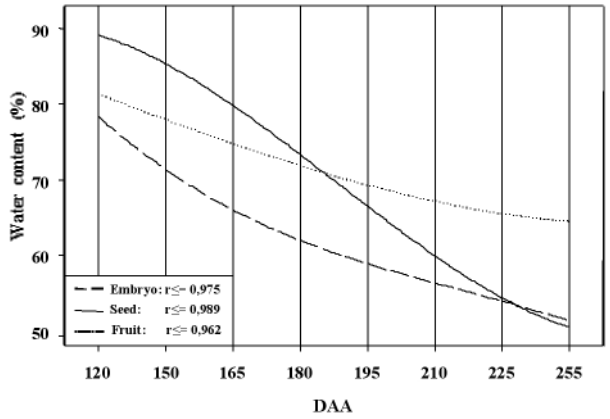


**Figure 2. Water content (fresh weigh basis) in whole coffee fruit, isolated seeds and isolated embryos during development. Due to the small sizes on the early stages of development, isolation of seeds and embryos was only possible from 120 DAA onwards.**

**Water content and dry weight**

The water content started to decrease in whole fruit from 90 DAA, revealing from 120 DAA onwards distinct patterns between the whole fruit and the isolated seed and isolated embryos (Figure 2). In accordance with Guimarães (1999), this variation is related to the species, cultivar and climatic conditions during development. Despite this variation, the water contents of the embryo and the seed appeared to be similar at the end of maturation, around 50%. However, the water content in the fruit was around 70%, as a result of the mucilaginous mesocarp, a characteristic of the ripe cherry fruit.

The developmental period of the coffee fruit and seeds is relatively long. Approximately 90% of the fruit were in the ripe cherry stage at 255 DAA (screen size: 30 and 32 mesh). The accumulation of dry weight in seeds was relatively slow from 150 DAA onwards, followed by a period of faster accumulation from 180 DAA until a maximum was reached around 235 DAA (Figure 3). The dry weight of the embryos reached also a maximum at 235 DAA, whereas the fruit still revealed accumulation of dry weight at 255 DAA. In general, the pattern of accumulation of fresh and dry weights and of water content for whole fruit during development was similar to those observed by Wormer (1964) and Cannell (1974).

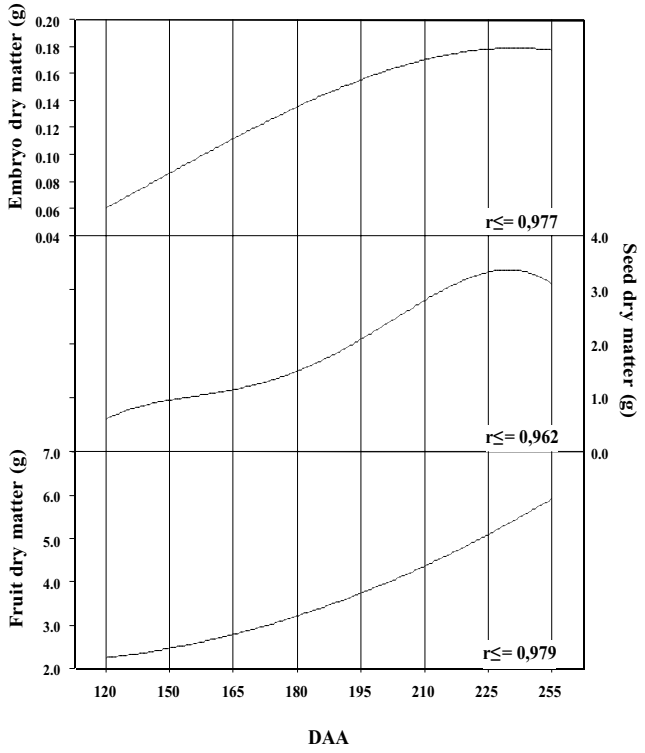


**Figure 3. Accumulation of dry weight in coffee fruit, isolated seeds and isolated embryos during development.**

In the case of seeds, the slight decrease in dry weight after 235 DAA (Figure 3) can be explained by an interruption of the translocation of photoassimilates from the plant to the fruit, and by substrate consumption necessary in the respiratory process during the full mature cherry stage as presumed by Carvalho and Nakagawa (1980). It could still be due to deterioration commonly noticed in the seeds that remain in the field after physiological maturation (Delouche, 1976). The continuous accumulation of dry weight in the fruit until 255 DAA may have resulted from the translocation, or exchange of photoassimilates between the fruit (pulp) and the seed, having a reflux from the seed to the fruit, as the fruit would be accumulating sugars in this phase.

**Characterization of fruit size and color**

The distribution profile of fruit size along the developmental time scale was obtained by means of classification and quantification of fruit retained in screens of different meshes (Figure 4). A minimization of the great initial fruit size variability was observed as development proceeded. At the end of maturation, 76.6% of the fruit were concentrated on screen 32, indicating the standardization of fruit size and synchronization of maturation. As for the coloration, a ratio of 39, 42 and 19% of green, yellowish-green, and ripe cherry fruit, respectively, was established at 210 DAA whereas at 255 DAA the distribution was 4% of green and yellowish-green fruit, and 92% of cherry fruit, respectively (Figure 5). Although several flowerings may occur, the distribution profile in the present study of fruit size and color for the cv. Acaiá indicates an ability of the plant to promote a general redistribution of metabolic activity among fruit throughout development, in order to diminish the inequality in favor of standardization and synchronization of the final maturation process of fruit and seeds in the plant as a whole. However, the data from Wormer (1964) does not confirm this assumption, indicating that different profiles of maturation may arise depending on cultivar and species of *Coffea*, and the local climatic conditions.

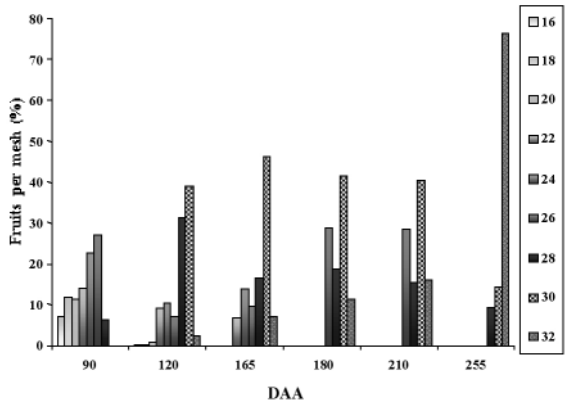


**Figure 4. Proportion of different coffee fruit sizes at different stages of development, from 16 to 32 meshes.**

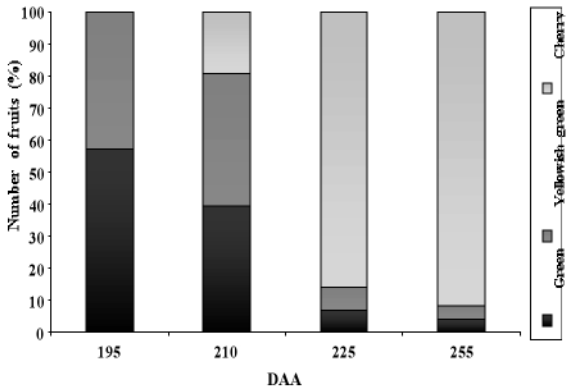


**Figure 5. Proportion of green, yellowish-green and cherry fruit during maturation.**

**Morphological analysis**

The images of Figure 6 show the development of the different tissues and organogênesis in coffee fruit from 60 DAA onwards, the time when favorable conditions induced full fruit development. The initial growth resulted from the growth of the maternal nucellus (future perisperm) tissue enclosing the still rudimentary embryo sac, and placed between the 2 integuments (Figure 6A). All these structures are protected by the layers of cells which would

give origin to the different layers of the fruit pericarp. Our observations provide evidence that the integuments degenerated during further accumulation of the nucellus and fruit growth (not shown). This aids to clarify the controversies whether the initial development of the fruit and the origin of the true reserve tissue in ripe coffee seeds were due to the growth of the integuments or of the nucellus (Houk, 1938; Graner, 1939; Fingerlind, 1939; Joshi, 1938; Mendes, 1941; Dedeca, 1957; Wormer, 1964).

The nucellus tissue was fully grown or already developing into a transient perisperm at around 90 DAA, giving rise to a 'false seed', which filled the cavity to be occupied by the 'true seed', which would result from the growth of the embryo sac enclosing the developing endosperm and embryo (Figure 6B). These observations corroborated those from Mendes et al. (1954), as the inner and larger portion of perisperm appeared as being degraded and/or consumed by the developing endosperm and embryo which appeared as a torpedo shape at around 120 DAA (Figure 6B, C). The growth and differentiation of endosperm tissue appeared to be completed by 150 DAA, possessing a milky consistency and occupying the total space previously occupied by the voluminous inner portion of the perisperm. Although not fully grown, the embryo at this stage already showed its final cotyledonary shape, as normally seen in mature seeds (Figure 6C, D). Malavasi (1997) cited the perisperm as a transient reserve tissue, which supplies the coffee embryo sac as a whole with nutritional substances during its development. The consumption of nucellus was cited as a normal process during the formation of the majority of seed species, for the origin and nutrition of the female gametophyte (Schmidt, 2000). Part of nucellus can remain without significant growth imprisoned between the integuments, being at least partially consumed as the ovule develops. This seems to be the case in coffee, as the transient perisperm may provide reserves for the differentiating endosperm and embryonic tissues. However, in some species it passes through a substantial development to form a definite perisperm, which will remain as the main reserve tissue in the ripe seed, as in *e.g.* sugarbeet (*Beta vulgaris*), *Euphorbia esula* and members of Caryphyllaceae (Malavasi, 1997; Schmidt, 2000).

Although apparently fully absorbed or consumed by the developing endosperm in coffee fruit, the outer and folded thin layer of the perisperm remained intact as a living tissue having an intense greenish color until the final stages of maturation, between 225 and 255 DAA, when it became grayish and transformed into the thin 'silver skin' which is normally seen enclosing the seeds in ripe fruit after desiccation (not shown). Little is known about the functionality of this thin greenish layer of perisperm during maturation. The greenish coloration indicates a possible extra photosynthetic contribution as to assist in the production and translocation of photoassimilates for fulfillment of the already differentiated endosperm with reserves during maturation. This hypothesis is supported by observations from assays which showed the incorporation of carbon isotope ($C^{14}$) by the cascade of photosynthetic reactions in this tissue (Mazzafera, personal communication). This means that light is capable of penetrating the fruit and seed tissues to reach the outer and the inner folded and greenish thin layer of perisperm that encloses the seed, meaning also that this tissue may be sensitive enough to perceive the minimum amount of light that may reach it.

The period between 150 and 225 DAA was characterized by the typical maturation related processes. There was a gradual decrease in water content, concomitant with the accumulation of dry weight in the whole fruit, as well as in isolated seeds and embryos (Figures 2 and 3), characterizing the filling and solidification of the endosperm, and filling and expansion growth of the cotyledonary embryo, which led to a gradual increase in fruit size and volume (Figure 4) and a turnover of the pericarp from green to yellowish-green, then to cherry-reddish color between 210 and 225 DAA (Figures 5 and 6D). In parallel, the endocarp became hard at around 210 DAA, then called 'parchment', when most fruit had reached the

30 mesh size. Some authors consider the constriction mechanics imposed by the hard endocarp a limiting factor to any further increase in seed size (Leon and Fournier, 1962; Wormer, 1964). Therefore, the growth of the seed apparently ceased at the immature stage of 210 DAA, whereas further growth of the fruit after this stage, into the 32 mesh size, would solely result from expansion growth of the mesocarp which swells due to its transformation into a mucilaginous tissue, making the whole pericarp thicker, as observed in the final stages of maturation, between 210 and 225 DAA (Figures 4, 5 and 6D).

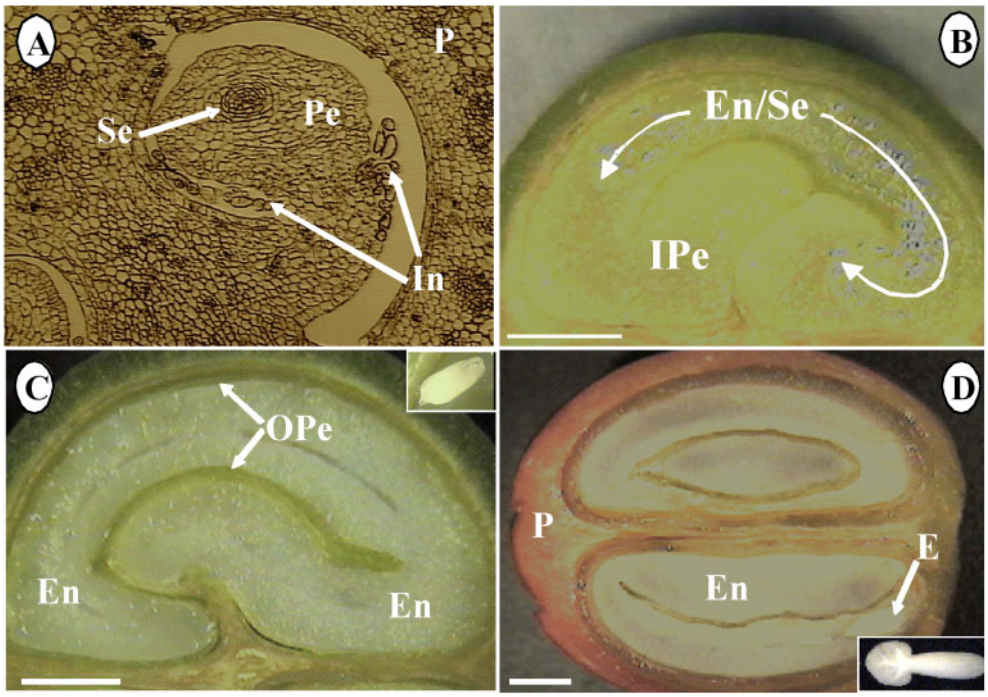


**Figure 6. Stereoscopic images of coffee fruit during development. Bars at B-D indicate 2 mm. A) An ovary after anthesis (0-60 DAA), *i.e.* after fertilization of the ovule, showing the growing nucellus / perisperm (Pe) tissue, the integuments (In), and the young embryo sac, *i.e.* the true seed (Se), surrounded by the layers of cells of the future fruit pericarp (P). (60 X). B) Transversal section of an immature fruit at 90 DAA, showing the gelatinous endosperm tissue (En) or the ´true seed´ (Se), which grows (arrows) uptaking the inner perisperm tissue (IPe). At this stage the embryo has a globular shape (not shown). C) Transversal section of immature fruit between 120 and 150 DAA, showing the remaining folded outer perisperm layer (OPe) surrounding what is then the complete milky endosperm (En). At this stage the still differentiating embryo has reached a torpedo shape (in detail at upper right rectangle). D) Longitudinal section of a mature cherry fruit at 225 DAA, showing the two fully developed mature seeds, enclosing fully developed cotyledonary embryos (E) inside the fully developed, solid and mature endosperms (En). The seeds are enveloped by the folded outer perisperm layer (future silver skin), which are then surrounded by the hard endocarps, the mucilaginous mesocarp and the reddish exocarp, which all together form the fruit pericarp (P). An isolated mature embryo shown on the lower right rectangle.**

**Germinability and desiccation tolerance**

The water contents of the fresh fruit varied from 79,4% at 150 DAA to 67.5% in cherry fruit at 225 DAA (Figure 2), similar to what Caixeta (1981) found in fruit of *Coffea arabica* L. cv. Mundo Novo (78,6% at 140 DAA and 66,5% at 220 DAA). Fruit were submitted to drying until stabilization of the water content of approximately 13%. Green immature fruit dried

faster than the more mature fruit (data not shown). Although still immature, cotyledonary embryos had acquired germinability between 120 and 150 DAA when isolated from the seed and directly incubated in MS culture medium (Table 1). The change of the developmental program into the germinative program in coffee embryos seems not to depend on previous desiccation as in bean seeds (Bewley et al., 1989), neither does it depend on the complete differentiation of the embryo as in tomato and maize (Berry and Bewley, 1991; 1992; Bochicchio et al., 1996; De Castro and Hilhorst, 2000). The seeds isolated from coffee fruit and submitted to germination in moistened paper only acquired germinability when isolated from yellowish-green fruit at 225 DAA, showing high total germination percentages without significant differences compared to seeds from cherry fruit (Table 2). The results demonstrate a reduction in the germination capacity of immature coffee embryos when enclosed by the whole seed, indicating the existence of a mechanism that keeps the embryo and the seed in the developmental mode while on the mother plant. In fact, the osmotic condition of the milky endosperm may contribute to keep the embryo in the developmental mode. In addition, hormonal control and/or mechanical impediment of the endosperm and surrounding fruit tissues may suppress embryo germination (Berry and Bewley, 1992). The precocious germination, before physiological maturity, in green or still immature stages, can also be observed in seeds of cotton, wheat, soybean, bean and tomato, when seeds are isolated and incubated in humid substrate, (Carvalho, 1974; Carvalho and Yanai, 1976; Jacinto and Carvalho, 1974; Silva, 1974; Berry and Bewley, 1991; De Castro and Hilhorst, 2000). In our study physiological maturity of the seeds occurred at around 135 DAA. However, this data should not be compared with those from Caixeta (1981), who considered physiological maturity a function of maximum dry weigh accumulated in the fruit.

**Table 1. *In vitro* germination percentage of isolated embryos before and after drying. FD – Fast Drying; SD – Slow Drying; YG – Yellowish Green; Ch – Cherry.**

| DAA | FRESH | FD | SD |
|---|---|---|---|
| 150 | 97 | 0 | 0 |
| 165 | 91 | 5 | 8 |
| 180 | 94 | 5 | 0 |
| 195 | 97 | 2 | 38 |
| 210 | 94 | 13 | 77 |
| 225 – YG | 97 | 83 | 97 |
| 225 – Ch | 100 | 58 | 94 |

*Scott-Knott test ($p < 0,05$). CV = 18,7.*

**Table 2. Germination percentage of seeds before and after drying. FD – Fast Drying; SD – Slow Drying; YG – Yellowish Green; Ch – Cherry.**

| DAA | FRESH | FD | SD |
|---|---|---|---|
| 150 | 0 | 0 | 0 |
| 165 | 0 | 0 | 0 |
| 180 | 0 | 0 | 0 |
| 195 | 0 | 0 | 0 |
| 210 | 0 | 3 | 21 |
| 225 – YG | 97 | 92 | 89 |
| 225 – Ch | 96 | 92 | 90 |

*Scott-Knott test ($p < 0,05$). CV = 10,5.*

Desiccation tolerance was tested by the capacity of isolated embryos and whole seeds to germinate after desiccation of the fruit. The capacity to tolerate desiccation became evident in

isolated embryos from 195 DAA and in whole seeds from 210 DAA, when submitting whole fruit to the process of slow drying (Tables 1 and 2). Isolated embryos and whole seeds only demonstrated full desiccation tolerance when submitted to fast drying when isolated from the yellowish-green fruit at 225 DAA onwards. The drying damage was not so evident in these advanced stages of development, since embryos and whole seeds from yellowish-green and cherry fruit demonstrated good desiccation tolerance either when fast dried or when slow dried (Tables 1 and 2). These results can be explained by events that occur at the end of the maturation period of diverse seed species. They may be associated with mechanisms of desiccation tolerance, as for example deposition of specific heat shock proteins (HSP), late embryo abundant proteins (LEA) and oligosaccharides, as well as alterations of vacuolar volume (Iljin, 1957; Farrant et al., 1997). However, coffee seeds usually present shorter longevity during storage when submitted to fast drying, as demonstrated in a number of other species (Roberts, 1973; Ellis et al., 1990; 1991; Black and Bewley, 1994; Guimarães, 2000). Fast drying has often been suggested to minimize deleterious effects associated with the dehydration of metabolically active tissues (Pammenter et al., 1991; Berjak and Pammenter, 1997; Pritchard and Manger, 1998). Presumably, the slow drying seems to be beneficial to the coffee seeds by allowing enough time for induction and operation of protection mechanisms and maintenance of cell viability, as also observed in other seed species and vegetative tissues tolerant of desiccation (Bewley and Black, 1994; Harada, 1997; Oliver and Bewley, 1997).

**Cell cycle activity**

We observed distinct profiles of β-tubulin accumulation for endosperm and embryos during development. The endosperm showed a decreasing pattern ahead of that of the embryo (Figure 7). In general, the relatively high contents of β-tubulin at the initial stages of development gradually decreased to almost undetectable levels until the final stages of maturation.

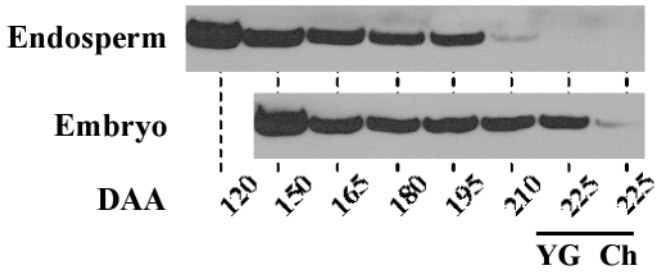


**Figure 7. Western blots showing β-tubulin (50 kD) accumulation profiles in the endosperm and embryo of coffee seeds at different developmental stages, between 120 and 255 DAA (Columns a-h). YG – Yellowish green; Ch – cherry. Embryos were not sampled at 120 DAA.**

The immunocytochemical analysis by means of immunofluorescence microscopy for the detection of polymerized tubulin and visualization of microtubules revealed an abundant network of cortical microtubular cytoskeleton, as well as cells in the process of division. Mitotic microtubule configurations were present in endosperm, as well as in embryos of seeds isolated from fruit harvested between 90 and 120 DAA (Figure 8A). As for the tubulin content (Figure 7), also a distinct pattern of degradation of microtubular cytoskeleton between endosperm and embryonic cells was observed. The degradation of microtubules in endosperm cells preceded the degradation in embryonic cells along development (Figure 8B), reaching undetectable levels at the final maturation stages (Figure 8C). At these stages only fluorescing agglomerates or single granules of tubulin were observed that probably were remnants of the depolymerization of microtubules.

The β-tubulin profiles correlate with the content and configurations of microtubular cytoskeleton during development (Figure 8).

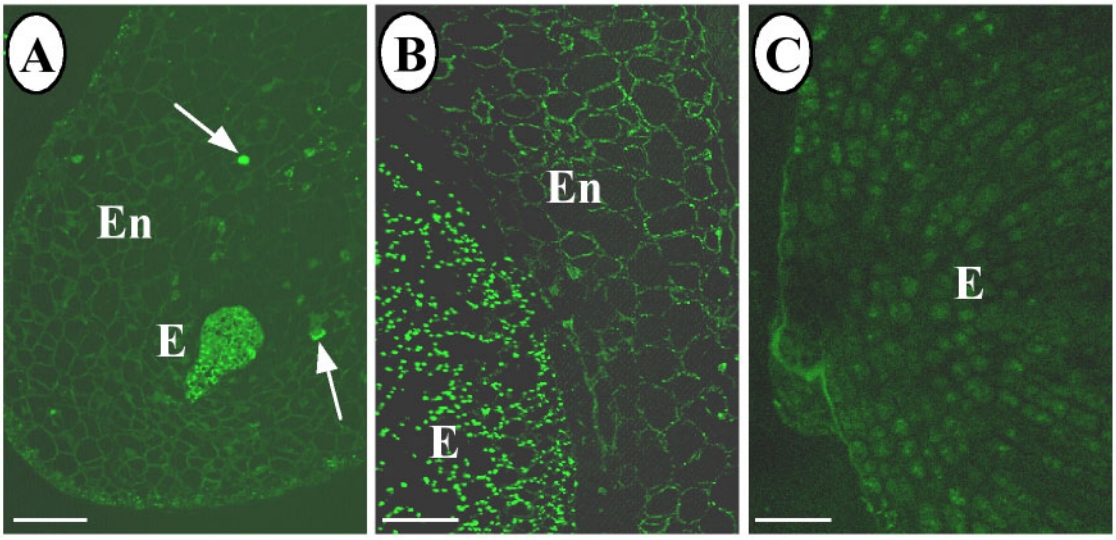


**Figure 8. Immunofluorescence (FITC) micrographs showing the microtubular cytoskeleton configurations in coffee seeds. Bars indicate 10 μm. A) Fruit at 90 DAA, showing the embryo sac and the initial development of the endosperm (En) which encloses a still undifferentiated globular embryo (E). Both tissues present an abundant network of microtubular cytoskeleton, representing expanding cells with cortical microtubular cytoskeleton as well as dividing cells with mitotic microtubular cytoskeleton configurations (arrows). A decreasing number of mitotic cells could be observed in endosperm and embryos until around 120 DAA (not shown). B) Fruit between 150 and 180 DAA, showing a large number of β-tubulin fluorescence granules as a result of the initial degradation of the cortical microtubular cytoskeleton in the embryo (E), whereas no microtubular cytoskeleton and only a few granules of β-tubulin are observed in the endosperm (En). C) Cherry fruit at 225 DAA, showing the complete absence of microtubular cytoskeleton and of β-tubulin granules in the radicle of the mature embryo (E).**

The high contents of tubulin correlated with the presence of cortical and mitotic microtubular cytoskeleton at the initial stages of endosperm and embryo development demonstrating that these coffee organs differentiate and grow through cellular division, as well as cellular expansion (Gunning and Steer, 1996; De Castro, 1998; De Castro et al., 2000). A decreasing number of mitotic configurations could be observed until around 120 DAA (Figure 8A), indicating this stage as the limit for endosperm and embryo histo-differentiation through cellular division. It is interesting to observe that this occurred in parallel with the consumption of the inner portion of the perisperm, yielding space to the growth of endosperm and the seed as a whole (Figure 6C).

From 120 DAA onwards only cortical cytoskeleton and granules of tubulin could be seen (Figure 8B, C), indicating the predominance of complementary morphogenetic events and growth of the endosperm and embryo through cellular expansion only. This appears to occur in consonance with the gradual process of dry weight accumulation and seed filling as maturation progresses (Figure 3). The advance of maturation led to the gradual degradation of the cytoskeleton and tubulin, reaching levels below the detectable limits as shown in the imunofluorescence micrographs (Figure 8B, C), as well as in the western blots (Figure 7, columns g-h). This pattern of microtubules and tubulin degradation in endosperm and

embryos during maturation of the coffee seeds was in general similar to those detected during the development of other seeds species, such as tomato, cucumber (*Lycopersicon esculentum* Mill) and neem (*Azadirachta indicates*. Juss.) (Sacandé et al., 1997; De Castro, 1998; De Castro and Hilhorst, 2000; Jing, 2000; Sacandé, 2000).

It has been reported that the degradation of microtubular cytoskeleton occurs, at least partially, as result of desiccation of recalcitrant seeds (Roberts, 1973; Berjak, 1989; Wesley-Smith et al., 1992; Mycock et al., 2000) and of vegetative tissues (Bartolo & Carter, 1991), when a critical water content is reached below which the reconstitution of the cytoskeleton is not possible after the re-hydration. As in tomato, cucumber and neem seeds, coffee seeds reach maturity with relatively high water content, at around 50%. Yet, functional microtubules and tubulin in coffee seeds are absent, as our data shows. As much as tomato and cucumber seeds, that are classified as orthodox (Roberts, 1973), coffee and neem seeds, that are considered as half-recalcitrant or of intermediate behavior (Ellis et al., 1990, 1991; Sacandé, 1997; Sacandé et al., 2000) tolerate forced desiccation after being isolated from ripe fruit, being capable to maintain viability and to reconstitute the microtubular cytoskeleton network after re-hydration (Roberts, 1973).

Therefore, the pattern of microtubular cytoskeleton and tubulin degradation during seed development seems to be independent of the orthodox, intermediate or recalcitrant character, but inherent to the proper developmental program of the different seed species. This program determines the integrity and cellular viability in desiccation conditions, in view of the capacity, at least partially, of reconstitution of the cytoskeleton after re-hydration, and full reactivation of cellular activity and maintenance of seed viability. This is reflected in the processes of differentiation and organogênesis, and in development itself. In this context, the coffee seeds that are classified as intermediate possess a program of degradation of the cytoskeleton and tubulin corresponding to that of orthodox seeds, which are capable of reconstituting it after re-hydration.

## CONCLUSIONS

The present study shows that the coffee fruit may not initiate growth immediately after fertilization of the ovule or flowering. They are capable of remaining viable in a state of latency for relatively long periods, up to 60 days in the present study, until favorable ambient conditions, *e.g.* water availability, can induce other flowerings and fruit growth. Despite the several flowerings and variations in the initial development of the fruit, the coffee shrub seems to make use of operating natural mechanisms during fruit development that favor the uniformity of size and synchronization of the maturation of the coffee fruit and seeds.

Initial growth of the fruit occurs as a result of the accumulation or growth of the maternal nucellus tissue giving rise to a transient perisperm, which seems to serve as source of tissue and reserves for the histo-differentiation of the endosperm and the embryo. The outer layer of the perisperm actually is not degraded. It remains as a greenish tissue and is apparently photossynthetically functional to function as an extra source of assimilates to be translocated to the seed during maturation. Once fruit maturity is achieved, this thin perisperm layer loses its functionality and remains as the ‘silver skin’ enclosing the seed after desiccation of the ripe cherry fruit. Apart from some biochemical and molecular studies of the perisperm of *Coffea canephora* seeds (Marraccini et al., 2001), not much is known with respect to the functionality of this transient tissue in coffee seeds, and probably nothing with respect to the functionality of the thin greenish perisperm layer during maturation.

Immature coffee embryos acquire germination capacity relatively early when isolated from the seed. However, they do not tolerate desiccation until the final stages of fruit maturation are reached, when whole seeds acquire germination capacity, as well as desiccation tolerance. These events occur in consonance with the accumulation of relatively high dry weight content as part of the developmental program in fruit and seeds when there is no functionality of cell cycle events.

The characterization of development in coffee fruit based on the analysis of cell cycle events (β-tubulin accumulation and configurations of microtubular cytoskeleton) allowed the distinct identification of the phases of histodifferentiation of the perisperm, endosperm and embryo, and of maturation of the coffee seed as a whole. Combined with the morphological data and with the physiological analysis of the pattern of water content, dry weight, germinability and desiccation tolerance, it became possible to produce a map and a profile of developmental functionality that can assist to unveil the molecular mechanisms and characteristics of the regulatory programs that act over the whole complexity of development of fruit and seeds in coffee plants. Over all, the present study precisely points out new relevant events, as well as complementary events to other biochemical and molecular studies of the development of coffee fruit (Marraccini et al., 2001; 2004), that may significantly contribute to understanding of the complex physiology behind development and maturation of coffee fruit and seeds. These aspects may be relevant not only in the perspective of coffee seeds as propagule or germplasm conservation, but also as a product for beverage, for which the fruit and seed developmental physiology is generally ignored by the research demanded by the coffee industry.

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# Generation and Analysis of a Coffee EST Database: Deductions about Gene Repertoire, Expression and Evolution

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## SUMMARY

An EST database has been generated for coffee, *Coffea canephora* var Robusta, based on sequences from approximately 47,000 cDNA clones derived from 5 different tissues/stages, with a special focus on developing seeds. When computationally assembled, these sequences corresponded to 13,175 unigenes, which are currently being analyzed for functional annotation, differential expression, single nucleotide polymorphisms (SNP), simple sequence repeats (SSR), and complete representation of biochemical pathways of interest. Here, we present a brief overview of how the sequences were generated, assembled, annotated, and compared to Arabidopsis and tomato. A relational database was created to hold the complete datasets, and a web interface was developed for easy access of the database. This infrastructure is a starting point for genomics research in coffee.

## RÉSUMÉ

Une base de données d'EST de Caféier, *Coffea canephora* var Robusta, a été créée à partir d'approximativement 47 000 clones d'ADNc de 5 types de tissus ou stade de développement différent plus particulièrement à partir de graines. Après assemblage informatique ces séquences représentent 13 175 unigènes qui sont en cours d'analyse pour leur annotation fonctionnelle, leur expression différentielle, le polymorphisme pour un nucléotide, les microsatellites, et la représentation des voies métaboliques et biochimiques d'intérêt. Nous résumons rapidement ci-après l'obtention des séquences, leur assemblage, leur annotation et leur comparaison avec Arabidopsis et la tomate. Une banque de données relationnelle a été créée pour stocker ces données et un accès internet aisé a été développé. Cette infrastructure constitue un point de départ pour la recherche génomique sur le caféier.

## INTRODUCTION

Despite its economic importance, coffee has received little attention with respect to molecular genetics and genomics research. As of August 2004, only 1535 nucleotide and 108 protein sequences from coffee had been deposited in GenBank. The majority of these sequences represent either leaf ESTs or sequences corresponding to enzymes in the caffeine biosynthesis pathway – the most extensively studied pathway in coffee (Moisyadi et al., 1998; Ogawa et al., 2001; Uefuji et al., 2003). Here, we report on the creation of an extensive EST database for coffee as a starting point for building the infrastructure for genomics research in this species.

Commercial coffee production relies mainly on two closely related species - *Coffea arabica* and *Coffea canephora,* accounting for approximately 70% and 30% of worldwide coffee production respectively (Herrera et al., 2002). Although C. *canephora* accounts for a lower total portion of the coffee market than does *C. arabica*, it is an important source for soluble coffee which is consumed widely throughout the world. *C. canephera* is a diploid (2n = 2x = 22), an out-crossing and highly polymorphic species native to central Africa (Wrigley, 1988). In contrast, *C. arabica* is a derived tetraploid (2n = 4x = 44) believed to be native to a small region of what is now Ethiopia.

The goal of the current project was to generate an EST database for coffee using high throughput single-pass 5' sequencing of cDNAs derived from leaf, pericarp and seed tissues derived from a set of *C. canephora* clones. Special emphasis was given to sequencing cDNAs from different stages of seed development, both to shed light on this important, but not well understood aspect of plant development and also to capture as many genes as possible involved in forming the final chemical composition of seeds which is the basis for the commercial product. The EST database reported here was used to derive a coffee unigene build, which was subsequently subjected to annotation and phylogenetic comparisons with the model species Arabidopsis as well as with a similar EST-derived unigene set for the closely related species, tomato.

## MATERIALS AND METHODS

### Source of tissues

Five tissue types were collected from field grown trees corresponding to different varieties and 5 different tissues/stages: leaf, pericarp, young cherry (seed and pericarp mixed), middle stage seed and late stage seed (Table 1). The maturation period of the *C. canephora* varieties under study is approximately 11 months, from pollination to ripening. The early stage cherry, middle stage seed and late stage seed were collected between 18 to 22 weeks, 30 weeks and 42 to 46 weeks after pollination, respectively.

**Table 1. *C. canephora* cDNA Libraries Constructed.**
**Overview of cDNA libraries constructed for *C. canephora*. 5 libraries were constructed, 3 of which were derived from seed, one from pericarp and one from leaf tissue as a reference.**

| Library Name | Tissue | Average Insert Size, kb | Good Quality ESTs |
|---|---|---|---|
| leaf | Leaves, young | 1.5 ± 0.6 | 8,942 |
| pericarp | Pericarp, all developmental stages | 1.4 ± 0.5 | 8,956 |
| early stage cherry | Whole cherries, 18 and 22 week after pollination | 1.4 ± 0.3 | 9,843 |
| middle stage seed | Endosperm and perisperm of seeds, 30week after pollination | 1.4 ± 0.3 | 10,077 |
| late stage seed | Endosperm and perisperm of seeds, 42 and 46 week after pollination | 1.4 ± 0.3 | 9,096 |

**RNA and mRNA Isolation**

Total RNA was extracted using phenol/chloroform (Rogers et al., 1999) and further treated with DNase I (RNase-free) and purified with RNeasy kit (Qiagen). mRNA was extracted from total RNA with PolyTrack mRNA isolation systems (Promega).

**cDNA Libraries**

Directional cDNA libraries were constructed with 3-5 ug of mRNA with ZAP-cDNA Gigapack III gold cloning kit (Stratagene). The average insert length was estimated by PCR in 36 randomly selected clones from each library. The average insert size of the libraries ranged from 1.2 to 1.5 kb (Table 1).

**Sequencing**

Bacteria were cultured in 384-well plates and cDNA inserts subjected to 5' end sequencing at the BioResource Center at Cornell University (www.brc.cornell.edu). The average size of quality reads was 613 bp with a maximum length of 1,037 bp.

**Sequence Quality Processing**

EST sequences were base-called and screened for vector sequences using PHRED software (Ewing et al., 1998). The longest stretch of overall high quality (PHRED score over 15 which corresponds to over 98% confidence) of each sequence was identified. PolyA repeats were trimmed to at most 20bp and any sequence past the PolyA (mostly low quality sequence) was discarded. After the trimming, the sequences were screened against the *E. coli* K12 genome to remove any bacteria contamination. The remaining sequences were screened for minimum length (150 bp) and maximum allowed ambiguity (4%) and low complexity (60% of the same nucleotide, or 80% of same two nucleotides which indicates error in sequencing).

**Unigene Assembly**

The sequences were first pre-clustered, and then assembled with CAP3 (Huang and Madan, 1999). Sequences were also checked for length, complexity and contamination, and chimera detection was performed.

**ANNOTATION**

**Annotation by sequence homology**

Sequence homology to Arabidopsis and Genbank NR sequences was used to functionally annotate the EST sequences using BLAST (Altschul et al., 1997). The NCBI (National Center for Biotechnology Information) non-redundant protein and dbest dataset are available http://www.ncbi.nlm.nih.gov and the Arabidopsis sequences were downloaded from the Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org). The tomato and potato sequences are available at the Solanaceae Genome Network (http://www.sgn.cornell.edu). In order to estimate ribosomal, chloroplast and mitochondrial genes, *C. canephora* unigenes were screened against *C. arabica* ribosomal and chloroplast sequences from NCBI, arabidopsis mitochondrial genome sequence from TAIR and tobacco chloroplast genome sequences from NCBI.

**Protein prediction**

The most likely coding frame and the corresponding transcribed peptide for each unigene was generated using ESTScan (Iseli et al., 1999), a program that detects coding region in EST sequences and corrects some sequencing errors (nucleotide substitution, deletion/insertion, erroneous stop codon, etc) base on bias in nucleotide composition of the species. As a HMM (hidden Markov Model)-based program, ESTScan requires a training set of high quality coding sequences to generate a model. However, very few high quality coffee coding sequences are publicly available (less than 50 of them in the GenBank). Therefore, we used as a training set of a total of 483 nuclear coding sequences from the closely related species, tomato (http://www.ebi.ac.uk/embl/).

**Functional annotation based on protein motifs and functional annotation using Gene Ontology (GO)**

ESTScan predicted peptides were subjected to InterPro Scan (Apweiler et al., 2001; Zdobnov and Apweiler, 2001) which integrates the most commonly used protein signature databases (PROSITE, PRINTS, Pfam, ProDom, etc) together with their associated scanning method for protein domain analysis. Based on the domain annotation, GO accession of the unigenes were assigned using interpro2go conversion file from the GO consortium (http://www.ebi.ac.uk/interpro).

**RESULTS AND DISCUSSION**

**Generation of *C. canephora* EST Database and Unigene Set**

A total of 62,829 cDNA clones, derived from mRNA from 5 different tissues/stages were subjected to 5' sequencing. After quality evaluation, the database was reduced to 46,914 high quality ESTs, averaging 613 bp in length. This corresponded to approximately 9,000 high quality ESTs from each of the 5 cDNA libraries (Table 1). Ribosomal, mitochondrial and chloroplast contaminations were minimal: at e value < 1e-10, only 0.1% of the unigenes matched to *Coffea arabica* chloroplast and ribosomal genes while 0.7% of the unigenes matched to arabidopsis mitochondrial and tobacco chloroplast whole genome sequences in combination.

These high quality ESTs were assembled into 13,175 unigenes with average length of 678 bp (ranging from150 to 2,714 bp), among which 7,272 (55%) were singletons and 5,903 (45%) were contigs. Of the contigs, the majority (87%) was represented by 2 to 10 ESTs while some (13%) were composed of over 10 ESTs.

**FUNCTIONAL ANNOTATION OF *C. CANEPHORA* EST-DERIVED UNIGENES**

**Coding Sequence Annotation**

ESTScan was able to identify protein coding sequences in 12,534 *C. canephora* unigenes (95.1% of total unigenes), among which 1,515 (11.5%) were complete (starting with ATG and ending with a stop codon). Due to the cDNA library construction method, the unigenes were biased to the 3' end – 57.0% of the unigenes covered the 3' end while only 35.9% covered the 5' end.

## Gene Ontology Annotation

Gene ontology annotations were made based on the InterPro domains using the interpro2go conversion file made by the InterPro consortium (http://www.geneontology.org and available at http://www.ebi.ac.uk/interpro). A total of 3,248 unigenes (24.7% of total unigenes and 74.4% of unigenes with InterPro domain match) had GO annotation. Among them, most (30.7%) were associated to 2 GO accessions, followed by 25.6% having 1 GO accession and the rest of 21.8% and 21.9% had 3 and 4 up to 12 GO accessions, respectively.

To get a better overview of the GO annotations, they were mapped to the plant GO Slim, a selected higher level categories of the GO ontology. The GO system comprises 3 distinct ontologies: the biological function, cellular component and molecular function. Since many membrane-anchoring sequences are in the 5' end while the C. *canephora* EST database was biased to the 3' end, it was not suitable for cellular component GO analysis. An overview of the GO Slim analysis of biological process categories and the corresponding analysis for the tomato EST set and the Arabidopsis proteome is given in Figure 1. Of the 57 plant biological process GO Slim categories, 32 were present in the *C. canephora* unigenes while 25 were absent. The tomato EST set has a distribution that is very similar to the coffee EST set, while the Arabidopsis dataset show some marked differences.

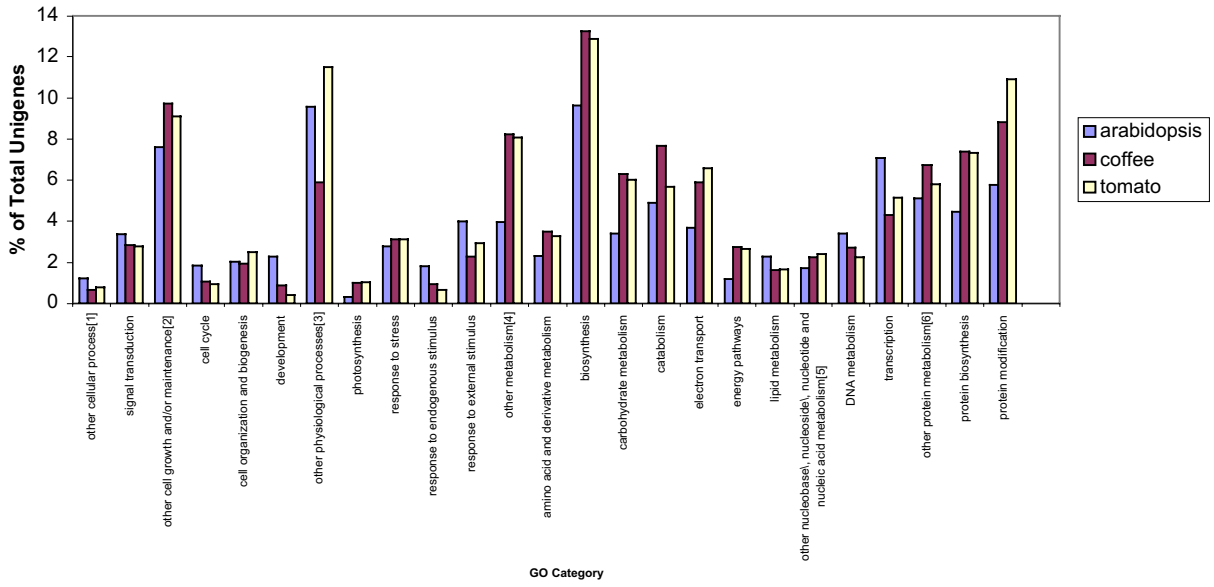


**Figure 1. Comparison of the gene annotation categories based on Gene Ontology in the coffee unigene set, the tomato unigene set and the Arabidopsis proteome. High level categories are shown on the x axis, and the number of genes in each category is shown on the y axis. The numbers of genes annotated to each categories is quite similar in the three datasets compared, indicating that the coverage of genes in the coffee library is good.**

## Highly-expressed unigenes

The most highly expressed unigenes, as defined by a maximal number of member ESTs, were determined for the coffee unigene set (Figure 2). While most of the top 20 unigenes corresponded to house keeping unigenes like SAM-synthase, ADP-ribosylation factor, peroxidase, rubisco etc, two did not have match in either arabidopsis proteome or GenBank nr databases. However they matched to multiple plant ests in GenBank dbest database.

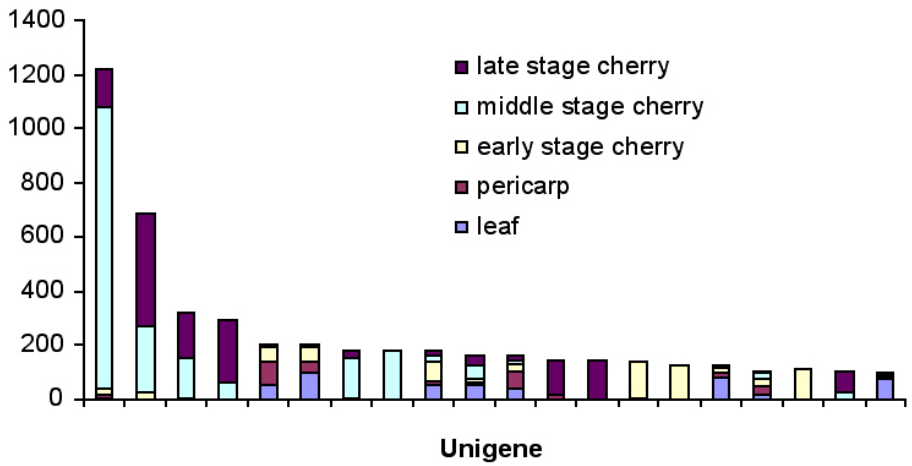


**Figure 2. Most highly expressed unigenes in the coffee EST set. The figure shows the twenty most expressed unigenes on the x axis, with the number of member ESTs given on the y axis, colored by library provenance. The most highly expressed genes in seed are storage proteins. See text for more detailed discussion.**

The top 2 unigenes are putative storage proteins dominated by late stage and middle stage seed library respectively. Together with 6 less abundant paralogs, the 2s storage proteins formed a gene family that is present in coffee but not arabidopsis or tomato. Other highly expressed genes belonged to the chitinase family, which is related to fungi resistance in many plants, including in coffee (Chen, 2003; Rojas-Herrera, 2002). They share low homology in the overall sequences and were dominated by leaf and pericarp library respectively. Interestingly, transcription factors, such as the WRKY family of transcription factors, were also among the highly expressed genes, and expressed specifically in early stage cherry. The WRKY protein family is a large and highly conserved gene family related to wound, stress, pathogen infection and senescence. In some recent studies, the WRKY family protein was found to be involved in sugar signaling (Sun, 2003) and seed development (Johnson, 2002).

**Comparisons of Coffee Gene Repertoire with those of Arabidopsis and Tomato/Potato**

Arabidopsis is the only dicot genome to be fully sequenced and hence provides a complete set of predicted genes against which to compare the EST-derived unigene set of coffee. However, coffee and Arabidopsis belong to different plant families (Rubiaceae and Brassiceae, respectively) which are distantly related phylogenetically. The best studied plant family that is most closely related to coffee is Solanaceae, and extensive EST databases have been developed for tomato/potato (Hoeven et al., 2002; Ronning et al., 2003) (see SGN, http://sgn.cornell.edu/). Rubiaceae and Solanaceae are closely allied families, both being members of the Asterid (I) clade. The close taxonomic affinities of coffee and tomato/potato are associated with a number of striking biological similarities, including the production of flesh berries, a similar genome content (C = 950 Mb and 640 Mb for tomato and *C. canephora* respectively), similar basic chromosome number (x = 12 for tomato and most other solanacaeae; x = 11 for coffee) and similar chromosome architecture with highly condensed pericentric heterochromatin and decondensed euchromatin at the pachytene stage of meiosis. For these reasons, the coffee unigene set was compared against both the Arabidopsis gene set (and derived predicted proteome) as well as the tomato and potato unigene sets.

**BLAST comparisons between coffee, Arabidopsis and tomato/potato**

To study the extent of gene conservation between the coffee, tomato and arabidopsis genomes, C. *canephora* unigenes were screened for sequence similarity against the tomato unigene set and the arabidopsis predicted proteome.

All 13,175 coffee unigenes were individually blasted against both the Arabidopsis predicted proteome and both the potato and tomato EST-derived unigene builds Currently, in the SGN database, there are more than 184,860 ESTs for tomato corresponding to 30,576 unigens and and 97,425 ESTs for potato corresponding to 24,931 unigenes. Tomato and potato are very closely related and share essentially the same genome; a recently generated combined tomato/potato unigene builds contained 45,000 unigenes. Of the 13,175 unigenes, 65% had significant matches (expect value <= 1e-5) in both arabidopsis and tomato database (http://www.sgn.cornell.edu) another 6% and 4% had matches only in arabidopsis or tomato respectively. Another 4% had matches to neither Arabidopsis nor tomato, but did have a significant match in the GeneBank or dbest databases.

## CONCLUSION

The coffee EST dataset that we have generated has allowed us to investigate the relationship of coffee with Arabidopsis, tomato and potato and other plant species. We have created an extensive database with sequence, assembly and annotation data that can be used to query for other topics of interest, such as the presence of biochemical pathways of interest. This database can also be used to search for molecular characteristics such as single repeat sequences and single nucleotide polymorphisms. Finally this EST set could also be used to create some coffee DNA chips for further differential display studies. The infrastructure that we have built will facilitate the advancement of coffee genomics research in the future.

## ACKNOWLEDGEMENTS

We also wish to thank Dr. Pierre Marraccini for his help in the early stages of this project.
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# Candidate Gene Strategy for the Study of the Chlorogenic Acid Biosynthesis

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## SUMMARY

Chlorogenic acid and other quinic esters are soluble phenolics that accumulate to substantial levels in green beans of some coffee species. If their diversity between species and during plant growth have been studied, very little is known about their actual biosynthetic pathway. A candidate gene strategy involving some genes of the phenylpropanoid pathway in *Coffea canephora* has been initiated. This study will be helpful to describe the metabolic pathway in coffee trees and highlight the major genes which regulated the CGA biosynthesis.

## RESUME

L'acide chlorogénique ainsi que d'autres esters de l'acide quinique sont des composés phénoliques solubles pouvant être accumulés de façon importante dans les grains verts de certaines espèces de caféier. Si la diversité entre espèces et durant la croissance des plantes a été étudiée, très peu d'informations sont disponibles sur la voie de biosynthèse complète de ces composés. Pour cette raison, une stratégie gène-candidat a été initiée, prenant en compte certains gènes de la voie des phénylpropanoïdes. Elle permettra de décrire la voie métabolique chez les caféiers ainsi que de mettre en valeur les gènes majeurs qui régulent la biosynthèse du CGA et des autres esters de l'acide quinique.

## INTRODUCTION

Chlorogenic acid (CGA, caffeoyl quinic acid, 5-CQA) is the major soluble quinic ester accumulated in plants. It is commonly considered as a storage form of cinnamic acid derivatives and is certainly involved in lignification in plants (Schoch et al., 2001). All the quinic esters, together with the 5-CQA, are often designated as chlorogenic acids (CGAs). These compounds are putatively involved in the protection of plant tissue from damages by oxidative stress, pathogen infection and wounding. They can also intervene in animal health: CGA, by its antioxidant activity, may prevent carcinogenesis (Niggeweg et al., 2004) and analogues of CGAs demonstrated a potent anti-viral activity (King et al., 1999). CGAs are biosynthetically derived from phenylalanine through the phenylpropanoid pathway (Figure 1) which leads to the synthesis of a wide range of compounds including flavonoids, isoflavonoid phytoalexins, coumarins and lignin (Hahlbrock and Scheel, 1989). From the p-coumaroyl-CoA, three possible pathways are proposed. Each involves the same types of enzymatic reactions: esterification and hydroxylation. In cultivated coffee trees, CGAs (CQA but also dicaffeoyl- and feruloyl- quinic acids) accumulate in beans and are mainly responsible for coffee bitterness by their degradation into phenols during roasting (Leloup et al., 1995). Their nature and their diversity between species as well as during plant growth have been widely studied (Anthony et al., 1993; Ky et al., 2001) but their synthesis, transport and accumulation

mechanisms are only partially described (Aerts and Baumann, 1994; Colonna, 1986). In particular, noting is known about the synthesis of the feruloyl quinic acids derived from caffeoyl quinic acids by a methylation. Coffee species can be a useful tool to study CGAs metabolism, particularly *C. canephora* and some wild species as C.*sp* N'Koumbala which largely accumulated CGAs. In this work, we used a candidate gene strategy to isolate some genes encoding enzymes from the phenylpropanoid metabolism. The analysis of their expression pattern and their possible co-location with already identified QTLs associated with CGAs accumulation in green beans will allow us to better describe the CGAs biosynthetic pathway in coffee trees.

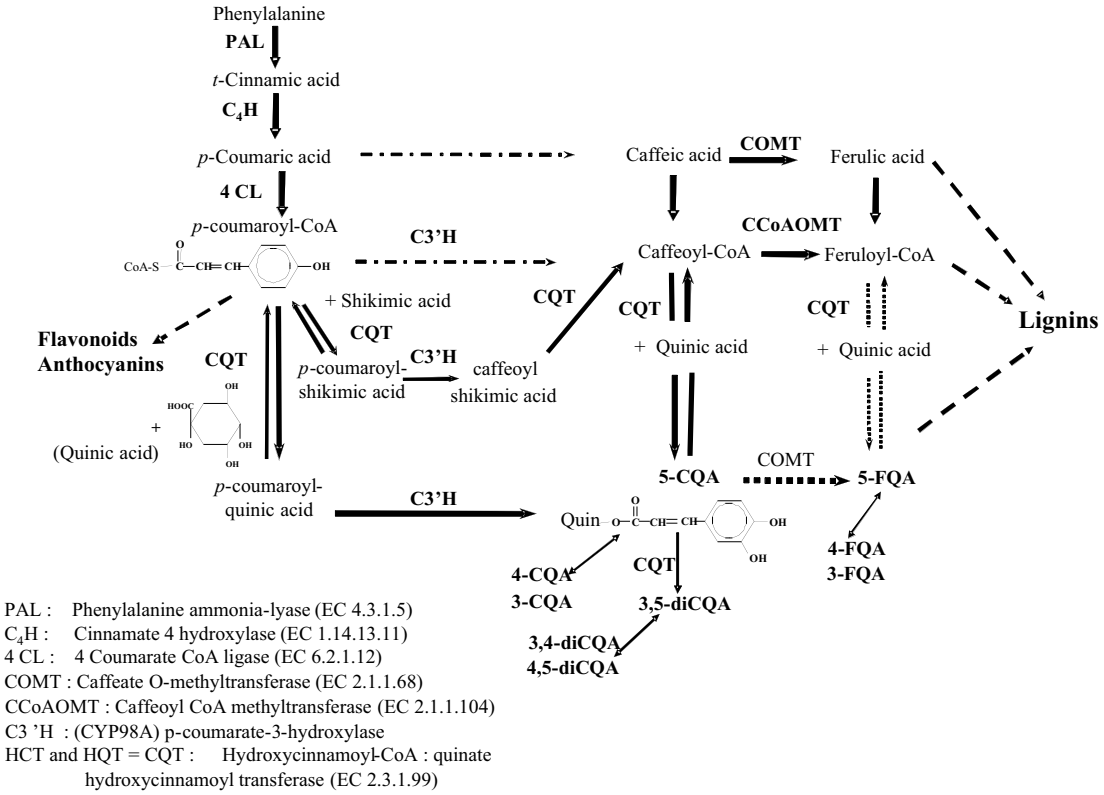


**Figure 1. Proposed pathways for the synthesis of chlorogenic acids in Coffee.**

## ABBREVIATIONS

PAL: phenylalanine ammonia-lyase, C3'H: CYP98A, *p*-coumarate hydroxylase; CQT: coumaroyl quinate transferase, COMT: caffeate methyltransferase, CCoAOMT: caffeoyl-CoA methyltransferase

## MATERIALS AND METHODS

Total DNA was purified according to the protocol of Ky et al. (2000), from leaves recollected on three trees of *Coffea canephora* Pierre (CAN) maintained in tropical greenhouses at the IRD research centre in Montpellier (France), and from trees of *C. liberica* var. Dewevrei (DEW), *C. pseudozanguebariae* (PSE), their F1 hybrids and the backcross progeny (BCDEW) maintained at the IRD research station in Man (Côte d'Ivoire).

CAN fruits were harvested on trees at the IRD research station. They were divided into three batches according to the husk colour: green (stage 1: F1), yellow (stage 2: F2) and red (stage 3: F3), immediately frozen in liquid nitrogen and maintained at –80°C until RNA extraction.

For most of the genes, consensus primers were designed after aligning corresponding sequences downloaded from GenBank. For *PAL* gene, primers were obtained according to the partial sequence of a *C. arabica PAL* gene (accession n° AF218454). For *C3'H*, semi-degenerated primers were designed according to the CODEHOP strategy (Rose et al., 1998). After amplification, the resulting fragments were purified using the EZNA gel extraction kit (Omega BioTek) and cloned into a pCR4-TOPO plasmid using the "TOPO TA for sequencing" kit (Invitrogen, Groningen, The Netherlands). Cloned fragments were sequenced by MWG-Biotech (Ebersberg, Germany).

Specific primers were designed when partial sequences were obtained from CAN. Genomic DNA from the other species was then amplified to determine gene organisation (exon/intron position) and interspecific polymorphism for genetic mapping.

Two *C. canephora* cDNA libraries were constructed, one from young leaves and the other from fruit (pool of immature and mature fruits), constructed with the "Zap Express cDNA synthesis kit" (Stratagene).

A leaf and a fruit EST libraries, derived from the previous cDNA libraries, were used to isolate the full length cDNAs of the candidates genes.

Total RNA was extracted from samples ground in liquid nitrogen. Extraction buffer (Corre et al., 1996) was added immediately to avoid thawing of the samples. After centrifugation (4.800 rpm, 30 min., 4°C), 7 mL of supernatant was ultracentrifuged at 20°C for 20h at 32.000 rpm (Beckman L7, SW41T1 rotor) in 3 ml of 5.7 M CsCl. Classical purification was then realised and the RNA was dissolved in water and stored at -80°C.

For RT-PCR analysis, 2 µg of total RNA from the different fruit samples were reverse-transcribed by AMV Reverse Transcriptase, with oligo dT primer (0.5 µg/µg of total RNA) using the kit Universal Riboclone cDNA synthesis-Promega. A second strand synthesis allowed to enrich the transcripts in the cDNA pool. A part (1/10) of the total cDNA was subjected to 35 cycles of PCR amplification.

Sequence similarities were investigated using the Blastx program maintained at NCBI. When a similarity greater than 90% was obtained for a protein sequence, it was considered that there was no ambiguity on the predicted gene function. Determination of ORFs and the derivation of predicted protein sequences, homology searches, multiple alignments, deduction of consensus sequences and primer design, were carried out using the DNASTAR package (Version 5.06 DNASTAR Incorporated, Madison, Wis., USA). Statistical analysis was done on Statistica.

Substrate specificity of C3'H was tested expressing the different *C3'H* genes in yeast. Activity analysis of the recombinant protein was done in presence of p-coumaric acid, p-coumaroyl shikimate or p-coumaroyl quinate and evaluated measuring by LS/MS the final product formed.

**RESULTS**

The first enzyme of the phenylpropanoid pathway is the phenylalanine ammonia-lyase (PAL). Analyses of EST sequences indicated that almost three genes encoding PAL are expressed in CAN fruits. The full length of two of them has been isolated: *PAL1* gene was obtained by screening the cDNA fruit library (*pF6*, accession n° AF460203) and *PAL2* by screening EST sequences. The open reading frame of *PAL1* and *PAL2* encode a polypeptide of 717 and 711

amino acids, respectively. The predicted molecular mass and theoretical pI (77.9 and 6.27; 77.2 kDa and 6.14, respectively) are consistent with the size determined for PAL polypeptides from other plants (72-83 kDa). The derived AA sequence showed the conserved motifs that are found in all PAL sequences, in particular the PAL active site: G-[STG]-[LIVM]-[STG]-[AC]-S-G-[DH]-L-x-P-L-[SA]-x(2)-[SAV]. *PAL1* and *PAL2* AA sequences shared only 84.6% similarity and the corresponding genomic sequences also displayed some differences. The two *PAL* genes differed by the length of their UTRs (3' and 5') and that of their intron (1800 and 1000 bp respectively).

The expression pattern of the two PAL genes was quite different. *PAL1* was expressed at all the observed fruit development and maturation stages and *PAL 2* was only expressed at the latest stage of fruit maturation (Figure 2).

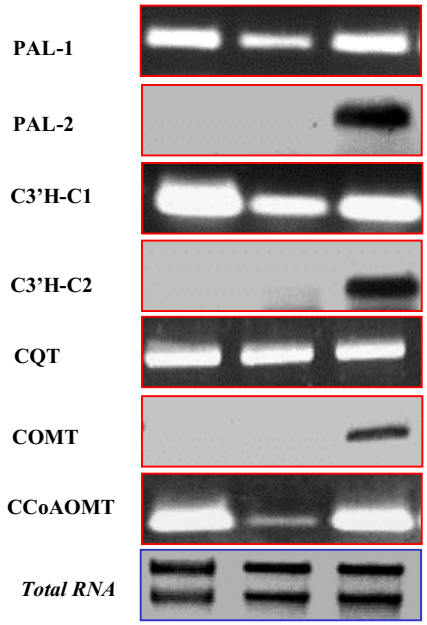


**Figure 2. Semi-quantitaive RT-PCR on C. canephora fruits at different stages of maturity.**

The esterification that leads to chlorogenic acids is performed by the reversible hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (CQT). It catalyses the esterification of p-coumaroyl-CoA with quinate or shikimate and of caffeoyl-CoA with quinate only. Little is known about this enzyme and only some gene sequences are available in data banks. One full length of the coding part of a *CQT* gene was found in the fruit EST library. It possessed a 1299 nucleotides long ORF, which encodes a 433 AA long protein.

The sequence contained the HXXXD and DFGWG motifs that are thought to comprise the active site of the acyl-CoA transferases. Analysis of its genomic sequence showed that this gene had no intron.

The expression pattern showed that this gene is transcribed at the same level at each stage of fruit maturation.

Hydroxylation, the other step leading to chlorogenic acids, is catalysed by the *p*-coumarate-2-hydroxylase (C3'H). This cytochrome P450 (group of the CYP98A) is a microsomal enzyme which hydroxylates coumaroyl-quinate or -shikimate, but also *p*-coumaric acid or p-coumaroyl-CoA. Two full lengths *C3'H* cDNA genes have been isolated using the

CODEHOP strategy and PCR screening of the fruit cDNA library. Both *C3'H-C1* and *C3'H-C2* had an ORF of 1524 bp. Each gene encodes a protein of 508 AA sharing only 75% identity between them. At the amino acid level, *C3'H-C1* and *C3'H-C2* showed 86% and 89% identity, respectively, with CYP98A13 from *Ocimum basilicum*. Genomic studies showed that these two genes had two introns of equal length (3000 and 100 bp).

Expression of each of these genes in yeast conducted to the synthesis of a recombinant protein. C3'H-C1 metabolised preferentially *p*-coumaroyl quinate instead of *p*-coumaroyl shikimate. The converse was observed with C3'H-C2. Both did not take coumaric acid as substrate. These data indicated that C3'H-C1 can be the enzyme that directly gives 5-CQA from the *p*-coumaroyl quinic acid.

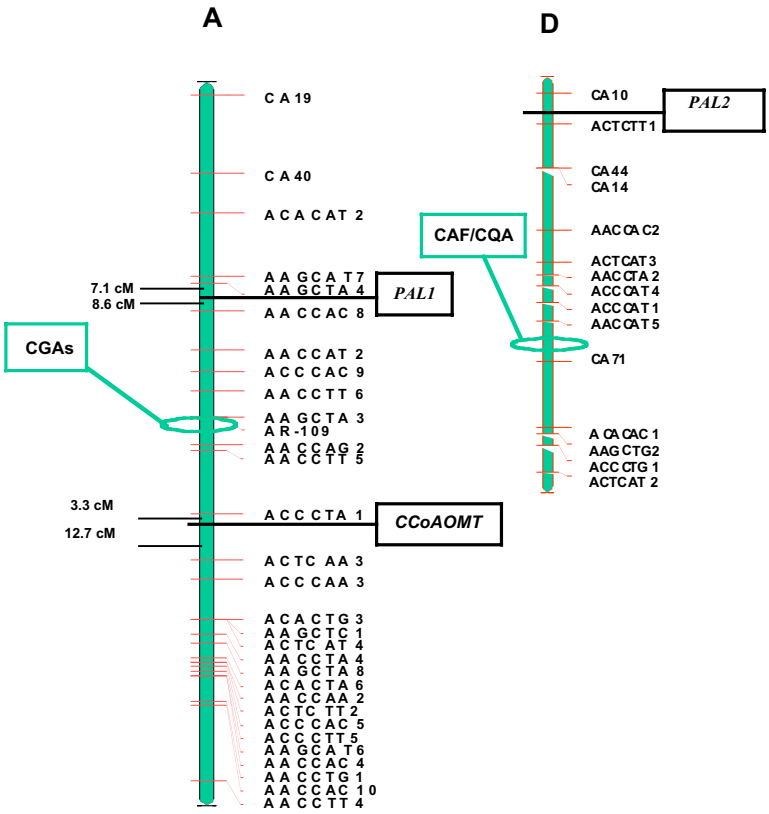


**Figure 3. Linkage groups A and D of a (PSE X DEW) W DEW genetic map showing the location of the CCoAOMT, PAL1 and PAL2 genes ant two QTL related to fruit content (CGAs: total CGAs content; CAF/CQA: ratio of caffeine.**

The pattern of expression of the two genes in fruits was quite different, *C3'H-C1* being always expressed and *C3'H-C2*, as *PAL2*, only clearly expressed in the last stage of maturation.

Biosynthesis of 5-FQA necessitates a methylation, which can occur on caffeic acid, catalyzed by the COMT, and on caffeoyl-CoA, catalyzed by the CCoAOMT (classical ways of lignification) or perhaps on the 5-CQA, catalyzed probably by a COMT. By screening the fruit cDNA library with specific PCR primers, a full length cDNA of COMT was isolated. The ORF was 1053 bp long, encoding a protein of 350 AA. The presence of an intron of about 700 bp near the 5'-end has been shown by genomic DNA analysis.

This gene was exclusively expressed in mature fruits, as observed for *PAL2* and *C3'H-C2*.

For the methylation of caffeoyl-CoA, analyses of EST sequences indicated that at least one gene encoded CCoAOMT and was expressed in CAN fruits. The coding part of the *CCoAOMT* gene was 744 bp long and encoded a protein of 247 AA. Analysis of genomic DNA showed the presence of a 500 bp intron.

Using specific PCR primers of *CCoAOMT*, it has been noticed that this gene is expressed preferentially in very young and mature fruits, similarly to *PAL1* and *C3'H-C1*.

A preliminary genetic mapping, using an offspring population from a backcross between PSE and DEW has been done with *PAL1*, *PAL2* and *CCoAOMT* genes. It showed that *PAL1* and *CCoAOMT* genes are located on the same linkage group (group A), in the vicinity of a QTL related to the fruit CGA content (Figure 3). *PAL2* is located on the linkage group D. As backcross heterozygous offspring displaying the PSE allele for *PAL1* and *CCoAOMT* showed a decrease of 15 and 11%, respectively for their CQA content in the fruits compared to the homozygous progenies for the DEW allele, the effect of *PAL1* and *CCoAOMT* genes on CQA content can be considered as very low.

## DISCUSSION

*PAL1*, *C3'H-C1* and *CCoAOMT* are three genes showing the same pattern of expression in CAN fruits. Interestingly, *PAL1* and *CCoAOMT* are located in the same region of the same linkage group and seemed to intervene weakly on the fruit CGA content. *PAL2*, *C3'H-C2* and *COMT* seemed to be exclusively expressed in mature fruits, and this expression cannot be related to the CGA level in fruits. The particular expression pattern of *CQT*, which is always expressed at the same level, can be explained by the number of different esterifications/de-esterifications that can be accomplished by the enzyme it encodes.

CGA biosynthesis *via* the phenylpropanoid pathway seemed to begin through the activity of a PAL encoded by the gene *PAL1* and to continue through p-coumaroyl quinic acid synthesis, by the activity of a CQT and then a C3'H encoded by *C3'H -C1*. These genes only explain 11% of the fruit CGA content. Other paths of biosynthesis can explain this weak effect of the genes on the CGA content. As suggested by Niggeweg et al. (2004), coffee plants, as other CGA accumulating species, can synthesised CGA in majority through caffeoyl-CoA, using the CQT esterase function between caffeoyl-CoA and CGA. Another reason could be that CGA content in fruit is not only due to their local synthesis. Accumulation mechanisms of CGA synthesized in other organs or modifications in catabolism can also be a source of increased content in fruits, without any relation to neosynthesis (Matsuda et al., 2003).

The effect of regulatory genes might be also more important to explain CGA content variation than the structural genes encoding the enzymes.

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# Sugar Metabolism during Coffee Fruit Development

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## SUMMARY

In this study, we investigated more particularly the sucrose synthase (Susy: EC2.4.1.13) at the biochemical and molecular levels during the development of coffee fruits. In addition, feeding experiments with $^{14}$C-sucrose and incubation of fruits with $^{14}CO_2$ were carried out. Our results suggest that Susy is the main enzyme responsible for sucrose metabolism in coffee fruits. In the pulp and endosperm of fruits at the last stage of maturation, a peak of Susy activity was observed and correlates with an increase of sucrose. At the molecular level, we cloned two cDNAs encoding different Susy isoforms. We also showed that both Susy-encoding genes were expressed in coffee fruits, with differences regarding their spatial and temporal expression. The $^{14}$C-experiments showed that sugars are not only transported from the leaves to the fruits, but also there is an intense communication among the tissues composing the fruits.

## RÉSUMÉ

Dans cette étude, nous avons analysé plus particulièrement la saccharose synthase (Susy: EC2.4.1.13) au niveau biochimique et moléculaire durant le développement des fruits de caféier. De plus, des expériences de nutrition utilisant du $^{14}$C -saccharose et d'incubation de fruits avec du $^{14}CO_2$ ont aussi été réalisées. Nos résultats suggèrent que la Susy est la principale enzyme responsable du métabolisme du saccharose dans les fruits de caféier. Dans la pulpe et l'endosperme de fruits au dernier stade de maturation, un pic de l'activité Susy est observé et se superpose avec l'augmentation de la quantité de saccharose mesurée. Au niveau moléculaire, nous avons cloné deux ADNc codant pour des isoformes différentes de Susy. Nous avons aussi montré ces deux gènes de Susy s'expriment dans les fruits de caféier, avec toutefois des différences spatiales et temporelles concernant leur expression. Les expériences de marquage au $^{14}$C ont montré que les sucres ne sont pas seulement transportés des feuilles vers les fruits, mais qu'il y a aussi des communications intenses entre les tissus qui composent les fruits.

## INTRODUCTION

In green coffee beans, the carbohydrate fraction represents about half of the dry weight, and participates in the extensive chemical changes associated to coffee roasting (Bradbury, 2001). Sucrose is considered as the major precursor of coffee flavor and aroma, because it is rapidly degraded during the roasting. Despite of that, little is known about sugar metabolism in coffee, particularly considering that coffee fruits take more than 30 weeks to reach maturation. Therefore, the aim of the present work was to increase our knowledge about sugar metabolism

in coffee, mainly to understand the sink-source relationships occurring between the different tissues present during coffee fruit development.

## MATERIAL AND METHODS

### *Plant Material*

Fruits of *Coffea arabica* cv IAPAR 59 were harvested from plants in the field (Sept. 2002/ April 2003) every 4 weeks from flowering until complete maturation. Fruit tissues (perisperm, endosperm, pulp) were separated and used independently to extract total RNA (Rogers et al., 1999a) or were subjected to sugars and enzyme activity analyses. In addition, leaves or fruits of branches bearing fruits were exposed to $^{14}CO_2$ (Carneiro et al., 1999 – see scheme Figure 2) for 4 h and then collected 24 h latter. Also detached fruits were fed with $^{14}$C-sucrose and collected after 24 h (Vitória and Mazzafera, 1999).

### Sugar determinations and enzyme analyses

Ethanolic extracts were used to determine soluble sugars by HPLC with pulse amperometric detection or by colorimetry (Buysse and Merckx, 1993; Van Handel, 1968). Susy activity and protein concentrations were determined according to Craig et al. (1999) and Bradford (1976), respectively. Susy activity was measured for sucrose synthesis. Ethanolic extracts were also obtained in the $^{14}$C experiments for determination of total radioactivity. For the distribution of radioactivity in the sugars, they were separated in HPLC using a radioactivity monitor.

### Nucleic acid manipulation

The partial SUS1 cDNA sequence is available in GeneBank under accession number AJ575256. For the expression analyses, northern blots were carried out using 15 µg of total RNA (Rogers et al., 1999a). Filters were hybridized independently with SUS1 and SUS2 partial cDNA fragments labeled with $^{32}$P.

## RESULTS

### Fruit growth, sugar concentrations and $^{14}$C distribution

Figure 1A shows the tissues growth during fruit development. In all tissues analyzed, reducing sugars (RS, mainly fructose and glucose) were in higher amount than sucrose (Figure 1B and C), except in the last stages of the endosperm development (205 and 234 DAF). The amount of RS was particularly important in the perisperm at 90 DAF, when the endosperm was too small to be separated. After 90 DAF, the amount of RS in the perisperm decreased rapidly, concomitantly with the endosperm growth. In this tissue, RS were not detectable from 120 DAF to 234 DAF. Sucrose accumulated gradually up to 6% at the time the maturation was completed. RS were in low concentration along the pulp development but increased rapidly in the latest stages of maturation, particularly when the fruit shifts green to red, between 205 and 234 DAF.

Branches bearing fruits at approximately 120 DAF were incubated with $^{14}CO_2$. The results showed that in addition to the leaves, $^{14}CO_2$ is actively assimilated in green fruits due to the photosynthesis. Photoassimilates (sucrose) are actively exchanged in all fruit tissues (Figure 2A). The highest accumulation in the perisperm shows its importance as a transfer tissue. Fruits fed with $^{14}$C-sucrose showed that indeed there is a transport from the pulp to the endosperm (Figure 2B). Already in the pulp a large fraction of the sucrose was converted to

reducing sugars (Figure 2C), what is in agreement with the data shown in Figure 1. The same was observed with the $^{14}CO_2$ incubation fruits (data not shown).

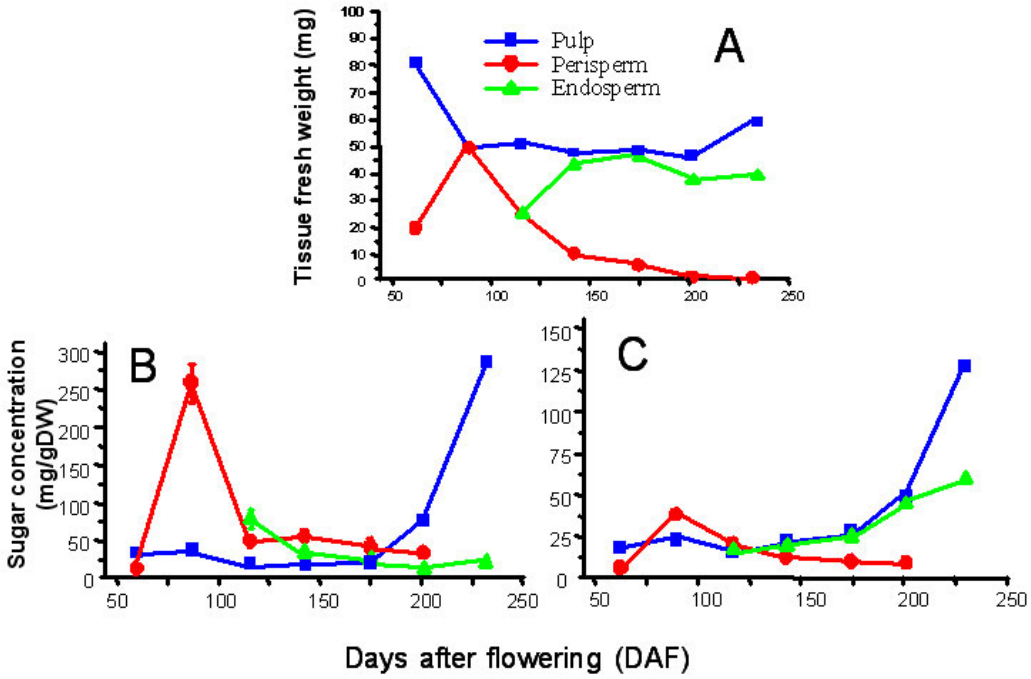


**Figure 1. Fruits aroxth (A), reducing sugars (B) and sucrose (C).**

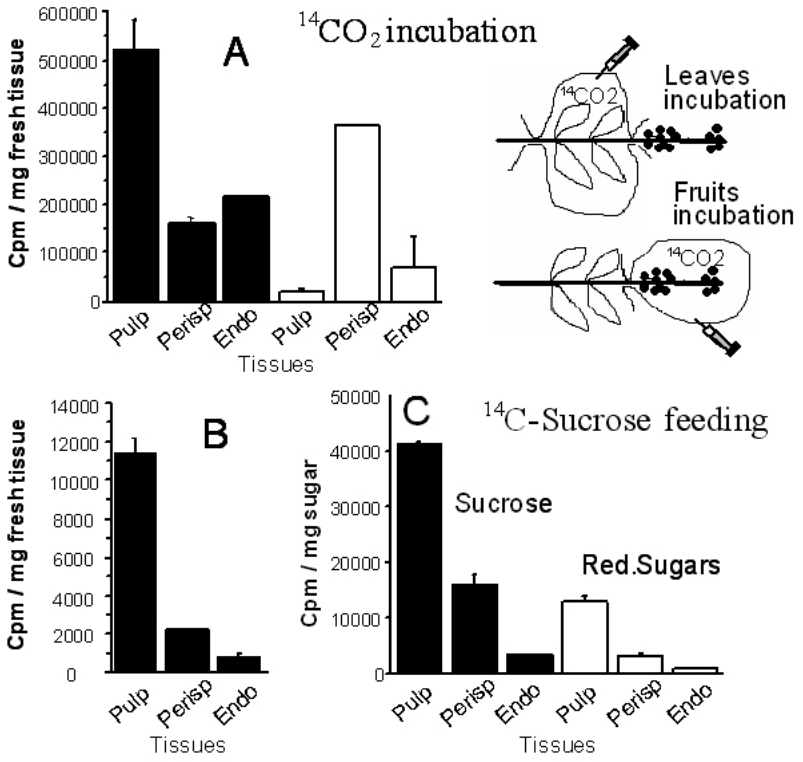


**Figure 2. Distribution of radioactivity in fruits of branches incubated with $^{14}CO_2$ (A-see scheme), in fruits fed with $^{14}$C-sucrose (B) and reducing sugars (white boxes) in these fruits (C).**

**Isolation of Susy cDNA sequences**

A partial Susy cDNA (SUS1) was cloned using degenerated primers in RT-PCR experiments with total RNA from coffee fruits. Its deduced protein shows 97% of similarity with the SUS2 protein from *S. tuberosum* (P49039). By Southern-blotting we showed that SUS1 was a member of a small gene family containing at least two genes (data not shown). Its corresponding full-length cDNA from *C. arabica* and equivalent gene from *C. canephora* were recently cloned and are under sequencing. Another Susy-encoding partial cDNA sequence (named SUS2) was also identified from the Brazilian Coffee Genome Project and presents only 59% of identity with SUS1 at the nucleic level and 74% at the protein level.

**Susy activity during fruit growth**

In all tissues analyzed, Susy always appeared to be more active than acid invertases (data not shown). However, because sugar analyses showed that there was no sucrose accumulation during the major part of coffee bean development, for example in the perisperm, we presumed that Susy functioned probably as a sucrose-degrading enzyme *in vivo*. The increase of Susy activity in the latter stages (205-234 DAF) of pulp and endosperm development was simultaneously accompanied by the increase of sucrose content in these tissues, supporting the conclusion the enzyme functions in the sucrose-synthesis sense that at these stages (Figure 3).

**Analysis of Susy gene expression**

The expression of Susy-encoding genes was checked in the fruit tissues using the SUS1 and SUS2 partial cDNA as probes (Figures 3 and 4). SUS1 mRNAs were detected in the perisperm at 90 DAF and no further observed. It was also observed at 125 DAF with a peak at 150 DAF in the endosperm, and with two expression peaks (at 60 and 150 DAF) in the pulp. In addition, SUS2 gene appeared to be expressed in the last stages of pulp development (200-230 DAF), overlapping the peak of Susy activity detected in this tissue (Figure 4).

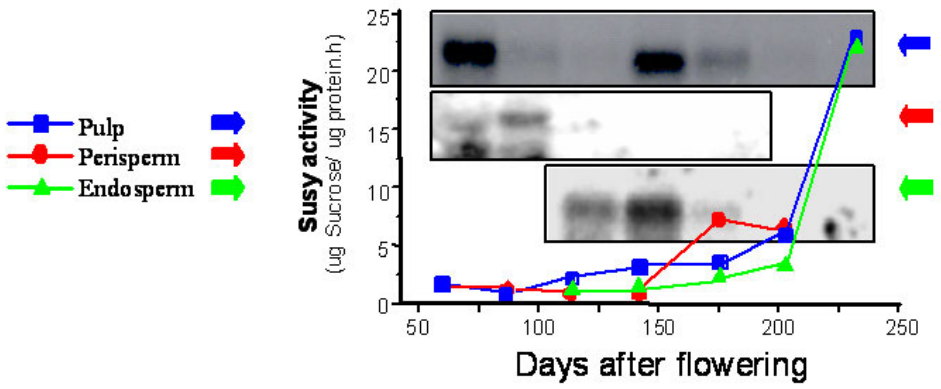


**Figura 3. Susy activities i separated tissues of coffee fruits. SUS1 gene expression (Northern-blots) is also presented.**

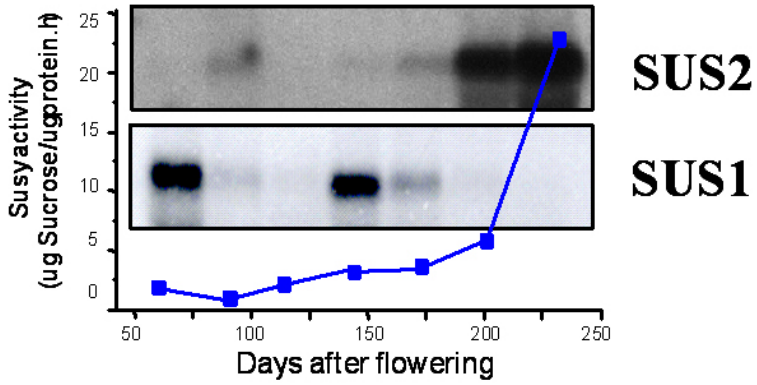


**Figure 4. Expression of SUS2 genes and Susy activity (from Figure 3) in coffee pulp under development.**

## DISCUSSION

Concentrations of RS and sucrose measured in fruit tissues confirmed previous information obtained on coffee (Rogers et al., 1999b). The data on $^{14}C$ also showed that the pulp plays an important role in fixing $CO_2$ and that the perisperm seems to behave as a passage tissue. Therefore, the decrease of RS sugars in the perisperm may also be due to a translocation to the endosperm. Indeed, incubating fruits with $^{14}C$-fructose showed a prompt transfer to the endosperm (data not shown).

From our results, we deduced that the transient accumulation of RS in the perisperm and endosperm probably is a consequence of the sucrose-degrading function of Susy rather than invertases. This was supported by the fact that no expression of invertase-encoding genes was detected in all these tissues (data not shown), whereas expression of Susy-encoding genes was observed in all tissues tested. This suggests that at least two Susy isoforms are implicated in sucrose metabolism in coffee fruits. In that sense, we proposed that the SUS1 functions mainly as a sucrose-degrading enzyme both in the perisperm and endosperm and that SUS2 should control the final concentration of sucrose found both in the pulp and in mature coffee beans.

## ACKNOWLEDGEMENTS

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# Survey of Endogenous β-glucuronidase (GUS) Activity in Coffee Tissues and Development of an Assay for Specific Elimination of this Activity in Transgenic Coffee Tissues

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## SUMMARY

The β-glucuronidase (GUS) gene (*uidA*/*gusA*) of *E.coli* is often used as a reporter gene to study the expression of foreign genes and genetic engineering of crop plants because of advantages over other reporter genes. However, the presence of endogenous GUS activity could cause difficulties in the assay and result in false positives. To avoid these difficulties we conducted a histochemical survey of endogenous GUS activity in various untransformed tissues of coffee (*Coffea arabica* and *C. canephora*) using X-gluc as substrate and compared with that of transformed embryogenic calli and somatic embryos carrying *gus*A-intron gene. We tested the effect of pH between 5 and 9 and methanol (20%) on GUS activity. Different untransformed tissues of coffee revealed various levels of endogenous GUS activity. Endosperm, integument, zygotic embryos, somatic embryos and stigmas exhibited a high level of endogenous GUS activity. Hypocotyl, ovary and pericarp showed medium level endogenous GUS activity, while the leaf, root, embryogenic and non-embryogenic calli, corolla tube and anthers showed low activity. Style did not exhibit any GUS activity. In all untransformed tissues, maximum GUS activity was noticed at pH 5 which was reduced with increasing pH. Methanol also reduced endogenous GUS activity in untransformed tissues. Contrary to the results obtained with untransformed tissues, the transformed tissues showed positive GUS reaction at all the pH tested and the intensity of GUS reaction was more in presence of methanol. Histochemical GUS assay reaction carried out at pH 9 in presence of 20% methanol did not detect endogenous GUS activity in untransformed coffee tissues but detected strong expression of transgenic bacterial GUS activity.

## INTRODUCTION

The β-glucuronidase (GUS) gene (*gusA*/*uidA*) of *E. coli* has been developed as a gene-fusion marker for higher plants (Jefferson et al., 1987). It has been widely used as a reporter gene in the study of foreign gene expression and in crop plant genetic engineering because of advantages over other reporter genes. One of the major reasons for the popularity of *gus* - fusion system was the general assumption that there was no detectable intrinsic GUS activity in higher plants. Jefferson et al. (1987) who tested several plants with a fluorescence assay did not find endogenous GUS activity. However, subsequently many plants were found to have endogenous GUS or GUS-like activity which can interfere with the activity originating from the introduced *gus* gene, especially if this level is low one. (Hu et al., 1990; Kosugi et al., 1990; Hodal et al., 1992).

Coffee is an extremely important woody, perennial agricultural crop. Among more than 50 species of *Coffea* reported, only *Coffea arabica* (2n = 44) and *C. canephora* (2n = 22) are economically important. In *C. arabica,* which produces high quality Arabica coffee and accounts for roughly 70% of the world market, genetic diversity is limited and the plants are

highly sensitive to pests and diseases. Although, some of the wild relatives of *C. arabica* have been identified as resistance source to several diseases and pests, the success in transferring the desirable traits to cultivated varieties has been limited due to reproductive barriers and frequent failures in the interspecific crosses. Also, conventional breeding of coffee is a low-efficiency and time consuming process. Genetic engineering could alleviate these problems by incorporating known genes into elite genetic backgrounds. Coffee transformation has been reported using the biolistic method (Van Boxtel et al., 1995), DNA electroporation using protoplasts (Barton et al., 1991) and various Agrobacterium systems (Spiral et al., 1999; Leroy et al., 2000; Hatanaka et al., 1999; Ogita et al., 2003).

In coffee, *gusA/uidA* gene is used to monitor transient expression (Van Boxtel et al., 1995) and stable transformation (Ocampo et al., 1991; Hatanaka et al., 1999; Leroy et al., 2000). Van Boxtel et al., (1995) observed disturbing endogenous light blue staining in control treatments of coffee somatic embryos. In this paper we report endogenous GUS-like activity in different tissues of coffee and the effect of pH and methanol on this putative endogenous GUS activity, as well as, introduced bacterial GUS activity. The objective of the study was to develop a GUS assay procedure suitable for assaying any transformed coffee tissue with the introduced bacterial *gusA* gene without interference from the endogenous GUS activity.

## MATERIALS AND METHODS

### Untransformed tissues

Different untransformed *in vivo*, as well as, *in vitro*-grown tissues of *C. arabica* and *C. canephora* were used (Table 1). Tender leaves, hypocotyl sections and roots of young (45 days-old) seedlings were used for assaying vegetative parts. Corolla tube, whole anthers and whole pistils from fully developed flower buds were used for assaying floral parts. Well differentiated zygotic embryos were isolated aseptically from 7-8 month-old, green fruits of *C. arabica* cv. Cauvery and *C. canephora* cv. CxR with hard endosperms. Either freshly isolated zygotic embryos were used for GUS assay or they were cultured on MS medium (Murashige and Skoog, 1962) with kinetin (0.1 mg/l) and the germinating zygotic embryos were assayed for GUS activity at 5, 10 and 25 days of culture. The same type of fruits mentioned above were cut transversely into 2-3 mm thick sections to get endosperm and pericarp tissues for the GUS assay. Transverse section of green, soft young fruits (3-4 month-old) were used for assaying integument (perisperm tissue).

Well-differentiated, white somatic embryos induced from the leaf explants of *C. arabica,* cv. Sln 9 and *C. canephora* cv. CxR were used. Non-embryogenic calli were induced from leaf explants of somatic embryo-derived aseptic plantlets of *C. arabica* cv. Sln 9 and *C. canephora* cv. CxR by culturing for 45 days on MS medium with 0.5 mg/l 2, 4-D. Yellowish, friable, embryogenic calli induced from the leaf explants of field-grown plants of *C. canephora* cv. CxR were used for the purpose.

### Transformed tissues

Transformed calli and somatic embryos of *C. arabica* S 4634, *C. canephora* cv. CxR were used. These transgenic tissues were obtained by co-cultivating the embryogenic calli with *Agrobcaterium tumefaciens* LBA 4404 (pSB1) harboring the binary plasmid pGSD2 containing hygromycin phosphotransferase gene (*hph*), β-glucuronidase gene (*gusA*) with catalase intron driven by CaMV35S promoter and tobacco osmotin gene in the T-DNA region. The transgenic calli and somatic embryos were selected in presence of 100 mg/l

hygromycin and integration of T-DNA was confirmed by Southern hybridization (Naveen et al., 2002).

**Table 1. GUS activity in different untransformed coffee tissues at different pHs in the absence (-me) or presence of 20% methanol (+me). (- = no activity, ++ = low, +++ = moderate, ++++ and +++++ = high GUS activity).**

| Tissues | | pH 5 | pH6 | pH 7 | pH 8 | pH 9 |
|---|---|---|---|---|---|---|
| Root | - me | ++ | + | - | - | - |
| | + me | + | - | - | - | - |
| Leaf | - me | ++ | + | - | - | - |
| | + me | + | - | - | - | - |
| Hypocotyl | - me | +++ | ++ | + | - | - |
| | + me | + | - | - | - | - |
| Corolla tube | - me | ++ | + | - | - | - |
| | + me | + | - | - | - | - |
| Anther | - me | ++ | + | - | - | - |
| | + me | + | - | - | - | - |
| Ovary | - me | +++ | ++ | - | - | - |
| | + me | ++ | + | - | - | - |
| Style | - me | - | - | - | - | - |
| | + me | - | - | - | - | - |
| Stigma | - me | ++++ | +++ | + | - | - |
| | + me | ++ | + | - | - | - |
| Integument | - me | +++++ | ++++ | +++ | ++ | + |
| | + me | +++ | ++ | + | - | - |
| Endosperm | - me | +++++ | ++++ | +++ | ++ | + |
| | + me | +++ | +++ | ++ | - | - |
| Zygotic embryo | - me | +++++ | ++++ | +++ | ++ | + |
| | + me | +++ | ++ | + | - | - |
| Somatic embryo | - me | ++++ | +++ | ++ | + | + |
| | + me | +++ | ++ | - | - | - |
| Non-embryogenic callus | - me | ++ | + | - | - | - |
| | + me | - | - | - | - | - |
| Embryogenic callus | - me | ++ | + | - | - | - |
| | + me | - | - | - | - | - |
| Pericarp | - me | +++ | ++ | + | - | - |
| | + me | ++ | + | - | - | - |

***Agrobacterium tumefaciens***

For checking the effect of pH and methanol on bacterial *uidA/gusA* gene, *Agrobacterium tumefaciens* LBA 4404 harbouring the binary plasmids pCAMBIA 1304 and pCAMBIA1201 (www.cambia.org) separately was used.

**Histochemical GUS activity assay**

All the tissues including bacterial cells were assayed for GUS activity with the indigogenic substrate X-gluc. The histochemical GUS assay was performed by modifying the established methods (Jefferson et al., 1987; Kosugi et al., 1990). GUS assay solution contained 1 mM X-gluc (Inalco, USA), 1 mM EDTA, 50 mM sodium phosphate buffer, 0.1 mM each of

potassium ferricyanide and potassium ferrocyanide. The assay solution was set to pH 5, 6, 7, 8 or 9 with NaoH or HCl in different experiments and used as such or supplemented with 20% methanol. The target tissues were incubated in the GUS assay solution for 18h at $37^0$C, washed with sterile distilled water and 70% alcohol. Chlorophyll containing tissues were washed additionally in a mixture of acetone and methanol (1:3). The intensity of blue staining which was considered proportional to GUS activity was scored visually in an arbitrary scale (Table 1). The results are based on three replications and for each treatment atleast 10 pieces of tissues were scored.

## RESULTS AND DISCUSSION

Endogenous GUS activity was determined histochemically in various untransformed tissues of coffee (Table 1) and compared with the GUS activity in transgenic coffee tissues. *A. tumefaciens* strain LBA 4404 harboring binary plasmids with intron-less *gusA* and *gusA*-intron gene served as control.

### Endogenous GUS activity in untransformed coffee tissues

In both arabica and robusta different untransformed tissues developed different intensities of blue colour indicating different levels of endogenous GUS activity (Figure 1 & Table 1). There were no significant differences between the two species. Endosperm, integument, zygotic embryos, somatic embryos and stigmas expressed high levels of GUS activity. Hypocotyl, ovary and pericarp showed moderate level of GUS activity, while the leaf, roots, embryogenic calli, non-embryogenic calli, corolla tube and anthers showed low activity (Table 1). GUS activity was not seen in style. Freshly isolated zygotic embryos showed strong GUS activity, but as they started germinating on the culture medium, the GUS activity was reduced with increasing culture period.

The endogenous GUS activity in all tissues was influenced by the pH of the assay solution (Table 1). Irrespective of the tissue, maximum GUS activity was noticed at pH 5 and it was reduced with increasing pH. In the tissues having low or moderate GUS activity it was completely eliminated at pH 9 (Figure 1). Methanol reduced endogenous GUS activity at all pH (Figure 1). At the highest pH tested (pH 9) in the presence of 20% methanol there was no detectable blue colour in any of the tissues tested, indicating full suppression of endogenous GUS activity. At pH 7 – the standard pH used for GUS assay -– in the absence of methanol, considerable GUS activity was noticed in some tissues, but in the presence of methanol at the same pH, GUS activity was eliminated except in the integument, zygotic embryo and endosperm tissues.

### GUS activity in transformed calli and somatic embryos of coffee

Transformed embryogenic calli and somatic embryos of arabica and robusta coffee carrying *gusA*-intron gene were assayed for GUS activity at different pH with or without methanol for comparison with untransformed tissues. The transformed tissues showed positive GUS reaction at all pH, either in the absence or presence of methanol (Figure 2A & B). The GUS activity was more in presence of methanol at all the pH tested in contrast to that of untransformed tissues. These findings indicate that methanol has positive effect and higher pH has no negative effect on the activity of introduced bacterial *gus* gene in coffee tissues.

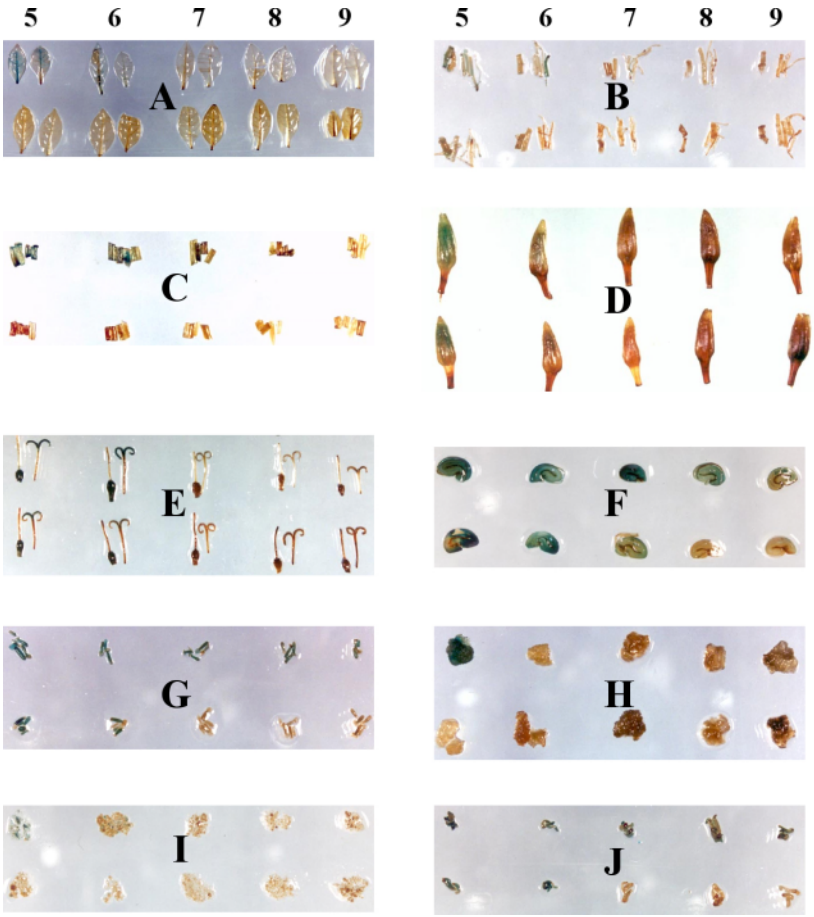


**Figure 1. Effect of pH (5, 6, 7, 8, 9) and methanol (in each figure, top row is assayed without methanol and bottom row with 20% methanol) on endogenous GUS activity in untransformed coffee tissues. A- leaf, B- root, C- hypocotyl, D- corolla tube, E- pistil, F- endosperm, G- zygotic embryos, H- non-embryogenic callus, I- embryogenic callus and J- somatic embryos.**

**Gus activity in *Agrobacterium tumefaciens***

*A. tumefaciens* LBA 4404 harboring the binary plasmid pCAMBIA 1304 showed positive GUS reaction at all pH tested (Figure 3A), irrespective of the absence or presence of methanol. There was no significant reduction in GUS activity with increasing pH from 5 to 9. The binary plasmid pCAMBIA 1304 has intron-less *gusA* gene in the T-DNA region. *A. tumefaciens* with pCAMBIA 1201 was negative to GUS reaction at all pH tested (Figure 3B), either in presence or absence of methanol. This could be expected because the plasmid has *gusA* gene with catalase intron and unlike eukaryotic plant cells, *Agrobacterium* lacks the necessary splicing mechanism to remove the intron for producing a functional GUS.

First observations on plant intrinsic GUS - like activities were described by Plegt and Bino (1989), Wenzler et al. (1989) and Terada & Shimamoto (1990). Hu et al. (1990) who conducted a survey on fifty two plant species detected such activities in certain part(s) of the fruit walls, seed coats, endosperm or especially, the embryos of the tested plants. Our results agree with the findings of Hu et al. (1990) and Hansch et al. (1995) that intrinsic GUS activity is mainly expressed during fruit and seed development.

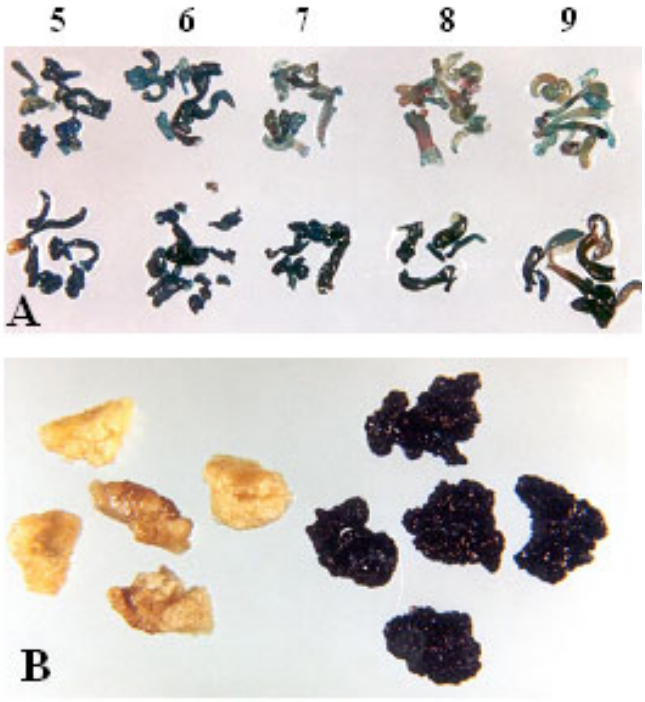


**Figure 2. GUS activity in transformed and untransformed coffee tissues. A- GUS activity in transformed SEs and embryogenic calli at different pH (5, 6, 7, 8 & 9) and absence of methanol (top row) or in presence of 20% methanol (bottom row), B- GUS activity in untransformed (left clump) and transformed (right clump) calli at pH 9 in presence of 20% methanol.**

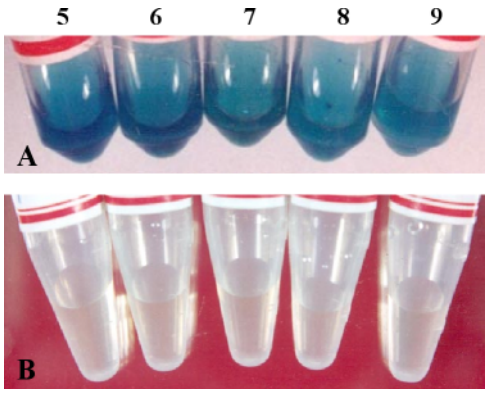


**Figure 3. GUS activity at different pH (5, 6, 7, 8 & 9) in *A. tumefaciens* LBA 4404/pCAMBIA 1304 (A) and LBA 4404/pCAMBIA 1201 (B).**

Van Boxtel et al. (1995) demonstrated transient expression of the *gus* marker gene following biolistic delivery of foreign DNA into coffee tissues. They reported intrinsic GUS-like activity producing pale blue colour in immature and mature somatic embryos, but did not observe this activity in the leaf tissues and cell suspensions without embryo structures. They did not use methanol and carried out the assay according to Jefferson et al. (1987) under standard conditions, at pH 7. Hatanaka et al. (1999) who performed histochemical GUS assay according to the method of Van Boxtel et al. (1995) did not notice intrinsic GUS activity in non-transformed embryogenic calli and leaf tissues, but noticed pale blue background in non-transformed somatic embryos. Leroy et al. (2000) have used classical X-gluc assay solution at pH 7 but with 20% methanol for GUS assay of transgenic coffee.

The present results indicate clearly that the disturbing endogenous GUS staining in coffee tissues could be eliminated by using 20% methanol and setting the assay solution to pH 9 (Figure 2B). Kosugi et al. (1990) demonstrated that 20% methanol included in the staining solution was optimal to suppress endogenous GUS activity in tobacco, rice, rape and maize. It lowered the endogenous activity of all plant extracts tested, while enhancing the activity of bacterial GUS. Our findings generally agree with the findings of Kosugi et al. (1990). However, in studies of Hansch et al. (1995) and Wilkinson et al. (1994) methanol did not specifically inhibit endogenous GUS activity. Alwen et al. (1992) and Hodal et al. (1992) demonstrated that the plant endogenous GUS - like activity had an acidic pH optimum of 4.7-5.0, while that of *E. coli* GUS is 6.5-7.0. We have got similar results in coffee. Ke et al. (2001) used pH 8 with 50% methanol for assaying GUS expression in transgenic rice and soya bean.

Our present findings indicate that a native GUS-like enzyme is expressed to different levels in different tissues and this activity differs from the introduced bacterial GUS activity in optimal pH requirement and sensitivity to methanol. Contrary to the endogenous GUS activity, introduced bacterial GUS activity was quite high in trangenic tissues even at pH 9 and in presence of methanol which suppress the endogenous GUS activity. Thus these assay conditions could be used safely to assay transgenic GUS activity in coffee tissues, and possibly in other species with high endogenous GUS activity.

**ACKNOWLEDGEMENTS**

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# Characterization of the Early Response of Coffee to *Hemileia vastatrix* using Real-time Quantitative RT-PCR

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## SUMMARY

Arabica coffee (*Coffea arabica* L) varieties may be subject to high losses in potential production due to the orange rust fungus *Hemileia vastatrix*. In some naturally resistant varieties, a specific resistance response called «hypersensitive reaction» is early elicited by fungal infection. To identify host genes involved in the hypersensitive reaction, subtractive cDNA libraries constructed at specific stages of infection were screened by differential hybridization and semi-quantitative RT-PCR experiments. Among the differentially expressed genes were three cDNA clones displaying sequence homology to (i) a transcription factor of the WRKY plant specific family (*CaWRKY*), (i) the *Arabidopsis* non-race specific disease resistance (NDR1) protein (*CaNDR1*) and (iii) a protein of unknown function (*CaR111*). Southern blotting experiments revealed that these genes are present as single-copy number in the genome of Arabica varieties and other Coffea species. mRNAs levels monitored during the early stages of the resistance reaction by real-time quantitative reverse-transcription Polymerase Chain Reaction (qRT-PCR) showed that the three genes were upregulated during pathogen infection. *CaWRKY* displayed the highest and earliest induction, starting as soon as 12 h after inoculation with a peak of induction around 18 h in the pathogen-treated resistant sample. Other genes were overexpressed within 12 h of inoculation, but to a lower extent than *CaWRKY*. These early responses begin prior to penetration of the host and may determine the outcome of the host-pathogen interaction. Further work will aim at understanding the role of selected clones in the mechanism of coffee resistance to parasites.

## INTRODUCTION

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* (Berkeley & Broome) is one of the most destructive disease of coffee (*Coffea arabica)*. Nowadays, the disease is widely distributed in all regions of the world where coffee is grown (Bettencourt and Rodrigues, 1988). In some naturally resistant coffee varieties, a specific plant resistance response called hypersensitive reaction (HR) is early elicited by fungal infection. At a microscopic scale, the HR is cytologically expressed by the plant cell death at infection sites (stomata) as soon as 48 h post inoculation (p. i.) (Martins and Moraes, 1996; Silva et al., 2002). Fungal growth usually ceased in the early stages of the infection process and HR lesions appears as chlorotic flecks visible at leaf surface (Silva et al., 2002; Rodrigues et al., 1975).

Coffee genes that are activated in the resistance response to CLR were identified by Expressed Sequence Tags (ESTs) analysis using the suppression subtractive hybridization (SSH) method (Diatchenko et al., 1996) to construct cDNA libraries enriched in HR-expressed genes (Fernandez et al., 2004). Classification of more than 400 non-redundant ESTs into functional categories based on their sequence homologies with known plant proteins showed that ca. 25 % ESTs may be associated with the early induction of defense responses in coffee (Fernandez et al., 2004). Differential screening of the SSH libraries and semi-quantitative RT-PCR experiments selected nine genes that showed enhancement of transcript accumulation at early stages of the HR (Fernandez et al., 2004). Based on their sequence homology, three ESTs were of potential interest regarding plant disease resistance. EST *CaNDR1* displayed high homology to the *Arabidopsis* non-race specific disease resistance (NDR1) protein, a key component of the signaling pathway governed by several resistance proteins (Century et al., 1997). *CaWRKY* putatively encoded a transcription factor of the WRKY plant specific family. Recent works evidenced that *WRKY* genes may be rapidly induced by pathogens or treatment with SA and may be involved in the activation of plant defense responses (Dong et al., 2003; Turck et al., 2004; Ülker and Somssich, 2004). Finally, *CaR111* has homology to a protein of unknown function, and may be a novel and interesting component of plant disease resistance.

The aim of this study was to monitor the differential expression patterns of *CaNDR1*, *CaWRKY* and *CaR111* during both compatible (susceptibility) and incompatible (resistance) interactions. Reverse-transcription (RT) combined to real-time quantitative PCR (qRT-PCR) is the most sensitive technique for mRNA detection and quantitation currently available (Heid et al., 1996; Livak and Schmittgen, 2001). We present here the development of a qRT-PCR assay to analyze the relative changes that occurred in coffee genes expression during infection by *H. vastatrix*.

## MATERIALS AND METHODS

### Plant variety, fungal strains, and inoculation

*Coffea arabica* var. Caturra were kept in the greenhouse. Sets of equally-aged plants were transferred to a growth chamber (Cryo-Rivoire, Saint-Gély du Fesc, France) under 100% relative humidity, at 25°C and under 12 h light before being inoculated with H. vastatrix. Plants were inoculated with *H. vastatrix* isolates either eliciting an incompatible interaction (race VI) or a compatible interaction (race II) as described in Silva et al. (2002). Leaves were collected at various times after inoculation and stored at –80°C until RNA extraction.

### Genomic DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blotting

Fresh leaves of *C. arabica* var. Caturra, *C. arabica* var. S4 Agaro, *C. canephora* T3561, *C. eugenioides* DA60, *C. congensis* CC54, *C. liberica* EB58, *C. racemosa* IA57 and *C. humilis* G59 were collected in the greenhouse and immediately frozen in liquid nitrogen. DNA extraction was performed using the DNEasy Plant minikit (Qiagen, France) following the manufacturer's recommendations. For each plant sample, 10 µg of genomic DNA was digested with 50-60 units of the restriction enzymes *Eco*RI or *Pst*I (Promega, France) with the addition of 5 mM spermidine per reaction for 16 h at 37°C. Restriction fragments were separated by electrophoresis in 0.8% agarose gels in TAE buffer. DNA fragments were blotted onto NylonN$^+$ membranes (Amersham, Les Ullis, France) by alkaline vacuum transfer (TE 80 TransVac, Hoefer Scientific Instruments, San Francisco, U.S.A.).

**Labelling of probes and hybridization conditions**

Hybridization probes used in this study were PCR-amplified cDNA fragments. Specific oligonucleotides were designed from the ESTs sequence and used to amplify plasmid cDNA inserts by PCR. After purification using QIAquick PCR purification kit (Qiagen, France) DNA probes (50 ng) were labelled with $^{32}$P-dCTP by random-priming, hybridized to membrane-bound DNA fragments and detected by autoradiography according to the manufacturer's specifications (Megaprime kit and hybridization buffer, Amersham, France).

**RNA extraction and RT-PCR**

Total RNAs were extracted from coffee leaves using the RNeasy Plant kit (Qiagen, France) completed by a DNAse treatment. Quality of RNA were checked on denaturing agarose gel. RNA samples were quantified using the RiboGreen RNA quantitation kit (Molecular Probes, Interchim, France) on a spectrofluorimeter (Hitachi F2500). A control PCR was run on extracted RNA samples to check the absence of genomic DNA.

First-strand cDNAs were synthesized from 1 µg of total RNA in 20 µl final volume, using Omniscript RT kit (Qiagen) and oligo-dT(18) primer (Eurogentec, Angers, France) following the manufacturer's instructions.

**Quantitative PCR assays**

Specific gene primers were designed from the cDNA sequence of each gene to be analyzed using the Primer Express 1.5 software (PE Applied Biosystems) following the manufacturer's guidelines. The *Ubiquitin* gene was used as endogenous reference (Genbank accession AF297089, Rojas-Herrera et al., unpublished). Primers and [5']6-FAM [3']TAMRA-labelled probes were synthesised by Eurogentec and used in qPCR experiments at 200nM final concentration. PCR reactions were performed in 25 µl final volume according to the manufacturer's instructions (qPCR Mastermix Plus, Eurogentec) and three replicates of the qPCR assay were used for each sample. Real-time qPCR was conducted on an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA, USA). PCR cycles were as follows: 1 cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles each of 15 s at 95°C, and 1 min at 60°C. Reactions performed without template did not result in any product.

**Quantitative PCR data analysis**

qPCR data analysis was achieved using the SDS software version 1.7 (Perkin-Elmer–Applied Biosystems). The threshold cycle (Ct) values of the triplicate PCRs were averaged and relative quantification of the transcript levels was performed using the comparative Ct method (Livak and Schmittgen 2001). Relative quantification relates the PCR signal of the target transcript in the infected sample to that of the control uninfected sample. The fold change in cDNA (target gene) relative to the endogenous control (Ubi) was determined by the following formula: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{Ubi})_{sample} - (Ct_{Target} - Ct_{Ubi})_{control}$.

**RESULTS AND DISCUSSION**

**Estimation of copy gene number in the Coffea sp. Genome**

To verify that the candidate ESTs corresponded to single genes, we conducted Southern-blots experiments on *C. arabica* genomic DNA. Genomic DNAs of other Coffea species were added to check for the presence of the gene and for detecting allelic polymorphisms. Coffee

DNA samples were digested either with the restriction enzyme *Eco*RI (no site in *CaWRKY*1 and *CaNDR1* ESTs, 1 site in *CaR111* EST), or with the *Pst*I enzyme (one site in the *CaWRKY*1 and *CaNDR1* ESTs, no site in *CaR111* EST). For all genes tested, a limited number (1-3) of digested-DNA fragments hybridized to the EST probe (Figure 1A, B and C).

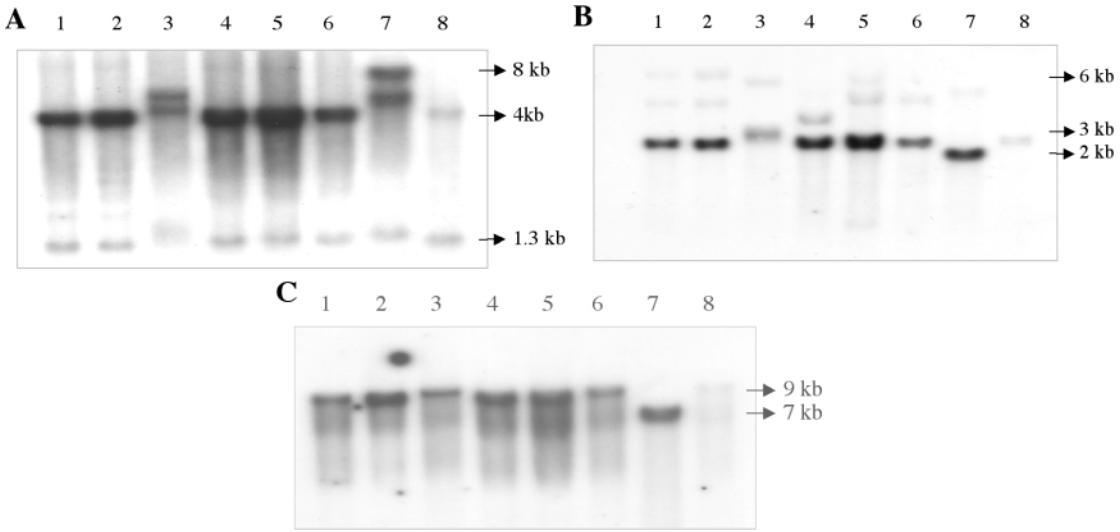


**Figure 1. Genomic DNA of *C. arabica* var.Caturra (1), *C. arabica* S4 Agaro (2), *C. liberica* (3), *C. congensis* (4), *C. canephora* (5), *C. eugenioides* (6), *C. racemosa* (7) *and C. humilis* (8) digested with *Eco*RI and probed with $^{32}$P-labeled DNA fragment of A) *CaWRKY1*, B) *CaR111* and C) *CaNDR1*.**

*CaWRKY* probe detected two high-molecular weight fragments in *Eco*RI-digested DNAs of *C. liberica* and *C. racemosa* and a sharp band in other Coffea species. *CaNDR1* and *CaR111* probes strongly hybridized to a single high-molecular weight-band in all species. Faint bands were also apparent after hybridization with the *CaR111* probe. In addition, restriction fragment length polymorphisms (RFLPs) were detected between the 7 Coffea species tested. For each probe tested, *C. liberica* (lane 3) and *C. racemosa* (lane 7) displayed different hybridization patterns than *C. arabica*, *C. canephora*, *C. eugenioides*, *C. congensis* and *C. humilis*. These data evidenced that the corresponding genes may be present as one copy (*CaR111*, *CaNDR1*) or two-copies (*CaWRKY1*) in the genome of all coffee species and that allelic variation may exist between species.

**Coffee gene expression during H. vastatrix infection**

For each gene tested, the PCR efficiency of the amplification reaction was first checked to ensure that the ΔCt between the endogenous reference gene and the gene of interest would not change when different amounts of total sample is added. PCR efficiencies were determined by generating a standard curve where the Ct values are plotted against the log of the initial quantities of the target gene in the PCR reaction. Serial dilutions of purified plasmid bearing the cloned EST fragment were prepared and calibrated amounts of 100, 500, $10^3$, $10^4$ and $10^5$ copies of plasmids were subjected to the PCR reaction. Copy number of each plasmid were calculated using the formula (copy number. $Pg^{-1}$ = (No. $10^{-12}$) / (M. X), with No: Avogadro number, M: average nucleotide molecular weight (650 $g.mole^{-1}$), and X: recombinant plasmid size (bp)). PCR efficiency (E) was calculated from the standard curve slope (S) as follow: $E = (10^{-1/S} - 1). 100$. For all genes tested, E varied from 93 to 100 % (data not shown).

To characterize the response of *C. arabica* to *H. vastatrix* infection, we monitored the mRNAs levels of the 3 candidate genes during the early stages of the plant - fungus

interaction. Time-course experiments were conducted using the Caturra variety challenged with *H. vastatrix* isolates either eliciting an incompatible interaction (resistance) or a compatible interaction (susceptible). Plants only sprayed with water were used as control. The *Ubiquitin* gene chosen as internal reference of gene expression was assayed in parallel with the candidate genes. The relative changes in gene expression estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) are presented in Table 1. Figure 2 illustrates the relative transcript level of the *CaWRKY* gene standardized with the *Ubiquitin* absolute transcript level in coffee leaves.

**Table 1. Relative quantification of *CaWRKY*, *CaNDR1* and *CaR111* mRNA levels in *C. arabica* leaves (noninoculated: C; *H. vastatrix* susceptible: S; *H. vastatrix* resistant: R) based on Ct values obtained in qRT-PCR experiments and using the *Ubiquitin* mRNA absolute levels as internal reference.**

| | | 12h | | | 20h | | | 24h | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **Sample** | **C** | **S** | **R** | **C** | **S** | **R** | **C** | **S** | **R** |
| *Ubiquitin* | Ct Ubi[a] | 26.45 | 25.84 | 26.59 | 26.36 | 26.35 | 25.92 | 26.05 | 25.91 | 25.95 |
| ***CaWRKY*** | Ct WRKY[a] | 31.20 | 28.69 | 26.31 | 30.62 | 25.49 | 23.82 | 28.62 | 24.37 | 23.91 |
| | ΔCt(WRKY-Ubi) | 4.75 | 2.85 | -0.27 | 4.26 | -0.86 | -2.10 | 2.56 | -1.53 | -2.04 |
| | ΔΔCt[b] | 0 | -1.89 | -5.02 | 0 | -5.12 | -6.36 | 0 | -4.1 | -4.61 |
| | **Fold change in gene expression[c]** | **1** | **3.71** | **32.55** | **1** | **34.97** | **82.51** | **1** | **17.14** | **24.44** |
| ***CaNDR1*** | Ct NDR1[a] | 29.89 | 28.88 | 28.65 | 30.05 | 29.81 | 28.35 | 31.66 | 32.38 | 31.25 |
| | ΔCt(NDR1-Ubi) | 3.44 | 3.04 | 2.06 | 3.68 | 3.45 | 2.43 | 4.395 | 4.225 | 2.95 |
| | ΔΔCt[b] | 0 | -0.40 | -1.37 | 0 | -0.22 | -1.25 | 0 | -0.16 | -1.44 |
| | **Fold change in gene expression[c]** | **1** | **1.32** | **2.59** | **1** | **1.17** | **2.38** | **1** | **1.12** | **2.72** |
| ***CaR111*** | Ct R111[a] | 28.07 | 26.99 | 25.57 | 28.11 | 27.74 | 26.65 | 29.23 | 32.06 | 30.05 |
| | ΔCt(R111-Ubi) | 1.62 | 1.15 | -1.01 | 1.75 | 1.39 | 0.72 | 1.97 | 3.90 | 1.75 |
| | ΔΔCt[b] | 0 | -0.46 | -2.63 | 0 | -0.35 | -1.02 | 0 | 1.93 | -0.21 |
| | **Fold change in gene expression[c]** | **1** | **1.37** | **6.21** | **1** | **1.28** | **2.03** | **1** | **0.26** | **1.16** |

[a] *mean of triplicates*
[b] *ΔΔCt = (Ctgene – CtUbi) sample – (Ctgene – Ctubi control*
[c] *fold change in gene expreion = $2^{-\Delta\Delta Ct}$*

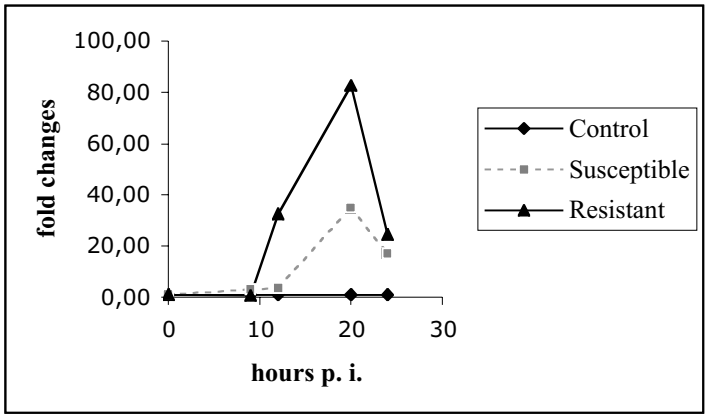


**Figure 2. qPCR analysis of *CaWRKY* gene expression changes during coffee / *H. vastatrix* infection.**

Monitoring of the mRNAs levels of the *CaWRKY* gene showed they were significantly increased (up to 82-fold) in the pathogen-treated coffee samples, as compared to control plants (Figure 2). Analysis of time-course experiments showed that the gene was transiently induced during the plant/fungus interaction. *CaWRKY* mRNA amounts increased from 12h p. i., reaching a peak around 18 - 20 h p.i. and returned to a basal level around 40-48 h p.i. (data not shown). Different induction levels between the resistant and susceptible samples were observed at early times of *H. vastatrix* infection (Figure 2). Induction of the gene in the resistant samples was higher (3- to 5- fold) from 12 h p. i. to 20 h p. i. and remained superior to the susceptible sample until 40 h p. i.

Concerning the *CaNDR1* and *CaR111* genes, different expression patterns were observed in qRT-PCR assays (Table 1). First of all, and contrary to the *CaWRKY* gene, *CaNDR1* and *CaR111* mRNA levels only displayed variation in the resistant samples. In the susceptible samples, mRNA amounts did not vary from the control plants sprayed with water. However, *CaNDR1* mRNAs levels of the resistant samples were never more than twice (2-2.7) those of the susceptible or the control samples. Concerning the *CaR111* gene, we observed a very transient increase of mRNA levels (up to 6-fold) that occurred at an early time point after inoculation but which was hardly reproducible in separate time-course experiments. As for the *CaWRKY* gene, induction of the *CaNDR1* gene was observed during early times (12-24 h p. i.) of the coffee – *H. vastatrix* resistant interaction. No difference in the *CaNDR1* mRNA levels could be detected after 30 h p. i. between the coffee samples (C, S or R) (data not shown).

For the three candidate genes tested, enhancement of the mRNA levels was observed during early times of pathogen infection (12-20h p. i). In several plant-rust interactions, host specific resistance responses are typically expressed concurrently with the formation of the first haustorium (Heath, 1997; Mould et al., 2003). In the coffee plant – *H. vastatrix* interaction, the haustorium stage may not be reached by *H. vastatrix* before 48 h p. i. (Martins and Moraes, 1996; Silva et al., 2002; Silva et al., 1999). Cytological observations of resistant coffee leaves revealed that in many infection sites (stomata) the fungus had stopped its growth at a pre-haustorium stage (Haustorium Mother Cell, HMC) (Silva et al., 2002). The induction of several genes that we observed around 12-20 h p. i. suggest that recognition of the pathogen may occur soon after penetration of the fungus into the substomatal chamber. Activation of these early transcriptional responses may determine the outcome of the host-pathogen interaction.

Microarrays analysis in model plants such as *A. thaliana* showed that a high number of plant genes are transcriptionnally regulated upon challenge by a pathogen (Maleck et al., 2000; Tao et al., 2003). Among them, components of resistance signalling pathways that are activated after the specific recognition of the pathogen may be used to achieve broad spectrum resistance in plants. By instance, the *NDR*1 gene confers enhanced resistance to virulent *P. syringae* isolates when artificially overexpressed in *A. thaliana* (Coppinger et al., 2004). Northern blot analysis showed that *NDR1* expression in *A. thaliana* is quickly induced (two times) in response to pathogen inoculation (Century et al., 1997). The qRT-PCR data we obtained here on the *CaNDR1* expression in the coffee plant – *H. vastatrix* interaction also showed a similar induction in the resistant samples. The exact role in disease resistance of the *A. thaliana* NDR1 protein is still unknown but its location in the plasma membrane suggests either NDR1 could directly interact with the pathogen or act as a transducer of pathogen signals (Coppinger et al., 2004).

Transcriptional activity of genes is modulated by the specific binding of transcription factors to DNA promoter sites. WRKY transcription factors are a major group of plant proteins

implicated in the regulation of several biological processes, including pathogen defense (Dong et al., 2003; Turck et al., 2004; Ülker and Somssich, 2004). In *A. thaliana*, 49 out of 72 *WRKY* genes were transiently activated upon pathogen-mimicking stimuli (Dong et al., 2003). In parsley, recent work evidenced that WRKY proteins are present as a complex pool of low-abundant preformed proteins in the cell nucleus and that *de novo* synthesis is quickly induced in response to a pathogen-derived elicitor (Turck et al., 2004). The promoter element (W-box) that binds WRKY proteins has been found in several plant defence – related genes (Turck et al., 2004; Maleck et al., 2000). Cloning and identification of the coffee *CaWRKY* gene which expression is early activated after *H. vastatrix* inoculation might offer new insights into the mechanisms that lead to coffee resistance to pathogens.

In conclusion, we developed a real-time reverse transcription (RT)-polymerase chain reaction (PCR) assay and used it to quantify resistance-candidate gene expression in coffee samples challenged with the rust fungus *H. vastatrix*. We showed that the candidate genes were upregulated at an early time after inoculation, suggesting that recognition of the parasite by the plant may occur soon after penetration in the substomatal chamber, leading to the activation of coffee resistance responses. Future work will aim at understanding the role of the *CaWRKY* , *CaNDR1* and *CaR111* gene in the mechanism of coffee resistance to parasites.

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# Genetic Variability of *Coffea arabica* L. Accessions from Ethiopia Evaluated with RAPDs

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## SUMMARY

The genetic diversity of 50 wild and semi-wild accessions of the *Coffea arabica* L. germplasm collection, gathered by the FAO and ORSTOM missions to Ethiopia, and maintained in Colombia by CENICAFE, was evaluated with RAPD markers. The evaluation was carried out in two phases: In phase one, the polymorphism of 8 Ethiopian accessions of different geographic origin, plus the cultivated variety 'Caturra', was assessed with the RAPD technique with forty-two 10-mer oligonucleotides. In phase two, 51 accessions were assessed with a set of 5 polymorphic primers that reproduced, with a correlation of 95%, the groups generated by the 24 polymorphic primers found in phase one. Principal Coordinate Analysis of molecular data revealed that a closely related group consisting of 86% of the Ethiopian *C. arabica* accessions evaluated are significantly different from the Caturra variety and could be used in a genetic breeding initiative to increase the variability of cultivated varieties. The results also indicate that a larger polymorphism is present in the Colombian replica of FAO Ethiopian coffee germplasm collection than previously reported

## INTRODUCTION

There have been limited studies of the genetic variability of *C. arabica* of Ethiopian origin. Except for the original ORSTOM evaluation of the collected materials (Charrier, 1978), and a recent diversity study by Anthony et al. (2001), who included a large sample of wild or semi-wild accessions, only a few accessions have been considered in most studies, thus under-representing the diversity that can be found in these materials (Lashermes et al., 1996; Orozco et al., 1994). There has also been a limited use of these materials for breeding purposes, and currently none of the coffee commercial cultivars available in the world come from introgressions from wild relatives.

In the study presented here, we determine the structure of the genetic variability of 50 wild or semi-wild accessions of *C. arabica* collected by the FAO (Meyer, 1968) and other missions to Ethiopia, using RAPD markers. We compare the variability among these accessions and the commercial cultivar Caturra, widely used in Colombia since the 1960s (Castillo, 1988) because its semi-dwarfness and adaptability to intensive cropping systems, but also open for improvement because of its susceptibility to several coffee diseases and pests.

### Plant Material

Forty-seven of the accessions studied were collected by the FAO mission to Ethiopia in 1964 (Table 1); accession Barbuk Sudan was collected in 1941 during an expedition to the Boma

Plateau in the South-East corner of Sudan; accession N-100 is a high-yielding Bourbon selection from the Lyamungu Coffee Research Station, which has been included in variety trials in Kenya (Jones, 1957) and finally, accession Adele Gummer Illubabor comes from the Experiment Station of Rwanda Urundi, in the former Belgian Congo (Sylvain and Cordoba, 1968).

**Table 1. List of coffee accessions evaluated with RAPD Markers.**

| Accesion | Origin | Accesion | Origin | Accesion | Origin |
|---|---|---|---|---|---|
| AdelleF | Illubabor | E-186 | Kaffa | E-388 | Kaffa |
| B.SudanF | Sudan | E-188 | Kaffa | E-389 | Kaffa |
| CaturraF A | Brasil | E-189 | Illubabor | E-391 | Kaffa |
| N-100 | Tanzania | E-208 | Illubabor | E-403 | Kaffa |
| E-018 | Sidamo | E-218 | Illubabor | E-405F | Kaffa |
| E-020 | Sidamo | E-225A | Kaffa | E-418 | Kaffa |
| E-030A M | Kaffa | E-238 | Sidamo | E-456 C | Illubabor |
| E-036M | Shoa | E-265 | Kaffa | E-471 | Kaffa |
| E-048A | Kaffa | E-277 | Kaffa | E-490F | Kaffa |
| E-059A | Kaffa | E-283 | Kaffa | E-496A | Kaffa |
| E-068F | Kaffa | E-286F | Kaffa | E-524 | Kaffa |
| E-084F | Kaffa | E-287 | Kaffa | E-563 | Gojjam |
| E-143 A | Kaffa | E-298 | Kaffa | E-566M | Gojjam |
| E-154 | Kaffa | E-337 | Kaffa | E-575A | Gojjam |
| E-177 | Kaffa | E-368 | Illubabor | E-576 | Gojjam |
| E-181 | Kaffa | E-374 | Illubabor | E-577F | Gojjam |
| E-182 | Kaffa | E-376 | Kaffa | E-579M | Eritrea |

## RESULTS AND DISCUSSION

### Polymorphic primers selectio

Among the subset of 9 accessions evaluated for RAPD markers, a total of 24 primers produced polymorphism (57% of the primers tested). In contrast, Anthony et al. (2001) indicate that only 16 out of 115 primers used for RAPDs (10.7%) detected polymorphism, even in a larger set of accessions (38) from the CATIE (Costa Rica) field collection. A total of 401 fragments were amplified for an average of 9,5 fragments per primer and 137 of those fragments were found polymorphic (34,2%). Again, the number of polymorphic bands was greater than the one reported by Anthony et al. (2001), who found only 29 polymorphic bands with the 16 primers evaluated.

### Allele distribution

The selected set of primers generated 58 polymorphic markers, with 39.6% of them present at high frequencies (0.8 or more), 20.7% as rare alleles in low frequency (0.2 or less), and 39.6% (23 markers) with frequencies between 0.2 and 0.8 (Figure 1). The marked differences between the levels of polymorphism found by Lashermes et al. (1996) and Anthony et al. (2001) and the genetic variability reported here, with accessions distributed by the same collecting

missions, deserves a closer look at the collection replicas, in particular the maintenance of the plants in the field.

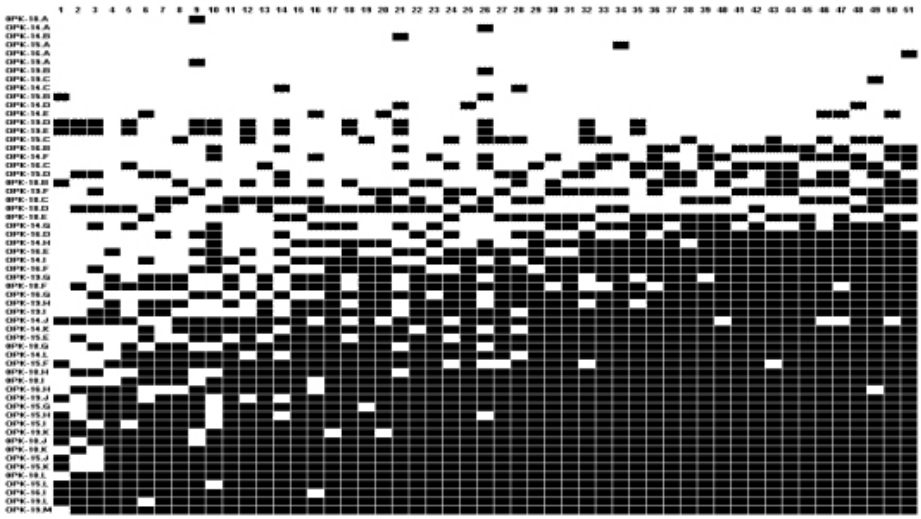

**Figure 1. Bandmap for all the polymorphic markers (rows) in the evaluated genotypes (columns). Black for present, white for absent. a) rare alleles (frequency between 0.02 and 0.2); b) very frequent alleles (frequency between 0.8 and 0.98).**

**Accessions clustering**

The analysis of diversity of wild and semi-wild *C. arabica* accessions with RAPD markers in this study was similar to that found by Moncada (2000) who reported that 68% of microsatellite markers detected polymorphism among 11 Ethiopian accessions. There was no association between the grouping and the origin site in Ethiopia, likewise the findings made by Lashermes et al. (1996) and Anthony et al. (2001) with different sets of accessions. It is also observed that the extreme diversity is exhibited by accessions gathered in the Kaffa province, although it also must be noted that 60.7% of the accessions were collected from this region.

Interestingly, commercial accessions Caturra and Bourbon (N-100) showed a high degree of genetic variability between them. This is surprising since Caturra is a dwarf mutant of the cultivar Bourbon and N-100 is a selection of the same cultivar made in Tanzania, therefore we would expect them to be genetically very similar. N-100 is part of the large group of 29 accessions clustered together in the PCOORDA analysis. The variability observed in these wild (collected directly in the forests), semi-wild (gathered from forests originated from abandoned cultivation plots) or domesticated accessions (found in familiar or commercial plantations, or collected in markets), do not support a concept of former dispersion being affected by domestication preferences, including characteristics such as yield, resistance to diseases, adaptability to soils, or others. This, as well as the observed lack of association to the geographic origin of collection, suggests a minor role of human settlers in the establishment of local selections in the early selection process towards cultivated varieties of *C. arabica* at the site of origin. It is possible that there were a large number of transfers of the best individuals between different populations, and not local selection of varieties.

For breeding purposes, our results indicate that a larger potential than previously reported is present in the germplasm collection of *C. arabica* from Ethiopia, which can be used to

improve popular commercial varieties such as Caturra. With the help of more elaborate molecular markers, such as the microsatellite set we are currently developing, new cultivars can be generated by introgression of wild alleles, oriented towards disease resistance and coffee specialty markets.

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# Genetic Maternal Map of Coffee

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## SUMMARY

Genetic maps are useful tools to speed up the selection process on plant breeding programs, particularly on perennials such as coffee (*Coffea* spp.). For its construction, a segregating population with its parents, and polymorphic markers are required. In order to develop a coffee map, one F2 tetraploid population already available, coming from the cross between Caturra variety and Timor hybrid was used. This population showed very low polimorphism (< 10%), when assessed with 40 microsatelites available at that moment and therefore it was discarded. Because of that, a new population was developed from the cross of two diploid species, *Coffea liberica* and *Coffea eugenioides*. This population theoretically coming from two known parental plants was scored using microsatellite markers in order to develop a map. However, some of the markers presented bands in high proportion on the F2 population that were not presented on the parents.

An exploratory analysis throughout amplification of DNA of neighbouring plants of that used as mother on the manual crossing and its posterior sequencing and comparison with those "rare" bands presented on the F2 population, showed coincidence of bands, which suggested contamination with pollen of those plants.

Based on those results and given the long period of time required to develop a new population it was decided to use this population to develop a maternal map, in which, only the maternal alleles are used. This strategy, allows the utilization of one already available population, which translates on time saving, particularly on perennials. The JoinMap algorithm allows the construction of a maternal map.

## INTRODUCTION

Genetic maps based on molecular markers, are a very useful tool to determine the heretability of agronomic traits important economically. To know the number of genes that influence a character, its location in the chromosomes, the effect of the copy number on the character expression, to study the transmition of specific genes or part of the genome from progenitors to progeny, to clone genes of importance based on their effects on the phenotype without knowledge of their specific functions.

To construct a map it is required to have available a segregating population, coming from two known progenitors. On coffee due to its perennial condition, to get a segregating population is a long process that could take at least 3 years. Based on this there is a necessity of using populations available. On this study, the results of the evaluation of two segregating populations for coffee mapping ar presented.

## MATERIALS AND METHODS

On first place one F2 tetraploide population coming from crossing Caturra x Timor hybrid was studied. Such population presented a very low level of polymorphism when microsatellite

markers were used for evaluation. Based on those results, development of new populations was initiated using diploid species. These diploid species are cross-pollinated and therefore assure a high degree of polymorphism. A F1 population was obtained by crossing *C. liberica* x *C.eugenioides* with 300 individuals. This population is being used to construct the genetic coffee map.

One hundred microsatelite markers were available for map construction.

## RESULTS AND DISCUSSION

After amplifying the DNA using some microsatellites the presence of bands different from those present on the parents were observed (Figure 1).

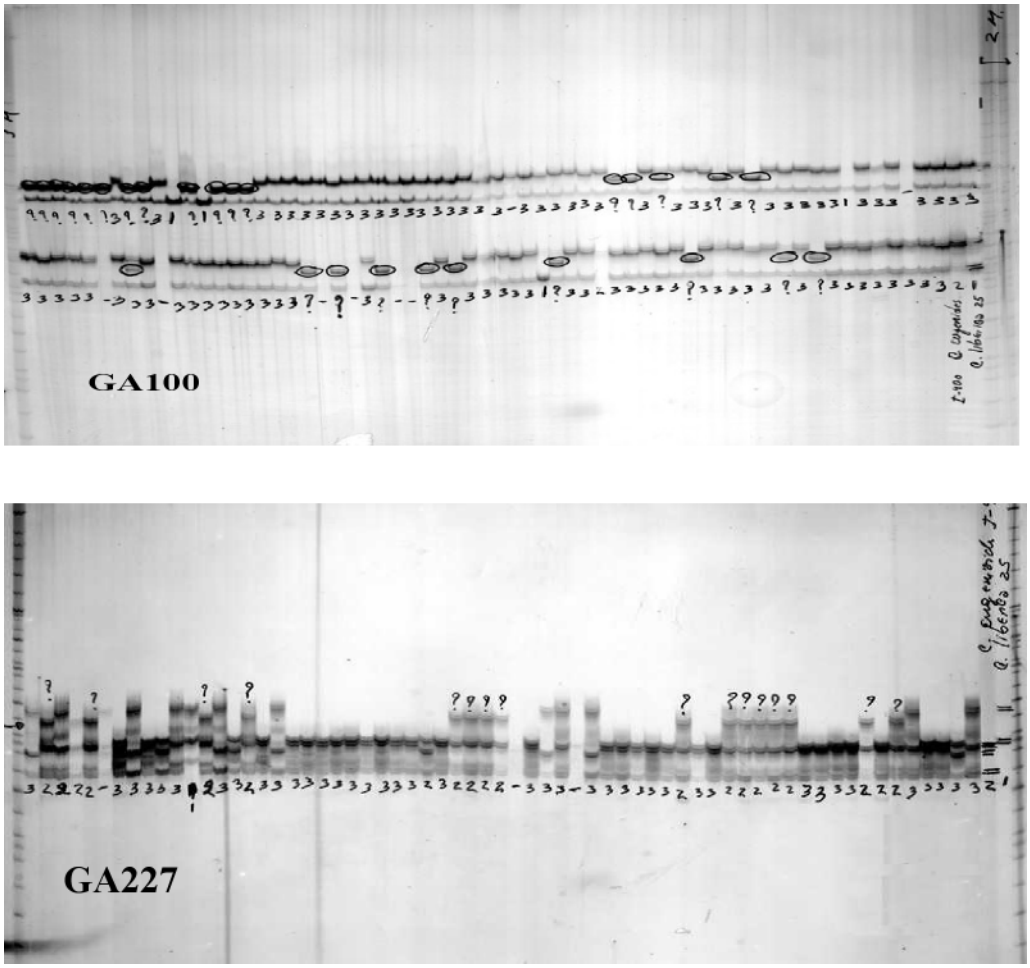


**Figure 1. Two different microsatellites showing the segregation patterns in an F1 population. Bands that are not present in the parents can be observed in the population. The segregation ratio is also very different from the expected one. To explain the "rare" bands appearing in the F1 population, the hypothesis of outcrossing or pollen contamination was proposed. To test this hypothesis, DNA from the neighboring plants around the plants used to make the crosses was extracted and checked for any evidence of out-crossing. In Figure 2, one can observe that the "extra" bands present in F1 population are also present in some of the neighboring plants planted close to the maternal parent.**

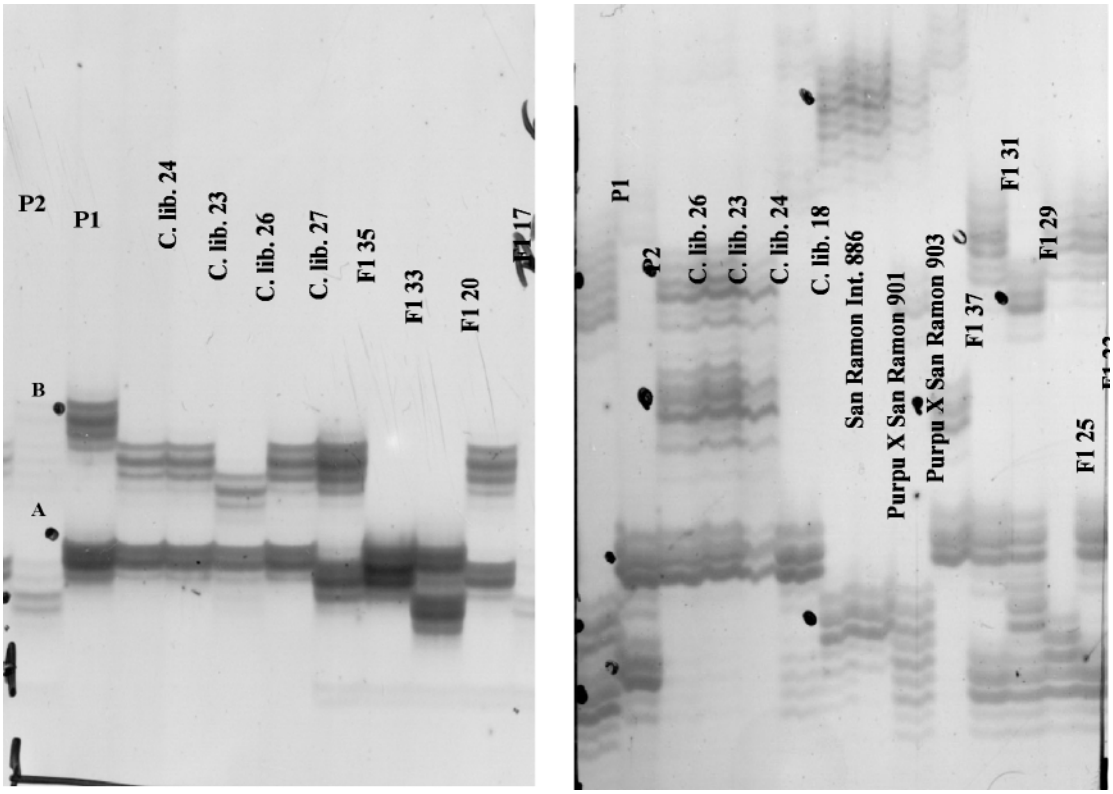


**Figure 2. Pattern of segregation of parents, F1 plants and neighbor plants for microsatellites.**

Sequencing of those "extra" bands, and bands from the F1, the parents and the neighbouring plants was done (Table 1), it can be seen that the parents differ from all the others on some nucleotides. The analysis of gels provide evidence on favour of the multi parental origin of the F1 population.

**Table 1. Sequences comparison among parents, F1 population and neighbor plants for microsatellite GA227.**

```
Clib-24-f
TTGAGAGGAGGAATAATCTAGATCATCTATGTTCAACACATAGAACTTACAAGGATAGAG 60
F1-17b-f
TTGAGAGGAGGAATAATCTAGATCATCTATGTTCAACACATAGAACTTACAAGGATAGAG 60
Clib-27-f
TTGAGAGGAGGAATAATCTAGATCATCTATGTTCAACACATAGAACTTACAAGGATAGAG 60
F1-35b-f
TTGAGAGGAGGAATAATCTAGATCATCTATGTTCAACACATAGAACTTACAAGGATAGAG 60
P1-b1-f
TTGAGAGGAGGAATAATCTAGACCATCTATGTTCAACACATAGAACTTACAAGGATAGAG 60
 ********************* ************************************

Clib-24-f
CATTCACCGAAAAGAGTATTCAAATCTTAAGTTGGTCATTGGAGAGAGAGAGAGAGAGAG 120
F1-17b-f
CATTCACCGAAAAGAGTATTCAAATCTTAAGTTGGTCATTGGAGAGAGAGAGAGAGAGAG 120
Clib-27-f
CATTCACCGAAAAGAGTATTCAAATCTTAAGTTGGTCATTGGAGAGAGAGAGAGAGAGAG 120
F1-35b-f
CATTCACCGAAAAGAGTATTCAAATCTTAAGTTGGTCATTGGAGAGAGAGAGAGAGAGAG 120
P1-b1-f
CATTCACTGAAAAGAGTATTCAAATCTTAAGTTGGTCATTGGAGAGAGAGAGAGATAGAG 120
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Due to the fact that during the sequencing project when microsatellite are present problems usually occur, the interpretation of the results need to wait until replication of sequencing be done.

## CONCLUSIONS

It is clear from this data that the "rare" bands may be coming from other C. liberica accessions. This suggests that accidental cross-pollination can not be ruled out as an explanation of the "non-parental" alleles in the mapping population. In order to use this population for mapping development, it is necessary to construct a maternal map using the JoinMap sofware JoinMap. This conclusion is based on the work of Chackett et al. (2000) who had a similar problem in tea.

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# Characterisation of Nigerian Robusta Coffee (C*offea canephora* Pierre ex. Froehner) Germplasm

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## SUMMARY

Thirty-seven *Coffea canephora* clones were characterised using forty-eight agro-botanical characteristics. The planting design was the Fisher blocks, in which five plants of each clone were established in contiguous rows. Spacing was 3.1 m by 3.1 m. Data were collected in three replicates. Characterisation data were analysed using multivariate analyses. Differences among the *C. canephora* clones were significant ($P < 0.05$) for thirty-five out of forty-eight agro-botanical characters. The coefficient of variation for the characters ranged between 1.11 and 28.47. The major discriminating characters were berry yield (kg/ha), number of berries on five bearing secondary branches and number of flowers per axil. Numerical taxonomy and multivariate system (NTSYS) effectively grouped the clones into four major clusters. Similarity coefficients among the clones ranged between 32.6% and 80.0%. The most divergent pair showing 32.6% similarity was clones T921 and C36, while clones T164 and T116 exhibited high similarity of 80.0%. The low coefficients of variation among many of the characters, the high level of overlaps and the low genetic distances among many of the clones as revealed by the similarity co-efficients, indicated that the genetic base of the Nigerian *C. canephora* germplasm is very narrow. Therefore, while existing clones in different clusters may be used for subsequent hybridisation and selection programme; it is recommended that the crop's germplasm should be expanded by introduction from other coffee growing countries. This will facilitate effective coffee breeding programme in the future.

## INTRODUCTION

The cultivated *Coffea* species are native of Africa (Chevalier, 1947). The six *Coffea* species in Cocoa Research Institute of Nigeria (CRIN), Ibadan are *C. arabica, C canephora* (robusta coffee), *C. liberica, C. excelsa, C. stenophylla and C. abeokutae*. In Nigeria, *C. arabica* and *C. canephora* are cultivated commercially. At CRIN, coffee breeding started in 1969 and it had led to the selection of some *C. canephora* clones. Information on the distinguishing characteristics among the different clones in Nigeria is not available. In CRIN's coffee germplasm, mislabelling of clones is common. Characterisation work will correct the anomaly and ensure an efficient coffee germplasm management. The objectives of this study was to carry out detailed agro-botanical characterisation of Nigerian *C. canephora* germplasm to determine range of existing genetic variability.

## MATERIALS AND METHODS

The study was carried out at the CRIN, Gambari, Ibadan (Lat. 7° 26'N Long. 3° 54'E; and 122 m above the sea level). Thirty-seven clones of *C. canephora* collected from different countries were used for this study. There were 2 clones from Ghana; 7 from Gabon; 5 from Java; 2 from Uganda; 4 from Benin Republic; and 17 from Zaire. The clones were planted in

1966. The planting design was Fisher blocks described by Montagnon et al. (1998). Spacing was 3.1m by 3.1m. Three plants were randomly selected per clone. The forty-eight agro-botanical characters studied were adapted from IPGRI (1996). Data were collected between 2000 and 2002, and analysed by multivariate techniques namely principal component analysis (PCA), similarity coefficient values (SCA) and four clustering techniques: complete linkage cluster analysis (CLCA), hierarchical cluster analysis (HCA), average linkage cluster analysis (ALCA) and numerical taxonomy system for statistics (NTYSYS).

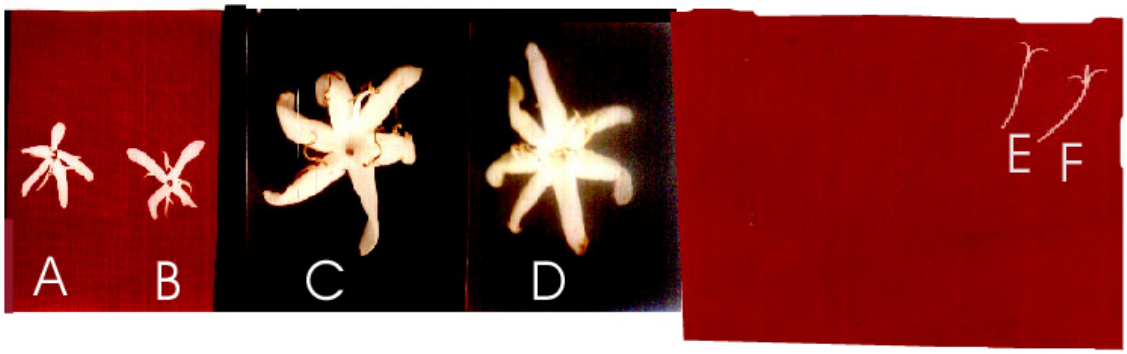


**Figure 1. Heteromorphism in the flowers of Coffea canephora and tri-headed style. A: Normal flower of Coffea canephora with five petals (x1/2); B: 4 petals (x1/2); C: 6 petals (x1) and D: 7 petals(x1). 4, 6 & 7 petals are rare occurrence found on clone M31; E: Normal style (x1); F: Detached tri-headed style (x1).**

## RESULTS AND DISCUSSION

### Agro-botanical characterisation

ANOVA showed that thirty-five agro-botanical characters were significant while thirteen were not. The growth habit of thirty-six clones were erect while that for clone T24 was compact and decumbent, thus, clone T24 could prevent rapid weed growth in a plantation and according to Montagnon et al. (1998) reduce herbicide use. The young leaf colours were three: purple, light green or green. Purple coloured young leaves were consistently observed in Niaollou robusta (M31) and E77; C108 had light purple young leaves while for all other clones the young leaves were green. The mature leaf colour could be categorised into three: green, yellowish green, and light green. The Quillou (C) and Java ex Gambari (T) clones had green and yellowish green mature leaves respectively. However the other clones had light green mature leaves. Coffee flowers are normally borne on the old leaf axils (Ferwerda, 1948). However, terminal inflorescence was observed in some of the *C. canephora* clones studied and, some of the terminal flowers found on clones G129, G87 and M31, finally developed into mature berry. The normal flower of *C. canephora* has five stamens and five petals (Cobley, 1956). In this study, about 5.0% of the flowers sampled on five clones namely: T93, T365, T45, M31 and M10 showed heteromorphism for stamens and petals (Figure 1). Four, six or seven petals and stamens were observed on few flowers of the five clones. The styles of coffee flower have two heads, however clones M31 had styles with tri-forked stigma (Figure 1). Coffee berry ripening could be classified into three viz: early in October/ November; normal in December; and late in January. Clones G129, G87, E106, E92, E130, G87, T169 and T921 ripened early. Also, clones C36, C96, C90, and C111are high yielding and ripened early. Early ripening ensures that the farmer is able to plan for berry harvesting within a short time frame at reduced cost (Ferwerda, 1948). Clones C36 and C111 with berry yield of 5670 kg/ha and 4720 kg/ha respectively are good selection for commercial planting. Though *C. canephora* exhibits biennial berry bearing (Ferwerda, 1948), clone C36

was regular in annual berry bearing. Clones with good productivity, early maturity and ripening that are suitable for commercial orchards are C36, C90, C96, C111 and T1049. The CV ranged from 0.0 to 28.47. The number of fruits in terminal node and the number of flowers with six petals had CV of 28.47 and 19.84 respectively. Many of the characters had low CV indicating low genetic variability.

**PCA and cluster analyses**

The PCA confirmed the low CV, in that the major discriminating characters were few, namely: yield (kg/ha), number of berries on five bearing secondary branches, number of flowers per axil, weight of 100 berries (g) and volume of 100 berries (ml). PCA clustered the 37 clones to four. The high yielding *C. canephora* clones (coded 3, 4, 5, 9 and 33, in Figure 2) are in cluster 4 and separated from the low yielding ones. The closeness of clones T197 (coded 33) and Quillou clones (coded 3, 4, 5 and 9) in the PCA, suggested that they are probably of the same ancestry. This classification would be useful in the choice of parent subsequent hybridization and selection programmes. There were overlaps of the clones suggesting that they are close genetically. SCV among the thirty-seven clones ranged from 32.6% to 80.0%. The most diverse pair showing 32.6% similarity was clones T921 and C36. Java Ex Gambari (T) clones showed high similarity co-efficients among themselves. The high similarity coefficients exhibited by the 'T' clones explained the high level of overlapping among the clones observed in the PCA graph. Since genetic distances among the "T" clones are very low, it implies any clone in the "T" group may be chosen for use in a breeding programme. The lowest and highest similarity levels recorded by each of the cluster techniques ranged from 21.0% to 52.0% for NTYSYS, 35.0% to 85.0% for both ALCA and CLCA, and 45.0% to 85.0% for HCA. The thirty-seven clones of *C. canephora* were clustered to four at similarity levels of: 34.0% by NTYSYS, 40.0% by ALCA, 50.0% by CLCA and 60.0% by HCA. Consequently, the most effective of the cluster techniques was NTYSYS. The results showed that, the Nigerian *C. canephora* population could be classified into four. Montagnon et al. (1998) observed that crosses between the two different clusters are generally more vigorous and productive than crosses within a cluster.

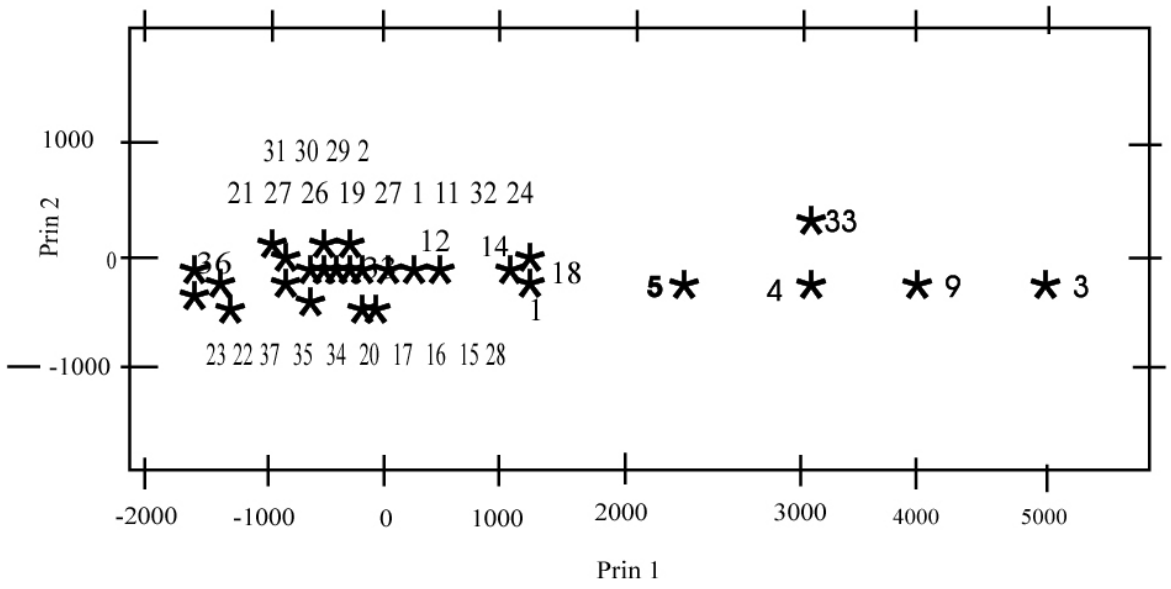


**Figure 2. Principal Component graph of axis 1 and 2 for thirty-seven clones of Coffea canephora. The figures represent individual clones.**

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# Determination of the Number of Self-incompatibility Alleles (SIA) in *Coffea canephora* and the Role of Pollen-stylar Protein in the Expression of SIA

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## SUMMARY

Four clones of *Coffea canephora* were utilized to determine the number of self-incompatibility alleles (SIA), and the role of pollen-stylar protein in the expression of SIA in the crop. The planting design was Fisher blocks, which five plants represent each clone. Spacing was 3.1 m by 3.1 m. Data were collected in three replicates. Fruit set data were subjected to diallel analysis; while the SIA was evaluated by stylar squash method and polyacrylamide gel electrophoresis. The self-compatibility index was 4.81, indicating that *C. canephora* is highly self-incompatible. Five self-incompatibility alleles: $S_1$, $S_2$, $S_3$, $S_4$ and $S_5$ were identified, thus a single gene with five alleles at its locus controls self-incompatibility in Nigerian *C. canephora* population. The protein band movement of the un-pollinated and cross-incompatible styles were restricted to the upper region of the gel (0.1-2.9 cm), while in cross-compatible combinations the thick bands were in both the upper and lower regions. Thus, the protein that is associated with the S-locus consists of those with low (lower region) and high molecular weights (upper region).

## INTRODUCTION

Low yield was attributed to the problem of self-incompatibility existing in robusta coffee. (Devreux et al., 1959) identified the nature of self-incompatibility in *C. canephora* as gametophytic; while it is controlled by a single gene (Berthaud, 1980) with multiple alleles (Lashermes et al., 1996). The number of the self-incompatibility alleles and S-allele genotypes within selected *C. canephora* population is important in the production of $F_1$ hybrid seed (Sanzol and Herrero, 2002). Hitherto, the Nigerian *C. canephora* population have not received attention in this respect. In an attempt to understand the molecular basis of incompatibility, efforts are being directed towards the identification of the components (protein, isozyme, DNA or RNA) associated with the S-allele expression in plants (Giles and Prakash, 1987). It is therefore considered desirable to investigate the role of pollen-stylar protein in S-allele expression in the Nigerian *C. canephora* population. The objectives of this study therefore was to determine the number of self-incompatibility alleles (SIA) in *C. canephora* and the role of pollen-stylar protein in the expression of SIA.

## MATERIALS AND METHODS

The study was carried out at the Cocoa Research Institute of Nigeria, Gambari, Ibadan. The experiments were carried out between 2000 and 2002.

**Estimation of the number of self-incompatibility alleles and electrophoresis**

Four plants from four different clones of *C. canephora*: A116 ($C^1$), C111 ($C^2$), T1049 ($C^3$) and M53 ($C^4$) were used. The clones have different self-incompatibility reaction. M53 is self-compatible while clones A116, C111, and T1049 are self-incompatibles (Omolaja, 1999). The individual S-genotypes and the number of alleles in the population were determined by stylar squash method and by assigning an arbitrary genotype of $S_{1.2}$ to plant $c^1$. Then the genotypes of other plants were revealed by means of its compatibility response (Ram and Sreenivasan, 1984) to plant $c^1$. Fifty percent of the pollinated ovaries were allowed to set seed. The same plants and cross design used for estimating the self-compatibility alleles of the styles above were also used in this study. The plant parts: anthers, un-pollinated and pollinated styles, were collected in two periods viz: before and after pollination. Homogenisation of the samples and protein extraction were done according to (Akinwusi and Illoh, 1995). The samples were loaded and electrophoresis was done in running tank: Hoefer SE 600 Series with a Pharmacia Biotech EPS 3500XL Power pack. After the protein had stacked in the lower gel the current was increased to 3mA per gel. The running took six hours. The gels were stained with 0.05% Coomasie brilliant blue for 15-20 minutes after which destaining was done at intervals of three hour for about 48 hours. The gels were observed by placing it on illuminated transparent glass. Then the distances of the bands from the origin, their thickness and intensity were recorded. The relative mobility (Rm) value of each band was calculated with transparent plastic ruler.

**RESULTS AND DISCUSSION**

**Estimation of the number of self-incompatibility alleles**

From the data (Tables 1 and 2) plant $C^2$ was compatible with $C^1$, which suggests that their two S-alleles were different. Hence $C^2$ should be $S_{3.4}$ in genotype. $C^3$ is partially compatible with $C^1$ and was given the genotype $S_{1/2}$ $S_x$*. Since plant $C^4$ was incompatible with $C^1$, it implies that the two plants have two common S-alleles, therefore $C^4$ should have $S_{1.2}$ genotype. Plant $C^3$ was compatible with $C^2$; since $C^2$ is $S_{3.4}$ genotype, $C^3$ would carry different alleles that is yet unknown ($S_{1/2}$ $S_x$). Plant $C^4$ that was incompatible with C1 was found to be compatible with $C^2$; since $C^2$ is $S_{3.4}$ genotype, the given alleles of S1.2 to $C^4$ properly agreed with this reaction. To determine the identity of Sx the remaining crosses are useful. Plant $C^3$ could have been assigned the same alleles as $C^4$ because of their common reactions in crosses with $C^2$. If that were so, then $C^4$ x $C^3$ should have been incompatible. $C^4$ x $C^3$ was however actually partially compatible (Table 2), this suggested that $C^4$ and $C^3$ only share same allele in only one locus. Then, $S_x$ of plant $C^3$ could be given the allele identity $S_5$. The hypothesis of plant $C^3$ carrying alleles $S_{1.5}$ fitted well into the model. Therefore, a total of five alleles $S_1$, $S_2$, $S_3$, $S_4$ and $S_5$ are available in the four plants studied. The seed set data corroborated the identity of the S-alleles involved as shown by the pollen tube count. Moreover, the S-alleles genotypes among the clones showed that a combination of clones T1049 and C111 or A116 and C36 in a seed garden would generate homogeneous hybrid seed. The clones are self-incompatible and high yielding which could be good candidates in bi-clonal seed garden. The observation of five alleles in this study confirmed earlier findings by (Berthaud, 1980), and (Lashermes et al., 1996) that a single gene with multiple alleles controls the gametophytic SI in *C. canephora*.

**Table 1. Compatibility responses of four selected plants in *Coffea canephora.***

| Cross | NPG | NPT | %G | NNPT | APT | %APT | %SS | CC |
|---|---|---|---|---|---|---|---|---|
| $C^2$ X $C^1$ | 42 | 22 | 52.38 | 19 | 3 | 1.36 | 62.89 | C |
| $C^3$ X $C^1$ | 35 | 9 | 25.71 | 4 | 5 | 55.55 | 12.83 | PC |
| $C^4$ X $C^1$ | 32 | 6 | 18.75 | 1 | 5 | 83.33 | 5.00 | IC |
| $C^3$ X $C^2$ | 46 | 34 | 73.91 | 32 | 2 | 5.88 | 62.82 | C |
| $C^4$ X $C^2$ | 67 | 14 | 20.89 | 13 | 1 | 7.14 | 43.11 | C |
| $C^4$ X $C^3$ | 21 | 8 | 38.09 | 4 | 4 | 50.00 | 30.23 | PC |

*NPG: Number of pollen grains; NPT: number of pollen tubes; G: germination; NNPT: number of normal pollen tubes; APT: abnormal pollen tubes; SS: seed set; CC: categories of compatibility. C: compatible crosses; IC: incompatible crosses; and PC: partially compatible crosses. $C^1$: A116; $C^2$: C111; $C^3$: T1049; $C^4$ : M53*

**Table 2. The Number and nature of S-alleles in selected clones of *Coffea canephora.***

| Compatibility | | Inferences | |
|---|---|---|---|
| Crosses | class | No. of alleles | Nature of the alleles |
| $C^2$ X $C^1$ | C | 2 + 2 | $S_{3.4}$ (1.2)** |
| $C^3$ X $C^1$ | PC | 2 + 1 | $S_{.1.5}$ (1.2) |
| $C^4$ X $C^1$ | 1C | 2 + 0 | $S_{1.2}$ (1.2) |
| $C^3$ X $C^2$ | C | 2 + 2 | $S_{1.5}$ (3.4) |
| $C^4$ X $C^2$ | C | 2 + 2 | $S_{1.2}$ (3.4) |
| $C^4$ X $C^3$ | PC | 2 + 1 | $S_{1.2}$ (1.5) |

*Plant $C^1$ was assigned an arbitrary genotype $S_1$ $S_2$. $S_1/_2$ : presence of $S_1$ or $S_2$; $S_3/_4$: presence of $S_3$ or $S_4$*

** *Figures in parentheses represent the genotypes of the male parent.*

**Electrophoretic analysis of anther and stylar protein**

The protein bands exhibited by the anther were few in number, fast in movement and thin in intensity. The reduced number as well as decrease in the intensity of protein bands from anthers of some of the *C canephora* clones studied is indicative of reduced protein accumulation in these anthers. The protein bands movement of the un-pollinated and cross-incompatible styles were restricted to the upper region (0.1-2.9 cm). In compatible crosses the bands were very thick and were distributed in both the upper and the lower regions of the gel. This suggests an association between the S-locus controlling cross-compatibility and the simultaneous presence of thick protein bands in the upper and lower regions of the gel. Thus, the protein that is associated with the S-locus consists of those with low (lower region) and high molecular weights (upper region). However there is need for more work in this regard in order to confirm the relationship between protein band and SI in *C. canephora*.

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# Some Genes Encoding Enzymes Involved in the Caffeine Bioynthesis of *Coffea pseudozanguebariae* Are Regulated by an Incomplete Splicing of their Pre-messenger RNA

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## SUMMARY

Three methylation steps occur at the end of caffeine biosynthesis. They are ensured by three N-methyltransferases (NMT), encoded by a multigene family. Using specific primers, the genes coding the NMT could be amplified, cloned and sequenced in some *Coffea* species having different caffeine contents. These genes have a very conserved structure (four exons, three introns).

The NMT genes expression had been studied in order to explain fruit caffeine content variations between two species namely: *Coffea canephora* and *C. pseudozanguebariae*, which present a quasi, null accumulation of caffeine in seeds. Analyses by RT-PCR on fruit RNAs of *C. pseudozanguebariae* at various stages of maturation indicated that all the genes of NMT were expressed. However, in this species, splicing of introns is disturbed in approximately 16% of the cases. This disturbance results in the skipping of the first and/or second intron, the partial excision of the second intron or the excision of an exonic fragment with the intron located downstream. The third intron is always correctly spliced. In all the cases, these aberrant excisions would lead to the synthesis of truncated proteins and consequently the synthesis of the final product could be affected.

## RÉSUMÉ

Trois méthylations interviennent dans la voie de biosynthèse de la caféine. Elles sont assurées par des N-méthyltransférases (NMT), codées par une famille multigénique. Grâce à des amorces spécifiques, les gènes codant les NMT ont pu être amplifiés, clonés et séquencés chez plusieurs espèces de caféiers ayant des teneurs en caféine différentes. Ces gènes ont une structure très conservées (quatre exons, trois introns).

L'expression des gènes de NMT à été étudiée pour tenter d'expliquer les variations de teneur en caféine dans les fruits chez deux espèces: *Coffea canephora* et *C. pseudozanguebariae* qui présente une accumulation quasi nulle de caféine dans les graines. Des analyses par RT-PCR sur fruits de *C. pseudozanguebariae* à différents stades de développement et de maturation ont indiqué que tous les gènes de NMT étaient exprimés. Cependant, chez cette espèce, l'épissage des introns est perturbé dans environ 16% des cas. Cette perturbation résulte en la non excision du premier et/ou deuxième intron, l'excision partielle du deuxième intron ou encore l'excision d'un fragment d'exon avec l'intron situé en aval. Le troisième intron est toujours excisé. Dans tous les cas, ces excisions aberrantes conduisent à la synthèse de protéines tronquées et la synthèse de produit final pourrait en être affectée.

## INTRODUCTION

Coffee is the first industrial crop in the world whose trade represents the second exchange amount of all commercial goods just after oil. Exclusively cultivated in the intertropical area, coffee is appreciated in many parts of the world, despite different cultures and habits, because of its aroma and its light stimulating properties, the late being mainly due to the caffeine contained in coffee beans.

Along with theobromine, its precursor in the metabolic pathway, caffeine is a purine alkaloid which biosynthesis is well known (Figure 1) and in which the last steps from xanthosine (or xanthine) to caffeine are accomplished by at least three distinct N-methyltransferases (Mösli Waldhauser et al., 1997; Kato et al., 2000; Ogawa et al., 2001; Mizuno et al., 2003; Uefuji et al., 2003; Kato et al., 2004). These enzymes are encoded by a multigenic family. Breeding programs were designed in order to lower the caffeine content as the demand for low (or nil) caffeine content is increasing. Interspecific crosses can be one way to go as some *Coffea* wild species do not accumulate caffeine in their beans. One of them is *C. pseudozanguebariæ*, originating from East Africa (Anthony et al., 1993). We surveyed in this study the presence and the expression of the genes encoding the N-methyltransferases in fruits, at different development and maturation stages of *C. pseudozanguebariae*.

## MATERIAL AND METHODS

RNA was extracted from *C. pseudozanguebariae* fruits at different ripening stage (Claire Bertrand et al., 2003) and gene expression was scored by RT-PCR. Primers used for RT-PCR were designed after sequence comparison to amplify specifically each NMT gene. RT-PCR products were verified by sequencing or restriction. RT-PCR were also performed on RNA extracted from *C. canephora* fruit and fruit cDNA libraies as control.

The percentage of RNA with unexpected length give an estimation of the proportion of wrongly processed transcripts.

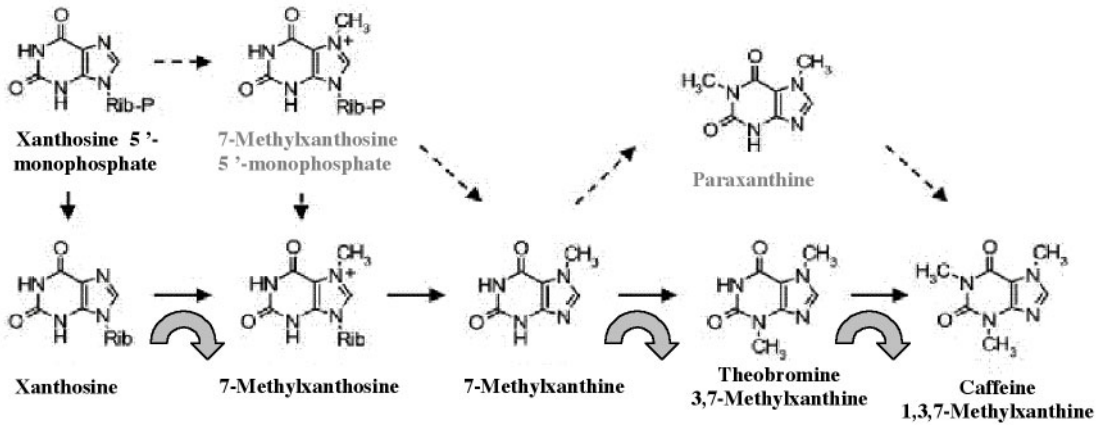


**Figure 1. Caffeine biosynthesis pathway (Uefuji et al., 2003). Grey arrows indicate the N-methylation steps of the majo.**

## RESULTS

Using gene specific primers, we showed that at least four different genes are present in *C. pseudozanguebariae* genome and are expressed in the fruit, whatever the ripening stage. Coding nucleotidic sequences of the cDNA didn't suggest any alteration of the deduced

amino acid sequence, which could explain the absence of caffeine in *C. pseudozanguebariae* beans.

The study of the expression showed the existence of a very interesting and unexpected phenomenon in *C. pseudozanguebariae*: the cloning of cDNAs with unexpected lengths lead to the observation that some transcripts of NMT genes are wrongly processed. (Figure 2, the first and/or second intron are totally or partially skipped, the third intron is never skipped).

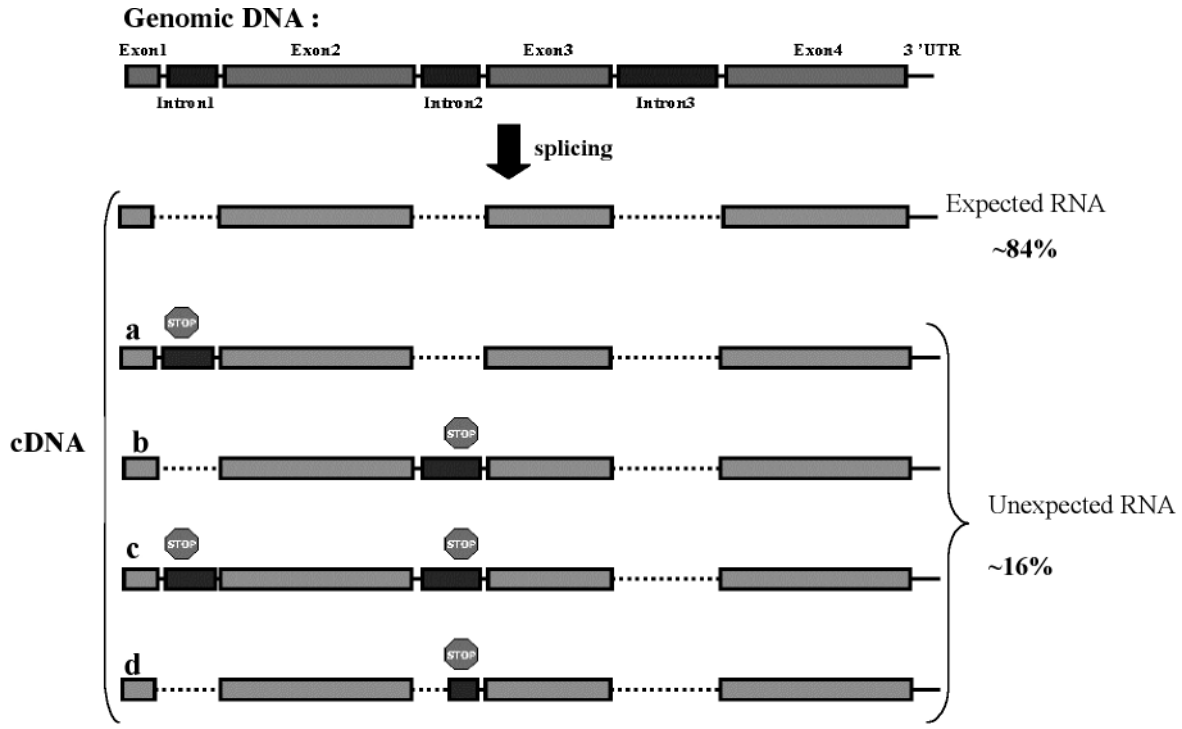


**Figure 2. Type of NMT transcripts in *C. pseudozanguebariae.* a: First intron skipped; b: second intron skipped; c: intron 1 and 2 skipped; d: partial excision of intron 2.**

Frequency of this phenomenon was estimated in the fruit of *C. pseudozanguebariae* and *C. canephora* at different ripening stage (Table 1) and indicated that a large population of primary transcripts is wrongly processed in *C. pseudozanguebariae* ($\geq$ 16%) even when this phenomenon occurs more rarely in *C. canephora* (< 4%).

All the RNAs containing a skipped intron encoded a truncated protein as stop codons were contained in the reading frame.

**Table 1. Percentage of skipped introns at different maturation stages.**

| Species | *C. pseudozanguebariae* | *C. canephora* |
|---|---|---|
| Stage 1 | 2/22 **(13,6%)** | 1/28 **(3,6%)** |
| Stage 2 | 4/23 **(17,4%)** | - |
| Stage 3 | 5/29 **(17,2%)** | - |
| Total | 12/74 **(16,2%)** | 1/28 **(3,6%)** |

## CONCLUSION

The absence of caffeine accumulation in *C. pseudozanguebariae* fruits doesn't depend on a missing gene or on a default of transcription of a gene involved in its biosynthesis nor on a mutation. The coding nucleotide sequences of NMT genes are highly conserved between *C. pseudozanguebariae* and *C. canephora* and the deduced proteins are even more similar (up to 100%).

The high frequency of skipped introns found at all development and maturation stages, and for all the NMT genes, in addition to the very diverse forms of resulting RNAs, makes believe that these RNAs do not arise from alternative splicing but from a deficient spliceosome. Furthermore, as the incompletely spliced RNAs generate truncated proteins these may interfere severely with the caffeine biosynthesis leading to its inactivation.

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# Genetic Differentiation between *Coffea liberica* var *liberica* and *C. liberica* var *dewevrei*. Comparison with *C. canephora*

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## SUMMARY

*C. liberica* var. *liberica* (LIB) and var. *Dewevrei* (DEW) belong to the complex *Pachycoffea* group (*Coffea*). Their taxonomical status often questioned, is still unelucidated. In this study, their diversity was characterised using morphological traits, molecular markers (AFLP) and reproductive isolation. There were differences in morphological traits, but not substantial enough to discriminate LIB and DEW. The clustering analysis of 93 AFLP markers clearly separated them into two distinct groups with high genetic differentiation ($G_{st}$ = 0.25). The pollen viability of DEW x LIB F1 hybrids (44.2%), similar to F1 hybrids between *C. canephora* (a well-defined species) and DEW or between *C. canephora* and LIB, showed marked reproductive isolation between. Molecular and F1 fertility data suggest revising the taxonomical status of LIB and DEW.

## RÉSUMÉ

*C. liberica* var. *liberica* (LIB) et var. *Dewevrei* (DEW) appartiennent au complexe des *Pachycoffea* (*Coffea*). Leur statut d'espèce ou de variété botanique a été mainte fois remis en cause. A ce jour, la question n'a toujours pas été élucidée. Dans cette étude, leurs diversités morphologique et moléculaire (marqueurs AFLP) ont été évaluées, de même que leur niveau d'isolement reproductif. Les caractères morphologiques ont montré des différences significatives, sans permettre une distinction nette. En revanche, une classification ascendante hiérarchisée, réalisée avec 93 marqueurs AFLP, a permis une séparation en deux groupes génétiques distincts, avec un niveau de différentiation élevé ($G_{st}$ = 0,25). La viabilité pollinique ($VP_{LIBDEW}$ = 44,2%) des hybrides LIBDEW est similaire à celle des hybrides F1 entre *C. canephora* (espèce bien définie) et DEW, ou entre *C. canephora* et LIB, montre un niveau élevé d'isolement reproductif. En somme, les données moléculaires et le niveau de stérilité des hybrides LIBDEW suggèrent de considérer LIB et DEW comme des espèces différentes.

## INTRODUCTION

The subgenus *Coffea* (*Coffea* genus) includes about a hundred species, endemic to Africa, the Mascarene Islands, Madagascar and Comoros (Charrier, 1978; Bridson and Verdcourt, 1988; Stoffelen, 1998). The *Eucoffea* section was split into five sub-sections, i.e. *Erythrocoffea, Pachycoffea, Nanacoffea, Melanocoffea* and *Mozambicoffea* (Chevalier, 1942). Presently, the only cultivated diploid species is *C. canephora* Pierre of *Erythrocoffea* subsection. Otherwise, only *C. liberica* Hiern of the *Pachycoffea* subsection is still cultivated to a minor extent, despite the fact that this species was of great economic importance during the 1930-50 periods. Currently, *C. liberica* gets interest in *C. canephora* breeding programmes for its clustered fruit maturation, high seed weight and low caffeine content. *C. liberica* was involved in diverse interspecific hybridisation programs (Louarn, 1992). Nonetheless, its taxonomic status is still complex (Portères, 1936; Lebrun, 1941; Chevalier, 1942; Bridson and

Verdcourt, 1988) and its genetic diversity is not known enough. Thus, our study was carried out in order to assess *C. liberica* genetic differentiation in comparison to that of the well-defined species *C. canephora.*

## PLANT MATERIAL AND METHODS

Plant material, maintained at the IRD Agricultural Station (Man, Côte d'Ivoire) consisted of *C. liberica* var liberica (LIB), *C. liberica* var dewevrei (DEW), *C. canephora* (CAN) and F1 hybrids: LIBCAN (= LIB X CAN), CANDEW (= CAN X DEW) et LIBDEW (=LIB X DEW).

Morphological notations were performed on leaves, flowers, fruits and seeds. Male fertility was evaluated throughout pollen viability and genetic differentiation was estimated using AFLP markers. Morphological data and pollen viability were analysed according to the nested ANOVA model and discriminant analysis. Clustering analysis was performed on molecular data.

## RESULT

Of the 19 evaluated morphological traits, 15 showed significant differences ($p < 0.001$) between LIB and DEW. In particular, DEW had larger leaves (leaf area index: 218.2 *vs.* 131.2 $cm^2$) and more inflorescences per node (7.9 vs. 5.4) than LIB. Conversely, LIB had thinner leaves (shape index: 2.4 *vs.* 2.2) and bigger fruits (fruit volume index: 4.0 *vs.* 2.9 $cm^3$) than DEW. Only five variables (number of flowers per inflorescence, leaves width, fruit width, number of flowers per node and leaves area) were kept as the most discriminate traits. Eighty height percent of the evaluated trees were well-classified but no discriminant factor allowed an accurate classification. Misclassified trees led to a partial overlap between LIB and DEW distributions (Figure 1). By contrast, the UPGMA cluster analysis gave a clear separation of LIB, DEW and CAN into three distinct genetic groups sharing only 40% similarity and there were no misclassified trees (Figure 2). Genetic distance between LIB and DEW ($d_{LD} = 0.62$), between CAN and DEW ($d_{CD} = 0.74$), and between LIB and CAN ($d_{LC} = 0.74$) showed that LIB and DEW were closer to each other than to CAN, but their differentiation was 50% higher than between the two CAN groups. Otherwise, Nei's $G_{st}$ value (= 0.25) showed great differentiation between LIB and DEW. F1 hybrid pollen viability between LIB and DEW was similar to that observed in LIB-CAN hybrids (45%) or DEW-CAN hybrids (54.6%) ($\chi^2 = 3.3$, $df = 6$, $p = 0.77$). These results suggest that genetic differentiation between LIB and DEW is higher than an intraspecific differentiation.

## CONCLUSION

This study on LIB-DEW differentiation – the first involving simultaneously morphological traits, molecular markers and hybrid fertility – clearly indicated that they are more differentiated than botanical varieties. This information could be useful in genetic resource conservation programs, and also in cultivated coffee improvement initiatives, by facilitating selection of parental genotypes. Similar studies should be extended to the whole *Pachycoffea* sub-section in order to assess genetic diversity and determine the taxonomy of this complex coffee group.

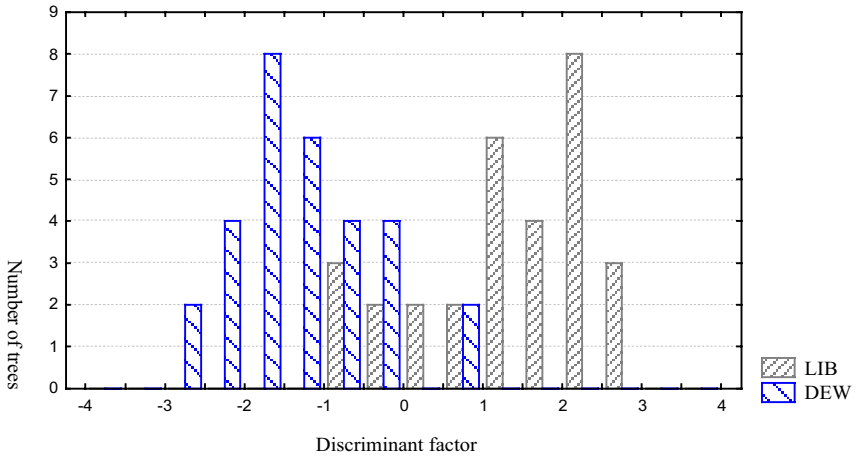


**Figure 1. Morphological differentiation between LIB and DEW (discriminant analysis based on 5 traits).**

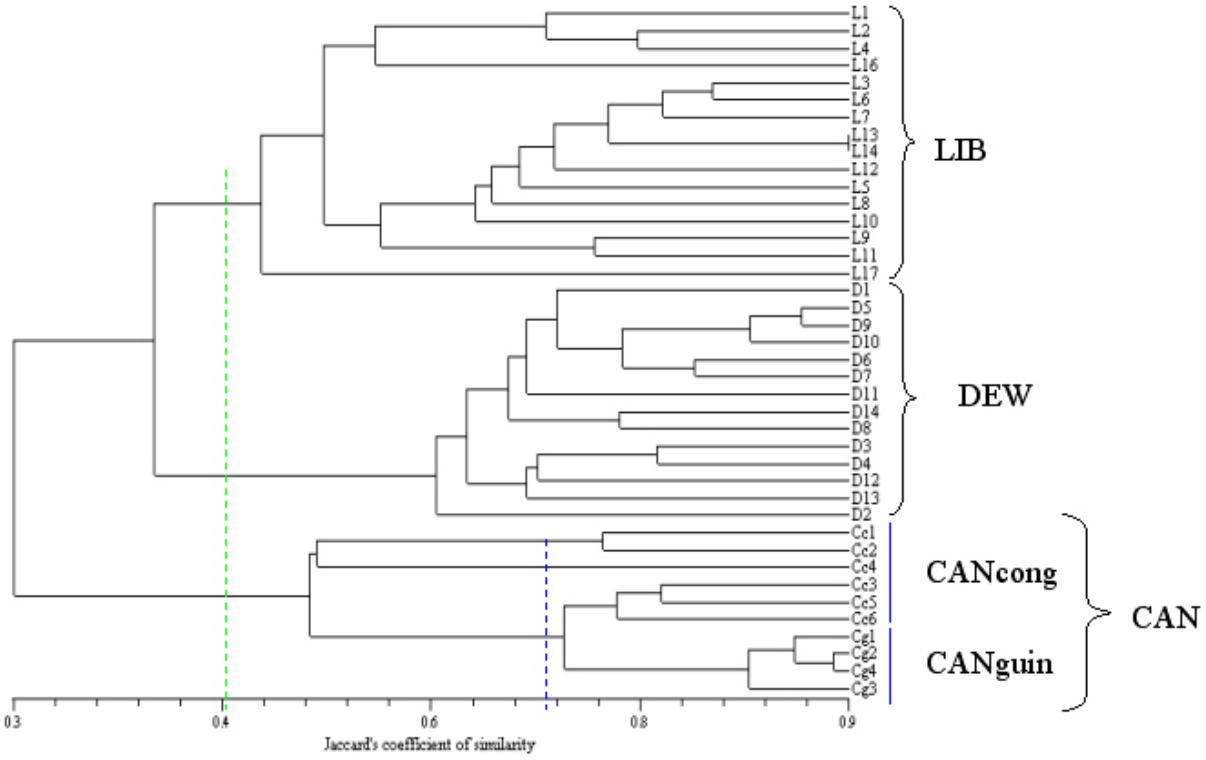


**Figure 2. Molecular differentiation between LIB, DEW and CAN (clustering analysis based on 93 AFLP markers).**

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# Coffee Tree Genome Size Variation: Its Relation with Retrotransposons Copy Number

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## SUMMARY

*Coffea* genus contains over 80 species and taxons among which only *C. arabica* is an allotetraploid. The genome size varies from 2C=1.03 pg to 2C = 1.76 pg with a constant chromosome number, 2n = 22. The genome size varies globally from East Africa, with the smallest genomes, towards Central and West Africa where the bigger genomes are found. *Coffea* species supposedly originated in East Africa. A hypothesis states that genome size differences resulted from an increase due to the presence of different types and copy numbers of repeated sequences among which retrotransposons. Gene duplication is also to consider but it may not be responsible for such a wide difference among species. Duplication of long chromosomal regions, instead of single genes, are also possible. Reduction of genome size cannot be discarded but it is a more complicated phenomena and with more dramatic consequences which can hardly be accounted for the present situation within *Coffea* genus.

Changes in genomic size were certainly accompanied by speciation as the success of interspecific crosses is correlated to the difference in the genome size of the parental species. We are presently analysing repeated sequences present in these genomes. We are estimating the copy numbers of different retrotransposons already identified. Paradoxically, one of the biggest genome, *C.heterocalyx* (2C = 1.73 pg) has apparently a low copy number of gypsy/Ty3 retrotransposon type, lower than *C. pseudozanguebariae*, which has a small genome (2C = 1,13 pg). Copia/Ty1 retrotransposon type seems to be more frequent than gypsy/Ty3 type in *Coffea* genomes. Nevertheless, increase of genomic sizes may depend not only on the copy number of retrotransposons but also on their genomic ubiquitous presence which might be at the origin of non reciprocal recombination due to the recognition of homologous sequences dispersed all over the genome.

## MATERIAL & METHODS

A library, enriched in repeated sequences, was established from total DNA isolated from F1 plants issued from a cross between *C. pseudozanguebariae* and *C. liberica* var *Dewevrei.* 184 clones from this library were sequences and blasted (n and x) against international data banks. A 350bp probe, homologous to gypsy-like reverse transcriptase and obtained through chromosome walking on *C. canephora* genomic DNA, was used as a radiolabeled probe for dot-blot analyses of genomes from 7 *Coffea* species covering the range of genome sizes: 0.9 to 1.8 pg/genome.

## BACKGROUND AND RESULTS

The genus *Coffea* originating from Africa and the Mascarene Islands, contains over 80 species and taxons. All, but one, species are diploid with 2n = 2x = 22. In order to take advantages of the genetic resources existing in the genus, inter-specific crosses have been realized involving wild species. Some combinations, mainly those involving East African X West African species, gave high levels of sterility in their offspring. This sterility is induced by the presence of univalent chromosomes during meiosis. Nuclear DNA content of diploid species varies from 0.9 pg to 1.8 pg. Sterility of interspecific hybrids increases accordingly to the genome size difference between parental species. As all the species have the same chromosome number, differences in DNA amount could be explained by variable abundance of repeated sequences. It is generally assumed that *Coffea* genus is originating from East Africa, it is interesting to note that genome size increases following the spread from East to West Africa (Figure 1). Active retrotransposons can lead to an increment of the genome size. We surveyed the presence of retrotransposons in *Coffea* genomes (Table 1) and estimated the copy number of gypsy-like elements in some species with different genomic DNA content (Table 2).


**Figure 1. Coffee center of origin and possible spread across the African continent.**

**Table 1. Blast results of 184 clones from a DNA library repeated sequences enriched.**

| | Transportable elements | | | Microsatellite sequences | No homology | Cytoplasmic DNA | Gene |
|---|---|---|---|---|---|---|---|
| | Gypsy | Other | Copia | | | | |
| **Number of clones** | 5 | 18 | 12 | 8 | 102 | 10 | 29 |
| **%** | 2,7 | 9,8 | 6,5 | 4,3 | 55,4 | 5,4 | 15,8 |
| **% Genomic repeated sequences** | 78,8 | | | | | | |

**Table 2. Estimation of gypsy-like element copy number.**

| Species | 2C value (pg) | Gypsy copy number |
|---|---|---|
| *C. arabica* | 2.61 | 1051 |
| *C. heterocalyx* | 1.74 | 47 |
| *C. canephora* | 1.43 | 128 |
| *C.* sp *moloundou* | 1.43 | 439 |
| *C.liberica* var**.** *Dewevrei* | 1.41 | 363 |
| *C. eugenioides* | 1.36 | 713 |
| *C. pseudozanguebariae* | 1.13 | 66 |

## CONCLUSION AND PERSPECTIVE

Coffee genome appears to be rich in repeated sequences. On a small DNA library sample, different types of such sequences were identified including several kinds of transposons and retrotransposons. Ongoing studies search to isolate a full-length gypsy-like element from *C. canephora* genome. An estimation of such element copy number in different *Coffea* species seems to indicate that there is no relation between genome size and gypsy-like element copy number. *C. heterocalyx* with a big genome size has a small number of gypsy-like copies and two species with identical nuclear DNA content, *C. canephora* and *C*. sp moloundou have very different copy numbers. Other retrotransposons were also identified. Copia-like elements appeared to be more numerous, a similar analysis of copy number estimation has started.

# AFLP and SSR Polymorphism in a *Coffea* Interspecific Backcross Progeny ((*C. heterocalyx* x *C. canephora*) x *C. canephora*)

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## SUMMARY

An interspecific cross (BC 1) involving a species with one of the biggest genomes in Coffea genus (*C. heterocalyx* (HET), qDNA = 1.74 pg) and a species with a medium genome size (*C. canephora* (CAN), qDNA = 1.43 pg) was studied using two types of molecular markers, AFLPTM and SSR. One hundred and eighty eight AFLP bands and 34 SSR primer pairs were suitable for the mapping. The total map length was 1360 cM with 190 loci distributed on 15 linkage groups.

The results were compared to the results obtained previously on an interspecific BC 1 progeny involving a species with a medium size genome (*C. liberica* var. *dewevrei*, DEW) and a species with one of the smallest size genome (*C. pseudozanguebariae*, PSE). They are discussed considering three main points: 1) the interest of the different markers type, 2) – the genomic distribution of AFLPs and SSR markers and 3) – the relation between AFLP polymorphism and the genome size.

## INTRODUCTION

Within *Coffea* sub-genus, all species but one (*C. arabica*) are diploid (x = 11) and the 2C nuclear DNA content (qDNA) ranges from about 1 to 1.8 pg. Moreover F1 interspecific hybrid fertility increases as the qDNA difference decreases. The genome size difference between *C. pseudozanguebariae* (PSE, 1.13 pg) and *C. liberica* var dewevrei (DEW, 1.43 pg) concerns all chromosomes. The genome size of their BC 1 hybrids increased as the chromosome number from DEW increased. Species-specific AFLPs mapped using this interspecific BC1 progeny were either clustered or not and distributed throughout the genome.
In this work we study an interspecific cross involving *C. heterocalyx* (HET, 1.74 pg) and *C. canephora* (CAN, 1.43 pg). AFLP and SSR bands were characterised according to polymorphism observed within-species and between-species.

## MATERIAL & METHODS

The BC1 progeny (72 plants) was derived from [CAN x HET] x CAN.

Genomic DNA isolation, AFLP and SSR protocols, data analyses are described in Coulibaly et al. (in press).

For both SSRs and AFLPs, HET bands were scored 1 for presence and 0 for absence.

**Table 1. AFLP primer combinations analysed and type and number of amplified bands produced per species.**

| Primers | Total CAN bands | Species-specific CAN/HET bands | Intra-CAN polymorphic bands (%) | Total HET bands | Specific HET/CAN bands |
|---|---|---|---|---|---|
| E1/M1 | 108 | 17 | 70 (64.8) | 65 | 14 |
| E1/M3 | 78 | 9 | 55(70.5) | 50 | 19 |
| E1/M4 | 84 | 9 | 53 (63.1) | 67 | 18 |
| E1/M5 | 85 | 14 | 44 (51.8) | 53 | 15 |
| E2/M1 | 141 | 12 | 87 (61.7) | 102 | 21 |
| E2/M2 | 103 | 16 | 61 (59.2) | 77 | 25 |
| E2/M3 | 95 | 9 | 63 (66.3) | 66 | 22 |
| E2/M7 | 84 | 10 | 57 (67.8) | 50 | 21 |
| E2/M8 | 130 | 20 | 78 (60) | 86 | 11 |
| E3/M1 | 107 | 12 | 70 (65.4) | 72 | 13 |
| E4/M8 | 90 | 10 | 65 (72.2) | 44 | 14 |
| E6/M1 | 75 | 9 | 49 (65.3) | 48 | 13 |
| total | 1180 | 147 (12.5) | 752 (63.7) | 780 | 206 (26.5) |

*X/Y: species comparison —X relative to Y. E1: AAC, E2: AAG, E3: ACA, E4: ACC, E6: ACT, M1: CAA, M2: CAC, M3: CAG, M4: CAT, M5: CTA, M7: CTG, M8: CTT.*

**Table 2. Characterisation of AFLP bands obtained in different genome comparisons. The total numbers of bands were calculated from the sole HET genotype and 10 genotypes for CAN, PSE and DEW.**

| | total monomorphic bands (%) | species-specific bands (%) | Within-species polymorphic bands (%) |
|---|---|---|---|
| CAN/HET | 428/1180 (36.3) | 147/1180 (12.5) | 752/1180 (63.7) |
| HET/CAN[a] | | *188/780 (24.1)* | |
| PSE/DEW[b] | 463/1259 (36.8) | 112/1259 (8.9) | 796/1259 (63.2) |
| DEW/PSE[b] | 506/1296 (39) | 123/1296 (9.5) | 790/1296 (60.9) |

*[a]: bands present in HET and absent in the 10 CAN genotypes are both species-specific and genotype-specific HET bands.*
*[b]: Unpublished data from Ky et al. (2000), calculated from the 12 primer combinations giving the highest species-specific PSE/DEW bands.*

**RESULTS**

87.4 and 80.5% of AFLPs and SSRs were mapped and distributed throughout the 15 linkage groups. The A/T content of +3/+3 selective nucleotides was neither correlated with the total number of bands nor with the number of polymorphic bands within-CAN species. When comparing species with the same mating system but different genome sizes, the total number

of bands was similar. The difference observed between CAN self-incompatible and HET self-fertile could more likely be explained by the heterozygosity level.

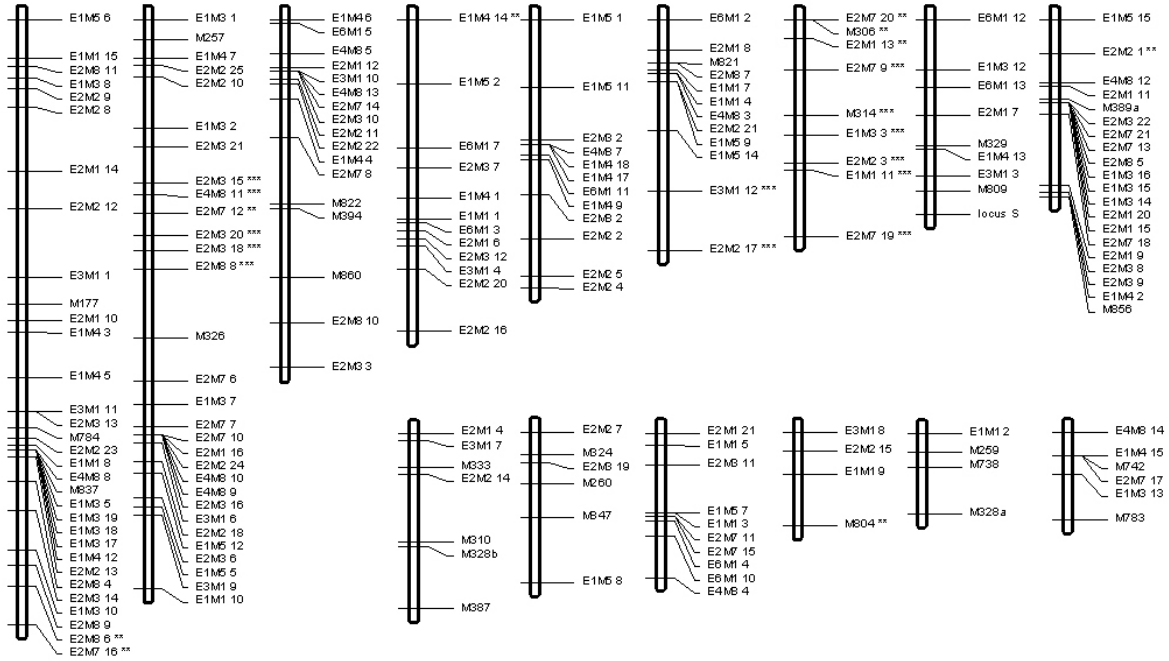


**Figure 1.**

## CONCLUSION

The AFLP polymorphism we observed herein was not correlated with the proportion of repeated sequences or with their AT richness.

Species-specific bands seemed to be similar in number irrespective of the genome sizes considered and their phylogenetic relation. Our results suggest two independent mechanisms involved: genomic differentiation affecting all species simultaneously, and directional DNA expansion/contraction in genomes over an east-to-west geographical distribution gradient.

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# Ethylene Receptors in *Coffea* and their Relationship with Fruit Ripening

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## SUMMARY

In the genus *Coffea*, fruit ripening time varies greatly between species. In *Coffea pseudozanguebariae* (PSE) the time period between anthesis and mature fruit is 9 weeks, while in others, it takes 36-38 weeks. Caffeine content in PSE fruits is very low, almost non measurable. In other species, like in *C. canephora* (CAN), it goes up to 2.8% dry matter basis (dmb). In order to study the relationship between ethylene perception, fruit ripening time and caffeine content in some species of the genus *Coffea*, ethylene receptors encoding genes have been analyzed. Two full-length cDNA were cloned (CoETR1 and CoEIN4) from CAN fruit and leaves cDNA libraries. The CoETR1 cDNA is 2,649 nucleotides long and it contains an open reading frame of 2,223 bp translated into 740 amino acids. The corresponding *CoETR1* gene has five introns, one of them shows an interspecific polymorphism. CoEIN4 cDNA is 2,906 bp in length. It contains a putative open reading frame of 2,297 bp translated into 765 amino acids. The expression of these two genes has been evaluated during fruit development in CAN and in PSE.

## RESUMEN

En el género Coffea, el período de maduración del fruto varía entre las diferentes especies. En *C. pseudozanguebariae* el tiempo entre la antesis y la maduración del fruto es de 9 semanas. En esta especie, el contenido de cafeina es muy bajo; mientras que en otras; *C. canephora*, por ejemplo este valor es de 3,3% en base a materia seca. Con el objetivo de estudiar la relación entre etileno, tiempo de maduración del fruto y contenido de cafeína en el genero *Coffea*; se han analizado varios genes receptores de etileno. Dos de ellos, de talla completa (CoETR1 y CoEIN4) han sido aislados de un banco de ADNc de fruto y hoja de *C. canephora*, respectivamente. El ADNc para ETR1 tiene 2649 nucleótidos, con una región codante de 2223 pb que trasladan en 740 aminoácidos. El correspondiente gen CoETR1 posee cinco intrones; uno de ellos, muestra polimorfismo entre varias especies. El ADNc de CoEIN4 posee 2906 pb, con una región codante de 2297 nucleótidos, codificando para 765 aminoácidos. La expresión de esos dos genes ha sido evaluada durante el desarrollo del fruto en las especies *C. canephora* y *C. pseudozanguebariae.*

## INTRODUCTION

The phytohormone ethylene plays a central role in physiological and developmental processes, such as germination, growth, flower initiation, senescence of leaves and flowers, organ abscission and fruit ripening (Abeles, 1992). It is also a major signal, mediating responses to a range of both biotic and abiotic stresses. At the level of gene expression,

ethylene has been shown to induce transcription of a wide range of genes involved in wound signalling (O'Donnell, 1996) and defence against pathogens (Ecker, 1987).

A family of five receptors mediates ethylene perception in Arabidopsis: ETR1, ERS1, ETR2, ERS2, and EIN4 (review in Wang et al., 2002). The five genes belong to two sub families: the first consists of ETR1 and ERS1. Their corresponding proteins have three hydrophobic transmembrane domains and a conserved hystidine kinase domain, whereas the second sub family of ETR2, EIN4 and ERS2 have four hydrophobic transmembrane domains and a degenerated hystidine kinase domain. The actual binding to ethylene by the receptor is mediated by a copper cofactor. Mutants that no longer bind copper are unable to binding ethylene (Rodriguez et al., 1999).

Species in the *Coffea* genus, show great differences in fruit ripening time. In *C. pseudozanguebariae* (PSE), a wild East African species, the fruit are ripe 9 weeks after anthesis, while in other species, this time is much longer, generally about 35-37 weeks. The caffeine content in PSE is very low too, it hardly can be measured, while in the two major cultivated species, *C. arabica* (ARA) and *C. canephora* (CAN) it varies from 1.2% to 2.8% (dmb) respectively.

Co-location of an ethylene receptor gene and a QTL related to the fruit quality has been established previously for melon (Périn et al., 2002). The location of a major gene related to fructification time was mapped on an interspecific genetic map (Akaffou et al., 2003) and this gene had an effect on the caffeine content. In order to study the possible relation between this two characters, fructification time and caffeine content, we characterised several ethylene receptor cDNAs and started that of their corresponding genes. The expression pattern of these genes was also investigated during fruit development in CAN and PSE.

## MATERIAL AND METHODS

### Plant material

The study of ethylene receptor genes was carried out on CAN, PSE and *C. liberica* var. *Dewevrei* (DEW). The trees were grown in a tropical green house at IRD in Montpellier, France. Leaf samples were recollected, frozen in liquid nitrogen and the genomic DNA immediately extracted.

### cDNA libraries construction

Two CAN cDNA libraries were constructed. One from a pool of immature and matured fruits and the other one from young leaves.

### Preparation of ethylene receptor gene-specific probes

Specific probes were generated by PCR amplification on CAN genomic DNA using degenerated primers designed after aligning corresponding sequences available in international databases. cDNA libraries were then screened using these probes.

### Amplification of full-length genes

Specific primers were designed for PCR amplification of the *CoETR1* gene in three species that differ in fruit ripening time (DEW, CAN and PSE).

## RT-PCR analyses

Total RNA was isolated at three and four different fruit ripening stages from CAN and PSE respectively, (from small green to red ripe). cDNAs were amplified by RT-PCR using specific primers for each gene. Amplification products were verified by sequencing or by their restriction pattern.

## RESULTS

### Characterisation of CoETR1

Five full-length cDNAs corresponding to the *CoETR1* gene were isolated from CAN fruit cDNA library. They were 2,649 bp; 2,683 bp and 3,162 bp in length. They contained a putative open reading frame of 2,223 bp translated *in silico* into 740 amino acids. The 5'-UTR was 114 bp long, but their 3'-UTR varied in length (313 bp, 347 bp and 826 bp).

The CoETR1 predicted polypeptide had a molecular weight of 82.48 kDa. The amino acid sequence of CoETR1 was 87.4% similar to the ETR1 gene of *Petunia x hybrida* (accession # AF145972). Its transmembrane domain was highly conserved. It had a similarity of 97.4%, with *Prunus persica* hydrophobic domain (Basset et al., 1999). The two cysteine residues (Cys-4 and Cys-6), that are responsible for an intermolecular disulphite-linked dimer formation (Schaller et al., 1995) were present in the CoETR1 AA sequence. Within the Histidine Kinase motif it had a conserved Histidine that serves as a presumptive site for autophosphorylation (His-352).

Within the genomic sequence obtained, we identified five introns. The position and the size of each of them is shown in Figure 1. The fifth intron had different size in the three studied species. This difference in size is basically due to the presence of indels as shown in Figure 2.

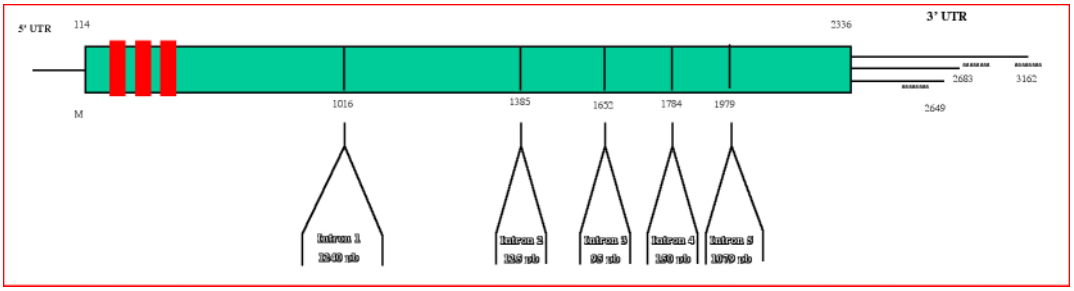


**Figure 1. C. canephora ETR1 gene. It shows the three trans membrain domains, five intron and three 3'UTR segment in three different cDNA clones.**

### Characterisation of CoEIN4

CoEIN4 cDNA has 2,906 bp. It contained a putative open reading frame of 2,297 bp translated into 765 amino acids polypetide whose N-terminal hydrophobic domain had a fourth membrane-spanning domain. This polypeptide had a deduced molecular weight of 85.63 kDa. The CoEIN4 amino acid sequence was 74.4% similar to that of LeETR5 of *L. esculentum* (Tieman and Klee, 1999), but it is only 35.3% similar to CoETR1. Similarly to LeETR5, ETR2 and ERS2 polypeptides, the putative autophosphorylated Histidine residue in the Histidine Kinase domain is not present in CoEIN4.

## Gene expression

Both genes: ***CoETR1*** and ***CoEIN4*** are identically expressed during fruit ripening in CAN and in PSE. This expression pattern indicates that possibly ethylene receptors genes would not play a major role in the determination of the fruit ripening time difference between these two species. However, possible post transcriptional or post traductional regulations may intervene and should be investigated. Alternative splicing of the primary transcript and putative alternative polyadenilation sites existing in the sequence of *CoETR1* last intron will be analysed and the relationship of their occurrence with differences in fructification processes will be analysed. Furthermore, quantitative studies of gene expression must be carried out, as a dosage effect might be more important than a qualitative difference.

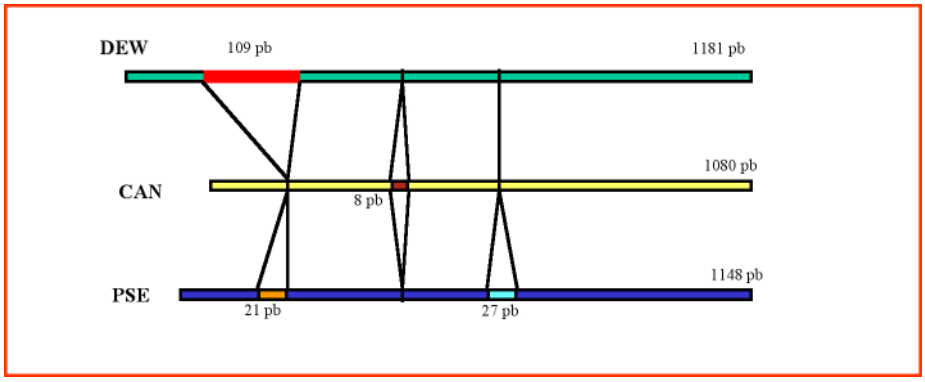


**Figure 2. Organisation of *CoETR1* gene 5th intron of in different *Coffea* species.**

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# Potential Use of D-Xylose for Coffee Plant Transformation

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## SUMMARY

An alternative approach involving xylose isomerase gene (*xylA*) as a positive selection marker system is being developed for coffee plant transformation. We report here that High Frequency Somatic Embryogenic calli derived from leaf tissue belonging to four different species of *Coffea* namely *C. arabica, C. canephora, C. heterocalyx* and *C. moloundou* cannot use D-xylose- a complex sugar as the sole carbon source ranging from 2 g/l to 32 g/l tried. Study conducted on mannose shown that it cannot be used as selective agent since coffee HFSE calli were able to use mannose as carbon source. The presence of xylose could not allow HFSE calli growth in all the four species of *Coffea.* Histological analysis by localizing protein, lipid and starch discriminated normal metabolically active positive control HFSE calli from xylose treated. However, plant cells were known to use the keto-isomer of xylose, D-xylulose (single chain molecule) as a carbon source. The significance of the present findings would enable to exploit the use of the gene encoding D-xylose ketol-isomerase, EC 5.3.1.5 as selectable marker gene that would interconvert D-xylose to D-xylulose. Xylose based positive selection would give us a biosafe method and eliminate undesired antibiotic or herbicide selection in production of transgenic plants of coffee.

## INTRODUCTION

The use of selective agents is necessary to increase the efficiency of transgenic plant recovery. Frequently herbicide or antibiotic resistance genes have been used as selective markers. Normal plant cell cannot utilize complex sugars such as xylose, mannose as carbon source. But in the presence of the gene that could breakdown these substrates into simple sugars, plants can utilize these complex sugar.

D-xylose is a hexose sugar like mannose, that frequently occur as a major residue in glycoproteins of the living system and that acts as a key component for most intermediary metabolism. These two sugars were used in our studies. In conventional antibiotic or herbicide resistance selection system the transgenic cells are stressed to survive by producing the protein conferring resistance to the selective agents while the non-transgenic cells are killed. Whereas, in xylose or mannose selection method the presence of the gene encoding xylose isomerase (*xylA*) or Phosphomannose isomerase (*manA/pmi*) in transformed cells can catalyze substrate xylose or mannose by reversible isomerization into simple sugars as xylulose or fructose-6-phosphate respectively that stimulates the growth and regeneration of transgenic cells while the non-transgenic cells are starved and not killed. Therefore, this method of selection is termed as "positive selection" (Joersbo and Okkels, 1996; Haldrup et al. 1998; Penna et al., 2002).

Haldrup et. al. (1998) using the gene *xyl*A isolated from *Thermoanaerobacterium thermosulfurogens* and *Streptomyces rubiginosus* had shown transformation frequency to be more efficient than kanamycin selection. Similarly, Phosphomannose isomerase (PMI) positive selection has been demonstrated in number of plant systems such as cassava (Zhang

et al., 2000), maize (Negrotto et al., 2000; Wang et al., 2000), rice (Lucca and Potrykus, 2001; Datta et al., 2003), sugarbeet (Joersbo et al., 1998) and wheat (Wright et al., 2001) using the gene *pmi*. Xylose-based selection had been so far demonstrated in potato, tobacco and tomato (Haldrup et al. 1998) but not on woody arborescent and plantation crops. Here we report our findings on the non-utilization of selective agent D-xylose by the untransformed HFSE cells from four *Coffea* species and their ability to use mannose. D-Xylose potentiality to be used as selective agent for positive selection in the transformation of Coffee trees is discussed.

## MATERIALS AND METHODS

Somatic embryogenic calli used in the investigation were derived from leaf explants. The genotype includes the two cultivated species namely *C. arabica; C. canephora* and two wild spcecies are *C. heterocalyx* and *C. moloundou*. The selected and washed healthy young leaves were disinfested by immersion in 70% alcohol for 1 min. then in 12° active Sodium hypochlorite (Javel Oxena – SH Pieri Chime) with 4% (w/v) ascorbic acid and 1% (v/v) Tween-20 as surfactant for 15 min. and washed thoroughly (3-5 rinses) with sterile water. The disinfested leaves were cut into segments of 1 $cm^2$ disc and five leaf segments were placed on 30ml of primary calli induction medium contained in 90 mm pre-sterilized petri dishes. The primary calli induction medium was composed of $NH_4NO_3$ 250 mg/l, $CaNO_3$ 300 mg/l, $KNO_3$ 400 mg/l as macronutrients, half strength of Murashige and Skoog (1962) (MS) micronutrients, thiamine-Hcl 15 mg/l, nicotinic acid 1 mg/l, pyridoxine-Hcl 1mg/l, FeEDTA half MS and supplemented with 30 g/l sucrose, 2, 4-D (Sigma) 0.5 mg/l and 2iP (Sigma) 1 mg/l in combination. The leaves were incubated in dark 26°C ± 1 on primary calli induction medium for 30 days and subsequently they were axenically transferred to HFSE induction medium 'E' of Van Boxtel and Berthouly (1996), maintained in 16h photoperiod cycle (cool diffused white fluorescent light) 10 μmol $m^{-2}s^{-1}$ and 20°C ± 1. After 30 days incubation in the 'E' medium, fresh yellow HFSE calli developed and proliferated on brown-turned primary calli.

About 50 mg of untransformed HFSE calli derived from the second medium ('E') were used to determine its ability to use D-xylose or mannose as carbon sources. The concentration of D-xylose and mannose ranging from 2, 4, 8, 12, 16 and 32 gm/l was employed. A positive control with 30 gm/l of sucrose and negative control without any carbon source was included in the study as reference. Fresh weights were used to measure the response of HFSE to various treatments. Each treatment had ten replicates. Fresh weight of all the treatment was measured in an interval of two weeks for six weeks. Arithmetic Mean, standard deviation and standard error were used throughout the experiments.

## RESULTS

The untransformed HFSE calli obtained from the leaf of four species of *Coffea* namely *C. arabica, C. canephora, C. heterocalyx* and *C. moloundou* were subjected to 2 to 32 g/l of D-Xylose and Mannose. The response of HFSE calli from these species to D-xylose and mannose are diverse and are shown Table 1 & 2.

### Response of HFSE calli from Coffee to D-xylose

All four species of *Coffea* showed inability to use D-xylose when supplemented as a sole carbohydrate source in the medium. HFSE calli placed on these mediums slowly turned beige and stopped proliferation. Therefore, there was no increase in their fresh weight and the cells did not show any necrosis or blackening of calli even after six weeks of culture on the same

medium. Nevertheless, concentrations used in these experiments were not lethal to the coffee tissue.

**Table 1 Mean fresh weight of xylose-starved HFSE callus of four *Coffea* species measured in the interval of 2 week for 6 weeks. Positive control ( with sucrose 30g/l) and negative control ( without any carbon source).**

| Xylose (gm/l) | Week interval | C. arabica **Fresh wg ± SD** | C. canephora **fresh wg. ± SD** | C. heterocalyx **fresh wg. ± SD** | C. mouloundou **fresh wg. ± SD** |
|---|---|---|---|---|---|
| **Control positive** | 0 | 50.8 ± 1.3 | 38 ± 2.0 | 53.1 ± 2.2 | 52 ± 1.6 |
| | II | 61.2 ± 1.3 | 131.3 ± 3.9 | 64 ± 3.3 | 96.6 ± 13.0 |
| | IV | 90.2 ± 1.5 | 213 ± 4.5 | 105.6 ± 6.0 | 178.8 ± 60.8 |
| | VI | 181 ± 2.5 | 336 ± 16.3 | 148.5 ± 12.8 | 248.4 ± 41.3 |
| **Control negative** | 0 | 52.4 ± 1.8 | 37 ± 2.5 | 53.9 ± 2.9 | 52 ± 2.0 |
| | II | 54.8 ± 1.3 | 35.2 ± 2.6 | 53.2 ± 3.7 | 51 ± 1.9 |
| | IV | 54.8 ± 1.3 | 34.6 ± 2.4 | 52.9 ±4.0 | 50.8 ± 1.6 |
| | VI | 54.4 ± 1.5 | 34.4 ± 2.4 | 52.4 ± 3.9 | 50.6 ± 1.8 |
| **2** | 0 | 51.4 ± 1.3 | 49 ± 0.1 | 52 ± 1.4 | 50.6 ± 0.9 |
| | II | 51.4 ± 1.3 | 49 ± 0.1 | 52.2 ± 1.5 | 57.6 ± 4.8 |
| | IV | 50 ± 1.0 | 47 ± 0.1 | 52.2 ± 1.5 | 55.4 ± 5.7 |
| | VI | 49.4 ± 0.5 | 47 ± 0.1 | 52 ± 1.4 | 55 ± 5.4 |
| **4** | 0 | 50 ± 0.0 | 39 ± 0.4 | 52 ± 2.0 | 50.4 ± 0.9 |
| | II | 49.4 ± 0.5 | 38 ± 0.3 | 50.8 ± 1.6 | 56.4 ± 0.5 |
| | IV | 49 ± 0.7 | 37 ± 0.4 | 50.6 ± 1.3 | 55.4 ± 0.9 |
| | VI | 48.8 ± 0.4 | 37 ± 0.5 | 50.4 ± 1.5 | 55.2 ± 0.8 |
| **8** | 0 | 51.2 ± 1.3 | 37 ± 0.5 | 52.2 ± 2.3 | 51.4 ± 1.7 |
| | II | 50.6 ± 0.8 | 35 ± 0.5 | 52.4 ± 2.3 | 58.8 ± 1.9 |
| | IV | 50.2 ± 0.8 | 35 ± 0.5 | 52.2 ± 2.3 | 58 ± 2.3 |
| | VI | 49.8 ± 0.8 | 34 ± 0.5 | 52 ± 1.9 | 57.6 ± 1.5 |
| **12** | 0 | 50.8 ± 1.3 | 39 ± 0.2 | 53 ± 1.6 | 51.8 ± 1.8 |
| | II | 48.8 ± 1.3 | 37 ± 0.3 | 53.2 ± 1.5 | 57 ± 2.0 |
| | IV | 48.6 ± 0.9 | 37 ± 0.3 | 53 ± 1.6 | 56.2 ± 3.0 |
| | VI | 48.4 ± 0.5 | 34 ± 0.3 | 53 ± 1.6 | 55.4 ± 3.0 |
| **16** | 0 | 50 ± 0.0 | 40 ± 0.8 | 52.2 ± 1.3 | 50.6 ± 0.5 |
| | II | 49 ± 1.0 | 39 ± 0.8 | 52.2 ± 1.3 | 55.2 ± 1.0 |
| | IV | 48.2 ± 1.3 | 38 ± 0.7 | 52 ± 1.2 | 54.2 ± 2.0 |
| | VI | 47.6 ± 0.9 | 35 ± 0.5 | 51.6 ± 1.1 | 53.8 ± 1.9 |
| **32** | 0 | 50.4 ± 0.5 | 35 ± 0.4 | 53.6 ± 1.5 | 50.8 ± 1.0 |
| | II | 45.6 ± 8.9 | 34 ± 0.3 | 53 ± 1.4 | 54 ± 1.4 |
| | IV | 44.8 ± 8.4 | 33 ± 0.2 | 53 ± 1.4 | 52.6 ± 0.9 |
| | VI | 43.6 ± 7.7 | 24 ± 0.7 | 52.6 ± 1.1 | 52.4 ± 1.1 |

*@ Values represent the means ± the standard deviation.*

**Response of HFSE calli to mannose**

Whereas in mannose, its presence in the induction medium substantially effected the HFSE calli response. It had promoted the proliferation of HFSE calli in all the four species of *Coffea.* A linear relationship between the concentration range (0.2% to 1.6%) of mannose in

the medium and the fresh weight of HFSE calli at the growth phase was observed. The maximum fresh weight growth of HFSE calli was observed at 16 g/l in *C.arabica* and *C. canaphora*; 12 g/l in *C. heterocalyx* and 8g/l in *C. moloundou* from six-week culture. The percentage of HFSE calli fresh weight over the positive control was 419%; 20%; 97% and 15% respectively. By comparison *C. moloundou* had shown less sensitive to mannose in relation to calli proliferation. It is interesting to note from our study that HFSE calli of *Coffea* species can use mannose as a sole carbon source even better than sucrose in the medium.

**Table 2. Mean fresh weight of mannose-grown HFSE callus of four *Coffea* species measured in the interval of 2 week for 6 weeks. Positive control ( with sucrose 30g/l) and negative control ( without any carbon source).**

| **Mannose (gm/l)** | **Week interval** | C. arabica **fresh wg ± SD** | C. canephora **fresh wg ± SD** | C. heterocalyx **fresh wg ± SD** | C. mouloundou **fresh wg ± SD** |
|---|---|---|---|---|---|
| **Control positive** | 0 | 50.8 ± 1.3 | 38 ± 2.0 | 53.1 ± 2.2 | 52 ± 1.6 |
| | II | 61.2 ± 1.3 | 131.3 ± 3.9 | 64 ± 3.3 | 96.6 ± 13.0 |
| | IV | 90.2 ± 1.5 | 213 ± 4.5 | 105.6 ± 6.0 | 178.8 ± 60.8 |
| | VI | 181 ± 2.5 | 336 ± 16.3 | 148.5 ± 12.8 | 248.4 ± 41.3 |
| **Control negative** | 0 | 52.4 ± 1.8 | 37 ± 2.5 | 53.9 ± 3.0 | 52 ± 2.0 |
| | II | 54.8 ± 1.3 | 35.2 ± 2.6 | 53.2 ± 3.7 | 51 ± 1.8 |
| | IV | 54.8 ± 1.3 | 34.6 ± 2.4 | 52.9 ± 4.0 | 50.8 ± 1.6 |
| | VI | 54.4 ± 1.5 | 34.4 ± 2.4 | 52.4 ± 3.9 | 50.6 ± 1.8 |
| **2** | 0 | 51 ± 0.7 | 51 ± 1.4 | 51.8 ± 2.4 | 54.4 ± 0.9 |
| | II | 59.4 ± 1.7 | 67.8 ± 7.8 | 68.7 ± 8.3 | 61 ± 1.9 |
| | IV | 82.6 ± 11.0 | 103 ± 7.6 | 96.4 ± 12.5 | 83.2 ± 3.6 |
| | VI | 110.4 ± 4.8 | 169.8 ± 15.0 | 151.2 ± 27.3 | 127.6 ± 4.9 |
| **4** | 0 | 50.8 ± 1.3 | 52 ± 2.5 | 51.9 ± 1.9 | 53.2 ± 1.0 |
| | II | 60.8 ± 2.6 | 73 ± 4.0 | 70.5 ± 3.8 | 62.4 ± 1.9 |
| | IV | 83.4 ± 3.3 | 131 ± 5.0 | 129.5 ± 6.13 | 83.8 ± 2.7 |
| | VI | 163.2 ± 3.6 | 234.2 ± 8.1 | 227.4 ± 15.3 | 158.2 ± 1.9 |
| **8** | 0 | 50.8 ± 0.8 | 50 ± 0.0 | 50.2 ± 4.5 | 53.4 ± 0.8 |
| | II | 64 ± 3.7 | 74.8 ± 1.6 | 73.8 ± 1.9 | 71.4 ± 2.5 |
| | IV | 108.8 ± 8.0 | 144.2 ± 0.8 | 141.6 ± 1.9 | 101 ± 3.2 |
| | VI | 182.6 ± 4.2 | 286.4 ± 2.0 | 282 ± 3.7 | 205.4 ± 9.5 |
| **12** | 0 | 50.8 ± 1.3 | 49.8 ± 0.8 | 49.6 ± 8.9 | 53 ± 1.6 |
| | II | 154.2 ± 24.0 | 82.4 ± 2.4 | 70.8 ± 4.2 | 61.6 ± 1.1 |
| | IV | 362.4 ± 72.0 | 165 ± 2.4 | 146.8 ± 5.2 | 96.4 ± 9.8 |
| | VI | 585 ± 27.5 | 321.6 ± 3.9 | 293.2 ± 10.2 | 180.2 ± 7.3 |
| **16** | 0 | 50.2 ± 0.4 | 52.2 ± 2.3 | 52.2 ± 2.7 | 51.8 ± 1.3 |
| | II | 232.6 ± 41.8 | 88.4 ± 2.5 | 70.6 ± 3.4 | 62.6 ± 2.2 |
| | IV | 734.4 ± 126.2 | 184.8 ± 4.3 | 133.2 ± 6.4 | 89.2 ± 2.1 |
| | VI | 938.8 ± 136.5 | 403.8 ± 4.7 | 252.2 ± 9.4 | 153.2 ± 2.7 |
| **32** | 0 | 50.6 ± 0.9 | 51.8 ± 1.3 | 52.2 ± 1.3 | 51.6 ± 1.5 |
| | II | 133.8 ± 3.6 | 89.2 ± 1.9 | 61 ± 1.6 | 71 ± 7.0 |
| | IV | 290.6 ± 9.9 | 208.4 ± 4.6 | 85.2 ± 2.2 | 99.8 ± 3.4 |
| | VI | 434.8 ± 5.9 | 382.2 ± 4.2 | 110.4 ± 3.7 | 104.4 ± 7.0 |

*@ Values represent the means ± the standard deviation.*

## DISCUSSION

We have evaluated the two selective agents D-xylose and mannose using principles based on carbon metabolism in plant cells. In our studies we have demonstrated that HFSE calli one of the target tissue for genetic transformation of coffee – cannot utilize complex sugars D-xylose as sole carbon source. Whereas, HFSE calli can utilize mannose as sole carbon source in all the four *Coffea* species experimented.

D-xylose had not shown any influence in the proliferation of untransformed cells from the beginning of introduction of D-xylose in the medium and in the wide concentration tried. Therefore the selection could well begin immediately after the transfer on the selection medium, unlike in antibiotic or herbicide resistance selection system that requires a time and change of medium to built tolerance of transformed tissue. This implies that xylose selection could be easy and quick. Thus D-xylose can be most promising positive selective agent in Coffee plant transformation. It did not allow HFSE calli proliferation in all four *Coffea* species uniformly. Whereas, mannose cannot be used as selective agent as it allow HFSE calli to proliferate.

The enzyme D-xylose isomerase catalyzes the reversible interconversions of D-xylose to D-xylulose and D-glucose to D-fructose (Whitaker et al., 1995). This enzyme is uncommon in plant kingdom and hence plants are unable to utilize D-xylose. To make a plant capable of utilizing D-xylose as carbohydrate source only one single gene - D-xylose isomerase gene (*xylA*) has to be introduced as the plants are known to express xylulokinase (Haldrup et al., 2001; Penna and Swennen, 2002).

Of the major concerns voiced about genetically engineered plants is the use of selective markers antibiotic or herbicide resistance genes of bacterial origin that might cause risk to both animals and plants. The advent of positive selection schemes helps to eliminate antibiotic or herbicide resistance selective markers. However, Haldrup et al. (1998) suggest that positive selection based on xylose is safe, efficient, inexpensive and faster. Recently, Kristo et al. (1996) had isolated the first eukaryotic xylose isomerase gene from barley. This provides an opportunity towards engineering plants with *xylA* gene of plant origin.

Though Phosphomannose isomerase (PMI) based selection is well demonstrated in number of crops such as maize, rice, wheat and sugarbeet, our studies with the selective agent mannose prove that all four species of *Coffea* can utilize mannose as sole carbon source and *C. arabica* had shown much pronounced response (419%) at 16 gm/l than the sucrose-grown. This implies a possible high activity of PMI in this coffee tree species. Also the present study confirms that higher level of mannose (32 gm/l) in the medium is inhibitory to HFSE calli proliferation. Based on these results it is possible to conclude that the optimal level of HFSE calli proliferation could be increased by substituting mannose in the coffee culture medium. Since coffee plant tissue can utilize mannose as carbon source, the use of phosphomannose isomerase positive selection becomes unfeasible.

We propose that xylose based positive selection could be a better alternative and safe method to genetic transformation. Further studies would focuse to investigate the regeneration potential of the *xylA* transformed HFSE calli on D-xylose containing medium.

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# DNA Polymorphisms in *Coffea arabica* L.

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## SUMMARY

Due to the tremendous developments in the field of molecular genetics, variety of techniques to analyze and utilize genetic variation has emerged during the last few decades. Genetic improvement of coffee (*Coffea arabica* L.) is particularly constrained by low genetic diversity, lack of genetic markers, lack of information on the genetic makeup and prolonged generation time. In this context, the use of DNA markers to detect and exploit the variation in the available genetic pool of *C. arabica* becomes highly imperative. Here, we describe three high genetic-resolution marker systems, viz., simple sequence repeats (SSR), sequence-specific amplification polymorphism (SSAP) and single nucleotide polymorphisms (SNPs) to possibly detect and exploit the genetic variation in the in *C. arabica* gene pool maintained in Italy. Analysis of 161 SSR markers, 20 retro-transposons based markers in various arabica genotypes though, have revealed low genetic variation in the arabica pool, it has led to the better understanding of the allo-tetraploid origin of arabcia genome. Analysis of 69 expressed sequence tag sequences (ESTs) have resulted to the detection of 4 new SNPs in arabica varieties and 30 polymorphic restriction pattern in other wild species, which intern could be used as potential markers for genotyping and genetic dissection of complex traits and disease. Furthermore, the data also suggests that arabica genome maintains a high level of heterozygosity particularly through paralogous genes.

## INTRODUCTION

Coffee tree belongs to genus *Coffea* in the family Rubiaceae. Although the genus *Coffea* is reported to comprise approximately 100 species (Bridson and Vedrcourt, 1988), commercial cultivation relies on only two species *Coffea arabica* L. popularly called as arabica and *C. canephora* Pierre, known as robusta. *Coffea arabica* L. is only self-fertile, allotetraploid species (2n = 4x = 44) in the genus while others, including *C. canephora* are diploid (2n = 2x = 22) and generally self–incompatible. Recent molecular-cytogenetic analysis established the fact that *C. arabica* is an amphidiploid formed by natural hybridization between *C. eugenioides* and *C. canephora* or ecotypes related to these diploid species (Lashermes et al., 1999). *C. arabica* is characterized by low genetic diversity which has been attributed to the allotetraploid origin, reproductive biology and evolution process of this species (Lashermes et al., 2000). On the other hand considerable variability was reported among diploid coffee species and some of these species form valuable gene reservoir, for different breeding purpose (Berthaud and Charrier, 1988). The conventional breeding efforts towards improving cultivars, especially in arabica coffee are often faced with constrains of low genetic diversity (Paillard et al., 1993), long pre bearing periods and difference in ploidy level between potential donors and recipient species.

Recent advances in DNA marker techniques offered new possibilities to overcome some of these limitations. These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. Therefore, the choice of most appropriate

genetic marker will depend on, e.g. the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints, and financial limitations. *C. arabica*, being the autogamous crop, detection of variation is a challenging task and therefore selection of marker system(s) is of particularly important. Considering the difficulties in detection of variation in *C. arabica* gene pool, we have selected three high resolution marker systems viz., microsatellites, retro-transposons and sequence based polymorphism.

Microsatellites have been the marker of choice for various reasons due to their high-information content, co-dominant nature, sensitivity and ease to analyse with minimal quantities of test samples (Powell et al., 1996). Microsatellite markers have been developed and utilized in many crop species, including rice (Wu and Tanksley 1993; Akagi et al., 1996), wheat (Song et al., 2002), potato (Ashkenazi et al., 2001) and coffee (Lashermes et al., 2000; Rovelli et al., 2000; Baruah et al., 2003).

Retro-transposons are mobile genetic elements found throughout the plant kingdom (Kumar and Bennetzen, 1999). Retro-transposons can be used in an AFLP (Amplified Fragment Length Polymorphism)-type reaction (Vos et al., 1995) termed Sequence- Specific Amplified Polymorphism (SSAP; Waugh et al., 1997). In the SSAP system, the target sequence is composed between the restriction site and the LTR (Long Terminal Repeats) sequence. Hence, the polymorphism that is visualized is for the presence of a retro-transposon at a given distance from a restriction site. In the SSAP technique, the selective bases added to the primers reduce the complexity of the amplified DNA, depending on the copy number of the retrotransposon targets. The use of SSAP has been described for barley (Waugh et al., 1997), wheat (Gribbon et al., 1999), pea (Ellis et al., 1998), and alfalfa (Porceddu et al., 2002).

Single nucleotide polymorphisms (SNPs) are the most abundant source of genetic variation among individuals of a species, which is estimated to be on an average 1 per 1000 base pairs. Although SNPs are mostly biallelic (less informative than short tandem repeats), they are more frequent and mutationally stable (Risch and Merikangas, 1996; Kruglyak, 1999) and therefore, it is considered to be one of the potential sources for genetic variation in arabica.

Keeping this view, the general objective of the present investigation was to examine and utilize the genetic variation in arabica gene pool with the particular interests were; (i) to validate a total of 161 microsatellite markers in a panel of arabica genotypes and examine the inheritance pattern in a cross, Caturra and E.typica; (ii) to assess the level of genetic variation in arabica types using SSAP and (iii) to detect and analyse SNPs from a 69 EST sequences.

## MATERIALS AND METHODS

### Plant materials and DNA extraction

The plant materials used in this study are 3 different set of panels involving arabica varieties, inter-specific hybrids and wild species, collected and maintained by University of Trieste, through international expeditions. A quantity of 3 to 4 grams of fresh leaves were collected from individual plants and were used for DNA isolation. DNA isolation was followed using CTAB procedure as described by Murray and Thompson (1980) with a little modification as required.

### Genotyping of SSR markers

A total of 235 microsatellite markers have already been developed from 2 enriched genomic libraries of *C. arabica* var. Caturra and Tekisic. These primers were initially standardized for

their polymerized chain reaction (PCR) conditions and then the working pairs were used for genotyping. For the later purpose, a total of 100 accessions comprising arabica genotypes and $F_2$ population of the cross Caturra and Etypica were used. PCR amplifications were typically performed in 25 µl reactions [containing: 10 ng genomic DNA, 1 pmole of each primer, 2 mM $MgCl_2$, 100 µm dNTPs, 1 x PCR buffer- II and 1 unit *Taq* DNA polymerase on a GeneAMP PCR 9600 Thermal-Cycler (Perkin Elmer Cetus), using a five-step program comprising: initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55–60°C (vary with the primer) for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min. All amplification products were checked on 2% agarose gel before resolving through GeneScan analysis on ABI-373$^{TM}$ DNA sequencer (Applied Biosystems) as per the manufacturer's instructions. Finally, the visualized microsatellite alleles were precisely sized using the software GS Analysis Ver 2.0 (Applied Biosystems) to calculate the number, range, and distribution of amplified alleles.

**SSAP genotyping**

Dissection of sequences encompassing part of the LTR from the Ty1-copia retro-transposon family was carried out using the protocol, adapted from Pearce et al. (1999). A quantity of 1 µg genomic DNA was restricted by Mse I (New England Biolabs) at $37^0$C. The fragments longer than 500 bp, were excised, purified and ligated to Msc adapters. Pre-selective amplification was performed using 1 µl of ligated DNA as a template in 50 µl reaction volume containing 1x PCR buffer (QIAgen); 0.125 units Taq DNA polymerase; 0.2 µM dNTPs; 250 nM Mse+0 primer (5'- GATGAGTCCTGAGTAA–3') and 1.5 mM $MgCl_2$. The PCR program consisted an initial denaturation of 72°C for 4 min followed by 25 cycles of 94°C for 30 min; 53°C for 30 min; 72°C for 1 min and a final elongation step of 72°C for 5 min. The PCR products were diluted 20-fold and 5 µl were used as a template for selective PCR. The selective amplification was performed in 25 µl reaction volume containing 1x PCR buffer (QIAgen); 0.125 units Taq DNA polymerase; 0.2 µM dNTPs; 150 nM LTR primer; 100 nM Mse+N primer (where N = A, C, G or T); 1.5 mM $MgCl_2$. The PCR program consisted of 94°C for 4 min followed by 25 cycles of 94°C for 30 min; 53°C for 30 min; 72°C for 1 min and a final elongation step of 72°C for 5 min. Four PCR reactions were carried out for each LTR primer, using Mse+A, Mse+C, Mse+G and Mse+T respectively. After a preliminary assay of PCR quality by electrophoresis on a 2% agarose gel, typically 25 µl of the PCR product was run on a 6% acrylamide denaturing gel. An ionic gradient was established using 1x TBE electrode buffer in the anode and 0.5x TBE buffer in the cathode followed by addition of sodium acetate to a final concentration of 1M after 2 hrs to retard migration of the shortest fragments. Gels were visualized by silver staining method (exposed for 20 min in 1% CTAB followed by 15 min in 0.3 $NH_3$, 20 min in fresh Tollen's solution (0.1% $AgNO_3$, 4 mM NaOH, 0.1% $NH_3$) and processed in 25% HCHOH and 2% $Na_2CO_3$. The polymorphic bands were excised from the gel, stored overnight in 1x *Taq* buffer (QIAgen) and re-amplified for further characterization.

**Analysis of ESTs**

From the library of about 2000 ESTs of *C. arabica*, we designed 69 primer pairs as described above and analysed on a set of genotypes. The presence of introns in the PCR amplified products was realized by the size longer than the corresponding ESTs. The PCR amplicons were digested with 30 different restriction enzymes. The selected amplicons were further sequenced for the detection of SNPs.

## RESULTS

### SSR polymorphism

Analysis of 161 microsatellites primers has resulted in varying level of polymorphism among the genotypes involved in different crosses. Of the 5 crosses analysed, Caturra x ET 30 (Ethiopica) was chosen as it gave maximum number of polymorphic markers. The $F_2$ population comprising 96 plants of the above cross was further analysed using the identified polymorphic markers. Table 1 shows the allelic distribution for the selected polymorphic markers for the parents and its $F_2$ population. Based on the segregation pattern, the parental types were determined (Figure 1).

**Table 1. $\chi^2$ Analysis for $F_2$ population of Caturra x ET30 with 96 individuals.**

| SSR Primer | Allele (bp) | | | | | Statistical Significance | |
|---|---|---|---|---|---|---|---|
| | Caturra | Etiopica-30 | $F_2$ population | | | Expected ratio | Observed ratio |
| CM 107 | 219 | 235 | 219 (19) | 219-235 (59) | 18: 235 | 1: 2: 1 | 0.79: 2.45: 0.75 – |
| CM 115 | 164-172 | 164 | 164-172 (71) | 164 (25) | 0 | 3: 1 | 2.96: 1.04 – |
| CM 129 | 204 | 200 | 200 (25) | 200-204 (51) | 20: 200 | 1: 2: 1 | 1.04: 2.13: 0.83 – |
| CM 120 | 174 | 180 | 180 (21) | 174-180 (43) | 32: 174 | 1: 2: 1 | 0.88: 1.80: 1.33 – |
| CM 119 | 170 | 176 | 170 (23) | 170-176 (50) | 23: 176 | 1: 2: 1 | 0.96: 2.08: 0.96 – |

*Values in parenthesis represent the number of individuals possessing the respective alleles*
*– : $\chi^2$ test - significant at 1% level*

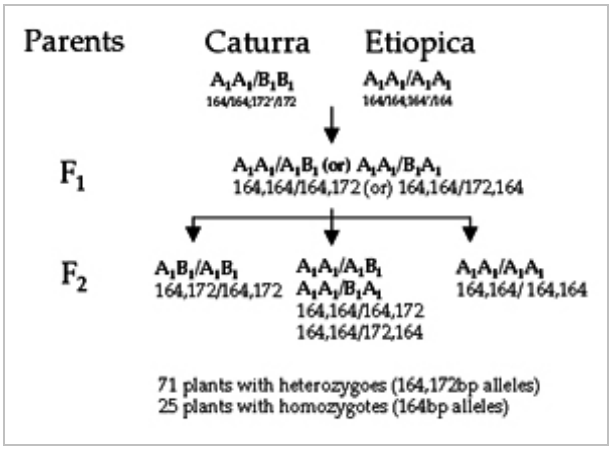


**Figure 1. Determination of parental genotype in Caturra x Etiopica cross.**

### Analysis of retro-transposon sequences

A total of 150 clones were sequenced as result of cloning the PCR products derived form LTR-based primers. Of the 150 sequences, 20 were confirmed to have poly-purine tract marking the start of 3' LTR. Primers were designed on this region. A total of 5 LTR primers in combination with 4 adapter primers (20 combinations) were optimised for their PCR conditions and were used in SSAP genotyping on a panel of *C. arabica*, *C. canephora* and *C. euginioides* accessions. Table 2 shows the summary of polymorphic bands obtained from

different genotypes. The total number of polymorphic bands was found to be 160 and the mean number of polymorphic bands per primer was 8. Of the 20 combinations, the pair 263+A gave the maximum number of polymorphic bands (14) in the analysed samples. A total of 70 polymorphic bands were excised from acrylamide gel and re-amplified for further characterization. Three primer pairs were designed in order to generate varietal-specific amplification of DNA traits encompassing a retro-transposon insertion site.

**Table 2. Summary of S-SAP genotyping.**

| Genotypes | 104 + A | 104 + C | 104 + G | 104 + T | 130 + A | 130 + C | 130 + G | 130 + T | 147 + A | 147 + C | 147 + G | 147 + T | 262 + A | 262 + C | 262 + G | 262 + T | 263 + A | 263 + C | 263 + G | 263 + T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maragogype | ■ | ■ | ■ | | ■ | | | | | | | | | | | | ■ | ■ | | |
| Maragogype | | | | | | | | | | | | | | | | ■ | | | | |
| Typica | | | ■ | | | | | | | | | | | | | ■ | | | | |
| Rume Sudan | ■ | | ■ | | | | ■ | | | | | ■ | | | | ■ | ■ | | | |
| Moka | | | | | | | | ■ | | | ■ | | | | | | | ■ | | |
| Etypica | | | ■ | | | ■ | | | | | ■ | | | ■ | | ■ | | | | |
| sarchimor | | | | | | ■ | | | | | | | | ■ | | | | | | |
| Catimor | | | | | | | | ■ | ■ | | | | | ■ | | | | ■ | | |
| Iapar59 | | | | | | | | | ■ | ■ | | | | ■ | | ■ | | ■ | | |
| Timor Hybrid | | | ■ | ■ | | ■ | | | | ■ | ■ | | ■ | ■ | | ■ | ■ | ■ | | ■ |
| Timor Hybrid | | | | | | | | | | | | | | | | ■ | ■ | | | |
| Bourbon Yellow | ■ | | ■ | | | | | | | | ■ | ■ | | | | | | | | |
| Bourbon LC | | | | | | | | | | | | | | | | | | | | |
| Catuai Yellow | | | | | | | | | | | | | | | | | | ■ | | |
| Caturra Red | | | | | | | | | | | | | | | | | | | | |
| Caturra | | | | | | | | ■ | | | ■ | | | | | | ■ | | | ■ |
| Caturra | | | | | | | | | | | | | | | | | | | | |
| Mundo Novo | | | | | | | | | | | | | | | | | | | | |
| Catuai | | | | | | | | | | | | | | | | | | | | |
| Bourbon Red | | | | | | | | | | | | | | | | | | | | |
| Catuai Red | | | | | | | | | | | | | | | | | | | | |
| Mundo Novo | | | | | ■ | | | | | | | | | | | | | | | |
| Laurina | | | | | ■ | | | | | | | | | | | | ■ | | | |
| Bourbon Tekisic | | | ■ | | | | | ■ | | | | | | | | | ■ | | | |
| Bourbon VD | ■ | | | | | | | | | | | | | | | | | | | |
| Pacas | | | | | | | | | | | | | | | | | | | | |
| Icatu | | ■ | | ■ | ■ | ■ | | | | | ■ | | ■ | ■ | | | | | | |
| Icatu | | ■ | ■ | | ■ | | | ■ | | | ■ | ■ | | | | ■ | ■ | | | |
| Tupi | ■ | ■ | | ■ | | ■ | ■ | | | | | | | | | ■ | | | | |
| AR(ET)5-06 | ■ | | ■ | ■ | | ■ | | | | | | | | | | | | | | |
| AR (ET)34B06 | | | ■ | | ■ | ■ | | | | | ■ | ■ | | | | ■ | | | | |
| Amphilo | ■ | | ■ | ■ | | | ■ | | | | | | | | | ■ | | | | |
| C. Arabica wild | | | ■ | ■ | ■ | ■ | | | | | ■ | | | | | ■ | | | | |
| C.canephorawild | ■ | ■ | | ■ | ■ | ■ | ■ | ■ | ■ | | | ■ | ■ | | ■ | | ■ | ■ | ■ | ■ |
| C.canephora | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | | | ■ | ■ | | ■ | | ■ | ■ | ■ | ■ |
| C.canephora | ■ | | | ■ | ■ | ■ | ■ | | | ■ | | ■ | ■ | | ■ | | ■ | ■ | ■ | ■ |
| Aramosa | ■ | | | | ■ | | | | | | | ■ | | | | | ■ | | | |
| C.eugenioides | | | | ■ | ■ | | | | ■ | | | | ■ | ■ | ■ | | ■ | ■ | ■ | |

■ Accessions showing at least one polymorphic band

## Restriction polymorphisms

A total of 69 ESTs were PCR amplified on a test Arabica sample, of which 25 were confirmed to have introns, identified by the size longer than the expected range. A total of 25 selected primer pairs were used to amplify a panel of 16 genotypes comprising 8 varieties and 8 species and further the amplicons were cleaved by 12-30 different restriction enzymes. A

total of 24 amplicons were found to have inter-specific polymorphic pattern while only 4 were polymorphic within Arabica varieties.

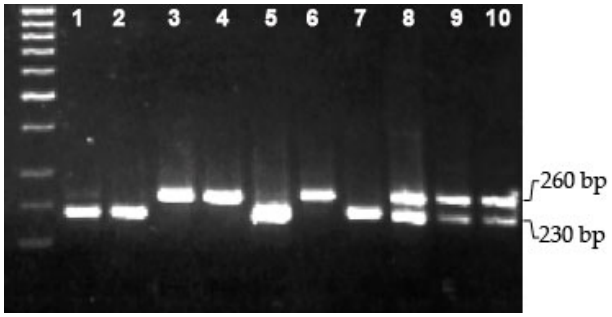


**Figure 2. PCR products generated from RM-00-O18 showing insertion polymorphism. 1) C. heterocalix; 2) C.mouloundu; 3) C. pseudozanguebarie; 4) C. racemosa; 5) C.liberica; 6) C.canephora; 7) C.eugenioides; 8) Laurina; 9) Caturra; 10) Aramosa. Marker corresponds to 1 kb ladder (GibcoBRL).**

The polymorphic amplicons were re-amplified, cloned, sequenced and further aligned to detect SNPs. As expected, the number of nucleotide polymorphisms was evident among the species, but surprisingly a very limited amount of heterozygosity was accounted. A total of 3 SNPs were found in the arabica varieties while the alignment showed 11 heterozygous loci. Figure 3 depicts the sequence polymorphism observed in arabica genotypes.

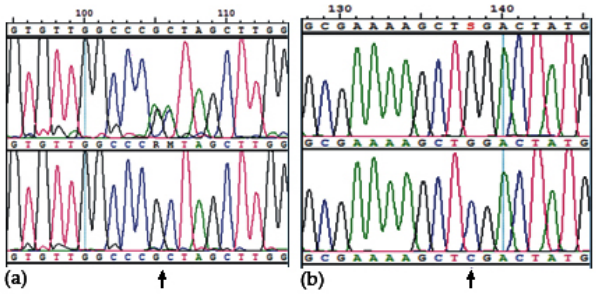


**Figure 3. Detection of SNPs in the Arabica genotypes. 3a) Heterozygous loci observed between Etiopica and Mundo Novo (arrow); 3b) SNP between Etiopica and Caturra.**

## DISCUSSION

In any tree improvement programme genetic variation is the backbone in order to exercise selection for isolation of superior plants. World coffee production relies mainly on two species, C. arabica and C. canephora. Superior quality is associated with C. arabica, which contributes 70% of the world coffee production. C. arabica has its primary centre of genetic diversity in the highlands of South western Ethiopia, the Boma Plateau of Sudan and Mount Marsabit (Kenya) (Lashermes et al., 1999).

In spite of their considerable economic importance, genetic research on coffee trees has been rather limited due to difficulties as mentioned above. As a prelude to any breeding programme, dissecting / understanding the variation existing species/varietal populations is crucial and most important as this helps in laying the foundation for crop improvement programmes. Though most of the wild Coffea species are out-breeding and panmictic, *C. arabica* is an autogamous crop with extremely limited genetic variation. This paper provides valuable information on the polymorphism in *C. arabica* using high-resolution genetic markers.

**Microsatellite analysis**

In principle, microsatellite markers are considered to be one of the powerful markers in detecting variation as it is based in hyper-variable region in the genome. In this study, a total of 235 microsatellite markers were screened for polymorphism against 5 different sets of parents with arabica background as step towards developing an arabica based genetic map. Of the 5 sets, Caturra x Ethiopica was found to be promising as it gave maximum number (13) of polymorphic markers. Caturra is a natural mutant from Bourbon types and Ethiopica is originally selected from its primary centers of origin, Ethiopia. Relatively more variation in the above cross is attributed to their pedigree.

Analysis of the $F_2$ population of the above cross-showed typical diploid-type segregation for all the polymorphic markers analysed. The locus CM115 is heterozygote for one of the parents, in which polymorphism is attributed to the mutation present in the unique flanking sequence. The $F_2$ population produced 71 heterozygotes and 25 homozygotes, marking a typical 3:1 ratio.

As shown in Figure 1, the heterozygote $F_2$ plants, in which either one of the 2 sets of ancestral chromosomes is assumed to have both the alleles (164 and 172 bp) and the possible genotypes were $A_1A_1/A_1B_1$, $A_1A_1/B_1A_1$ and $A_1B_1/A_1B_1$. In the case of homozygotes with 164 bp alleles, the genotype was assumed to be $A_1A_1/A_1A_1$, where the primer pair might have amplified the locus simultaneously all the 4 chromosomes.

Based on this, the parental genotypes were assumed to as $A_1A_1/A_1B_1$ for Caturra and $A_1A_1/A_1A_1$ for Etiopica. In the scenario of low level of polymorphism in arabica gene-pool, our analysis shows that above cross can be potentially used by extending the analysis with reasonable number of polymorphic markers for the use in developing a low density genetic map.

**Retro-transposon based genotyping**

Retro-transposons, in principle, have high copy number, observed over relatively short evolutionary time scale due to its peculiar mode of transposition and thus assumed to be potential markers for hunting polymorphism in *Coffea* genome. In the present study, 20 LTR-primer combinations were analysed against 40 *Coffea* accessions. The number of polymorphic bands were ranging from 3 to 14 with the mean number of polymorphic bands was found to be 8 per primer combination. Considering the limited amount of variation in the Arabica gene pool, the above results indicate that, though intra-specific variation is relatively less, retro-transposons can be potentially used to develop varietal specific fingerprints. About 70 polymorphic bands were excised from acrylamide gels and re-amplified for further characterization. Three primers have been designed (data not shown) in order to give varietal-specific amplification of DNA traits encompassing a retro-transposon insertion site. The preliminary attempts to type varietal-specific pattern for Tupi and Bourbon Tekisic cultivars were successful.

In the present study, SSAP genotyping was carried out using poly-acrylamide gel electrophoresis following silver-staining based visualisation. Although, in theory, silver staining the gels allows visualization of the 'undesired' Mse-Mse amplicons, the different primer concentrations and annealing temperature actually prevent formation of such products, as ascertained by the absence of common bands, when two LTR primers were used in combination with the same Mse+N primer.

**Restriction analysis and Detection of SNPs**

As expected, the restriction cleavage of EST amplicons showed low level of variation in arabica varieties (accounted to a number of 3 in the case of arabica varieties and 29 in the case of wild species). In principle, the sum of restricted products length is expected to be equal to the original amplicon length. But in many cases, the cleaved product length was twice or more than twice to that of the mother amplicons, which explains the fact that the corresponding PCR product contained heterozygous loci.

Interestingly, the arabica genotypes showed more number of such products (data not shown) than the diploid wild species. Sequencing and further aligning the PCR products resulted in detection of 3 new SNPs and 11 heterozygous loci in arabica varieties. The heterozygous loci were identified by the presence of overlapping peaks (bases) in the electropherogram (Figure 3a). Again, the overlapping peaks (heterozygous loci) were more prevalent in arabica types. C. arabica is basically an allo-tetraploid species, having the two ancestral genomes, descended form two different species. Allopolyploidy is a well-known process in plant speciation involving the duplication of an interspecific hybrid genome. However, evolution of the two duplicated parental genomes (i.e., the homoeologous subgenomes) in the allopolyploid remains obscure in most cases because of the lack of information on the initial conditions (Wendel, 2000). The high level of heterozygosity in arabica is attributed to its allo-tetraploid behavior and source of variation between the two ancestral genomes could be paralogous, i.e., the paralogous genes diverged after duplication events.

## CONCLUSION

Despite of the difficulties in hunting for polymorphism in arabica genome, the present study provides useful information on coffee polymorphism using 3 high resolution marker systems. The cross Caturra x Etiopica was selected to be the potential for the use in arabica genetic map construction using microsatellite markers. The retro-transposon based genotyping was considered to be the promising for genetic analysis of coffee, especially its secondary gene-pool for conservation, management, resolving taxonomic relationships, and even more importantly for their use as efficient, informative genetic landmarks. Analysis of ESTs in arabica types has resulted in detection of 4 SNPs, which further provide the scope for extending the analysis with more number of ESTs for the detection of new SNPs. Furthermore, the data also suggests that arabica genome maintains a high level of heterozygosity particularly through paralogous genes.

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# Development of Microsatellite Markers for the Identification of Brazilian *Coffea arabica* Varieties

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## SUMMARY

Microsatellite markers, also known as SSRs (Simple Sequence Repeats), have proved to be excellent tools for variety identification and determining genetic relationships. A set of 130 SSR markers was used to analyze the genetic similarity among twenty-five *Coffea arabica* varieties. These were composed of nineteen commercially important Brazilian varieties, six Indian hybrids of *Coffea arabica*, *Coffea canephora* and *Coffea liberica.* The set of SSR markers used consisted of 54 newly developed markers derived from microsatellite enriched libraries, 57 markers designed on the basis of coffee SSR sequences available from public databases, six markers already published and thirteen universal chloroplast microsatellite markers. Only 22 markers were polymorphic, detecting 2-7 alleles per locus, with an average of 3.5 alleles. The level of polymorphism was positively correlated with the number of repeats. Based on the band patterns generated by the polymorphic SSR loci, the set of twenty-five coffee varieties were clustered in two main groups. One group composed of the largest part of the Brazilian varieties and a second one composed of the Indian hybrids and a few Brazilian varieties. Color mutants could not be separated. The clustering was in accordance with the genealogy of the varieties and showed the high similarity among the Brazilian varieties.

## INTRODUCTION

Coffee is an important cash crop in many countries. Among of the existing species, *Coffea arabica* L. is the most widely grown, due to its reduced caffeine content in the beans and smoothness of the beverage produced. The species accounts for 73% of the world production and almost the total production of the Latin American countries (Orozco-Castilho et al., 1994). In 2001 the species *Coffea arabica* was included in the Brazilian list of species from which varieties can be protected, however without the stable and homogeneous markers required for effective protection. In the past years DNA based markers have been used to study genetic diversity of many plant species. This type of marker allows the analysis of variation present in the DNA itself and can be used for the identification of varieties. In addition, they are independent of the environment and may be detected in any sort of tissue and developmental phase of the plant (Ferreira and Grattapaglia, 1998). Analysis of the *C. arabica* varieties in Brazil has shown that the material utilized is derived from a few ancestors' varieties (Typica, Bourbon and Sumatra), which experienced spontaneous mutations and crossings among them (Mendes and Guimarães, 1998). The nuclear DNA variation in coffee has been evaluated using, SSRs (Vascoto et al., 1999; Combes et al., 2000; Anthony et al., 2002). These studies have showed that genetic variation in the genus *Coffea* is low, especially among the cultivated varieties. The chloroplast DNA (cpDNA) non-coding regions have been used as a source of molecular markers in studies concerning the relationship within and among species of the genus *Coffea* (Orozco-Caltillo et al., 1996; Cros

et al., 1998). Despite the existing of microsatellite markers developed specifically for coffee, the number of these markers currently available is still limited. As *Coffea arabica* is the most important, there is an urgent need for additional microsatellite makers that will allow the unique identification of closely related varieties. Therefore the aims of this study are to develop and characterize more microsatellite markers for *Coffea arabica* and evaluate these markers for use in the identification of the varieties of commercial interest in Brazil.

## MATERIAL AND METHODS

*Plant material and DNA isolation:* A group of 19 *Coffea arabica* varieties was selected to represent the complete range of varieties most grown in Brazil. Six Indian hybrids of *C. arabica, Coffea liberica* and *Coffea canephora* were included in this work.

*SSR sequences from public databases and primer design:* Coffee microsatellite sequences were extracted from the NCBI database.

*Microsatellite isolation:* Additional microsatellites were isolated from two enriched small-insert genomic libraries constructed according to Van de Wiel et al. (1999).

*Microsatellite analysis:* Microsatellite were amplified by PCR, separated on 6% polyacryilamide gels and visualized with silver staining.

*Data analysis:* For the polymorphic microsatellite loci the number of alleles per locus and the number of allelic phenotypes were counted. A presence/absent (1/0) allele matrix was built using the Jaccard similarity coefficient. The data generated were subjected to cluster analysis with the UPGMA algorithm.

## RESULTS

*Microsatellite enrichment from C. arabica:* Of the 135 recombinant clones coming from the first enrichment (low stringency) library, 33% (45) contained microsatellite sequences and eighteen inserts had flanking regions suitable for primer design. From the 397 recombinant clones characterized in the second (high stringency) enriched library 22% (89) contained microsatellite sequences and Thirty-five inserts had flanking regions suitable for primer design. (Table 1).

**Table 1. Results of microsatellite cloning and sequencing of two enriched libraries (EL) of *C. arabica*. Two elution conditions were used: 1) low-stringency (0.5xSSC) and 2) high-stringency (0.2xSSC). Positive clone indicates the number of clones hybridizing to a labeled oligo probe mixture. SSR indicates the number of clones containing a microsatellite. Primers designed indicates the number of clones on the basis of which primers could be designed for amplification of the microsatellite.**

| EL | Screened clones | Positive clones | Sequenced clones | SSR | Primers designed | Polymorphic markers |
|---|---|---|---|---|---|---|
| 1 | 3572 | 135 | 110 | 45 | 18 | 2 |
| 2 | 3840 | 397 | 192 | 89 | 35 | 5 |

*Markers from public database sequences and literature:* Screening of public databases (http://www.ncbi.nlm.nih.gov) for microsatellite sequences (July 2002) resulted in 57 accessions.

*Marker characterization and allelic variation:* A total of 130 primer pairs were tested for the degree of polymorphism and pattern quality on a set of 25 coffee varieties. One hundred and twenty five primers amplified the expected DNA fragments and only 22 were polymorphic. The majority are markers containing the GT. The polymorphic primers all gave a pattern quality of 1 or 2 (Smulders et al., 1997) and could be scored unambiguously. A total of 77 alleles were detected (Table 2) using the 22 polymorphic SSR loci and the number of alleles per locus ranged from 2 to 7, being detected on average 3.5 alleles per locus.

**Table 2. Number of alleles, number of effective alleles (ne) and number of allelic phenotype of the 22 polymorphic microsatellite markers.**

| SSR marker | No. of alleles | No. of effective. alleles (ne) | No. of allelic phenotype |
|---|---|---|---|
| M20a | 4 | 1.3 | 4 |
| M24 | 4 | 1.9 | 6 |
| 119-5CTG[b] | 2 | 1 | 2 |
| 62-6CTG[b] | 2 | 1 | 2 |
| 49-6CTG[b] | 3 | 1.1 | 2 |
| 45-6CTG[b] | 2 | 1 | 2 |
| 39[c] | 3 | 1 | 2 |
| 47[c] | 3 | 1.9 | 4 |
| 53[c] | 3 | 1.8 | 2 |
| 59[c] | 7 | 3.9 | 6 |
| 63[c] | 3 | 1.4 | 3 |
| GTG10[d] | 2 | 1 | 2 |
| CTT[d] | 3 | 1 | 2 |
| 6[e] | 7 | 1.8 | 5 |
| 10[e] | 3 | 1.2 | 3 |
| 15[e] | 4 | 1.5 | 3 |
| 17[e] | 5 | 3.4 | 6 |
| 20[e] | 7 | 1.8 | 7 |
| Ccmp3[f] | 3 | 1 | 2 |
| Ccmp6[f] | 2 | 1.2 | 2 |
| Ccmp10[f] | 3 | 1 | 2 |
| NTCP8[f] | 2 | 1 | 2 |
| Total number of alleles | | | 77 |
| Average alleles/locus | | | 3.5 |

*[a]:Primer sequences published by Combes et al. (2000), [b]: Primers developed from clone sequences publishes by Rovelli et al. (2001) in NCBI database; [c]: Primers developed from clone sequences publishes by Cristancho et al. (2002) in NCBI database; [d]: Primers obtained in the first genomic library; [e]: Primers obtained in the second genomic library; [f]: chloroplast markers.*

*Variety identification:* The UPGMA dendrogram showed that most of the Brazilian varieties were placed in a group with a high bootstrap value (81.7%), indicating a reliable clustering. The Indian hybrids and the remaining Brazilian varieties were placed in groups, which showed bootstrap values, bellow 50%. Some varieties could not be distinguished, being most of them color mutants. Varieties-specific alleles were amplified in the loci 59, 17 and 20 for the varieties Bourbon Vermelho, Icatu Amarelo and Vermelho, respectively, being possible the separation of the last two varieties. The loci 59 and 17 were the most discriminating, both with six allelic phenotypes and number of effective alleles of 3.9 and 3.4, respectively (Table 2). Although locus 47 does not show a high number of effective alleles and has only four

allelic phenotypes in the material tested, it was one of the more discriminating markers for Brazilian varieties.

## DISCUSSION

*Enriched libraries:* In terms of efficiency, the second enriched library (high stringency wash of the filter) generated a higher frequency of microsatellite containing clones, which on average were longer and consisted of higher percentage of perfect repeats (results not shown). This result can be explained by the release of more tightly bound long perfect repeats from the membrane. It was found that most of the di-nucleotide microsatellite repeats were of the GT motif, which is in agreement with previous microsatellite retrieval efforts in coffee (Vascotto et al., 1999) and with the *C. arabica* microsatellite sequences present in the NCBI database.

*Allelic variation:* Using our set of 25 varieties, only 22 out of the 130 markers (17%) tested showed polymorphism. Remarkably, several of these markers (6 from the 22 (excluding the cp markers) could only be scored dominantly, sowing just one allele that was either present or absent. This clearly reflects the narrow genetic base of coffee. There is hardly any diversity among the material tested, in particular among the Brazilian varieties. The number of alleles per locus ranged from 2 to 7, which is in agreement with previous work in coffee (Vascotto et al., 1999; Anthony et al., 2001; Anthony et al., 2002).

*Variety identification:* The Indian hybrids were clustered far from the most part of the Brazilian varieties probably because the presence of *C. canephora* and *C. liberica* in the genealogy of Indian genotypes. This could be confirmed using chloroplast markers. The clustering of the Brazilian varieties is in accordance with the genealogical data. The varieties Acaiá Cerrado and Mundo Novo showed a 100% similarity, which can be explained by the fact that Acaiá Cerrado is a selection inside Mundo Novo. In spite of the high genetic similarity among these varieties, they are phenotypic different. Acaiá Cerrado shows trees with cylindrical top and low diameter when compared with Mundo Novo trees. In contrast to our findings, Teixeira et al. (1999) found low genetic similarity between Acaiá Cerrado and Mundo Novo. Their study was based on RAPD markers. The genetic similarity among Ouro Verde, Rubi and Topázio is high probably because the latter two varieties have Catuaí as a parent and Ouro Verde is a selection from Catuaí Amarelo. Therefore, Ouro Verde and Rubi show red fruits and Topázio shows yellow fruits. Ouro Verde shows green young leaves and Rubi shows tanned young leaves. The impossibility to separate color mutants like Catucaí Amarelo and Vermelho and Catuaí Amarelo and Vermelho is expected as mutants usually are the result of very few mutations that are difficult to pick up with molecular markers (Weising et al., 1995; Vosman et al., 2004). According to the pair wise similarity matrix, the varieties Obatã, Caturra Amarelo and Vermelho, Oeiras, Catuaí Amarelo and Vermelho and Bourbon Vermelho showed at least a genetic similarity of 0.860. Many of these varieties are mutants and were obtained from selections or crosses among. The distance between Bourbon Vermelho and Amarelo probably is due to the fact that the last one is not only a genetic mutation but also a natural cross between Bourbon Vermelho and the variety Amarelo de Botucatu (Mendes and Guimarães, 1998). Variety IAPAR 59 is a selection out of Sarchimor which one has *C. canephora* in its genealogy. Probably because of this IAPAR 59 was clustered nearly the Indian hybrids. For the same reasons the varieties Icatu Amarelo and Vermelho and Tupi, who have *C. canephora* as an ancestor, clustered among the Indian material. In conclusion, in this study we developed and tested 130 new microsatellite markers for *C. arabica*. These markers combined with other published ones, provide a powerful tool in coffee research, including variety identification and marker-assisted selection. Also, we have conclusively shown that the coffee genepool used in Brazil for the development of new

varieties is very narrow. Coffee breeding could benefit from the introduction of new breeding material from Ethiopia or India.

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# Chlorogenic Acid Content Swap during Fruit Maturation in *Coffea pseudozanguebariae.* Qualitative Comparison with Leaves

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## SUMMARY

Chlorogenic acid content (CGAs: 5-CQA and related quinic esters) was evaluated during fruit and leaf development of *Coffea pseudozanguebariae*, a wild caffeine-free species showing low CGAs content in green beans. In both organs, CGAs content decreased during growth and feruloylquinic acids constituted most of the CGAs. In fruits, a critical step was emphasised at the end of maturation, as 5-CQA content drastically increased. Changes were attributed to the increase, inside the fruit, of the seed size, which differently accumulates CGAs.

## RÉSUMÉ

La teneur en acides chlorogéniques (ACGs : 5-CQA et autres esters quiniques) a été évaluée pendant le développement de fruits et feuilles de *Coffea pseudozanguebariae*, une espèce sauvage sans caféine, montrant une faible accumulation d'ACGs dans les grains verts. Pour les deux organes, la teneur en ACGs diminue pendant la croissance et les acides férulylquiniques sont les ACGs majoritaires. Dans les fruits, un seuil critique est observé en fin de maturation, lorsque la teneur en 5-CQA augmente. Les changements sont attribués à l'augmentation, dans le fruit, de la taille de la graine qui accumule différemment les ACGs.

## INTRODUCTION

Chlorogenic acids (CGAs) are products of the phenylpropanoid metabolism. CGAs not only include quinic esters with caffeic acid, i.e. caffeoylquinic acids (CQA) and dicaffeoylquinic acids (diCQA), but also other hydroxycinnamoyl conjugates, such as ferulic or *p*-coumaric acid derivatives. In green coffee beans, 98% of CGAs belong to three classes, i.e. CQA, diCQA and FQA (feruloylquinic acids). Each class includes three isomers according to the acylating residue positions. A great diversity in green bean CGAs content exists between coffee species (Anthony et al., 1993). A wild caffeine-free species, *Coffea pseudozanguebariae* (PSE), native of East Africa, has a low CGAs content in green beans (1.2% dmb) and is proposed as a gene donor to improve *C. canephora* cup quality. In this work, time-course variations of CGAs content have been surveyed in leaves and fruits of PSE in order to compare CGAs content with beans.

## MATERIAL AND METHODS

*Coffea pseudozanguebariae* fruits were harvested on field-grown trees (CNRA, Divo, Côte-d'Ivoire). They were taken at four maturity stages (F1, F2, F3 and F4) corresponding to 40, 60, 80 and 100% respectively of the fructification time: from flowering to bean maturity (9-10 weeks for PSE). Leaves were sampled on tropical greenhouse-grown trees (IRD, Montpellier,

France), at the tip (stage L1), on non-ligneous (stage L2) or ligneous branches (stage L3). Samples were immediately frozen in liquid nitrogen and stored at –80° C.

CGAs extraction (three extractions for each leaf stage and four for each fruit stage) and determination by HPLC-DAD were carried out according to Ky et al. (1997). CGAs isomer content was expressed in percentage of dry matter basis (% dmb).

Dry matter was evaluated by desiccation of powdered samples (15 h, 100 °C).

All results were analysed using one-way ANOVA with fixed effect and Newman and Keuls-test to compare maturity levels and means. The CGAs clustering analysis was done with Ward's criteria and Euclidian distance as parameters (Statistica software package). CGAs were presented according to the IUPAC (1976) numbering system. Abbreviations proposed by Clifford (1985) were adopted.

The primary variables were:

3-CQA, 4-CQA, 5-CQA: 3-,4- and 5-caffeoylquinic acid;
3,4-diCQA, 3,5-diCQA, 4,5-diCQA: 3,4-, 3,5- and 4,5-dicaffeoylquinic acid;
3-FQA, 4-FQA, 5-FQA: 3-, 4- and 5-feruloylquinic acid.

Secondary variables were defined on the basis of primary variables:

CQA = 3-CQA + 4-CQA + 5-CQA; DiCQA = 3,4-diCQA + 3,5-diCQA + 4,5-diCQA; FQA = 3-FQA + 4-FQA + 5-FQA; CGAs = CQA + DiCQA + FQA.

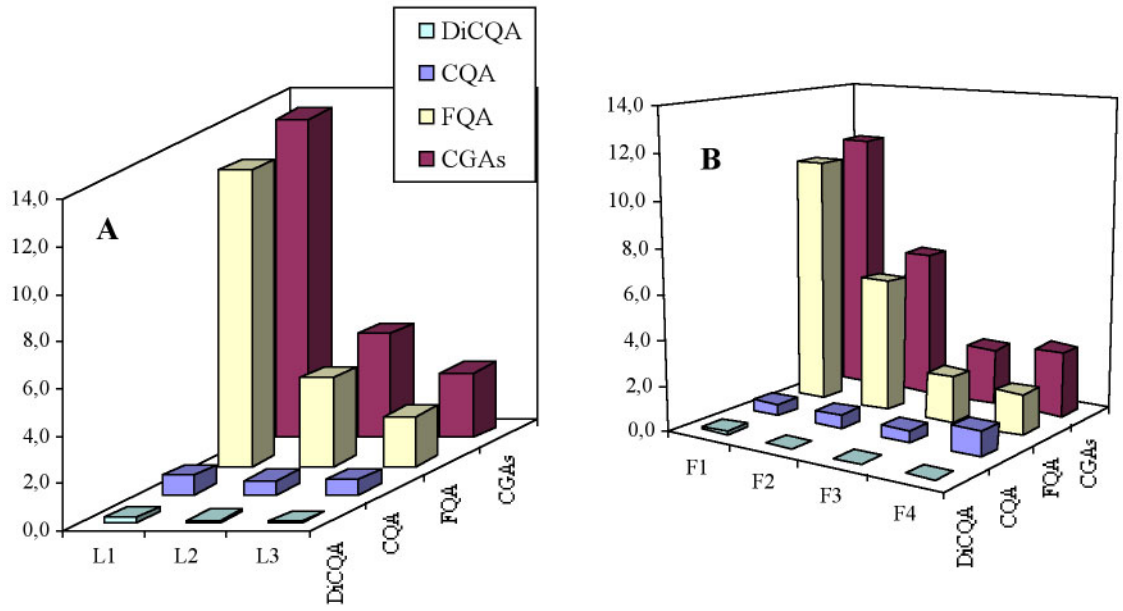


**Figure 1. Chlorogenic acid content in growing leaves (A) and fruits (B) of *Coffea pseudozanguebariae*.**

## RESULTS

In leaves and in fruits, CGAs content decreased from stage 1 to stage 3, due to a general decrease in quinic ester content (Figure 1). FQA appeared as the major CGAs, as CQA and diCQA were poorly accumulated (less than 0.8% and 0.2% dmb respectively).

All the CGAs isomers were not detected. Only 5-CQA, 5-FQA and 4-FQA were present whatever the organ and the growth stage studied. A close linear relationship existed between 5-FQA and 4-FQA content in fruits and in leaves except for stage F4. Concerning diCQA class, 3,5-diCQA was the major isomer. The other two compounds were not accumulated from the stage 2.

In fruits, stage 4 seemed to be a critical step, as CQA content drastically increased (2-fold higher than at stage 3) when compared to the other CGAs and 5-FQA/4-FQA was modified.

The clustering analysis using CQA, FQA and diCQA content as variables showed that samples are grouped according to the growth stage. Cluster G1 included all the F1 and L1 stages and G2 pooled the other stages divided in two sub-cluster, one containing all the stages 2 and the other including stages 3 and 4, in which mature fruits (stage 4) are clearly defined.

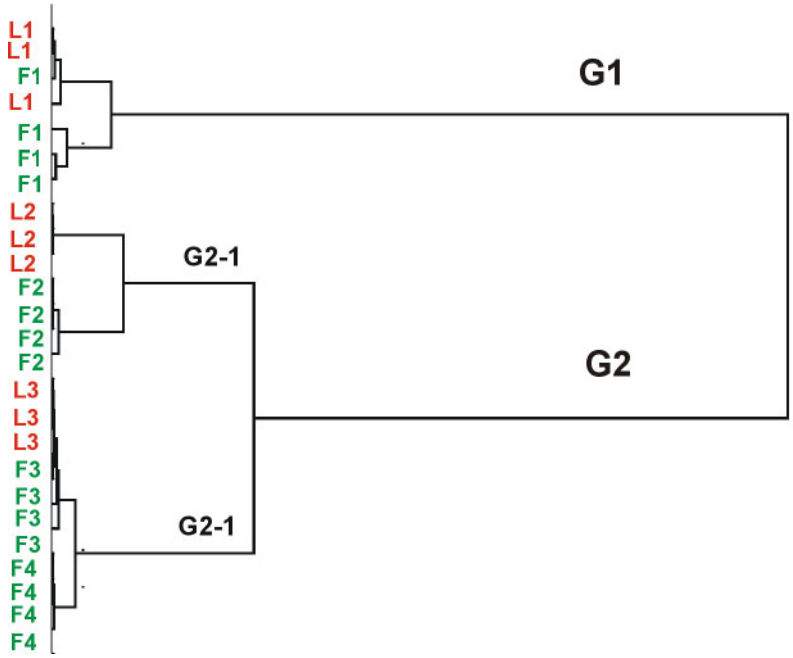


**Figure 2. Clustering tree showing similarities between CGA profiles in PSE fruit and leaves at different stages of maturity.**

## DISCUSSION

In young fruits, the interlocular space is essentially occupied by the perisperm, which, as leaf tissue, has a sporophytic origin (Rogers et al., 1999). This common origin may explain the resemblance in evolution of CGAs content between the two organs. By contrast, fruits at stage 4 are principally formed by endosperm in which the CGAs metabolism may be different. Previous CGAs evaluations in seeds have shown that FQA is residual and that CGAs metabolism seemed to be completely oriented towards CQA biosynthesis and accumulation (Ky et al., 1999).

These results indicate the presence of a critical stage when fruits become mature, with some expression changes in seeds, orienting CGAs metabolism towards CQA biosynthesis and accumulation instead that of FQA.

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# The Use Gibberellic Acid ($GA_3$) for Induction of Direct Somatic Embryogenesis in the New *Coffea Arabica* Hybrid

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## SUMMARY

Using greenhouse raised coffee seedlings (six-month old) the effect of $GA_3$ on induction of direct somatic embryogenesis was assessed. $GA_3$ at either 0.5 or 5 μM supported embryogenesis in the new hybrid. The embryos produced were light pink in colour as compared to the cream-white coloration of coffee somatic embryos produced on media containing other types of growth regulators. $GA_3$ at 0.5 μM produced the highest percentage of embryogenic cultures (90%) and the highest mean numbers of embryos per explant (21.1) while $GA_3$ at 5 μM produced the lowest frequency (10%) of embryogenic cultures and the lowest mean number of embryos per explant (1.7). Regenerated embryos were capable of developing into plants.

## INTRODUCTION

Disease control is the most important constraint to sustainable and economic production of arabica coffee in Kenya. Of major concern is the control of Coffee Berry Disease (CBD) caused by *Collectrotichum Kahawae* sp Nov., Coffee Leaf Rust caused by *Pseudomonas syringae* pv garcea. The major crop losses in the late sixties led to the launch of a breeding program undertaken as joint project between the Kenyan and Netherlands governments in 1971. The breeding programme resulted in the release of a new improved *Coffea arabica* Ruiru 11 in 1985. Ruiru 11 is resistant to CBD and CLR is high yielding and has good cup quality. The propagation of Ruiru 11 is by F1 hybrid seed produced by hand artificial cross-pollination (Van der Vossen and Walyaro, 1981). This method is not only limiting in the number of seeds produced in any given season but also it is also cumbersome, labour intensive and time consuming. Tissue culture methods such as somatic embryogenesis present an alternative method for propagating the new variety. A great amount of coffee tissue culture work has been reported on induction of somatic embryogenesisin coffee using a combination of auxins and cytokinins (Sondhal and Sharp, 1977, 1979; Pierson et al., 1983; Scopke et al., 1987; Sanatana et al., 1988; Kahia and Owour, 1990; Neuenschwander and Baumann, 1992; Ascanio and Arcia, 1994; Boxtel and Berthouly, 1996). There are a few reports where cytokinins alone have been used to induce somatic embryos in coffee (Hatanaka et al., 1991; Yasuda et al., 1995; Kahia, 1999). Gibberellic acid has generally been found to inhibit somatic embryo formation (Ammirato, 1974; Fujimura and Komamine, 1975; Tisserat and Murashige, 1977 b, c; Kochba et al., 1978) but recent reports have indicated that it enhances somatic embryogenesis (Rabbani, 1992; Hanault and Maatar, 1995 and Patnaik and Debata, 1997). In coffee, Priyono 1992 reported the induction of somatic embryos (indirect) using $GA_3$ and an auxin. During the work being reported, when leaf explants were cultured on $GA_3$ and IAA callus was obtained, which was not desirable thus, prompting the study of the effect of $GA_3$ on induction of somatic embryos.

## MATERIALS AND METHODS

Leaves collected from six-month old Ruiru 11 seedlings grown in the Wye College greenhouse. The greenhouse temperature varied diurnally by seasons. Temperature ranged from 15 to 20 in Winter (November-March) and 20-30 in summer (April-October). The photoperiod and light intensity in the green house also varied according to season and presence or absence of direct sunshine. Leaf explants were transported from the greenhouse in polythene bags and kept under running tap water for 30 min. They were then sterilized using 20% bleach solutions for 15 min. they were then rinsed twice in sterile double distilled water followed by a quick immersion (30 sec) in 70% (v/v) ethanol and then rinsed in double distilled water. The leaf explants were induced for 14d on Modified Linsmaeir and Skoog (MLS) medium supplemented with 100mg/l inositol, 30 mg/l cystein HCL 2% sucrose one of the five concentrations of $GA_3$ being tested (0, 0.5, 5, 10, 20). $GA_3$ stock solutions were filter through 0.22 μm membrane filters (Millipore, UK) mounted on 47 mm diameter Swinex disc holders. The filtered solutions were added to the autoclaved media after cooling to 60°C and mixed in well before the media were dispensed to 25-cell replidishes. This was followed by a transfer to MLS medium without $GA_3$. For induction and regeneration of somatic embryos, the cultures were incubated in the dark at a temperature of 25°C. Germination of the embryos was done in test tube of dimensions 75x25 cm containing MS medium and incubated at 25°C and 16h photoperiod.

## RESULTS

All leaf explants turned brown and after four weeks globular structures appeared from the cut edges of leaf explants on media containing low levels of $GA_3$ at either 0.5 or 5 μM. The embryos produced were light pink in color as compared to the cream-white coloration of coffee somatic embryos produced on media contain other types of growth substances. $GA_3$ at 0.5 μM produced the highest percentage of embryogenic culture (90%) and the highest mean number of embryos per explant (21.1) while $GA_3$ at μM produced lowest frequency (10%) of embryogenic cultures and the lowest mean numbers of (1.7) (Table 1). There were no embryos formed in the controls (0 μM $GA_3$) and on media contain $GA_3$ in the range 10-20 μM. Development of somatic embryos in the responsive cultures progressed though the typical globular, heart and torpedo-shaped to cotyledonary stages of embryo development. The globular and torpedo and cotyledonary stages could be seen frequently in all cultures but occurrence of fully developed heart-shaped stage was rare. The regenerated embryos had a conversion efficiency of 60%, when placed on a growth regulator-free MS basal medium supplemented with 2% sucrose.

**Table 1. Effect of $GA_3$ on induction and regeneration of coffee somatic embryos.**

| $GA_3$ conc. (μM) | Embryogenic cultures (%) | Mean No. of embryos /explant (±SE) |
|---|---|---|
| 0 | 0 | [x]0.0 |
| 0.5 | 90 | 21.1±2.02 |
| 5 | 10 | 1.7±0.93 |
| 10 | 0 | 0 |
| 20 | 0 | 0 |

[x] *The zero responses are not included in the statistical analysis.*

## DISCUSSION

Regeneration of plants *in vitro* can be accomplished by organogenesis or somatic embryogenesis. To reduce the risks of somaclonal variation during multiplication of plant

material, direct somatic embryogenesis is preferred to indirect somatic embryogenesis. During the current study, and attempt to culture leaf explant on a medium supplemented with $GA_3$ and auxins resulted information of callus. Somatic embryogenesis was induced in the lower concentrations of $GA_3$ (05 and 5 μM). We were able to establish that $GA_3$ could be used alone to induce somatic embryos in the new hybrid Ruiru 11. The embryos regenerated using $GA_3$ were bigger than those regenerated using other growth regulators and had a higher conversion ratio. This is the first report on the use of $GA_3$ to induce somatic embryos of coffee.

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# RAPD Marker Analysis Reveals High Genetic Stability in High Frequency Somatic Embryogenesis-derived Plants of Robusta Coffee (*Coffea canephora*)

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## SUMMARY

This paper reports high frequency somatic embryogenesis, plant regeneration and high genetic stability in somatic embryo-derived plants (SEPs) of CxR cultivar of robusta coffee (*Coffea canephora* Pierre) as assessed by RAPD markers. High frequency embryogenic calli were induced from the leaf explants from a 15 year-old superior plant (TL1) on MS medium supplemented with IAA (1 mg/l) and kinetin (5 mg/l). Somatic embryos were matured on MS medium with ABA (1 mg/l) and germinated on half strength MS medium supplemented with kinetin (0.1-1 mg/l). After hardening in netpots in a polytunnel and further growth in polybags in nursery, the SEPs were planted in the field. There was no detectable morphological variation in the SEPs. Fourteen SEPs established in the field were used for RAPD analysis. Genomic DNA was isolated from the fresh leaves of the mother plant (TL1) and one year-old SEPs by a modified SDS method. After initial screening of 25 arbitrary 10-base primers, 16 primers that gave distinct RAPD bands were used for further study. In total, 121 RAPD markers were obtained from 16 primers, out of which 120 markers were monomorphic across all the SEPs and the mother plant. One polymorphic marker of 1.4 kb size was obtained with the primer OPA-14 in one SEP.

## INTRODUCTION

CxR (Congensis x Robusta) is a popular robusta cultivar developed at Central Coffee Research Institute, India through interspecific hybridization between *C. congensis* (congensis) and *C. canephora* (robusta). The cultivar shows variation in the seed progeny, due to high heterozygosity and gene segregation. Micropropagation could be useful for rapid clonal multiplication of superior genotypes of this cultivar. Somatic embryogenesis is preferred for micropropagation of coffee due to recovery of high frequency somatic embryos (HFSE) from adult plants. However, *in vitro* cultured plants might exhibit somaclonal variation (Larkin and Scowcroft 1981), which is often heritable and is therefore unwanted in clonal propagation. DNA markers can be used to characterize somaclonal variation with precision. Among different DNA markers available, RAPD markers (Williams et al., 1990) are popular since technology is simple, quick to perform and requires very little plant material. Recently, different DNA markers including RAPD are used to test the genetic stability of somatic embryo-derived plants of *C. arabica* (Rani et al., 2000). Here we report a procedure for generating somatic embryo-derived plants (SEPs) in a superior genotype of CxR cultivar of *C. canephora* and the high genetic stability observed in the SEPs as assessed by RAPD markers.

## MATERIALS AND METHODS

### Production of somatic embryo-derived plants (SEPs)

Fully expanded, tender leaves collected from a 15 year-old superior plant (TL1) of C x R cultivar of *C. canephora* were disinfected for 10-15 min with 1% sodium hypochlorite solution and thoroughly rinsed with sterile distilled water. Explants cut from the lamina excluding midveins, margins and apical and basal portions were cultured on modified MS medium (Murashige and Skoog, 1962) (Table 1).

**Table 1. Composition of modified MS medium used for somatic embryogenesis and plant regeneration.**

| Medium | Composition |
|---|---|
| Callus induction medium (CIM) | MS + IAA (1 mg/l) + Kn (5 mg/l) + PVP (100 mg/l) + L-cysteine HCl (50 mg/l) |
| Embryo induction medium (EIM) | MS + IAA (1 mg/l) + Kn ( 5 mg/l) |
| Embryo maturation medium (EMM) | MS + ABA (1 mg/l) |
| Embryo germination medium 1 (EGM1) | 1/2 MS + Kn (1 mg/l) |
| Embryo germination medium 2 (EGM2) | 1/2 MS + Kn (0.5 mg/l) |
| Embryo germination medium 3 (EGM3) | 1/2 MS + Kn (0.1 mg/l) |

The explants were cultured upside down on CIM (Table 1) for callus induction. After four weeks' culture on CIM, callusing explants were transferred to EIM for induction of embryogenic calli (EC). Clumps of EC were transferred to EMM for 4 weeks for maturation of somatic embryos (SEs). Clumps of well differentiated SEs were transferred to EGM1 for 4-6 weeks to induce germination. Then germinating SE clumps were subcultured on EGM2 for 4-6 weeks and then subcultured repeatedly on EGM3 at 4-6 weeks interval till they developed 3-4 pairs of leaves and 3-4 cm long roots. These plantlets were used for *ex-vitro* hardening.

For hardening, the plantlets were planted in Soilrite in plastic netpots and grown in polytunnels under high humidity and low light intensity. After hardening for 2 months in netpots, the plants were transferred to polybags containing soil and grown in nursery at reduced humidity and slightly higher light. After growing in the nursery for 5-6 months, the plants were established in the field.

### RAPD analysis of SEPs and mother plant

DNA was extracted from fresh leaves collected from the mother plant and 14 one year-old SEPs in the field using a modified SDS method. Each 20 µl PCR reaction mixture for RAPD comprised of 1X assay buffer, 14.5-20 ng decamer primer (Operon, USA), 1.25 mM of each of the dNTPs, 1.2 units of Taq DNA polymerase and 12.5 ng template DNA. Amplification was achieved in a thermocycler with the following program: initial denaturation at 94°C for 3 min, followed by 45 cycles of 1 min for 94°C, 36°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 5 minutes. The reaction mixture was electrophoresed and DNA bands were visualized by ethidium bromide.

## RESULTS AND DISCUSSION

### Production of somatic embryo-derived plants (SEPs)

On CIM the explants developed about 1.5 mm thick, compact, greenish-yellow primary callus along their edges in about a month. On EIM more than 90% of the explants developed cream coloured, highly friable, embryogenic calli (EC) from the primary calli in about 3 months (Figure 1A). The EC proliferated vigorously on EIM and differentiated high frequency somatic embryos (HFSE) in another 3 months (Figure 1B). When clumps of EC along with early stage somatic embryos (SEs) were cultured on EMM for 1 month, the SEs differentiated further, but differentiation of SEs was highly asynchronous.

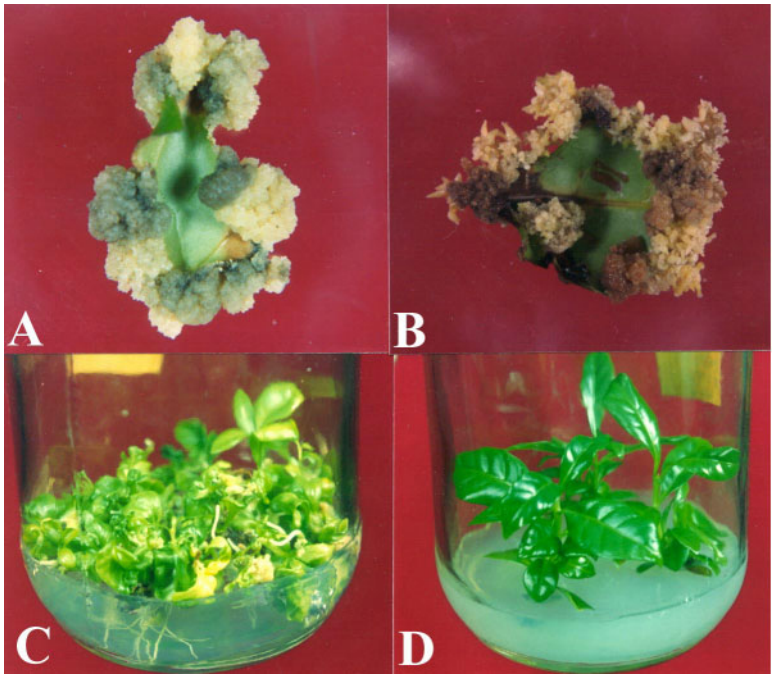


**Figure 1. Plant regeneration through somatic embryogenesis. A-embryogenic callus, B-SE differentiation, C-SE germination, D-SE- derived plants.**

The SEs germinated slowly on germination media (EGM1 to EGM3). By the end of 3rd subculture on EGM 3 many plantlets with 3-4 pairs of leaves and 3-4 cm long roots were produced (Figure 1C & D), which were used for *ex-vitro* hardening. After hardening for 2 months in Soilrite in netpots, the plants were transferred to soil in polybags and grown in nursery for 5-6 months. Then these plants were established in the field where they showed normal vegetative growth without any detectable morphological change.

### RAPD analysis

After initial screening of 25 arbitrary 10-mer primers, 16 primers that gave distinct bands were used to produce RAPD profile of the mother plant and 14 SEPs. The number of bands obtained with different primers ranged between 6 and 10. The 16 primers used in this analysis yielded 121 distinct bands, out of which 120 were monomorphic across all the 14 SEPs and the mother plant (Figure 2A), while one band of 1.4 kb produced with the primer OPA-14 was polymorphic in one SEP (Figure 2B).

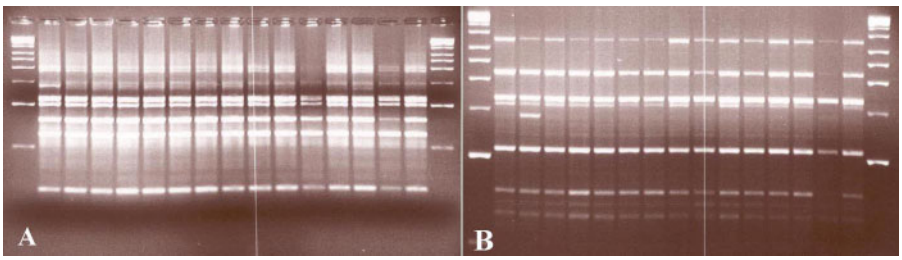


**Figure 2. RAPD profile of the mother plant & SEPs. A-monomorphic markers in all samples, B-one polymorphic marker in a SEP.**

We have used leaf tissues for regeneration of a CxR clone through HFSE. Rani et al. (2000) who studied somatic embryo-derived plants of *Coffea arabica* using RFLP, RAPD and ISSR markers found relatively low level of polymorphism in the nuclear genome, higher polymorphism in the mitochondrial genome and no polymorphism in the chloroplast genome. Our results indicate a low risk of genetic instability for micropropagation of robusta coffee using the protocol described here.

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# *Coffea arabica* L - A Compilospecies: Implications for Breeding

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## SUMMARY

There are two schools of thought regarding the origin and evolution of *Coffea arabica* L. the world's most important non-alcoholic stimulant beverage. The first or conventional school of thought based their inference on the evidence raised from observations on morphological, cytological, biochemical and reproductive biological features. The school proposes that *C. arabica* evolved by spontaneous hybridization of *C. eugenioides* with *C. canephora*, *C. congensis* or *C. liberica* and doubling of the chromosomes in the natural hybrid. An interesting offshoot of this thought is the belief that *C. arabica* is a segmental allotetraploid. The second or technological school of thought hypothesizes that natural hybridization of *C. eugenioides* or a sub-species by an unreduced gamete of *C. canephora* and spontaneous stabilization of chromosome number in the progeny of triploid hybrid resulted in the evolution of *C. arabica* on the basis of evidence from molecular marker studies. Evidence from some of the marker studies also suggests that *C. arabica* may be sharing considerable genomic homologies with *C. racemosa* and *C. congensis*. Molecular cytogenetic evidence also supports that *C. eugenioides* and *C. congensis* are the probable evolutionary parents of *C. arabica*. The distribution of *C. arabica* outside the area of distribution of all diploid species was attributed to the events of Pleistocene. Both schools agree that *C. eugenioides* or a sub-species of it is the most probable female progenitor of *C. arabica.* While they differ in the matter of male progenitor, in that, a species of the canephoroid group or liberio-excelsoides group is considered to be the likely male progenitor of *C. arabica*. Considering the genetical evidence from plant breeding studies, the disease resistance genes of *C. liberica* and *C. canephora* are inherited by *C. arabica*. All these available evidence points to the possible compilospecies nature of *C. arabica*. This has strong implications for the breeding practices, as inheritance patterns in compilospecies are considerably different from those observed in diploids and allopolyploids. These aspects are discussed and a possible breeding model with integration of vegetative selection to maintain traits of interest in the commercially exploited arabicoid derived materials is suggested. Utilization of unique tetraploid hybrids of interspecific origin like Ligenioides, Racemusta and Robarbica are proposed for use in breeding for resistance to coffee berry disease, insects and nematodes on the basis of their cytoplasmic genetic endowments.

## INTRODUCTION

Coffee is perhaps the single Third World commodity that has attained distinction in world trade of being the second largest traded commodity. While this is a position of pride, coffee trade in the recent times has suffered serious setbacks due to global surpluses (Van der Vossen, 2000), causing consternation in the small grower sector all over the world, as the cost of production could not be realized from the proceeds of sale. In this situation, reducing the cost of cultivation is most important. Present paper briefly reflects on the Arabica coffee breeding programmes and discusses the possibility of evolving materials that are likely to be less demanding by way of crop husbandry, on the basis of available evidence pointing to the possible compilospecies nature of *C. arabica*.

**What is a Compilospecies?**

Harlan and De Wet (1963) defined compilospecies as a species that is capable of annexing the genetic endowments of related species, sometimes to the extent of driving the species being annexed out of existence.

This definition implies that compilospecies are aggressively adaptable. The cultivation history of *C. arabica* is a pointer indicating the wide adaptation of this species. Thus, even though *C. arabica* is a native of the Southwestern highlands of Ethiopia, it survived the hostile climate of Yemen where the Arabs who controlled the world coffee trade for over three centruries took it. Arabica not only survived but flourished in the various colonies of almost all European powers spread across the globe from Far East, South East Asia and Latin America and the Oceania besides the home continent of Africa contributing to the present day crisis caused by over production.

**Origin of *Coffea arabica***

Various early studies indicated the possible involvement of *C. eugenioides* and *C. canephora*, *C. congensis*, *C. stenophylla, C. dewevrei* or *C. liberica* as the parent species in the origin of *C. arabica* (Carvalho et al., 1969; Narasimhaswamy, 1962; Narasimhaswamy and Vishveshwara, 1967; Ram and Sreenivasan, 1981; Reddy et al., 1984; Charrier and Berthaud, 1985; Eskes, 1989). Molecular marker studies confirmed that *C. eugenioides* or a closely related sub-species is the most likely female progenitor of *C. arabica* on the basis of organellar DNA analysis (Berthou et al., 1983; Lashermes et al., 1996b) and *C. canephora* or a sub-species is strongly suggested to be the probable male contributor on the basis of DNA markers (Lashermes et al., 1995, 1996a, 1997, 1999; Orozco-Castillo et al., 1996). *C. eugenioides* and *C. congensis* were considered to have contributed genomes to *C. arabica* on the basis of genomic in situ hybridization analysis (Raina et al., 1998). RAPD and microsatellite marker studies also indicated that many other species of *Coffea* possess DNA that is homologous/ homeologous that of *C. arabica* (Combes et al., 2000; Ram and Sreenath, 2000a, b). Ligenioides, an amphiploid derived from an $F_1$ hybrid of *C. liberica* and *C. eugenioides* by natural doubling of chromosomes is observed to have large genetic similarity with Hibrido de Timor (Ram et al., 2000, 2002). Considering the diverse origin of these two interspecific hybrids it was inferred that *C. liberica* and *C. eugenioides* also carry substantial genomic homology with *C. canephora* and *C. arabica* the parents of HDT. Arabica was shown to have inherited the rust resistance genes of *C. liberica* ($S_H$3) and *C. canephora* ($S_H$6,7,8,9) (Rodrigues et al., 1975; Eskes, 1989; Bettencourt et al., 1992) and support this inference.

The foregoing discourse indicates that *C. arabica* has the capacity to assimilate genes from many species of *Coffea* diploid gene pool and thus can be classified as a compilospecies that has emerged in a contemporary climatic situation from a species complex with low adaptive and survival value in the location of its origin, the Southwestern high lands of Ethiopia. A recent origin in the quaternary period was suggested for *C. arabica* on the basis of ribosomal DNA (rDNA) sequence data (Lashermes et al., 1995, 1997) and its distribution outside the area of distribution of diploid species on the basis of Pleistocene events (Lashermes et al., 1999).

Free intercrossing of different species of *Coffea* to form reasonably fertile hybrids was reported (Narasimhaswamy and Vishveshwara, 1967; Carvalho and Monaco, 1967; Charrier, 1978; Louarn, 1993). Cases of natural doubling of chromosomes in a hybrid of *C. liberica* x *C. eugenioides* and spontaneous occurrence of tetraploids in the descendants of the

interspecific hybrids of *C. liberica* x *C. eugenioides*, *C. excelsa* x *C. eugenioides* and *C. racemosa* x *C. canephora* were also reported (Narasimhaswamy, 1962; Narasimhaswamy and Vishveshwara, 1967; Reddy et al., 1991). These phenomena are important in theorizing the origin of *C. arabica* as many of these tetraploids resemble *C. arabica* and some intercross with *C. arabica* giving rise to moderately fertile hybrids (Ram et al., 2002). These events point to the possible participation of more than two species in the evolution of *C. arabica* either at the time of its birth or subsequently by the inheritance of their characters through interspecific hybridization. Genetic conversion reported in the interspecific hybrids of *C. liberica dewevrei* x *C. pseudozanguebariae* (Ky et al., 2000) is a significant contribution towards understanding the evolution of *C. arabica*. Given the present knowledge that *C. arabica* is of recent origin by interspecific hybridization and allopolyploidy in the vegetation flux of Pleistocene, the following hypothesis appears to be appropriate.

Prior to Pleistocene, all species of *Coffea* were probably diploid and occupied a focus area in what is present equatorial East Africa. From this focus, the young genus would have started undergoing the various processes of organic evolution such as migration, adaptation and different modes of isolation (Stebbins, 1950) to give rise to the presently recognized species. Molecular evidence points to the possible differentiation of the main clusters of evolution in the genus *Coffea* before the appearance of *C. arabica* (Berthou et al., 1983). At this point of time in evolution, the violent events of Pleistocene would have been experienced by these evolving species, some of which probably came to exist together in environments that are not very conducive for their survival (such as Southeastern Sudan and Southwestern Ethiopia). Thus, a new trend of evolution through interspecific hybridization would have got initiated and followed by polyploidy to give rise to diverse tetraploids whose interbreeding in the new habitat would have resulted in the formation of *C. arabica*.

The great variability of Ethiopian Arabicas recorded by the earlier investigators (Monaco, 1965; Sylvain, 1955) and confirmed by the modern molecular studies (Lashermes et al., 1995, 1996c) is consistent with the possibility of *C. arabica* being a compilospecies.

**Implications for Breeding**

*C. arabica*'s being a compilospecies has important implications for the methodology of breeding this very important species that is sustaining the economies of many developing countries.

Several genes of *C. arabica* were shown to follow a disomic inheritance as is typical of all diploid species (Krug and Carvalho, 1951; Carvalho et al., 1991). This behaviour probably holds good to the extent of selection and breeding utilizing the many biotypes of *C. arabica* without crossing the boundaries of this species. However, this could not be so on account of the relatively narrow genetic base of *C. arabica* and possible limited variability concerning the traits of interest such as pest and disease resistance. Prospecting for resistant types revealed the presence and variability of these traits in the various species of diploid constellation and the spontaneous tetraploid interspecific hybrids prompted the temptation to transfer these characters from diploid species to *C. arabica*.

Recently, tetrasomic inheritance of characters, using molecular markers, was reported in Arabusta hybrids (Lashermes et al., 2000). This is positive evidence that *C. arabica* would not behave like a diploid when interspecific hybrids are involved in breeding. The foregoing discussion indicates that simple methods of plant breeding are not adequate for breeding *C. arabica* as it manifests diverse behaviour in different genetic contexts. The following model is suggested to take care of the compilospecies behaviour.

**Breeding Strategies**

At present, Arabicoid germplasm carrying diverse traits of interest from the species of the secondary and tertiary gene pools are available in various countries where research efforts are devoted towards the development of interspecific hybrids. It is important that these be deployed in breeding programmes to derive types suitable for niche environments. Indian experience with three Robusta-Arabica hybrids is that when pure Arabicas are to be improved by involving them it is best to exploit the $F_1$ hybrids or the immediate next generation derived from selected $F_1$ individuals to counteract the effects of modifier and epistatic genes and loss of target genes through genetic conversions. This was advocated by other breeders also (Charrier, 1982; Van der Vossen, 1985).

Besides the above, in India, there are novel tetraploid hybrids that can be of potential application in breeding Arabicas resistant to diseases and pests (Ram et al., 2004, in preparation). Thus, Ligenioides was found to cross freely with a variety of Arabicas to give rise to fertile hybrids. Ligenioides manifested considerable resistance to CLR and white stem borer (Ram et al., 2002; Ram, 2003). $F_1$ hybrids of Ligenioides x HDT manifest high resistance to CLR, improved bean size and cup quality (Ram et al., 2004, in preparation). In $F_2$, segregation towards susceptibility was recorded. Thus, exploiting $F_1$ hybrid by vegetative selection is envisaged for this material. Another interesting material is a hybrid derived from the cross of *C. racemosa* and *C. canephora*. $F_1$ hybrids of these species are diploid (2n = 22) and highly sterile (Ram et al., 1981). However, a few open pollination derived seed gave rise to Arabica-like tetraploid plants (Reddy et al., 1991), which are also self-compatible. It is observed that this hybrid also crosses freely with Arabicas and Arabicoids to yield hybrids. These are being studied and are a potential source of resistance to CLR, nematodes, leaf miner and possibly abiotic stress. Several tetraploids were identified in the open pollination derived progenies of *C. excelsa* x *C. eugenioides*. These plants are a potential source of resistance against a variety of adversaries. These are now being studied for their possible utility in Arabica breeding.

Another interesting possibility of the use of these novel materials is in the context of breeding for resistance against coffee berry disease (CBD) that is presently endemic in the African countries. Most of the Arabica coffee grown in these countries is of the varieties derived from Kents and Bourbon. These varieties are pure Arabicas without any introgression of genes from diploid species. Considering the possible maternal ancestry of *C. eugenioides* in the evolution of *C. arabica*, all pure Arabicas are expected to carry a large cytoplasmic contribution from this species. Thus, the very first appearance of CBD on *C. eugenioides* and its subsequent spread to *C. arabica* (Mogk, 1975; Van der Vossen, 1985) reflect a case of cytoplasmic uniformity rendering the coffee plant varieties vulnerable to CBD pathogen very similar to that of T-cytoplasm and Southern blight of Corn (Levins III, 1990). Novel Indian coffee hybrids carry the cytoplasmic components of *C. liberica* (in Ligenioides), *C. racemosa* (in Racemusta) and *C. canephora* (in Robarbica) and offer a new alternative in breeding for CBD resistance.

From the foregoing discourse, it is evident that tetraploids can be obtained from the hybridization of various diploid species of Erythrocoffea (Canephoroid group) and Pachycoffea (Liberio-Excelsoid group) with several Mozambicoffea (*C. eugenioides* and related species). These tetraploids offer considerable natural protection against adversaries like diseases (CLR and CBD), pests (nematodes and insects) and abiotic stress by integrating them in the Arabica breeding programmes. Inherent resistance of the materials emanating from such breeding programmes renders Arabica cultivation relatively less demanding and more attractive.

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# Discontinuous Distribution of Caffeine Content among Wild *Coffea* Species Beans

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## SUMMARY

Previous evaluations of caffeine content have already focused on wild species of coffee trees, but this assessment included six new taxa from Cameroon and Congo and involved a simplified method that generated more accurate results. This new study allowed to show that discontinuities existed in the caffeine content range and that Cameroon and Congo can be a centre of diversity for caffeine content.

## RÉSUMÉ

Cette nouvelle étude de diversité de teneur en caféine chez des espèces sauvages comporte six nouveaux taxons provenant du Cameroun et du Congo et a été réalisée à l'aide d'une méthode d'extraction simplifiée. Elle permet de montrer qu'il existe une discontinuité dans le contenu en caféine et que le Cameroun et le Congo pourraient être un centre de diversité pour le contenu en caféine.

## INTRODUCTION

Caffeine is a secondary metabolite accumulated in green coffee beans. This purine alkaloid is of great interest in coffee because of its impact on beverage quality. This explains current research initiatives aimed at identifying genes involved in its biosynthesis (Mizuno et al., 2003; Uefuji et al., 2003) or obtaining genetically modified varieties with low caffeine content (Ogita et al., 2003). Coffee trees *stricto sensu* belong to the sub-genus *Coffea.* More than 80 wild species have been described, including two cultivated species, i.e. *C. arabica* L. and *C. canephora* Pierre. In green coffee beans, caffeine content (CAF) varies markedly between species, from 0% dry matter basis (dmb) in *C. pseudozanguebariae* to 2.5% dmb in *C. canephora* (Anthony et al., 1993). CAF also varies within species (Ky et al., 2001). Most of the variation (about 94%) is under genetic control (Barre et al., 1998; Montagnon et al., 1998).

In this paper, we present a new biochemical evaluation of caffeine content diversity using 21 diploid species (six new undescribed taxa from central Africa were added) and a simplified method for caffeine extraction.

## MATERIAL AND METHODS

Coffee cherries were harvested at full maturity on four accessions of 15 diploid species and six undescribed taxa of different geographical origin (Table 1) which are field maintained at the IRD coffee research station (Man, Côte d'Ivoire). They were depulped using the wet processing method, desiccated on silicagel and 50 green beans per tree were frozen in liquid

nitrogen, prepared for caffeine extraction and analysed by HPLC-DAD as already described (Campa et al., 2004).

All results were analysed using the Statistica software package (5.1 version, 1997 for Microsoft Windows). Each tree was represented by its mean caffeine content. The statistical analysis only concerned between-species variations which were tested using a one-way ANOVA. A Newman and Keul's test was carried out for multiple mean comparisons.

**Table 1. Geographical origin of *Coffea* species and taxa.**

| **Species and taxa** | **Geographical origin** |
|---|---|
| *C. brevipes* | Cameroon |
| *C. Canephora* | Côte-d'Ivoire |
| *C. congensis* | Congo Democratic Republic |
| *C. eugenioides* | Kenia |
| *C. heterocalyx* | Cameroon |
| *C. humblotiana* | Comores |
| *C. humilis* | Côte-d'Ivoire |
| *C. kapakata* | Angola |
| *C. liberica dewevrei* | Central African Republic |
| *C. liberica* Koto | Cameroon |
| *C. liberica liberica* | Côte-d'Ivoire |
| *C. pseudozanguebariae* | Kenia |
| *C. racemosa* | Tanzania |
| *C. salvatrix* | Tanzania |
| *C. pocsii* | Tanzania |
| *C. stenophylla* | Côte-d'Ivoire |
| *C.* sp Bakossi | Cameroon |
| *C.* sp Congo | Congo Democratic Republic |
| *C.* sp Ncongo 2 | Congo Democratic Republic |
| *C.* sp Moloundou | Congo Democratic Republic |
| *C.* sp N'koumbala | Cameroon |

## RESULTS

For the 21 species analysed, caffeine content in green beans ranged from trace amounts (< 0.01%) in *C. pseudozanguebariae* to 2.64% in *C. canephora* (Figure 1). The detection of trace amounts of caffeine (<0.01%) in green beans of species previously classified as caffeine-free (Charrier et Berthaud, 1975) was certainly due to the simplified extraction method used (Barre et al., 1998), which limits yield loss and permits detection of lower caffeine concentrations.

ANOVA showed significant between species differences ($F_{20,61} = 327$; $p < 0.001$), representing 94.3% of the total variance. Transformed data were used in the analysis [$y = \log(x + 0.03)$], as the variance was related to the mean.

Four classes were distinguished. The first class (CAF1) corresponded to the group of caffeine-free species described previously. Classes CAF2, CAF3 and CAF4 included species whose caffeine content was formerly considered as a quantitative trait, with a continuous distribution ranging from 0.5% dmb in *C. eugenioides* to 2.4% dmb in *C. canephora*. This new qualitative

distribution in four classes could be due to the increased number of species taken for this analysis.

Caffeine content was found to increase two-fold from CAF2 (0.54% dmb) to CAF3 (1.07% dmb) and from CAF3 to CAF4 (2.31% dmb). This kind of evolution cannot be explained by the bottleneck hypothesis. The best hypothesis seemed to be a genetic control of the caffeine content, as genes controlling CAF are known to have multiplicative effects (Barre et al., 1998). This was confirmed here by the relationship between variance and mean.

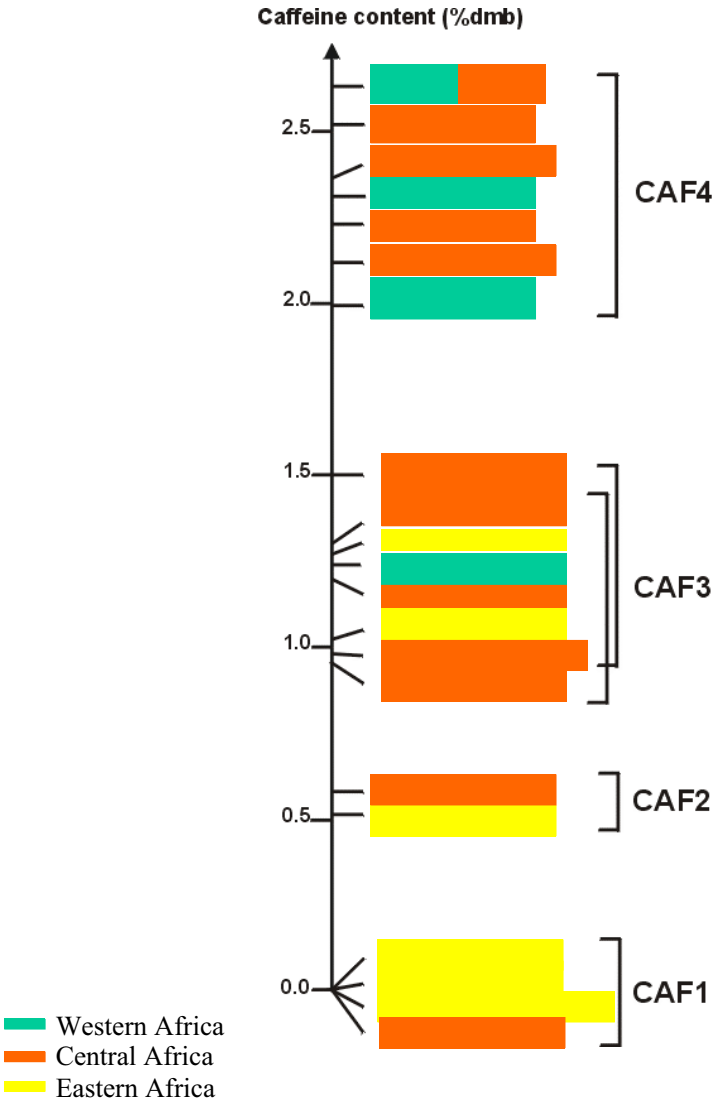


**Figure 1. *Coffea* clusters for caffeine content (expressed in %dmb). Brackets indicate Newmann and Keuls test results.**

In East Africa, caffeine content (CAF) in beans ranged from trace amounts in *C. pseudozanguebariae* to 1.3% in *C. pocsii*. In West Africa, CAF ranged from 1.25% in *C. liberica* subsp. *liberica* to 2.6% in *C. canephora*. By contrast, in Cameroon and Congo, CAF ranged from trace amounts in *C.* sp. Bakossi to 2.6% in *C. canephora* and seemed to cover the whole variation range. This clearly shows that Central Africa is a centre of diversity for caffeine content.

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# Fingerprinting of Indian Coffee Selections and Development of Reference DNA Polymorphism Panels for Creating Molecular IDs for Variety Identification

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## SUMMARY

There are two main cultivated species of coffee namely, tetraploid *C. arabica* and diploid *C. canephora* (also called robusta coffee). A number of breeding programs were initiated in India at Central Coffee Research Institute, Chikamagalur, with main thrust being building resistance coupled with productivity without affecting quality in arabica and improvement of bean quality and yield in robusta. Such efforts spread over many decades resulted in the development of 16 superior selections of coffee (14 arabica and 2 robusta selections). The present study was undertaken to fingerprint these station-bred selections using high-resolution DNA marker approaches for their individualization and to ascertain their genetic base. DNA typing was performed using fluorescence-AFLPs, RAPDs and a large number of in-house developed coffee-specific microsatellite markers. The data revealed very limited variability in arabica genotypes, which could be visualized for their discrimination only by employing a very large number of DNA markers. The fingerprinting data were analyzed to create reference 'DNA polymorphism panels' (Microsatellite, AFLP, RAPD-based) which can readily be used for: a) generating 'Molecular IDs' needed for genotype individualization/registration/IPR protection; b) selecting genetically diverse genotypes and suitable/efficient marker approaches for breeding programs; and c) other genetic studies. In our opinion, this study involving DNA fingerprinting of Indian coffee selections, in persepctive strongly suggests the need for creation of molecular data banks (enumerating DNA marker efficiencies and DNA polymorphism status) for globally available elite coffee germplasm for use as reference resources. Availability of such resources would increase the practical utility and efficiency of the DNA typing approaches that are exorbitantly costly but are highly desired in coffee genetic improvement programs, especially on arabica coffee that inherently suffers with low genetic base.

## INTRODUCTION

Coffee, a commodity of great importance in global trade, is cultivated in over 50 countries and revenue from this beverage crop contributes substantially to the national exchequer of the producing countries. The commercial coffee production relies on only two species *C. arabica* (Arabica coffee) and *C. canephora* (Robusta coffee), which accounts for 70% and 30%, respectively, of world production. *C. arabica* is the only self-compatible tetraploid species of the genus characterized by low genetic diversity which has been attributed to its allotetraploid origin, reproductive biology and evolution process (Lashermes et al., 1996). In contrast, considerable variability was reported among diploid species that form valuable gene reservoir for different breeding purposes (Berthaud and Charrier, 1988). Indian coffee improvement programs to develop elite cultivars of both arabica and robusta, initiated in early 20$^{th}$ century

at the Central Coffee Research Institute (CCRI), Balehonnur, have resulted in many superior coffee selections (14 of arabica and three cultivars of robusta) that are widely grown in the country.

High cost of development and need for germplasm protection, have increased the need for reliable methods of cultivar/variety identification, which in turn has served as the driving force for development of more reliable and cost effective approaches for genotype individualization. Traditionally, it is the morphological markers that have been for cultivars identification and purity. However, in recent past, molecular characterization (DNA fingerprinting) using DNA markers has become popular as it provides quicker and accurate assessment of genetic structure of the individual genotype without the confounding effect of environment, and high-resolution of discrimination and reliability. In coffee, potential of DNA marker technology is now being increasingly realized viz., germplasm characterization and genetic diversity analysis (Lashermes et al., 1996; Anthony et al., 2002; Steiger et al., 2002), analysis of alien genome introgression (Prakash et al., 2002; Herrera et al., 2002) and identification of markers linked to the resistance genes (Noir et al., 2003;P Prakash et al., 2004). In this paper we report the molecular characterization of Indian coffee selections using RAPD (Randomly Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and in-house developed microsatellite markers for generating molecular IDs for their individualization.

## MATERIAL AND METHODS

The plant material used for analysis comprised of 16 station-bred selections (14 arabica and 2 robusta) developed at the Central Coffee Research Institute, India. Genomic DNA was isolated from fresh leaves as described by Aggarwal et al., (2002), and used for molecular characterization using three DNA typing approaches i.e., RAPD, f-AFLP and microsatellites. A total of 37 random decamer primers, 7 f-AFLP primer-pairs (each primer having 3-selective bases) and 150 in-house developed microsatellite markers were used for analysis. The procedures followed and PCR conditions used for AFLP and Microsatellite markers were as described by Aggarwal et al. (2002) and Baruah et al. (2003) respectively.

## RESULTS AND DISCUSSION

The DNA typing of the Indian-bred selections revealed that, irrespective of the marker approach used (RAPD, SSR and AFLP), the DNA profiles were very distinctive between diploids and tetraploids and the polymorphism was always more for diploid robustas. The RAPD analysis with 37 random decamer primers generated a total of 690 markers of which 80% markers were polymorphic among the 16 genotypes tested. The AFLP assays with 7 primer pairs generated 320 markers out of which 69% were polymorphic. Majority of the polymorphism revealed with these two multi-locus DNA typing approaches was accounted by diploid robusta selections (61% for RAPDs and 51% in case of AFLPs) as against only 18-19% in tetraploid arabica selections (Figure 1). Almost similar level of polymorphism was seen with microsatellite markers, wherein 23% markers were found to be polymorphic when only the tetraploid selections were considered compared to 57% being informative in case of the 2 diploid robustas (Figure 1).

Significant differences were observed between different RAPD primers, AFLP-combinations and microsatellite markers with respect to the informative polymorphism revealed by them. A number of these marker/primer combinations were found to be completely monomorphic (and thus uninformative) for the materials analyzed (especially arabica selections) and thus of low to negligible utility for the purpose of germplasm characterization.

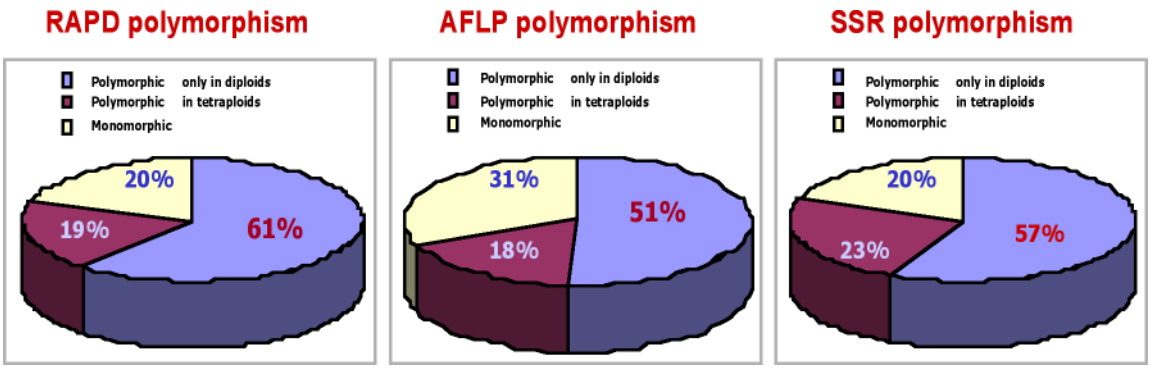


**Figure 1. Distribution of DNA polymorphism seen in station-bred arabica (tetraploid) and robusta (diploid) selections using 3 different DNA typing approaches.**

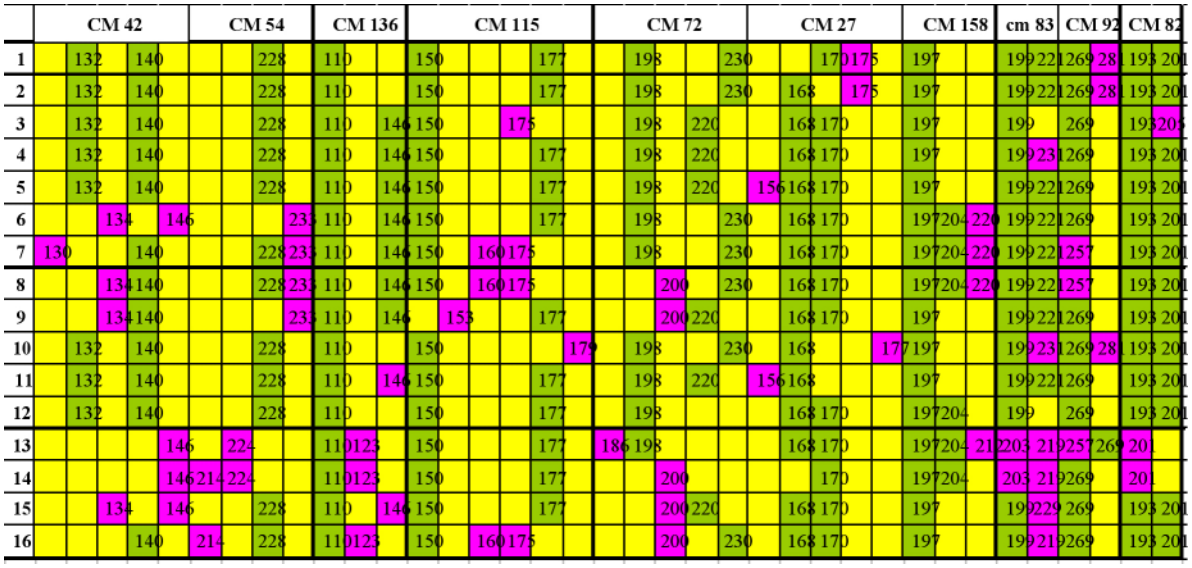


**Figure 2. Part of 'reference polymorphism panel' showing allelic distribution (across 10 in-house developed coffee specific microsatellite markers) for 16 station-bred coffee selections developed in India. Note that each of the selection can be uniquely individualized in the panel. Sixteen coffee selections are: S.288, S.795, Sln-4 (Agaro), Sln-4 (Ciocee), Sln-5A, Sln-5B, Sln-7.3, Sln-8, Sln-9, Sln-10, Sln-11, Sln-12, S-274, CxR, Sln-4 (Tafarikela) and Sln-6.**

Although genetic variation among arabica cultivars was limited, the DNA polymorphism obtained was found to be sufficient to distinguish and individualize the arabica genotypes studied. The fingerprinting data were also analyzed to identify the most informative markers (primer combinations/approaches) that could distinguish the coffee selections especially the arabicas. Of the many informative RAPD primers, a sub-set of 6 primers could be identified that discriminated all the coffee selections. Similarly, 4 of the 7 AFLP primer-combinations were identified that could resolve all the genotypes. Further, compared to RAPDs and AFLPs, although relatively a lower proportions of the ~150 tested microsatellite markers were informative for arabicas, but these were relatively more efficient to discriminate all the 16 selections (e.g., a sub-set of 6 of the ~30 microsatellites that showed some polymorphism among the 14 arabica selections, could reliably distinguish all of them).

To increase the long-term utility of the DNA fingerprinting analysis, all the DNA marker data were used to create 'Reference DNA polymorphism Panels', cataloguing allelic/marker distribution revealed by different DNA typing approaches for all the coffee genotypes. These

panels can be used to generate 'Molecular IDs' for genotype individualization and for other advantages in programs of genetic improvement, such as, selection of suitable/diverse parental materials and also most informative DNA marker(s) for linkage analysis avoiding the need for 'parental survey'. A representative 'Reference panel' based on the allelic polymorphism data across 10 coffee specific microsatellite loci (developed at CCMB, Hyderabad) suitable for identifying/distinguishing the CCRI station-bred selections is shown in Figure 2.

## CONCLUSIONS AND PERSPECTIVES

The present study demonstrated that DNA markers could be used reliably for identification of the coffee germplasm and that the microsatellites are the most efficient and desirable markers for the purpose. The data obtained can be used for ascertaining the genetic diversity in the available cultivated and wild exotic germplasm of coffee. The present study also highlights the overall low efficiency of these otherwise high-resolution approaches for coffee genepool characterization. The data clearly establish that despite their high-genetic resolution, DNA typing need to be carried out at large scale to resolve the low variation inherent in the coffee genepool especially of arabica, thus making the whole exercise resource intensive and practically non-viable. The later constrain warrants development of ways and means to increase the efficiency of DNA markers based expensive but unavoidable approaches for coffee genetic analysis. An easy way to achieve this can be creation of molecular data banks enumerating DNA marker (approach) efficiencies and more importantly, DNA polymorphism status (using defined markers/guidelines) for the analyzed germplasm, for use as reference resource. Based on our experience, we propose construction of 'Reference DNA polymorphism databases' of elite coffee germplasm available world-wide using standard repeatable markers (such as microsatellites) that can then be used by the coffee geneticists/breeders community for various advantages namely, for: a) better management, utilization, registration and IPR protection of elite coffee germplasm; b) selection of suitable material/genotypes and informative DNA markers for breeding and linkage analysis; c) exchange of germplasm, etc.

## ACKNOWLEDGEMENTS

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# Development and Characterization of Coffee Specific Microsatellite Markers for Use as Potential Genetic Markers

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## SUMMARY

Availability of informative genetic markers is prerequisite for effective genetic-linkage studies. Recent advances in molecular biology have led to the development of a plethora of DNA variation based efficient marker systems that promise-impetus, dependability and directionality to the genetic improvement efforts. Among various types of DNA markers the short-sequence repeat (SSR) based microsatellite markers have proven to be the most desirable for genetic studies due to their codominant nature, stability, abundance, sensitivity, ease and speed of analysis, minimal sample requirements and suitability for automation. Despite their potential and desirability, very few such markers have been developed and described in the literature for coffee tilldate. Under the first Indian initiative on Coffee Genomics, we have been able to develop 150+ new coffee microsats that provide potential genetic markers for germplasm characterization and molecular linkage studies in coffee.

For development of microsatellite markers, small-insert, partial, genomic libraries (comprising ~ 75,000 plasmid clones) were constructed from total DNA of *Coffea arabica* var.HdeT (Hibrido-de-Timor) and *C. canephora*. These libraries spotted on high-density nylon filters were screened for SSR positive clones through Southern hybridization with different synthetic oligonucleotide repeat probes. Based on hybridization signals, a large number of putative repeat-positive clones were selected and sequenced. The sequence data revealed relatively low abundance of SSR motifs in coffee genome, with AG di-repeat being most frequent. The clone sequences having 18+ bp repeat-motifs were used for designing PCR primers, which were subsequently validated using panels of elite coffee genotypes for their suitability as genetic markers. In total > 170 informative markers could be developed that also showed broad cross-species transferability. It is hoped that this new set of markers would serve as an important resource for coffee genomics.

## INTRODUCTION

Coffee is an important plantation crop belonging to the genus *Coffea* of the family Rubiaceae. Although, > 100 species of coffee are known, its commercial cultivation relies only on two species, amphidiploid *Coffea arabica* L. (2n = 4x = 44) and diploid *C. canephora* Pierre (2n = 22). Considerations like sustainability over ever-changing demands of agro-climatic conditions and the commercial markets calls for continuous efforts to develop better coffee genotypes. The conventional breeding efforts to this end, especially in arabica coffee have been seriously constrained mainly due to the narrow genetic base of the arabica genepool, lack of efficient breeding tools and linkage maps, long pre-bearing periods and difference in ploidy level. Recent advances in DNA marker technology offer possibilities to overcome

some of these limitations by providing highly informative genetic markers and marker assisted breeding.

Out of various kinds of DNA markers, PCR-based microsatellites are most desirable for germplasm characterization and crop improvement and are widely used for genetic analysis, development of linkage maps and population improvement by employing marker aided selection techniques (Gupta and Varshney, 2000). Despite these advantages, only ~150 microsatellite markers have been reported till to date for coffee (Baruah et al., 2003; Bhat et al., 2004), signifying the need for expanding the repertoire of these genetically highly informative markers for efficient management and improvement of coffee germplasm resources. Under the first Indian initiative on Coffee genomics, at our center we have initiated efforts for development of microsatellite markers to complement genetic studies on coffee and this paper deals with the leads achieved in this regard.

## Material and Methodology

We have used the conventional approach involving construction and screening of genomic library for development of SSR-markers (Sambrook et al., 1989). For the purpose, small-insert partial genomic libraries were constructed from genomic DNA of two coffee cultivars, *C. arabica* var.HdeT and *C. canephora* var.sln-274. Genomic DNA used for library construction was isolated from leaves as detailed by Aggarwal (2002). Ten micrograms of total genomic DNA was restriction digested with *Sau*3aI (NEB) in case of *C. arabica* and double digested with *Rsa* I and *Hae* III (NEB) restriction enzymes in case of *C. canephora*. Digested DNA samples were size-fractionated on agarose gel and fragments in the range of 500 to 1500 bp were eluted using GFX column (Amersham Pharmacia). The eluted DNA fragments were cloned in plasmid vectors pBKS(+) and/or pMOS (Amersham Pharmacia) and transformed into electro-competent *E. coli* (DH10B) cells by electroporation. The recombinant clones were selected by blue-white selection using X-gal (@ 40 μg/mL) and IPTG (@ 20 μg/mL). The individual white recombinant colonies were then curetted into 96 or 384 well microtitre plates in 10% glycerol LB-ampicillin medium, grown overnight and stored at –70°C till further use. The curetted clones were spotted on nylon membrane using 96-/384- pin replicators (Nunc Technologies) to prepare high-density library filters for screening and identification of SSR-positive clones by Southern hybridization with labeled-oligo probes.

The genomic libraries were screened using different combinations of di-, tri- and tetra-repeat SSR oligos of 30-mer lengths that were synthesized in-house (on DNA/RNA Oligosynthesizer394, Applied Biosystems) and 5`-end labeled using $\gamma$–$P^{32}$-dATP radionucleotide using Polynucleotide Kinase (NEB). The oligos used for library screening were selected based on their relative abundance in the coffee genome (our unpublished data). All library filters were screened using 9 oligoprobes, namely, $(CA)_{15}$, $(GA)_{15}$, $(CAA)_{10}$, $(GGT)_{10}$, $(ATT)_{10}$, $(AGG)_{10}$, $(AGA)_{10}$, $(ACT)_{10}$ and $(CATA)_8$, but *C. canephora* library was also screened using 5 additional oligoprobes i.e., $(GAC)_{10}$, $(CAT)_{10}$, $(CTG)_{10}$, $(CGG)_{10}$, and $(GATA)_8$. Hybridizations were performed in 6 x SSC at 55 °C for 12-14 hrs and filters were washed at relatively high increasing stringency till the counts have reduced significantly. The hybridized filters were exposed to phosphor imager for different lengths of time (usually 4-8 h) and scanned to identify probe-hybridized, SSR-positive clones. Plasmids were isolated from the positive clones using alkaline lysis method, and the cloned inserts were amplified and sequenced using M13 universal primers. Each clone was sequenced for both strands using BigDye terminator kit (ABI) on automated DNA sequencer ABI3700 (Applied Biosystems).

The sequences were searched using microsatellite search tool MISA (Theil et al., 2003) for ascertaining frequency and distribution of SSRs having a minimum number of 12 repeat for mono, 6 for di, 5 for tri, tetra, penta and hexamer motifs. The clones found positive for SSR repeat length of at least 18-mer were used for designing primers and marker development. The primers were designed using Primer 3 (http://www-genome.wi.mit.edu/genome_software/other /primer3.html) or GeneTool Lite version 1.0. All primer pairs were used to standardize the PCR-amplification conditions and then the working pairs were tested for their utility as potential genetic markers using panels of elite genotypes/accessions of *C. arabica*, *C. canephora* (robusta) and 17 species of *Coffea* and related genera *Psilanthus*. In general, PCR amplifications were carried out using ~10 ng genomic DNA at 2 mM $MgCl_2$ and annealing temperature ranging between 55 - 60°C. Microsatellite alleles were resolved by GeneScan analysis on ABI-377 DNA sequencer and characterized using the software Genotyper 2.1 (Applied Biosystems).

## RESULTS AND DISCUSSION

More than 1300 genomic clones putatively positive for SSR-motifs were identified in Southern hybridization based screening of the two libraries, of which > 1000 clones were sequenced for both the strands. The summary of the marker development efforts is summarized in Table 1, which clearly shows that the genomic-library approach is resource intensive but reasonably good success can be achieved if the hybridization based screening is done appropriately.

*In silco* analysis of the sequenced clones for ascertaining frequency and distribution of the SSR repeats revealed that the di-repeats were the most abundant in the coffee genome followed by the mono-, tri- and tetra-/higher-order repeats in the decreasing proportion. Significantly, the spread of repeats were broadly comparable in the two species despite their ploidy differences; the only exception being a relatively higher proportion of tri-repeats in the diploid *C. canephora*. Overall, AG repeats were the most abundant among the di-repeats whereas poly-A characterized the mononucleotide stretches (Figure 1). Among the tri-repeat motifs for which both the libraries were screened, ACT, AAT and ACC were more abundant than the others, but in case of *C. canephora* library which was additionally screened for three other trirepeat motifs, the AGC repeats were the most abundant. The data also revealed many SSRs in the sequenced clones that were not used for screening the genomic libraries, e.g., AT repeats, stretches of mononucleotides etc. In general, these were seen in clones that also had parts of targeted repeats, thus indicating random overlapping distribution of SSRs in the coffee genome and the good quality of the genomic libraries generated in the study.

Hybridization based screening was quite efficient as 55% putatively selected clones were found positive for SSRs on sequencing. Of these, a large number had a repeat core length of > 18 bp, an important attribute indicative of the suitability for possible conversion to an informative microsatellite marker. These short-listed clone sequences were used to design 219 primer pairs of which 173 pairs could be successfully converted to microsatellite markers. Detailed validation studies carried out using panels of arabica/robusta genotypes and related species representatives indicated their suitability for use in genetic studies, as well as broad cross-species transferability. In general, the newly developed microsatellites revealed an overall low heterozygosity among arabica genotypes but considerable allelic variability in the robustas genepool. These observations are strongly in agreement with the earlier studies on the genetic status of the coffee genepool that clearly show a much narrow genetic base of the arabicas compared to robustas. Also, the genetic diversity seen using the new microsats is largely comparable with that seen using other DNA marker approaches (our unpublished data). Further, in comparison to the cultivated genepool, the new markers were found much

more polymorphic in the related taxa (*Coffea* and *Psilanthus* spp.) with very broad cross-species specificities. The data thus suggest the potential of the new markers for genetic analysis of coffee especially its secondary genepool for conservation, management, resolving taxonomic relationships, and even more importantly for their use as efficient, informative genetic landmarks on interspecific molecular linkage maps being developed for genetic improvement of coffee.

**Table 1. Summary statistics of the work done for development of microsatellite markers using genomic libraries of coffee.**

| | Parameters | *C. arabica* | *C. canephora* |
|---|---|---|---|
| 1 | Total number of clones picked -up from HDT library | 50,000 | 18,000 |
| 2 | Total no.of clones screened | 50,000 | 15,744 |
| 3 | Total no.of clones selected from primary screening | 5,400 | 1200 |
| 4 | Total no.of clones selected from secondary screening | 850 | 446 |
| 5 | No. of Clones sequenced | 711 | 310 |
| | Size of genome sequenced | 580686 $_{bp}$ (~0.58 $_{Mb}$) | 266600 $_{bp}$ (~0.27 $_{Mb}$) |
| | Range of the clone size | 300 $_{bp}$ to 3.0 $_{kb}$ | 280 $_{bp}$ to 2.6 $_{kb}$ |
| | Average size of the clones | 830 bp | 860 bp |
| 6 | No. of clones with repetitive motif >18bp | 320 | 148 |
| 7 | No. of clones used for primer design flanking the repeats | 220 | 59 |
| 8 | No.of primer pairs synthesized (till date) | 160 | 59 |
| 9 | No. of primer pairs validated as SSR markers | 122 | 51 |

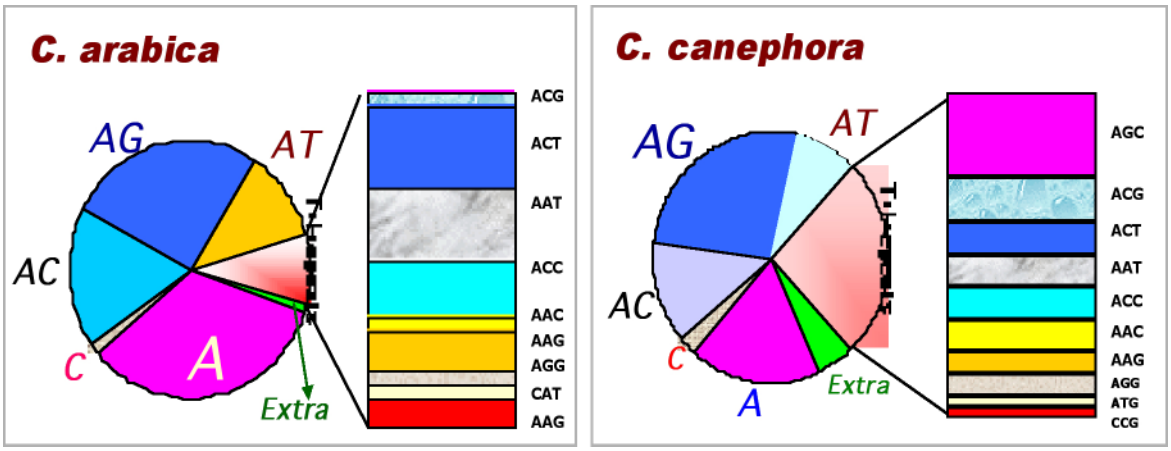


**Figure 1. Distribution of various repeats among the sequenced clones of two coffee species.**


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# Phenotypic and Genetic Analysis of Root and Associated Physiological Traits in *Coffea canephora* Accessions

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## SUMMARY

In the present study, 31 accessions of *Coffea canephora* were assessed for variability in root traits by gravimetric method and also genetic analysis with Random Amplified Polymorphic DNA (RAPD) markers for drought tolerance/ resistance. A significant genetic variability in root characteristics such as total root biomass, root to shoot ratio and total dry matter was observed among the accessions. A significant positive association was recorded between root weight and total biomass production under stress and non-stress conditions indicating low Genotypic X Environmental interactions for these traits. The robusta accessions were categorized in to different groups based on normal Z-distribution *viz.,* high root, low root, high WUE and low WUE types. Categorization of accessions into different groups using 18 selected 10 mer Operon series primers indicated maximum linkage distance of 135 units. Based on these results, the contrasting accessions with high and low root biomass types were crossed and mapping populations were established for construction of genetic linkage map in robusta coffee.

## INTRODUCTION

Coffee is an important plantation crop grown predominantly in the peninsular India. Growth and productivity of this crop is constrained by the water availability particularly during rain free periods (November to March). From this context, exploring the available variability in root characteristics is highly relevant to extract water from deeper profiles of soil. Deeper root systems can significantly increase the total biomass and yield. Large genotypic differences in growth and productivity of many species in response to water deficit in many species are found to be associated with differences in efficiency of root system. In fact, the significance of roots has been shown by grafting studies in perennial plant species (White and Castillo, 1989).

## MATERIALS AND METHODS

Gravimetric approach was followed for determination of root, WUE and associated traits among the *C. canephora* accessions at Central Coffee research Institute (CCRI). As robusta is a cross-pollinated and heterozygous species, the single nodal cuttings were collected and raised according to Anil kumar, (1994). One year after establishment of suckers/clones at nursery conditions, 31 robusta accessions were chosen from different accessions existing at CCRI and transplanted to battery containers having soil, FYM and sand mixture (6:2:1). The seedlings were maintained at 80 and 60% Field capacity. Observations on Cumulative water added (CWA), Whole plant leaf area – Initial and Final, Root traits like root biomass, number of secondary roots and root length, Total dry matter (TDM) accumulated – Initial and Final,

Gas exchange parameters and $\Delta^{13}C$ signature were carried out in battery grown seedlings. Using these data, the parameters such as Cumulative Water Transpired (CWT), Leaf Area Duration (LAD), Mean Transpiration Rate (MTR), Net Assimilation Rate (NAR) and Water Use Efficiency (WUE) were computed.

The leaf sample for DNA extraction was processed according to the method of Tai and Tanksley (1990). The method of Porebski et al. (1997) which was modified by Bhat et al. (2002) was used for DNA extraction from dried coffee leaf samples. The molecular aspect of the coffee was carried out at Dept. of Crop physiology, UAS, GKVK, Bangalore by using 18 Operon series RAPD primers.

## RESULTS AND DISCUSSION

Genetic variability in root characteristics and associated physiological traits of *C. canephora* accessions were determined both under non-stress and stress conditions. Among the accessions, maximum root dry weight of 37.06 g/plant was noticed in BR.9 and the low root biomass of 3.63 g/plant was observed in S.4048. However, the difference in root biomass between stress and non-stress conditions was non significant. Significant variation was observed in total biomass among the 31 accessions. The maximum biomass of 113.23 g/plant was observed in S.3339. The biomass accumulation ccwas decreased under moisture stress conditions in coffee accessions compared to non-stress condition (Table 1). Similar significant genetic variability was shown in perennials like cashew by adopting gravimetric approach (Anil Koushik, 1999). The effect of stress was mainly on shoot growth rather than on root growth. Genetic variability in root to shoot ratio was also cccobserved.

There are several reports where positive significant association was shown between root characteristics and drought resistance. An increase in the root growth when plant experiences water stress has been well documented as an adaptive strategy evolved by the plants to avoid stress effect (Sharp and Davies, 1989). The high total biomass without reduction in root growth observed in the present study indicates the desirable trait for stress tolerance. This might be due to genetic make up of the plants, as the developmental pattern of coffee roots depends on the plant genotype (Franco and Inforzato, 1946).

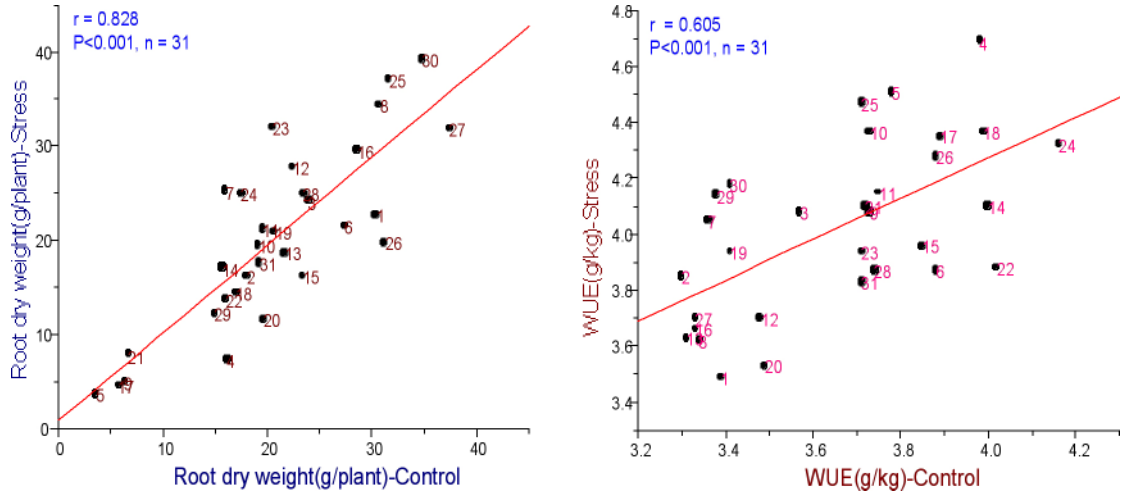


**Figure 1. Relationship of root dry weight and WUE of *C. canephora* accessions between non stress and stress conditions.**

**Table 1. Genetic variability in root characteristics, total dry matter and root to shoot ratio in *C. canephora* accessions grown at two moisture regimes.**

| Sl. Nos | Accessions | Root wt. (g/plant) | | Mean | TDM (g/plant) | | Mean | Root to shoot ratio | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | Stress | | Control | Stress | | Control | Stress | |
| **1** | **S.3317** | 30.40 | 22.75 | **26.58** | 105.35 | 70.69 | **88.02** | 0.39 | 0.44 | **0.42** |
| **2** | **Kagnalla** | 18.05 | 16.24 | **17.15** | 79.37 | 59.00 | **69.19** | 0.28 | 0.35 | **0.32** |
| **3** | **S.4041** | 23.94 | 24.13 | **24.04** | 105.38 | 99.63 | **102.51** | 0.28 | 0.31 | **0.30** |
| **4** | **S.3309** | 16.18 | 7.32 | **11.75** | 53.31 | 34.44 | **43.88** | 0.42 | 0.25 | **0.34** |
| **5** | **S.4048** | 3.57 | 3.69 | **3.63** | 19.25 | 22.08 | **20.67** | 0.23 | 0.18 | **0.21** |
| **6** | **S.4046** | 29.37 | 21.51 | **25.44** | 124.96 | 74.05 | **99.51** | 0.29 | 0.39 | **0.34** |
| **7** | **S.4045** | 15.97 | 25.19 | **20.58** | 52.81 | 85.30 | **69.06** | 0.37 | 0.38 | **0.38** |
| **8** | **BR.12** | 30.76 | 34.39 | **32.58** | 108.37 | 112.87 | **110.62** | 0.37 | 0.41 | **0.39** |
| **9** | **$L_1$ valley** | 6.33 | 4.95 | **5.64** | 21.89 | 15.49 | **18.69** | 0.34 | 0.36 | **0.35** |
| **10** | **S.3349** | 19.12 | 19.45 | **19.29** | 67.31 | 77.91 | **72.61** | 0.37 | 0.31 | **0.34** |
| **11** | **S.4040** | 19.56 | 21.28 | **20.42** | 72.72 | 78.87 | **75.80** | 0.34 | 0.35 | **0.35** |
| **12** | **S.3332** | 22.40 | 27.82 | **25.11** | 89.17 | 95.99 | **92.58** | 0.31 | 0.38 | **0.35** |
| **13** | **S.3327** | 21.68 | 18.65 | **20.17** | 74.76 | 65.27 | **70.02** | 0.38 | 0.37 | **0.38** |
| **14** | **S.3329** | 15.69 | 17.05 | **16.37** | 65.07 | 69.23 | **67.15** | 0.30 | 0.31 | **0.31** |
| **15** | **S.3322** | 23.35 | 16.12 | **19.74** | 114.86 | 69.85 | **92.36** | 0.25 | 0.28 | **0.27** |
| **16** | **C x R** | 28.61 | 29.51 | **29.06** | 84.48 | 86.62 | **85.55** | 0.47 | 0.48 | **0.48** |
| **17** | **S.3325** | 7.00 | 4.55 | **5.78** | 24.19 | 16.88 | **20.54** | 0.33 | 0.30 | **0.32** |
| **18** | **S.3330** | 16.99 | 14.51 | **15.75** | 52.45 | 40.19 | **46.32** | 0.42 | 0.47 | **0.45** |
| **19** | **S.3347** | 20.73 | 20.84 | **20.79** | 61.68 | 65.33 | **63.51** | 0.44 | 0.41 | **0.43** |
| **20** | **Tetraploid** | 19.69 | 11.70 | **15.70** | 75.60 | 43.35 | **59.48** | 0.34 | 0.35 | **0.35** |
| **21** | **S.3318** | 6.70 | 7.83 | **7.27** | 19.25 | 18.38 | **18.82** | 0.46 | 0.57 | **0.52** |
| **22** | **S.274** | 16.01 | 13.76 | **14.89** | 68.34 | 53.03 | **60.69** | 0.29 | 0.32 | **0.31** |
| **23** | **S.4042** | 20.44 | 32.09 | **26.27** | 69.13 | 98.39 | **83.76** | 0.40 | 0.46 | **0.43** |
| **24** | **S.3311** | 19.51 | 24.92 | **22.22** | 73.41 | 73.60 | **73.51** | 0.32 | 0.47 | **0.40** |
| **25** | **S.3339** | 33.62 | 37.01 | **35.32** | 106.09 | 120.37 | **113.23** | 0.43 | 0.43 | **0.43** |
| **26** | **Village** | 31.25 | 19.73 | **25.49** | 99.59 | 62.49 | **81.04** | 0.44 | 0.44 | **0.44** |
| **27** | **S.3334** | 37.40 | 31.81 | **34.61** | 115.1 | 106.77 | **110.94** | 0.47 | 0.41 | **0.44** |
| **28** | **S.4044** | 23.45 | 25.04 | **24.25** | 82.49 | 74.56 | **78.53** | 0.38 | 0.48 | **0.43** |
| **29** | **S.3316** | 14.91 | 12.26 | **13.59** | 36.38 | 34.54 | **35.46** | 0.57 | 0.45 | **0.51** |
| **30** | **BR.9** | 34.85 | 39.26 | **37.06** | 99.13 | 116.75 | **107.94** | 0.46 | 0.44 | **0.45** |
| **31** | **BR11** | 19.22 | 17.62 | **18.42** | 64.80 | 59.40 | **62.10** | 0.39 | 0.39 | **0.39** |
| | **Average** | **20.86** | **20.10** | | **73.76** | **67.78** | | **0.37** | **0.39** | |
| **CD at 1%** | Clones (C)<br>Treatment (T)<br>C x T | **5.376**<br>**NS**<br>**7.602** | | | **16.130**<br>**4.096**<br>**22.811** | | | **0.056**<br>**0.014**<br>**0.080** | | |

**Table 2. Classification of *C. canephora* accessions based on absolute root weight under non stress conditions.**

| Groups | Root weight (g/plant) | Accession numbers | Accessions |
|---|---|---|---|
| Group I | > 30 | 1, 8, 25, 26, 27 and 30 | S.3317, BR.12, S.3339, Village robusta, S.3334 and BR.9 |
| Group II | 25-30 | 6 and 16 | S.4046 and C x R |
| Group III | 20-25 | 3, 12, 13, 15, 19, 23 and 28 | S.4041, S.3332, S.3327, S.3322, S.3347, S.4042 and S.4044 |
| Group IV | 15-20 | 2, 4, 7, 10, 14, 18, 20, 11, 22, 24, and 31 | Kagnalla robusta, S.3309, S.4045, S.3349, S.3329, S.3330, S.4040, Tetraploid robusta, S.274, S.3311 and BR.11 |
| Group V | <15 | 5, 9, 17, 29 and 21 | S.4048, $L_1$ valley robusta, S.3325, S.3318 and S.3316 |

Accessions were classified into five groups based on absolute root weight under non-stress conditions. The accessions with high root biomass under non-stress also showed high root biomass production under moisture stress condition (Table 2).

A strong positive relation ship was observed between the root weight (r = 0.828) under stress and non-stress conditions and also WUE (r = 0.605) under non-stress and stress conditions (Figure 1) signifies a low G X E interaction among the robusta accessions. A very low G x E interaction and high heritability (Ismail and Hall, 1993) renders WUE as a potential physiological trait for crop improvement.

Two hundred and fifty six bands were produced from selected 18 RAPD primers used in estimating the genetic diversity among 31 robusta accessions. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963). Cluster analysis of 31 accessions of *C. canephora* revealed maximum linkage distance of 135 units on the dendrogram. Three major clusters were observed on the dendrogram and selected S.3334 and $L_1$ valley robusta, based on genetic distance couple with high and low root biomass characteristics for developing mapping population. However a low genetic variability was observed among the robusta accessions. Bhat (2002) also reported low genetic diversity based on analysis of RAPD markers in arabica coffee. Similarly, RAPD markers have been successfully employed to analyze the genetic diversity among the cultivated and sub-spontaneous accessions of *C. arabica* by Lashermes et al. (1996).

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# Identification of Putative Resistance Gene Analogues in *Coffea* and Related *Psilanthus* Taxa

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## SUMMARY

Biotic stress related damage in plants could be managed by building disease resistance in cultivated genepool through breeding for plant resistance genes (R-genes). Recent studies show that R-genes conferring resistance to diverse pathogens in different plant species share a number of common functional motifs and are frequently clustered in the genome. This considerable conservation at the DNA sequence/protein level in R-genes has led to the development of novel PCR-based molecular approaches to isolate putative resistance gene analogues (RGAs) from new plant sources with considerable ease. We have successfully used one such approach to amplify putative RGAs from *Coffea* and four related endemic *Psilanthus* taxa with degenerate primer pairs designed from conserved P-LOOP and GLPL region of NBS-LRR type resistance gene.

To isolate RGAs, we tested both the total genomic DNA, as well as cDNA as templates in coffee and only genomic DNA for the four related taxa. The PCR-amplified products of expected size range of 500 bp were cloned in to pMOS plasmid vector. The cloned fragments were amplified from the recombinant plasmid DNA using M13 universal primers and were sequenced for both strands on an automated DNA sequencer ABI3700 using fluorescence-dideoxy terminator chemistry. The sequences were partially characterized by BLASTn homology searches. Of the 434 sequences, thus analyzed, there were twenty-seven sequences that had all the characteristic features of the known NBS-LRR type of R-genes. Of these 27 putative RGAs, 24 had complete uninterrupted reading, while remaining 3 sequences showed one or more stop codons. The sequence comparisons with known reference RGAs of coffee and other plant species revealed considerable variability in the putative RGAs amplified in the study, especially for those from related, wild *Psilanthus* taxa. The data thus suggest the potential of the secondary genepool as donors of possible resistance genes to the present day cultivated species of coffee.

## INTRODUCTION

The commercial cultivation of coffee (family: Rubiaceae) relies mainly upon two species, namely, *Coffea arabica* L. (2n = 4X = 44) and *C. canephora* Pierre (2n = 2X = 22). *C. arabica* accounts for ~70% of world production of high quality coffee but is susceptible to several pests and disease causing pathogens. The latter remains one main concern in arabica coffee cultivation that results in considerable losses to the global coffee industry. Breeding for disease resistance thus, has been one of the top priorities in coffee cultivar improvement programs worldwide. Conventional breeding efforts in this direction face limitations such as, lack of good resistance sources, poor understanding of the inheritance of resistance traits and need for long breeding cycles for transfer of genes to commercial cultivars. In this context, the new genetic tools in the form of molecular marker technologies provide promising

alternatives to expedite the process of identifying, integrating and tracking of genes responsible for the biotic stress in crop plants.

Characterization of plant resistance genes is an important step in understanding plant defense mechanisms to combat biotic stress caused by a wide array of phytopathogens. Classical genetic and molecular data show that genes (R-genes) determining disease resistance in plants are frequently clustered in the genome. Data on such genes conferring resistance to diverse pathogens cloned from several species in recent years show that these encode proteins that share some common functional motifs. Most of these R-genes are found to encode a Leucine Rich Repeat (LRR) region comprising a highly conserved backbone of amino acid motifs. This considerable conservation at the DNA sequence/protein level in majority of disease resistance genes makes it possible to isolate resistance gene analogues (RGAs) from new plant sources via PCR approach with degenerate primers.Using similar PCR approach Noir et al. (2001) isolated 27 RGAs from cultivated coffee species. In the present study we have attempted to amplify and isolate RGAs from four wild related taxa of coffee belonging to the genus *Psilanthus* (endemic to India), in addition to the genomes of cultivated coffee and cDNA (originally isolated from moisture and high light-stressed leaves) of *C. arabica* var. Sln.12. The results on characterisation and diversity analysis of the putative NBS-LRR type RGAs and their relationship with few other known R-genes are reported and discussed.

## MATERIALS AND METHODS

Genomic DNA samples representing two cultivated varieties (*C. arabica* var. Sln12 and *C. canephora* var. BR9) and four endemic wild *Psilanthus* species viz., *P. bengalensis, P. khasiana, P. travencorensis, P. weightiana*, isolated from leaf materials originally obtained from Central Coffee Research Institute, Balehonnur, India, and cDNA obtained from light-drought stressed leaves of *C. arabica* var. Sln-12, formed the source material for this study. The genomic DNA was isolated from fresh leaf samples according to Aggarwal et al. (2002). Two sets of coffee specific non-degenerate primers (Noir et al., 2001) were used to amplify NBS containing RGA sequences from the source materials. PCR amplifications were performed in 20 µL reactions each comprising: 25 ng of genomic DNA/cDNA, 1 µM each primer, 150 µM dNTPs, 1 x PCR buffer II (Perkin Elmer), 2mM $MgCl_2$ and 1 unit of Ampli*Taq* DNA polymerase, on a PTC-200 thermolcycler (MJ Research). Amplification profile used was: initial denaturation step at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec and elongation at 72°C for 1.5 min, and a final elongation step of 5 min at 72°C. PCR products were checked and sized on agarose gel before cloning in plasmid vectors pMOS (Amersham) and pCR2.1-TA (Invitrogen). Recombinant plasmid DNA was isolated manually by alkaline lysis method and cloned amplicons were amplified individually using universal M-13 primers for further sequencing. The amplified inserts falling in the size range of 450-600 bp were sequenced on the automated DNA sequencer ABI PRISM-3700 for both strands using the BigDye dideoxy terminator cycle sequencing chemistry (Applied Biosystems).

## RESULTS AND DISCUSSION

In total, 434 clones were sequenced and analyzed for their genetic content by sequence homology search. Majority of the amplified sequences were found to have no motifs characteristic of the target R-genes. However, BLASTn search revealed 27 sequences that showed high similarity to the NBS domains of R-genes. The 24 of these sequences could be translated to polypeptides without any stop codons, and they showed strong overall similarities to several plant R-gene sequences and many RGA sequences cloned from plant species using similar PCR-based approaches. The remaining 3 sequences showed similarity to

cloned disease resistance genes at the DNA level, but contained one or more stop codons or frame shifts resulting in interrupted ORFs.

Comparative sequence analysis (performed using Clustal-X) of the newly amplified coffee RGAs with the reference R-gene nucleotide as well as peptide sequences retrieved from the NCBI database revealed that these shared high similarity with them. Of the 24 new translatable RGAs, 9 were found to be of considerable smaller size and thus not used in further comparative analysis. Sequence analysis of the 15 newly amplified coffee RGAs with 12 already reported coffee RGAs and 5 RGAs from other plant taxa (from NCBI database) revealed high similarity especially, across the four motifs P-loop, kinase-2, kinase-3a and GLPL as also reported by Noir et al. (2001). These results suggest that newly isolated RGAs from *Coffea* and *Psilanthus* belong to the NBS-LRR resistance-gene super family. Further, these new RGAs of coffee and related *Psilanthus* species identified in this study showed considerable sequence variation within them. Despite the variation, all the RGAs were found closely related to one or several plant R-genes identified earlier in coffee and several other plant species.

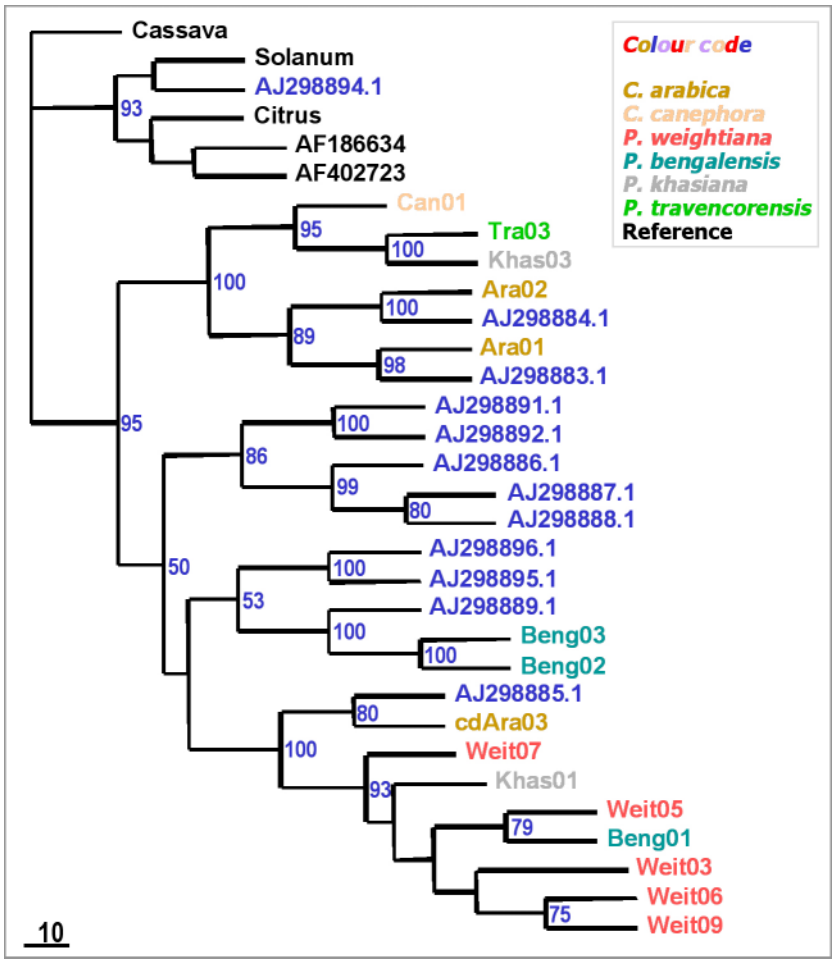


**Figure 1. Neighour-Joining phenetic tree showing relationship of new RGA nucleotide sequences isolated from the cultivated coffee varieties (Sln12 and BR 9) and wild related Psilanthus spp., with the reference RGA sequences (identified by their GenBank accession numbers). The bootstrap values (only > 50%) are shown at nodes of the clusters.**

Phylogenetic analysis was performed to evaluate the relationship among the coffee RGAs and other plant R genes. The phylogenetic trees were constructed using both distance- and

character-based algorithms for both nucleotide as well as putative peptide sequences using Phylip software package. The clustering outputs were also tested for reliability and confidence limits using bootstrapping. The analysis revealed: 1) new RGA to be most related to NBS-family reference RGA sequences described for coffee and other plant taxa; 2) considerable variation in the newly identified RGAs at the nucleotides level; and c) comparable similar relationships using peptide level variation that was generally lower than that seen in the nucleotide sequences. More significantly, the phenetic clustering revealed relatively many unique RGAs from the related the endemic *Psilanthus* spp species compared to that of cultivated varieties of *C. arabica* and *C. canephora* (Figure 1). The RGAs isolated from *P. weightiana* were found to be very distinct from others because of large deletions.

One RGA sequence could be isolated using cDNA source that was originally obtained from the abiotic stressed leaves of coffee. To our understanding, this observation is novel and may be suggestive of a role for RGA like gene(s) in abiotic stress tolerance, an interesting possibility that need to be explored further.

Many of the clones that didn't show homology to any known R-genes, showed homology to a number of important regulatory proteins like calcium binding proteins, GTP pyrophosphokinases, retro-transposons etc, which may be useful by-products of the RGA search by PCR approach.

## CONCLUSION AND PERSPECTIVES

The study demonstrates that new RGA can be successfully isolated by the indirect PCR-approach. More importantly, the results suggest that the related endemic taxa of coffee can serve as donor of disease resistance genes to present day cultivated varieties. Finally, identification of a transcribed RGAs from cDNA from abiotic stressed coffee leaves suggest newer interesting possibilities and need for exploring the role of R-genes abiotic stress tolerance in addition to their implied role in biotic resistance.

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# Post Transcriptional Gene Silencing for Down Regulating Caffeine Biosynthesis in *Coffea canephora* P. ex Fr.

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## SUMMARY

RNAi, co-suppression and antisense technologies are powerful tools of post transcriptional gene silencing in sequence specific manner. *Agrobacterium* mediated genetic transformation was achieved in *Coffea canephora* for silencing of N-methyl transferase involved in caffeine biosynthesis. In the present study, we report the transformation and direct regeneration of transformants through secondary embryos in the presence of selection pressure and hormonal regimes. Three different constructs were made for silencing caffeine biosynthesis in *Coffea canephora*. pSAT 201 is a co-suppression construct, consists of a fragment of methyl transferase in sense orientation with respect to the promoter. pSAT 202 is an antisense construct, consists of a fragment of methyl transferase in antisense orientation with respect to the promoter. pSAT 222 is an invert repeat construct, which is expected to induce target gene silencing through RNAi. These constructs were mobilised to *A. tumefaciens* and sonication assisted *Agrobacterium* mediated transformation method was adopted for plant transformation. Transformation efficiency of 0.5-3.0% was obtained depending on the regeneration medium. The putative transgenic secondary embryos were selected under 20mg/L hygromycin for at least 8 months. The transgenic nature was confirmed by PCR and southern blots. The purine alkaloids were extracted and estimated by HPLC and characterized by LCMS. Transformation using pSAT 222 construct was found to be most efficient and the caffeine content in one transformant was 3.8% of the control value. Transformants with antisense construct resulted in caffeine levels 10% of the control value, transformants with co-suppression construct resulted in 0.78% caffeine, when compared to control with 0.8% caffeine. These data clearly demonstrated reduction in caffeine levels. However the extent of silencing varied in independent transformants.

## INTRODUCTION

Coffee is an extremely important agricultural crop belonging to family Rubiaceae. Commercial coffee production relies mainly on two species: *Coffea arabica* (Arabica) and *C. canephora* (Robusta). Coffee breeding by conventional methods is a low efficiency and time consuming process due to their long cycles. Hence, genetic transformation is a desirable technique for genetic improvement of coffee (Hatanaka et al., 1999). Genetic transformation of coffee plants was reported by several groups. (See review Ogita et al., 2002). Excess intake of caffeine, which is an important constituent in coffee, is thought to induce functional diseases such as insomnia and dizziness. Unpleasant short term side effects from caffeine include palpitations, gastrointestinal disturbances, anxity, tremor, increased blood pressure etc (Ashihara et al., 2001). Caffeine is synthesized through three sequential methylation of xanthosine. First xanthosine is converted into 7- methyl xanthine and then into 3,7 dimethyl xanthene (theobromine), finally into 1, 3, 7 trimethyl xanthine (caffeine). These steps are

catalyzed by N- methyl transferases that use *S*-adenosyl –L-methionine as the methyl doner (Ashihara and Crozier, 2000).

RNAi, co-suppression and antisense technologies are powerful tools of post transcriptional gene silencing in sequence specific manner. In this report we demonstrate the efficiency of these strategies in down regulating caffeine biosynthesis.

## MATERIALS AND METHODS

*Coffea canephora* genotypes S-274 and CxR (obtained from CCRI, India) were used for these experiments. Somatic embryos were induced as reported by Berthouly and Michaux-Ferriere (1996). The somatic embryos were transformed as described by Trick and Finer (1997). The selection and regeneration medium comprised either of 0.5 $mgl^{-1}$ thidiazuron (TDZ), 60 μm AgNO3, 1 $mgl^{-1}$ 2ip and 5μM triacontanol with hygromycin for selection. *Agrobacterium rhizogenes* agropine type strain A4 and *Agrobacterium tumefaciens* strain EHA 101 carrying different constructs for post transcriptional gene silencing was used to transform *Coffea canephora*. The binary vector pCAMBIA 1301 was used as the primary vector and sense(pSAT 201), antisense (pSAT 202), and inverse repeat (pSAT 222) vectors containing a portion of N methyl transferase was used for transformation of coffee. The transformation efficiency was recorded by counting the number of putatively transgenic secondary embryos selected on 20 $mgl^{-1}$ hygromycin after a period of minimum 8 months of culture. The putative transformants were first checked with primers for the presence of *hpt* gene, *rol* gene and then by southern hybridisation using the *hpt* gene fragment as probe. Caffeine levels were estimated by HPLC and identified by LCMS.

## RESULTS AND DISCUSSION

The hormonal regimes used in the regeneration medium significantly enhanced transformation efficiency (Table 1, Figure 2a, b, c). The best transformation efficiency of 3% was obtained when 0.5 $mgl^{-1}$ TDZ or 60 μM silver nitrate was used along with EG medium (Table 2). Hairy roots were induced when hypocotyls of *Coffea* were infected with wild *A. rhizogenes* (Figure 2d). No embryos were regenerated when control untransformed explants were cultured under 5mg/l hygromycin (Figure 2e). Hairy root induction was suppressed and direct transgenic secondary embryo regeneration occurred possibly due to the hormonal regimes used in the selection medium along with hygromycin (Figure 2f). Scanning electron micrographs showed prominent micro wounds on the surface of the mature somatic embryos caused by ultrasonication. Colonisation of *Agrobacterium* was observed on the periphery of the wounds during the co-cultivation period (Figure 3 a,b,c).

**Table 1. Response for secondary embryo induction in EG medium supplemented with TDZ, Triacontanol, $AgNO_3$ and 2ip.**

| Media | Percentage response | Number of sec.embryos |
|---|---|---|
| EG control | 64 | 9-10 |
| EG+ $AgNO_3$ 60uM | 25-30 | 25-30 |
| EG+TDZ 0.5 $mgl^{-1}$ | 83 | 20-25 |
| EG +Tria.11uM-IAA | 96 | 15-20 |
| EG+2ip | 80-90* | 300-500** |

*EG=MS salts half strength+B5 vitamins+0.45 $mgl^{-1}$ IAA+0.25 $mgl^{-1}$ BA.*
**Embryogenic callus. **high frequency embryos from callus.*

**Table 2. Influence of media composition in induction of transgenic secondary embryo induction under hygromycin selection.**

| Media | Transformation Frequency (percentage) | Nature of secondary embryos |
|---|---|---|
| EG control | 0 | No secondary embryo induction |
| | | White globular clustures |
| EG+ $AgNO_3$ 60uM | 2-3 | Minute clustures |
| EG+TDZ 0.5 $mgl^{-1}$ | 2-3 | Tubular creamish |
| EG +Tria.11uM – IAA | 1-2 | Very minute globular and |
| EG+2ip | 2 (callusing prior to embryogenesis) | transluscent |

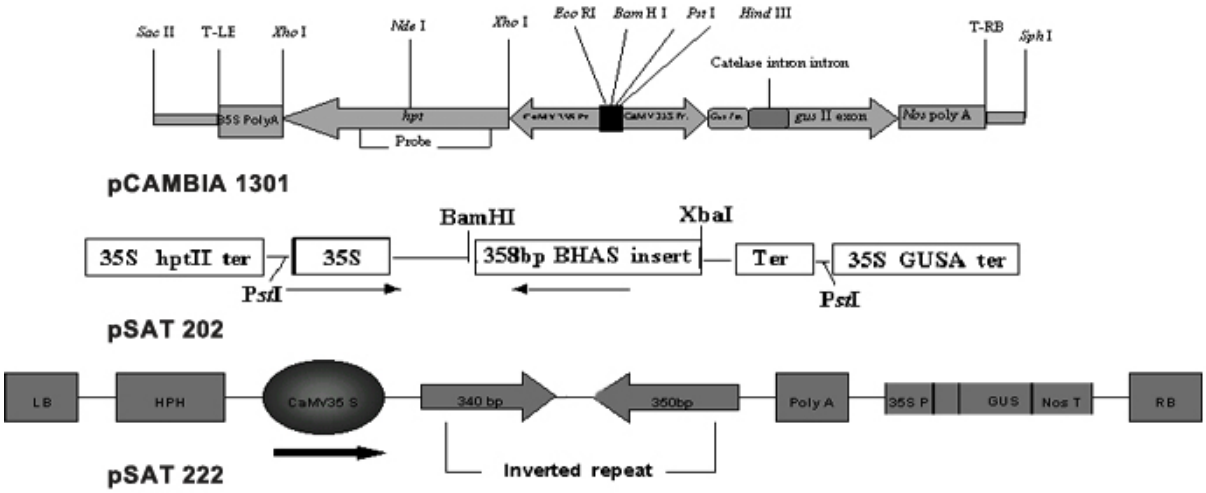


**Figure 1. Constructs for post transcriptional gene silencing using conserved sequences of N methyl transferase.**

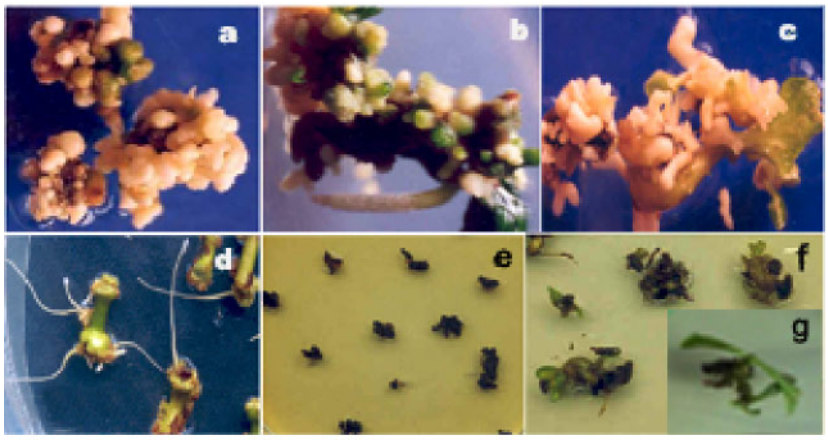


**Figure 2. a: Secondary embryos under 40 mM silver nitrate; b: Secondary embryos under 0.5 mg $l^{-1}$ thidiazuron; c: Secondary embryos under 5mM triacontanol; d: Induction of hairy roots; e: Control explants under 10mg $l^{-1}$ hygromycin; f: Selected transgenic plants under 20mg $l^{-1}$ hygromycin; g: 18 month old transgenic plant.**

PCR using the *hpt* primers provided molecular evidence of the transgenic nature of plants resistant to hygromycin. The expected size fragment of 479 bp of the hygromycin phosphotransferase gene was obtained in all transgenic lines and was absent in non-transgenic

plants (Figure 3a). Integration of *rol* A genes along with integration of T DNA from the binary vector when *A. rhizogenes* was used for transformation was shown in three of the five plantlets tested. DNA from two plantlets did not support amplification of the *rol* A gene (Figure 3b). Further investigation is needed to understand the nature and mechanism of integration of T DNA from Ri and binary plasmids in a single cell and in different cells. Secondary embryogenesis or direct regeneration methods help avoidance of culture induced somaclonal variations which is common in callus derived tissue culture plants. We have analysed these five transgenic plants for variation using ISSR primers. There were differences in the pattern of amplification by ISSR primers among the five plants (Figure 3c). This may be mainly due to random integration of T DNA from Ri plasmid and the binary vector into the host genome.

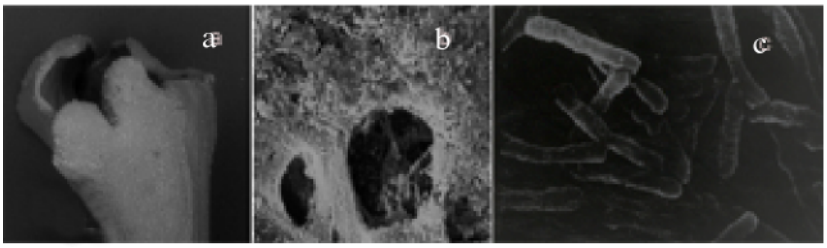


**Figure 3. Scannining Electron Micrographs of somatic embryos, wounding by sonication and *Agrobacterium* colonization on wounded regions of co-cultivated explants.**

Southern blot analysis with a probe for *hpt* gene fragment provided evidence for independent transgene transfer and integration into the plant chromosomal genome. The presence of several fragment of variable size in some of the analysed plantlets indicates insertion of multiple copies of the T DNA into the plant genome (Figure 4).

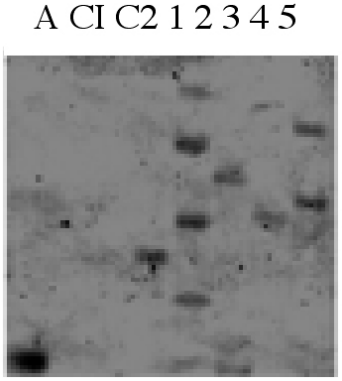


**Figure 4. Southern blot analysis of PCR positive transformed plants. Lanes: A-pCAMBIA 1301 vector, C1 and C2 untransformed *Coffea canephora* plantlets plants, 1 to 5-DNA from PCR positive *Coffea canephora* transformed with pCAMBIA 1301.**

In subsequent experiments only *A. tumefaciens* was used to transform *Coffea* with constructs for post transcriptional gene silencing (PTGS). Approximately 350bp fragment was cloned by PCR from the second axon of the conserved region of the N-methyl transferase. This fragment was used to make following constructs. pSAT 201 is a co-suppression construct, consists of a fragment of methyl transferase in sense orientation with respect to the promoter. pSAT 202 is an antisense construct, consists of a fragment of methyl transferase in antisense orientation with respect to the promoter. pSAT 222 is an invert repeat construct, which is expected to induce target gene silencing through RNAi. All the co cultivated somatic embryos were subjected to secondary embryogenesis under hygromycin selection. The selected

secondary embryos were grown on up to 20 mg/l hygromycin containing medium for at least 8 months period to eliminate untransformed lines completely. These somatic embryos were analysed for caffeine content. Transformation using pSAT 222 construct was found to be most efficient and the caffeine content in one transformant was 3.8% of the control value. Transformants with antisense construct resulted in caffeine levels 10% of the control value, transformants with co-suppression construct resulted in 0.78% caffeine, when compared to control with 0.8% caffeine. These data clearly demonstrated reduction in caffeine levels (Figure 6). However the extent of silencing varied in independent transformants. Work is under progress to study N-methyl transferase transcript levels by RT PCR in different PTGS strategies in individual transformants and to regulate PTGS in tissue specific manner.

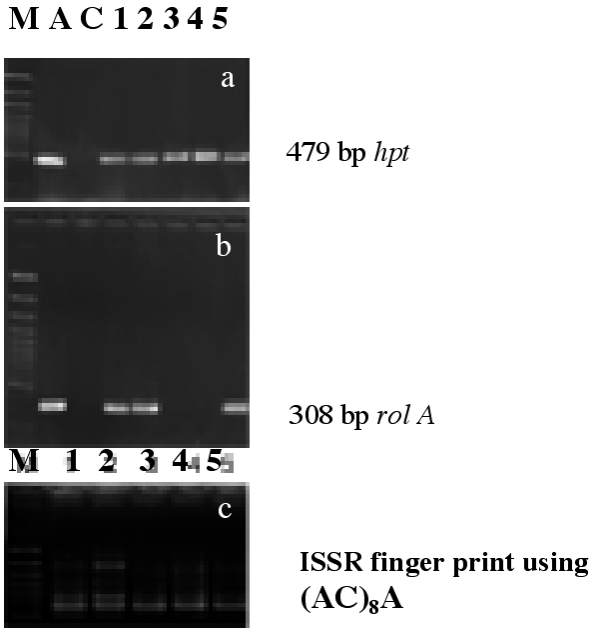


**Figure 5. a and b: are analysis of genomic DNA from putative transformants of *C. canephora* after 10 months of selection on hygromycin. PCR was carried out using gene specific primers for 479 bp of *hpt* gene and 308 bp of *rol* A gene. Lanes; M- 100bp marker, A- DNA from *A. rhizogenes* harboring the binary vector, C-DNA from the untransformed *C. canephora*. 1 to 5- DNA from independent transformants selected under hygromycin; c: ISSR fingerprints of these transformants showing very less variations among the regenerants derived through secondary embryogenesis.**

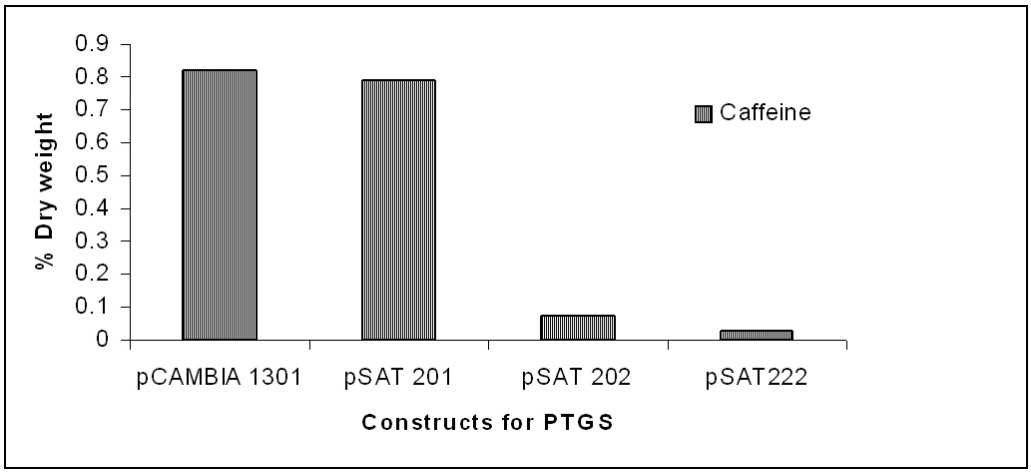


**Figure 6. Caffeine levels in transgenics using different PTGS constructs.**

## ACKNOWLEDGEMENTS

We are thankful to CCRI, India for providing seeds of *Coffea canephora.* VK and SKV are grateful to CSIR, Government of India for Research Fellowship. We thank Dr. V. Prakash, Director, CFTRI for his encouragement. This work was funded by Department of Biotechnology, Government of India.

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# Control of Chlorogenic Acid Formation in Leaves and Endosperm of Coffee Fruit of *Coffea arabica*

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## SUMMARY

EST sequences of genes coding for enzymes involved in chlorogenic acid (CGA) biosynthesis were identified in the Brazilian Coffee Genome Project databank and used to study the control of the content of this phenol in leaves and fruits of *C. arabica* cv. Mundo Novo. RT-PCR analyses showed reduction in the expression of PAL, C4H, C3H, 4CL and CQT with tissue age. In the fruit endosperm a marked decrease in the final maturation stage was observed. Etiolated seedlings obtained from seeds germinated in the dark and transferred to light showed a significant increase of CGA after 24 h. The increase was transient and paralleled by the expression of PAL, C4H, C3H, 4CL and CQT. Therefore, our results suggest that CGA formation can be manipulated by altering the expression of these genes.

## RÉSUMÉ

Les séquences EST des gènes codant pour les enzymes impliquées dans la voie de biosynthèse des acides chlorogéniques (CGA) ont été identifiées dans la base de données du projet Brésilien de génomique du caféier, et utilisées pour étudier les modes de contrôle de l'accumulation de ces composés phénoliques dans les feuilles et les fruits de *C. arabica* cv. Mundo Novo. Des analyses par RT-PCR ont montré une réduction de l´expression des gènes PAL, C4H, C3H, 4CL et CQT avec l´âge des tissus. Dans l'endosperme, on a observé une nette diminution au stade final de maturation. Des plantules étiolées obtenues à partir de graines ayant germé à l´obscurité et transférées à la lumière présentent une augmentation des CGA après 24 h. Cette augmentation est transitoire et est observée en même temps que l'expression des gènes PAL, C4H, C3H, 4CL et CQT. Par conséquent, nos résultats montrent que la formation des CGA peut être manipulée en alterant l´expression de ces gènes.

## INTRODUCTION

Chlorogenic acids (CGAs) are products of phenylpropanoid metabolism and significant amount of these compounds are found in leaves and fruits of coffee. In seeds of the species *C. arabica* and *C. canephora* it may reach 4-6% and 7-11%, respectively (Clifford, 1985). Among several possible isomers, 5-caffeoylquinic acid (5CQA) is the most abundant in coffee seeds. It has been suggested that CGAs play a role as intermediates for the biosynthesis of lignin during seedling growth (Aerts and Baumann, 1994). They may also protect plants against herbivores and pathogens (Shadle, et al., 2003). Recently, several reports have shown that CGAs may have a beneficial effect on human health (Nardine et al., 2002; Lee et al., 2003). CGAs in coffee beverage are usually linked to the acidity of the beverage (Clifford, 1985) and the proportion between monoisomers and diisomers seems to be important for cup quality (De Menezes, 1994).

Qualitative and quantitative alterations in the CGA content are observed during the development of leaves and fruits of coffe (Clifford and Kazi, 1987; Bertrand et al., 2003). At some stages of the fruit development these alterations are very significant suggesting the existence of a metabolic regulation. So far there is no study on the control of CGA biosynthesis in this plant. In this study, using semiquantitative RT-PCR we studied the expression of genes coding for enzymes involved in the biosynthesis of CGAs in plants (Figure 1).

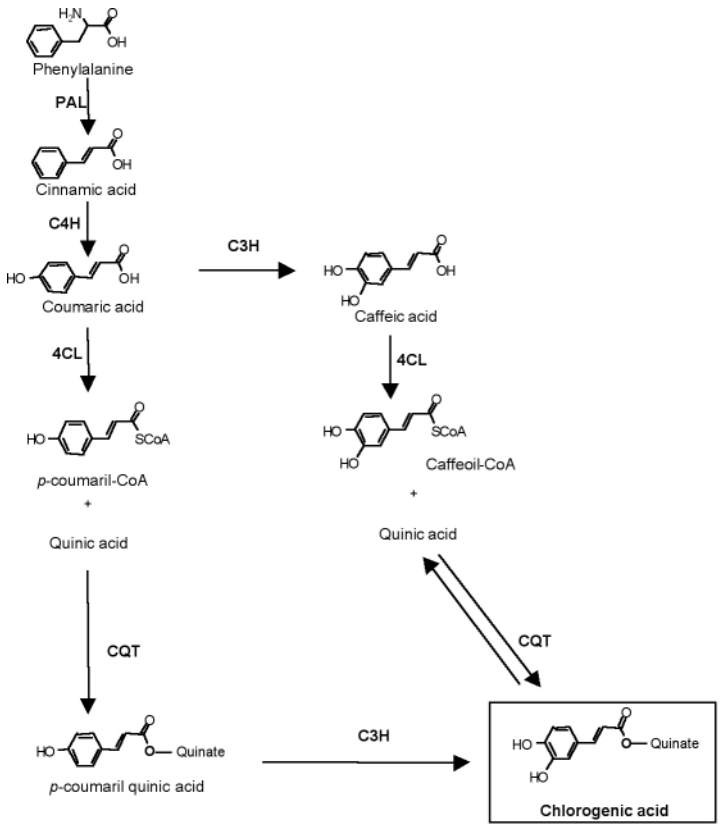


**Figure 1. Biosynthesis of chlorogenic acid in plant.**

## MATERIAL AND METHODS

*C. arabica* cv. Mundo Novo: 1) leaves of etiolated seedlings transferred to light; 2) 1st, 2nd, 3rd and 4th leaf pairs of adult plants; 3) endosperm from fruits at four different developmental stages (from green to mature). ESTs for PAL (phenylalanine ammonia-lyase), C4H (cinnamate 4-hydroxylase), C3H (*p*-cumarate 3-hydroxylase), 4CL (4-hydroxycinnamoil-CoA ligase) e CQT (hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase) were identified in the databank of the Coffee Genome Project and were used to design forward and reverse primers, which were used in semiquantitative RT-PCR (Berdy et al., 2001). Actin was used as reaction loading control. Total RNA was used to produce the first strand cDNA. 5CQA was quantified in methanolic extracts by reversed phase HPLC analysis and UV detection using a diode array detector.

## RESULTS AND DISCUSSION

Expression analyses by RT-PCR showed that expressions of PAL, C4H, C3H, 4CL and CQT are reduced as coffee tissues aged (Figures 2a and 2b). 5CQA also decreased with tissue aging indicating that its content might be associated with the expression of the genes analyzed.

Leaves from etiolated seedlings and transferred to the light showed marked alterations in the content of 5CQA as well as the genes coding for the five enzymes of the biosynthetic pathway (Figure 2c). These results support the suggestion that 5CQA accumulation is coordinated by the expression level of PAL, C4H, C3H, 4CL and CQT.

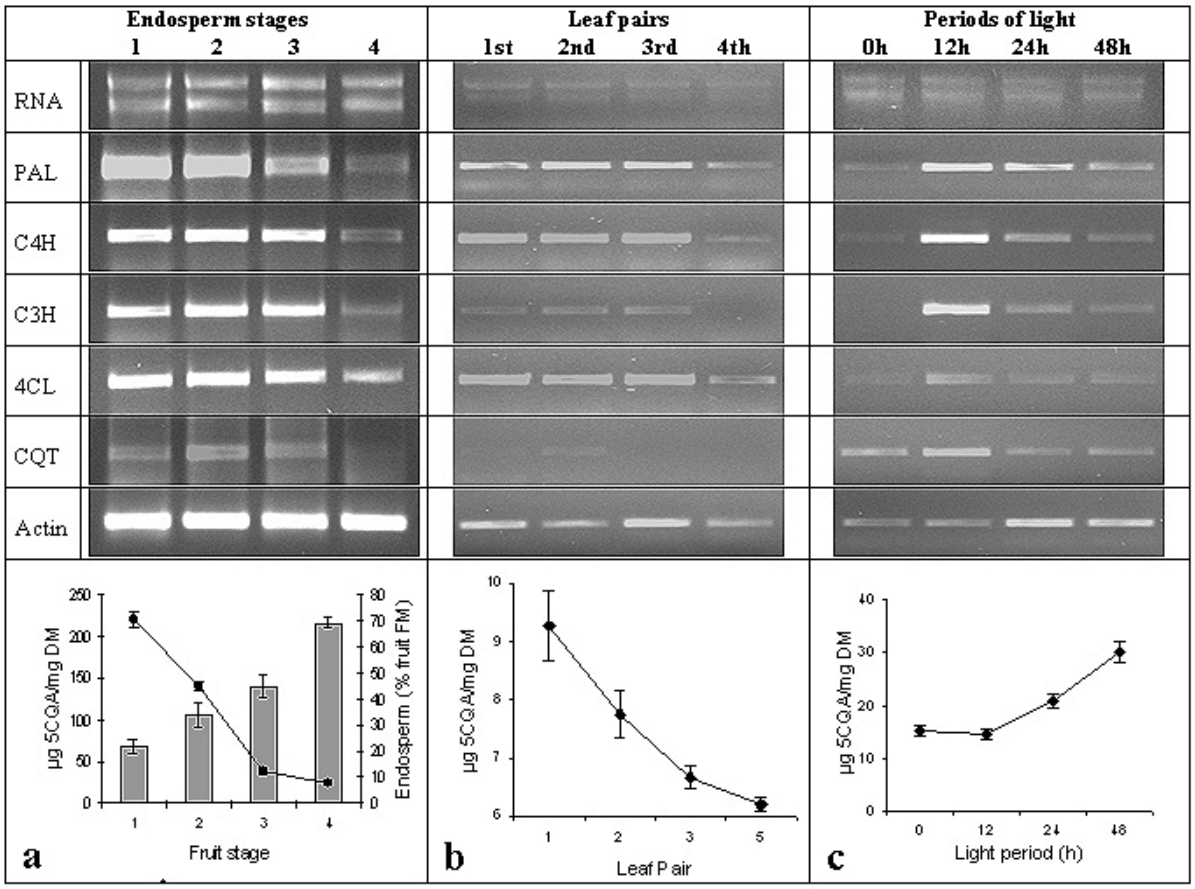


**Figure 2. a: RT-PCR for PAL, C4H, C3H, 4CL, CQT and actin in the endosperm at stages 1-4, and 5CQA contents (line) and % endosperm FM in the fruit; b: RT-PCR for PAL, C4H, C3H, 4CL, CQT and actin in developing leaves, and 5CQA contents; c: PCR for PAL, C4H, C3H, 4CL, CQT and actin in etiolated leaves exposed to light for 0, 12, 24 and 48 h, and 5CQA contents.**

In the endosperm (Figure 2a), the expression observed at the last stage (mature fruit) was clearly lower than the previous stage (green fruit with hard endosperm), yet the 5CQA decrease was not intense as in the other stages. Although we have not analyzed the CGAs isomers, this leveling may have be linked with the qualitative alterations occurring at the end of fruit maturation (Clifford and Kazi, 1987; Bertrand et al., 2003).

Coordination of transcription seems to be common in metabolic pathways related to induced responses in plants (Hahlbrock et al., 2003), which appear to be a strategy developed by plants to protect against herbivory and disease. CGAs are included among the compounds produced for this purpose (Shadle et al., 2003). Our results show that CGA formation in coffee is under the coordination of the enzymes studied here. Therefore, it should be possible to manipulate CGA formation in coffee by altering the expression of PAL, C4H, C3H, 4CL and CQT genes.

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# Randomly Amplified Microsatellites (RAMs) Approach to Assess Molecular Diversity in Robusta Coffee in Relation to Coffee Wilt Disease in Uganda

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## SUMMARY

Coffee wilt disease (CWD), which is currently devastating *Coffea canephora* (Robusta coffee) in Uganda is a vascular wilt disease caused by a fungus called *Fusarium xylarioides*. Studies conducted in the screen house from 1997 by artificially inoculating the plants, and observation made under field condition revealed deferential response to the disease by different coffee lines. In order to assess the molecular basis of the observed variation, coffee lines with known response to CWD were categorized as susceptible, moderately resistant and resistant. Total genomic DNA was isolated from representative number of plants from each category and amplified by polymerase chain reaction (PCR) using four (TG, CA, ACA and GT) randomly microsatellite primers. The products were run on agarose gel by electrophoresis and photographed under UV light. Only CA and TG primers produced polymorphic bands and their products used in the analyses. Cluster analysis with unweighted pair-group method using arithmetic averages (UPGMA) and weighted pair-group method using arithmetic averages (WPGMA) produced no major clusters but the sub-clusters formed fairly grouped different coffee lines according to their response to the disease. ANOVA analysis showed significant different between products of the primers but no significant different between different coffee lines. The limited primers used have revealed the function of the genetic makeup of different robusta coffee lines to their response to CWD. More detail work is planned with more primers in-order to locate the possible loci where the resistance lies for construction of a probe to enhance the present conventional breeding method through marker assisted selection and to initiate breeding strategies for durable resistance in the robusta coffee through genetic engineering.

## INTRODUCTION

Coffee is one of the major agricultural export commodities for Uganda and robusta coffee contributes about 80% of coffee export. Coffee wilt disease (CWD) caused by a fungus called *Fusarium xylarioides* (Steyaer) is currently the most devastating production constrain. The use of resistant cultivars is the most effective control measures. However, knowledge on the molecular diversity of breeding materials is essential for development of resistant cultivars. Although there is substantial germplasm collection present at the Coffee Research Institute (CORI), its molecular diversity is unknown. Breeding on progress at CORI has indicated that different coffee lines respond differently to CWD (Musoli et al., 2001). This study was therefore designed to assess the molecular diversity of the germplasm collections in relation to CWD. The identification of a probe that targets only resistant material would greatly enhance conventional breeding strategies through marker assisted selection. Despite its economic and agricultural importance coffee has not benefited extensively from the technological developments which have been applied to other cash crops. In this manuscript we demonstrate that randomly amplified microsatellites (RAMs) can be used for the genetic

characterization of coffee germplasm, and the estimation of relatedness to their response to CWD.

## MATERIAL AND METHODS

### Plant selection

The coffee genotypes studied comprised the following categorized as resistant, partially resistant and susceptible **(**Table 1).

**Table 1.**

| Source | Resistant | Moderately resistant | Susceptible |
|---|---|---|---|
| (Catimors) | NG9258, NG 1960 | – | – |
| Screen house screening | 202/63, 202/62, J24/13/20. | – | – |
| Germplasm under field evaluation | Q/3/4[a], J/1/1[a], | C/6/10 | P/3/6 |
| Arubusta (arabica x robusta) | RUME 14/12 x 236/26cs19 (RUME 14/) and RUME 14/1 x 236cs14 | – | – |
| From 'hot spots' in farmers fields | Kanayatorogo tree 4, Kato K. tree 1 | – | Sekamate Tree 1 |
| Commercial clones | – | 1s/3, 258/24 | 1s/2, |

## TOTAL GENOMIC DNA ISOLATION

Three youngest fully opened leaves were washed with sterile distilled water, blotted dry, put together and then punched into 1.5 ml eppendorf tube using locking cups. Samples were immediately put on ice before transportation to the laboratory. DNA was isolated from fresh leaf material using a modification of the method described by Orozco-Castilo et al. (1994). The DNA was re-suspended in 50 µl of 1x TE buffer (10mM Tris-HCl (pH7.5), 1mM EDTA) and stored at –20$^{o}$C. To test for the quality and quantity of DNA, the sample was run on 2% agarose gel along side digested λDNA of concentration 25 ng/µl, visualized by ethidium bromide fluorescence under UV light and photographed.

## DNA AMPLIFICATION (RAMS)

The amplifications were carried out in 25 µL mixtures using the procedure described by Orozco-Castilo et al., 1994.

## DATA ANALYSIS

A phenetic analysis was conducted, based on the CA and TG RAMs primers that produced amplified polymorphic bands of DNA. The presence or absence of polymorphic bands was scored 1 and 0, respectively. The results obtained for both CA and TG primers were pooled in a single linkage cluster analyses with unweighted pair-group method using arithmetic averages (upgma) basing on the number of common bands shared.

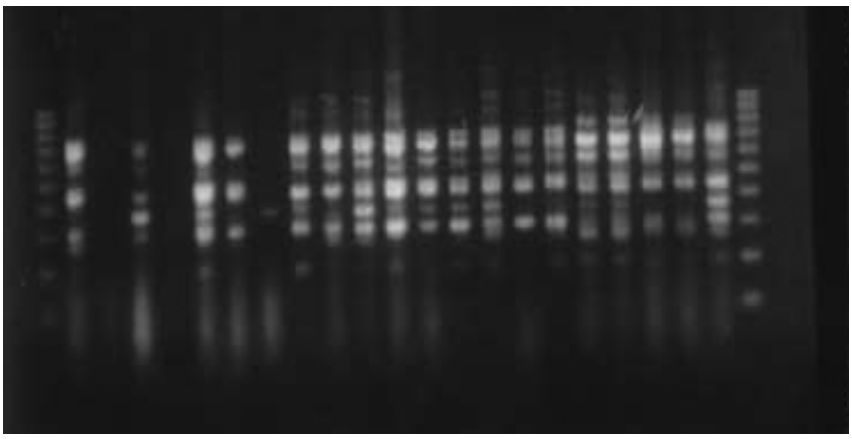

**Figure 1. Amplification products of generated with TG primer. Lane 1 and 23 contained standard 100 bp molecular size marker. Lane 5 contained water as a negative control.**

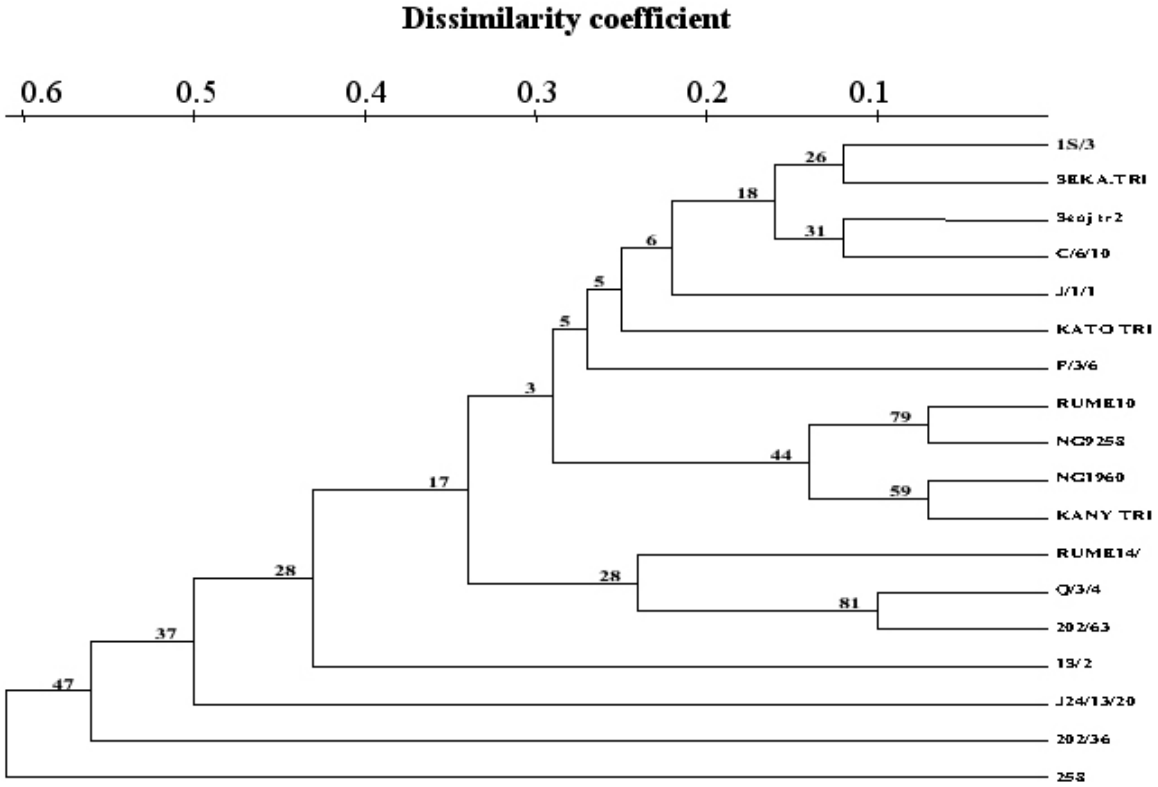


**Figure 2. Dendogram generated with upgma. A total of 183 polymorphic amplified bands were used in the analysis. The scale shown above the dendogram is the genetic dissimilarity. Number at the nodes of major clusters represent bootstrap values (%). The coffee cultivars are indicated on the right side of the dendogram. The dendogram was constructed using treecon 1.3 computor programme.**

## RESULTS AND DISCUSSION

A total of 201 reproducible polymorphic bands were generated with TG and CA primers. 183 of which were polymorphic while 18 were not polymorphic and were not used in the analys. A dendogram (Figure 2) was constracted that depicts mean similarities between groups on coffee lines with identical RAMs patterns. Five subclusters (groups) were produced since no major clusters was produced due to low dissimilarity coefficient less than 0.95 (95% confident interval).This grouping was supported by the low bootstrap values ranging from

only 3 to 47. This shows high similarities among the coffee lines used. Bootstrap value is a percentage value showing percentage dissimilarity (distance analysis) between two or group of coffee lines.

Group 1 contains only susceptible and moderately resistant lines. Group 2 had only resistant coffee except P/3/6 which is susceptible. Group 3 had catimors and arubusta that are resistant except Kanyatorogo tree 1 which is moderately resistant. Group 4 also contains only ressistant varieties. Group 5 separated out 258/24 as being completely different from the rest. 258/24 is one of the unimproved commercial coffee clones in Uganda. Analysis of variance (ANOVA) showed significant difference in the products of different primers ($P = 0.0003$), and no significant difference amongst different coffee lines ($p = 0.4023$). This is supported by dissimilarity coefficient less than 0.95 and low bootstrap values between different groups.

## CONCLUSION AND RECOMENDATIONS

The study has shown that RAMs analyses provides accessible and relatively inexpensive data which are useful entry points for coffee that is still poorly known at the DNA level in relation to CWD. No common band (s) appearing in resistant lines may suggest that resistance is due to additive effects of genes but not just effect of a single gene. The study has also shown that variation in response to CWD by the different coffee lines is more attributed to their genetic makeups. Future work aims at identification of a marker and Quantitative Trait Linkages (QTL) associated with resistant varieties and that would accelerate the breeding process by allowing indirect selection of resistant individuals based on marker genotype through marker assisted selection strategy.

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# Cloning and Characterisation of Promoter for N-Methyl Transferase Gene from Coffee

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## SUMMARY

A PCR based method for walking in uncloned genomic DNA was adopted to successfully isolate and clone the 5' regulatory region of a coffee N-Methyl Transferase (NMT) gene. Sequence analysis performed with the aid of the Plant CARE, PLACE and PATCH programs, part of the TRANSFAC database, resulted in the identification of several core fragments of great importance for promoter function. The whole fragment was fused translationally in frame to the reporter gene β-glucuronidase (*uidA*) to study the ability of this genomic DNA fragment to direct gene expression. The resulting promoter + *uidA* translational fusion construct was used in *Agrobacterium tumefaciens* mediated transformation of *Nicotiana tabacum*. GUS assays showed that the isolated promoter directed the expression of the reporter gene in transgenic tobacco plants. A series of deletions in the NMT promoter region linked to the β-glucuronidase (*uidA*) coding region are being constructed and these will be analyzed for GUS activities by stable expression in transformants of *Nicotiana tabacum*.

## INTRODUCTION

Caffeine and Theobromine are purine alkaloids, which are produced by a variety of plant species, mainly coffee, tea and cacao. Caffeine is synthesized by sequential methylation of xanthosine at 7-, 3- and 1- *N* via: Xanthosine —> 7- methylxanthosine (7mX) —> 7-methyl xanthine —> Theobromine —> Caffeine; the first, second and fourth steps being catalyzed by N-methyl transferases (NMTs) that use S- adenosyl-L-methionine (SAM) as the methyl donor (Ashihara and Crozier, 2001). It is speculated that coffee plants possess multiple sets of enzymes, which are necessary for constitutive production of caffeine in relevant tissues (Uefuji et al., 2003). The NMT genes isolated so far resemble each other in nucleotide and amino acid sequences and the differences in their spatial and temporal expression may be attributed to the 5' regulatory regions of these genes. To study the sequences responsible for the regulated expression of coffee NMTs, 5' upstream region of one of the NMT gene was isolated by PCR based walking technique. In this report, we describe the successful isolation of promoter for one of the coffee N- Methyl Transferase gene and its ability to drive the expression of the GUS reporter gene in transformed tobacco.

## RESULTS AND DISCUSSIONS

Using a PCR method for walking in uncloned genomic DNA (Siebert et al., 1995) the 5' regulatory region of Coffee N-Methyl Transferase (NMT) gene was successfully isolated (Figure 1). The 774 bp PCR walking product was cloned and sequenced. DNA alignments revealed that the 5' untranslated region (UTR) and the first exon sequence of the walked DNA fragment correspond to the cDNA sequence of Theobromine synthase CaMXMT-1 (Accession No. AB048794). The isolated promoter fragment contains a commonly occurring

TATA box at –256nt (numbering related to start ATG), and two putative CAAT boxes located at –295 and –328 nt (Figure 2). The transcriptional start site (TSS) consensus sequence was located 215 nt upstream of start ATG of the coding region.

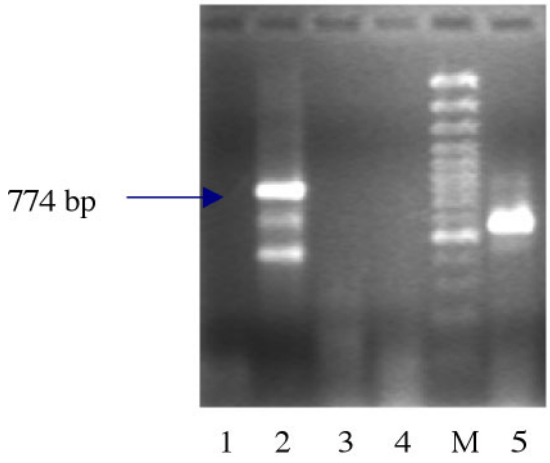


**Figure 1. Agarose gel electrophoresis of PCR walking products. Products were generated by nested PCR with adapter ligated DNA libraries using adapter and gene specific primers: lane 1 D*ra* I library; lane 2 E*co*R V library; lane 3 H*inc* II library; lane 4 S*ca* I library; lane M 100 bp DNA ladder; lane 5 S*sp* I library.**

Sequence analysis of DNA fragment located 692 nt upstream to first ATG of CaMXMT- 1, using PLACE program (Higo et al., 1999) based on TRANSFAC database, revealed the presence of several putative motifs known to be of great importance in the promoter function. The most frequently reported domains in the promoter sequence are those recognized by Dof proteins. Nine domains with AAAG core sequence at –608, –593, –557, –549, –539, –513, –491, –438 and –395 nt were found. Another GT-1 cis- element (GRWAAW) was found at five positions –611, –572, –479, –398, and –325 nt. Among other cis- elements that are recognized are WRKY factor binding sites (TTGAC at –655, –554 and –142 nt); E box CANNTG (–623 and -484 nt), GATA and I boxes involved in light regulation at (GATA, –434, –429 and –424 nt) and (GATAA, –434 and –429 nt) respectively; –300 element (TGHAAARK, –480 nt); -10 PEHVPSBD (–445) and 2SSEEDPROTBANAPA (–415 nt).

The 774 bp fragment spanning the - 691 bp to + 83 bp region (numbering related to ATG of the Theobromine Synthase cDNA sequence, Accession No. AB 048794) was fused translationally in frame with a cassette containing the GUS gene and the NOS terminator in a binary vector pCAMBIA 1381. Plasmids pCAMBIA 1301 as positive control (containing CaMV35S promoter), pCAMBIA 1381 as negative control (containing promoterless GUS cassette) and pPCTS774 (containing NMT promoter) were used in *Agrobacterium tumefaciens* mediated tobacco transformation. The cultures were selected on 10mg/l hygromycin and the selected callus lines were subjected to GUS assay to confirm the expression of the reporter gene. Histochemical staining of GUS activity was performed using a procedure previously described (Jefferson et al., 1987). GUS activity was not detected in the transgenic calli transformed with the vector pCAMBIA 1381. However, GUS activity was detected in transgenic calli transformed with the vector pCAMBIA 1301, confirming the expression of CaMV 35S promoter. Similarly, GUS activity was detected in transgenic calli transformed with the NMT promoter/GUS fusion construct pPCTS774, confirming the ability of the isolated promoter to drive gene expression (Figure 3).

```
                                                          WRKY
-662                                                a gttgatTTGA Cattgaatag
                                 E-Box                GT-1 Dof
-642 actccagctt cttcctttCA TTTGttgaga GGAAAAagaa acagacggga
     Dof                      GT-1                  Dof WRKY Dof
-592 aagaaatagg aatgaagcaG AAAAAtggtg ggctaaaAGT CAaaagaagc
       Dof                                Dof
-542 ggaaagtgat agtgtagagg aagaagggaa agaggcggaa gaacaggagg
      Dof    E-box GT-1
-492 aaaggtaCAG GTGAAAAAtc aaaattctgc ccaaatagta agctactatt
         Dof                                          GT-1 Dof
-442 ctgaaagcct gttatgtcta gaaggacaaa cacgtacttt taaGGAAAAg

-392 gacaaaaatg ccctagaata gttatacact gtttagacca gggtacactt
                       GT-1
-342 tatgaggtta ttgctGGAAA Aaacgaaaca tagtggctgc ttctctccca
                                                        CAAT
-292 tcttttgcaa cgtgggagaa gaagccttag gttttTATAT ATAtatgtaa
                                                  TATA box
-242 gatgtaagat aagataagat atttcatccc cgcatctcaa cttctgattt
                                  TSS
-192 tatcattcgt gtctggttcc cgttggctgt gcgctttctt tctgacgaaT
     WRKY
-142 TGACagactt ttctacgcac ggaggtagct ggttagcata cgcatctatg

 -92 aaattttcgc tacttaagcc cgaaattttg cacaattaat cattaacaga
                                                    +1
 -42 caccttcttt agcagtcgca attcgattgt cctgcatatg aATGgagctc
                                                      M  E  L
  10 caagaagtcc tgcatatgaa tgaaggtgaa ggcgatacaa gctacgccaa
       Q  E  V   L  H  M   N  E  G  E   G  D  T   S  Y  A
  61  gaatgcatcc gacaatgtct gtct
      K  N  A  S   D  N  intron1
```

**Figure 2. Sequence of the CaMXMT-1 promoter is given along with the first exon. The A of the first codon of ATG is assigned +1. The transcription start site (TSS), putative TATA box and CAAT box sequences are italicized and underlined. The, E box, GT-1 box and WRKY sequences are bold and enlarged; Dof protein binding sites are underlined. The deduced amino acid sequence of the first exon is given the nucleotide sequence.**


**Figure 3. Detection of GUS activity in transformed tobacco calli.**

The callus lines, which were positive for GUS assay, were selected for further plant regeneration. Plantlets were regenerated from the transgenic callus and the plant tissues were subjected to GUS assay. Gus activity was observed in the leaf margins, trichomes of the transformed tobacco plant (Figure 4). This is in accordance with the previous reports (Ashihara and Crozier, 2001; Frischknecht et al., 1986) that caffeine and related purine alkaloids are present in young growing tissues, and offer resistance against pests feeding on those tissues. GUS activity was also observed in transformed plant with CaMV 35S promoter, used as a positive control.

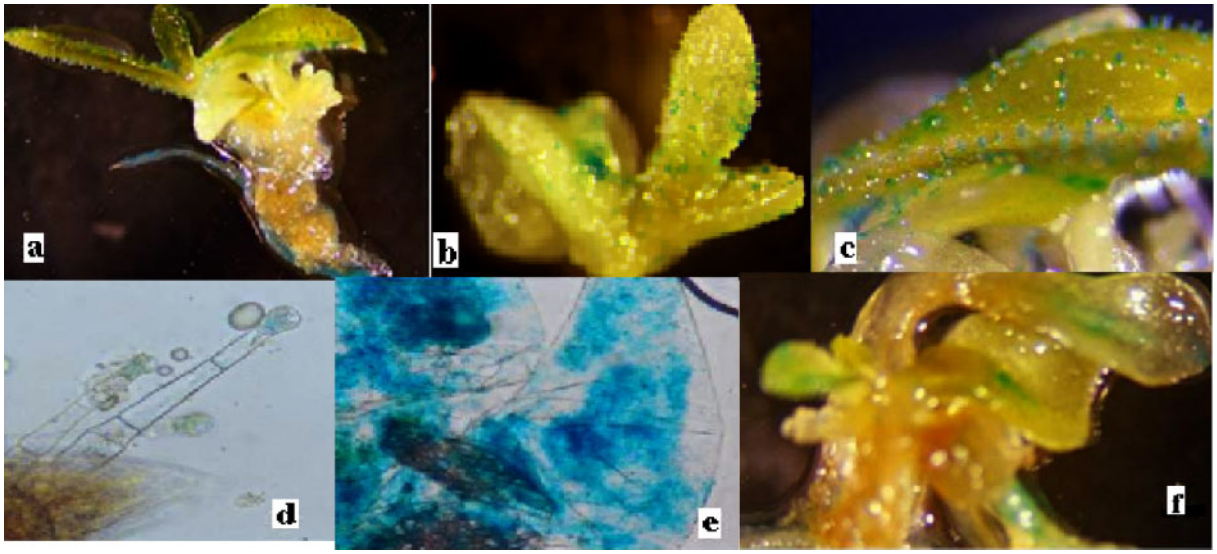


**Figure 4. Histochemical localization of GUS activity in the regenerated transgenic tobacco plants. (a) Tobacco plant transformed with coffee NMT promoter driven cassette, (b) GUS activity in leaves, (c) and (d) GUS activity in leaf trichomes, (e) GUS localization in cells from calli, (f) Tobacco plant transformed with CaMV35S promoter driven cassette.**

Another fragment of ~930 bp containing the 5' upstream region of coffee NMT gene has been obtained. A series of deletions in this NMT promoter region are being constructed and these will be analyzed for GUS activity by stable expression in transformed tobacco. These studies are likely to provide newer insights into the regulation of coffee NMT genes.

## ACKNOWLEDGEMENTS

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# Robusta-like Coffee Plants with Arabica-like Cup Quality- Myth or Possibility?

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## SUMMARY

Presently arabica coffee plantations suffer serious damage due to ravages of white stem borer (*Xylotrechus quadripes* Chev.) and leaf rust *(Hemileia vastatrix,* B.& Br.) resulting in substantial crop loss whereas robusta coffee is relatively less affected by both these pests. The breeding and release of C x R variety from CCRI, India is a step in developing coffee plants that look like robusta but with improved cup quality. In the present study, a natural triploid of C x R was crossed with Catimor arabica and the progeny segregated into 70% tetraploid C x R types and 30 % Catimor types with C x R types showing freedom from rust, self pollination upto 50 % and cup quality characterized by fair to good body, slight to fair acidity and light + flavor notes indicating taste nuances of both arabica and robusta.

## INTRODUCTION

Robusta coffee is known for its general robustness with less susceptibility to leaf rust (*Hemileia vastatrix* B & Br.) and white stem borer (*Xylotrechus quadripes* Chev.), thick plump beans producing a strong, bitter brew lacking in aroma and acidity. Arabica coffee on the other hand is susceptible to the attack of leaf rust (to varying degree on different varieties) and white stem borer and produces thinner, broader beans yielding a thin brew with characteristic aroma and acidity. A question often asked by planters and researchers is whether it is possible to produce coffee plants that look like robusta with its natural resistance/ tolerance to leaf rust and white stem borer but with a cup quality similar to arabica. Breeding programs involving crossing of arabica with robusta initiated in several countries with this objective (Capot, 1977; Cramer, 1957; Monaco et al., 1974; Srinivasan and Vishveshwara, 1980) have not given desired success because of backcrossing with arabica which drives the hybrid more towards arabica making it susceptible to both the enemies.

The breeding and release of Congensis X robusta (C x R) hybrid from CCRI, India (Anonymous, 1998) which was earlier known as Congusta in Dutch East Indies (present Indonesia) (Cramer, 1957) is a step in developing coffee plants with robusta phenotype but with cup quality described as "one of sweetness and soft buttery notes- cup nuances which are indeed rare in the robusta strain" (Sunalini N. Menon, 2000). However the cup quality of C x R, although better than that of other robusta, is not nearer to arabica because it lacks acidity and flavor. Hence an effort was made to cross C x R with arabica, the result of which is reported in this article.

## MATERIAL AND METHODS

C x R is a diploid with 2n = 22 chromosomes like robusta. It cannot be easily crossed with arabica which is a tetraploid with 2n = 44 chromosomes. Even if crossing is possible, the

resulting progeny will be triploid with 2n = 33 chromosomes and will be sterile yielding no seed. Sometimes shortcuts are possible in breeding if desirable plants are tailor made in nature. A weak looking plant with no fruit or seed set was identified in a plot of C x R at Coffee Demonstration Farm of the Coffee Board at Gonicoppal in south Coorg (Figure 1). Its chromosome number was checked and found to be a triploid with 2n = 33. It was used in crossing with Catimor arabica. Fruit and seed set was observed only when it was used as pollen parent. Resulting progeny of 33 plants was planted at Coffee Research Sub-station of the Board at Chettalli in 1998. The progeny segregated into 22 CxR like plants and 11 Catimor like plants (Figure 2 & 3). CxR types however appeared like tetraploids with thicker and broader leaves. Chromosome number confirmed their tetraploid (2n = 44) status. Flowering was observed in 2001. During 2002 all the plants were selfed by watering and bagging before general blossom. Percentage fruit and seed set, out-turn (fruit to clean coffee), grade percentage and cup quality were assessed during 2002 and 2003. Next generation was established at 4 farms of the Board.

**Figure 1. Natural Triploid of C x R.**

**Figure 2. Catimor Plant.**

**Figure 3. $F_1$ of Catimor x Triploid C x R.**

## RESULTS AND DISCUSSION

Progeny showed fruit set under self-pollination ranging from 2.8 to 51.2% in CxR types and from 21.1 to 77.4% in Catimor types. Percentage of floats was 13.8 % in CxR types and 10% in Catimor types (Table 1). Out-turn ratio (fruit to clean coffee) was 7.5:1 in CxR types and 5.5:1 in Catimor types. Clean coffee grade percentages showed 27.2% peaberry and 46.6% 'A' grade beans (retained on 6.65 mm screen) in CxR types. The combined percentage of 'A' and 'B' (retained on 6.0 mm screen) grade beans was 59.1%. This grade profile was similar to that of standard CxR with normal bold beans and superior to that of ordinary robusta. All the CxR type plants were free from rust.

**Table 1. Out-turn (Fruit to clean coffee) in Catimor x (CxR).**

| Description | Fruits (Kg) | Floats % | Wet Parchment (Kg) | Dry Parchment (Kg) | Clean coffee (Kg) | Out turn ratio |
|---|---|---|---|---|---|---|
| Arabica-like plants | 10 | 10 | 3.85 | 2.05 | 1.80 | 5.5: 1 |
| Robusta-like plants | 12 | 13.8 | 3.50 | 1.80 | 1.60 | 7.5: 1 |

**Table 2. Clean coffee grade percentages.**

| Description | Peaberry % | 'A" grade % | 'B' grade % | 'C' grade % | Blacks,bits & browns % |
|---|---|---|---|---|---|
| Arabica-like plants | 9.1 | 69.9 | 12.5 | 6.8 | 1.7 |
| Robusta-like plants | 27.2 | 46.6 | 12.5 | 5.7 | 8.0 |

Cup quality analysis revealed 'Fair to good body; a neutral, fairly soft and smooth cup with hints of slight to fair acidity and flavor indicating taste nuances of both arabica and robusta' (Table 3).

**Table 3. Quality assessment of bean samples from Catimor x (CxR).**

| Year | Lab. | Raw | Roast | Cup |
|---|---|---|---|---|
| 2002 | Private Lab. | Medium to small. Oval. | Fairly even roast. Fair swelling. Sl. dull.Many open centers. Fairly even surface. Small % of chaff adhering. | Fair body. Fair+ to good acidity. Light+ flavor notes. Clean. Aftertaste is one of acidity. FAQ to Sl.above FAQ. |
| 2002 | Board's Lab. | ---------- | ---------- | Good Body, Sl.acidity<br>Fairly Soft<br>Fairly Neutral |
| 2003 | Private lab. | Greyish with a hint of brown | Fairly even roast. Good swelling. Slightly dull. Hint of shine. Many with open centers. Fairly smooth surface. Fair % of chaff adhering to the beans. | Fair+ to good. Neutral. Fairly soft & smooth. Clean. Hint of bitterness. Presence of acidic notes. A neutral, Fairly soft & smooth<br>Cup. Rating: Good |
| 2003 | Board's Lab. | Greenish grey. Oval oblong beans. | Even roast, medium-good swelling. | Medium to good body. Fair acidity. Slight flavor. Rating: FAQ+(Beans appear like robusta but tastes like arabica) |

Segregation of open pollinated progeny from CxR types in the next generation of 2 years old plants has shown a proportion of 70% CxR types and 30% Catimor types out of a population of 346 plants which is similar to that of parental population. Pattern of segregation in the selfed progeny needs to be studied.

The above results have given an indication that it should be possible to evolve a population of coffee plants that phenotypically resemble robusta but with seed quality that has a mix of arabica and robusta.

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# High – Efficiency *Agrobacterium* – Mediated Transformation of Coffee (*Coffea canephora* Pierre ex. Frohner) Using Hypocotyl Explants

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## SUMMARY

An efficient regeneration and *Agrobacterium* mediated transformation system was developed for two commercially important cultivars (c.v S.274 and CxR) of robusta coffee. Hypocotyl explants obtained from the *in vitro* grown seedlings and embryogenic calli gathered from the hypocotyl explants were co-cultivated with *Agrobacterium tumefaciens* strain *EHA 101/p BECKS* that harbored genes for β-glucuronidase (GUS), kanamycin (*npt II*) and hygromycin (*hpt*) resistance. Prior to co-cultivation the target explants were incubated on MS medium containing 0.1 mg/l 2,4-dichlorophenoxyacetic acid, 1 mg/l Indole -3 Acetic Acid and 4 mg/l Kinetin for 3 days in darkness. Several factors which significantly affected the t-DNA transfer were evaluated and optimized co-cultivation was performed at 22°C in an acidic medium (pH 5.5) in the presence of 50 μM acetosyringone for 3 days. After 2-3 months of culture on hygromycin (30 mg/l) supplemented selection medium, hygromycin resistant somatic embryos were isolated and separately cultured on germination medium containing 0.5 mg/l IAA and 2 mg/l kinetin and 50mg/l hygromycin. Putative transformed plantlets of two cultivars were obtained after rooting on half strength MS medium supplemented with 0.5 mg/l kinetin.and 40 mg/l hygromycin and subsequently hardened in the polybags containing soil mixture. Stable transformation of the recovered plants was verified by histochemical GUS assay and molecular analysis.

## INTRODUCTION

Genetic improvement of coffee by conventional breeding is slow and tedious due to long generation time. This makes coffee an ideal target plant species for gene transfer technologies which can provide a direct route for the introduction of a specific genetic change within a short period of time. Improvement of genetic engineering requires an efficient gene delivery system, reliable selection of transformants and high frequency regeneration of transformed tissue.

Among various transformation methods available, *Agrobacterium* transformation is favored because it introduces few transgene copies with less fragmentation and also the usual Mendelian inheritance of transgenes. *Agrobacterium* transformation is influenced by many variables such as plant genotype, target tissue, *Agrobacterium* strains, binary vecor system and transformation and culture conditions (Hiei et al., 1994; Zhang et al., 1997 and Mishra et al., 2002). Although *Agrobacterium* -mediated transformation in coffee is reported, the efficiency of transformation was very low (Hatanaka et al., 1999; Leroy et al., 2000).The aim of this investigation reported here was to develop an efficient regeneration and transformation system for *Coffea canephora* using hypocotyl explants. The development of a reliable

transformation protocol for this crop will form the basis for its future genetic enhancement by complimenting classical breeding program.

## MATERIALS AND METHODS

### Plant Material and culture medium

Seeds of *C. canephora* (cv. S.274 and CxR) was collected and stored in a refrigerator at 4ºC till use. After disinfection, seeds were sown in culture bottles containing 40 ml of germination medium consisting of half strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 2% sucrose and agar and the pH of the medium was adjusted to 5.8. The cultures were kept in dark for 5-6 weeks till the germination of seedlings following which they were transferred to light with a 16-h photo period at a 40-50 µmol $m^{-2}5^{-1}$ provided by cool fluorescent tubes.

Hypocotyl segment of the germinated seedlings were cut in to small pieces (1 cm length) and incubated in MS medium supplemented with different growth regulators for callus induction and somatic embryogenesis (Table 1). All the cultures were maintained in dark till the development of distinct somatic embryos. Germination of somatic embryos was carried out in presence of light in half strength MS medium supplemented with 0.5% kinetin.

**Table 1. Effect of growth regulators on callus induction and SE from the hypocotyl explants.**

| Sl No. | Basal MS medium + Growth regulators ($mg^{-1}$) | Genotype | % explants with callus | % explants with SE |
|---|---|---|---|---|
| 1 | IAA(1)+ Kn(5) | S-274 | 64.16 ± 4.36 | 49.22 ± 1.76 |
| | | C x R | 66.10 ± 7.88 | 52.14 ± 1.38 |
| 2 | IAA (1)+BA(5) | S-274 | 76.62 ± 3.42 | 48.38 ± 3.84 |
| | | C x R | 69.58 ± 5.62 | 44.26 ± 5.22 |
| 3 | NAA(1)+Kn(5) | S-274 | 67.18 ± 2.89 | 64.36 ± 2.18 |
| | | C x R | 54.34 ± 5.13 | 52.76 ± 1.83 |
| 4 | NAA(1)+BA(5) | S-274 | 66.52 ± 5.32 | 61.28 ± 2.56 |
| | | C x R | 72.86 ± 4.36 | 50.08 ± 1.81 |
| 5 | 2,4-D(0.5)+Kn(5) | S-274 | 97.40 ± 0.48 | 14.18 ± 5.26 |
| | | C x R | 92.52 ± 1.38 | 22.46 ± 2.48 |
| 6 | 2,4-D(0.1)+IAA(1)Kn(4) | S-274 | 81.44 ± 3.86 | 78.36 ± 1.48 |
| | | C x R | 78.76 ± 4.22 | 76.48 ± 1.06 |

### Transformation & Selection

Disarmed *A. tumefaciens* strain EHA 101 (pEHA101) (Hood et al., 1986) containing the binary vector pBECKS Gus intron/hph (McCormac et al., 1997) was used for hypocotyl transformation. This construct contains chimeric gusA gene with a plant intron in the protein coding region. The CaMV35pro-hph-Nos ter in the t-DNA of pBECKS permit hygromycin selection.

Bacterial cells were cultured on an orbital shaker at 28ºC at 180 rpm in LB medium (Sambrook et al., 1989) containing 100 mg/l spectinomycin. Bacterial cells were pelleted at 3500 rpm for 10 min, re-suspended and diluted in liquid co-cultivation medium containing MS salts and vitamins, 0.1 mg/l 2, 4-D, 0.5 mg/l IAA and 3 mg/l kinetin, 3% (wt/vol.) sucrose, pH5.4. Hypocoty segments of 1cm length and embrygenic calli gathered from the

hypocotyl were separately incubated in bacterial suspension for 15 min. The infected explants were placed on solid co-cultivation medium and kept in dark for 1-5 days. After co-cultivation, explants were washed in cefotaxime solution (250 mg/l) and either assayed foe gusA expression or transferred to somatic embryogenesis medium as described. Selection of transformed tissue was carried out in the presence of 30 mg/l hygromycin B at monthly intervals till somatic embryos were prominently developed. Putatively transformed somatic embryos were incubated in the germination medium with 40-50 mg/l hygromycin. After the formation of 3-4 pars of leaves, plants were transferred to net pots with soilrite for hardening.

**Histochemical GUS Assay**

Gus activity in the co-cultivated hypocotyl explants were assayed histochemically following the method of Jefferson (Jefferson, 1987).

**Genomic DNA isolation and Molecular Analysis**

High molecular weight genomic DNA was isolated from the control as well as transformed tissue using CTAB procedure (Doyle and Doyle, 1987). After RNase A treatment the mixture was extracted once with phenol/chloroform (1:1) and once in chloroform. DNA was precipitated by the addition of 1/10 vol of 3M sodium acetate and 2 vol 96% EtOH, washed and finally resuspended in TE buffer.

PCR was performed in a MJ Research thermolcycler. The primers used for amplification of *gusA* gene were 5'-AGTGTACGTATCACCGTTTGTGTGAAC-3' and 5'-ATCGCCGCTTT GGACATACCATCCGTA-3' and those used for amplification of *hpt* gene were 5'-AAGCCTGAACTCACCGCGACG-3' and 5'-AAGACCAATGCGGAGCATATAC -3'. Standard PCR reaction was carried out in 50µl reactioncontaining 100 ng of DNA, 200 µM DNTPs, 0.25 mM Magnesium chlorideand 1X triton X reaction buffer containing 50 mM KCL, 10 mM Tris HCL, 0.1% v/v triton x and 2 unit taq polymerase enzyme. The PCR conditions were 95°C for 3 min followed by 35 cycles of 0.5 min at 95°C, 0.5 at 35°C and 1,25 min at 72°C with a final extension of 5 min at 72°C. PCR products were separated on agarose gels and visualized with ethidium bromide.

**RESULTS AND DISCUSSION**

The morphogenic response of hypocotyl explants of S.274 and CxR to various combinations of auxins and cytokinins was examined. The auxin 2,4-D in combination with Kn induced massive watery callus from the hypocotyl explants compared to any other combinations (Table 1). However, somatic embryogenesis was considerably low in the presence of 2,4-D. Use of 2, 4-D in lower concentration (0.1 mg/l in presence of IAA (1 mg/l) significantly increased the responsiveness of the hypocotyl explants to callusing as well as somatic embryogenesis (Table 1) and therefore this combination was used later for transformation experiments.

The influence of co-cultivation period on transformation frequency using histochemical *gusA* assay revealed a positive co-relation between co-cultivation days and transient *gusA* expression (Figure 1). However co-cultivation of hypocotyl explants beyond 4 days led to an uncontrollable bacterial growth resulting explant necrosis and death. The hypocotyl as a suitable target explant for *Agrobacterium* transformation was already tested in coffee during an optimization protocol (Mishra et al., 2002).

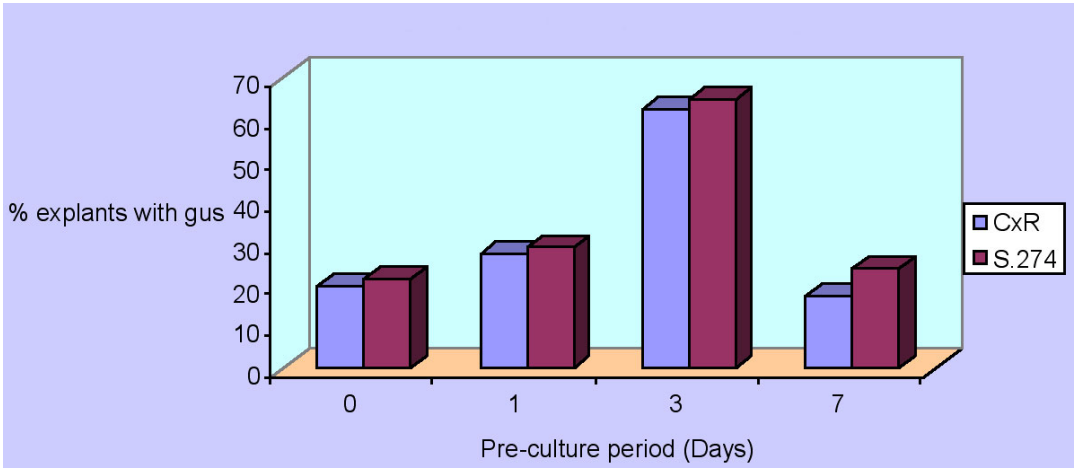


**Figura 1. Effect of Pre-culture on gus expression.**

Similarly the effect of pre-culture of explant prior to co-cultivation was also tested and it was observed that 3 day of preculture of explants in callus induction medium (CIM) increased transformation frequency (Figure 2). Pre-culre of explants prior to *Agrobacterium* co-cultivation has been shown to improve transformation frequencies in many woody plantsin cluding coffee (Srikandarajah & Goodwin 1998, Mishra et al., 2002).

In the present experiment hygromycin B was effectively used to isolate transformed tissue from the nontransformed ones in the selection medium. In earlier studies Hatanaka et al. (1999) used a higher concentration of hygromycin and a combination of hygromycin and kanamycin (50 mg/l each) for elimination of nontransformed tissue. However, in the present study, hygromycin at 30 mg/l completely inhibited non-transformed tissue growth without interfering with the transformed tissue proliferation.

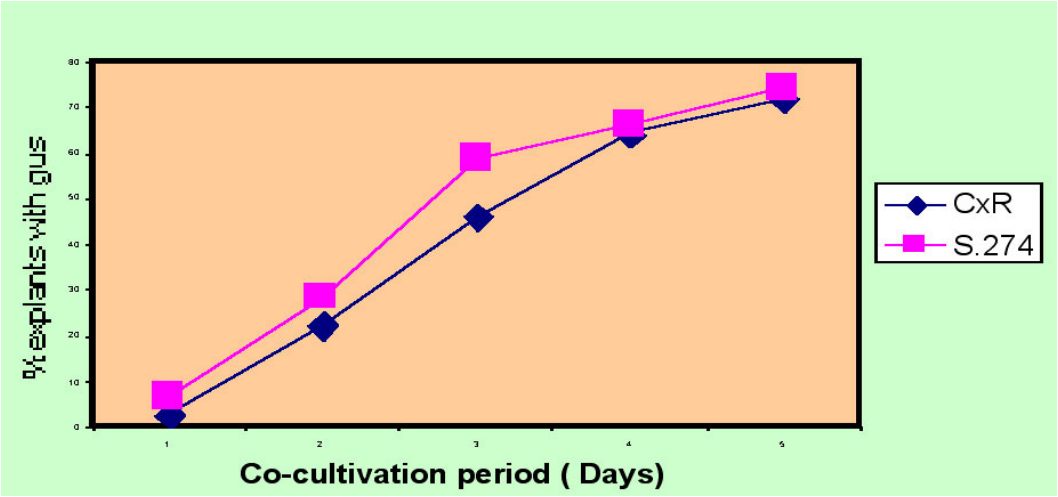


**Figure 2. Effect of Co-cultivation period on gus expression.**

The putatively transformed somatic embryos exhibited a strong gusA expression. With the development of cotyledonary leaves gusA expression was gradually shifted to the emerging cotyledonary leaves and terminal portion of the hypocotyl, which is a characteristic features of CaMV 35s promoter. Plantlets were obtained in high frequency and integration of transgenes (gusA and hpt) were confirmed through PCR analysis.

In conclusion, we have developed a high efficiency *Agrobacterium* transformation system in *C.canephora* using hypocotyl explants.

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# Micropropagation of *Coffea arabica* cv. Cauvery via Somatic Embryogenesis

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## SUMMARY

Plant regeneration through somatic embryogenesis is reported in three genotypes of *Coffea arabica (Cauvery* / Catimor) viz., S.4347, S. 4348 and S. 4350 from cultured leaf tissues. In all the experiments, MS medium supplemented with different combinations and concentrations of auxins (2,4-D and NAA) and cytokinins (Kn, BAP and TDZ) supplemented with 50 $mgl^{-1}$ of L-cystiene HCl were used initially for callus induction. The calli were subcultured at 30-40 days intervals onto fresh medium with 1 $mgl^{-1}$ each of 2,4-D and BAP and also different concentrations of BAP alone. After 4 months of incubation of the callus induction medium, embryogenic calli developed in S. 4348 genotype followed by S. 4347 and S. 4350 genotypes. Initially, low frequency of somatic embryogenesis was achieved in all the genotypes tested. However, after subculture on half-strength MS medium with 0.1 $mgl^{-1}$ of Kn, high frequency as well as more uniform somatic embryos were obtained. After 4-5 subcultures at monthly intervals, these somatic embryos were germinated and converted into plantlets on the same medium. Subsequently, plantlets grew further, hardened and established in the field. Regenerated plants and seedlings were compared for different morphological and agronomic characteristics.

## INTRODUCTION

Coffee is one of the most important agricultural products in the international market. *Coffea arabica* was the first species to be commercialized in Asia and Europe and accounts for about 70% of the coffee consumed in the world because of its superior beverage quality. Commercial production of coffee has been greatly improved by plant breeding.

Recent advances in plant cell, developmental and molecular biology will lead to further improvement in coffee agriculture and bean quality. Pioneer work on coffee tissue culture was first published by Staritsky (1970). He was successful in inducing somatic embryos and plantlets from orthotropic shoots of *Coffea canephora.* Later, there are number of reports on somatic embryogenesis and plant regeneration from different tissues. In the present study, we report micropropagation via somatic embryogenesis, hardening and subsequent field performance in three genotypes of *C. arabica* cv. Cauvery/ Catimor.

## MATERIALS AND METHODS

The leaves were collected from the field grown plants of *C. arabica* cv. Cauvery/ Catimor (S. 4347, S. 4348 and S. 4350) from the farm of Central Coffee Research Institute (CCRI), India. They were sterilized in 1 percent sodium hypochlorite solution for 7-8 minutes and washed 4-5 times with sterile distilled water. The leaf discs were made (5-6 mm in diameter) in 0.1% sterile solution of ascorbic and citric acid for avoiding phenolic oxidation and placed with abaxial side up as described by Sondahl and Loh (1988) on agar solidified nutrient media.

MS (Murashige and Skoog, 1962) media with L-cysteine HCl (50 $mgl^{-1}$); PVP (100 $mgl^{-1}$), sucrose (3 and 4%), pH was adjusted between 5.6-5.8 and solidified with 0.8% bacteriological agar were used. This basal media were modified by supplementing with different concentrations and combinations of auxins (2,4-D, IAA and NAA) and cytokinins (Kn and BAP). The primary cultures were incubated in growth room under natural light conditions. Whereas, the embryogenic cultures, somatic embryos and plantlets were incubated under light at an intensity of 30 μmol $m^{-1}$ $s^{-2}$.

Embryogenic calli and somatic embryos were transferred on to MS medium supplemented with 1 $mgl^{-1}$ of ABA for 4-5 weeks for embryo multiplication and maturation and subcultured to medium with 0.5 $mgl^{-1}$ of Kn initially for one month and then reduced concentrations of Kn (0.1 $mgl^{-1}$) for embryo germination and plantlet development. Regenerated plantlets having long shoots with 2-4 pairs of expanded leaves and good roots were planted in netpots containing soilrite mixture for hardening. Two months hardening, they were transplanted into polybags containing soil mixture, established in the field and morphological and yield data was recorded.

## RESULTS AND DISCUSSION

High rate of loss was noticed in Monsoon (80-90%), followed by winter (50-60%) even after treating the leaves with systemic fungicide (Bavistin). However, less contamination (20-30%) was observed in summer. Callus proliferation started after 12-15 days of inoculation from the cut edges of the leaf explants. Moderate to massive watery callus was obtained in presence of IAA, NAA in combinations with Kn and BAP but inhibited the embryogenesis. Whereas, moderate, light brown to massive calli was obtained in presence of 1 $mgl^{-1}$ of 2,4-D and 4 $mgl^{-1}$ of Kn and it has influenced the induction of somatic embryos (Table 1). When these calli along with explants were subcultured on to medium with 1 $mgl^{-1}$ each of 2,4-D and BAP and induced somatic embryogenesis.

**Figure 1. Plant regeneration via somatic embryogenesis from the leaf cultures of *Coffea arabica* cv. Cauvery genotypes. A. High frequency of somatic embryos produced from leaf explants of S. 4348 on half strength MS medium with 0.1 $mgl^{-1}$ of Kn. B. Hardened plants in net pots containing soilrite mixture.**

Initially somatic embryogenesis was achieved in a very low percentage and low frequency. Later, high frequency was achieved (Figure 1A). When the embryogenic calli and somatic embryos of all genotypes of *C. arabica* were subcultured with reduced concentrations of Kn (0.1 $mgl^{-1}$) with 4% sucrose for 2-3 times at monthly intervals, the embryos started germinating and converted into plants. Plants grew further with long shoots having 2-3 pairs of expanded leaves and good roots of 2-3 cm long, they were planted in net pots containing soilrite mixture for hardening (Figure 1B).

Regenerated plants and seedlings of same mother plants were planted in field and established in two estates of Karnataka for evaluation. Morphologically, regenerated plants of Cauvery

genotypes showed more vigor than seedlings initially. Further, the morphological growth, yield, bean grade and cup quality of the regenerated plants was found to be almost on par with the seedlings. However, accumulation of further data on growth index, yield and cup quality rating of micropropagated plants and seedlings in subsequent years is needed to confirm the specific advantage over seedlings.

**Table 1. Effect of growth regulators in MS media on callus induction and embryogenesis from leaf tissues of *C. arabica* cv. Cauvery (data recorded after 3 months).**

| Genotypes | Growth regulators tested ($mgl^{-1}$) | %. of explants callused | Type of calli | % of explants turned embryogenic |
|---|---|---|---|---|
| S.4347 | **2,4-D + Kn** | | | |
| | 0.1 + 4.0 | 48.6 | 2-10 mm, light brown | --- |
| | 1.0 + 4.0 | 41.3 | 5-20 mm wide, massive | 1.2 |
| | **NAA + Kn** | | | |
| | 1.0 + 5.0 | 34.7 | 6-10 mm, moderate | |
| S.4348 | **IAA + BAP** | | | -- |
| | 0.1 + 5.0 | 36.1 | 4-8 mm, yellowish | |
| | **NAA + Kn** | | | |
| | 0.05 + 0.25 | 70.8 | 6-10 mm, moderate | ---- |
| | 1.0 + 5.0 | 38.7 | 6-15 mm,light brown | 2.8 |
| | **2,4-D+ BAP** | | | |
| | 0.1+ 4.0 | 55.7 | 2-10 mm, light brown | ---- |
| | 1.0+ 4.0 | 38.5 | 5-25 mm, light brown | 1.2 |
| S.4350 | **NAA + Kn** | | | |
| | 1.0 + 5.0 | 37.4 | 5-10 mm, moderate | 5.8 |
| | **2,4-D + Kn** | | | |
| | 1.0 + 5.0 | 40.3 | 4-20 mm, massive | 0.7 |
| | **BAP alone** | | | |
| | 1.0 | 44.6 | 1-3 mm wide, whitish | --- |
| | 2.0 | 41.1 | 1-3 mm, light brown | 4.3 |
| | 5.0 | 46.4 | 1-3 mm wide, whitish | ---- |

During the present study, percentage of leaf explants that, produced somatic embryogenesis was less. However, high rate of multiplication of embryogenic calli and somatic embryos was achieved in all the genotypes of *C. arabica* cv. Cauvery. This protocol for plant regeneration through somatic embryogenesis and hardening could be useful for large scale multiplication of elite plants.

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# Genetic Diversity in Indian Arabica Coffee (*Coffea arabica* L) Germplasm Collection through RAPD Markers

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## SUMMARY

Genetic diversity was assessed through RAPD markers in 32 arabica coffee germplasm collections available in India along with three diploid species. Total 284 markers were produced by 43 random primers tested out of which 138 were found polymorphic between the arabica populations studied. RAPD markers revealed less diversity among arabica collections available in India. Genetic distance based UPGMA tree resulted two major groups one being pure arabica genotypes and the second is robusta / HdeT introgressed lines. Kent, S-288, Bourbon, Dilla & Alghe, S-12 Kaffa, Geisha, Blue Mountain, San Ramon, Cioccie, Agaro, Tafarikela and hybrids like S-795, S-5A, S-10, S-11, S-2800 and S-2803 formed group one representing pure arabicas wherein S-2790, S-2794, S-7.3, Cavimor, Sarchimor, Ruiru II, S-12 (Catimor), HdeT, S-6, Devamachy and Rume Sudan formed second group. Three diploid species separated from all arabica populations.

## INTRODUCTION

Coffee (*Coffea arabica* L.) being an important plantation crop of India plays significant role in earning foreign exchange. Crop was benefited by newer varieties through conventional breeding mainly focusing on rust resistance, high productivity, adaptability and quality through out coffee growing regions of the world. Most of the world's coffee improvement programmes are centered on arabica coffee to overcome challenges poured by rust fungus without exception to India. In this context Central Coffee Research Institute, Coffee Board, Govt. of India is enriched with great number of arabica collections introduced from different regions of the world. Further 12 elite selections were released for cultivation after successful hybridization and evaluation of the same (Anonymous, 2002). Present study was undertaken to assess genetic diversity and relatedness of cultivars in arabica germplasm collections and station bred selections through RAPD markers.

## MATERIALS AND METHODS

43 coffee genotypes were selected for the present study which includes 32 arabica cultivars/hybrids, 8 clones of the cultivars and three diploid species (Table 1). Each cultivars / hybrid was represented by single plant. For DNA isolation two step method (Ky et al., 2000) was followed with slight modification. PCR amplification was carried out having 1X PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP's each, 0.5 μM primer and 0.6 units of Taq DNA polymerase (Bangalore Genei) in a final 20 μL reaction volume having 40 to 60 ng of genomic DNA and overlaid with 15 μL of mineral oil. Amplification was performed with 38$^{o}$C annealing temperature. Total 45 random primers reported earlier (Anthony et al., 2001; Lashermes et al., 1996; Santa Ram et al., 2002; Sera et al., 2003) giving good amplification in coffee was used for PCR amplification.

**Table 1. List of coffee genotypes selected for RAPD studies.**

| Sl No | Genotype | Specification |
|---|---|---|
| 1 | Kent | Pure arabica selection |
| 2 | S-288 | Pure lines from S-26 |
| 3 | S-288 33/1 23 | Rust differential clone |
| 4 | S-795 | S-288 x Kent |
| 5 | Cioccie | Ethiopian arabica |
| 6 | Agaro | Ethiopian arabica |
| 7 | Agaro 110/5 | Ethiopian arabica (Rust differential clone) |
| 8 | Tafarikela | Ethiopian arabica |
| 9 | S-5A | S-881 (Rume Sudan) x Devamachy |
| 10 | S-5B | F2 of S-333x Devamachy |
| 11 | Devamachy | Natural hybrid of robusta and arabica |
| 12 | S-881 | Rume Sudan (Parent of 5A) |
| 13 | S-6 | BC2 of S-274 x Kent |
| 14 | S-274 | Pure robusta |
| 15 | San Ramon | Dwarf mutant of arabica |
| 16 | S-7.3 | {(San Ramon x 795) x Agaro} x HdeT |
| 17 | HdeT | Pure line parent used for hybridization |
| 18 | HdeT 832/1 | Rust differential clone |
| 19 | HdeT 1343/269 | Rust differential clone |
| 20 | S-2790 (S-9) | HdeT x Taferikela |
| 21 | S-2800 | Bourbon x HdeT |
| 22 | Bourbon 16/1 | Parent of S-2800 |
| 23 | Bourbon 63/1 | Rust differentials clone |
| 24 | S-2794 | HdeT x Geisha |
| 25 | Geisha 87/1 | Rust differential |
| 26 | S-2803 | HdeT x S-12 Kaffa |
| 27 | S-12 Kaffa | Parent of 2803 |
| 28 | S-12 Kaffa 134/4 | Rust differential clone |
| 29 | S-10 | (Caturra x Cioccie) X (Caturra x 795) |
| 30 | S-11 | *C. liberica* x *C. eugenioides* |
| 31 | *C. liberica* | Diploid species |
| 32 | *C. eugenioides* | Diploid species |
| 33 | S-12 Catimor | Caturra (Bourbon mutant) x HdeT |
| 34 | HdeT x Catuai | HdeT x Catuai (Caturra x Mundo Novo) |
| 35 | Ruiru II | Introduction |
| 36 | MundoNovo | Bourbon X Typica |
| 37 | Sarchimor 13682/42 | Villasarchi (Bourbon derived) X HdeT |
| 38 | Dilla & Alghe 128/2 | Rust differential |
| 39 | DK 32/1 1/6 | Rust differential clone of Kent |
| 40 | 34/13 353 4/5 | Rust differential clone of S-795 |
| 41 | Blue Mountain | Typica derived |
| 42 | Natural Variant | San Ramon mutant |
| 43 | Cavimor 13727/18 | Catimor x (Villasarchi x HdeT) |

PCR products were resolved on 1to 1.2% agarose gel. PCR fragments were scored manually for presence / absence (1/0) and genetic distance was calculated according to Nei (1978).

PHYLIP 3.6a3 computer package was used for data analysis. Binary data was boot-strapped for 200 data sets and distance matrix was generated. A strict consensus tree was generated from 200 UPGMA trees obtained from distance matrices and tree was viewed with Treeview 1.6.6 package.

## RESULTS AND DISCUSSION

Out of 45 random primers tested 43 produced reproducible banding patterns with a total of 284 fragments out of which 138 were found polymorphic. Among 138 polymorphic bands 32 were recorded only in S-5B, S-6, Devamachy, Rume Sudan and HdeT genotypes. Seven unique markers were recorded in Rume Sudan. Six markers differentiated two HdeT genotypes ('A' and 'R' type). One marker was found to be unique for HdeT 832/1 ('A' type plant).

UPGMA tree showed two groups among arabica collections. Pure arabicas and few hybrids formed one group where robusta / HdeT introgressed lines formed other group. First group includes Kent, S-288, Bourbon, Dilla & Alghe, S-12 Kaffa, Geisha, Blue Mountain, San Ramon, mutant genotype, Cioccie, Agaro, Tafarikela and hybrids like S-795, S-5A, S-10, S-11, S-2800, S-2803 and HdeT x Catuai. All these varieties are pure arabicas except for S-288, which is selection from S-26 population, which in turn liberica introgressed line. Advanced progenies of S-288 might have segregated and might be the reason for closeness with pure arabicas. In case of hybrids though HdeT was involved as one of the parent in S-2800, S-2803 and HdeT x Catuai these three hybrids showed closeness with pure arabicas indicating less or no introgression from HdeT parent.

The other group comprised hybrids involving either robusta (Spontaneous) or HdeT as one of the parent. In case of S-2790, S-2794, S-7.3, Cavimor, Sarchimor, Ruiru II and S-12 Catimor, HdeT was one of the parent which contributed resistance against leaf rust (later resistance was breakdown in some cases with the evolve of new rust races). As like HdeT, Devamachy is also a spontaneous hybrid of robusta and arabica and was used for selection 5 crosses (S-5A and S-5B) where S-5A exhibited closeness with pure arabicas and S-5B was found closer with S-6. S-6 is an artificial cross between robusta and Kent arabica with repeated back cross to Kent.

S-5A and S-11 clustered with Ethiopian arabicas. S-11 is an interspecific hybrid of *C. liberica* and *C. eugenioides* with arabica characters, which is resulted from fertile tetroploid sucker on sterile F1 plant. Advanced progenies of this sucker exhibited high percentage of cross compatibility (Annon, 1965; Reddy, 1985) and might have received pollen from nearing Cioccie/ Taferikela plants and later might have acquired and stabilized resembling Ethiopian arabicas. In case of S-5A (Devamachy x Rume Sudan) Rume Sudan is native of southwest Ethiopia and might be due to this S-5A clustered with Ethiopian arabicas.

HdeT 1343/269 ('R' type) plant was found closer with HdeT parent and HdeT introgressed lines indicating R type plant as parent, whereas 'A' type plant (HdeT 832/1) was completely separated from others. Out of eleven Indian selections S-1, S-3 and S-10 clustered with pure arabicas. S-7, S-8, S-9 and S-12 formed one group of HdeT introgressed lines. Ethiopian arabicas (S-4) formed one group along with S-5A and S-11 wherein S-6 and S-5B is found to be closer to each other. Surprisingly two cultivars MundoNovo and S-12 Kaffa are included along with the second group. Although both are pure arabicas their position in the dendrogram is uncertain.

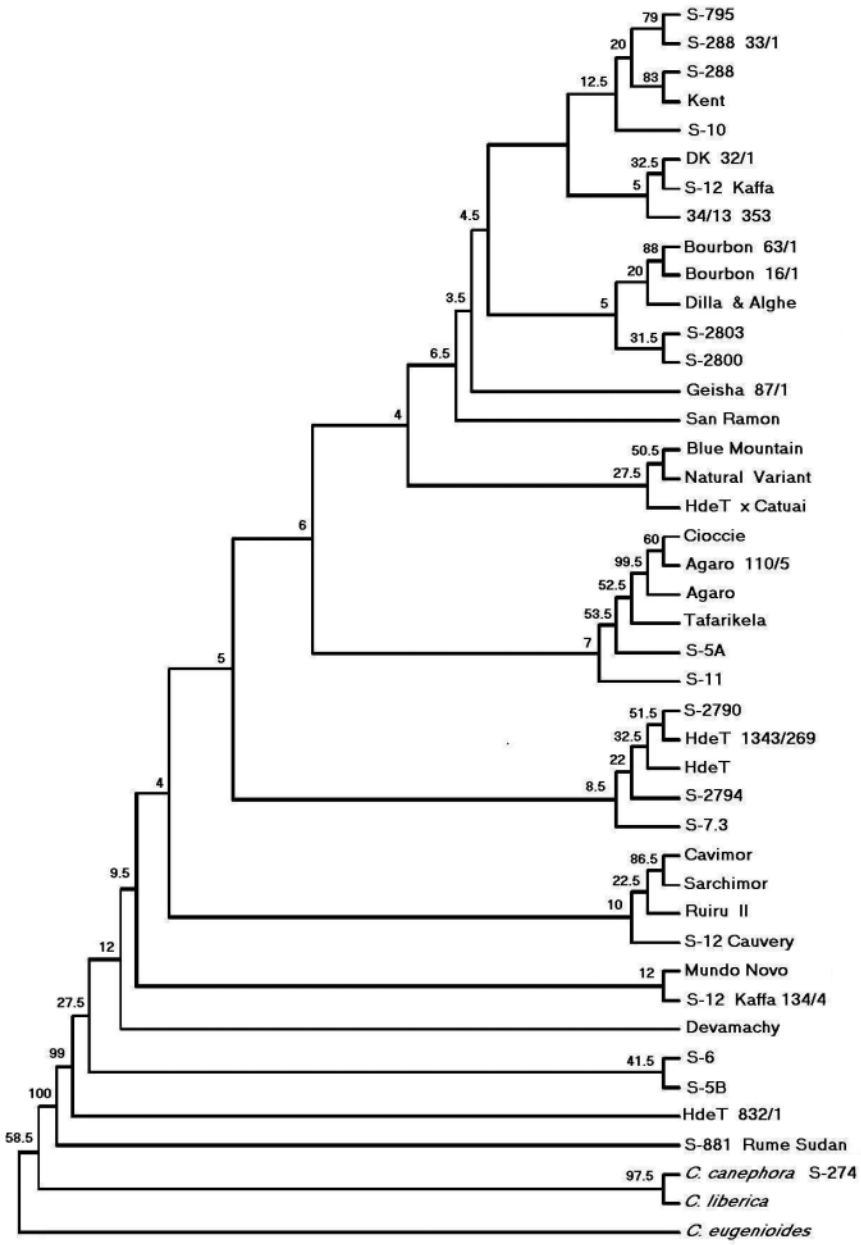


**Figure 1. UPGMA tree coffee genotypes based on genetic distance from 45 random primers. Values on the brancehs are the Bootstrap values from 200 replicates.**

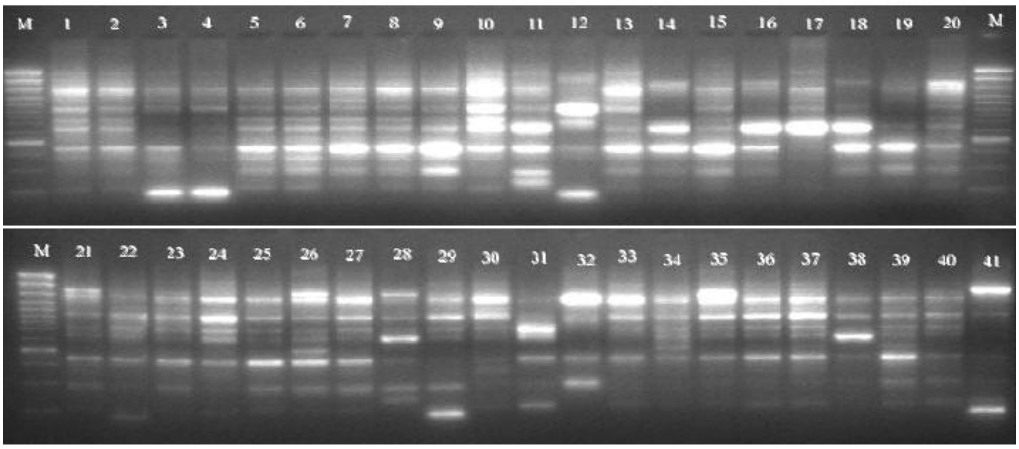


**Figure 2. RAPD profile of coffee genotypes with OPC-15 primer.**

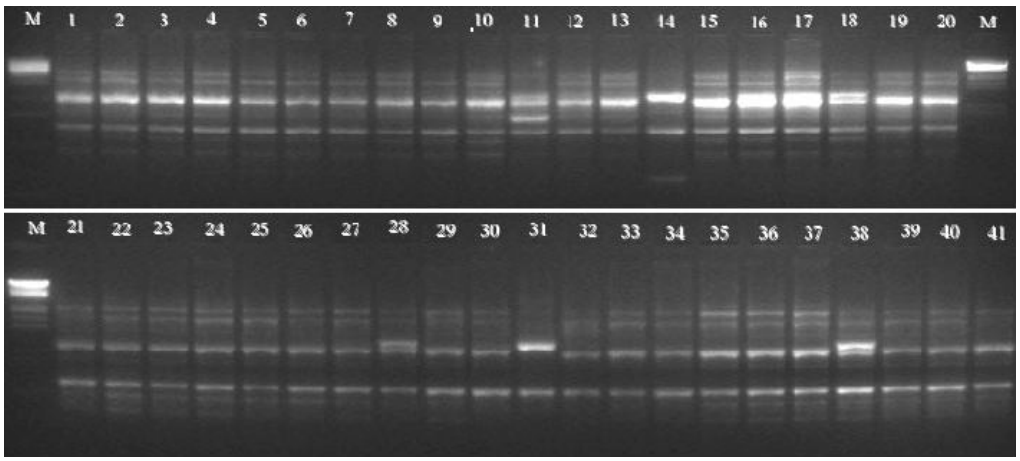


**Figure 3. RAPD profile of coffee genotypes with UBC-220 primer.**

Present study indicated low level of genetic diversity in arabica germplasm collections available in India. Though crosses were made between different genotypes, finally all hybrids showed resemblance with either pure arabica group or with robusta / HdeT introgressed lines. This might be due to the high level of segregation in Coffee. Within the available germplasm Devamachy, Rume Sudan, HdeT 832/1 were found to be highly diverse from other genotypes and as like HdeT, these two can be exploited for future breeding strategies.

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# Pyridine Nucleotide Cycle and Trigonelline Synthesis in Developing Leaves and Fruit of *Coffea arabica*

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## SUMMARY

We examine the biosynthesis of trigonelline in leaves and fruits of Arabica coffee (*Coffea arabica*) plants. [$^{3}$H]quinolinic acid, which is an intermediate of *de novo* pyridine nucleotide synthesis, and [$^{14}$C]nicotinamide and [$^{14}$C]nicotinic acid, which are degradation products of NAD, were converted to trigonelline as well as pyridine nucleotides. These tracer experiments suggest that the pyridine nucleotide cycle, nicotinamide —> nicotinic acid —> nicotinic acid mononucleotide (NaMN) —> nicotinic acid adenine dinucleotide (NaAD) —> NAD —> nicotinamide mononucleotide (NMN) —> nicotinamide, operates in coffee plants, and trigonelline is synthesized from nicotinic acid formed in the cycle. Trigonelline is synthesized and accumulated in leaves and fruits during development. The trigonelline synthesis in pericarps is much higher than that in seeds, but its content in seeds is higher than pericarps, so that some of the trigonelline synthesized in the pericarps may be transported to seeds. Trigonelline synthesis from [$^{14}$C]nicotinamide was also found in *Theobroma cacao* plants, but instead of trigonelline, nicotinic acid-glucoside was synthesized from nicotinamide in tea and tobacco plants.

## INTRODUCTION

Trigonelline (*N*-methylnicotinic acid) is a secondary metabolite derived from pyridine nucleotides. Coffee beans contain a large amount of trigonelline, and food chemical studies indicate that this compound is thermally converted to nicotinic acid and some flavour compounds during roasting. The direct precursor of trigonelline is nicotinic acid, which appears to be produced as a degradation product of NAD. We have reported that trigonelline and its synthetic ability from [$^{14}$C]nicotinic acid are distributed in all parts of coffee seedlings (Zheng and Ashihara, 2004).

Here we studied the metabolism of [$^{3}$H]quinolinic acid, [$^{14}$C]nicotinamide, and [$^{14}$C]nicotinic acid in leaves of *Coffea arabica*. We found that the *de novo* and salvage pathways of NAD synthesis are operative, and that nicotinamide and nicotinic acid, which are formed as products of the pyridine nucleotide cycle give rise to trigonelline.

The content and concentrations of trigonelline and the metabolic profiles of [$^{14}$C]nicotinamide were studied in leaves and fruits of *Coffea arabica* at different stages of growth. Based on our findings, we discuss possible mechanisms of accumulation, transport and re-utilization of trigonelline in leaves and fruits of coffee plants. For comparison, metabolism of [$^{14}$C]nicotinamide was also examined in other plants.

## MATERIALS AND METHODS

Leaves of *Coffea arabica* were obtained from 3-year-old trees grown in a greenhouse at Ochanomizu University. Leaves at six different stages were used: Stage **I**, expanding buds; stage **II**, small-size young leaves; stage **III**, almost fully developed young leaves; stage **IV**, mature leaves from flash shoots; stage **V**, aged leaves from 1-year-old shoots; and stage **VI**, fallen yellow leaves. Fruits of *Coffee arabica* c v. KA18 (yellow catuai) and cv. KO34 (typica), and fruits of *Theobroma cacao*, were obtained from the Experimental Station of Hawaii Agriculture Research Center, Kunia Station. Fruits of *Coffea arabica* were categorised as follows: Stage **A**, young small size fruits; **B**, developing medium size fruits; **C**, developing large size fruits; **D**, mature green unripe fruits; **E**, ripe fruits coloured yellow (KA18) or red (KO34).

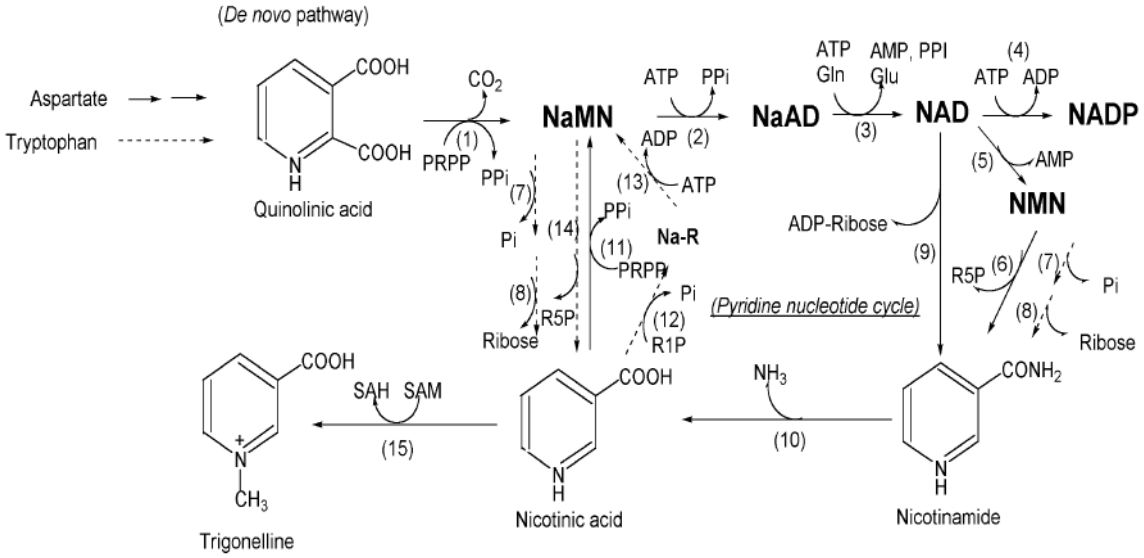


**Figure 1. Possible biosynthetic pathway of trigonelline in coffee plants.**

## RESULTS AND DISCUSSION

Metabolism of [$^3$H]quinolinic acid, [carboxyl-$^{14}$C]nicotinic acid and [carbonyl-$^{14}$C]nicotinamide was investigated using *Coffea arabica* leaves (stage **III**). Significant amounts of radioactivity from [$^3$H]quinolinic acid was recovered as pyridine nucleotides (43%) and trigonelline (36%). Nicotinic acid and nicotinamide taken up by the segments was converted to nucleotides and trigonelline, but incorporation of radioactivity into trigonelline was greater. Nicotinamide taken up by leaf segments was readily converted to nicotinic acid. Nicotinic acid is used preferentially for nucleotide synthesis, and the remainder may be used for trigonelline synthesis. Similar patterns of metabolism were found in young fruits of *Coffea arabica.*

To study the pyridine nucleotide cycle, experiments on the time course of [carbonyl-$^{14}$C]nicotinamide were carried out using leaf segments of *Coffea arabica*. The results suggest that the cycle, nicotinamide —> nicotinic acid —> NaMN —> NaAD —> NAD —> NMN —> nicotinamide operates in *Coffea arabica* plants.

Fig. 2 summarises the distribution of trigonelline in leaves and fruits at different growth stages. A high concentration of trigonelline was found in leaves at stages **I** and **II** (27-28 μmol $g^{-1}$ fresh weight). However, the total content of trigonelline per leaf was highest in stage **III** leaves (18 μmol $leaf^{-1}$). The trigonelline content fell markedly during maturation and

senescence. In young fruits (**A**-**C**), the concentrations of trigonelline were between 8 and 13 µmol $g^{-1}$ fresh weight, but the trigonelline content per fruit increased with fruit development. At stages **D** and **E**, the pericarp and seeds were separated. Although the contents in pericarps of KA18 and KO34 fruits were different, the contents in seeds were quite similar in the two varieties. After ripening, the total trigonelline content in seeds had increased, but that in the pericarp was reduced.

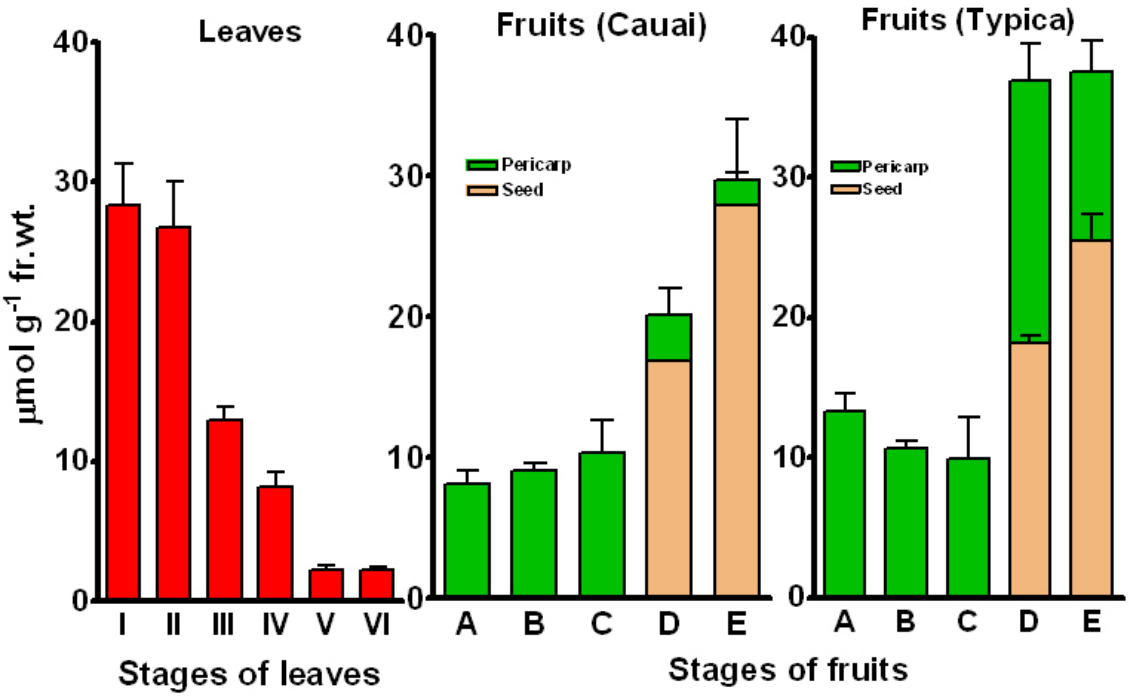


**Figure 2. Endogenous levels of trigonelline in leaves and fruits of *Coffea arabica* plants.**

Figure 3a shows the metabolic fate of [$^{14}$C]nicotinamide in *C. arabica* leaf segments at different developmental stages during 4 h incubation. At every stage, trigonelline was the most heavily radiolabelled compound. Figure 3b-3c show the metabolic fate of [carbonyl-$^{14}$C]nicotinamide in the pericarps and seeds at stage D and E of cultivars KA18 and KO34. In unripe fruits (stage **D**) there was pronounced trigonelline synthesis in the pericarps, where nearly 70% of [carbonyl-$^{14}$C]nicotinamide was converted to trigonelline. Trigonelline synthesis activity declined in the pericarps of ripe fruits. In stage E, almost all trigonelline synthesis activity was gone in the seeds, but the highest accumulation of trigonelline occurs in the seeds during ripening.

We investigated the metabolism of [$^{14}$C]nicotinamide in cacao, tea and tobacco. In cacao plants, in addition to trigonelline, a small portion of the radioactivity was found in nicotinic acid-glucoside. In tea leaves and roots, after 1 h-incubation of [$^{14}$C]nicotinamide, the heavily labelled compounds were nicotinic acid-glucoside. On further incubation, most radioactivity was found as $^{14}CO_2$. No radioactivity was found in trigonelline. A similar metabolic profile of nicotinamide was also found in leaves of tobacco.

Our present data suggest that, in addition to the *de novo* pathway for NAD synthesis, the six membered pyridine nucleotide cycle (PNC VI), NAD —> NMN —> nicotinamide —> nicotinic acid —> NaMN —> NaAD —> NAD operates in coffee plants. Some minor pathways may also be operative in part; for example, nicotinamide may be also produced from NAD by an ADP-ribosylation reaction (step 9 in Figure 1), and NaMN may be formed

by an alternative route from nicotinic acid (steps 12 and 13 in Figure 1). Other alternative routes are shown in Figure 1 by dotted arrows.

Trigonelline accumulates in fruits of *Coffea arabica* during growth, and finally accumulates in seeds. In stage D, the bean dry matter accumulation stage, high biosynthetic activity of trigonelline is found in the pericarps, although some activity was also found in seeds. In stage E**,** the fruit ripening stage, biosynthetic activity decreased markedly. This suggests that net biosynthesis of trigonelline takes place in the young pericarps, and trigonelline may be transported from the pericarps to seeds. Baumann and Wanner (Baumann and Wanner, (1972) demonstrated the translocation of caffeine from the pericarps to seeds in *Coffea arabica*. A similar translocation mechanism for trigonelline may be present in coffee plants. Trigonelline accumulated in seeds is converted to nicotinic acid during germination, and is used for the NAD synthesis as shown by Shimizu and Mazzafera (Shimizu and Mazzafera, 2000).

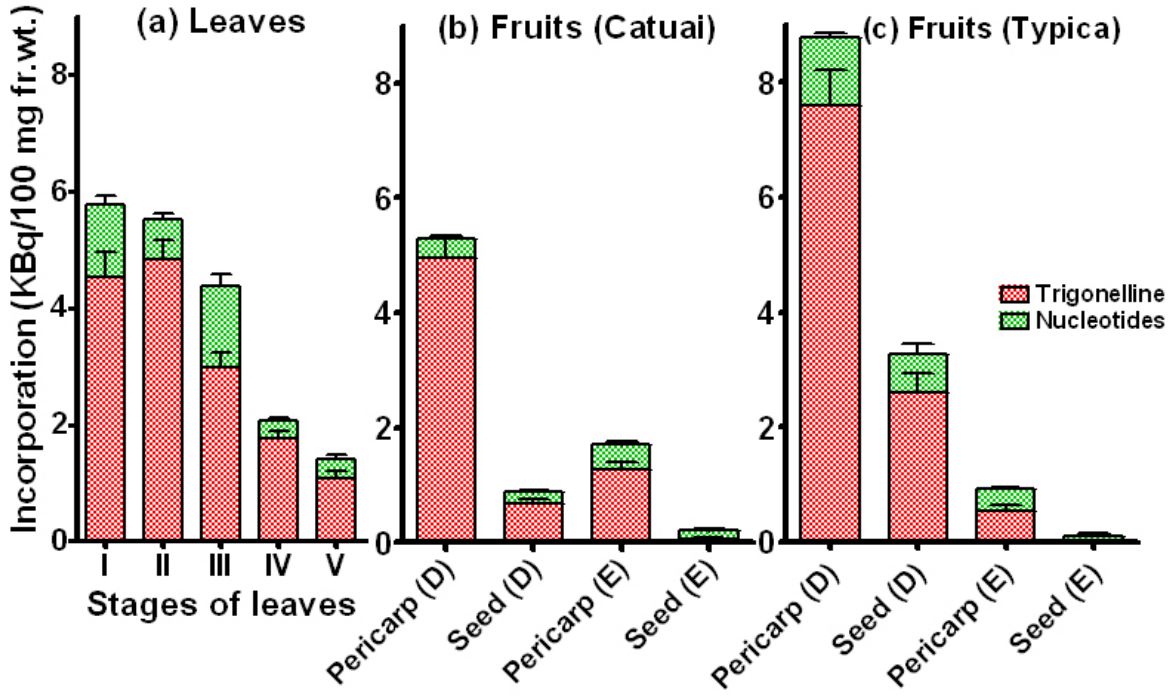


**Figure 3. Biosynthesis of trigonelline and pyridine nucleotides in leaves and fruits of *Coffea arabica* plants.**

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