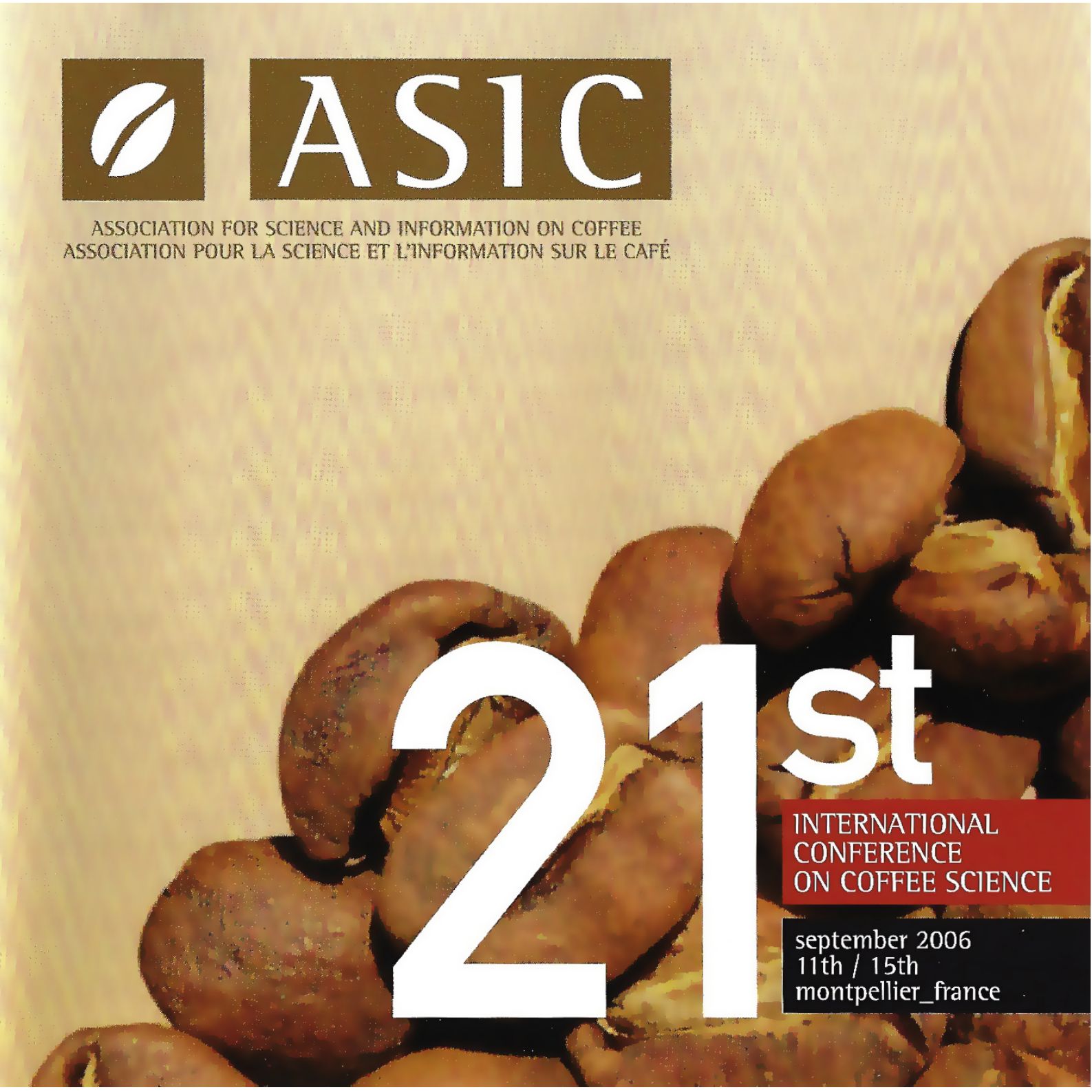
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Coffee and Cancer Risk: an Update

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SUMMARY

Epidemiological studies on coffee and cancer risk published after 1990 were reviewed. For colorectal cancer, most case-control studies reported relative risks (RR) below unity. However, cohort studies failed to show any consistent relation. Coffee drinking favourably affects serum levels of liver enzymes, and is inversely related to the risk of cirrhosis. At least four case-control and three cohort coffee studies reported RRs of liver cancer between 0.5 and 0.7 for coffee drinkers as compared to nondrinkers. Coffee drinking has also been favourably related to the risk of oral and esophageal cancers, but the evidence is scanty. Coffee drinkers have higher RRs of bladder cancer as compared to nondrinkers, in the absence however of any dose or duration risk relation. Available evidence on pancreatic, as well as on several other cancers including lung, breast, ovary, prostate and lymphoid neoplasms allows to exclude any consistent relation with coffee drinking.

INTRODUCTION

The possible relation between coffee and cancer risk has been extensively considered in epidemiologic studies. The main cancer sites of interest are colorectal and liver on one side, for which inverse relations had been reported, and bladder and pancreas on another side, for which direct relations had been suggested. Available evidence on these sites will be reviewed here, together with a short mention on other relevant cancer sites. Focus will be given to data published after 1990, i.e. after the Monograph 51 of the International Agency for Research on Cancer on coffee (IARC, 1991).

COLORECTAL CANCER

The 1990 IARC Working Group (IARC, 1991) concluded that in humans "there is some evidence of an inverse relationship between coffee drinking and cancer of the large bowel".

The literature from 1990 to 2005 on the relation between coffee, decaffeinated coffee, and colorectal cancer risk is summarized here. At least four cohort (total cases number of 1694) and nine case-control studies (7555 cases) analysed colon cancer; four cohort (568 cases) and four case-control studies (2704 cases) rectal cancer; six case-control studies (854 cases) colorectal cancer combined (Tavani and La Vecchia, 2004). For colon cancer most case-control studies found risk estimates below unity; the results are less clear for cohort studies. No consistent relation emerged for rectal cancer. Decaffeinated coffee was not related to either colon or rectal cancer in one cohort and three case-control studies (Tavani and La Vecchia, 2004). One cohort study found an inverse relation for decaffeinate coffee only (Michels et al., 2005).

There are various possible reasons for the apparent inverse association between coffee intake and colon cancer risk observed in case-control but not in cohort studies. One of these is the presence of some bias or residual confounding in case-control studies. However, any obvious

methodological bias is unlikely to account for the consistent results in different countries and settings. Likewise, major publication bias is unlikely, since also negative results on the issue have attracted interest over the last few years. Most papers allowed for several potential confounding risk factors, but multivariate analyses did not substantially modify the results.

A biological interpretation of the potential inverse association between coffee and large bowel cancer has been related to reductions of cholesterol, bile acid and neutral sterol secretion in the colon by coffee, since bile acids are promoters of colon carcinogenesis (Potter, 1992). This would also be consistent with the association being stronger for (or restricted to) colon rather than rectal cancer. Furthermore, coffee intake increases colonic motility, which has been inversely related to colon cancer risk by influencing the exposure to colonic carcinogens (Brown, 1990). Coffee beans also contain several phenolic compounds with antioxidant properties (such as caffeic acid and chlorogenic acid)Daglia et al., 2000; Anese and Nicoli, 2003)) and diterpenes (such as cafestol and kahweol) with anticarcinogenic activity (Cavin et al., 2002).

LIVER CANCER

Several data on a potentially favourable effect of coffee on liver function and liver diseases have accumulated over the last two decades. These span from liver enzymes, to cirrhosis and to hepatocellular carcinoma (HCC), and therefore constitute a continuous not only of epidemiological data, but also of biological and clinical evidences.

Coffee consumption, in fact, has been inversely related to gamma-glutamyltransferase (GGT) and alamine aminotransferase (ALT) activity in studies from Norway, Italy, Finland, France, Japan and the United States. Such inverse relations were particularly strong in high risk subjects, including heavy alcohol drinkers (La Vecchia, 2005). Coffee drinking has also been inversely related to the risk of cirrhosis in studies from North America and Europe (La Vecchia, 2005).

Cirrhosis is a major correlate of HCC, and the relation between coffee drinking and the risk of primary liver cancer has been examined in at least six studies. An Italian case-control study based on 151 cases of hepatocellular carcinoma reported a multivariate relative risk (RR) of 0.78 for drinkers of ≥ 3 cups of coffee per day, compared to non coffee drinkers (Gallus et al., 2002). In a Greek case-control study (Kuper et al., 2000), including 333 cases, the age- and sex-adjusted RR was 0.7 for drinkers of ≥ 20 cups of coffee per week compared to non drinkers.

In an other Italian study (Gelatti et al., 2005), compared with non coffee drinkers, the RRs were 0.8 for drinkers of 1-2 cups per day, 0.4 for those of 3-4 cups, and 0.3 for drinkers of ≥5 cups per day.

In a prospective study of 344 cases from Japan, subjects who consumed coffee on a daily basis had lower HCC risk than those who almost never drank coffee (RR = 0.49 [95% confidence interval, CI = 0.36 to 0.66]). The risk decreased with the amount of coffee consumed (compared with nondrinkers, the RR for 1-2 cups per day = 0.52; for 3-4 cups per day = 0.48; for ≥ 5 cups per day = 0.24) (Inoue et al., 2005). A pooled analysis of two other Japanese studies including a total of 117 cases gave a RR of 0.58 (95% CI 0.36-0.96) for drinkers of ≥ 1 cups/day compared to never drinkers (Shimazu et al., 2005).

The favourable effect of coffee consumption on HCC may be due to its inverse relation with cirrhosis, but allowance for clinical history of cirrhosis did not totally account for the inverse

association. Given its effects on liver enzymes and cirrhosis, and the weight of epidemiological evidence, coffee appears therefore to have a real effect in reducing the risk of hepatocellular carcinoma, as suggested also by data on rodents (Tanaka et al., 1990). Various components of coffee have been related to such a favourable effect, including caffeine, coffee oils kahweol of cafestol, and antioxidant substances from coffee beans, but no definite evidence is available.

BLADDER CANCER

The IARC Monograph 51 evaluated 22 studies published before 1990 (IARC, 1991). Of these, 16 found moderately increased RRs of bladder cancer in coffee drinkers compared to nondrinkers; in seven of these, the association was significant and in three there was also evidence of a dose-risk relation. No relation was observed in six other studies. Because smoking is an important risk factor far bladder cancer, lifelong nonsmokers were also considered separately to obtain information on the potential distorting effect of tabacco. Some relation with coffee was still observed, although it was less clear, possibly because of smaller numbers.

After the publication of the IARC Monograph, at least four cohort (Mills et al., 1991; Chyou et al., 1993; Stensvold and Jacobsen, 1994; Zeegers et al., 2001) and 13 case-control studies (Clavel, 1991; Nomura et al., 1991; Kunze et al., 1992; D'Avanzo et al., 1992; Escolar Pujolar et al., 1993; McGeehin et al., 1993; Vena et al.,1993; Momas et al., 1994; Brown et al., 1995; Bruemmer et al., 1997; Donato et al., 1997; Pohlabeln et al., 1999; Geoffroy-Perez et al., 2001) provided information on coffee and bladder cancer.

Three cohort studies found moderately increased risk of bladder cancer in coffee drinkers, in the absence of a dose-risk relation. In the Californian Seventh-Day Adventists cohort, which included 52 cases, the smoking-adjusted RR was 1.99 (nonsignificant) for ≥ 2 cups/d of coffee compared with non coffee drinkers, with a stronger relation in never smokers (Mills et al., 1991). In a cohort of Japanese-Americans living in Hawaii (including 92 cases), a high consumption of coffee nonsignificantly increased risk (Chyou et al., 1993), and, in a Norwegian cohort of 43,000 men and women, the RR for drinkers of ≥ 7 cups/d of coffee was 1.5 in men (based on 40 cases) and 2.4 in women (based on 13 cases) (Stensvold and Jacobsen, 1994). In the Netherlands Cohort Study based on 569 cases, the RR for an increment of 1 cup/day of coffee was 1.03 in men and 0.84 in women (Zeegers et al., 2001).

In a pooled analysis of 10 European studies restricted to 564 non smokers, there was no excess risk in coffee drinkers (OR 1.0) (Sala et al., 2000). Another meta-analysis, which included 3 cohort and 34 case-control studies, estimated that coffee consumption increased urinary tract (mainly bladder) cancers risk by approximately 20%: the RR, adjusted for age, sex, and smoking was 1.18 (95% CI 1.03-1.36) for coffee and 1.18 (95% CI 0.99-1.40) for decaffeinated coffee (Zeegers et al., 2001).

Thus, the large amount of epidemiological data on coffee and bladder cancer risk allow to exclude a strong association. It is not clear whether the moderate association reported in many studies is causal. The major potential confounding factor is cigarette smoking, which is related to both coffee consumption and bladder cancer risk; however, misclassification of smoking status or residual confounding are unlikely to completely explain the association. Other possible sources of residual confounding include diet or occupational exposure to bladder carcinogens, although the similar associations found in men and women suggest that occupation is unlikely to be a major confounder.

PANCREATIC CANCER

In the early 1980s, a case-control study from North America showed a direct association between coffee consumption and pancreatic cancer risk (MacMahon et al., 1981). Overall, 21 case-control studies on pancreatic cancer relationship were reviewed in the IARC Monograph 51 (IARC, 1991). Among them, 10 found moderate positive associations, which were weaker after allowance for smoking, and the remaining studies found no association.

The results of at least seven cohort studies have been published after the IARC Monograph 51 (IARC, 1991). No association emerged in a cohort of 17,633 American men (RR 0.9 for intake of ≥ 7 cups/d, based on 56 cases) (Zheng et al., 1993); in a Norwegian cohort of 43,000 men and women (RR 0.6 in men for ≥ 7 cups/d and 1.2 in women for > 6 cups/d, based, respectively, on 26 and 13 cases) (Stensvold and Jacobsen, 1994); in a cohort of nearly 14,000 residents of a retirement community from the USA (RR 0.88 for ≥ 4 cups/d, based on 65 cases) (Shibata et al., 1994); in the Health Professionals Follow-up Study (RR 0.37 for > 3 cups/d of coffee and 0.99 of decaffeinated coffee, based on 130 cases) (Michaud et al., 2001); in the Nurses' Health Study (RR 0.88 for for > 3 cups/d of coffee and 0.85 for decaffeinated coffee, based on 158 cases) (Michaud et al., 2001) and in the Japan collaborative cohort study for evaluation of cancer risk, where no trend in risk with number of cups emerged (Lin et al., 2002). Conversely, in the Iowa Women's Health study cohort on nearly 34,000 women and including 66 incident cases of pancreatic cancer, the RR was 2.15 for drinkers of more than 17.5 cups/week of coffee. The association was not significant in never smokers (Harnack et al., 1997).

Most case-control studies published after the IARC Monograph found no significant association between coffee and pancreatic cancer risk (Tavani and La Vecchia, 2004; 2000), and one study found an inverse association (Bueno de Mesquita et al., 1992).

It is possible therefore that any relation between coffee and pancreatic cancer is not causal but explainable through selection or recall bias, residual confounding with cigarette smoking (the major recognized risk factor far pancreatic cancer) or other sources of confounding. In any case, a strong association between coffee and pancreatic cancer can now be excluded.

OTHER CANCERS

The IARC Monograph 51 included data on coffee and gastric cancer from five case-control studies (IARC, 1991). There was no evidence of association in any of them. More recently, a Norwegian cohort study observed no association between coffee intake and gastric cancer risk (RR 0.5 in men and in women, not significant, based on 78 cases) (Stensvold and Jacobsen, 1994). A cohort study of Japanese residents in Hawaii, including 108 cases, found that men who drank one cup of coffee per day had an elevated risk of gastric cancer compared to non coffee drinkers (RR = 2.5) (Galanis et al., 1998). No association between coffee drinking and gastric cancer risk was found in four case-control studies: a Spanish study based on 354 cases (Agudo et al., 1992), a Swedish study based on 338 cases (Hansson et al., 1993), a Japanese one based on 893 cases (Inoue et al., 1998), and a Polish study based on 464 cases (Chow et al., 1999). Thus, it is now clear that coffee is unlikely to have any major effect on gastric carcinogenesis.

Six studies providing data on cancers of the oral cavity, pharynx, and esophagus were considered in the IARC Monograph (IARC, 1991). There was no evidence of association with coffee consumption in any of them. Since then, at least one cohort (Stensvold and Jacobsen, 1994) and five case-control studies (La Vecchia et al., 1990; Franceschi et al., 1991; Pintos et

al., 1994; Bundgaard et al., 1995; Castellsague et al., 2000) found no association for these cancers, whereas a case-control study based on 749 cases of oral and pharyngeal cancer and 395 of esophageal cancer from Italy (Tavani et al., 2003) found that risk approximately halved in the highest compared to the lowest quintile of coffee intake.

With relevance to breast cancer, of the seven case-control studies considered in the IARC Monograph (IARC, 1991), none found an association with coffee consumption (IARC, 1991). More recently, three cohort and four case-control studies, including an Italian one with nearly 6000 cases (Tavani et al., 1998), found no association (Tavani and La Vecchia, 2000; 2004).

Likewise, no consistent relation was observed between coffee, ovarian, laryngeal, lung, prostate, cervical, endometrial and thyroid cancers, Hodgkin's and non Hodgkin lymphomas, sarcoma, multiple myeloma and skin melanoma (Tavani and La Vecchia, 2004; Tavani et al., 2003; Naldi et al., 2004).

CONCLUSIONS

The large amount of epidemiological data on bladder cancer allow to exclude a strong association with coffee intake, and the lack of dose-response relation does not support causality. Likewise, an association between coffee and pancreatic cancer risk can now be excluded. For colorectal cancer, most case-control but not cohort studies reported an inverse association. Epidemiological data indicate an inverse relation between coffee, liver and probably oral and esophageal cancers.

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Coffee and Risk of Type 2 Diabetes

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SUMMARY

It is well known that coffee is one of the most widely consumed beverages in the world. During the last decades research has attempted to clear health benefits or harms received from coffee drinking. Knowledge on both positive and negative health effects of coffee is important to allow individuals to make informed choices regarding coffee consumption. Coffee consumption has been extensively studied in relation to various diseases, but not until recently has it been examined in relation to risk of type 2 diabetes. Most of the recent population-based studies have been revealed positive health effect of coffee consumption on the risk of development type 2 diabetes. Our most recent data showed that coffee consumption may also be somehow protective in diabetes serious complications. This article will review the present state of the association between coffee consumption and type 2 diabetes and our last findings.

INTRODUCTION

Type 2 diabetes, 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. It is estimated that in 2000 there were approximately 161 million individuals with the disease and that this number is likely double by 2030 (Wild et al., 2004). In Europe their number will increase from approximately 16 million in1994 to 24 million in 2010 (Amos et al., 1997).

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.

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. Evidence increasingly indicates that healthy lifestyle and dietary habits can prevent most cases of type 2 diabetes. The prevention of type 2 diabetes has become a major issue both significantly and from public health view (Tuomilehto et al., 1997). Therefore investigating on effects of dietary constituents and in particular common part of them will be an important issue. Coffee with a large number of consumers and numerous constituents has been provided good grounds on this issue.

COFFEE AND ITS CHEMICAL COMPOSITION

Probably coffee is one of the most consuming beverages in the world and Finns have the highest rate in the world (11.3 kg) which is more than twice of average rate in Europe (5 kg). Although coffee has been consumed for centuries, the chemical composition of coffee has not yet completely known.

In fact coffee is a complex compound of potential neutriceuticals. Agricultural factors, roasting, blending, and brewing determine coffee's chemical composition. Coffee is the major source of phenolic polymers, chlorogenic acid and also caffeine (Clifford, 2000). Phenolic compounds are known as antioxidants in vitro and might reduce the risk of cardiovascular disease as well as other degenerative diseases (Rice-Evans et al., 1996; Olthof et al., 2001). 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 major component of this class is caffeic acid, which occurs in food mainly as esters called chlorogenic acid. Daily intake of chlorogenic acid in coffee drinkers being 0.5-1 g, whereas coffee abstainers typically ingest < 100 mg/day (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. Some of the chlorogenic acid can also transform in quinides that have been shown to enhance insulin action (Shearer et al., 2003).

COFFEE CONSUMPTION AND RISK OF TYPE 2 DIABETES AND ITS COMPLICATIONS

The association of coffee consumption and the risk of type 2 diabetes were evaluated in a population-based cohort of 17111 Dutch men and women aged 30-60 years (Van Dam and Feskens, 2002). During follow-up, 306 new cases of type 2 diabetes were reported. 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. Therefore, higher coffee consumption was associated with a lower risk of type 2 diabetes (Table 1). Data from large U.S. cohorts of men and women followed 41 934 men from 1986 to 1998 and 84 276 women from 1980 to 1998. During follow-up 1333 new cases of type 2 diabetes in men and 4085 new cases in women were documented. They found 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. The overall results in this study showed that long-term coffee consumption of coffee and total caffeine intake was significantly associated with a reduced risk of type 2 diabetes (Salazar-Martinez et al., 2004).

A Japanese study comprising 1916 men and 2704 women aged 40-59 years found that coffee intake or caffeine intake from coffee was inversely associated with the prevalence of fasting hyperglycemia (fasting plasma glucose ≥ 6.1 mmol/l) (Table 2) (Isogawa et al., 2003).

In 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, without prior diabetes or cardiovascular disease from 1979-1981. They found 74 new cases of diabetes. They achieved the same inverse association between coffee consumption and incidence of type 2 diabetes (Rosengren et al., 2004).

Our recent prospective population based study of 6974 Finnish men and 7655 women, 35 to 64 years of age without any history of stroke, coronary heart disease, or diabetes at baseline (Tuomilehto et al., 2004) revealed an strong and graded inverse association between coffee consumption and risk of development of type 2 diabetes (Table 3).

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
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
Adjustment for CVD, hypertension, & 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 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.*

Table2. 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.*

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.*

Table 4. Adjusted linear regression analysis of the association between coffee consumption and glucose and insulin levels among non-diabetic Finnish men and women.

	Regression coefficient (95% CI)		
Coffee consumption, cups/day	Fasting glucose	2-hour glucose	Fasting insulin
Men			
Adjustment for age and study year	-0.18 (-0.39 to 0.03)	-0.55 (-1.14 to 0.04)	-0.14 (-0.24 to -0.03)‡
Multivariate adjustment*	-0.33 (-0.56 to -0.10)‡	-0.64 (-1.28 to -0.01)‡	-0.22 (-0.33 to -0.12)‡
Women			
Adjustment for age and study year	-0.10 (-0.35 to 0.15)	-1.34 (-1.99 to -0.68)‡	-0.10 (-0.23 to 0.03)
Multivariate adjustment*	-0.26(-0.50 to -0.01)‡	-1.77 (-2.43 to –1.12)‡	-0.26 (-0.38 to -0.14)‡
Men and women combined†			
Adjustment for age and study year	-0.15 (-0.31 to 0.01)	-0.89 (-1.33 to -0.45)‡	-0.12 (-0.21 to -0.04)‡
Multivariate adjustment*	-0.29 (-0.45 to -0.12)‡	-1.16 (-1.61 to -0.70)‡	-0.24 (-0.31 to -0.16)‡

**Adjusted for age, study year, body mass index, systolic blood pressure, education, occupational and leisure time physical activity, walking or cycling to/from work, cigarette smoking, alcohol and tea consumption. †Adjusted also for sex. ‡P value < 0.05.*

Table 5. Hazard ratios of total, cardiovascular, coronary heart disease, and stroke mortality by volume of coffee consumption among subjects with type 2 diabetes.

	Daily coffee consumption, cups				P for trend
	0-2	3-4	5-6	≥7	
Total mortality					
No. of deaths	247	384	529	311	
Person-years	11 772	20 551	29 927	17 406	
Adjustment for age, sex, and study year, HR (95% CI)	1.00	0.79 (0.67-0.93)	0.72 (0.62-0.85)	0.79 (0.67-0.94)	.001
Multivariate adjustment, HR (95% CI) *	1.00	0.77 (0.65-0.91)	0.68 (0.58-0.80)	0.70 (0.59-0.85)	<.001
Cardiovascular mortality					
No. of deaths	146	241	337	185	
Adjustment for age, sex, and study year, HR (95% CI)	1.00	0.81 (0.66-0.99)	0.75 (0.62-0.92)	0.79 (0.63-0.98)	.041
Multivariate adjustment, HR (95% CI) *	1.00	0.79 (0.64-0.97)	0.70 (0.57-0.86)	0.71 (0.56-0.90)	.006
Coronary heart disease mortality					
No. of deaths	96	160	231	111	
Adjustment for age, sex, and study year, HR (95% CI)	1.00	0.81 (0.63-1.05)	0.77 (0.61-0.98)	0.71 (0.54-0.94)	.092
Multivariate adjustment, HR (95% CI) *	1.00	0.78 (0.60-1.01)	0.70 (0.54-0.90)	0.63 (0.47-0.84)	.014
Stroke mortality					
No. of deaths	35	54	69	52	
Adjustment for age, sex, and study year, HR (95% CI)	1.00	0.75 (0.49-1.14)	0.62 (0.41-0.94)	0.90 (0.59-1.40)	.081
Multivariate adjustment, HR (95% CI) *	1.00	0.77 (0.50-1.19)	0.64 (0.41-0.99)	0.90 (0.56-1.45)	.12

**Adjusted for age, sex, study year, body mass index, systolic blood pressure, total cholesterol, education, alcohol and tea consumption, and smoking status. †Abbreviations: CI, confidence interval; HR, hazard ratio.*

There was no association between coffee consumption and 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. Because 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 the reasons for lack of association in previous Finnish study (Tuomilehto et al., 2004; Reunanen et al., 2003). Recent systematic review of the literature by Van Dam et al. (2005) revealed that coffee consumption has been associated with a substantially lower risk of developing type 2 diabetes.

In a cross-sectional study of middle-aged Finnish men and women we found an inverse association between coffee consumption and several markers of glycemia and diabetes. We found that coffee consumption was associated with lower values of fasting glucose, 2-hour glucose and fasting insulin among non-diabetic subjects. Coffee consumption was significantly and inversely associated with impaired fasting glucose, impaired glucose regulation, and hyperinsulinemia among both men and women and with isolated impaired glucose tolerance only among women (Table 4) (Bidel et al., 2006).

Type 2 diabetes is a well known risk factor for cardiovascular disease (CVD) morbidity and mortality (DECODE Study Group, 2001; Hu et al., 2005; Haffner et al., 1999; Brand et al., 1989; Pan et al., 1986; Balkau et al., 1998). However, in a large prospective study we found an inverse association between coffee consumption and the risk of total, CVD and coronary heart disease (CHD) mortality among patients with type 2 diabetes. These associations were independent of other CVD risk factors: age, BMI, systolic blood pressure, total cholesterol, physical activity, alcohol drinking, and smoking (Table-5) (Bidel et al., 2006). [29]

In the other study we evaluated the association between liver enzyme γ- glutamyltransferase (GGT) and coffee drinking on the risk of type II diabetes. This study confirmed our previous finding that coffee consumption was inversely and significantly associated with type II diabetes (Tuomilehto et al., 2004). We also found that the results were modified by baseline GGT levels. In the high GGT levels (≥ 75% percentile), coffee drinking was more strongly inversely associated with type II diabetes than in lower GGT levels (< 75% percentile). (Unpublished data)

SUGGESTED MECHANISMS BY WHICH COFFEE MAY EXERT ITS EFFECTS

Coffee contains many compounds other than well-known caffeine which may have potential to influence glucose regulation at the intestinal, hepatic and also peripheral delivery stages of the glucose metabolism. At the intestinal stage inhibition or retardation of the action of α− Glucosidase by chlorogenic acid is one of the possible mechanisms. The inhibition of this enzyme is an effective approach to control hyperglycemia (Matsui et al., 2001). It has also been reported that chlorogenic acid inhibits glucose transporters (Na^+ -dependent glucose transporter) at the same stage (Kobayashi et al., 2000). In addition coffee may also 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 reduced Glucose-6-phosphatase (Glc-6-Phase) hydrolysis or its inhibition by chlorogenic acid may reduce plasma glucose output leading to reduce plasma glucose concentration (Newgard et al., 1984; Youn et al., 1986; Arion et al., 1997; Andrade-Cetto and Wiedenfeld, 2001). At the peripheral delivery

stage, lower fasting insulin values and lower risk of hyperinsulinemia among coffee consumers that we found it in the second part of this study, may be interpreted as an improvement in insulin sensitivity by coffee consumption. Acute effects of caffeine to decrease insulin sensitivity have also been reported (Keijzers et al., 2002). However we believe these effects might be modified during long period of coffee consumption as it has already seen in cardiovascular effects (Denaro et al., 1991) and glucose metabolism may follow different pattern among heavy and chronic coffee consumers. Also beneficial effects of coffee's components other than caffeine on insulin sensitivity should be considered (Clifford, 2000). As we reported in our recent study; diabetic patients who drink coffee have lesser cardiovascular complications than non-drinker. We speculated that coffee may prevent excessive oxidative stress which is responsible for developing type 2 diabetes and its cardiovascular complications (Bidel et al., 2006).

CONCLUSION

In a series of studies we found positive effects of coffee consumption on the risk of developing type 2 diabetes and its complications. Our knowledge about mechanisms or process by which coffee contents may exert their beneficial effects on diabetes has yet to be completed.

It is well known that type 2 diabetes is potentially preventable or if it occurs the complication could be delayed by changing some lifestyle measures. Weight control, physical activity, and particularly diet are the most important issues in type 2 diabetes. Nutritional management is one of the most important tasks in treatment of diabetes. Dietary guidelines design for preventing diabetes, improve the treatment and prevent or delay diabetes complications.

Coffee is part of the diet of most of the people which has been shown to have positive healthy effects on overall glucose metabolism. However we still believe that these positive effects cannot be solely achieved by coffee drinking without considering any other lifestyle measures. In addition the association between coffee consumption and type 2 diabetes may be affected by important factors such as type of the coffee, duration of coffee drinking and so on, which are usually, vary in different populations. So, they should be carefully considered in any further investigations.

As a final word based on overall findings, it may be concluded that coffee is a safe and useful drink in association with type 2 diabetes. Expanded investigations require in order having better knowledge of coffee and its complex components and their effects on human body in health and disease status.

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Mechanistic Aspects of DNA – and Cancer – Protective Effects of Coffee

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SUMMARY

A number of epidemiological studies indicate that consumption of coffee is inversely related to the incidence of specific forms of cancer in the liver and in the gastrointestinal tract. In order to provide mechanistic explanations for this phenomenon, we evaluated the literature concerning the prevention of DNA damage (which plays a key role in the formation of malignant cells) and cancers by adduct forming genotoxic carcinogens in the human diet and reactive oxygen species (ROS). Furthermore, we conducted animal experiments and human intervention trials to substantiate putative protective effects. We failed to find protective effects towards induction of DNA damage by heterocylic aromatic amines (which are pyrolysis products of amino acids) in animal and human studies but a strong reduction of formation of aflatoxin B_1 induced preneoplastic lesions was seen with caffeinated and decaffeinated metal filtered coffee preparations in rats. Furthermore, a pronounced reduction of BPDE (the reactive metabolite of benzo[a]pyrene) induced DNA-damage in human lymphocytes was found in a human intervention study. These effects are probably due to induction of glutathione-S-transferases – in particular the GSTP isoform is induced. Results of animal and human experiments indicate that coffee consumption causes a pronounced induction of these detoxifying enzymes. Another important mechanism of protection elicited by coffee is the inactivation of ROS, which was observed in a number of earlier *in vitro* studies. We could demonstrate in a human intervention trial (i.e. in single cell gel electrophoresis assays) that consumption of coffee results in a significant reduction of endogenous formation of oxidised bases and reduces the sensitivity of lymphocytes towards ROS (hydrogen peroxide) induced DNA-damage. It can be tentatively assumed that the reduced incidence of liver cirrhosis and hepatocellular carcinomas in coffee drinkers may be causally related to the antioxidant properties effects of coffee, since it is assumed that ROS mediated damage plays a key role in the aetiology of these diseases.

INTRODUCTION

Coffee is among the most widely consumed beverages worldwide, and its production increased substantially over the last years (Clarke and and Vitzthum, 2001). A number of epidemiological studies indicate that its consumption is inversely related with the incidence of specific forms of cancer, for example in the colon (Tavani and C. La Vecchia, 2004) and in the liver (La Vecchia, 2005). In addition, there is increasing evidence that coffee intake leads

to a reduced rate of liver cirrhosis (Corrao et al., 2001; Klatsky and Armstrong, 1992; Klatsky et al., 1993; Gallus et al., 2002). Also the correlation between liver cirrhosis and hepatocellular cancer (HCC) is well established and it was found in several studies that coffee consumption is inversely related to the incidence of HCC (Gallus et al., 2002; La Vecchia et al., 1998; 1989; Gelatti et al., 2005; Inoue et al., 2005). Other organs have been less intensely studied, but evidence is increasing that also oesophageal cancer rates may be lower in coffee consumers (Tavani and C. La Vecchia, 2000).

Descriptive epidemiological studies do not provide mechanistic explanations and in order to explain phenomena seen in such investigations further studies are required which provide information concerning the mode of action of putative protective dietary factors. The present article gives a short overview on the DNA and cancer protective mechanisms of coffee, which have been identified so far, including some results of experiments, which have been conducted recently at the Institute of Cancer Research (Medical University of Vienna, Austria).

DNA-STABILITY AND CANCER

The development of cancer is a multi-step process, and it is well documented that one of the key events that leads to formation of malignant cells are DNA-alterations in cancer-related genes which encode for signalling and cell division (Pitot, 2002). Furthermore, it is well documented that DNA-instability is causally related to other diseases (such as neural disorders), reduced fertility and accelerated ageing (Harman, 1981; Ames et al., 1993; von Zglinicki et al., 2001).

In the last decade, strong efforts have been made to identify constituents in the human diet, which improve the DNA-integrity. Many investigations concerned protective effects of food components against damage caused by genotoxic carcinogens, contained in human foods such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines, heterocyclic aromatic amines (HCAs) and aflatoxins. The latter two groups are of particular interest in regard to coffee studies; the amines are considered to be important aetiological factors for colon cancer while the mycotoxins causes liver cancer in humans. It is assumed that the high prevalence of HCC in Africa and China is due to consumption of contaminated foods together with a significant contamination by the hepatitis virus. Another important issue is the detection of dietary components, which protect against oxidative DNA-damage caused by reactive oxygen species (ROS), which play a key role in the onset of many forms of cancer (Hussain et al., 2003).

PROTECTIVE EFFECTS OF COFFEE AND ITS CONSTITUENTS AGAINST DNA-REACTIVE CARCINOGENS CONTAINED IN THE HUMAN DIET

Table 1 summarizes the results of some investigations in which protective effects of coffee and its constituents were observed either in *in vitro* or animal studies. It can be seen that prevention of cancer induction and DNA-damage against a broad variety of carcinogens was found, which are considered to be important etiological factors in humans.

Table 1: Protective effects of coffee and coffee constituents against adduct forming dietary carcinogens (*in vitro* and animal studies)

Compound	Observation	Proposed Explanation	Ref.
Aflatoxin B_1	▪ reduced formation of adducts in rat hepatocytes after fee-ding coffee specific diterpe-noids (C+K) ▪ reduced induction of rever-tants in Salmonella/micro-some-assays with enzyme ho-mogenates prepared from cof-fee fed rats	▪ induction of GST which detoxifies AFB_1-meta-bolites ▪ inhibition of activating enzymes	(Cavin et al., 1998; 2001)
Polycyclic aromatic hydrocarbons (PAHs)	▪ inhibition of induction of tumour formation by DMBA in feeding experiments with coffee beans and also with C+K in rats ▪ prevention of benzo[a]pyrene DNA-adduct formation *in vitro* by C+K	▪ induction of GST, i.e. increased GST-activity in the liver of mice after feeding with green beans (active principle C+K palmitate) ▪ inhibition of CYP1A1 (which activates PAHs) by C+K in human Beas-2B cells	(Lam et al., 1982; Cavin et al., 2003)
Heterocyclic aromatic amines (HAs)	▪ inhibition of PhIP DNA-adduct formation in the colon of rats after administration of C+K ▪ prevention of PhIP-induced DNA-damage by C+K in a human derived liver cell line ▪ deacrease of bacterial muta-genicity of PhIP in the salmonella/microsome assay ▪ decreased PhIP adducts in the liver (50%) of rats	▪ inhibition of activating enzymes (i.e. CYP1A2) ▪ induction of detoxifying enzymes (UDPGT, GST)	(Huber et al., 2004; Majer et al., 2005; Turesky et al., 2003)
Nitrosamines	▪ prevention of genotoxic effects of NDMA in human derived liver cell lines by C+K ▪ inhibitory effect of coffee against NDEA-induced hepa-tocarcinogenesis in male Wis-tar rats	▪ it can be excluded that the effect is due to the inhibition of activating enzymes such as CYP-2E1 (p. c. W. Huber) ▪ possible explanation: in-duction of O^6MGMT- a DNA-repair enzyme re-moving methyl groups	(Lam et al., 1982; Majer et al., 2005; Huber et al., 2003; Li , 1991)

Abbreviations: C+K: cafestol and kawheol, DMBA: 7,12-dimethyl-benz[a]an-thraene, PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine, UDPGT: UDP-glucuronosyl transferase; GST: glutathione-S-transferase, NDMA: N-nitrosodime-thylamine, NDEA: N-nitrosodiethylamine, MGMT: O^6-methyl-guanine-DNA methyltransferase, p.c.: personal communication

In order to find out, if coffee prevents DNA-damage caused by **heterocyclic aromatic amines** (HAs), which are formed as pyrolysis products of amino acids in meats during cooking (Jagerstad and Skog, 1991), we conducted a model study with rats, in which prevention of DNA-damage by coffee was monitored by single cell gel electrophoresis (SCGE) assays. These experiments are based on the determination of DNA-migration in an electric field (Sasaki et al., 2002) and we showed earlier that prevention of DNA-migration of heterocyclic aromatic amines is paralleled by reduction of the formation of preneoplastic foci in the liver and in the colon (Kassie et al., 2002). To find out if there is any difference between the efficiency of different coffee preparations, we used metal filtered (UC) and paper filtered (FC) coffee. The results of a representative experiment are shown in Figure 1. It can be seen that no protective effect was detectable in livers and colons of animals, which received coffee. One possible explantation might be the fact that coffee was found to be (in contrast to cafestol and kawheol) a inducer of the isozyme CYP1A2, which catalyses the formation of DNA reactive metabolites of heterocyclic aromatic amines (HAs).

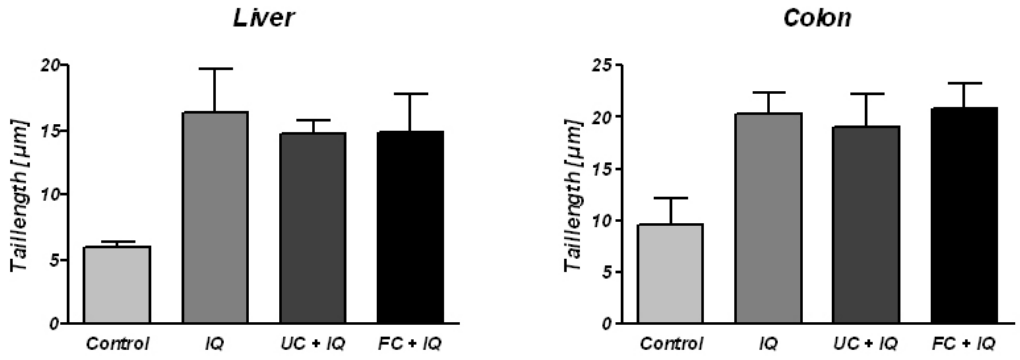

Figure 1. (a, b) Effect of coffee on DNA-migration induced by the heterocyclic aromatic amine IQ (2-amino-3-methylimidazo[4,5-f]quinoline) in livers (1a) and colons (1b) of rats. Coffee (Brand: "Brasil Sanft") was prepared by boiling 50 g in 600 ml water for 5 min and was subsequently filtered through a paper filter (Melitta, FC) or through a metal mesh (Phillips, UC) and was administered orally to male OFA rats over three days by gavage (1% of BW/animal/day). On the last day, the animals were treated orally with IQ (suspended in corn oil, dose 50 mg/kg BW). Four hrs later, the animals were killed, the organs removed and DNA-migration monitored as described in detail in the article of Kassie et al. (2002). From each organ, three slides were prepared and 50 cells were analysed for DNA- migration. In addition, viability of cells was determined with the trypan blue exclusion test. Bars indicate means ± SD of four animals per experimental point.

In another animal experiment the effect of coffee consumption on the formation of **aflatoxin B_1** induced GST-P+ foci was investigated. These foci are premalignant lesions which are likely to develop into hepatocellular carcinomas (Ehrlich et al., 2004). It can be seen that AFB_1 caused a strong induction of foci frequencies (87-fold over the background). Partial replacement of the drinking water by the different coffee preparations resulted in highly significant protective effects. With caffeine containing paper and metal filtered coffee more or less similar protective effects were observed, and the foci frequencies decreased in both cases by approximately 75%. The effect seen with decaffeinated French press coffee (which contains in contrast to the other preparations high amounts of coffee diterpenoids) was less pronounced but still highly significant (reduction by 43%). Furthermore, our findings indicate that the coffee diterpenoids play no or only a marginal role as similar protective effects were seen with paper filtered coffee (which contain very low concentrations of C+K) and metal filtered coffee (which contains much higher concentrations). Since the protection seen with

the decaffeinated sample (which was prepared from the same brand) was much weaker, we conclude that caffeine (which is known to induce glutathione-S-transferase) accounts for approximately 40% of the protective activity of coffee towards AFB_1 (Huber and Parzefall, 2005).

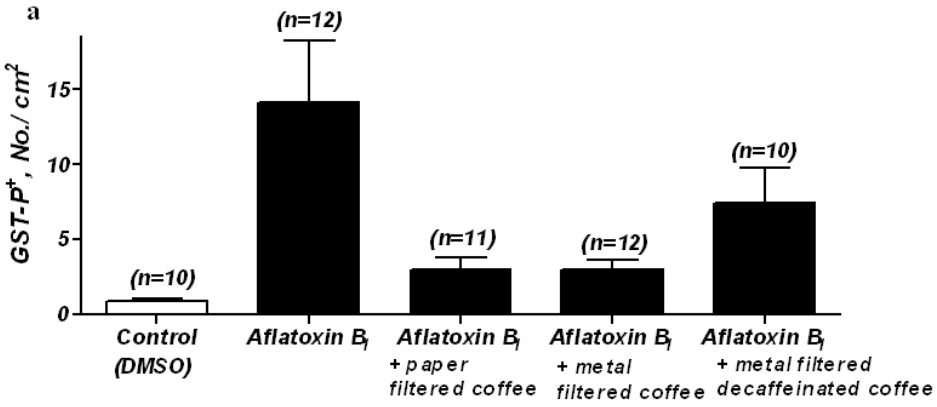

Figure 2. Effect of different coffees on the induction of GST-P$^+$ foci in the liver of rats by AFB_1. The drinking water of the animals was supplemented with the coffees as follows: 25% (18,7 mg/ml coffee per day/ rat) were given during the first three days followed by five days supplementation with 50% (37,5 mg/ml coffee per day/ rat). The animals were treated on the last day with AFB_1 (*i.p.* injection of 0.57 mg/kg BW). 10 wks after the treatment the animals were killed and the livers removed. Subsequently, liver tissue sections were stained and the number of GST-P$^+$ foci evaluated as described by Grasl-Kraupp et al. (1993).

PREVENTION OF DNA-DAMAGE BY ADDUCT FORMING CHEMICALS IN HUMANS

Only few data are available which concern the prevention of DNA-damage by dietary carcinogens in humans. For example, Steinkellner et al. (2005) reported a substantial reduction (45 %) of BPDE ((+/-)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide)-induced DNA-damage after the intake of 1 liter (50g coffee/l) of metal filtered coffee over a period of 5 days. BPDE is the main metabolite of benzo[a]pyrene, which has been used in many studies as a model compound of the PAHs. The protective effect seen in the human trial could be explained by induction of GST, and in this context it is notable that results of earlier studies with laboratory rodents showed that coffee beans protect rats against DMBA induced mammary cancer. Also this PAH forms DNA reactive diolepoxides, which are detoxified by GST; and it is conceivable on the basis of the results described above that coffee protects humans against cancer induction by PAHs

In contrast, we found no evidence for protective effects against PhIP, one of the most abundant heterocyclic aromatic amines, in a further human intervention trial, (see Figure 3) while Huber et al. (2004) observed strong protective effects (prevention of PhIP adduct formation) with high doses of the coffee diterpenoids in experiments in rats.

In a further recent study (Hoelzl et al., unpublished data), the impact of coffee intake on induction of DNA-damage by alkylating agents was studied in an SCGE-intervention trial. No clear evidence for protective effects against methylmethane sulfonate (MMS), methylnitrosourea (MNU) and nitrosodi-methylamine (NDMA) -induced DNA-damage was found in this experiment.

The latter findings indicate that the levels of diterpenoids – which caused significant effects against amines and alkylating agents in animal experiments and in in vitro studies were not sufficiently high in the coffee preparations used in the human stuides to cause significant protection

ANTIOXIDANT EFFECTS OF COFFEE AND COFFEE CONSTITUENTS

It is well documented that reactive oxygen species (ROS) have a strong impact on the health status of humans, and that they play an important role in the aetiology of cancer and ageing (Ames, 1993; Hussain, 2003). Numerous *in vitro* investigations have been carried out concerning the antioxidant properties of coffee and its constituents, such as Maillard products, chlorogenic acids and caffeine (Azam et al., 2003; Daglia et al., 2004; Somoza et al., 2003; Yanagimoto et al., 2002). In many studies with coffee, protective effects were observed with low dose levels while with increased concentrations oxidant effects were found which where attributed to formation of hydrogen peroxide resulting from the reduction of atmospheric oxygen by polyphenols in presence of large amounts of transition metals and high oxygen tension. However, such a prooxidant activity *in vitro* has been documented with other dietary polyphenols and antioxidants, for example from wine and black tea (Stadler, 2001). Therefore, the biological significance of the pro-oxidant and mutagenic activity of coffee has to be interpreted with caution since the *in vitro* assays do not reflect adequately conditions present in physiological situations, and in the case of coffee it was postulated that the effects which were observed at high dose levels may be due to experimental conditions, which are not relevant for man (Stavric, 1992). Table 2 summarizes the results of human studies in which the impact of coffee consumption on oxidative DNA-damage was investigated.

Table 2. Results of human studies concerning antioxidant properties of coffee.

Study type	Results	Ref.
Questionnaire based SCGE-study with lymphocytes (measurement of FPG-sensitive sites) n=71 (48m+23f)	_ increased levels of oxidised purines in the group of coffee drinkers (19-23%)	Giovannelli et al., 2002)
Questionnaire based measurement of 8-hydroxy-deoxy-guanosine in leucocytes, n=102, (51m+51f)	_ reduced formation of oxidised DNA-bases (10% effect, not significant)	(van Zeeland et al., 1999)
Measurement of GSH levels in plasma of coffee drinkers. Randomised intervention study n=64, (31m+33f)	_ significant increase of GSH-levels in the colorectal mucosa by 8% and in plasma by 15%: indirect evidence for an anti-oxidant effect of GSH-which is a potent antioxidant	(Grubben et al., 2000)
Measurement of GST in plasma (in particular GST-Pi) Intervention trial n=10, (3m+7f), 1000 ml/d, 5d	_ significant increase (8%) after 5 days of coffee consumption (1000 ml/d) of the overall GST-levels, and 2-fold induction of GSTP, indirect evidence for antioxidant effect as GST protects against ROS; in the same study also a significant reduction of DNA-damage caused by BDPE (a me-tabolite of benzo(a)pyrene) was obser-ved	(Steinkellner et al., 2005)

Abbreviations: m: males, f: females, GSH: glutathione, GST: glutathione-S-transferase.

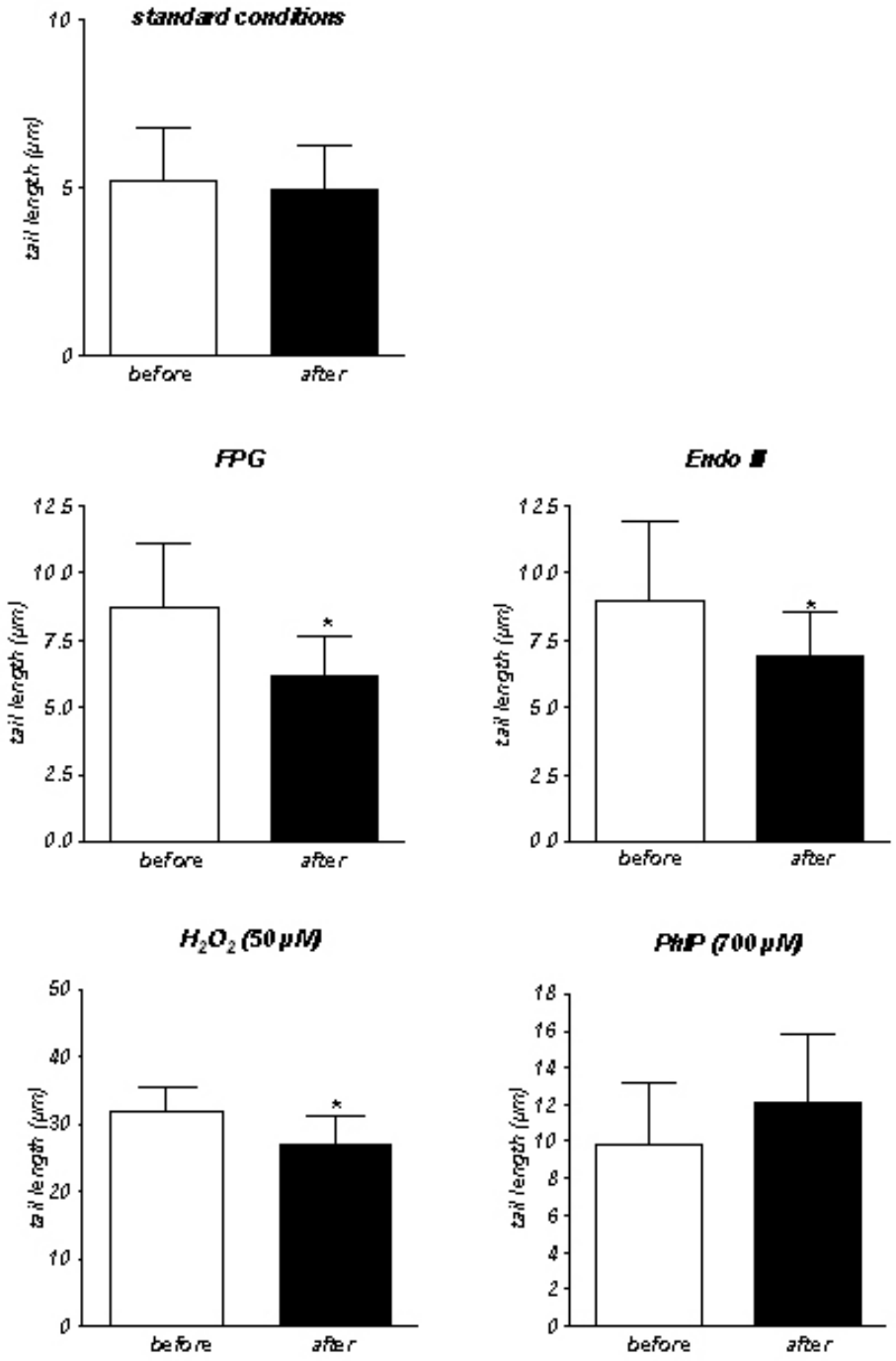

Figure 3. Effect of coffee consumption on endogenous and chemically induced DNA-damage. Eight individuals participated in the intervention trial. Each of them consumed 600 ml (400 ml paper filtered, 200 ml metal filtered coffee) coffee over a period of five days. Before and after the intervention, lymphocytes were isolated from blood and analysed for DNA-migration in SCGE experiments. Figure 3a shows the results obtained with standard electrophoresis conditions. In experiments with H_2O_2 (d) and PhIP (e), three cultures per participant were prepared with PBS (pH = 7.4) and the cells were treated on ice with H_2O_2 (50 µM) for 5 minutes or with PhIP (700 µM) for 30 minutes. To monitor endogenous formation of oxidised bases, the nuclei on the slides were treated with FPG (b) for 30 min and with ENDO III (c) for 45 min. Bars indicate means ± SD of results obtained with the eight participants. White bars: DNA-migration before coffee consumption, black bars: DNA-migration after coffee consumption. * indicate statistical significance (p-value ≤0.05, analysis of variance ANOVA).

The results of a recent human intervention study, conducted by Bichler et al. (submitted 2006), are summarized in Figure 3. In this experiment, the extent of DNA-migration was monitored before and after consumption of coffee in single cell gel electrophoresis assays under standard conditions. In addition, also alterations of the sensitivity of the DNA towards ROS (H_2O_2) induced DNA-damage were investigated by treatment of lymphocytes with hydrogen peroxide, and the increase of DNA-migration due to endogenous formation of oxidised DNA-bases was monitored by use of DNA specific lesion enzymes (formamidopyrimidine glycosylase - FPG and endonuclease III - Endo III). These results indicate that coffee consumption causes significant antioxidant effects in humans.

Further experiments indicated that the protective properties of coffee are not only due to direct scavenging effects (which were seen in *in vitro* experiments with hydrogen peroxide) but also to induction of the antioxidant enzyme superoxide dis-mutase. After the intervention, the activity of this enzyme was increased signi-ficantly (38%), whereas the levels of glutathione peroxidase (another important antioxidant enzyme) were not altered.

In this context it is notable that no protective effect was seen in a similar study, in which the participants consumed 600 g of different fruits and vegetables per day (Moller et al., 2003). This comparison might be taken as an indication that coffee consumption has in this test system a higher antioxidative potential than a mixed fruit and vegetable diet. This assumption is further enhardened by investigations in which the antioxidant capacities of coffee and other dietary constituents were determined on the basis of *in vitro* experiments, and used for the calculation of the total antioxidant intake of humans (Pellegrini et al., 2003). However, due to the small number of the participants in the aforementioned coffee study further follow up experiments are required to support our results. In addition it has been shown that coffee is one of the major source in the average daily antioxidant intake in humans (Svilaas et al. 2004).

CONCLUSIONS

The currently available data indicate that coffee consumption protects against specific classes of genotoxic carcinogens such as aflatoxin B_1 and PAHs, while the assumption of protective effects toward heterocyclic aromatic amines and nitrosamines could not be confirmed. Furthermore, we found pronounced prevention of oxidative DNA-damage in humans after coffee consumption (600 ml/d over 5 days). This observation might explain the results of several epidemiological studies, which indicate that coffee intake is inversely related to the incidence of hepatocellular carcinomas, liver cirrhosis and colon cancer as it is well documented that reactive oxygen species are causally involved in all this diseases.

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Coffee and Health: the Holistic Approach

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SUMMARY

The assessment of risk to human health of foods, food ingredients/contaminants and nutrients has historically been conducted independently of possible health benefits. In addition, different scientific approaches have been used to estimate health risks and benefits of foods and their constituents. When a food or food constituent is associated with both potential health risks and benefits, and when the health effects appear to be dependent on the level of intake, there is a need to determine a consumption range with an acceptable balance of risks and benefits. The extensive database on the health effects of global coffee consumption developed over the past 30 years provides an excellent opportunity to investigate this balance between risks and benefits. While the health risks of coffee and its key ingredients, particularly caffeine, have been the focus of thousands of published scientific/medical studies addressing almost all known animal toxicity endpoints and human disease outcomes, more recent research attention has been focussed on investigating the possible health benefits of coffee consumption. As evidenced by the conclusions of the other expert presenters in this symposium, a growing literature appears to be concluding that moderate coffee consumption may be associated with reduced risk of type 2 diabetes, coronary heart disease, Parkinson's disease and even some cancers. There is also a well-established database on the benefits of human coffee and caffeine consumption in improving both physical and mental performance. In concert with this "good news" about coffee consumption are extensive investigations underway in many laboratories throughout the world to determine the biochemical mechanisms by which these beneficial health effects may be operating. One of the most exciting areas of mechanistic research is the possible cancer-protective role of coffee's naturally occurring polyphenolic antioxidants (chlorogenic acid derivatives) and heat-produced antioxidants (Maillard Reaction Products, including volatile heterocyclic compounds and melanoidin polymers). Studies in many countries have shown that coffee is actually the major individual source of dietary antioxidant potential, and laboratory studies have shown that some coffee constituents can induce the formation of carcinogen-detoxifying enzymes. Therefore, while trace levels of many animal carcinogens can be found in coffee as consumed, there are also many compounds now identified in coffee that may reduce the risk of cancer. In conclusion, the dietary cancer risk of coffee consumption cannot properly be assessed by examining just the concentrations and potencies of the individual animal carcinogens contained in the product. Instead, coffee should be evaluated using the "Holistic Approach" that also takes into account the simultaneous presence of natural, health-protective compounds, the beneficial effects produced by the Maillard reaction, and most importantly the extensive human epidemiologic database clearly showing that coffee consumption reduces cancer risks.

INTRODUCTION

Diet and health concerns

There have been dramatically increased concerns about diet and health during the past 20 years, with the constant flow of anxiety-provoking headlines and media stories linking individual foods/beverages and ingredients to diseases such as cancer, heart disease, obesity, diabetes, reproductive effects and others. In particular, what we eat (or don't eat) is oftentimes linked to an increased risk of human cancer, where we witness the "carcinogen-of-the-month" syndrome. However, a great deal of this information can be preliminary in nature and based largely on high-dose animal cancer studies and small epidemiologic studies, but once a larger body of studies has been accumulated, there is often a near absence of any real health concern. The information that does build up during the intervening years can lead to much nutrition nonsense and "food faddism," producing nations of "avoiders" of specific foods and ingredients such as salt, fat, meat, carbohydrates and even coffee.

Historical perspective on coffee

This has been especially true for several decades related to the avoidance or reduction in daily use of coffee and caffeine by some consumers because of perceived health concerns. But why have coffee and caffeine been so maligned for so long? In fact, perhaps no substances have been the subject of more conflicting media and scientific reports in recent decades than coffee and caffeine. For both, there has been a long history of adverse health studies and regulatory issues beginning in the 1960's, and this has led to distorted media treatment of these issues and the generation of many adverse health *myths.* This history has been complicated by the fact that there are over 2,000 known chemical components in a cup of coffee and over 20,000 published, chemical and health-related studies appearing in the literature.

But the current *facts,* supported by much scientific study performed by universities and the industry over recent years, demonstrate many coffee and caffeine *positive* health attributes, leading to the conclusion that coffee and caffeine can actually be *good* for your health and can be accepted by consumers without worry and guilt. These conclusions are gaining attention in the global press, and in fact are beginning to influence physicians and other health professionals to advise people about the real health benefits of moderate coffee consumption. This paper will address some of the significant positive news and the need to take a "Holistic Approach" [a "risk vs. benefit" approach] in determining the health role of coffee in today's diet, especially in relation to the possible protective effect of coffee in human cancer.

CONTROVERSIES IN RISK EVALUATION OF FOODS AND FOOD CHEMICALS

There are four key areas of controversy in evaluating the risk of foods and food chemicals, and these have all applied to the risk evaluation of coffee and its components during the past decades. Much of what has led to the demonstration of coffee/caffeine's adverse effects in the past 30 years is now known to have resulted from methodological weaknesses occurring in the published animal toxicology and human epidemiologic research. The four areas which have been the greatest sources of controversy are as follows:

1. Methodological weaknesses in epidemiologic research. Human epidemiologic studies of coffee and caffeine have often suffered from several methodologic weaknesses: (a) erroneous hypotheses have often been tested: (b) there have been numerous problems with study design; (c) there are inherent difficulties in studying human subjects; (d) authors have given biased interpretations of earlier findings that have falsely supported their

current findings; (e) known confounders of coffee consumption have often not been accounted for (the best example was cigarette smoking confounding coffee's relationship to bladder cancer); (f) the difficulty of accurately trying to assess daily intake of coffee, where different cup sizes and varying brew strengths and types of coffee have complicated exposure estimation and assessment; and (g) the failure to give proper weight to negative epidemiology studies where coffee has been shown not to be associated with a specific disease.

2. Interpretation of rodent cancer bioassays of very extreme chemical doses. Animal toxicology studies of coffee and its many individual, trace components have also suffered from key methodologic deficiencies, the two most prominent being: (a) the treatment of laboratory animals with unrealistically excessive doses over their lifetimes, most notably the "Maximum Tolerated Dose" (MTD) quantities used in carcinogen bioassay testing protocols; and (b) the use of straight-line, no-threshold extrapolation of animal tumor results to humans, a paradigm that many toxicologists are now considering may be unjustified. Thus, much of the negative, adverse findings for coffee components (particularly on trace-level animal carcinogens found in roasted coffee) have come from the failure to heed the interpretive advice laid down centuries ago by the Paracelsus (1493-1541), the "Father of Modern Toxicology:"

   "*The Dose Makes the Poison:* All things are poison and there is none which is not a poison. Solely the dose differentiates a poison from a remedy."

   Many eminent toxicologists are now beginning to question the human relevance of tumors seen in these high-dose rodent bioassays, and some believe it is time to stop doing chronic MTD studies altogether. In addition, toxicologists make two possibly flawed assumptions about the chronic bioassay's tumor findings. The first is the *"Dose Extrapolation"* assumption, wherein effects seen at high doses are assumed to also occur at much lower human doses. And the second is the *"Species Extrapolation"* assumption, that if tumors are observed in rodents, then similar tumors probably occur in humans. However, toxicologists are now trying to better understand the tumor mechanisms and biochemical modes of action for a chemical before using the bioassay's tumor results for regulatory or warning purposes.

3. Assessing the risk of individual chemical components in a food or beverage instead of assessing the safety of the whole food. This area is especially controversial when it relates to the assessment of risk of animal carcinogens formed during the processing and heating of foods and beverages, including coffee and its components. An important report (National Research Council., 1996) on the carcinogenicity of individual food chemicals published by the U.S. National Academy of Sciences' National Research Council in 1996 came to several key conclusions about the cancer potential of food chemicals: (a) "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;" (b) "The varied and balanced diet needed for good nutrition also provides significant protection from natural toxicants;" (c) "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 (d) "Closing Remarks…Most naturally occurring minor dietary constituents occur at levels so low that any biologic effect, positive or negative, is unlikely."
   Carcinogens from heated foods have been a health concern since the 1970's, including those produced by the "Maillard Browning Reaction" between carbohydrates and amino

acids. However, an important question in assessing individual heat-induced food chemicals and their safety in humans in general (and coffee chemicals' safety in particular) is why there is still such an intense interest in trace level carcinogens, when there is little human epidemiologic evidence linking these foods and beverages with disease risk, including cancer.

4. The health benefits of a food or beverage are often neglected by health and regulatory authorities. While it is important to evaluate the toxicological risks of heat-induced chemicals in foods, it is equally important to fully evaluate the safety of whole foods and beverages using modern epidemiologic techniques. It is becoming increasingly important to recognize that health-beneficial food chemicals occur naturally, including many in coffee, as do other health-protective compounds produced during heat processing and cooking. Thus, it is critical to also evaluate these beneficial health effects of heated foods/beverages, such as coffee, and then to undertake a thorough risk-benefit evaluation of the whole food or beverage [see the discussion of the "Holistic Approach" below]. Such an evaluation must carefully consider how best to interpret animal toxicology results for individual coffee chemicals, as well as recent information that coffee may actually be cancer protective. While the regulatory and health authorities that assess food safety are obliged to focus great attention on the occurrence and potential risk of suspected toxicants and carcinogens in foods, they should also be encouraged to systematically investigate the health benefits of specific components in foods as well as the whole foods.

WHAT DO WE KNOW ABOUT COFFEE AND CANCER RISK?

Coffee contains trace levels of over 30 identified animal carcinogens, including acrylamide, furan, caffeic acid, various aldehydes, polycyclic aromatic hydrocarbons, ochratoxin A and many others. While a few of these chemicals are naturally occurring in green coffee, many of the others are chemicals formed at trace levels during the roasting of coffee by means of the Maillard browning reaction. But, after three decades of research, most health authorities across the globe now agree that coffee drinking is not a human cancer risk. In 1991 the International Agency for Research on Cancer (IARC) published a monograph on coffee, tea, caffeine and related substances (IARC, 1991). IARC concluded that caffeine was not carcinogenic and that coffee was only very weakly associated with an increased risk of bladder cancer. IARC also concluded that coffee may be related to reduced risk of colorectal cancer, and this conclusion has been strongly supported by many recent studies (reviewed in Tavani and La Vecchia, 2004). Recent studies have also shown that coffee drinking may be associated with reduced risk of liver and breast cancer.

One of the most exciting areas of coffee's mechanistic research is the possible cancer-protective role of its naturally occurring polyphenolic antioxidants (chlorogenic acid derivatives) and its heat-produced antioxidants (Maillard reaction products, including volatile heterocyclic compounds and brown melanoidin polymers). Studies in many countries have now shown that coffee is actually the major individual source of dietary antioxidants (exceeding wine, tea, chocolate, and individual fruits and vegetables), and in-vitro studies have also shown that some coffee constituents can induce the expression of carcinogen-detoxifying enzymes. Ironically, then, coffee actually contains natural and heat-induced "protective" chemicals that may help to reduce the potential carcinogenic risk of other coffee chemicals that are reported to be animal carcinogens. Consequently, it may well be that the anti-carcinogenic effects of these coffee components outweigh any risk from the known carcinogens, leading to overall cancer protection at the organ sites showing reduced human cancer risk among coffee drinkers.

OTHER HEALTH EFFECTS ADDRESSED IN THIS SYMPOSIUM

As evidenced by the presentations and conclusions of the other expert scientists speaking in this symposium, a growing literature appears to be concluding that moderate coffee consumption may be associated with reduced risk of type 2 diabetes, cardiovascular disease, Parkinson's disease and, as noted above, some forms of human cancer (Dórea and da Costa 2005; Higdon and Frei 2006; van Dam, 2006).

Reduced risk of type 2 diabetes

Habitual consumption of 5 or more cups/day has been associated with improved glucose regulation and tolerance and a substantially lower risk of type 2 diabetes (35-75%) in diverse populations in the United States, Europe, and Japan. Consumption of 3-4 cups/day has also been associated with a reduced risk when compared to fewer cups. Caffeine-containing and decaffeinated coffees have been shown to give similar protective effects, which may be attributed to natural polyphenolic antioxidants, lignans and magnesium.

Reduced risk of liver disease

Coffee consumption has been associated with a clinically significant reduced risk of cirrhosis and liver cancer in several study populations. These data suggest that the reduced risk of alcohol cirrhosis may be associated with coffee constituents such as polyphenolics and related substances. Possible mechanisms for this observation may be lower activity of selected aminotransferases, possible inhibition of inflammatory transcription factors and perhaps increased expression of detoxifying enzymes.

Reduced risk of Parkinson's disease

Epidemiologic studies have strongly linked the neuroprotective effect of caffeine consumption by men and postmenopausal women with a reduced risk of developing this disease. Some research in neuropharmacology suggests that just one cup of coffee/day (80-140 mg caffeine) can halve the risk of the disease, since caffeine's adenosine-blocking power may be one mechanism through which the brain cells in Parkinson's disease are protected or conserved.

Cardiovascular disease risk

Many cohort studies have not found a significant association between coffee consumption and coronary heart disease. Some case-control studies have suggested an increased risk of coronary heart disease among those with higher coffee consumption compared to those who consume moderate or low levels of coffee. However, a recent comprehensive 20-year prospective cohort study with approximately 130,000 men and women without a history of cardiovascular disease or cancer did not provide any evidence that coffee consumption increases the risk of coronary heart disease (Lopez-Garcia et al., 2006).

Other beneficial health effects

Numerous other studies have shown that coffee drinking increases mental alertness, cognitive functions, wakefulness, and physical stamina, while it also reduces the risk of Alzheimer's disease, asthma, kidney stones, gallstones, depression/anxiety and suicidal behavior.

CONCLUSION: THE "HOLISTIC APPROACH"

The dietary cancer risk of coffee consumption cannot properly be assessed by examining only the trace concentrations and carcinogenic potencies of the individual animal carcinogens contained in the product. Instead, coffee should be evaluated using the "Holistic Approach" that also takes into account the simultaneous presence of natural, cancer-protective compounds, the beneficial effects of those chemicals produced by heating via the Maillard browning reaction, and most importantly the extensive human epidemiologic database clearly showing that coffee consumption reduces cancer risks. Additionally, while the "Holistic Approach" can be useful in addressing the risk/benefit assessment specifically related to coffee and cancer endpoints, its use could also be developed to assess the overall health effects of coffee consumption, i.e., weighing the potential risks vs. benefits across all disease outcomes that have been reported for coffee.

Furthermore, it is important to encourage health and regulatory authorities globally to make two important changes in their historic approach to food safety assessment: (1) to carefully assess the risks and benefits of whole foods and beverages using the "Holistic Approach," not just assessing the well-studied, individual chemicals in these products; and (2) to give much greater public health consideration to the potential health benefits of heated foods and beverages, such as coffee.

More than 30 years of research and a very extensive database on the health effects of coffee provide the industry and regulatory/health professionals with an excellent opportunity to reexamine the risks and benefits of this widely consumed beverage. The controversies and purported health risks surrounding coffee and its component chemicals have been the focus of thousands of studies addressing a litany of animal toxicity and human disease outcomes. Today, however, many negative health myths about coffee drinking have been transformed into validated health benefits as a result of more recent mechanistic and epidemiologic research studies. In conclusion, the preponderance of scientific and clinical evidence suggests that moderate coffee consumption (3-5 cups/day) may be associated with the reduced risk of numerous disease conditions.

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Chlorogenic Acids – Their Characterisation, Transformation During Roasting, and Potential Dietary Significance

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SUMMARY

This paper selectively reviews the literature on chlorogenic acids (cinnamoyl-quinic acids) and related substances in coffee. Attention is drawn to advances in analytical procedures and to the novel substances found in green and roasted beans. Recent data for absorption and metabolism of chlorogenic acids and their transformation products are presented along with a consideration of their possible biological effects.

INTRODUCTION

Chlorogenic acids are a major component of the green coffee bean and much has been written about them and their contribution to the unique properties of the coffee beverage. An electronic search (August 2006) of PubMed identified over 1000 publications concerned with chlorogenic acids, with some 650 of these published since this author's previous contribution to ASIC (Clifford, 1998). This manuscript is a selective review of this literature and other recent reviews should be consulted for additional information specific areas (Clifford, 2003; 2000).

CHEMISTRY AND ANALYSIS

Classically, chlorogenic acids are a family of esters formed between quinic acid and certain *trans* cinnamic acids, including caffeic, *p*-coumaric, ferulic and 3,4-dimethoxycinnamic acid (Clifford, 2000; 1999; IUPAC, 1976). Their structures are shown in Figure 1. In the IUPAC system (–)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid. However, Eliel and Ramirez (1997) recommend 1α,3*R*,4α,*5R*-tetrahydroxycyclohexane carboxylic acid to avoid ambiguity when describing quinic acid itself, or any 1,4-disubstituted quinic acids where the two substituents are identical. Although the subtle differences in structure that are described are not easy to visualise, an appreciation of them has become important since it is now recognised that quinic acid epimerisations may occur during coffee roasting, producing novel quinic acids and quinic lactones (quinides) (Scholz-Böttcher et al., 1991; Scholz and Maier, 1990), plus chlorogenic lactones (Farah et al., 2006; 2005; Frank et al., 2006; Ginz, 2001; Ginz and Engelhardt, 1995) and presumably novel chlorogenic acids. There is, not surprisingly, evidence that structure influences taste (Frank et al., 2006) and thus potentially other physiological properties that might impact upon health. The epimeric *muco*-quinic acid structure is shown in Figure 2.

In the classic chlorogenic acids, based upon quinic acid as shown in Figure 1, four mono-acyl isomers are possible, and three of these are well known in coffee (3-caffeoylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid). Similarly, six *homo*-diacyl chlorogenic acids occur and three are well known in coffee (3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid). In the case of *muco*-quinic acid (Figure 2) only two isomers

are likely to be observed in coffee extracts unless chiral columns are used because 3*S*-caffeoyl-*muco*-quinic acid and 5*R*-caffeoyl-*muco*-quinic acid are coeluting enantiomers: 4-caffeoyl-*muco*-quinic acid would be resolved.

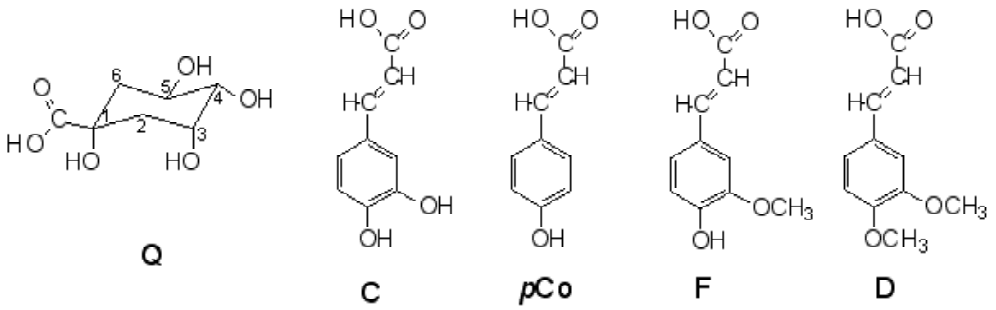

Figure 1. Structure of Quinic Acid and Cinnamic Acids commonly found in Chlorogenic Acids (IUPAC numbering. Q = quinic acid; C = caffeic acid; *p*Co = *p*-coumaric acid; F = ferulic acid; D = 3,4-dimethoxycinnamic acid.

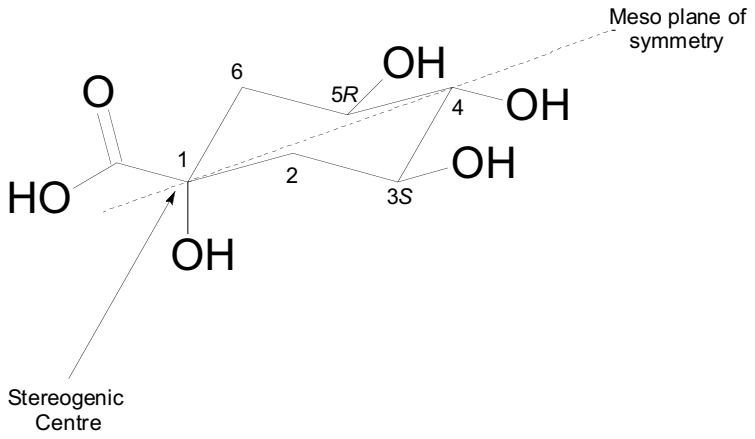

Figure 2. *Muco*-quinic acid is pseudo-asymmetric.

The multiple chiral centres present in chlorogenic acids creates a potential for overlapping signals makes it difficult to determine the precise structure of chlorogenic acids by NMR. It has been recognised that the effects of solvent and temperature are greater than once anticipated and special precautions are necessary to minimise the risk of incorrect assignments of structure (Pauli et al., 1999; 1998). These fundamental complications are made more severe by the lack of authentic commercial standards (Clifford, 2003), and the difficulty of isolating sufficient of a minor component to attempt definitive NMR studies.

Some of these problems have been circumvented by the development of powerful LC–Ion Trap MS (LC–MS^n) procedures for chlorogenic acids analysis (Clifford et al., 2006; 2005; 2003). These procedures characterise a chlorogenic acid not only by its molecular mass, but also by the nature and intensity of the fragments produced after a particular ion has been trapped. By working initially with pure compounds it was possible to relate the fragmentation pattern to structure and elucidate plausible mechanisms to account for the differences observed. These relationships have proven sufficiently robust to allow the methods to be transferred between laboratories, and used for the characterisation of novel compounds without isolation. This approach has identified many new chlorogenic acids in green coffee beans. Relevant mass spectra have been published elsewhere (Clifford et al., 2006; 2005; 2003; Clifford and Knight, 2004), in some cases accompanied by hierarchical keys to aid

interpretation of spectra (Clifford et al., 2005; 2003). The full complement of 45 compounds, incorporating the 27 recent additions, is summarised in Table 1.

Table 1. The Chlorogenic acids of green coffee beans.

• Three isomers of *p*-coumaroylquinic acid
• Three isomers of caffeoylquinic acid
• Three isomers of feruloylquinic acid
• Three isomers of dimethoxycinnamoylquinic acid*
• Three isomers of di-*p*-coumaroylquinic acid*
• Three isomers of dicaffeoylquinic acid
• Three isomers of diferuloylquinic acid*
• Six isomers of caffeoyl-feruloylquinic acid
• Six isomers of *p*-coumaroyl-caffeoylquinic acid*
• Three isomers of caffeoyl-dimethoxycinnamoylquinic acid*
• Three isomers of feruloyl-dimethoxycinnamoylquinic acid*
• Three isomers of *p*-coumaroyl-feruloylquinic acid*
• Three isomers of *p*-coumaroyl-dimethoxycinnamoylquinic acid*

**Recently reported.*

Precise quantitative data are not available for the content of these novel chlorogenic acids in green coffee beans, but a comparison of the A_{325} values with the value for 5-caffeoylquinic acid, that is commonly present at approximately 5% dry matter basis (dmb), suggests that in total they will not exceed some 0.7 to 1% dmb in a robusta and about half that in an arabica.

The significance of these minor chlorogenic acids is not clear. However, it is of interest from a biosynthetic perspective that for some classes of *hetero*-diacyl chlorogenic acids six isomers are produced whereas for other classes only three are produced. This suggests that the ability of the chlorogenic acid:chlorogenate cinnamoyl transferase enzyme(s) to attach a second cinnamoyl residue is influenced in some cases by the identity and position on the quinic acid moiety of the first cinnamic acid residue. With regard to the *p*-coumaroyl-feruloylquinic acids the constraints observed in coffee are absent from *Aster ageratoides* which produces the theoretical six isomers (Clifford et al., 2006).

The use of similar LC-MS methods has led to the discovery also of several new cinnamoyl-amino acid conjugates (*p*-coumaroyl-tyrosine, feruloyl-tyrosine, feruloyl-tryptophan and caffeoyl-phenylalanine) in green coffee beans (Clifford and Knight 2004). Quantitative data obtained using isotopically-labelled standards have been reported for some of these plus the novel caffeoyl-aspartic acid and *p*-coumaroyl-aspartic acid conjugates in roasted coffee (Stark et al., 2006). Caffeoyl-tryptophan dominates (ca 3 mg/kg) followed by *p*-coumaroyl-tryptophan (ca 0.5 mg/kg), caffeoyl-tyrosine (ca 100 μg/kg), caffeoyl-aspartic acid and feruloyl-tryptophan (ca 40 μg/kg) and *p*-coumaroyl-aspartic acid and *p*-coumaroyl-tyrosine (ca 10 μg/kg) (Stark et al., 2006). These compounds have been reported to be astringent, with their astringency threshold being a function primarily of the amino acid residue (Stark and Hofmann, 2005). Some are considered to contribute to the puckering astringency of cocoa (Stark et al., 2006).

Ongoing and as yet unpublished investigations from the author's laboratory suggest the presence in green coffee beans also of at least one caffeic acid glycoside, several caffeoyl-glucose esters and several chlorogenic acid glycosides. In addition coffee leaves clearly contain *cis*-5-caffeoylquinic acid whereas this is normally undetectable in the beans, presumably as a consequence of the leaf's exposure to UV light.

Coffee roasting destroys a significant percentage of the chlorogenic acids and creates a series of transformation products that may be unique to coffee. Recent studies have focussed on the chlorogenic lactones because of their bitterness and possible biological effects (Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995; Bennat et al., 1994; Schrader et al., 1996). Table 2 lists those known to be present in the roasted bean.

Table 2. The Chlorogenic Lactones of Roasted Coffee

Chlorogenic lactones	Roasted coffee (%d.b.)	References
3-*O*-caffeoyl-γ-quinide	0.08-0.24	Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995; Bennat et al., 1994; Schrader et al., 1996*
4-*O*-caffeoyl-γ-quinide	0.02-0.14	Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995; Bennat et al., 1994; Schrader et al., 1996*
5-*O*-caffeoyl-*epi*-γ-quinide		Frank et al., 2006; Farah et al., 2005
5-*O*-caffeoyl-δ-quinide		Ginz and Engelhardt, 1995
4-*O*-caffeoyl-*muco*-γ-quinide		Frank et al., 2006; Farah et al., 2005
5-*O*-caffeoyl-*muco*-γ-quinide		Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995
3-*O*-feruloyl-γ-quinide		Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995
4-*O*-feruloyl-γ-quinide		Frank et al., 2006; Farah et al., 2005; Ginz and Engelhardt, 1995*
3,4-*O*-dicaffeoyl-γ-quinide		Frank et al., 2006; Farah et al., 2005*
3,5-*O*-dicaffeoyl-*epi*-δ-quinide		Frank et al., 2006; Farah et al., 2005
4,5-*O*-dicaffeoyl-*muco*-γ-quinide		Frank et al., 2006; Farah et al., 2005
At least two more feruloylquinides		*

* *Clifford, unpublished data.*

COFFEE CONSUMPTION

Several databases are now available for the content of various phenols and polyphenols in foods and beverages, but generally these focus on flavonoids and report values of the (poly)phenolic aglycones measured after release from their conjugating moieties (Clifford and Brown, 2006). While these provide valuable data with which to seek epidemiologic links between diet and health, it is important not to overlook the fact that biological properties may be determined by the nature of the conjugate rather than solely by the aglycone. For example, it is now well-established that the absorption of quercetin-glycosides differs with the identity and location of the sugar residue(s) (Day et al., 2003; Graefe et al., 2001). Such differences

have yet to be investigated for the chlorogenic acids, but there is evidence that their sensory properties are influenced in this way (Farah et al., 2006; Frank et al., 2006). and it is not unreasonable to anticipate that other biologically-significant properties might also be.

Studies at the University of Surrey (Gosnay et al., 2002; Woods et al., 2003) indicate very clearly that chlorogenic acids consumption may be substantial. For example, the mean daily intake for 103 women (aged 20 to 30 years) was estimated at 350 mg while that for a group of males (aged 27 to 57) was 670 mg, supplying respectively 47% and 64% of their mean daily intake of total (poly)phenols. A study of a German population estimated mean daily chlorogenic acids intakes (as caffeic acid) at 229 mg for women and 179 mg for men (Radtke et al., 1998). For these, and many other populations, coffee is the richest dietary source of chlorogenic acids (Clifford, 2000; 1999) demonstrating clearly that if dietary (poly)phenols are associated with a health benefit, coffee can make a significant contribution.

ABSORPTION AND METABOLISM

Some studies report that after volunteers consumed chlorogenic acid-rich foods or beverages (including coffee) or pure 5-caffeoylquinic acid, intact chlorogenic acid can be found in plasma(Bugianesi et al., 2004) or urine (Cremin et al., 2001; Olthof et al., 2001; 2003; Ito et al., 2005), whereas other similar studies have failed to demonstrate its absorption intact (Nardini et al., 2002; Wittemer et al., 2005). Plasma concentrations are low (19-45 nM) (Nardini et al., 2002). It would appear that more than one isomer of caffeoylquinic acid is excreted in human urine (Ito et al., 2005), but the relative bioavailability of isomers and whether or not they can by inter-isomerised *in vivo*, have not been thoroughly investigated so far. Data are available for both 5-caffeoylquinic acid (Lafay et al., 2006; Azuma et al., 2000) and 1,5-dicaffeoylquinic acid (Yang et al., 2006; 2005) administered to rats. There are currently no data for the chlorogenic lactones.

Absorption of 5-caffeoylquinic acid is biphasic reflecting some absorption in the stomach, and some in the large bowel (Wittemer et al., 2005).

Only some 2% of the dose has been recovered in urine (Olthof et al., 2001; Ito et al., 2005) and the majority of the dose is exposed to the gut microflora in the large bowel (Gonthier et al., 2003). Transformations made by human-associated gut microflora include hydrolysis to cinnamic acid and quinic acid, dehydroxylation, side chain hydrogenation, and side chain shortening (Scheline, 1978). These microflora metabolites can be (partially) absorbed from the colon, some actively by the monocarboxylate transporter (Nardini et al., 2002; Takanaga et al., 1994; Tsuji et al., 1994; Tamai et al., 1997; Konishi and Shimizu, 2003; Konishi et al., 2003; Konishi and Kobayashi, 2004), followed by mammalian metabolism, including methylation, and conjugation with glycine, glucuronate and sulphate. Gut flora metabolites of caffeic acid and / or chlorogenic acid found in plasma include caffeic, ferulic, isoferulic, dihydrocaffeic and vanillic acids as glucuronide and sulphate conjugates, with caffeic and isoferulic also detected as the aglycones. Hippuric acid, produced by a combination of microbial and mammalian metabolism is probably the major chlorogenic acid metabolite (Olthof et al., 2003).

BIOLOGICAL EFFECTS

Seeking to demonstrate health-promoting effects of minor dietary components has been an area of intense activity for more than a decade. Many investigations have used cultured cells and high doses of test substance, a protocol that is difficult to relate to real life. If the test substance is chemically ill-defined (for example coffee brew) rather than a pure substance it is

impossible to identify the active principal for any effect observed. Applying a pure substance direct to liver cells, for example, does not reflect what happens in real life if the substance is either not absorbed, or is extensively metabolised before reaching that tissue. Even if the test substance does indeed reach the tissue in question, the dose used *in vitro* might be much higher than would ever occur *in vivo*, and a linear dose–response should not be assumed since the effect may be thresholded, or biphasic. Animal studies avoid some of these problems, but species differences in metabolism may still complicate extrapolation to humans. The examples of possible beneficial effects discussed below have been selected from the literature having regard to these limitations so far as is possible (Clifford and Brown, 2006; Clifford, 2004).

The large bowel has a complex, concentrated and intensely active anaerobic microflora. Poorly absorbed dietary (poly)phenols can reach mg/ml concentrations (ca 0.1 to 1 μM) (Jenner et al., 2005) in the large bowel and there is a potential for prebiotic effects (Clifford et al., 2005). These might be due to direct inhibition of certain gut microorganisms by the (poly)phenols consumed, or indirectly by the phenolic acid metabolites that some species are able to produce but which others cannot tolerate.

The concentrations of certain phenolic acids in human faecal water have been reported to reach concentrations in the range 40 nM to >400 μM (Jenner et al., 2005), and in some cases exceed the concentration required *in vitro* to inhibit *Listeria monocytogenes* (Fallingborg, 1999). Polyphenol-rich diets consumed by humans, pigs and sheep have lowered the colonic pH value, suppressed *Clostridium perfringens*, and increased the proportion of bifidobacteria without inhibiting lactic acid bacteria (Clifford et al., 2005; Goto et al., 1998; Hara, 1997; Okubo et al., 1992).

The gut microflora-generated phenolic acids once absorbed may have endogenous biological activity (Clifford and Brown, 2006). For example, 3,4-dihydroxyphenylacetic acid has *in vitro* anti-proliferative activity (Gao te al., 2006); and 2-hydroxybenzoic acid is an effective anti-inflammatory (Patrignani, 2000) used clinically in the form of low-dose aspirin. Animal studies have indicated that phenolic acids produced by gut flora metabolism, provided they reach the tissues without mammalian conjugation, may inhibit platelet aggregation (Kim et al., 1998; Yasuda et al., 2003), and reduce hepatic cholesterol synthesis through inhibition of HMG-CoA reductase (Bhat and Ramasarma, 1979; Lee et al., 2001). The cholesterol lowering has been demonstrated in animals (Lee et al., 2001; Bok et al., 1999; Kim et al., 2003; Lee et al., 2001; Matsumoto et al., 1998; Yamakoshi et al., 1999) and volunteers (Kurowska et al., 2000) given foods containing the microbial substrates, and while this does not prove the mechanism, it does demonstrate plausibility, and *in vivo* efficacy.

Over the last few years epidemiological evidence has accumulated that higher coffee consumption might be associated with a lower risk of developing Type II diabetes (van Dam and Feskens, 2002; Saremi et al., 2003; Agardh et al., 2004; Rosengren et al., 2004; Salazar-Martinez et al., 2004; Tuomilehto et al., 2004; Andersen et al., 2006). Such statistical relationships do not prove cause and effect, and do not immediately suggest mechanisms, and there has been much speculation about possible mechanism(s) involving effects on insulin and associated peptide hormones (GIP and GLP-1) (Atanasov et al., 2006; van Dam, 2006; McCarty, 2005; Shearer et al., 2003; Johnston et al., 2003).

We have shown in volunteers under controlled clinical conditions, by observing a reduced secretion of glucose-dependent insulinotropic peptide (GIP) and an increased secretion of glucagon-like peptide (GLP-1), that glucose consumed in coffee beverage is absorbed more slowly and lower down the gastro-intestinal tract than when the same quantity of glucose is

consumed in the same volume of water (Johnston et al., 2003). Thus, when glucose is consumed in coffee, the peak plasma concentration is lower than when consumed in water. Current wisdom suggests that a high post-prandial surge in plasma glucose concentration is an independent risk factor for the development of Type II diabetes and other (vascular) diseases (Ludwig, 2002), and thus its suppression may confer some protection against disease development *In vitro* studies have demonstrated that the sweet-tasting 1,5-γ-quinide (Shamil et al., 1987; Kellard et al., 1988) can inhibit active glucose transport in cell culture (Clifford et al., 2004) at a concentration that might reasonably be expected to occur in the duodenum after drinking coffee. It is possible that this interaction might explain our observations in volunteers and, at least in part, the epidemiological relationships. Figure 3 illustrates the rationale and assumptions made in estimating concentrations of 1,5-γ-quinide that might occur in the gut.

- Quinide in medium roasted coffee up to *ca* 1% (Scholz-Böttcher et al., 1991; Scholz and Maier , 1990; Scholz-Böttcher, 1991)
- Assume 25 to 33% total solids extraction during 'domestic' brewing (50% for commercial instant) and no losses of quinide
- ∴ quinide 3 to 4% of total solids (2% of instant)
- Assume coffee beverage 2% solids as drunk
- ∴ quinide 60 to 80 mg / 100 ml beverage (40 mg / 100 ml instant)
- Allow a 10-fold uncertainty factor
- ∴ quinide in beverage 0.35 to 0.46 mM (0.23 mM in instant)
- Allow a 10-fold dilution in stomach and duodenum
- ∴ quinide in duodenum 35 to 46 μM (23 μM for instant)
- *In vitro*, even 1 μM delayed glucose uptake ($p = 0.013$)
- Could quinide explain delayed glucose uptake in volunteers?
- Post-prandial hyperglycaemia important risk factor in CVD (Coutinho et al., 1999)

Figure 3. Quinide Inhibition of Glucose Transport.

Some green coffee beans are steam treated (dewaxed) to produce 'stomach-friendly' roasted coffees. One effect of steam treatment is to favour progressive isomerisation of 5-caffeoylquinic acid to 4-caffeoylquinic and 3-caffeoylquinic acid (König and Sturm, 1983; Clifford, 1985). The feruloylquinic acids and dicaffeoylquinic acids are similarly affected. Some hydrolysis of dicaffeoylquinic acids to caffeoylquinic acids and caffeic acid, and of the monoacyl chlorogenic acids to cinnamic acid and quinic acid also occurs. These changes in composition favour a greater production during roasting of the sweet-tasting 1,5-γ-quinide and the lactones of the 3-acyl and 4-acyl chlorogenic acids. It has been reported that 4-caffeoylquinic-1,5-γ-lactone binds *in vitro* to the μ-opioid receptor with a K_i value of 4.4 ± 0.4 μM a value that could possibly be achieved in the gut lumen after coffee consumption. Figure 4 illustrates the rationale and assumptions made in estimating concentrations 4-caffeoylquinic-1,5-γ-lactone that might occur in the gut after coffee consumption. Pharmaceutical preparations with μ-opioid activity are used to alleviate the symptoms of diarrhoea by reducing gut motility and it may be that the stomach-friendliness of certain coffees can be explained by a greater content of opioid-active lactones. If motility were also

reduced in the upper gastro-intestinal tract then this effect might also slow glucose uptake by delaying gastric emptying.

- 4-CQ-γ-L can reach 0.14% in roasted coffee (Bennat et al., 1994; Schrader et al., 1996)
- Assume 25 to 33% total solids extraction during 'domestic' brewing (50% for commercial instant) and no losses of 4-CQ-γ-L
- ∴4-CQ-γ-L 0.4 to 0.55% of total solids (0.27% of instant)
- Assume coffee beverage 2% solids as drunk
- ∴4-CQ-γ-L 8 to 11 mg / 100 ml beverage (5.5 mg / 100 ml instant)
- Allow a 10-fold uncertainty factor
- ∴4-CQ-γ-L in beverage 0.024 to 0.033 mM (0.016 mM in instant)
- Allow a 10-fold dilution in stomach and duodenum
- ∴4-CQ-γ-L in duodenum 2.4 to 3.3 μM (1.6 μM for instant)
- ∴for 4-CQ-γ-L 4.4 ± 0.4 μM for the μ-opioid receptor (de Paulis et al., 2004)

- Could this reduce gut motility and partly explain delayed glucose uptake in volunteers?
- Might this also explain stomach friendliness of some coffees?

Figure 4. 4-Caffeoylquinic-1,5-γ-lactone and Opioid Effects.

The quinides and chlorogenic lactones may well be unique to coffee beverage. If either or both of these effects prove to be real, then optimising them through manipulation of bean selection, processing and roasting technology presents both an opportunity and challenge to the industry, complicated possibly by the need also to balance the content of sweet tasting 1,5-γ-quinide and the bitter tasting chlorogenic lactones.

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Bioavailability of Chlorogenic Acid

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SUMMARY

Absorption and metabolism chlorogenic acid (5-*O*-caffeoylquinic acid, CGA), the main phenolic acid in the human diet, was studied in rats in order to determine where and under which form this ester of caffeic acid is absorbed. Nutritional intervention, *in situ* intestinal perfusion and *in situ* gastric infusion studies were carried out**.** CGA was found stable in the stomach and small intestine contents, whereas it was hydrolysed into caffeic acid in the cecum. CGA and caffeic acid appeared early at 1.5 h in plasma and urine, suggesting an absorption of CGA in the upper part of the gastro-intestinal tract. Using the *in situ* intestinal perfusion rat model with cannulation of biliary duct, the net absorption of CGA was determined and accounted for 8%. Part of CGA was recovered in the gut effluent as caffeic acid showing the presence of trace esterase activity in the gut mucosa. No CGA was detected in either plasma or bile and only low amounts of phenolic acids were secreted in the bile. The present results show that CGA is absorbed with hydrolysis in the small intestine but does not explain the rapid appearance of CGA in aorta blood showed in the first experiment. Consequently, by infusing gastric buffer supplemented with CGA in the ligated stomach of food deprived rats during 30 min, intact CGA was found in the gastric vein and aorta. No other metabolites could be detected. These results show that CGA is quickly absorbed in the rat's stomach in its intact form, absorbed with hydrolysis in the small intestine and hydrolysed into caffeic acid by the microflora in the cecum. Moreover, in contrast to numerous flavonoids, absorbed CGA is poorly excreted in the bile or in the gut lumen.

INTRODUCTION

CGA, an ester of caffeic acid with quinic acid is found in a wide range of fruits and vegetables and is particularly abundant in coffee. Caffeic and chlorogenic acids have antioxidant properties illustrated by their ability to scavenge various free radicals when tested *in vitro* (Phytochemistry, 1994; Free Radical Biology and Medicine, 1995; Clinical Science, 1996; Free Rad. Biol. Med., 1996; IUBMB Life, 1999). *In vivo*, they increase the plasma antioxidant capacity, the concentrations of endogenous antioxidants such as vitamin E and the *ex vivo* resistance of lipoproteins to oxidation (J Agric Food Chem, 2002; J Agric Food Chem, 2002; Int J Vitamin and Nutrition Research, 2005). Moreover, they have been reported to prevent different cancers and cardiovascular diseases in several experimental studies on animal models (Cancer Lett, 1986; Basic Life Sci, 1990; Carcinogenesis, 1993; Journal of Clinical Biochemistry and Nutrition, 1993; Hypertension Research, 2002).

However, the biological properties of chlorogenic or caffeic acids depend on their absorption in the gut and on their metabolism. The absorption of caffeic acid in the small intestine has been well characterized in both experimental animals (*in vivo* and *in situ*) and humans studies (Arch Int Physiol Biochim, 1971; J. Pharm. Biomed. Anal., 1988; J Agric Food Chem, 2000; J. Nutr. 2001; J Agric Food Chem, 2001a), however, the bioavailability of CGA is more controversial. In some studies, CGA has been detected in urine with recoveries varying from

0.3 to 2.3 % suggesting absorption without structure modification (J. Nutr. 2001; J Agric Food Chem, 2001; Quimica Nova, 2004; Br J Nutr, 2005). Others authors failed to detect CGA in plasma in both rats and humans after ingestion as a pure compound or in coffee (J Agric Food Chem, 2002; J Agric Food Chem, 2000; Free Radic Biol Med, 1999). Caffeic acid and its *O*-methylated metabolites are commonly found in plasma and urine after CGA ingestion in rats and humans showing that CGA is hydrolysed in the body (J Agric Food Chem, 2002; J Agric Food Chem, 2000; Free Radic Res, 2001; Phytomedicine, 2005). Such a reaction could take place either in the gut mucosa or be catalysed by the gut microflora. No esterase activity able to hydrolyse CGA could be detected in human tissues (intestinal mucosa, liver) or biological fluids (plasma, gastric juice, duodenal fluid) in rats or humans (J Agric Food Chem, 2000; J. Nutr. 2001; J. Sci. Food Agric., 1999; J Agric Food Chem, 2001; Free Radic Biol Med, 2001). On the other hand, microflora in the large intestine possesses esterase activity towards CGA (J Appl Microbiol, 2001; Free Radic Biol Med, 2004). These results suggest that caffeic acid found in plasma originates from the hydrolysis of CGA in the colon. However, this would be inconsistent with a rapid detection (30 min after CGA administration) of caffeic and ferulic acids in the plasma of rats (J Agric Food Chem, 2000).

The aim of this work was to explore the stability of CGA in the gut, the form under which it is absorbed through the gut mucosa and the site of absorption along the gastrointestinal tract. Rats were fed a diet supplemented with CGA and phenolic acids were estimated in the stomach, small intestine and cecal contents at different time points along the meal as well as in bladder urine and plasma. Absorption of CGA through the small intestine and the stomach mucosa was also examined by using an *in situ* intestinal perfusion and an *in situ* gastric infusion models in food-deprived rats.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats, weighing approximately 180 g, were housed, one per cage, in temperature-controlled rooms (22 °C), with a dark period from 8 a.m. to 20 p.m. and with access to food from 8 a.m. to 16 p.m. They were fed a semipurified control diet for 7 days. Rats were maintained and handled according to the recommendations of the National Institute for Agricultural Research Ethics Committee, in accordance with Decree 87-848. Sixteen rats were then fed during three days the same diet supplemented with CGA (0.25% w/w). Diet intake was measured on the last day when each animal was killed and CGA consumption was calculated accordingly.

The third day of CGA supplementation, rats were anaesthetised with sodium pentobarbital 1.5 h, 3 h, 4.5 h and 7 h (n = 4 per sampling time) after the beginning of the meal. Stomach, small intestine and cecum contents, aorta blood and bladder urine were collected at each time. The gastric and intestinal contents were weighed separately and immediately frozen at –20 °C until analysis. Blood was drawn into heparinized tubes and centrifuged. Urines and plasma were acidified with acetic acid 10 mmol/L. All samples were stored at –20 °C until analysis. Fourteen other rats were deprived of food for 24 h before *in situ* intestinal perfusion (n= 8) and in situ gastric infusion (n = 6) experiment.

Rats were anaesthetized with sodium pentobarbital 24 h after the end of the last meal by intraperitoneal injection, maintained at 37 °C on a heat plate and kept alive during the perfusion or the infusion periods.

In situ gut perfusion

After cannulation of the biliary duct, a perfusion of a jejunal and ileal segment of intestine (from the flexura duodenojejununalis to 5 cm distal from the valvula ileocoecalis) was prepared by installing cannulas at each extremity. This segment was continuously perfused *in situ* with a physiologic buffer supplemented with 50 µmol/L CGA (n = 4). A flow rate of about 0.75 mL/min and a temperature of 37 °C were maintained during the 45 min of the perfusion (Am J Physiol, 1999; J Nutr, 2004; Br J Nutr, 2004; FEBS Lett, 2000; J Nutr, 2003). Average flow rate was determined for each experiment over the 45 min of perfusion. These quantities of CGA perfused would correspond to an intake for humans of about 500 mg of CGA. This amount of CGA corresponds to 3 or 4 cups of coffee (Cancer Causes and Prevention, 2001). CGA was quantified in the buffer at the end of the experiment and found to be stable in the buffer throughout the perfusion period. The effluent at the exit of ileum and the bile were collected throughout the 45 min of the perfusion period for analysis of phenolic acids. At the end of the experiment, blood samples were withdrawn from the mesenteric vein. Perfusate, bile and plasma samples were acidified with 10 mmol/L acetic acid and stored at –20 °C.

Determination of the intestinal and biliary fluxes

All the calculated fluxes were expressed in nmol/min. The fluxes in the effluent have been calculated by taking into account the intestinal absorption of water as previously described (Am J Physiol, 1999). For a given phenolic acid the net transfer in the enterocyte was evaluated by the difference between the perfused flux and the flux of perfused molecules recovered in the non hydrolysed effluent at the end of the perfusion. The secretion of conjugates back into the gut lumen was determined as the difference between the flux of molecules perfused measured in the non hydrolysed effluent and the flux of total phenolic acids in hydrolysed effluent. The net absorption was calculated as the difference between net transfer into the enterocyte and the intestinal secretion of metabolites (*O*-methylated conjugated forms). The biliary secretion of conjugated forms was determined as the product between the biliary flow rate (µL/min) and the concentrations of the phenolic acids measured in the bile after enzymatic hydrolysis (µmol/L).

In situ gastric infusion

After cannulation of the biliary duct, the pylorus was ligated and the stomach was filled *in situ* through the cardia with a 5 mL CGA solution buffered to mimic the osmotic and pH conditions found in the stomach during a meal. This buffer (pH 3) was maintained at 37 °C and contained 7 mmol/L of CGA. CGA was stable in buffer under the experimental conditions (data not shown). Thus, the stomach contained 35 µmol of CGA. The cardia sphincter was ligated to prevent any gastroesophageal reflux. The amount of CGA infused into the stomach was established in order to fit in the quantities found in the stomach during the CGA feeding experiment. Stomach content was collected 30 min later and blood was withdrawn from the gastric vein and abdominal aorta into heparinized tubes and centrifuged. Two more rats were used as control, infused with the same buffer without CGA, and blood treated in identical conditions. Plasma samples were acidified with 10 mmol/L acetic acid. All samples were stored at –20°C before analysis.

Analysis of phenolic acids

Treatment of gut content samples

Contents of stomach, small intestine and cecum collected from rats fed the CGA-supplemented diet were spiked with sinapic acid as internal standard. Nine volumes of methanol/H_2O/conc.HCl (70:28:2; v/v/v) containing sinapic acid was added to one volume of each content. The mixture was vortexed for 30 sec, sonicated at 0°C and centrifuged for 10 min at 4°C and 3000 × g. The supernatant was diluted with water (1:1, v:v), 1 mL of hexane was added and the mixture was vortexed. After centrifugation, the upper organic phase was removed and the lower was diluted 25 times in methanol/H_2O/conc.HCl (70:28:2 v/v/v) except for cecal samples which were directly analysed.

For the gastric infusion experiment, stomach contents were centrifuged, filtered on fritted glass and directly analysed. Phenolic acid concentrations in the stomach lumen were corrected for variations in volume due to gastric secretion. PEG, a compound not absorbed in the stomach, was added to the gastric buffer, and its concentration in the gastric buffer determined at the beginning and at the end of the experiment (Am J Physiol, 1968). The ratio between these two concentrations reflects the intensity of the gastric secretion.

Treatment of other samples

Perfusate, bile, plasma and urine samples were spiked at 5 µmol/L with sinapic acid or vanillic acid as internal standard. Before analysis urine samples were diluted with 0.5 mol/L sodium acetate containing 2 g/L ascorbic acid to reach pH 6.8. Perfusate, bile and plasma samples were adjusted to pH 6.8 with 0.1 volume of 0.5 mol/L sodium acetate containing 2 g/L ascorbic acid. Urines and plasma samples were incubated for 4 h at 37°C, perfusate and bile samples were incubated for 45 min at 37°C in absence or in the presence of $2x10^8$ units/L β-glucuronidase from *Escherichia coli*. This enzyme was selected rather than *Helix pomatia* enzyme preparation as we noticed that CGA was partially hydrolysed by this last enzyme mixture (Br J Nutr, 2005) but stable when treated by *E. coli* enzyme showing the absence of esterase able to hydrolyze CGA in this enzyme preparation. The presence of sulfatase activity in the *E. coli* enzyme preparation was checked, using a rat plasma sample containing known sulfate esters of quercetin (data not shown).

Phenolic acids, in treated samples, were extracted by adding 2.85 volumes of methanol/H2O/conc.HCl (70:28:2 v/v/v) and centrifuged for 4 min at 12,000 × g. The resulting supernatant was analysed by HPLC as described below.

Chromatographic conditions

Phenolic acids in gut contents, bile, plasma and urine were analysed by HPLC coupled to an 8-electrode CoulArray Model 5600 detector (Eurosep, Cergy, France). The system was fitted with a 5 µm C-18 Hypersil BDS analytical column (150 × 4.6 mm; Life Sciences International, Cergy, France). Mobile phase A was 5% acetonitrile in 30 mmol/L NaH_2PO_4 at pH 3, and mobile phase B, 50% acetonitrile in 30 mmol/L NaH_2PO_4 at pH 3. The separation was performed at 35 °C. The flow rate was 0.8 $mLmin^{-1}$.

Plasma samples, collected in the gastric vein and abdominal aorta after the gastric infusion experiment, were analysed by HPLC-electrospray ionization-tandem mass spectrometry with triple quadrupole MS detection (HPLC-ESI-MS/MS; API 2000, Applied Biosystem, Canada). Plasma were treated as above and directly injected (40 µL) into the LC-ESI-MS/MS system, fitted with a YMC-Pack ODS-AM column (250x3.0 mm I.D, 5 µm particle size, YMC

Europe GmbH, Schermbeck, Germany) and a YMC-Pack ODS-AM guard column (10x4 mm I.D, 5 μm particle size, YMC Europe GmbH, Schermbeck, Germany). The mobile phase consisted of water/acetonitrile/formic acid (94.5:4.5:1, v/v/v) (solvent A) and water/acetonitrile/formic acid (49.5:49.5:1, v/v/v) (solvent B). The eluent was delivered at a 200 μL min^{-1} flow rate.

Data analysis

Numerical values are mean +/- SEM. Data were analysed using XLStat ver.7.5 Addinsoft 2004. Significant differences were determined by one-way or by two-way ANOVA. When significant differences were detected we used Tukey's Multiple Comparisons Test to assess difference among the means. Differences with $p < 0.05$ were considered significant.

RESULTS

Nutrional intervention study

Stability of chlorogenic acid in the gastro-intestinal contents

In the stomach, similar quantities of CGA were found at the end of each time period. In the small intestine, CGA content increased significantly after 3 h. Content values at 4.5 and 7 h were not significantly different from that found at 3 h. Only traces of caffeic acid (1.0 +/- 0.1 % of total phenolic acids) could be detected in both the stomach and the small intestine. Much lower contents of CGA were found in the cecum. Maximal value was observed 4.5 h after the beginning of the meal. It was significantly higher than the contents found at the other time points (mean 0.13 μmol). Caffeic acid was also found in the cecum in significant amount, accounting respectively for 15, 32, 21 and 26 % of total phenolic acids measured at each time point respectively.

Plasma kinetics of chlorogenic acid and its metabolites

No CGA or other phenolic acids were detected in aortic plasma of control group. In supplemented group, both chlorogenic and caffeic acids were detected early (1.5 h) after the beginning of the meal in similar concentrations (Table 1). No significant variations in the concentrations were observed along the meal.

Table 1. Phenolic acid concentration in the plasma of rats fed a diet supplemented with chlorogenic acid.

Time after the beginning of the metal, h	1.5	3	4.5	7
Phenolic acids				
Ceffeic acid	0.31+/– 0.15	0.50+/– 0.21	0.19+/– 0.02	0.28+/– 0.04
Chlorogenic acid	0.33+/– 0.07	0.53+/– 0.21	0.39 +/– 0.11	0.50+/– 0.11

Values are means +/– SEM, n = 4. Ferulic and isoferulic acids were detected at 0.06 and 0.08 μmol/L respectively.

Concentrations of phenolic acids in urine taken directly into the bladder and repartition of excreted metabolites

No CGA or other phenolic acids were detected in urine of the control group. In supplemented group, CGA was detected in urine from 1.5 h after the beginning of the meal together with

caffeic, ferulic and isoferulic acids. CGA excretion tends to decrease over time in proportion to the amount ingested. Caffeic acid was also excreted early after the beginning of the meal; its relative excretion over other phenolic acids increased at 3 h as compared to 1.5 h ($p < 0.001$).

The early appearance of **CGA** in plasma and urine suggested an absorption in the upper part of the gastro-intestinal system.

In situ intestinal perfusion of chlorogenic acid in rats

When CGA was perfused at 40.4 +/– 0.8 nmol/min into the upper intestinal tract, both chlorogenic and caffeic acids were recovered in the effluent at the end of perfusion. Their respective fluxes in the non hydrolysed effluent were 36.7 +/– 1.1 nmol/minand 0.23 +/– 0.02 nmol/min. These phenolic acids fluxes correspond to 90.8 % and 0.6 % of the perfused flux of CGA respectively (Figure 1).

The net transfer of CGA into the entérocytes was 3.7 +/– 0.1 nmol/min corresponding to 9.2 % of the perfused flux. When the effluent was treated with deconjugating enzymes, the flux of CGA was not changed: 36.7 +/– 0.3 *vs* 36.7 +/– 1.1 nmol/min in non hydrolysed effluent, indicating the absence of intestinal secretion of conjugates of CGA. After enzymatic hydrolysis, the flux of caffeic acid significantly increased (0.48 +/– 0.02 nmol/min; $p < 0.05$ *vs* caffeic acid in non hydrolysed effluent) indicating the presence of caffeic acid conjugates released in the lumen. Free caffeic acid and conjugated derivatives found in the effluent were due to intestinal activity and their secretion in the lumen represented 1.2 % of perfused flux.

Thus, net absorption of CGA was 3.2 +/– 0.8 nmol/min and accounted for 8% of the perfused flux.

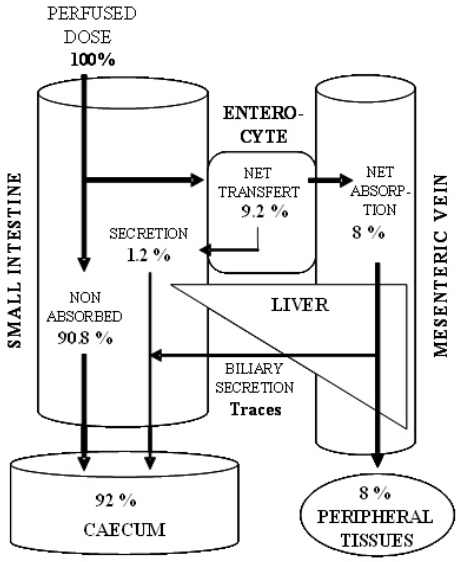

Figure 1. Fate of chlorogenic acid at the splanchnic level after *in situ* intestinal perfusion.

Analysis of the plasma samples collected after CGA perfusion showed the presence of isoferulic acid in mesenteric plasma (0.12 +/– 0.02 µM). In the bile, only caffeic acid was recovered in low concentration (3.0 +/– 1.3 pmol/min).

In situ gastric infusion of chlorogenic acid in rats

A significant proportion of CGA was absorbed as indicated by its disappearance in the stomach after a 30 min infusion (Table 2**)**. Only traces of caffeic acid were observed in the stomach content after incubation. Phenolic acids and their metabolites were looked for in the plasma collected from the gastric vein and aorta by LC-ESI-MS/MS after the 30 min infusion period. Two peaks with the same m/z transition as CGA were detected. They were absent in plasma when rats are infused with control buffer. One of the two peaks has a retention time identical to that of the commercial standard and is attributed to CGA (5-*O*-caffeoylquinic acid). This shows for the first time that CGA is absorbed in its intact form in the stomach. The second peak has not been identified and is more likely an isomer of CGA. No other metabolites could be detected in the plasma.

Table 2. Chlorogenic acid absorption and plasma a concentrations after its infusion into the stomach of rats during 30 min.

	Chlorogenic acid – μmol
Injected into the gastric lumen	35.0 +/– 1.0
Recovery from the gastric lumen at the end of infusion	29.3 +/– 0.7* % of injected dose
Absorption from the gastric lumen	16.3 +/– 1.9
	μmol/L
Gastric vain	3.3 +/– 1.3
Aorta	1.6 +/– 0.3

*Value are mean +/– SEM, n = 6. *Different from "Injected into the gastric lumen", p < 0.001.*

CONCLUSION

This study shows for the first time that CGA is not hydrolysed in the stomach and the small intestine, but absorbed in the stomach in its intact form and as hydrolysed forms such as caffeic and (iso)ferulic acids in the small intestine. Once reaching the cecum, CGA is hydrolysed into caffeic acid and further metabolised into other aromatic acids.

Different authors reported that caffeic acid was detected early in plasma, 30 to 60 min after consumption of pure CGA or coffee by rats or humans (J Agric Food Chem, 2002; J Agric Food Chem, 2000). They suggested that CGA was hydrolysed in the upper digestive tract as they could not detect any CGA in plasma. The absence of CGA would most likely be explained by a too rapid transit through the empty stomach in these two studies carried out with CGA solutions or brewed coffee in fasting rats or fasting subjects. In contrast, when volunteers consume coffee with a whole breakfast, CGA was present in urine (Br J Nutr, 2005). Thus, the role of the stomach in polyphenol absorption and the influence of the food matrix on absorption should be further investigated.

Moreover, further studies will be required to understand the exact mechanisms of absorption and identify the transporters involved in the different parts of the gastro-intestinal tract.

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Chlorogenic Acids from Coffee are Absorbed and Excreted in Human Digested Fluids

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SUMMARY

This study aimed at investigating human fluids as bioavailability and excretion markers of chlorogenic acids from coffee. Three caffeoylquinic acids and other non-esterified cinnamic acid derivatives were identified in human digestive fluids and plasma. In addition, three dicaffeoylquinic acids were identified in plasma. Our results show, for the first time, that chlorogenic acids from coffee are absorbed in humans, circulate in their blood stream and are partially excreted via saliva and gastro-intestinal fluids.

RESUMEN

Este estudio tuvo como objetivo investigar fluidos humanos como marcadores de biodisponibilidad y de excreción de los ácidos clorogenicos presentes en café. Tres ácidos cafeoilquinicos y otros derivados de ácidos cinámicos no esterificados fueron identificados en fluidos digestivos y en plasma. Además, tres ácidos dicafeoilquinicos fueron identificados en plasma. Nuestros resultados muestran, por primera vez, que los ácidos clorogenicos del café son absorbidos en humanos, circulan en su corriente sanguínea y son parcialmente excretados a través de la saliva y fluidos gastrointestinales.

INTRODUCTION

For years, coffee has been a target of scientific interest due to the presence of important bioactive compounds including chlorogenic acids (CGA), which are esters of *trans*-cinnamic acids (such as caffeic – CA, ferulic – FA and *p*-coumaric – CoA acids) with (–)-quinic acid (Figure 1) (Farah and Donangelo, 2006). In fact, coffee is considered to be one of the main dietary sources of CGA and, because of that, one of the main sources of antioxidants in a number of countries (Svilas et al., 2004; Vinson, 2005). 5-caffeoylquinic acid (5-CQA), the major CGA in coffee, is almost a ubiquitous compound in the plant kingdom, participating in the composition of most plant-derived foods (Farah and Donangelo, 2006).

Despite the promising research results on antioxidant and other biological properties of CGA, data on their absorption and metabolism in the human body are still scarce. Among the various CGA isomers present in coffee, only trace concentrations of 5-CQA have been identified in rat plasma and urine, even though urine has been considered to be a non-preferential excretion pathway for CGA, at least in rats (Choudhury et al., 1999). After 5-CQA consumption, trace amounts of 3-CQA, 4-CQA and 5-CQA were also identified in human urine (Olthoff et al., 2001). However, no intact CGA has been identified in human

fluids after coffee consumption, which has lead researchers to believe that in humans most CGA from coffee are hydrolyzed before or upon absorption.

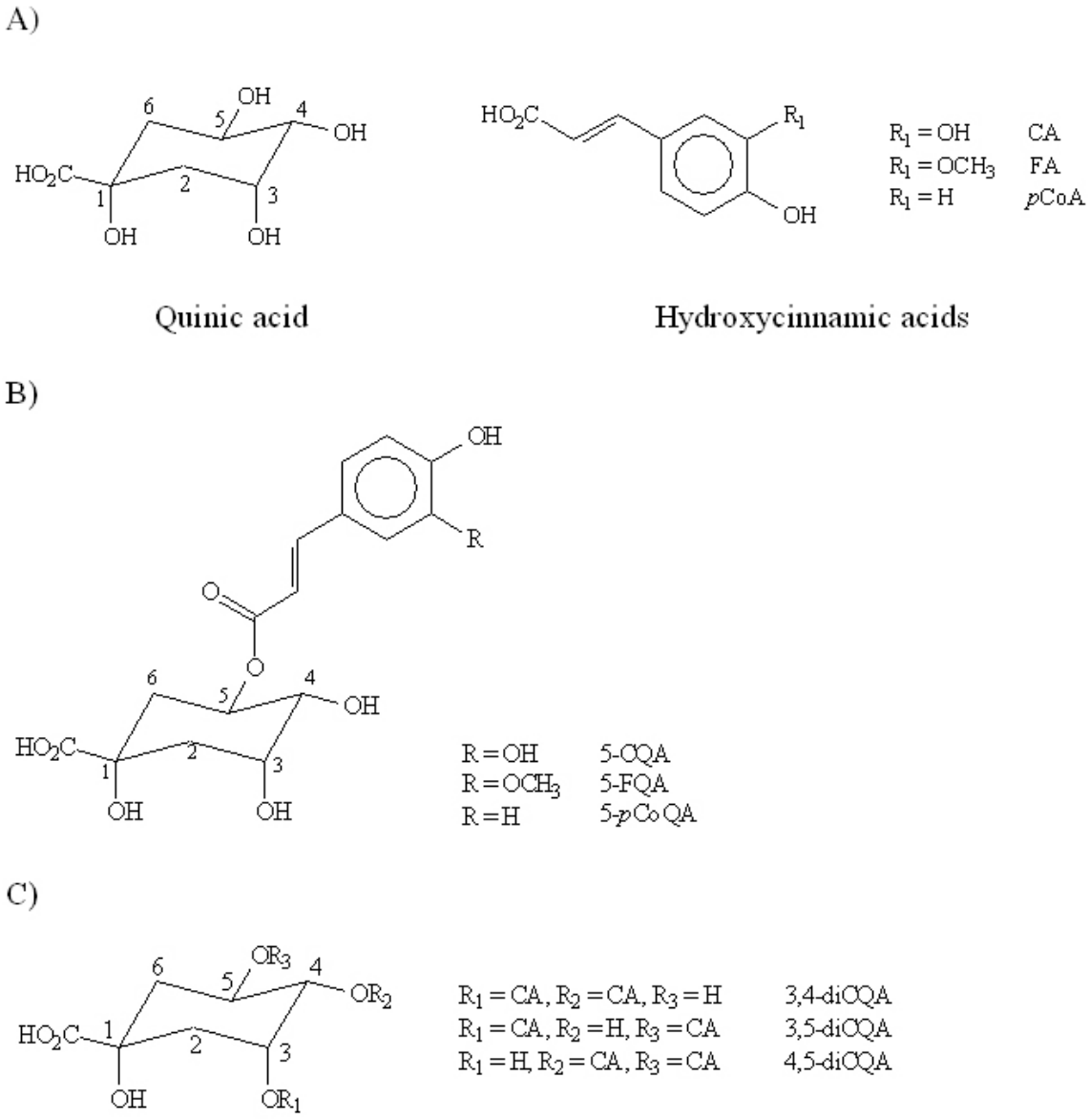


Figure 1. Chlorogenic acids and related compounds. (A) Chlorogenic acids immediate precursors; (B) monoesters of quinic acid with hydroxycinnamic acids (example of 5-isomers); (C) diesters of quinic acid with caffeic acid.

Considering that CGA metabolites as well as other phenolic compounds have previously been identified in bile (Das and Sothy, 1971), we investigated human gastrointestinal digestive fluids (GIF) and saliva as possible CGA bioavailability and excretion markers. As a significant fraction of the world population carries *Helicobacter pylori* (HP) in their stomach, we also compared fluids containing and not containing, this bacteria. Finally, we investigated the distribution of CGA major isomers in human plasma after coffee consumption.

MATERIAL AND METHODS

Eighty seven GIF samples were obtained from male and female healthy adults and elders (regular and non regular coffee drinkers), after at least 12 h fasting during upper endoscopy exams. GIF samples were divided into HP (–) and HP (+) and also into samples with pH < 6

(with high concentration of gastric fluid) and pH ≥ 6 (high concentration of pancreatic fluid and bile) (Figure 2)**.** Saliva samples were collected from male and female healthy adults (n = 15) directly into glass tubes after 12h fasting. Samples were clarified and analyzed by HPLC and LC-MS for phenolic compounds as previously described for coffee (Farah et al., 2005). Statistical analyses were performed by STATISTICA®, version 7.0 (USA) using LSD Test and Student t test for independent samples.

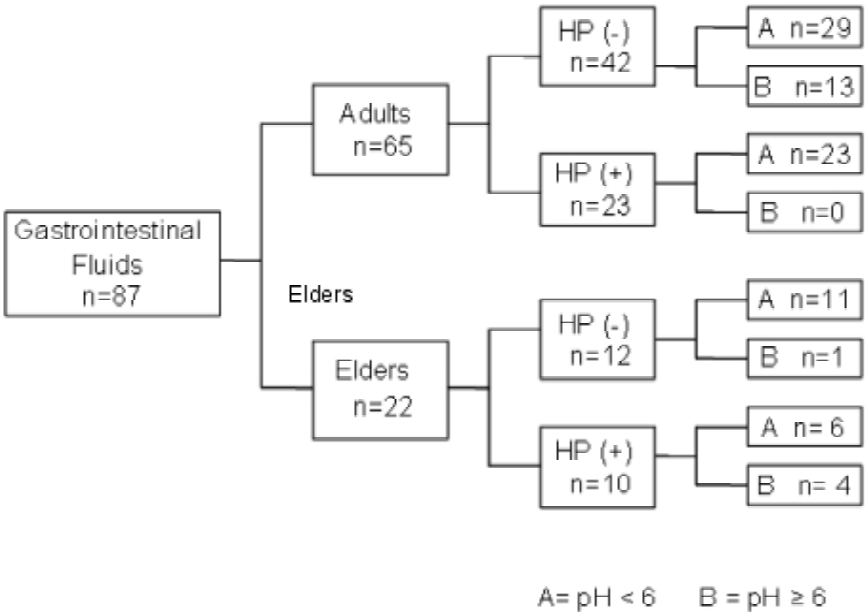
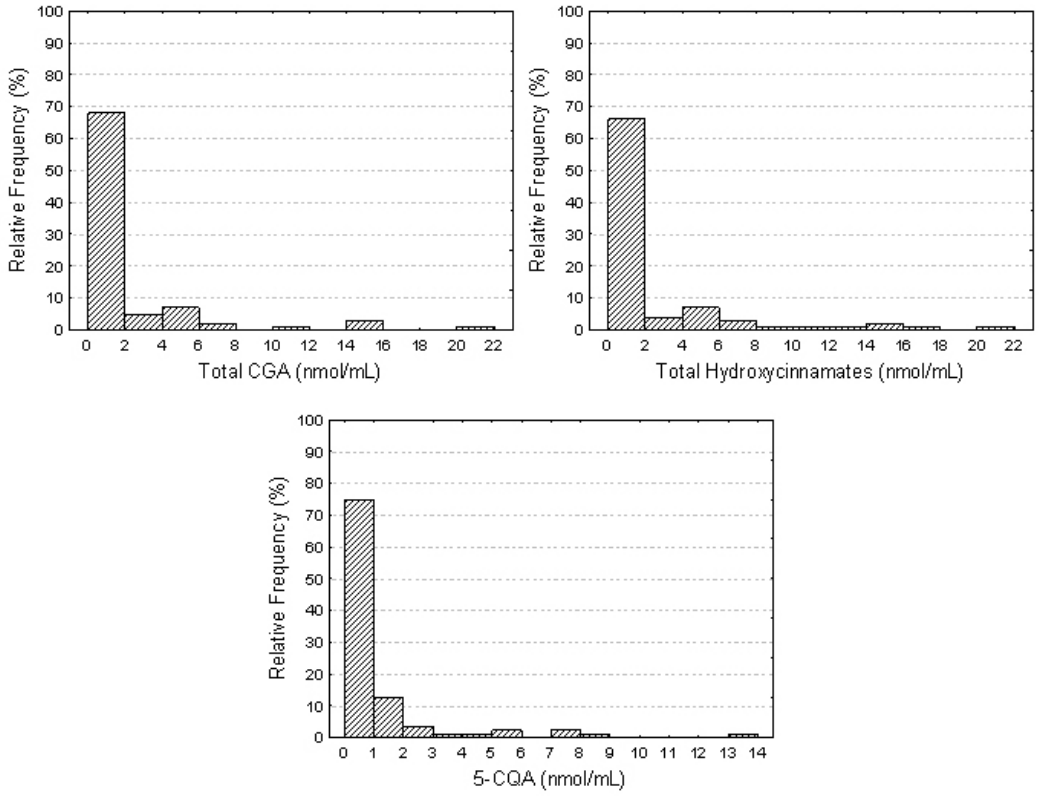


Figure 2. Characterization of human gastrointestinal fluids investigated in the present study.

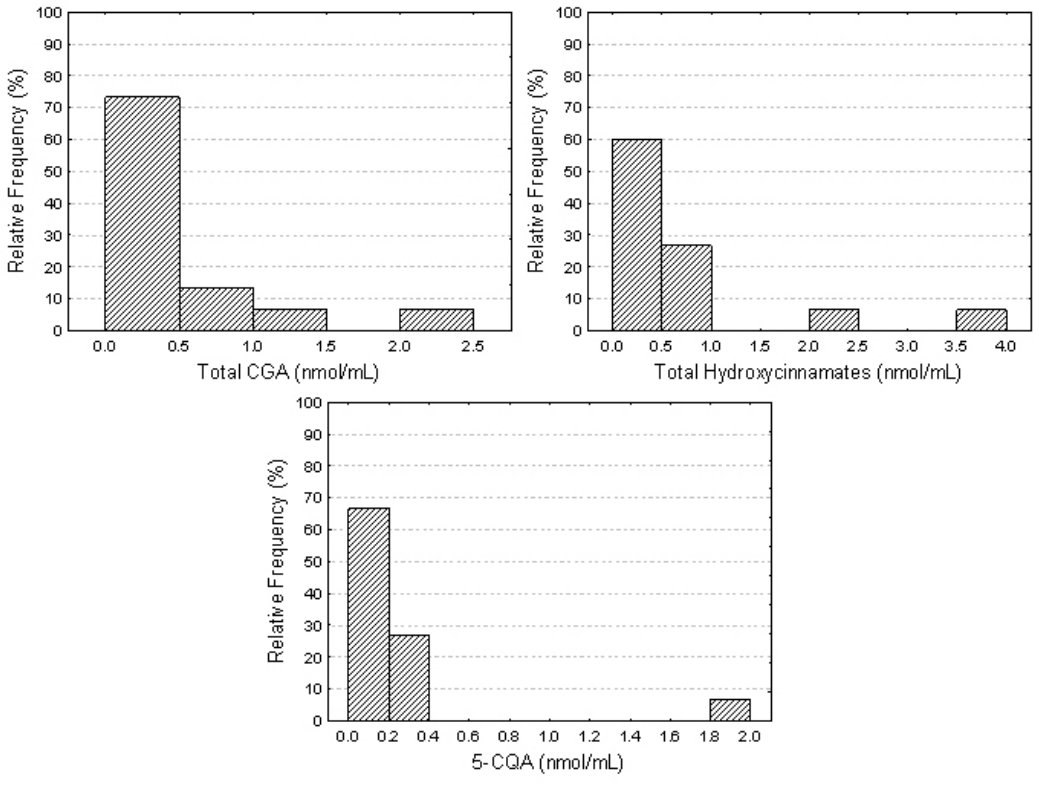
RESULTS AND DISCUSSION

HPLC results showed that the three main CGA isomers in coffee (5-CQA, 4-CQA and 3-CQA) as well as other cinnamic acids were present in both GIF and saliva. About 97% of GIF samples contained 5-CQA, 66% contained 3-CQA, 55% 4-CQA and 55% CA. Only 5% and 3% of the GIF samples contained FA and *p*-CoA, respectively. Similarly, 93% of the saliva samples contained 5-CQA; 87% contained 3-CQA; 53% 4-CQA; 7% FA and 27% *p*-CoA. CA was not identified in saliva samples.

There was a large inter-individual variation in the concentrations of CGA and non-esterified cinnamic acids measured in digestive fluids, probably due to the inter-individual variability in habitual CGA intake. Despite the inter-individual variability, most volunteers had up to 2.0 nmol/mL of total CGA and hydroxycinnamates in GIF and up to 0.5 nmol/mL of total CGA and hydroxycinnamates in saliva (Figure 3). The average concentrations of total CGA in GIF and saliva were, respectively, 2.21 ± 0.50 nmol/mL and 0.49 ± 0.24 nmol/mL, being 5-CQA responsible for about 50% of CGA in both GIF and saliva. The average concentration of total hydroxycinnamates (including CA, FA and p-CoA) was 2.22 ± 0.62 nmol/mL and 0.72 ± 0.39 nmol/mL, respectively.



a)



b)

Figure 3. a) Relative frequency of total chlorogenic acids (CGA), total hydroxycinnamates and 5-CQA concentrations in the gastrointestinal fluids of the 87 volunteers. b) Relative frequency of total chlorogenic acids (CGA), total hydroxycinnamates and 5-CQAconcentrations in the saliva of the 15 volunteers.

No significant differences were observed between individuals of different sex and age, HP carriers and non-carriers, and between gastric and enteric fluids. On the other hand, a significant difference was observed between the phenolic composition of saliva and GIF. While CQA and CA were preferentially excreted in GIF, saliva contained higher amounts of FA and *p*-CoA, which is reflected in the average concentrations of total hydroxycinnamic acids in GIF and saliva. Figure 4 presents the average distribution of chlorogenic acids and other hydroxycinnamates in gastrointestinal fluids and saliva of all volunteers – adults and elders, *Helicobacter pylori* carriers and non-carriers.

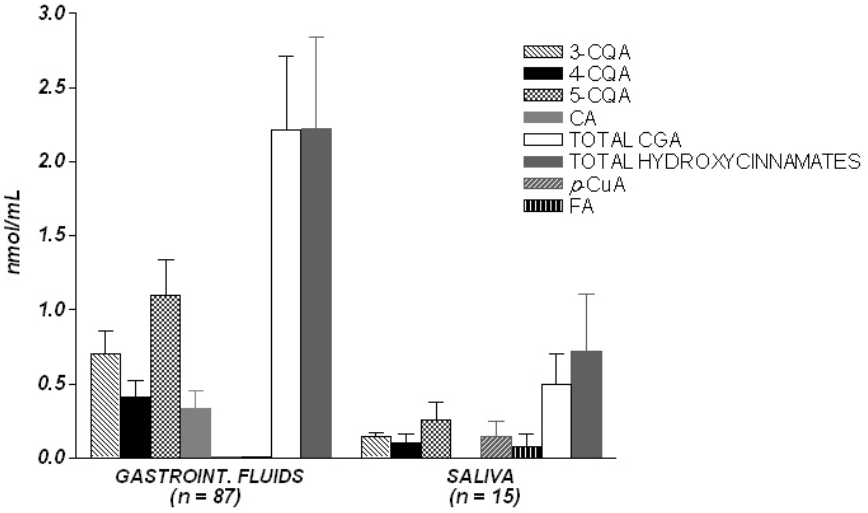


Figure 4. Average distribution of chlorogenic acids and other non-esterified hydroxycinnamic acids in gastrointestinal fluids and saliva of adults and elders (*Helicobacter pylori* carriers and non-carriers).

The presence of non-esterified hydroxycinnamates in human and/or rat plasma and urine has been previously reported (Bourne and Rice, 1998; Olthof et al., 2003; Nardini et al., 2002; Cremin et al., 2001). Considering that in the analytical tests 5-CQA recovery was very high, with no isomerization or hydrolysis, the identification of 3-CQA, 4-CQA and 5-CQA in the investigated digestive fluids indicates that not only 5-CQA but other CGA isomers circulate in the bloodstream, being excreted via digestive fluids. Since most hydroxycinnamic acids previously identified in plasma were conjugated with either glucuronic acid, sulfates or other compounds, investigation of conjugated forms of CGA in digestive fluids is under way.

In order to confirm the bioavailability of CGA isomers from coffee in humans, a coffee infusion containing 1.31 g of CGA (3.08 mmol CQA; 0.24 mmol FQA; 0.25 mmol diCQA) was orally administered to female and male adult volunteers (n = 6) after 12 h overnight fasting period. Plasma and urine samples were collected before and after coffee ingestion for 4 h. In addition to 3-CQA, 4-CQA and 5-CQA, three dicaffeoylquinic acids and CA were identified in the plasma of all volunteers, mostly in conjugated forms hydrolyzed by a pool of enzymes from *Helix pomatia* with β-glucuronidase and sulfatase activity. Also, two feruloylquinic acids were identified in one volunteer. Figure 5 shows the concentrations of total CGA, CQA, diCQA and CA in human plasma samples during four hours after coffee consumption.

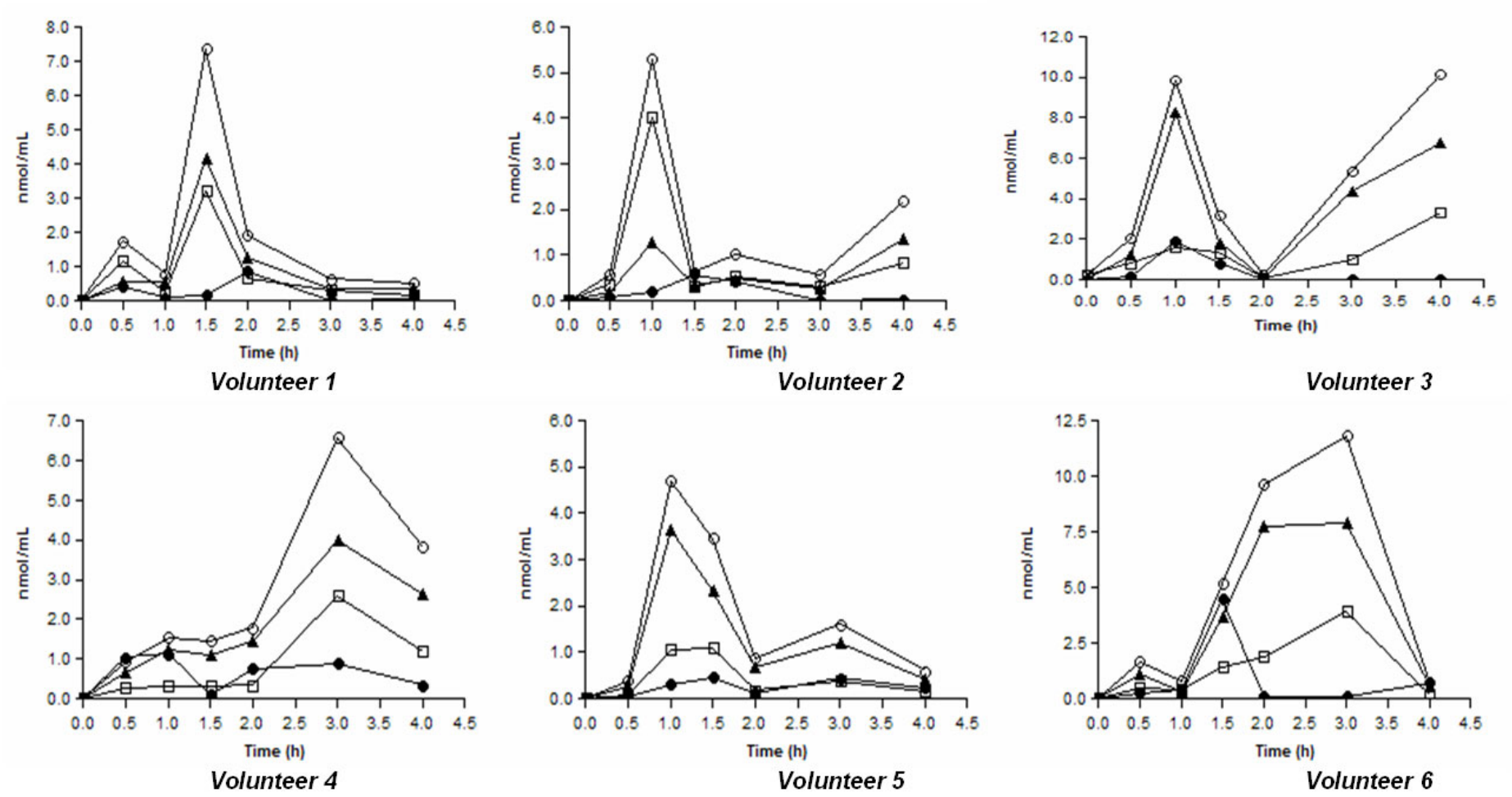


Figure 5. Concentrations of total CGA (—○—), CQA (—▲—) and diCQA (—□—) and CA (—●—) in human plasma samples after coffee consumption.

As for digestive fluids, a large inter-individual variation was observed in CGA concentrations and kinetics in plasma (Figure 5). Two absorption peaks were identified - the first and minor one 0.5-1 h after coffee consumption, suggesting that some absorption occurs in the stomach, and the second one 1.5-4 h after coffee consumption, suggesting absorption throughout the small intestine. Maximum plasma concentrations of CQA ranged from 3.6 to 7.9 nmol/mL, while maximum plasma concentrations of diCQA ranged from 1.1 to 5.0 nmol/mL. CQA plasma concentrations are in agreement with those observed in the investigated digestive fluids from different individuals.

The main CQA identified in plasma was 5-CQA and the ratios 5-CQA/3-CQA and 5-CQA/4-CQA were higher in plasma then in the coffee beverage consumed by the volunteers. Considering that 5-CQA recovery in our analytical tests was very high, 3-CQA and 4-CQA cannot derive from isomerization of 5-CQA, even if we consider some 5-CQA derived from partial hydrolysis of 3,5-diCQA and 4,5-diCQA.

The only CGA identified in the urine of all volunteers was 5-CQA, which is in accordance with previous results (Olthof et al., 2003). Other phenolic compounds - CA, dehydrocaffeic acid, FA acid, isoFA, gallic acid, vanillic acid, sinapic acid – were identified in the urine of all volunteers, being gallic and dehydrocaffeic acids the major urinary metabolites. *p*-CoA and *p*-hydroxybenzoic acid were also identified although not in the urine of all volunteers. These are compounds described in the literature as primary and secondary metabolites of 5-CQA. Some of them, such as 5-CQA, FA, isoFA were also observed in plasma before coffee consumption.

Our results indicate that besides 5-CQA, other chlorogenic acid isomers from coffee are bioavailable in humans.

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Bioassays to Evaluate the Physiological Functional Properties of Chlorogenic Acid and of an Instant Coffee Extract with High Levels of Chlorogenic Acids

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SUMMARY

Coffee is the major source of chlorogenic acid on human diet and *in vitro* studies show that this phenolic compound is a very specific inhibitor of glucose-6-phosphatase, a key enzyme of the two main hepatic metabolic ways that release glucose. Accordingly, for the first time, as much as we know, it was observed that an instant coffee extract with high levels of chlorogenic acids (37.8%), inhibited up to 50% glucose-6-phosphatase activity on microsomal fraction of hepatocytes. However, a lack of effect of chlorogenic acid on hepatic production of glucose from glycogenolysis and on glycogenolysis and glycolysis rates on liver perfusion was observed and could be explained by the fact that possibly this compound hasn't reached intracelular compatible levels with the inhibition of glucose-6-phosphatase. Intravenous or oral administration of the instant coffee extract also didn't imply in reduction of blood glucose levels, plasmatic concentration of total cholesterol and triacylglycerols. Chlorogenic acid, however, promoted a significant reduction (up to 21,76%) on 10 and 15 minutes of the glycemic peak after the oral glucose tolerance test, that characterizes it as a potential glycemic index reductor agent, possibly by attenuating the intestinal glucose absorption, highlighting it as a compound of interest to help in risk reduction of type 2 diabetes development.

INTRODUCTION

Chlorogenic acid is a natural phenolic compound and coffee is its major source on human diet (Clifford, 1999; Nardini et al., 2002; Olthof et al., 2001). On instant coffee commercially available, the level of total chlorogenic acids may vary from 0.7 to 5.9% (Nogueira and Trugo, 2003).

In vitro studies show that chlorogenic acid is a very specific inhibitor of glucose-6-phosphatase (G-6-Pase) (Arion et al., 1997; Hemmerle et al., 1997; Schindler et al., 1998), enzyme that catalyses the final reaction of glycogenolysis and gluconeogenisis, the two main hepatic metabolic ways that release glucose. It was also demonstrated that synthetic chlorogenic acid derivatives are effective not only in inhibit glucose-6-phosphatase in *in vitro* studies, but also in inhibit hepatic glucose output and reduce blood glucose levels (Hemmerle et al., 1997; Herling et al., 1999; 1998; Parker et al., 1998). However, there is only one known study were it was observed a reduction of blood glucose levels of fed type 1 diabetic rats, from 1 to 3 hours, after oral administration of chlorogenic acid (Andrade-Cetto and Wiedenfeld, 2001).

Once there is no available data on literature about the effects of chlorogenic acid on hepatic production of glucose, its effects on intestinal absorption of glucose are not conclusive and, as much as we know, there are no studies about the specific effects of instant coffee's chlorogenic acids on glucose-6-phosphatase activity, blood glucose levels and plasmatic total cholesterol and triacylglycerols concentrations, in the present study, these physiological events were analysed in the presence of either chlorogenic acid (5-caffeoylquinic acid – 5-CQA) or an instant brazilian coffee extract with high levels of chlorogenic acids (37.8%) and caffeine absence.

MATERIAL AND METHODS

Main Chemicals

Chlorogenic acid (5-cafeoylquinic acid, C16H18O9, MM 354.3) was purchased from Sigma Chemical Company and the instant brazilian coffee extract with high levels of chlorogenic acids (37.8%) and caffeine absence was produced as described by Bicchi et al. with modifications (1995) and was compounded by 19% of 5-CQA and 4-CQA, 16.2% of 3-CQA and 2.6% of 3-feruloylquinic acid (3-FQA), 20% of carbohydrates, 13.61% of proteins, 6.1% of moisture, absence of caffeine and absence or maximum levels of lipids similar to instant coffee (0.2%).

Animals

For the experiments, male Wistar rats (body weight 160-230 g), fed or submitted to 24 hours of alimentary privation, depending on the metabolic parameter to be investigated, were used.

Bioassays

Glucose-6-phosphatase activity

The effect of the instant coffee extract with high levels of chlorogenic acids on glucose-6-phosphatase activity was evaluated in rat liver intact microsomes obtained by differential centrifugation (Bracht and Ishii-Iwamoto, 2003).

Liver perfusion

The studies of the effect of chlorogenic acid 1 mM on hepatic metabolic parameters (glucose output from glycogenolysis, glycogenolysis and glycolysis rates) were performed in *in situ* liver perfusion (Kelmer-Bracht et al., 1984), conducted as follows:

Time interval (min)	0-10	10-40	40-50
Control group*	**KH****	**KH + Glucagon 1 nM**	**KH**
Experimental group*	**KH**	**KH + Glucagon 1 nM + Chlorogenic acid 1 mM**	**KH**

**Fed animal; ***KH= Krebs-Henseleit (perfusion fluid).*

Glycogenolysis was calculated as the sum of hepatic production of glucose + $^1/_2$ of the sum of hepatic production of L-lactate + pyruvate and glycolysis as the sum of hepatic production of L-lactate + pyruvate (Lopes et al., 1998). The differences between the area under the curves of both control and experimental groups were analysed by an appropriate statistical test.

Intravenous administration

For the analysis of the acute effects of chlorogenic acids naturally found in instant coffee on blood glucose levels and plasmatic concentrations of total cholesterol, phosphate buffer 5 mM pH 7.4 (control group) or instant coffee extract (70 mg/kg) dissolved in the same buffer (experimental group) were administrated intravenously (bolus injection) and blood samples were collected on 5; 15; 30 and 60 minutes after the administration for biochemical determinations.

Oral administration

For the analysis of the sub-chronic effects of chlorogenic acids naturally found in instant coffee on blood glucose levels and plasmatic concentrations of total cholesterol and triacylglycerols, water (control group) or instant coffee extract (100 mg/kg/day) (experimental group) were administrated orally by gavage during 15 days and at the end of this experimental period blood samples were collected for biochemical determinations.

Oral glucose tolerance test

To evaluate the effect of oral administration by gavage of chlorogenic acid on oral glucose tolerance, the animals received orally a pre-treatment with water (control group) or chlorogenic acid 3.5 mg/kg (experimental group). Then, the blood glucose base-line was determined (0 minutes) and it was administrated a 200 mg/kg glucose load on the lack (control group) or presence of chlorogenic acid 3.5 mg/kg (experimental group). Blood samples were collected from the tip of the tail in distinct moments (0; 5; 10; 15; 30; 45; 60 and 90 minutes) for glucose determinations. Once chlorogenic acid solution had an acid pH (2.8), an experiment to control the possible interference of the pH was also conducted by adding HCl to the glucose solutions in order to reach a pH similar to that found in the experiment with chlorogenic acid.

Statistical analysis

To analyse the results, accordingly to the necessity, the statistical tests one-way ANOVA followed by Tukey, unpaired Student's *t* test or Mann-Whitney test were employed. Results are expressed as mean ± standard deviation and the significance level adopted was 5% ($p < 0.05$).

RESULTS AND DISCUSSION

The instant coffee extract, which contains chlorogenic acids, inhibited up to 50% ($p < 0.05$) glucose-6-phosphatase activity on microsomal fraction of hepatocytes (Figure 1), where the enzyme is accessible, and chlorogenic acid does not have to get into the cell. As much as we know, this is the first evidence that chlorogenic acid's natural isomers from commercially available instant coffee showed the functional property of inhibit G-6-Pase.

However, a lack of effect of chlorogenic acid 1 mM on hepatic production of glucose from glycogenolysis and on glycogenolysis and glycolysis rates on liver perfusion was observed (Figure 2) and could be explained by the fact that possibly this compound has not reached intracellular compatible levels with the inhibition of G-6-Pase, once chlorogenic acid is a very specific inhibitor of G-6-Pase (Hemmerle et al., 1997; Schindler et al., 1998) and its synthetic derivatives inhibit glucose-6-phosphatase in *in vitro* studies and hepatic glucose output in liver perfusion studies and even reduce blood glucose levels (Hemmerle et al., 1997; Herling

et al., 1999; Herling et al., 1998; Parker et al., 1998; Herling et al., 2002; Van Dijk et al., 2001).

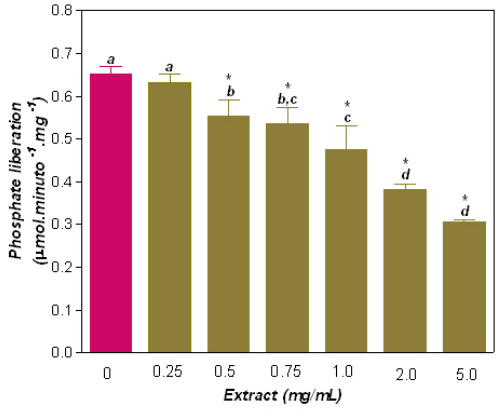

Figure 1. Effect of the instant coffee extract with high levels of chlorogenic acids on G-6-Pase activity. Livers from 24 h fasted rats were homogenized and submitted to differential centrifugation as described in Methods. The microsomal fraction was used to do the G-6-Pase activity assay in the lack (■) or presence (■) of different concentrations of the instant coffee extract with high levels of chlorogenic acids. Each bar represents the mean ± SD of 3 to 5 determinations. Results analyzed by ANOVA followed by Tukey. Distinct letters represent significant differences ($p < 0.05$). * $p < 0.05$ versus control.

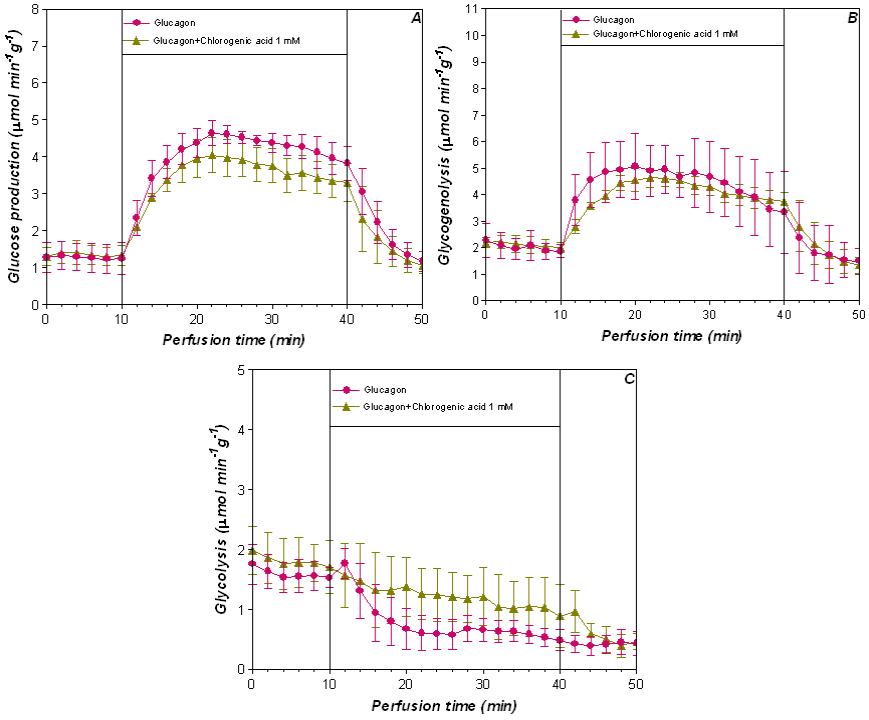

Figure 2. Hepatic production of glucose (A), glucogenolysis (B) and glucolysis (C) on the presence of glucagon. Livers from fed rats were submitted to *in situ* perfusion with Krebs-Henseleit buffer. Glucagon 1 nM (●) or Glucagon 1 nM + chlorogenic acid 1 mM (▲) were infused from 10 to 40 minutes. Samples to glucose, L-lactate and pyruvate determinations were collected each 2 min. Each point represents the mean ± SD of 3 to 5 experiments. Differences between the AUCs of control and experimental groups were analyzed by unpaired Student's *t* test.

Intravenous administration of the instant coffee extract also did not imply in reduction of blood glucose levels and plasmatic concentration of total cholesterol on 5, 15, 30 and 60 minutes after the administration (Table 1).

Table 1. Plasmatic concentrations of glucose and total chlolesterol after intravenous administration of the instant coffee extract to fed rats. The blood glucose levels (mg/dL) and total plasmatic cholesterol concentration (mg/dL) were evaluated on 5; 15; 30 and 60 minutes after the intravenous administration of the instant coffee extract with high levels of chlorogenic acids (70 mg/kg).

Groups	Blood glucose levels			
	5 min	15 min	30 min	60 min
Control	131.25±19.29 (7)	126.33±10.62 (9)	119.33±11.03 (9)	99.65±8.14 (9)
Instant coffee extract	137.03±10.39 (7)	122.02±11.30 (10)	115.51±8.50 (11)	106.92±10.58 (10)
Groups	Total cholesterol			
	5 min	15 min	30 min	60 min
Control	55.17±8.64 (8)	60.44±7.73 (10)	62.51±13.40 (11)	52.00±12.98 (9)
Instant coffee extract	47.99±5.00 (8)	68.92±19.62 (8)	59.26±13.14 (9)	52.29±11.15 (6)

Data reported as mean ± SD. Results, within fixed time, analyzed by unpaired Student's t test (A) or by the equivalent non-parametric test of Mann-Whitney, when necessary (B). ( ) Number of animals.

The oral administration, instead, promoted a slight increase in blood glucose (Table 2) and, once in another experiment of oral administration of chlorogenic acid with the same conditions (data not showed) it was not observed an increase in blood glucose levels, the result observed with the instant coffee extract could be explained by physiologic and metabolic alterations promoted by other extract's components that not chlorogenic acid.

Table 2. Plasmatic concentrations of glucose, total cholesterol and triacylglycerols and body weight gain after oral administration of the instant coffee extract to fed rats. The blood glucose levels (mg/dL), plasmatic concentration of total cholesterol (mg/dL) and triacylglycerols (mg/dL) and the rat's body weight (g) were evaluated after 15 days of oral administration of the instant coffee extract with high levels of chlorogenic acids (100 mg/kg/day).

Groups	Glucose	Total cholesterol	Triacylglycerols
Control	114.53±7.67 (9)	44.59±8.55 (10)	62.52±20.97 (8)
Instant coffee extract	128.91±13.67 (8) *	57.31±11.37 (8) *	70.74±14.74 (8)

Groups	Inicial weight	Final weight	Weight gain
Control	163.5±11.80 (10)	226±15.24 (10)	62.5 (10)
Instant coffee extract	160±13.54 (10)	235±20.41 (10)	75 (10)

*Data reported as mean ± SD. Results analyzed by unpaired Student's t test. * $p < 0.05$ versus control. ( ) Number of animals.*

The oral administration of the extract also promoted a slight increase in total cholesterol levels (Table 2), and this represents a contribution to literature, once the issue "effects of chlorogenic acid on cholesterol" is still controversial (Frank et al., 2003; Sotillo and Hadley, 2002) and needs to be elucidated.

The triacylglycerols levels and the weight gain of the animals were not changed by the oral administration of the instant coffee extract (Table 2).

Chlorogenic acid, however, provoked a significant reduction (up to 21.76%) ($p < 0.05$) on 10 and 15 minutes of the glycemic peak after the oral glucose tolerance test (Figure 3), possibly by attenuating the intestinal glucose absorption, and this effect should not be attributed to the pH of the solution.

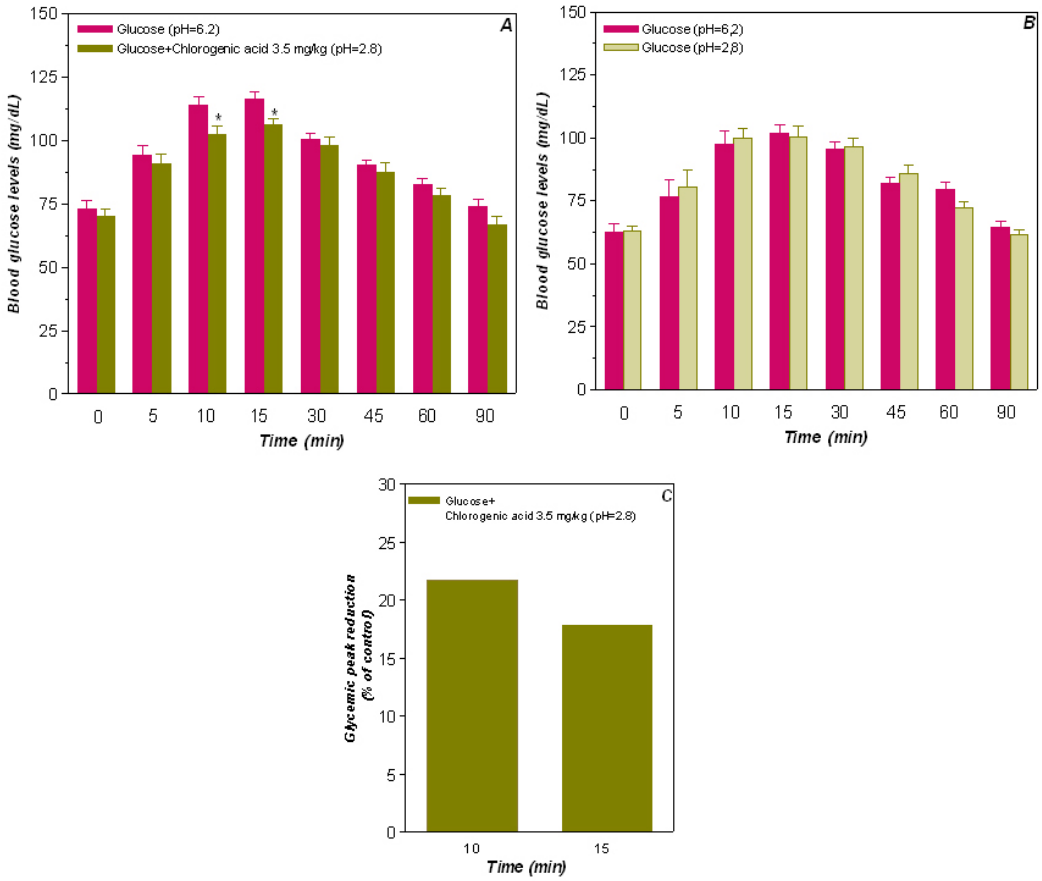

Figure 3. Effect of chlorogenic acid (A) and pH (B) on the oral glucose tolerance test and percentage of blood glucose levels reduction on 10 and 15 minutes of the glycemic peak (C). 24 h fasted rats were submitted to oral glucose tolerance test in the lack (■) or presence (■) of chlorogenic acid (A) or in the presence of glucose solutions with pH 6.2 (■) or 2.8 (■) (B). Blood glucose levels were evaluated on 0 (basal); 5; 10; 15; 30; 45; 60 and 90 minutes after the oral load of glucose. Each bar represents the mean ± SD of 6 to 10 experiments. Differences between control and experimental groups, within fixed time, were analyzed by unpaired Student's *t* test. * $p < 0.05$ versus control.

In fact, Welsch et al. (1989) described that chlorogenic acid reduced 80% of the glucose transport capacity in brush border membranes vesicles isolated from rat small intestine and it was also described that the consumption of glucose with decaffeinated coffee by fasted humans attenuated glucose absorption on small intestine and resulted in a tendency to reduce blood glucose levels (Johnston et al., 2003). There is even evidence that consumption of instant coffee promoted blood glucose levels reduction during oral glucose tolerance tests with humans [Mccarty, 2005a; 2005b; Van Dam et al., 2004; Yamaji et al., 2004)

In this sense, the results obtained in the present study characterizes chlorogenic acid as a potential glycemic index reductor agent, possibly by attenuating the intestinal glucose absorption, highlighting it as a compound of interest to help in risk reduction of type 2 diabetes development.

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High Molecular Weight Components from Coffee Beverages – Degradation by Human Intestinal Bacteria

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SUMMARY

Polysaccharides, proteins and melanoidins make up at least 30% of the dry matter of coffee beverages, but little is known about their physiological effects. In this study the fermentability of high molecular weight fractions obtained from filter coffee beverages (*C. arabica*, light, medium and dark roasted) by human intestinal bacteria was investigated. Within 24 h of fermentation polysaccharides were extensively degraded (75-90%) and short chain fatty acids were produced in molar ratios of about 6:3:1 for acetate, propionate and butyrate, respectively. Whereas galactomannans were fermented rapidly, arabinogalactans were degraded at a slower rate. The rate of arabinogalactan fermentation varied for fractions differing in the range of molecular weight. Structural changes of polysaccharides in the course of fermentation were analyzed by methylation analysis. A faster decrease in (1→3)-linked galactosyl residues than in (1→6)-linked galactosyl residues accompanied by debranching the galactan backbone was shown. (1→5)-Linked arabinosyl residues were the least degradable structural units of arabinogalactans. From a decrease in brown color during fermentation it is assumed that melanoidins were also degraded or modified by human intestinal bacteria.

INTRODUCTION

Coffee beverages contain remarkable amounts of high molecular weight substances, including polysaccharides, proteins and melanoidins. Polysaccharides and proteins make up about 30% of the dry matter. Whereas structural features of polysaccharides from coffee infusions have been investigated in several studies (Navarini et al., 1999; Nunes and Coimbra, 2002), detailed structures of coffee melanoidins are still unknown. However, they should not be neglected, because they contribute to the high molecular weight fraction of coffee infusions and may affect human health.

Little is known about physiological effects of high molecular weight components from coffee beverages. Melanoidins show antioxidant properties and possibly bind nutritionally important metals. Being part of the dietary fiber complex galactomannans and type II arabinogalactans present in coffee infusions may have an influence on human health. However, information about their physiological properties is rare, e.g. effects related to their fermentation by colonic microbiota have not been investigated yet. Apart from polysaccharides also coffee melanoidins may be utilized by human intestinal bacteria. Fermentation studies using model melanoidins have been carried out *in vitro* and *in vivo* (rat) (Ames et al., 1999; Dell´Aquila et al., 2003; Horikoshi et al., 1981; Jemmali, 1969; Wynne et al., 2001). Results indicate that melanoidins may be partially degraded by human colonic bacteria and affect faecal bacterial populations. Information about the fermentability of melanoidins from real food systems is

limited. Up to now only bread crust melanoidins were shown to be utilized by human gut bacteria *in vitro* (Borrelli and Fogliano, 2005).

The aim of this study was to get information about the fate of high molecular weight material from coffee infusions in the human colon. For this high molecular weight fractions were obtained from filter coffee beverages (*C. arabica*, light, medium and dark roasted) using ultrafiltration. The fractions were chemically characterized and subjected to fermentation with human faeces bacteria in batch culture. Bacterial growth was monitored and products from microbial fermentation, e.g. short chain fatty acids, were determined. The extent of polysaccharide degradation as well as structural changes were determined by detailed carbohydrate analysis. Furthermore changes in brown color, that would indicate microbial fermentation or modification of melanoidins, were analyzed.

MATERIALS AND METHODS

Preparation of coffee beverages

Ground Arabica coffee (Columbia; light, medium and dark roasted) was kindly provided by Tchibo (Hamburg, Germany). Filter coffee beverages were prepared with a commercially available coffee machine (50 g ground coffee/L water).

Fractionation of coffee beverages

After removal of material insoluble at room temperature, the filter coffee beverages were fractionated by sequential ultrafiltration (Vivaflow 50, Sartorius AG, Goettingen, Germany) with molecular weight cut-offs of 100, 50, 10 and 3 kDa. The obtained fractions >100 kDa, 50-100 kDa, 10-50 kDa und 3-10 kDa were freeze-dried and analyzed as described below.

Batch culture fermentation

Human faecal samples were washed and bacteria were subsequently separated by centrifugation (10000 g, 6 min). The obtained pellet was used for the fermentation of high molecular weight fractions (1.8 mg/mL), which was carried out for 24 h in a minimal medium under anaerobic conditions. Controls included glucose as substrate and blanks without substrate or without bacteria. Aliquots were taken after 0, 6, 12 and 24 h of fermentation and analyzed as described below.

Determination of optical density

Aliquots of each batch culture sample were centrifuged (14000 g, 5 min). The pellet was resuspended in a defined volume of phosphate buffered saline and applied to optical density measurement at 600 nm (Beckman DU-640 Spectrophotometer, Beckman, Krefeld, Germany).

Analysis of short chain fatty acids (SCFA)

SCFA were determined by GC-FID (HP 5890 Series II GC, Hewlett-Packard, Waldbronn, Germany) using a HP-FFAP capillary column (30 m length, 0.53 mm i.d., and 1.0 µm film thickness). Isobutyric acid was used as internal standard.

Determination of total carbohydrates

The total carbohydrate content was determined using the phenol-sulfuric acid method (Dubois et al., 1956). Calibration data were obtained on the basis of mannose and arabinose (working range 10-150 µg/mL). The total sugar content was calculated as sum of anhydrosugars.

Analysis of monosaccharides

Neutral monosaccharides were released by Saeman hydrolysis, modified by Englyst et al. (Englyst et al., 1994) and analyzed as their alditol acetates by GC-FID (Blakeney et al., 1983) (GC Focus Series, Thermo Electron S.p.A., Milan, Italy) using a DB-5 capillary column (30 m length, 0.32 mm i.d., and 0.25 µm film thickness).

Methylation analysis

Methylation analysis was carried out as described by Nunes and Coimbra (2001) with minor modifications. Partially methylated alditol acetates were characterized by GC-MS (HP 5890 Series II GC, HP 5972 Series Mass Selective Detector, Hewlett-Packard, Waldbronn, Germany) using a DB-5MS capillary column (30 m length, 0.25 mm i.d., and 0.25 µm film thickness). Quantitation of partially methylated alditol acetates was accomplished by GC-FID (GC Focus Series, Thermo Electron S.p.A., Milan, Italy) using a DB-5 capillary column (30 m length, 0.32 mm i.d., and 0.25 µm film thickness). Molar response factors of Sweet et al. (1975) were used for quantitation.

RESULTS AND DISCUSSION

Starting material

500 mL of filter coffee beverages prepared from light, medium and dark roasted Arabica coffee contained about 2 g of high molecular weight material (soluble at room temperature). The major part obtained by ultrafiltration was present in the fractions 3-10 kDa and >100 kDa.

Polysaccharides were major components of the high molecular weight material. The total carbohydrate content was comparable for the fractions from coffees with different roasting degrees (Table 1). The fractions 3-10 kDa were all characterized by a lower content of carbohydrates than fractions >10 kDa. The polysaccharides were mainly composed of mannose, galactose and arabinose, which are the main constituents of galactomannans and arabinogalactans. Their molar proportions vary to some degree for the different high molecular weight fractions and also depend on the degree of roast. Compared to fractions from medium and dark roasted coffee, fractions obtained from light roasted coffee are characterized by higher proportions of arabinose and galactose, which is in accordance with the results obtained by Nunes and Coimbra (2002).

For all three coffees, the fractions 10-50 kDa showed the highest relative amounts of mannose and as a consequence higher relative amounts of galactomannans. However, for the dark roasted coffee, the four fractions >3 kDa were very similar in their monosaccharide composition.

Table 1. Total sugar content, monosaccharide composition and degradation of total sugar after 24 h of fermentation.

coffee	fraction	total sugar[a] [g/100g]	monosaccharide composition [mol%]					total sugar degradation [%]
			Rha	Ara	Man	Glc	Gal	
light roasted	>100 kDa	49.6	4.8	21.5	15.8	1.4	56.6	81
	50-100 kDa	40.6	3.3	17.8	19.6	1.7	57.6	85
	10-50 kDa	41.8	2.3	13.8	37.8	1.3	45.0	80
	3-10 kDa	28.6	2.8	21.1	27.2	2.5	46.5	80
medium roasted	>100 kDa	53.7	4.5	15.9	26.7	3.9	49.0	80
	50-100 kDa	50.0	2.3	11.1	44.2	3.0	39.3	82
	10-50 kDa	56.7	2.1	8.4	57.2	2.2	30.1	90
	3-10 kDa	33.2	3.6	18.5	26.9	4.0	47.1	77
dark roasted	>100 kDa	47.0	3.0	14.2	39.8	0.8	42.2	75
	50-100 kDa	47.0	1.4	14.0	38.4	0.7	45.4	81
	10-50 kDa	42.4	1.6	12.3	46.6	0.8	38.8	82
	3-10 kDa	30.2	2.5	15.6	34.7	2.0	45.2	80

[a] *anhydrosugar.*

Fermentation experiments

Distinct increases in optical density, caused by bacterial growth, indicated that all high molecular weight fractions obtained from light, medium and dark roasted coffee beverages were utilized as a sole carbon source by human faecal bacteria. Polysaccharides, are probably the preferred substrates of colonic bacteria. After 24 h of fermentation a substantial decrease (75-90%) in total carbohydrate content was observed for all fractions indicating that polysaccharides were extensively degraded (Table 1). Presumably, the presence of melanoidins or structural units deriving from the Maillard reaction did not affect the fermentability of polysaccharides.

As a result of carbohydrate utilization SCFA were produced by intestinal bacteria. The fermentation of all subjected fractions led to the production of acetate, propionate and butyrate in molar ratios of about 6:3:1. The degree of roast did not significantly affect molar ratios of produced SCFA. Compared to dietary fiber from most other sources, the fermentation of polysaccharides from coffee beverages led to higher relative amounts of propionate. This is in accordance with results obtained by *in vitro* fermentation studies using galactomannans (from guar gum, tara gum, locust bean gum) or arabinogalactans (from larch) (Amado, 2005; Englyst et al., 1987). It is generally accepted that SCFA play an important role in maintaining human gut health (Plaami, 1997). Butyrate is preferred as a luminal nutrient by the colonic mucosa, whereas propionate is only partially metabolized by colonocytes and preferably transported to the liver. The production of SCFA is associated with a decrease of colonic pH thus reducing the conversion of primary to secondary bile acids that are regarded as cytotoxic. Lower pH-values also impede growth of pathogenic bacteria whereas growth of bifidobacteria and other lactic acid bacteria is supported.

After 24 h of fermentation the monosaccharide composition of residual carbohydrates changed, indicating that galactomannans and arabinogalactans were degraded to a different extent and/or at a different rate (Figure 1). The molar proportion of mannose decreased in all fractions, whereas the relative amount of arabinose increased. Taking into account the low total sugar content after 24 h of fermentation it can be concluded that mannans were almost completely degraded. Arabinogalactans, however, were degraded to a lesser extent.

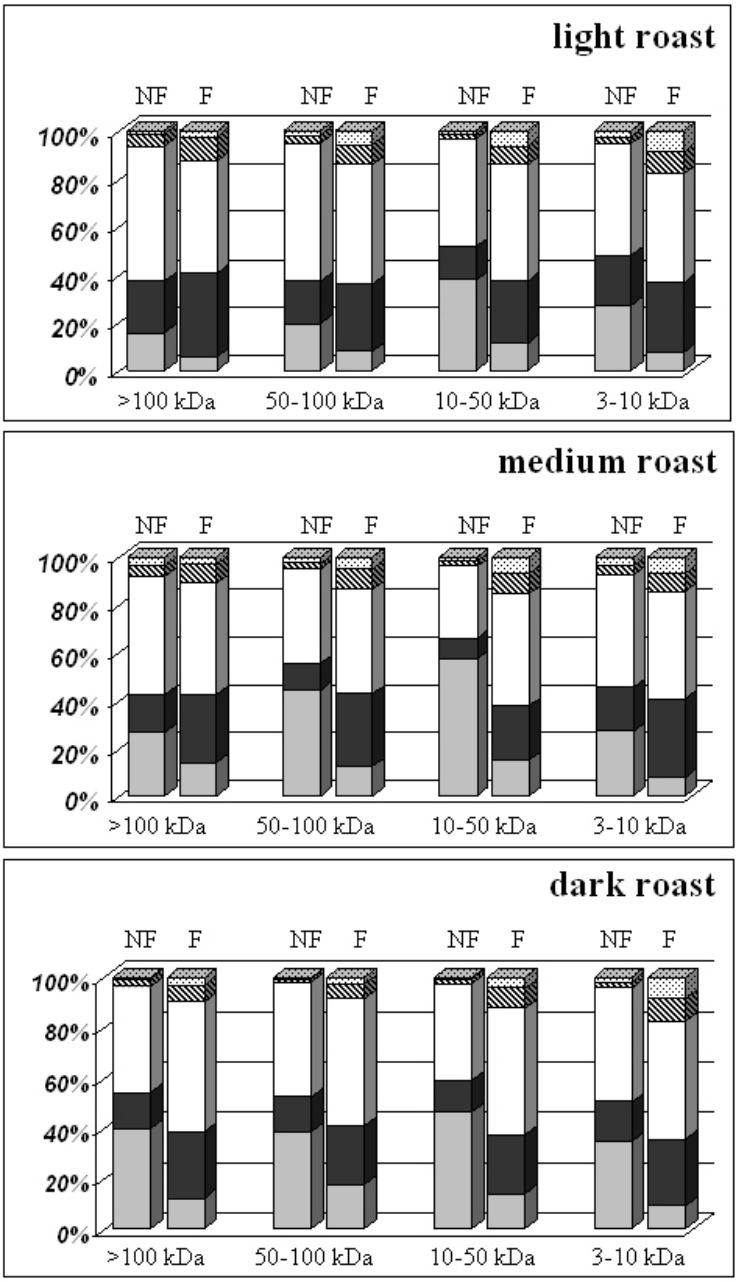

Figure 1. Monosaccharide composition of polysaccharides present in non-fermented (NF) and fermented (F) coffee fractions.

In order to investigate the rate of degradation of individual monosaccharides the residual material was analyzed after 0, 3, 6, 12 and 24 h (fractions from medium roasted coffee). Mannose was shown to be degraded rapidly between 6 and 12 h of fermentation, whereas the degradation of arabinose and galactose was decelerated. Probably due to their highly branched nature arabinogalactans are not as rapidly degraded as mannans. For mannans, the rate of degradation did not depend on the molecular weight fraction, whereas arabinogalactans of different molecular weights where fermented at varying rates. This might be due to a varying complexity of arabinogalactans present in coffee beverages and hence in fractions of different molecular weights. Although an influence of Maillard reaction products on the polysaccharide fermentation is not likely, we cannot fully rule out that they affect the fermentation process of arabinogalactans. However, for a better estimation of the effects of Maillard reaction products on the fermentation of polysaccharides it is necessary to learn more about possible linkages between polysaccharides and products deriving from Maillard reaction.

Methylation analysis gave more detailed information about arabinogalactan degradation and time dependent structural changes. A decrease in the ratio branched/non-branched galactosyl residues with increasing fermentation time allowed the conclusion that side chains were removed from the galactan backbone. It was also shown that the relative amounts of (1→3)-linked galactosyl residues decreased faster than these of (1→6)-linked galactosyl residues. A decrease in the ratio terminally/(1→5)-linked arabinosyl residues might indicate that arabinosyl residues terminating side chains composed of galactosyl residues are better degradable than those terminating arabinan side chains. The least degradable structural units of arabinogalactans were made of (1→5)-linked arabinosyl residues.

For all fractions a slight decrease in brown color (determined as absorbance at 405 nm) was observed during fermentation. After 24 h of fermentation the absorbance at 405 nm decreased about 20% compared to blanks (coffee fraction in culture medium without bacteria). Although polysaccharides probably are the preferred substrates for colonic microbiota, it seems possible that also melanoidins were partially degraded or modified. In order to investigate the fate of melanoidins in the human colon, it is necessary to get a better insight into their structural properties. This requires the development of suitable separation procedures. Due to the complexity of these high molecular weight components a lot of research has to be done in this area.

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Evaluation of the Nutritive Value of Soluble Coffee

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SUMMARY

The European Union is considering to making nutritive labeling mandatory for all food, including coffee. Food legislation has defined methodologies to evaluate nutritive values, which are not adapted to roasted products such as coffee. The evaluation of the energy value and dietary fibers were revisited for soluble coffee. The energy value of soluble coffee should be determined from its detailed composition. Applying this approach, soluble coffee provides 67 kCal/100 g powder compared to the 350 kCal/100 g established with the official method. This difference is due to a systematic over-evaluation of the protein and carbohydrate contents in soluble coffee with the official method. The nutritive value is fairly independent of the blend characteristics but increases for soluble coffee obtained from severe extraction processes (e.g. 98 kCal/100 g). The contribution of soluble coffee to the energy balance of a diet is minute, i.e. ~1 kCal/cup brought by 0.1 g of proteins and < 0.2 g of carbohydrates. However, soluble coffee is an important source of soluble dietary fibers (mannans, arabinogalactans), i.e. ~30%, which correspond to 0.6 g/cup of fibers. The possible contribution of melanoidins to dietary fibers needs further clarification.

RÉSUMÉ

La déclaration nutritionnelle pourrait devenir obligatoire dans l'union européenne pour tous les aliments, y compris le café. Les méthodes officielles d'évaluation de la valeur nutritionnelle ne sont cependant pas adaptées aux produits torréfiés comme le café. La méthode d'évaluation de la valeur énergétique et du contenu en fibres du café soluble est discutée dans cette étude. La valeur énergétique du café soluble devrait être déterminée à partir de sa composition détaillée. Ainsi le café soluble apporte 67 kCal/100 g de poudre contre 350 kCal/100 g selon la méthode officielle. La différence est le résultat d'une surévaluation systématique des teneurs en protéines et glucides du café soluble par la méthode officielle. La valeur nutritionnelle dépend peu des caractéristiques du blend, mais augmente dans les cafés solubles fabriqués dans des conditions d'extraction sévères, 98 kCal/100 g. Une tasse de café soluble contribue de façon marginale à la balance énergétique ~1 kCal/tasse et apporte 0.1 g de protéines et moins de 0.2 g de glucides. Le café soluble contient ~30% de fibres solubles (arabinogalactanes, mannanes) ce qui représente 0.6g de fibres par tasse. La contribution des mélanoidines aux fibres alimentaires mériterait d'être étudiée plus précisément.

BACKGROUND

The European Union is considering making the nutritive labeling mandatory for all food, including coffee. Methodologies to evaluate nutritive values described by EU directives (Directive E 90/496/CEE 24.09.1990) are based on the global evaluation of water, ash, protein, fat, carbohydrates, organic acids and dietary fibres. Although this approach may be appropriate in most cases, such crude evaluation leads to systematic over-evaluation of the nutritive value when dealing with thermally treated products such as roasted coffee and

soluble coffee. The purpose of this work was to assess the detailed chemical composition of soluble coffees and propose a more adapted way for calculating its nutritive value.

EXPERIMENTAL

Green & Soluble Coffees

- Three green coffees blends (i.e. pure *Arabica*, pure *Robusta*, 50/50 *Arabica/Robusta*) were prepared from commercial grade coffees. Each green coffee blend was roasted in an industrial roaster at four different roast levels (i.e. CTN 110, 92, 73, 55CTN). From each coffee roast, a soluble coffee was prepared by applying identical industrial extraction conditions. Twelve soluble coffees covering the range of commercially available soluble coffees were thus obtained.
- A commercial soluble coffee prepared from a medium roasted mixed blend (i.e. 65% *Arabica*, 85CTN) was used for the dietary fibre evaluation.

Analytical Procedures

- The green coffee blends were analysed as previously described (Leloup et al., 2004). An extensive characterisation of the soluble coffees was performed as described in Figure 1.
- The nutritive values in kilojoules or kilocalories were calculated from the amounts of the energy-supplying constituents, i.e. protein, fat and available carbohydrates as defined above, multiplied by the corresponding factors and subsequent summation. The factors are those from EU directive on the nutritive values of food ingredients:

Fat	37kJ/g	or	9kCal/g
Protein/available carbohydrates	17kJ/g	or	4kCal/g
Available organic acid	13kJ/g	or	3kCal/g

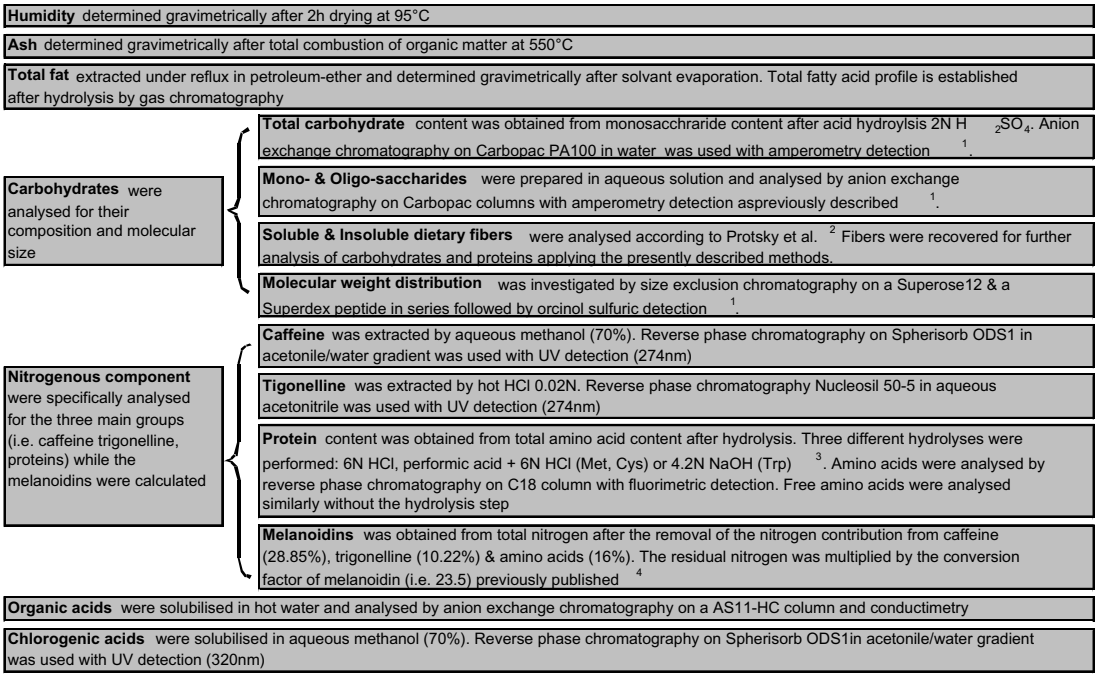

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Figure 1 . Analytical approach for the detailed characterization of soluble coffee.

RESULTS & DISCUSSION

Coffee Processing and Impact on Coffee Chemistry

Green coffee is composed of carbohydrates (~50%), proteins (~12%), fat (9-15%) and ash (~4%) Additionally green coffee also contains significant amounts of other nitrogen components (~2-3%) (i.e. caffeine, trigonelline) and organic acids (~10-12%) such as chlorogenic acids (Table 1).

However, green coffee is not consumed as such. With a view of producing a flavourful and soluble beverage in coffee manufacturing, green coffee is basically submitted to two successive thermal treatments.

- ***Roasting*** consists in heating up coffee beans at high temperature to initiate complex chemical and physical changes. Chemically, roasting induces the degradation of proteins and polysaccharides into more reactive compounds (i.e. carbonyl, amino group), which further participate to complex Maillard-type reaction pathways. Melanoidins are one of the end products of the Maillard reaction and are generally described as high molecular weight, nitrogenous, coloured compounds (Figure 2).
- ***Extraction*** consists in submitting the roasted coffee to successive water extraction steps in percolators at temperatures ranging approximately between 100-180 °C. Chemically, the water extraction results in a selective solubilization of coffee solids. Depolymerization and degradation mechanisms may occur during the extraction at high temperature resulting in the generation of low molecular weight compounds (e.g. oligosaccharides).

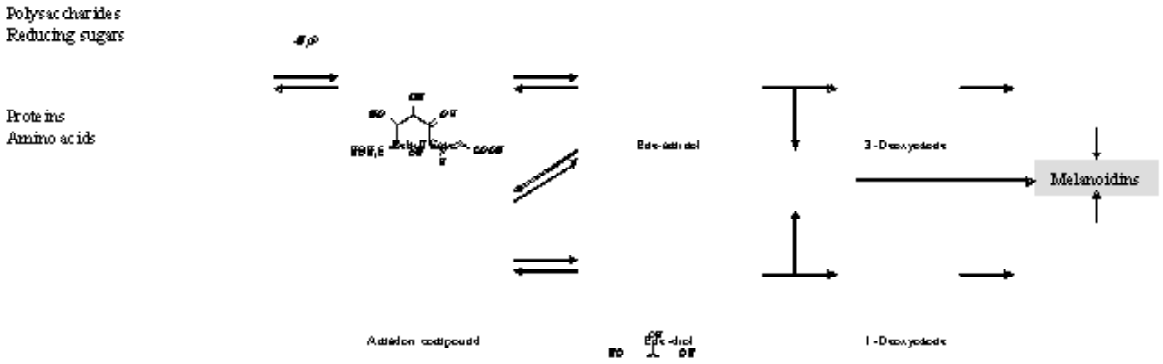


Figure 2. Maillard reaction pathways.

Therefore, in order to determine a meaningful value of the nutritive value of soluble coffee, one should determine both quantitatively and qualitatively the solids actually extracted during the process. The extensive evaluation of the twelve soluble coffees is presented in Table 1 and compared to those of the initial green coffees. The chemical entities (e.g. proteins, melanoidins) and their relative ratio in soluble coffee highly differ from that found in green coffee. The next paragraphs will focus at evaluating (1) the energy value and (2) the dietary fibre content of soluble coffee.

Table 1. Chemical composition of green coffee bean blends and soluble coffees obtained by industrial manufacturing (roasting, extraction, drying).

		Blend pure Arabica					Blend 50/50 Arabica/Robusta					Blend pure Robusta					Mean	stdev
Arabica	%	100	100	100	100	100	50	50	50	50	50	0	0	0	0	0	-	-
Roast colour	CTN	Green	110	92	73	55	Green	110	92	73	55	Green	110	92	73	55	-	-
H2O	**%**	**11.10**	**4.25**	**4.26**	**3.10**	**3.56**	**11.07**	**3.76**	**3.98**	**3.62**	**3.83**	**11.37**	**3.60**	**3.91**	**2.07**	**3.14**	**3.59**	**0.60**
Ash	**% d.b.**	**3.86**	**8.06**	**7.91**	**7.99**	**7.77**	**4.40**	**7.82**	**8.01**	**7.77**	**7.89**	**4.36**	**7.37**	**7.21**	**7.43**	**7.67**	**7.74**	**0.27**
Lipids	**% d.b.**	**15.21**	**0.13**	**0.17**	**0.11**	**0.18**	**12.22**	**0.15**	**0.09**	**0.15**	**0.26**	**9.44**	**0.16**	**0.18**	**0.26**	**0.27**	**0.18**	**0.06**
Saturated fat	% d.b.	n.d.	0.05	0.06	0.04	0.06	n.d.	0.05	0.03	0.05	0.09	n.d.	0.06	0.07	0.09	0.10	0.06	0.02
Total Nitrogen	**% d.b.**	**2.32**	**2.94**	**2.89**	**2.84**	**2.90**	**2.58**	**3.18**	**3.21**	**3.15**	**3.27**	**2.78**	**3.44**	**3.37**	**3.32**	**3.43**	**3.16**	**0.22**
Caffeine	**% d.b.**	**1.26**	**2.51**	**2.51**	**2.48**	**2.40**	**1.88**	**3.33**	**3.26**	**3.19**	**2.97**	**2.27**	**3.89**	**3.74**	**3.80**	**3.75**	**3.15**	**0.57**
Caffeine nitrogen	*% d.b.*	*0.36*	*0.72*	*0.72*	*0.71*	*0.69*	*0.54*	*0.96*	*0.94*	*0.92*	*0.86*	*0.65*	*1.12*	*1.08*	*1.10*	*1.08*	*0.91*	*0.16*
Trigonelline	**% d.b.**	**0.82**	**1.21**	**0.91**	**0.67**	**0.41**	**0.74**	**0.91**	**0.73**	**0.52**	**0.32**	**0.67**	**0.74**	**0.55**	**0.37**	**0.19**	**0.63**	**0.29**
Trigonelline nitrogen	*% d.b.*	*0.08*	*0.12*	*0.09*	*0.07*	*0.04*	*0.08*	*0.09*	*0.07*	*0.05*	*0.03*	*0.07*	*0.08*	*0.06*	*0.04*	*0.02*	*0.06*	*0.03*
Total Amino Acids	**% d.b.**	**11.11**	**6.91**	**6.50**	**6.19**	**6.25**	**11.41**	**6.79**	**6.56**	**6.26**	**7.01**	**11.82**	**7.33**	**6.45**	**6.05**	**6.14**	**6.54**	**0.40**
Amino acid nitrogen	*% d.b.*	*1.78*	*1.11*	*1.04*	*0.99*	*1.00*	*1.83*	*1.09*	*1.05*	*1.00*	*1.12*	*1.89*	*1.17*	*1.03*	*0.97*	*0.98*	*1.05*	*0.06*
NH4	% d.b.	0.26	0.09	0.08	0.07	0.08	0.27	0.07	0.08	0.06	0.08	0.28	0.11	0.10	0.09	0.07	0.08	0.01
Non polar apliphatic	% d.b.	3.78	2.50	2.39	2.24	2.25	3.95	2.49	2.43	2.31	2.57	4.10	2.79	2.38	2.24	2.27	2.41	0.17
Aromatic	% d.b.	0.96	0.64	0.62	0.62	0.58	1.01	0.74	0.70	0.67	0.71	1.11	1.09	0.74	0.69	0.68	0.71	0.13
Polar uncharged	% d.b.	0.95	0.29	0.21	0.14	0.14	1.02	0.25	0.22	0.14	0.17	1.07	0.33	0.18	0.11	0.11	0.19	0.07
Sulfured	% d.b.	0.00	0.12	0.12	0.13	0.15	0.00	0.12	0.12	0.12	0.12	0.00	0.11	0.10	0.10	0.11	0.12	0.01
Acidic	% d.b.	3.48	3.04	2.88	2.83	2.87	3.55	2.88	2.79	2.76	3.15	3.59	2.55	2.71	2.62	2.71	2.82	0.17
a-amino	% d.b.	1.67	0.22	0.20	0.18	0.18	1.62	0.24	0.22	0.20	0.22	1.68	0.35	0.23	0.19	0.20	0.22	0.04
Free Amino Acids	% d.b.	0.37	0.76	0.69	0.64	0.67	0.44	0.71	0.71	0.63	0.72	0.51	0.79	0.68	0.65	0.64	0.69	0.05
Melanoidins	**% d.b.**	**2.2**	**23.2**	**24.2**	**25.1**	**27.5**	**3.1**	**24.4**	**26.8**	**27.6**	**29.6**	**3.8**	**25.2**	**28.3**	**28.6**	**31.5**	**26.8**	**2.5**
Melanoidin nitrogen	*% d.b.*	*0.09*	*0.99*	*1.03*	*1.07*	*1.17*	*0.13*	*1.04*	*1.14*	*1.17*	*1.26*	*0.16*	*1.07*	*1.20*	*1.22*	*1.34*	*1.14*	*0.11*
Total carbohydrates	**% d.b.**	**49.90***	**37.3**	**37.0**	**37.1**	**36.4**	**51.90***	**37.2**	**37.0**	**36.6**	**34.9**	**48.86***	**36.4**	**35.6**	**35.1**	**34.3**	**36.2**	**1.0**
Mannitol	% d.b.	0.43	0.36	0.39	0.43	0.41	0.40	0.31	0.32	0.35	0.35	0.35	0.25	0.27	0.28	0.30	0.34	0.06
Total Arabinose	% d.b.	3.92	4.15	3.55	3.07	2.91	4.73	4.33	4.11	3.46	3.25	4.82	4.84	4.08	3.57	3.32	3.72	0.58
Total Galactose	% d.b.	10.37	15.63	14.14	12.97	13.20	12.23	15.80	15.22	13.89	13.24	12.76	16.78	15.04	14.13	13.14	14.43	1.24
Total Glucose	% d.b.	9.35	1.16	1.06	1.06	0.92	9.06	1.14	1.08	1.08	0.94	8.93	1.12	1.07	1.05	0.96	1.05	0.08
Total Xylose	% d.b.	0.22	0.15	0.13	0.13	0.12	0.26	0.14	0.12	0.11	0.11	0.27	0.13	0.11	0.09	0.08	0.12	0.02
Total Mannose	% d.b.	19.85	15.30	17.11	18.81	18.33	19.82	14.87	15.56	17.05	16.57	18.96	12.76	14.37	15.34	15.90	16.00	1.69
Mono-saccharides	**% d.b.**	**0.17**	**5.46**	**5.50**	**5.50**	**4.51**	**0.22**	**5.54**	**5.23**	**5.25**	**4.31**	**0.16**	**5.32**	**5.50**	**5.57**	**4.83**	**5.21**	**0.43**
Free Arabinose	% d.b.	0.00	1.34	1.00	0.67	0.61	0.00	1.36	1.06	0.82	0.55	0.00	1.54	1.14	0.80	0.54	0.95	0.34
Free Galactose	% d.b.	0.08	1.29	1.33	1.37	1.09	0.06	1.36	1.33	1.34	1.08	0.03	1.32	1.41	1.40	1.22	1.30	0.11
Free Glucose	% d.b.	0.00	0.31	0.32	0.34	0.27	0.00	0.30	0.29	0.31	0.26	0.00	0.27	0.31	0.35	0.31	0.30	0.03
Free Mannose	% d.b.	0.00	1.94	2.23	2.46	2.05	0.00	1.92	1.96	2.17	1.92	0.00	1.64	2.03	2.35	2.18	2.07	0.22
Free Fructose	% d.b.	0.09	0.58	0.62	0.66	0.49	0.16	0.60	0.58	0.61	0.50	0.13	0.55	0.61	0.67	0.59	0.59	0.06
Di-saccharides	**% d.b.**	**3.63**	**3.93**	**3.96**	**3.96**	**3.00**	**2.96**	**4.00**	**3.70**	**3.72**	**2.81**	**1.68**	**3.79**	**3.96**	**4.03**	**3.31**	**3.68**	**0.41**
Sucrose	% d.b.	3.63	0.00	0.00	0.00	0.00	2.96	0.00	0.00	0.00	0.00	1.68	0.00	0.00	0.00	0.00	0.00	0.00
Di-saccharides**	% d.b.	-	3.93	3.96	3.96	3.00	-	4.00	3.70	3.72	2.81	-	3.79	3.96	4.03	3.31	3.68	0.41
Organic acids	**% d.b.**	**2.33**	**7.53**	**7.96**	**8.09**	**8.44**	**1.99**	**6.86**	**7.39**	**7.77**	**8.34**	**1.71**	**6.39**	**7.41**	**7.87**	**8.37**	**7.70**	**0.62**
Quinic	% d.b.	0.63	3.55	4.11	4.59	4.88	0.47	3.75	4.34	4.88	5.20	0.27	3.82	4.91	5.49	5.85	4.61	0.72
Pyroglutamic	% d.b.	0.00	0.45	0.53	0.62	0.77	0.00	0.42	0.51	0.59	0.82	0.00	0.40	0.50	0.59	0.75	0.58	0.14
Formic	% d.b.	0.02	1.03	1.04	0.99	1.03	0.01	0.85	0.85	0.87	0.85	0.02	0.79	0.83	0.79	0.77	0.89	0.10
Malic	% d.b.	0.46	0.75	0.68	0.57	0.50	0.34	0.48	0.44	0.39	0.38	0.26	0.29	0.26	0.23	0.22	0.43	0.17
Oxalic	% d.b.	0.04	0.17	0.20	0.20	0.22	0.09	0.19	0.19	0.20	0.22	0.14	0.19	0.19	0.18	0.18	0.19	0.01
Citric	% d.b.	1.08	1.54	1.36	1.07	0.99	0.94	1.12	1.02	0.81	0.81	0.89	0.84	0.68	0.54	0.52	0.94	0.31
phosphate	% d.b.	0.11	0.05	0.05	0.05	0.06	0.13	0.05	0.05	0.05	0.06	0.14	0.05	0.05	0.05	0.08	0.05	0.01
Chlorogenic acids	**% d.b.**	**8.13**	**5.21**	**3.79**	**2.56**	**1.77**	**9.08**	**4.80**	**3.49**	**2.38**	**1.84**	**9.93**	**4.80**	**3.14**	**1.62**	**1.35**	**3.06**	**1.36**
Other unknown	**% d.b.**	**5.04**	**8.05**	**9.20**	**9.77**	**9.08**	**3.07**	**7.95**	**6.74**	**7.98**	**7.11**	**6.98**	**7.84**	**7.64**	**9.14**	**6.70**	**8.10**	**1.01**

** Total carbohydrate in green coffee also includes uronic acids*

*** di-saccharide of mannose, galactose and/or arabinose evaluated from a calibration curve relating mono- and di-saccharide levels*

Energy Value Assessment

From these analytical data, the nutritive value of soluble coffee was further calculated by applying the factors for the four energy-supplying constituents. These values were further discussed with those obtained when applying the analytical methodology recommended by Food legislation (Directive E 90/496/CEE 24.09.1990; Souci.Fachmann.Kraut).

- **Fat** is hardly extracted during conventional industrial extraction process. The total fat content in soluble coffees is low and on average **0.18 ± 0.06%**. It has a marginal contribution to energy (i.e. **1.6 kCal/100 g**). Saturated fatty acids accounted for about 45% of total fatty acids, resulting in about 0.06 ± 0.02% saturated fat in the soluble coffee.
- **Nitrogenous components** in soluble coffee are a complex mixture of chemical families including proteins (**6.5 ± 1.4%**), caffeine (**3.2 ± 0.6%**) and trigonelline (**0.6 ± 0.3%**) from green coffee and melanoidins (**26.8 ± 2.5%**) formed upon soluble coffee manufacturing. The latter compounds do not contribute significantly to the energy balance (Dworkschack, 1980).
- Food legislation defines protein as being N x 6.25, the removal of other nitrogen sources being not explicitly mentioned. Table 2 provides for the twelve soluble coffees the different ranges that can be proposed for proteins in soluble coffee. Protein

evaluation based on total nitrogen is over-evaluated and should be replaced by a protein content determined from the total amino acid content.

Table 2. Protein content and energy depending on analytical methodology.

Method	**N x 6.25** N by kjeldhal	**$N_{corrected}$ x 6.25 *** Corrected N	**Amino acids** by HPLC
"Proteins" in g/100g	19.8±1.4%	13.7±0.6%	**6.5±0.4%**
"Energy " in kCal/100g	79	55	**26**

* *N by Kjeldhal corrected for N contained in caffeine and trigonelline.*

- **Carbohydrates** in soluble coffee are essentially composed of two non-digestible polysaccharide families, i.e. arabinogalactans, galactomannans. Initial sucrose is entirely degraded during roasting, but variable amounts of mono- and disaccharides (e.g. mannobiose) may be generated by thermal hydrolysis of coffee polysaccharides during the extraction process. Their amount increase at higher thermal load as shown in Figure 3.
-

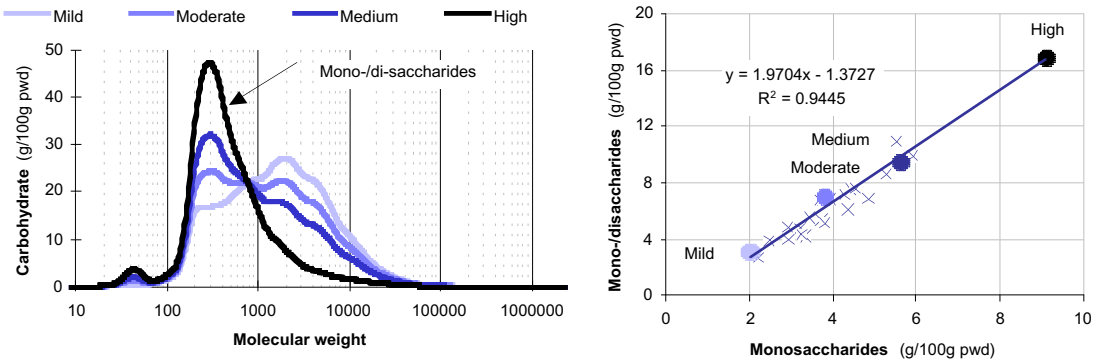


Figure 3. Impact of thermal load on soluble coffee carbohydrates (left) Molecular weight profile (right) Mono-/disaccharide content evaluated by HPLC for ~30 soluble coffees.

There is no legal obligation for a detailed analysis of carbohydrates, which may be evaluated by difference to 100 of all other quantified components (i.e. humidity, ash, proteins, fat, dietary fibers, available organic acids). This approach applied to soluble coffee is misleading, as many compounds (e.g. chlorogenic acids: ~1-5%, melanoidins: ~23-31%) will be considered as available carbohydrates. It results in over-evaluated available carbohydrate levels reported for soluble coffee, i.e. 72%[3]. A revisit of carbohydrate assessment on the basis of biochemical determination was already discussed within the AOAC (International AOAC, 2005) but is still not adopted.

The available carbohydrates should be determined from the mono-/disaccharide content in soluble coffee. In the present soluble coffee range, available carbohydrates account for **8.9 ± 0.8%**, but could be lower or higher depending on the thermal load applied during the extraction process (Table 3).

Table 3. Available carbohydrate content and process severity. Available carbohydrates [$CHO_{available}$] evaluated from monosaccharides [CHO_{Mono}]
[$CHO_{available}$](g/100g)=1.97[CHO_{Mono}]-1.37.

Thermal load	**Mild**	**Medium**	**High**
Available Carbohydrates in g/100g	2.6	**8.9**	16.4
"Energy " in kCal/100g	10	**36**	65

- **Available organic acids** are those that can be used by the human organism (i.e. lactic, malic, citric). They account for **1.4±0.5%** in soluble coffees and have a marginal contribution to energy (i.e. **4 kCal/100 g**).

Energy value of the soluble coffees was further calculated by integrating the proposal for protein and carbohydrate assessment (**Table 4**). A soluble coffee provides on average 277 kJ/100g powder or 67 kCal/100g powder. The nutritive value is almost independent of blend composition and roast colour. A cup of coffee (2g serving) delivers ~**6 kJ/cup** or **~1 kCal/cup** brought by ~0.1g of protein and 0.2g of mono-/disaccharides.

Depending on the severity of extraction conditions, the energy value may be modulated towards lower values, i.e. ~4 kJ/cup or < 1 kCal/cup, for mild processes or higher values, i.e. ~8 kJ/cup or ~2 kCal/cup, for severe processes. Most commercial soluble coffees, however, belong to the mild/medium process category.

In conclusion, the energy value of soluble coffee calculated from its detailed chemical characterization is five times lower than the data obtained by applying the analytical methodology recommended by Food legislation, i.e. 67 kCal/100g powder vs. 350 kCal/100 g (Souci.Fachmann.Kraut). The contribution of soluble coffee to the energy balance of the diet is thus minute (**~1 kCal/cup**). In conclusion, the analytical methodology should be reviewed to take into account the particularities of soluble coffee composition.

Table 4. Nutrient content and energy value of soluble coffees based on detailed chemical analyses.

Nutrient	Unit	Mild Process	Medium Process	Severe Process
Fat	g/100g		0.18±0.06	
Protein	g/100g		6.54±0.40	
Carbohydrates	g/100g	2.6	8.89±0.84	16.4
Organic acids	g/100g		1.37±0.48	
Energy in pwd	kCal/100g (kJ/100g)	**42 (173)**	**67±4 (277±16)**	**98 (400)**
Energy per cup	kCal/cup (kJ/cup)	**0.8 (3.5)**	**1.3±0.1(5.5±0.3)**	**1.9 (8.0)**

Cup size : 2g soluble coffee powder in 100mL.

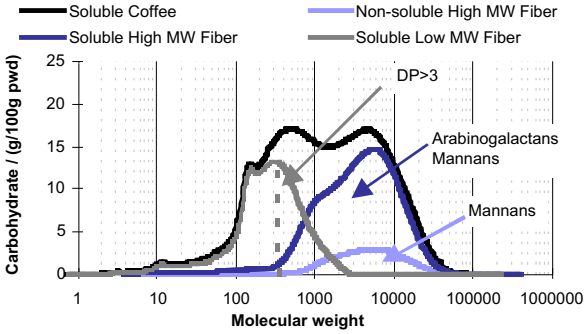
Dietary Fibres in Soluble Coffee

Dietary fibers are primarily defined as the sum of all high molecular weight (MW) substances, which cannot be enzymatically degraded in the human digestive system. Basically, they include polysaccharides (e.g. cellulose, hemicellulose) as well as phenolic polymers (e.g. lignin) (Souci.Fachmann.Kraut). Recently, non-digestible oligosaccharides with a degree of polymerization DP≥3 and substances associated with fibers (e.g. waxes, modified cellulose) have also been suggest to count as dietary fibers (Tungland, D. Meyer, 2002).

Coffee melanoidins are products resulting from the degradation of carbohydrates and proteins through Maillard-type reactions. Coffee polysaccharides bear substantial amounts of low molecular weight units generated by Maillard-type reactions (Nunes et al., 2006). Therefore, on the basis of both the dietary fiber definition and the structural association between coffee polysaccharides and Maillard reaction products, the possible classification of melanoidins into the dietary fiber category should be reviewed.

Dietary fibers in soluble coffee were evaluated by the classical gravimetric and enzymatic method (Prosky et al., 1017). In such methods, proteins or derived compounds (e.g. melanoidins) are removed from the final fiber balance resulting in the quantification of high MW carbohydrates. The carbohydrates composition and MW profile of the fibers were analyzed. The low MW soluble fibers were further derived from the detailed carbohydrate analysis of soluble coffee and fibers (Figure 4).

Soluble coffee contains **~20%** of high MW fibers composed of mannans and arabinogalactans. A small proportion (~3%) made of mannans is non soluble. Low MW fibers account for ~10% and are composed of oligosaccharides of mannose, galactose and/or arabinose. Summing the different contributions, carbohydrates contributing to the dietary fibers account for **~30%** in soluble coffee. The contribution of melanoidins to dietary fibers should be investigated in more details as it could contribute significantly to the dietary fiber balance (i.e. > 20%).



	High MW Fiber		Low MW Fiber
	Non soluble	Soluble	
Arabinogalactans	0.2	11.3	5.2
Mannans	3.1	6.5	4.6
Fiber content	3.3	17.9	**9.8**
Total Fibers	**21.2**		
Melanoidins	**19.9**		

Figure 4. Molecular weight distribution and composition of carbohydrates in the different dietary fiber fractions i.e. non-soluble high MW, soluble high MW, soluble low MW.

CONCLUSIONS

The energy value of soluble coffee should be determined from its detailed composition. Applying this approach, soluble coffee provides 67 kCal/100 g powder. The nutritive value is fairly independent of the blend characteristics. The contribution of soluble coffee to the energy balance of a diet is minute, i.e. ~1 kCal/cup brought by 0.1 g of proteins and < 0.2 g of carbohydrates. However, soluble coffee is an important source of soluble dietary fibers (mannans, arabinogalactans), i.e. ~30%, which correspond to 0.6 g/cup of fibers.

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Mono- and Dicaffeoylquinic Acids from Coffee Are Absorbed by Humans

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SUMMARY

Despite the innumerous potential pharmacological properties of chlorogenic acids reported in the literature, little is known about their bioavailability in humans. The goal of the present study was to evaluate the distribution profile of the major chlorogenic acid isomers and their metabolites in human plasma and urine, after oral coffee administration. Three caffeoylquinic acid isomers and three dicaffeoylquinic acid isomers were identified in the plasma of all six volunteers after coffee consumption, while two feruloylquinic acids were identified in only one volunteer. The main chlorogenic acids metabolites identified in urine were: isoferulic, gallic, dihydrocaffeic, ρ-hydroxybenzoic, vanillic, ρ-coumaric, sinapic, caffeic and ferulic acids. Our results show that the major chlorogenic acid isomers from coffee are bioavailable in humans.

RESUMEN

A pesar de las innumerables propiedades farmacológicas de los ácidos clorogénicos reportadas en la literatura, poco se sabe sobre su biodisponibilidad en humanos. El objetivo de este estudio fue evaluar el perfil de distribución de los principales isómeros de ácidos clorogénicos y sus metabolitos en plasma y orina de humanos, después de la administración oral de café. Tres isómeros del ácido cafeoilquínico y tres isómeros del ácido dicafeoilquínico fueron identificados en el plasma de los seis voluntarios del estudio después de la ingestión de café, mientras que dos ácidos ferruoilquínicos fueron identificados en apenas un voluntario. Los principales metabolitos de los ácidos clorogénicos identificados en orina fueron los ácidos isoferrúlico, gálico, dihidrocafeico, ρ-hidroxibenzoico, vanílico, ρ-cumárico, sináptico, cafeico y ferrúlico. Nuestros resultados muestran que los principales isómeros de los ácidos clorogénicos del café son biodisponibles en humanos.

INTRODUCTION

Chlorogenic acids (CGA) are a family of phenolic compounds formed by the esterification of certain *trans* cinnamic acids, such as caffeic (CA), ferulic (FA), and ρ-coumaric (ρ-CoA), with quinic acid. The main CGA subgroups in coffee are caffeoylquinic acids (CQA), feruloylquinic acids (FQA) and dicaffeoylquinic acids (diCQA), with at least three isomers per group. These compounds have been receiving much attention due to their pharmacological properties observed *in vitro* and *in vivo*, such as antioxidant and hypoglycemiant (Moreira et al., 2005; Shearer et al., 2003). However, studies on CGA bioavailability in humans are limited, mainly due to the inexistence of commercial standards for all CGA isomers. CA, a primary metabolite of CQA, and diCQA, have been detected in human plasma and/or urine, especially in conjugated forms (Cremin et al., 2001; Nardini et al., 2002; Rechner et al., 2001;

Olthof et al., 2003; Wittemer et al., 2005). From all CGA in coffee, only 5-CQA – the major CGA in coffee – has been detected in rat plasma (Azuma et al., 2000). In humans, only traces of 5-CQA, its isomers 3-CQA and 4-CQA, and metabolites have been detected in urine after oral supplementation with 5-CQA (Booth et al., 1957; Olthof et al., 2001). In the present work, we investigated the presence of the major CGA isomers in human plasma, and metabolites in urine, after the oral administration of coffee.

MATERIAL AND METHODS

A coffee infusion prepared with light medium roasted Robusta coffee and containing 1.31 g of CGA (3.08 mmol CQA; 0.24 mmol FQA; 0.25 mmol diCQA) was offered to six adult volunteers. Blood samples were collected after 12 h fasting and 0,5; 1.0; 1.5; 2.0; 3.0 and 4.0 h after coffee administration. Plasma was deproteinized, treated with ß-glucuronidase, extracted with a methanolic solution, clarified and analyzed by reverse-phase (RP) HPLC. Identification of peaks was perfomed by LC-MS and standards (Trugo and Macrae, 1984; Farah et al., 2006). Urine samples were also collected prior and after coffee administration, clarified and analyzed by RP-HPLC and LC-MS. Statistical analyses were performed by Statistica® (version 7, Tulsa, OK, USA), using Student t test for independent samples. Molar ratios of isomers, calculated from the areas under the curve (AUC) of CGA isomers in plasma and from the content of CGA isomers in the beverage, were compared. Differences were considered when $p \leq 0.05$.

RESULTS AND DISCUSSION

Characterization of the brewed coffee offered to the volunteers

Eight CGA isomers (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, 4-FQA and 5-FQA) were identified and quantified in the coffee beverage, from which CQA represented the great majority (86%) (Table 1).

Table 1. Content of the main CGA isomers in the coffee beverage offered to the volunteers during the study*.

Compound	Content (_mol/200 mL beverage)
3-CQA	903
4-CQA	1055
5-CQA	1117
4+5-FQA	239
3,4-diCQA	82
3,5-diCQA	75
4,5-diCQA	91
Total CGA	**3562**

**Analyses performed in triplicates, with variation coefficient < 4%.*

Plasma samples

No CGA or CA was detected in plasma before coffee administration. After oral coffee administration, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were identified in the plasma of all volunteers (Figure 1), while 4-FQA and 5-FQA were detected in the plasma of only one volunteer. Maximal plasma concentration (C_{max}) of CGA was observed at 2,25 h after coffee administration (Table 2). CA – a precursor and immediate

metabolite of CQA and diCQA – was also present in all plasma samples after coffee administration, with maximum concentration 1,4 h after coffee consumption (Table 2).

The presence of free and conjugated forms of 5-CQA in rat plasma after intraperitoneal administration of 5-CQA has been previously reported (Azuma et al., 2000). Conjugated CA has also been identified in rat and human plasma after coffee digestion (Cremin et al., 2001; Nardini et al., 2002; Rechner et al., 2001; Olthof et al., 2003; Wittemer et al., 2005; Azuma et al., 2000). 3-CQA, 4-CQA and 5-CQA have been previously identified in human digestive fluids (Farah et al., 2006). The presence of 5-CQA and other CGA isomers from coffee in human plasma has not been described in the searched literature. The low amount of CA (4%) formed during analytical recovery tests in our study suggests that most CA identified in plasma would come either from a high uptake and/or from degradation of CGA inside the human body.

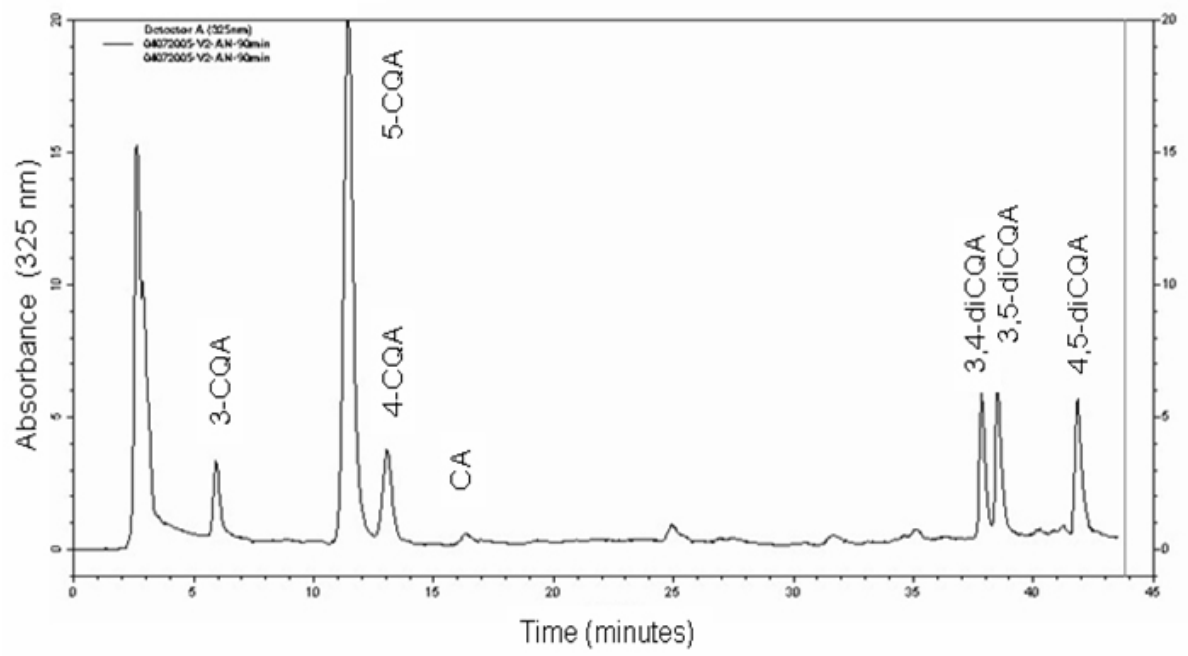

Figure 1. Typical chromatogram of human plasma obtained 2 h after oral coffee consumption.

Table 2. T_{max}, C_{max} and AUC of CGA isomers and CA identified in human plasma after coffee administration*.

Compound	T_{max} (h)	C_{max} (nmol/mL)	AUC (nmol/mL.h)
3-CQA	1,75 ± 0,99	1,00 ± 0,75	1,65 ± 0,96
4-CQA	2,08 ± 1,20	1,04 ± 0,68	1,85 ± 1,24
5-CQA	2,33 ± 1,17	3,14 ± 1,64	8,10 ± 5,05
3,4-diCQA	2,25 ± 1,25	0,92 ± 0,32	1,75 ± 0,58
3,5-diCQA	2,33 ± 1,17	1,17 ± 0,95	1,85 ± 0,83
4,5-diCQA	2,33 ± 1,17	1,11 ± 0,36	2,04 ± 0,67
Total CQA	1,75 ± 0,99	5,00 ± 2,53	11,48 ± 7,12
Total diCQA	2,33 ± 1,17	3,63 ± 1,28	5,63 ± 1,83
Total CGA	2,25 ± 1,25	8,00 ± 2,50	17,11 ± 8,41
CA	1,42 ± 0,38	1,56 ± 1,52	2,81 ± 1,75

*C_{max} - Maximum Concentration; T_{max}, - Time Corresponding to $C_{max;}$- AUC- Area under the curve; * Mean results from six volunteers.*

A large interindividual variability was observed in the kinetics of all CGA isomers identified in plasma. Individual differences in the absorption and metabolism of 5-CQA, CA and other phenolic compounds have been previously reported in the literature (Farah, 2004; Rechner et al., 2004).

The average molar ratio CQA:diCQA was about six times higher in coffee beverage than in plasma, suggesting different mechanisms of absorption and/or metabolization, where the absorption of diCQA would be favored over CQA or where tissue uptake of CQA after absorption would be favored over that of diCQA.

Similarly, the molar ratios 5-CQA:3-CQA and 5-CQA:4-CQA were about four times higher in plasma than in the coffee beverage, suggesting a higher absorption of 5-CQA compared to 3-CQA and 4-CQA isomers, or that 3-CQA and 4-CQA are quickly metabolized and/or stored in liver and/or other organs, compared to 5-CQA.

Urine samples

The only CGA isomer identified in urine after coffee administration was 5-CQA, the major CGA in coffee. CGA metabolites identified in urine after coffee administration were: isoferulic, gallic, dihydrocaffeic, _-hydroxybenzoic, vanillic, _-coumaric, sinapic, caffeic and ferulic acids. Gallic acid was the phenolic compound with the highest increment after coffee administration in the urine of all the volunteers, followed by dihydrocaffeic acid. On average, the amounts of gallic acid and dihydrocaffeic acid, represented together about 56% of the total amount of phenolic compounds identified in urine after coffee administration. All these phenolic compounds have been previously identified in urine after coffee consumption **(**Cremin et al., 2001; Olthof et al., 2003; 2001). As with plasma, there was a large interindividual variability in the concentration and kinetics of all compounds identified in urine.

CONCLUSIONS

Our results show that not only 5-caffeoylquinic acid but also other major chlorogenic acid isomers from coffee are absorbed and metabolized by humans.

ACKNOWLEDGEMENTS

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The Effect of Human Digestive Fluids on Chlorogenic Acid Isomers from Coffee

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SUMMARY

The aim of this study was to investigate the effect of human digestive fluids on chlorogenic acids (CGA) from coffee in an *ex vivo* digestion model. While gastric fluids did not affect CGA concentration in coffee during incubation for up to 8 h, most digestive fluids with higher concentration of enteric fluids produced isomerization and partial hydrolysis of CGA. These effects increased with increasing pH and incubation time, although they were highly variable among individual samples. While average 5-CQA loss after 8h incubation was ~9%, total average CGA loss was ~6%. Therefore, a large amount of CGA is probably available for absorption after human digestion.

RESUMEN

El objetivo de este estudio fue investigar el efecto de fluidos digestivos humanos sobre los CGA del café en un modelo de digestión *ex vivo*. Mientras que los fluidos gástricos no afectaron la concentración de CGA del café durante una incubación de 8 h, la mayor parte de los fluidos digestivos con alta concentración de fluidos entéricos produjeron isomerización e hidrólisis parcial de CGA. Estos efectos aumentaron con el aumento del pH y del tiempo de incubación, a pesar de que fueron altamente variables entre las muestras individuales. A pesar de la perdida promedio de ~9% en 5-CQA después de 8h de incubación, la pérdida total de CGA no excedió 6%.

INTRODUCTION

Chlorogenic acids (CGA) are non-flavonoid phenolic compounds abundant in coffee. The major CGA groups in coffee are the caffeoylquinic acids (CQA); feruloylquinic acids (FQA) and dicaffeoylquinic acids (diCQA), with at least three isomers per group. In the last decade, a series of pharmacological properties have been attributed to these compounds (Farah et al., 2005). Although CGA-rich coffee has a potential use as a functional food, data on the bioavailability of CGA is scarce. Literature reports on human studies suggest a small absorption of 5-caffeoylquinic acid (the only commercially available CGA) in the intestinal portion of the gastrointestinal tract. Despite the brief contact of food with saliva, gastrointestinal transit time may take from 2-8 h (Davenport, 1980). Since coffee may be ingested between and after meals, and considering variations in transit time, CGA may remain in contact with digestive fluids for many hours. Artificial gastric fluids did not succeed in hydrolyzing 5-CQA (Olthof et al., 2001). The activity of esterases in the small intestine mucosa and lumen has been suggested (Andreason et al., 2001; Nardini et al., 2002) and an intense esterase activity was observed in the gut (Couteau et al., 2001; Dupond et al., 2002). But the question whether 5-CQA undergoes hydrolysis in the digestive tract has not been

answered yet. Therefore, our aim was to investigate the effect of human digestive fluids on CGA from coffee in an *ex vivo* digestion model.

MATERIAL AND METHODS

Eleven gastrointestinal fluid samples, six with pH < 6 (higher concentration of gastric juice) and five with pH ≥ 6 (higher concentrations of pancreatic juice and bile), were obtained from male and female adult volunteers during upper endoscopy exams. A concentrated aqueous green coffee extract containing: 22.9 ± 0.2 μg of 3-CQA; 25.5 ± 0.1 μg of 4-CQA; 136.6 ± 0.4 μg of 5-CQA; 4.2 ± 0.1 μg of 4-FQA; 10.2 ± 0.2 μg of 5-FQA; 8.1 ± 0.1 μg of 3,4-diCQA; 8.5 ± 0.3 μg of 3,5-diCQA and 8.1 ± 0.3 μg of 4,5-diCQA was incubated in triplicates for 2 and 8 h with water and human digestive fluids, in the dark, at 37°C and under mild agitation. After the experiments, samples with pH > 3 were acidified with HCl 3N. Samples were then clarified with Carrez solutions and analyzed by HPLC (Farah et al., 2005). Statistical analyses were performed by Statistica®, version 6.0, 2000 (USA), using ANOVA and Student t test for independent samples. Differences were considered when $p < 0.05$.

RESULTS AND DISCUSSION

Neither water nor digestive fluids with pH < 6 affected CGA isomers concentrations during incubation for up to 8 h. These results confirm those previously reported in the literature after incubation of 5-CQA with human gastric juice for 2 h (Olthof et al., 2001), and strongly suggest the absence of esterases able to hydrolyse CGA in the stomach. In contrast, most digestive fluids with pH ≥ 6 produced isomerization of the phenolic acid moiety attached to the 5-position of the (-)-quinic acid to the 3- and 4- positions and partial hydrolysis of the CGA isomers, in general. These effects tended to increase with increasing pH and incubation time, although they were non-linear and highly variable among individual samples (Figure 1**)**. For example, while a sample with pH 6,3 produced a 9.3% decrease in 5-CQA concentration after 8 h incubation, a sample with pH 7.2 produced a 7.2% decrease. The differences observed among the effects of digestive fluids on CGA could most probably be attributed to differences in ionic forms concentration in the fluids, and not to the presence of esterases, since an increase was observed in the concentrations of 3- and 4- isomers and not so much of CA. However, specific experiments should be performed in order to investigate the presence of esterases in the fluids.

Despite losses of up to 25% in experiments using 5-CQA alone after 8 h incubation (data not shown), losses of 5-CQA in coffee ranged from 2.1 to 7.8% after 2 h incubation, with average loss of 4.7%, and from 7.2 to 10% after 8 h incubation, with average loss of 8.7%. Total CGA loss ranged from 1.8% to 4.3% after 2 h incubation and from 4.2% to 8.0% loss after 8h incubation, with average losses of 2.9% after 2h incubation and 5.7% after 8h incubation. These lower losses in CGA compared to losses in 5-CQA are mostly due to the increase in 3-CQA and 4-CQA concentrations produced in consequence of the isomerization of 5-CQA during incubation.

CONCLUSIONS

Our results suggest that all CGA isomers from coffee consumption remain intact in the stomach, and that despite some loss after contact with intestinal fluids, a large amount of CGA remains available for potential absorption in the small intestine.

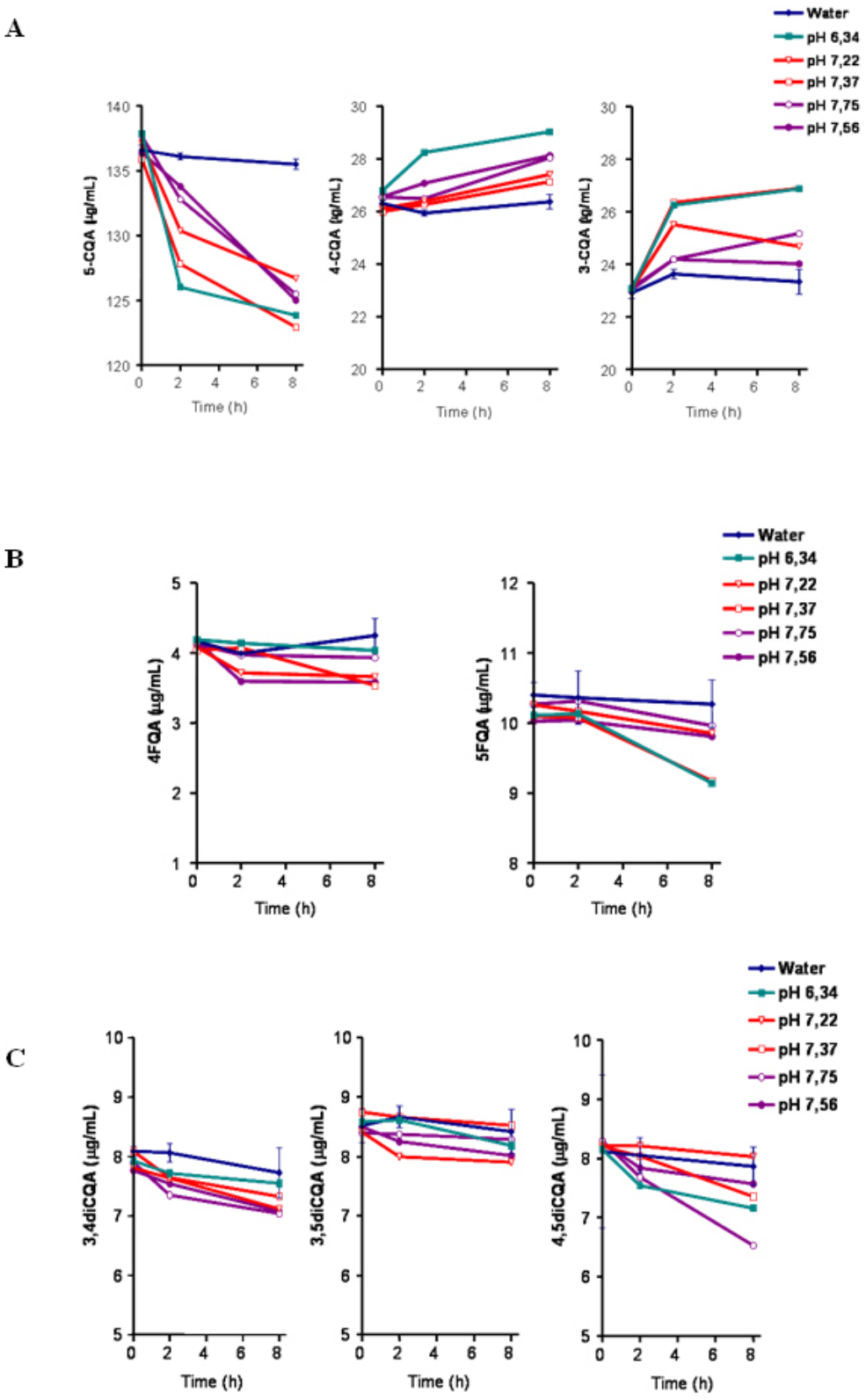

Figure 1. Effect of individual natural digestive fluids with pH ≥6 on caffeoylquinic (a) feruloylquinic (b) and dicaffeoylquinic (c) acids from coffee, during 8 h incubation at 37 °C, in the dark, under mild agitation. Average results from triplicates of experiments.

ACKNOWLEDGEMENTS

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5-Caffeoylquinic Acid Digestibility in Human Digestive Fluids

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SUMMARY

In this study, we investigated the effect of artificial and human gastrointestinal fluids on 5-caffeoylquinic (5-CQA) acid. Neither water nor artificial and natural digestive fluids with pH < 6 affected 5-CQA concentrations during incubation for up to 8 hs. On the other hand, artificial and natural fluids with pH ≥ 6 produced isomerization and hydrolysis of 5-CQA, as pH and incubation time increased. A large variation in the natural fluids responses within this pH range was observed, with average 5-CQA losses of ~20% after 8 h incubation. The comparison of natural and artificial fluids suggests the absence of esterases able to hydrolyze 5-CQA in gastric fluids. The possibility of existence of esterases able to produce mild hydrolysis of 5-CQA in the intestinal portion of some individuals cannot be discarded yet.

RESUMEN

En este estudio, investigamos el efecto de fluidos gastrointestinales humanos y fluidos artificiales, sobre el ácido 5-cafeoilquinico (5-CQA). Ni el agua ni fluidos artificiales y naturales, con pH < 6, afectaron las concentraciones de CQA durante incubación hasta 8h. Por otro lado, los fluidos artificiales y naturales con pH ≥ 6 produjeron isomerización e hidrólisis del 5-CQA, a medida que el pH y el tiempo de incubación aumentaron. Fue observada una gran variación en las respuestas de los fluidos naturales dentro de este rango de pH, con perdidas promedio de 5-CQA de ~20% después de 8h de incubación. La comparación entre fluidos naturales y artificiales sugiere la ausencia de esterasas capaces de hidrolizar 5-CQA en fluidos gástricos. Por otro lado, la posibilidad de la existencia de esterasas capaces de producir hidrólisis parcial de 5-CQA en la porción intestinal de algunos individuos no puede ser todavía descartada.

INTRODUCTION

Chlorogenic acids (CGA) are important phenolic constituents of coffee, accounting for 6-14% of green beans composition (Farah and Donangelo, 2006). Despite their thermolability, light to medium roasted coffees still contain considerable amounts of these compounds. 5-caffeoylquinic acid (5-CQA) is the main CGA in coffee, being responsible for about 50-60% of total CGA content. Various medicinal properties of 5-CQA have been observed in *in vitro* and animal models, some of them related to their natural antioxidant properties (Moreira et al., 2005; Farah et al., 2005). However, data on digestibility and bioavailability of 5-CQA in humans is limited. The occurrence of a small absorption, mostly in the jejunum portion of rats intestine is reported (Spencer et al., 1995). Evidences of 5-CQA absorption in human are still insufficient. Only caffeic acid has been identified in plasma after coffee consumption (Nardini et al., 2002), although traces of intact 5-CQA have been identified in human urine (Olthof et al., 2001). The action of esterases in the intestinal mucosa has been reported (Andreason et al., 2001). The possibility of partial hydrolysis in intestinal lumen is not discarded, especially considering that despite the brief contact of food with saliva in the

mouth, gastrointestinal transit time may take from 2-8 hs (Davenport, 1980) and 5-CQA from coffee or other food sources may remain in contact with digestive fluids for many hours. In the present study, our aim was to investigate the effect of human digestive fluids on 5-CQA in an *ex vivo* digestion model. We also compared the results obtained with human digestive fluids with those from artificial digestive fluids in order to distinguish between the effects of the ionic forms – present in both fluids – from the enzymatic effects present only in the natural fluids. As a considerable percentage of the population carries *Helicobacter pillory* (HP) in their digestive tract, we included in our investigation some fluids containing this bacteria.

MATERIAL AND METHODS

Digestive fluid samples were obtained from thirty adult volunteers during upper endoscopy exams. Ten samples were Hp (+) and twenty were Hp (-). Samples were also divided according to pH (< 6 and ≥ 6) in order to separate those with higher concentration of gastric fluid from those with higher concentrations of pancreatic fluid and bile. pH groups were determined based on intestinal pH reported in the literature (Figure 1).

Gastric and pancreatic juices (pH 1.3 and pH 8.3, respectively) were prepared with the mixture of HCl, $NaHCO_3$, NaCl and KCl, according to literature data (Tunio et al., 1997; Code , 1975; Berne and Levy, 1998) (Figure 1).

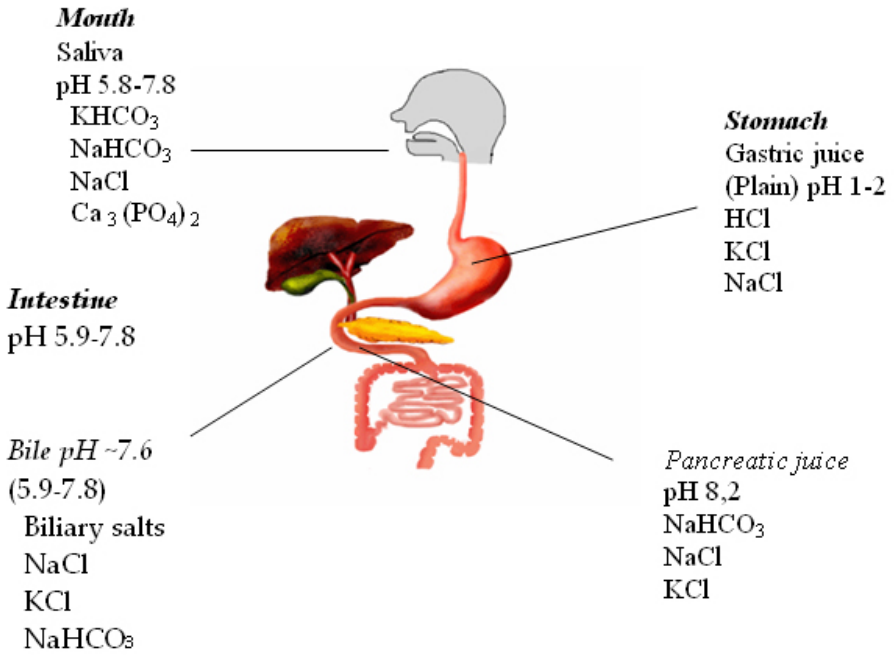

Figure 1. Characterization of digestive fluids in the human digestive tract according to literature data (Tunio et al., 1997; Code , 1975; Berne and Levy, 1998).

Mixtures with different pH were made representing the pool of digestive fluids in the gastrointestinal tract. A concentrated aqueous solution of 5-CQA (Sigma-Aldrich, USA) was incubated with water, artificial fluids or human digestive fluids (final concentration of 60 µg/mL) for 2 and 8h, and for 2, 4, 6 and 8h, depending on the available volume of fluid, at 37 °C, under mild agitation. Samples were then clarified and analyzed by HPLC (Farah et al., 2005). Statistical analysis was made by Statistica® software, version 6.0 (2000) using LSD test and Stuart t test for independent samples. Results were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Figure 2 shows the interactive effects of pH, time and the presence of *Helicobacter pylori* in natural digestive fluids on 5-CQA, during 8 h of incubation in the dark, at 37 ºC, under mild agitation. Neither water nor digestive fluids with pH < 6 affected 5-CQA concentrations during incubation for up to 8 h. On the other hand, artificial and natural fluids with pH ≥ 6 produced isomerization and hydrolysis of 5-CQA, as pH and incubation time increased, with production of 3-CQA, 4-CQA and caffeic acid. A large variation in the natural fluids responses within this pH range was observed, with losses of up to ~60% after 8 h incubation. Average 5-CQA loss in this pH range was ~20% after 8 h incubation. The comparison between the results obtained from artificial and natural human digestive fluids strongly suggests the absence of esterases able to hydrolyze 5-CQA in gastric fluids. In the enteric fluids, the increase in $NaHCO_3$ concentration – and not necessarily in pH – seemed to be associated with isomerization and loss of 5-CQA during incubation. This was concluded after comparison of natural fluids results with those from artificial fluids with same pH but different concentrations of $NaHCO_3$. Moreover, considering the behavior of a few individual samples, the existence of esterases in enteric fluids able to hydrolyze this compound in some individuals could not be discarded and needs further investigation.

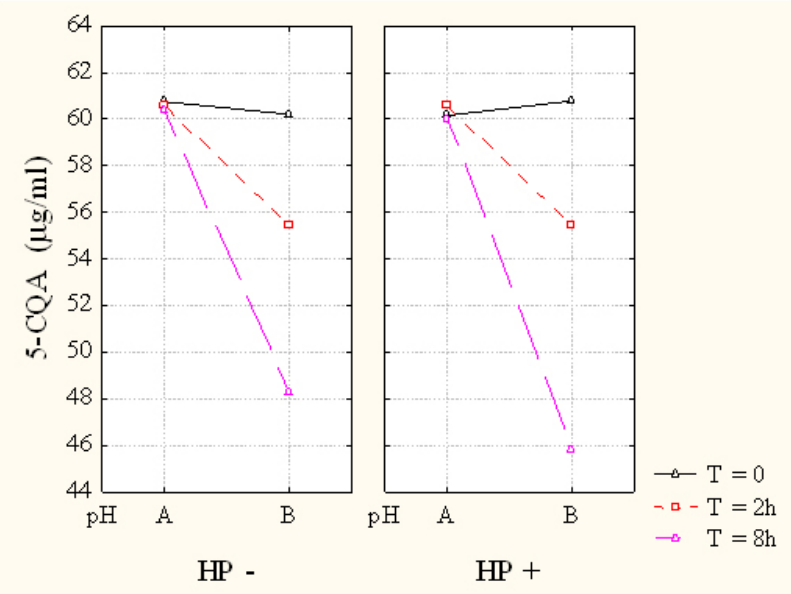

Figure 2. Interactive effects of pH, incubation time (0, 2, 8 h) and the presence of *Helicobacter pylori* (HP) in natural digestive fluids on 5-CQA, at 37 °C, under mild agitation. Initial 5-CQA concentration was 60 µg/mL. Results are means of triplicates of experiments. pH A < 6; pH B ≥ 6.

No differences were observed between the average results obtained with Hp (+) and Hp (-) fluids after 2 h of incubation. After 8 h incubation, only a small difference of ~3% was observed between the average results obtained for Hp (+) and Hp (-) fluids. However, looking at individual values, this difference was not consistent among samples, being caused by the large individual differences that are more likely due to a large variation in concentrations of ionic forms and/or a mild action of esterases in some individuals.

CONCLUSIONS

The results obtained in this study strongly suggest that the 5-caffeoylquinic acid orally consumed by humans remains intact in the stomach, and that despite of large differences in

the action of intestinal fluids from different individuals on 5-caffeoylquinic acid, a considerable portion of this compound is still available for absorption in the intact form or for the action of esterases in the small intestine mucosa.

ACKNOWLEDGEMENTS

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Chlorogenic Acid Isomers from Coffee Are Differentially Uptaken by HepG2 Human Hepatoma Cells

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SUMMARY

The uptake of chlorogenic acids from coffee extracts by HepG2 human hepatoma cells was investigated. HepG2 cells were incubated with a commercial instant decaffeinated coffee solution (1.5% and 4% w/v) for 1.5-7.5 hours. Results from incubation with coffee at 1.5% showed that although the concentration of 5-caffeoylquinic acid (5-CQA) in cell culture media was about 1.5 times higher than that of both 3-CQA and 4-CQA, the uptake of the latter two isomers by HepG2 cells was 5 and 6 times higher, respectively, than the uptake of 5-CQA. Similar results were observed for 3- and 4-feruloylquinic acids (FQA) compared to 5-FQA. FQA uptake was also facilitated compared to CQA uptake. Increasing CGA concentration in cell media caused a relative decrease in the uptake of feruloylquinic acids in comparison with caffeoylquinic acids and the uptakes of 3- and 4-substituted isomers, especially FQA isomers, while the uptake of 5-CQA and 5-FQA was proportional to their concentration increase in the cell culture media. Our results suggest differentiated transport systems for CGA isomers, where the uptake of some isomers is facilitated – at least in part – by a carrier system(s). Further investigation is under way.

INTRODUCTION

Chlorogenic acids (CGA) are esters of hydroxycinamic acids with (-)-quinic acid. These compounds are largely distributed in the plant kingdom, with especially high amounts in coffee seeds, where they play an important role as antioxidants. The main groups of CGA in coffee are the caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA), with at least three isomers per group. During coffee roasting, quinolactones are produced through dehydration of CGA and formation of intramolecular bonds (Farah and Donangelo, 2006). A number of pharmacological properties have been attributed to CGA such as hepatoprotective, anti-inflammatory and the ability to increase hepatic glucose utilization and to lower blood glucose (Farah and Donangelo, 2006; Moreira et al., 2005; Jonston et al., 2003; Shearer et al., 2003; Basnet et al., 1996). However, studies on CGA absorption and metabolism are scarce. CGA isomers have recently been identified in human plasma, urine and digestive fluids (Olthof et al., 2003; Farah, 2004; Monteiro, 2006), mostly in conjugated forms. The partial absorption of CGA *via* portal system and storage in the liver prior to systemic distribution has also been suggested (Baer-Dubowska and Szaefer, 1998). Despite this, no reports are found on CGA isomers uptake by liver cells.

The human hepatome HepG2 cell line has been largely used in studies investigating the liver uptake of different food components, including phenolic compounds such as flavonoids (Yi et al., 2006). In the present study, we used HepG2 human hepatocytes to investigate CGA isomers uptake during incubation with coffee beverage.

MATERIAL AND METHODS

HepG2 cells were incubated with a commercial instant decaffeinated coffee infusion added directly into the cell culture media (1.5% and 4% w/v) for 1.5-7.5 hours. After the incubation period, cells were washed with saline and the phenolic fraction was extracted with a mixture of methanol/water, clarified by filtration and analyzed by reverse-phase HPLC (Farah et al., 2005). Aqueous cell extracts were also incubated with *ß*-glucuronidase type II from *Helix pomatia* (Sigma-Aldrich, USA) to investigate the existence of conjugated forms. Cell culture media was also analyzed at each time point.

RESULTS AND DISCUSSION

Six chlorogenic acid isomers and two lactones were identified and quantified in the media used in this study. Only traces of 3,4-diCQA and caffeic acid were identified in the media, probably due to diCQA degradation during the roasting process. The thermolability of CGA is well known (Farah and Donangelo, 2006) and the coffee used in this study could be classified as very dark roasted coffee. CGA concentration in the cell media remained relatively stable during the 7.5 h of incubation (Figure 1).

Maximum CGA uptake was at 5.5 h of incubation. After this point, the cells started to loose viability probably due to the presence of one or more toxic substances in the culture media. One possibility could be the low amount of caffeine remaining in decaffeinated coffee (0.3 g %), since caffeine was previously shown to be toxic to neuroblastoma cells (Jang et al., 2002). Although HepG2 cells incubated with up to 10 mM of 5-CQA and caffeic acid in our laboratory were viable even after 24 h incubation, other phenolic compounds have previously shown toxicity for HepG2 cells (Yi et al., 2006; Ramos et al., 2005), which raises the possibility of application of such compounds in cancer prevention and therapy. Therefore, the toxicity of CGA and cinnamic acids other than 5-CQA and CA for HepG2 cells should be studied.

Results from incubation with coffee at 1.5% showed that although the concentration of 5-CQA in cell culture media was about 1.5 times higher than that of 3-CQA and 4-CQA, the uptake of the latter two isomers by HepG2 cells was 5 and 6 times higher, respectively, than the uptake of 5-CQA. Similar results were observed for FQA - intracellular concentrations of 3-FQA and 4-FQA were 5 and 7 times higher, respectively, than that of 5-FQA, while in the cell culture media concentrations of 3-, 4- and 5-substituted isomers were similar. FQA cell uptake was also higher than CQA uptake (Figure 2).

CA was identified inside the cells, which indicates degradation of CQA inside the cell or high uptake of CA from the media. Ferulic acid was not identified inside the cells, even though FQA were uptaken, which increases the possibility of a high uptake of CA from the media rather than degradation of CQA. Lactones levels inside the cells were low at all time points, suggesting either low uptake, degradation or return to the chlorogenic acid form inside the cells.

Increasing CGA concentration in cell media (4% coffee infusion) reduced, relatively speaking, the uptake of 3- and 4-substituted CGA isomers, especially FQA, which could, perhaps, be inhibited by larger amounts of CQA while the uptake of 5-CQA and 5-FQA was proportional to their concentration increase in cell culture media. Toxicity was also increased.

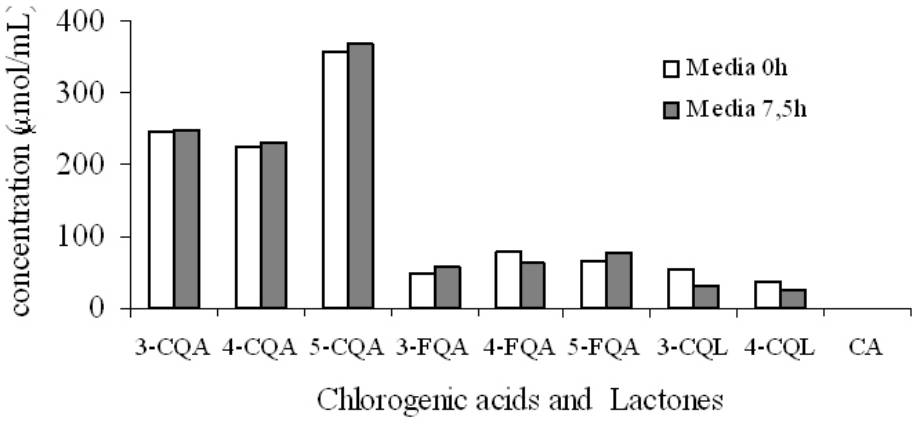

Figure 1. Chlorogenic acids (CQA and FQA) and caffeoylquinic lactones (CQL) concentration in cell culture media before and 7.5 h after incubation with 1,5% coffee solution. Results are mean of duplicates of determinations, from three experiments.

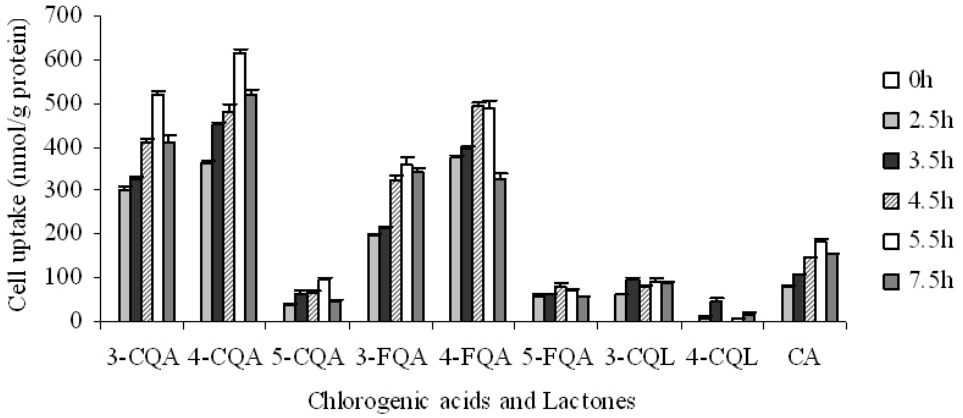

Figure 2. Chlorogenic acids (CQA and FQA) and caffeoylquinic lactones (CQL) uptake by HepG2 cells after incubation with decaffeinated instant coffee at 1,5%. Average of triplicate of experiments ± standard deviation.

No glucuronated forms were identified in HepG2 cell extracts.

Results suggest that HepG2 uptake of CGA isomers with substituents in the 3- and 4-positions of the quinic acid is mediated – at least in part – by a carrier system. Isomers with substituents in 5- position seem to be uptaken by passive diffusion. Further investigation is under way.

ACKNOWLEDGMENTS

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Study of Chlorogenic Acid Functional Properties: Effects on Hepatic Glucose Output, Blood Glucose Levels and Plasmatic Lipids

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SUMMARY

In vitro studies show that chlorogenic acid is an inhibitor of glucose-6-phosphatase, enzyme that catalyses the final reaction of glycogenolysis and gluconeogenisis. Accordingly, in this study, chlorogenic acid inhibited approximately 40% of glucose-6-phosphatase activity, but had no effect on hepatic production of glucose from gluconeogenesis. Since there were indications of lack of hepatic uptake of chlorogenic acid, it is possible that this compound hasn't reached intracellular compatible levels with the inhibition of the target-enzyme, what was reinforced by the fact that intravenous and oral administration of chologenic acid also didn't imply in reduction of blood glucose levels and plasmatic concentration of total cholesterol and triacylglycerols.

INTRODUCTION

Chlorogenic acid, a phenolic compound found in coffee, was identified as an inhibitor of glucose-6-phosphatase in rat liver microsomes (Arion et al., 1997; Hemmerle et al., 1997; Schindler et al., 1998). It was also demonstrated that synthetic chlorogenic acid derivatives are effective not only in inhibit glucose-6-phosphatase in *in vitro* studies, but also in inhibit hepatic glucose output and reduce blood glucose levels (Hemmerle et al., 1997; Herling et al., 1999; 1998; Parker et al., 1998). However, there is no available data on literature about the effects of the natural compound chlorogenic acid on hepatic production of glucose and its effects on blood glucose levels and lipidic metabolism are not conclusive. The present work's objective was to study the effect of chlorogenic acid on glucose-6-phosphatase activity, hepatic glucose output, blood glucose levels and plasmatic total cholesterol and triacylglycerols concentrations. Hepatic uptake of chlorogenic acid and its effects on hepatic catabolism of L-alanine were also evaluated.

MATERIAL AND METHODS

For the experiments, male Wistar rats, fed or submitted to 24 hours of alimentary privation, depending on the metabolic parameter to be investigated, were used. The glucose-6-phosphatase activity was evaluated in rat liver intact microsomes (Bracht and Ishii-Iwamoto, 2003). The studies of hepatic metabolic parameters (glucose output from gluconeogenesis, L-alanine catabolism and hepatic uptake of chlorogenic acid) were performed in *in situ* liver perfusion (Kelmer-Bracht et al., 1984) and chlorogenic acid levels were determined by HPLC (Ky et al., 1997). For the analysis of the chlorogenic acid effects on blood glucose levels, plasmatic concentrations of total cholesterol and triacylglycerols, chlorogenic acid was

administrated intravenously (unique injection of 70 mg/kg) or orally (100 mg/kg/day-during 15 days) and blood samples were collected for biochemical determinations. To analyse the results, accordingly to the necessity, the statistical tests one-way ANOVA followed by Tukey, unpaired or paired Student's *t* test, Mann-Whitney or Wilcoxon tests were employed. Results are expressed as mean ± standard deviation and the significance level adopted was 5% ($p < 0.05$).

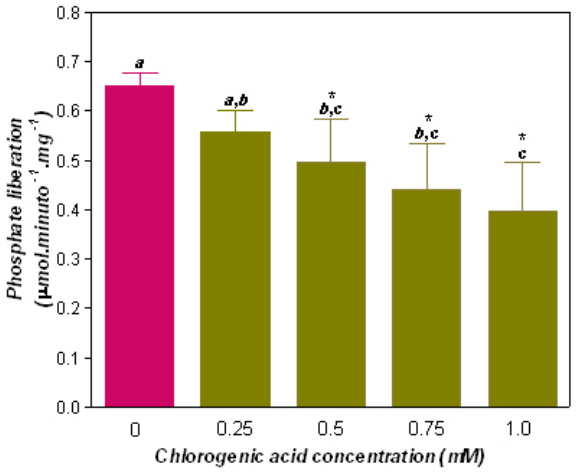

Figure 1. Effect of chlorogenic acid on G-6-Pase activity. Livers from 24 h fasted rats were homogenized and submitted to differential centrifugation. The microsomal fraction was used to do the G-6-Pase activity assay in the lack (■) or presence (■) of different concentrations of chlorogenic acid. Each bar represents the mean ± SD of 5 determinations. Results analyzed by ANOVA followed by Tukey. Distinct letters represent significant differences ($p < 0.05$).* $p < 0.05$ versus control.

RESULTS AND DISCUSSION

Chlorogenic acid 1 mM inhibited approximately 40% ($p < 0.05$) of glucose-6-phosphatase activity on microsomal fraction of hepatocytes (Figure 1), but a lack of effect of the tested doses of chlorogenic acid (0.33 mM; 0.5 mM and 1 mM) on hepatic production of glucose from gluconeogenesis and L-alanine catabolism on liver perfusion was observed (Figures 2, 3 and 4).

Since there were indications of lack of hepatic uptake of chlorogenic acid (Table 1), it is possible that this compound hasn't reached intracellular compatible levels with the inhibition of the target-enzyme.

Accordingly, intravenous (Table 2) and oral administration of chologenic acid (Table 3) also didn't imply in reduction of blood glucose levels. In these studies, it was still observed that plasmatic concentration of total cholesterol and triacylglycerols were not reduced by chlorogenic acid (Tables 2 and 3).

So, studies were higher intracellular levels of chlorogenic acid can be reached will be necessary to verify its physiological properties and to analyse coffee potential to modulate high hepatic production of glucose in type 2 diabetes.

Financial support: Companhia Iguaçú de Café Solúvel and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

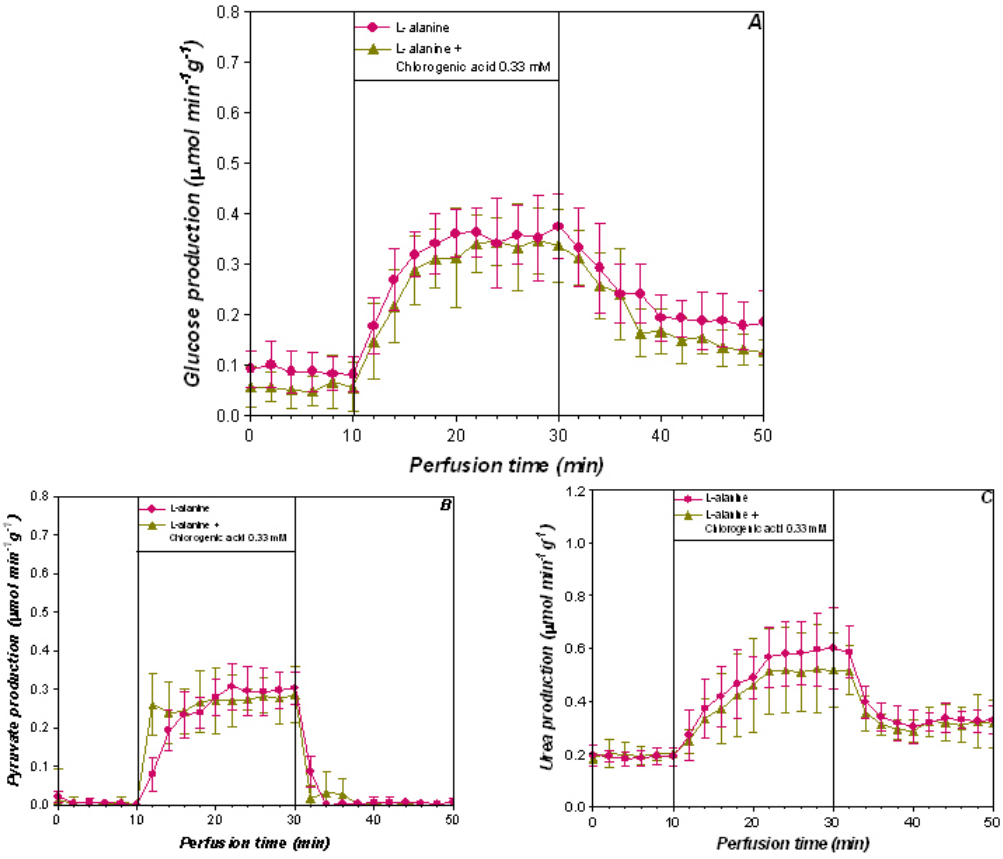

Figure 2. Hepatic production of glucose (A), pyruvate (B) and urea (C) from L-alanine. Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs-Henseleit buffer. L-alanine 2.5 mM (●) or L-alanine 2.5 mM + chlorogenic acid 0.33 mM (▲) were infused from 10 to 30 minutes. Samples to glucose, pyruvate and urea determination were collected each 2 min. Each point represents the mean ± SD of 4 or 5 experiments. Differences between the AUCs of control and experimental groups were analyzed unpaired Student's *t* test.

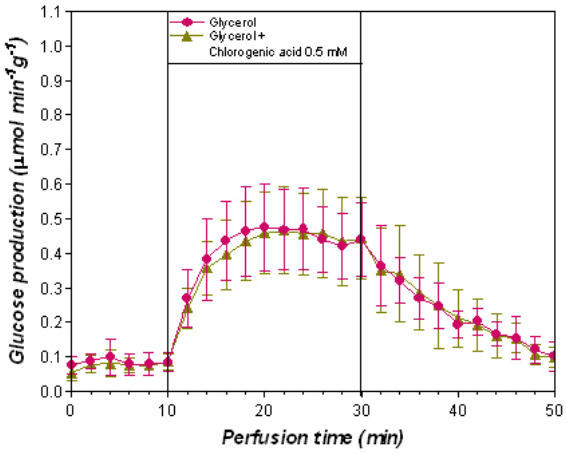

Figure 3. Hepatic production of glucose from glycerol. Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs-Henseleit buffer. Glycerol 2 mM (●) or glycerol 2 mM + chlorogenic acid 0.5 mM (▲) were infused from 10 to 30 minutes. Samples to glucose determination were collected each 2 min. Each point represents the mean ± SD of 4 or 5 experiments. Differences between the AUCs of control and experimental groups were analyzed by unpaired Student's *t* test.

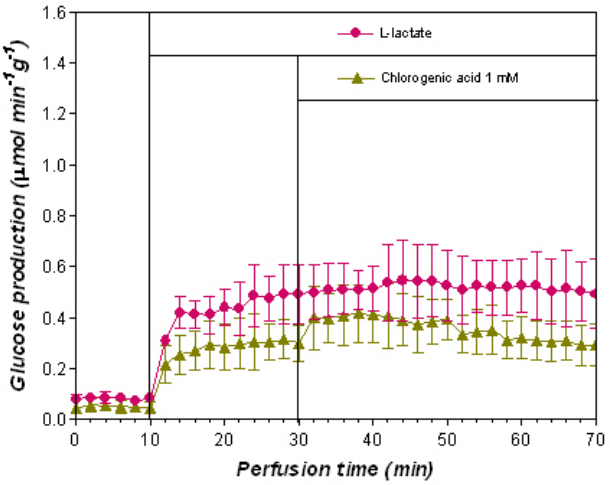

Figure 4. Hepatic production of glucose from L-lactate. Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs-Henseleit buffer. L-lactate 2 mM was infused from 10 to 30 minutes and L-lactate 2 mM (●) or L-lactate 2 mM + chlorogenic acid 1 mM (▲) were infused from 30 to 70 minutes. Samples to glucose determination were collected each 2 min. Each point represents the mean ± SD of 3 experiments. Differences between the AUCs of control and experimental groups were analyzed by unpaired Student's *t* test.

Table 1. Chlorogenic acid concentrations before and after passing through liver. Different concentrations (0.33; 0.5 or 1 mM) of chlorogenic acid were infused in liver (before get into the liver) and samples of the effluent perfusate (after passing through liver) were collected on 10; 20; 30 or 50 minutes to chlorogenic acid concentration (_g/mL) determination.

Chlorogenic acid before get into the liver	Chlorogenic acid after passing through liver			
	10 min	20 min	30 min	50 min
59.32±6.22 (0.33 mM)	61.49±6.15	----	----	----
96.77±15.69 (0.5 mM)	94.40±5.17	----	----	----
261.76±19.57 (1 mM)	259.02±26.14	----	276.99±8.34	267.77±6.52
247.41±26.33 (1 mM)	----	228.95±31.91	----	----

Each data represents the mean ± SD of 3 experiments. Results analyzed by paired Student's t test and confirmed by the non-parametric test of Wilcoxon, when necessary.

Table 2. Plasmatic concentrations of glucose and total chlolesterol after intravenous administration of chlorogenic acid to fed rats. The blood glucose levels (mg/dL) and total plasmatic cholesterol concentration (mg/dL) were evaluated on 5; 15; 30 and 60 minutes after the intravenous administration of chlorogenic acid (70 mg/kg).

Groups	Blood glucose levels			
	5 min	**15 min**	**30 min**	**60 min**
Control	131.25±19.29 (7)	126.33±10.62 (9)	119.33±11.03 (9)	99.65±8.14 (9)
Chlorogenic acid	134.32±7.97 (6)	115.08±16.04 (10)	123.22±9.25 (9)	103.70±10.41 (10)

Groups	Total cholesterol			
	5 min	15 min	30 min	60 min
Control	55.17±8.64 (8)	60.44±7.73 (10)	62.51±13.40 (11)	52.00±12.98 (9)
Chlorogenic acid	59.74±9.28 (8)	55.47±12.94 (8)	65.34±9.73 (7)	61.28±16.55 (6)

Data reported as mean ± SD. Results, within fixed time, analyzed by unpaired Student's t test (A) and confirmed by the non-parametric test of Mann-Whitney, when necessary (B). ( ) Number of animals.

Table 3. Plasmatic concentrations of glucose, total cholesterol and triacylglycerols after oral administration of chlorogenic acid to fed animals. The blood glucose levels (mg/dL), plasmatic concentration of total cholesterol (mg/dL) and triacylglycerols (mg/dL) were evaluated after 15 days of oral administration of chlorogenic acid (100 mg/kg/day).

s	Glucose	Total cholesterol	Triacylglycerols
Control	114.53±7.67 (9)	44.59±8.55 (10)	62.52±20.97 (8)
Chlorogenic acid	120.54±9.13 (9)	61.87±11.35 (8) *	75.95±25.02 (8)

*Data reported as mean ± SD. Results analyzed by unpaired Student's t test. * $p<0.05$ versus control. ( ) Number of animals.*

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In vitro Antimicrobial Activity of Coffee Extracts on Enterobacteria

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SUMMARY

The *in vitro* antimicrobial activity of commercial coffee extracts was investigated on 9 human safety relevant strains of enterobacteria: *Citrobacter freundii, Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli, Klebsiela oxytoca*, *Proteus hauseri*, *Proteus mirabilis*, *Salmonella choleraesuis* and *Serratia marcescens*. The antimicrobial activity was investigated by the disc diffusion method. The three coffee extracts showed similar inhibitory effects on all tested strains, except for two strains which responded differently, with *C. freundi* showing the least sensitivity of all strains and *P. hauseri* showing the highest.

INTRODUCTION

Coffee is one of the most popular and widely consumed beverages throughout the world due to its pleasant taste and aroma and to its stimulant effect. Recently, a number of beneficial health properties have been attributed to coffee (Yen et al., 2005), among them, the antimicrobial activity. Dogazaki et al. (2002) and Furuhata et al. (2002) reported antibacterial activity of brewed coffee against *Legionella pneumophila.* According to Daglia et al. (2002) and Almeida et al. (2004), the growth of *Streptococcus mutans*, the major causative agent of dental caries in humans, was inhibited by coffee extracts.

Enterobacteria are ubiquitous microorganisms, but have a predominant habitat in the gastrointestinal tract of humans and animals. Because of the survival under adverse environmental conditions, they can colonize diverse ecological niches, being found in a large number of foods (Diekma et al., 1999). Furthermore, there is concern regarding their antibiotic resistance and the presence of virulence factors.

Therefore, the objective of this study was to investigate the antimicrobial activity of coffee extracts against enterobacteria.

MATERIALS AND METHODS

Coffee samples and standards

Three different brands of coffee (*Coffea arabica* L.) were purchased in Belo Horizonte, state of Minas Gerais, Brazil. The grains were ground by means of a domestic mill (Braun, USA) and passed through a 20 mesh sieve. Coffee extracts were obtained by using 8.0 g of coffee per 40 mL of boiling water. The mixture was kept in a boiling water bath for 3 min under agitation and filtered through qualitative filter paper. Chlorogenic acids [5-caffeoylquinic acid, Aldrich C44206; 3- and 4-caffeoylquinic acid (Farah et al., 2006)], trigonelline (Sigma T5509) and caffeine (Reagen) were used as standards.

Characterization of coffee extracts

The CIE color characteristics of the ground roasted coffees were determined using the ColorTec colorimeter PCM (Accuracy Microsensor). The pH of the extracts was obtained by means of a pH meter. The levels of chlorogenic acids (Farah et al., 2006), trigonelline and caffeine (Farah et al., 2006) were determined. The samples were clarified and analyzed by HPLC using two different systems.

Determination of the antimicrobial activity

The antimicrobial activity of the coffee extracts was determined by the disc diffusion method (NCCLS, 1993). The inoculum was standardized by transferring colonies from Nutrient Agar to sterile saline up to 10^8 cfu/mL. 200 μL of the suspensions were placed onto the surface of Mueller Hinton Agar in 150 mm Petri dish and spread homogeneously with a Drigalski spatula. Discs (6.0 mm diameter) were impregnated with 20 μL of the coffee extracts and placed on the surface of the agar containing each bacterium. The plates were incubated at 36.5 ± 1.0 °C for 48 h. The inhibition zones were measured with a caliper considering the total diameters. Similarly, each plate carried a blank disc containing 20 μL sterile distilled water and an antibiotic disc containing 30 μg of chloramphenicol.

Statistics

Each experiment was performed in triplicate. The results were submitted to analysis of variance and the means were compared by the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Characteristics of the coffee samples

There was no significant difference on a* values and hue angles of the three coffee samples (Table 1). However, L*, b* and c* varied significantly, with higher values observed for samples B and C. The significant inverse correlation between L* and the degree of roasting is well known (Farah et al., 2006; Borges et al., 2002). It suggests that sample A was roasted to a higher degree compared to samples B and C, which did not differ statistically.

The pH of the coffee extracts varied significantly, with higher values observed for brand A, followed by brand C which differed from brand B. According to Sabbagh and Yokomizo (1976), the stronger the roasting degree, the higher the pH, which is in agreement with luminosity results for sample A.

The levels of the chlorogenic acids varied significantly among coffee brands. Since chlorogenic acids are partially degraded during the roasting process (Monteiro and Trugo, 2005) and sample A showed to be darker than samples B and C, one would expect lower levels of chlorogenic acids in sample A. These results are in accordance with the levels of chlorogenic acids described by Farah et al. (2006) for samples classified as very dark regarding roasting degree. The levels of caffeine and trigonelline in the coffee samples were similar to values reported in the literature (Ky et al., 2001). Higher levels of caffeine and lower levels of trigonelline were found in sample A. According to Macrae (1989), even though high temperatures can be reached (~230 °C) during coffee roasting, the loss in caffeine is insignificant. The degradation of trigonelline during the roasting process is well known (Monteiro and Trugo, 2005; Macrae, 1989).

Table 1. CIE L*a*b* color characteristics of the ground roasted coffee samples and pH and levels of trigonelline and chlorogenic acids of different brands of coffee extracts.

Parameter	Values / Brands		
	A	B	C
CIE L*a*b* color			
L*	20.92 ± 1.18 [b]	29.4 ± 0.37 [a]	26.4 ± 1.08 [a]
a*	5.98 ± 2.99	10.3 ± 1.36	8.53 ± 2.16
b*	11.7 ± 2.08 [b]	26.0 ± 0.77 [a]	20.8 ± 2.17 [a]
chroma	13.3 ± 2.26 [c]	28.0 ± 0.24 [a]	22.6 ± 1.37 [b]
hue	63.4 ± 12.9	68.4 ± 3.16	67.5 ± 7.07
pH	5.29 ± 0.03 [a]	4.88 ± 0.03 [c]	5.02 ± 0.01 [b]
Trigonelline (mg/mL)	0.49 ± 0.05 [c]	1.63 ± 0.04 [a]	1.50 ± 0.02 [b]
Caffeine (mg/mL)	2.23 ± 0.03 [a]	2.18 ± 0.03 [a,b]	2.11 ± 0.04 [b]
Chlorogenic acids (mg/mL)			
3-CQA	0.08 ± 0.00 [c]	0.88 ± 0.01 [a]	0.71 ± 0.01 [b]
4-CQA	0.12 ± 0.00 [c]	1.11 ± 0.02 [a]	0.90 ± 0.01 [b]
5-CQA	0.09 ± 0.01 [c]	1.79 ± 0.03 [a]	1.46 ± 0.01 [b]

CQA = caffeoylquinic acid. Mean of triplicates. Means (± standard deviations) with different letters in the same line (a,b,c) are significantly different (Tukey test, $p \leq 0.05$).

Even though physico-chemical characterization indicated a difference on the degree of roasting among samples, they could be all categorized according to the Brazilian Association of the Coffee Industries-ABIC reference color system as very dark regarding roasting degree.

***In vitro* antimicrobial activity of coffee extracts against enterobacteria**

There was no significant difference on the inhibitory effect of the different coffee brands on the strains investigated, even though physico-chemical characteristics varied among samples **(**Table 2**)**.

Table 2. Inhibition zones obtained with coffee aqueous extracts on enterobacteria.

Strains	Inhibition zones (mm) / coffee brands		
	A	B	C
C. freundii (ATCC 8090)	7.1 ± 0.6 [b]	6.9 ± 0.5 [b]	7.0 ± 0.5 [bc]
E. aerogenes (ATCC 13048)	8.4 ± 0.6 [ab]	8.2 ± 0.7 [ab]	8.7 ± 0.3 [ab]
E. cloacae (ATCC 23355)	9.0 ± 1.0 [a]	9.3 ± 1.3 [a]	9.7 ± 1.3 [ab]
E. coli (ATCC 25922)	8.1 ± 0.4 [ab]	8.1 ± 0.3 [ab]	8.2 ± 0.5 [ab]
K. oxytoca (ATCC 49131)	7.5 ± 0.7 [ab]	7.7 ± 0.3 [ab]	7.5 ± 0.5 [b]
P. hauseri (ATCC 13315)	9.2 ± 1.1 [a]	9.3 ± 1.1 [a]	9.8 ± 0.6 [a]
P. mirabilis (ATCC 25933)	8.1 ± 0.5 [ab]	8.8 ± 0.3 [ab]	8.75 ± 0.4 [ab]
S. enterica (ATCC 14028)	8.1 ± 1.3 [ab]	7.9 ± 1.2 [ab]	8.3 ± 1.56 [ab]
S. marcescens (ATCC 8100)	9.2 ± 0.3 [a]	9.2 ± 0.8 [ab]	9.1 ± 0.1 [ab]

Mean of triplicates. Mean values (± standard deviation) with different letters (a,b) in the same columns are significantly different (Tukey Test, $p \leq 0.05$). Positive control: 30 µg chloramphenicol/disk formed inhibition zones with mean (± standard deviation) diameters in mm of: C. freundii – 32.7 ± 1.7, E. aerogenes – 25.0 ± 2.3, E. cloacae – 32.2 ± 1.2, E. coli – 28.3 ± 1.5, K. oxytoca – 30.8 ± 0.8, P. hauseri – 20.9 ± 1.1, P. mirabilis, – 21.2 ± 0.8, S. enterica – 27.3 ± 0.9, S. marcescens– 27.4 ± 1.6.

On the other hand, when comparing the susceptibility of the different strains to the coffee extracts, differences were observed. Larger diameters of the inhibition zones were observed for *P. hauseri* indicating the higher sensitivity of this specie to the coffee extracts. Smaller diameters were observed for *C. freundii*, which indicated that it was less sensitive to the coffee extracts. The other strains of enterobacteria showed intermediate sensitivity to these compounds.

The present work demonstrates that the three coffee extracts included in this study showed antimicrobial activity for the nine enterobacteria investigated. However, the enterobacteria responded differently to the coffee extracts.

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Arabinogalactan Isolated from Coffee Beans Indicates Immunomodulating Properties

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SUMMARY

Coffee bean polysaccharides, consisting mostly of arabinogalactan, galactomannan, and cellulose, make up about 50 % of the dry weight of the bean. The bioactive properties of arabinogalactan from several plant sources have been reported in relation to their prebiotic, cholesterol-lowering and immunomodulating properties. To investigate whether arabinogalactan from coffee beans also has bioactive properties, we examined the proliferation or production of cytokines in murine, splenocytes and bone marrow-derived dendritic cells (BMDCs) in response to arabinogalactan from coffee beans, and immunomodulating activity in mice. In the presence of arabinogalactan from coffee beans the proliferation of macrophage and splenocyte was observed. Furthermore, murine BMDC and splenocytes treated with arabinogalactan from coffee beans exhibited the production of T helper-1-type cytokines, IL-12p40 and IFN-gamma. In the *in vivo* study, the production of IL-12p40 in mice that ingested arabinogalactan from coffee beans (2.5 mg/day) increased as compared to control mice. These results show that arabinogalactan from coffee beans can stimulate immunocytes and enhance Th1 immune responses.

INTRODUCTION

Type II arabinogalactan (AG) composed of beta- (1→3)-linked galactan main chain with frequent arabinose and galactose residues containing side chains (Clarke and Vitzthum, 2001). AG from coffee beans is contained in the coffee beans up to 17% (Redgwell et al., 2006). Experimental analysis has determined molecular weights of AG from coffee beans ranging from 150,000 to 2,000,000 (Redgwell et al., 2002).

Many immune responses are controlled by the proportion of T helper (Th) 1 to Th2. Interleukin (IL)-12 produced by macrophages and dendritic cells (DCs) induces Th1 development and interferon (IFN)-gamma production by T cells. Th1 response has been shown to play an essential role in defense against tumors and microbial infections.

It is reported that various polysaccharides potentiate Th1 immune responses. However, the processes of immunoenhancing in AG from coffee beans is not yet understood.

In the present study, to investigate whether AG from coffee beans are activated the immune cells, we analyzed the proliferation of mouse splenocytes and macrophages stimulated with AG from coffee beans, IL-12 or IFN-gamma secretion of mouse splenocytes and bone marrow derived dendritic cells (BMDCs) stimulated with AG from coffee beans, and the effect of the administration of AG on IL-12 production *in vivo*.

MATERIALS AND METHODS

Preparation of Arabinogalactan from coffee beans

AG from coffee beans was prepared according to the modified methods of Hashimoto et al. (1971), Wolfrom et al. (1960), and Bradbury et al. (1990). The commercial quality (Indonesia AP-1) coffee beans obtained in 2004 from Java Island, Indonesia, were used. The powdered beans (100 g) were extracted for 2 h in 1000 ml water at 121 °C and sediment was removed by centrifugation (5000 rpm, 30 min). The hot water extract (1000 ml) was extracted with 1000 ml of 0.2 M NaOH (25 °C, 6 h). Then, the extract was adjusted to pH 7.5 and sequentially extracted with chloroform (1 h), ethyl acetate (1 h), diethylether (1 h). Then, the aqueous layer was adjusted to pH 8.0, and 300 mg of Trypsin (Nacalai Tesque Inc. Kyoto, Japan) was added while stirring continuously for 72 h at 50 °C. After 72 h, the mixed solution was filtered and the supernatant was dialyzed in water for several days. Then, 100 ml of NaClO was added to the solution at 60 °C for the purpose of degrading polyphenolic compounds and ethanol (98%) was added and stirred constantly until it reached a final concentration of 75%. The supernatant and precipitate was centrifuged, the supernatant was discarded and the precipitate was dialyzed in water for 2 weeks and freeze-dried.

Molecular weight distribution

A size-exclusion chromatography was performed on a column (100 cm x 16 mm) of Sephadex G-200 (GE Healthcare Bio-Sciences Co.Ltd., NJ). A 10 mg of sample was eluted with a 50 mM phosphate buffer (pH 6.8) containing 100 mM NaCl at a flow rate of 20 ml/h.

Preparation of splenocytes and peritoneal macrophages

7-10 weeks aged specific pathogen-free BALB/c mice splenocytes and peritoneal macrophages were prepared as described previously (Choi et al., 2005) with some modifications. Briefly, splenocytes were suspended in RPMI 1640 supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 x 10^{-5} M of 2-mercaptoethanol and placed at densities of 5 x 10^5 cells /well in 96-well plates and 30 x 10^5 cells /well 24-well plates for proliferation and cytokine determination, respectively.

Peritoneal exudates cells (PECs) were obtained with ice-cold Hank's balanced salt solution (HBSS) and plated at densities of 1 x 10^5 cells/well in 96-well plates. PECs were incubated for 2 hours at 37 °C. Non-adherent cells were removed by washing four times with cold HBSS and adherent peritoneal macrophages were cultured with RPMI 1640 medium.

Isolation of bone marrow derived dendritic cells (DCs)

A modification of the methods of Inaba et al. (1992) was used. The bone marrow cells (10^6 cells/well) were placed in 24-well plates in 500 μl of RPMI 1640 complete medium supplemented with 4 ng/ml of IL-4 and 10 ng/ml of GM-CSF. Every 2 days, cells were washed with medium to remove non-adherent granulocytes. After 6-day cultures, cells were analyzed for the effects of AG from coffee beans on cytokine production.

Administration procedure

BALB/c mice (9 week old) were used in the oral administration study. Two group of study were established: a control group with water to drink, and a group receiving a beverage that

provided them with 2.5 mg/day of AG from coffee beans. After 1-week administration, the plasma samples from the mice were collected for cytokine assay.

Proliferation assay

Splenocytes and macrophages were stimulated with AG from coffee beans (0.125, 0.25, 0.5 μg/ml), LPS (20 μg/ml), Con A (20 μg/ml) or medium as a negative control. The simulation was a period of 1 hour for splenocytes and of 4 hours for macrophages. These proliferations were measured by the WST-1 reagent (Takara, Otu, Japan) according to manufacturer's instructions using Microplate Reader Model 550 (Bio-Rad Laboratories, Alfred Nobel Drive Hercules, California) at 450 nm (605 nm reference filter). Proliferation activity was calculated as a ratio of the control.

Cytokine production by ELISA

Splenocytes and BMDCs were stimulated with 0.25 μg/ml of AG from coffee beans and medium as negative control for 20 hours at 37 °C in 5% CO_2. The plasma samples and the culture supernatants were collected and stored at –80 °C until needed for the assay. The plasma samples and the culture supernatants were assayed for IL-12+p40 and IFN- gamma using commercially available ELISA kits (BioSource International, Inc., Camarillo, California) according to manufacturer's instructions.

RESULTS

Molecular weight distribution

Analysis by gel-filtration chromatography on Sephadex G-200 column using standard pullulan markers showed that the AG from coffee beans indicated the presence of a single peak and had an apparent molecular weight distribution of 90.3-197 kDa. The molecular weight range of AG from coffee beans samples is a fairly narrow. Redgwell et al. reported that AG from coffee beans with a wide range of molecular weights, from 500 to 2000 kDa (3). However, Leloup et al. (1993) reported that the molecular weight range of water extractable carbohydrates (predominantly galactomannans and AGs) from green arabica beans was found to be up to 200 kDa, and their results agreed with ours.

Carbohydrate composition of AG from coffee beans

The galactose / arabinose ratio of AG from coffee beans was 2.6. This result was similar to the major AGP fraction isolated and purified from green coffee beans in a resent study (Redgwell et al., 2002; 2005). Based on the sugar composition of AG from coffee beans samples, it is speculated that this population consists of AG.

Proliferation of splenocytes and macrophages stimulated with AG from coffee beans

The proliferation of splenocytes and macrophages stimulated with AG from coffee beans at concentrations ranging from 0.125 to 0.5 μg/ml was significantly increased (Figure 1A, B).

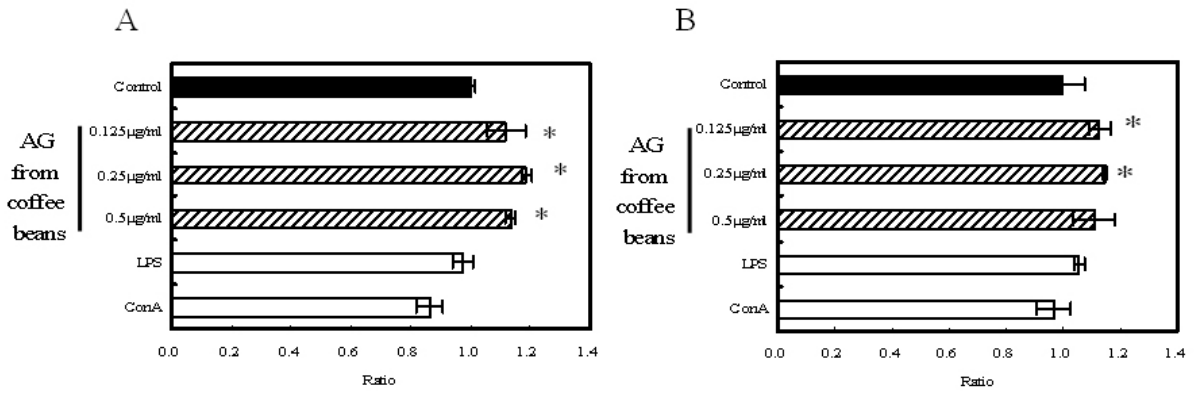

Figure 1. Proliferation of splenocytes (A) and macrophages (B) treated with AG. Proliferation is expressed as the ratio of absorbance relative to the control absorbance at 450 nm. The results are expressed as means ± SD (n = 3). $^{*}p < 0.05$ v.s. Control. Results were analyzed using ANOVA and multiple comparison tests.

Cytokine production of splenocytes and BMDCs stimulated with AG from coffee beans

Th1 type cytokine IL-12 of splenocytes incubated with AG from coffee beans was 1.2 times greater than the control (data not shown) IL-12 and IFN-gamma production in BMDCs stimulated with AG from coffee beans was 1.6 times and 25 times higher, respectively, compared with the control ($p < 0.01$) (Figure 2A, B).

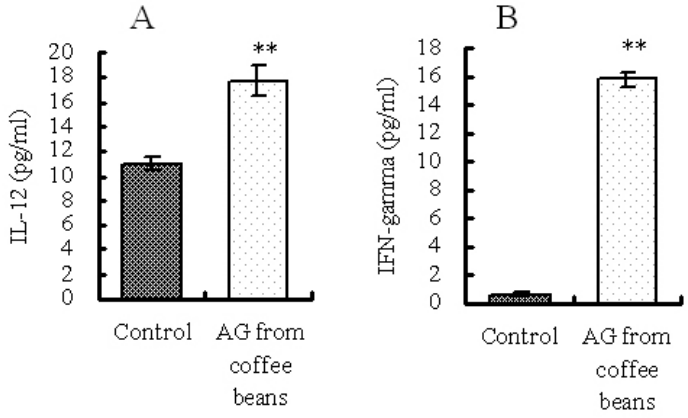

Figure 2. Quantitative analysis of IL-12 and IFN-gamma production from DCs. DCs were stimulated with AG for 20 hours. We used the cells, which were stimulated by only medium, as control. The results are expressed as means ± SD (n = 3). $^{**}p < 0.01$ v.s. Control. Results were analyzed using Student's t-test.

In vivo effects of AG from coffee beans on production of cytokines

A significant increase in IL-12 production was observed in the mice treated with AG from coffee beans compared with the control ($p < 0.01$) (Figure 3).

DISCUSSION

In this study, we reconfirmed that AG is contained in coffee beans and obtained the fact that the molecular weight of our purified AG from coffee beans ranges from 90.3-197 kDa. This finding is larger than AG from other plants. Furthermore, we have demonstrated that AG from

coffee beans enhanced proliferation of splenocytes and peritoneal macrophages and activated production of Th1-type cytokines, IL-12 and IFN-gamma. We also showed that AG from coffee beans raised the level of IL-12 in mouse plasma. Our results suggest that AG from coffee beans may be immunomodulating properties.

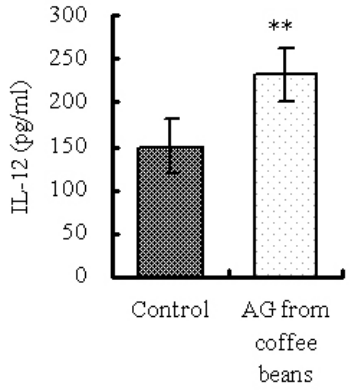

Figure 3. Quantitative analysis of IL-12 production in plasmas from mice, which were administered with AG (2.5 mg/day). We used the plasma, which was from mice administered with only water, as control. The results are expressed as means ± SD (n = 3). **p < 0.01 v.s. Control. Results were analyzed using Student's t-test.

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Coffee and Sexuality

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SUMMARY

Coffee consumption has played various and controversial roles in human sexuality since the advent of its use without much in the way of scientific validation. Contemporary science has clarified the relationship of coffee (caffeine) to sexuality. Its actions include central nervous system stimulation, glucagon enhancement, and phosphodiesterase-5 inhibition. Despite intriguing findings, there is no conclusive evidence that coffee might aid in the treatment of sexual dysfunction. Nevertheless, science supports long held beliefs and that coffee's role in sexuality may be the promotion of endurance, positive behavior, and an alert response.

INTRODUCTION

Kaldi was a mythical Ethiopian goat herder believed to have discovered coffee. Arabians and Western Europeans subsequently adopted its use. Its rise in popularity incited reaction based on the hypothesis that coffee along with tea was an intoxicant and influenced social behavior including sexuality.

Twentieth century science supported the thesis that coffee (caffeine) was related to sexuality biochemically and behaviorally. This report reviews some elements of social history and scientific inquiry relating to coffee and sexuality. Our review includes political and religious historical texts, and twentieth century science.

HISTORY OF SOCIAL DEVELOPMENT

According to the myth, Kaldi found his flock prancing around after grazing on the bright red, cherry-like fruit of a shrub native to Kaffa, Abyssinia in northeastern Africa where it grew wild. After indulging, Kaldi was noted by pilgrims on their way to Mecca to be dancing among his herd. The pilgrims were delighted to discover that the fruit enabled longer prayers and greater concentration, especially when the seeds were roasted, ground and steeped in hot water. They called it "Kahwah" or "Cahuah". Yemen Mocha was the first coffee to be commercially cultivated (Ukers, 1922).

Kiva Han, the world's first coffee shop, opened in Constantinople in 1475. From there, its use spread throughout Arabia in the 16th century (Bersten, 1999). However, conservative Islam discouraged its use regarding it as an intoxicant and therefore a product of the devil's work. The early sixteenth century Turkish siege of Vienna introduced coffee to the Viennese. Thereafter, its use spread across Europe. Once again, reaction occurred. This time it involved the church. Pope Clement VIII is credited for saving the day for us by saying "Why, this Satan's drink is so delicious that it would be a pity to let the infidels have exclusive use of it" (Ukers, 1922).

Bach's Coffee Cantata, 1732-1735, was a most popular secular composition (Ukers, 1922). It addressed a recurring theme relating coffee to sexuality. "Ah! How sweet coffee tastes! Lovelier than a thousand kisses." It concluded with the aria "no wooer may come to the

house, unless he promises me himself, and has it put in the marriage contract that he will allow me to make coffee whenever I will!"

The coffee craze hit London in mid 16th century. Once again reaction occurred. Charles II, who was incidentally no model of decorum himself with his many mistresses, regarded coffeehouses as centers of sedition (Bersten, 1999). In response to the "Women's Petition Against Coffee," he closed all coffee houses in Britain. The proclamation read, in part, "Whereas it is most apparent that the multitude of coffee houses of late years set up and kept within this kingdom ... and the great resort of idle and disaffected persons to them, have produced very evil and dangerous effects; His Majesty hath thought it fit and necessary; that the said coffee houses be put down and suppressed." Public protest forced him to reverse his decision two weeks later. Frederick the Great of Prussia also tried to block coffee with a protective tariff against importation (Ukers, 1922). At the same time, concerns were raised about coffee and sexuality.

In the 1670's, British wit zeroed in on coffee. The Huntington Library and Gardens in San Marino, Californa houses a collection of such pamphlets. The language is unauthored, raw Elizabethan street talk. One writer's image of a coffee house character is that "He is so refractory to divinity that morality itself cannot hold him, he affirms human nature knows no such things as principles of good and evil, and will swear all women are whores, though his mother and sister both stand by" (Anonymous, 1673). This was answered with a medically interesting vindication that coffee was good for "expelling wind; fortifying the liver, refueling the heart, corroborating the spirits, both vital and animal, quickening the appetite, afflicting digestion, helping the stone, taking away rheums and dysreflexias" (Anonymous, 1673). Finally, *A Lash for the Parable-Maker under the Allegory of Apes and Monkeys* read in part: "But this discourse was interrupted by a She-monkey of a Mercury, that cried about the Coffee-House, the restored Maidenhead; What would not some She-monkeys about this town give to have their Maidenheads restored, that they might lose them as often?" (Anonymous, 1691). With each era, coffee was perceived as an intoxicant as well as a sexual stimulant for better or for worse.

CONTEMPORARY SCIENCE

Biochemical and behavioral scientific investigations of caffeine undertaken in the later half of the 20th century helped to define coffee's influence on human sexuality. Earl W. Sutherland, Jr. with T. W. Rall discovered cyclic adenine monophosphate (cAMP). For this, Sutherland won the Nobel Prize in physiology and medicine. In the 1960's, his group identified phosphodiesterase-5 (PDE5) inhibitors, which were deemed responsible for mediating energy to actively relax smooth muscles such as those in the pulmonary bronchioles and genital cavernosal tissue. This was a brilliant but counter-intuitive concept. The first agent they studied in this regard was caffeine. Indeed their group has stated that caffeine was the first PDE5 inhibitor to be identified (Corbin and Francis, 2003). They also found that caffeine enhanced glucagon thereby causing accumulation of cAMP and conversion of stored glycogen to glucose.

Nitric oxide is involved in the nonadrenergic, noncholinergic neurotransmission that leads to the smooth-muscle relaxation in the corpus cavernosum thereby permitting penile erection (Rajfer et al., 1992; Goldstein et al., 2002). Sildenafil blocks breakdown of cyclic guanosine monophosphate (cGMP) and drives the reaction towards dilation (Corbin and Francis, 1999). Today's list of PDE inhibitors includes sildenafil, caffeine (dipyridamole), an experimental peripheral vasodilator (3-isobutyl-1-methylxanthine, IBMX) and theophylline (a common agent for treating asthma). Sildenafil has the highest relative potency of these PDE5 inhibitors

with caffeine falling in the middle followed by IBMX and theophylline. Caffeine and sildenafil both have similar structural sites capable of binding to allosteric cGMP binding sites (Figure 1).

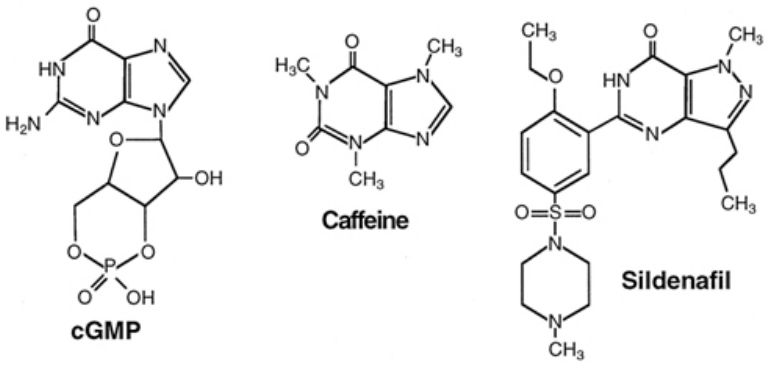

Figure 1. Structure comparison of PDE5 inhibitors with cGMP.

It appears that we may have come full circle, that science may have ratified the past, that "Coffee, Tea *And* Me" may have scientific as well as historical and social significance.

Caffeine is said to be the most widely used stimulant in the world (Guarraci and Benson, 2005). In athletics, it has been said that caffeine may be of ergonogenic benefit during endurance exercise performance, especially when glycogen depletion would be rate limiting to performance (Tarnopolsky, 1994). Moderate doses of caffeine might have influenced the behavior of female Texas rats as well as the women of Charles II and Frederick the Great. A recent Texas study showed that caffeine altered locomotor mating behavior of the female rat in a dose related fashion by decreasing the latency to return to the male following ejaculation (Guarraci and Benson, 2005). In the 1950's, similar findings were reported for male rats: decreased latency to first sexual activity and increased rate of copulation (Zimbardo and Barry, 1958). In the Texas study of female rats, caffeine also influenced sexual motivation. It selectively increased entries of female rats into the male chamber. The controls were pregnant females. The authors concluded that the effects of caffeine on female mating behavior, like the male, might have reflected an increase in both locomotor activity and sexual motivation.

CONCLUSIONS

Coffee consumption has historically played various and controversial roles in human sexuality since the advent of its use. Until now, however, despite suggestive findings, there is no conclusive evidence that coffee might aid in the treatment of sexual dysfunction. Notwithstanding, science supports long held beliefs and that coffee's role in sexuality may be the promotion of endurance, positive behavior, and an alert response.

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Untargeted Metabolomics as a Novel Tool in Coffee Research

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SUMMARY

In this report we introduce metabolomics as a promising approach to study the metabolite composition of the coffee plant and its products in a comprehensive manner. Using both gas chromatography and liquid chromatography coupled to high resolution mass spectrometry, we were able to detect and compare thousands of mass signals likely representing hundreds of compounds simultaneously. Here, we studied the alterations in the metabolite profiles upon open air-drying of fresh ripe fruit of *Coffea arabica* (50% moisture) towards fully-dried green coffee beans (11% moisture). Various patterns of alterations in relative metabolite abundance upon drying could be discriminated, suggesting differential and specific effects on metabolites and biosynthetic pathways. The example provided clearly indicates that metabolomics can provide novel insights in coffee metabolism. The wide-ranging analyses approaches will greatly enhance our possibilities to find novel markers for quality traits or genotypes, to monitor and control changes occurring upon pre- and post-harvest treatments, and to unravel coffee biology.

INTRODUCTION

Metabolomics is the latest – omics tool and aims to study the metabolic composition of organisms, tissues or cells in an as detailed as possible manner (Hall et al., 2002; Hall, 2006; Kopka et al., 2004; Bino et al., 2004). By comparing the metabolomes of different samples detailed insight in the similarities and/or differences in metabolic pathways between the samples under investigation can be obtained. This information can be coupled to specific characteristics or traits such as genotype, plant growth, disease resistance, after-harvest processing, product quality, etcetera. As such, metabolomics is fully complementary to genomics, tanscriptomics and proteomics. Since plants are particularly rich in chemically diverse metabolites, which can be present at a large range of concentrations, no single analytical method is currently capable of extracting and detecting all metabolites. Several methods suitable for large-scale analysis and comparison of metabolites in highly complex extracts have been described (Hall, 2006). Yet, gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are currently the most widely used techniques in plant metabolomics.

GC-MS based applications mostly involve derivatization of metabolites after extraction from plant tissues. This approach covers of large variety of non-volatile metabolites, mainly those involved in primary metabolism, including organic acids, amino acids and sugars (in polar extracts) as well as lipophilic compounds (in apolar extracts) (Lisec et al., 2006; Roessner-Tunali et al., 2003). A different GC-MS based metabolomics application, without

derivatization, is profiling compounds that are naturally volatile, such as volatile esters, alcohols, aldehydes and mono- and sesquiterpenes (Tikunov et al., 2005). The preferred technique for profiling of semi-polar compounds is definitely LC-MS. Compounds that can be detected by LC-MS involve a number of plant primary metabolites, like citric acid and phenylalanine, but more specifically the large group of plant secondary metabolites, such as alkaloids, phenolic acids, phenylpropanoids, flavonoids, polyamines, saponins and all kinds of derivatives thereof (Moco et al., 2006; Verhoeven et al., 2006).

Since standards are commercially available of most primary metabolites, GC-MS can produce quantitative data for up to a few hundreds of compounds involved in central cellular metabolism (Kopka et al., 2004; Lisec et al., 2006; Roessner et al., 2000). For LC-MS, however, the number of commercially available standards of secondary metabolites is still very limited per plant species or tissue. Thus, an essentially untargeted approach in which all mass signals present in the datasets are taken into account will provide sample comparison at a more comprehensive level (Vorst et al., 2005). In such approach, all mass signals from both known and unknown compounds are extracted and aligned across all samples. After applying appropriate statistics and multivariate analyses tools, mass signals correlating with a specific quality trait or genotype are then identified as far as possible, e.g. by using accurate mass calculations, MS/MS fragmentation and comparison with standards (if available). Examples of such metabolomics approaches in plant research are the comparison of Arabidopsis (*Arabidopsis thaliana*) plants (von Roepenack-Lahaye et al., 2004), mutants of tomato (*Solanum lycopersicum*) fruit (Bino et al., 2005), tubers of potato (*Solanum tuberosum*) genotypes and different harvest times (Vorst et al., 2005), tissue-specificity of metabolic pathways in tomato fruit (Moco et al., 2006), and identifying quantitative trait loci (QTL's) controlling metabolite composition in Arabidopsis (Keurentjes et al., 2006).

Raw (green) coffee beans are known to contain a high diversity of metabolites including natural volatiles, polar compounds such as sugars and amino acids, semi-polar compounds like alkaloids, chlorogenic acids and other phenylpropanoids, and apolar compounds like fatty acids and sterols. The exact metabolite composition of the raw beans depends on various factors including genetic origin, growth conditions, (a)biotic stress, fruit and seed development, and coffee processing treatments. In fact, most studies on coffee metabolites aimed to describe the relationship between chemical composition of the raw beans and the quality of the final beverage (Campa et al., 2005; Clifford, 1985) or to detect differences between genotypes (Andrade et al., 1998; Casal et al., 2003; Martín et al., 1998). During the last decades, these studies provided relevant information on the levels and quality importance of these specific compounds. However, large-scale comprehensive metabolomics approaches may greatly enhance the possibilities to find novel markers for quality traits and genotypes, to establish unforeseen but relevant alterations upon pre- and postharvest-treatments and to unravel coffee biology.

In this paper, we introduce metabolomics as a novel tool in coffee research. As an example of its potential power, we investigated the alterations that take place in metabolome of *Coffea arabica* seeds upon sun-drying from fresh fruits towards the final dry state, using both GC-TOF MS and LC-QTOF MS techniques.

MATERIAL AND METHODS

Plant material

Ripe fruits of *Coffea arabica* L. (variety Topasia) were selected and processed in the Federal University of Lavras, Brazil. The dry-processing method was used and drying was carried out

in open air in the yard. During drying samples were taken by immediately freezing entire fruits in liquid nitrogen. Subsequently, the exocarp and endocarp were manually removed from the frozen fruits, avoiding contamination and thawing of the endosperm. Samples of fresh seeds (non-dried, 50% moisture) and at moisture contents of about 40, 30, 20 and 11% were sent to Plant Research International, The Netherlands, on ice and stored at -80ºC before analyses.

Metabolite extraction

Frozen seeds were ground into a fine powder in liquid nitrogen. For LC-MS analyses, frozen powder corresponding to 100 mg dry matter was weighed and extracted with 4 ml of 75% aqueous-methanol acidified with 0.1% formic acid, by taking into account the exact water content of each sample. After sonication for 15 min and centrifugation for 10 min, the samples were filtered over a 0.2 µm teflon filter. For GC-MS, an equivalent of 50 mg dry matter was weighed, and methanol and water were added such that the water content in all samples was 4% (v/v) in a total volume 1.4 ml. A two-phase extraction method was used to separate polar and apolar compounds (Lisec et al., 2006), using ribitol as an internal standard. The polar water-methanol extracts were taken and completely dried under vacuum.

Metabolic profiling

Two different metabolite profiling techniques were applied, i.e. soft-ionization LC-MS of semi-polar extracts and electron impact GC-MS of derivatized polar extracts. In case of LC-MS, metabolites were separated by reversed phase HPLC on a Luna C_{18} column (150 x 2.0 mm; Phenomenex) and detected online firstly by a photodiode array detector and secondly by a high resolution quadrupole time-of-flight mass spectrometer (QTOF-MS Ultima) equipped with a separate lock mass spray for accurate mass calculation (Moco et al., 2006). Mass spectra of ions (mass 100 to 1500) were collected every second. In case of GC-MS, dried polar extracts were derivatized by methoximation and trimethylsilylation (Lisec et al., 2006) using a CombiPal robot for on-line derivatisation. Samples were injected in a GC6890N gas chromatograph (Agilent Technologies), compounds separated on a ZB50 capillary column (30 m x 0.32 mm; Phenomenex) and detected by a Pegasus III time-of-flight mass spectrometer (LECO) at a scanning rate of 20 spectra per second (mass 50-600).

Data processing

Metalign™ software (www.metalign.nl) was used to extract per analytical method all mass signals detected, to align these signals across the samples and to visualize differential mass peaks between (groups of) samples (Moco et al., 2006; Vorst et al., 2005; Bino et al., 2005). Mass signal intensities were nlog-transformed and loaded into GeneMaths (Applied Maths, Belgium) software for multivariate analyses and data-clustering.

RESULTS AND DISCUSSION

Coffee extracts were analyzed by both GC-(TOF)MS and LC-(QTOF)MS. Visual inspection of the LC-MS chromatograms (Figure 1) revealed a number of different metabolites involved in secondary metabolsim, many of which have been previously reported to be important in coffee quality. Thus, a number of phenylpropanoids (including several mono- and dicaffeoylquinic acids) and alkaloids appeared as prominent peaks in the LC-MS profiles.

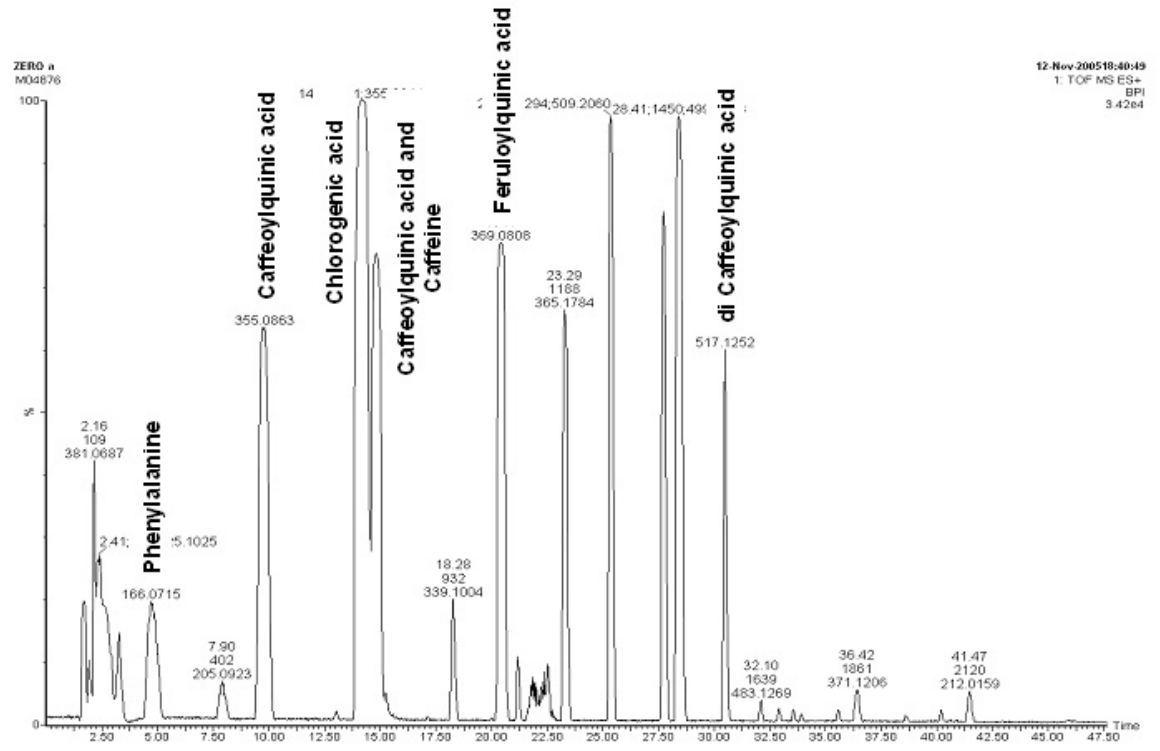

Figure 1. Example of LC-QTOF MS chromatogram of green coffee. Sample (fresh beans, 50% moisture) was extracted with aqueous-methanol and analyzed in ESI-positive mode. Compounds indicated are some examples of metabolites identified based on their accurate mass and absorbance spectra.

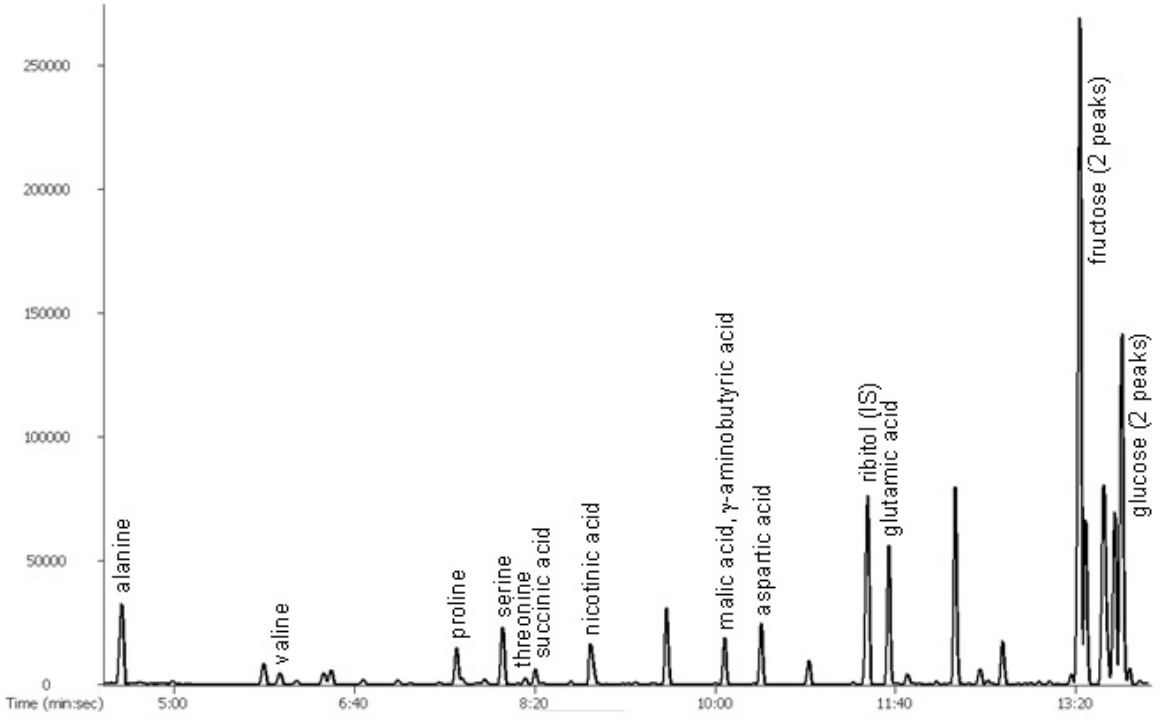

Figure 2. Example of GC-TOF MS chromatogram of a derivatized polar extract of green coffee (fresh beans; 50% moisture). Some compounds identified are indicated. Ribitol is used as internal standard (IS).

In the GC-MS chromatograms (Figure 2) metabolites mainly involved in primary metabolism, such as sugars including sucrose, organic acids and amino acids, were visible, next to the coffee-specific compounds trigonelline and caffeine.

Using the dedicated Metalign-software, per sample about 2,000 relevant mass signals (signal to noise ratio > 3) could be extracted from the LC-MS chromatograms. Though LC-MS is a so-called soft-ionization technique meant to produce ions from the entire molecule (parent ions), other signals such as natural isotopes and unintended fragmentation and adduct formation from the parent ion are frequently observed as well. Thus, the total number of coffee metabolites detected by LC-MS is less. Based on results obtained with other plant species (Moco et al., 2006; Vorst et al., 2005; Bino et al., 2005; Keurentjes et al., 2006) we estimate that the total of 2,000 LC-MS signals represent a few hundred of metabolites, mainly of semi-polar nature. Likewise, more than 20,000 mass signals were extracted by GC-MS analysis of polar extracts. As the GC-MS approach involves electron impact ionization meant to obtain many fragments per metabolite, the number of (polar) metabolites detectable by GC-MS is much lower and likely also in the order of a few hundred (Lisec et al., 2006). Anyway, the number of different compounds detectable by these two complementary metabolomics techniques is substantial and may cover a significant part of the coffee bean metabolome.

Using standards, metabolites detected by GC-MS or LC-MS can be quantified. However, facing the facts that the amount of commercially available standards for coffee metabolites is rather limited, especially for secondary metabolites, and that many compounds detected by metabolomics techniques are still unknown (i.e. novel compounds) (Hall, 2006; Vorst et al., 2005; von Roepenack-Lahaye et al., 2004; Bino et al., 2005; Moco et al., 2006), we usually regard all mass peaks as separate signals in data processing and multivariate analyses. Since the same amounts of dry matter were extracted, the (semi-quantitative) data obtained can be directly exploited to compare samples based on the measured intensity of each mass signal. GeneMath software was used to cluster the metabolite signals across the samples (Figure 3A). Next to isotopic forms and other redundant signals from the same metabolite, highly correlated mass signals may reveal metabolites from the same biosynthetic pathway. Mass signals were subsequently grouped according to their overall response upon coffee drying (Figure 3B) and some examples of drying-induced effects on metabolite abundance are shown in Figure 4. As shown by targeted analysis of free amino acids in green coffee (Bytof et al., 2005), the stress related amino acid gamma-aminobutyric acid (GABA) is markedly higher in dry-processed coffee as compared to fresh seed and wet-processed coffee. In our metabolomics experiments on dry-processed coffee, it appeared that a number of compounds, most of which still have to be identified, clearly increased upon seed drying (left pattern, second raw of Figure 3B). Among these was GABA: this compound increased continuously upon drying from 50% to 11% moisture (Figure 4A). Also a specific dicaffeoyl-quinic acid isomer was found to increase upon drying, though its relative level did not increase further from 20% to 11% moisture (Figure 4B). This result suggests that phenylpropanoid dimerization can continue up to water contents as low as 20%. As is obvious from the different patterns observed (Figure 3B), not all metabolites respond to seed drying: citric acid, for example, was not markedly affected (Figure 4C).

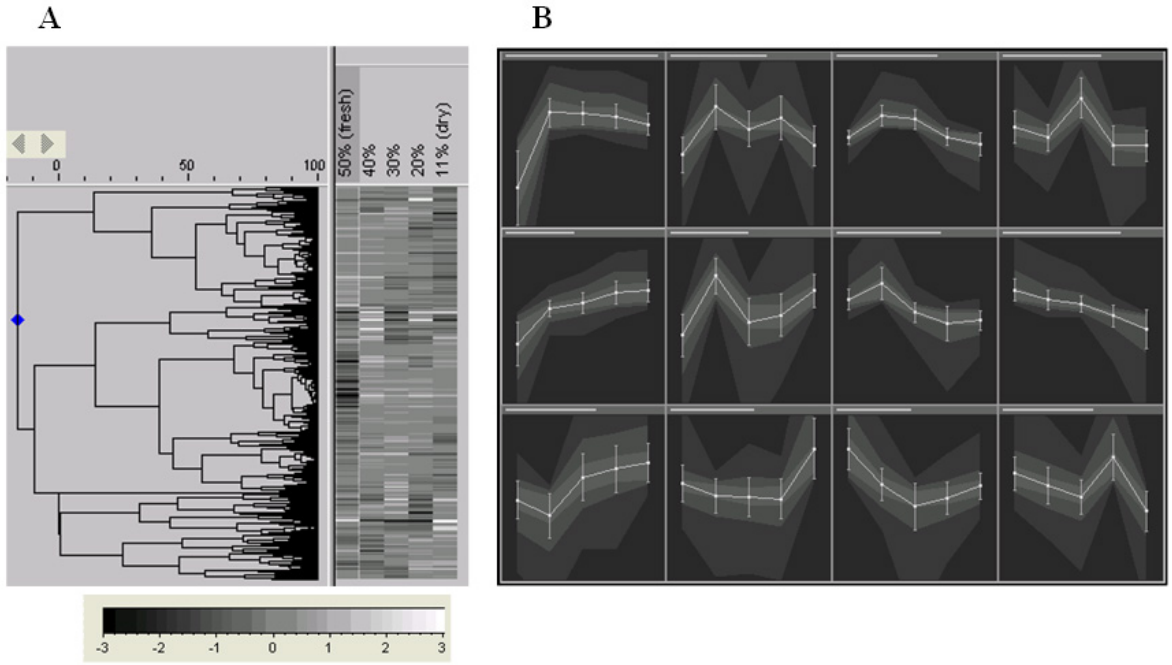

Figure 3. Multivariate analyses of metabolomics data of coffee samples upon sun-drying. Dry-processed C. arabica seeds were allowed to dry in the sun in open air, and samples were taken. Samples at indicated seed moisture levels (% of wet basis) were analyzed in 3 replicates using both GC-TOF MS and LC-TOF MS. Mass peaks were extracted and aligned using Metalign software, mean values of replicate analyses calculated and loaded into GeneMaths software for multivariate analyses. A: Hierarchical clustering (Pearson correlation) of mass signals across samples. Relative abundance of mass signals (n-log data, normalized to raw average) is indicated in grey-scale. B: Self-organizing map of mass signals (using 12 groups) indicating different patterns of response upon coffee drying. Note that the order of samples in each group is the same as in A, i.e. 50%, 40%, 30%, 20% and 11% moisture (from left to right). Error bars indicated at each moisture level indicate the overall variation in signal patterns within each cluster.

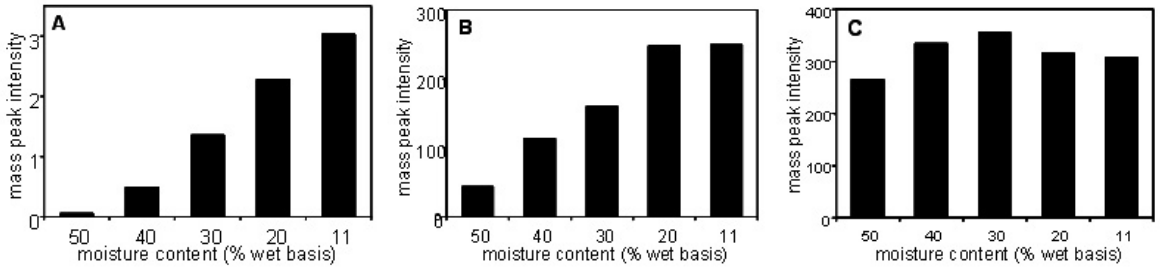

Figure 4. Some examples of metabolite patterns observed upon drying of C. arabica beans. A: gamma-aminobutyric acid (GABA); B: dicaffeoyl-quinic acid (isomer eluting at 30.5 min); C: citric acid

In conclusion, our data indicate that in potential metabolomics is a powerful tool in coffee research. The results obtained with metabolomics approaches are well comparable with those obtained by dedicated analysis targeted towards only a few compounds or a single group of compounds. However, the key advantage of metabolomics is the simultaneous ("multi-parallel") detection of hundreds of compounds from different metabolic pathways. As such, metabolomics approaches provide a more comprehensive view of the metabolome of the coffee plant, which may result in novel insights in the effects of, amongst others, genetic

variation, growth environment, diseases, fruit development, harvest and post-harvest treatments, drying and roasting on the metabolic composition of coffee and finally on the quality of its beverage. In fact, using LCMS- and GCMS-based metabolomics approaches we are currently studying in detail the effects of different post-harvest processing and drying treatments on the green coffee metabolome (manuscript in prep.).

ACKNOWLEDGEMENTS

The authors kindly thank Francel Verstappen and Bert Schipper (Plant Research International) for their excellent assistance in GC-MS and LC-MS analyses, respectively. The preparation of this paper and the work described herein were made possible through funding from the Centre for BioSystems Genomics (which is part of the Netherlands Genomics Initiative and the Netherlands Organisation for Scientific Research).

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A Contribution to the Occurence and Contents of Carboxylic Acid-5-hydroxytryptamides (C-5-HT) in Green and Processed Coffee Beans

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SUMMARY

It is well known that the carboxylic acid-5-hydroxytryptamides (C-5-HT) in coffee wax are on a certain scale decomposed during roasting or steam treatment of coffee beans. Additionally, a decrease in C-5-HT contents can be observed when decaffeinating the beans with dichloromethane. Aside from the influence of the processing methods roasting, steam treatment and decaffeination upon the total C-5-HT contents in coffee beans, the effects of these methods on the up to now known 5-HT, consisting of saturated, unsaturated and hydroxy fatty acids, are presented in this paper. Regarding all three of the processing methods, different impacts on the various C-5-HT were observed. Additionally, the increasing decomposition of C-5-HT in relation to the roasting degree is presented. Whereas varying the steaming parameters has no influence on the total C-5-HT reduction, different decaffeination methods with carbon dioxide and dichloromethane result in distinct differences.

ZUSAMMENFASSUNG

Aus der Literatur ist bekannt, dass Carbonsäure-5-Hydroxytryptamide aus Kaffeewachs bei der Röstung und der Behandlung mit Wasserdampf in gewissem Maß abgebaut werden. Ebenso ist bei der Entkoffeinierung mit Dichlormethan eine Abnahme im C-5-HT-Gehalt zu beobachten. In der vorliegenden Arbeit wurde der Einfluss der Behandlungsverfahren Röstung, Dämpfung und Entkoffeinierung auf die Gesamtgehalte der C-5-HT untersucht. Die Auswirkung der genannten Verfahren auf die mittlerweile bekannten 5-HT mit gesättigten, ungesättigten und Hydroxyfettsäuren wird beschrieben. Es zeigt sich bei allen drei Behandlungsverfahren ein abweichendes Verhalten der verschiedenen C-5-HT. Darüber hinaus wird gezeigt, dass bei steigendem Röstgrad ein vermehrter Abbau von C-5-HT auftritt. Während die Variation von Dämpfungsparametern keinen Einfluss auf die C-5-HT Reduzierung aufweist, werden Unterschiede bei der Entkoffeinierung mit verschiedenen CO_2-Methoden und Dichlormethan deutlich gemacht.

INTRODUCTION

The surface of green coffee beans is covered by a thin waxy layer. The amount of the surface wax is between 2% and 3% of the total coffee lipids. Depending on the solvent, this layer is more or less removed by treating green coffee beans with organic solvents. Some of the main constituents of the coffee wax are the so called carboxylic acid-5-hydroxytryptamides (C-5-HT). This substance group, amides of serotonin (5-hydroxytryptamine, 5-HT) and fatty acids with different chain lengths, was first introduced by Wurziger et al. (1968). They isolated and characterised the three dominant 5-HT with arachic, behenic and lignoceric acid (Figure 1).

Lateron, Folstar described stearic acid-5-HT as well as 20-hydroxy-arachic- and 22-hydroxy-behenic acid-5-HT (Folstar et al., 1980). Recently, apart from palmitic acid-5-hydroxytryptamide, our working group identified the unsaturated components octadecadienoic and eicosenoic acid-5-HT and the odd numbered henicosanoic and tricosanoic acid-5-HT in green coffee wax (Kurzrock et al., 2004; Hinkel and Speer, 2005). The latter C-5-HT can be found in coffee wax in very small amounts, only.

The occurrence of the odd-numbered C-5-HT was confirmed by Lang and Hofmann (2005).

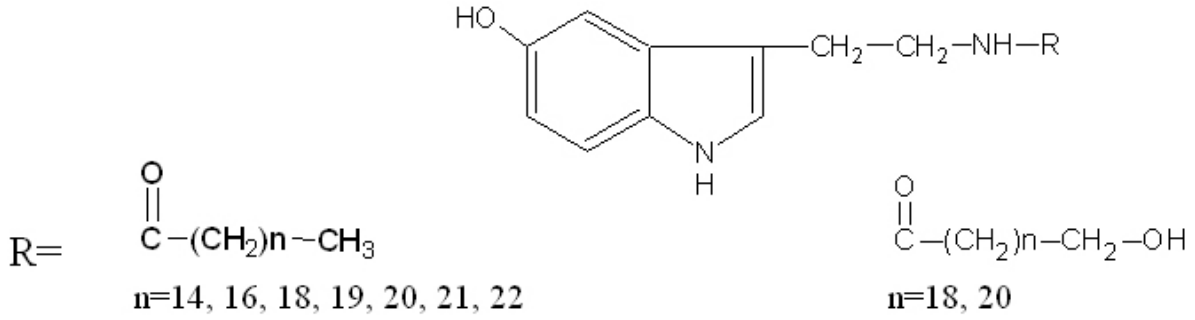


Figure 1. Structural formulae of carboxylic acid-5-hydroxytrypamides.

Aside from the distribution of the up to now known C-5-HT in green coffee beans, the influence of processing methods such as roasting, steam treatment and decaffeination on the 5-HT connected with saturated, unsaturated and hydroxy fatty acids is presented in this paper.

EXPERIMENTAL

Frozen coffee beans were ground in a mill and afterwards sieved. The powder obtained with a particle size of less than 630 µm was used for accelerated solvent extraction (ASE). After dilution, 2 ml were used for clean up by means of solid phase extraction (SPE). To increase the repeatability the RapidTrace system (Caliper) for automated SPE was used. A portion of the purified extract was evaporated and the same amount of mobile phase was added to the residue. After filtration, this solution was used for injection. For the HPLC analysis a Merck-Hitachi LaChrom 7000 system with a fluorescence detector was used. The fluorescence detector was operated at an excitation wavelength of λ_{ex} = 281 nm, and the emission wavelength was set at λ_{em} = 330 nm.

For quantification, standard solutions made of various synthesised reference C-5-HT were used. Since the difference in the concentration levels of minor and major components in the coffee was up to 200 times, two calibration curves were used for the different levels. The contents of the single C-5-HT were calculated on the basis of the synthesised standard and then related to the dry weight of the samples.

RESULTS AND DISCUSSION

In the working range, the run of the calibration curves was linear (Figure 2). The recovery rate of the method was determined to be about 90%, and the limit of detection was 2.5 µg/g at a signal to noise ratio of 3. The variation coefficients were below 5%.

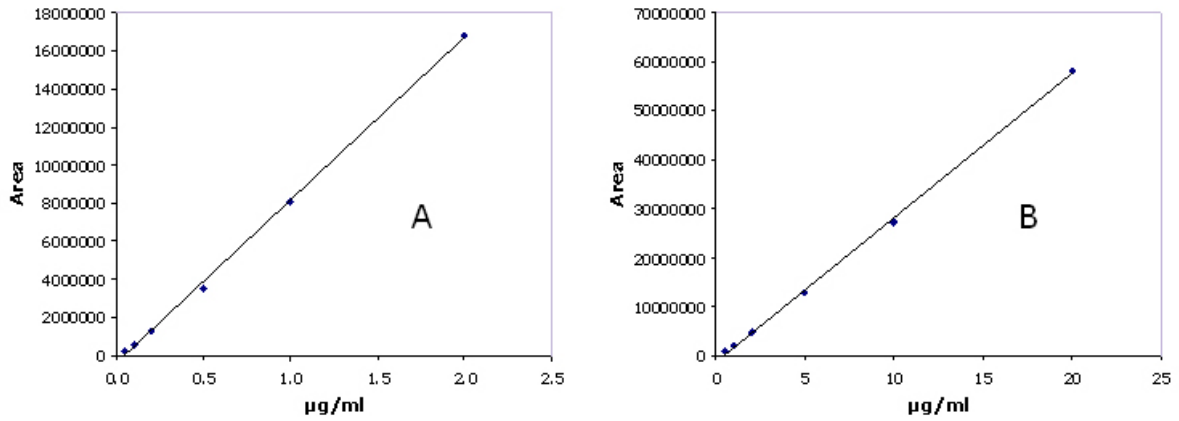


Figure 2. Calibration curves for quantification of the C-5-HT for minor (A) and major components (B)

In Figure 3, a HPLC-chromatogram of a green Robusta coffee is shown. Whereas the minor constituents can be observed in the first fifteen minutes of the chromatogram, the major components C20- and C22-5-HT can be found at retention times greater than 20 minutes.

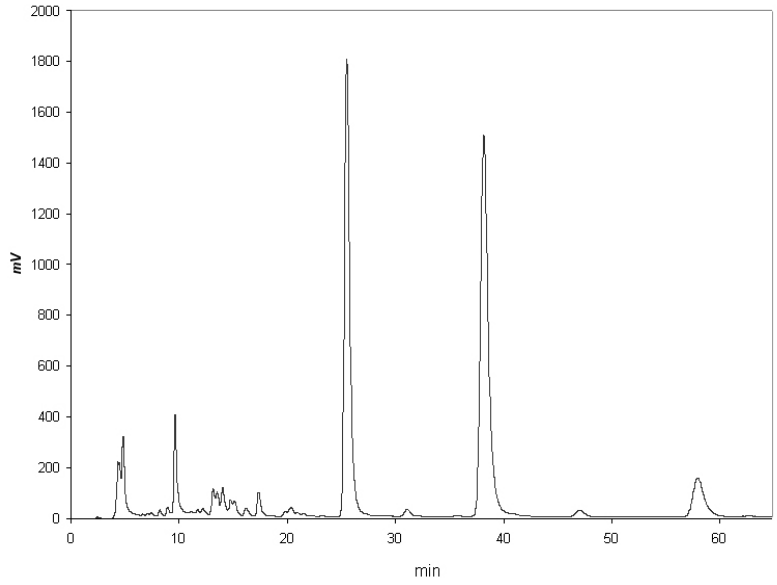


Figure 3. C-5-HT in green Robusta coffee.

C-5-HT in green coffee beans

For determination of the C-5-HT in green coffee beans, 17 Arabica and 22 Robusta coffees were available. Whereas about 90% of the Robusta coffees analysed had total C-5-HT contents below 1200 µg/g, 90% of the Arabica coffees showed values above 1400 µg/g (Figure 4).

Concerning the total content of the C-5-HT in Arabica coffees, it was nearly twice the value of the Robusta coffees (Figure 5). When comparing the peak-area-ratio of the two major components behenic and arachic acid-5-HT, this once again resulted in a factor of two. Whereas in Robusta coffees the signal area ratio of C22- to C20-5-HT is equivalent, it is about 2:1 in Arabica coffees (Figure 6). Hunziker et al. also found some indications of differences in the fatty acid distribution of C-5-HT. But due to the limited number of samples analysed, they could not specify these findings (Hunziker and Miserez, 1979).

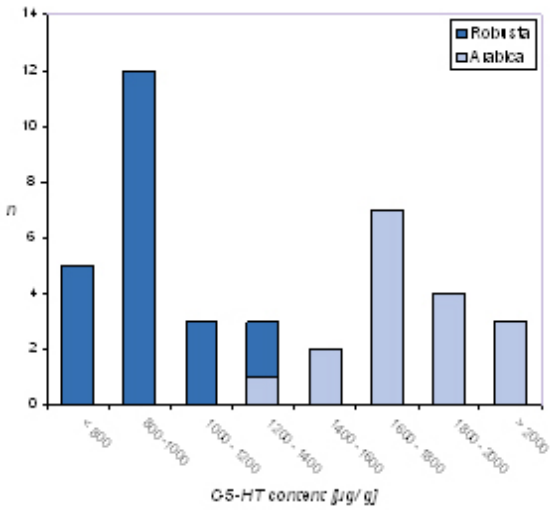


Figure 4. Number of green coffees related to the concentration intervals of the C-5-HT.

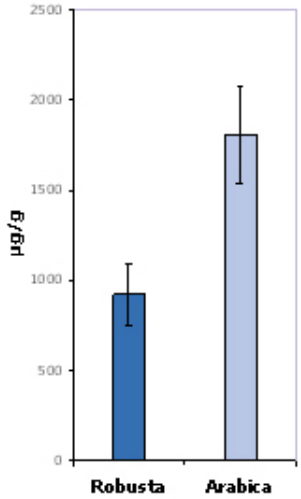


Figure 5. Total C-5-HT content in green coffee*.

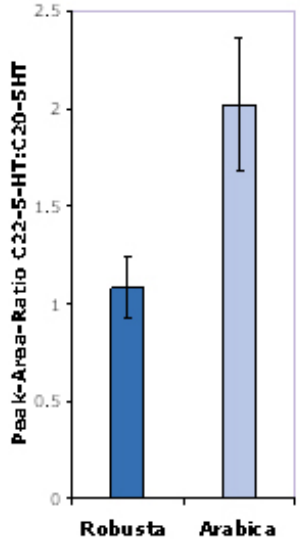


Figure 6. Peak-area-ratio of behenic and arachic acid-5-HT in green coffee beans*.

Although there are clear differences in the total C-5-HT content and the peak-area-ratio of the two major homologues, the percentage of the C-5-HT in Arabica and Robusta is similar. The major part of the C-5-HT in green coffee is represented by the amides with saturated even-numbered fatty acids (94%, D). The percentage of the minor components is 6%, segmented in saturated odd-numbered fatty acids (2%, C), unsaturated fatty acids (1%,B) and in 3% (A) of 5-HT with hydroxy fatty acids (Figure 7).

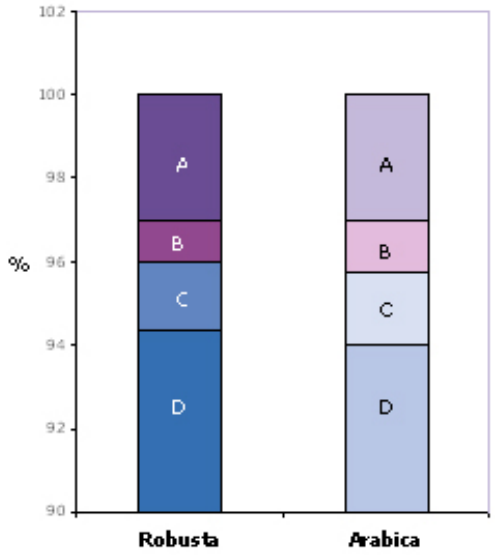


Figure 7. Percentage of C-5-HT groups in green Robusta and Arabica coffees.

C-5-HT in roasted coffee beans

The influence of roasting on the total content of C-5-HT in coffee beans was already described by van der Steegen et al. and Wurziger et al. 30 years ago (van der Steegen and Noomen, 1977; Wurziger, 1969). To get additional information about the roasting impact on the C-5-HT, an industrially roasted Colombia coffee was available at three different roasting degrees.

The influence of low, medium and strong roasting is shown in Figure 8.

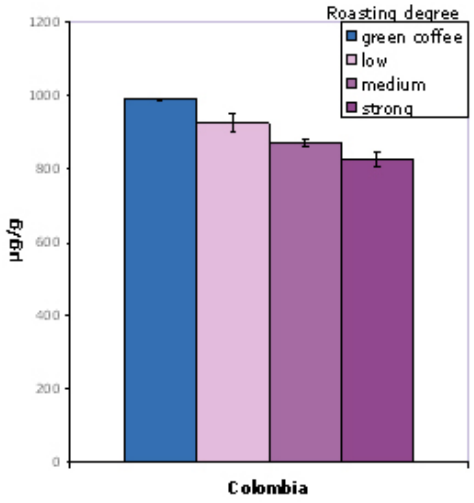


Figure 8. Influence of the roasting degree on the total C-5-HT content (n = 2)*.

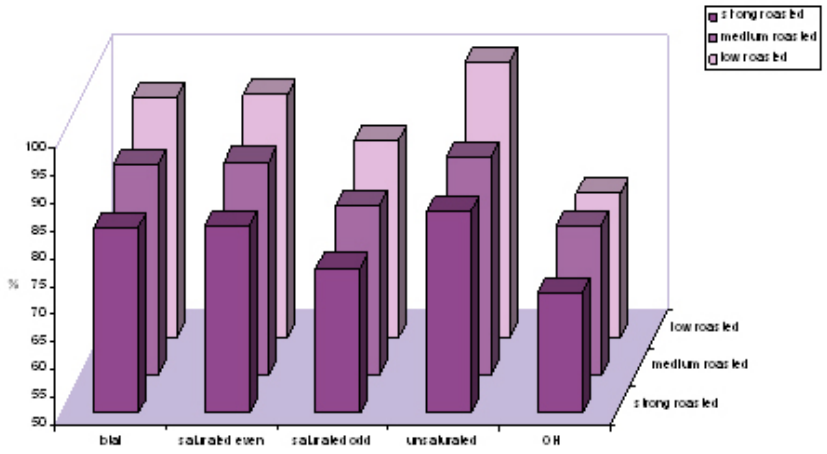


Figure 9. Roasting effects on different C-5-HT groups.

The different roasting degrees are represented by the ratio of the coffee diterpenes cafestol and dehydrocafestol. After roasting, dehydrocafestol is found as a decomposition product of cafestol. The stronger the roasting, the smaller are the values: 36 for the mild roasting, 26 for a medium degree of roasting and 17 for strong roasting conditions.

Depending on the roasting degree, the total C-5-HT content in the analysed Colombia sample is reduced from 7% to 12% and 17%. Regarding the influence of roasting on the different C-5-HT groups, the greatest effect is observed in the 5-HT with hydroxy fatty acids (Figure 9). Whereas the 5-HT of the other groups are reduced in a similar way with increasing roasting conditions, the amount of the hydroxy fatty acids-5-HT is minimized to approx. 20% at mild roasting conditions.

C-5-HT in steam-treated coffee beans

For consumers with a sensible stomach, the coffee brew can have certain irritating effects. Since the Lendrich patent 70 years ago, steam treatment of coffee beans is an appropriate method to reduce these irritating effects (Lendrich et al., 1933).

Aside from the removal of until now not clearly identified irritating substances, the waxy layer of the coffee bean is partially removed when steam-treating the coffee. As a result, a decrease in the total C-5-HT content was described earlier by Wurziger et al. (1973). Apart from similar steamed samples and additionally roasted ones, a sample with varying steaming parameters was analysed.

When comparing the total C-5-HT content of green and treated beans, the loss of C-5-HT is, in accordance to Wurziger et al. (1973), equivalent to 30% (Figure 10).

A variation in the processing parameters time and temperature resulted in a maximum reduction of 30%. In Figure 11 the effects of the steam treatment at two different temperatures for 30 and 120 minutes respectively on the total C-5-HT content are shown. Both at 120 °C and 132 °C, there is hardly any influence on the total C-5-HT from the prolongation of the steaming time. Additionally, the variation of the steaming temperature shows no differences in the amounts of C-5-HT. Therefore, independent from the applied steaming parameters, there is always the same reduction of about 30% of the total amount of the tryptamides.

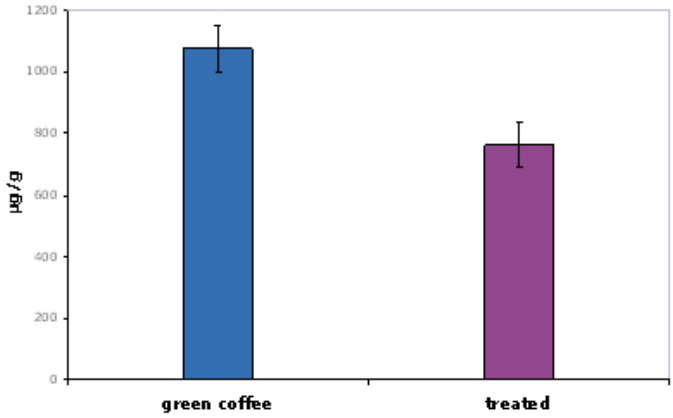


Figure 10. Effect of steam treatment on the total C-5-HT content in green coffee beans (n = 5)*.

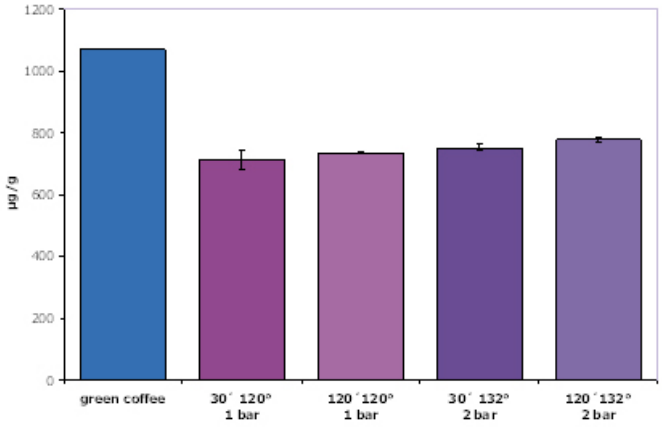


Figure 11. Variation of steaming parameters (n=2)*.

Similar to roasting, 5-HT with hydroxy fatty acids are decomposed to an above average degree (Figure 12). When steaming, the reduction is 40%. The effect of combined steaming and roasting is shown in Figure 13.

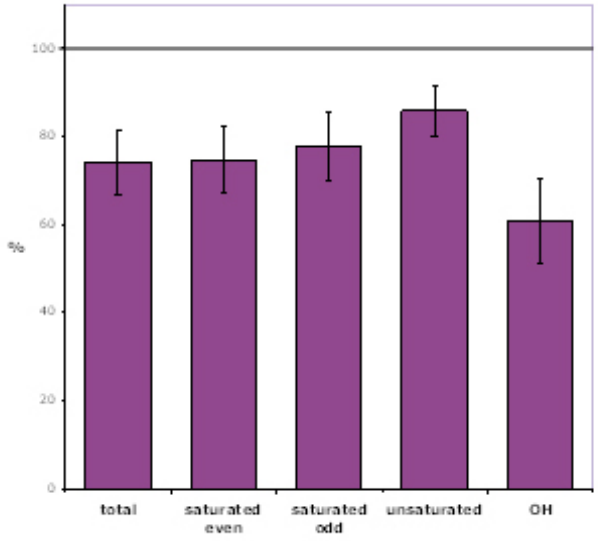


Figure 12. Percentage of remaining C-5-HT after steaming (n = 10)*.

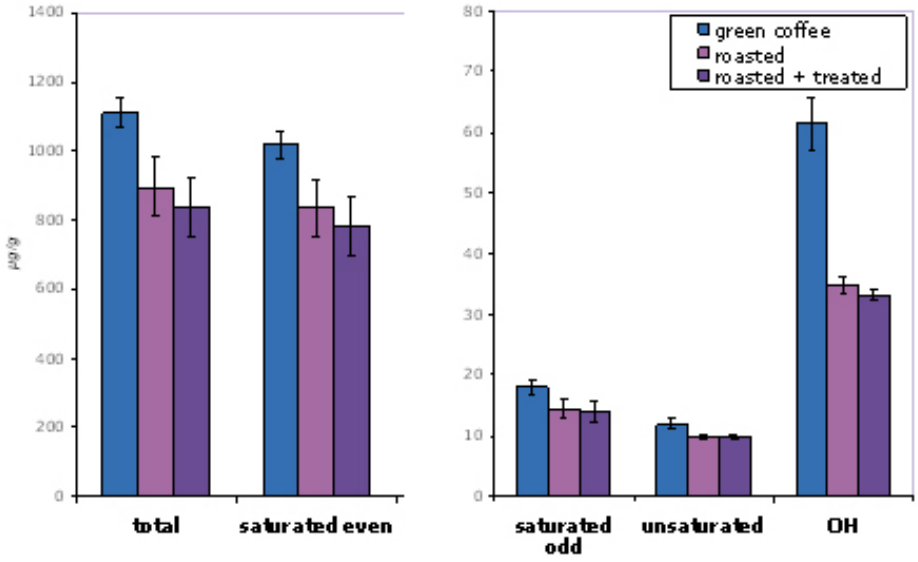


Figure 13. Effects of a combined roasting and steaming on the C-5-HT content of green coffee beans (n = 4)*.

When roasting the green coffee beans, the total amount of C-5-HT is reduced by 20%. An additional steaming of the beans prior to roasting results in a decomposition of 25%. However, the decrease of the hydroxy fatty acids-5-HT is about 40%, which is comparable to the single processing steps of steaming and roasting.

C-5-HT in decaffeinated coffee beans

Nowadays, the removal of caffeine from the coffee beans is carried out either by using supercritical carbon dioxide, water, or the application of organic solvents like ethyl acetate or dichloromethane (DCM). Different green coffee beans and their corresponding decaffeinated counterparts were investigated. The effects of two different decaffeination methods with carbon dioxide as well as the decaffeination of a Robusta and Arabica coffee with dichloromethane were analysed.

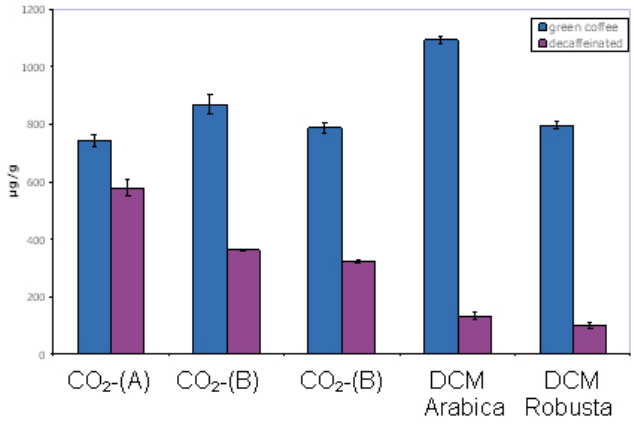
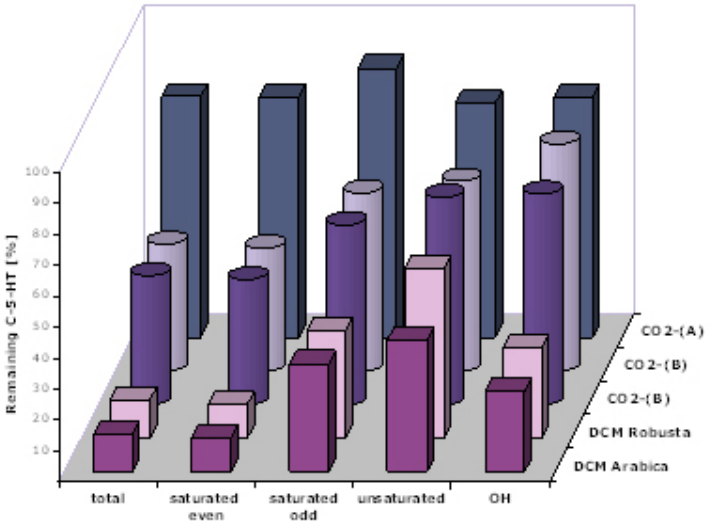


Figure 14. Impact of decaffeination on total C-5-HT content (n = 2)*.

Regarding the influence of the decaffeination method used, there were differences in the effects on the different C-5-HT groups. As is shown in Figure 15, the single C-5-HT groups act in a similar way when applying decaffeination method CO_2-(A). When using method CO_2-

(B) the 5-HT with hydroxy fatty acids are less affected than those with saturated even-numbered fatty acids. A similar picture is obtained when taking a look at the decaffeination with dichloromethane. While the fatty acid amides with saturated even-numbered fatty acids are reduced to 10% of the total C-5-HT content, those with saturated odd-numbered and unsaturated fatty acids show a percentage of 40%.

Whereas method CO_2-(A) leads to a reduction of about 20%, the other method CO_2-(B) decreases the total C-5-HT content by 60% (Figure 14). The greatest effect on the total amount of C-5-HT in the coffee beans can be noticed in reference to decaffeination with dichloromethane. In both, Arabica and Robusta coffee, a decrease of 85% can be observed. This correlates with data from Wurziger et al. (1973).



* *(± S.D.)*

Figure 15. Influence of different decaffeination methods on the percentage of C-5-HT groups.

CONCLUSIONS

The green Arabica coffees analysed here show twice the C-5-HT contents the Robusta coffees. Whereas 90% of the Arabica coffees have values greater than 1400 µg/g, almost the same percentage of Robusta coffees has total C-5-HT contents lower than 1200 µg/g.

The decomposition of the C-5-HT is connected to the roasting degree. With an increasing thermal load, the decomposition of fatty acid-5-hydroxytryptamides rises. Steam treatment of the analysed coffee beans leads to an overall reduction in C-5-HT contents of 30%, independent of the applied temperature and time. The 5-HT with hydroxy fatty acids are more strongly influenced by roasting and steam treatment than those with saturated and unsaturated fatty acids. Regarding decaffeination, the greatest effect in reduction of the C-5-HT can be observed when using dichloromethane, although not all groups are removed in comparable percentages.

ACKNOWLEDGEMENTS

We would like to thank the German Coffee Industry for providing the green and processed coffee samples.

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NIR-based Determination of Differences in Green Coffee Chemical Composition Due to Geographical Origin

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SUMMARY

The final aroma and taste of roasted coffee depends on the content of flavor precursors in green coffee beans, which can be influenced many factors such as coffee variety, growing conditions, and harvesting (post-harvest) conditions. Thus, a good knowledge of the chemical composition of green coffee is indispensable for the development of premium quality products. Classical wet chemistry analysis is time-consuming and costly. Near InfraRed (NIR) spectroscopy is a rapid alternative method that can be employed to assess green coffee composition. A NIR calibration was developed for the main chemical compounds (i.e. moisture, lipids, proteins, sucrose, caffeine, trigonelline, chlorogenic acids, organic acids) on 120 green coffee samples with a wide distribution in geographical origin. The calibration was subsequently used to determine the composition of another 1200 green *Arabica* and *Robusta* coffee samples and the results were statistically interpreted for determination of compositional differences based on geographical origin. Classification models can be established based on geographical origin but they do not validate if applied to other crop years. The results clearly show the potential of the NIR to assess a large number of coffee varieties by using a small but representative reference set. However, they also show the danger of using a predictive instrument for derivation of functional relationships.

RÉSUMÉ

La qualité finale d'une infusion de café dépend de teneur en précurseurs d'arômes et de flaveurs dans le café vert. La balance de ces derniers dépend de facteurs génétiques (espèce, variété), agronomiques (climat, sol) et technologiques (traitement post-recolte). En conséquence, une bonne connaissance de la composition chimique du café vert est indispensable pour développer des produits de haute qualité. Les méthodes chimiques classiques sont longues et coûteuses. Le développement de méthodes rapides telle que la spectroscopie proche infra-rouge (NIR) présente une alternative intéressante pour évaluer la composition chimique du café vert. Une calibration NIR a été développée pour les principaux composés chimiques (humidité, lipides, protéines, saccharose, caféine, trigonelline, acides chlorogéniques, acides organiques) sur la base des données analytiques obtenues pour 120 cafés verts (*Arabica*/*Robusta*) de diverses origines géographiques. La calibration a été utilisée pour déterminer la composition de 1200 cafés verts *Arabica* et *Robusta* et les résultats analysés statistiquement. Des classifications on pu être établies sur la base de l'origine géographique mais. Ces résultats montrent le potentiel du NIR pour évaluer rapidement la composition chimique du café vert à partir d'une sélection restreinte d'échantillons représentatifs. Mais ils montrent également le danger à utiliser le NIR comme outil prédictif de relations fonctionnelles.

INTRODUCTION

The final aroma and taste of roasted coffee depends on the flavor precursors content of the green coffee beans, which in turn depend on many factors such as coffee variety, growing conditions, and harvesting (post-harvest) conditions. Thus, a good knowledge of the green coffee chemical composition is indispensable for the development of premium quality products. Classical wet chemistry analysis is time-consuming and costly. Near InfraRed (NIR) spectroscopy is a rapid alternative method that can be employed to assess green coffee composition.

EXPERIMENTAL

1. **NIR –** NIR reflectance spectra were collected using a scanning monochromator FOSS NIRsystems spectrophotometer (model 6500, Gerber Instruments, Effretikon, Switzerland), using the software WinISI II (Foss-Tecator, Infrasoft International, LLC). The analyses were performed on green coffee beans in a full size rectangular cell. For each sample 16 scans were recorded in reflectance mode in the 1100-2500 nm range in 2 nm steps.
2. **Chemical Analyses** – Moisture, lipids, proteins, carbohydrates, caffeine, trigonelline, chlorogenic acids, organic acids were determined by classical analytical means (Leloup et al., 2004).
3. **Statistical Processing** – Calibration models (PLS) were developed with WinISI II. Discriminant models (PLS-DA) were developed with the SIMCA P11 (Umetrics, Sweden).
4. **Samples** – For calibration and validation 120 samples of green coffee beans were used. The calibrations were used to assess the composition of 1200 samples comprising of both Arabica and Robusta genotypes from South America (Brazil, Colombia, Costa Rica, Guatemala, Mexico), Africa (Ethiopia and Kenya) and Asia (Vietnam and Thailand). The samples comprised of crops from the years 2001 to 2005. Samples from the crop years 2002 to 2004 were used to develop discriminant models while the samples from crop year 2005 were used for the purposes of validating the models.

RESULTS AND DISCUSSION

NIR calibrations to assess green coffee composition parameters are well described in the literature (Esteban-Diez et al., 2004; Vaast et al., 2004; Bertrand et al., 2005) and it will not be insisted upon here. Main statistics presented in Table 1 give an estimate of the calibration goodness of fit and the level of errors that can be expected for determinations of new samples.

The calibration was used to determine the parameters listed in Table 1 for 1200 green coffee samples (both *Arabica* and *Robusta* types). Chemometric analyses were then carried out with the purpose of obtaining classification models based on a wide geographical distribution on both the NIR determined composition and the 2nd derivative NIR spectra.

Table 1. NIR calibration statistics. N = number of samples, STDEV= standard deviation.

Parameter	Calibration Error	Prediction Error	R^2	Mean	Maxim	Minim	STDEV	N
Moisture	0.19	0.23	0.98	9.96	14.33	5.58	1.47	343
Lipids	0.60	0.66	0.93	12.10	18.72	5.47	2.23	113
Caffeine	0.09	0.10	0.98	1.76	3.46	0.05	0.57	116
Trigonelline	0.05	0.06	0.82	0.73	1.09	0.36	0.12	119
Chlorogenic Acids	0.36	0.44	0.93	8.69	12.96	4.42	1.44	119
Organic Acids	0.14	0.16	0.88	2.01	3.20	0.82	0.39	122
Sucrose	0.55	0.63	0.95	6.57	13.64	0.00	2.39	116
Total Nitrogen	0.06	0.07	0.96	2.52	3.38	1.66	0.28	122
protein	0.32	0.39	0.90	12.01	15.05	8.96	1.00	122

PCA carried with the above parameters as variables for the *Arabica* type on the crop years 2002-2004 shows a good separation between samples from South America and Africa (Figure 1). Lipids and protein stand out as the variables with the highest influence on the separation of the two groups. Moreover, in the African group, Ethiopian and Kenyan samples are also clearly distinguishable. An average compositional difference shows that coffee from Ethiopia is higher in lipid contents, while coffee from Kenya is closer to the South American counterparts. A modeling of only the South American samples (Figure 2 with only the clusters for Colombia and Brazil shown for clarity) clearly distinguishes between Brazil, Colombia, Salvador and Ecuador, while samples from Nicaragua, Costa-Rica, Guatemala and Mexico form a mixed cluster. Again an average compositional difference shows that the green coffees from Brazil and Salvador have the higher protein content, green coffee from Ecuador has the lowest (Table 2). Coffee from Ecuador has the highest sucrose content, Brazil and Colombia the lowest. Coffees from Brazil and Colombia also have the highest lipid content.

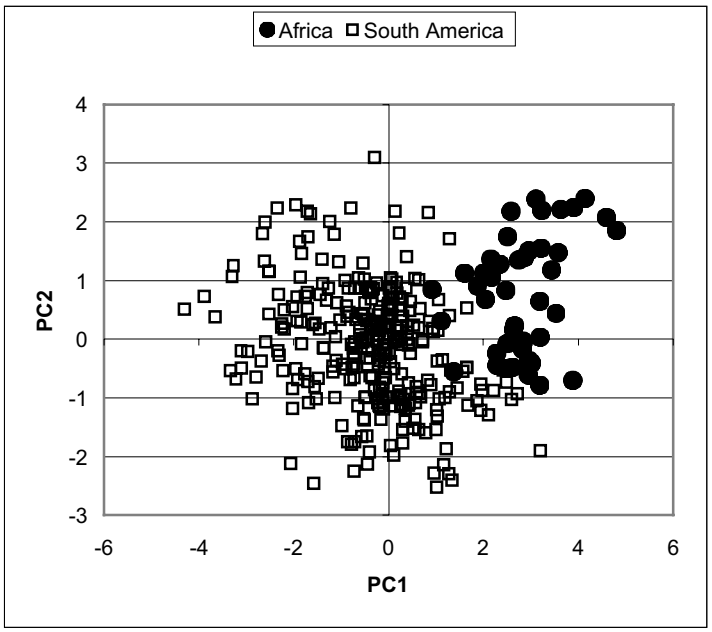

Figure 1. PCA on compositional parameters – Africa and South America, Arabica type.

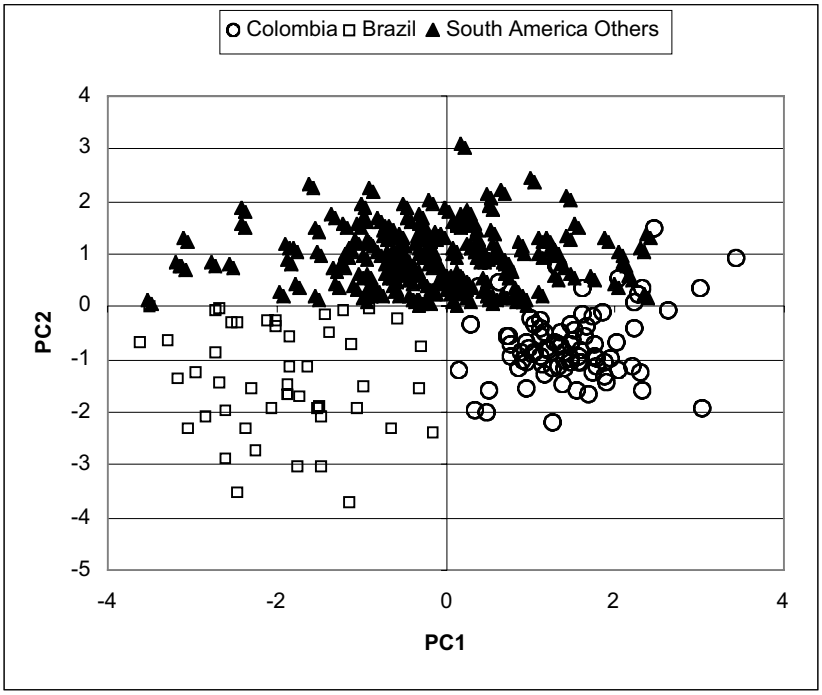

Figure 2. PCA on compositional parameters – South America, Arabica type.

Table 2. Average Compositions, crop years 2002-2004 (blue: Max, Pink: Min).

	Dry Matter	Lipids	Caffeine	Trig.	Chlorogenic Acids	Organic Acids	Sucrose	Total N	Protein
Costa Rica	90.57	14.20	1.25	0.82	7.14	2.28	7.89	2.32	11.47
Guatemala	90.13	14.30	1.25	0.81	7.35	2.24	7.81	2.31	11.47
Salvador	90.68	14.10	1.22	0.83	7.21	2.34	8.02	2.38	11.93
Ecuador	91.64	14.13	1.36	0.72	7.54	2.32	8.95	2.23	10.99
Brazil	90.60	14.73	1.30	0.90	7.58	2.33	6.93	2.39	12.15
Colombia	89.00	14.74	1.31	0.81	7.56	2.23	7.28	2.28	11.24
Nicaragua	89.39	14.21	1.26	0.79	7.08	2.27	7.87	2.34	11.59
Ethiopia	91.26	15.17	1.26	0.84	8.21	2.22	8.54	2.15	10.71
Kenya	90.78	14.79	1.19	0.82	7.88	2.24	8.28	2.20	10.98

In order to verify the clustering results and obtain models that can be used for prediction purposes, a discriminant analysis has to be carried out using the whole NIR spectra since our calibrations do not take into account all the chemical composition of the coffee beans. For this purpose the samples were divided in two sets. One set comprising of two third of the samples was used to develop the models. The other set comprising of one third of the samples was used in an effort to validate the discriminant models obtained. A PLS-DA separates well the South American and African samples (Figure 3). Similar with the compositional PCA, Ethiopian and Kenyan samples are also clearly clustered (figure not shown). An inspection of the loadings reveals that the absorption bands with the highest influence correspond to the lipids bands at 1728 and 1624 nm. For the South American samples, again a good separation is obtained between Brazil, Colombia (Figure 4, with only the clusters for Colombia and Brazil shown for clarity), Costa-Rica, Guatemala, Ecuador and Salvador, while samples from Mexico, Nicaragua, and Honduras were in the same group.

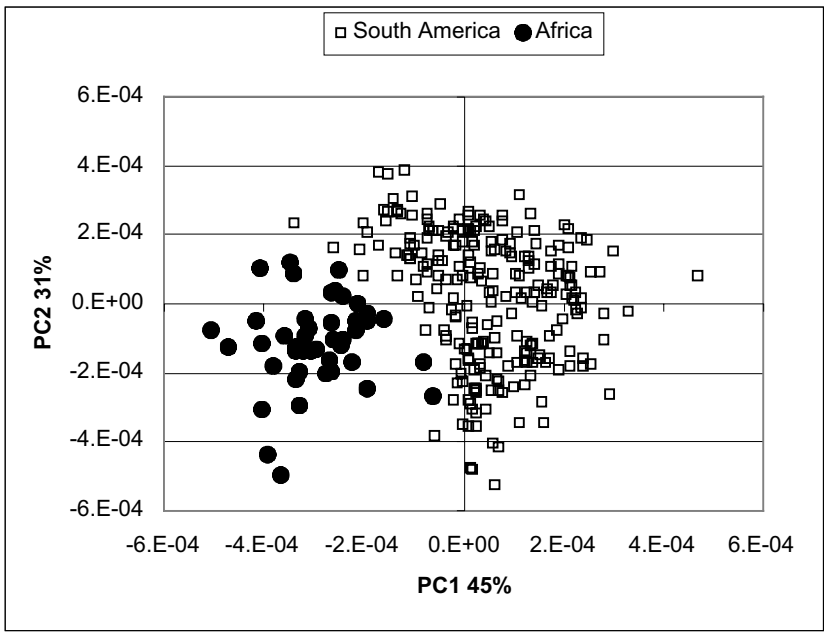

Figure 3. PLS-DA on NIR spectra – South America and Africa, Arabica type.

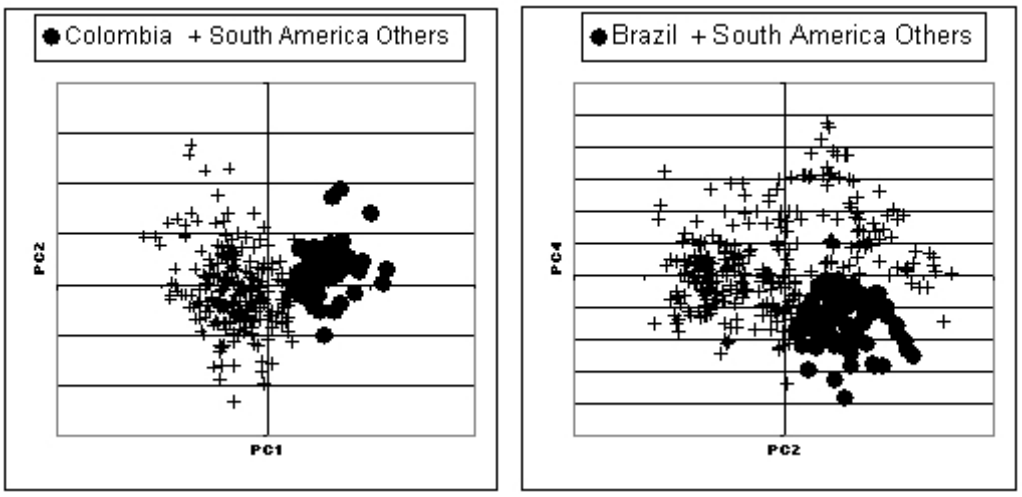

Figure 4. PLS-DA on NIR spectra - Brazil, Colombia and South America, Arabica type.

For the crop year 2005 the situation is similar with good separation of the samples based on their geographical origin (Figure 5). The obtained classification models for the crop years 2002-2004 perform well when validated with the classical methods: cross validation and separation of one third of the samples into a validation set. Based on the validation data presented in Table 3, one can conclude that the obtained models could be used to predict the geographical origin of other samples.

However, if the samples from crop 2005 are used as a validation set of the 2002-2004 PLS-DA model it can be observed that the prediction fails. Half of the Brazilian samples are correctly predicted as coming from Brazil, however the other half is predicted to be from the rest of South America. Similar results are obtained for the 2005 predictions for Columbia and Guatemala. The situation does not improve when trying to predict the origin of crop year 2005 African coffees. While Ethiopian coffees are correctly classified, Kenyan coffees show strong resemblance with the previous years crops from Colombia. (Tables 4**).** The composition NIR calibration instead is still perfectly valid for the 2005 samples.

Table 3. PLS-DA green coffee classification validation for the 2002-2004 crop years and the results for the 2005 crop, N is the total number of samples divided into 2/3 calibration and 1/3 validation.

	Correctly classified %	Correctly classified %	Total N
Colombia	95.6	90.0	120
Brazil	96.2	92.3	104
Costa Rica	100	95.7	63
Guatemala	90.7	83.7	57
Ecuador	91.1	85.7	75
Salvador	91.2	88.2	91
Others	88.8	88.4	90

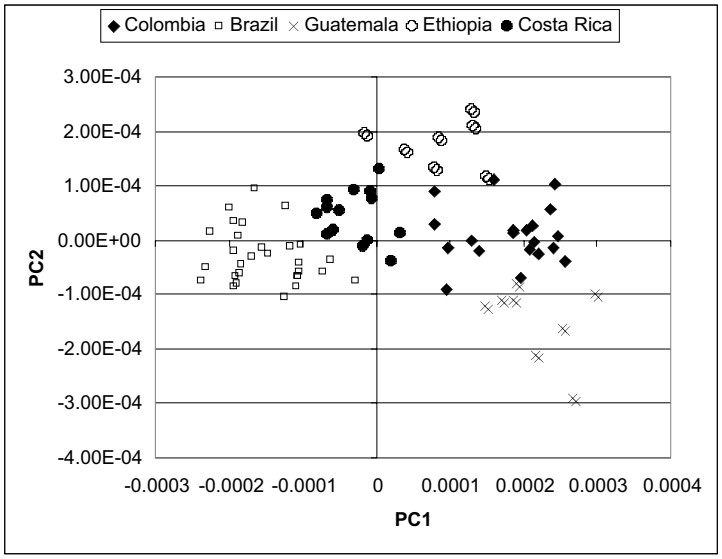

Figure 5. PLS-DA on NIR spectra – Brazil, Colombia, Guatemala, Costa-Rica and Ethiopia, crop 2005

Table 4. Predicted class membership for 2005 crop based on the 2002-2004 PLS-DA model.

	Members	Colombia	Brazil	Costa Rica	Guatemala	Correctly classified
Colombia	30	**17**	5	6	2	56.7
Brazil	26	0	**12**	6	0	46.2
Costa Rica	16	0	0	**15**	0	93.8
Guatemala	14	0	0	6	**5**	35.7

The explanation for the failure of the models lies in the predictive nature of the relationships that can be established between NIR spectra (chemical composition) and geographical origin. The functional variables, which can include rainfall levels, soil characteristics, altitude distribution, type of agrochemicals used, are not included. Plots of rainfall versus soil characteristics are likely to yield similar graphs with well-defined clusters based on geographical origin. While weather patterns might be similar for a few years in a row, they are still unpredictable. NIR calibrations, once established, can provide large data bases in a

short period of time. It is very tempting to create such databases and derivation of predictive relationships follows easily. However, the ease with which a NIR instrument can generate such relationships can also prove misguiding. Predictive relations only work when all the other functional variables not accounted for (commonly called the matrix effect) are not changing. This study, however, does not dismiss similar publications in which classification based on geographical origin based on NIR absorptions were performed on a smaller scale (i.e. regions with closer distances) and where other possible functional variables were taken into account. Actually, predictive models of this type are likely to work for new samples if they come from the same crop years as the samples used for model development. In view of the results obtained for classification trials for the *Arabica* genotype, it was felt by the authors that it was not necessary to extend a similar approach to the *Robusta* type. The failure of NIR predictive models is also a common factor for the lack of trust sometimes encountered for this technique. In most of these cases, the developed models are misused for systems where a matrix change is clearly involved.

CONCLUSIONS

NIR spectra can be used to develop discriminant models based on green coffee geographical origin. Calibrations based on a small set can be used to assess significant differences between average compositions. However, using these relationships for determining a sample of unknown geographical origin was not possible if the sample was from another crop year. This is because the obtained relationships between NIR spectra and geographical origin are, mathematically, of a predictive type, and not of a functional type. A host of other variables should be taken in consideration for viable discriminant models (e.g. rainfall, altitude, soil characteristics). In view of the results obtained for classification trials for the *Arabica* genotype, it was felt by the authors that it was not necessary to extend a similar approach to the *Robusta* type. However, NIR calibrations for compositional parameters can be reliably used for crop years that are not included in the calibration development.

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HR-MAS ^{1}H NMR Study of Hydration Water in Arabica Green Coffee: Relation to the Final Beverage Quality

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SUMMARY

The goal of this study was to determine the ability of high-resolution magic angle spinning (HR-MAS) NMR spectroscopy to assess the quality of green coffee before roasting. To this purpose, ten Arabica green coffee samples, half of which had produced good quality coffees (G samples) while the others resulted in worse tasting beverages (B samples), were analysed by HR-MAS ^{1}H NMR spectroscopy at high field (600 MHz) to determine their chemical composition and water hydration. From the proton NMR spectrum of each coffee powder a metabolic profile was obtained and a comparison between the average profile of G and B samples was made using different statistical methods. Principal component analysis (PCA) gave a good separation of G and B samples and this separation was mainly due to the amount of bound water. Two water signals, attributed to internal and external water, were in fact observable in the ^{1}H NMR spectrum at different chemical shift, after the addition of a small amount of water. Thus, more detailed information on the water structure was obtained by studying the position, intensity and shape of the NMR resonances as a function of the hydration. Finally, pulsed field gradient (PFG) HR-MAS ^{1}H NMR spectroscopy was applied to measure self-diffusion coefficients of water and oil molecules in the coffee powder. We showed that the externally added water diffuse in large pores within the powder particles whereas the fatty acids diffuse in a restricted environment within the coffee matrix.

INTRODUCTION

Water content and distribution is a crucial factor for green coffee quality, shelf-life and determines finally also the quality of roasted coffee. Information on the amount and state of water can be obtained by dry weight and water activity measurements (Clarke, 1985; Reh et al., 2006). However, more detailed information on the behaviour of water molecules is needed to better correlate water properties with coffee quality. More accurate data on the distribution and dynamics of water in the green coffee can be obtained by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy. Similar to solid state NMR, the use of magic angle spinning (MAS) effectively reduces spectral line broadening due to chemical shift anisotropy, homonuclear dipolar interactions and variations in magnetic susceptibility (Andrew and Eades, 1959; Garroway, 1982). This technique produces narrow lines in heterogeneous samples such as tissues or whole cells (Cheng et al., 1997; Weybright et al., 1998). Thus, this approach can be used to determine the chemical composition and water hydration of green coffee during different steps of the coffee production chain.

HR-MAS ^{1}H spectroscopy can also be applied to measure the diffusion coefficient (D) of water and oil molecules in the complex matrix of green coffee. With pulsed field gradient

(PFG) NMR, the self-diffusion coefficient is determined as a function of the effective diffusion time (Δ) and the mean distance travelled by the diffusing molecules during the diffusion time can be calculated (Stejskal E.O. and Tanner, 1965; Callaghan, 1991). For biological heterogeneous systems such as coffee, the displacement of the diffusing molecules depend on the interactions with the porous matrix and may be restricted by pore walls. Thus, diffusion measurements can provide information on the structure of the bean's cellular matrix

MATERIALS AND METHODS

Coffee samples. Ten samples of Arabica green coffee beans (*Coffea arabica*), half of which had produced good quality coffees (G samples) while the others resulted in worse tasting beverages (B samples), were ground using an M20 Universal mill (IKA Werke GmbH). For each powder, the water activity was determined by an AquaLab instrument provided by Decagon Devices Inc.

NMR measurements. HR-MAS ^{1}H NMR spectroscopy was performed on a Bruker 600 Avance NMR instrument at a spinning rate of 6 kHz and at a temperature of 300 K. ^{1}H NMR spectra were obtained from coffee powders with grain size between 250 and 500 μm without any solvent addition. No lock signal was used during the NMR measurements. Diffusion experiments were performed on the coffee powder after addition of a small amount of water (50% by weigh) by applying a PFG stimulated echo sequence at different gradient strengths. The duration of the magnetic field pulse gradients (δ) was 0.5 ms while their separation (Δ) ranged from 50 to 800 ms. For each value of Δ, the apparent diffusion coefficient was obtained by fitting the echo decay curve as a function of gradient strength to a Gaussian function. Spectral manipulation and fitting were accomplished by the Bruker Topspin 1.3 software package.

Statistical analysis. To evaluate the possibility to discriminate between high and low quality coffee from the NMR spectra, different statistical approaches such as principal component analysis (PCA), linear discriminant analysis (LDA) and tree cluster analysis (TCA) were used. To reduce the number of variables and take into account for small resonance shifts, integrals were calculated on selected spectral regions (bucketing). From each bucket, the correlation matrix was calculated and all the signals displaying a correlation higher than 0.1 was added together. By this procedure, the number of variables was reduced to four. All statistical analyses were accomplished by a home-developed software using Python and R statistical routines (R Development Core Team, 2005).

RESULTS AND DISCUSSION

Figure 1 shows the HR-MAS ^{1}H NMR spectrum of a powder sample of green coffee. The high field resonances (0-3 ppm) are typical of the aliphatic chain of fatty acids. Olefinic signals and glycerol resonances are visible at 5.7 ppm and 4.2 ppm, respectively. The single broad signal at 4.78 ppm is due to the natural bound water in the coffee beans

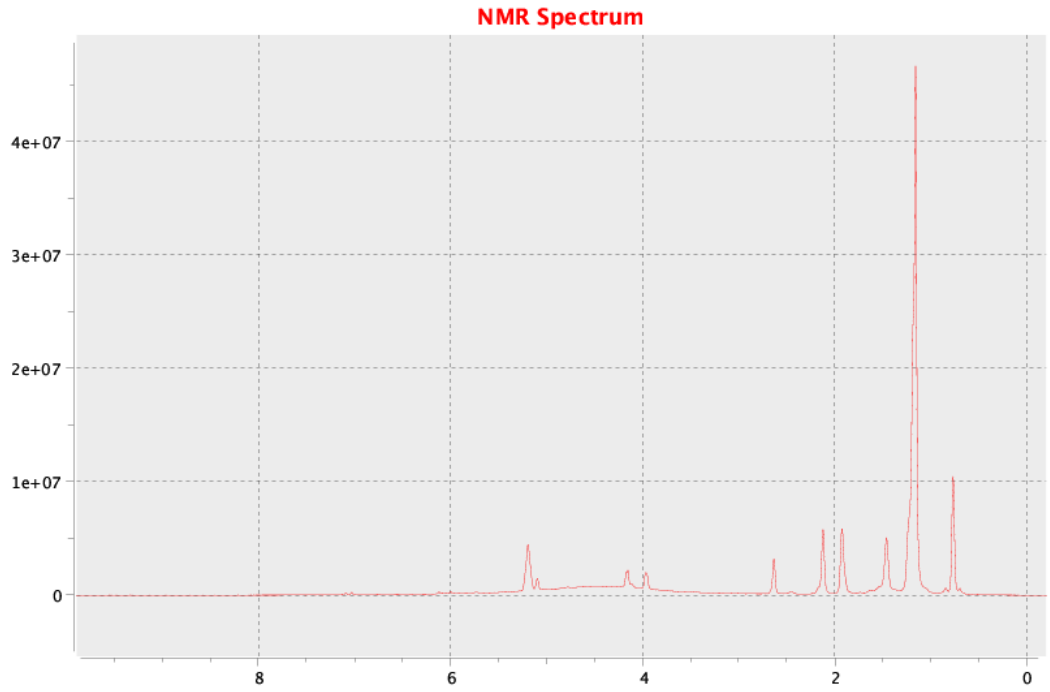

Figure 1. HR-MAS ^{1}H NMR spectrum of a powder sample of green coffee (*Coffea arabica*).

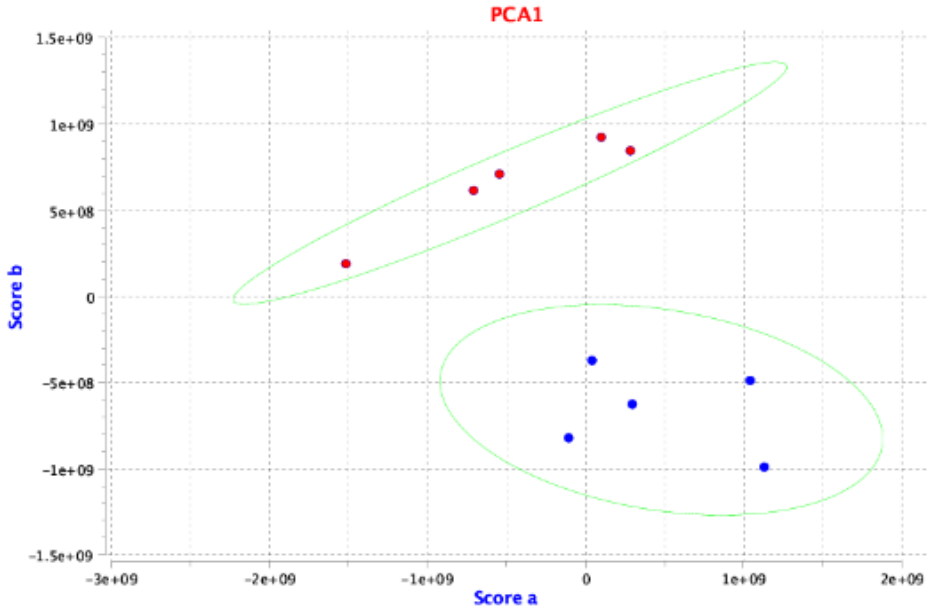

Figure 2. PCA scores plot produced from the NMR spectra of ten Arabica green coffee samples. Red circles represent good quality coffee whereas blue circles represent bad quality coffee.

Principal component analysis (PCA) of the four reduced variables obtained after the correlation analysis is summarized in Figure 2, where the scores plot of the first and third principal components is reported. As can be seen, two groups are well defined in the scores plot and represent the high and low quality coffee samples. The ellipse represents the 95% of confidence. From the analysis of PCA loads, it results that the separation of the two groups is mainly due to the amount of bound water. On average, the G samples have a higher content of

internal water than the B samples. This feature is confirmed by the dry weight and water activity measurements that showed lower water content and activity in the B coffee samples.

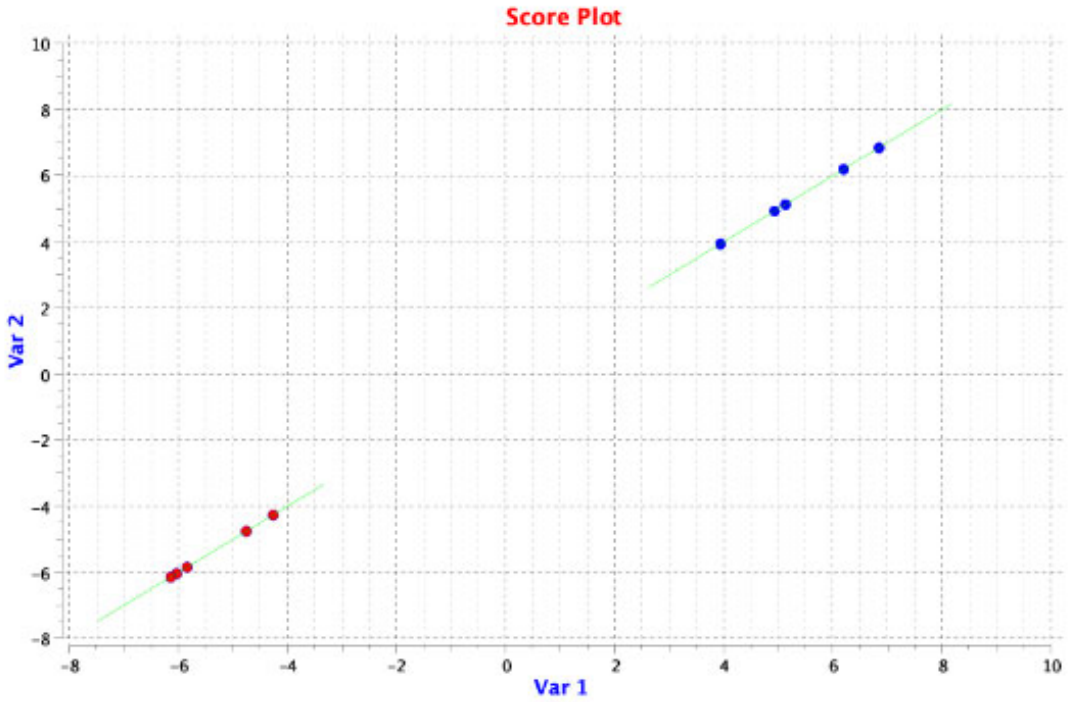

Figure 3. LDA projections of NMR data from ten Arabica green coffee samples. Red circles represent good quality coffee whereas blue circles represent bad quality coffee.

The same four variables were also used for linear discriminant analysis (LDA) and tree cluster analysis (TCA). The corresponding LDA plot is reported in Figure 3 where it is again clear the separation between the two coffee groups. Also TCA with Canberra distance was able to discriminate between the two classes of coffee samples as shown in the TCA plot depicted in Figure 4.

More detailed information on the water structure was obtained by studying the position, intensity and shape of the NMR resonances as a function of the hydration. From the analysis of proton spectra as a function of the amount of externally added water (data not shown) it appears that the water fraction observed in the raw coffee samples does not exchange with the added water fraction and thus it is probably confined into cell compartments.

In Figure 5, the diffusion coefficient of externally added water and fatty acids is separated as a function of the diffusion time. Unfortunately, the diffusion coefficient of the bound water could not be measured due to the extreme large line-width of its resonance. Our data indicate that the diffusion behaviour of water and fatty acids is very different. The diffusion coefficient of water appears independent from the diffusion time (Δ) and is about one half of that of pure water at the same temperature. On the other hand, fatty acids are very slowly diffusing having a diffusion coefficient two orders of magnitude lower than that of pure water, which displays a strong dependence on the observation time (Δ).

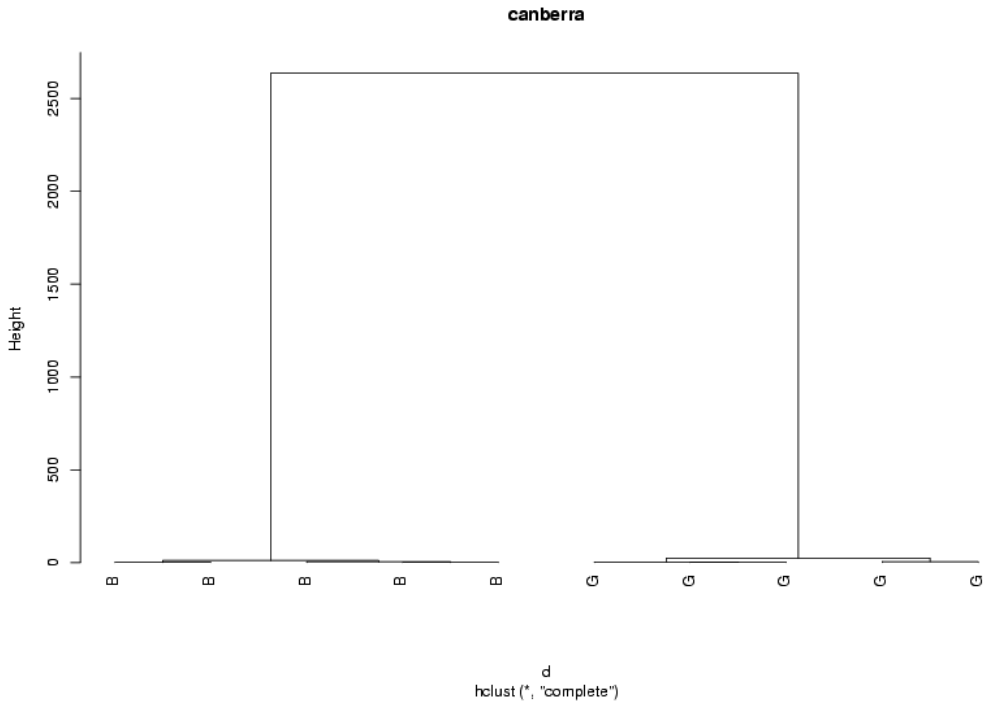

Figure 4. TCA plot produced from the NMR spectra of ten Arabica green coffee samples: (G) good and (B) bad quality coffee samples.

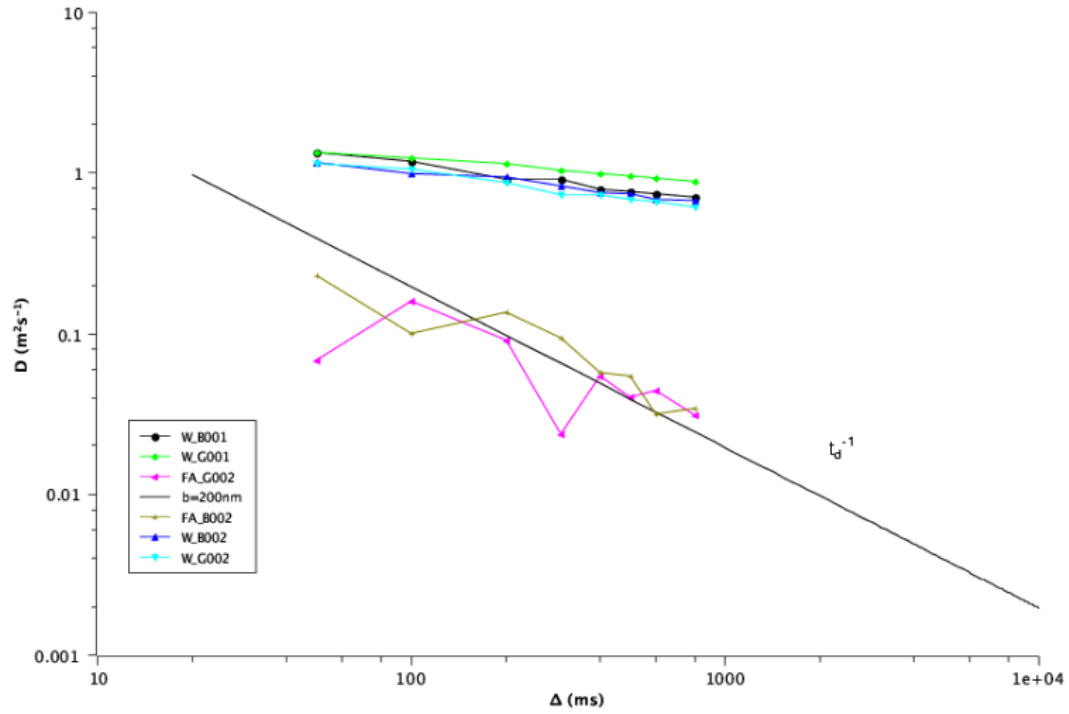

Figure 5. Diffusion coefficients of water (W) and fatty acids (FA) for selected Arabica green coffee samples as a function of the observation time (Δ). The restricted diffusion behaviour of fatty acids is reported for a barrier of 200 nm (solid line).

In the case of fatty acids, the behaviour of *D* versus *t* is typical of diffusion in the presence of obstructing barriers. In fact, at long observation times (Δ) in the presence of a barrier of the

dimension d, the apparent diffusion coefficient depends on the diffusion time as follows: $D_{app} = d^2/2t$. Hence, the longer the molecules diffuse the more restricting barriers will be encountered. The restricted diffusion t^{-1} behaviour is reported in Figure 5 for a barrier of 200 nm (solid line). From the diffusion measurements it results that the externally added water molecules diffuse in large pores within the powder particles whereas the fatty acids diffuse in a restricted environment within the porous matrix.

This preliminary study indicates that HR-MAS ^{1}H NMR spectroscopy is a promising technique for studying water hydration and chemical composition in green coffee. This methodology could be applied for the determination of the changes occurring in green coffee in order to identify the potential damages and quality loss.

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Isolation and Characterization of High Molecular Weight Coffee Melanoidins

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SUMMARY

High Mw melanoidins populations from coffee brew were separated based on charge and ethanol solubility. It was found that the obtained populations differed in their composition and color. All fractions contained nitrogen and only 83% of the total nitrogen content was found to be originating from amino acids, and the remainder (17%) of the nitrogen is likely to be part of melanoidins. A correlation between the protein content and the melanoidin content was found which allowed us to conclude that proteins (fragments) are probably part of the melanoidin complex. Another aspect was that melanoidins possess a negative charge as shown by anion-exchange chromatography, although quite some distinction could be made in the anionic character of different melanoidin populations.

INTRODUCTION

Melanoidins are compounds present in roasted coffee beans and coffee brew. They are expected to have beneficial effects on health as they show anti-oxidant activity. Next to this, they are reported to bind flavor compounds, which is important as flavor is one of the most important qualities of roasted coffee.

Melanoidins are Maillard reaction products that are formed in the final stage of the Maillard reaction. Melanoidins are generally referred to as brown macromolecular nitrogenous compounds and are often measured by measuring the absorbance at 405 nm, which is basically measuring the brownness. In the past several attempts have been made to characterize the molecular structure of these compounds. This resulted in three propositions for the structure of these molecules. Heyns and Hauber (1970) and Tressl et al. (1998) stated that Melanoidins are polymers built up of repeating units of furans and/or pyrroles, formed during the advanced stages of the Maillard reaction and linked by polycondensation reactions. Hofmann (1998) detected low Mw colored substances, which were able to cross-link proteins via _-amino groups of lysine or arginine to produce high Mw colored melanoidins. Kato and Tsuchida (1981), and more recently the group of Cämmerer (2002; 1995) suggest that the melanoidin skeleton is mainly built up of sugar degradation products, formed in the early stages of a Maillard reaction, polymerized through aldol-type condensation, and possibly linked by amino compounds.

Most of the research on the structure of melanoidins was conducted making use of model reactions in which, for example, a reducing monosaccharide and an amino acid were heated to form melanoidins. The outcomes of these model reactions provide useful information on how

melanoidins might look like. However, one has to realize that the composition of a real food system, like coffee beans, is far more complex. Because of this, the melanoidins formed will be far more complex than the melanoidins formed in a model reaction.

When looking e.g. to coffee roasting, the Maillard reaction could be complicated by side-reactions caused by esterification-, degradation-, and polymerization reactions of other coffee components. It is known that the chlorogenic acids content decreases rapidly upon processing of the green bean into roasted beans and it is likely that part of these chlorogenic acids become incorporated into the melanoidin complex (Montavon et al., 2003). Furthermore, caramelization reactions might play a role as well.

Until now, the melanoidin content of coffee is determined by "by difference". This is the percentage that is left after having subtracted all the known compounds present in a coffee (carbohydrates, proteins, caffeine, etc.) from 100% starting material. The remaining percentage that cannot be accounted for is than called melanoidins. However, carbohydrates and/or proteins might be part of the melanoidin complex as well, and such "by difference" calculations may therefore result in an under-estimation of the amounts of melanoidin complex present.

Up to now, the precise structure of melanoidins present in coffee remains unknown and a better understanding of the structural properties of melanoidins is needed to understand and control the functional properties of melanoidins. Therefore, in this study, the structure of melanoidins in coffee was studied by separation of coffee melanoidins into several fractions by using various separation techniques followed by characterization of the obtained fractions. By doing so, we hope to end up with information on what melanoidins look like in coffee brew.

MATERIAL AND METHODS

High Mw melanoidins, obtained by diafiltration of a coffee brew using a 3 kDa hollow fiber ultrafiltration column, were further fractionated using sequential ethanol precipitation steps and fractions obtained were characterized as described by Bekedam et al. (2006). High Mw melanoidins were also subjected to anion-exchange chromatography on a 5 mL HiTrap Q Fast Flow column (Amersham Biosciences, Uppsala, Sweden) as described by Bekedam et al. (1993).

RESULTS AND DISCUSSION

High molecular weight material was isolated from a coffee brew by diafiltration yielding 16% (w/w) of the brew's dry matter. The high molecular weight fraction "Brew HMw" was subjected to sequential ethanol precipitation at 20, 40, 60 and 80% (w/w) ethanol. Five fractions were isolated: the precipitates of the four ethanol precipitation steps and the supernatant of the final (80%) ethanol precipitation step. All fractions were present in significant amounts (Table 1) and these fractions were analyzed for their composition.

The melanoidin content was determined by measuring the absorbance at 405 nm (Table 1). It was found that the melanoidin content increased with increasing ethanol concentration. Proteins are one of the major HMw compounds in coffee beans, and they are expected to be involved in the Maillard reaction by either reacting of an amino side chain or by providing amino acids due to degradation upon heating. The protein levels were determined by amino acid analysis with the aim to determine in which fraction most proteins end up (Table 1).

Table 1. Total Nitrogen Content, Protein Nitrogen Content, and 405 nm absorption of Brew HMw and Brew HMw Ethanol Precipitation Fractions.

Sample	Yield (%, w/w)	Total Nitrogen Content (%, w/w)	Protein Nitrogen Content (%, w/w)	Absorption 405 nm (-)
Brew HMw	100	1.4	1.1	0.6
EP20	19	1.1	0.8	0.2
EP40	11	0.7	0.5	0.4
EP60	28	0.7	0.4	0.3
EP80	17	1.4	1.0	0.6
ES80	13	4.4	3.6	1.2

It was found that the ethanol precipitate and supernatant of 80% ethanol had the highest nitrogen level. This was surprising since the most abundant water-soluble protein in green coffee beans is globulin, which tends to precipitate readily at low (~25%) ethanol. The presence of high levels of proteins at high ethanol concentration confirms that proteins are degraded and/or chemically modified upon roasting, resulting in a higher solubility in ethanol. As melanoidins are mentioned to be nitrogenous compounds, due to the integration of an amino acid moiety in this Maillard product, it was interesting to determine the total nitrogen content next to the protein nitrogen content (nitrogen from amino acids/proteins). It was found that proteins accounted for 83% (w/w) of the total nitrogen content, in all fractions (Table 1). Thus 17% (w/w) of the nitrogen was present as non-protein (or non-amino acid) nitrogen (NPN). Since this was the case for all fractions it was shown that a clear correlation exists between the total nitrogen content, the protein nitrogen content and the NPN content. The NPN is likely to originate from amino acids that were integrated in the melanoidin complex during the Maillard reaction. Proteins are an important supplier of amino acids for the Maillard reaction since the free amino acid content is very low in green beans. The value of 17% NPN allows to suggest that around 1 out of 6 amino acids present in proteins is involved in melanoidin formation. The melanoidin content (absorbance at 405 nm) was plotted as a function of the total nitrogen content, and a correlation was found between the melanoidin content and the total nitrogen content. As the total nitrogen correlated with the protein content it can be stated that the melanoidin content is correlating with the protein content. This allowed us to suggest that proteins are probably part of the melanoidin complex. Whether the amino acids, present as recognizable units within the melanoidin complex, should still be referred to as proteins or should be encoded e.g. peptide fragments is questionable. However, the incorporation of these protein/peptide fragments in the melanoidin complex explains the solubility of the protein/peptide fragments.

Another part of our research dealt with the ionic charge of the melanoidins. It was previously reported by Tomlinson et al. (1993) that melanoidins, made by the model reaction of 5-hydroxymethylfurfural with glycine were negatively charged. Morales (2002) reported that UV_{280} absorbing compounds present in HMw coffee brew showed negative charge as well. Both Tomlinson et al. (1993) and Morales (2002) used capillary electrophoresis in their studies, making use of the charge present in the material they studied. From the capillary electrophoresis results they concluded that their material was negatively charged. As we were interested in the charge of melanoidins, we applied the Brew HMw fraction on anion exchange columns. The starting buffer had a very low ionic strength, which was subsequently increased stepwise to a very high ionic strength. It was found that no Brew HMw material bound when it was applied on a strong cation exchange column; all material eluted in the unbound fraction (Figure 1). It was concluded that coffee melanoidins do not expose a positive charge. On the other hand, almost all (> 95%) of the melanoidins as based on its 405 nm absorption, bound to a strong anion exchange column (Figure 1). It was concluded that

coffee melanoidins are negatively charged. The melanoidins present in the Brew HMw eluted partly at high and partly at low ionic strengths of the eluent, showing that melanoidins are heterogeneous in their anionic behavior.

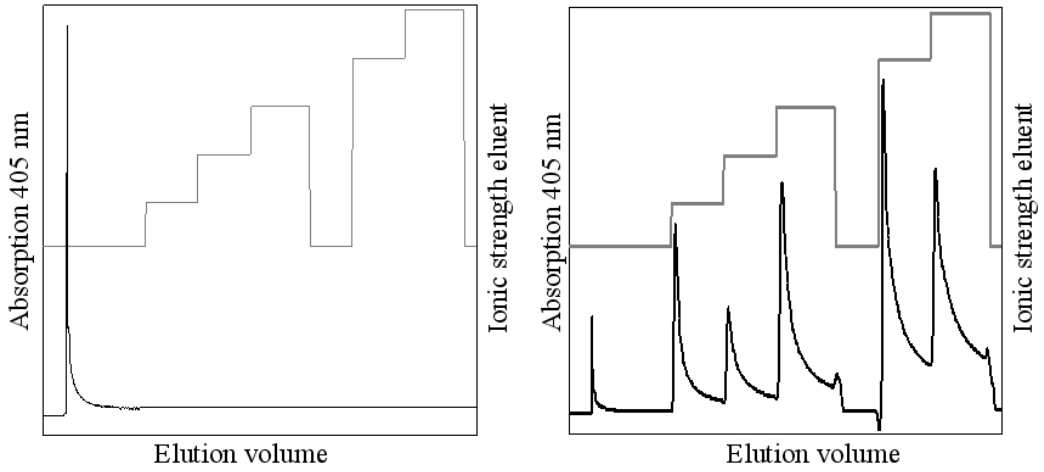

Figure 1. Cation exchange elution pattern of Brew HMw (left); Anion exchange elution pattern of Brew HMw (right).

The six fractions differing in charge were collected and analyzed for their composition. It was found that all fractions differed in composition (total nitrogen content, phenolic group level (Folin-Ciocalteau assay), melanoidin content, and sugar content and composition). Most sugars bound to the column, which was surprising since galactomannans, which makes up a large part of the carbohydrates in coffee brew, are neutral polymers in the green bean (Oosterveld et al., 2004). This suggests that the carbohydrates can obtain a negative charge due to reactions that occur upon roasting. It was also found that the phenolic group level, which was determined by the Folin-Ciocalteau assay, changed with the melanoidin content. This was also found to be the case for the ethanol precipitation fractions. This suggests that phenolic groups are incorporated in the melanoidins complex.

CONCLUSIONS

Various coffee melanoidin populations were isolated, differing in ethanol-solubility and charge properties. It was found that part of the nitrogen is not present as protein structures anymore. The protein content was proportional to the melanoidin content, indicating that proteins are incorporated in melanoidins. Whether the peptide bonds between all amino acids is still intact remains unknown, but in that case it might be well possible that part of the protein serves as ‘backbone’ of the melanoidin complex. The other option is that protein fragments (peptides) are part of he melanoidin complex. With respect to the charge of melanoidins it was found that melanoidins are negatively charged. The differently charged melanoidin populations were found to be different with respect to their composition and it seems that phenolic groups are related to melanoidins.

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Lipidomics and the Influence on Quality of Coffee

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SUMMARY

Lipid oxidation compounds in organisms are involved in important signalling pathways correlated to stress and disease but also to attack defence and physiological response. Current works in brewery science evaluate the influence of germination and storage on lipoxigenase generated hydroperoxides and other lipid degradation products and their influence on staling. In coffee the correlation between LOX (EC 1.13.11.12) generated precursors and aldehyde potential has not been assessed so far. We report here the results of sensible precursor quantification and the generation of free aldehydes (esp. E2-nonenal) in coffee in the processing chain monitored by TDS-GC-SIM analysis for pentafluorobenzylhydroxylamine (PFBHA) derivatives.

INTRODUCTION

Fresh roast and ground coffee and the beverages thereof are highly appreciated for their delicious aroma, which is to a great part generated during roasting. Depending on the preparation and conservation this aroma has relative short shelf-life (Cappuccio et al., 2001). As part of the volatiles from the raw bean survives the roasting process they contribute to the origin typical aroma and, in some cases, also to the off-flavour already present in the raw material (Boosfeld and Vitzthum, 1995). Most of the currently known off-flavours are correlated to post-harvest damages (TCA, geosmin see Holscher et al., 1995) and especially storage and transport conditions have strong influence on shelf-life or viability of green coffee seeds (Dussert et al., 2006). Cleavage of triglycerides (TG) could be identified as a major change in the chemistry of green coffee (Speer et al., 2004) and oxidation of TG or the free fatty acids (FFA) are proposed as pathway for the fatty, oily off-flavour of long stored green coffee (Boosfeld and Vitzthum, 1995).

Analytically only sum parameters like POV (peroxide values) were used to describe the oxidative damage mostly in roast and ground coffee (Huynh-Ba et al., 2001) and no direct correlation between the hydroperoxioctadienic acids (HpODE) precursors and the sensorial relevant unsaturated aldehydes has been made so far in coffee.

In malt (Hubke et al., 2005) and other seeds (Feussner et al., 1995; Ridolfi et al., 2002) however the oxygenation of storage lipids has been assessed and for beer and olive oil a correlation to volatile compounds or residual nonenal potential (malt-RNP, Guido et al., 2005) and changes during aging of the finished beverage (Liegeois et al., 2002) have been made. Also in medicinal chemistry identification of oxidation products of membrane poly unsaturated fatty acids (PUFA) and their products (Schneider et al., 2005) is important. Recent propagation of LC-MS instrumentation has made it possible to directly quantify HpODE without prior derivatization and separation of the stereo-isomers or even optical isomers and to identify the enzymatic or chemical pathway of their formation.

EXPERIMENTAL

Material and Method

Oil extraction

Oil was squeezed out of fresh green coffee beans directly after harvesting in the country of origin (Brazil). In the laboratory gram quantities of the oil were extracted by soxhlet extraction with pentane and kept under inert gas before oxidation or analysis.

Extraction of hydroperoxides

For the extraction of the thermo-labile HpODE freshly ground green coffee beans (liquid nitrogen) were treated three times by pentane at natural pH. In case of aqueous extracts these were acidified to pH 1.5 by addition of HCl and extracted by pentane at room temperature. Solvent was blown off under a flow of nitrogen at room temperature and an aliquot of the lipidic phase was re-dissolved in mobile phase.

HPLC-MS/MS conditions

The LC system consisted of a HP1100 liquid chromatograph (Agilent Waldbronn, Germany) equipped with a vacuum degasser, a quaternary solvent delivery compartment with low pressure mixing, an auto-sampler, and a column compartment. The separation was performed on a Gemini (Phenomenex, Aschaffenburg, Germany) column (150 mm x 3 mm, 3 um) using a flow of 0.4 ml/min at 30 °C. The mobile phase was LC-MS grade acetonitrile and water under addition of 0.1 % of formic acid in the ratio 55 to 45 volume percent.

For optimization, an HPLC mix of the HpODE (9/13) and 3 HpOTE was used by syringe injection (Cayman chemical, Michigan, USA). The mass spectrometer Q Trap (Applied Biosystems/MDS Sciex, Darmstadt) operated in ESI negative mode at 250°C (source temperature). The resulting compound conditions for the measurement of MRM (311/113) and (293/113) for the 13-HpODE and 9-HpODE (MRM 311/185 and 293/185) were IS -4500, DP -20, EP -10, CE -20 at dwell times of 150 msec.

TDS-GC-MS

Direct thermal extraction of the volatile and semi-volatile compounds by TDS-GC-MS as described for olive oil by Gerstel Application Note 09/2000. By directly injecting 1 ul of the green coffee oil into an empty glass tube followed by thermo desorption under inert atmosphere the volatile compounds are trapped into a cooled injector. After quick heating the compounds are transferred into the GC capillary, separated and identified by mass spectrometer.

TDS: glass wool 20°C ramped 60°C/min to 60°C for 5 min, 50 ml/min Helium
PTV: -120°C ramped 720°C/min to 250°C for 3 min, solvent vent at 0.5 min with 25 ml/min. Linear filled with silanized glass wool
GC: 60 m HP-Wax (0.25 mm ID, 0.25 um DF). 35°C(3min)-4°C/min-220°C. 2.4 ml helium
MS: EI 70 eV, Scan 41-250 m/z

SBSE

Aqueous green coffee extracts were prepared using 15 g of milled green coffee and 100 ml of boiling water. After 5 minutes the sample was filtrated and 10 ml were extracted by SBSE at 1000 rpm for 30 min. Derivatization took place on the stir bar for 30 minutes using diluted PFBHA solution at 1000 rpm. Thermo desorption was performed automatically by TDSA2 (Gerstel, Muehlheim an der Ruhr, Germany) in selected ion monitoring (SIM) mode with electron ionization (70 eV). Ions monitored were m/z m/z 181, 250, 335 for E-2-nonenal, m/z 181, 264, 333 for E,Z-2,6-nonadienal and E,E-2,4-nonadienal, and m/z 181, 276, 347 for E,E-2,4-decadienal.

SDE

Milled green coffee was extracted for 2 h by a Likens-Nikerson simultaneous distillation extraction using pentane/diethylether as organic solvents.

RESULTS AND DISCUSSION

Sun light stressing of green coffee oil produces the typical volatile compounds responsible for the rancid impression in various food materials upon heating (Fish, Haugen et al. 2002, Rice, Lam et Proctor 2003).

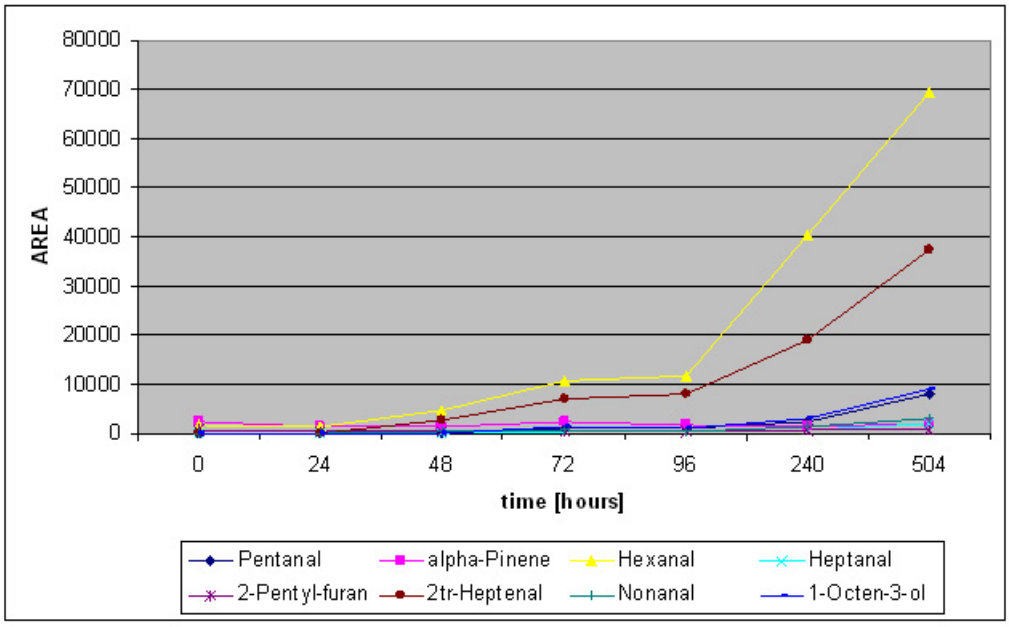

Figure 1. volatiles generated in TD-GC-MS from non-volatile precursors.

In Figure 1, the effect of oxidative stress on green coffee oil and the volatile compounds formed upon heating in inert atmosphere directly in the thermo desorption unit of the GC-MS is demonstrated. According to similar observations in roast and ground coffee (Huynh-Ba et al., 2001) the volatile aldehydes are formed via a non-volatile precursor which liberates these compounds only upon heating or enzymatically in analogy to other lipid rich seeds (Seyhan et al., 2002). The pathways of formation of these reactions are widely agreed upon in literature and are summarized in Figure 2 (for a review see Quarantelli et al., 2003). Separating the antioxidants (mainly CQA) present in the green coffee by pressing the beans enhances the autoxidation behaviour of this oil, resulting in a sensorially rancid oil after only a few weeks even at ambient temperature.

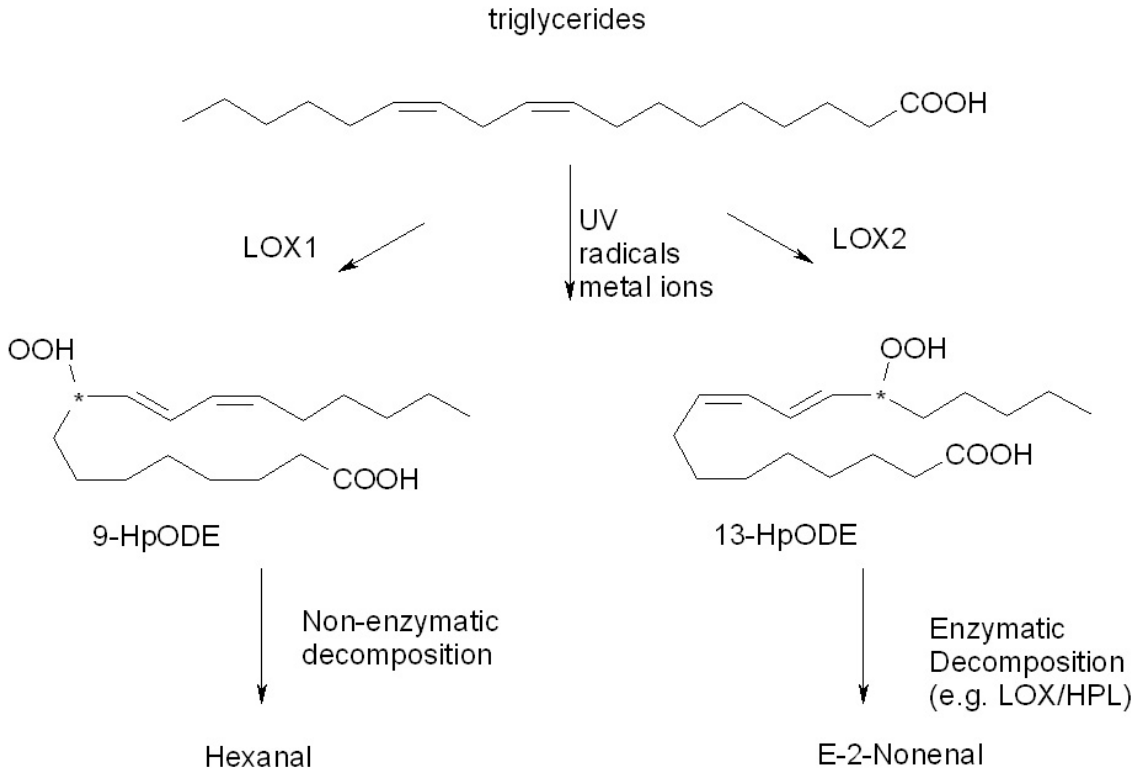

Figure 2. scheme lipid oxidation reaction.

In the case of accelerated oxidation tests hexanal can be used as an indicator substance, but only upon heating under inert atmosphere, so after liberation from the precursors.

To our knowledge the identification of the HpODE as responsible precursors in coffee has not been described in literature before. The first aim was to setup a method able to identify and quantify these products via MS infusion and fragmentation experiments. In Figure 3 the MS/MS/MS spectra ($QqQ_{LinearTrap}$) of 9- and 13- HPODE from a commercial standard are shown. The fragmentation patterns have been confirmed by MacMillan and Murphy 1995 using stable isotope labelled compounds and are displayed together with their spectra and fragmentation schemes in Figure 4.

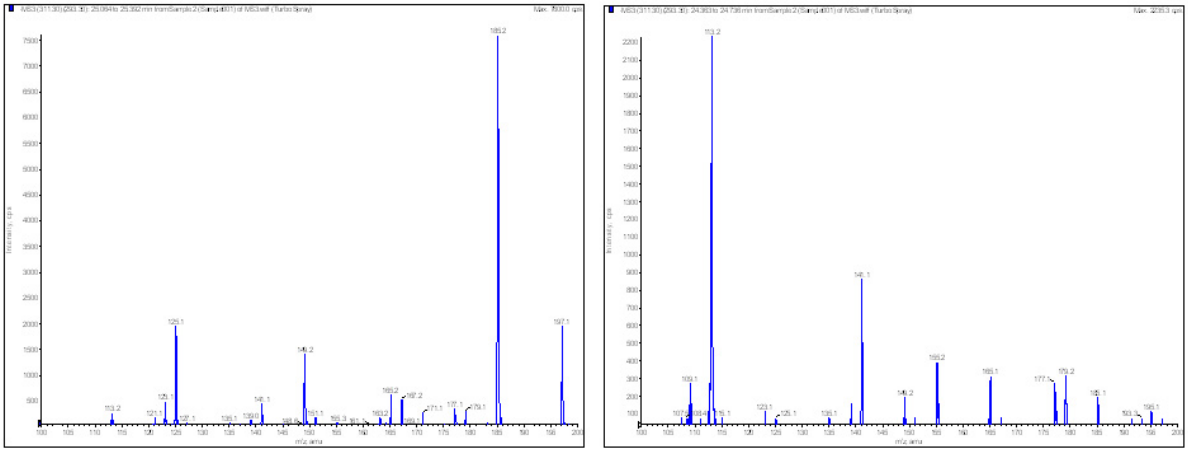

Figure 3. MS^3 spectra ($QqQ_{LinearTrap}$) of 9- and 13-HPODE.

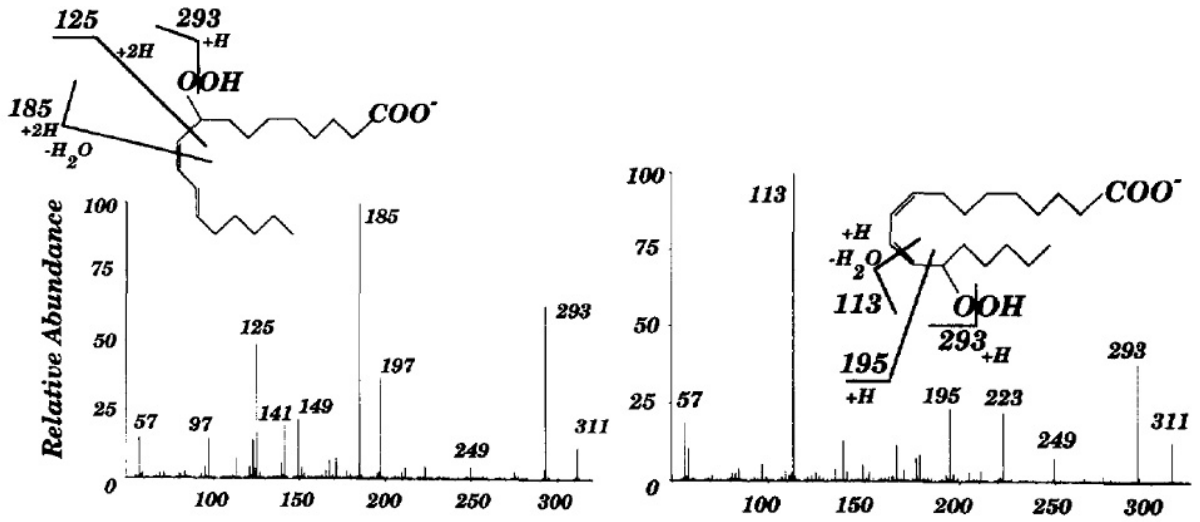

Figure 4. fragmentation patterns of 9- and 13-HpODE according to MacMillan and Murphy 1995.

In direct infusion the molecular mass of this HpODE (311 m/z) could be easily identified in stressed oil and was nearly absent in oil carefully extracted from perfect quality raw coffee. The separation of the two stereo-isomers (9/13 HpODE) is possible using a stable C18 column, but DAD detection at wavelength of 235 nm is rather insensitive compared to the proposed MRM detection. For the same lipidic extracts of coffee beans the amount of hydroperoxides was measured using the method described (data not shown) and correlated with the amount of aldehydes present in SDE or Twister aqueous extracts of the same material. On bar derivatization of the aldehydes with PFBHA resulted in a pair of oximes which could be sensitively detected by standard GC-MS (compare Figure 5).

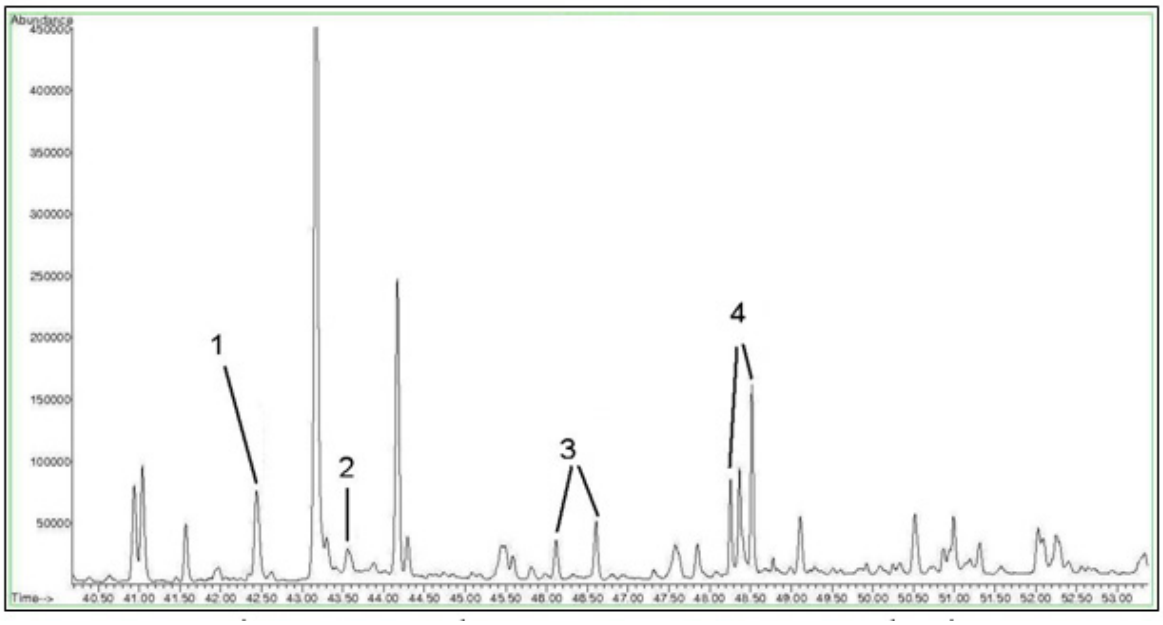

Figure 5. SIM chromatogram:1. E-2-nonenal oxime, 2. 2,6 nonadienal oxime, 3. 2,4 nonadienal oximes, 4. 2,4 decadienal oximes.

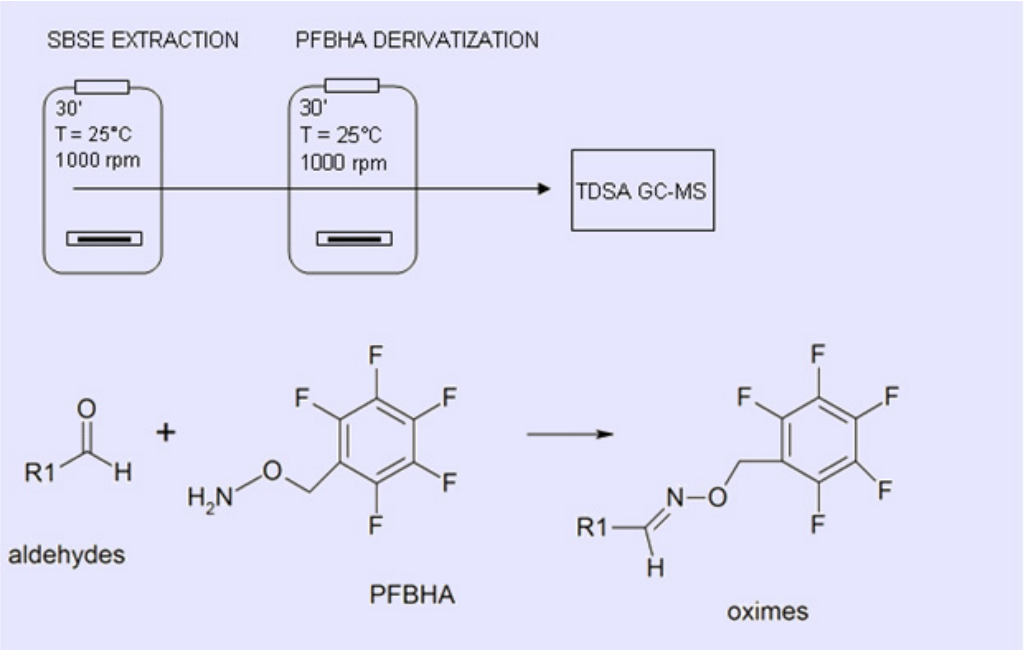

Figure 6. sample preparation and derivatization scheme oxime determination GC-MS.

The amount of these low threshold off-flavor aldehydes was correlated in sensorial evaluation to the cup quality in coffee. Profiles of the decadienal and E-2-nonenal concentrations determined in coffee extracts are demonstrated in Figure 7 in comparison to the sensorial intensity of woody off-flavor (rated from 1-5, with 5 as strongest impact).

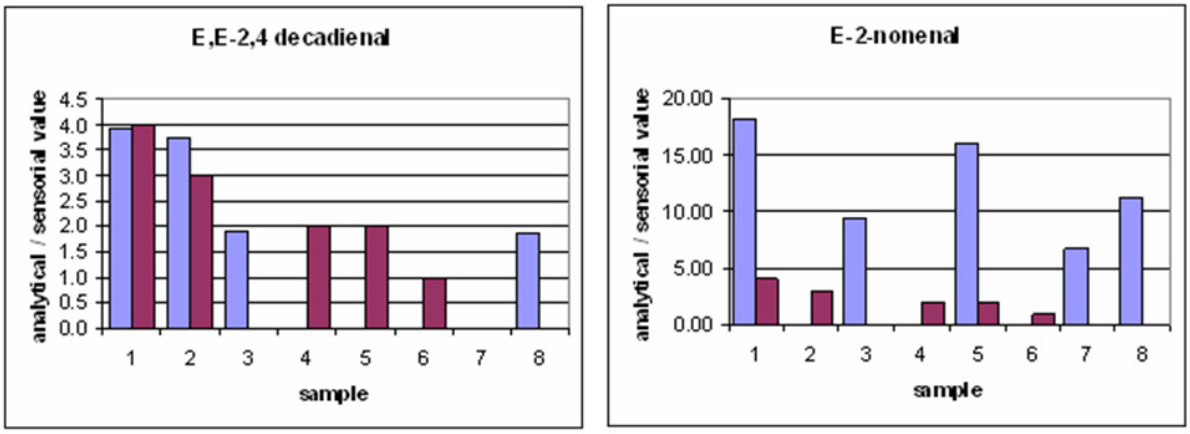

Figure 7. matching of analytical and sensorial results (arbitrary units of AREA compound / AREA of int. standard blue bars, sensory score woody defect right bars, 1-5 with 5 most intense).

Currently under investigation is the ratio and chirality of the two hydroperoxides to get further inside knowledge of the pathways (LOX1/LOX2/chemical) responsible for the formation of these off-flavor precursors.

CONCLUSIONS

The presented work gives some insight in the oxidative changes of the lipidic fraction of the coffee bean. The hydroperoxides of octadienoic acid (HpODE) could by identified by MS/MS experiments in coffee beans. In coffee oil the kinetics of the aldehyde generation in the course of oxidation of the coffee oil were followed by TDSA-GC-MS and a sensible method for the

determination of the aldehydes responsible for woody off-flavor could be setup by SBSE followed by on-bar derivatization to the oximes and GC-MS SIM quantification. Still ongoing is the correlation of the quantification results of the HpODE isomers and stereoisomer to the biological or chemical pathways.

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Neuroactive Beta-Carbolines in Arabica and Robusta Coffee

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SUMMARY

The concerns about potential coffee health risks have contributed to inumerous reports in literature, mostly based on epidemiological studies. However, in most cases, the results are conflictive and general conclusions are still difficult to take. On the other hand, coffee consumption has been recently associated with reduction in the risk of several chronic diseases, namely Parkinson's disease. However caffeine does not seem to be the key factor in this case. The possible involvement of beta-carbolines found in coffee, a class of neuroactive compounds, is a new possibility. Based on above facts, our purpose was to determine the beta-carboline amounts in arabica and robusta coffee beans from several geographical origins. Several roasting degrees were evaluated in all samples, since the amounts of these compounds are known to increase with temperature. A total of 10 arabica and 7 robusta coffee samples were analyzed, both in green beans and after three different roasting degrees, in a total of 68 samples. The compounds (harman and norharman) were extracted by SPE and quantified by HPLC/FLD with a suitable internal standard. Adequate statistical treatments were applied to the data obtained. The results clearly demonstrate that these compounds are formed during the roasting procedure since their content is low in the green beans and increases with the roasting intensity. Robusta beans always presented higher amounts of the two compounds than arabica ones. The mean levels found were 6 and 2 mg/kg for norharman and harman, respectively, and they show that coffee is, probably, one of the most important human sources of these compounds.

INTRODUCTION

The beta-carbolines harman and norharman are part of the human diet since they are naturally found in several vegetables and medicinal plants in the low nanogram level. Recent studies have shown that tobacco smoke is the major contributor to the human intake of harman and norharman and that several micrograms of these compounds are formed by pyrolisis per cigarette (Totsuka et al., 1999; Pfau and Skog, 2004). Another major source has been recently reported with their detection in roasted coffee beans and brewed coffee (Herraiz, 2002).

Together with the smoking habit, the regular coffee intake has also been reported as being protective in the development of neurodegenerative diseases, namely Parkinson (30% risk reduction against 60% in the former) (Hernán et al., 2002; Martyn and Gale, 2003; Tan et al., 2003; Ascherio et al., 2004). Their major alkaloids, nicotine and caffeine, respectively, have been implicated, but they do not seem to be the only nor the strongest compounds contributing to these observations. The high amounts of beta-carbolines in tobacco smoke and roasted coffee place them at the top of the suspected list. Also, while caffeine and nicotine are reported to act as antagonists for the adenosine A2 receptor, preventing the suppression of the dopaminergic activity by adenosine, these beta-carbolines are potent MAO inhibitors, in both rat and humans (Chen et al., 2001; Herraiz and Chaparro, 2006; 2005) what seems to be the main mechanism for the reduction rate in Parkinson disease.

Aware of the chemical differences between arabica and robusta coffee, these work objectives were to investigate the influence of the coffee specie and the degree of roast on the beta-carboline levels. In order to eliminate the variations due to the beverage preparation method, our study focused on the coffee beans exclusively.

MATERIAL AND METHODS

A total of 17 coffee samples (10 arabica and 7 robusta) from different geographical origins were collected and roasted at three different temperatures in a Probat Duett Roaster, simulating light, medium and dark roasts.

The beta-carbolines were extracted with hot diluted hydrochloric acid and the extracts purified by SCX-SPE (Herraiz, 2002), with harmaline as internal standard. Their separation was performed by reversed-phase HPLC with a gradient of TFA in formate buffer and acetonitrile with fluorescence detection.

RESULTS AND DISCUSSION

The roast intensities requirements can vary extensively from country to country accordingly with regional preferences, coffee specie, roaster type, and beverage preparation method. The "lighter" roasts, for instance, with organic roast losses (ORL) from 13 to 16% (dw) are usually preferred for filter coffee, while an "expresso" demands stronger roasts (17-20%). It is also known that, due to their inherent characteristic, namely their reduced size, the robusta beans roast deeper than the Arabica ones under the same roasting conditions.

When all samples are discussed together (Table 1) it can be observed that the green beans contain only small amounts of both compounds. An extensive formation of norharman during roast is easily observed, with mean values almost duplicating between the 240 ºC and the 280 ºC roasts. The harman levels are always smaller and their mean levels are almost constant in the three roasts types. Both compounds are statistically higher in the robusta samples for all the temperatures assayed.

Table 1. Mean levels (n=17) of the beta-carbolines analysed.

μg/g	green	240 ºC	260 ºC	280 ºC
Norharman	0.04 ± 0,00	3.87 ± 1.24*	5.01 ± 0.61*	6.99 ± 2.08*
Harman	0.05 ± 0,01	1.20 ± 0.36*	1.23 ± 0.39*	1.39 ± 0.53*

**Significant at $p < 0.01$.*

Important observations can also be detailed when the samples are grouped by specie, independently of their roast degree. The mean values in the arabica roasted samples (n = 30) were 3.1 ± 1.6 μg/g for norharman and 0.6 ± 0.3 μg/g for harman while in the robusta roasted samples the values were 7.8 ± 3.1 μg/g and 1.9 ± 10.8 μg/g, respectively Even considering all samples together, the higher amounts in the robusta samples presented a high statistical significance ($p < 0.01$).

The betacarboline amounts grouped by specie and roast degree are detailed in Table 2. The results also show that while the norharman amounts increase in both species with the roast temperature, those of harman decrease in the arabica samples. These observations suggest that harman might be present as an intermediate, being norharman the final compound in this

reaction formed by demethylation of harman. The different amounts in the two species also suggest the presence of different precursor amounts.

Table 2. Beta-carboline contents in Arabica and robusta samples.

	μg/g	green	240 ºC	260 ºC	280 ºC
Arábica (n= 10)	Norharman	0.03 ± 0.00	2.43 ± 0.77	2.61 ± 10.17	3.26 ± 0.79
	Harman	0.03 ± 0.00	0.83 ± 0.12	0.53 ± 0.15	0.44 ± 0.12
Robusta (n=7)	Norharman	0.06 ± 0.01	5.31 ± 1.72	7.40 ± 1.04	10.72 ± 3.38
	Harman	0.07 ± 0.02	1.57 ± 10.61	1.93 ± 0.64	2.34 ± 0.94

Tryptophan is usually reported as being harman precursor in several other matrices and in the human organism. This situation might also be applied to roasted coffee since the robusta beans also present higher amounts of tryptophan when compared to the arabica ones, as previously analyzed in group (Herraiz, 2002).

Special attention must be given to the high standard deviation observed in the content of both carbolines. It was observed that the final roast degree was not always similar even for samples from the same specie and geographical origin. In same cases, extensive variations, both in color readings and ORL's were found. Besides the intrinsic beans characteristics, a reference must also be made to the roaster since, being regulated for a specific air temperature (240, 260 or 280 ºC), the first roasts were usually lighter than expected when roasting in a sequence. This situation made it necessary to repeat some roasts occasionally. The lighter roast presented ORLs (dw) between 5% and 12% and the darker were between 13% and 26%. Some of these last samples were already outside the usual roasts, even for espresso coffee, but were used in order to better understand the roast influence on the two compounds.

A possible correlation between the beta-carbolines amounts and the roasting degree was investigated. Generally, a correlation is observed when the species are taken separately. The increase is more noticeable for norharman, with a 0.72 correlation in arabica and 0.75 in robusta. When the samples are analyzed together the coefficient is even lower. Therefore, one must conclude that the beta-carboline levels depend not only on the coffee specie, roasting degree, and geographical origin, but also on several other factors that influence physical and chemically the bean.

These results show that beverages obtained from mixture of arabica and robusta beans will produce beverages with higher beta-carbolines content. Also, the darker beans will transfer higher beta-carbolines amounts into the beverages, especially norharman. Therefore, the human intake of these beta-carbolines through coffee beverages is highly variable.

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Imaging Mass Spectrometry as an Innovative Tool to Study Coffee Fruit Development

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SUMMARY

The coffee fruit development covering the time between anthesis and full ripening is variable from few weeks to more than a year. *Cofea arabica* requires 6 to 8 months to maturate. Due to several flowerings that may occur in C. arabica at each production season, fruit growth is asynchronous during development, with different proportions of various fruit-developmental stages in the same plant, which may affect cup quality. Except for the large number of studies concerning caffeine metabolism in coffee, very few data are available concerning proteins and metabolic pathways important for coffee cup quality. Therefore, studies on protein expression associated with tissue localization may hold the key to a better understanding of the role of molecules during coffee fruit development. Molecular ion-images acquisition by mass spectrometric methods has opened a novel frontier on detection of molecules on tissue samples. This so called imaging mass spectrometry has been successfully used to perform direct spatial mapping of peptides and proteins in several tissue samples. In the present study, we show the spatial mapping of molecules during several developmental stages of coffee fruits. The *C. arabica* embryos were collected and stored in liquid nitrogen following dissection. After such procedures the embryos were immediately immersed on 1 μL of matrix (alpha-cyano-4-hydroxy-cinnamic acid, 10 mg/ml in acetonitrile/water/3% trifluoroacetic acid 5:4:1). After 20 min, the embryos were air dried for 10 minutes, mounted onto a target plate by a double-sided adhesive tape, and submitted to mass spectrometric analyses. Ion profiling was performed using an UltraFlex II TOF/TOF (Bruker Daltonic, Germany) operating on positive mode. Automatic scanning steps were accomplished using FlexImaging software. The pulsed laser was rastered across the sample surface in 100 μm single steps. Spectra were generated for each image from 30 laser shoots at each position in the global maps consisting of 4170 spectra. Intensity-based ion maps of the molecular components of embryos surface were acquired ranging from 2000 to 20000 Da. Despite the presence of several molecular components exhibiting similar spatial distribution profiles during all developmental stages, other components seem to be expressed only on specific stages or specific regions of the evaluated tissues. These observations coupled to the possible spatial co-localization of molecular components could be used as starting points for functional studies aiming at the elucidation of important metabolic steps of coffee fruit development.

INTRODUCTION

The coffee fruit development covering the time between anthesis and full ripening is variable from few weeks to more than a year. *Coffea arabica* requires 6 to 8 months to maturate. Due to several flowerings that may occur in *C. arabica* at each production season, fruit growth is asynchronous during development, with different proportions of various fruit developmental stages in the same plant, which may affect cup quality (De Castro and Marraccini, 2006). Except for the large number of studies concerning caffeine metabolism in coffee, very few data are available concerning proteins and metabolic pathways important for coffee cup

quality. Therefore, studies on protein expression associated with tissue localization may hold the key to a better understanding of the role of molecules during coffee fruit development. Here we report the spatial mapping of molecular components in coffee tissues by using imaging mass spectrometry (IMS), a technique initially used to distinguish the proteomics of specialized tissues in mammalian organ sections. The identity of one detected component was determined and primary sequence similarity showed that this protein belongs to the lipid transfer proteins (LTP's) family. The presence of some posttranslational modifications according to tissue region were also detected.

MATERIALS AND METHODS

Coffee fruits

C. arabica fruits were obtained at different developmental stages and beans with distinct quality features.

Tissue dissection

Coffee embryos and endosperms were stored in liquid nitrogen and sectioned for imaging mass spectrometry approaches. Crude extracts were also obtained by homogenizing coffee fruits with an acetonitrile:water (1:1) solution.

Imaging mass spectrometry

Coffee tissue sections (3-4 mm) were air-dried under a glass slide for 10 minutes, photographs were captured, and by using a forceps the tissue pieces were directly attached to a MALDI plate by a double-sided adhesive tape. After the tissue was placed on the target well, 1 μL of matrix (alpha-cyano-4-hydroxycinnamic acid, 10 mg/ml in acetonitrile/water/ 3% trifluoroacetic acid (5:4:1) was deposited with a pipette and spread over the skin fragment. In some instances tissue sections were directly immersed in matrix after sectioning. After air-drying for 20 min, the tissue sections were submitted to mass spectrometric analyses. Molecular ions profiling was performed using an UltraFlex II TOF/TOF (Bruker Daltonics, Germany) operating on linear positive mode. Automatic-scanning steps were accomplished by using FlexImaging software. The laser was rastered across the sample surface in 100-200 _m single steps of 30 laser shoots at each position in the global maps consisting of some thousand spectra. FlexImaging was used to generate intensity-based ion maps of the molecular components of the plant-tissue surface ranging from 2000 to 20000 Da.

RP-HPLC

Reverse-phase HPLC analysis was performed using a C18 column at a constant flow rate of 1 mL/min using a gradient spanning from 0 to 65% of acetonitrile:TFA (99.9:0.1) in 40 minutes. Molecular masses (MS) were determined by MALDI-TOF/MS.

Trypsin digestion

RP-HPLC fractions displaying the expected molecular masses of searched proteins were trypsin digested aiming at sequencing experiments.

De novo sequencing

Molecular masses of the major detected peptides were also determined by MALDI-TOF/MS using the UltraFlex II controlled by the FlexControl 2.4.30.0 software. The MS/MS spectra were carried out in the positive mode precursor ion fragmentation (LIFT/CID) at a laser frequency of 50 Hz with external calibration, using the Peptide Calibration Standard-Starter Kit 4. Resulting data were analyzed using FlexAnalysis.

Peptide sequence analysis

Similarity searches were performed using the FASTA 3 program on the Expasy Molecular Server (http://ca.expasy.org/). tBlastn analysis were also performed against the Brazilian coffee EST database (Vieira et al., 2006) (www.genome.cenargen.embrapa.br). Sequence alignments were performed using ClustalW multiple alignment tool available on Bioedit v. 7.0.4.1 software.

RESULTS

Despite the presence of several molecular components exhibiting similar spatial distribution profiles during all developmental stages (Figure 1d-1e), other components seem to be expressed only at specific stages (Figure 1c and 1f). These observations coupled to the possible spatial co-localization of molecular components could be used as starting points for functional studies aiming at the elucidation of important metabolic steps of coffee fruit development and mainly its quality. This fact led us to investigate the molecular components of the endosperm of good vs. bad quality beans (EM vs. DU). The presence of a consistent repeat of +42 Da (some possible posttranslational modification) in a molecular component (m/z 9271) led us to search for the identity of this protein (Figure 2). After some traditional proteomics approaches (Figure 3) we identified this protein as a novel lipid transfer protein (LTP) sharing high similarity to previously described LTPs (Figure 4). Since LTPs have a crucial role during seed development we hypothesize that such molecular mass increment could be associate with some important developmental stage-dependent metabolic pathway.

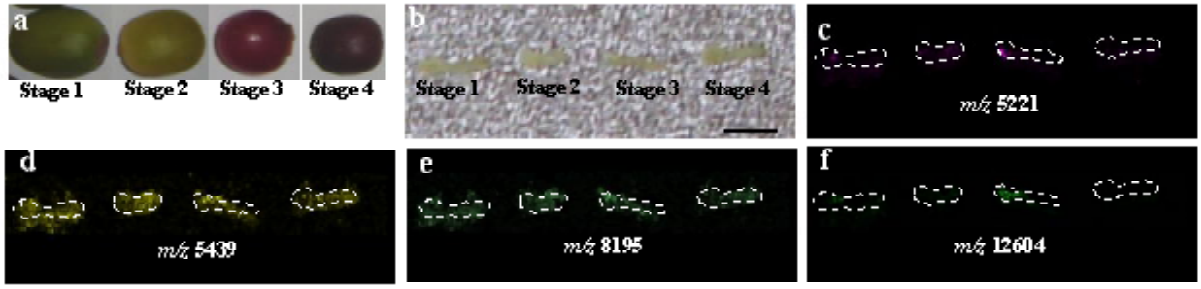

Figure 1. (a) Coffee fruits in different developmental stages; (b) Embryos photography. Bar = 3 mm.; (c-f) Molecular images. Dashed lines show embryos contour.

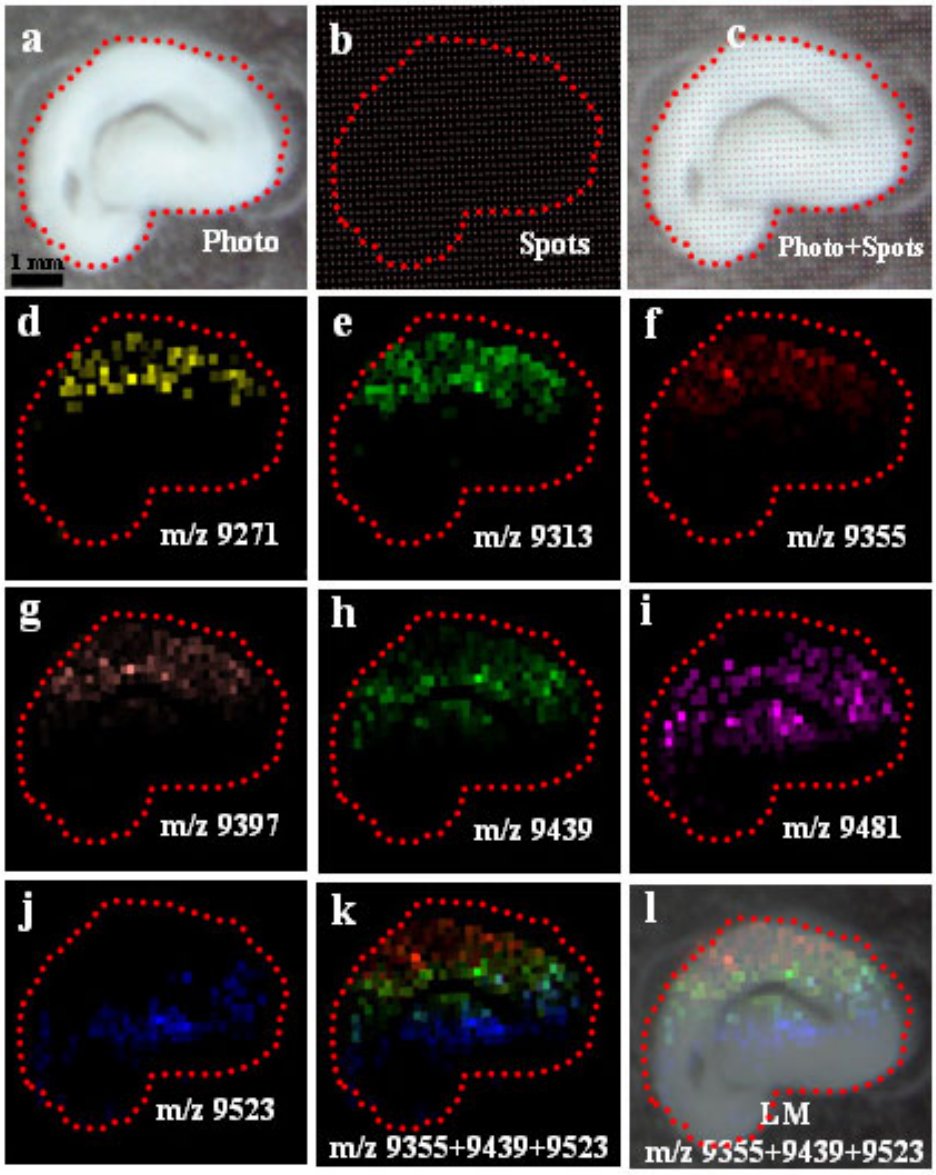

Figure 2. EM – (a) Coffee endosperm transversal section in stage 4 of development; (b) Scanned area displaying ionized spots; (d-j) Molecular images of components detected with a +42 Da increase. (k) Co-localization of some molecular components; (l) Comparison of the co-localized components with endosperm section photo. Dashed lines show endosperm contour.

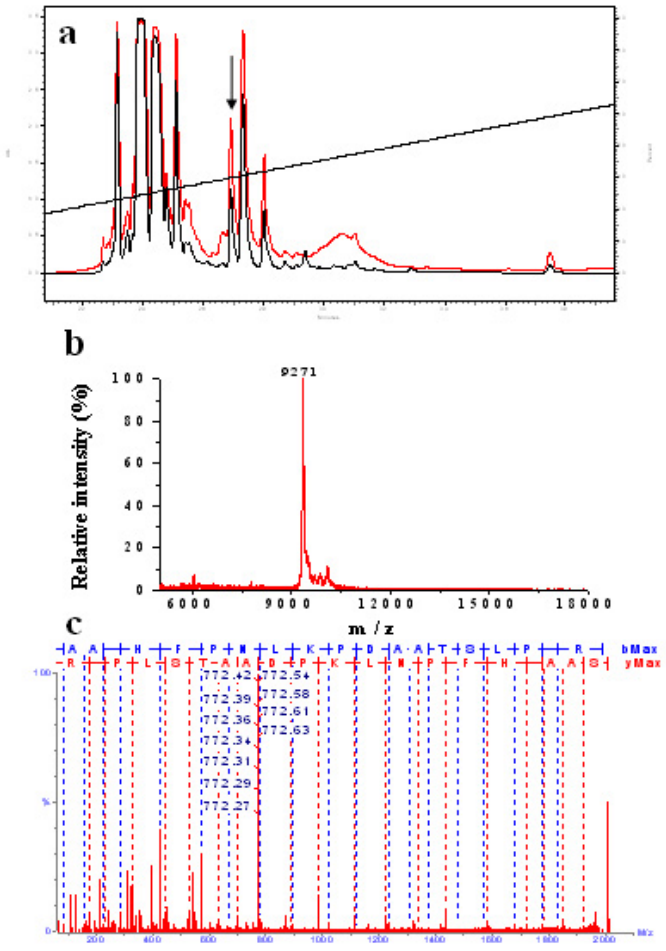

Figure 3. (a) RP-HPLC purification step of the 9 ka protein. Arrow points the fraction containing the coffee endosperm 9 kDa protein; (b) MS spectrum of the fraction labeled by arrow in (a); (c) Representative MS/MS spectrum of a trypsin-digest fragment of the coffee 9 kDa protein.

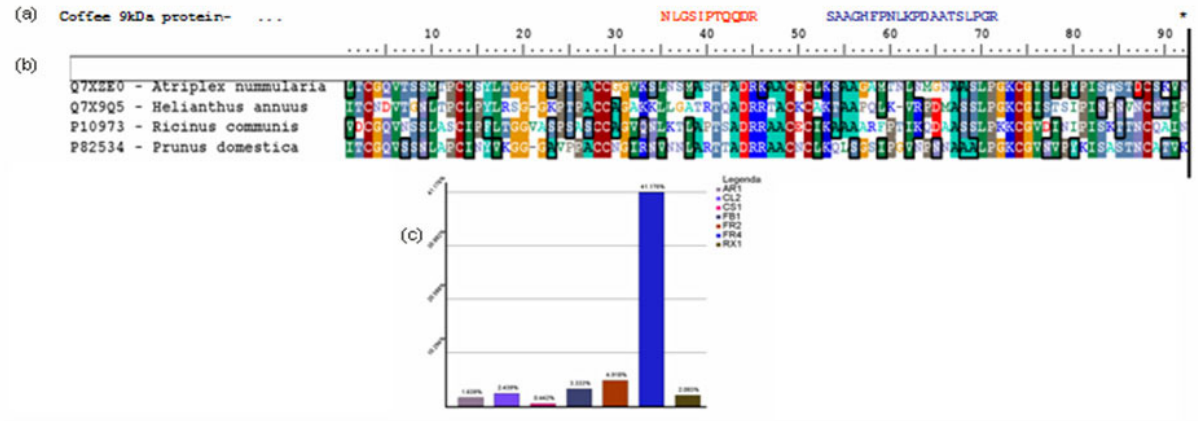

Figure 4. (a) Peptídeos trípticos correspondentes à proteína do café de 9 KDa, identificada por analises de tBLASTn contra o banco de dados Coffee EST. Os aminoácidos em azul foram obtidos por seqüenciamento *de novo*, e os em vermelho foram deduzidos por MALDI-MS. (b) Alinhamento demonstrando a similaridade entre as seqüências de aminoácidos das proteínas de transferência de lipídeos de diferentes espécies. (87-94% de similaridade); (c) Expressão eletrônica de LTP (cDNAs) em diferentes bibliotecas de cDNAs de café.

DISCUSSION AND CONCLUSION

We have shown in the present study that IMS can be an useful tool, alone or in conjunction with traditional biochemical techniques, to investigate coffee proteins in coffee beans, involved or associated with important metabolic activities determinant of quality. Further development of this technique may allow it to be used in screening programs for quality assessment.

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Sugars in Green Coffee Beans – Important Aroma Precursors an their Correlation to Post Harvest Method

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SUMMARY

Quantitative analyses of low molecular weight **sugars** in green **coffees** (Coffea Arabica) have been carried out by **LC-MS** to check either **processing** method and **storage** conditions. Coffees that have been processed by wet or dry traditional methods showed a relationship between the kind of post harvesting and the fructose and glucose contents. It has been seen that wet treatment leads to low levels of these sugars and the dry method leads to higher amounts. A comparison with the fresh controls indicates that the low levels of both sugars could be the consequence of a decrease of glucose and fructose concentration during the dry processing. For the first time cryo-conserved samples showed that dry processed coffee retains initial fructose and glucose levels, whereas semi-washed or wet processing reduces the amount of mono-saccharides present in the beans. At the same time we stored batches of same green coffees with different moisture content (MC) (7%, 10%, 13%) in fridge (10 °C) at room-temperature (25 °C) and in stove (40 °C) for nine months. Also in these cases we analysed fructose, glucose and sucrose and the conclusion is that high moisture or high temperature during storage of green coffee seams to cleave sucrose in fructose and glucose in parallel to sensorial deterioration and allows the use of monosaccharide content as quality marker. According to Knopp et al. (2005) and Bucheli et al. (1998) in both cases the glucose level is a good green coffee quality marker, instead sucrose, the major low molecular sugar in green coffee beans, is not significantly affected by coffee processing, but here we found even complete cleavage of sucrose under the drastic conditions.

INTRODUCTION

Green coffee is usually processed by two methods: wet and dry (sometimes called *natural*) processing. They are used to convert the freshly harvested coffee cherries into green coffee beans. The following roasting process leads to products that are differently characterized in aroma and taste. Wet process coffee brews usually show a fine aroma and a higher acidity; in contrast dry process coffee brews show full body and sweetness. To evaluate the differences brought by the kind of cherries used in wet (fully ripe cherries) and in dry (fruits of all stages) process, we planned our study starting from a unique coffee batch and adopted careful hand-selection for the dry process samples.

The second part of the study is related to check a coffee quality indicator. Appropriate storage conditions are important to avoid raw material spoiling, and the MC measurement is not sufficient obtained information about the raw product quality. Only the use of sensitive and reliable markers could fill this gap and could help to detect early stages of coffee deterioration that are currently detected by cup tasting (Bucheli et al., 1998) or labour intense extraction and GC-MS analysis of the green bean volatiles. Glucose is present only in traces amounts in good quality coffee (Wolfrom et al., 1960; Viani, 1986), and is generated in higher amounts upon poor storage as a result of sucrose hydrolysis (Bucheli et al., 1996). Small increases of glucose can be precisely determined by LC-MS (Nielsen et al., 2006).

EXPERIMENTAL

Material and Method

Batch processing

For the processing experiments only fully ripe and sound coffee fruits were used. This was ensured by thorough manual sorting of single batches of freshly harvested coffee cherries. The experiments were undertaken in parallel (wet and dry, plus control) and in three repetitions.

Wet processing

The coffee fruits were pulped using a drum pulper, and submitted to 22 hours of underwater tank fermentation. Then the washed parchment coffee beans have been dried on a sun terrace. After six days the final water content of 12% was achieved.

Dry processing

The coffee cherries of the same batch used for the wet processing have been dried as whole fruits on the terrace sun. After sixteen days the final water content of 12% was achieved.

Control samples

Green coffee cherries of the same batch were "killed" using a liquid nitrogen bath. In such way we blocked the initial sugar content to the original level even before every processing treatment.

Storage conditions and sampling

First of all we shared the original batch in three parts, the first one was kept as it was, the second was lyophilized to obtain a MC of 7%, the third part was treated in exccicators filled with water under the sieves and heated to about 35 °C to avoid the direct contact between liquid water and green coffee, but permit green coffee to absorb water up to a MC of 13%. Samples were then put in hermetic bags and closed under vacuum pressure. Each MC condition was put in fridge (10 °C), in room (25 °C) and in oven (40 °C). Storage went on for 9 months.

Moisture Analysis

MC was determined by GAC 2100 (Dickey John; Auburn, IL, USA) using the determination of capacitance in triplicate.

Sample preparation

5 g of the raw coffee were ground after freezing in liquid nitrogen and the power was suspended in 100 ml of boiling water for 5 minutes. After centrifugation, to separate sugars from other components, 1 ml of supernatant was passed through a two-steps SPE extraction column sequence: a hydrophobic C18 and a SAX (strong anionic exchange) cartridges were connected in series and conditioned each with 1ml of methanol followed by 1 ml of milli-Q water. This procedure was done in triplicate.

Cup quality Evaluation

The green coffee was roasted, ground and brewed in hot water. All samples were blindly tested every three months during the storage trial with a panel usually composed by the same four to six persons. Cup tasting was carried out against a freshly roasted coffee from industrial production line (illycaffé S.p.A.)

Chromatographic conditions

Instrumentation

The LC system consists of a HP1100 liquid chromatograph (Agilent Technologies, USA) equipped with a vacuum degasser, a quaternary solvent delivery compartment with low pressure mixing, an auto-sampler and a column compartment. Separation was performed on a Shodex column (Asahipak NH2 250 x 4.6 mm) at 40 °C using 0.1% formic acid and acetonitrile in 40/60 isocratic mode with a flow rate of 0.6 ml/min.

Detection was performed by MRM multiple reaction monitoring of the ions 383 $[2M+Na]^+$ to 203 for glucose and fructose and 365 $[M+Na]^+$ for saccharose in ESI-MS/MS mode on an Applied Biosystems QTRAP instrument. Calibration was performed against external standards.

Alternatively evaporative light scattering (ELSD, Sedere, France) could be used for detection using an isocratic 75/25 mixture using water and acetonitrile. The detector was operated at 3.5 bar air pressure at 42 °C, using a gain setting of 12 in this case.

Figure 1 shows an schematic drawing of separation and detection principle.

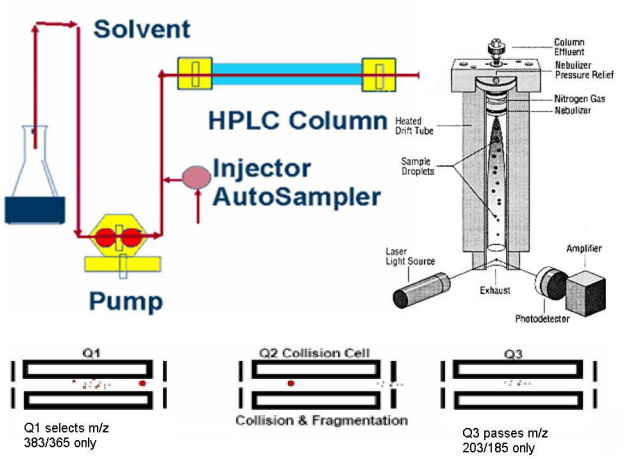

Figure 1. Schematic drawing of instrumentation and detection principle.

RESULTS AND DISCUSSION

A typical distribution pattern for an unwashed Arabica from Brazil for the low molecular carbohydrates present in green coffee is shown in Figure 2. Sucrose accounts for more than 90% of total low molecular carbohydrates. Fructose and glucose levels are about 20 times lower; others are even less than 100 parts of the sucrose levels (Knopp et al., 2005).

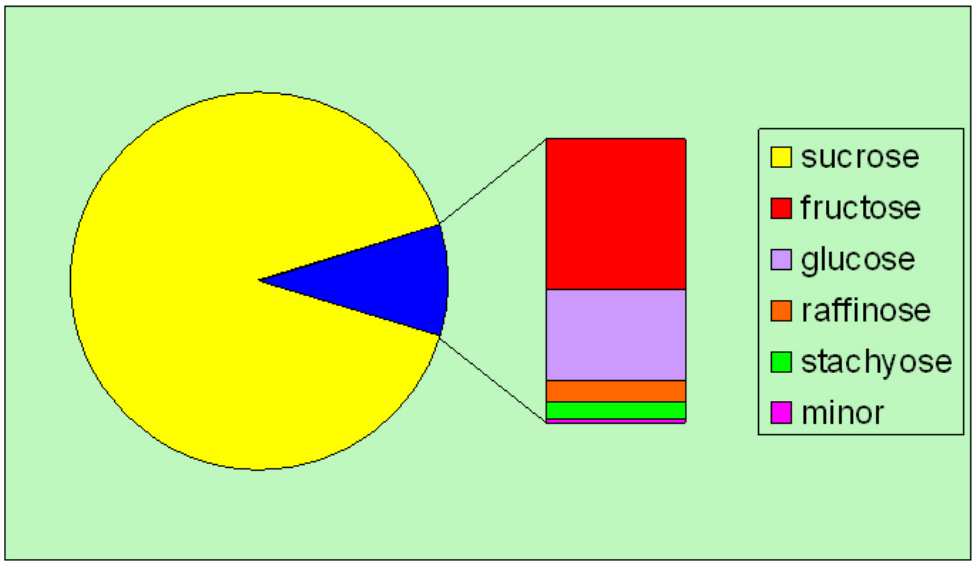

Figure 2. Distribution profile of oligosaccharides present in green coffee.

As can be seen in Figure 3 sucrose is not affected by the way of processing treatment. On the other hand fructose and glucose show a clear influence by the post-harvesting process:

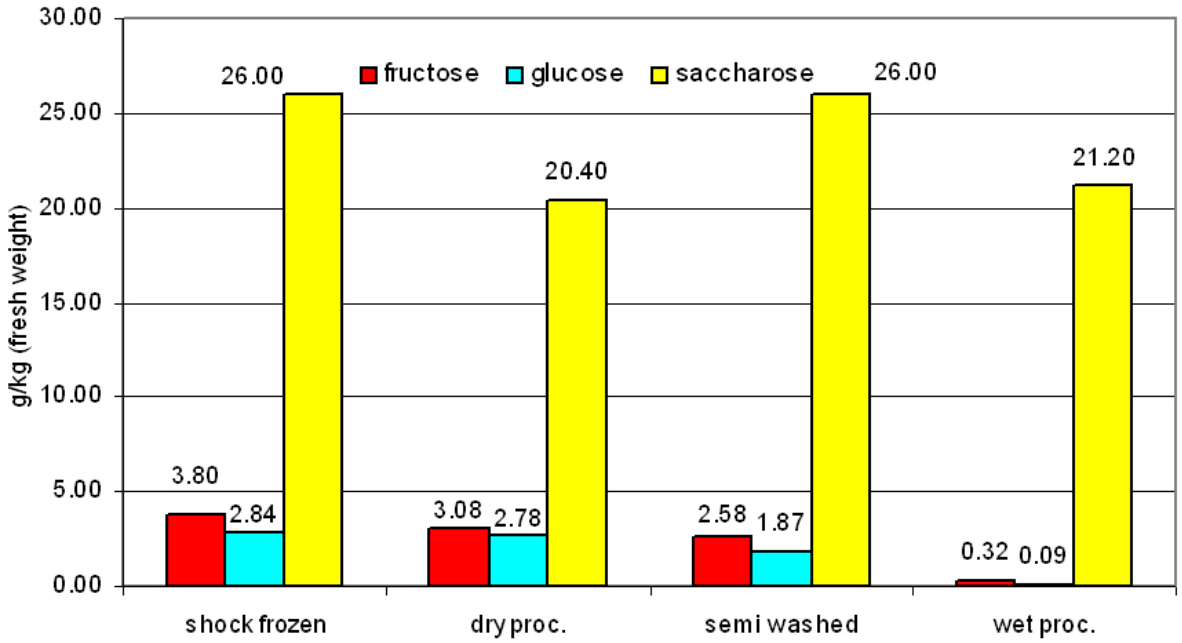

Figure 3. Influence of post harvest treatment on mono saccharide and sucrose levels.

The mono saccharides vary and show high levels in shock frozen samples and dry processed samples, lower concentrations in semi-washed samples and lowest in wet processed samples. In comparison to literature the sucrose levels here determined are quite low and may be due to incomplete extraction or under estimation due to non-linear calibration curves.

In Figure 4 is reported the fructose behaviour shown by a coffee stressed by high temperature (40 °C) and low MC (7%).

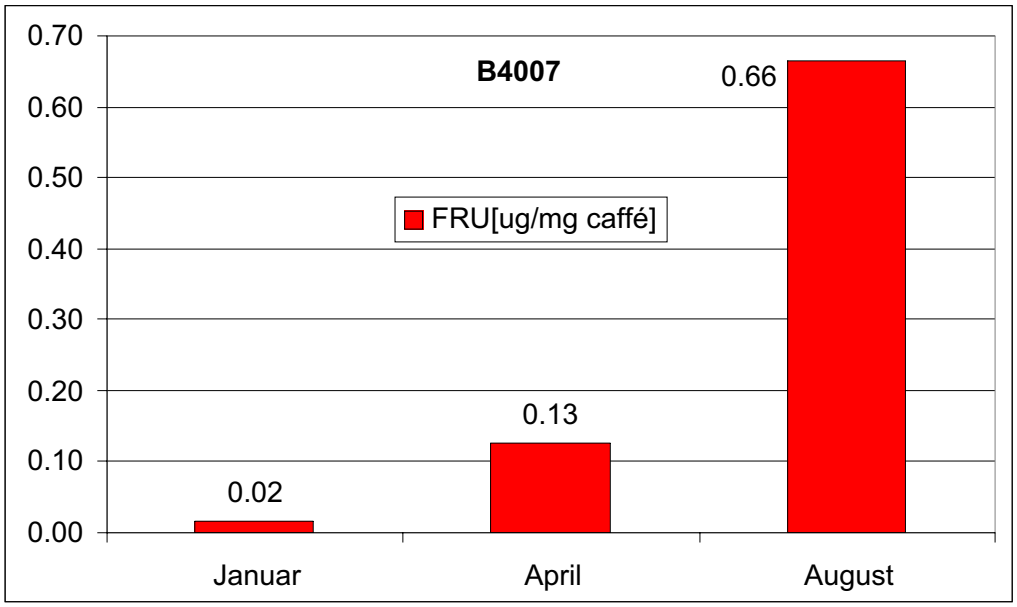

Figure 4. High temperature storage conditions on 2006 samples fructose content.

Figure 5 shows the saccharose pattern for the same storage condition showing a slow decrease, which was going to zero for high temperature and high moisture storage (dat not shown here):

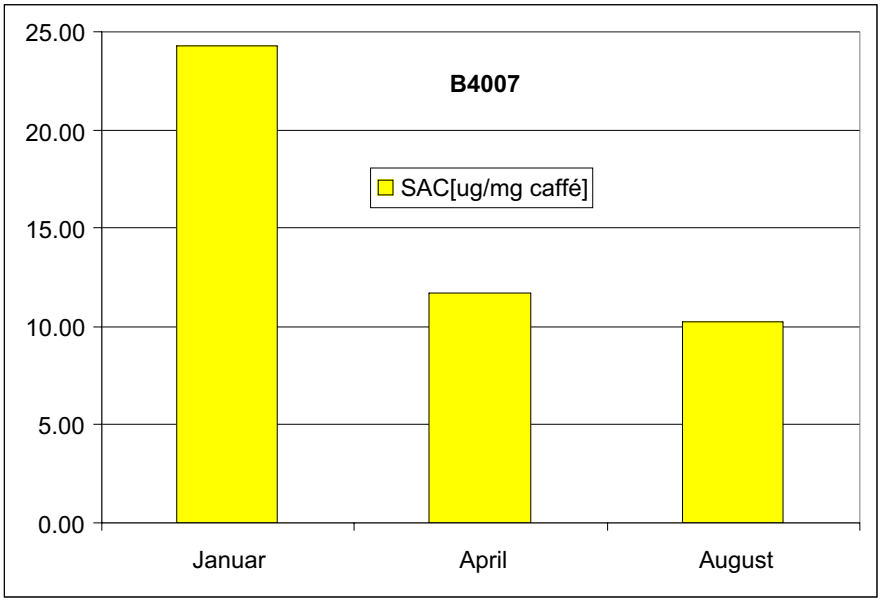

Figure 5. Reduction of sucrose levels under high temperature storage.

Sensory evaluation done in parallel showed a deterioration of the quality in cup as expected by these harsh storage conditions.

CONCLUSION

The influence of post harvest treatment on mono-saccharide concentration in green coffee beans could be confirmed by LC-ELSD and LC-MS/MS as a universal technique. For the first time cryo-conserved samples showed that dry processed coffee retains initial fructose and glucose levels, whereas semi-washed or wet processing reduces the amount of mono-

saccharides present in the beans. High moisture or high temperature during storage of green coffee seams to cleave sucrose in fructose and glucose in parallel to sensorial deterioration and allows the use of monosaccharide content as quality marker.

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Caffeine, Phenol and Protein Analyses of Thirty-seven Clones of Nigerian Robusta Coffee (*Coffea canephora* Pierre ex. Froehner)

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SUMMARY

A study was carried out to characterise thirty-seven *Coffea canephora* clones using three biochemical characteristics, namely caffeine, phenol and protein contents. The phenol and caffeine contents were determined following extraction while protein was studied by polyacrylamide gel electrophoresis (PAGE) of floral bud. The clones were planted using the Fisher blocks, in which five plants of each clone were established in contiguous rows. Data were collected in three replicates. Characterisation data were analysed using analysis of variance and mean separation was done using Duncan's Multiple Range Test. Phenol content in the berry pulp of the clones ranged from 2.56% to 15.63%. The average percent of phenol in the berry pulp was 9.51, which was significantly higher ($p < 0.05$) than what was obtained in the leaves (4.54). The coefficient of variation for pulp phenol was high (35.26), thus indicating that, rapid selection for favourable phenol percentage is feasible. The high level of phenol found in some of the clones studied may be valuable in breeding for resistance to some major diseases and insect pests of coffee. Caffeine content among the clones ranged from 1.10% to 1.53% on dry matter basis (dmb). Clone C36 is high yielding and relatively low in caffeine content, hence it is a suitable clone that could be included in any breeding programme for low caffeine coffee in Nigeria. All the Niaollou (M) clones had high caffeine content. There were differences in the mobility and intensity of protein bands in thirty-three clones; four clones – E1, M36, T155 and T921 had no bands, suggesting that these clones have low protein content. In terms of bands` mobility, there were three categories: the fast (6.0-9.0 cm), the intermediate (3.0-5.9 cm) and the slow bands (0.1-2.9 cm.). The three categories of band occurred at four different levels of intensities: very thick, thick, thin and faint. Majority of the "T" clones showed thin and faint bands. The variation in the protein banding patterns of the different *C. canephora* clones observed in this study provides further information on the existing genetic diversity of the coffee clones in addition to that provided by agro-botanical characters.

Keywords: Caffeine, phenol, protein, *Coffea canephora,* Nigeria

INTRODUCTION

Coffee, a beverage crop, is the second most important commodity in the international market after petroleum (ICO, 1995). Among the important chemical compounds that are found in coffee, three of interests to breeders are caffeine, phenol, and protein (ICO, 1999).

Phenol, when present in significant quantity in coffee has been observed to be associated with resistance to leaf rust and coffee berry disease (Walyaro, 1983). Ram *et al*. (1982) reported significant positive correlation between phenolic compounds in coffee varieties and their resistance to leaf rust disease. Romanenko (1985) reported that the polyphenol content of coffee leaves is a good criterion for evaluating breeding material and selecting donors for resistance to the insect *Aphis humili*.

Caffeine (1, 3, 7-trimethylxanthine) is a methylated purine alkaloid (Vasudeva et al., 1981) that is widely distributed in the family Rubiaceae (Bonner, 1950). Its presence in different members of the genus *Coffea* is well known (Raju and Gopal 1979; Raju et al., 1981). Caffeine (C_8 H_{10} O_2 N_4) has molecular weight of 194.19 and is also found in kola, cocoa and tea. Caffeine increases alertness and improves attention (Nguyen-van Tam and Smith, 2001). It increases both speed and accuracy of retrieval of information and also improves short-term memory in reasoning or calculation tasks. Roasted coffee beans have between 1 and 2% caffeine but the level in the beverage is highly dependent on the method of preparation. Normal caffeine concentration in a cup of coffee varies between 50 mg and 125 mg. Caffeine stimulates the synthesis or release of the catecholamine, epinephine and norpinephrine hormones (better known as adrenalin and nor-adrenalin respectively) into the blood stream. Though caffeine consumption has been exonerated so far from causing either birth defects or low birth weights, the stimulant is not beneficial during pregnancy (Borea et al., 2001). For many consumers, the answer to the real or imaginary adverse effects of caffeine consumption is to consume decaffeinated instant coffee. Indeed, demand for decaffeinated coffee is increasing rapidly in the world market. In the USA alone, one-fifth of the wholesale value of coffee products, which exceeds $6 billion, is decaffeinated (Sreenath, 1997). Coffee is industrially decaffeinated before the beans are roasted, by the use of organic solvents or carbon dioxide, which is not acceptable to consumers due to reasons like residues of organic solvents in the coffee powder. Consequently, there is an urgent need to develop caffeine-free coffee cultivars (Sreenath, 1997). Caffeine content is greater in robusta than in arabica (Sylvain, 1969; Raju and Gopal, 1979). The direction of the international market on caffeine content in C. c*anephora* is towards its reduction (Montagnon et al., 1998; Ashihara and Crozier, 2001). Consequently, one of the breeding objectives for improved coffee quality in the world today is low or no caffeine content (Sreenath, 1997).

The protein band variation from different crop plant is based partly, to differences in the molecular weight of protein Crop traits are the expression of genes, and proteins are the primary products of genes. Agro-botanical variation reflects diversity in genetic structure. Genetic diversity in a crop germplasm is therefore better understood through the analysis of the primary product of the gene i.e. protein.

Gottlieb (1971) reported that variation in banding pattern could be equated to variation in genes coding for various proteins. Protein electrophoresis therefore offers an efficient means of identifying and quantifying genetic variation in crop germplasm (Davis, 1964; Carrens et al., 1997). The advantage of biochemical markers such as protein, is that they are genotypic markers and hence, will reflect the actual genetic distances between accessions and their common ancestry more accurately than phenotypic markers (Gepts, 1995). Berthou and Trouslot (1979) used isozyme analysis to separate the Guinean group from the Congolese group of *C. canephora* populations. The objectives of this study therefore were to carry out biochemical characterisation of Nigerian *C. canephora* germplasm.

MATERIALS AND METHODS

The study was carried out at Cocoa Research Institute of Nigeria, Gambari, Ibadan. The *Coffea canephora* clones studied and their geographical origin are A110, A116 (Ghana), C36, C90, C96, C105, C107, C108, C111 (Gabon/Zaire), E1, E77, E92, E106, E1, (Java), G87, G129 (Uganda), M10, M31, M36, M53 (Benin Republic), T4, T24, T45, T93, T116, T164, T169, T204, T314, T365, T921, T1049, T197, T155, T176, T395 and T220 (Zaire) The clones were planted in 1966. The plants were coppiced in 1997 and 2000. Three biochemical compounds namely phenol, caffeine and protein contained in each of the thirty-seven clones were determined.

Determination of phenolic content

Sample collection, sample preparation and determination of the total phenol

Two plant parts (fresh leaves and the pulp of seven months old mature berry) were investigated for their phenolic content. The plant parts were collected randomly from each of the thirty-seven clones. 100 g of fresh leaves or berry pulp were washed and dried in an oven at 50-60 °C. The samples were milled to powder using Moulinex electronic grinder and stored in a conical flask in a dessicator at 4 °C until used. A sample of 2.0 g of oven dried and milled leaves or berry pulp was taken into a conical flask containing 100 ml of 80.0% ethanol and boiled for eight hours. The ethanol solvent was changed by decanting three times (3 x 100 ml) during the extraction of the total phenol. The ethanol extractions were then concentrated to 30-40ml in a vacuum circulating evaporator. The residue was refluxed for 1hour, filtered hot and diluted to 100 ml with distilled water. Then the filtrate was dried in the oven at a temperature of 80 °C. The percent phenol for each clone was determined from the weight of the dried residue. The percent phenol was calculated by dividing the weights of the dried residue with the initial 2.0 g of oven dried sample and multiplying by 100. The phenol extraction for each clone was repeated three times. The procedure followed in this study was as described by Bate-Smith (1962) and used by Odebode (1995). The data obtained were subjected to analysis of variance, while significantly different means were compared using Duncan's Multiple Range Test.

Determination of caffeine content

Sample preparation, caffeine extraction and determination

Fruit that were used for caffeine determination were harvested at complete maturity and depulped using the wet processing method (Omolaja and Williams, 1997). Caffeine content was determined by modifying the extraction methods of Horwits (1970) and Barre et al. (1998). Caffeine content was evaluated using 40 randomly selected coffee beans from freshly harvested berries per clone. The samples were milled to fine powder using Moulinex electronic grinder and stored in plastic containers in a dessicator at 4 °C until used. Each batch of beans was crushed for eight minutes. One gram of dried coffee powder was weighed into a conical flask.

Then 50 ml of 0.5 N boiling or hot H_2SO_4 were added. The mixture was then refluxed on water-bath for 30-50 minutes. Afterwards it was allowed to cool, then centrifuged and filtered. 40 ml was taken into a separating funnel and 30ml chloroform was added, and vigorously shaken. Two layers were formed at the top (organic) and bottom (chloroform) part of the separating funnel. The chloroform layer was collected. Another 30 ml chloroform was added to the organic content remaining in the funnel, which was shaken properly and the chloroform layer was collected as previously done. Then 40 ml of the chloroform fraction was dispensed into a pre-weighed 100 ml flat-bottomed flask. This was distilled off at between 60-80 °C. The distillate was dried in the oven to obtain caffeine, and the weight of caffeine was determined. The percent caffeine for each clone was obtained by dividing the caffeine weight by the initial weight of sample, and multiplying by 100. The extraction for each clone was repeated three times. Analyses of variances for caffeine content among the clones were carried out (Barre et al., 1998). Significantly different means were compared using Duncan's Multiple Range Test.

Electrophoretic analysis of floral bud protein

Unopenned floral buds collected from each of the thirty-seven *C. canephora* clones were used for the analysis. Five grams of the floral bud sample were used per clone.

Protein extraction

Floral bud protein was extracted by grinding 1g of the floral bud with mortar and pestle. 7.5 ml of 0.9% sodium chloride (NaCl) was added during grinding. The mixture was allowed to settle inside the test tube immersed inside an ice bath for one hour (Illoh, 1990). Then it was centrifuged at 4000 revolution per minute for 15 minutes. The clear supernatant was then poured into the test tube and stored inside the refrigerator. On the day of use, sucrose crystals (5 grains), three drops of each of 2.0% mercapto-ethanol and sodium dodecyl sulphate (SDS) were added to 1ml of the supernatant in the test tube and the mixture was boiled for 15 minutes in the water-bath. After cooling down to room temperature, a drop of 0.05% bromophenol blue was added to the sample as front marker (Akinwusi and Illoh, 1995).

Preparation gels are as indicated in Table 1. The loading of gels, electrophoretic run, staining, destaining, and measurement of the gels were carried out according to Omolaja and Fawole (2004). Photographs and drawings of the position of the different bands were done. Each protein band was considered as a qualitative character and coded as 1(presence) versus 0(absence) (Carrens et al., 1997). Characterisation data were subjected to Analysis of Variance and significantly different means were compared by Duncan's Multiple Range Test.

Table 1. Preparation of gel for the electrophoresis of floral bud protein, anther, pollinated and un-pollinated styles protein.

Stock solutions/ components	Upper gel	Lower gel
Floral bud protein		
40% Acrylamide	1.0 ml	10.0 ml
Upper gel buffer	2.5 ml (0.5 M; pH 6.8)	-
Lower gel buffer	-	7.5 ml (1.5 M; pH 8.8)
Distilled water	6.33	19.5
10% Sodium dodecyl sulphate	1.0	0.4
10% Ammonium persulphate	0.05	0.1
TEMED (N N N 'N'—Tetramethyl ethylenediamine), 'Electran'	20.0 ul	50.0 ul
Anther, pollinated and un-pollinated styles protein		
Acrylamide	1.35 ml	10.0 ml
Upper gel buffer	2.50 ml	-
Lower gel buffer	-	7.50 ml
Distilled water	6.00 ml	11.20 ml
10.0% Sodium Dodecyl Sulphate	0.10 ml	0.30 ml
Ammonium persulphate	0.01 ml	0.30 ml
TEMED	0.03 ul	0.10 ul

Table 2. Phenol and caffeine content of thirty-seven clones of *Coffea canephora* clones.

Clone	Phenol %		Caffeine%
	Pulp	Leaves	
A110	3.26d*	3.23b	1.36a
A116	15.00a	3.16cb	1.20b
C36	3.26a	4.06b	1.10b
C90	10.83b	4.30b	1.16b
C96	9.97b	3.03cb	1.26b
C105	10.80b	6.26a	1.43a
C107	7.70cb	4.10b	1.40a
C108	6.60c	3.61b	1.26b
C111	11.40b	6.20a	1.23b
E1	3.40d	4.10b	1.36a
E77	7.41c	4.13b	1.33a
E92	8.30cb	5.03a	1.30a
E106	4.40d	5.06a	1.53a
E130	2.56d	3.06cb	1.10b
G87	9.60b	3.81b	1.40a
G129	9.46b	3.66b	1.36a
M10	4.03d	4.26b	1.36a
M31	12.53b	3.36b	1.33a
M36	11.40b	5.13a	1.46a
M53	10.53b	4.66b	1.43a
T4	15.63a	5.43a	1.33a
T24	11.56b	5.22a	1.16b
T45	11.62b	5.11a	1.30a
T93	10.40b	5.03a	1.30a
T116	11.71b	5.41a	1.20b
T164	11.62b	5.62a	1.30a
T169	10.53b	4.98a	1.36a
T204	2.63d	4.61b	1.23b
T314	9.64b	4.62b	1.20b
T365	12.56b	4.63b	1.33a
T921	10.71b	5.22a	1.20b
T1049	12.60b	3.46b	1.30a
T197	8.44cb	3.44b	1.43a
T155	9.23b	4.81a	1.43a
T176	10.44b	5.22a	1.50a
T395	10.06b	5.11a	1.26b
T220	10.52b	4.48b	1.43a
Mean	9.51	4.54	1.31

Values with similar letters are not significantly different from each other by Duncan's Multiple Range Test at $P < 0.05$.

RESULTS

Phenol analysis of thirty-seven *Coffea canephora* clones

The percent phenol in the berry pulp ranged between 2.56 and 15.63 among the clones studied. The percent phenol of clones C36, E1, E130, T204 and A81 were significantly less

than those of the other clones at $p < 0.5$ (Table 2). Clones A116, M10, M31, T365, T1049 and T4 had the highest percent of phenol.

Table 3. Relative mobilities of protein bands in thirty-seven clones of *Coffea canephora.*

Clone	Slow bands	Intermediate bands	Fast bands	Total no of bands
	0.1-2.9cm	3.0-5.9cm	6.0-9.0cm	
A110	1	-	1	2
A116	-	-	1	1
C36	-	-	2	2
C90	1	-	-	1
C96	-	-	3	3
C105	1	1	1	3
C107	1	-	2	3
C108	-	-	1	1
C111	-	3	3	7
E1	-	-	-	-
E77	-	-	2	2
E92	1	-	-	1
E106	-	1	-	1
E130	1	1	2	1
G87	2	1	1	4
G129	2	-	-	2
M10	-	-	2	2
M31	1	2	1	4
M36	-	-	-	-
M53	1	1	-	2
T4	2	-	1	3
T24	1	-	1	2
T45	2	-	-	2
T93	2	-	-	2
T116	1	2	2	5
T164	1	-	2	3
T169	-	-	1	1
T204	-	1	-	1
T314	1	-	-	1
T365	-	-	1	1
T921	-	-	-	-
T1049	1	-	1	2
T197	1	-	1	2
T155	-	-	-	-
T176	-	1	-	1
T395	1	-	1	2
T220	1	-	-	1

-- *Band absent.*

The average percent of phenol in the berry pulp was 9.51, which was significantly higher ($p < 0.05$) than what was obtained in the leaves (4.54). The range of phenol in the leaves was between 3.03% and 6.26%. The coefficient of variation for pulp phenol was high (35.26), thus indicating that, rapid selection for favourable phenol percentage is feasible.

Caffeine analysis of thirty-seven *Coffea canephora* clones

The caffeine content among the clones ranged between 1.10% and 1.53% on dry matter basis (dmb) (Table 2). Clones T24, T395, E130, C36 and T365 had caffeine contents that were significantly lower than the other clones ($p < 0.5$). Clones E106 and T176 recorded the highest caffeine contents of 1.53% and 1.50% dmb respectively. Clone C36 that was among the clones that recorded the lowest caffeine content of 1.10% is a regularly bearing and high yielding clone. All the Niaollou (M) clones had high caffeine content.

Electrophoretic analysis of floral bud protein in *C. canephora*

The protein band variations as observed in thirty-seven clones of *C. canephora* are presented in Table 3. Marked differences were observed on the number, combination and intensity of bands among the clones.

In terms of bands` mobility, there were three categories: the fast (6.0-9.0 cm), the intermediate (3.0-5.9 cm) and the slow bands (0.1-2.9 cm.) (Table 3). The protein bands at 0.1 to 2.9cm and 6.0 to 9.0cm were exhibited by twenty-one clones. The intermediate bands were observed in only nine of the clones studied. Some bands at 1.0, 7.0 and 8.0 cm were unique to clones C111, E130 and T24. The highest number of bands was found in clone T116, while clones E1, M36, T921 and T155 did not show any band. The three categories of bands occurred at four different levels of intensities: very thick, thick, thin and faint. Majority of the "T" clones showed thin and faint bands.

DISCUSSION

The coefficients of variation among many of the characters were low, indicating that the genetic base of the Nigerian *C. canephora* germplasm is very narrow. The thirty-seven clones studied showed different levels of phenol content. According to Bate-Smith (1962) high level of phenol in plants are associated with their tolerance/ resistance to some diseases and insect pests. The high level of phenol found in some of the clones studied may be valuable in breeding for resistance to some major diseases and insect pests of coffee. Romanenko (1985) observed that Asian coffee clones with high phenolic contents were resistant to attack by the leaf sucking insect *Aphis humilis*. Studies on intra-specific variation in caffeine content in many *Coffea* species including *C. canephora* showed caffeine free coffee was yet to be found (Barre et al., 1998). Low caffeine coffee is however, increasingly being demanded in the international market (Sreenath, 1997). The caffeine content among the clones ranged between 1.10 and 1.53% . Clone C36 is high yielding and relatively low in caffeine content, hence it is a suitable clone that could be included in any breeding programme for low caffeine coffee in Nigeria.

The variation in the protein banding patterns of the different *C. canephora* clones observed in this study provides information on the existing genetic diversity of the coffee clones. Isozyme studies showed that diploid *Coffea* species are characterised by a high level of genetic diversity with a polymorphism index of 0.05 compared with 0.03 for the amphidiploid and self-fertile *C. arabica* (Berthou and Trouslot, 1977).

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Is the GC Analysis of Cafestol and Kahweol a Problem?

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SUMMARY

When they analyzed the coffee diterpenes in the unsaponifiable matter using GC/MS, Guerrero et al. identified a number of different cafestol and kahweol derivatives in green Arabica and Robusta coffee beans. Aside from cafestol, kahweol, 16-0-methylcafestol and 16-0-methylkahweol they described the degradation products of cafestol and kahweol as e.g. dehydrocafestol and dehydrokahweol, previously known as components of roasted coffee, exclusively. Our investigations with a cafestol and kahweol standard solution demonstrated that using the GC-analysis without silylation, decomposition products of these diterpenes could be determined as well. However, after silylation of the diterpenes such products were not detectable, leading to the supposition that the GC conditions applied by Guerrero et al. may not be suitable for analyzing the heat-labile diterpenes cafestol and kahweol.

INTRODUCTION

Using GC/MS, Guerrero et al. identified a number of different cafestol and kahweol derivatives in green coffee beans, e.g. 15,16-dehydrocafestol and 15,16-dehydrokahweol (Guerrero et al., 2004). These findings were surprising.

Using HPLC, our working group analyzed only cafestol and kahweol in green Arabica coffees and in addition, 16-O-methylcafestol and 16-O-methylkahweol in green Robusta coffees (Kölling-Speer et al., 2001). 15,16-dehydrocafestol and 15,16-dehydrokahweol were detected in roasted coffees, exclusively, but not in green coffees nor even in strongly steam-treated coffees (Kurt and Speer, 2001). Therefore, we examined the behavior of cafestol and kahweol during GC analysis.

METHODS

GC conditions (HP 5890 GC-FID): column: DB 5, 30 m, 0.25 mm ID, 0.1 µm film (J&W Scientific); oven temperature: 1 min at 60 °C, 15 °C/min to 280 °C, 15 min at 280 °C; detector: 290 °C; injection: 1 µl; injector temperature for silylated diterpenes: 280 °C.

GC/MS conditions (Varian-GC, MAT 95): column: DB 5, 30 m, 0.25 mm ID, 0.1 µm film (J&W Scientific); injector: 300 °C; oven temperature: 1 min at 60°C, 10°C/min to 280 °C, 15 min at 280 °C; injection: 1 µl.

RESULTS

In previous studies we determined the diterpenes in coffee with GC as well (Speer, 1989; Speer and Kölling-Speer, 2006). However, we analyzed the diterpenes after silylation, not unsilylated. In Figure 1 a GC chromatogram of a silylated cafestol/kahweol standard solution, injected at 280 °C, is presented. Usually, we obtain two signals, one for the persilylated cafestol und one for the persilylated kahweol. In the case of a roasted coffee, cafestal, kahweal and the two dehydro compounds are normally detected in the range marked with a bracket.

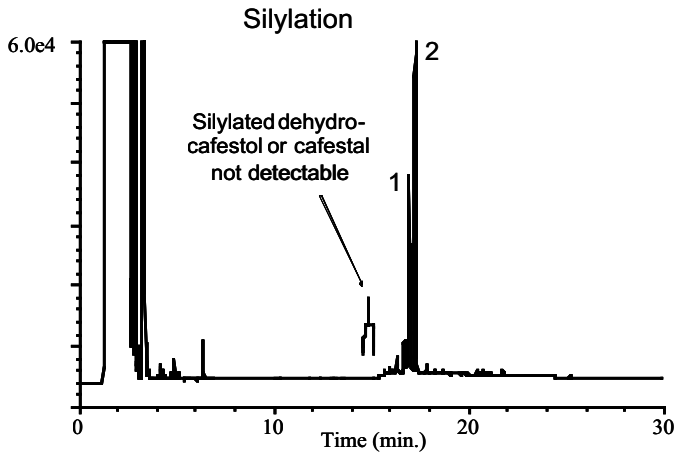
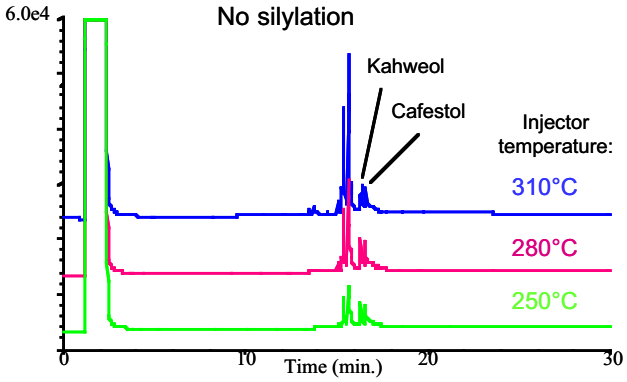


Figure 1. Chromatogram (GC/FID) of a silylated cafestol/kahweol standard solution, 1 kahweol, 2 cafestol, 200 ng/µl each

Analyzing the solution without derivatisation we obtained the chromatograms shown in Figure 2. Depending on the chosen injector temperature cafestol und kahweol were decomposed. The number and the intensity of the components formed increased with rising injector temperature.

In order to obtain more information about the degradation products we used GC combined with MS (Figure 3). Injecting the cafestol/ kahweol standard solution at 300 °C injector temperature, we detected, in addition to the two diterpenes, a number of degradation products. The first group with m/z 296 belong to kahweol, the latter derivatives with m/z 298 to cafestol. Furthermore, there was one component with m/z 370 and one with m/z 372.



Figrure 2. Chromatograms (GC/FID) of an unsilylated cafestol/kahweol standard solution, 200 ng/µl each, injected at different injector temperatures.

Comparing Figure 2 and Figure 3 it becomes obvious that not only the injector temperature influences the analysis but also the injection port used (GC/FID: Agilent; GC/MS: Varian).

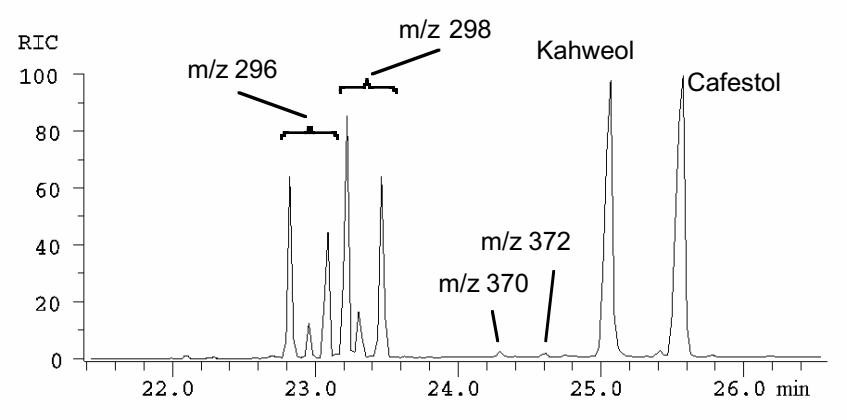


Figure 3. GC/MS chromatogram of the unsilylated cafestol/kahweol solution, 200 ng/µl each, injected at injector temperature 300 °C.

CONCLUSIONS

When analyzing heat-labile substances such as cafestol and kahweol, the conditions of the analyzing method have to be adapted so that artifacts cannot be produced and, thus, not detected.

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Diterpenes in Green Coffees Harvested in Different Years

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SUMMARY

Diterpenes were analyzed in 53 Arabica coffees and 37 Robusta coffees from various cultivation areas obtained in 2000, 2004 and 2005. In the Arabica coffees the content of cafestol ranged from 3.6 to approx. 10.2 g/kg; kahweol was determined from 1.9 to approx. 4.5 g/kg dry weight of coffee. In the Robusta coffees the contents of the main diterpenes, cafestol and 16-OMC, ranged from 1.9 to 6.8 g/kg and from 0.8 to 2.5 g/kg dry weight. The contents of the minor component kahweol were in most cases smaller than 0.1 g/kg, and the amounts of 16-OMK were below 0.03 g/kg dry weight.

INTRODUCTION

The two most important commercial coffee species, Coffea Arabica and Coffea Canephora var. Robusta, differ in their pentacyclic diterpenes (Figure 1): Arabica coffee beans contain the diterpenes cafestol and kahweol, whereas in Robusta coffee beans cafestol, small amounts of kahweol, and in addition, 16-O-methylcafestol (16-OMC) and 16-O-methylkahweol (16-OMK) are found (Speer et al., 1991; Kölling-Speer et al., 2001).

Green Arabica and Robusta coffees obtained in three different years from various cultivation areas in Africa, Asia, Central and South America were analyzed for their diterpene contents.

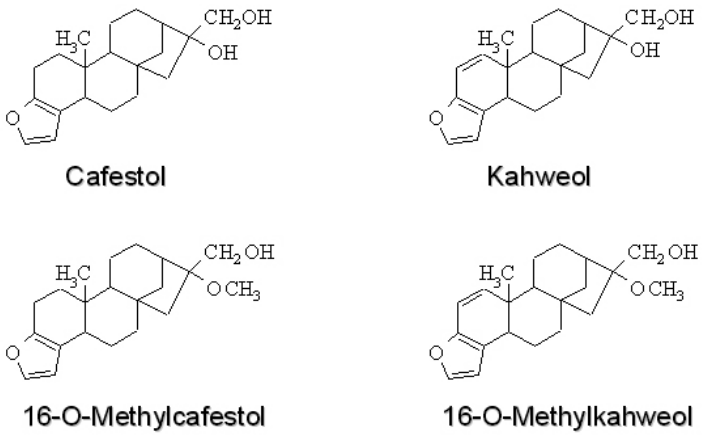

Figure 1. Structural formulae of cafestol, kahweol, 16-OMC, and 16-OMK.

METHOD

The determination was carried out using DIN method No. 10779 (1999). The quantification of the diterpenes was performed by RP-HPLC with UV-detection at 220 nm and 290 nm on the

basis of external calibration using standard substances. The contents of kahweol and 16-OMK were calculated with the factor of Nackunstz et al. (1987). The detection limits of all four diterpenes were approx. 0.010 g/kg.

RESULTS

53 Arabica coffees and 37 Robusta coffees from various cultivation areas obtained in 2000, 2004 and 2005 were investigated.

In the Arabica coffees (Figure 2), only cafestol and kahweol were found with more cafestol than kahweol in each case. 16-OMC was not detected, which had been expected because 16-OMC is the reliable indicator substance for Robusta coffee. The content of cafestol ranged from 3.6 to approx. 10.2 g/kg, and kahweol was determined from 1.9 to approx. 4.5 g/kg dry weight of coffee.

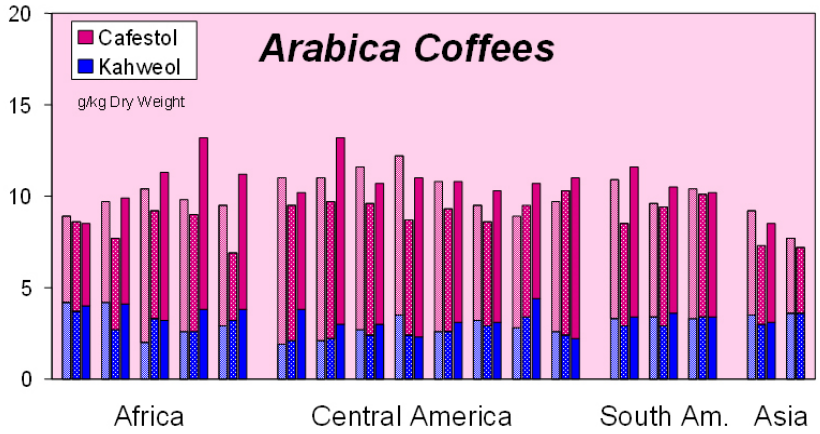

Figure 2. Contents of cafestol and kahweol in Arabica coffees.

In the Robusta coffees (Figure 3), in addition to cafestol and the expected 16-OMC, kahweol and 16-OMK were detected as well. The contents of the main diterpenes cafestol and 16-OMC ranged from 1.8 to 6.8 g/kg and from 0.8 to 2.5 g/kg dry weight. The median value and the mean value for 16-OMC were equal with approx. 1.7 g/kg dry weight.

The contents of kahweol and 16-OMK as minor components were significantly lower. The values of kahweol were in most cases smaller than 0.1 g/kg, and the amounts of 16-OMK were below 0.03 g/kg dry weight.

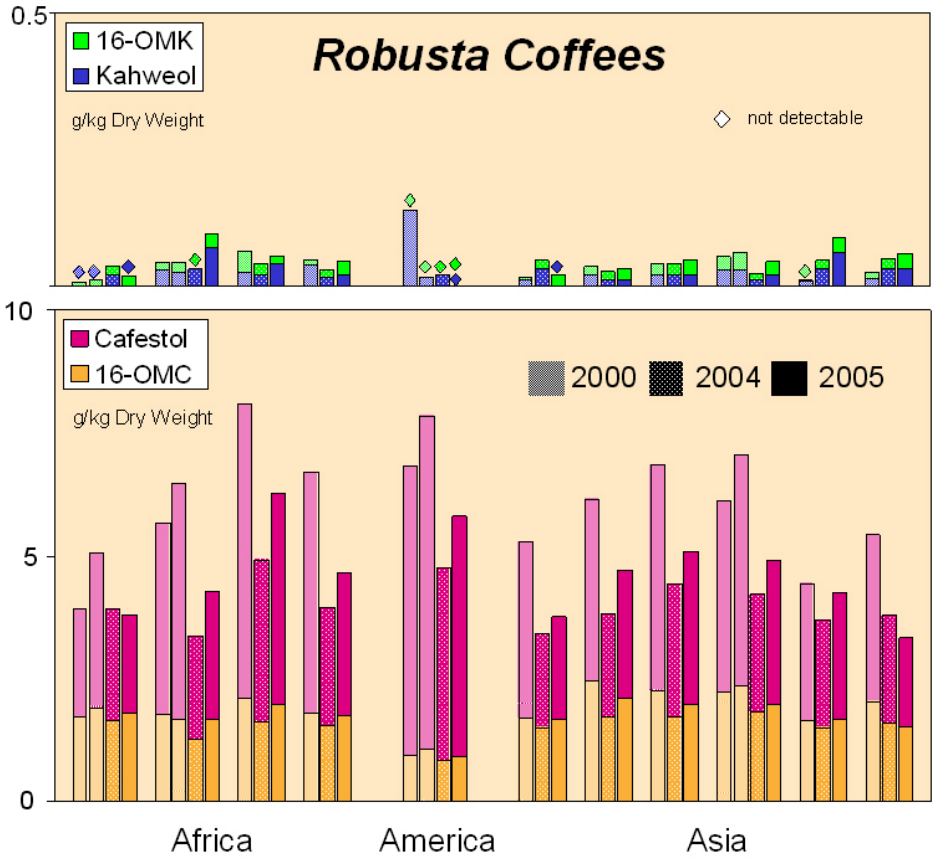

Figure 3. Contents of cafestol, 16-OMC, kahweol, 16-OMK in Robusta coffees.

ACKNOWLEDGEMENT

We would like to thank the German Coffee Industry for providing the green coffee samples.

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Tocopherols in Green Coffees Harvested in Different Years

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SUMMARY

Tocopherols were analyzed in 34 Arabica coffees and 22 Robusta coffees from various cultivation areas obtained in 2004 and 2005. In the Arabica coffees the total content of α- and β-tocopherol ranged from 124 to 166 mg/kg dry weight. In the Robusta coffees the contents of α-tocopherol and β-tocopherol showed amounts from 53 to 78 mg/kg dry weight. The portion of α-tocopherol was between 25% and 30% in regard to the Arabicas and between 42% and 50% regarding the Robustas. In the Robusta coffees additionally γ-tocopherol could be detected in traces.

INTRODUCTION

The published data about the amounts and the distribution of the individual tocopherols for the two most important coffee varieties are inconsistent (Folstar et al., 1977; Ogawa et al., 1989). Therefore, green Arabica und Robusta coffees harvested in the last years from the most important cultivation areas in Asia, Africa, Central und South America were analyzed. In addition, we examined a small number of roasted coffees as results recently presented by González et al. (2001) reported higher amounts of γ- tocopherol in roasted coffee than in green coffee.

METHOD

Saponification was carried out using the DIN method No. 10779 (1999). The quantification of the tocopherols was performed by normal phase HPLC with fluorescence detection (Ex 295 nm, Em 330 nm) on the basis of external calibration using standard substances.

RESULTS

34 Arabica coffees and 22 Robusta coffees, both obtained in 2004 and 2005, were analyzed. In Figure 1 and Figure 2 the HPLC chromatograms of an Arabica and a Robusta coffee are presented. In agreement with literature data, α- and β-tocopherol were detected. So far, the occurrence of γ-tocopherol had not been firmly established and could only be assumed (Figure 2). By means of GC-MS, we were able to clearly detect and identify γ-tocopherol in some Robusta coffees (Figure 3) (Kölling-Speer and Speer, 2005).

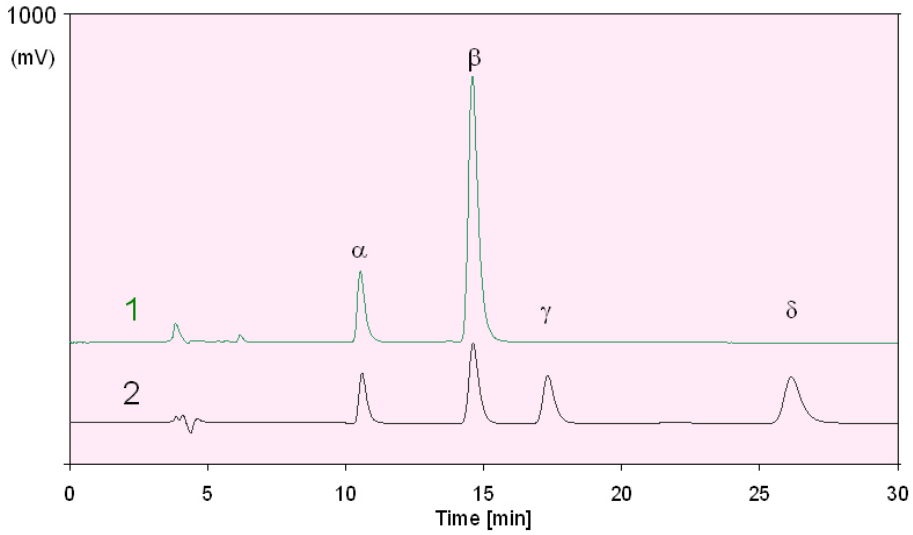

Figure 1. HPLC chromatograms of tocopherols 1 in a green Arabica coffee and 2 of a standard solution (1.1 µg/ml).

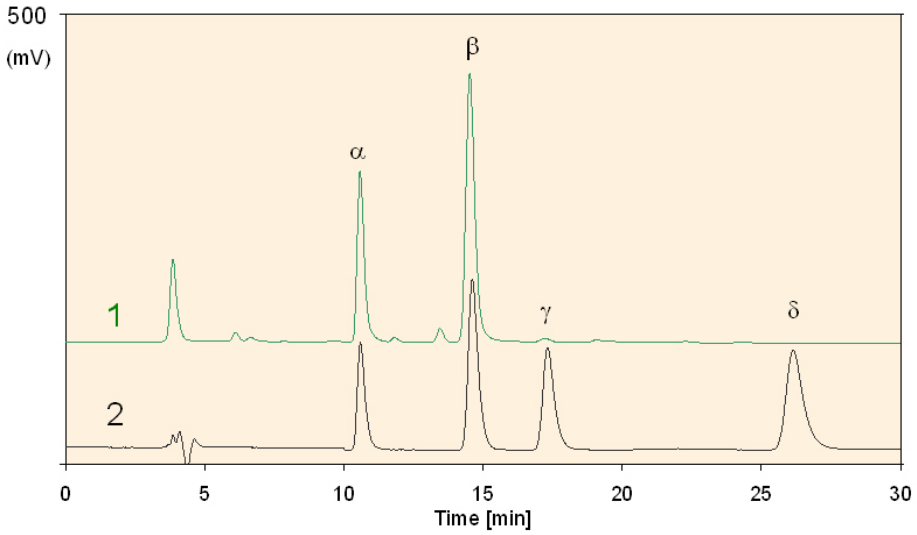

Figure 2. HPLC chromatograms of tocopherols 1 in a green Robusta coffee and 2 of a standard solution (1.1 µg/ml).

Due to the extremely small amounts of γ-tocopherol (below 1 µg/kg), only the results for α- and β-tocopherol are presented here. Arabica coffees contained more tocopherols in sum than Robusta coffees did (Figures 4 and 5). The latter showed amounts from 53 to 78 mg/kg dry weight and the Arabicas had amounts from 124 to 166 mg/kg dry weight. The portion of α-tocopherol was between 25% and 30% in regard to the Arabicas and between 42% and 50% regarding the Robustas.

In addition to the green coffees, a small number of roasted coffees were analyzed as well. The results by González et al. who found higher amounts of γ-tocopherol in roasted than in green coffee could no be confirmed. The reason may be that different methods were employed: We analyzed the tocopherols in the unsaponifiable matter, González et al. injected the extracted coffee oil directly without further clean-up.

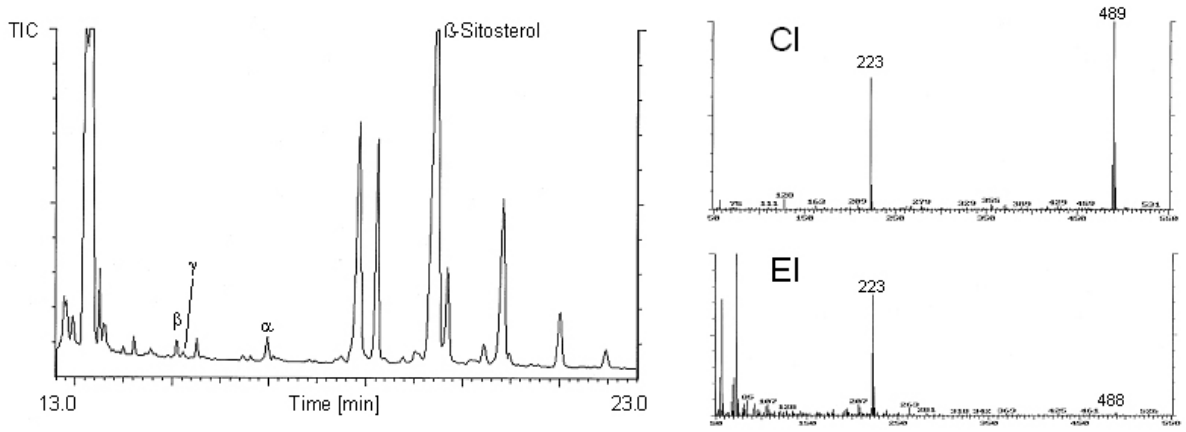

Figure 3. GC/MS chromatogram of tocopherols in green Robusta coffee with CI and EI mass spectrum of γ-tocopherol, respectively.

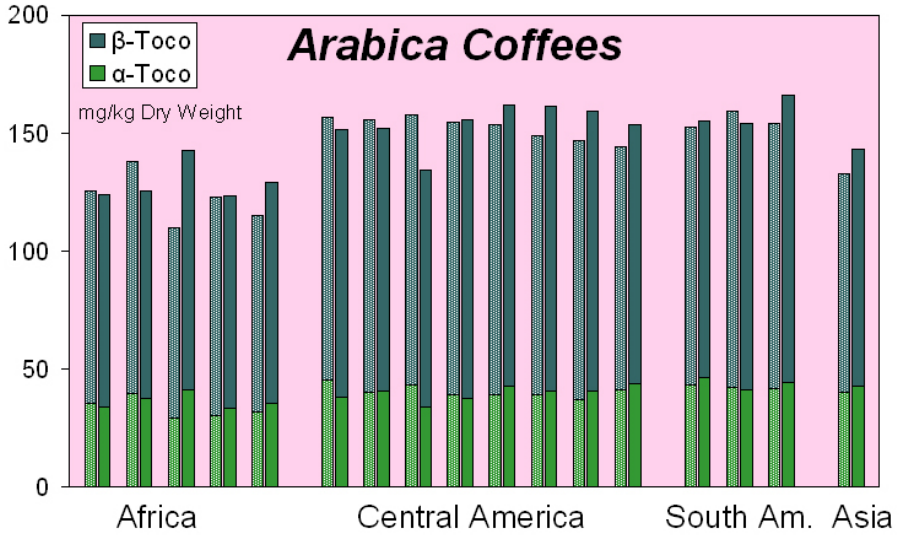

Figure 4. Contents of α- and β- tocopherol in Arabica coffees.

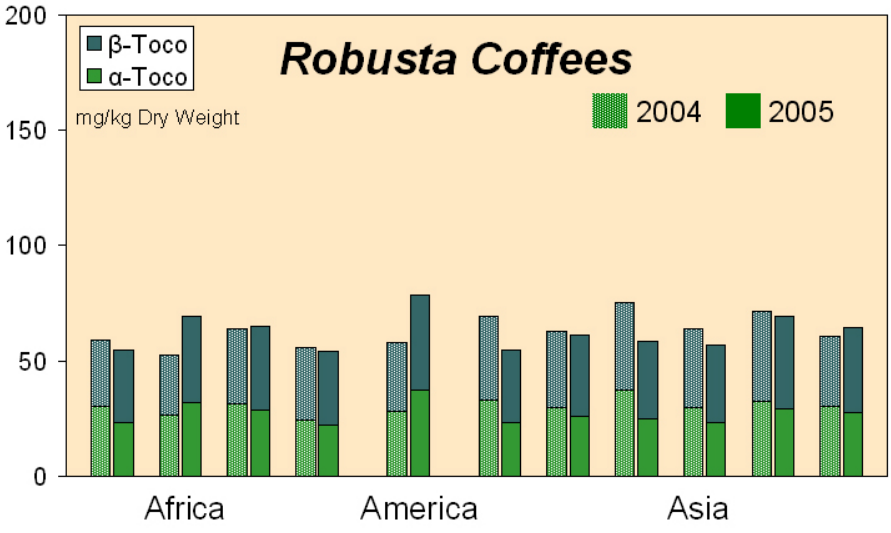

Figure 5. Contents of α- and β- tocopherol in Robusta coffees.

ACKNOWLEDGEMENT

We would like to thank the German Coffee Industry for providing the green coffee samples.

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16-O-Methylcafestol Determination in Coffee Using Different HPLC Columns and Eluents with Diode Array and MS Detection

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SUMMARY

The diterpene 16-O-methylcafestol (16-OMC), the characteristic indicator substance for Robusta coffee, is determined according DIN 10779 by RP-HPLC with an acetonitrile-water mixture as eluent. Regarding the new stationary phases as well as monolithic columns, a number of different RP-HPLC columns were tested (Nucleosil® 120-3 C_{18}, Nucleosil® 100-5 C_{18} AB, Purospher® RP-18e, Synergi 4µ Max-RP™, Polaris® C18-Ether 5 µ, Synergi 4 µ Fusion-RP™, Chromolith® Performance RP-18e, Chromolith® SpeedROD RP-18e). In consideration of the economical aspect, acetonitrile or methanol were used in isocratic compositions with water as eluent.

INTRODUCTION

The diterpene 16-O-methylcafestol (16-OMC) is the characteristic indicator substance for Robusta coffee in mixtures with Arabicas (Speer et al., 1991; Kölling-Speer et al., 2001). The analysis of 16-OMC is usually performed by using DIN method 10779: After lipid extraction and subsequent saponification, the unsaponifiable matter (mainly the diterpenes) is separated by HPLC on a C_{18} column with an acetonitrile-water mixture (50:50) (DIN 10779, 1999). Detection can be carried out with DAD or MS. A mass spectrum of 16-OMC is presented in Figure 1.

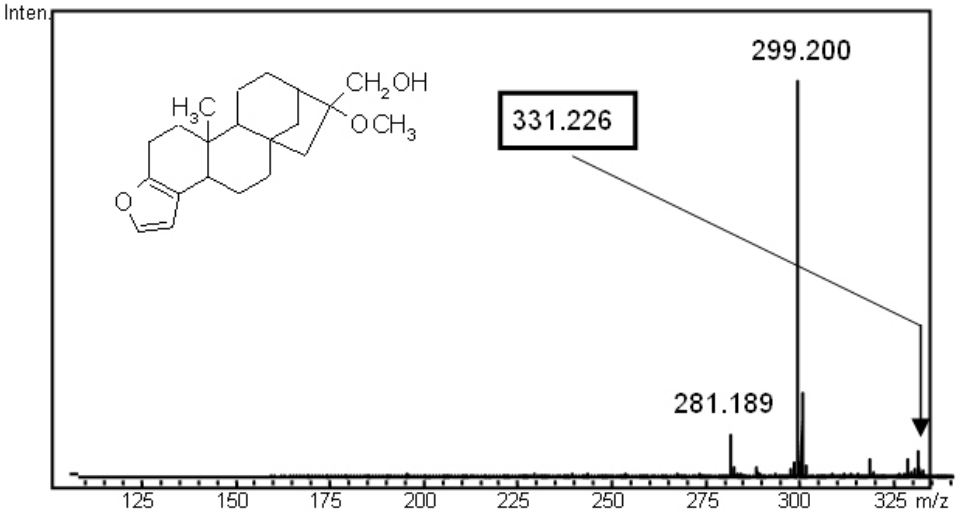

Figure 1. Mass spectrum of 16-OMC aquired using "microTOF" MS (Bruker, Germany) ESI pos. mode.

In our working group we generally use a Nucleosil® 120-3 C_{18} column (Macherey&Nagel, Germany) (Figure 2) which separates cafestol and kahweol, solely differing in a double bond, nearly down to base line. However, one disadvantage is the long run time of approx. 70 minutes.

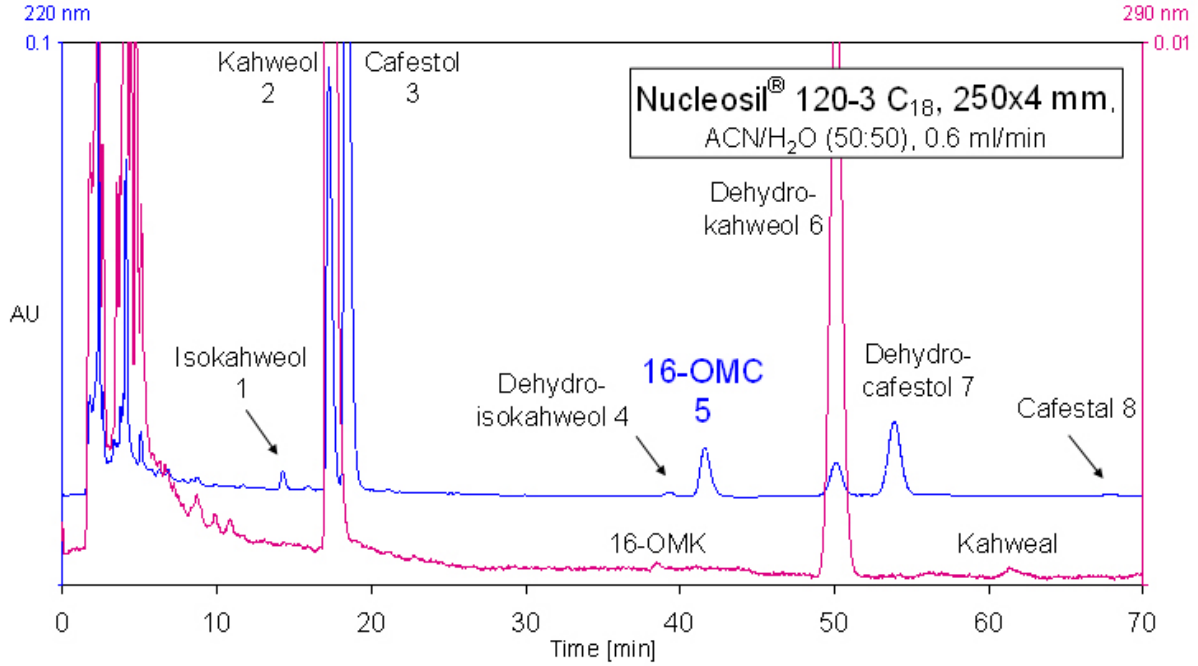

Figure 2. HPLC chromatograms of an Arabica/Robusta coffee mixture.

Therefore, a number of new stationary phases offered by different companies were tested for the diterpene analysis. The chromatograms and a short description of the assets and drawbacks are summarized below.

METHOD

In each case, the same sample (Arabica/Robusta coffee mixture) prepared according DIN 10779 was injected with 20 or 5 µl depending on the column diameter, and the chromatograms were acquired with DAD.

RESULTS

Using Nucleosil® 100-5 C_{18} AB (Macherey&Nagel, Germany) and Purospher® RP-18e (VWR, Germany), cafestol and kahweol were separated only coarsely, whereas the other diterpenes could well be determined in a run time of 45 minutes or 25 minutes, respectively. The column Synergi 4µ Max-RP™ (Phenomenex) separated well all of the diterpenes; however on isocratic conditions, the run time was too long with 100 minutes.

With 50 minutes, the analysis took half of the time on Polaris® C18-Ether 5µ (Varian), and each diterpene could be determined well (Figure 3). Here, as well as on Purospher® and Synergi Max™, dehydroisokahweol eluated behind the 16-OMC.

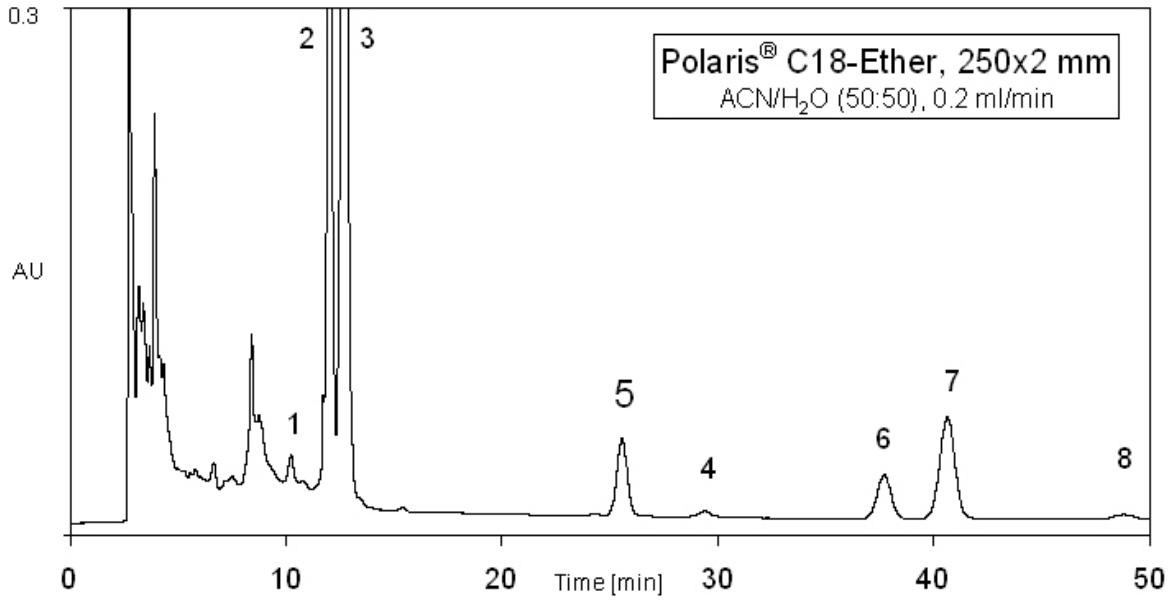

Figure 3. HPLC chromatogram of the same coffee sample, UV 220 nm.

Using Synergi 4µ Fusion-RP™ run time for diterpenes ended after approx. 55 minutes (Figure 4). It could be accelerated by gradient elution, but the fast consecutive series of the dehydro compounds and cafestal reduces this possibility.

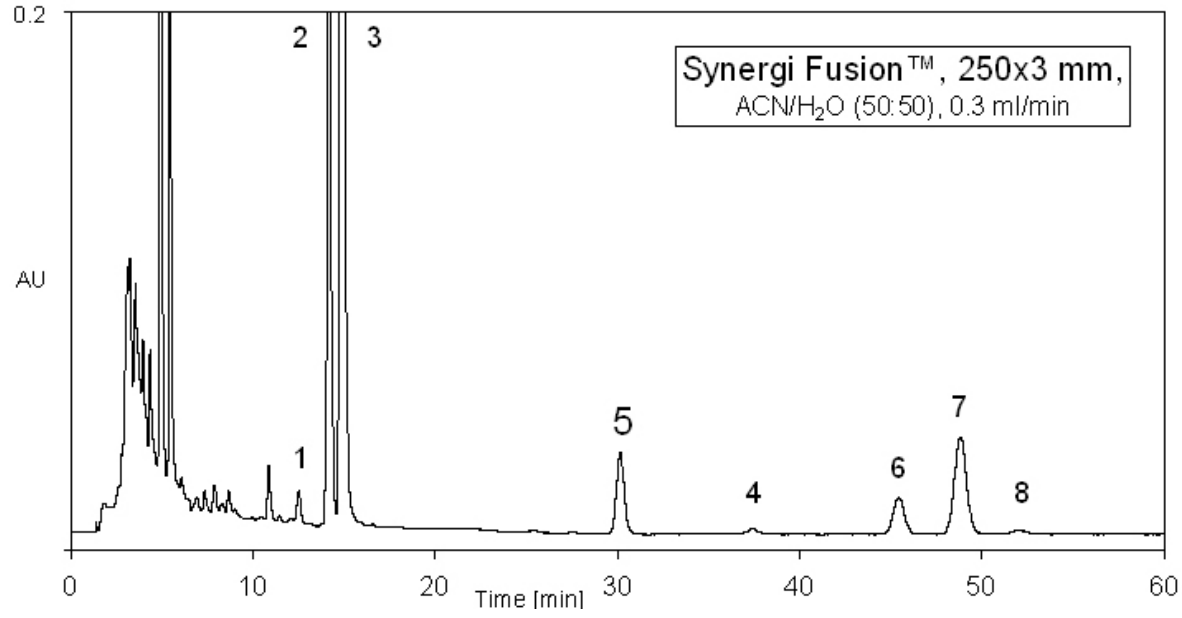

Figure 4. HPLC chromatogram of the same coffee sample, UV 220 nm

Besides the conventional particulate RP-columns, with Chromolith® Performance RP-18e and Chromolith® SpeedROD RP-18e (VWR, Germany) two monolithic silica columns were applied for the diterpene analysis (Figures 5-7). Run time could be shortened up to 6 minutes on Chromolith® Performance. However, due to the higher flow rate, sensitivity decreased and the roasting compounds kahweal and cafestal could hardly be detected.

Noticeable was the influence of the eluent: using methanol/water, separation was better and faster than with acetonitrile/water (Figures 5, 6).

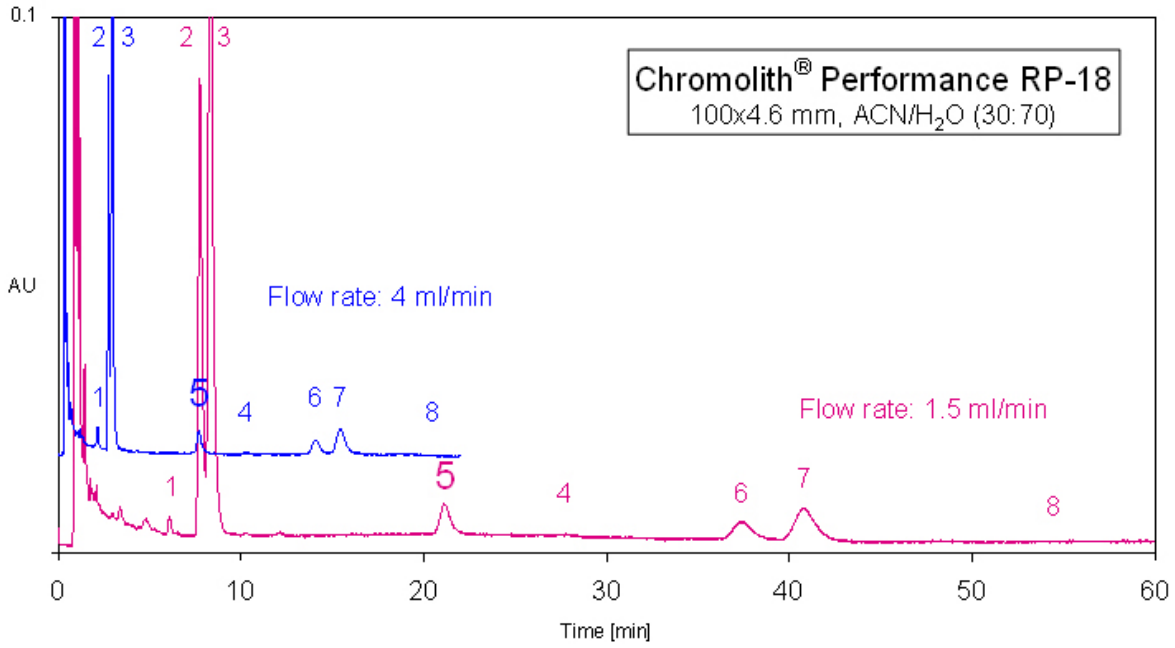

Figure 5. HPLC chromatograms of the same coffee sample, UV 220 nm.

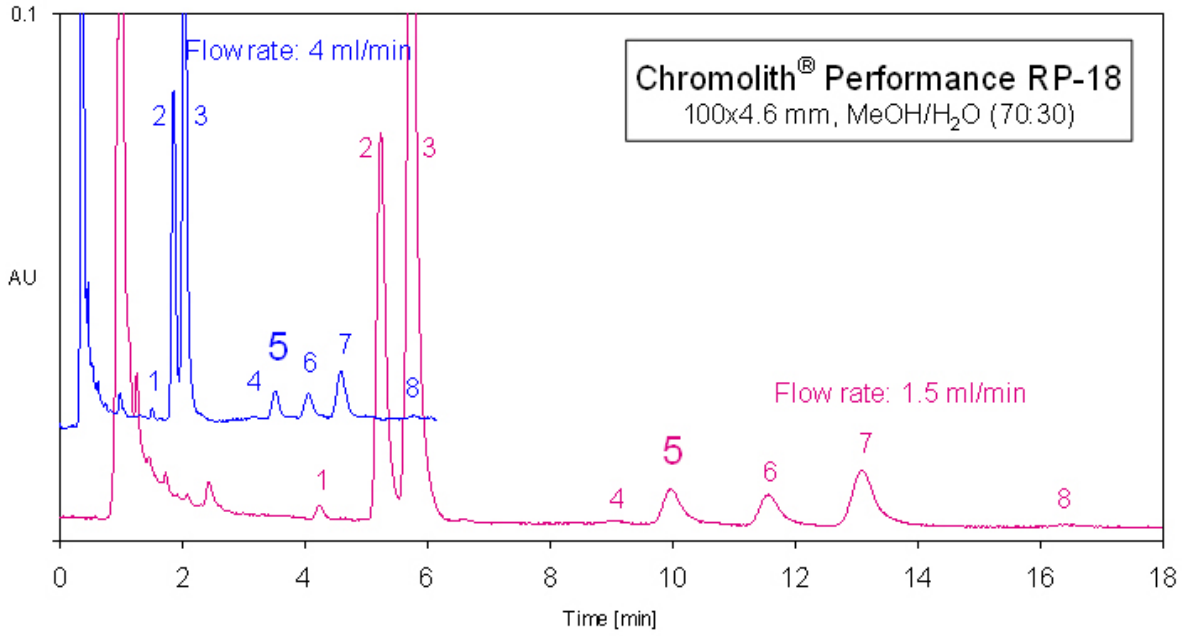

Figure 6. HPLC chromatograms of the same coffee sample, UV 220 nm.

Especially with a flow rate of 1 ml/min, the Chromolith® SpeedROD RP-18e is very suitable for analyzing coffee samples with LC/MS (Figure 7).

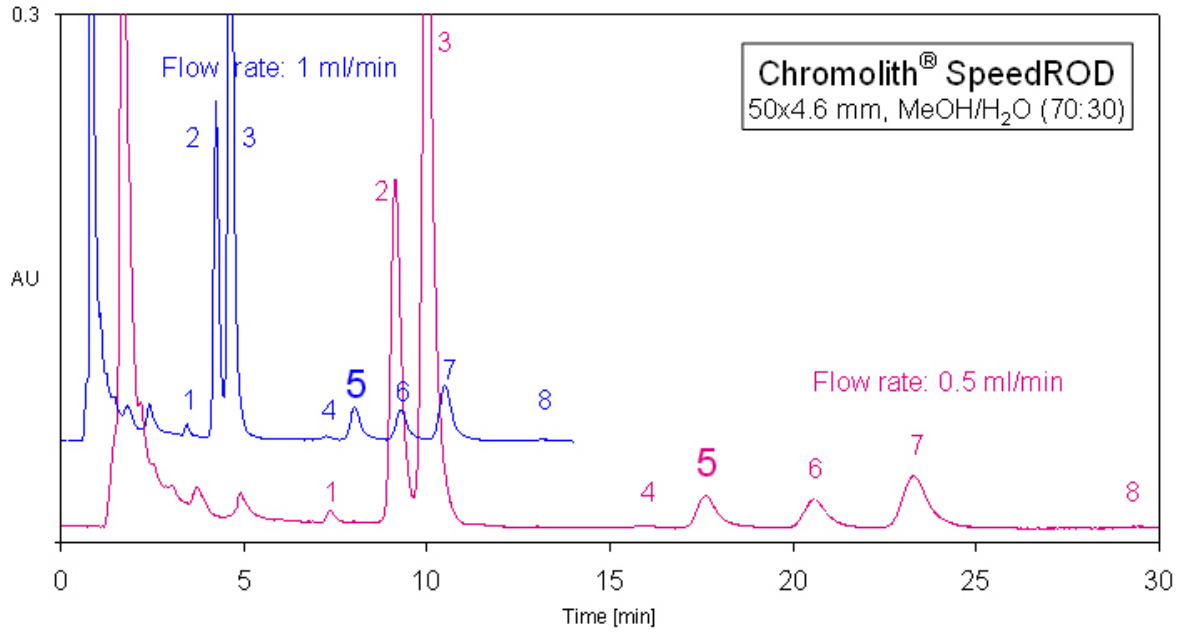

Figure 7. HPLC chromatograms of the same coffee sample, UV 220 nm.

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Comparison of Different Staining Methods for Coffee Proteomic Analysis

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SUMMARY

Proteomic methods, such as bidimensional electrophoresis (2-DE) and mass spectrometry, have been extensively used for the study of protein differential expression in several plants including *Arabidopsis thaliana*, rice and wheat. Specifically in the 2-DE method, deep attention must be given to the protein staining technique, which often involves silver nitrate or Coomassie Brilliant Blue (CBB). Silver staining is usually preferred over CBB due to the higher sensitivity obtained. Nevertheless, silver-staining resolution could significantly vary according to the studied organism and more specifically to the researched tissue. In *Coffea* spp., 2-DE analysis has been rarely employed. Some studies of protein expression have been reported in this culture mainly involving the biosynthesis of caffeine and metabolism during seed germination. The study of the global protein expression in coffee plants in response to biotic stress conditions had not been reported until now. Phytonematode infection can be considered one of the most important biotic stresses that affect coffee production and *Meloidogyne paranaensis* is one of the major nematode species that infects coffee plants. In this report, the protein expression of infected- and non-infected roots of coffee (*Coffea canephora*) were analyzed and the protein pattern determined by 2-DE. Gels were stained with silver nitrate or CBB, in order to obtain an optimized method for proteomic analysis of plant-nematode interaction. The 2-DE analysis revealed an enhanced number of protein spots, as well as differentially expressed proteins, when CBB was used. A total of approximately 70 and 100 spots were observed in silver and CBB stained gels, respectively. Moreover, 18 differentially expressed proteins were observed in CBB gels, and only 8 in the silver stained gels. This report showed that the staining method was crucial for an optimized protein analysis of coffee. Similar results were obtained for cotton roots and therefore these results may be extended to other plant species in order to better understand the host-pathogen interaction.

INTRODUCTION

Proteomic methods, such as bidimensional electrophoresis (2-DE) and mass spectrometry, have been extensively used for the study of protein differential expression in several plants. Specifically in the 2-DE method, deep attention must be given to the protein staining technique, which often involves silver nitrate or Coomassie Brilliant Blue (CBB). Silver staining is usually preferred over CBB due to the higher sensitivity obtained. Nevertheless, silver-staining resolution could significantly vary according to the studied organism and more specifically to the researched tissue.

In *Coffea* spp., 2-DE analysis has been rarely employed. Some studies of protein expression have been reported in this culture mainly involving the biosynthesis of caffeine and metabolism during seed germination. The study of the global protein expression in coffee plants in response to biotic stress conditions had not been reported until now.

Phytonematode infection can be considered one of the most important biotic stresses that affect coffee production and *Meloidogyne paranaensis* is one of the major nematode species that infects coffee plants. Several attempts have been made in order to control nematode infection, including the use of nematicides and crop rotation; however, the control of this pathogen using these methods is generally inefficient. The use of resistant genotypes and also genetically modified plants with enhanced resistance has been pointed as an alternative, which could contribute to a significant reduction in economic losses caused by *Meloidogyne* spp. Therefore, the knowledge of plant resistance genes and the elucidation of plant-pathogen interaction mechanisms are of great importance to control phytonematodes.

OBJECTIVE

The objective of this study was to analyze the protein expression of infected- and non-infected roots of coffee (*Coffea canephora*) by 2-DE and compare different staining methods in order to maximize the number of proteins being analyzed.

MATERIAL AND METHODS

Plant material

Coffee (*C. canephora*) plants were grown in sterile soil and maintained in green house. After six months, coffe roots were used for nematode infection. Non-infected plant roots were colected, washed and frozen in liquid nitrogen for further protein extraction and considered as the control condition.

Nematode culture and inoculation

Tomato roots were washed and homogenized in a blender with 0.5% (v / v) sodium hypochloride in order to obtain nematodes. The resulting material was sifted and the eggs were removed. After eclosion, juveniles were collected by centrifugation for 30 min at 2,500 x g and counted for plant infection. Each plant was infected with approximately 10,000 juveniles of *M. paranaensis*. Infected roots were collected 6 and 10 days after inoculation.

Protein extraction and bidimensional electrophoresis

Total proteins and isoelectric focusing were performed as described by Mot & Vanderleiden (1989). Approximately 150 ug of proteins were loaded onto the gel after a pre-run. Molecular mass separation was performed according to Laemmli (1970). After running, 2D gels were fixed overnight in a solution containing 30% ethanol and 10% acetic acid. Silver staining was carried out according to Blum et al. (1987). For CBB staining, a Coloidal Coomassie solution (0.1% Coomassie G250; 2% phosphoric acid; 10% ammonium sulphate and 20% methanol) was used.

RESULTS AND DISCUSSION

In the present report, coffee roots infected with *M. paranaensis*, were used for protein extraction and analyzed by 2-DE. Gels were stained with silver nitrate or CBB, in order to obtain an optimized method for proteomic analysis of plant-nematode interaction.

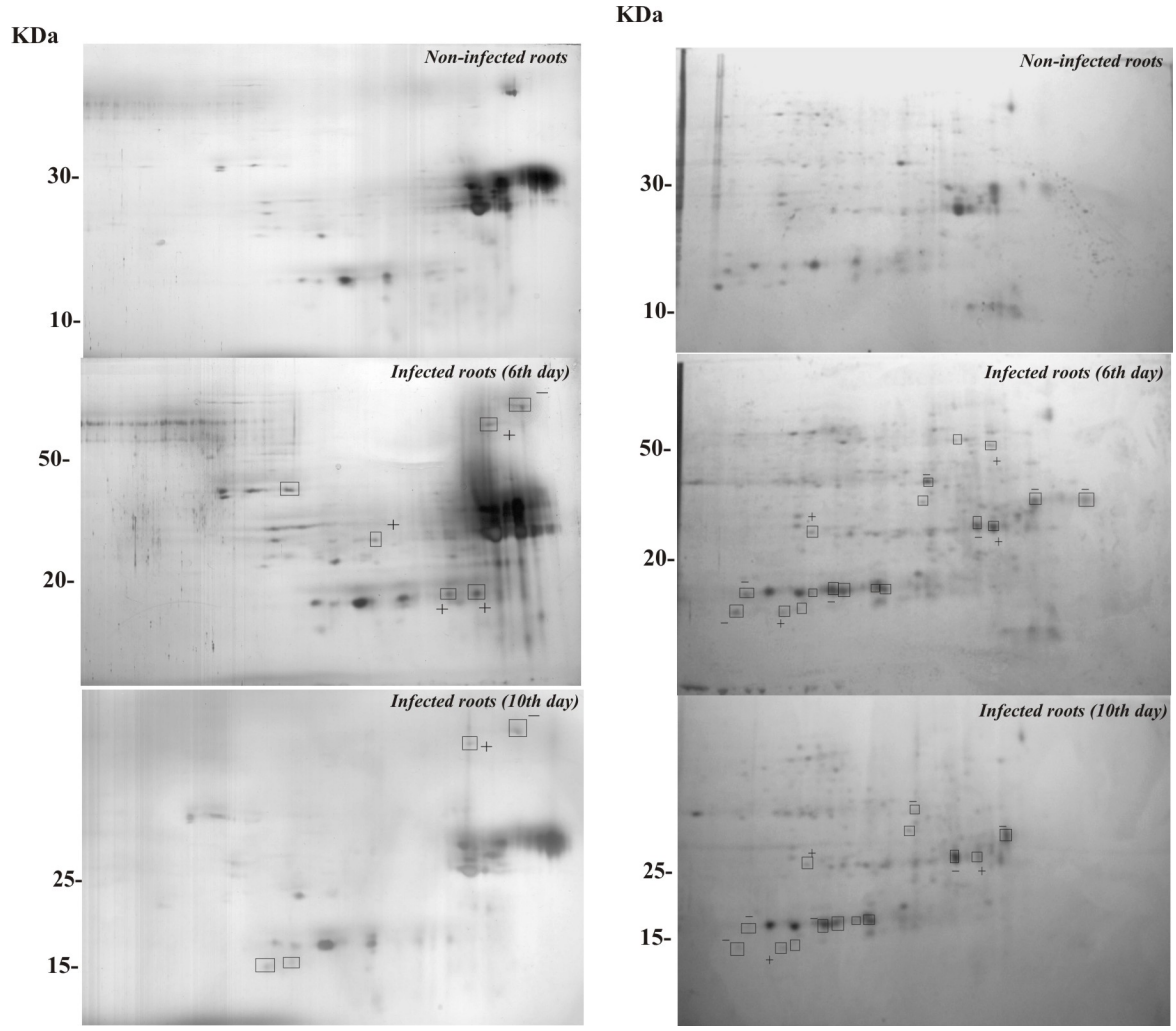

Figure 1. Bidimensional gels of coffee roots stained with silver nitrate (right) and CBB (left) of non-inoculated roots and infected roots 6 and 10 days after inoculation, as indicated. The pH of the gels ranged from 4-8. Squares indicate differentially expressed proteins in infected roots when compared to non-infected roots. The symbols + and – represent up- and down-regulated proteins, respectively, squares without symbols indicate new proteins and arrows point to differential spots observed in silver and CBB stained gels.

A total of approximately 70 and 100 spots were observed in silver and CBB stained gels, respectively. In order to identify differentially expressed proteins, the protein maps of the 6th and 10th day after nematode infection were determined and further compared to maps obtained from non-infected roots. CBB colored gels revealed at least 18 differentially expressed proteins at 6 days after inoculation including 4 up-, 7 down-regulated and 7 totally new and 14 differential spots at 10 days after inoculation, including 3 up-, 6 down- regulated and 5 new. A surprising result was obtained when gels were silver stained. By using this staining procedure, only 6 different proteins (4 up-, 1 down-regulated and 1 new) were visualized at the 6th day and 4 spots (1 up-, 1 down-regulated and 2 new) at the 10th day after inoculation (Figure 1). Another unexpected result showed that most differentially expressed

proteins identified in the silver stained gels were not observed in the CBB gels. Only 2 differential spots were common to the gels colored with both methods (Figure 1). Vediyappan et al. (2000) obtained similar results in the comparison of different staining methods for the analysis of *Candida albicans* proteins. It is possible that differences in the staining capacities of silver nitrate and CBB are responsible for the revelation of different proteins. In order to standardize this procedure, higher protein amounts were loaded onto the 2-D gel stained with silver nitrate. However, this modification lead to the presence of more pronounced stripes and background (data not shown), clearly reducing gel visibility and spot identification.

CONCLUSION

The results obtained herein showed that the staining method is crucial in order to maximize the number of proteins observed and that in the analysis of root proteins in coffee, CBB staining is more efficient. Similar results were obtained for cotton roots and therefore this conclusion may be extended to other plant species in order to better understand the host-pathogen interaction. We conclude that more than one coloring method should be employed when screening for proteins involved in specific biological processes.

ACKNOWLEDGEMENTS

This research was supported by Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café.

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Black *Aspergillus* Species, OTA Producers. Conditions for their Growth and Toxigenesis in Coffee

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SUMMARY

Water activity (Aw) and temperature play a major role in the development of *Aspergillus* species and their toxigenesis in coffee. At a temperature of 28 °C, optimum growth for *A. carbonarius* and *A. niger* was obtained for an Aw of 0.99. Below 0.85, there was no growth in 21 days. For both species, OTA production only occurred above an Aw of 0.85. OTA only appeared after 14 days with an Aw of 0.99, except for *A. carbonarius,* for which it occurred after 7 days. Caffeine and chlorogenic acids had an inhibitive effect on OTA production, and production was totally inhibited by caffeine at 2.5 $mg.ml^{-1}$

RÉSUMÉ

L'activité de l'eau (Aw) et la température jouent un grand rôle dans le développement des *Aspergillus* et leur toxinogénèse dans le café. A 28 °C la croissance optimale est obtenue à une Aw de 0,99 pour *A. carbonarius* et *A. niger*. En dessous de 0,85 il n'y a pas de croissance à 21 j. Pour les 2 espèces, la production d'OTA n'a lieu qu'au dessus d'une Aw de 0,85. L'OTA n'apparaît qu'après 14 j à une Aw de 0,99 sauf pour *A. carbonarius* où elle apparaît après 7 j. La caféine et les acides chlorogéniques ont un effet inhibiteur sur la production d'OTA, celle-ci étant totalement inhibée par la caféine à 2,5 $mg.ml^{-1}$

INTRODUCTION

Ochratoxin A (OTA) is a toxin produced by moulds belonging to the genera *Aspergillus* and *Penicillium,* which contaminate numerous foodstuffs, including coffee (Abarca et al., 2001). This toxin is absorbed in the gastro-intestinal tract and distributed via the blood to various organs, including kidneys, liver and muscles. OTAappear to have immunotoxic, nephrotoxic, teratogenic and carcinogenic effects (Pfohl-Lezkowics et al., 2002). Three species in particular of the genus *Aspergillus* are OTA producers: *A. niger, A. carbonarius* and *A. ochraceus*. The black *Aspergillus* species, *A. niger* and *A. carbonarius,* frequently contaminate green coffee, cereals and grapes (Joosten et al., 2001; Cabañes et al., 2002; Pardo et al., 2003). Given their great resistance to ultraviolet light and sunlight, these black *Aspergillus* species develop on foodstuffs dried in the sun in producing countries. *A. niger* would seem to be found most frequently in foodstuffs, but that might be due to a problem of differentiating between the two species, which leads to *A carbonarius* being identified as *A. niger* (Abarca et al., 2001). Indeed *A. niger* and *A. carbonarius* are two very similar species. Their differentiation is mostly morphological under a light microscope, with *A. carbonarius* displaying rougher and larger spores than *A. niger*. They have similar physiological characteristics but *A. carbonarius* develops at lower temperatures than *A. niger.* Its maximum

growth temperature is 40 °C and the optimum is between 32-35 °C. Likewise, its growth is more limited at a lower Aw and only germinates with an Aw of at least 0.82 at 25-30 °C. On the other hand, *A. niger* and *A. carbonarius* cannot germinate at 37°C. *A. niger* can germinate from an Aw of 0.77 at 35 °C and develops optimally at quite a high temperature, with a maximum of 45-47 °C, a minimum of 6-8°C and an optimum of 35-37 °C. Its growth rate varies little on a basic medium containing sugars, NaCl or glycerol at different water activities. *A. niger* can develop with pH values below 2.0 and at low Aw values (Abarca et al., 1994). Of the moulds described in foodstuffs, *A. niger* predominates in a tropical climate in the field or on stored foodstuffs. It is the species of *Aspergillus* that is most frequently responsible for post-harvest rotting of fruits. *A. carbonarius* is reported much less frequently. These black *Aspergillus* species can develop during drying or during green coffee storage (Bucheli et al., 2000). Moisture and temperature are the main factors conducive to green coffee contamination and OTA production (Northolt et al., 1979). The effect of water activity (Aw) and temperature on growth and OTA production have been described, including on coffee, primarily for *A. ochraceus* (Delas et al., 1995; Taniwaki et al., 2001; Suarez-Quiroz et al., 2004). Some compounds contained in coffee would seem to inhibit mould growth and toxin production (Buchanan et al, 1981; Hasan, 1999; Soliman, 2002; Suarez-Quiroz et al, 2004). Such an effect was reported for caffeine and chlorogenic acids on *A. ochraceus* by Suarez-Quiroz et al (2004).

The aim of our work was to detect Aw, caffeine and chlorogenic acids effects on the growth and OTA production of black *Aspergillus* spp found in coffee, *A. carbonarius* and *niger.*

MATERIAL AND METHODS

Strains

Aspergillus strains were isolated from OTA contaminated green coffees: Robusta green coffee from Ivory Coast (dry process) with 0.34 µg OTA.kg^{-1}, and Robusta green coffee from Indonesia, also (dry process) and with 1.3 µg OTA.kg^{-1}. The ability to produce OTA was checked on a BNM medium (Moss and Frank, 1985).

Coffee

The Arabica coffee cherries used as the substrate came from Kenya (2004 harvest) and were prepared by the dry process. They did not contain any trace of OTA.

Culture media

Aspergillus strains were grown on PDA medium (Difco) at 28 °C for 5 days. The media used to study the effect of caffeine and chlorogenic acids were prepared supplementing PDA with caffeine (Merck ref. 2584) at 0.0, 1.0, 1.5, 2.0 and 2.5 mg.ml^{-1} (w/v) respectively, and with chlorogenic acids (40CQA International Development and Manufacturing Inc.) at 0.0, 2.0, 4.0, 6.0 and 8.0 mg.ml^{-1} (w/v).

Inoculation and culturing on coffee cherries

We used the protocol described by Suarez-Quiros et al. (2004).

OTA quantification

We used the protocol described by Suarez-Quiros et al. (2004)

Ergosterol quantification

Mould growth was monitored by quantifying ergosterol (Saxena et al., 2001).

RESULTS AND DISCUSSION

Study of Aspergillus growth at different Aw values

At a temperature of 28 °C, maximum growth was obtained in both cases for an Aw of 0.99 after 21 days of incubation, and only began with an Aw of 0.85. *A. niger* was found to develop better than *A. carbonarius* (Figure 1). Almost 3 times more biomass was obtained.

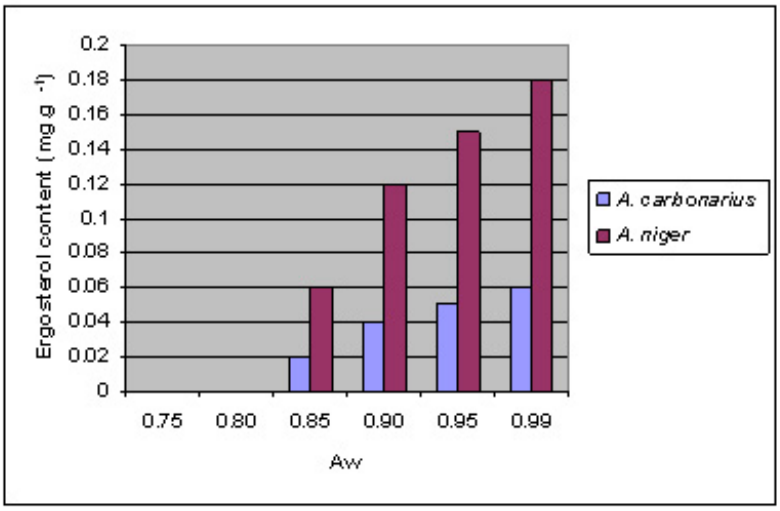

Figure 1. Effect of water activity on growth and OTA production by *A. carbonarius* and *A. niger* at 28 °C.

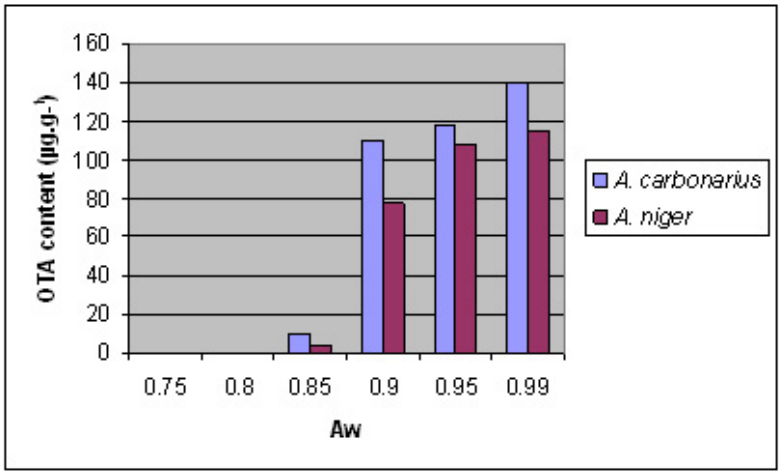

Figure 2. Effect of water activity on growth and OTA production by *A. carbonarius* and *A. niger* at 28 °C.

Study of OTA production

At 28 °C, OTA production for *A. carbonarius* was found to start at an Aw of 0.85 with an optimum at 0.99. For *A. niger,* despite effective growth at an Aw of 0.85, toxin production was virtually zero at that Aw. The optimum was also reached at an Aw of 0.99 (Figure 2).

These results show that at 28 °C the two moulds had optimum growth and toxigenesis at an Aw of 0.99 and that water activity was a factor that limited growth and OTA production. These results are very similar to those described for *A. ochraceus* (Suarez-Quiroz et al., 2004) as the optimum growth and toxigenesis for the two black *Aspergillus* species was at an Aw of 0.99 and that of *A. ochraceus* was at 0.95. For both *Aspergillus* species, at the Aw values studied, the OTA/biomass ratio was constant: 2.7 ± 0.2 for *A. carbonarius* and 0.7 ± 0.1 for *A. niger*. This shows that *A. carbonarius* was more toxigenic than *A. niger,* under those development conditions.

Kinetics of OTA production

OTA production remained negligible over time, even after 21 days' incubation at an Aw of 0.85, for both *Aspergillus* species (Figure 3). For an Aw of 0.99, OTA production by *A. carbonarius* was almost maximum from as early as 14 days, unlike *A. niger* for which production was slower and only reached its maximum after 21 days. Changes in the Aw during traditional sun drying of coffee were studied (Mburu, 1999). The Aw decreased slowly and the coffee remained for a long time under conditions conducive to mould development and OTA production: 5 to 7 days between 0.85 and 0.99, including 2 to 4 days at 0.95. To reduce the risks of OTA contamination, this critical period would have to be reduced as much as possible using rapid techniques to reduce the water content. Of course, protection from remoistening during storage will also be required.

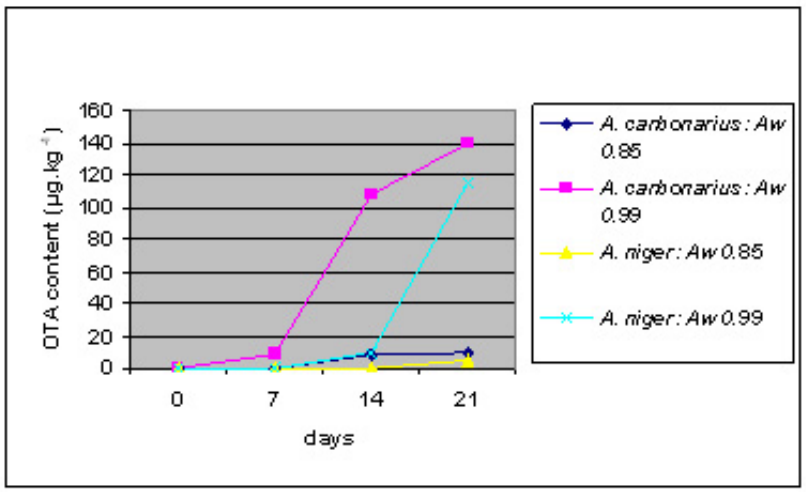

Figure 3. OTA production kinetics for an Aw of 0.85 and 0.99 for *A. carbonarius* and *A. niger.*

We measured the effect of caffeine and chlorogenic acids on the growth and OTA production of black *Aspergillus* species on a supplemented synthetic medium (PDA). The compounds studied were added to obtain concentrations equivalent to those found naturally in Arabica and Robusta green coffees: 1.0 to 2.6% for caffeine and 7 to 15% for chlorogenic acids (Hasan, 1999). The lowest caffeine and chlorogenic acid concentrations corresponded to the Arabica coffee and the highest to the Robusta coffee. At 28 °C, chlorogenic acids, even at maximum concentration, had little effect on *Aspergillus* growth rates, whilst caffeine reduced it by half at a concentration of 1 $mg.ml^{-1}$ (Figures 4 and 5). The presence of these two compounds significantly reduced OTA production by the two *Aspergillus* species (Figures 6 and 7). The effect was greater on *A. niger*. However, caffeine played a greater inhibitive role than chlorogenic acids, since at 2 $mg.ml^{-1}$, there was no further OTA production by either *Aspergillus* species.

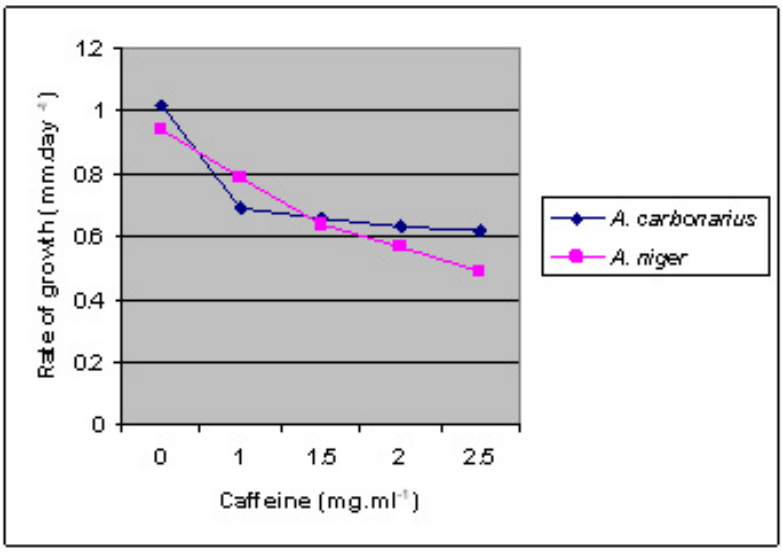

Figure 4. Effect of caffeine and chlorogenic acids on *A. carbonarius* and *A. niger* growth.

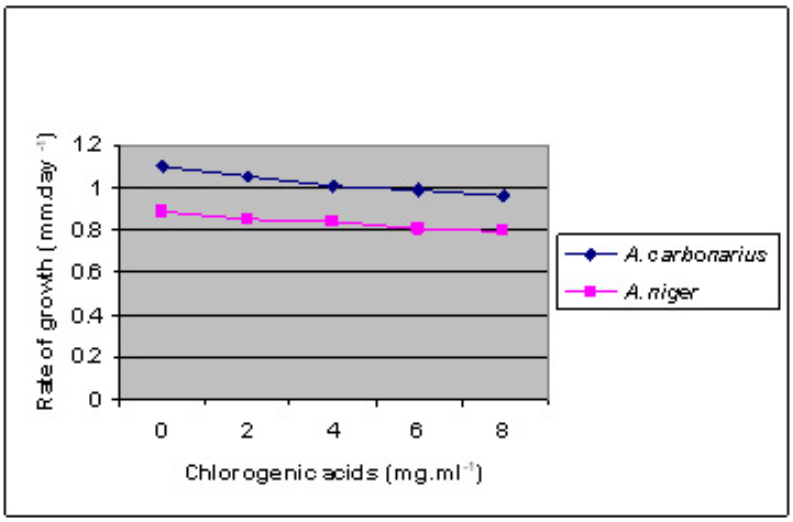

Figure 5. Effect of caffeine and chlorogenic acids on *A. carbonarius* and *A. niger* growth.

Effect of caffeine and chlorogenic acids on growth and OTA production

These results show that, in theory, Robusta coffees would seem to be less likely to enable ochratoxin A production. However, the dry processing of Robusta coffees entails long drying that takes at least 21 days with a critical Aw for the first 6 days. Such conditions are conducive to mould development on the shells and subsequent contamination. To limit contamination, the critical period must be reduced by improving drying conditions. Lastly, although OTA production can be limited, the best solution, in addition to respecting optimum drying and storage conditions, remains early prevention by protecting from fungus contamination and development, including in the field.

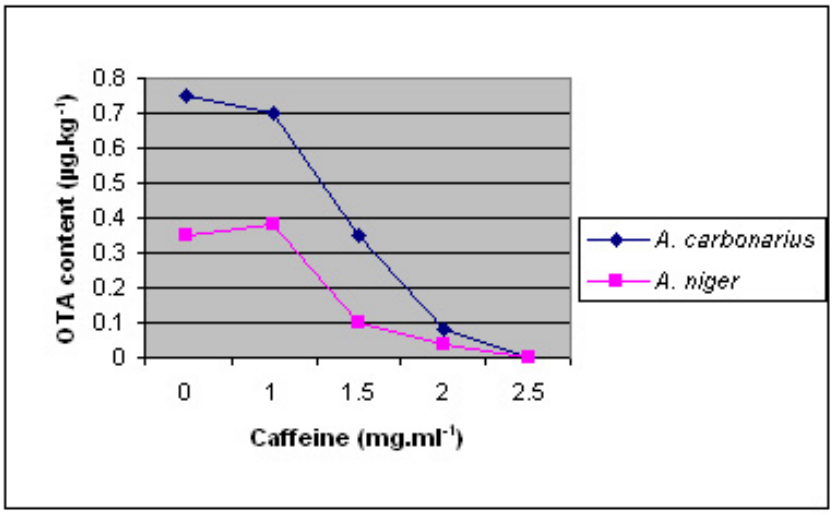

Figure 6. Effect of caffeine and chlorogenic acids on OTA production by *A. carbonarius* and *A. niger*.

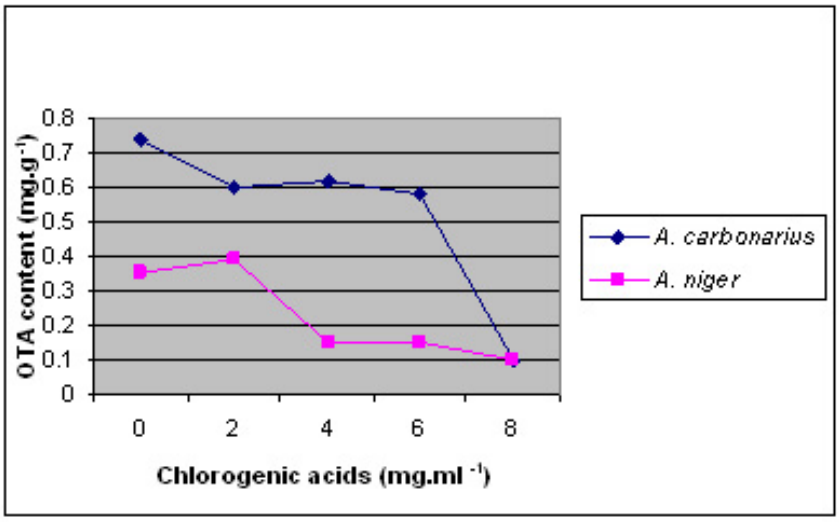

Figure 7. Effect of caffeine and chlorogenic acids on OTA production by *A. carbonarius* and *A. niger*.

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Fungal Metabolites in Coffee

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SUMMARY

The presence of fungi in coffee and the influence of these microrganisms in the final beverage, from two Brazilian regions, Patrocínio (Minas Gerais State) and Pirajú (São Paulo State) were investigated. Fifty beans were analyzed from each sample and the fungal species were isolated and identified. To study beverage quality, raw coffee beans were roasted and ground. The samples were evaluated in three different tasting tests: infusion, diluted espresso and espresso. A total of 547 strains were isolated. Coffee samples from Patrocínio had a low percentage of fungal infection with an average of 8.2%. *Eurotim sp* and *Fusarium sp* were the genera isolated the most. Coffee samples from Pirajú showed a high infection of *Penicillium brevicompactum.* The average infection was 29.3%. *Penicillium palitans, Fusarium lateritium*, *Fusarium oxysporium*, *Aspergillus niger* and *Aspergillus ochraceus* were the other fungi isolated from this region. Some coffee samples had a good clean beverage, with distinct aromas such as: chocolate, caramel and floral. These samples were more infected with: *Cladosporium* spp, *Fusarium* spp., *P. brevicompactum* and *P. palitans*. On the other hand, the most common fungi isolated from raw coffee beans which had fermented, stinker and rioy taste were *A. niger, A. ochraceus, Fusarium* spp and *P. brevicompactum*. Raw coffee samples were analysed to obtain the profile of volatile metabolites. Studies comparing the fungal infection to chemical metabolites are being carried out.

INTRODUCTION

Coffee flavour is affected by a number of factors including genetic (specie, variety and strain); ecological (region, area and climatic conditions); processing (harvesting, drying techniques, storage and transportation). Besides these factors, the presence of microrganisms on coffee fruit and the intensity of the proliferation during drying and storage, can also be important in the definition of final beverage quality.

The coffee volatiles were studied by several authors (Amstalden et al., 2001, Spadone et al., 1990, De Maria et al., 1996, Sanz et al., 2002) but the correlation with the presence of fungi was not studied in great detail.

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

Coffee samples (*Coffea arabica*) were collected in two Brazil regions: Pirajú, in São Paulo State and Patrocínio, in Minas Gerais State (Cerrado Mineiro), at two different processing:

pulped natural coffee and natural coffee (whole fruit). The natural coffee were dried floater, immature and sweeping fruits (from the soil).

Mycological analyses

Coffee beans were desinfected with 0.4% chlorine solution for 1min. Ten coffee beans were plated on 5 Petri dishes containing 18% Dichloran Glicerol Agar (DG18) with cloranfenicol. Petri dishes were incubated at 25 ºC from 5 to 7 days. The percentage infection was calculated according to the Pitt and Hocking (1997) method. The isolates were inoculated in Malt Extract Agar (MEA) and identified according to Pitt (2004), for *Penicillium* species and Klich and Pitt (1988) for *Aspergillus*. The other species were identified based on Pitt and Hocking (1997), Samsom et al. (1996) and other classification keys.

Sensorial analyses

To study drinking quality, raw coffee beans from each sample were roasted and ground. Samples were evaluated by three different tasting tests: 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.

Volatile analyses

To capture the raw and roasted coffee volatiles, the SPME technique and a polar fiber (DVB/Carboxen™/PDMS) were used. One gram of ground sample was extracted by headspace at 65 ºC during 30 min. GC/FID Shimadzu equipment equipped with a fused-silica capillary column (Supelcowax ^{tm} 30 m x 0.25 mm x 0.25 μm film) was used with nitrogen as the carrier gas. Temperature was programmed to rise from 40 to 230 ºC at 4 ºC/min. The profile of volatile metabolites was obtained and in future studies, the most important compounds are going to be identified by GC/MS.

RESULTS AND DISCUSSION

Tables 1 and 2 show the percentage of fungi in coffee (from Pirajú/SP and Patrocínio/MG) and its influence on sensorial evaluation.

The contamination level of coffee from Pirajú/SP was higher than Patrocínio/MG. In general, samples from Minas Gerais did not have high fungal contamination (less than 20%) and the most isolated fungi were *Eurotium rubrum* and *Fusarium* sp. Natural coffee (samples 5, 6, 7 and 8, Table 2) had higher fungal contamination and negative sensorial characteristics compared to pulped natural coffee.

In respect to the Pirajú samples, a high fungi infection was verified and the mycobiota was very different to those from Patrocínio. Out of 11 samples from Pirajú, five had 100% infection, even those with positive sensorial evaluation. The most isolated species were: *Penicillium brevicompactum, Penicillium palitans*, *Aspergillus niger, Aspergillus ochraceus* and *Fusarium lateritium*. Samples which had higher contamination with *P. brevicompactum* and *P. palitans* had positive sensorial characteristics such as caramel, and floral with slightly adstringent. On the other hand, samples with higher than 16% of *A. niger* and *A. ochraceus* infection had negative characteristics such as, rioy and mouldy flavour.

Table 1. Fungi percentage in coffee from Pirajú/SP and its influence on sensorial evaluation.

	Nº ISOLATES (% INFECTION)								
Fungi species	**(Pulped natural coffee)**				**(Natural coffee – sweeping)**		**(Natural coffee-dried floater)**		**(Natural coffee-immature)**
	sample 1	**sample 2**	**sample 3**	**sample 4**	**sample 5**	**sample 6**	**sample 7**	**sample 8**	**sample 9**
P. palitans	4(8)			14(28)		17(34)		5(10)	
P. brevicompactum	13(26)	35(70)	5(10)	31(62)	24(48)	5(10)	4(8)	7(14)	9(18)
A. ochraceus	2(4)	1(2)			15(30)		2(4)		2(4)
A. niger	3(6)	3(6)	8(16)	15(30)	8(16)	32(64)	9(18)	11(22)	20(40)
A. carbonarius				2(4)					
F. lateritium	14(28)	19(38)	2(4)	6(12)	25(50)			5(10)	5(10)
F. oxysporium	13(26)	6(12)	1(2)						1(2)
F. semitectum	2(4)				2(4)				1(2)
Cladosporium sp		3(6)							
Total isolates (% infected beans)	51(100)	67(100)	15(30)	69(100)	74(100)	54(100)	15(30)	28(56)	38(76)
Sensorial analyses(flavours)	*- Chocolate -Adstringent*	*-Caramel -Slightly adstringent*	*-Floral -Caramel*	*-Caramel -Slightly adstringent*	*-Mouldy -Rioy*	*-Slightly fermented -Strong immature*	*-Slightly fermented -Strongly immature -Strongly adstringent*	*-Mouldy -Stinker*	*-Strongly fermented*

Table 2. Fungi percentage in coffee from Patrocínio/MG and its influence on sensorial evaluation.

	Nº ISOLATES (% INFECTION)							
Fungi species	**(Pulped natural coffee)**				**(Natural coffee)**			
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample6**	**Sample 7**	**Sample 8**
E. rubrum	3(6)	3(6)	1(2)	2(4)	2(4)	1(2)	1(2)	3(6)
E. repens							1(2)	
E. amstelodami								2(4)
E. chevalieri		1(2)	1(2)					1(2)
Fusarium sp.	1(2)		1(2)		5(12)			
F. semitectum								1(2)
F. lateritium						9(18)		3(6)
Cladosporium sp.				1(2)				
P. brevicompactum					1(2)			
Total isolates (% infected beans)	4(8)	4(8)	3(6)	3(6)	9(18)	10(20)	2(4)	10(20)
Sensorial analyses(flavours)	*-Strong stinker*	*- Sweet - Caramel*	*- Sweet - Caramel*	*-Slightly bitter*	*-Fermented -Adstringent*	*-Immature -Fermented*	*-Immature -Fermented*	*-Stinker -Immature*

The comparison of chromatographic profiles of raw coffee, from different qualities and regions, was carried out and are shown in Figures 1 and 2 respectively. The presence of different peaks and GC profiles can be noticed. The most relevant compounds will be identified in raw and roasted coffee, using GC/MS.

Figure 1. Raw coffee chromatographic volatile profiles comparing 2 Pirajú (SP) samples.

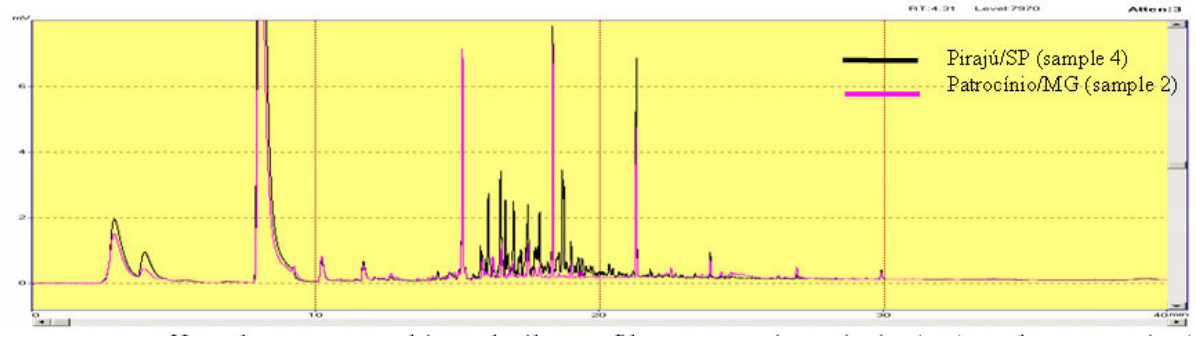

Figure 2. Raw coffee chromatographic volatile profiles comparing Pirajú (SP) and Patrocínio (MG).

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Toxigenic Fungi and Ochratoxin A in Defective Coffee Beans

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SUMMARY

Defective coffee beans may contain toxins such as ochratoxin A (OTA) and other compounds which affect health. The objectives of the present study were to evaluate the mycobiota and the presence of ochratoxin A in the two principal coffee species: *Coffea arabica* (arabica) and *Coffea canephora* (robusta) in sound (good) and defective coffee beans. The coffee defects were separated manually into: black, immature, black green and sour beans. Samples were surface disinfected and plated directly onto Dichloran Glycerol agar, and incubated at 25 ºC for 5 to 7 days. After incubation, plates were examined and all fungal species were first isolated onto malt extract agar plates. After growth, other culture media were used for identification. The coffee samples were analyzed for OTA. The toxin was detected and quantified by High Performance Liquid Chromatography (HPLC) equipment. The detection limit of the method was 0.2 μg/kg OTA. Robusta coffee presented more contamination by toxigenic fungi than arabica. *Aspergillus niger* was the most common potentially toxigenic species found, followed by *Aspergillus ochraceus* and *Aspergillus cabonarius.* However, OTA production varied from 6 to 20%; 66 to 100% and 60 to 100% in these species, respectively. The levels of ochratoxin A in arabica, residue of arabica, robusta and residue of robusta coffee were: < 0.2, 0.62, 1.56 and 9.01 μg/kg, respectively. The coffee beans with sour defect had a higher ochratoxin A concentration (11.35 μg/kg) than those with other defects.

INTRODUCTION

The defects in beans can be of intrinsic or extrinsic nature. They can be produced in the field, during harvest, processing, transport, storage, originating from physiological or genetic grain modification. The defects of intrinsic nature are associated with beans irregular in visual appearance (black, sour, immature, black green, spongy beans and others), or associated to bean shape (shell, shell core, broken, insect damaged, pulper-nipped and others). The defects of extrinsic nature are those represented by foreign matter, such as husks, sticks, stones and clods. All these defects depreciate the coffee price on the market. Furthermore, some of these defects alter considerably the sensorial beverage characteristic, adding off taste such as fermented, woody, rotten fish, stinker and others, and lowering the quality.

Since the presence of defective grains in a coffee lot is a reality, a more detailed study of these defects is important not only in financial terms, but also from the public health point of view.

With the exception of physiological and genetic defect origin, most result from inadequate agricultural practice. Among them, is the permanence of coffee fruits in contact with the soil, which brings microrganism contamination and formation of black and sour defects. Some microrganisms can spoil the fruit, cause defects in the grain and produce toxins.

A toxin which has been studied in coffee is ocratoxin A (OTA). It is produced principally by *Aspergillus ochraceus*, *Aspergillus niger* and *Aspergillus carbonarius*, (Urbano et al., 2001; Joosten et al., 2001; Taniwaki et al., 2003). OTA is nephrotoxic, teratogenic and mutagenic in several animals. It has been detected in coffee samples from all over the world (Studer-Rohr et al., 1995; Patel et al., 1997; Van der Stegen et al., 1997; Taniwaki 2006).

Considering these facts it is very important to investigate the presence of OTA in each defective bean, but especially on the defects: black, immature, black green and sour as well as their mycobiota.

Therefore, the objectives of the present study are: (i) To verify the presence of OTA in defective coffee beans: black, immature, black green and sour; (ii) To verify the mycobiota of these defects.

MATERIAL AND METHODS

Samples

- *Coffee arabica* (arabica) type 3, from Patrocinio-Minas Gerais;
- Residue of arabica, from Patrocinio-Minas Gerais;
- *Coffee canephora* (robusta), type 6, from Rondônia;
- Residue of robusta, from Rondônia

Mycological analysis

Samples of coffee beans were surface disinfected with 0.4% chlorine solution for 1 min (Pitt and Hocking 1997), and then a total of 50 beans was plated directly (10 particles per plate) onto Dichloran 18% Glycerol agar (Hocking and Pitt 1980). The plates were incubated at 25 ºC for 5 to 7 days, then inspected for colony growth visually and with the aid of a stereomicroscope.

Identification of fungi

Each isolate was grown on standard identification media and identified according to Klich and Pitt (1988), Pitt and Hocking (1997). Numbers of isolates identified as *A. ochraceus* or closely related species, *A. carbonarius* and *A. niger* were counted for each sample, and percentages infection of the coffee fruit calculated.

Test for OTA production by isolated fungi

The isolates identified as *A. ochraceus* or closely related species*, A. niger* and *A. carbonarius,* were grown on yeast extract 15% sucrose agar at 25 ºC for 7 days and evaluated for the production of OTA by the agar plug technique as described by Filtenborg et al. (1983).

Analysis of OTA in coffee

Coffee samples were analyzed for OTA according to Vargas et al. (2005). 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 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 detection limit of this method was 0.2 μg/kg OTA.

Separation of coffee defects

The coffee defects were separated manually in: black, immature, black green and sour beans by the Assicafé team.

RESULTS AND DISCUSSION

The percentage of fungal infection in arabica and robusta coffee is shown in Tables 1. Robusta coffee had higher fungal infection than arabica coffee. Even the robusta considered good showed high infection of potentially toxigenic fungi. Apparently, the defects were responsible for the increase of *Aspergillus carbonarius* in arabica coffee from 0 to 10%; *A. niger* infection from 6 to 20% and *A. ochraceus* from 0 to 18%. *A. niger* was the most common potentially toxigenic species found, followed by *Aspergillus ochraceus* and *Aspergillus cabonarius.* However, OTA production varied from 6 to 20%; 66 to 100% and 60 to 100% in these species, respectively.

Table 2 shows the ochratoxin A content in arabica and robusta coffee. Only the residue of robusta coffee had high OTA contamination (9.01 μg/kg). Arabica considered good, residue of arabica and robusta considered good, showed reduced OTA content. Robusta coffee showed higher infection by toxigenic fungi than arabica.

Table 1. Percentage of fungal infection in arabica and robusta coffee.

Fungal species	Percentage of infection (%)			
	Arabica	Residue of arabica	Robusta	Residue of robusta
Aspergillus carbonarius	-	10	-	-
Aspergillus flavus	-	2	-	6
Aspergillus niger	6	20	62	76
Aspergillus ochraceus	-	18	14	6
Eurotium chevalieri	-	8	8	-
Eurotium rubrum	6	8	4	2
Fusarium sp.	2	2	4	6
Mucor sp.	2	4	-	-
Rhizopus sp.	-	-	-	-
Dematiaceous fungi	2	-	-	2

Table 2. Ochratoxin A (μg/kg) content in arabica and robusta coffee.

Samples	Ochratoxin A (μg/kg)			
	Arabica	Residue of arabica	Robusta	Residue of robusta
1	ND[1]	0.62	1.36	8.83
2	ND	0.63	2.34	9.18
3	ND	0.62	0.97	9.03
Mean	ND	0.62	1.56	9.01

[1]*ND: not detected by the method (limit of detection: 0.2 μg/kg).*

Table 3 shows the OTA content in each arabica coffee defect separated as: half black, black, immature, dark green, black green, and sour beans. The sour defect showed the highest

concentration of OTA (11.35 μg/kg), while in the other defects the content was lower then 0.5 μg/kg.

Table 3. OTA (μg/kg) content in arabica coffee defects.

Coffee defects	OTA (μg/kg)
Control (sound beans without defects)	ND[1]
Half black	0.45
Black	0.49
Immature	ND
Dark green	ND
Black green	0.39
Sour	11.35

[1]*ND: not detected by the method (limit of detection: 0.2 μg/kg).*

CONCLUSION

There was an increase in ochratoxin A concentration in arabica and robusta coffee with defects. Residue of robusta coffee showed the highest infection by OTA producing fungi. The reduction of defects in coffee lowers the level of infection by toxigenic fungi, reduces ochratoxin A content and improves considerably beverage quality.

ACKNOWLEDGEMENTS

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Influence of the Fermentation Stage on the Ochratoxin A Production in Coffee

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SUMMARY

Strains potentially producers of Ochratoxin A (*A. ochraceus* and *A. niger*) represent 3 to 25 % of fungal contamination of Mexican Arabica coffee. No strains of *Aspergillus carbonarius* and *Penicillium verrucosum* were isolated on different coffees. The study of the impact of the process ways on contamination and OTA toxinogenesis showed that *A. ochraceus* is sensitive to competition with the autochthonous flora of coffee that is not true for *A. niger* which establishes easily itself. However, this one does not produce the toxin so faster and in higher quantity than *A. ochraceus*. The dry and wet processes with mechanical pulping and mucilage removal gave results lightly different from those of the traditional wet process using microbial mucilage removal: this last is less favourable to fungal growth even if there was not really significant difference on the Ochratoxin A production ability between the processes studied. The presence of a microbial fermentation stage is however a factor of limitation of OTA toxic risks as well as quick diminution in Aw during the drying.
Keywords: Coffee, Processing, Ochratoxin A, *Aspergillus ochraceus, Aspergillus niger*

RÉSUMÉ

Les souches productrices d'Ochratoxine A (*A. ochraceus* et *A. niger*) représentent 3 à 25 % de la contamination fongique du café Arabica mexicain. Aucune souche d'*Aspergillus carbonarius* et de *Penicillium verrucosum* n'a été isolée sur les différents cafés. L'étude de l'impact du type de traitement post-récolte sur la contamination et la toxinogénèse pour l'OTA a montré qu'*A. ochraceus* est sensible à la compétition avec la flore autochtone du café, alors que ce n'est pas le cas pour *A. niger* qui colonise le milieu facilement. Cependant *A. niger* ne produit pas la toxine aussi rapidement et en aussi grande quantité qu'*A. ochraceus*. Les procédés "sec" et "humide" avec dépulpage et démucilagination mécaniques donnent des résultats sensiblement différents de ceux obtenus avec le procédé humide traditionnel utilisant une démucilagination microbienne: ce dernier procédé est moins favorable à la croissance fongique même s'il y a peu de différence sur l'aptitude à la production d'Ochratoxine A. La présence du stade de fermentation microbienne est cependant un facteur de limitation de risque au même titre que l'obtention d'une baisse rapide de l'Aw pendant le séchage.
Mots-clé: Café, Procédés post-récolte, Ochratoxine A, *Aspergillus ochraceus, Aspergillus niger*

INTRODUCTION

Ochratoxin A (OTA) is the main mycotoxin known in coffee. OTA occurrence in coffee beans can be due to both environmental conditions, and processing conditions (Romani et al., 2000). OTA was present before storage, indicating the possibility that harvesting and post-harvest handling of coffee cherries could be the critical steps leading to contamination (Bucheli et al., 1998; 2000). There is currently little information available on the presence of OTA-producing moulds in coffee beans in the wet and mechanical processes and the impact of these processes on the production or presence of OTA. Ochratoxin can be due to cherry contamination "in the field" by toxigenic moulds, with toxin production occurring before or after harvesting and, in this case, during or after the process, which presupposes the persistence of OTA producing moulds. It may also be due to recontamination after the process. In 1999, Frank observed that there was usually a reduction in the number of filamentous fungi during fermentation, to the benefit of yeasts (Frank, 1999). On the other hand it was assumed that when pulp (mesocarp) was removed, an excellent substrate for the development of OTA producing strains was also eliminated (Joosten et al., 2001). The purpose of this work was to study if the fermentation stage in the wet processing affected mould occurrence and toxigenesis up to the green coffee stage, using artificial contamination by OTA producing strains.

MATERIALS AND METHODS

Coffee samples

Coffee cherries (*Coffea arabica*) were harvested in a plantation from the Coatepec area (Xalapa, Mexico).

Coffee processing

Three coffee processing methods, wet (mucilage by fermentation), mechanical (mucilage was removed mechanically) (Penagos Hnos & CIA LTDA, Colombia) and dry, were tested on 40-Kg batches of coffee cherries. The trials were repeated 4 times. An air dryer at 45 °C was used for the three methods. The first two processes gave parchment coffee and the other gave dry cherry coffee. Parchment and husk were removed by mechanical hulling.

Post-harvest processes tested in model system

The processes were studied in the laboratory on batches contaminated (2 kg) with OTA producing strains *A. ochraceus* Wilhelm (MULC 44640) and *A. niger* van Tieghem (MULC 44639): For the dry method, the cherries were dried in the sun. For the wet and mechanical processes the coffee was then washed and oven-dried at 45 °C.

Mycological analysis

Samples (300 g) were collected for each method of coffee processing at different stages and spread-plated onto DRBC agar (Samson, 1991). The results were expressed in CFU/g. Subsamples (50 beans) of parchment coffee, dry cherry or green coffee were also plated directly onto DG18 agar (Hocking and Pitt, 1980; Guiraud, 1998). The plates were incubated at 25 °C for 5 to 7 days, the results being expressed as a percentage of infected beans (% infection). Predominant moulds were identified according to Samson et al. (1985). Isolates identified as *A. ochraceus* and *A. niger*, were counted and percentages occurrence were calculated as proportion of total fungi.

OTA quantification

From coffee beans, the coffee was dried at 70 °C, frozen at –80 °C then ground. Samples were analysed according to Nakajima et al. (1997). OTA was detected and quantified by HPLC (Shimadzu Corporation, Japan).

RESULTS AND DISCUSSION

Evolution of mould flora during the processes

Independently of the coffee processing used, a decrease in the mould count was found. For the viable count methodology it was not possible to prove the existence of ochratoxigenic strains in parchment and dry cherry coffee. Nevertheless, by the direct plating technique, several strains of *A. niger* and *A. ochraceus* were isolated from beans. It can be seen that the average rate of parchment and dry cherry coffee infection by these fungi was low (3% to 25%). This method made it possible to determine the percentage of beans contaminated by moulds, which was high in parchment coffee (71%-92%) decreasing later on in green coffee (15%-34%).

Variation in contamination by OTA producing strains

Contamination with the strains studied revealed differences. *A. ochraceus* was unable to establish itself properly in the presence of native flora, and was therefore susceptible to competition, which was not the case with *A. niger* which established itself easily. However, this one does not produce the toxin so faster and in higher quantity than *A. ochraceus*. For *A. ochraceus,* its non-establishment in terms of abundant development does not mean an absence of danger, as conidia might remain alive during the process and keep their post-process development potential.

The type of process used affected the evolution of the flora studied, but overall the results were similar for both strains (Table 1). The OTA content produced increased in 48h from 0 to 1.9 mg/kg for *A. ochraceus* inoculated batches, for *A. niger* the OTA contents were much lower than *A. ochraceus*. In terms of variation in the OTA content resulting the mucilage removal was much less effective, which is compatible with the fact that the toxin was able to migrate or be synthesized in deeper zones of the bean, through mycelium establishment. This was also true for hulling in the dry method and in the wet method with fermentation. In the first case, this could also be explained by neosynthesis from toxigenic contaminating strains. In the second case, this could be explained by bean wetting, which promoted diffusional transfer. Lastly, it was confirmed that the best way to limit the development and impact of contaminating toxigenic flora in the field remains the physical wet method.

Little work had previously been undertaken in this perspective, in particular none on the impact of using the physical wet method, as differences between the processes had particularly been considered in terms or economics, environmental impact and the consequences for flavour quality. It is clear that "susceptibility" to OTA production and its health consequences need to be taken into account in a global discussion on the choice of process.

Table 1. *A. ochraceus* and *A. niger* development and ochratoxin A (OTA) production (between parenthesis) at different stages of coffee post-harvest processing (assayed in triplicate).

Treatment stage	Wet method		Physical wet method		Dry method	
	A. ochraceus	***A. niger***	***A. ochraceus***	***A. niger***	***A. ochraceus***	***A. niger***
	Count CFU/g[a] or % of infection[b] and **OTA content (µg/kg)[c]**					
Cherry at t=0[a]	1.7±0.1 x 10^5 **(n.d.)**	1.8±0.2 x 10^5 **(n.d.)**				
Cherry at t=48h[a]	1.2±0.1 x 10^5 **(1.9±0.3)**	1.6±0.8 x 10^5 **(trace)**				
Pulped coffee[a]	1.4±0.5 x 10^5 **(2.1±0.4)**	1.6±0.7 x 10^5 **(trace)**				
Fermented coffee[a]	2±0.2 x 10^4 **(1.9±0.3)**					
Coffee minus mucilage[a]			1±0.1 x 10^2 **(1.7±0.04)**	3±0.2 **(trace)**		
Parchment coffee[b]	25% **(2.2±0.2)**	60% **(trace)**	5% **(1.7±0.04)**	15% **(trace)**		
Dry cherry[b]					40% **(2.5±0.5)**	60% **(trace)**
Green coffee[b]	25% **(0.7±0.1)**	40% **(trace)**	0% **(trace)**	0% **(n.d.)**	30% **(1.5±0.3)**	40% **(trace)**

[a]Dilution and surface inoculation technique. [b]Direct inoculation of bean with results expressed as % infected beans (used for samples giving a negative results by plate count due to the limited of detection, i.e. <50 CFU/g). [c]HPLC. n.d. not detected

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Evaluation of the Effect of Roasting on Reducing OTA Contamination in Arabica Coffee

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SUMMARY

The present study aimed at an evaluation of ochratoxin A (OTA) degradation during coffee roasting. Two roasting levels were evaluated, based on the average final temperature of the coffee bed: mild roast (190 °C) and dark roast (210 °C). OTA contamination of each coffee lot was based on average results from three sub-samples, varying from 7.5 to 20.3 μg/kg coffee. Results obtained for the roasted sub-samples showed average OTA degradations of 63% and 82% for mild and dark roasts, respectively. Data variability found in the roasted coffee data was compatible with the determinations for green coffee. The obtained results showed that roasting reduces OTA levels in coffee. However, such reduction was found to be quite heterogeneous.

INTRODUCTION

Literature data on the influence of roasting and brewing on OTA decomposition presents several inconsistencies. Studies have employed naturally or artificially contaminated coffee samples, and also samples inoculated with OTA producing fungi and have reported OTA degradation levels ranging from 0 to 100%. One should expect an increase in OTA degradation as roasting conditions become more severe. However, this was not the case in all studies. Van der Stegen et al. (2001) did not find significant differences in OTA degradation after varying both roasting time (2.5 to 10 minutes) and temperature (425 to 470 °C). Romani et al. (2003) reported a general tendency of increase in OTA degradation for darker roasts, but also reported inconsistent degradation data for both medium and light roasts. The discrepancies between some findings have been associated to differences in the method of introducing the toxin into the ground beans, lack of precision and selectivity of analytical techniques (Whitaker and Park, 1994; Pittet et al., 1996), variations in roasting conditions (Romani et al., 2003) and toxin/substract interaction (Pittet et al., 1996; Tsubouchi et al., 1987). However, the major factor affecting OTA determination is related to an appropriate sampling procedure, since OTA distribution in coffee is quite heterogeneous. It has been reported that inappropriate sampling could lead to up to 90% error in OTA detection (Whitaker and Park, 1994). Usually, only a small percentage of coffee beans are contaminated in a lot and thus OTA contamination results are not representative of the entire lot. Furthermore, some literature data for OTA detection in roasted coffee are presented per mass of roasted coffee or do not specify the basis used. In that case, they do not take into account the overall dry matter loss that occurs during roasting, resulting on underestimation of OTA degradation values. In view of the above, the present study aimed at an evaluation of OTA degradation during coffee roasting, based on a previously validated sampling method (Vargas et al., 2004; 2005), taking into account the effect of weight loss during roasting.

METHODOLOGY

Arabica coffee was provided by DECAF/PR (Paraná, Brazil) in four lots previously identified as potentially contaminated with OTA. Sampling was based on a previously validated procedure (Vargas et al., 2004; 2005) in order to constitute a 16 kg representative sample for each lot. Each sample was subdivided into 1 kg subsamples, and nine sub samples were employed in the present study. Three subsamples were separated for OTA analysis and the remaining were roasted in a lab-scale coffee roaster (rotating cylinder). The roaster, working at a rotation speed of 80 rpm, was pre-heated during 10 min and then loaded with 1 kg coffee. Two roasting levels were evaluated, based on the average final temperature of the coffee bed: mild roast (190 °C) and dark roast (210 °C). Roasting time varied from 12 to 16 minutes. Coffee samples were weighted after roasting, in order to evaluate weight loss. Roasting tests were performed in triplicate. OTA analysis was based on the HPLC methodology described by Vargas et al. (2005).

RESULTS AND DISCUSSION

Results obtained for OTA determination in green coffee are shown in Table 1. These results show that OTA distribution is not uniform in a given lot and reinforce the need for an appropriate and validated sampling procedure.

Table 2. OTA levels in green coffee.

Lot	Subsamples	OTA levels (µg/kg)	Lot average level (µg/kg)
	A1	19.75	
1	A5	9.34	16.3
	A9	19.80	
	A1	23.20	
2	A5	8.25	11.7
	A9	3.52	
	A1	7.51	
3	A5	3.32	20.3
	A9	50.20	
	A1	6.62	
4	A5	12.03	7.5
	A9	3.72	

The effect of roasting on OTA levels is shown in Figure 1. Results show that OTA contamination decreases with roasting and that degradation will be more effective upon increasing roasting time and temperature. This behavior is in agreement with some of the literature data (Romani et al., 2003; Blanc et al., 1998; Nehad et al., 2005). Average values observed for OTA reduction were 63% and 82% for mild and dark roasts, respectively.

Degradation values are within literature data (Romani et al., 2003; Blanc et al., 1998). According to Blanc et al. (1998), OTA destruction during roasting can be attributed to both thermal destruction and chaff removal. Romani et al. (2003) evaluated OTA degradation at three different roasting levels and observed that OTA reduction increased with roasting time. Furthermore, these authors reported that OTA reduction seems to be related to the original OTA content, and that higher degradation values were observed for the sample that originally presented higher OTA content. The same behavior was observed in the present study, for which higher OTA degradation levels were observed for Lot 3.

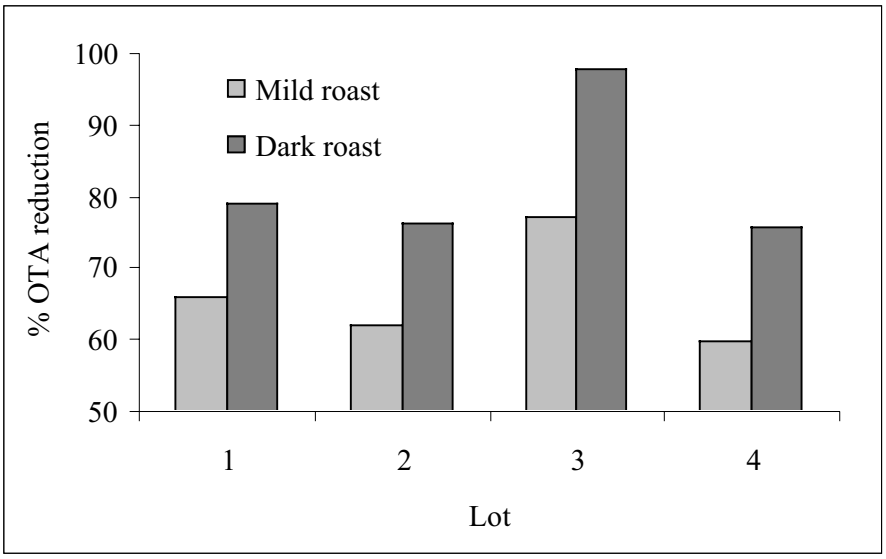

Figure 1. Average OTA degradation values.

CONCLUSIONS

It can be concluded that roasting reduces OTA levels in coffee. However, such reduction can be heterogeneous. Therefore, industrial roasting will achieve some reduction in OTA levels which, in some cases, could be quite significant. Thus, in view of the heterogeneity observed in both OTA distribution in green coffee and degradation upon roasting, one should not rely on roasting for OTA elimination and should avoid processing contaminated coffee samples. It is noteworthy to mention that in our study, 100% degradation only occurred for samples that were effectively burned and thus one could find residual OTA in contaminated samples, even for intense roasting conditions.

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Chlorogenic Acids Distribution in Brazilian Defective Coffee Beans

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SUMMARY

The goal of this study was to evaluate the chlorogenic acids (CGA) content and distribution in different types of Brazilian defective coffee beans and to compare the CGA composition of good quality beans with that of "PVA", a commercial mixture of defective and non-defective coffee beans sold in the Brazilian coffee market. Among all defective beans, dark-green beans presented the highest levels of CGA (87 g/kg, dry weight), while the lowest levels were observed in black defective beans (13 g/kg, dry weight). Total CGA content in PVA mixture was similar to that of non-defective beans. The high content of CGA in immature defective beans raises the possibility of future commercialization of this type of defective bean for pharmaceutical and cosmetics use.

RESUMO

Este estudo objetivou avaliar os teores e a distribuição dos ácidos clorogênicos (CGA) de diferentes tipos de defeitos do café Brasileiro, bem como comparar a composição de ácidos clorogênicos em grãos de boa qualidade com a de uma mistura de PVA vendida no mercado brasileiro. Entre todos os defeitos, grãos verde-escuros apresentaram os teores mais elevados de CGA (87 g/kg, base seca), enquanto os menores teores foram observados nos defeitos pretos (13 g/kg, base seca). O teor total de CGA no PVA foi semelhante ao da amostra de boa qualidade, o que faz com que teores de CGA sozinhos não possam ser utilizados como indicadores de qualidade. Os altos teores de CGA em defeitos verdes despertam a possibilidade de comercialização desses grãos para finalidades relacionadas com a indústria farmacêutica e de cosméticos.

INTRODUCTION

Coffee plays an important part in the world's economy, especially in producing and exporting countries such as Brazil. The non-discriminative harvest method used in Brazil commonly results in crops with defective beans that considerably affect the sensorial aspects of the beverage. Only about 80% of the arabica coffee production in Brazil meets the international market standards. The remaining 20% are classified as defective beans and commercialized in the national market under the name of PVA (P-black or over-riped beans, V – immature beans and A – sour or fermented beans) (Mazzafera, 1999; Farah et al., 2006). Chlorogenic acids (CGA) are relevant phenolic compounds in coffee due to their potential biopharmacological activity in humans, including antioxidant properties, and contribution to flavor. The main subgroups of CGA found in coffee are: caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA). Despite their importance to flavor, high levels of CGA have been associated with undesirable flavor characteristics (Farah et al., 2006). Considering the limited knowledge about the chemical composition of PVA, the goals of this

study were to evaluate the CGA content and distribution in different types of defective coffee beans constituent of PVA and to compare CGA content and distribution of a PVA mixture with good quality non-defective beans.

MATERIAL AND METHODS

Brazilian arabica coffee samples were obtained from Minas Gerais state producers and classified by ASSICAFÉ (São Paulo, Brazil) as excelent quality; good quality; black beans, black-immature beans; light immature and dark immature beans, sour beans and a commercial mixture of PVA containing ~ 60% of defective beans, in which light and dark immature beans prevailed, accounting for ~ 48% of defects (Table 1). Samples were analyzed by reverse-phase HPLC according to Farah et al. (2006; 2005).

Table 1. Percentage distribution of defective and healthy beans in PVA mixture.

Type of bean	*Percentage (%)*
Black	7.14
Half-black	3.53
Sour	9.85
Immature	21.95
Dark-green	8.55
Black-green	5.08
Other	3.08
Non-defective	40.82

RESULTS AND DISCUSSION

Eight CGA isomers were quantified in the studied samples. A large variation among CGA contents of defective and good quality beans was observed. The highest CGA content was observed in dark immature beans (87 g/kg, dry weight), which decreased with maturation. Black defective beans showed the lowest CGA content (13 g/kg, dry weight).

The contents of 5-CQA, the main CGA in coffee, varied from 56 g/kg (dw), in dark immature beans, to 6 g/kg (bs), in black beans. 5-CQA content in good and excelent samples, which were practically free of defects, was 40 g/kg (bs). Our results are in accordance with those from Mazzafera (1999), who observed higher total phenols and 5-CQA contents in immature beans compared with good quality beans. Franca et al. (2004) also found lower 5-CQA levels in black beans compared to immature and good quality beans, even though such differences were not as dramatic as those observed in this study.

Although the percent distribution of total CQA, total FQA and total diCQA was similar for all defective and non-defective samples, their individual isomers distribution was quite different (Tables 2a and 2b). Higher amounts of 3-CQA, 4-CQA and 4-FQA isomers were observed in black and sour defective beans, compared to other defective beans and with good quality beans, probably due to isomerization of 5-CQA and 5-FQA during the fermentative process of sour beans and aging of black beans. Slightly higher contents of 3- and 4-substituted isomers were also observed in the PVA mixture, probably due to the presence of black and sour defective beans in the mixture.

Table 2. Total (2a) and individual (2b) chlorogenic acid contents in green arabica samples of excelent quality (E), good quality (G), PVA mixture (PVA).; Immature (I); dark-green (DG); black-green (BG); Black (B); Sour (S). CQA = caffeoylquinic acids; FQA = feruloylquinic acids; diCQA = dicaffeoylquinic acids. Results of triplicate analyses expressed in g/ kg, dry base.

2a)

Sample	Total CQA	% CQA	Total FQA	%FQA	Total diCQA	%diCQA	Total CGA
E	50.28±0.45	84.6	2.71±0.08	4.6	6.43±0.12	10.8	59.42±0.53
G	53.24±0.81	84.0	2.76±0.06	4.4	7.05±0.06	11.0	63.05±0.90
PVA	54.57±0.77	85.0	3.25±0.03	5.1	6.24±0.20	9.7	64.05±0.94
I	65.59±0.17	86.0	3.66±0.05	4.8	7.06±0.11	9.3	73.32±0.32
DG	73.90±0.31	85.4	4.37±0.05	5.0	8.24±0.06	9.5	86.51±0.30
BG	65.55±0.54	85.4	3.44±0.09	4.5	7.79±0.24	10.1	76.78±0.69
B	11.05±0.32	83.0	0.87±0.03	6.5	1.38±0.01	10.3	13.30±0.34
S	57.90±0.67	83.4	3.58±0.20	5.1	7.92±0.03	11.4	69.40±0.84

2b)

Samples	3-CQA	4-CQA	5-CQA	4-FQA	5-FQA	3,4-diCQA	3,5-diCQA	4,5-diCQA
E	4.34±0.05	5.99±0.08	39.95±0.32	0.48±0.04	2.23±0.06	1.44±0.03	3.81±0.11	1.18±0.04
G	5.60±0.17	7.15±0.26	40.49±0.41	0.49±0.03	2.27±0.03	1.98±0.02	3.42±0.03	1.65±0.02
PVA	7.02±0.15	8.85±0.10	38.70±0.53	0.74±0.05	2.50±0.03	1.95±0.05	2.13±0.06	2.16±0.11
I	5.74±0.01	8.42±0.05	48.44±0.11	0.66±0.02	3.01±0.03	1.95±0.03	2.91±0.03	2.20±0.05
DG	7.65±0.09	10.60±0.05	55.64±0.17	0.79±0.01	3.58±0.04	2.48±0.01	3.28±0.04	2.48±0.03
BG	5.93±0.11	8.66±0.42	50.96±0.19	0.60±0.01	2.83±0.09	2.10±0.07	3.46±0.10	2.23±0.07
B	2.25±0.09	2.57±0.07	6.24±0.18	0.34±0.02	0.53±0.01	0.47±0.01	0.45±0.01	0.45±0.02
S	8.53±0.06	10.49±0.08	38.88±0.53	0.90±0.06	2.68±0.15	2.58±0.02	2.65±0.03	2.69±0.06

Generally speaking, CGA content in PVA mixture was quite similar to that of non-defective beans (Table 2a), probably due to the contribution of the various types of defective beans with different CGA contents and to the high percentage of non-defective beans in the mixture (Figure 1).

CONCLUSIONS

Despite the large differences observed in CGA content and distribution of the various types of defective beans, the CGA composition of PVA mixture and healthy beans was quite similar. This means that CGA content alone cannot be used for coffee quality prediction. Also, the low quality of PVA - at least in this case- cannot be attributed to CGA contents. A larger number of PVA samples will be investigated in order to confirm these results.

Considering the various potential pharmacological applications of chlorogenic acids, the high content of CGA in green defective beans raises the possibility of commercialization of green beans for pharmacological use.

AKNOWLEDGMENTS

The authors greatly acknowledge the financial support of CBP&Dcafe-EMBRAPA, Conselho Nacional de Pesquisa (CNPq) and Fundação de Amparo a Pesquisa do Estado Rio de Janeiro (FAPERJ). We also would like to thank Dr Aldir Teixeira, from ASSICAFÉ, Dr. Marta Taniwaki (ITAL), Dr. Rosires Deliza, and Ana Gonçalves (EMBRAPA) for samples classification.

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Kinetic Behavior of Chlorogenic Acids and Lactones during Roasting of Brazilian *Coffea arabica* and *Coffea canephora* Cultivars

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SUMMARY

The isomerization and degradation kinetics of chlorogenic acids (CGA) and the formation and degradation kinetics of chlorogenic acid lactones (CGL) during coffee roasting were studied in Brazilian *C. arabica* and *C. canephora* coffee samples. Caffeoylquinic and feruloylquinic acids showed a similar kinetic behavior, with isomerization in early stages of roasting, followed by degradation and formation of lactones. Degradation of dicaffeoylquinic acids was observed, with concomitant formation of caffeoylquinic acids and lactones. Caffeoylferuloylquinic acids isomers showed similar degradation kinetics, with the exception of one isomer that was formed during coffee roasting. Maximum CGL values in both *C. arabica* and *C. canephora* species were observed in light medium roast, ~15 and ~13% weight loss, respectively.

INTRODUCTION

Chlorogenic acids (CGA) are a family of esters formed between certain *trans* cinnamic acids, such as caffeic (CA), ferulic (FA) and *p*-coumaric (*p*-CoA) acids, and (–)-quinic acid. The main subgroups of chlorogenic acid isomers in coffee are the caffeoylquinic acids (CQA), feruloylquinic acids (FQA), dicaffeoylquinic acids (diCQA) and, in smaller amounts, *p*-coumaroylquinic acids (*p*-CoQA) and caffeoylferuloylquinic acids (CFQA). Green coffee beans may contain as much as 15% of CGA and during coffee roasting, partial degradation of these components is observed, generating the corresponding lactones (CGL). Both CGA and CGL are known to contribute to coffee flavor and may be of potential biopharmacological importance in humans (Variyar et al., 2003; Moreira et al., 2005; Farah et al., 2005). The study of these compounds in Brazilian coffee cultivars is still incipient but important, since Brazil is the first world coffee producer and exporter (Brazilian Coffee Industries Association, 2005). In the present work, we studied the isomerization and degradation kinetics of CGA and the formation and degradation kinetics of CGL during coffee roasting in three commercially important coffee varieties of Brazil.

MATERIAL AND METHODS

Two *C. arabica* samples (cv. Mundo Novo and cv. Catuaí Vermelho) and one *C. canephora* sample (cv. Conillon) were roasted in a commercial bed fluid roaster (i-Roast® Model No. 40009, Hearthware Home Products, USA) at a maximum temperature of 190 ^{o}C for increasing periods of time in order to provide a wide range of roasting degrees. Roasting degrees were determined by percent weight loss during roasting and by comparison with the color disks from the "Roasting Color Classification System" (Agtron-SCAA, Reno, NV, 1995), following the standards used by the Brazilian Coffee Industries Association (ABIC). Analysis of CGA and CGL were performed by liquid chromatography – eletronspray ionization – mass

spectrometry (LC–ESI–MS). CFQA isomers identification was suggested by comparison of our chromatograpic profile and relative intensity with that of Clifford et al. (2003).

RESULTS AND DISCUSSION

The kinetic study of CQA and FQA groups in both species showed that during the early phase of roasting (~8% weight loss), 5-substituted isomers were converted into 3- and 4-substituted isomers, with no significant change in the total amount of CGA and with minor formation of CGL. The next phase of roasting (~ 8-14% weight loss) was characterized by the concurrent degradation of CGA and formation of CGL. During the final phase of the roasting process (~14-25% weight loss) degradation of both CGA and CGL was observed (Figure 1).

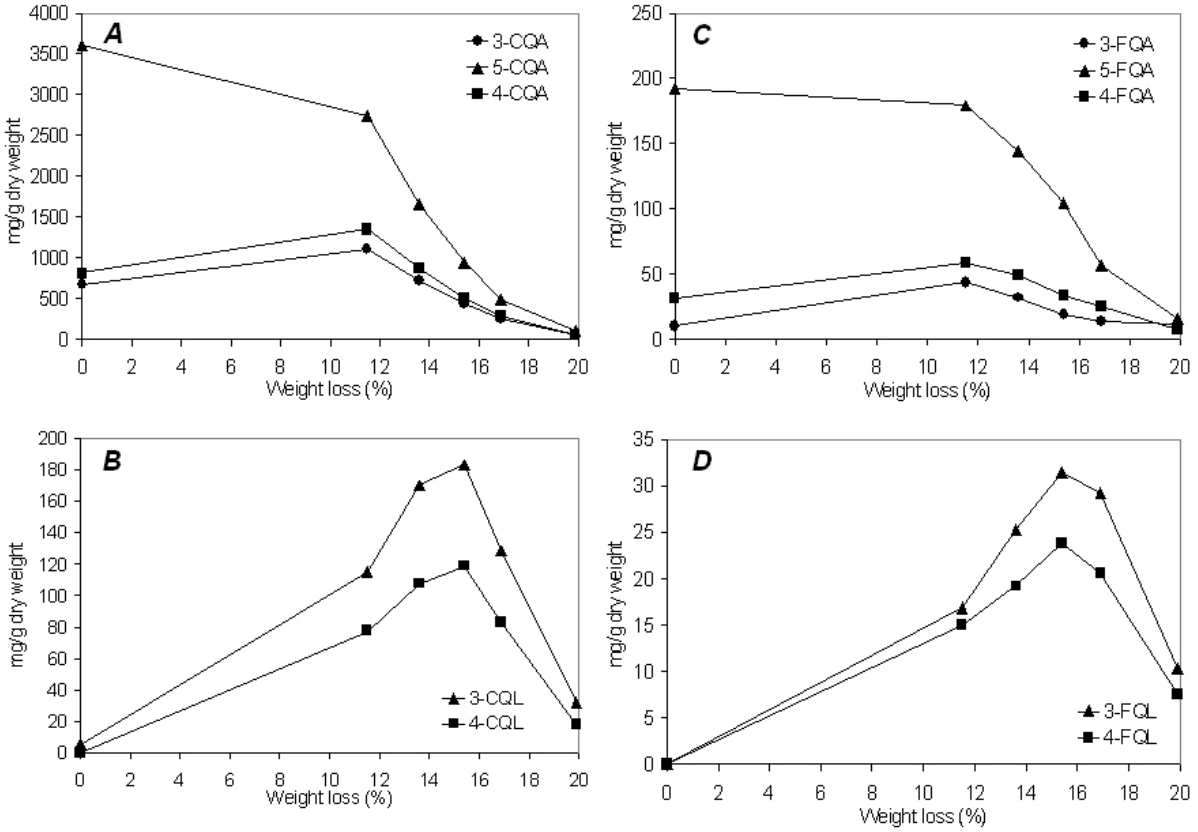

Figure 1. Kinetic behavior of CQA (A), CQL (B), FQA (C) and FQL (D) groups (mg/g dry weight) during roasting of *C. arabica* Mundo Novo cultivar (expressed as percentage weight loss).

The kinetic behavior of the diCQA group (Figure 2) differs from that of the CQA and FQA groups only in the early phase of the roasting process, which is characterized by the isomerization and degradation of diCQA, with no significant formation of the corresponding CGL. In that phase, the degradation of diCQA leads to the formation of CQA isomers with loss of a caffeic acid residue, as well as other derivatives, as previously observed by Leloup et al. (1995).

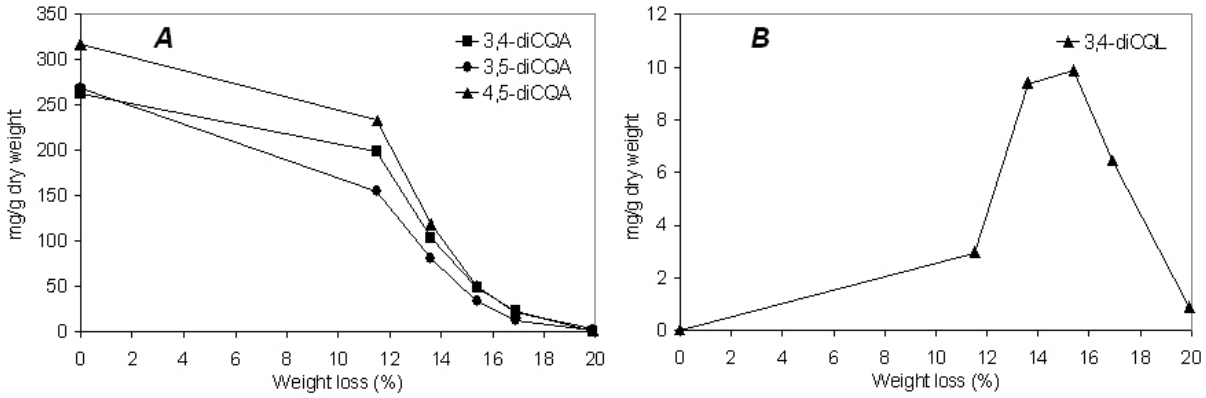

Figure 2. Kinetic behavior of diCQA (A) and diCQL (B) groups (mg/g dry weight) during roasting of *C. arabica* Mundo Novo cultivar (expressed as percentage weight loss).

Although CFQA isomers identified in this study were quantified as a whole, the comparison between their individual relative signal intensities could be accomplished using LC-MS. CFQA peak areas were normalized by the 5-CQA standard peak area to eliminate within-run ionization differences. Among the seven CFQA isomers identified in green coffee, six were degraded during roasting in a similar fashion, while one of them was formed during the early phase of roasting, being the most abundant by the end of the process (Figure 3).

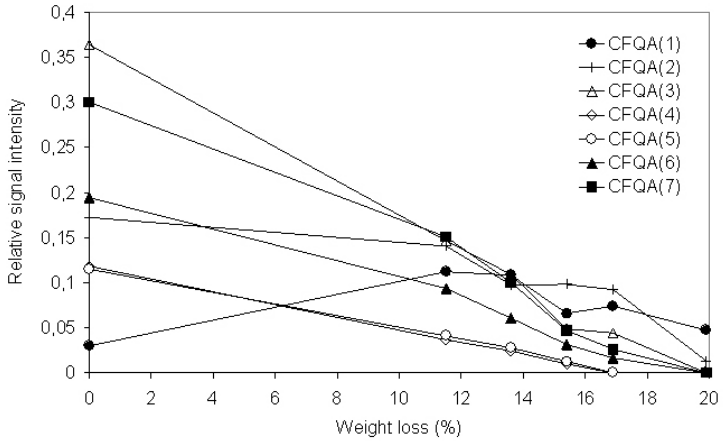

Figure 3. Kinetic behavior of CFQA isomers 1–7 (relative intensity) during roasting of *C. arabica* Mundo Novo cultivar (expressed as percentage weight loss).

Figure 4 shows the degradation kinetics of CGA and the formation and degradation of CGL during roasting of *C. arabica* and *C. canephora* samples. In *C. arabica* samples, the highest amounts of CGL in Catuaí Vermelho and Mundo Novo cultivars (431.0 mg/100 g and 375.6 mg/100 g dry weight, respectively) were observed at ~15% weight loss (light medium roast). These results are in conformity with data recently published for *C. arabica* cultivars from Ethiopia and Brazil (Farah et al., 2005). The highest content of CGL in *C. canephora* (619.8 mg/g dry weight) was observed at ~13% weight loss (light medium roast). This amount is not only higher than all reported *C. arabica* CGL amounts but also higher than the amount observed by Farah et al. (2005) when analyzing a *C. canephora* Robusta cultivar from Uganda. Even after subtraction of some CGL isomers that we quantified in this study, such as 3-FQL, 3-CoQL and 4-CoQL, which were not previously quantified in Robusta (Farah et al., 2005) due to co-elution with other compounds, the Brazilian *C. canephora* cultivar still showed higher content of CGL in comparison with the African cultivar.

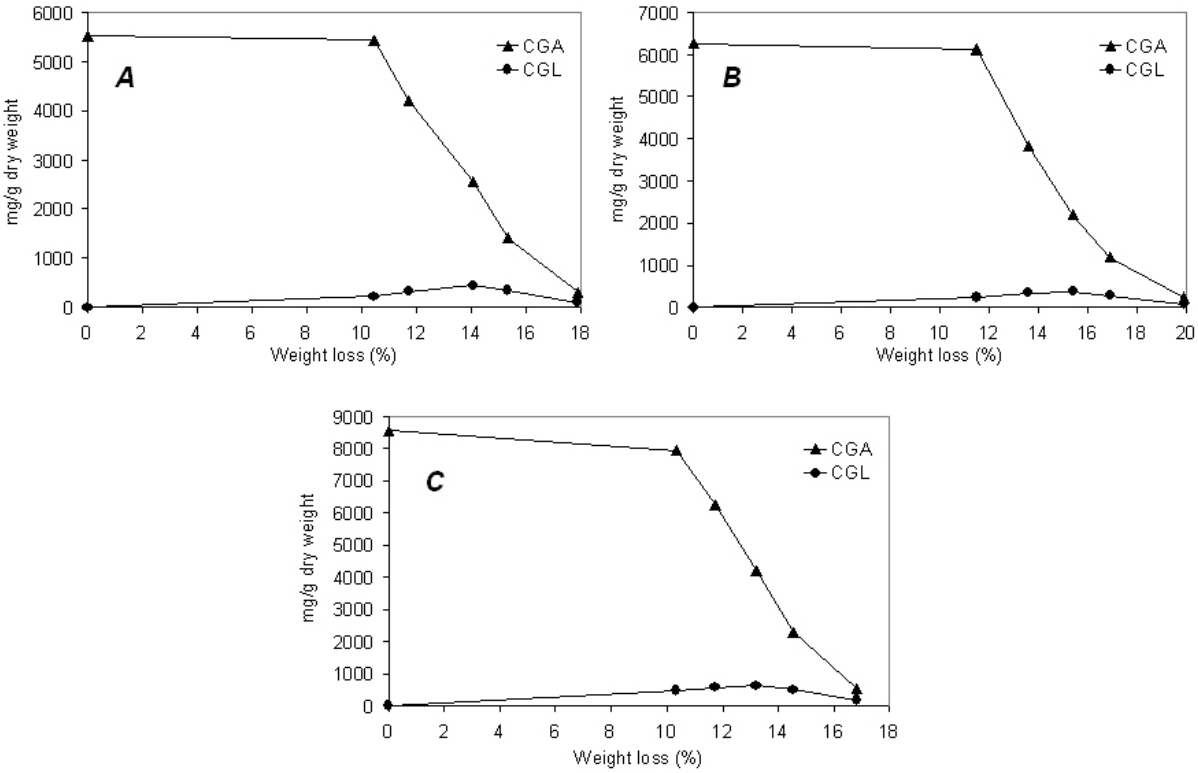

Figure 4. Kinetic behavior of both CGA and CGL during roasting of Catuaí Vermelho (A), Mundo Novo (B) and Conillon (C) coffee samples.

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Investigation of Novel Chlorogenic Acids and Lactones in Brazilian *Coffea arabica* and *Coffea canephora* Cultivars by LC-ESI-MS

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SUMMARY

Major and minor chlorogenic acids and lactones were investigated in green and roasted Brazilian *C. arabica* and *C. canephora* samples by LC–DAD–ESI–MS. In addition to 18 major chlorogenic acids and lactones identified in green coffee, 25 to 38 – depending on cultivar and species – minor potential chlorogenic acids and lactones isomers were observed in green and roasted coffee samples. Epimerization of (–)-quinic acid and *trans-cis* isomerization of cinnamic acids are considered.

INTRODUCTION

Coffee is one of the most consumed beverages in the world, with 2005 production reaching approximately 7 million tons worldwide (Brazilian Coffee Industries Association, 2005). In this scenario, Brazil remains the first world producer and exporter. Coffee beans contain a wide variety of phenolic compounds, mainly chlorogenic acids (CGA), which are a family of mono- and diesters of certain *trans* cinnamic acids, such as caffeic (CA), ferulic (FA) and *p*-coumaric (*p*-CoA) acids, and (–)-quinic acid. The main subgroups of chlorogenic acid isomers in coffee are caffeoylquinic acids (CQA), feruloylquinic acids (FQA), dicaffeoylquinic acids (diCQA) and, in smaller amounts, *p*-coumaroylquinic acids (*p*-CoQA) and caffeoylferuloylquinic acids (CFQA) (Figure 1). CGA are known for their potential biological importance in humans (Farah and Donangelo, 2006) and for being important for the development of flavor in coffee beverage (Farah et al., 2006). Their lactones (CGL), formed during roasting of the beans (Figure 2), also contribute importantly to bitterness and, despite their low concentrations their impact on the final cup quality may be significant (Ginz and Enhelhardt, 1995). Moreover, considering that CGA distribution profile in coffee may be used as a means to identify coffee species (Gerrero et al., 2001), we should not discard the possibility of using CGA profile also for discrimination between cultivars. In the present work, we looked for novel minor CGA and CGL isomers in green and roasted economically important Brazilian *C. arabica* and *C. canephora* cultivars.

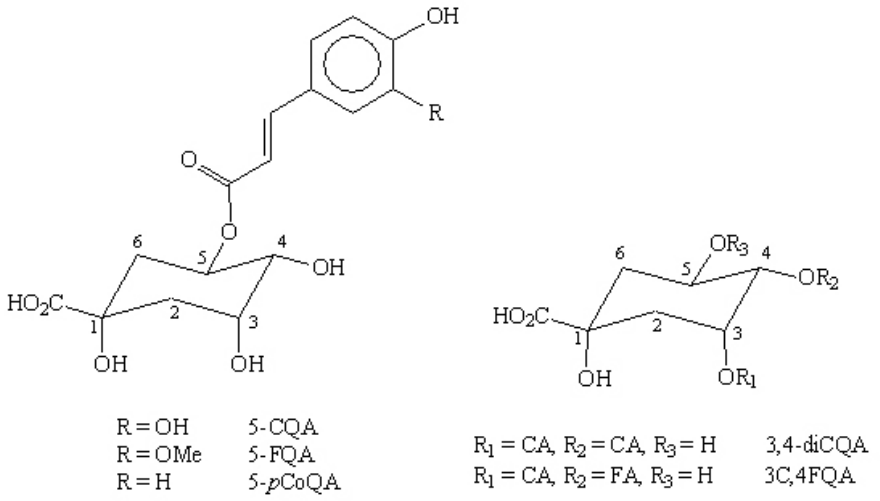

Figure 1. Main chlorogenic acid goups found in coffee: CQA, FQA, *p*CoQA, diCQA and CFQA. Different combinations of esterification also occurs in carbons 3, 4 and 5 of quinic acid.

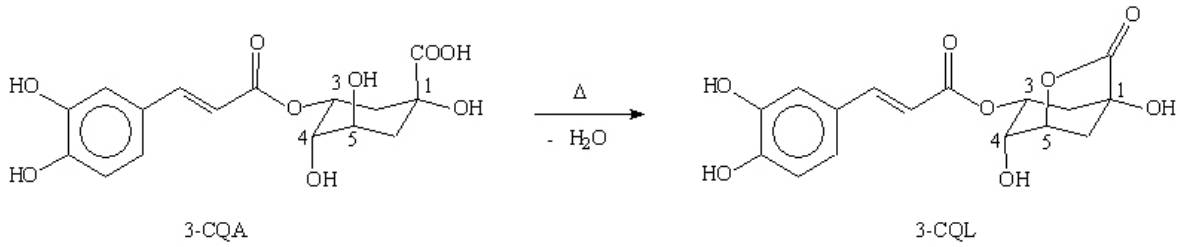

Figure 2. Generation of 3-CQL from 3-CQA through the loss of a water molecule and the formation of an intramolecular ester bond during roasting process. Although under IUPAC rules the numbering systems for chlorogenic acids and lactones are different, to avoid confusion, in this work the same numbering of the carbons was used for both the lactones and their precursors.

MATERIAL AND METHODS

Green samples of *C. arabica* cv. Mundo Novo, *C. arabica* cv. Catuaí Vermelho and *C. canephora* cv. Conillon were obtained directly from reliable producers in Guaxupé, Minas Gerais, Brazil. Samples were roasted from light to very dark roasting degrees in a bed fluid roaster (i-Roast® Model No. 40009, Hearthware Home Products, USA) with a maximum temperature of 190 ºC. Liquid chromatography – diode array detector – electronspray ionization – mass spectrometry (LC–DAD–ESI–MS) analyses of coffee samples were performed by monitoring of the ions with *m*/*z* corresponding to the main groups of CGA and CGL, according to Farah et al. (2006). CFQA isomers identification was suggested by comparison of our chromatograpic profile and relative intensity with that of Clifford et al. (2003).

RESULTS AND DISCUSSION

Chromatograms of green and roasted C. canephora coffee recorded at 320 nm are shown in Figure 3 and 4, respectively. This cultivar was chosen to exemplify major CGA and CGL chromatographic profile since it showed the highest number of peaks.

Light medium roasted samples (~14% weight loss for cv. Catuaí Vermelho and cv. Mundo Novo, and ~12% weight loss for cv. Conillon) presented the largest variety of peaks. Table 1 shows the number of individual CGA and CGL isomers observed in both green and roasted coffee cultivars. In total, 43 CGA and CGL isomers were found in cv. Catuaí Vermelho, 48 in cv. Mundo Novo and 56 in cv. Conillon, with some minor isomers being apparently observed for the first time in coffee. Although a higher number of isomers was observed for cv. Mundo Novo and Conillon in comparison to cv. Catuaí Vermelho, isomers were not necessarily the same among different coffee samples. Since there were no available standards for minor CGA and CGL, the unequivocal identification of such minor isomers was not possible.

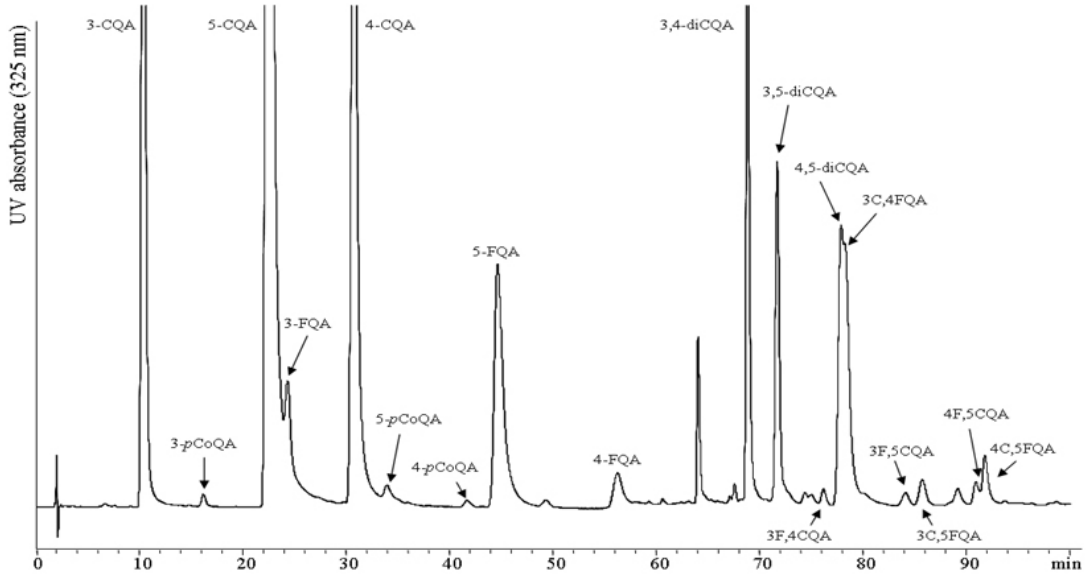

Figure 3. HPLC separation of major chlorogenic acids in green *C. canephora*, cv. Conillon. 3-CQA, 4-CQA, 5-CQA and 3,4-diCQA peaks are shown off scale to highlight small peaks.

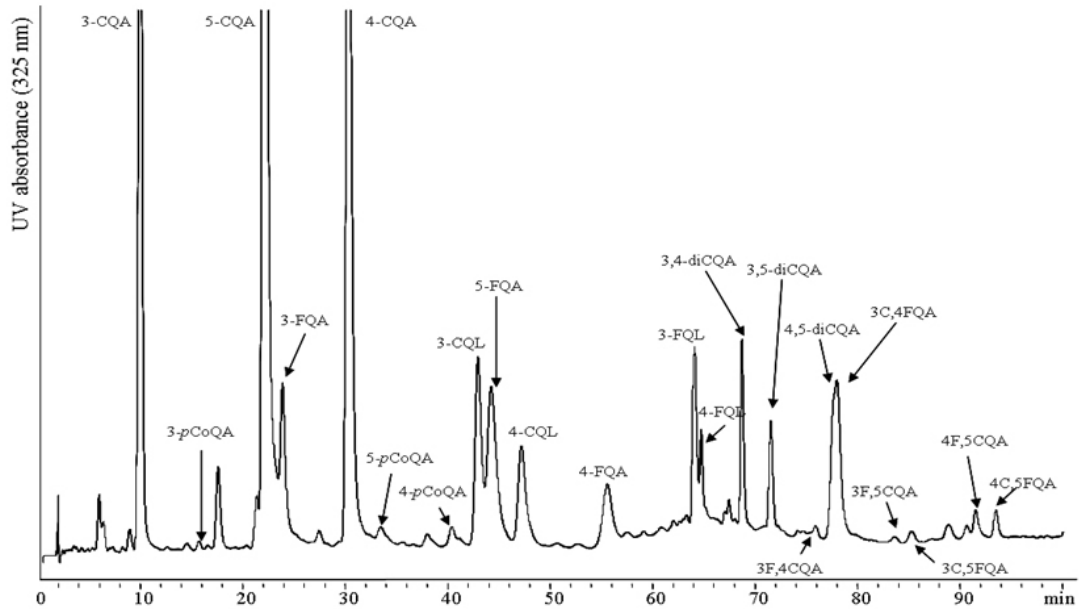

Figure 4. HPLC separation of major chlorogenic acids and lactones in roasted (~13.2% weight loss) *C. canephora*, cv. Conillon. 3-, 4- and 5-CQA peaks are shown off scale to highlight small peaks.

In general, minor CGA isomers may be stereoisomers of the major CGA formed by *cis*-isomers of cinnamic acids and/or different conformational forms of (–)-quinic acid. It has already been reported that, during coffee roasting, racemization of (–)-quinic acid takes place, generating at least six different stereoisomers (Scholz-Böttcher and Maier, 1991). The formation of six stereoisomeric quinic acids (four *meso* forms and two pairs of enantiomers) was also reported by Maier et al. (1991) when (–)-quinic acid was refluxed in strong acid. 5-*cis*-CQA has been previously tentatively identified in eggplants (Whitaker and Stommel, 2003).

Table 1. Number of individual CGA and CGL isomers observed in green and roasted Brazilian coffee samples.

CGA/CGL group	Catuaí Vermelho		Mundo Novo		Conillon	
	Green	Roasted[a]	Green	Roasted[a]	Green	Roasted[a]
CQA	3	6	3	9	6	9[b]
FQA	5	2[b]	6	5	7	13
diCQA	4	5[b]	4	4[b]	3	8
p-CoQA	3	3	3	3	3	3
CFQA	11	8	11	7	7	8[b]
CQL	1	5	1	5	1	5
FQL	0	2	0	2	1	3
diCQL	0	2	0	2	2	2[b]

[a]Light Medium roast (14% weight loss for Catuaí Vermelho and Mundo Novo and 12% weight loss for Conillon). [b]One ore more isomers different from those found in the same green sample.

Considering CGL isomers, in general, because the elimination of a water molecule from the six-member ring of the (–)-quinic acid requires a *syn*-1,3-diaxial configuration of the hydroxyl and carboxyl groups to form lactones, only those isomers that are not substituted in the 5-position of the (–)-quinic acid are able to form a 1,5-γ-quinides during roasting (Farah et al., 2005). However, other lactones may arise from CGA that have undergone racemization of their (–)-quinic acid moiety. For those CGA stereoisomers, 1,5-*epi*-γ-, 1,5-*muco*-γ- and 1,5-*neo*-γ-lactones could possibly be formed. Moreover, stereoisomers not substituted in the 4-position of the quinic acid may suffer an intramolecular esterification generating 1,4-*epi*-δ-, 1,4-*scyllo*-δ- and 1,4-*neo*-δ-lactones (Maier et al., 1991).

Related compounds such as caffeoyltryptophan, caffeic, ferulic and quinic acids were also identified in this study. In addition, we observed small amounts of compounds with *m*/*z* of 543 and 525, consistent with diFQA isomers that were previously reported by Clifford et al. (2006), and never reported diFQL isomers, respectively.

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Investigation of Radical Scavenging Properties of *Espresso* Brews

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SUMMARY

A Trolox-equivalent antioxidant capacity assay which is based on the decolorization of $ABTS^{\bullet+}$ radical ions has been adapted to HPLC and GPC separation systems. The method permitted the elucidation of radical scavengers in coffee brews and espresso percolates. Results clearly indicate that the radical scavenging effectiveness of coffee brews is intensely dependant on the grind size. Analyses conducted on espressos revealed that more than 70% of the overall radical scavenging activity is recovered within the first 30 sec of percolation. Molecular weight distribution and anti-radical effectiveness of espresso melanoidins were determined by means of combined gel permeation chromatography with $ABTS^{\bullet+}$-analysis (GPC-$ABTS^{\bullet+}$). The results point out the importance of melanoidins as effective radical scavengers in coffee brews. Furthermore it is shown that the radical scavenging activity of melanoidins depend on the molecular weight.

INTRODUCTION AND OBJECTIVES

Coffee is probably the most frequently consumed beverage worldwide. Recent knowledge suggests potential health benefits from coffee consumption due to its antioxidant nutrients (Ranheim and Halvorsen, 2005. The aim of this paper is to assess the influence of the coffee brewing process with view to the radical scavenging effectiveness of the coffee beverage. A special consideration was given to the percolation of espresso coffees where brewing is a crucial step to achieve optimum sensory quality.

METHODS AND MATERIALS

Coffee Samples. Cameroun *Arabica* and Vietnam *Robusta* Coffees were roasted on a Probat drum roaster model 'Probatino', batch size 1 kg, to a medium "European" roast colour. Brews were prepared from ground coffees by means of a household drip filter machine model Krups AromaPro. The standardized ratio of coffee weight to water volume was 50 g/L.

Three different kind of commercial **Espresso-Pad** samples were obtained from Illycaffé (Trieste, Italy). All samples were *Arabica* E.S.E.7 g tampered coffee pods labelled as *"Dark Roast", "Light Roast",* and *"Decaffeinated Medium Roast"*. Espresso brews were made on a Francis X5 espresso machine (Francis, Italy).

Particle size distribution measurements were performed by a LASER diffraction instrument (Sympatec Helos, Claustal-Zellerfeld, Germany; Figure 1).

Chemical analysis of **non-volatile constituents** and **radical scavenging capacities** was accomplished by on-line HPLC-$ABTS^{\bullet+}$-analysis. This method is described somewhere else (Cassano et al., 2006). Radical scavenging capacities are expressed in Trolox equivalents

(TE). Trolox is a synthetic antioxidant used as a reference for a highly antioxidative substance.

In a novel approach **GPC (*gel permeation chromatography*)** analysis was directly coupled with $ABTS^{\bullet+}$-analysis, thereby enabling the simultaneous determination of high-molecular weight melanoidins, including their molecular weight distribution and radical scavenging activities. For this purpose a HEMA BIO 40 - HPLC column (MZ Analysentechnik, Mainz, Germany) was used which is characterized by an exclusion limit of 40000 Da, average particle size of dp = 10 µm, and pressure resistance up to 20 bar. This column is compatible with aqueous solvents. Melanoidins were monitored at 405 nm (Figure 3). The GPC system was combined with the $ABTS^{\bullet+}$-assay (*on-line GPC- $ABTS^{\bullet+}$*) to evaluate radical scavenging properties of separated melanoidins.

INFLUENCE OF GRIND SIZE

Figure 1 shows brew yields and radical scavenging capacities in relationship to the average particle size.
It is evident that the grinding degree has a distinct effect on brew yields (left diagram). The increased surface of finer coffee particles facilitates a more efficient transfer of soluble and emulsifiable substances. This finding collaborates well with the progressive rise of radical scavenging capacities at smaller grind sizes (right diagram).

Grinding has a stronger impact on antioxidant capacities in comparison with the roasting process (see Cassano et al., 2006).

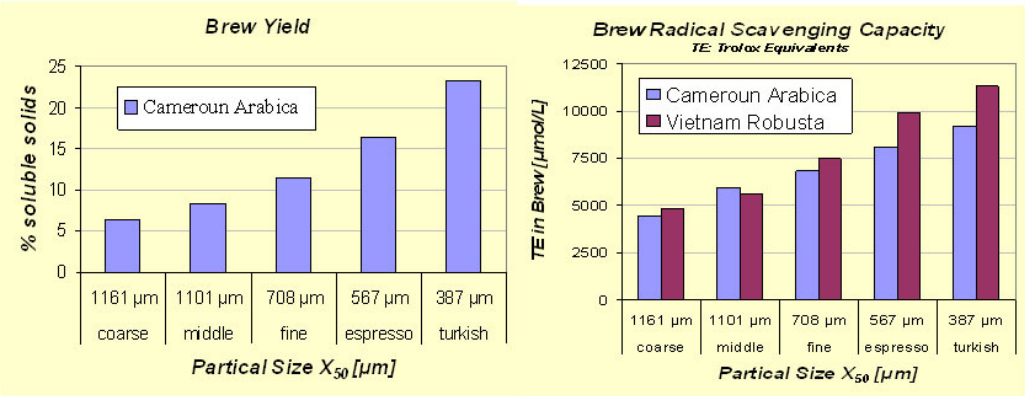

Figure 1. Extractable solids ("brew yield", left) and overall radical scavenging capacities (right) as a function of grind size.

ESPRESSO PERCOLATION KINETICS

In order to investigate the influence of percolation time, an espresso machine was directly connected to an HPLC fraction collector. The extraction kinetics of radical scavengers and other non-volatile constituents (caffeine, chlorogenic acids) was determined in thereby obtained fractions by means of HPLC-$ABTS^{\bullet+}$ and FIA-$ABTS^{\bullet+}$.

Figure 2 provides an example of HPLC-$ABTS^{\bullet+}$ chromatograms that are obtained from the analysis of an Espresso brew. The $ABTS^{\bullet+}$-bleaching profile at 650 nm exhibits the predominant radical scavenging effectiveness of the chlorogenic acids which are selectively detected at 320 nm.

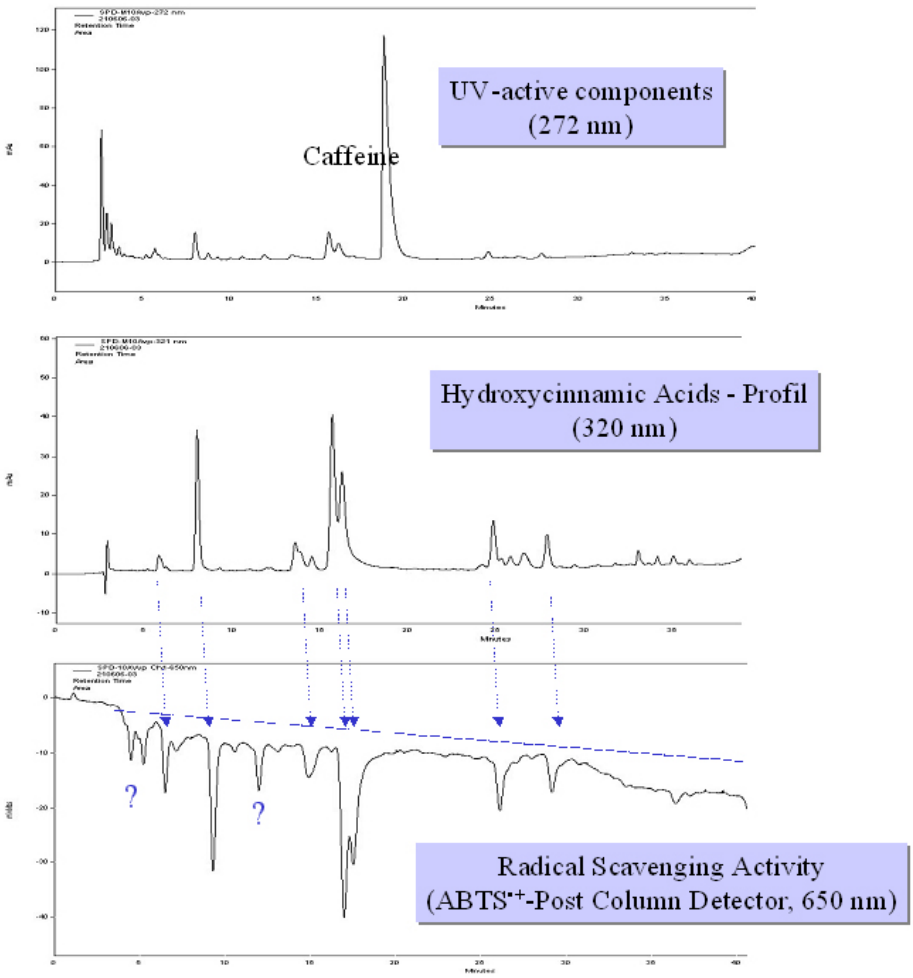

Figure 3. Exemplary chromatograms obtained from HPLC-ABTS$^{\bullet+}$-separation of an Espresso fraction.

The percolation kinetics of the investigated Espresso samples is shown by Figure 3. As a result the majority (> 70%) of the antioxidants is recovered within the first 30-40 sec, which is the optimum brewing time for espresso coffees.

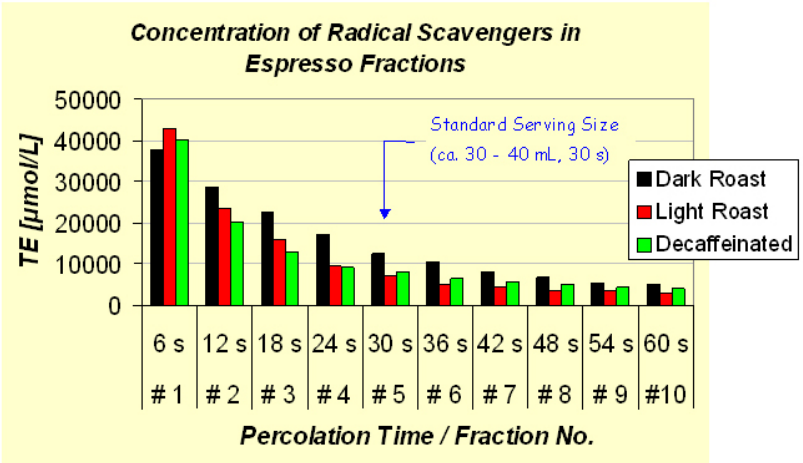

Figure 4. Extraction kinetics of radical scavenging capacity (FIA-ABTS$^{\bullet+}$). Fraction size: 6 sec.

ESPRESSO MELANOIDINS

Melanoidins in espresso brews and fractions that were collected during espresso percolation were analysed by GPC-ABTS$^{\bullet+}$ (Figure 5). The brown-coloured melanoidins were detected at 405 nm. It must be pointed out, that trigonelline was co-eluted due to an ion exclusion effect. However, this compound does not interfere with the quantification of melanoidins as it has no absorbance at 405 nm. Furthermore trigonelline does not react with ABTS$^{\bullet+}$. Chlorogenic acids are not eluted due to irreversible binding on the stationary phase.

As a matter of principle, retention times are inversely correlated with molecular weights in GPC separations. Due to lack of suitable molecular weight standards, we can provide only preliminary information on absolute molecular weight distributions at this point of time (Figure 6). The bar charts show only small differences in composition and radical scavenging effectiveness of melanoidins of dark roasted, light roasted and decaffeinated espresso brews. Obviously the specific antioxidative capacity of melanoidins is somewhat dependent on the molecular weight.

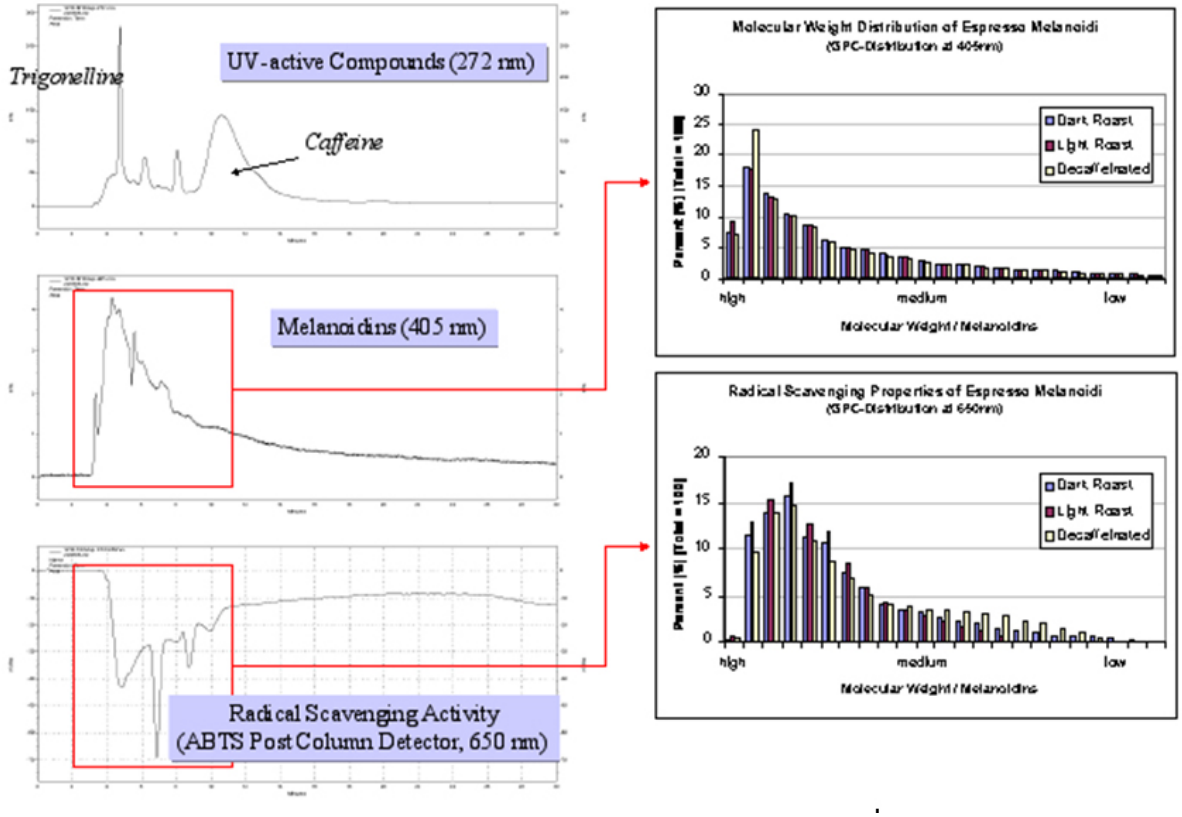

Figure 5. Exemplary chromatograms obtained from GPC-ABTS$^{\bullet+}$-separation of an Espresso sample (left).Semi-quantitative molecular weight distribution of Espresso-melanoidins and appropriate radical scavenging capacities (right).

CONCLUSIONS

Grind size of roasted coffee significantly affects the level of extractable antioxidants in the final coffee beverage. A smaller grind size, as typically used for Espressos, leads to considerably higher radical scavenging loads in the brew. This parameter surpasses the effects of varying roasting degrees (Cassano et al., 2006).

Simultaneous determination of non-volatile compounds and radical scavengers was accomplished by means of on-line HPLC-$ABTS^{\bullet+}$-analysis. It is evident from extraction kinetics measurements that approx. 70% of total radical scavenging activities were extracted within typical espresso percolation times of 30 seconds. This makes Espresso an excellent source of antioxidants!

The radical scavenging potential of coffee melanoidins could be elucidated by means of *gel permeation chromatography* (GPC) with on-line $ABTS^{\bullet+}$-postcolumn derivatization. GPC-$ABTS^{\bullet+}$ was applied to investigate the radical scavenging properties of espresso melanoidins in relationship to percolation times and molecular weights.

ACKNOWLEDGEMENT

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Abbreviations:

ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid);
FIA: Flow Injection Analysis;
GPC: gel permeation chromatography;
HPLC: high-performance liquid chromatography.

Correlating Chemical Compounds with Antioxidant Capacity in Torrefacto Roast Coffees

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SUMMARY

Antioxidant capacity of coffee is dramatically determined by roasting process. Torrefacto coffee is characterized by the addition of sugar at the end of the roasting process. The aims of the present work are to characterize commercial torrefacto roasted coffees and to try to elucidate the potential antioxidant capacity of coffee chemical compounds by means of multivariate statistical techniques (Correlation and Principal Components Analyses). Only high significant ($p < 0.01$) and excellent ($r > 0.75$) correlations between both antioxidant capacity parameters (DPPH and potential redox) and melanoidins (0.876 and -0.836, respectively) and trigonelline (0.889 and -0.797) have been found. Moreover, 5-CQA (0.633 and –0.573), hexanal (0.471 and –0.875) and caffeine (0.414 and –0.687) showed significant ($p < 0.05$) but moderate correlation. Furthermore, Principal Component Analysis allowed separation of torrefacto roast blends by PC1 (58.8%) characterised by antioxidant capacity and their related coffee chemical compounds. Antioxidant capacity of coffee could be mainly explained by the Maillard reaction and caramelization products enhanced by the addition of sugar in roasting process. However, there are other compounds such as trigonelline that may contribute to the antioxidant capacity of coffee.

INTRODUCTION

The predominance of coffee beverages over the other hot drinks is due essentially to consumer preference for their physiological effects, characteristic taste and agreeable aroma. Moreover, this choice is reinforced by recent studies that show the high contribution of coffee to the intake of antioxidants in the diet (Svilaas et al., 2004; Pulido et al., 2006). Antioxidant capacity of coffee is dramatically determined by roasting process. Torrefacto coffee, which plays an important role in consumption in some Southern European countries and in South America, is characterized by the addition of sugar at the end of the roasting process (no more than 15% by weight). Naturally occurring reactions in roasting process like Maillard and caramelization reactions are enhanced by sugar addition. A recent study (López-Galilea et al., 2006) has reported that when the percentage of torrefacto coffee is increased, an increase of the antioxidant capacity is observed. So, the aims of the present work were to characterize commercial torrefacto roasted coffees and to try to elucidate the potential antioxidant capacity of coffee chemical compounds by means of multivariate statistical techniques (Correlation and Principal Components Analyses).

MATERIALS AND METHODS

Materials

Three conventional roasted coffee blends (0T), two blends with 30% torrefacto roasted coffee (30T), two blends with 50% torrefacto roasted coffee (50T) and two 100% torrefacto roasted

coffees (100T) were purchased in a local market. Pure reference standards were from the suppliers given in parentheses: pentoxiphylline caffeine, trigonelline, 5-caffeoylquinic acid, caffeic acid, ferulic acid and 4-vinylguaiacol, acetaldehyde, propanal, 2-methylpropanal, hexanal, (Aldrich, Saint Quentin Fallavier, France), 3-methylbutanal, 2, 3-butanedione, 2, 3-pentanedione, (Acros organic, Noisy le Grand, France).

Caffeine and trigonelline

Extract preparation, cleanup and HPLC analysis have already been described by Maeztu et al. (2001) HPLC analysis was achieved with an analytical HPLC unit (Hewlett-Packard 1100). A reversed-phase Hypersil-ODS (5 μm particle size, 250 x 4.6 mm) column was used. The mobile phase was acetonitrile/water (15:85) in isocratic conditions at a constant flow rate of 2.0 mL min^{-1} at 25 ºC. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 280nm.

5-Caffeoylquinic acid (5-CQA). Extraction of 5-CQA, cleanup and HPLC analysis were carried out according to the method of Bicchi et al. (1995). The HPLC equipment has been described above. The conditions of the gradient solvent system used were 100% citrate-acetic acid buffer solution (pH 3.0) for 2 min, 85:15 buffer/methanol for 8 min, both at a flow rate of 0.8 mL min^{-1}, and 85:15 buffer/methanol for 5 min at a flow rate of 1.2 mL min^{-1}. The wavelength of detection was at 325 nm.

Hydroxycinnamic acids (caffeic acid and ferulic acid) and 4-vinylguaiacol

Extract preparation, cleanup and HPLC analysis were carried out according to the method of Álvarez-Vidaurre et al. (2005) with HPLC equipment described above.

Melanoidins (A420 nm)

50 mL coffee extract, obtained by solid-liquid extraction 10/100 (g/mL) using deionised water at 100 ºC for 10 min, were diluted up to 2 mL with deionised water. Absorbance was measured at 420 nm after exactly 1 min, in a 3 mL capacity glass cuvette (1 cm length) at 25 °C with a spectrophotometer Lambda 25 UV-VIS (Perkin-Elmer Instruments, Madrid, Spain).

Volatile Compounds

Volatile compounds extraction and GC analysis were carried out with the method described by Sanz et al. (2001). Volatiles were extracted using a static headspace sampler (Hewlett-Packard model 7694). GC analysis was achieved with a capillary column DB-Wax (60 m x 0.25 mm x 0.5 mm film thickness, J&W Scientific) in a HP 6890 gas chromatograph (Hewlett-Packard). Mass spectrometry was performed with a mass selective detector HP 5973 (Hewlett-Packard) operated in the electron impact ionization mode (70 eV). Seventeen key odorants were identified by comparing their mass spectra with those of the pure reference compounds and Wiley library and in addition by comparison of their Retention Index with those of standard compounds and data from the literature. Areas of peaks were measured by calculation of the total ionic Current (TIC).

Statistical analysis

Analysis of Variance (ANOVA) was applied to the results. T Tukey was applied as the test *a posteriori* with a level of significance of 95%. Pearson Correlation and Principal Component

Analyses were applied to the obtained results and antioxidant capacity measured by DPPH and potential redox in the previous work (López-Galilea et al., 2006). All statistical analyses were carried out using SPSS v.11.0 software package.

RESULTS AND DISCUSSION

Results for coffee compounds are shown in Table 1. 100 Torrefacto coffees, that are robusta variety, showed the highest values of caffeine and 5-CQA. In torrefacto coffee blends (30T, 50T, 100T) trigonelline content is significantly higher than in conventional ones (0T). Melanoidins increased with the percentage of torrefacto coffee, maybe due to a higher formation of Maillard reaction products (MRPs), but also to caramelization enhanced by sugar addition in torrefacto roasting process.

Table 1. Chemical compounds of ground roasted coffees.

	0T (n=9)	30T (n=6)	50T (n=6)	100T (n=6)
Caffeine (g/100g d.m.)	1.64 ± 0.16 ^{a}	1.58 ± 0.16 ^{a}	1.82 ± 0.05 ^{b}	1.95 ± 0.01 ^{b}
Trigonelline (g/100g d.m.)	0.38 ± 0.02 ^{a}	0.57 ± 0.08 ^{b}	0.61 ± 0.05 ^{bc}	0.68 ± 0.02 ^{c}
5-CQA (g/100g d.m.)	0.31 ± 0.02 ^{a}	0.38 ± 0.03 ^{b}	0.29 ± 0.01 ^{a}	0.43 ± 0.01 ^{c}
Caffeic Acid (mg/100g d.m.)	0.39 ± 0.05 ^{a}	0.63 ± 0.06 ^{a}	1.12 ± 0.41 ^{b}	0.58 ± 0.04 ^{a}
Ferulic Acid (mg/100g d.m.)	1.60 ± 0.14 ^{ab}	1.93 ± 0.58 ^{b}	1.28 ± 0.12 ^{a}	1.88 ± 0.37 ^{b}
4-Vinylguaiacol (mg/100g d.m.)	6.61 ± 1.74 ^{c}	4.86 ± 0.89 ^{b}	5.76 ± 0.19 ^{bc}	3.06 ± 0.47 ^{a}
Melanoidins (Abs 420nm)	0.32 ± 0.04 ^{a}	0.48 ± 0.03 ^{b}	0.54 ± 0.02 ^{c}	0.62 ± 0.02 ^{d}

All values are shown as means ± standard deviations. In each row, different superscripts indicate significant difference ($p < 0.05$) among coffee samples. d.m.: dry matter.

Results of seventeen identified and quantified key odorants are shown in Table 2. The presence of aldehydes and ketones is, in most of cases, negatively affected by torrefacto roasting process in the line with Sanz et al. (2002). The increase in 2-ethyl-6-methylpyrazine with torrefacto roast suggests that pyrazines formation in roasting process is favoured by sugar addition. Moreover Barcarolo et al. (1996) presented pyrazines as chemical products of the MR, which is positively affected by sugar addition.

Relating to the study about antioxidant capacity of coffee samples affected by torrefacto roasted, only high significant ($p < 0.01$) and excellent ($r > 0.75$) correlations between both antioxidant capacity parameters (DPPH and potential redox) and melanoidins (0.876 and -0.836, respectively) and trigonelline (0.889 and -0.797) have been found. Moreover, 5-CQA (0.633 and –0.573), hexanal (0.471 and –0.875) and caffeine (0.414 and –0.687) showed significant ($p < 0.05$) but moderate correlation.

Furthermore, Principal Component Analysis allowed separation of torrefacto roast blends by PC1 (58.8%) characterised by antioxidant capacity and their related coffee chemical compounds. In fact, the left half graphic includes both, the torrefacto coffee samples and the antioxidant coffee compounds reported by several authors (Somoza et al., 2003; Fujioka and Shibamoto, 2006; Shibamoto, 1980; Azam et al., 2003; Delgado-Andrade and Morales, 2005). Taking into account correlation analysis, Maillard reactions and caramelization products such as melanoidins are the main responsible for the strong antioxidant properties of coffee. However, other coffee compounds such as 5-CQA, hexanal, caffeine and mainly trigonelline may contribute to the high antioxidant capacity to torrefacto coffee. On the other hand, the right half graphic includes both the conventional coffee samples and the majority of volatile compounds. Thus torrefacto coffee had a higher antioxidant capacity than conventional one, but was less aromatic.

Table 2. Chromatographic areas (x10^{-6}) of some key odorants in ground roasted coffee samples[1].

KI[2]	Rel[3]	Compound	0T (n=9)	30T (n=6)	50T (n=6)	100T(n=6)
635	B	Methanethiole	0.02 ± 0.03^{a}	n.d.	n.d.	0.63 ± 0.43^{b}
645	A	Acetaldehyde	4.39 ± 0.48^{c}	2.73 ± 0.95^{b}	2.17 ± 0.37^{b}	1.06 ± 0.61^{a}
712	A	Propanal	5.17 ± 0.80^{c}	2.43 ± 0.43^{b}	2.36 ± 0.30^{b}	1.44 ± 0.67^{a}
747	A	2-Methylpropanal	16.03 ± 2.40^{b}	7.15 ± 1.09^{a}	7.62 ± 1.48^{a}	13.00 ± 7.93^{b}
839	B	Butanal	0.45 ± 0.13^{a}	0.19 ± 0.12^{a}	0.18 ± 0.04^{a}	0.44 ± 0.46^{a}
880	B	2-Methylbutanal	15.59 ± 2.48^{b}	8.34 ± 1.21^{a}	9.85 ± 0.35^{a}	10.33 ± 5.66^{a}
884	A	3-Methylbutanal	20.47 ± 3.22^{b}	10.72 ± 1.09^{a}	10.82 ± 2.54^{a}	11.21 ± 7.12^{a}
962	A	2,3-Butanedione	6.44 ± 1.09^{b}	3.39 ± 0.51^{a}	2.83 ± 0.51^{a}	2.33 ± 1.51^{a}
1058	A	2,3-Pentanedione	7.37 ± 1.03^{b}	4.12 ± 0.66^{a}	3.10 ± 0.38^{a}	3.27 ± 1.72^{a}
1084	A	Hexanal	0.10 ± 0.02^{a}	0.11 ± 0.02^{a}	0.14 ± 0.02^{a}	0.39 ± 0.22^{b}
1252	B	Furfurylmethylether	0.25 ± 0.03^{a}	0.21 ± 0.04^{a}	0.19 ± 0.03^{a}	0.19 ± 0.12^{a}
1283	B	3(2H)-Furanone, dihydro-2-methyl	1.60 ± 0.53^{ab}	1.85 ± 0.61^{b}	1.09 ± 0.12^{a}	1.17 ± 0.26^{a}
1359	B	2-Ethylpyrazine	1.00 ± 0.22^{a}	0.69 ± 0.06^{a}	0.74 ± 0.12^{a}	0.82 ± 0.70^{a}
1411	B	2-Ethyl-6-methylpyrazine	0.18 ± 0.05^{a}	0.11 ± 0.01^{a}	0.13 ± 0.03^{a}	1.23 ± 0.76^{b}
1432	B	2-Ethyl-3-methylpyrazine	0.21 ± 0.03^{a}	0.20 ± 0.01^{a}	0.19 ± 0.02^{a}	0.15 ± 0.14^{a}
1516	B	2-Furfurylmethylsulfide	0.16 ± 0.09^{a}	0.09 ± 0.01^{a}	0.11 ± 0.02^{a}	0.14 ± 0.13^{a}
1536	B	2-Acetylfuran	0.47 ± 0.10^{a}	0.42 ± 0.13^{a}	0.38 ± 0.05^{a}	0.42 ± 0.25^{a}

[1]All values are shown as means ± standard deviations. In each row different superscripts indicate significant difference ($p < 0.05$) among coffee samples. [2] Retention Index calculated on HP-Wax capillary column. [3] Identification proposals is indicated by the following: A, mass spectrum agreed with standards injected in the same conditions; B, tentative identification by comparing mass spectrum with Wiley mass spectral database and retention indices with literature data. n.d. not detected.

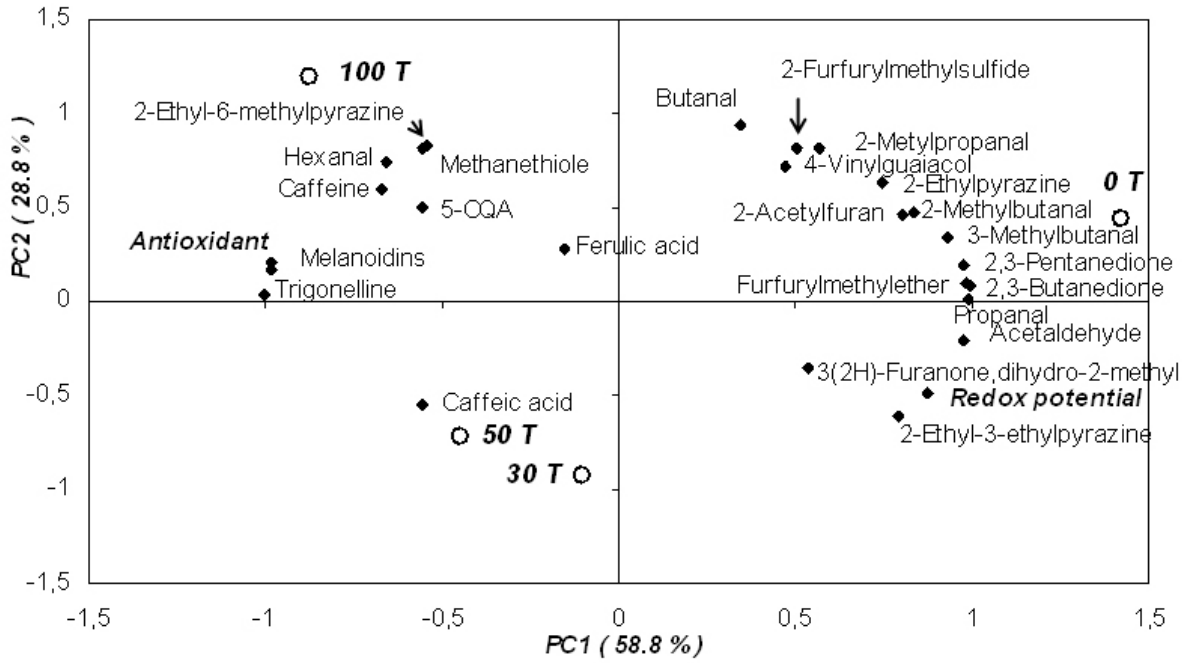

Figure 1. Principal Component Analysis of ground roasted coffee.

CONCLUSIONS

In conclusion antioxidant capacity of coffee could be mainly explained by the Maillard reaction and caramelization products enhanced by the addition of sugar in roasting process. However, there are other compounds such as trigonelline that may contribute to the antioxidant capacity of coffee.

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Determination of Radical Scavenging Capacities in Differently Roasted Coffees by On-line Combination of High Performance Liquid Chromatography with ABTS$^{\bullet+}$-Decolorization

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SUMMARY

The ABTS$^{\bullet+}$ stable radical bleaching assay has been combined with HPLC for on-line separation and elucidation of radical scavenging activities. The method is suitable for the identification and quantitative determination of radical scavengers in coffee. Results clearly show that *roasting degree* and *roasting speed* influence the radical scavenging properties of coffee brews. The *roasting method* (*drum* vs. *rotating fluidized bed roasting*) has no effect at set degree of roast.

INTRODUCTION AND OBJECTIVES

The status of knowledge on antioxidant components in roasted coffee was recently reviewed by Hofmann and Somoza (2005). Procedures for the identification of antioxidative compounds in complex food matrices are usually based on time-consuming and laborious fractionation procedures after chromatographic separation.

We have designed a reversed-phase HPLC method with on-line post-column ABTS$^{\bullet+}$ bleaching assay for fast and automated identification of radical scavenging capacities (Zapp and Moretti, 2005). The analytical concept follows a procedure first described by Koleva et al. (2001).

The objective of this paper is to demonstrate the feasibility of HPLC-ABTS$^{\bullet+}$ for the elucidation of antioxidant properties (radical scavenging capacities) of coffee beverages. Furthermore, the impact of the (commercial-scale) roasting process on coffee antioxidants is investigated. In particular, roast degree, roast method (*drum roaster* vs. *rotating fluidized bed roaster*), and roasting speed (*fast/high yield roasting vs. conventional roasting*) are studied.

EXPERIMENTAL

Instrumentation

The instrumental set-up for HPLC analysis with on-line post-column ABTS$^{\bullet+}$-derivatization is shown by Figure 1. After HPLC separation the reagent solution which contains the stable blue-coloured ABTS$^{\bullet+}$-radical ion is mixed with the eluate from the column. Selectively, compounds with radical scavenging properties decolorize the ABTS$^{\bullet+}$ and thereby develop negative peaks (indirect detection). In the simplified *flow injection analysis* mode (FIA-ABTS$^{\bullet+}$) the same instrumentation is used without separation column and HPLC detector. Trolox, a synthetic vitamin E analogue, is used for calibration. Radical scavenging capacities of coffee samples are calculated from peak areas and expressed in terms of Trolox-equivalent (TE) concentrations in µmol/L, or as TE-yield related to the mass of coffee powder in µmol/g.

Sample Preparation

Roasted coffee beverages was ground to a medium/fine grind size (X_{50} ca. 700 µm) and brewed with a household coffee machine (Krups Aroma Pro; Melitta filter paper). The coffee-to-water ratio was 50 g/L. The coffee samples were diluted with deionized water prior to analysis.

Roasting

Brazil and Colombian *Arabicas* were roasted on a Gothot "Ideal Rapid", drum roaster (Gothot GmbH, Mühlheim, Germany). Alternatively a RFB-30 rotating fluidized bed roaster (Neuhaus-Neotec, Ganderkersee, Germany) was used. Batch sizes were usually 30 kg of green coffee beans.

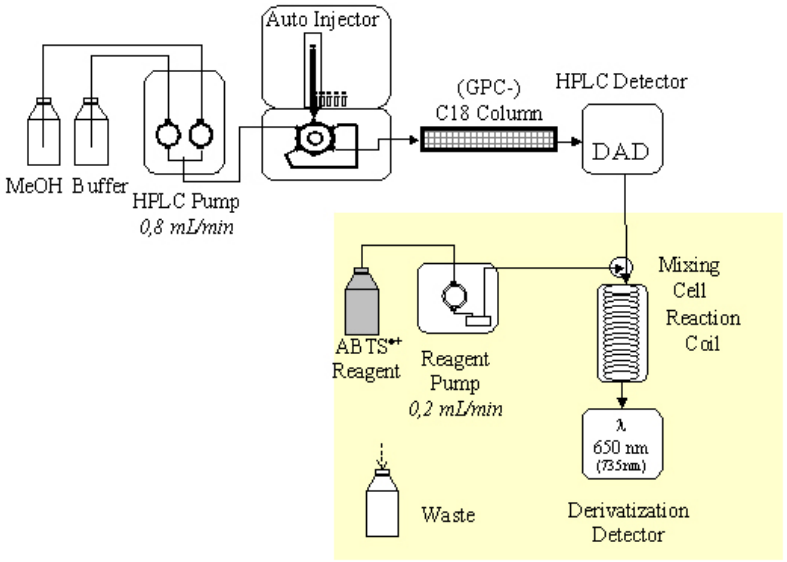


Figure 1. Instrumental set-up for on-line HPLC- $ABTS^{\bullet+}$- analysis.

ON-LINE IDENTIFICATION OF RADICAL SCAVENGING PROPERTIES

Figure 2 shows chromatograms obtained by on-line HPLC-$ABTS^{\bullet+}$-analysis of a medium roasted coffee (Honduras *Arabica*). Non-antioxidative substances like caffeine and trigonelline are not visible in the post-column bleaching profile at 650 nm!

ROAST DEGREE

In tendency, more intense roasting results in a decline in radical scavenging capacities in the initial phase of roasting (Figure 3, left). Differences between "light", "medium" and "dark" roast extent are fairly small, however. TE values tend to increase again when coffees were over-roasted towards a burnt-black colour.

The relationship between radical scavenging activity and roast degree cannot entirely be explained with the progressive degradation of chlorogenic acids during roasting (Figure 3, right). It can be hypothesized that the destruction and/or transformation of chlorogenic acids results in products which are effective antioxidants (Bonnländer et al., in press).

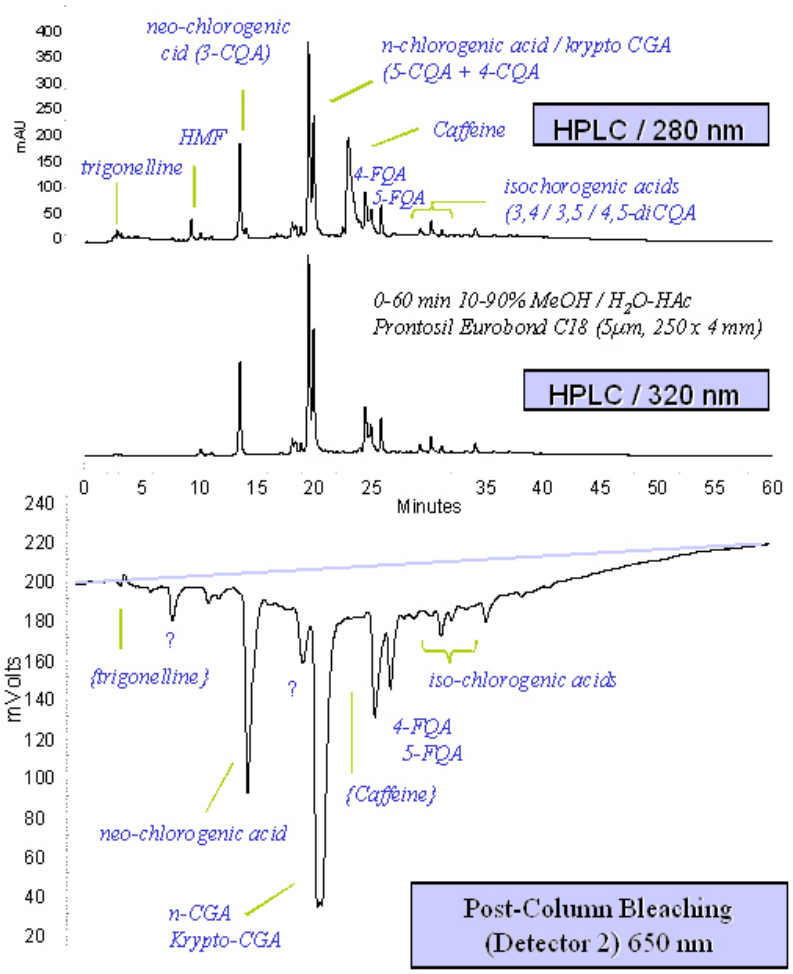


Figure 2. Analysis of a medium-roasted Arabica coffee (here: Honduras).

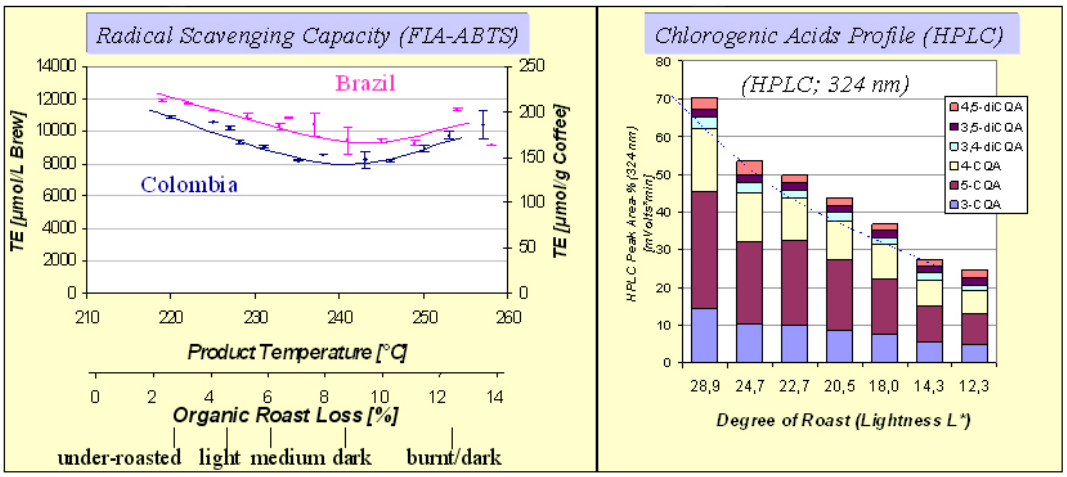


Figure 3. Effect of roasting degree on overall radical scavenging capacities of coffee brews (roasting method: RFB / water quench).

ROASTING PRINCIPLE: DRUM- VS. RFB-ROASTING

Two degrees of roast ("medium/light" and "espresso/dark") were produced from Colombian and Brazil green coffees using a drum and a RFB roaster. Experimental conditions as well as results from basic physical, chemical and sensory analysis are given by Tables 1 and 2.

Table 1. Roasting Profiles (Roaster Comparison Study).

Sample description	Roaster type	Batch size [kg]	Quenching	Approx. hot air temperature [°C]	Approx. product end temperature [°C]	Roasting time [s]
Colombia "*medium/light*"	Drum	15	Air	200	196	450
	RFB	35	Air	380/350/330	215	430
Colombia "*Espresso/dark*"	Drum	30	Air	210	202	500
	RFB	35	Air	360/340	229	490
Brazil "*medium/light*"	Drum	30	Air	210	189	430
	RFB	30	Air	360/330	221	360
Brazil "*Espresso/dark*	Drum	30	Air	303	202	530
	RFB	30	Air	370/340/300	232	390

Table 2. Results of Basic Physical, Chemistry, and Sensory Analysis (Roaster Comparison Study).

Bean origin	Roast degree	Color (CTn-units)	Roasted whole bean density[3] [kg/L]		Organic roast loss [%]		pH (TA)[1] [mmol/100g i.d.m.]		Extractable solids[1] [%]		Sensory analysis[2]
		Drum/R FB	Drum	RFB	Drum	RFB	Drum	RFB	Drum	RFB	Drum vs. RFB
Colombia	***"medium/light"***	115±1	0,674	0,680	6,30	5,12	4,95 (20,1)	4,95 (20,4)	25,02	25,30	No difference
	"Espresso/dark"	81±1	300,607	0,594	8,41	7,74	5,25 (15,5)	5,20 (18,1)	25,80	26,10	No difference
Brazil"	***"medium/light"***	115±1	0,597		7,67	6,89	5,15 (17,9)	5,15 (18,1)	25,90	26,10	No difference
	"Espresso/dark"	87±0	0,594	0,586	9,35	8,79	5,45 (13,8)	5,35 (14,8)	25,70	26,70	No difference

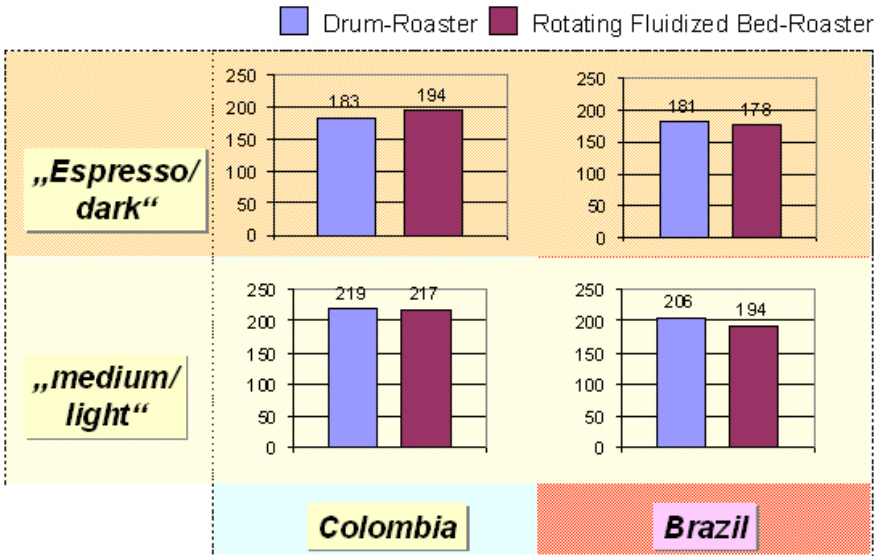
[1]Household filter brewer Krupps AromaPro, Melitta filter-paper, medium-fine grind size, 50 g/L; [2]Taste panel evaluation (triangle test); N > 37; 3Green Coffee densities are 1,27 kg/L for Brazil and 1,36 kg/L for Colombians.

In order to compare between the two roasting methods, samples from the same origin which were roasted to the same degree were submitted to a sensory evaluation by a student taste panel. The result of triangle tests was that no significant difference between the roasting methods was perceivable.

However, organic roasting losses were lower when RFB-roasting was applied (Table 2).

The roasting method did not affect the radical scavenging capacity of the corresponding coffee brews (Figure 4).

In accordance with results discussed above, the radical scavenging properties were higher at lighter roast colours.



TE in [µgmol/g].

Figure 4. Effect of the roasting method on radical scavenging capacities of coffee brews at set degree of roast.

RFB ROAST SPEED

Hot air temperatures o and/or green coffee batch sizes were systematically varied in order to produce samples of the same degree of roast in different *roasting times*. Two series were made from Brazil beans corresponding to a light and dark degree of roast.

As expected, extraction yields were higher in case of shorter roasting times (Figure 5, right). The same tendency was observed with look at the results from FIA-ABTS$^{\bullet+}$-measurements of brew radical scavenging activities (Figure 5, left). Shorter roasting times preserved higher loads of radical scavengers in the analysed coffee brews.

In contradiction to the extraction yields, which were higher for darker roasts at a given roasting time, the TE-levels are noticeably lower at darker roast degree.

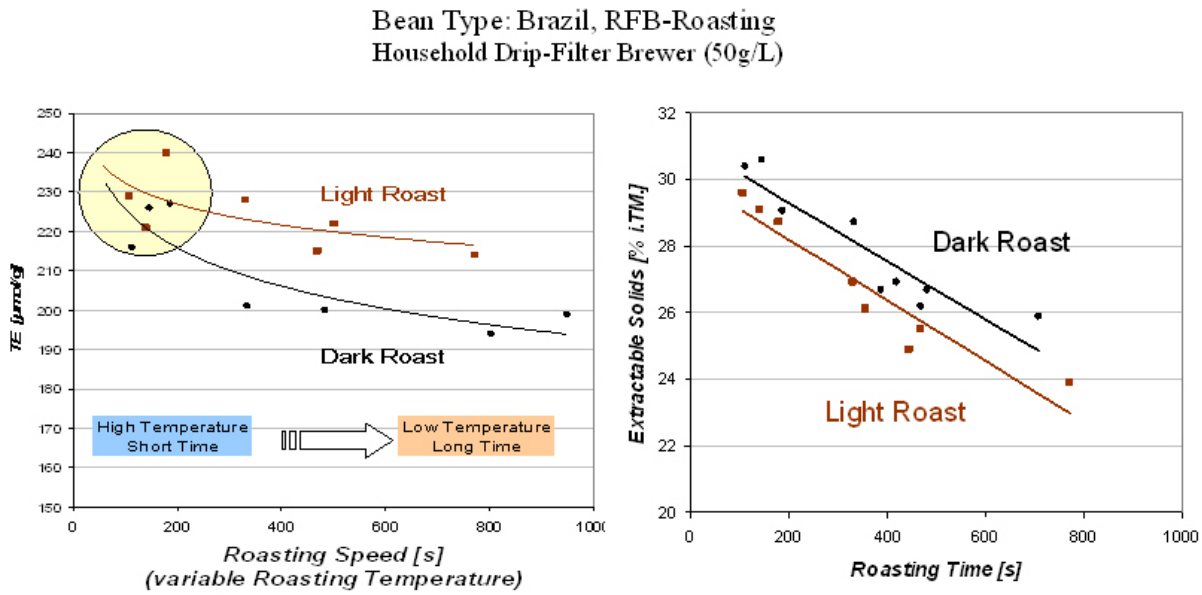


Figure 5. Effect of roasting speed on extraction yield (right) and radical scavenging capacity (left) at two degrees of roast.

CONCLUSIONS

HPLC with post-column $ABTS^{\bullet+}$-derivatization is a suitable tool for the identification and quantification of antioxidants in coffee. Chlorogenic acids and related hydroxycinnamic acids are among the key players for antioxidant activities of coffee beverages. The process of roasting resulted in a significant decline of this class of compounds. To a lesser extent, total radical scavenging capacities decrease from "light" over "medium" to "dark" roast colours. There is experimental evidence that thermal degradation products of hydroxycinnamic acids are antioxidants (Bonnländer et al., in press).

Only minor differences between *drum-* and *RFB-roasting* were observed with regard to sensory characteristics and scavenging activities towards the $ABTS^{\bullet+}$-radical. However, organic roast losses were lower for RFB-roasting.

At set degree of roast the *roasting speed* has some influence on total radical scavenging capacities. Very fast roasting results in higher antioxidant capacities. This finding can be explained mostly by better extractabilities (yield) of the fast roasted coffees.

ACKNOWLEDGEMENT

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Profile and Levels of Bioactive Amines in Regular, Decaffeinated and Organic Instant Coffee

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SUMMARY

The levels of ten free bioactive amines, pH and color characteristics were determined in different types of instant coffee. The amines were extracted with TCA and quantified by ion-pair HPLC, post-column derivatization with OPA and fluorimetric detection. The pH varied from 4.86 to 5.15, with higher levels in decaffeinated coffee. The color characteristics varied among types of instant coffee. Nine amines were detected (except tryptamine). Tyramine was present in every sample, followed by cadaverine and serotonin. Total amine levels varied from 0.28 to 2.76 mg/100 g. Serotonin was the predominant amine followed by cadaverine, tyramine and spermidine. Significantly higher tyramine levels were found in decaffeinated coffee and higher cadaverine levels were detected in decaffeinated and organic coffee. Investigations are needed to ascertain the impact of these amines on coffee flavor and on human health, and to determine the factors which affect amine formation and accumulation in instant coffee.

INTRODUCTION

Coffee is one of the most popular and widely consumed beverages throughout the world due to its pleasant taste and aroma and to its stimulant effect. Furthermore, recently, a number of beneficial health properties have been attributed to coffee (Farah et al., 2006). Instant coffee has become popular because of the ease and speed of preparation and long shelf life (Nogueira and Trugo, 2003).

In recent years, the number of studies on the composition of coffee has increased significantly, mainly phenolic acids and nitrogenous compounds such as caffeine and trigonelline. However few studies were undertaken on bioactive amines, which play important roles in plant development and also in human health. In plants, the amines are required for growth, control of intracellular pH, response to stress, and defense responses to pathogens, insects and predators (Glória, 2005; Kalac and Krausová, 2005). The profile and levels of bioactive amines in a product can be used as a quality index, reflecting the quality of the raw materials or the hygienic conditions prevalent during processing. The levels of bioactive amines in food can affect its sensory properties. With regard to human health, some amines are required for normal development and growth, response to stress, inhibition of lipid peroxidation, stabilization of membranes, maturation of the gastrointestinal tract, whereas others are vasoactive or psychoactive (Glória, 2005; Wang et al., 1975).

Green coffee was reported to contain mainly putrescine, spermidine and spermine. Putrescine was the prevalent amine followed by spermidine and spermine. The presence of other amines was also reported in green coffee, among them serotonin, tyramine, histamine and cadaverine. Serotonin was found at high levels whereas tyramine, histamine and cadaverine were present at low levels (Cirilo et al., 2003; Casal et al., 2004; Vasconcelos et al., 2006). The levels of

putrescine were lower and of tyramine were higher in *Coffea canephora* var. robusta compared to *Coffea arabica* L. (Casal et al., 2004). Studies undertaken by Oliveira et al. (2005) indicated that coffees of low cup quality (Rio) contained significantly higher levels of putrescine compared to high cup quality (Soft). Furthermore, histamine and tryptamine were also detected in coffees of low cup quality. Vasconcelos et al. (2006) detected uncommon amines in defective coffee beans: histamine in black, immature and sour beans; tryptamine in immature and sour beans; and cadaverine in black beans.

During roasting of coffee, there is a significant decrease on the levels of amines. The higher the temperature or the time used during roasting the lower the levels, up to a point in which no amines are detected (Cirilo et al., 2003; Casal et al., 2004; Amorim et al., 1977). Furthermore, during roasting of coffee for 12 min at 300 ºC, Cirilo et al. (2003) detected the formation of agmatine. It was suggested that the formation of this amine was possible by decarboxylation of arginine or by the release of this amine from conjugated forms (Cirilo et al., 2003; Casal et al., 2004).

No information was found on the levels of bioactive amines in instant coffee. Therefore, due to the scarcity of data and to the relevance of these compounds to food quality and human health, the objective of this study was to investigate the profile and levels of bioactive amines in instant coffee.

MATERIAL AND METHODS

Sixty-eight samples of instant coffee were purchased at grocery stores in Belo Horizonte, MG, Brazil (July 2002 until December 2003). The samples comprised three types (regular, decaffeinated and organic), 12 brands and five different lots from each brand. The samples were analyzed for the profile and levels of bioactive amines, pH and CIE L a* b* color characteristics (Cirilo et al., 2003; Borges et al., 2002). The amines were extracted with 5% trichloroacetic acid and determined by ion-pair HPLC, post-column derivation with *o*-phthalaldehyde and fluorimetric detection (Cirilo et al., 2003). The data were submitted to analysis of variance and the means were compared by the Duncan test at 5% probability.

RESULTS AND DISCUSSION

pH and color characteristics

Table 1. pH values and CIE L*a*b* color characteristics of the different types of instant coffee.

Characteristics	Mean value ± sd / Type of instant coffee		
	Regular	Decaffeinated	Organic
pH			
	5.05 ± 0.07 b	5.15 ± 0.14 a	4.86 ± 0.04 c
Color characteristics			
L*	14.61 ± 2.20 c	20.61 ± 4.69 b	33.48 ± 3.02 a
a*	6.61 ± 2.03 b	7.91 ± 3.63 b	11.53 ± 4.01 a
b*	3.73 ± 2.33 c	12.56 ± 8.03 b	33.51 ± 6.73 a
chroma*	9.01 ± 5.15 c	15.35 ± 8.00 b	36.24 ± 3.99 a
Hue angle º	32.89 ± 17.47 b	54.03 ± 15.02 a	67.15 ± 9.28 a

Mean values with different letters in the same line are significantly different (Duncan test, $p \leq 0.05$).

The pH and the color characteristics of the samples are indicated on Table 1. Lower pH values were found in organic coffee, compared to regular, which was lower than the decaffeinated coffee. Overall, higher color characteristics were observed for organic instant coffee, followed by decaffeinated, and by regular coffee.

Levels of free bioactive amines

Nine of the ten amines investigated were detected in the samples, except for tryptamine. Tyramine was the present in 100% of the samples, followed by cadaverine (88%), serotonin (72%), spermidine (65%), phenylethylamine (57%), putrescine and histamine (54%). Agmatine and spermine were only detected in 29 and 18% of the samples.

The levels of amines detected in the coffee samples are indicated in Figure 1. Total amine levels varied from 0.28 to 2.76 mg/100 g. The amines found at higher levels were serotonin, cadaverine, tyramine and spermidine. Higher cadaverine levels were detected in decaffeinated and organic instant coffee, whereas higher tyramine levels were detected in decaffeinated samples.

No information was found in the literature on bioactive amines in instant coffee. However, it should contain the same types of amines found in roasted coffee. Roasted coffee contains mainly serotonin, which seems to be the most resistant to the roasting process. Other amines like putrescine, spermidine and spermine could be detected in light roasts, whereas when submitted to a dark roast, agmatine could be formed (Cirilo et al., 2003; Casal et al., 2004; Amorim et al., 1977).

The levels of serotonin, spermidine and putrescine were similar to those found in roasted coffee. However cadaverine, tyramine, histamine and phenylethylamine, which were not present in roasted coffee, were found at significant levels.

According to Nogueira and Trugo (2003), the composition of instant coffee can be affected during processing and also by the species and varieties of coffee used in the blends. Therefore, the presence of different types of amines in instant coffee could indicate that they were introduced during its processing.

The amines could have been formed during the extraction, concentration or dehydration processes. Furthermore, they could have been present in the roasted coffee at the conjugated form which was hydrolyzed during instant coffee processing. Robusta green coffee was observed to contain higher tyramine levels; therefore, the use of this coffee variety at a higher proportion in the blend could provide a coffee with higher tyramine levels (Casal et al., 2004). Studies are necessary to ascertain the factors affecting the formation and accumulation of these amines in instant coffee.

Considering an intake of 5 cups of the beverage with the highest levels of tyramine and serotonin, the ingestion of these amines would be respectively 24.5 and 39.0 μg daily. According to Glória (2005), high levels of tyramine (10 and 6 mg/100 g of food) can be hazardous to the health of normal and individuals under monoaminoxidase inhibitor drugs, respectively. Therefore, the levels of tyramine in the coffee samples would not represent any risk to consumers. No information was found regarding serotonin, however the presence of this amine in coffee could be interesting since it has physiological roles in humans as a neurological mediator (Casal et al., 2004).

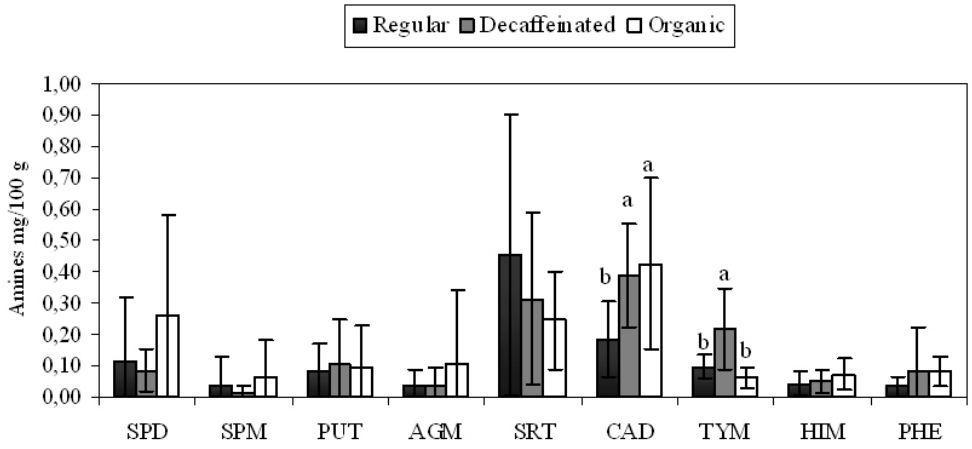

Figure 1. Levels of free bioactive amines in different types of instant coffee (SPD = spermidine; SPM = spermine; PUT = putrescine, AGM = agmatine; SRT = serotonin; CAD = cadaverine; TYM = tyramine; HIM = histamine; PHE = phenylethylamine. Mean values for each amine with different letters are significantly different (Duncan test, $p \leq 0.05$).

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Free and Conjugated Biogenic Amines in Green and Roasted Coffee Beans

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SUMMARY

Biogenic amines are present in a wide range of vegetables, either free or conjugated, but reports in coffee are rather limited and restricted to the free ones. In order to determine the composition of arabica and robusta species in terms of free and conjugated biogenic amines and to study their usefulness as discriminators for coffee variety and post-harvest processing method (dry or wet), a total of 9 arabica and 11 robusta samples from several geographical origins were analysed. The amines (putrescine, cadaverine, serotonin, tyramine, spermidine, and spermine) were determined by RP-HPLC/fluorescence after derivatization with dansyl chloride. Putrescine was the main biogenic amine present in the green beans either free or conjugated. With roasting, a significant loss in the free and conjugated biogenic amines levels was observed, especially the free ones. Free putrescine could be used in the discrimination of arabica and robusta green beans with high statistical significance. There is also some evidence that these compounds can be used for discrimination between green coffees subjected to dry and wet processing, but a higher number samples is necessary if definitive conclusions on the significance of the differences caused by the two post-harvest processes are intended.

INTRODUCTION

Biogenic amines are known to be present in a wide range of food products including fish, meat, dairy products, vegetables, fruit, nuts, chocolate, wine, and beer, being either natural endogenous constituents or formed during food processing by the action of decarboxylase-positive microorganisms (Santos, 1996). References on the biogenic amine contents of coffee are very scarce. Amorim et al. (1977) reported free polyamines contents of arabica coffee for the first time, aiming to find correlations between their levels and beverage quality. More recently, preliminary studies on free biogenic amines levels in some arabica and robusta coffee samples were carried out in our laboratory, gathering some evidence that these compounds could be used as chemical markers for the two coffee species (Casal et al., 2002). A recent work by Cirilo et al. (2003) complemented these studies on Arabica green and roasted samples. On the basis of these observations and following our works on the discrimination of coffee varieties (Casal et al., 2000; 2003) the aims of the present work were: i) to determine the composition of green coffees of arabica and robusta species in terms of free and conjugated biogenic amine and, ii) to study the usefulness of this compounds as discriminators between the most representative coffee species and, eventually, open the way for further authenticity studies for the assessment of coffee geographical origin, or even the post-harvest processing method applied to coffee beans (either wet or dry process).

MATERIAL AND METHODS

Coffee samples

A total of 30 coffee samples (11 arabicas and 19 robustas) from diversified geographical origins were analyzed, being representative of the coffees generally consumed in Portugal, usually for espresso blends. Among the arabicas, 5 samples came from Central America, and 6 from South America. As regards robusta, 16 samples came from Africa and 3 from Asia. All samples were submitted to a standard roast procedure for 15 min, beginning at about 160ºC and finishing at 220 ºC, with an industrial Probat roaster.

Two selected green coffee samples – one arabica (Brasil, dry-processed) and other robusta (Ivory Coast, dry-processed) were roasted in a laboratory oven for 15 minutes at several temperatures ranging from 140 to 220 ºC.

Sample preparation

The biogenic amines extraction was based on published methodologies (Tiburcio et al., 1985; Armas et al., 1999). Three extracts were obtained per sample, corresponding to the free, the acid-soluble conjugates and acid-insoluble conjugates. The extracts were submitted to an ion-pair clean-up method with bis-2-ethylhexylphosphate (BEHPA) and dansylated (Casal et al., 2002) before their chromatographic separation on a Jasco (Japan) liquid chromatograph equipped with a multiwavelenght diode array detector and a fluorimetric detector. A reversed-phase Tracer-Excel 120 ODSA (250 x 4 mm i.d., 5 μm) column (Teknokroma, Spain), operating at 40 ºC, with a gradient of 0.05M phosphoric acid and methanol/acetonitrile. Except for serotonine, that was quantified based on the UV reading at 254 nm, all other amines were quantified by the fluorescence signal response, using 1,7-diaminoheptane as internal standard.

RESULTS AND DISCUSSION

The composition of robusta and arabica green coffee samples, in terms of these three fractions, is presented in Table 1. It can be observed that putrescine was the most abundant amine in both species, followed by spermidine, spermine, and latter serotonine. Cadaverine and tyramine were present in very low levels. Except for spermine, all biogenic amines were present mainly in the free form. Putrescine was statistically different between the two species, both free and as acid-insoluble conjugates, with significantly higher values in the arabica samples in both cases. All other statistical differences were of reduced significance, namely the higher levels cadaverine and tyramine in robusta samples.

The levels of tyramine in robusta coffees varied substantially, although the median value was very close to the arabica's one. A closer inspection of results showed that the large variations were due to the five robusta coffees from Angola (Amboim and Ambriz), being statistically different ($p < 0.0001$) from the other robustas. This observation might be correlated with the high levels of caffeoyltyrosine, reported as a possible marker for Angolan robusta's (Correia et al., 1995).

Table 1. free and conjugated biogenic amines in Arabica and Robusta green coffee (mg/kg dw).

	Free					Acid-soluble conjugates					Acid-insoluble conjugates				
	Arabica		Robusta			Arabica		Robusta			Arabica		Robusta		
Biogenic amine	mean	SD	mean	SD	*t* -test[a]	mean	SD	mean	SD	*t* -test[a]	mean	SD	mean	SD	*t* -test[a]
Putrescine	47.9	11.4	11.1	3.6	***	3.7	2.8	3.2	2.4	ns	2.9	0.8	0.6	0.3	***
Cadaverine	0.2	0.2	0.4	0.2	*	0.3	0.2	0.4	0.3	ns	0.1	0.1	0.1	0.1	ns
Serotonine	2.5	0.7	2.1	0.8	ns	0.8	0.6	1.2	0.9	ns	nd		nd		ns
Tyramine	0.2	0.1	3.1	4.6	*	0.2	0.2	2.4	3.2	*	0.4	0.3	0.9	0.8	ns
Spermidine	9.0	3.0	8.3	2.8	ns	3.5	2.7	2.6	3.0	ns	0.5	0.2	0.4	0.2	ns
Spermine	5.5	3.3	6.0	3.8	ns	2.0	2.6	3.6	3.4	ns	5.8	1.7	5.2	1.7	ns

*Nd – not detectable; SD – Standard deviation; a * significant differences ($p < 0.05$), ** significant differences ($p < 0.01$), *** significant differences ($p < 0.001$), ns – not significant differences.*

Putrescine values were significantly higher ($p < 0.001$) in the wet-processed Arabica samples (58.6 ± 5.9 mg/kg dw) when compared to the dry-processed ones (38.9 ± 4.7 mg/kg dw) which must be correlated with the fermentation step but the material used is too small in range for any definitive conclusions on significant differences between the two post-harvest processes. When the dry processed arabicas and robusta samples are compared, the putrescine levels still statistically higher in the Arabica samples.

After a standard roast procedure (160-220 ºC, 14 min) applied to all 30 samples, all the major amines were detectable only in minor amounts, mainly in the free form, without significant statistical differences.

The selected Arabica and robusta samples roasted at several temperatures were analysed for putrescine, spermidine and spermine in the free and conjugated form. The total polyamine contents are presented in the Figure 1.

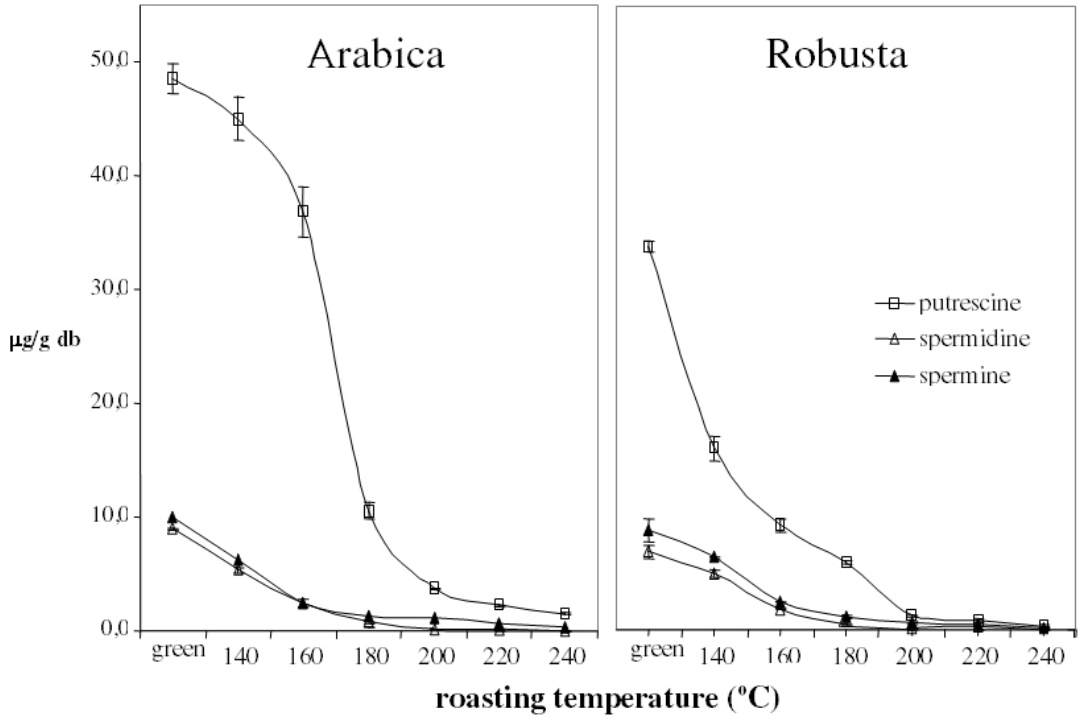

Figure 1. Total polyamine content (S+SH+RH fractions).

By comparing the total PAs amount (S + SH + RH), as represented in Figure 1, it can be observed that both samples presented a similar behavior, with almost linear reductions on all PAs until 180-200 ºC. The roasted coffees present only minor amounts of these polyamines, being the conjugated forms more resistant to the temperature effects. Nevertheless, Robusta Put began to decrease at the lower temperature tested (140 ºC), while in Arabica that observation was more significant only after 160 ºC, which can be due to intrinsic beans characteristics, both physical and chemical. The fate of all the PAs lost during coffee roasting has yet to be elucidated, but they can be volatile precursors of important aroma compounds or potential precursors of carcinogenic N-nitroso compounds.

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Determination of Acrylamide in Coffee and Coffee Products by GC-MS using an Improved SPE Clean-up

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SUMMARY

An improved GC-MS method to determine acrylamide (AA) in coffee and coffee products was developed. The method was based in two main purification steps, the first one with ethanol and Carrez solutions in order to precipitate polysaccharides and proteins, respectively, and the second with a layered Solid Phase Extraction (SPE) column which proved to be efficient to eliminate the main chromatographic interferences. The developed method is applicable to a wide range of coffee products. Thirty three different samples were analysed. The results obtained were in the range of 11.4-36.2 μg AA L^{-1} for "espresso coffee" and 200.8-229.4 μg AA L^{-1} for coffee blends with cereals. The results indicated that the presence of cereals increases remarkably the observable AA levels.

RÉSUMÉ

Une méthode améliorée de GC-MS a été développée pour déterminer l'acrylamide (AA) dans le café et des produits de café. La méthode s'est basée sur deux étapes principales de purification, la première avec de l'éthanol et les solutions de Carrez afin de précipiter des polysaccharides et protéines, respectivement, et la seconde avec une colonne superposée de l'extraction de phase solide (SPE) qui s'est avérée efficace pour éliminer les interférences chromatographiques principales. La méthode développée est applicable à une large gamme de produits de café. Trente trois échantillons différents ont été analysés. Les résultats obtenus étaient dans la gamme de 11.4-29.4 μg AA L-1 pour le «café expresso» et de 200.8-229.4 μg AA L-1 pour le mélange de café avec des céréales. Les résultats ont indiqué que la présence des céréales augmente remarquablement les niveaux observables d'AA.

INTRODUCTION

The occurrence of acrylamide (AA) in foodstuffs particularly in certain baked and fried products such as potato chips and French fries was first reported by Tareke et al. in 2002. AA is now known to be formed by the Maillard reaction during industrial food processing, retail, catering and home food preparation (Mottram et al., 2002; Stadler et al., 2002; Becalski et al., 2003). The foodstuffs that contribute most to AA exposure vary depending upon the population's nutritional habits and the way the food is prepared and processed (Dybing et al., 2005). Generally, the most important sources of AA appear to be potato products (potato chips, French fries, and potato snacks), cereals (breakfast cereals, roasted cereals), baked goods (bread, cookies, biscuits), and brewed coffee. Recent reports showed that coffee is among the highest contributors to the AA intake in some countries in Europe (Granby et al., 2004; Boon et al., 2005).

In Portugal, coffee is highly consumed as "espresso" which has a peculiar brewing technique: a small amount of hot water (± 30 mL) is percolated in a very short time at high pressure

through a layer of ground roasted coffee (± 6-7 g), the coffee cake, to produce efficiently a very concentrated brew (≈200 g of ground coffee per litre) comparing with the 20-60 g of ground coffee per litre related in other European countries (Nunes et al., 1997).

Several methods to determine AA in food have been developed and their success is very dependent of the extraction and clean-up steps. The most used procedures usually fail when dealing with coffee products, due to their inability to avoid the presence of interferences that co-elute with the analyte thus preventing its correct quantification (Delatour et al., 2004; Pittet et al., 2004; Zhang et al., 2005).

The objective of this work was to develop a method to determine AA in several coffee products using GC-MS as analytical technique. The method was successfully applied to determine acrylamide in ready to drink products and ground coffee that was analysed as "espresso" (Soares et al., 2006).

EXPERIMENTAL

Twenty four samples of roasted coffee beans, five samples of instant coffee, two samples of coffee blends with cereals, one sample of roasted cereals and one soluble "cappuccino" were analysed by the developed method.

To 10 mL of "espresso" coffee or 2 g of other coffee product dissolved in 10 mL of water, 15 mL of absolute ethanol were added. Proteins were precipitated with Carrez solutions. The SPE clean-up was performed in columns prepared by adding a 1g layer of C18 sorbent to the Isolute Multimode 3 g column. For instant coffee it was necessary to perform a previous SPE cleaning with a 3 g C18 columns home made prior to the Isolute cleaning. The collected extract was derivatized with bromine and afterwards extracted with 10 mL and 5 mL portions of ethyl acetate/n-hexane 4:1 (v/v). The volume was reduced to 0.5 mL under a gentle stream of nitrogen. The solutions were then injected in the gas-chromatograph.

RESULTS AND DISCUSSION

The addition of absolute ethanol to coffee samples intended to control the great quantity of foam that was constantly being formed during almost all steps of the sample preparation and resulting in severe losses of the analyte and contamination of the used instruments. The adopted solution was an adaptation from the work of Nunes and Coimbra (1998), where the precipitation of polymeric polysaccharides, responsible for the "foam stability" in espresso coffees, was achieved by adding 55 and 75 % ethanol solution at 4 °C during one hour.

The SPE clean-up was a challenge in the sense that it was difficult to achieve the correct ratio between the two used sorbents. The use of a single purification step with C_{18} revealed to be useful to eliminate the coffee colour but it was not effective to remove other compounds that overlapped the 2,3-DBPA peak no matter what quantity of sorbent was used. When using Isolute Multimode cartridges (0.5 or 1 g) it was verified that the sorbent layer was completely saturated with very small quantities of coffee samples (0.5-1.0 ml). Because it was difficult to achieve such small sample volumes during the evaporation step, the rejection of an important portion of the extract was necessary, with the inherent loss of sensitivity of the overall method. The solution found was to increase the quantity of sorbent material instead of decreasing the sample volume. The use of higher quantities of Isolute Multimode (3 g) resulted in an increase of the analytical signal but it was not effective to eliminate the coloured compounds and suppression of the MS response was observed. The addition of both sorbents in a single step was the solution found to surpass both problems. It was found that a

ratio of 1:3 of C_{18}: Isolute was ideal to eliminate the most relevant contaminants present in "espresso" coffee.

Soluble coffees, revealed to be a troublesome matrix due to its high percentage of polysaccharides. The addition of ethanol was not enough to eliminate the majority of the polysaccharides that increased the formation of foam and the 1:3 ratio between the sorbents was incapable of eliminate all the interferences observed in the analytical signal. Besides, these samples provoked contamination of the injector, what demanded constant changing of the liner sometimes just after few injections. The addition of a bigger top layer of C_{18} was impracticable due to the dimensions of the used cartridges. The use of a previous clean-up step with a C_{18} sorbent, in which the majority of the coloured compounds were eliminated, followed by a second clean-up step with the "C_{18} layered" Isolute column was the best solution found.

The recovery of the method was tested using an instant coffee surrogate solution (containing 200.8 μg L^{-1} of AA) prepared with 8 g of powder dissolved in 40 ml of water and divided into four aliquots of 10 ml each. AA was added at each aliquot at the levels from 0 to 2 μg corresponding to 0-200 μg L^{-1}. The AA recovery from the spiked solutions varied between 97.4 to 108.4 %.

The limit of detection (LD) and the limit of quantification (LQ) of the method were calculated from de calibration curve parameters. So, LD = 3.3 x (s/S) and LQ = 10 x (s/S) where s is the standard deviation of the intercept of the regression line and S is the slope. The results obtained were LD = 1.5 μg L^{-1} and LQ = 4.5 μg L^{-1}.

The results obtained for the 33 coffee samples analysed are presented in Table 1.

Table 1. Acrylamide levels measured in 33 coffee products. The minimum and maximum values are presented and also the mean level of AA in the different coffee products analysed.

Samples	n	AA found μg L^{-1}		
		Min	Mean	Max
"Espresso"	24	11.4	21.0	36.2
Soluble coffee	5	47.4	72.4	95.2
Coffee blends with cereals	2	200.8	215.2	229.4
100 % Roasted cereals	1	-	137.0	-
Cappuccino	1	-	6.4	-

Accordingly AA levels ranged from 11.4 to 36.2 μg L^{-1} AA in ground roasted coffee analysed as "espresso", 47.4 to 95.2 μg L^{-1} AA for instant coffee and 200.8 to 229.4 for coffee blends with cereals. Roasted cereals with no added coffee present 137.0 μg L^{-1} AA. Soluble "cappuccino" contained 6.4 μg L^{-1}. Considering that 6 g of ground coffee extracted with ~30 mL water are used to prepare an "espresso", the concentration of AA per cup is therefore 0.32 to 1.46 μg/30 mL. To prepare a cup of instant coffee the usual measure is 2 g per individual doses making 0.47 to 0.95 μg per 30 mL. Considering that the blends with cereals were prepared as soluble coffee (2 g/ 30 mL cup) the concentration of AA per cup is therefore 2.01 to 2.09 μg per 30 mL cup. "Cappuccino" as analysed is a soluble mixture of coffee, milk powder, cocoa and sugar and each individual dose correspond to 14 g, resulting in an AA concentration of 0.45 μg per 30 mL cup. These results highlight the fact that the addition of cereal products to coffee increase the amount of AA observed. This in agreement with studies

reporting that cereal products present substantial amounts of AA due to its chemical constitution and processing (Yusà et al., 2006).

CONCLUSION

GC/MS methods usually suffer from the presence of co-extractives which rapidly contaminate the chromatographic system, degrading the respective performance and avoiding the correct quantification of AA.

This manuscript describes a GC/MS method based on an improved sample preparation procedure that enables the correct quantification of AA in coffee extracts without the problems generally associated with that kind of samples. The high levels of sensitivity, and reproducibility achieved recommend it when dealing with this type of food matrices. The method was applied to several coffee products and the quantity of AA was found to be dependent on the coffee processing and preparation, namely the addition of cereals.

ACKNOWLEDGEMENTS

The authors are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004.

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Fate of ^{14}C-Acrylamide in Roasted Coffee During Storage

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SUMMARY

Acrylamide (AA), which can be formed during heating of carbohydrate rich foods depending on the amount of precursors, has carcinogenic potential. In coffee, AA is formed during roasting. Storage experiments with roasted coffee (ground or whole beans) have shown that free AA concentration decreases during storage depending on time and temperature. In this study we investigated the fate of AA during storage by tracing ^{14}C-AA, added to roasted and ground coffee. Radiolabel was measured in coffee brew, filter residue and volatiles. In the brew, total ^{14}C-label decreased during storage of roasted ground coffee, while it concomitantly increased in the filter residue. ^{14}C-AA was the only ^{14}C-related compound found in the brew. No volatile ^{14}C-AA related compounds could be detected. According to this, the decrease of the concentration of free AA in coffee is a result of AA binding to material that remains in the filter residue.

INTRODUCTION

As evidenced by Delatour et al., 2004, Hoenicke & Gatermann, 2005, and Lantz et al. (personal communication) AA contents in roasted coffee decrease during storage in a time and temperature dependent manner. The current study investigates the fate of acrylamide during storage. Roasted and ground coffee was spiked with ^{14}C-radiolabeled acrylamide and stored (at room temperature (RT) / 37 °C). Radiolabel was traced in coffee brew, filter residue and volatiles. Measurement was accomplished in intervals of a few weeks up to several months. Total radioactivity of the brew was measured by liquid scintillation counting. The brew was further investigated for potential ^{14}C-acrylamide reaction products by high performance liquid chromatography coupled with a radio flow detector. The activity bound to coffee components remaining in the filter residue was monitored by liquid scintillation counting after combustion. In addition potential formation of volatile radioactive compounds related to acrylamide was investigated. (Figure 1).

MATERIALS AND METHODS

Chemicals

[2,3-^{14}C]-AA (5 mCi/mmol, 1mCi/ml) was purchased from American Radiolabeled Chemicals.

Coffee

Roasted and ground coffee “Santos” prepared by Tchibo Manufacturing GmbH.

Analysis

Liquid scintillation counters were used for the measurement of the total radioactivity in the brew and in the filter residue after combustion (Biological Oxidizer, Zinsser). ^{14}C-AA and related compounds were analyzed by RP-HPLC coupled with a radio flow detector as described below.

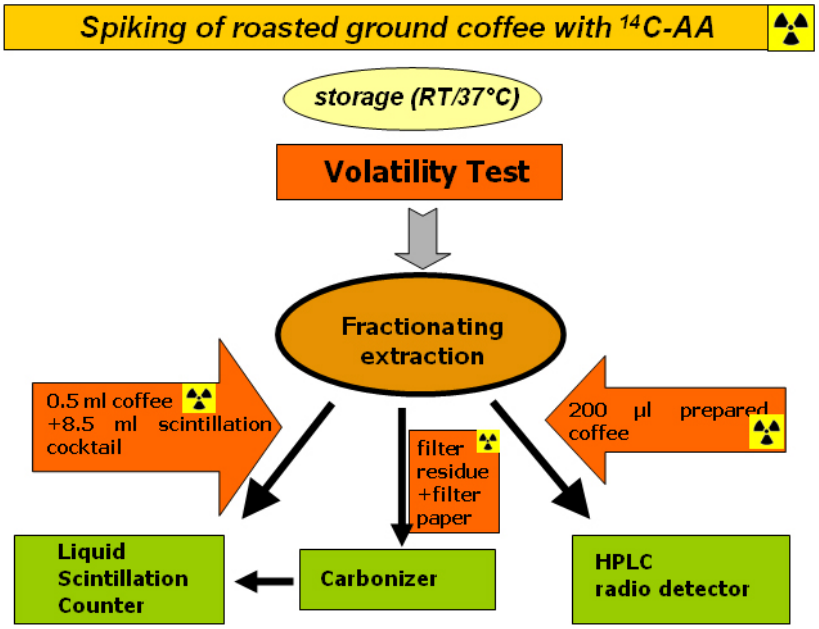

Figure 1. Concept to investigate the fate of Acrylamide in roasted coffee during storage.

Preparing of (^{14}C-)AA spiked coffee

Roasted ground coffee was slurried in a methanolic solution of (^{14}C-)AA for 10 minutes. Then the solvent was removed by rotary evaporation. The total amount of AA in 3 batches of spiked coffee (natural AA + added (^{14}C-)AA) was about 370 µg/kg ("**low**"), 830 µg/kg ("**medium**") and 6900 µg/kg ("**high**").

Volatility test

Radiolabelled components eventually volatilized during brew preparation were screened for using an all glass closed system equipped with an activated carbon filter. Radioactivity monitoring was achieved by charcoal extraction with dichloromethane and methanol and by wiping test.

Fractionating extraction

0.5 g ^{14}C-AA spiked coffee ("high" and "medium") or 1 g coffee ("low") was extracted with 15 x 1.5 ml water (90-95°C). Fractions 1, 2 and 3 were collected separately, whereas fractions 4-9 and 10-15 were pooled.

RP-HPLC

Column: Purospher STAR RP-18 end-capped (5 µm) 250-4 (Merck). Radio-flow detector: Radiomatic Flo-one®Beta Radio-Chromatography Detector Series A-500 (Packard). Mobile

phase: water/methanol 40-100%; 0-20 min. Centrifuged brew was injected after precipitation of proteins by acetone (200 µl).

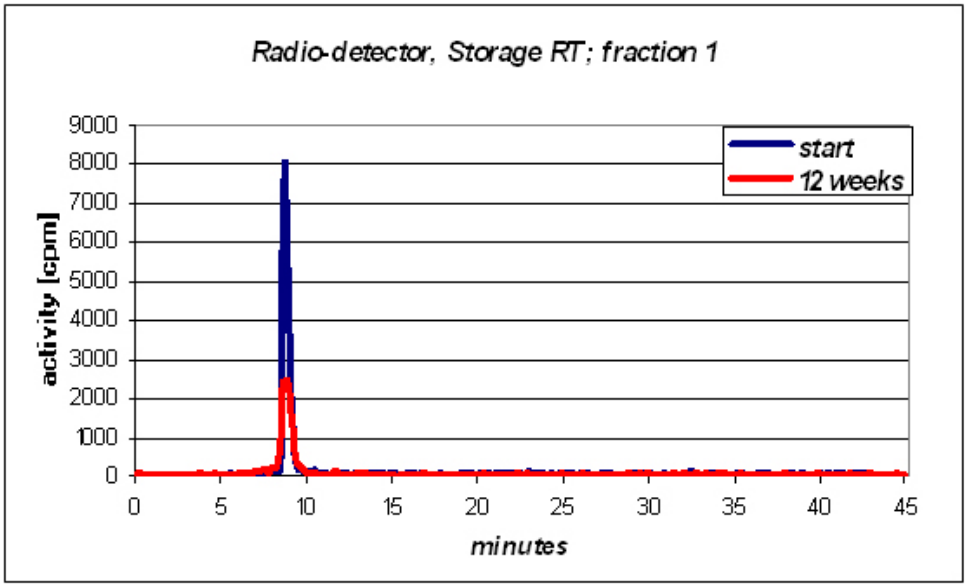

Figure 2. Radio-Chromatogram of coffee brew.

Combustion: The filter residue and filter paper were combusted in an oxidizer of the BASF AG agricultural center Limburgerhof. The formed $^{14}CO_2$ was trapped in scintillation cocktail and measured by LSC.

RESULTS

In a pilot experiment the fate of AA in coffee during 60 days storage under non-vacuum and vacuum was compared. It was found, during this time period the different storage conditions did not significantly influence the disappearance rate of AA. In another 4 weeks storage experiment with ^{14}C-AA spiked coffee we found that no volatile ^{14}C-compounds evaporated during storage at 37 °C. Furthermore no volatile radioactive material escaped during preparation of coffee brew. As evidenced by fractionating extraction of the coffee the ^{14}C extraction profile did not change during storage. .

The brew was analysed for ^{14}C-related compounds by HPLC and radiodetection. Only free ^{14}C-AA was detected. There was no indication for any other water soluble AA reaction products.

In the filter residue a time and temperature dependent build up of ^{14}C label was observed.

After 28 weeks a steady decrease of ^{14}C-AA down to 50% (37 °C) and 70% (RT) was observed in the brew, while the ^{14}C label was concomitantly build up in the filter residue. The ^{14}C distribution kinetics between brew and filter residue are apparently not influenced by the amount of ^{14}C-AA label added intially. This indicates that within the ^{14}C-AA amounts used, the potential AA reaction partners in coffee are available in excess.

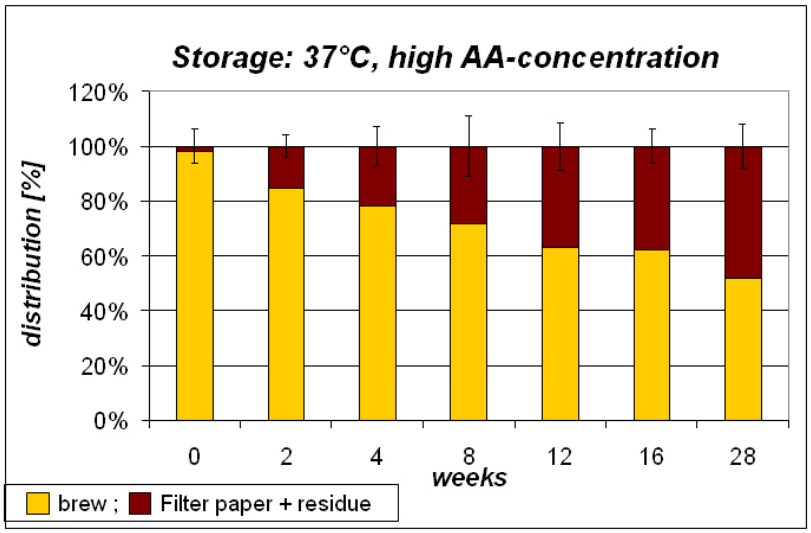

Figure 3. Distribution of total ^{14}C label between brew and filter residue at 37 °C up to 28 weeks

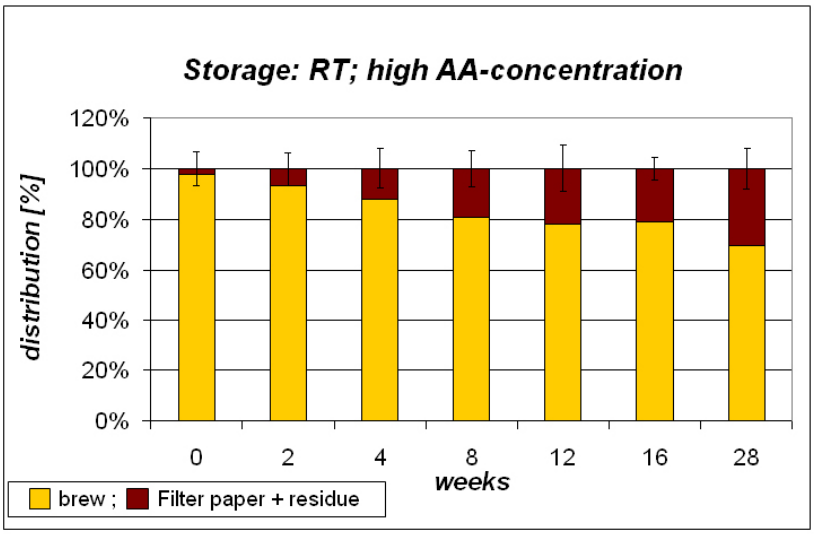

Figure 4. Distribution of total ^{14}C label between brew and filter residue at RT up to 28 weeks

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Prediction of Coffee Roasting Degree by Near Infrared Spectroscopy

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SUMMARY

A possible application of FT-NIR spectroscopy was tested in order to evaluate different roasting degrees in coffee beans. Relationships between some physico-chemical changes during coffee roasting and near infrared spectra of raw and roasted coffee were investigated in order to test the effectiveness of non-destructive NIR measurements for the prediction of roasting degree. Pure and blend samples of several Arabica and Robusta coffee varieties were roasted in a pilot plant roaster for 3, 4, 5, 5.5, 6 and 6.5 minutes and analysed for conventional roasting parameters (weight loss, colour, moisture and density) and NIR spectroscopy. Data results of conventional roasting analysis were used as reference method for the calibration of the NIR system. Using the partial least square algorithm, good statistics were obtained for the calibration of moisture, weight loss and density ($R^2 > 0.9$) with root mean square error of cross-validation ranging from 0.02 to 1.32. Furthermore, it was possible to discriminate the different roasting degrees of coffee beans by cluster analysis on the basis of the obtained NIR spectra. Validation results allowed to well predict the data obtained by conventional measurements.

RÉSUMÉ

On a testé une possible application de la spectroscopie FT-NIR afin d'évaluer les degrés differents de torréfaction du café en grains. On a étudié les relations entre certains changements physiques et chimiques provoqués après la torréfaction et les spectres NIR des échantillons de café crus et torréfiés.Tout cela a le bout d'évaluer l'efficacité des mesures pas destructives, effectuées par le FT-NIR, pour la prédiction du niveau de torréfaction. Les échantillons de cafés et de mélanges de Arabica et Robusta ont été torréfiés dans une torréfacteur-pilote pendant 3, 4, 5, 5.5, 6 et 6.5 minutes et ils ont été analysés selon les paramètres de torréfaction conventionnels (diminution de poids, couleur, humidité et densité). En plus par chacun d'eux on a été obtenu les spectres au NIR. Les résultats des mesures conventionnelles ont été utilisés comme méthode de référence pour la calibrage des spectres NIR. La validation externe effectuée avec un *partial least square* algorithme a permis de bien prédir les résultats obtenus par les mesures conventionnelles.

INTRODUCTION

The quality of coffee used for beverages is strictly related to the chemical composition of the roasted beans, which is affected by the composition of the green beans and post-harvesting processing conditions (drying, storage, roasting and grinding) (Franca et al., 2005a). Roasting is a complex process from a chemistry point of view, since hundreds of chemicals reactions take place simultaneously (Franca et al., 2005b). Several chemical descriptors were proposed as indicators of the degree of roasting. The analytical methodologies employed in the quantification of these compounds are time consuming and sometimes complex (Dutra et al.,

2001). In this way, there is a need to find simple, fast and reliable methods for tackling food authentication problems. One such technique is near infrared spectroscopy which has been shown to be useful for the rapid, non-invasive analysis of a wide range of foodstuffs (Kemsley et al., 1995).

The aim of this research is a study on a possible application of FT-NIR spectroscopy in comparison to results of some traditional physico-chemical analysis (moisture, density, colour and weight loss) in order to evaluate different roasting degrees in coffee beans.

MATERIALS AND METHODS

The dataset used in this study was composed of 105 green and different roasted coffee samples (*C. Arabica*, *C. Robusta* and industrial blends). The roasting process was performed in a pilot plant (STA impianti, Zola Predosa, Italy) for 3, 4, 5, 5.5, 6 and 6.5 minutes. After each roasting step coffee samples were analysed for the following physical and chemical roasting parameters: weight loss, density (Lerici et al., 1980), colour (L*, a*, b*) and moisture (A.O.A.C., 2000). The FT-NIR spectra were obtained in reflectance by a Bruker VECTOR 22/N spectrometer; each spectrum was recorded with a resolution of 8 cm^{-1}, averaged over 32 scans, within the wavelength range 4000-12000 cm^{-1}, with five replicates for each individual sample. An average spectrum was subsequently computed from the collected replicates. All spectra were subjected to an optimization process, in order to evaluate the best pre-treatment and spectral region in terms of root mean square error of cross validation (RMSECV). The best pre-treatment obtained was first derivative applied in the wav. range from 5446 to 6101 cm^{-1}. Qualitative discriminant analysis was carried out through cluster analysis (Mahalanobis' distances). Moreover, a quantitative analysis was carried out on all spectral data throught cross validation (CV); then an external validation (Partial Least Square Regression) was performed dividing all samples into calibration (70 samples random) and validation (35 samples random) sets. All spectroscopic data analysis were performed by a NIR dedicated software (OPUS^{TM}, version 4.2).

RESULTS AND DISCUSSION

The results obtained from traditional measurements well discriminated the green and roasted at different degrees coffee samples (Figure 1).

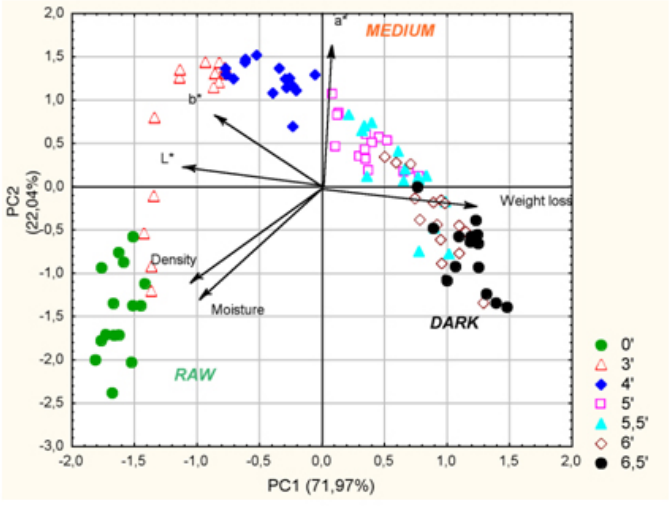


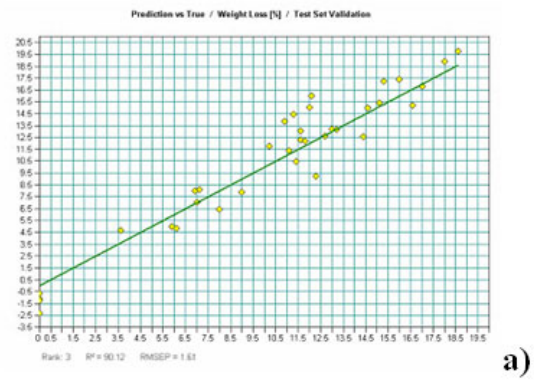
Figure 1. Principal component analysis (biplot) from 105 green and differently roasted coffee samples.

The qualitative spectroscopic analysis (cluster results – data not showed) was able to well discriminate coffee samples on the basis of roasting degree and kind of varieties.

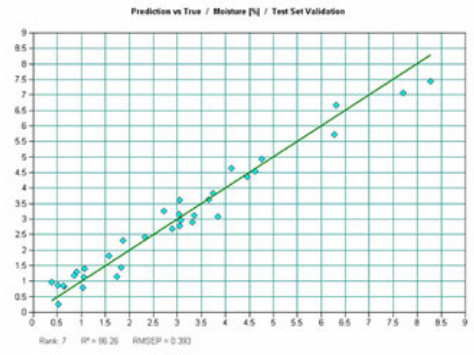
Good calibration statistics were obtained by cross validation (Table 1). Moreover, a good predictive model was tested for the prediction of weight loss, moisture and density data by external validation (PLSR – Figures 2a, b, c).

Table 1. Determinations coefficient (R^2) and statistical error of cross validation.

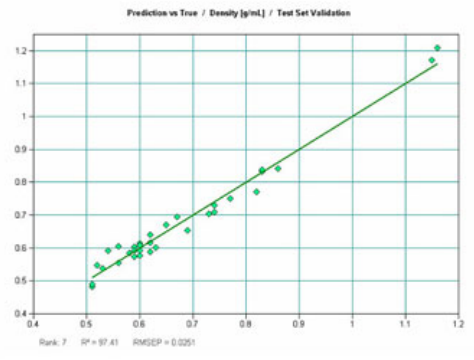
Parameter	CV (R^2)	RMSECV
Weight loss	94.70	1.32
Moisture	96.04	0.51
Density	97.81	0.03



a)



b)



c)

Figure 2. External validation results: mesured vs predicted weight loss (a), moisture (b) and density (c) data for the PLS model calculated from the roasted coffee data set.

CONCLUSIONS

The FT-NIR results may be considered reliable and comparable to those obtained from standard analysis.

The high determination coefficients obtained from calibration and validation equations allowed predicting the traditional roasting parameters.

FT-NIR could be a very powerfull technique for on-line and routine applications to predict different roasting degrees of coffee.

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Application of NIR for Caffeine Analysis Development of a Global Calibration

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SUMMARY

Near-Infrared Spectroscopy (NIRS) is a rapid analysis technique which has been widely applied in the food industry. Once calibrated, NIRS is robust and does not require skill operators for routine operation. Therefore, it is an ideal tool for QA departments when robustly calibrated versus the existing reference methods. However, development of robust calibrations often requires extensive analysis of a representative sample set, and can be the rate-limiting step in implementation. In addition, once developed, calibrations must be maintained to ensure reliable performance. In order to reduce the time required for development of NIRS calibrations for caffeine analysis in roast and ground coffee, a global calibration was developed, which can then quickly be applied at multiple locations/on multiple instruments. To assess the status in each location, a sample population of about about 50 samples (spread over the expected process and product variation and population) was analysed using the reference method and NIRS. Any outlier samples were added to the calibration, and the instrument was standardized against a reference instrument. Results indicate similar performance of the NIR analysis versus the reference test method in terms of reproducibility, precision and accuracy. The calibration will be maintained using a `centralized' approach.

CONCEPT OF CENTRALIZED NIR

Centralized NIRS seeks to deal with some of the disadvantages of NIR by minimizing the time needed to set up a calibration. It achieves this by using already-established (global) databases and extending them as necessary to suit local needs. The global database is managed and updated centrally. The following approach was used to test centralized NIR in practice. An initial calibration for caffeine containing 467 samples of roast and ground coffee was put in place. The initial master database was transferred to instruments at various Kraft sites and supplemented with local samples. This means that, during the implementation phase, when many samples are added from different plants, databases at the different sites will vary slightly. The databases are then merged to create the global or central database and calibration(s). All sites then use the same central database. The database is managed centrally.

DETAILS OF THE INITIAL MASTER DATASET

467 samples with caffeine levels ranging from 0.02-0.68 g/100g were in the database. The standard error of prediction (SEP) was approximately 0.015 (Figure 1).

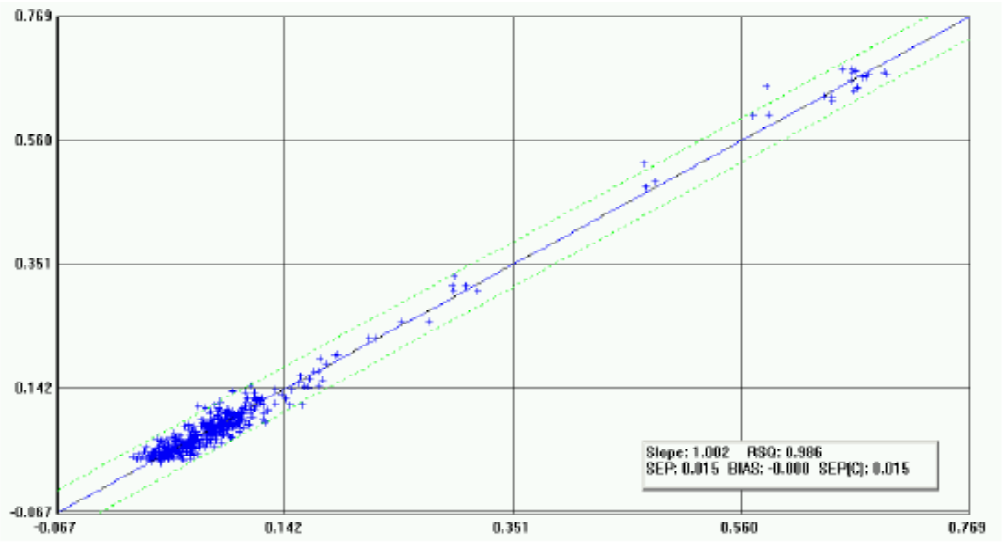

Figure 1. Master calibration for caffeine measurements in R&G coffee.

PROCESS OF TESTING CENTRALIZED NIR

The process of testing centralized NIR included:

- Selection of one test site which is using same NIR instrument as master NIR instrument
- Use of ~ 50 local samples for initial evaluation
- Scanning of all samples on the NIR instrument and analysis using reference method
- Selecting ~ 10 samples based on their spectral properties and caffeine levels to standardization the local NIR instrument with the master instrument
- Transferring master database updated with local samples
- Criteria of success: Accuracy should be comparable to master calibration (SEP = 0.015)

INITIAL EVALUATION OF ACCURACY

50 local samples were added to the database and predicted (Figure 2). The SEP for the local test site was 0.008, clearly below project target of 0.015.

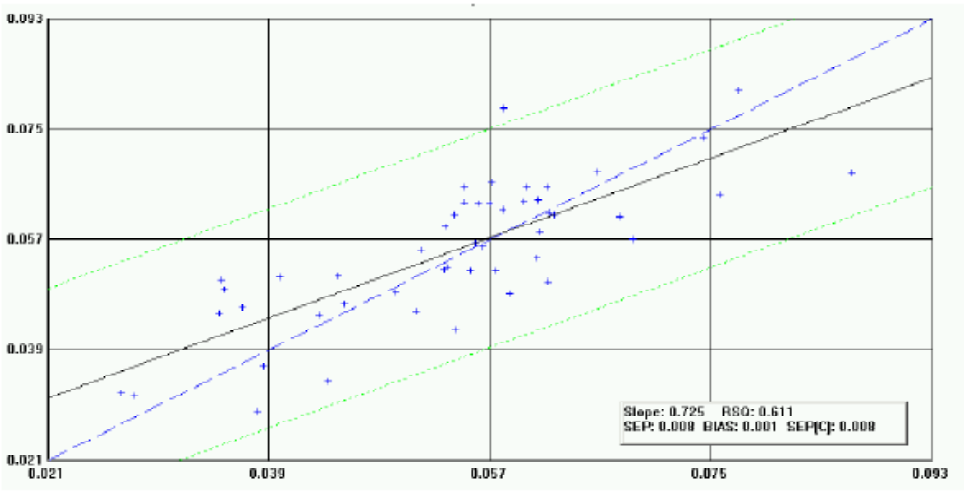

Figure 2. Updated master calibration with local samples for caffeine measurements in R&G coffee.

CONCLUSIONS

The concept of centralized NIR calibration was successfully applied to the analysis of caffeine in roast and ground coffee. Accuracy of the NIR analysis compares well to the reference method. The benefits have been a significant reduction in the data required to develop robust calibrations, leading top faster implementation.

Caffeine Determination in Single Beans by LC-MS/MS DAD

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SUMMARY

Caffeine is one of the compounds the inspiring effect of coffee is correlated to and for which coffee besides its aroma is highly appreciated (Spiller, 1998). Depending on cup size, mode of preparation and variety the average cup of coffee contains about 80 mg of caffeine, but its variability is high in different breads of coffee beans (Baumann et al., 1998). Various methods like bio-sensing, capillary electrophoresis, (near) infrared und ultraviolet spectroscopy are used for quantification. Here we like to give results on the variation of caffeine content in single green beans of commercial lots of Santos coffee by LC-DAD and LC-MS/MS comparing natural variability and method proficiency (Kolb and Hippich, 2005) using stable isotope dilution analysis (SIDA) to correct for extraction errors in small quantity of single bean material.

INTRODUCTION

Caffeine is the main methylxanthine that occurs in coffee, tea, cocoa, matè and cola beverages (Spiller, 1998). It is the most studied compound in coffee, since it has well known physiological effects. The caffeine content in Arabica and Robusta has widely been studied, and varies from approx. 3 % in Robusta species to 1.2-1.5 % in Arabica, depending on type and origin (Baumann et al., 1998). A cup of regular espresso prepared of Arabica coffee contains about 80 mg of caffeine, distributed in about 50 beans. Despite the use of various methods of analyses (UV-spectroscopy, Thin-Layer Chromatography (TLC), Capillary Electrophoresis (CE), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) as well as bio-sensing, (NIR) Near-InfraRed spectroscopy), there is little known on its content and distribution in single raw coffee beans. The biological aspects of caffeine distribution in the plant have been studied by Baumann (1998) and on last years ASIC meeting Desobgo (2004) suggested that caffeine is not evenly distributed in the bean, being more concentrated in the outer part of the bean and less in the inner, but using a not very precise method. Here we investigate the caffeine content in single beans with a LC-MS/MS DAD technique using stable isotope dilution analysis (SIDA).

EXPERIMENTAL

From a batch of Santos green coffee from Brazil, several beans of different weight were selected, varying from 110 to about 200 mg each. The beans were frozen in liquid nitrogen in order to render them more fragile, they were broken using a pistil and mortar and extracted with boiling water under addition of magnesium oxide to avoid complex formation especially with chlorogenic acids (CQA). Samples were sonicated in ultrasonic bath at 80 °C for 10 minutes, and the supernatant was analyzed after centrifugation, using a LC/MS/MS instrument in series with an diode array detector (DAD).

The analysis conditions are summarised here:

HPLC Agilent 1100

Column	*Phenomenex Luna C8 150 x 3.0 mm 3 u*
Column temperature:	*30 °C*
Detector:	*UV (272, 254 nm)*
Mobile phase:	*Acetonitrile/H2O (LCMS grade) 80/20*
Flow rate:	*0.4 ml min-1*

Applied Biosystems QTRAP Parameters ESI positive:

Curtain Gas	*(CUR)*	*35 psi*
Collision Gas	*(CAD)*	*4*
IonSpray Voltage	*(IS)*	*5500 V*
Temperature	*(TEM)*	*380 °C*
Ion Source Gas1	*(GS1)*	*15 psi*
Ion Source Gas2	*(GS1)*	*55 psi*

MRM 194.8/138.3 and 194.8/110.1
MRM 198.0/140.1 and 198.0/112.2 for C13-caffeine

RESULTS AND DISCUSSION

Results were obtained by LC-DAD and confirmed by LC-MS/MS (positive ESI) analysis. To correct for the caffeine recovery from the samples caffeine C13 was added and quantified via MRM using the transitions obtained from syringe injection in accordance to the literature (Kolb and Hippich, 2005).

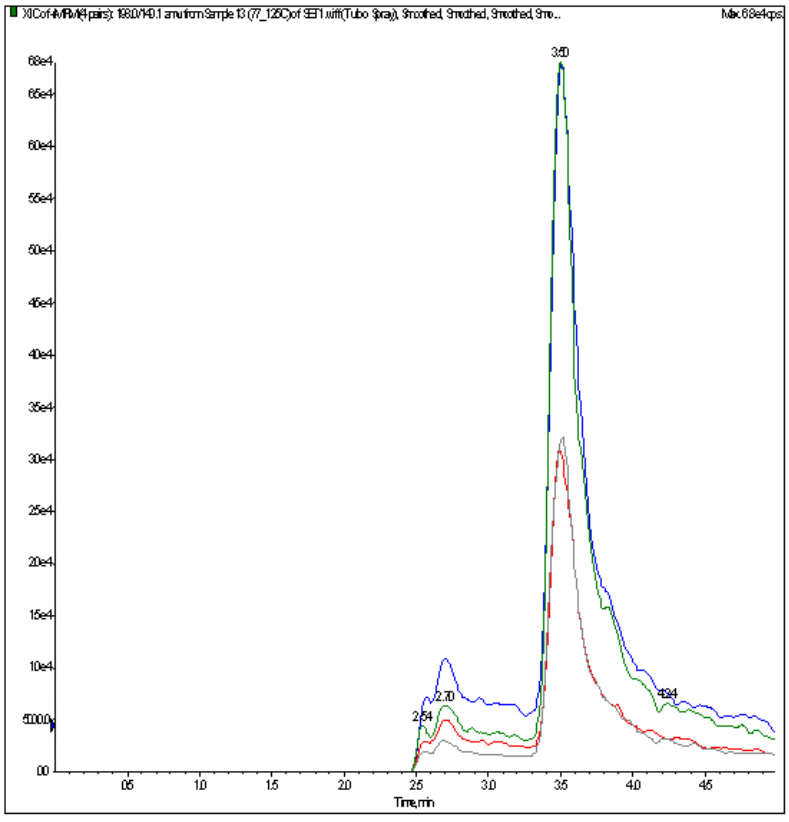

Figure 1. LC-MS ESI positive chromatogram of single bean extract spiked with 13C-caffeine.

The analysis of caffeine content of 48 single beans of green coffee from Brazil was performed. The average caffeine content (dry weight) for the lot was 1.19%. Nevertheless, the caffeine is not equally distributed in all the beans, as can be seen in Figure 2, where the content varies from 0,73 to 1.52% for most of the beans, and being the range 1.13-1.32% the most frequent interval of data.

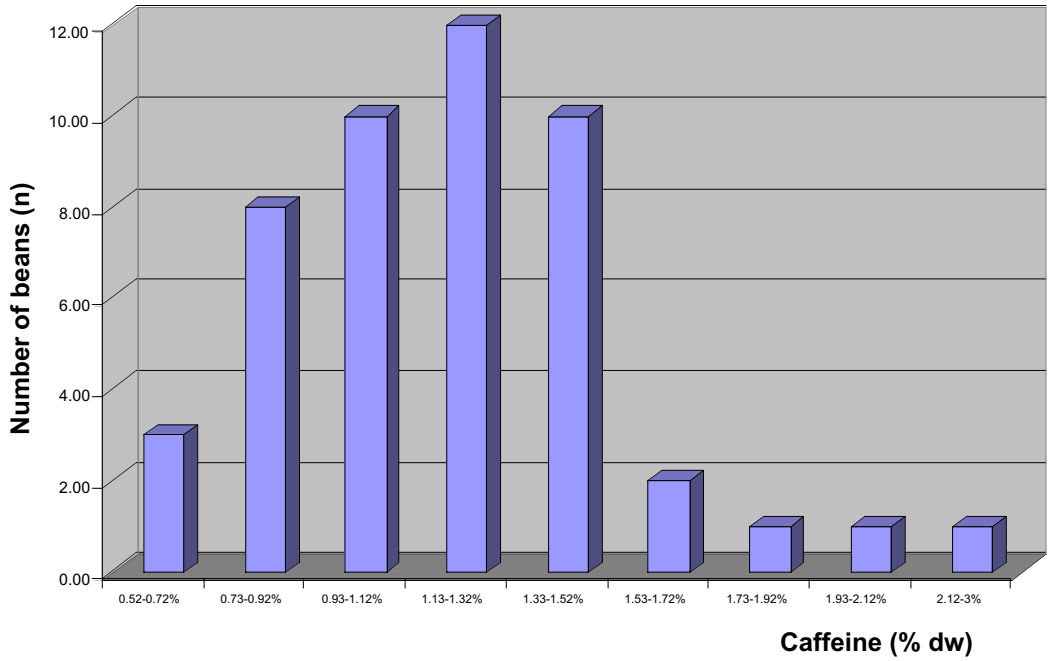

Figure 2. Distribution of caffeine in green Santos coffee beans.

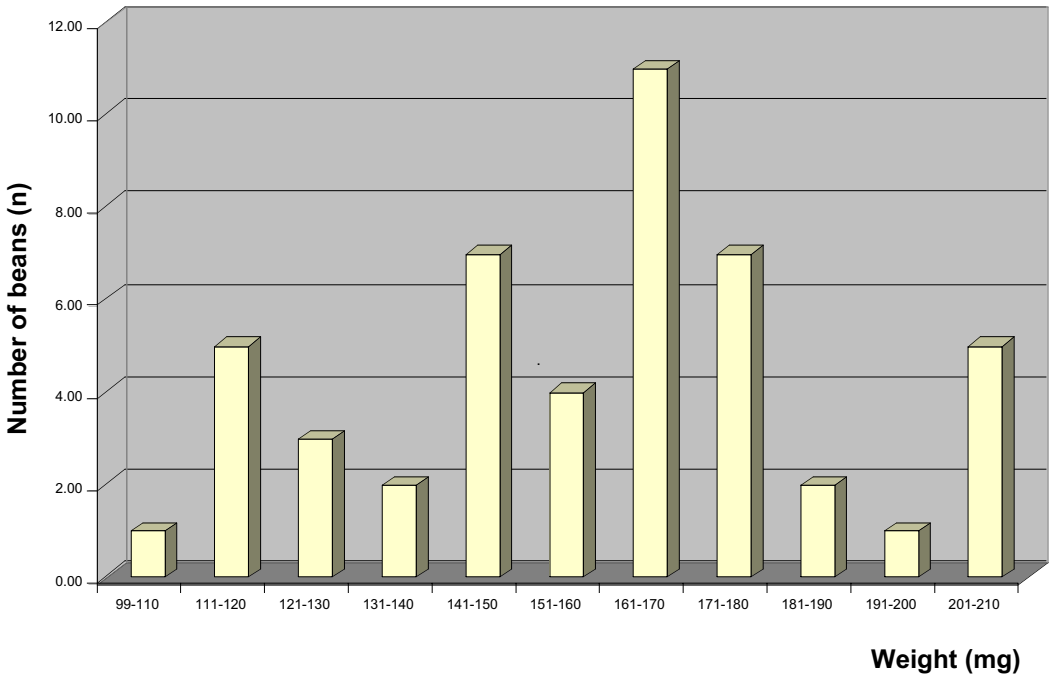

Figure 3. Weight distribution of green single beans from Santos coffee.

Samples were accurately weighted on analytical balance and corrected for average moisture content; the average weight was 159 mg. In Figure 3 is shown the weight distribution of the beans.

Table 1. Caffeine content in single beans.

Bean	Weight (mg)	Caffeine per mg
1	153	0.52
2	117	0.68
3	210	0.70
4	202	0.74
5	170	0.75
6	117	0.87
7	159	0.87
8	166	0.88
9	173	0.89
10	202	0.91
11	195	0.91
12	134	0.93
13	175	0.93
14	210	0.96
15	190	0.99
16	167	1.14
17	166	1.16
18	134	1.16
19	157	1.20
20	165	1.22
21	173	1.23
22	175	1.36
23	167	1.46
24	164	1.55
25	113	2.02
26	113	2.90
27	125	1.50
28	166	1.09
29	143	1.26
30	119	1.41
31	127	1.44
32	173	1.17
33	161	1.12
34	177	1.06
35	143	1.53
36	152	1.43
37	169	1.25
38	109	1.79
39	146	1.36
40	209	0.94
41	148	1.28
42	125	1.38
43	170	1.08
44	147	1.39
45	148	1.24
46	145	1.37
47	180	1.21
48	189	1.03
Average	159.13	1.19

CONCLUSION

Coffee is appreciated for its aroma and its stimulating effect. Caffeine as responsible principle is not equally distributed in beans (2). Various methods like bio-sensing, capillary electrophoresis, (near) infrared and ultraviolet spectroscopy are used for quantification of caffeine. The content of caffeine in singles beans was determined in this work by LC-DAD and LC-MS/MS using Stabile Isotope Dilution Analysis (SIDA) after extraction under addition of magnesium oxide. Results on the variation of caffeine content and weight of single beans of a commercial lot in green bean are given.

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Genetic Diversity, Nirs, Fruit Biochemical Analyses and Cup Testing in Cultivated *Coffea canephora* Pierre from Different Districts in Uganda

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SUMMARY

Research for genetic improvement still lacks adequate core germplasm for improving *C. canephora* quality, consequently farmers lose more of their incomes particularly in severe world coffee price crisis. Also the great range of variation within the Ugandan *C. canephora* has clearly been recognised but no attempt, however, has been made to differentiate the components at DNA level. In our study, samples were collected as seed and cuttings from farms Kawanda germplasm collection. Species diversity was evaluated using Sequence Repeats (SSR), Near Infra Red spectroscopy, (NIRS), biochemical titrations and cup testing after roasting. Controls were from known genetic diversity groups of *C. canephora*. A diversity tree was constructed with SSR polymorphism by Neighbour Joining (NJ) analyses from dissimilarity matrix. DNA results pointed out three major groups of farm trees with one group constituting of entries from closely located districts and controls a distinct group of their own. Four groups were derived from NIRS analyses of fruits with Erecta types forming own group and collections from mainly one district comprising another. Ugandan genotypes were also noted to have high sucrose and fat content with cup test analysis confirming that Ugandan robustas are better than most other robustas. No significant difference between NIRS and biochemical analysis for caffeine offer opportunity for analysing samples from more districts and Kawanda collection using NIRS because it is fast and cheaper. Use of DNA, NIRS, biochemical and cup test analyses in correlation with the environmental information offer opportunity for selecting entries with good quality that will promote sustainable development of the coffee growing areas by generating a reliable market.

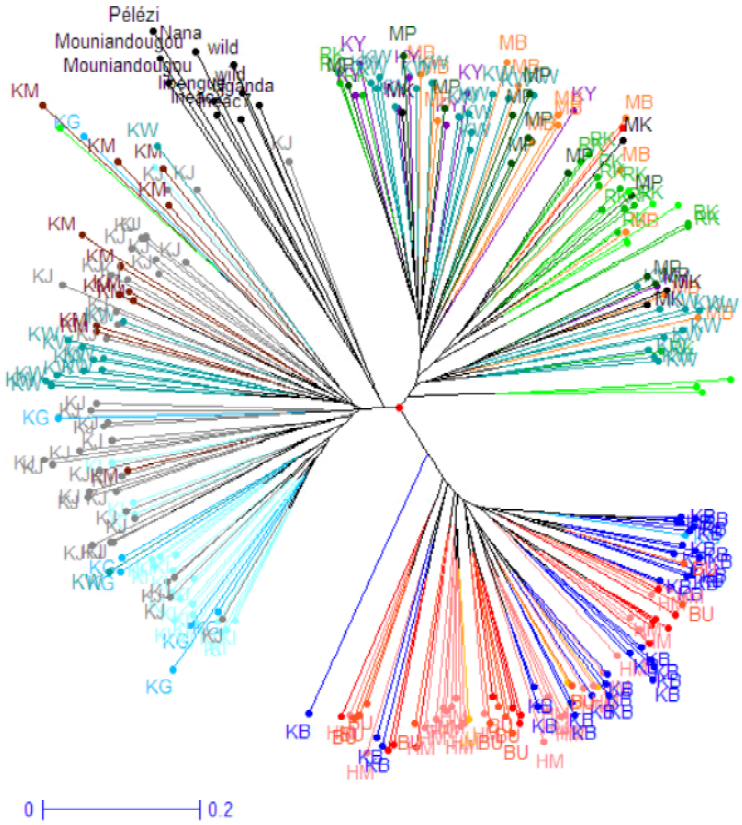
INTRODUCTION

With the exception of *C. arabica* (2n = 44), all coffees in the genus *Coffea* are diploid (2n = 22), with gametophytic self in-compatibility. *C. canephora* constituting 90% of Ugandan production, is a major source of foreign exchange, local revenue and employment (UCDA, 2002/03). Over 2.5 million people are involved in its cultivation, processing and trade. Research for genetic improvement still lacks adequate core germplasm. Consequently, low quality production has made small farmers lose more of their revenue particularly in severe world coffee price crisis. The purpose of this study is to understand robusta genetic, biochemical and organoleptic biodiversity at the small farm level in all traditional producing areas in Uganda. With this knowledge, we hope that markers related to coffee quality will be pointed out and used for breeding varieties producing high quality robustas.

MATERIALS AND METHODS

C. canephora cuttings and seed were collected from farms in traditional growing areas and Nganda, Erecta and hybrids from Kawanda germplasm collection. SSR was used to evaluate 250 DNA samples from 10 districts after DNA extraction from leaves. A diversity tree was constructed with SSR polymorphism by Neighbour Joining (NJ) analyses from dissimilarity matrix (Prakash et al., 2005). NIRS electromagnetic radiations discriminated and grouped 93 fruit samples from 5 districts based on their seed chemical composition and fingerprint (Davrieux et al., 2003). Sixteen genotypes representing groups determined by Malahanobis distance were evaluated by biochemical titrations for dry matter and caffeine. Also cup testing was conducted on 40 samples after roasting. Controls used were from known genetic diversity groups of the same species.

RESULTS/DISCUSSIONS



Key: Letter codes= represent different districts.

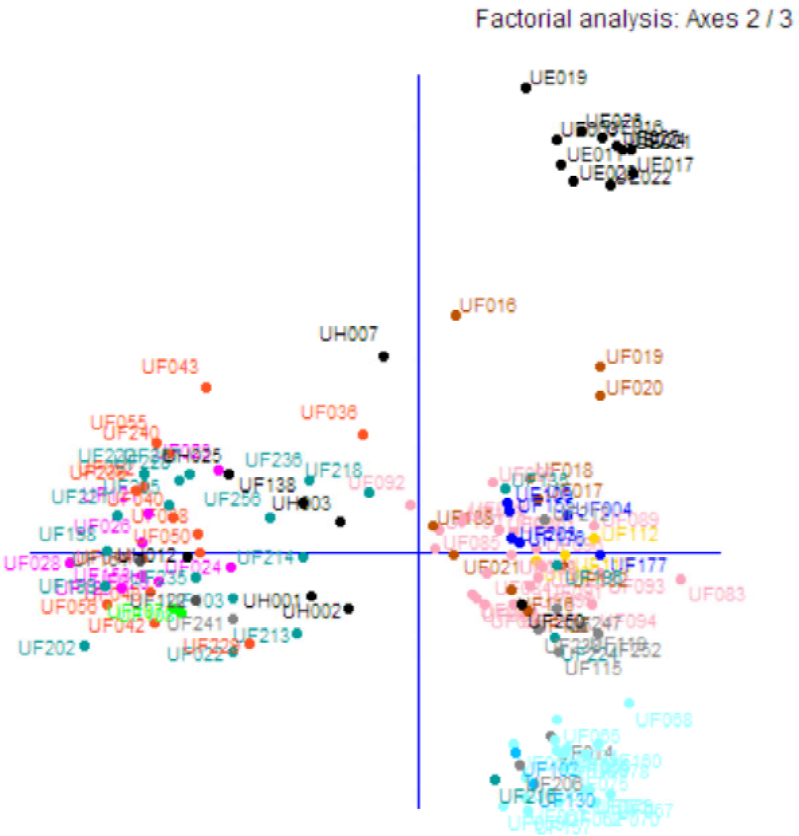
Figure 1. Darwin NJ tree and AFTD for Districts on DNA analysis with 18 SSR markers.

DNA results pointed out three major groups of trees. One group was constituted of entries from closely located districts while the other two are composed of individuals that necessarily do not come from neighbouring districts. Controls constituted a group of their own.

Four groups were derived from NIRS analyses of fruits. Erecta types stand out on own group as well as some farm collections from mainly one district. The hybrids were in another group with some farm collections.

Also NIRS results indicated that Ugandan genotypes have high sucrose and fat content. While cup test analysis confirmed that Ugandan robustas are of high organoleptic quality, with some qualities (acidity) comparable with some arabicas.

For caffeine analysis, no significant difference from NIRS and biochemical analysis offer opportunity for analysing more samples using NIRS that is fast and cheaper.



Key: UE = Erecta types; UH = Hybrids; UN = Nganda types ; UF = Farm collections

Figure 2. Fruit analyses with Near Infrared Spectroscopy.

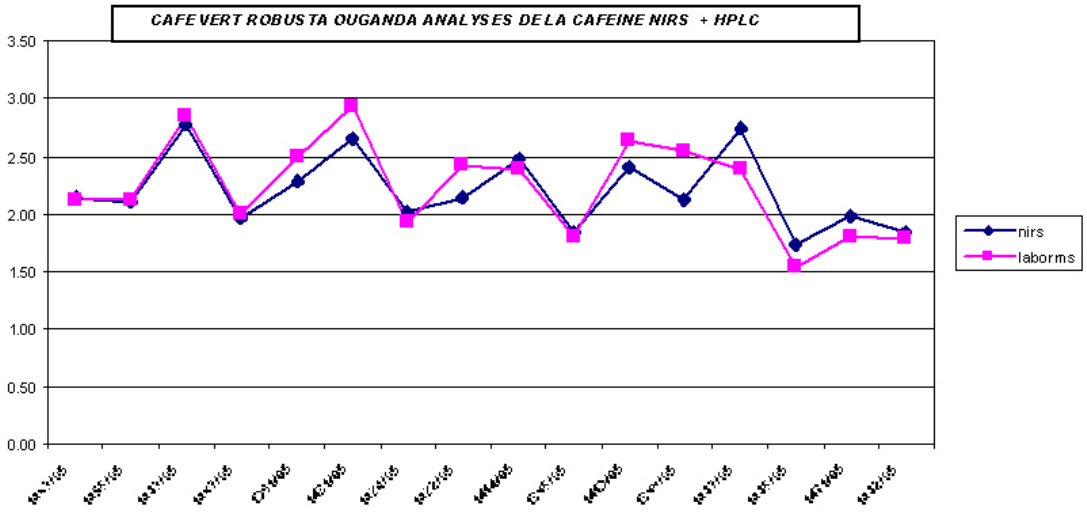


Figure 3. Green robusta caffeine analysis using NIRS and HPLC.

CONCLUSION AND RECOMMENDATIONS

DNA, NIRS and biochemical analyses revealed species diversity within Ugandan farms and collections. *C. canephora* in Ugandan farms is genetically diverse providing opportunity for desirable trait selection. First results obtained pointed out that some of the collected are of very good quality. Unfortunately due to the low number of samples regarding to the coffee area in Uganda, it is difficult to have a complete image of robusta quality in Uganda. For good representation and comparison, need to sample more districts and increase numbers of nganda, erecta and hybrids for NIRS and cupping. There will be need to collect and relate environmental information with NIRS, biochemical and cup test results to identify genetic contribution to quality that can be used for crop improvement. Eventually relate NIRS, biochemical and cup test results with DNA fingerprints to detect specific DNA sequences associated with a good level of robusta quality.

ACKNOWLEDGEMENT

Grateful to USDA for funding the research through ICRAF and CIRAD for technical expertise.

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Quality Determination and Group Discrimination of Shade Grown Coffee Using Solid Phase Microextraction

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SUMMARY

We hypothesized that in order to link chemistry to the organoleptic assessment, the coffee should be analyzed in a form that is routinely used by cuppers to assess quality. In this study, chemical profiles of the volatile organics in the head-space of brewed coffee, brewed in the same manner and ratios used for organoleptic assessment, were obtained. Volatile organic compounds were extracted using a solid phase microextraction fiber. After desorption onto a GC column with FID detector, this resulted in a profile containing over 80 peaks. This method was used to determine the volatile organic profiles of roasted coffee sub-samples obtained from an experiment evaluating the effects of shade on coffee quality and ecophysiology. Peak area data from selected marker peaks in the profiles of different treatments were compared. Marker peaks were used as variables in canonical discriminate analysis to determine the effects of shade treatments and location on the chemical profile.

INTRODUCTION

Traditionally, coffee quality has been assessed using trained cuppers. The movement towards a more objective means of quality assessment has led to experiments that alter cultivation practices to elucidate changes in the green coffee chemistry and cupping profile. Vaas et al. (2006) have shown that when comparing 45% shaded and full sun growing conditions for dwarf coffee (*Coffea arabica* L. cv. Costa Rica 95), there is a statistical difference in the biochemical composition of the beans and on the cupping quality. However, this study did not directly link the specific compound concentrations and cupping quality in any empirical way. Our previous study (Jackson et al., 2004) linked the concentration of specific green bean compounds (chlorogenic acids, organic acids and free sugars) to location, variety and year of harvest. However, we could not find a statistically significant link between green bean content and cupping scores.

An alternative approach to chemically characterizing green coffee is to analyze the volatile components of a roasted coffee brew. A number of methods have been described to achieve this including flushing freshly ground coffee with helium directly onto a GC and separating the components (Murota, 1993) and using various forms of solid phase microextraction (SPME) matrices either in the coffee brew or the headspace (Bicchi et al., 1997, 2002; Meija et al., 2003; Rocha et al., 2003) for selective extraction followed by GC profiling.

The study described here is part of a larger study (posters PA170, PA171) of the effects of shade on coffee quality and ecophysiology of a single coffee variety grown in two locations. Chemical profiling was used to determine if the shade treatments had an effect on brewed coffees from these treatments and whether this profile could be used solely to discriminate between the treatments and locations.

MATERIALS AND METHODS

Coffea arabica cv Typica was grown in two locations in Hawaii, USA. The trees were grown under full sun, 40% black shade cloth, 40% aluminum shade cloth, kaolinite spray, or macadamia trees. Coffee samples were harvested, processed and roasted in the same fashion. The day after roasting, the coffee was cupped. A 3.3 g sub-sample of each coffee used for the cupping was sealed in an airtight vial. The following day, the coffee was brewed in a 150 ml headspace vial with 60 ml of 90° C water and sealed. After brewing for 5 minutes, a PDMS/CAR/DVB SMPE fiber was injected into the headspace and held there for 5 minutes. The fiber was inserted into an HP 5890 GC injection port (250 °C). Chromatographic conditions were: Temperature program: 0-4 min: 40 °C, 4-45 min: 3°/min increase to 163 °C, 45-51.7 min: 20°/min increase to 230 °C, 51.7-61.7 min: 230 °C; Injection: splitless; Head pressure: 10 PSI; Detector: FID; Carrier gas, helium. The column used was a Stabilwax DB, 30m length, .53 mm ID. Field replicates of the treatments were analyzed once. The FID detector signal was monitored using PeakSimple™ software to integrate individual peaks. Peak areas were subjected to canonical discriminate analysis.

RESULTS

A typical GC/FID analysis revealed more than 80 separate peaks (Figure 1). Of these, 43 had a signal to noise ratio greater than 5:1. These were selected for discriminate analysis.

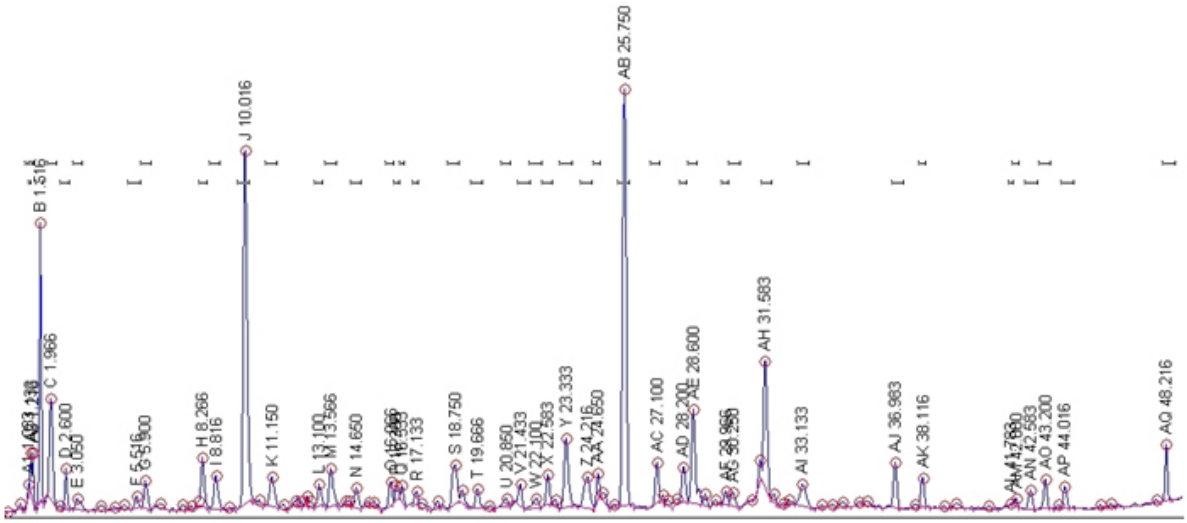

Figure 1. A typical GC/FID Profile.

Using 43 of the volatile compound peaks in the shade treatment discrimination yielded complete group identification with no mis-classification (Figure 2). The first eigenvector accounted for 100% of the variation.

Stepwise variable selection of the data yielded perfect classification of treatments with 14 aroma compounds (Figure 3). Two eigenvectors accounted for 98% of the variation.

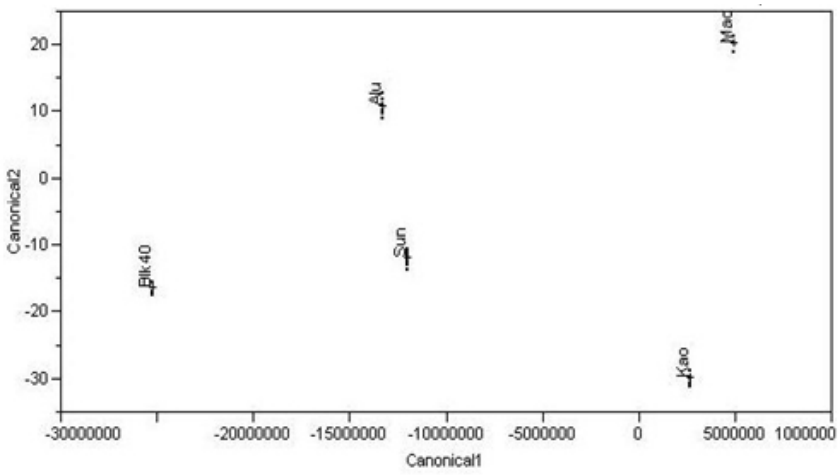

Figure 2. Discrimination of shade treatment based on 43 volatiles (combined locations).

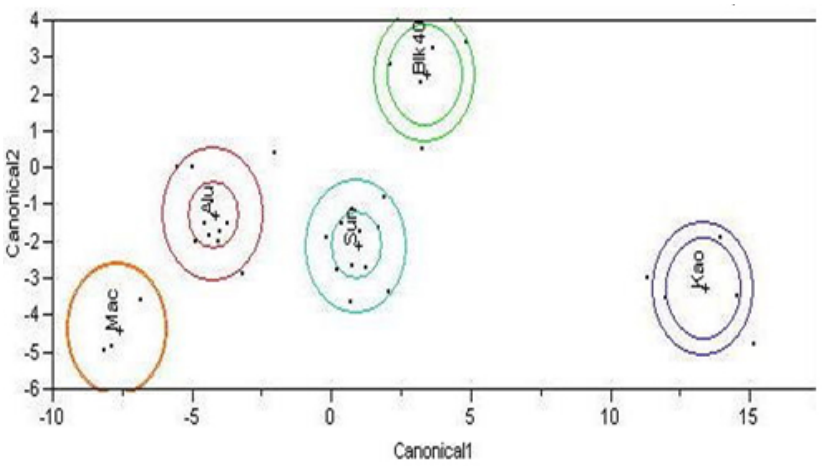

Figure 3. Discrimination of shade treatment based on 14 volatiles (combined locations).

Correct classification of samples by location, with no misclassification, required only 4 aroma compounds (Figure 4). The first eigenvector accounted for 100% of the variation.

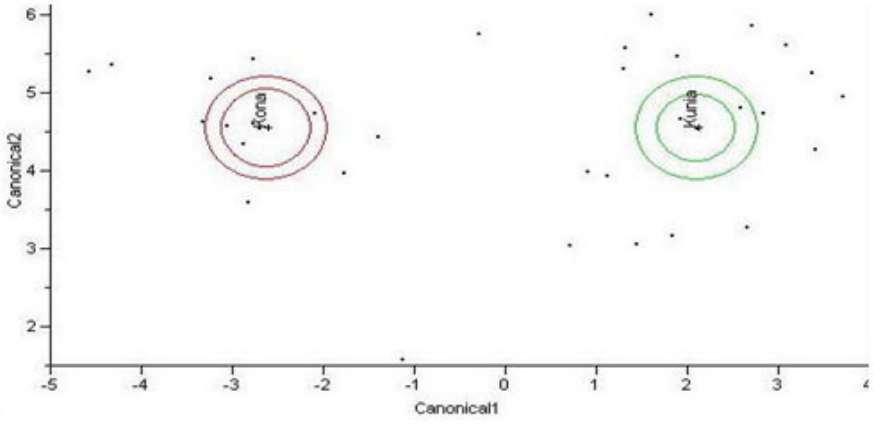

Figure 4. Discrimination of location based on 4 volatiles (treatments combined).

Chemical profiling was able to discriminate between treatments and locations more accurately than cupping alone.

DISCUSSION

Discrimination of coffees based upon analysis of brewed coffee volatiles by SPME proved to be a very powerful technique. Very few compounds were required to accurately discriminate based upon treatment and location. Discrimination between very similar treatments, for example black shade cloth versus aluminum shade cloth, was easily achieved with a high degree of accuracy. Since not all treatments are replicated at both locations, location may play a role in this discrimination. However, the two replicated treatments from both locations were accurately placed in the same groups. Specific chemicals needed for treatment discrimination are independent of those affected by location differences.

Chemical profiling provided a more accurate means of discriminating between treatments and locations than cupping (data not shown). Classification of coffees by this method likely can be used in detecting adulteration of coffee and in authentication of coffee origin. This technique appears to be very powerful in distinguishing coffee's response to even small environmental differences.

Of possibly greater importance is the relevance of SPME headspace analysis of brewed coffees as a screening method for determining the types of metabolic pathways associated with responses to environmental differences. In the future, attempts will be made to identify those compounds responsible for the discrimination observed in this study.

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Lipoxygenase Activity and Hydroperoxide Formation in Coffee (*Coffea arabica* L.) Cherries Cultivated by Different Agronomic Techniques

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SUUMARY

It is widely accepted that biotic stress induces acidic lipoxygenase (LOX) activity mainly due to the effects of methyl jasmonate (Buzi et al., 2004). The activation of LOX pathway leads to the production of a large amount of oxidation products, which may affect the coffee in organoleptic profile (Kohlmann et al., 1999). Hitherto the presence of LOX in coffee (*Coffea arabica* L.) plants is still scarcely examined (Rojas et al., 1993), therefore the aim of this study was to investigate the possible involvement of this enzyme in oxidative stress of cherries from plants cultivated in organic and conventional manners. Primary and secondary oxidation products were evaluated in three separate cherry fractions: pulp, parchment seed and green coffee. Hydroperoxides, measured by HPLC, were highest in parchment seed and lowest in green coffee, but no significant difference was found between the samples obtained from the two cultivations. However, the pulp fraction from organic cultivated plants had a larger amount of primary peroxidation products, when compared to that from conventionally cultivated plants. The presence of LOX was then examined in all cherry fractions by immunochemical analysis. Only the pulp fraction showed an 84 kDa protein that exhibited a cross-reactivity against a pea LOX antibody. Subsequently, the presence of LOX was confirmed in heavy (28000 g) and light (100000 g) membrane pulp fractions by an enzymatic assay, immunochemical method and hydroperoxide formation. In both organic and conventional cultivation systems, the light membrane showed the highest LOX activity, measured as conjugated diene formation, at pH 8.0. Instead, the heavy membrane fraction highlighted a different pH optimum, which was found to be 5.5 in pulps from organic cultivations and 8.0 in the conventional ones, respectively. All the activities reported were inhibited by nordihydroguaiaretic acid (NDGA) and caffeic acid, well known LOX inhibitors. The NDGA-sensitive hydroperoxide production was simultaneously confirmed by RP-HPLC analysis. Oxidative stress was also detected by evaluating the antioxidant activity in all fractions. A lipophilic extraction, performed using dioxane:water (95:5, v/v), revealed that parchment seeds possessed the lowest antioxidant capacity. On the other hand, a significant difference in the antioxidative capacity was detected in parchment seeds from organic and conventionally cultivated plants. Taken together, these results show, for the first time, the presence of LOX in coffee cherries, whose activity was associated to membrane fractions. Furthermore, the cultivation techniques seem to induce the activation of different isoenzymes. In particular, the organic ones were subjected to an oxidative stress in coffee fruits leading to the expression of an acidic LOX activity, detectable in the pulp fraction.

INTRODUCTION

Lipoxygenase (LOX) is induced in plants, by biotic stress through a mechanism mediated by methyl jasmonate (Buzi et al., 2004). The activation of LOX pathway leads to the production of undesired volatile secondary products (mainly aldehydes and terpens), which interfere with the coffee organoleptic profile ((Kohlmann et al., 1999). Up to date, few evidences have been collected about LOX presence in coffee (Rojas et al., 1993), so this study was undertaken to investigate the possible involvement of the enzyme during oxidative stress in cherries from organic (identified as STRESS) or conventional cultivations (identified as CTRL) in Brazil.

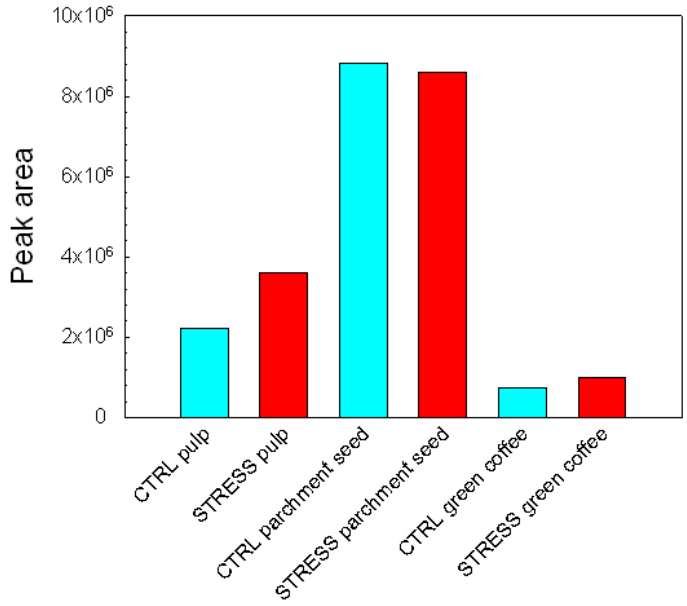

Figure 1. HPLC hydroperoxide determination on different coffee cherry portions cultivated in Brazil and milled after treatment with liquid N_2.

Acid extraction (pH 2) was performed using hexane as solvent. Samples were then dried with N_2 flux and resuspended in mobile phase (acetonitrile, water and acetic acid, 80:20:0,1). Linoleic acid hydroperoxide was used as an internal standard.

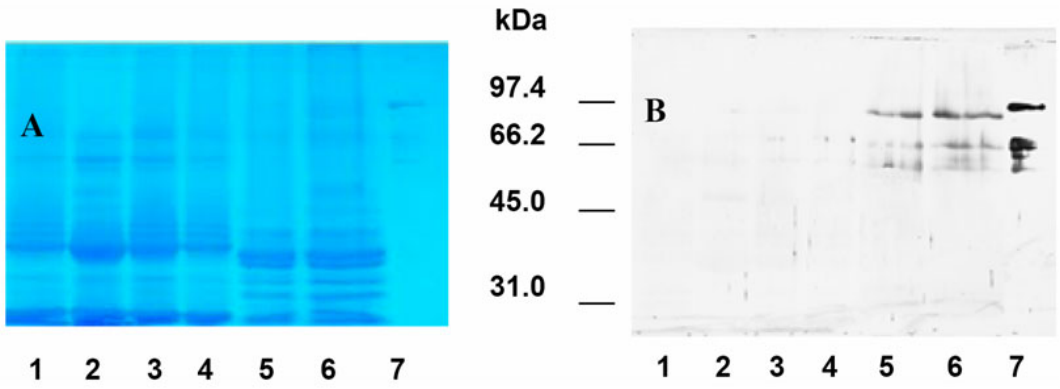

Figure 2. SDS-PAGE and Western-Blot of coffee protein obtained from different parts of the fruit.

Panel A shows protein pattern in cold acetone extracts. Each lane was loaded with 30 µg of protein. Panel B indicates cross-reactivity with pea LOX Ab.

1: CTRL seeds
2: STRESS seeds
3: CTRL parchment seeds
4: STRESS parchment seeds
5: CTRL pulp
6: STRESS pulp
7: soybean LOX

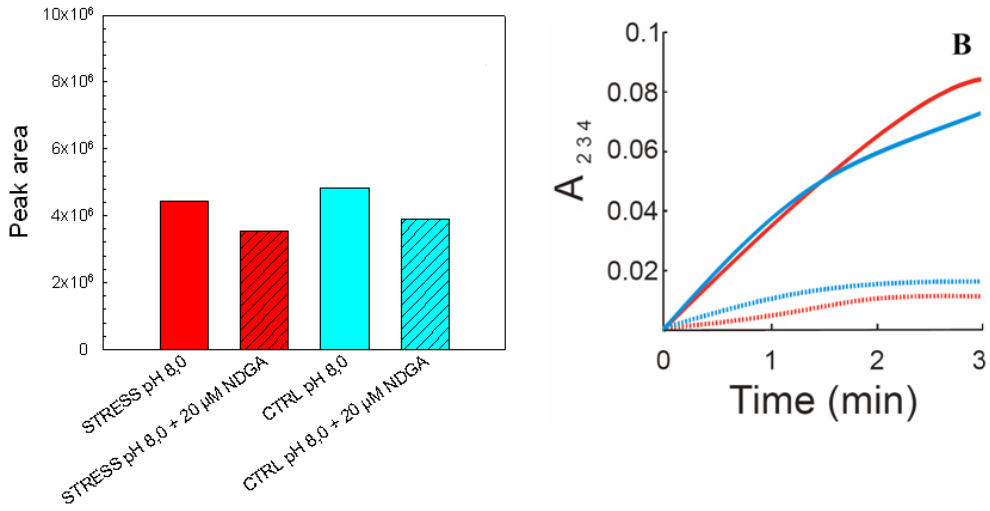

Figure 3. Lipoxygenase activity measured by hydroperoxide (HPLC) determination (panel A) and by conjugated diene formation (panel B) in coffee pulp "light fraction" membranes (microsomes).

Procedures for hydroperoxide extraction and HPLC evaluation were as in Figure 1. Conjugated diene formation was measured as absorbance increase at 234 nm. Red lines or columns= STRESS; cyan lines or columns = CTRL. Solid lines = FFA; dashed lines = FFA + 20 µM NDGA.

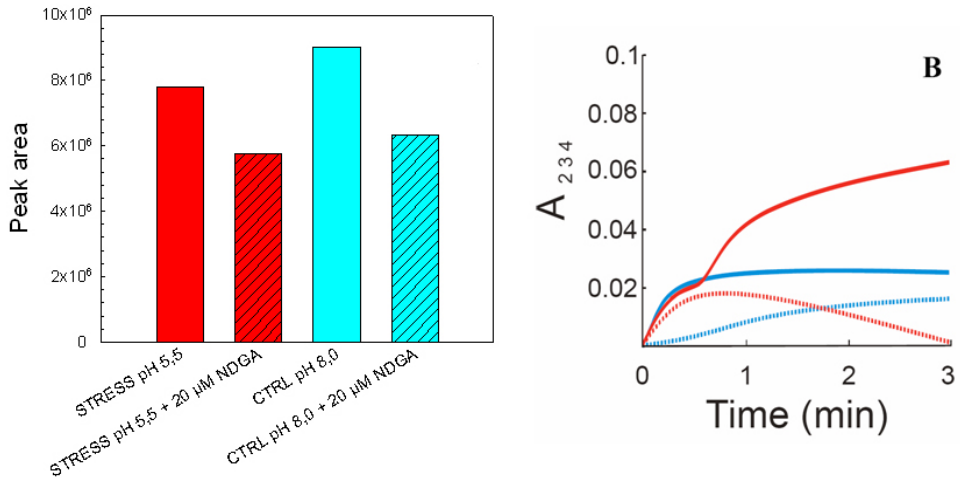

Figure 4. Lipoxygenase activity measured by hydroperoxide (HPLC) determination (panel A) and by conjugated diene formation (panel B) in coffee pulp "heavy fraction" membranes (mitochondria).

Procedures for hydroperoxide extraction and HPLC evaluation were the same as in Figure 1. Conjugated diene formation was measured as absorbance increase at 234 nm. Red lines or columns = STRESS; cyan lines or columns = CTRL. Solid lines = FFA; dashed lines = FFA + 20 µM NDGA.

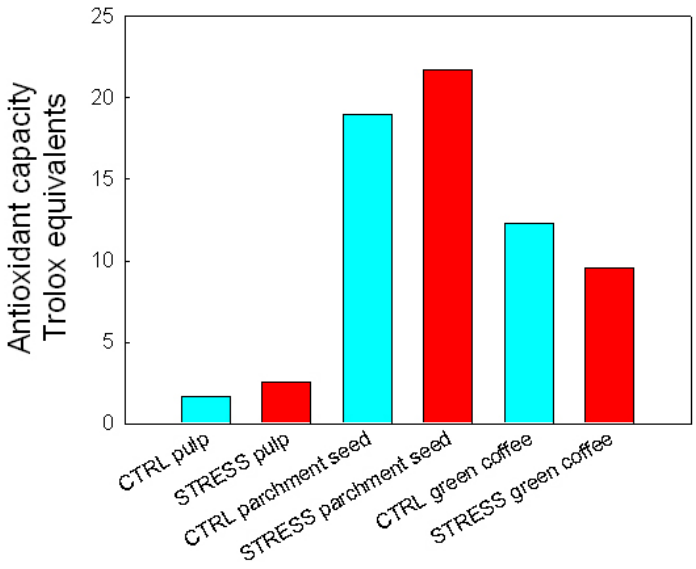

Figure 5. Antioxidant activities of acqueos extracts (pH 7.5) determined by crocin kinetic competition on different portions of coffee cherries, treated with liquid nitrogen.

Peroxy radicals are generated *in situ* by diazocompound decomposition. The bleaching of crocin is directly correlated to radical production and happens with a constant speed. When part of the peroxyl radicals are quenched by other antioxidants the bleaching rate is lower, and it is correlated to the concentration of the other antioxidants. The ratio between the bleaching speed of crocin without or with other antioxidants yields the antioxidant capacity of the sample.

The slope of a known antioxidant, such as Trolox C, can be calculated. Other substances, even complex mixtures, such as coffe extracts, can be measured, and their values related to that of Trolox C. The result will be an equivalent millimolar concentration of Trolox C.

CONCLUSIONS

Above exposed data demonstrate, for the first time, the presence of LOX in coffee cherries, whose activity is associated to membrane fractions. Furthermore, the expression of different isoenzymes, characterized by different pH optima, seems to be related to the cultivation techniques. In particular, organic methods cause an oxidative stress in coffee fruits, leading to the synthesis of an acidic LOX activity in fruit pulp, detectable both as hydroperoxide production and conjugated diene formation. Instead pulp fruit from conventional cultivations show a comparable LOX activity, but with an higher pH optimum. Acidic LOX activity could represent a typical physiological response against biotic stress. These results could represent a starting point to better understand the evolution of undesired volatile compounds linked to the LOX pathway, which may affect coffee quality and flavours.

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Aspects of Milk and Milk Foams on Dairy Based *Espresso* Coffee Hot Drinks

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SUMMARY

Cappuccino, *macchiato*, *latte* and *cafè au lait* are very popular coffeehouse beverages. Most of *espresso* coffees are consumed every day by adding milk and particularly milk-foam, originating the endless family of *cappuccino*-like drinks. The milk foaming, therefore, is an important quality determinant in the manufacture of dairy-based *espresso* hot drinks. In addition to *espresso* quality, milk type and its ability to form a stable foam with desired texture and rheological properties play a fundamental role in determining the quality of these products. Steam frothing of milk is used to produce a proper foam for many dairy-based *espresso* hot drinks. Chemical composition and processing such as protein and fat contents, heat treatments and technologies used, temperatures and operative conditions used to obtain the foam may strongly affect the foaming properties of milk. In the present work, milk steam frothing has been studied under strictly controlled experimental conditions. Data reproducibility has been obtain by fixing and optimizing the whole set of variables that may influence the final foam characteristics. We have then evaluated the importance of milk temperature on the foaming properties measured in terms of overrun values. The effect of two consecutive milk steam frothing processes on the overrun values and chemical composition have been also evaluated. Experimental results put in evidence the necessity of standardization of the foaming technique to get repeatable overrun values in the framework of comparison among different types of milk. Moreover, the overrun value have to be considered just as one of the features which makes a milk foam ideal for *cappuccino*. Steam-frothing determines a not negligible (up to 10% w/w) milk dilution as evidenced also by HPLC analysis of protein fractions after two consecutive foaming processes. Performance differences between pasteurized and UHT milks has been also carried out. Preliminary data suggest no significant differences, particularly if UHT milk are obtained by direct steam exchange method.

INTRODUCTION

Steam frothing of milk is used to produce a proper foam for many dairy-based *espresso* coffee hot drinks such as *cappuccino*, *macchiato*, *latte* and *cafè au lait* . In spite of its extensive use, detailed investigations on steam frothing process are far to be abundant (Buchanan, 1965,; Deeth & Smith, 1983; Levy, 2003; Levy et al., 2003), and, as far as we know, mainly addressed to milk technologists. On the contrary, a number of commonly-held beliefs about factors affecting steam frothing can be easily found on trade and technical literature although most of them have been shown to be untrue (Deeth & Smith, 1983).

In order to assess milk foaming performance as a function of several factors, including, milk composition and processing, heat treatment, storage conditions etc., a so called Steam Frothing Value (SFV) has been used (Deeth & Smith, 1983; Levy, 2003). This parameter, also known as overrun, is calculated by using the following formula:

$$SFV = (TV - LV)/LV \times 100$$

TV is the total volume of liquid plus froth, whereas LV is the liquid volume of the milk, both measured at known time after preparation (typically 3 min). Unfortunately, several steam frothing procedures and equipments can be used and this renders any comparison of literature data very difficult if not impossible. What's more, independently on adopted procedure, it is clear that by changing the initial milk volume (500 mL in Buchanan, 1965 or 200 mL in Deeth & Smith, 1983) different SFVs can be obtained.

Moreover, due to the fact that water is incorporated during the steam frothing (up to 12% depending on initial milk volume and temperature) and pouring the milk froth in a measuring container (typically a graduated cylinder) is necessary to get experimental data, the calculated SFV is affected by an error assumed to be negligible but not yet reported. This view is additionally complicated by the difficulty to accurately read the top level of the foam layer into the measuring container.

As far as reported SFVs is concerned, it is possible to observe very high values, up to 150, related to "excellent" frothing assessment (Deeth & Smith, 1983). From a milk technological point of view this is obvious, but from a dairy-based *espresso* coffee cup quality point of view lower SFVs appear close to be excellent. As a matter of fact, the milk foam volume has to be paralleled by milk foam rheology (milk foam has to be liquid-like, pourable possibly without yield stress) and visual texture (in terms of air bubbles size and its distribution and gloss) to be appropriate for dairy-based *espresso* coffee hot drinks and this is generally achieved when relatively low air content is incorporated into the foam.

In the present study, different experimental set up have been used to characterize several milk samples with the scope to individuate optimal SFV for dairy-based *espresso* coffee hot drinks. Resorting to the experience of professionals and *cappuccino*'s masters, appropriate experimental conditions have been selected. Attention has been paid to assess the effect of fat content, heat treatment, initial milk temperature and double steam frothing on SFVs.

EXPERIMENTAL

Materials

Commercial fresh pasteurized/homogenized whole milk (3.6% fat content) and semi-skimmed milk (1.6% fat content) samples, commercial UHT whole, semi-skimmed and skimmed (0.1% fat content) milk samples and UHT (by direct steam exchange method) milk samples with a fat content ranging from 3.6% to 5.0% (supplied by Centrale del Latte di Brescia, Italy) have been used to evaluate the milk steam frothing performance. Commercial fresh pasteurized/homogenized whole milk was used to evaluate the effect of a double steam frothing on the SFV and on the milk protein denaturation.

Methods

Professional milk steam frothing method

Preliminary steam frothing tests have been performed by F. Storm (World Barista Champion, 2002) and by G. Milos and M. Pauletic (Università del Caffè, Trieste, Italy) by using a professional *espresso* coffee machine ("La Marzocco", Italy) in order to determine the proper SFV range to ensure excellent milk foam quality. Milk was place in a stainless steel pitcher

and the relative position of the steam arm and the pitcher was modified in the course of the steam frothing test in accordance with the experience of the skilled operator.

Adapted milk steam frothing method

The professional milk steam frothing method has been subsequently adapted to avoid any manual intervention.

Milk (250 g at 5 ± 2 °C) was placed in a 500 mL graduated glass beaker properly positioned under the steam arm ("La Marzocco", Italy) of an experimental steam generator (Monopak, Italy) or of a professional *espresso* coffee machine ("La Marzocco", Italy), so that the 4 holes in the steam nozzle were approximately 5-6 mm below the surface of the milk. Geometrical constrains for the steam arm and the milk beaker have been fixed and no manual intervention has been used during test. Steam from the generator, preset to operate at constant pressure of, was injected into the milk at a constant rate (milk thermal gradient in the range 4.20 _ 4.30 °C/sec) for a fixed time of 14 sec (procedure a), or until a fixed milk temperature of 65 °C (procedure b). The milk with froth was directly measured into the graduated glass beaker within 3 minutes after preparation. At least 3 tests per condition have been carried out.

Milk protein analisys

Total milk protein content was determined by Kjeldhal method (FIL-IDF 20B, 1993).The quantitative analysis of the whey protein was achieved by the method of Resmini et al. (1989), using a Varian Model 230 Pro Star HPLC equipped with a Rheodyne Model 7725i injector (40 μL loop), a Varian Model 330 Pro Star UV-VIS spectrophotometer detector set at 205 nm and a PLRP-S (polystyrene divinyl benzene) column from Polymer Laboratories Ltd (UK), 150 x 4.6 mm, 5μm particle size, 300Å pore size.

RESULTS AND DISCUSSION

Preliminary steam frothing tests

The standard professional milk steam frothing method consists of two different phases:

i. air incorporation (below 35 °C)
ii. spinning (35 °C – 65 °C)

the relative position of the steam arm and the pitcher are different in the course of the two phases. In the first one, small up and down movements are performed, in the second one, the steam arm is positioned to induce a circular turbulent flow to the milk. The milk temperature is detected by hand. The whole set of operation strictly requires the professionality of a skilled operator to get result reproducibility.

Independently on milk heat treatment (pasteurization or UHT) and milk fat content (up to 4.3%) the average SFV determined by using this method falls in the range 55-65. As expected, the proper overrun for dairy-based *espresso* coffee hot drinks is relatively low if compared with SFV literature data. However by including the water incorporated during the process (8-10% in the present case) the SFV range reduces to 45-55. These results strongly suggest that the error due to water incorporation is by far to be negligible and the comparison with literature data may be misleading.

Milk steam frothing performance

On the basis of the preliminary tests our efforts have been devoted to individuate experimental conditions without the necessity to resort to manual intervention during steam frothing process. Particularly a geometrical constriction of the steam arm was fixed and the steam nozzle was kept approximately 5-6 mm below the surface of the milk. In such a way an objective comparison of milk steam frothing performance has been obtained. In Table 1 corrected for water incorporation SFV average and standard deviation data are reported.

Table 1. Averege SFV experimental data obtained by adapted milk steam frothing method (procedure a).

Milk sample	Professional *espresso* coffee machine	Experimental steam generator
Fresh pasteurized whole (brand A)	43 ± 6	57 ± 2
Fresh pasteurized whole (brand B)	45 ± 4	58 ± 2
Fresh pasteurized whole (brand C)	40 ± 3	57 ± 3
UHT whole	40 ± 5	
UHT semi-skimmed	47 ± 8	60 ± 2
UHT skimmed	53 ± 6	
UHT (4.3% fat content)	49 ± 5	
UHT (5.0% fat content)	38 ± 6	
Fresh pasteurized semi-skimmed	39 ± 9	57 ± 3

It has to be stressed that within the same experimental conditions the differences in the obtained SFVs are not statistically significant (Tukey's multiple comparison test, ($p \leq 0.05$). This is in full agreement with previous studies (Deeth & Smith, 1983; Levy, 2003; Levy et al., 2003) and confirm, once again, that heat treatment and fat content do not have a significant effect. As previous research has shown, it has to be underlined that skimmed milk tends to produce a lighter, airier foam over whole milk leading to differences in the rheology and in the visual texture of the corresponding foam. The obtained SFVs are close to those obtained in the preliminary tests suggesting that both the experimental set up realistically mimic the standard professional milk steam frothing method. The differences between the two set of data may mainly reflect the different steam pressure in the boiler (1.5 bar for the professional *espresso* coffee machine; 3 bar for the experimental steam generator).

Effect of milk temperature prior to frothing on SFVs

It is well known that milk cooled to low temperature (below 5°C) shows better steam frothing than milk at higher temperature, and this is related to the amount of steam required (air incorporation) to raise milk temperature to 65 °C (Deeth and Smith, 1983). Unfortunately, the bad practice to leave milk at room temperature close to the *espresso* coffee machine is still dominant in the great majority of coffee shops and bars. By monitoring the temperature of 1 L of fresh pasteurized whole milk (in its Tetrapak original packaging) initially at 5 °C, maintained at room temperature (22 °C) close (20 cm.) to the experimental steam generator, the temperature _ time profile (data not shown) reveals that a time of 2 or 3 hours is sufficient to raise the milk temperature to 10 °C or 15 °C. As far as SFV is concerned, in Table 2, a comparison between procedure a) and b) has been performed to study the effect of the initial milk temperature on SFV data. Fresh pasteurized whole milk and the experimental steam generator have been used. In spite of the fact that within the same procedure the differences are not statistically significant, the SFV obtained by applying procedure b) are more scattered.

It has to be mentioned that the visual texture of the foams appears to be influenced by the adopted procedure, however, this aspect is out of the scope of the present study.

Table 2. Averege SFV experimental data as a function of initial milk temperature.

Initial Milk Temperature (°C)	Procedure a	Procedure b
1	68 ± 2	76 ± 5
5	65 ± 4	72 ± 4
10	68 ± 2	80 ± 5

Effect of a double steam frothing on the SFV and on the milk protein denaturation

In a previous work, it has been found that double frothing, with intermediate cooling, markedly improved the foaming behaviour of milk (Deeth & Smith, 1983). By using fresh pasteurized whole milk, double frothing has been performed with the experimental steam generator and procedure b). The intermediate cooling at 4°C has been performed by an ice bath. The steam frothing has been paralleled by chemical analysis of the protein component, in order to assess eventual changes induced by the process.

The obtained average SFVs: 74 ± 4 (first frothing) and 71 ± 5 (second frothing) are not statistically different and thus the present study does not confirm previous data, within the experimental conditions presently used. Differences in visual texture and smell, however, have been obtained.
Regarding the chemical data, Table 3 shows the comparison of protein components before and after steam frothing. Data was expressed as relative percentage of whey proteins to exclude the mistake induced by water incorporated during the steam frothing (ca. 10% in present case). Proteose peptone component have been included due to its possible role in the milk foaming (Corradini and Innocente, 1994). Not significant changes in the whey protein profile are showed during two frothing test. BSA data only show a trend, albeit very modest (see Table 3).

Table 3. Relative milk protein composition before and after steam frothing. Mean values within the same row followed by the same letters do not differ significantly at $p \leq 0.05$ according to Tukey's test.

	Before process	After first frothing	After second frothing
Total proteins %	100	100	100
Whey proteins %	16.00a	15.22a	15.90a
Total albumins %	13.21a	13.72a	13.38a
_ – lactalbumin %	2.77a	2.87a	2.68a
BSA %	0.54a	0.49b	0.48b
_ – lactoglobulin %	9.90a	10.36a	10.22a
Proteose peptone %	2.85a	2.80a	2.82a

CONCLUSIONS

Experimental procedures able to carry out steam frothing without any manual intervention have been individuated and tested. This is an important step to carry out objective comparison of milk steam frothing performance. The SFV range appropriate for dairy-based *espresso* coffee hot drinks has been determined to be in the range 55 - 65. Milk samples differing in fat content and heat treatment have shown to adequately perform for dairy-based *espresso* coffee

hot drinks, since in terms of SFV no statistically significant differences have been obtained. This is in agreement with previous studies (Deeth and Smith, 1983; Levy, 2003; Levy et al., 2003). It has to be taken into the account, however, the fundamental role played by characteristics other than SFV, like milk foam rheology and visual texture, in selecting optimal milk for dairy-based *espresso* coffee hot drinks. In this regards, the fat content is important. In fact, skimmed milk, in spite to its acceptable SFV, is less indicated to prepare excellent foam for *cappuccino*, however, when abundant, light and airy foam is required, as in the case of cold drinks, skimmed milk represents the first choice. Experimental data put also in evidence that the milk temperature prior frothing (up to 10 °C) and a double steam frothing don't affect the SFVs, but result in a different visual texture and smell of a foam obtained. Therefore the organoleptic properties of the milk after steam frothing and the foam rheology and visual texture, can constitute a fertile territory for further studies.

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Physicochemical Characterization of the *Espresso* Coffee Foam

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SUMMARY

The foam layer of small bubbles with a particular tiger-tail pattern on top of the small cup volume beverage, make *espresso* coffee very different from the other pure coffee based beverages. Conditions normally used to brew *espresso* coffee enhance several surface tension-related phenomena such as foam formation and stabilisation. Any error in grinding or in percolating, in temperature or extraction level is immediately denounced by the colour, the volume, the texture and the persistence of foam. Moreover, it has also to be stressed that the ingredients, including water, may strongly affect the whole set of foam characteristics. The importance of the foam as a very positive connotate in recognising quality of the *espresso* coffee brew may be witnessed by the term "*crema*" used in Italy to name the foamy layer. In facts, the term derives from the identical name of a dessert preparation highly appreciated by people and it is often used as synonymous of a "delicious food texture". Several studies have been aimed at elucidating *espresso* coffee surfactants and surface properties, however, little is known about foam characteristics and foam formation mechanism. This is particularly true from a quantitative point of view and it is mainly due to the experimental difficulties which have to be overcome to study this peculiar food foam. The present study reports on the physicochemical characterization of the *espresso* coffee foam. Several experimental approaches have been followed in order to obtain quantitative data. The role played by carbon dioxide release in *espresso* coffee foam is discussed in detail and emphasized.

INTRODUCTION

Foam appearance is the most important characteristic that a consumer uses to ascertain the quality of *espresso* coffee. In spite of its relevant role, there are few reports on this food foam, and they mainly focused on its chemical aspects and interfacial properties (Navarini et al., 2004; Nunes et al., 1997; Petracco et al., 1999; D'Agostina et al., 2004). On the other hand, a huge number of detailed investigations have been reported on beer, Champagne and sparkling wines foams.

Although foam physics is a very complex and challenging topic, it is possible to simplify to the following four key events, the high number of phenomena involved in foam formation and retention (Bamforth, 2004; Evans and Sheehan, 2002):

- Bubble formation
- Bubble rise
- Drainage
- Coalescence and Disproportionation

The most important mechanism for bubble formation is nucleation. In beverages like beer and Champagne wine, although are supersaturated solutions of carbon dioxide, bubbles do not nucleate spontaneously. A nucleation site must be present, which may be a particle, scratch on the glass or a pre-formed micro bubble. The bubble radius (Rb) that is generated is proportional to the radius of nucleation site (Rn) through the following equation:

$$Rb = (3R\eta\gamma/2\rho g)^{1/3} \quad [1]$$

Where γ is the surface tension (mN/m), ρ is the relative density (kg/m^3) and g the acceleration due to gravity (9.8 m/s^2). To achieve the smallest possible bubble, it is necessary to have the smallest possible nucleation site. When a bottle of Champagne wine is opened, classic nucleation theory tells us that the critical radius (or, simply, the size) below which bubble production is impossible is around 0.2 micrometers (Liger-Belair, 2004). According to eq. 1, the influence of surface tension and density on bubble size is very low.

After bubbles are formed and released from their nucleation site, they rise toward the liquid surface in bubble trains and grow in size during their ascent. This event is important as an appealing spectacle in beer (Bamforth, 2004) and in Champagne wine (Liger-Belair, 2004) but it is also important because the bubble growth rate determines the final average size of the bubbles. In carbonated beverages, the bubble growth observed during ascent is caused by a continuous diffusion of dissolved carbon dioxide through the bubble interface into the bubble itself (Liger-Belair, 2004). The growth rate, k, of bubbles ascending in a Champagne wine has been theoretically derived and connected with some physicochemical parameters of the liquid medium as follows (Liger-Belair et al., 2003):

$$k = dR/dt \approx 0.63(k_B T/P)D^{2/3} (2\alpha\rho g/9\eta)^{1/3} \Delta c \quad [2]$$

where R is the bubble radius, k_B is the Boltzmann constant, T is the absolute temperature, P is the pressure into the bubble assumed to be equal to the atmospheric pressure, D is the diffusion coefficient of CO_2 molecules dissolved in the liquid medium, ρ is the liquid density, η is its dynamic viscosity, g is the gravity acceleration, α is a numerical factor close to 0.75 and Δc is the difference in CO_2 concentrations between the liquid bulk and the close vicinity of the bubble surface in equilibrium with the gaseous CO_2 into the rising bubble.

As soon as foam is formed liquid starts to drain from it. The main driving force for drainage is gravity, which acts directly on the liquid in a non-horizontal film, and indirectly through suction of the Plateau border (the meeting point of three liquid films). In drainage, the liquid phase properties (viscosity of film liquid and density) play a major role (Dickinson, 1992).

Bubble coalescence results from the rupture of the film between bubbles to make larger ones whereas, disproportionation, often described as the most important type of instability in foams, is the diffusion of gas from small bubbles into big bubbles. The driving force for disproportionation is the Laplace pressure difference over a curved bubble surface which results in a higher pressure within a small bubble than within a large one. As gas solubility increases with pressure (Henry's law), more gas dissolves near the small bubble than near the large one, and so the latter grows at the expense of the former (Dickinson, 1992). This phenomenon is governed by the De Vries equation (Bamforth, 2004):

$$Ri^2 = R0^2 - (4RTDS\gamma t/P\theta) \quad [3]$$

Where Ri is the bubble radius at time t, R0 the bubble radius at start, R the gas constant, T absolute temperature, D the gas diffusion coefficient in the liquid, S the solubility of the gas, γ the surface tension, t the time, P the pressure and θ the film thickness between bubbles. It is evident the importance of the gas phase in this key event. The enormous benefit to beer foam stability of nitrogen gas is due to its solubility in respect to that of carbon dioxide (Bamforth, 2004).

Coming back to *espresso* coffee, the intriguing question is: may, the above mentioned physics, be of help in the understanding of its foam? To answer this it is necessary to determine the chemical nature of the gas phase in the *espresso* coffee foam.

It is well known that carbon dioxide is the most quantitatively relevant gaseous reaction product generated during coffee roasting and entrapped within the cell structure. It has been reported that immediately after grinding of the beans, carbon dioxide content ranges from 4.0 to 8.6 mg (corresponding to 2.0 to 4.4 mL at STP) per gram of coffee (Anderson et al., 2003). It has also been reported that two additional carbon dioxide sources can be individuated in the *espresso* coffee brewing method, both deriving from bicarbonate ions contained in the water (Rivetti et al., 2001). In particular, carbon dioxide from bicarbonate thermal decomposition and from bicarbonate neutralization by coffee acids.

Carbon dioxide has been obviously suggested as the main constituent of the gas phase of *espresso* coffee foam (Illy and Viani, 2005), however, as far as we know, no data have been yet obtained to corroborate this view.

In the present work, the carbon dioxide content of roasted and ground Arabica blend has been determined and in parallel the corresponding *espresso* coffee foam has been quantitatively determined. Possible foam formation mechanism has been proposed.

EXPERIMENTAL

Materials

Roasted and ground pure *Coffea arabica* coffee blend was produced by illycaffè S.p.A. (Trieste, Italy) in roasting grade N (medium). In order to prepare samples differing in carbon dioxide content, roasted coffee beans as well as ground coffee samples were equilibrated and/or degassed at room temperature at varying time. Different production lots and Easy Serving Espresso^{TM} (ESE) coffee powder samples were used. Drierite (anhydrous calcium sulphate) (W.A. Hammond Drierite Company Ltd., Xenia, USA) and Ascarite II, e.g. NaOH coated silica gel (Thomas Scientific, Swedesboro, USA) were used. All chemicals were of analytical grade (Sigma-Aldrich, Italy).

Methods

Carbon dioxide content was determined by the method of Hinman (1993) in an apparatus shown and described in detail elsewhere (Anderson et al., 2003).

Roasted coffee beans were ground using a professional coffee grinder (Luigi Mazzer s.r.l., Italy) to obtained a particle size distribution close to that mentioned as “fine grind” in Anderson et al. (2003).

Espresso coffee beverages were prepared by using a professional *espresso* coffee machine (La Marzocco s.r.l., Italy) under the following conditions: coffee powder 13.5 g, extract (beverage) mass 50 ± 5 g g (double shot) according to *espresso* coffee preparation standard (Petracco, 2001).

Ion exchange softened water (bicarbonate: 200 mg/L residual total hardness: 4 °F) was used. Two different methods were used to quantitatively determine the *espresso* foam:

1) the whole beverage (double shot) was percolated into a 100 mL graduated cylinder and the foam volume recorded after 15 s at rest.
2) The whole beverage (double shot) was percolated into a 100 mL separatory funnel. After 15 s at rest, the liquid was drained off, and the foam was gravimetrically determined (foam weight). (see Figure 1)

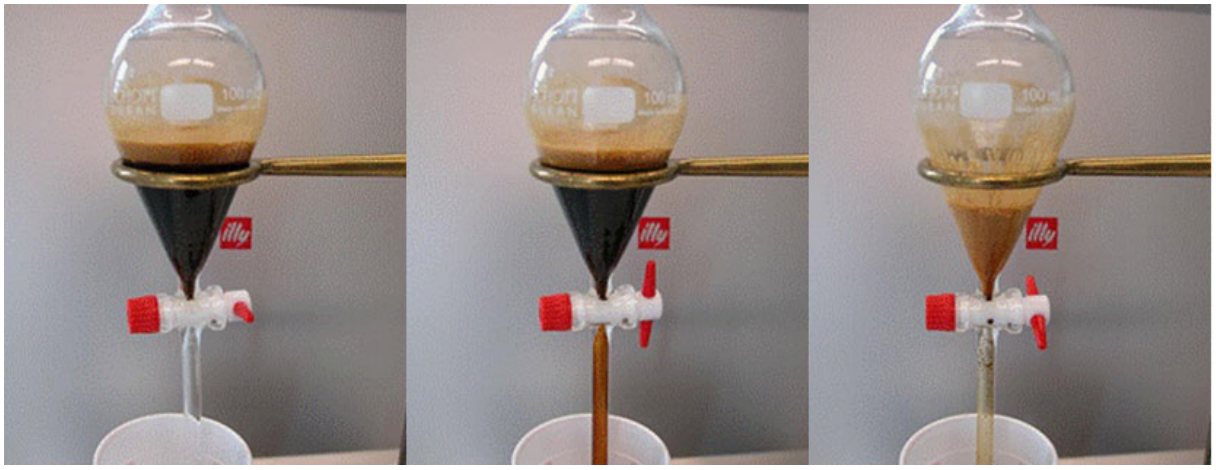

Figure 1. Experimental set up and sequence for *espresso* foam weight determination.

Carbon dioxide content determination (in duplicate) was paralleled by foam weight and foam volume determination (5 replicates) for each sample. Care was taken in order to standardize the experimental procedure and to reproduce the thermal history during measurements.

RESULTS AND DISCUSSION

In order to assess the proper range of percolation time and particle size distribution to maximize foam quantity, a preliminary test was carried out. Roasted coffee beans were ground in a wide range of particle size distribution and the corresponding *espresso* beverages (50 ± 5 g) were prepared. Foam weight was determined for each prepared sample. In Figure 2, the foam weight as a function of flow (in terms of beverage mass/percolation time) is reported. High foam weight values were obtained by selecting experimental conditions which ensure a percolation time range 25-28 s.

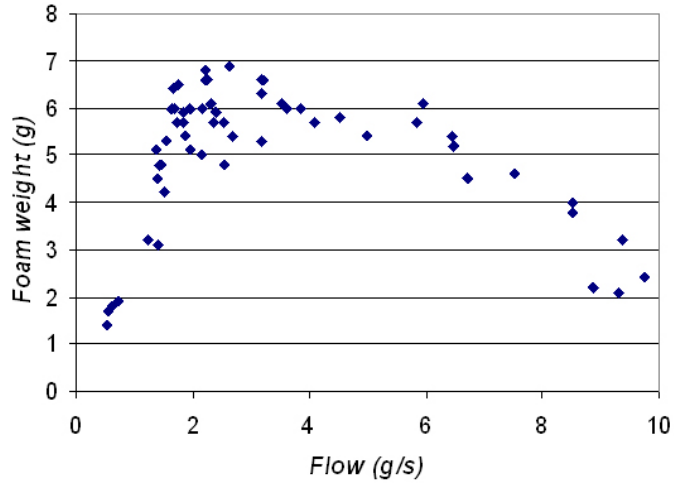

Figure 2. *Espresso* coffee foam weight as a function of beverage flow.

These findings strongly confirm the importance of the coffee/water contact time as far as the foam is concerned. Under-extracted or over-extracted *espresso* coffee beverages, due to coffee cake porosity, are characterized by low amount of foam. This is in full agreement with reported observations (Illy and Viani, 2005).

In Figures 3 & 4 foam weight and foam volume are reported as a function of carbon dioxide content, respectively. As clearly shown in the figures, the correlation is very good. In spite of different experimental conditions, the measured carbon dioxide content is within the range reported by Anderson et al. (2003) for Arabica coffee. Experimental data put in evidence the importance of the carbon dioxide content as far as the *espresso* coffee foam is concerned.

It has been reported that the most rapidly varying property of the *espresso* coffee after brewing is gas content (Illy and Viani, 2005). Gas release occurs owing to the sudden pressure drop undergone by the beverage when issuing out from the *espresso* machine. Dissolved gases, mainly carbon dioxide, quickly effervesce in the cup, and bubble up to build a layer of froth. (Illy and Viani, 2005).

This view, however, seems far to be a conclusive mechanism description for *espresso* coffee foam formation.

Taken into the account the carbon dioxide present in the coffee dose, only, it may be hypothesised that during *espresso* coffee brewing, the CO_2 water solubilization at high pressure and temperature lead to a carbon dioxide supersaturation conditions into the beverage. In fact, assuming about 61 mg of carbon dioxide per dose (13.5 g of roast & ground coffee) available to be solubilized into about 50 mL of pure water it is possible to obtain a CO_2 concentration equal to 1.22 g/L which is ca. three times lower than the carbon dioxide solubility at a pressure of 9.2 bar and at a temperature of 100°C (Muller et al. 1988; Duan and Sun, 2003) but ca. 2 times higher than the solubility at a pressure of 1 bar and at a temperature of 60 °C (Perry, 1984). Under this hypothesis, the effervescence observed during *espresso* brewing could be related to bubble formation by nucleation and bubble rise. Micronic solid particles and sub-micronic cell wall fragments, present in the beverage (Illy and Viani, 2005), may act as nucleation site. Moreover, differently from beer and Champagne wine, the small volume of an *espresso* beverage offers a limited length (about 1.5 - 2 cm in a standard *espresso* coffee cup) for bubble rise. This two conditions could be related to the size of the tiny bubbles characterizing *espresso* coffee foam.

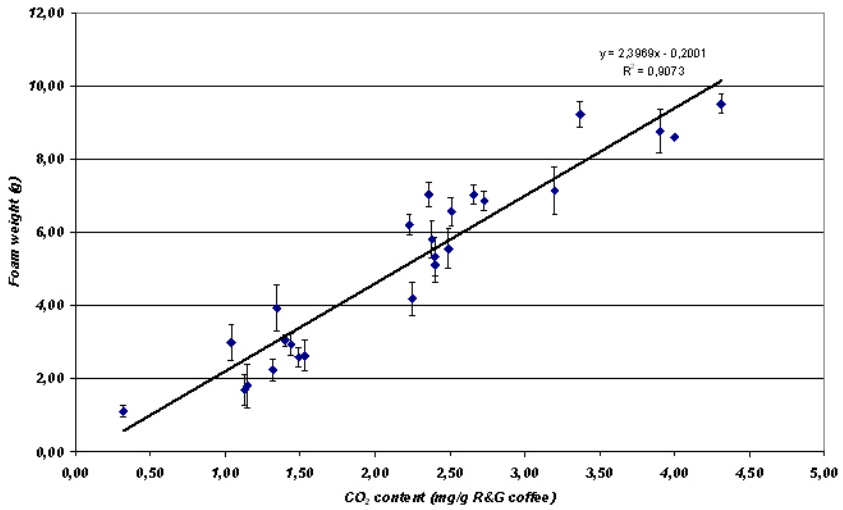

Figure 3. *Espresso* coffee foam weight as a function of carbon dioxide content.

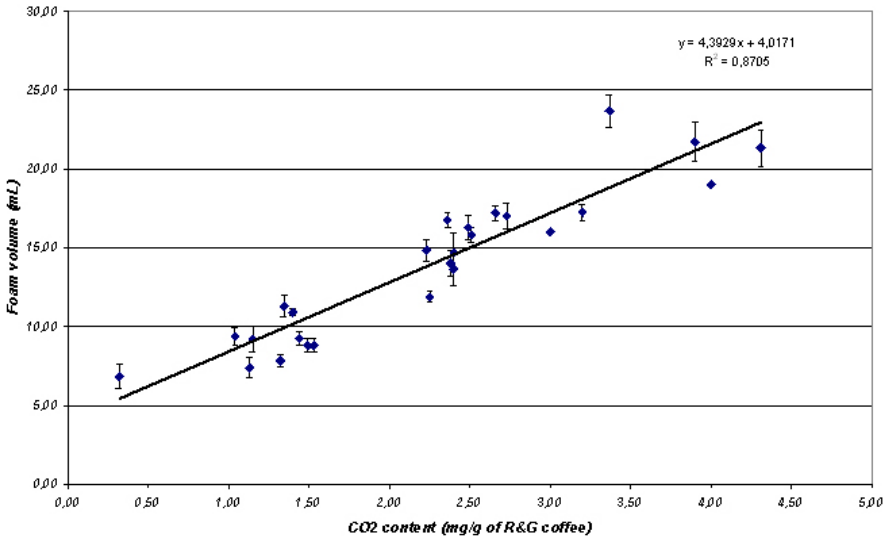

Figure 4. *Espresso* coffee foam volume as a function of carbon dioxide content.

As far as the instability of the *espresso* coffee foam is concerned, it has to be stressed that the relatively high temperature of the beverage may induce the reduction of the film thickness between bubbles by evaporation of water. It is conceivable that the foam rupture on the top of an *espresso* coffee is probably mainly ascribed to this mechanism rather than disproportionation.

The experimental data, expressed in terms of foam density as a function of carbon dioxide content, are reported in Figure 5.

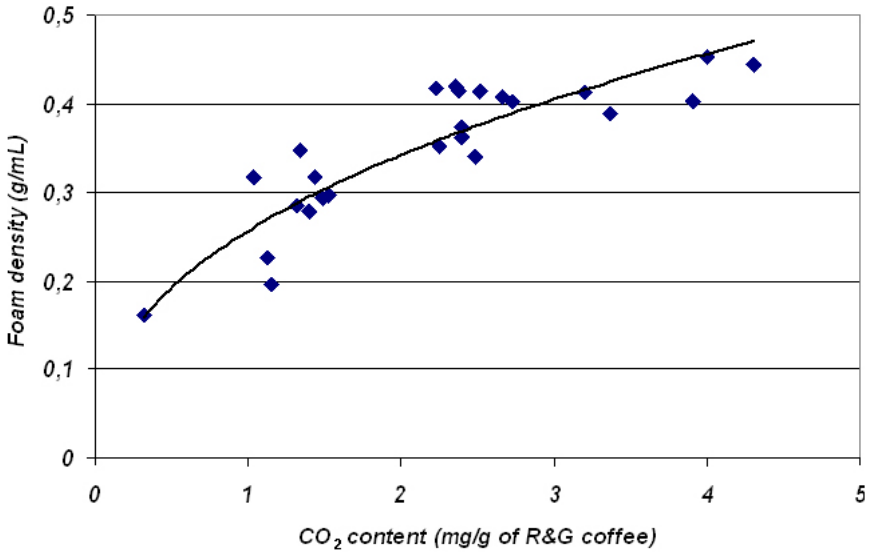

Figure 5. *Espresso* coffee foam density as a function of carbon dioxide content.

Foam density values at carbon dioxide content higher than 2.5 mg/g are within the range of foam density, 0.40-0.60 g/mL, reported for whipped cream (Campbell, 2003). In all cases, *espresso* coffee foam density values are lower than those reported for milkshake (0.90-0.95 g/mL) (Campbell, 2003).

CONCLUSIONS

By using two different foam quantitative methods, it has been possible to "measure" the importance of carbon dioxide as main gas phase of the *espresso* coffee foam.

The experimental data are consistent with the possibility of carbon dioxide supersaturation conditions in the beverage as the driving force for the *espresso* coffee foam formation. This hypothesis permits to explain several features like, for instance, the foam reduction observed by using very soft waters, the foaming behaviour of Robusta blends (containing almost the double of carbon dioxide according to Anderson et al., 2003), the foaming behaviour of darker roasting degree and the importance of the foam as a signature of optimal preparation conditions in terms of pressure, temperature, particle size etc.

Although food foam density is dependent on temperature, composition and processing factors, the preliminary comparison of the present data with those reported in literature suggests a possible role played by *espresso* coffee foam in the texture perception. In this perspective, a rheological characterization of the *espresso* coffee foam could be of interest.

Of course, the present investigation is just a preliminary work towards the understanding of the *espresso* coffee foam formation mechanism. In fact, in the lack of further studies, the formulated hypothesis and subsequent discussion have to be considered as a speculative activity.

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Review of Coffee Aroma Chemistry

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SUMMARY

The state-of-the art in coffee aroma chemistry will be reviewed. This includes the discussion of publications and patents in the area of aroma analytics, character impact flavour compounds and other key ingredients that relate to product quality as well as of flavour formation and chemical reactivity of odorants during processing and shelf-life. Coffee aroma is a complex mixture of more than 1000 volatile compounds (e.g. Nijssen et al., 1996; Vitzthum, 1999). The application of the 'Odor Activity Value' concept resulted in 23 character impact compounds, for which a contribution to the overall coffee aroma has been shown using reconstitution and omission experiments (Grosch, 1999). Coffee aroma reconstitutes have been established exhibiting a good base coffee character (Mayer et al., 2000). However, they cannot mimic the character of various coffee types nor describe the whole coffee aroma sensation. Recently, a predictive mathematic model was developed showing a good correlation between sensory profiling and analytical differences in chemical composition by means of PTR-MS (Lindinger et al., 2005). This novel approach has been proven capable in differentiating the aroma profiles of various espresso coffees. Other approaches considered the identification of new aroma and taste ingredients as well as certain physico-chemical phenomena to better understand cup quality (Kerler et al., 2004; Navarini et al., 2004; Frank et al., 2006). Sulfur compounds are known to be responsible for the fresh, roasted character of coffee. Their formation upon roasting (Milo et al., 2001) and degradation during processing and shelf life will be covered. The latter aspect relates to thiol binding sites in coffee solids (Müller and Hofmann, 2005), aroma-aroma reactions and ways how to preserve them in coffee products. Sulfur compounds are also excellent markers that can be used to monitor the freshness of coffee products as a function of the presence of oxygen during processing and/or shelf life as well as the quality of the packaging material.

COFFEE AROMA ANALYTICS & SENSORY-ANALYTICAL CORRELATION

Coffee aroma is a complex mixture of more than 1000 volatile compounds (e.g. Nijssen et al., 1996; Vitzthum, 1999) and thus makes aroma analysis a challenging task. This is especially true when it comes to correlating analytical with sensory data. The Aroma Value Concept (OAV) developed by Grosch and co-workers has been the most comprehensive approach to evaluate coffee aroma, resulting in 23 key aroma compounds for which a contribution to the overall coffee aroma has been shown (e.g. review by Grosch, 2001). Coffee aroma models that have been established with these key aroma compounds were found to be close to the aroma of a filter brew (Mayer et al., 2000; Audouin, 2002). However, models could not yet mimic the character of various coffee types nor describe the whole coffee aroma sensation.

One constraint of the OAV concept is that psychophysical parameters such as aroma-taste and aroma-matrix interactions, in-mouth aroma release and aroma burst are not taken into account. The significance of these parameters has been illustrated by several examples such as the importance of the viscosity of the espresso coffee in the mouthfeel sensation (Navarini et al., 2004), the significance of organic acids for the attenuation of the Robusta-like coffee character (Zehentbauer et al., 2004), as well as the dependence of the overall intensity on the

initial aroma burst (Buettner and Montserrat, 2004; Zeller et al., 2004). These psycophysical phenomena are important factors in the understanding of the whole complexity of coffee flavour perception.

Using multivariate statistical analysis of data obtained by PTR-MS measurements of coffee headspace, a mathematical model has been developed for the first time, which can predict the sensory profile of various espresso coffees (Lindinger et al., 2005). Chemometrics combined with the metabonomics approach make use of a similar concept and aims at identifying marker compounds that can be linked to quality characteristics of coffee. Using various complementary analytical techniques such as GCxGC/TOF-MS, LC-QTOF-MS, and PTR-MS, a large data set of both volatiles and non-volatile compounds is targeted.

In conclusion, sensory-analytical correlation remains still a challenge, especially when it comes to identifying causal links. Investigations into classical aroma and taste chemistry as well as new trends such as chemometrics are required to face this challenge.

FLAVOUR FORMATION

Biomimetic in-bean experiments have been performed to study the formation of key aroma compounds from precursors in a 'natural' coffee bean environment (Milo et al., 2001). Correlations between various water soluble precursors and odorants could be established. An example is the group of guaiacols that was found to be highly correlated with the amount of feruoylquinic acids in the green coffee. In contrast, furfurylthiol was the only aroma compound which was generated from non-water soluble precursors.

An overview of formation pathways of key aroma compounds in coffee was presented. Besides the study of Milo et al. (2001), a large number of model studies conducted under roasting conditions was taken into account (e.g. Holscher et al., 1992; Amrani-Hemaimi et al., 1995; Yaylayan and Keyhani, 1999; Hofmann et al., 2000; Hofmann and Schieberle, 2001a). It can be concluded from these studies that detailed knowledge exists about flavour formation during roasting processes. However, as was shown for 2-furfurylthiol as an example, some formation pathways that were validated in model reactions may not occur during coffee roasting. This is due to the fact that the coffee bean environment is much more complex as compared to food models. As a consequence, additional precursor studies under 'real' coffee bean conditions are required. For example, the use of isotope labeled precursors that are incorporated into the green coffee bean prior to roasting would be a powerful approach to increase knowledge about flavour formation during coffee roasting.

AROMA STABILITY & SHELF LIFE

Sulfur compounds are known to be responsible for the fresh, roasted character of coffee. Chemical reactions that are involved in the degradation of odour active thiols were emphasized. Such reactions concern the binding of thiols to the coffee matrix through nucleophilic addition either to phenolic compounds (Kraehenbuehl et al., 2004; Müller and Hofmann, 2005) or melanoidins (e.g. CROSSPY identified by Hofmann and Schieberle, 2001b). In addition, thiols can undergo radical induced oxidative reactions (Hofmann et al., 1996; Munro et al., 2003).

Charles-Bernard et al. (2005) studied the impact of various additives (e.g. nucleophiles, antioxidants) on the rate attenuation of thiol degradation in coffee brew models. The authors showed that the strongest attenuation can be achieved by using a combination of a competitor nucleophile (e.g. hydroxylamine) and storage of the brew model under inert atmosphere.

Sodium sulfite that has both nucleohpilic and antioxidative properties also showed a strong stabilizing effect on thiols. In addition, they found that the reactivity of aromatic and aliphatic thiols differs, the former group being more susceptible to oxidative degradation, whereas the latter group mainly reacts via nucleophilic addition.

Based on their importance to the aroma of coffee and their high sensitivity, aroma-active thiols (e.g. methanethiol, 2-furfurylthiol, 3-methyl-2-buten-1-thiol) as well as certain aldehydes and ketones were found to be good freshness markers used to monitor coffee aroma shelf life (e.g. Holscher and Steinhart, 1992; Czerny and Schieberle, 2001; Cappuccio et al., 2001). Poisson et al. (2006) recently showed that dimethyl sulfide is a very sensitive freshness marker that can be linked with rates of flavour fading in single sealed coffee portions that were filled at different residual oxygen levels.

OUTLOOK

Further research in the area of coffee aroma chemistry is suggested to focus on the following aspects:

- Sensory-analytical correlation: additional studies should address a deeper understanding of perceptual and cognitive interactions, the biochemistry of receptors for odour and taste molecules, investigations into taste chemistry as well as new analytical approaches (e.g. chemometrics, identification of new key compounds).
- Flavour formation: the use of isotope labeled precursors in biomimetic in-bean experiments would be a powerful approach to increase knowledge about the contribution of precursors to the formation of certain key aroma compounds in the complex coffee bean environment.
- R&G ageing is well understood. Challenges exist in view of improving shelf life of e.g. canned liquid coffee as well as of more detailed understanding of prevailing degradation reactions.

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Studies on Coffee Aroma Precursor Compounds

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SUMMARY

This study attempts to elucidate the contribution of single flavor precursor compounds to coffee aroma by using model roasting experiments in simple glass reaction vials. A set of precursor compounds that is necessary to produce a fine R&G-like coffee aroma upon model roasting has been identified. By reducing the number of precursors used in the set the fine coffee-like aroma turned into roasted and burned aroma notes. The model system was extended by using extracted coffee beans as reaction chamber for the precursor compounds. The influence of different precursors on the flavour profile was tested and omission experiments of single precursor compounds were performed. Different sets of precursor compounds have been identified leading to Arabica and Robusta-type flavour profiles, respectively. pH of green coffee was identified as one of the main drivers for the flavour differences between Arabica and Robusta in addition to precursor compound composition.

INTRODUCTION

The generation of flavor in roasted foods and the development of brown color is due to chemical reactions between carbonyl groups in reducing sugars and amino groups, the Maillard reaction. Ho et al. (1993) have shown that the majority of potent aroma compounds in coffee stem from the Maillard reaction. A look at data of green coffee chemical composition gives a hint at important precursors for coffee aroma (Table 1). These precursors are consumed during coffee roasting while other compounds nearly remain unchanged such as caffeine. The elucidation of the individual contribution of single precursor compounds is subject of this study. The approach chosen is dual: first model roasting trials with dry mixtures of precursor compounds in glass vials have been performed, secondly extracted green coffee beans (depleted of flavor precursor compounds) have been used as a more realistic reaction chamber into which dissolved mixtures of precursor compounds have been re-infused and finally roasted to produce “real” roasted coffee beans.

MATERIALS AND METHODS

Green coffee extraction

Green coffee (Arabica) was extracted for 4 h at 80 °C with 3 times replacement of extraction water. The pooled extracts have been freeze-dried. Extracted beans were dried in a convection drying oven at 41 °C for 22 h.

Table 1. Green coffee chemical composition (Ho et al., 1993; De Maria et al., 1996; Clarke and MacRae, 1985; Flament, 2002; Parliament, 2000; including own unpublished results).

	% Content (dry weight)		
	Robusta green	Arabica green	Arabica roasted
Alkaloids			
Caffeine	2.0	1.0	1.0
Trigonelline	1.0	1.0	0.5
Minerals	4.5	4.5	4.5
Acids			
Chlorogenic	9.0	7.0	3.0
Quinic	0.5	0.5	1.0
Aliphatic	2.0	2.0	3.0
Sugars			
Sucrose	4.0	7.0	0.2
Others	0.5	0.5	0.3
Polysaccharides			
Arabinogalactan	18.0	15.0	13.0
Mannan	22.0	22.0	20.0
Cellulose	8.0	8.0	8.0
Lignin	2.0	2.0	2.0
Protein			
Polymer	12.0	11.0	8.0
Peptides/ Amino acids	1.0	8.0	0.0
Lipids	10.0	15.0	17.0
Melanoidins	0.0	0.0	18.0

Model roasting in reacti vials

Small vials designed for the production of reaction flavours ("reacti vials") were filled with 100 mg of a precursor mixture and closed with Teflon septa. They were placed in a preheated drying oven for 10 min (225-230 °C).

Preparation of precursors

Chlorogenic acid fraction and a crude protein fraction

150g Amberlite XAD-7 HP is soaked in ethanol for ca. 1 hour and filled into a glass column (50 * 5 cm), then rinsed with ca. 2l of distilled water. 300 g of green coffee extract was adjusted to pH 4.5 with diluted phosphoric acid and applied onto column. Subsequently, the column is rinsed with ca. 1L of distilled water (note: the aqueous eluate is kept for the isolation of the protein fraction). The column is then eluted with ca. 1.2l of ethanol. The ethanolic eluate (chlorogenic acid containing fraction) is then freeze-dried.

Crude protein fraction

Isolation of protein from the aqueous eluate of the chlorogenic acids isolation:

- the aqueous eluate from the isolation of chlorogenic acids (each ca. 1l volume) is stirred with 2 l of ice-cold ethanol
- the precipitate is allowed to settle, the liquid decanted.

- the precipitate is centrifuged and freeze-dried (= crude protein fraction)

Mannan

Mannan is prepared by enzymatic cleavage of a commercially available galactomannan in order to reduce the chain length of the carbohydrate molecules. 20 g galactomannan (Gum Locust Bean from seeds of Ceratonia siliqua) are dissolved within ca. 10 min under constant stirring in a solution of 50ml gamanase (1ml conc. enzyme from Novo Nordisk A/S; in 100ml dist. H_2O) and ca. 500ml of water, the solution is then heated on a hotplate until boiling and filtrated. The filtrate is freeze-dried. Yield: ca. 16 g.

Trigonelline maleate

Application of ion exchange chromatography to transform the trigonelline*HCl into the maleate form in order to remove chloride: 225 g of Amberlite Ira-68 are immersed in ca. 500 ml of ethanol, transferred into a glass column and rinsed with ca. 1l of water. A solution made up of 30 g DL-malic acid, 25.2 g KOH and 600 ml H_2O is then eluted through the column dropwise. The column is further rinsed with 2l of water. Now, a solution of 22.5 g trigonelline*HCl in 400 ml water is eluted through the column, rinsed with 750 ml of water and all eluates are freeze-dried. Yield: 22.5 g. To improve the purity of the freeze-dried eluate, the residue is stirred with acetone, filtrated over a Büchner funnel and freeze-dried. Yield: ca. 20 g of crystalline white substance (= trigonelline maleate).

Composition of amino acid mixture: 10 g of the mixture is made of: 1.63 g aspartic acid, 0.52 g serine, 2.85 g glutamic acid, 0.59 g glycine, 0.17 g histidine, 0.31 g arginine, 0.21 g threonine, 0.59 g alanine, 0.55 g proline, 0.21 g cysteine, 0.28 g tyrosine, 0.28 g valine, 0.14 g methionine, 0.28 g lysine, 0.21 g isoleucine, 0.28 g leucine, 0.45 g phenylalanine and 0.45 g tryptophane.

Preparation of re-infused coffee beans

Preparation of artificial precursor mixes:
1. weigh amounts from Tables 3 and 4 into glass beakers; make up to 120 g with water (resulting pH ca. 2.7 to 2.8)
2. adjust pH to 5.0 or 6.2 with KOH
3. re-infuse into 120 g of depleted Arabica beans for 2 h at 80 °C
4. wash with 50 ml of water
5. dry in convection oven at 41 °C for 22 h
6. roast to ca. 10 Lange for Arabica-type or 20 Lange for Robusta-type beans.

Measurement of aroma compounds by GC-MS

Isolation of aroma compounds from the coffee brew is achieved by liquid/liquid extraction with dichloromethane. Subsequently the extract is analyzed by GC/MS. Quantification is achieved using internal standards. Instrumentation used: GC-MS system consisting of: Cooled Injection System KAS 3 (Gerstel), autosampler MPS 2 (Gerstel), gas chromatograph 6890 (Agilent), mass spectrometer 5973 (Agilent), GC column: HP1701 (Agilent); length 30 m; ID 0.32 mm; film thickness 1.00 µm, Carrier gas: Helium. GC-MS parameters: Flow mode: constant flow (2.0 ml/min column flow), Temperature program: Initial temperature: 35 °C (1 min), Rate A: 6 °C/min to 230 °C (15 min). List of analytes according to Blank et al. (1992).

RESULTS AND DISCUSSION

Model roasting trials in reacti vials

For the evaluation of the aroma generation by various precursors, model roasting trials were employed and the odour generated was evaluated by "sniffing". It could be demonstrated that even under simple reaction conditions a good and differentiated coffee aroma can be produced from a range of naturally occurring precursor compounds. The success of this approach made it possible to study the impact of single precursors and their collaborative effects. A fine R&G-like coffee aroma was produced with the set of precursors as outlined in Table 2a. After roasting, the samples were allowed to cool down to room temperature for ca. 15 min, opened and sniffed in an informal tasting session. The taste of the samples was not evaluated. Trial A W1 (cf. Table 2a/b) is a replicate of A and showed that reproducible results are obtained with the model roasting system.

Trial A uses the full set of precursor compounds which has been suggested by the green coffee chemical composition (cf. Table 1).

- caffeine
- trigonelline
- chlorogenic acid fraction (isolated from green coffee extract)
- sucrose
- mannan
- arabinogalactan
- protein
- (free) amino acids

Table 2. a: Model roasting trials with reacti vials (A W1-W7). b: Sensory impact of precursors (A W1-W7).

a)

	A	A W1	A W2	A W3	A W4	A W5	A W6	A W7
Trigonellin - maleat	40 mg	40 mg	40 mg	40 mg	40 mg	40 mg	40 mg	**0**
Caffeine	50 mg	50 mg	50 mg	50 mg	50 mg	**0**	**0**	**0**
CGA - Fraction	225 mg	225 mg	**0**	225 mg	**0**	**0**	**0**	**0**
Sucrose	650 mg	650 mg	650 mg	650 mg	650 mg	650 mg	650 mg	650 mg
Arabinogalactan	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg
Mannan	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg	**0**	**0**
free Amino Acids	75 mg	75 mg	75 mg	75 mg	75 mg	75 mg	75 mg	75 mg
Proteinfraction	200 mg	200 mg	200 mg	**0**	**0**	**0**	**0**	**0**
Total	1440 mg	1440 mg	1215 mg	1240 mg	1015 mg	965 mg	865 mg	825 mg

b)

	A	A W1	A W2	A W3	A W4	A W5	A W6	A W7
	fresh R&G coffee, espresso-like	like A	roasty, spicy, less coffee character	roasty, spicy, less coffee character	roasty, spicy, sweet, less coffee character	like A W4	like A W4	like A W4

It can be seen in Table 2 a/b that only A and A W1 with the full set of precursors provided a good fine coffee-like aroma with dark Espresso-like characteristics. The model roasting trials reported here are a proof-of-principle that by using the set of green coffee aroma precursors previously identified, a good quality R&G-like coffee aroma is generated even in the absence of the bean environment.

Trials A W2, W3 gave roaste and spicy aroma characters with less coffee-like aroma than A. The omission of the chlorogenic acid fraction which potentially also contains glycosidically bound precursors (Weckerle et al., 2002; Weckerle et al., 2003; Degenhardt et al., 2004) resulted in a less fine coffee character. However, the roastiness was still considerable. The contribution of the chlorogenic acid fraction with the glycosides to the coffee aroma was found to be relevant for the coffee aroma since it imparted a fine aroma. From these findings it is unclear whether this effect must be attributed to the chlorogenic acids, glycosides or other precursor compounds present in this complex fraction isolated from green coffee.

Another step away from the typical coffee aroma was done when both chlorogenic acids as well as protein were omitted from the precursor mixture (A W4, cf. Table 2 a/b). Additional sweet aroma notes which are untypical for coffee have been perceived.

A W5-W7 in comparison to A W4 (cf. Table 1 a/b) proved that caffeine, trigonelline and mannan are relatively ineffective precursors in this model system. Especially caffeine is generally not regarded as an aroma precursor.

As a next step to "closer to reality", depleted (= extracted) green coffee beans have been used as a reaction chamber for coffee flavor precursors. Depleted beans (green Arabica extracted at 80°C for 4h with 3 times replacement of extraction water) have been re-infused with an "artificial precursor mix" aiming at an Arabica-type flavour profile (cf. Table 3). The pH value of the mixture was adjusted to 5.0 prior to re-infusion into the bean matrix. After drying of the beans, the green coffee was roasted to a roast color of ca. 10 Lange. The pH adjustment is required to direct the flavour generation pathways towards distinct flavour profiles.

Informal sensory analysis of the respective coffee brews showed a relatively good match with an average Arabica-type coffee. Aroma analysis data show that most of the aroma relevant compounds correlate well with a typical Arabica-type flavour profile.

The next goal was the shift of the flavour profile towards a Robusta-type flavour (harsh, rubbery). To achieve this some changes have been made to the mixture. The main differences are (cf. Table 4):

1. increase of caffeine
2. presence of chlorogenic acid fraction together with ferulic acid
3. reduction in organic acids
4. presence of methyl isoborneol (MIB)
5. pH level of 6.2 of the mixture

Most important is the pH level of the infusion mix. In the case of the Robusta mix, it was adjusted to pH 6.2 in comparison to 5.0 for the Arabica mix. This difference in green extract pH accounts for the majority of the flavour differences. The Maillard reaction proceeds in different reaction pathways at different pH levels. The addition of methylisoborneol increased the Robusta recognition by imparting musty aroma notes which are normally not typical for Arabicas. Aroma analysis data show that most of the aroma relevant compounds correlate well with a typical Robusta-type flavour profile (Figure 1).

Table 3. Precursor mix composition for Arabica-type (for experimental conditions refer to Materials and Methods section).

Components	[g]
Alkaloids	
Trigonelline maleate	0.80
Caffeine	1.00
Sugars	
Arabinose	0.15
Sucrose	13.00
Polysaccharides	
Mannan	4.00
Protein / Amino acids	
Free amino acids	1.50
Other acids	
Citric * H2O	1.91
Malic	0.89
Quinic	0.70
Phosphoric 85%	0.20

Table 4. Precursor mix composition for Robusta-type (for experimental conditions refer to Materials and Methods section; abbreviation: MIB = methyl isoborneol).

Components	[g]
Alkaloids	
Trigonelline maleate	0.40
Caffeine	2.00
CGAs	
CGA-fraction	6.50
Sugars	
Arabinose	0.15
Sucrose	5.00
Polysaccharides	
Mannan	4.00
Protein / Amino acids	
Free amino acids	1.50
Other acids	
Ferulic acid	0.05
Citric * H2O	0.98
Malic	0.45
Quinic	0.36
Phosphoric 85%	0.10
Mib	Trace

To further study the impact of single precursor compounds on the overall flavour, compounds from Table 3 have been omitted from the mixture and their sensory impact has been shown. The mix was reduced to only sucrose and organic acids. The resulting coffee brews were thin and acidic and not roasted, coffee-like. An analysis for aroma compounds by GC based on liquid-liquid partitioning of a coffee brew with dichloromethane showed that the content of pyrazines, furfurylthiol and other sulfides are low in the present coffee. These classes of compounds seem to be main drivers for the roasted character of coffee.

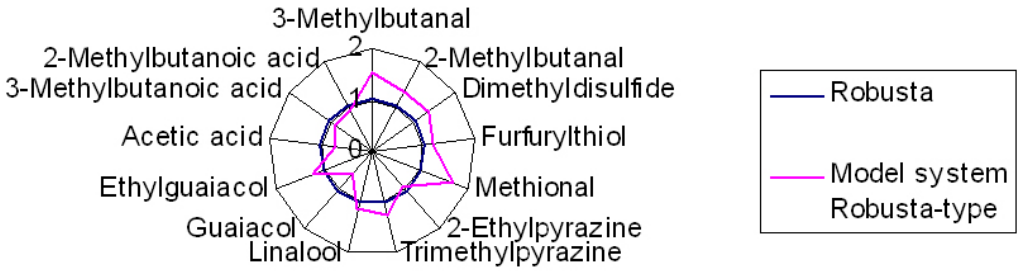

Figure 1. Aroma analysis results of Robusta coffee (20 La) in comparison to model roast mixture with composition from Table 4.

The addition of the chlorogenic acid (CGA) fraction to the Arabica-type precursor mix in Table 3 showed relatively low impact on the aroma quality of the Arabica-type model system. The resulting brews appeared a little more "dirty". There was a difference dependent on the type of chlorogenic acid material used. When a CGA fraction which was isolated from green coffee extract was used, more phenolic aroma compounds (guaiacols) are generated. This is explained by the fact that the CGA fraction contains 5-FQA (5-feruoyl quinic acid), the main precursor for phenolic aroma compounds via ferulic acid (Flament, 2002). By replacement of the CGA fraction with commercially available neo-chlorogenic acid (n-CGA), the content of phenolic compounds is reduced. The content of guaiacols are equal for n-CGA as well as without CGAs. n-CGA is not a precursor for guaiacols under the conditions of the test.

The omission of amino acids from the precursor mixes in Tables 3 and 4 resulted in a disappearance of the roasted character. The significance of amino acids for the generation of pyrazines and Strecker aldehydes such as the methylbutanals and methylpropanals has been demonstrated. Without the presence of amino acid-type precursors, the typical roastiness of coffee can not be achieved.

In summary, the presented model roasting proved that the set of precursor compounds required to generate coffee flavour is known. Furthermore, the fine tuning of green coffee pH levels is necessary to direct the flavour towards Arabica or Robusta-types of coffee. This model systems offers excellent opportunities to study the impact of single flavour precursor compounds by performing omission trials with single compounds or groups of compounds. Green coffee pH levels have been identified as major drivers for flavour differences found in different coffees.

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Key Aroma Compounds of Soluble Coffee

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SUMMARY

Relatively few data on the volatile composition and sensorial relevant fraction of soluble coffee has been published, reinforcing the importance of a better understanding of its aroma composition, obtaining more details on the subtle differences of applicable productive techniques and among products. Considering an approach that understands both analytical and sensorial techniques, this work aims to identify the main components of soluble coffee aroma and their sensorial impacts, enabling the focused development of technological alternatives for aroma enhancement, potentially reflecting on the economics of its commercialization. The volatile components were isolated via HS-SPME technique and then identified by GC-MS. The GC-FID/O technique was used for the volatiles qualitative sensorial description by the Free Choice Profile method. A comparison with data obtained using an electronic nose proved it to be a functional tool to classify instant coffees. On the seven samples tested, up to 414 volatile compounds were analytically detected, being 24 quantified, 132 detected on the sensorial analysis, indicated to be of sensorial impact, amidst which 22 were quantified.

INTRODUCTION

Generally speaking, several volatile components present in foodstuffs have little sensorial relevance and the recent research methodologies applied have made possible to researchers the identification and quantification of a comparatively reduced number of key aroma components, decisive of the odour finally sensed (Blank et al., 1992; Semmelroch et al., 1995). On this particular, among the employed techniques, gas chromatography coupled with olfactometry has been applied for several foods, including roasted coffee (Bassoli, 2006). Also, special sensors array instruments, called electronic noses, have been used, attempting to help or reproduce, notwithstanding partially, the answer of the human sense of smell to a composed volatile (Bassoli, 2006). During soluble coffee production, several transformations and aromatic losses happen, which combined to the intrinsic thermal processes, modify quantitative and qualitatively its volatile fraction composition, differentiating it from a reference brewed coffee. Coffee aroma is composed of a great variety of functional chemical groups, where the composition depends on factors such as species and variety, growth conditions and crop, storage, roasting degree, let alone all other process conditions (Clarke and Macrae, 1985). More than eight hundred volatile compounds have been identified in roasted coffees, positioning the coffee beverage as a high complexity drink in terms of volatile composition (Shibamoto, 1992). However, contrarily to roasted coffee, the volatile and sensorial relevant fraction composition of soluble coffee has been studied, so far, at a lesser extent. This reinforces the need of a better understanding of its aroma composition, helping to clarify even the subtle but important differences among products and processing techniques that guide consumers` perception and preference (Haesselbarth and Ullrich, 2002). The present work adopted an integrated approach to study soluble coffee aroma relevance, by combining sensorial analysis with instrumental methodology, based on the identification of

the volatile fraction composition and the respective aromatic impacts. This was achieved using a HS-SPME (Viegas et al., 2004) extractive technique, gas chromatography coupled with mass detection (GC-MS) as identification technique and GC-FID/O olfactometry technique, for qualitatively describing the sensorial impact. Results were compared to the ones obtained with an electronic nose.

MATERIALS AND METHODS

Soluble coffee samples

Seven samples were tested as described in Table 1, all distinct amongst themselves, according to an expert sensorial panel initial evaluation.

Table 1. Soluble coffee samples analysed.

Sample	Origin	Remark	Raw materials	Roasting intensity
1*	Brazil	experimental	arabica	medium
2*	Brazil	experimental	arabica	medium
3*	Brazil	experimental	conilon	medium
4	Brazil	retail market	conilon / arabica	dark
5	Brazil	retail market	---	dark**
6	England	retail market	---	medium**
7	England	retail market	---	medium / light**

**Produced at Café Iguaçu R&D laboratory. **Estimated by sensorial analysis.*

Volatiles extraction – HS-SPME

2.8 grams of sample were directly weighted in a 20 ml vial. After proper sealing, it was heated for 10 minutes at 70 ºC, exposing then a SPME (DVB/CAR/PDMS) 50/30 fibre for 30 minutes in the headspace, followed by a desorption step, made directly at the GC injector port.

Chromatography conditions – GC-MS

A 6890N gas chromatograph coupled to a 5973 selective mass detector (Agilent) was used. Injection volume of 1 µl on splitless mode and with He (5.0) as carrier gas, injector temperature and time respectively of 250 ºC and 10 minutes were the analytical conditions adopted. A polar capillary column INNOWax (60 m x 320 µm x 0.25 µm) was used, with an oven temperature profile starting at 40 ºC then held for 5 min, raised by 4 ºC/min to 60 ºC, held for 5 min, raised by 8 ºC/min to 250 ºC, held for 3 min. The volatile components identification was primarily based on the NIST library v.2.0a (2002), followed by mass spectra interpretation. The quantitative determination used 25 external standards considered of aromatic impact in the consulted literature (see Table 2).

Sensorial analysis – GC-FID/O

For olfactometry (Ruth and Connor, 2001), a GC-FID chromatograph was connected to an ODO II olfactometer system (SGE). Retention times were monitored for comparison against the analytical determination. The OSME technique was used (Mcdaniel et al., 1989), with a semi-trained sensorial panel, applying the Free Choice Profile technique (Williams and Langron, 1984). Each of the 5 panel members selected described the aroma quality, properly logged, and registered the aroma intensities thru time in a direct time-intensity scale from 0 to

100. Retention times, peak areas, odoriferous intensity and time for each component of the Osmegrams were compiled. Multidimensional analysis was used for a statistical evaluation, performed by the Generalised Procrustes Analysis (GPA) (Oliveira and Benassi, 2003), Senstools v. 2.3.28 (Op & P Product Research).

Electronic nose analysis

2.8 grams of sample were directly weighted in a 40 ml vial. After proper closure, it was processed by a PEN2 e-nose model (Airsense Analytics) equipped with a 10 sensors array, sampling directly the headspace of each product. Total analysis time was 2 minutes per sample.

RESULTS AND DISCUSSION

Instrumental data

In the seven analysed samples, there were from 378 to 429 chromatographic peaks, totalising 414 different compounds. Those detected under a certainty level of 50% or above (Bassoli, 2006; National Institute Of Standards And Technology, 2002) accounted respectively for about two thirds of the total chemical classes and around half of the total number of compounds referenced to roasted coffees (Clarke and Macrae, 1985; Moreira et al., 2000). Under a certainty level of 60% or above (Bassoli, 2006; National Institute Of Standards And Technology, 2002) combined to mass spectra interpretation, a total of 160 compounds could be identified, ranging from 69 to 126 compounds each sample, compatible with the range of 84 to 116 reported for green coffees (Monroy, 2005). Twenty four volatile compounds were quantified (Table 2).

Furthermore, comparing with roasted and ground coffee data (Supelco, 2001) where 57 compounds were identified, 2-butanone, 3-methyl-butanal, 2-ethyl-hexanol, dimethyl disulfide, phenol, pyridine, pyrazine, 4-methyl-thiazole, 2,5-dimethyl-pyrazine, 2,6-dimethyl-pyrazine, 2,3-dimethyl-pyrazine and 2,3,5-trimethylpyrazine were identified on the soluble coffees tested as well. Twenty four volatile compounds were quantified, as shown in Table 2, highlighting 4,5-dimethyl-thiazole, identified with the employed technique. In general, the quantifiable components are also present in both green and roasted coffees, but, although concentrations found were coincident in some cases, they cannot be objectively compared with quoted references. By correlating raw materials composition to the experimental samples, it could be noticed that 1-methyl-piperidine, methional, 2-ethyl-1-hexanol, 3,4-dimethyl-2,5-furanedione, benzyl alcohol and maltol were characteristic of sample 1, obtained from arabica coffee. For both experimental samples produced with arabica coffees, ethyl-benzene and 3-methyl-2-buten-1-ol were commonly detected. The conilon based sample 3, presented 2-pyrrolidone and 2,4-ditert-butyl-phenol compounds. In the Brazilian retail samples, sample 4 could be associated to ethyl acetate and 1,5-dimethyl-1H-pyrrol-2-carbonitrile, while butanal, decane, 4,5-dimethyl-oxazole and 3-hexen-2-one were characteristic of sample 5. About English market samples, besides several components individually detected, associations were with acetone, 2,3-dimethyl-isoxazole, 1-(2-methyl-1-propenil)-pyrrolidone, ethyl-pyrazine and 1-(2-furanil-methyl)-1H-pyrrol. The retail samples had in common 3-methyl-butanal, 1-hydroxi-2-propanone, 4,5-dimethyl-thiazole, 2-methoxy-methyl-furane and 2n-propyl-pyrazine.

Table 2. Volatile compounds found in the soluble coffee samples related with literature.

Compound	Concentration range (mg/kg)	Referenced concentrations - green and roasted coffees (mg/kg)
2,3-butanedione	8.5 to 19.6	50 Indonesian (Grosch, 1996), 1.8 Colombian * (Ho et al., 1993), 50 Colombian * / 58.7 Kenyan (Grosch et al., 1996)
2,3-pentanedione	15.2 to 37.3	40 arabica / 50 robusta (Grosch et al., 1996)
Pyridine	14.3 to 46.9	49 roasted (Stofberg and Stoffelsma, 1981)
Pyrazine	1.9 to 6.6	2.8 roasted and ground (Ho et al., 1993)
4-methyl-thiazole	4.2 to 7.3	0.5 to 1.0 arabica / 0.8 to 1.5 robusta (Tressl, 1989)
2,5-dimethyl-pyrazine	18.8 to 36.8	25 to 35 roasted (Silwar et al., 1987), 17 roasted (Stofberg and Stoffelsma, 1981)
2,6-dimethyl-pyrazine	50.4 to 126	30 to 35 roasted (Silwar et al., 1987), 19 roasted (Tressl, 1989)
2,3-dimethyl-pyrazine	4.9 to 11.6	2.5 roasted (Ho et al., 1993), 5.3 roasted (Silwar et al., 1987)
4,5-dimethyl-thiazole	1.7 to 3.2	Not found
2,3,5-trimethyl-pyrazine	12.6 to 38.5	5.0 roasted (Hashim and Chaveron, 1996)
2-ethyl-3-methyl-pyrazine	16.9 to 41.2	1.1 roasted and ground (Ho et al., 1993)
2-furfuriltiol	3.2 to 29.9	1.1 arabica / 1.7 robusta (Semmelroch et al., 1995)
Acetic acid	288 to 442	Traces arabica (Van Der Stegen and Van Duijn, 1987)
Methional	2.7 to 12.1	0.2 Colombian / 0.1 Indonesian (Semmelroch et al., 1995), 0.2 roasted (Czerny and Grosch, 2000)
Furfural	70.3 to 438	22.5 roasted (Ho et al., 1993)
2,3-diethyl-5-methyl-pyrazine	4.2 to 10.6	0.06 to 0.1 arabica * / 0.3 robusta * (Grosch, 1998)
Linalool	5.3 to 11.0	0.73 Colombian (r) (Ho et al., 1993), 0.02 green (Holscher and Steinhart, 1995)
Isovaleric acid	21.4 to 52.3	13 green / 21 roasted (Wöhrmann et al., 1997), 16 to 41 arabica * (Schröder et al., 1997)
Guaiacol	19.3 to 65.7	4.2 arabica / 28.2 robusta (Semmelroch et al., 1995), 4.0 roasted (Czerny and Grosch, 2000)
Furaneol	26.7 to 71.4	109 arabica / 57 robusta (Semmelroch et al., 1995)
4-ethyl-guaiacol	6.0 to 26.8	21 arabica / 4.1 arabica * (Czerny and Grosch, 2000)
Cis-isoeugenol	2.9 to 16.1	0.12 Colombian * (Ho et al., 1993)
Hydroxi-methyl-furfural **	14.4 to 183	Not found
Vanillin **	0.1 to 2.1	4.8 Colombian / 16.1 Indonesian (Semmelroch et al., 1995)

**Roasted. ***Not detected by sensorial analysis.*

Sensorial analysis

The results showed that all samples were properly characterized and discriminated (Figure 1). Multivariate studies in sensorial analysis are generally two-dimensional. In this work, the choice was to study the first 3 dimensions, responsible for the largest variances, respectively 20.62%, 12.30% and 11.17%. When using the Free Choice Profile, the variability explained usually is smaller than the one observed in a conventional quantitative descriptive analysis, for instance. The total variance explained of 44.09% is comparable to Narain et al. (2003), where the explained variability found was 25%. The repeatability of each panelist was verified through its residual variance, in accordance with the value considered acceptable, close to 1.0% (Op & P Product Research). Correlations with the three defined dimensions for the GPA were quite distributed, heterogeneous and relatively small, being all samples were very well separated (Figure 1). The panelists sniff detected 132 compounds of aroma impact, being 22 quantified (Table 2). The consensus for the terms used among the panelists for all the seven samples resulted in 71 sensorial descriptors, not shown. Reviewing the literature references for roasted coffee (Bücking, 1999; Schenker et al., 2002; Grosch, 2001) and coffee beverage (Mayer et al., 2000) (Table 3), the amount of key aroma compounds varied between 15 and 40, equivalently to the 69 key aroma compounds herein identified, also comparable to data earlier obtained (Sanz et al., 2002), that indicated 40 key aroma compounds combining both soluble and filtered coffees. Paralleling with 45 compounds of high aromatic impact in coffee (Blank and Sen, 1992; Semmelroch et al., 1995; Moreira et al., 2000; Semmelroch and Grosch, 1996; De Maria et al., 1999; Rowe, 2000; Jameson, 2001; Schenker et al., 2002; Rowe, 2002), 27 of those were identified in the samples tested in this work. Nevertheless, vanillin and hydroxi-methyl-furfural were not detected, probably due to their intrinsic retention times that lead them into a particularly abundant chromatogram region, consequently troubling sensorial identification.

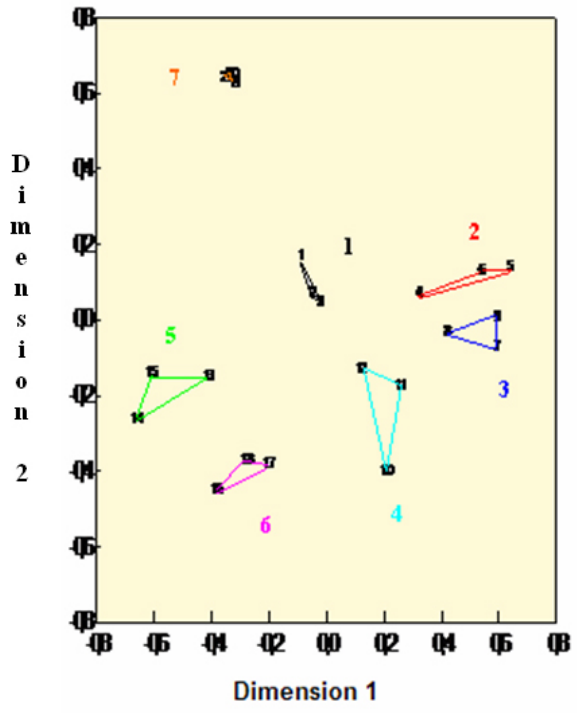

Figure 1. Consensual configuration for the panelists.

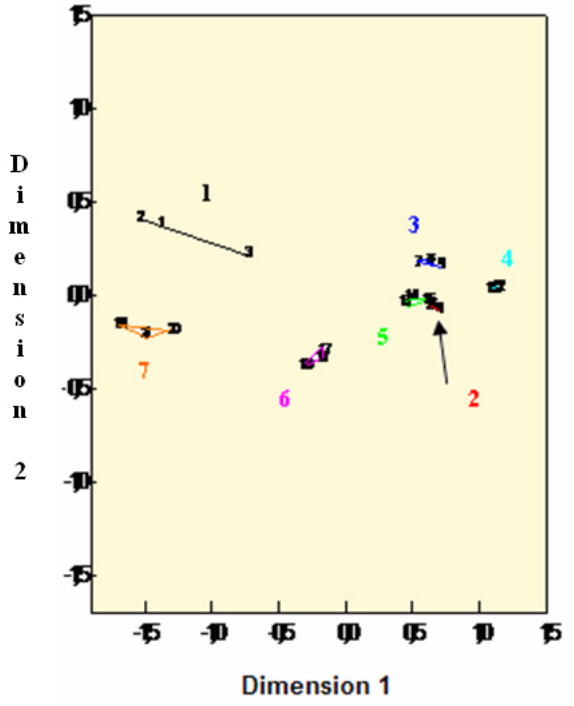

Figure 2. Electronic nose consensual configuration.

Table 3. Volatile components identified and their respective sensorial descriptors.

		S	A	M	P	L	E	S
COMPONENT	**Sensorial descriptors**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
2,3-butanedione	vanilla, sweet potatoes, caramel	X	X	X	X	X	X	X
2,3-pentanedione	hazelnut, sweet, almonds, vanilla	X	X	X	X	X	X	X
Pyridine	dry peas, sweet, burnt	X	X	X	X	X	X	X
Pyrazine	solvent, rubber, oat, ether, mouldy	X	X	X	X	X	X	X
4-methyl-thiazole	burnt, acetone, mouldy, cooked meat, peanuts	X	X	X	X	X	X	X
2,5-dimethyl-pyrazine	hazelnut, corn, roasted coffee, almonds, fruity	X	X	X	X	X	X	X
2,6-dimethyl-pyrazine	cooked, hazelnut, corn, alcohol, potatoes, earthy	X	X	X	X	X	X	X
2,3-dimethyl-pyrazine	popcorn, corn, mouldy, alcohol, nuts, fruity	X	X	X	X	X	X	X
4,5-dimethyl-thiazole	popcorn, corn, rubber, caramel, plastic				X	X	X	X
2,3,5-trimethyl-pyrazine	potatoes, beans, popcorn	X	X	X	X	X	X	X
2-ethyl-3-methyl-pyrazine	corn, sweet, alcohol, passion fruit, papaya			X			X	X
2,3,5-trimethyl-pyrazine	potatoes, beans, popcorn	X	X	X	X	X	X	X
2-ethyl-3-methyl-pyrazine	corn, sweet, alcohol, passion fruit, papaya			X			X	X
2-furfuriltiol	alcohol, solvent, vinegar, sweet	X	X	X	X	X	X	X
Acetic acid	vinegar, acetic acid	X	X	X	X	X	X	X
Methional	potatoes, cooked potatoes	X	X	X	X	X	X	X
Furfural	hay, grass	X	X	X	X	X	X	X
2,3-diethyl-5-methyl-pyrazine	fried potatoes, mouldy	X	X	X	X	X	X	X
Linalool	passion fruit, green leaves, grass, figs	X	X	X	X	X	X	X
Isovaleric acid	corn bran	X	X	X	X	X	X	X
Guaiacol	corn, smoked, medicinal, camphor, peppery	X	X	X	X	X	X	X
Furaneol	alcohol, sweet, caramel, vanilla	X	X	X	X	X	X	X
4-ethyl-guaiacol	smoked, corn, cheese, spicy, peppery	X	X	X	X	X	X	X
Cis-isoeugenol	smoked, corn	X	X	X	X	X	X	X

Electronic nose results

The best discrimination region ranged from 8 to 12 seconds of run time (with 1 second intervals), defined in preliminary tests with soluble coffees (Bassoli, 2006; Airsense, 2002). Total variance explained was 98.99%, respectively 98.51% and 0.48% in the two considered dimensions. Each interval had its repeatability verified through the residual variance (Op & P Product Research), the largest observed being 0.19% at 8 seconds. As there was consensus, one hypothesis to be lately explored would be to consider only this specific time for such analysis. Obtained results allowed the characterization and discrimination of all samples (Figure 2), and a good discrimination power was observed. This indicates that the electronic nose technique, being a fast and simple method, should be regarded as a potential powerful tool for classification of soluble coffees, complementarily to sensorial evaluation.

CONCLUSIONS

Combining the HS-SPME extraction technique with GC-MS, between 378 and 429 chromatographic peaks were detected on the seven soluble coffee samples analysed, with a total of 414 different compounds detected. From these, 24 components were quantified. Using GC-FID/O olfactometry and Free Choice Profile techniques, 132 compounds of aromatic impact were detected, being 22 quantified. Sensorial data was processed by GPA, being all samples discriminated properly. Similar results were obtained when submitting the samples to an electronic nose. It is suggested that the electronic nose could be a powerful auxiliary tool to classifying soluble coffees. The identification of the main aroma components and their odour relevance may help either in the maximization of those compounds considered positive for the beverage or reduction of those regarded as not desirable to quality, in a selective way. It also widens insights about concentration and interaction effects on the final sensorial sensation. Furthermore, targeting key aroma compounds allows the development of technological alternatives for soluble coffee aroma improvement.

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Coffee Aroma is Perceived Differently by Consumers and by Trained Panelists

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SUMMARY

It is commonly accepted that coffee aroma can only be reliably described by experts or trained panels as coffee aroma results from a complex mixture of 20-40 key volatile compounds and their interaction. Without sensory training, it is unlikely that consumers can describe coffee aroma as precisely and consensually as a trained panel. But this does not mean that consumers are not able to detect and describe sensory difference between coffees. The aim of this study is to investigate the descriptive ability of consumers based on odour only (aroma above the cup) and to compare their description with those obtained by a trained panel. Odour description of eight different instant coffees was carried out by 1) 10 trained subjects according to the classical Quantitative Descriptive Analysis (scoring of the intensity of the different attributes of each product); 2) 40 coffee consumers using a sorting task (product grouping according to their similarities with a free description of each group). The analysis of sorting data showed that consumers grouped the eight coffees consensually but also differently from the trained panel. The present study highlighted a gap between sensory information collected from a trained panel and from consumers. This gap may be explained by differences between consumers and sensory panel in terms of evaluation approach, which may influence coffee perception. Indeed consumers have a holistic approach considering product sensory properties as a whole, which promotes the impact of cognitive processes (liking, interaction between senses, familiarity or expectation) on perception. On the contrary, trained panelists evaluate products with an analytical approach. They describe individually and independently each attribute of the sensory glossary following a procedure learnt during training. This training reduces therefore the impact of cognitive processes on perception.
***Keywords*:** organoleptic properties, cognitive processes, sensory training, consumer

RÉSUMÉ

Il est communément reconnu que l'arôme de café ne peut être décrit de manière fiable que par des experts ou un panel entraîné, l'arôme de café résultant d'un mélange complexe de plus de 20-40 composés d'arôme clé et de leurs interactions. Sans entraînement, il est peu probable que des consommateurs puissent décrire les arômes de café de façon aussi précise et consensuelle qu'un panel entraîné. Mais cela ne signifie pas que les consommateurs ne sont pas capables de détecter et décrire des différences sensorielles entre différents cafés. Le but de cette étude est d'explorer la capacité des consommateurs à décrire des cafés en se basant sur l'odeur uniquement et de comparer leur description à celle obtenue avec un panel entraîné. L'odeur de huit cafés instantanés a été caractérisée par 1) 10 sujets entraînés suivant la méthode classique de l'analyse descriptive quantitative (notation sur une échelle de l'intensité des différents attributs de chaque produit); 2) 40 consommateurs de café par la méthode de tri (groupement des produits en fonction de leurs similarités avec une description libre de chaque groupe). L'analyse des données de tri a montré que les consommateurs groupent les huit cafés de façon consensuelle mais qui diffère du panel entraîné. Cette étude a mis en évidence des différences entre les informations sensorielles provenant du panel entraîné et des

consommateurs. Ces divergences pourraient être expliquées par les différentes approches utilisées. En effet, les consommateurs ont une approche holistique considérant les propriétés sensorielles d'un produit comme un tout et donc fortement influencée par les processus cognitifs (appréciation, interaction entre sens, familiarité, attente). Au contraire, le panel de sujets entraînés évalue les produits avec une approche analytique. Ils décrivent individuellement et indépendamment chaque attribut du glossaire sensoriel en suivant une procédure apprise durant l'entraînement. Cet entraînement réduit donc l'impact des processus cognitifs sur la perception.
***Mot clés*:** propriété organoleptique, processus cognitif, entraînement sensoriel, consommateur

INTRODUCTION

As for wine, it is commonly accepted that coffee aroma can only be reliably described by experts or trained panels. Coffee aroma is a complex mixture of 20-40 key volatile compounds and their interaction different volatile compounds and about 36 olfactory notes describing it are reported in "Le Nez du Café" (J. Lenoir, 2006). Without sensory training, it is unlikely that consumers can describe coffee aroma as precisely and consensually as a trained panel. But this does not mean that consumers are not able to detect sensory difference between coffees. On the contrary they can be very sensitive to aspect, olfactory, taste or texture properties. However the hedonic and cognitive aspects (interaction between senses, familiarity) may modulate consumer description whereas descriptive methodology used by a trained panel reduces the impact of cognitive mechanisms on perception.

Pioneer psychophysic studies demonstrated interactions between independent senses (Murphy et al. 1977, Murphy and Cain 1980). Especially, subjects attribute a taste to aqueous solution flavoured with an odorant. Indeed an odour can acquire a taste quality when the odour-taste pair is perceived in food commonly experienced by consumers. Sensory interactions are reported to result from associations experienced and memorized through food exposure without any explicit attention or learning (Köster et al., 2004; Köster, 2005). Product familiarity also strongly modulates the strength of interactions and therefore perception (Labbe et al., 2006).

According to Prescott (1999) and Prescott, Johnstone and Francis (2004), the impact of cognitive mechanisms on perception depends on the exposure strategy applied to subjects. A synthetical exposure, encouraging subjects to evaluate, for instance, olfactory and taste stimuli as a unique flavour modality promotes olfactory/taste integration. Conversely, an analytical exposure, encouraging subjects to dissect their perception into independent sensory dimensions, limits interactions between senses.

The objective of the present study was to compare aroma grouping and description of a set of instant coffees between a group of consumers and a trained panel. To achieve this goal, aroma description of a same set of coffees was done by consumers and by a trained panel using methodologies mimicking best how they usually taste products: 1) a synthetical (or holistic) procedure for consumers, the sorting task with verbal description which consists in grouping samples according to their similarities and differences and in describing each group 2) an analytical method, the quantitative descriptive analysis, which is generally used by trained panel to specify the nature and the intensity of the sensory characteristics perceived when a product is evaluated (Stone et al., 1974).

MATERIAL

The set of products consisted in eight soluble coffees varying in origin (X and Y) and aroma strength and balance (four conditions, 1, 2, 3 and 4 for each origin). In the present study, coffee samples will be therefore named according to these two factors i.e. from coffee X1 to X4 and from coffee Y1 to Y4.

METHODOLOGY

Sorting procedure with verbalisation task (holistic approach)

Subjects

40 consumers used to drink black coffee participated to the evaluation

Sensory procedure and tasting condition

During a first session, the panelists were informed about the principle of the sorting procedure. Within a second session, the panelists received the entire set of coffees at once. They were asked to sort the products served in cup at 65°C into groups having similar odour properties without tasting samples but only by smelling. No other recommendation was given, except that they had to make at least two groups. Then they were invited to describe their groups with their own vocabulary. Evaluation was conducted in an air-conditioned room (20 °C) and under white light in separate booths.

Data analysis

The analysis of sorting data consisted in three steps (Cartier et al., 2006):

a) Product map using multidimensional scaling (MDS):
   An individual binary dissimilarity matrix indicating whether two samples were grouped together was constructed for each panelist. The 40 individual matrices were summed and the resulting dissimilarity matrix was submitted to the classical metric multidimensional scaling MDS (Togenson, 1952) using NCSS software (Hintze, 2001).
b) Product description using the vocabulary elicited to describe groups:
   The vocabulary generated to describe the groups was used to build a contingency table for the products. Each term used to describe a group of samples was reallocated to each product of the group. We therefore assumed that all the products belonging to the same group could be described by the same terms. The resulting contingency table was then reduced and simplified: (1) Terms having similar meanings were grouped by the Sensory Analyst; (2) only terms having a quotation frequency higher or equal to 10% for the sample with highest elicitation rate were considered.
c) Projection of product descriptions on MDS-map
   The items describing products were projected on the MDS map using the correlation structure of product coordinates on the map and product descriptions in the contigency table.

Quantitative descriptive analysis (analytical approach)

Subjects and training

A 10-subject panel used to evaluate coffee samples was used. The panelists received an updated training during 4 sessions on four odour attributes (overall intensity, roasty, earthy

and fruity). Overall intensity and roasty attributes were proposed to the panel because they are prevalent criteria for coffee evaluation. Fruity and earthy were generated by the panel to completely cover the product space. During training each panelist had 1) to memorize the attributes and to detect them in coffee. References were presented for each attribute and simple discrimination tests were performed and 2) to rank products for each attribute and then to rate intensity levels on the scale. For each attribute, two products with two intensity levels (weak and strong) were presented.

Sensory procedure and tasting condition

The eight samples were evaluated according to the quantitative descriptive analysis based on a randomized experimental design. Each sample was evaluated monadically using a 10 cm unstructured linear scale, anchored at the extremities with “not at all intense” and “very intense”. Data acquisition was realized on paper form generated by FIZZ software (Biosystemes, Couternon, France). Four samples were evaluated per session. Two sessions were therefore carried out. The presentation design, based on Williams Latin squares (Williams, 1949), balanced position and order effects. Samples were coded with 3-digit random numbers and served at 65 °C in a 100 ml cup. Rinsing was done between samples with water and unsalted crackers. Tests were conducted in an air-conditioned room (20 °C), under white light in individual booths.

Data analysis

Firstly, data were submitted to two-way ANOVAs (origin and process aroma technology) on the product means (scores averaged across pnalists) to identify significant impact on coffee aroma description of the two factors, oigin and technology (differences were considered as significant at p-value = 0.05). Secondly, trained panel sensory attributes were projected on the MDS map resulting from consumer sorting using the correlation structure of product coordinates on the MDS map and product mean sensory profiles. Finally, a principal component analysis (PCA) was conducted on product means in order to obtain a sensory map, independent of consumers.

RESULTS

Sorting task with sensory procedure

Figure 1 shows that consumers grouped consistently coffees according to their origin (axis 1) and also according to the aroma strengh and balance opposing conditions 1 and 2 to conditions 3 and 4 (axis 2). This map represents well the initial data, as shown by low stress (= 0.10), and high Pearson's correlation between actual and MDS dissimilarities (r = 0.93).

Projection of items that were elicited by at least 10% of consumers for a same sample showed a consistent textual characterisation among the panel (Figure 2). Coffees X were perceived “light” and coffee Y were perceived “burnt” and “roasted”. In addition coffees X1 and X2 were described as coffee, flat, mild and sweet and opposed to coffees Y3 and Y4 described as chemical, intense, bitter and acid. These results showed that consumers also used taste attributes (sweet, acrid, bitter) to describe olfactory perception.

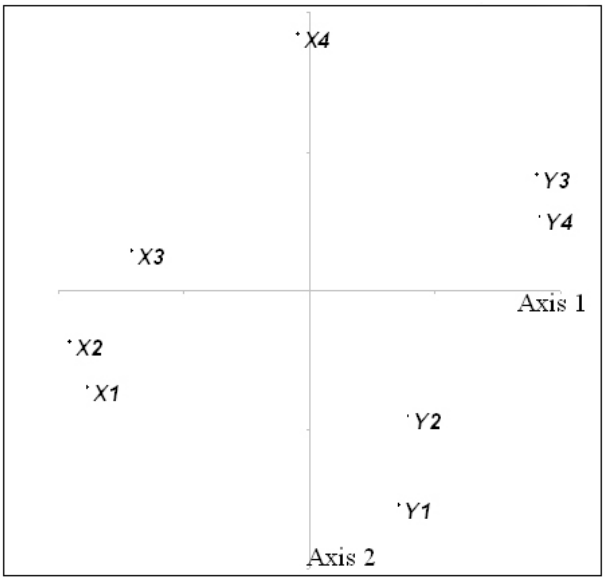

Figure 1. First factorial maps of MDS issued from sorting with consumers.

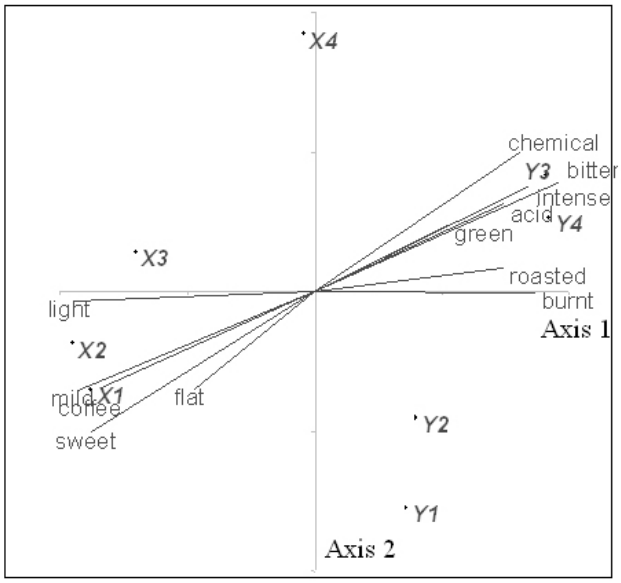

Figure 2. First factorial maps of MDS issued from sorting with consumers with projection of consumer items describing the products.

Quantitative descriptive analysis

Projecting on the consumer map the four sensory attributes (underlined) based on QDA results (Figure 3) shows that trained panel description tended to account for only one dimension of the consumer space. Indeed three highly correlated attributes (overall intensity, roasted and earthy) differentiated aroma strength and balance conditions 3 and 4 and conditions 1 and 2 (two way ANOVAs p-values for aroma strength and balance factor of 0.03, 0.05 and 0.01 for overall, roasty and earthy attributes respectively). But trained panel attributes did not enable to discriminate samples according to the coffee origin (two way ANOVAs p-values for origin factor 0.20, 0.17, 0.59 and > 0.99 for overall aroma, roasty, earthy and fruity attributes respectively). Fruity attribute is not well represented on the consumer map.

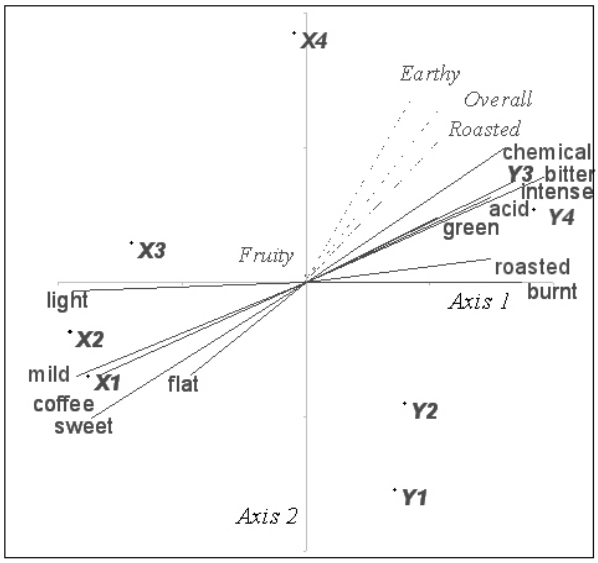

Figure 3. First factorial maps of MDS issued from sorting with consumers with projection of consumer items describing significantly the products and of trained panel attributes (italic font).

PCA of the trained panel scores (Figure 4) shows that, contrary to the consumers, origin was not the main factor of discrimination. X samples are central with very close sensory peoperties whereas Y samples are more scattered on the PCA map with larger different sensory differences. Even if fruity differences were not significant among Y samples (two way ANOVAs p-value for process aroma technology factor of 0.25), the fruity attribute had a tendancy to separate two couples of samples that were not discriminated by consumers: coffees Y1 vs. Y2 and coffees Y4 vs. Y3.

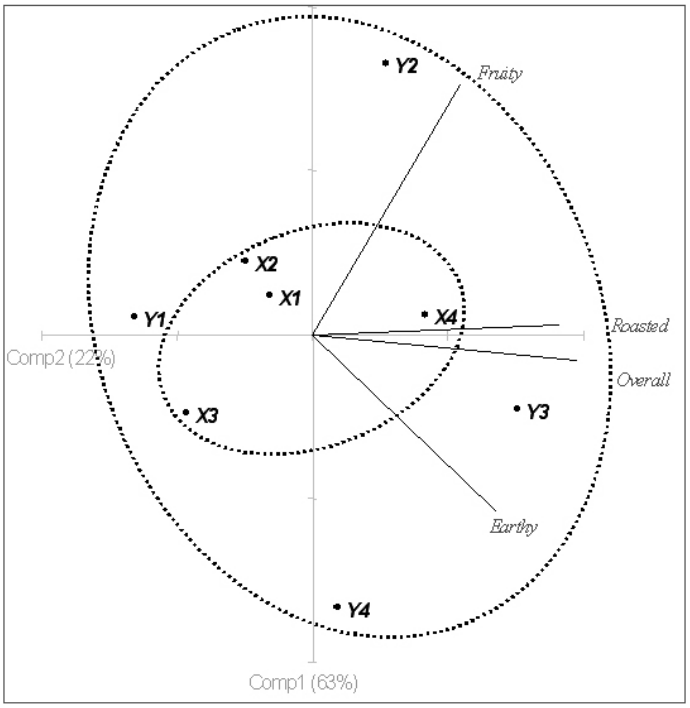

Figure 4. First factorial maps of PCA issued from quantitative descriptive analysis with trained panelists.

DISCUSSION

Consumer description of aroma characteristics of the eight instant coffees differed largely from the sensory panel description. Indeed consumers distinguished coffees according to their origin (coffees X opposed to coffees Y) with a detailed description for each group. A different level of familiarity between the two coffee origins could explain this opposition.

For the trained panel, coffees X had no specific features (samples were close from each other and centred on the PCA map) and more diversity was perceived within coffees Y compared to consumers. Indeed the coffees Y were mainly differentiated in terms of fruitiness by the trained panel with a tendency to oppose samples Y1 and Y2 as well as the samples Y4 and Y3. Consumers did not consider this fruity characteristic, which seemed to be less important than other criteria to differentiate these coffees.

Within the X origin, consumers also differentiated the coffees according to the different technologies whereas the sensory panel did not. Preference may explain these differences especially in the present study where aroma differences, known to drive liking, were strong. Indeed pleasantness or rejection induced by coffee aroma may modulate perception.

Results of consumer verbalization showed that consumers used taste attributes to describe coffee aroma highlighting confusion between olfaction and taste and suggesting the role of perceptual associations acquired through food experience.

Our findings suggested that coffee aroma perception of consumer seems to be strongly modulated by hedonic and cognitive mechanisms. On the contrary for the trained panel, the impact of these factors may be reduced due to training where subjects learn to evaluate independently each sensory dimension (analytical strategy).

As a consequence of these differences, the common task of investigating the sensory drivers of consumer liking should always start with the holistic consumer liking to be explained using analytical sensory attributes (e.g. internal preference mapping) instead of investigating how analytical differences might influence consumer liking (e.g. external preference mapping).

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Impact of Post-Harvest Treatment on the Aromatic Quality of "Bourbon Pointu" Coffee in Reunion Island

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INTRODUCTION

The Reunion island is looking for agricultural productions of diversification. One of them could be the specific coffee of Reunion called "Bourbon pointu". This coffee is the object of an experimental program begun in 2002 and financed by European union, the "Region Reunion" and Cirad. The program aim is to evaluate the interest to develop "Bourbon pointu" coffee and to study the viability of this production. This study investigates the influence of the fermentation conditions of post-harvest treatments on the aromatic profile of "Bourbon pointu" coffee.

MATERIALS AND METHODS

Sampling

9 coffee samples "Bourbon pointu" collected into 2006 underwent three post-harvest treatments whose fermentations conditions are as follows (Table 1):

Table 1. Fermentation conditions of the three post-harvest treatments.

	Fermentation of green coffee	Treatment 1			Treatment 2			Treatment 3		
Treatment. (h)	without water	12	12	12	12	12	12	24	24	24
	under water	24	24	24	36	36	36	24	24	24
	renewal water	no	no	no	12	12	12	12	12	12
Fermentation. Time (°C)	dry beginning	22°7	23°2	23°0	25°1	25°2	26°5	28°8	27°0	28°1
	dry end/wet beginning	19°4	19°6	19°9	23°3	23°0	23°1	27°6	28°1	27°9
	wet end	20°8	20°5	20°3	24°1	23°8	24°0	22°3	25°0	26°6

All the samples were roasted (initial temperature of 200-210 °C and finale of 160-170 °C) during 11 to 13 minutes and an identical grinding.

Protocol

An optimisation of extraction conditions of volatile compounds of coffee "Bourbon pointu" by SPME was carried out. The following conditions were thus used: extraction temperature 50 °C, equilibrium time 10min, adsorption time 45 min.

The volatile fraction of 2 g of coffee (added with 3 ml of water and 50 µLof internal standard (butan-1-ol with 0.5 g/l)) is extracted by SPME thanks to a fibre CARBOWAX/PDMS with the extraction conditions described above. The aromatic compounds were analysed in CPG using a chromatograph Hewlett Packard 5890. Each sample was analysed three times.

The aromatic compounds identification of coffee “Bourbon pointu” was carried out by CPG-SM of Agilent with respective reference 6890 and 5973 N.

The quantitative information of the aromatic compounds gathered in chemical classes was treated statistically by Anova, ACP and AFD with the software Xlstat v.6.

RESULTS AND DISCUSSION

Identification of aroma compounds

120 aromatic compounds of coffee " Bourbon pointu" were identified and gathered in chemical family: furans (28), pyrazines (21), pyrroles (11), ketones (11), sulphur compounds (10), aldehydes (9), terpenic compounds (6) and phenolic (6), esters (5), alcohols (3) acids (3) and pyridines (3), benzene compounds (2), lactone (1) et miscellaneous (1).

Statistical analyse on the treatment influence

After identification and quantification of flavour compound, a statistical study (ANOVA) on sums of relative surfaces by classes of compounds shows significant differences (95%) between treatments for all the classes. Therefore, a principal components analysis was carried out and discriminated correctly only the treatment 1. As the Anova results showing significant differences between treatments 2 and 3, a discriminating factorial analysis was carried out on the same data file so as to know if a discrimination between the three treatments were obtained.

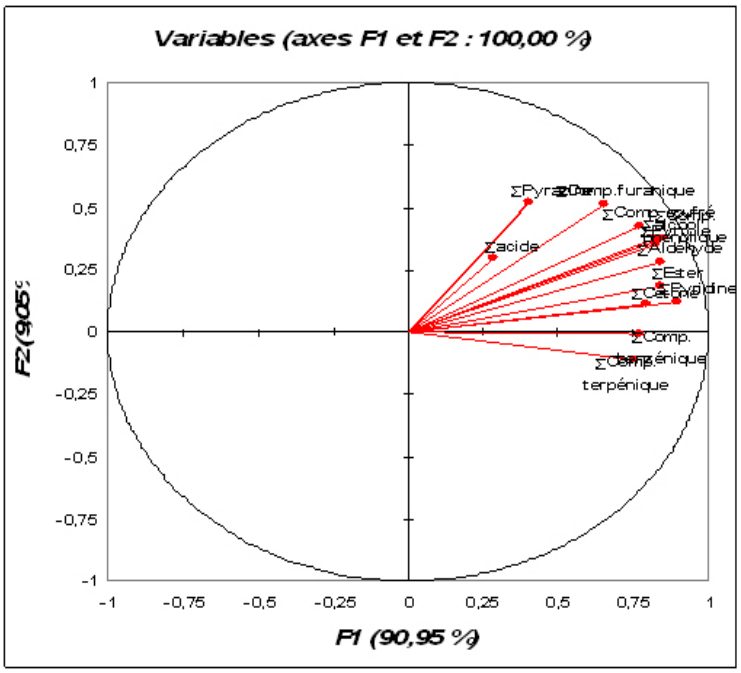

Figure 1. Loading score on first plan of AFD of the chemical classes.

The F1 axis of AFD (Figure 1) explains 90.9% of information and almost all the chemical families are correlated positively. The F2 axis is correlated positively with the contents of furans and pyrazines. The samples discrimination after AFD according to the post-harvest treatment is complete (Figure 2). It is mainly based on the contents of chemical classes of compounds, correlated with axis 1, which are weak for treatment 1, average for the treatment 2 and strong for the treatment 3. The concentrations of pyrazines and furans, correlated with axis 2, discriminate treatment 2 of the others.

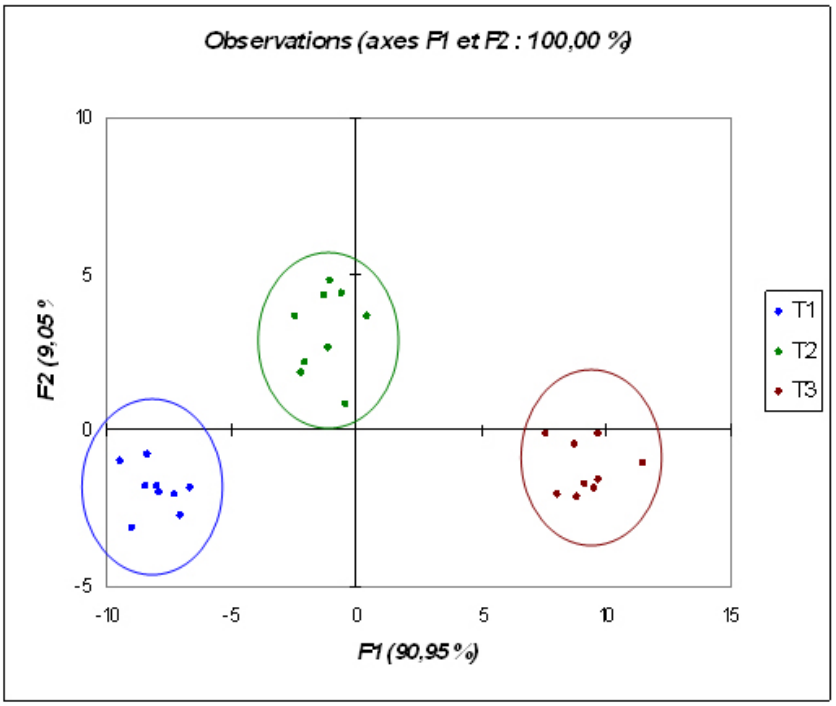

Figure 2. Score plot of "Bourbon pointu" coffee treated by different post-harvest treatments on first plan of AFD.

The weak concentrations in aromatic compounds resulting from treatment 1 could be explained by the lowest initial and final fermentation temperature of this treatment. These conditions could induce delay in fermentation leading to a biochemical composition in potential aromatic precursors weaker at the end of fermentation. The treatment 3 leads to the strongest concentrations of flavour compound. This can be explained by a longer time of fermentation for this treatment (24 h), inducing a more important production of flavour precursors. A diffusion of biochemical compounds (polyphenols, sugars) present in the coffee bean could also be at the origin of these differences. This diffusion would be less important in treatment 3 than in the treatment 2 which time of underwater fermentation is longest (36 h).

CONCLUSION

The statistical analysis of the chemical classes discriminates the three types of post-harvest treatments. It is based on different quantitative contents rather than on a qualitative difference in composition. The conditions of treatment 3 seem to be more adapted to the development of coffee flavour "Bourbon pointu". However, these results have to be confronted to the results of the sensory analysis to determine if these higher concentrations are also connected to a better appreciation.

Chemical and Organoleptic Characteristics of Yellow and Red Coffee Cultivars

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SUMMARY

Coffee cultivars producing yellow and red fruits selected by IAC Breeding Program descend from Bourbon. Although they were improved through several generations and may differ in some characteristics, all of them have at least half of Bourbon gene pool. Information on plant productivity, adaptability and stability in different regions and resistance to biotic stress are available, however, there is a lack of data comparing organoleptic and chemical properties. The aim of this study was to compare sensory and chemical characteristics of coffee beans from red and yellow fruit cultivars. There was no clear relationship between fruit color and beverage quality of coffees prepared by dry and wet processes and results suggested interaction among processing method and variety. Comparing grains from yellow and red fruits of the same cultivars, trigonelline content was not significantly different in 58% of treatments. Total chlorogenic acids concentration was higher in grains of yellow fruits in 42% treatments. Glucose, fructose and sucrose were higher in grains from yellow fruits in 83, 67 and 42% of treatments, respectively.

INTRODUCTION

Technically, the commercial value of Brazilian green coffee is based on the number of defected beans, overall bean aspect and size and also on the cup quality. Some factors such as cultivar, microclimate, agricultural care and post-harvest treatment are determinant of these characteristics. Currently, special attention has been done to cup quality of coffees and in this context it is peremptory that proper cultivar selection and grain processing are accomplished.

Cup quality results of a combination of volatile and non-volatile components in roasted beans, which are in the last instance defined by green beans composition. Green bean components such as chlorogenic acids, carboxylic acids, carbohydrates, proteins, trigonelline and lipids are generally related to cup quality and their concentrations are used in preliminary judgment of the potential cup quality of a cultivar.

There are no reports comparing chemical composition of green beans or beverage sensory characteristics of cultivars with yellow and red fruits. Therefore, the objective of this work was to evaluate 5 cultivars selected in IAC through these parameters.

MATERIAL AND METHODS

Plant Material

Bourbon and other 4 *C. arabica* cultivars developed in IAC from crosses with Bourbon were evaluated. Coffee plants were grown at 669 m altitude, except Bourbon which was at 1,100 m. Table 1 shows the varieties studied and percentage of Bourbon genome in each one.

Table 1. Yellow and red fruit cultivars of *C. arabica* evaluated.

CULTIVAR	BOURBON GENOME	CULTIVAR	BOURBON GENOME
Icatu Vermelho* IAC 4040	~50%	Obatã Vermelho IAC 4275	~62,5%
Icatu Amarelo** IAC 3282	~62%	Obatã Amarelo IAC 4836	~69%
Catuaí Vermelho IAC 99	~75%	Bourbon Vermelho	100%
Catuaí Amarelo IAC 62	~75%	Bourbon Amarelo	100%
Ouro Verde Vermelho IAC 4395	~62,5%		
Ouro Verde Amarelo IAC 4397	~62,5%		

**Vermelho = Red fruits. **Amarelo = Yellow fruits.*

Sample Preparation

Coffee samples were processed by wet and dry methods. Red and yellow fruits of Catuaí and Icatu cultivars were also left in trees until almost completely dry. Sun drying to about 11% moisture was carried out in sieves. After hulling, chemical analyses were performed in green beans ground to less than 0.5 mm.

Sucrose, Glucose and Fructose

Were extracted in water at 70°C, cleaned in disposable C_{18} column, filtered and quantified. Dionex column CarboPack PA1 and pulsed amperometric detector were used in HPLC analyses. Water and 300 mM NaOH were used in the system.

Trigonelline and Total Chlorogenic Acids

Were extracted in 70% aqueous methanol at 60°C. C_{18} Shim-pack CLC-ODS(M) column and UV detector set at 272 nm were used. Elution was performed with methanol/water/AcOH (50/49.5/0.5 v/v/v) at 1ml/min.

Sensory Analysis

Brews prepared with 10 g mediun-light roasted coffee and 100 ml of hot water were tasted by 3 experts. Aroma, astringency, body, cleanliness, acidity, sweetness, bitterness and overall quality were used for cup quality assessment. Scores ranged from 0 up to 5.

RESULTS AND DISCUSSION

There was not a regular relationship between chemical composition of green beans and fruit color. Results showed herein suggest interaction between processing method and cultivar.

Therefore, there was no statistical difference between yellow and red cultivars regarding to trigonelline content in green beans prepared by dry method excepting Bourbon, in which the concentration was higher in grains from red fruits (Table 2).

Table 2. Trigonelline and total chlorogenic acids (CGA) content in green beans of red and yellow fruits of *C. arabica* cultivars processed by dry and wet methods.

Variety	Trigonelline (%db)		CGA (%db)	
	Dry	Wet	Dry	Wet
Icatu Vermelho*	1.18	1.18	4.36a	4.18a
Icatu Amarelo**	1.09	1.10	4.07b	4.09a
Catuaí Vermelho	1.06	1.19a	4.05b	4.68a
Catuaí Amarelo	1.08	1.05b	4.54a	4.04b
Ouro Verde Vermelho	1.03	1.07a	4.11a	4.09a
Ouro Verde Amarelo	1.06	0.96b	4.06a	4.05a
Obatã Vermelho	1.04	1.02	4.33b	4.50a
Obatã Amarelo	1.00	1.09	4.64a	4.31b
Bourbon Vermelho	1.06a	1.09	4.35b	4.54b
Bourbon Amarelo	1.01b	1.07	4.97a	5.10a

**Vermelho = Red fruit. ** Amarelo = Yellow fruit.*

A tendency to higher trigonelline content in grains from red fruits was clearer in wet processed grains.

If fruits are left dry in trees trigolline content is higher in grain of Icatu Vermelho, 1.34%, than in Icatu Amarelo, 1.23%. On the other hand, grains from yellow fruits of Catuaí cultivars, exhibited more trigonelline, 1.22%, than those from red fruits, 1.06%.

A more regular pattern was observed with total chlorogenic acid concentration. A tendency to higher concentration of CGA in red fruits was observed in dry and wet processed grains. Higher CGA content was also measured in grains of fruits left dry in trees. CGA concentrations in these grains were 4.51% in Icatu Vermelho and 4.17% in Icatu Amarelo, and 4.59% in Catuaí Vermelho and 4.06% in Catuaí Amarelo.

Differences in sucrose concentration were more evident when wet process was employed. Although there is a tendency to higher concentrations in grains from yellow fruits there was not completely clear relationship between fruit color and sugar concentration (Table 3). Most of cultivars showed higher fructose and glucose concentrations in grains from yellow fruits. Thus, glucose was higher in grains from yellow fruits in 10 of 12 treatments, including dried in trees, and fructose was higher in grains from yellow fruits in 8 of 12 treatments.

Figure 4 shows significant differences in beverage sensory characteristics of yellow and red fruit cultivars. In general, no clear relationship between fruit color and quality attributes was observed. Considering, for example, Overall Quality, dry processed grains of Bourbon Amarelo promoted higher quality beverage than Bourbon Vermelho, however the opposite was observed in cultivar Ouro Verde.

Superiority of yellow coffee beverages was, however, clear when fruits were left in trees.
Table 3**.** Fructose, glucose and sucrose content in green beans of red and yellow fruits of *C. arabica* cultivars processed by dry and wet methods.

Table 3.

CULTIVAR	Fructose (%db)		Glucose (%db		Sucrose(%db)	
	Dry	Wet	Dry	Wet	Dry	Wet
Icatu Vermelho*	0.12b	0.15b	0.06b	0.15a	10.13a	10.99a
Icatu Amarelo¨**	0.24a	0.23a	0.16a	0.12b	8.78b	8.41b
Catuaí Vermelho	0.14b	0.13b	0.13a	0.06b	8.54a	10.31a
Catuaí Amarelo	0.23a	0.26a	0.04b	0.16a	7.81a	8.15b
Ouro Verde Vermelho	0.29a	0.29a	0.02b	0.07b	7.65a	8.00b
Ouro Verde Amarelo	0.23b	0.22b	0.08a	0.09a	8.71a	10.07a
Obatã Vermelho	0.12b	0.15b	0.03b	0.07b	9.35b	10.40b
Obatã Amarelo	0.20a	0.20a	0.08a	0.09a	12.66a	12.02a
Bourbon Vermelho	0.17b	0.12a	0.04b	0.02b	9.26a	7.87b
Bourbon Amarelo	0.20a	0.13a	0.07a	0.05a	9.37a	10.28a

**Vermelho = Red fruit. **Amarelo = Yellow fruit*

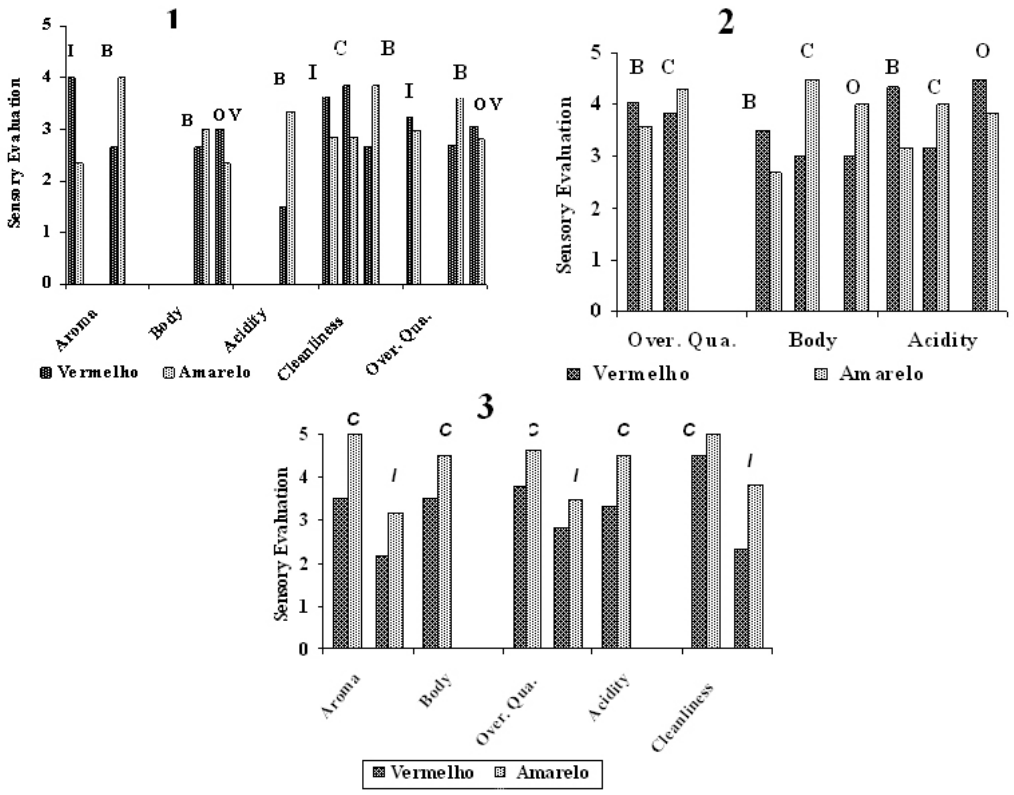

Figure 1. Significant differences in attribute scores of beverages. Dry (1) and wet methods (2) and left dry in trees (3). Letters are the 1st letter of cultivar name.

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Consumer Detection Limit of Defective Beans in Brazilian Coffee

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SUMMARY

PVA is a low quality green coffee mixture containing large amounts of seeds from immature, over-ripe and fermented fruits, which is sold in the Brazilian internal market. The proportion of each type of defective bean may change among different PVA mixtures but in general, the addition of such defective beans to coffee blends is known to decrease coffee quality. This study aimed at investigating the role of PVA composition on the consumer detection threshold for defects in coffee beverage, estimating the point at which coffee consumers would be able to perceive the difference between the control sample and those samples containing different percentages of PVA. The analysis of results from two independent trials revealed that the percentage of actual defective beans in the mixture of PVA was determinant for the consumer perception, no mattering the proportions of each individual type of defective bean. Additional controlled trials should be performed in order to confirm these results.

INTRODUCTION

Brazil is the first world coffee producer and exporter. Because most coffee in Brazil is harvested by the stripping method, significant amounts of immature, over-ripe and fermented seeds are observed after primary processing. These intrinsic types of "defective" beans are mechanically sorted in cooperatives, along with other types of defective beans and non-defective beans. For economical reasons, this low quality mixture is sold in the internal market under the name of PVA, which stands for black (P), immature (V) and sour (A) defective beans (Pereira, 2003). Therefore, the practice of adding a small proportion of PVA to good and medium quality coffee is common in the Brazilian coffee market. The presence of defective beans in coffee blends may change sensory characteristics, yielding a more astringent and acidic beverage (França et al., 2005) and, for trained panelists, different types of defects may produce different undesirable notes. The proportion of each individual defect in PVA may vary depending on several factors, such as climate, and post harvest methods, and therefore, PVA composition may also be reflected in the quality of the beverage (Pereira, 2003). Considering that variations in taste attributes have an impact on hedonic responses (Shepherd, 2001), and that consumers may have different flavour perception from experts trained for sensory analysis (Prescott et al., 2005), this study aimed at investigating the role of PVA composition on the consumer detection threshold for defects in coffee beverage, estimating the point at which coffee consumers would be able to perceive the difference between the control sample and those samples containing different percentages of PVA.

MATERIAL AND METHODS

The study was comprised of two independent trials, using two PVA samples, from different regions of Brazil. PVA-1 contained about 10.7% of black beans, 30.5% of immature beans and 9.8% of sour beans (total of 51% defective beans). PVA-2 contained about 2.0% of black beans, 8.0% of immature beans and 4.4% of sour beans (total of 14.4% of defective beans). The Triangle Test design was used for both trials (Meilgaard et al., 1991).

In both trials, good quality beans and PVA mixtures were roasted to dark-medium roasting degree – a traditional roasting degree in the Brazilian market –, in a bed fluid roaster, using 225 °C for 15 min for good quality beans and at 222 °C for 12.5 min for PVA, respectively, in order to obtain the same roasting degrees for both types of coffee. Following, they were grounded using a coffee grinder (LEOGAP M-50, sieve no.1 – Brazil).

In the first trial, fifty regular coffee consumers aged between 18-55 years evaluated the control sample (good quality) vs. five blends with different percentages of PVA-1 (5, 10, 20, 30 and 40%). In the second trial, one hundred regular coffee consumers aged between 18-65 years evaluated the control sample (very good quality) vs. five blends with different percentages of PVA-2 (1, 3, 5, 10, and 20%).

Consumers tasted three coffee samples presented in 50mL porcelain cups coded with three digit numbers, and indicated the odd one. They repeated the process until they had tried all percentages of PVA. The order of presentation was randomised among and within series. The evaluation were carried out inside the sensory booths under red light in order to mask the beverage appearance Water and biscuits were provided to consumers as a cleanser between samples. Binomial distribution table for Triangle Tests was used for data analysis (Roessler et al., 1978).

RESULTS AND DISCUSSION

Figure 1 shows the Triangle test results for the first trial, considering the five blends with different percentages of PVA-1 (5, 10, 20, 30 and 40%). Results from the first trial showed that consumers perceived the beverage with 5% of PVA (corresponding to 2.5% of actual defective beans) as being different from the control. In the second trial (Figure 2), considering the five blends with different percentages of PVA-2 (1, 3, 5, 10, and 20%), the beverage with 20% of PVA was perceived as different from control, and the detection threshold was estimated at the 16% level (corresponding to 2.3% of actual defective beans) (Figure 3).

It is interesting to note that a slight improvement on the quality of control sample (second trial) produced a slight decrease in the perception limit (0,2%).

The comparison of both independent trials revealed that the percentage of actual defective beans in the mixture of PVA was determinant for the consumer perception, no mattering the proportions of each individual type of defective bean. Further controlled trials should be performed in order to confirm these results.

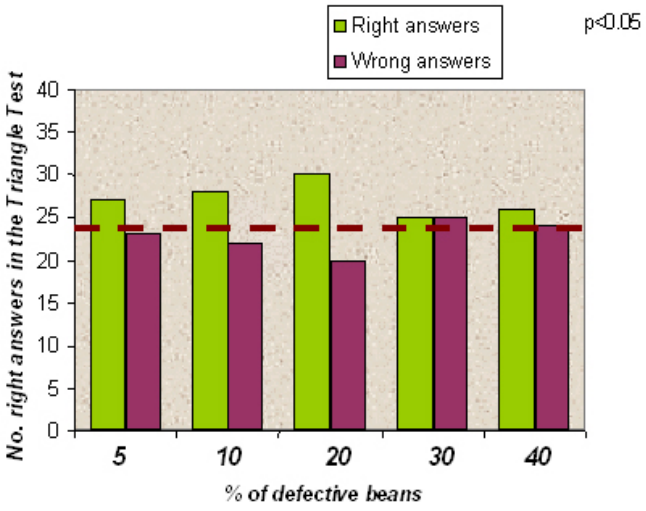

Figure 1. Triangle Test results from the 1st trial.

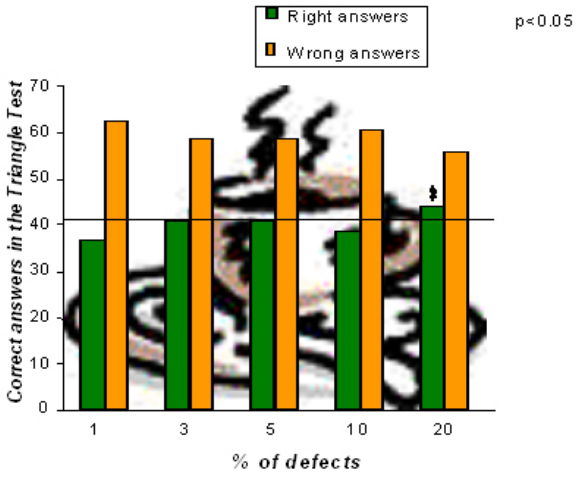

Figure 2. Triangle Test results from the 2nd trial.

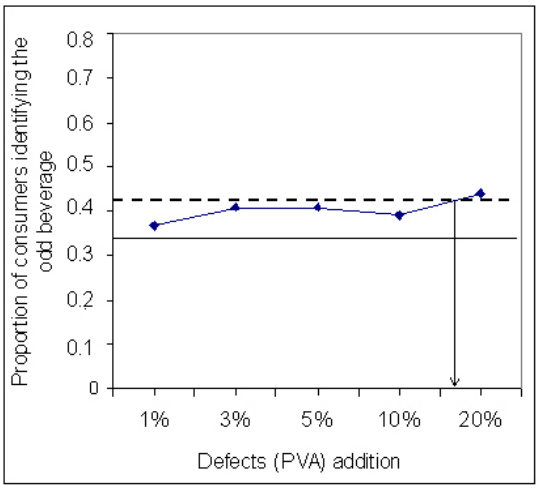

Figure 3. Proportion of consumers identifying the coffee beverage with added defects (PVA). The solid line (0.33) represents a selection of the odd sample in the Triangle Test by chance, and the dotted line indicates the 5% significance criterion (0.42) using the binomial distribution for triangle tests (N=100), which is reached at ~ 16% defects (CDT).

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The Effect of Black Bean, Black-Green Bean and Immature Bean Defects in *Espresso* Coffee: One Single Bean Can Spoil One Cup

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SUMMARY

A cup of *espresso* coffee is the result of the percolation of hot water under pressure through a bed of approximately fifty roasted and ground coffee beans. Due to the extremely high yield, the beverage is richer in flavour, when compared to other brewing methods, but it is also so sensitive to possible defects that it is said that "one single defective bean can spoil one cup". We investigated this statement by means of sensory analysis. Therefore we took into considerations some of the worst visual defects - black beans, black-green beans, immature beans and brown (sour) beans - and evaluated cups with different concentrations of each defect by means of sensory analysis. A lot of care was paid selecting both the defective and non defective beans, in order to start the experiment in controlled conditions. A trained panel evaluated *espresso* cups according to a complete block experimental design with replicates. Statistical analysis showed that, in case of black bean and black-green bean defects, 2% are enough to be clearly perceived and therefore modify the flavour of the *espresso*. This percentage corresponds to one bean per cup. Furthermore, the qualitative description of the cup changes according not only to the type of defect (being black bean and black-green bean very different in their sensory profile), but also to the concentration at which the defect is present in the cup. This means that single compounds chemical analyses are not exhaustive in *espresso* flavor description, unless a pattern recognition approach is used instead of a peak to flavour one to one matching
Key words: *espresso*, cup quality, sensory analysis, black bean, immature bean, black-green bean

INTRODUCTION

Espresso coffee is a traditional Italian brewing method which extracts roasted and ground coffee at 90 °C water temperature and 9 Bars water pressure (Petracco, 2005), leading to an elixir, higher in body (Navarini et al., 2004) and flavour, when compared to all other coffee preparations: this is the result of the higher extraction yield of *espresso*, which brings into the cup a higher amount of volatiles, compared to other brewing styles (Petracco, 2001). In a perfect blend this causes the delight of the consumer; on the other hand the system is very susceptible to defects (Teixeira and Teixeira, 2005). It is said that sometimes a single defective bean can spoil the quality of an *espresso* cup.

Therefore, the selection and classification of raw coffee during purchasing step by a roasting company is fundamental to minimise the possibility of having defective beans in the blend.

Different producing countries adopt different coffee classification systems, used to assign to each coffee lot a commercial value (Pallotti, 2000). Dimension and aspect of the beans, along with producing region, altitude, bean size, number of defective beans, and beverage quality are characteristics to establish the commercial standard.

Defects can be identified by observing the colour of the silverskin (perisperma) or the bean (endosperma). Few countries adopt a system comparable to Brazilian one, which takes into consideration the number of defective beans in order to establish the commercial type of the coffee. According to the Brazilian Official Classification System black beans and immature beans are between the worst types of defects; no reference is taken to another kind of defect named black-green, nor in other countries this distinction between black and black-green is made. This defective bean is however different from the black bean: the silver skin of a black–green bean is of a shining black colour, while the endosperm of a black bean is black opaque. Moreover, their odour, when scratched on a sandpaper is different, being the first fermented and the second ashy and mouldy. Since several studies proved the difference between black and black-green beans, this has been accepted and recognised in the revised version of ISO standard 10470 – Green Coffee – Defect Reference Chart (2004).

LITERATURE REVIEW

Coffee defects have been extensively studied since the '30s under all aspects, from the possible cause of their origin, to the influence on the cup quality on sensory, microscopy, chemical points of view. Already in the '30s Perrier (1932) stated that only when maturation stage is perfect coffee reaches its maximum quality: before and after quality is compromised. According to several studies (Bernegg Specher von, 1938; Graner and Godoi, 1959; Carvalho et al., 1970; Dentan and Illy, 1985; Dentan, 1991; Full et al., 1999), only ripe fruits must be picked in order to obtain a first quality product, because in that stage the minimum amount of defects is present. Also the drying temperature is important (Teixeira et al., 1982) and should not be higher than 40 °C in order to minimise black-green beans.

Lazzarini & Moraes (1958) reported, "the beverage quality of a coffee sample depends on the proportion of deteriorated beans and on the state of deterioration of these beans"[1]. Recently Mazzafera [16] came to the same conclusions after a deep chemical investigation of immature and black-green coffee. Many researchers (de Camargo and Queiroz Telles, 1953; Raposo, 1959; Dentan, 1989) recognised the importance of black beans in spoiling coffee beverage: "it can be considered as a rotten bean and therefore spoils a good coffee beverage turning it into a disgusting one"[2].

Attention has been put in the '60s on the processing of coffee in order to eliminate the defects (Tosello, 1962; Regitano et al., 1965; Sampaio, 1967; Jordão, 1968; Teixeira et al., 1971). Observing the silver skin, which is green, does the characterisation of the immature bean defect. Unfortunately, if the silver skin is removed, then it become impossible to recognise the defect, and the bean is classified as normal. Therefore the commercial type (and value) of the coffee is increased, but its sensory quality does not (Teixeira and Fazuoli, 1985). Electronic sorting machines cannot eliminate completely this problem despite the progresses and developments in the field in the last years (Bee and Suggi Liverani, 2005).

Several studies, mainly by Teixeira and co-workers, showed a correlation between amount of defect and flavour, finding a threshold for an evident deterioration of the cup quality. These are 10% for black beans (Gomes et al., 1967), 15% for light-green immature beans (Teixeira et al., 1970), and 2% of black-green defect (Teixeira and Figueiredo, 1985).

Starting from the '70s, when efficient GC/MS systems and later GC-olfactometry system were available, many volatile compounds in defective beans were identified, responsible for

[1]Originally in portuguese: "a qualidade da bebida de uma amostra de café depende da proporção de grãos deteriorados e do grau de deterioração desses grãos"

[2] Originally in portuguese:"o preto, tanto da roça como do terreiro, é um dos defeitos mais graves do café; é o grão que pode ser considerado apodrecido e que, por isso mesmo, prejudica realmente a bebida do café, tornando-a a pior possível"

the off-flavours (Barel et al., 1973; Vincent et al., 1975; Guyot et al., 1982; Bade-Wegner et al., 1997; Full et al., 1999) even if none of the studies reports sensory analysis of the cup.

SCOPE

Black, black-green, immature and brown (sour) beans defects are extremely important to spoil cup quality. Up to now no correlation between visual defects and perception in the cup has been carried out, being all sensory studies based on a semi-qualitative method. Most of them have been performed by Brazilian researchers, therefore following the traditional Brazilian cupping technique (*prova da xicara*), which is 5-10 g of coarse ground coffee per 100ml of water in infusion for approximately 5 minutes, before tasting. Samples are judged according to a hedonic scale which goes from very smooth (*estritamente mole*, in portuguese), to smooth (*mole*) to almost smooth (*apenas mole*), hard (*duro*), rioy (*riado*) and rio (*rio).*

The scope of the present study was to get more knowledge on the effect of each of these defects in the cup and particularly on *espresso* coffee, both on the quantitive point of view, i.e. the detection threshold and the relation between percentage of defect in the cup and perceived intensity, and on the qualitative point of view, i.e. the perception they evoke, with different percentages. We will demonstrate that in some cases one single bean can spoil the cup's quality.

Five steps have been followed:

1. "Good", non-defective coffee has been visually selected
2. Defects have been selected one by one, keeping only beans which clearly showed a defect
3. A medium roasting degree (16% weigh loss) has been chosen, and great care has been taken in uniformly roasting the batches.
4. Sensory thresholds for black, green and black-green defects have been established for *espresso*
5. The effect of increasing concentrations of each defect in the cup has been evaluated

MATERIAL AND METHODS

Sample preparation

A highly trained staff has visually separated the defects, keeping only those defects to which a univoque identity could be given. All coffee was *Coffea arabica* from Brazil.

Defective coffee came from reject of electronic sorting machines. Coffee used as reference was sampled and tasted before to ensure absence of defects.

Defects have been then roasted separately from non-defective coffee, in order to have a 100% defective sample in case of immature beans. Since roasting is very difficult with black-green and black beans, but they are easily recognizable also after roasting, they have been roasted together with non defective beans with percentage 40% defect 60% non defective, and separated after roasting. Non-defective beans have been then discarded.

Non-defective beans to constitute the reference have been roasted before defective ones, to avoid possible contaminations from the roasters. Coffee has been roasted to a medium roasting degree (approximately 16% weight loss) with a Probat model BRZ4 (220 °C for 5-6 minutes for 100 g sample).

Espresso coffee has been brewed using a professional machine (La Marzocco) with water temperature of 94 °C and pressure of 9 bars.

Cups of non-defective coffee (called clean or good coffee) were brewed, accordingly to *espresso* preparation rules (13 g coffee powder – which comes from approximately 100 roasted beans – to make 50 ml beverage in 30s) on one percolation group of the *espresso* machine. One the other group a 100% defective cup was brewed. To prepare a defective cup with a given percentage of defects, that amount of coffee was removed with a syringe from the clean cup and substituted by defective coffee. The error on the weigh is 0.1 g.

Detection threshold

In order to establish detection thresholds for each defect, a set of 3-AFC (Alternative Forced Choice) tests (Meilgaard et al., 1999) were performed on each judge, with concentrations spaced by a factor 2.

The amount of defects has been varied geometrically from one sample to the other (double/half).

The BET (Best Estimate Threshold) is calculated as the geometric mean between the minimum amount correctly perceived and the first error. The panel's BET is the geometric mean of the individual BETs. Confidence interval of 95% has been calculated as well.

Coffee descriptive profile

After determining thresholds, the second step of the study consisted in the characterisation of samples with different percentages of defects. Five sessions with 10 samples per session have been run. In each session at least 4 clean samples were present, in random order, to check against type I errors, for a total of 21 clean cups, while defects were present in random order.
In the following table 1 the defective samples with their percentage in the cup is presented. The number in brackets is the number of replicates of that particular sample.

Table 1. scheme of the samples used in the experiment, with name of defect, quantity in the cup, number of repetitions in brackets, and sample code.

Name of Defect	Black bean	Black-green bean	Immature bean
percentage defect per cup	2 (1) B2	2 (3) BG2	4 (2) LG4
	4 (1) B4	4 (3) BG4	8 (2) LG8
	8 (1) B8	6 (3) BG6	12 (2) LG12
	12 (1) B12	8 (3) BG8	16 (2) LG16
	16 (1) B16		20 (2) LG20
			32 (2) LG32

Sample were presented monadically in random order. Assessors were instructed to rinse their mouth after each sample with abundant water.

Time interval between two sections was approximately 1:30 min.

Assessors were asked to evaluate the samples and mark a score for the intensity of the defect on a semi-quantitative scale with semantic anchors (1 = absent 2 = low 3 = medium 4 = high). Half scores were permitted. They were also asked to describe the sensantion if a defect was present.

Before starting the session a clean cup was provided as a reference. As a result of preliminary tests, this procedure was adopted because it helps minimizing type I errors, due to expectation.

Data analysis

Data were analysed with Systat 8.0 and FIZZ 2.10.

Since the scale was a discrete scale with semantic anchors, data can be considered as ordinal but qualitative. In this case the scores provide information regarding the order of intensity of the perception, and the interval between two successive values will not necessarily have the same amplitude. In such kinds of test this approach is probably more honest, even if it lacks of accuracy. The median, and not the mean value, is to be preferred as an estimate of the expected value. A Kruksal Wallis test has been chosen to evaluate possible differences between samples.

Data have been then modelled according to a Stevens' law model, in order to evaluate the increase in the perceived intensity with increasing quantity of defect in the cup.

RESULTS AND DISCUSSION

Determination of detection thresholds

Mean value and confidence interval are calculated as geometric mean of the average values for each taster. The 95% CI is skewed being antilog of the CI for the mean of the Log data.

In the following table 2 the best estimate threshold for the panel (expressed in percentage for one cup) for each defect is given.

Table 2. best estimate thresholds (BET) and 95% confidence intervals for black, black-green and immature bean defects in *espresso*.

Name of defect	Black bean	Black-green bean	Immature bean
BET	3.7%	2.0%	7.4%
95% CI Upper	11.8%	5.6%	18.8%
95% CI Lower	1.1%	0.7%	2.9%

Thresholds for black and black-green are very low, less than 4%, that is 1 g of defect per cup of *espresso* (25 ml). The confidence interval for these defects is very narrow as well. In the case of black-green, 2% represents exactly 1 bean per cup, which is the affirmation we did in the title of this paper.

The threshold for immature beans is almost double, that means 2 g per cup, and its confidence interval is wider, because of different sensibilities towards this defect. The upper limit is approximately 19% that is a little bit less than 5 g per cup. The threshold for brown (sour) bean defect was higher than the others ones and had big differences between the assessors, that's why we had discarded the results. One of the causes for this variability could be the different origin of this defect. More studies about this defect could be developed.

These thresholds cannot be taken for general. As reported in sensory literature (Meilgaard et al., 1999), perception thresholds can vary by a factor of 100 or more from person to person and hundreds of tests would be required to get significative results. Nevertheless, they can be used as a first indication, and served as a starting point for the next step of the research.

Type I error and carryover effect

When performing sensory tests in which the presence or absence of a defect is questioned, there is a tendency by the assessors to commit type I error (in this case to perceive a defect

when it is not present). Furthermore, when dealing with *espresso* coffee, the long lasting flavour of the beverage and the difficulty to rinse properly the oral cavity, set both a temporal limit and a limit to the number of samples per session. Thus the number of samples per session cannot be too large, in order to prevent the assessors from reaching their saturation. A sufficient time interval between samples is required, during which the assessors have to carefully rinse their mouth, in order to prevent carryover effect of a defect on a successive non defective sample.

For these reasons some training sessions have been performed, in order to determine the maximum number of samples per session, the time interval between samples and to make the assessors acquainted to the random order of "clean" and defective samples. The number of samples per session was set to 10. During the first training sessions type I error has been high (because of expectation problems). It is interesting to notice that when a clean sample was preceded by other two consecutive clean samples, the third one was perceived as defective. Coming the coffee from the very same powder, this represents an evidence of expectation error.

To overcome this problem a "clean" reference has been given before starting the test. This precaution reduced the number of false positive answers.

Type II error and sensibility

Table 3 reports type II errors (i.e. the assessor does not perceive a defect when it is present). They can be explained by the different sensitivities of the assessors to different defects.

Table 3. type II error, for each session and for each assessor.

Defect	Assessor 1	Assessor 2	Assessor 3	Assessor 4	Assessor 5	Panel
Black bean	0/5	0/5	1/5	0/5	0/5	1/25
Black-green bean	0/10	2/10	1/10	0/10	0/10	3/50
Immature bean	1/11	3/11	2/11	3/11	2/11	11/55

Assessor 2 did not recognise twice the black-green bean defect when in concentration of 1%; no assessor judged as defective immature bean defect when in very low concentration (2%). This consitutes a confirmation that the detection threshold is above this concentration. Skipping that concentration type II error drops to 4%.

Kruskal Wallis test

In order to compare the different intensities of defect between themselves and with the clean cup, a Kruskal-Wallis test for each type of defect has been calculated. As stated before, we prefer to consider the dataset as consisting of scores on an ordinal scale. Therefore, the analysis of variance would be inappropriate, being the median the proper estimator of the statistics. In order to establish which were different a multiple comparison for treatment was used (Siegel and Castellan, 1991).

In the following the results within each group of different defect is reported.

As far as black beans are regarded, even the difference between the clean cup and B2 is significative ($p < 0.05$). B2 is also perceived as less intense than B12 and B16. From B4 on, no significative difference is found even if B16 is scored as intense (score 4) by the whole panel. It is interesting to note that B1 is almost half the percentage compared to previously

calculated BET. That could be explained with an increase of sensitivity of the assessors when subjected to multiple tests with the same defect.

Also for black-green bean defect the difference between the clean cup and BG2 (lowest concentration) is significative ($p < 0.05$). In its turn BG2 is significantly less intense than BG4, BG6 and BG8, while these three do not differ. The immature bean defect, when at lowest concentration (LG4) is under detection threshold, and therefore perceived as a clean cup. Sample LG16 deviates from the expected trend: there is an inversion in the perceived intensity between it and LG12. It should be remarked, however, that they have not been tested in the same session and therefore a direct comparison has not been done by the assessors: it can be explained as noise due to fluctuations in a discrete scale with few values.

Data modelling: Stevens' law

Data have then been fitted a Stevens' law model ($y = 1 + k * x^n$), where y is the perceived intensity and x is the percent of defect in a cup. Since the scale used starts from 1 an additive term has been added. Results are given in figure 1, where the fitting of the data is plotted, and in table 4, where also a threshold corresponding to y = 2, that is clearly perceivable defect has been calculated. This threshold is in agreement with the values found with the BET for immature bean and black-green bean defects. As far as black bean defect is concerned, again the threshold is half the value found with the 3-AFC method, thus supporting what hypothesed after calculating the KW tests.

Table 4. constant for the Stevens' law model for each defect and threshold corresponding to the clearly perceivable defect.

Defect	Stevens' law			
	R (observed vs predicted) [2]	k	n	x | y=2
Black bean	0.94	0.98	0.43	1.0
Black-green bean	0.96	1.03	0.54	0.9
Immature bean	0.70	0.44	0.59	4.0

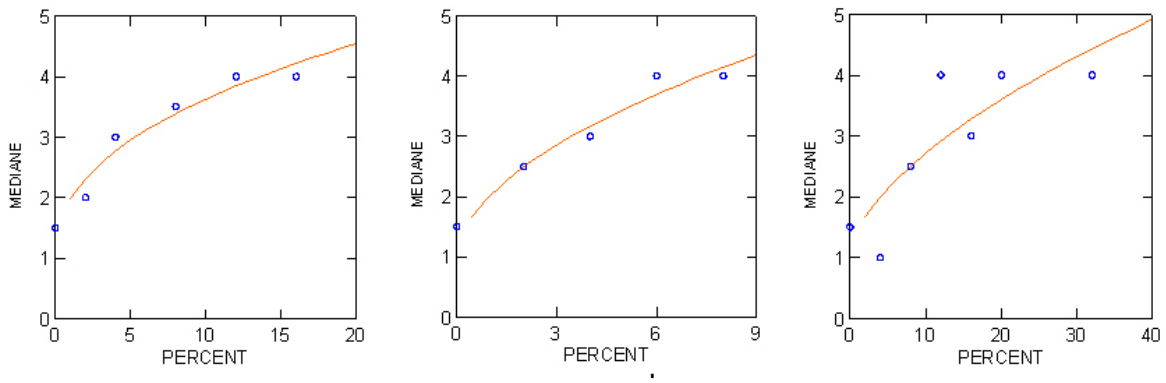

Figure 1. Stevens' law model for the three investigated defects. From left to right black, black-green, and immature beans.

Description of the defects according to increasing percentages

During profile sessions, the assessors were asked to evaluate the intensity of the defect, if present, and to describe the sensation they perceived. The analysis of the descriptions provided by the assessors to the defects is very intriguing. First of all we must note that while in some cases the description (i.e. the sensation) is not affected by the intensity, in other cases

the assessors change their description of the same (visual) defect with increasing percentage of the defect itself.

Results are summarised in table 5. For sake of clarity we provide results for descriptions concerning low intensity (i.e. a score 2 on the scale) and high intensity (i.e. a score equal or higher than 3.5 on the scale).

Table 5. description of the sensation evoked by the defect present in the cup at different concentrations.

	Low intensity (2)		
Defect	grams per cup	percentage	Description
Black bean	0.5g	2%	Mouldy/Earthy/Woody
Black-green bean	0.5g	2%	Fermented
Immature bean	1.5g	6%	Green/Immature
	High intensity (>3.5)		
Defect	grams per cup	percentage	Description
Black bean	2g	8%	Mouldy
Black-green bean	1.5g	6%	Stinker
Immature bean	3g	12%	Fermented/Stinker

Black beans give a mouldy/ashy/woody sensation. As the percentage of defect increase in the cup, the mouldy sensation prevails, but the description does not change substantially with the intensity. Coffee with black-green beans in very low concentration is perceived as fermented. When the concentration increases the cup is described as "stinker". As the name suggests, this is the worst defect found in coffee from a sensory point of view.

Immature (light green) beans in low concentration provoked a perception of immature/green defect in the cup. Also in this case, as the percentage of defect in the cup grows, the description changes to fermented, and even stinker.

The results obtained with immature (light-green) and black-green defects are not of straightforward interpretation, but they are definitely intriguing. In facts, for both defects the perceived intensity increases with increasing percentage of defect in the cup, but also the attributes which assessors use to describe the type of perceived defect change. Thus, at first glance the results appear incongruent, because people describe differently the sensations arising from the same compounds, even if in different concentrations.

Moreover, chemical studies to characterise coffee aroma, mainly by gas-cromatography and sometimes coupled to a sniffing port, have never put in evidence this phenomenon.

Often a biunique correspondence between odour molecule and perceived sensation has been given, without taking into account the role of concentration. Therefore, ethyl hexanoate and ethyl tiglate are thought to be responsible for a green-immature note, and ethyl α-methylbutyreate and ethyl isovalerate for fermented (Flament, 2002). We should point out that none of the studies which lead to the identification of off-flavour key compounds take into account the possible change of perceived sensation due to different concentrations and the interactions and synergies of the odorous molecules in building an "odour pattern". These works describe one molecule at the time, as it comes out of the gas-cromatographer column, and not as a whole, like our olfactive system perceive them (Full et al., 1999;Barel et al., 1973; Vincent et al., 1975; Guyot et al., 1982; Bade-Wegner et al., 1997). On the other hand, neurophysiology can provide and explanation to the apparent mismatch we pointed out: odour molecules (both via orthonasal or retronasal) interact with olfactive receptors, located in the

olfactory epithelium with a sort of key-lock mechanism. In a cup of *espresso* we can count approximately one thousand different odour molecules. The chemical signal of the coffee flavour is thus transduced into a set of electrical pulses, which travel to specific targets in the olfactory bulb, called glomeruli, according to the different types of receptor. Pulses patterns are thus generated and successively elaborated in the orbitofrontal cortex, letting us recognise an "odour" (Cappuccio, 2005). The different sensitivity of the single glomerulus produces different patterns and different mappings for different odorant concentrations (Rubin and Katz, 1999); odour molecules and their concentrations can be coded by the different spatial activation pattern of the glomeruli.

CONCLUSIONS

One cup of *espresso* coffee (25 ml) comes from the extraction by hot water under pressure of approximately 50 roasted and ground beans.In some cases a very low percentage of defect can spoil the cup, that is alterate significantly its flavour. We took into consideration three of the most important defects, as regnised by ISO, that is black, black-green and immature beans. We asked ourselves what was the percentage of each defect necessary and sufficient to spoil one cup, and what was the sensation evoked by each of these defects. In the case of black and green-black defects results from sensory analysis show that 2% are enough to be clearly perceived and therefore ruin the flavour. This 2% is exactly one bean in one cup which we hypotised in the title. Furthermore, the description of the cup changes according not only to the type of defect, but also to the concentration at which the defect is present in the cup.

It would be interesting to correlate these results to chemical ones. One way could be the analysis of the beverage itself by recently developed techniques, as SBSE, which allows to analyse the same sample used for sensory analysis.

The study put in evidence the importance of training, even in case of experts, in order to minimise type I and type II errors.

It would also be interesting to repeat the test with a wider scale (e.g 0-10) in order not to compress results at the higher end of it, thus gaining in discriminative power. At this stage of the research, being the panel well trained to the detection of defects but lacking experience and practice in the use of wide scales the Authors preferred to adapt the scale to the common use of the panel to avoid generating confusion.

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Influence of Water Quench Cooling on Properties of Roasted Coffee

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SUMMARY

Four different cooling methods (passive, air quenching, water quenching, water quenching with increase of water content) were applied to coffee of the same roast degree. Gas desorption, firmness, and average pore radius were determined. In addition, losses of volatile compounds during storage were analyzed using SPME-GC-MS. It was shown that water quenched coffees exhibit higher degassing which is probably due to higher permeability of cell wall structure. Aroma loss in water quenched coffees is in the same range as in air quenched coffees. Therefore chemical degradation of aroma compounds rather than aroma stripping is supposed to be the main factor for coffee staling.

INTRODUCTION

In the technology of coffee roasting rapid cooling is judged to be crucial to coffee quality in order to prevent potential over-roasting and aroma losses caused by slow cooling. Water quenching presents an efficient way to achieve fast cooling. However, Illy and Viani (1995) found this method critical for roasted coffee quality due to possible oxidation reactions on coffee surface and opening of pores allowing stripping of volatile substances. These authors also mentioned that water quenching provokes cell wall cracking and therefore more pronounced structure collapse in cells leading to faster degassing, an assumption which is corroborated by Shimoni and Labuza (2000). Spadone and Liardon (1989) showed that concentrations of hexanal, several branched aldehydes, ketones and alkylfurans in coffee cooled by air and by water quenching changed in a similar way during storage. As the two cooling methods resulted in different moisture content, they concluded that lipid oxidation as well as at least some chemical reactions involved in coffee ageing were independent of water content in roasted coffee.

The aim of this study was to determine whether water quench cooling with and without increase of water content implies significant effects on aroma stability, degassing, oxidative stability, and roast coffee structure.

MATERIALS AND METHODS

Roasting

Wet processed Colombian *Coffea Arabica* was roasted with a fluidized-bed hot-air laboratory roaster in batches of 200 g green beans. A low-temperature long-time process (LTLT, 228 °C, 12 min, (Schenker et al., 2000) was chosen. Four different cooling methods were applied (Table 1).

Table 1. Cooling Methods.

Method	Procedure based on 200 g batches	Final water content [g/100 g wb]
Passive Cooling	Ambient air (unforced) (45 min)	1.7
Air Cooling	Ambient air stream (4 min)	1.9
Water Cooling 1	8.7 g water (20 s) and ambient air stream (4 min)	2.1
Water Cooling 2	35 g water (12 s) and ambient air stream (4 min)	4.2

Gas Desorption Measurement

Batches of 80 g coffee beans were placed in 500 mL septum flasks immediately after roasting. Headspace pressure was measured periodically, whereby the flasks were vented after each measurement.

Firmness

Firmness of coffee beans was determined using a shearing test in a Kramer cell. A single layer of roasted coffee beans was placed in the cell and the maximum force was measured at a deformation rate of 100 mm/ min.

Mercury Porosimetry

Mercury intrusion porosimetry was applied for measuring average pore radius (Schenker et al., 2000).

SPME-GC-MS Analysis of Coffee Aroma

Ground coffee was extracted with boiling water during 10 min. After cooling, standards were added and coffee aroma compounds were sampled with solid phase microextraction at 40 °C during 10 min using a DVB/CAR/PDMS-Fibre. Separation was carried out on a medium polar OV-1701 column and detection was done on a quadrupole mass spectrometer using single ion monitoring (SIM). 3 aldehydes (2-methylbutanal, 3-methylbutanal, hexanal), 2 ketones (2,3-butanedione, 2,3-pentanedione), 2 sulfides (dimethyl sulfide, dimethyl trisulfide), 1 pyridine (pyridine), 6 alkylpyrazines (2-ethyl-3-methylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-3,5-dimethyl-pyrazine, 2-ethyl-3,6-dimethylpyrazine) and 1 phenolic compound (4-vinylguaiacol) were analyzed. Aroma compounds were quantified with an isotope dilution assay using isotopically labelled standards.

RESULTS

Water quenched coffees exhibited considerably higher degassing rates than air quenched coffees (Figure 1). Firmness measurements with the Kramer cell showed that roasted coffee beans with higher water content were less brittle (Figure 2) and that maximum force was in good linear correlation to water content in light roasts. Compared to light roasts, the increase of maximum force at higher water content was less pronounced in dark roasted coffees. No clear correlation between water content and average pore radius was found (Figure 3). These facts corroborate the assumption that higher degassing rates of water quenched coffees are due to higher permeability of cell wall structure rather than increased porosity or defects in cell wall integrity.

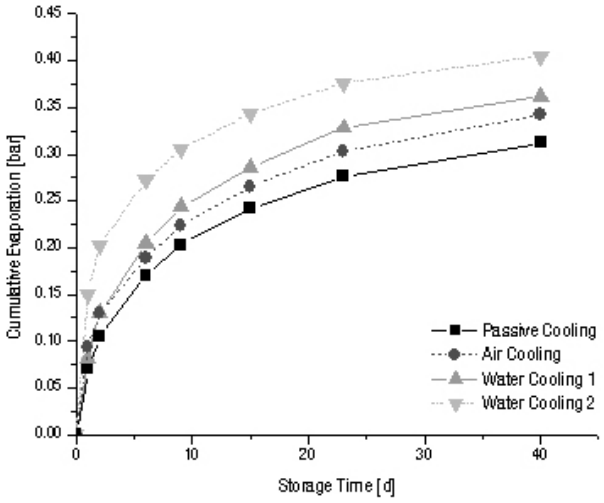

Figure 1. Cumulative evaporation during storage of roasted coffee.

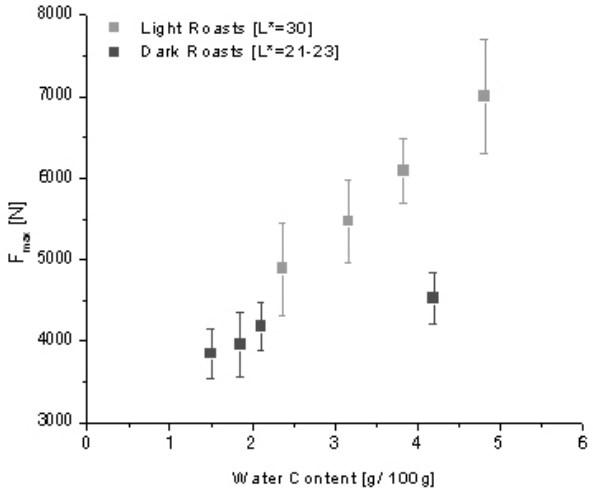

Figure 2. Firmness in relation to water content and roast degree.

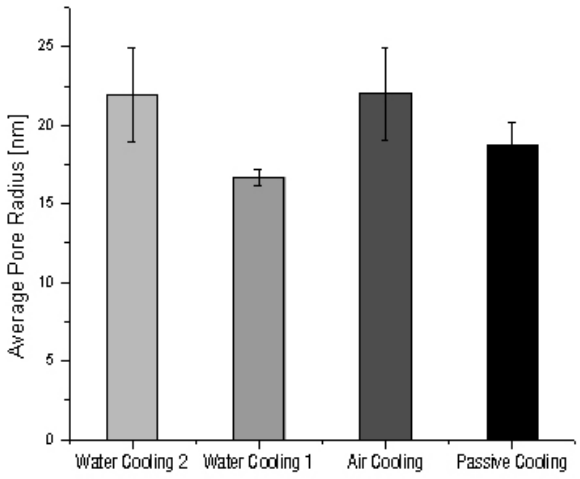

Figure 3. Average pore radius of roasted coffee.

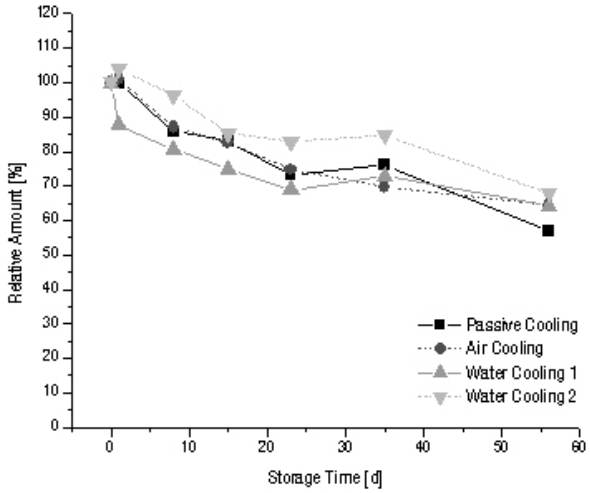

Figure 4. Evolution of 3-methylbutanal content during storage.

Although degassing was considerably higher in the water quenched coffee with higher water content (Water Cooling 2), loss of volatile aroma compounds was in the same range as in air quenched coffees as shown for 3-methylbutanal (Figure 4). This implies that degassing contributes only little to aroma loss which is in agreement with the findings of Spadone and Liardon (1989) but unlike the results of Nicoli et al. (1993) where an equal behaviour of carbon dioxide desorption and loss of volatiles was found. Dimethyl trisulfide however showed a strikingly different behaviour upon storage in the different coffees. Coffee with high water content (Water Cooling 2) and slowly cooled coffee (Passive Cooling) both exhibited a more pronounced increase in the concentration of dimethyl trisulfide during the first 3 weeks of storage (Figure 5). Since this compound is an oxidation product of methanethiol, faster thiol oxidation is assumed in these two coffees. On the other hand no increase in hexanal content was observed and no substantial differences were found between the samples (Figure 6). Therefore the extent of coffee lipid oxidation was low and comparable in all samples.

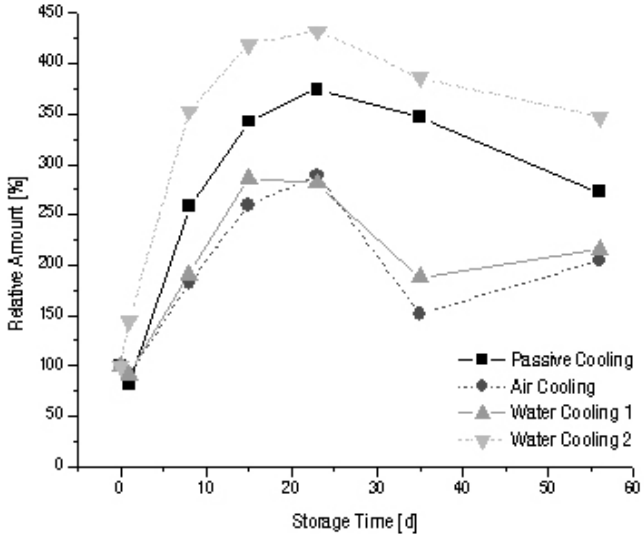

Figure 5. Evolution of dimethyl trisulfide content during storage.

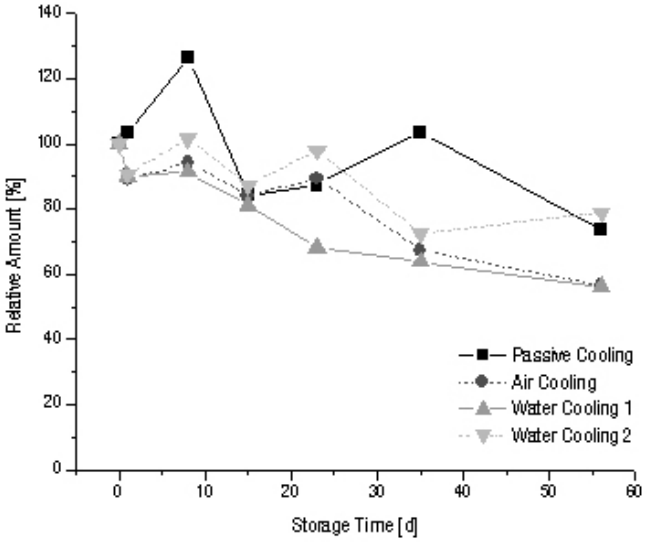

Figure 6. Evolution of hexanal content during storage.

CONCLUSIONS

* Water quenched coffees with increased water content exhibit markedly higher degassing which is probably due to higher permeability of cell wall structure. Average pore radius does not seem to be a determining factor.
* Aroma losses in air and water quenched coffees are comparable although degassing is higher in water quenched coffees. Chemical degradation of aroma compounds rather than aroma stripping seem to present the main reason for coffee staling.
* Lipid oxidation does not take place in the initial period of storage and may contribute to coffee staling at a very late stage only.
* Dimethyl trisulfide presents an interesting indicator for oxidative changes in relation to cooling methods and water content.

ACKNOWLEDGEMENTS

Financial support by G. W. Barth AG (Freiberg/Neckar, Germany) and assistance in aroma analysis by Nestlé PTC Orbe (Orbe, Switzerland) are gratefully acknowledged.

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Modified Coffee Roasting as a Means of Acidity Increase

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SUMMARY

Organic acids which impact sourness perception of roast and ground coffee have been studied thoroughly in the past. Malic, citric, phosphoric and acetic acids have been identified as the major contributors to coffee sourness. Malic and citric acids, e.g., are already present in the green bean, while other acids are being formed during roasting (Clark and MacRae, 1985). The formation of acids during roasting of coffee beans follows well-established chemical reaction pathways and precursors of acids have been identified. Sucrose is considered as a major precursor for organic acids such as acetic acid and formic acid. Formation kinetics have been studied in detail with isotopically labeled precursor compounds (Ginz et al., 200). In the present study, our trials have shown that an additional amount of acidity is created by applying roasting parameters with modified time-temperature curves and subsequent extraction techniques. The flavor development was monitored by comprehensive aroma analysis and supported by sensory studies of the roasted and ground coffees. Acid extracts obtained by the presented roasting method have been characterized by pH, TA and organic acid measurements. The sensory impact of the extracts was studied after add-back to both soluble and roast and ground coffees and a significant enhancement of sourness perception was demonstrated for soluble coffee.

INTRODUCTION

Fine acidity is a positive characteristic of high quality Arabica coffees. Also, acidity is one of the key attributes for distinguishing of soluble and roast coffee brews.

Organic acids, e.g. citric and malic acids, are present in green coffee and degrade during roasting. Additional acids, e.g. formic, acetic acids are generated from carbohydrate degradation in the early stages of roasting. Most aroma compounds, however, are generated in the later pyrolysis stage of roasting. The hypothesis was established that partially roasted, yellow to light brown coffee beans (30-40 La) would be a good source of water-extractable acidity. Interrupting the roasting process at a very early stage is not expected to have a negative impact on the quality of the finished product after roasting to a final roast colour of 6-16 La.

This study should add to understanding the potential of de-coupling coffee aroma and taste.

MATERIALS AND METHODS

2kg of Arabica coffee (Colombia Excelso) were roasted on a fluidised bed drier (Neuhaus Neotech RFB 10) at 255 °C for 175s to achieve a roast colour of 30La. Partially roasted beans were extracted with water (bean:water 1:2) for 2h at 20 °C in a rotary evaporator. Beans and liquid were separated using a sieve and beans were dried in an fluidised bed drier. The dried beans were re-roasted at 259 °C for 200 s (roast colour 12 La). A standard coffee, directly

roasted to 12 La at 259 °C, and a process control sample were also produced. The latter was made according to above described procedure, omitting the extraction and drying parts.

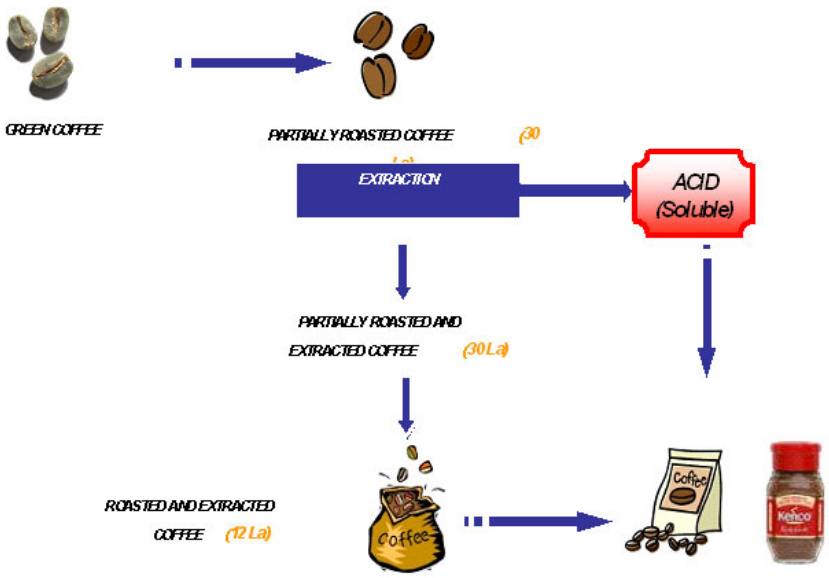

Figure 1. Process concept.

Organic acids were analysed by capillary zone electrophoresis, as described in Weers et al., 1995).

RESULTS

Partial roasting of green coffee

As illustrated in picture 1, 'roasting acids', e.g. Formic and Glycolic acids are found in very lightly roasted coffee beans and therefore generated early in the roasting process. Contrary, acids present in green coffee, e.g. Citric and Malic acids have not been decomposed at this roasting stage:

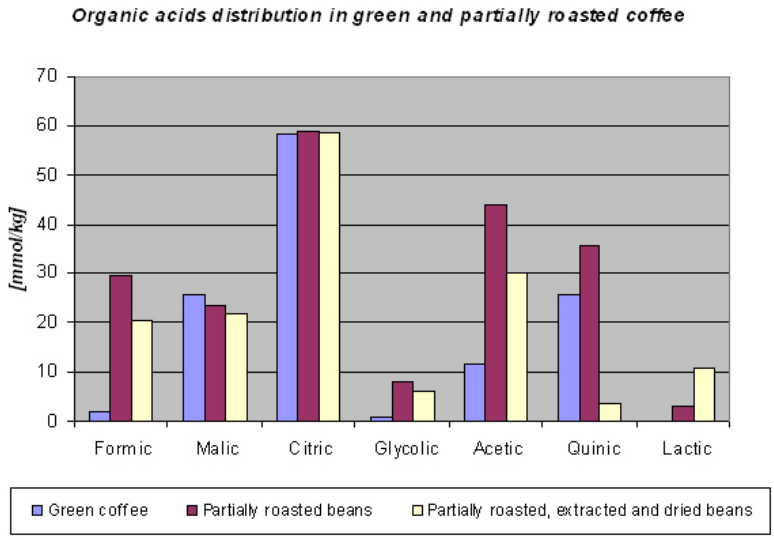

Figure 2. Organic acids contents in green and partially roasted coffees with and without extraction (in mmol/kg).

Extraction of acids

From 1kg of green coffee roasted to 30La, 510g of extract with a soluble solids content of ~2% were obtained. Extraction results in an acidic, sour tasting solution. Extraction yield of acids is 15-20%, based on acids present in the partially roasted coffee. Roughly 25% of the extract solids are organic acids, the pH value is 5.3. The organic acids distribution is illustrated in picture 2.

Chlorogenic acids and free carbohydrates are found in traces, only (< 1% of the solids). However, extraction and drying result in a significant loss in aroma compounds already present at the early roasting stages.

Effect of partial roasting/extraction on finished product

Acid removal mid-way through the roasting process does not have significant effects on organic acids content of finished products (all at 12 La).

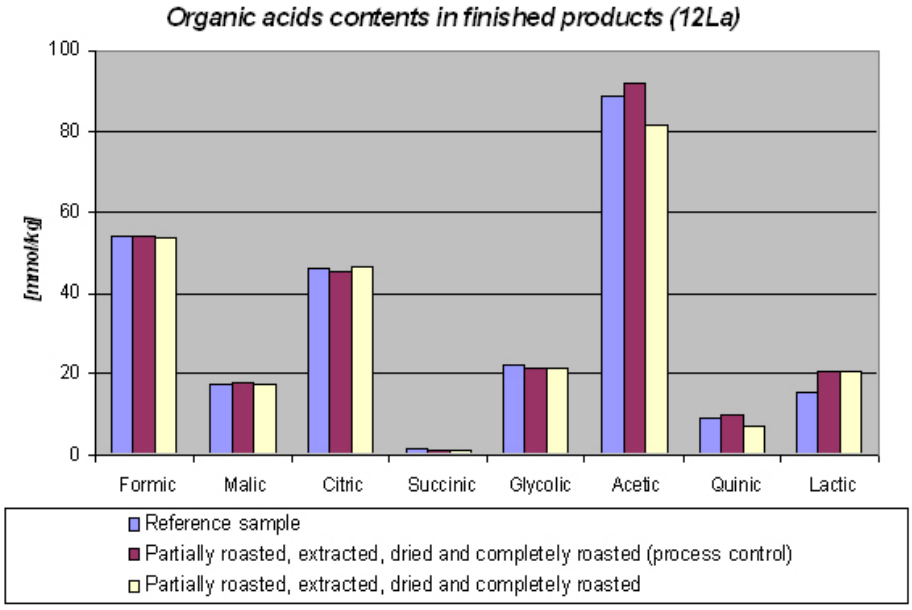

Figure 3. Organic acids contents in reference and process control samples and partially roasted, extracted and completely roasted coffees (in mmol/kg).

Sensory evaluation of the coffees revealed that reference and process control samples are very close. The extracted sample was perceived slightly weaker – although soluble solids measurement in the brews suggested otherwise – but was surprisingly coffee-like.

Sensory effect of acid add-back

Aliquots of the above extract were added back to roast and ground and soluble coffees in stochiometric ratios. In case of roast and ground coffee, 22 ml of aqueous solution was added to 40 g of coffee grounds in filter of a household coffee brewer. The coffee was then brewed similar to controls. In case of soluble coffee 16 ml of acidic extracts were added to 15 g of coffee solids and than made up to 1000 ml with boiling water.

In both cases amounts of acidic extract below and above the stochiometric ratios were also tested (data not shown).

Table 1: Sensory descriptors of acid enhanced soluble coffee; * on 1-11 scale (no-extreme difference).

Sample	Sensory Description	Average Rating vs Reference*
Reference	Dark, caramelly, slightly smoky, grainy, slightly bitter, charred	--
+16ml acidic extract	Citrus/fruity, green, more acidic, slightly more aromatic, slightly winey note, less bitter	4

A perceivable increase in pleasant acidic/citrus notes was achieved in soluble coffee, with the overall above-cup aroma profile of the coffee little affected. However, some off-notes ('winey') were also introduced. Addition of acidic extract was unanimously judged to not improve beverage quality of roast and ground Robusta coffee. For roast and ground Colombian coffee, results were inconclusive.

CONCLUSION

It was successfully shown that removal of organic acids generated early in the roasting process is fully compensated during later stages of roasting. It was also shown, that cold water extraction removes organic acids without affecting other flavour precursors, e.g. free carbohydrates and chlorogenic acids to a significant extent.

Soluble coffee acidity is enhanced by adding back such acidic extract to coffee brew. The acidity enhancement has been judged to improve soluble coffee flavour.

For roast and ground Robusta coffee, no significant changes have been observed. The changes observed for Colombian coffee are perceivable, but have not been clearly linked to a quality improvement

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The Analysis of Coffee Aroma by Liquid and Headspace Methods Using a Single Column, Liner and GCMS

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SUMMARY

Liquid and headspace injection techniques are common tools in the analysis of coffee aroma; each presenting different advantages and drawbacks. Methods using the same column and liner have been developed for the two techniques, which enables switching from one to the other by the simple expedient of changing the syringe in the autosampler. In addition, the required sensitivity has been achieved for both methods in full scan mode with a runtime of 10 minutes. A novel selective splitting technique has been developed to enable this sensitivity to be obtained.

INTRODUCTION

In the GCMS analysis of coffee aroma, it is usual to measure the headspace above a coffee brew or soluble coffee. Static headspace analysis presents two advantages: being a good tool for the distal analysis of very volatile compounds such as short chain aldehydes or ketones and requiring minimal, or no, sample preparation.

On other occasions, however, it is necessary to focus more on the less volatile coffee aroma compounds or to employ a different sample preparation technique, for example, liquid-liquid extraction. Liquid extraction methods, such as simultaneous distillation extraction or liquid-liquid extraction, extract the aqueous coffee brew or solution with a volatile organic solvent; which is subsequently injected into the GCMS.

It is usually the case that these two injection techniques, headspace or liquid, have very different chromatographic requirements and it is necessary to use different columns for each of them. This normally means that it is necessary to have systems dedicated to each type of analysis, limiting the flexibility, efficiency and, therefore, the throughput of the systems.

MATERIALS AND METHODS

Sample preparation

Samples for headspace analysis were weighed into 20mL headspace vials. These were incubated and agitated at 60 °C using a GERSTEL MPS 2 autosampler configured for headspace analysis with a 2.5 mL heated headspace syringe.

Liquid samples were prepared by membrane assisted solvent extraction (MASE) in the same MPS 2 autosampler configured for liquid preparation with a 1 mL syringe.

MASE uses a hydrophobic polypropylene membrane; into which the extraction solvent is injected. It replicates liquid-liquid extraction, but has the advantage of yielding a dry, injectable sample without the need for using chemical drying agents, such as anhydrous sodium sulphate. The MPS 2 fully automates the process, in batches of up to 6 samples at a time (limited to this by the agitator capacity), with a total sample capacity of 96 samples. Tertiary butyl methyl ether (MTBE) was the extraction solvent used for this application.

Gas chromatography and mass spectroscopy

An Agilent 6890 gas chromatograph coupled with an Agilent 5975 mass spectrometer, operated in full scan mode, were used for the method development. Varian Factor Four™ columns were chosen due to claimed lower bleed. The following phases and dimensions were used:

- VF-17 0.25 µm i.d. 0.25 µm film thickness
- VF-17 0.25 µm i.d. 1 µm film thickness
- VF-1701 0.25 µm i.d. 0.25 µm film thickness
- VF-1701 0.25 µm i.d. 1 µm film thickness

For the liquid injection, the oven parameters used were: 40 °C with no hold, rising to 270 °C at a rate of 30 °C/minute, holding for 1.33 minutes. For the headspace injection, the oven parameters used were: 40 °C, holding for 1.5 minutes, rising to 270 °C at 30 °C/minute holding for 0.33 minutes.

Samples were injected into a peltier cooled GERSTEL CIS 4+ temperature programmable injector with a Tenax TA™ liner installed.

RESULTS AND DISCUSSION

Method development, phase I: Choice of column

The columns were evaluated according to the resolution they were able to achieve and the speed of analysis.

Under the specified conditions; it was found that, although the separation for most compounds was acceptable for GCMS analysis, one critical pair could be problematic. The resolution of 3-methylbutanal and 2-methylbutanal could only be achieved using the higher polarity phase, VF-1701, with the thicker 1 µm film.

As the speed of analysis was not dramatically different between the four columns, all of them could elute the last problematic compound, caffeine, within 10 minutes; as a result, the VF-1701 column with the 1 µm film thickness was chosen since it satisfied the 3-methylbutanal/2-methylbutanal resolution requirement.

Method development, phase II: Optimisation of the liquid injection method

The splitless injection of 1 µL of liquid coffee extract (obtained by MASE) yielded a chromatogram with good separation of all the analytes of interest and achieved the required sensitivity. Figure 2 shows the total ion chromatogram for a 1 µL injection.

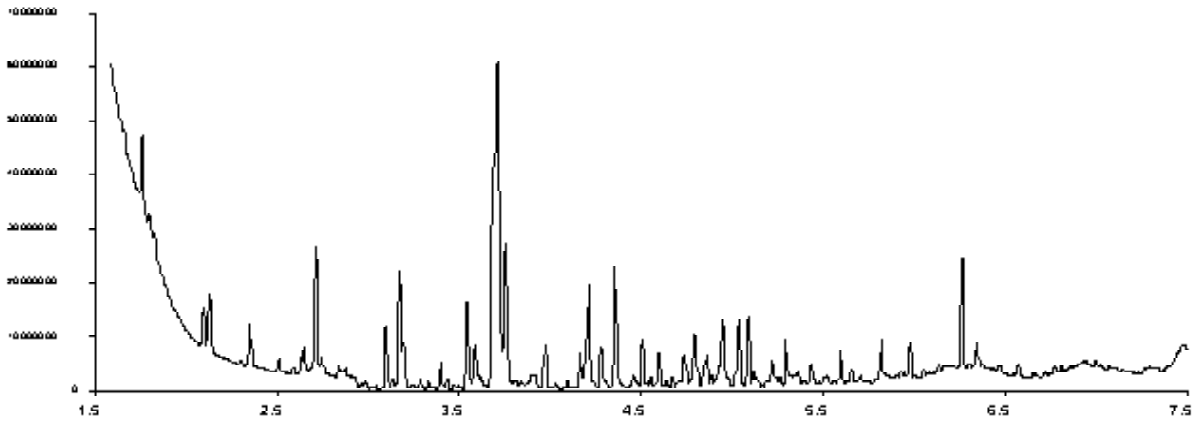

Figure 2. Total ion chromatogram of 1 µL MASE extract.

Method development, phase III: Optimisation of the headspace parameters

For static headspace analysis, our original method used two consecutive 2.5 mL injections into a Tenax TA packed liner, cooled to –30 °C with liquid nitrogen. This was not a cost-effective method, and by moving to a method that permits the use of a peltier cooled inlet we remove the hazard of using liquid nitrogen in the laboratory. Two consecutive 2.5 mL headspace injections into a Tenax TA™ liner, peltier cooled to 15 °C in solvent vent mode were still required to maintain sensitivity. The split valve was then closed and the CIS temperature increased at a rate of 12 °C/second to 240 °C to desorb the analytes onto the column.

This resulted in poor peak shape and resolution of the volatile compounds due to column overload as these analytes are present in much larger amounts than the less volatile compounds in the incubated headspace. Figure 3 shows a solvent vent-splitless headspace injection.

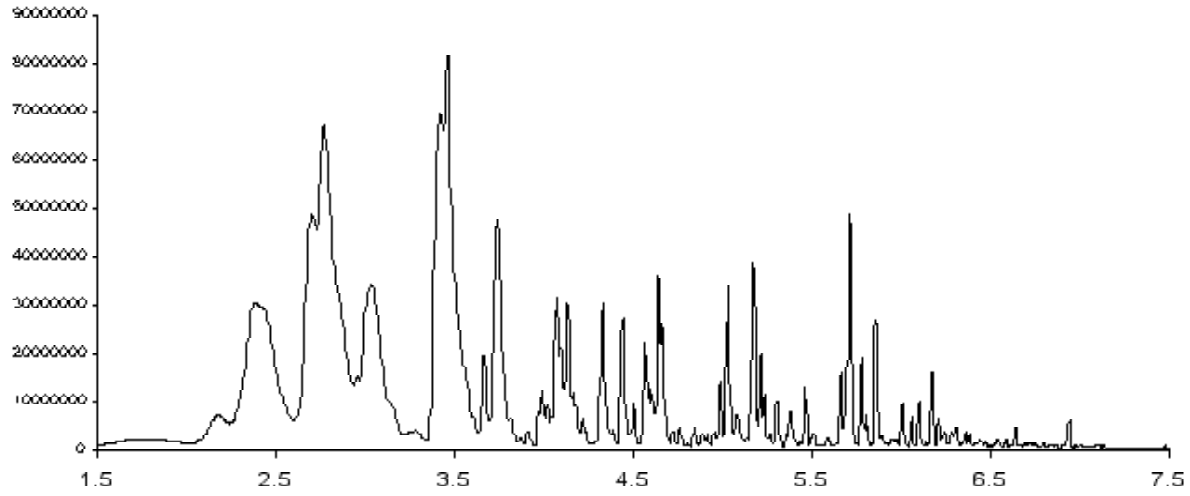

Figure 3. Solvent vent-splitless headspace injection.

To solve this, the injection mode was changed to a split injection to allow the purge of the excess volatiles and improve the column performance. However, this resulted in a lack of

sensitivity for the least abundant semi-volatile compounds. Figure 4 shows a split headspace injection.

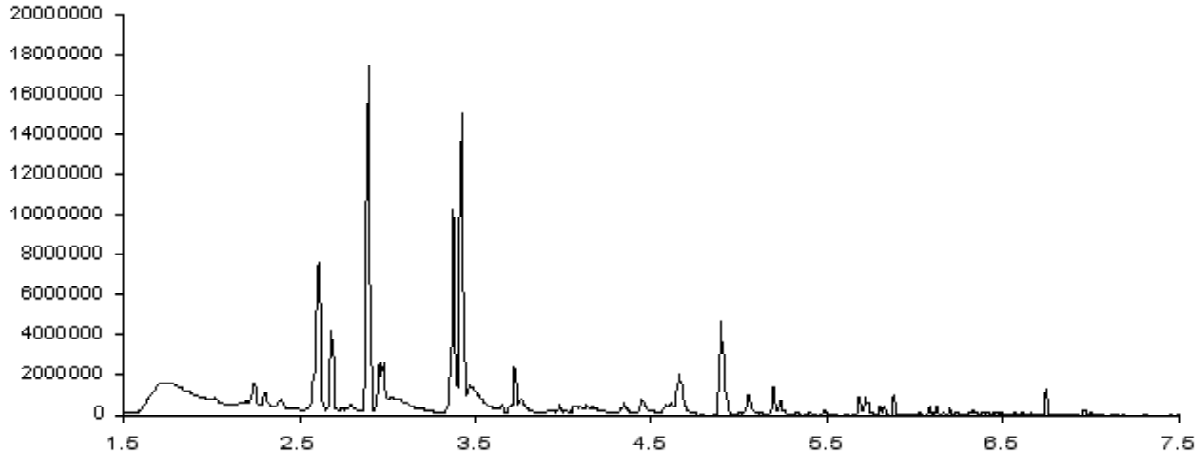

Figure 4. Split headspace injection.

By developing a "selective splitting" method, we were able to use a large split ratio to avoid column overload of the abundant volatile analytes, then close the split valve to achieve a splitless transfer of the less abundant semi-volatile analytes. This was achieved by focusing the compounds at 15 °C in solvent vent mode, keeping the split valve open until the most volatile compounds, those that desorb from the trap at a lower temperature, were desorbed. The split valve is then closed and the CIS is heated to 240 ºC to facilitate the desorption of the less volatile compounds. Figure 5 shows a selective split headspace injection.

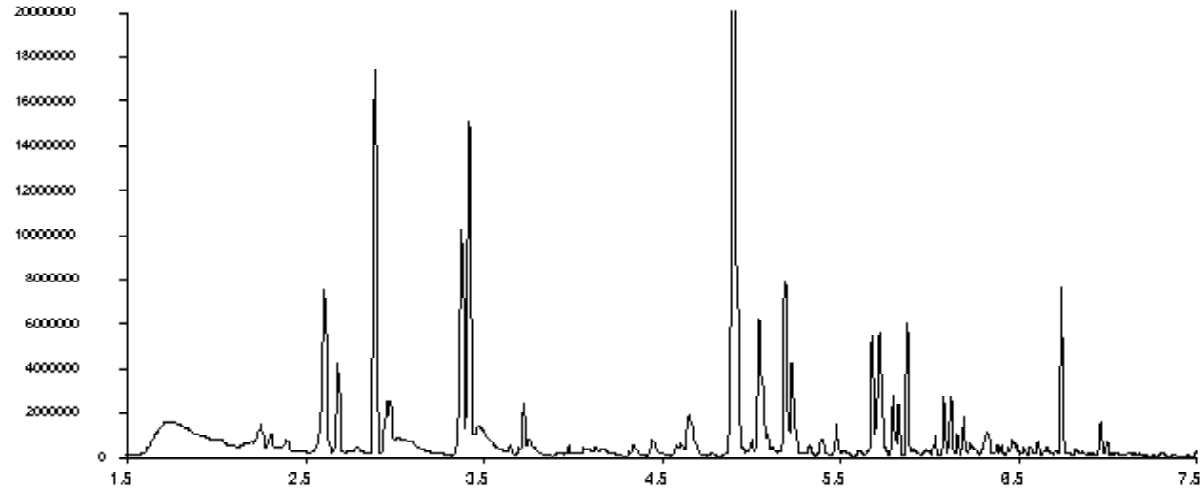

Figure 5. Selective split headspace injection.

CONCLUSIONS

Liquid and headspace injection techniques are now able to be run on the same instrument. The only change needed when moving from one type of injection to another is to change the syringe in the MPS 2 autosampler. In addition, the runtimes have been reduced to 10 minutes from 30 minutes and are run in full scan mode instead of SIM, improving the qualitative data produced.

By using MASE instead of liquid-liquid extraction, the analysis is fully automated and more reproducible than the manual extraction method that this technique has replaced. This method has enables us to use 250 μL of MTBE per sample rather than 1 mL of dichloromethane.

The "selective splitting" headspace method is now being implemented to increase the productivity, sensitivity and instrument reliability.

These changes allow quick re-configuration of the GCMS instruments to meet varying demands in workload without the need for venting instruments to change columns, allowing greater productivity without the need to increase the number of GCMS instruments.

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Characterization of Espresso Coffee Aroma by Static Headspace GC-MS and Sensory Flavor Profile. Maeztu et al., J. Agric. Food Chem. 2001, 49, 5437-5444

Membrane-Assisted Solvent Extraction of Triazines and Other Semivolatile Contaminants Directly Coupled to Large Volume Injection / GCMS. Hauser et al. GERSTEL application note.

Photoionization Detection.
A Rapid Method to Measure Aroma in Packaged Coffee Products

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SUMMARY

In-pack aroma is a key driver of consumer liking for soluble coffee. Currently, headspace gas chromatography (HSGC) method is commonly used to determine Total Aroma Counts (TAC's) in packaged coffee products. The method is time consuming and complex and must be conducted by skilled analysts, and so cannot easily be used at-line. To overcome these disadvantages, a rapid method using a photoionization detector (PID) has been developed. The PID uses an ultraviolet light source to break down volatile organic compounds to positive and negative ions that are counted by the detector. Calibration of the PID versus the HSGC reference method showed a high correlation (> 0.99). Benefits of the new system are reduction of analysis time, increase of testing capacity, ease of use and lower capital and maintenance costs.

PRINCIPLE OF THE NEW METHOD

The PID uses an ultraviolet light source to break down volatile organic compounds to positive and negative ions that are counted by the detector (Figure 1). The subsequent charge detected is proportional to concentration of volatile components in the gas stream.

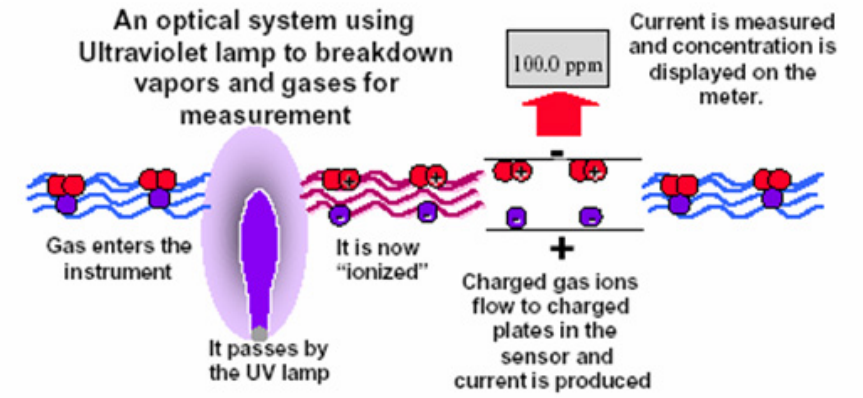

Figure 1. Principle of photo ionisation detection.

ADVANTAGE / DISADVANTAGE OF THE NEW METHOD

The PID is an easy-to-use, portable and hand-held system. Therefore it can be used at-line. Due to its simplicity, maintenance requirements and costs are low. Measurements take only 10 seconds and calibration is quick and simple.

A disadvantage of the system is that it only reports a total aroma value, and cannot identify single analytes.

COMPARISON BETWEEN PID AND HSGC

A comparison between the HSGC and PID method was carried-out. 125 ml serum bottles were spiked with aroma oil equivalent to 0.005 g to 0.045 g aromatized coffee oil / 100 g coffee jar. All samples were packed in a packing tent under nitrogen atmosphere to prevent aroma decay. Aroma was measured in all samples with the PID and by HSGC (Figure 2).

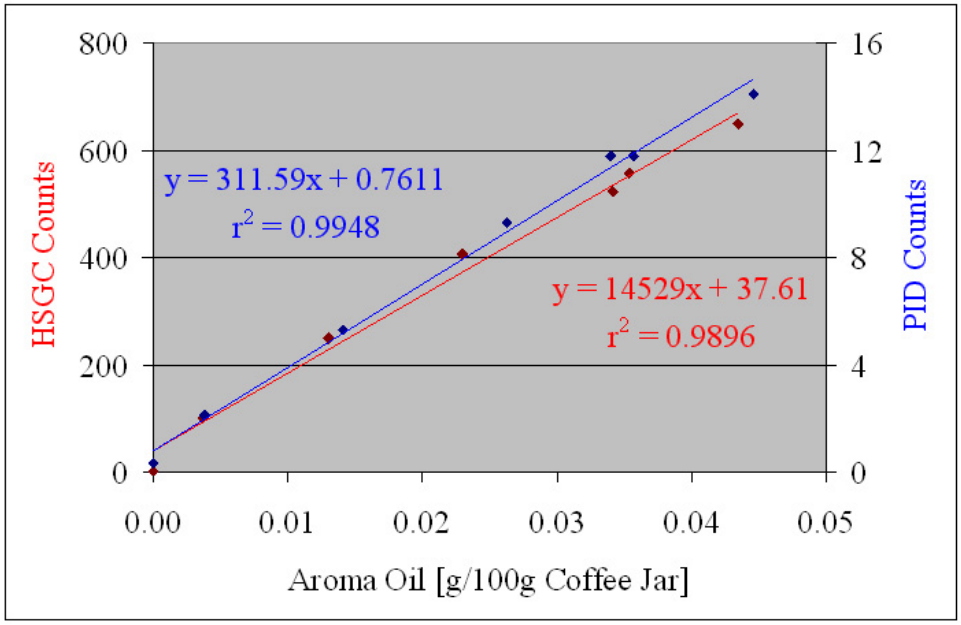

Figure 2. Correlation between PID and HSGC methods.

High correlations were observed for PID and HSGC method versus the amount of aromatized coffee oil spiked into the coffee jars.

COMPARISON BETWEEN PID AND HSGC - VARIABILITY

Variability of the PID and HSGC method were tested by measuring identical samples in duplicate on three different days. Both methods showed comparable variations (CV)
Table 1).

Table 1. Variability of PID and HSGC method.

	PID Counts	HSGC Counts
Day 1	61.1	2683.9
	64.5	2695.9
Day 2	58.3	2279.8
	68.6	2597.1
Day 3	65.4	2768.9
	66.8	2381.2
Avg.	**64.1**	**2567.8**
StdDev.	**3.8**	**194.4**
CV	**5.9**	**7.6**

The variability of PID and HSGC method was similar when determined on three different days.

CONCLUSIONS

The PID provides a rapid total in-jar aroma measurement with a high correlation (> 0.99) to the Headspace Gas Chromatography reference method. The benefits of the new system are: reduced analysis time, ease of use and lower capital and maintenance costs.

Coffee Freshness Alteration of Roasted Coffee Beans and Ground Coffee in the Presence of Oxygen and under Protective Conditions

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SUMMARY

The alteration of coffee freshness in roasted coffee beans as well as roast and ground coffee (R&G) was assessed by using two complementary flavour isolation techniques (SPME and SPE) for the quantification of volatile freshness and oxidation markers. It was shown that rapid degradation of freshness markers as well as formation of oxidation markers occurs as soon as the R&G coffee gets in contact with oxygen. The whole coffee beans also do not provide an adequate protection against quality alteration. Our quantitative data clearly revealed that the oxidative reactions are just delayed. Best protection of coffee freshness throughout shelf life was only achieved when R&G coffee was stored in single portions filled under inert atmosphere at low residual oxygen level.

INTRODUCTION

It is well known that the desirable fresh aroma of coffee alters rapidly when it is exposed to oxygen and/or humidity and light. Packaging materials and storage conditions that prevent coffee being in contact with oxygen and humidity are indispensable for a prolonged shelf life. However, the protection is suspended once the packing has been opened. Packaging of roast and ground (R&G) coffee in single sealed portions circumvents this problem and guarantees freshness and thus coffee aroma quality throughout shelf life.

The aim of this work was to study the alteration of the freshness of roasted coffee in adequately sealed portions as compared to R&G coffee and whole roasted beans exposed to air (but not to light). Freshness and oxidation markers were monitored during a period of twenty days for unprotected R&G coffee and twenty weeks for unprotected roasted beans as well as single portions.

Furthermore the impact of oxygen present during the filling of single portions on the coffee shelf life was studied. The same freshness and oxidation markers as mentioned before were evaluated throughout a shelf life of nine months in single portions filled under inert gas atmosphere (residual oxygen level at 2 and 5%) as well as under air (21% oxygen).

All together, 11 freshness markers (sulfur compounds and aldehydes) and 1 oxidation marker (hexanal, formed by lipid oxidation) have been selected for the assessment of coffee aroma stability based on two criteria. First, studies were considered that linked analytical to sensory data in a causal manner (Mayer et al., 2000; Audouin, 2002). They showed that sulfur compounds (dimethyl sulfide, 2-furfurylthiol and methional) as well as the Strecker aldehydes (2- and 3-methylbutanal) are key contributors to the coffee aroma. Secondly, these compounds are also known to be sensitive and, thus, degrade during shelf life of coffee (Grosch , 2000; Holscher and Steinhart, 1992; Czerny and Schieberle, 2002; Cappuccio et al., 2002; Fischer and Schieberle, 2005).

MATERIALS AND METHODS

The analytical method implied the use of stable isotope labelled molecules as internal standards to determine absolute amounts (mg/kg coffee solids) of markers in each sample. The analytes were isolated either by means of Solid Phase Micro Extraction (SPME) or Solid Phase Extraction (SPE) followed by subsequent measurements by GC/MS and/or GC-GC/MS.

Quantification of markers by Solid Phase Extraction (SPE)

5 g of R&G coffee were suspended with 20 ml hot water and stirred during 5 min. Defined quantities of labelled compounds were added (2-furfurylthiol, methional, furaneol, sotolone, maltol and 3-methylbutanoic acid) and stirred to equilibrate during 10 min. The suspension was filtrated and 15 ml methanol was added in order to precipitate polysaccharides. After filtration, the filtrate was diluted with 100 ml water. LiChrolut® EN cartridges (Merck, 500 mg, 6 ml, 40-120 μm) were conditioned with each 6 ml of dichloromethane, methanol and methanol/water (10/90 v/v). The sample solution was loaded onto the cartridge, washed with 2 x 6 ml methanol/water (10/90 v/v) and the analytes were then eluted with 2 x 1 ml dichloromethane. The organic phase was dried under sodium sulfate and the aroma extract was analysed by means of GC/MS or GC-GC/MS.

Quantification of markers by Solid Phase Micro Extraction (SPME)

R&G coffee was suspended in hot water (Tc 5% for dimethyl sulfide, methanethiol, hexanal and 3-mercapto-3-methylbutyl formate and Tc 1% for 2-methylpropanal, 2- and 3-methylbutanal, 2,3-butanedione, 2,3-pentanedione, N-methylpyrrole, pyridine and 4-vinylguaiacol), stirred during 10 min and cooled down. Defined quantities of labelled standards were added and the solution was stirred for 10 min. After decanting of the solids, 7 ml were pipetted in 20 ml vials. The coffee solutions were equilibrated for 60 min at 20 °C in the sealed vials and the aroma compounds were then extracted from the headspace during 10 min at 40 °C using SPME (2 cm fibre coated with PDMS/DVB/Carboxen). Aroma compounds were thermally desorbed at 240 °C and injected into a GC/MS apparatus.

High Resolution Gas Chromatography/Mass Spectrometry (HRGC/MS)

Mass spectrometry was performed on a MD800 (Quadrupol, Fisons Instruments, Brechbühler, Zürich, Switzerland) in tandem with a polar silica capillary (ZB-Wax, 60 m X 0.25 mm; film thickness, 0.25 μm; Zebron, Brechbühler, Switzerland). The samples were applied by split/splitless injector technique at 40 °C. After 6 min, the temperature of the oven was raised by 4 °C/min to 240 °C and held for 10 min. Specific fragments of analyte and labelled standards were recorded at 70 eV in the electron impact (EI) mode.

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, Switzerland) were inter-connected via a MCSS (moving capillary stream switching) system. In both gas chromatographs, capillaries with different stationary phases were installed (GC1: ZB-Wax; 30 m X 0.32 mm; film thickness, 0.25 μm; and GC2: ZB-1701, 30 m X 0.25 mm; film thickness, 0.25 μm; both columns from Zebron, Brechbühler, Switzerland). The time windows for cutting of the eluate at GC1 were determined by means of reference substances.

Mass spectra in the EI mode (MS/EI) were generated at 70 eV on an MD800 (Fisons Instruments, Brechbühler, Zürich, Switzerland).

RESULTS AND DISCUSSION

Shelf life of single sealed R&G coffee compared to unprotected coffee

The results of the quantification of freshness markers (sulfur compounds and aldehydes) and one oxidation marker during shelf life of unprotected roasted coffee (R&G, whole beans) as well of R&G coffee in single portions are shown in Figure 1.

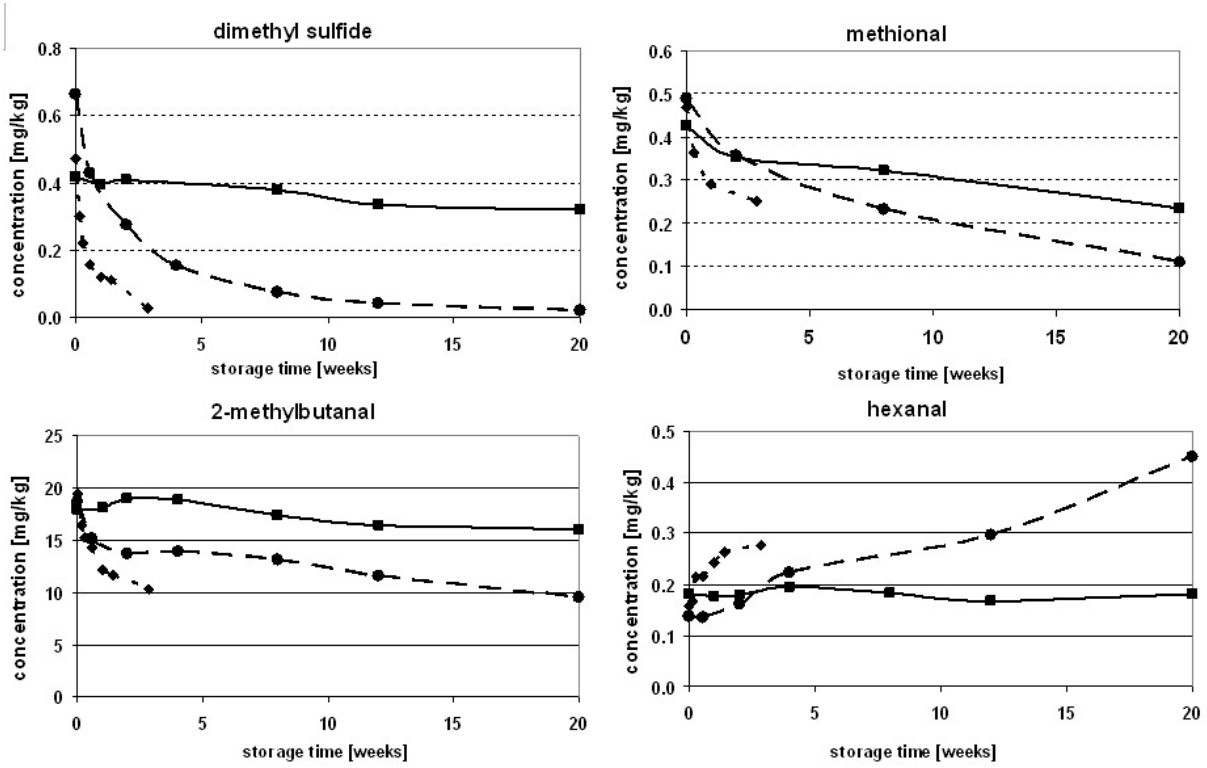

Figure 1. Alteration of freshness and oxidation markers during 20 weeks (20 days) in single sealed R&G coffee —■—, unprotected roasted coffee beans —●—, and unprotected R&G coffee —◆—.

The quantification of freshness markers revealed that degradation of sulfur compounds proceeds rapidly in roasted coffee already during a short storage period when it is exposed to air. Particularly in R&G coffee, a decrease in the concentration of dimethyl sulfide from 0.47 mg/kg to 0.12 mg/kg and methional from 0.47 mg/kg to 0.29 mg/kg was observed during the first seven days of storage.

In roasted whole beans the amounts of dimethyl sulfide and methional decreased from 0.66 mg/kg to 0.28 mg/kg and from 0.48 mg/kg to 0.36 mg/kg, respectively, during storage for two weeks. In comparison, these compounds were significantly more stable in portioned coffee during the whole storage period, where concentrations of dimethyl sulfide only slightly changed.

In protected R&G coffee 2-methylbutanal was present in a concentration of 17.9 mg/kg. After twenty weeks of storage a concentration of 16 mg/kg was measured. Changes were more pronounced in unprotected roasted coffees, where quantities in R&G coffee decreased from

19.5 mg/kg to 12.2 mg/kg after seven days and in whole beans from 18.6 mg/kg to 9.5 mg/kg during storage of twenty weeks.

In addition, analytical results showed an immediate generation of hexanal in roasted coffee stored under non-protective conditions. In R&G coffee the concentration of hexanal rose from 0.16 mg/kg up to 0.26 mg/kg during ten days of storage, whereas in whole beans its amount was found to be 3 times higher after storage for twenty weeks. No formation of hexanal was determined in single portions where the concentration remained constant during the whole storage period.

The concentrations of low volatile compounds like 4-vinylguaiacol, sotolone and 2-ethyl-3,5-dimethyl pyrazine remained quite constant in all products during the whole storage period (data not shown).

It can be concluded that during the shelf life of coffee, especially when exposed to oxygen, important aroma compounds (sulfur compounds and Strecker aldehydes) decrease, thus resulting in fading of coffee freshness. In parallel, oxidation products like hexanal increase and several low volatiles (pyrazines, phenols and furanones) remain constant. All together, these phenomena lead to a relevant alteration of the coffee aroma balance.

Impact of oxygen present during filling of R&G coffee

The influence of oxygen present during filling of R&G coffee in single portions on its shelf life is summarized in Figure 2.

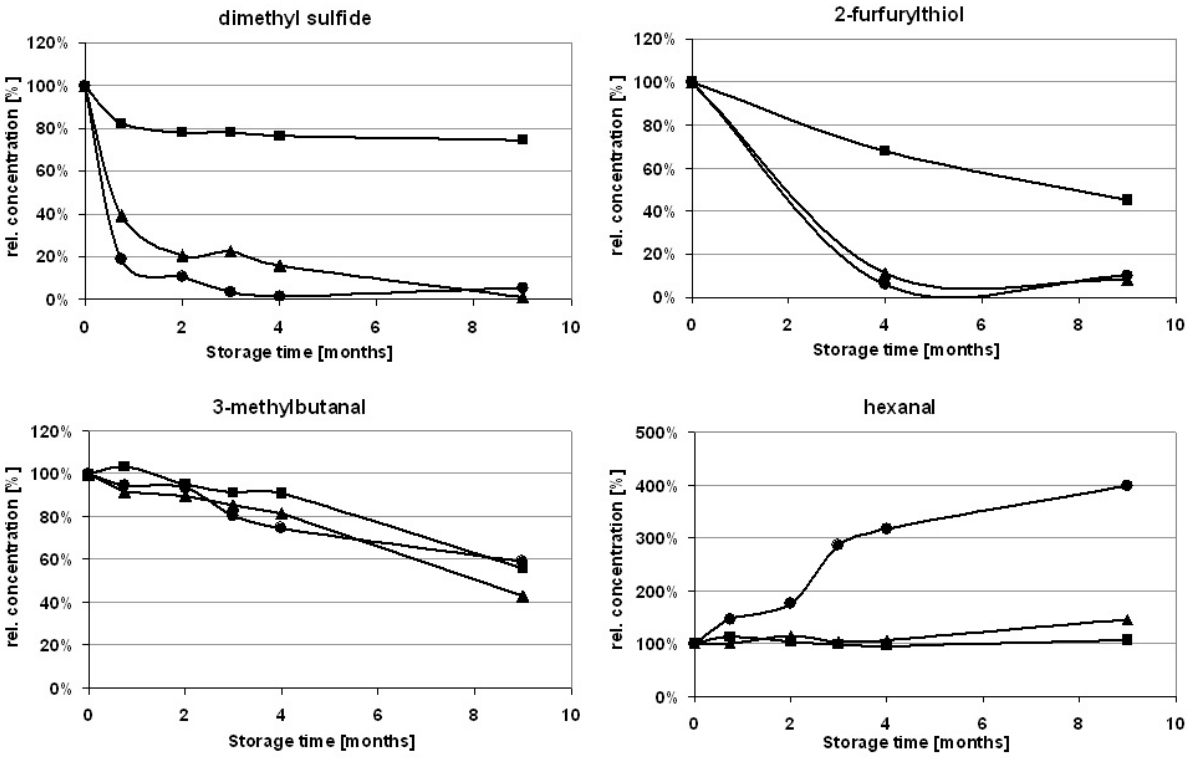

Figure 2. Storage test during 9 months on single sealed R&G coffee filled under different oxygen levels. Relative concentrations of freshness markers are given in % (t_0 set as 100%; —■— filled at 2% oxygen; —▲— filled at 5% oxygen; —●— filled at 21% oxygen).

The amounts of sulfur compounds, especially of dimethyl sulfide and 2-furfurylthiol, were shown to decrease rapidly towards 0% in the samples that contained 5% or more oxygen. At lower oxygen level of 2% degradation reactions were significantly slowed down. After nine months of storage 80% of dimethyl sulfide and about 50% of 2-furfurylthiol was still present in the product.

The amount of 3-methylbutanal decreased in all coffee samples to a level of 40% to 60% during nine months storage. It can be assumed that its degradation is not oxygen-related. Other reactions might occur as the aldehydes are quite electrophilic molecules susceptible to different kind of chemical reactions.

Concentrations of hexanal increased significantly when the coffee was filled under non-protective atmosphere (21% oxygen) resulting in a four time higher content. Lipid oxidation was shown to be delayed when oxygen level is at 5% in the single portion. No formation of hexanal was observed in R&G coffee when oxygen was reduced to 2%.

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Studies on the Orthonasal and Retronasal Aroma Profiles of Freshly Brewed *Espresso* and Caffè Latte

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SUMMARY

Studies on fresh and pleasant aroma released from espresso provide useful information for developing attractive beverage products of coffee. However, fresh espresso aroma changes quickly, and a sampling method to collect a sufficient amount of headspace volatiles in a short time is required for this study. A versatile analytical method using solid-phase microextraction (SPME) was developed for evaluating orthonasal (headspace) aroma and retronasal aroma of espresso. Volatiles from the headspace above freshly brewed espresso in a beaker were collected with SPME for the evaluation of headspace aroma, and volatiles in a retronasal aroma simulator (RAS), a model mouth developed by Roberts and Acree (1995), were collected for the evaluation of retronasal aroma of espresso. Among several types of SPME fibers, divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS) showed the highest overall sensitivity. Experiments to optimize exposure time of SPME fiber showed that sampling headspace aroma for 1 min and retronasal aroma for 2 min were within linear range for most espresso volatiles respectively. Volatiles collected with SPME fiber were analyzed by gas chromatography/mass spectrometry (GC/MS) and GC/olfactometry (GC/O, CharmAnalysis™). The 64-fold GC/O dilution analysis was achieved by varying length of SPME fiber and by splitting of the injected sample in different ratio in the GC injection port. Attractive aromas released from freshly brewed espresso and caffè latte were evaluated using this optimized reproducible method.

INTRODUCTION

In our previous studies (Akiyama et al., 2003; 2005; on submitting), we developed an SPME-based headspace sampling method for flavor analysis and evaluation of the characteristic coffee aroma generated during grinding and from freshly brewed drip coffee. This sampling method proved to be simple, rapid, reproducible and suitable to collect sufficient amount of headspace volatiles in a short time for GC/MS and GC/O analysis, which were effective tools for the characterization of the aroma profile of fresh coffee.

This study is aimed at the examination of volatile composition and odorants that contribute to characteristic orthonasal and retronasal aromas of freshly brewed espresso, and the influence of the addition of milk to the both fresh espresso aromas. Therefore, applying the optimized SPME sampling method suited to capture headspace volatiles, the method of analyzing the volatiles released from freshly brewed espresso using RAS (Roberts and Acree, 1995; Deibler et al., 2001) was developed in order to investigate profile of the retronasal aroma. In addition, we adopted a 64-fold GC/O dilution analysis by varying length of SPME fiber and

by splitting of the injected sample in different ratio in the GC injection port (Deibler et al., 2004) in this study. Odor intensities of the aromas released from freshly brewed espresso and caffè latte, which is made from fresh espresso and milk, could be evaluated in detail using this optimized and reproducible method. The GC/O analysis showed different aroma profiles for headspace (orthonasal) aroma and RAS (retronasal) aroma of espresso or caffè latte, and the influence of the addition of milk to their aroma profiles.

MATERIALS AND METHODS

Coffee and Milk Sample

Ethiopia G2 and Guatemala SHB arabica coffee (*Coffea arabica*) beans were roasted to dark roast (roast degree L value; 18) using a Probat G-12 roaster (Emmerich, Germany). The former was used for comparison of the GC/O results from the 64-fold dilution analysis and conventional 8-fold dilution analysis (Figure 3) and the latter was used for the other experiments. Commercial UHT-pasteurized (135 °C, 2 sec) milk manufactured by Morinaga Milk Industry was used in the milk addition experiments. The milk contained more than 3.5% fat and more than 8.3% solid of non-fat.

Espresso Brewing

The automatic espresso machine (Saeco Royal Professional, Nihon Saeco K. K., Kawasaki, Japan) was used for grinding roasted coffee beans and brewing espresso. Roasted coffee (ca. 7.5 g) were ground, and brewed with ion exchange hot water with pressure of 15 atm for ca. 15 sec. It took ca. 30 sec from grinding to brewing. The espresso was characterized as follows; volume ca. 65 g, Brix ca. 2.5 °, temperature of espresso ca. 70 °C, temperature of headspace air ca. 55 °C.

SPME Device

DVB/Carboxen/PDMS fiber with 50/30 _m thickness (Supelco Co., Bellefonte, PA, USA) was used for sampling headspace and RAS volatile compounds.

Headspace SPME Sampling

A headspace sampling method was used as a sampling method for orthonasal aroma. The espresso was collected into 300 mL-volume beaker, in which hot water and milk (ca. 70 °C, total 130 g (Table 1)) heated by a microwave oven had been prepared, and covered with aluminum foil after mixing. The SPME fiber, the stainless steel housing of which was set into the flask as shown in Figure 1, was pushed out of its housing just after the espresso was brewed, and exposed to the headspace air.

Table 1. Sample composition of espresso (milk 0%) and caffè latte (milk 20%, milk 60%).

	Espresso	Milk	Water	Milk solid of non fat (SNF)	Milk fat	Coffee (in terms of green beans)
Milk 0%	65 g	?/	130 g	?/	?/	5.0%
Milk 20%	65 g	39 g	91 g	1.7 g	0.7%	5.0%
Milk 60%	65 g	117 g	13 g	5.0 g	2.1%	5.0%

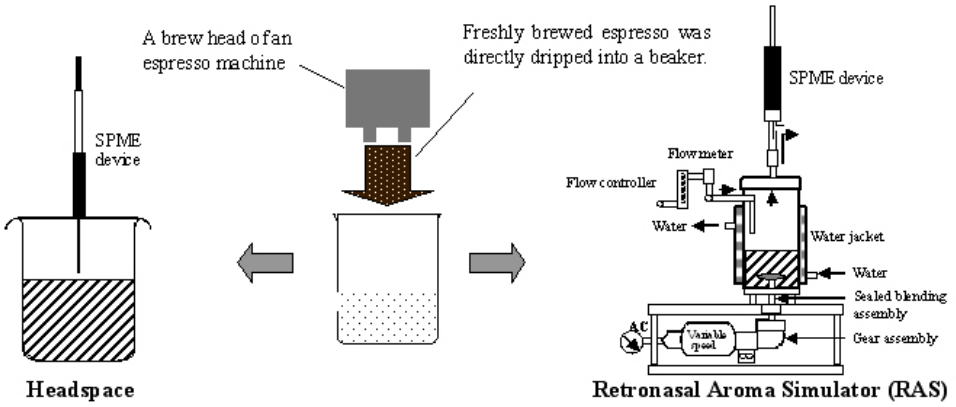

Figure 1. Sampling method and apparatus used to trap headspace and RAS volatile compounds of freshly brewed espresso and caffè latte.

RAS Parameters and SPME Sampling using RAS

A method using RAS was used as a sampling method for retronasal aroma. The RAS was composed of a 1-L stainless steel blender container and assembly, a voltage controller and high torque-speed motor to give precise control of blender speed (650 rpm) to simulate a model mouse, a controlled nitrogen gas supply (1000 mL/min) to sweep over the freshly brewed espresso (milk 0%) or caffè latte (milk 20%, milk 60%), and a water jacket to control the temperature (38 °C) (Figure 1). The RAS volatiles were trapped by exposing the SPME fiber to the effluent gas.

Dilution Analysis by Varying Length of SPME Fiber and GC Split Ratio (Injector Splitting Dilution Method) and SPME-GC/O Evaluation of Volatile Compounds

Volatile compounds of the headspace were collected with two different SPME fiber lengths (20 mm and 0.25 mm), respectively. After sampling, the fibers was placed into the injection port of the GC/O, and thermally desorbed for 10 min at 250 °C with dilution ratios (1, 1/2, 1/4, 1/8) using four different split ratios (1:0, 1:1, 1:3, 1:7) as shown in Figure 2. Odor activities of volatile compounds obtained by GC/O dilution analyses were represented as charm values (Acree et al., 1984) and the relative importance of component odorants were represented as odor spectrum value (OSV). OSV is the normalized charm value modified with an approximate Stevens' law exponent (n = 0.5) (Acree, 1997).

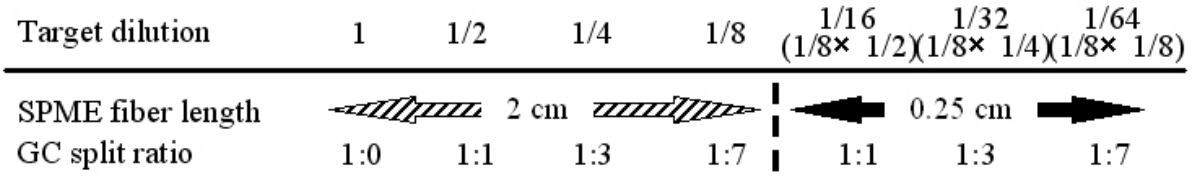

Figure 2. Schematic diagram of GC/O dilution analysis by varying length of SPME fiber and GC split ratio.

RESULTS AND DISCUSSION

Parameters for Headspace and RAS SPME Sampling

DVB/Carboxen/PDMS fiber, which had previously been found to be optimum among six SPME fibers to sampling headspace volatile compounds from brewed drip coffee (Akiyama

et al., on submitting), was adopted for sampling headspace volatile compounds from espresso and caffè latte in this study.

Firstly, for determination of the appropriate SPME sampling time for studying headspace aroma, the SPME fiber was exposed to the headspace of freshly brewed espresso for 0.5, 1, 2, 3, and 4 min respectively. To make analysis more effective it is favorable to collect volatiles in larger quantity, but a longer sampling time can lead to changes in the fresh coffee aroma, or a constitutional difference from 'real headspace' caused by competition phenomena on the SPME fiber. In the case of 2 min sampling, some compounds such as 2- and 3-methylbutanals showed smaller increase in peak abundance and lost linearity, which led to a change in the component proportion of headspace volatiles. From these results, 1_min exposure, which maintained high coefficients of determination (R^2 > 0.80) between each peak area and exposure times, was chosen for the sampling time for headspace aroma. In this manner, optimum sampling time was determined to be 1 min for headspace aroma and 2 min for RAS aroma. Relative standard deviations (RSDs) of the peak area of each component in three repetition experiments under the same sampling condition were small enough (RSDs < 10%) in both cases.

GC/O Dilution Analysis by Varying Length of SPME Fiber and GC Split Ratio (Injector Splitting Dilution Method)

The 8-fold GC/O dilution analysis using varying length of SPME fiber (20, 10, 5, and 2.5 mm) was successfully applied to the GC/O analysis of coffee and we verified the reliance in previous studies (Akiyama et al., 2003; Akiyama et al., on submitting). In this study, a new 64-fold GC/O dilution analysis obtained by varying length of SPME fiber and GC split ratio (Deibler et al., 2004) was adopted in order to obtain more detailed information on odor intensity.

The GC/O results from the 64-fold dilution analysis using the injector splitting dilution method were compared with those from conventional 8-fold dilution analysis method by varying length of SPME fiber as shown in Figure 3. The results gave more detailed information and new comprehension, with 8 odorants (guaiacol (phenolic), 4-ethylguaiacol (phenolic), 2-furfurylthiol (smoke-roast), 4,5-diemethyl-3-hydroxy-2(5*H*)-furanone (sweet-caramel), 2-ethyl-3,5-dimethylpyrazine (nutty-roast), furufryl methyl sulfide (smoke-roast), 2,3-diethyl-5-methylpyrazine (nutty-roast), and skatole (phenolic)) showing larger intensities in the 64-fold dilution analysis than in the 8-fold dilution analysis.

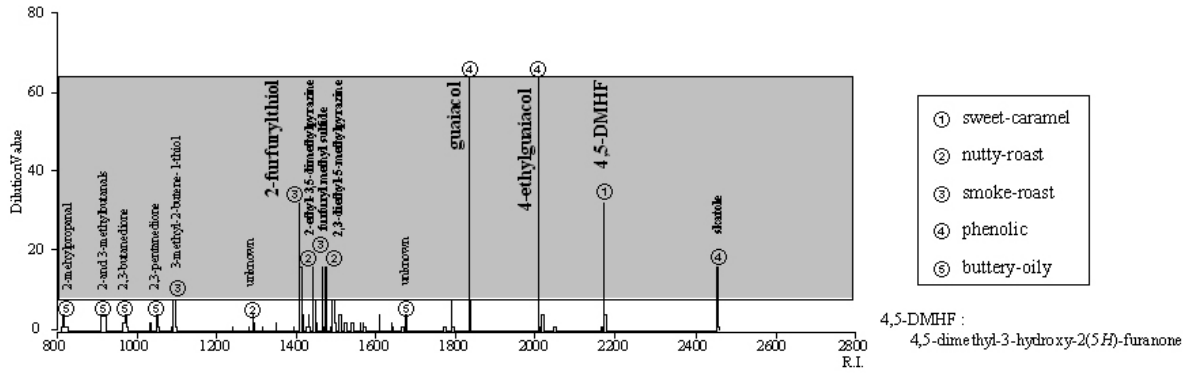

Figure 3. Comparison of the charm chromatogram from the 64-fold dilution analysis using the injector splitting dilution method and conventional 8-fold dilution analysis method by varying length of SPME fiber.Gray background: range of GC/O dilution analysis from 8- to 64-fold.

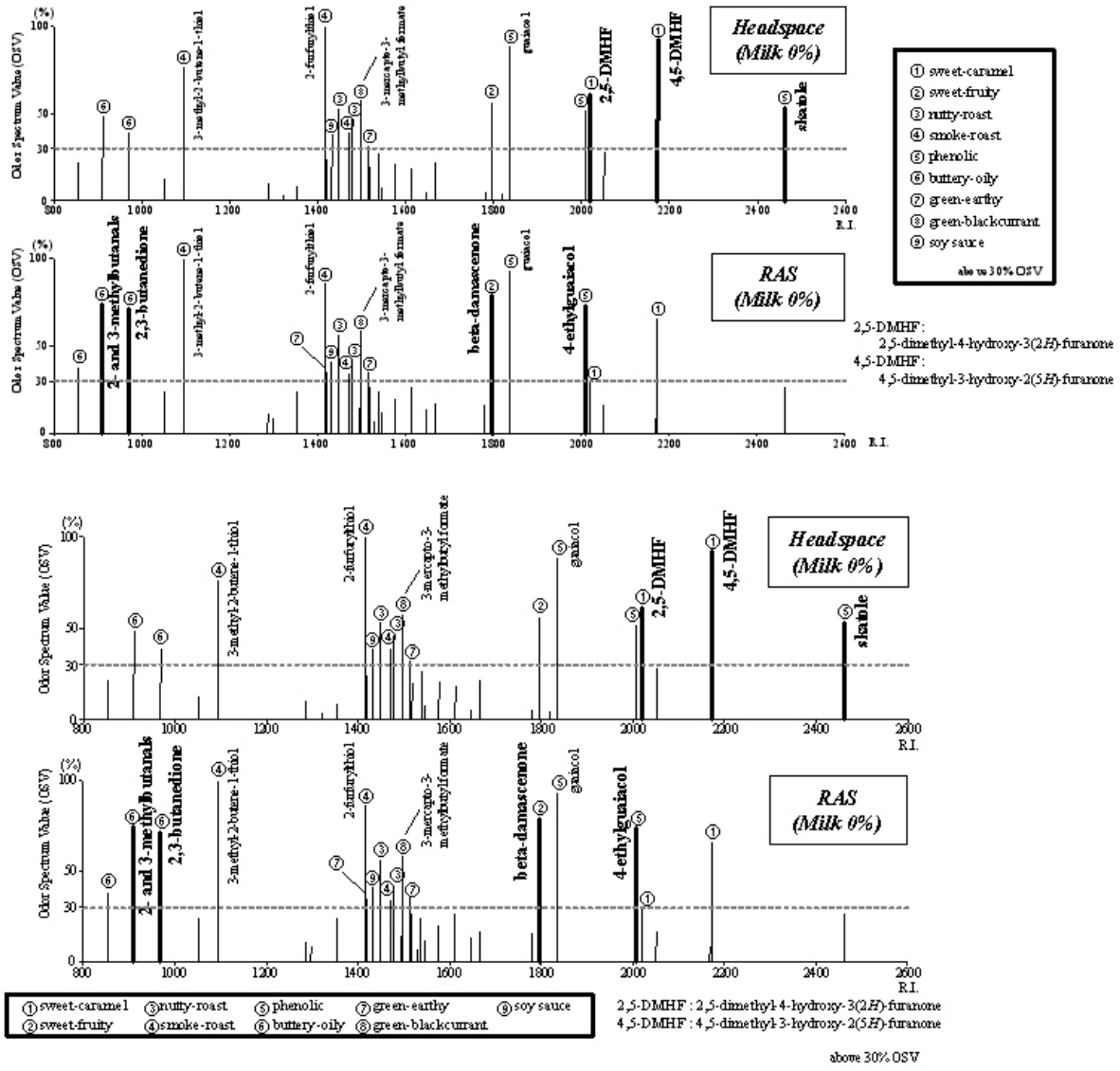

Figure 4. Comparison of the odor spectrum of headspace (orthonasal) aroma and RAS (retronasal) aroma.

Comparison of the Odor Spectrum of Headspace Aroma and RAS Aroma

The odor spectrums from GC/O analyses of headspace aroma and RAS aroma were compared in Figure 4. From the comparison of the odor profiles, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (sweet-caramel), 4,5-dimethyl-3-hydroxy-2(5*H*)-furanone (sweet-caramel), and skatole (phenolic) showed higher intensity ratio in headspace aroma of espresso. On the other hand, 2- and 3-methylbutanals (buttery-oily), 2, 3-butanedione (buttery-oily), beta-damascenone (sweet-fruity), and 4-methylguiaiacol (phenolic) had higher OSV in RAS aroma.

Influence of Milk Addition on Headspace Aroma and RAS Aroma

Commercial UHT-pasteurized milk was added to freshly brewed espresso with ratios of 20% or 60% as the composition shown in Table 1. The changes of aroma profiles by adding milk to the espresso were examined. The total intensity (charm values), and in particular the headspace aroma intensities of 3-methyl-2-butent-1-thiol (smoke-roast), beta-damascenone

(sweet-fruity), and skatole (phenolic) became smaller as a content ratio of milk increased (milk 0% → 20% → 60%). Similar influences from milk addition were observed for RAS aroma (data not shown).

Comparison of the Headspace Aroma and RAS Aroma Profiles from Espresso and Caffè latte

The odor compounds detected by GC/O were classified to ten odor descriptions. The charm value ratios of each description versus total charm values were shown in Figure 5. In espresso (milk 0%), headspace aroma showed higher ratio of sweet-caramel than RAS aroma, whereas RAS aroma had higher ratio of buttery-oily than headspace aroma. In caffè latte (milk 60%), headspace aroma showed a similar ratio for each description as espresso, however, RAS aroma had higher ratio of phenolic description than espresso. From these results, it was suggested that milk had little influence on the headspace aroma, but a large influence on the RAS aroma, in particular to phenolic odor.

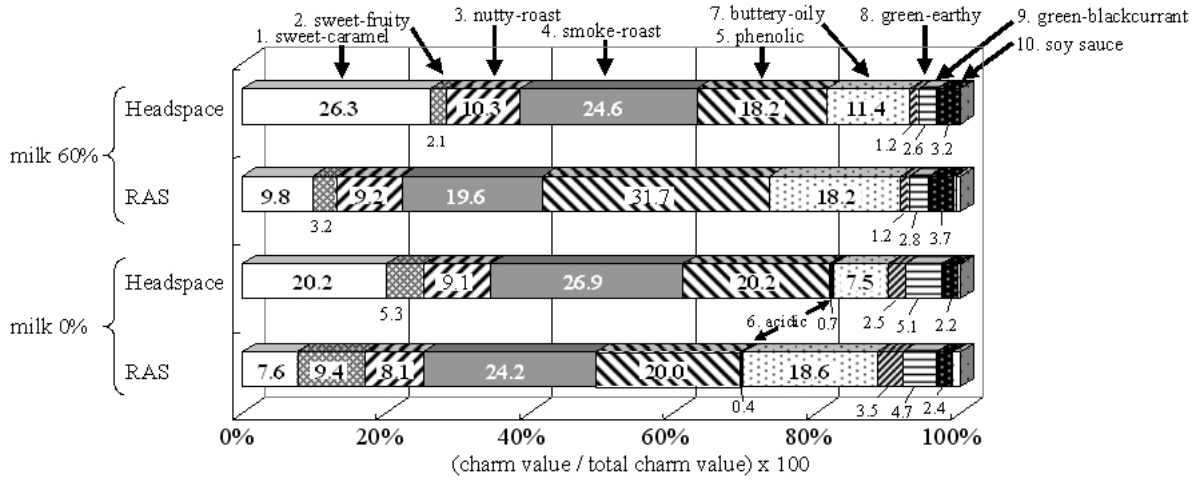

Figure 5. Comparison of the headspace aroma and RAS aroma profiles from espresso and caffè latte.

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Flavour Perception of White Coffee Beverages – Influence of Milk Processing

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SUMMARY

The popularity of coffee beverages is based on its energising effect and the special flavour. This flavour is undoubtedly affected by the addition of milk products. The influence of milk processing and ingredients on sensory properties of white coffee beverages has not yet been thoroughly investigated. In order to gain further insights into these interactions, analysed milk products processed in a defined manner were added to a standardised fresh coffee beverage. The resulting odour, taste and retronasal odour perception were measured by sensory evaluations (intensity tests), and selected volatiles were analysed by static headspace (SHS) GC/MS. The effects of varying fat contents (3.5 and 1.5%) and fat dispersion were studied. The milk with the lower fat content and with smaller fat globules, resulting from double homogenisation (250/50 bar each), induced a more intense coffee-related retronasal odour perception whereas the milk-related impression was similar to the whole milk. An addition of casein increased the creamy and milky odour perception and reduced the coffee-related taste and retronasal odour, respectively. Using instrumental analysis, it could be observed that the effect of an addition of twice homogenised, twice pasteurised low-fat milk and an addition of whole milk were similar.

INTRODUCTION

The coffee flavour of milky coffee beverages is not only determined by the coffee but it also develops an interplay with the added milk product. For the coffee specialties such as “cappuccino” or “café au lait” which are in increasing demand, the several consumers’ milk products are used as additives. Up to now the influence of the milk treatment and of the milk constituents on odour, taste and retronasal odour perception of white coffee beverages are relatively unknown. Numerous investigations have already been performed on the pure roasted coffee. E.g. Bücking and Steinhart used milk products for their studies, but those were limited to commercial products (Bücking and Steinhart, 2002). In order to determine the relevant influencing factors for the flavour of milky coffee beverages in this project, the components and those parameters important in the industrial production and manufacturing, resp., of coffee and the milk products were to be varied. The effects of those variations were to be sensorically and analytically monitored by the changes in descriptors (Parat-Wilhelms et al., 2005; Denker et al. 2006). Variations in the fat content of a food matrix are known to affect aroma release. The influence of fat is expressed by the hydrophilic and/or hydrophobic interdependency (de Roos, 1997). The lipophilic flavour compounds are bound to the fat molecules by reversible van der Waals forces (Plug and Haring, 1993). In the presence of proteins, the flavour release is reduced due to the proteins' capability to form covalent and reversible hydrogen bonds with the flavour substances (Leland, 1997).

MATERIAL AND METHODS

Coffee

Arabica coffee beans from Kenya were dark roasted (roasting degree: 70 scale divisions; 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

Low-fat milk (1.5% fat) and whole milk (3.5% fat) were made of fresh raw milk (on pilot plants). The whole milk was homogenised once at a pressure of 200/50 bar. The low-fat milk samples were homogenised twice at 250/50 bar and 60 °C. Both milk variants were heated up to 73 °C for 20 s.

Sensory Evaluation

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 panelists had previously received a special training in intensity sensory evaluations of coffee beverages. 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 (Bücking 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).

Static Headspace Analysis

For the external static headspace the parameters and the modified device according to Bücking and Steinhart were used (Bücking and Steinhart, 2002; Parat-Wilhelms et al., 2005). The volatiles were trapped on Tenax® TA and were desorbed from the Tenax® TA tubes by use of a thermal desorber auto sampler system TDS A (Gerstel, Mühlheim a. d. R., Germany) and injected into a cold injection system CIS 3 (Gerstel) with liquid nitrogen cooling (–150 °C). The studied volatiles were identified by their mass spectra, odour impressions and retention times in comparison with standard substances. GC analysis was performed with a Hewlett-Packard model series II gas chromatograph (HP 5890 GC series II) coupled with an HP 5971A mass selective detector (MSD) and a sniffing port for GC/O analysis.

RESULTS AND DISCUSSION

The low-fat milk should always undergo a more intense homogenisation, in order to get a fat globule surface and a full body comparable to the whole milk. A short-time pasteurised whole milk was served as control. The resulting volume-related mean fat globule diameter d[4.3] was 0.47 µm. When the average value was related to the fat globule surface, d[3.2]-values resulted in approx. 0.30 µm. The double homogenisation of the low-fat milk resulted in a d[4.3]-value of approx. 0.23 µm and a d[3.2]-value of approx. 0.14 µm (Figure 1). The specific volume-related surface (SV) can be calculated in m^2 per ml fat from the d[3.2]-values (Buchheim et al., 1986). For the whole milk (d[3.2] = 0.30 µm) the fat globule surface was approx. 750 m^2 per kg milk. For the twice homogenised low-fat milk only a slightly smaller surface of 690 m^2 was calculated. However, it has to be considered that with the decrease of fat content and fat globule diameter the casein micelles increasingly enter into the calculation. The bimodal distribution curve shows that the demarcation of casein and fat particles could only be estimated. If a border is set at 0.195 µm, which does not seem completely unrealistic

for the process of the distribution curves, the calculated d[3.2]-value increases from approx. 0.14 to 0.32 µm. Consequently, the fat globule surface is reduced to approx. 300 m^2/kg milk. According to a rough estimation of Walstra, the fat globule surface is covered by approx. 10 mg protein per m^2 fat (Walstra et al., 1999). This means that whole milk needs about 0.7-0.8% protein and low-fat milk only about 0.3 % for the emulsification of the fat globules.

The question as to whether the addition of twice homogenised low-fat milk samples to the coffee beverage gave different effects in comparison to the use of whole milk was examined using triangular tests. Differences were significantly confirmed.

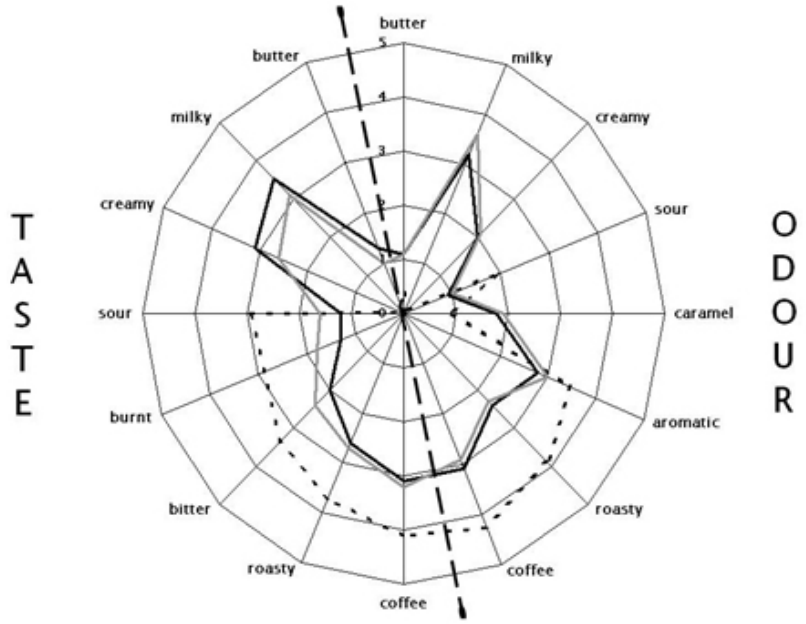

Figure 1. Aroma profile: odour and taste intensities of coffee beverages with additions of differently treated milk samples.

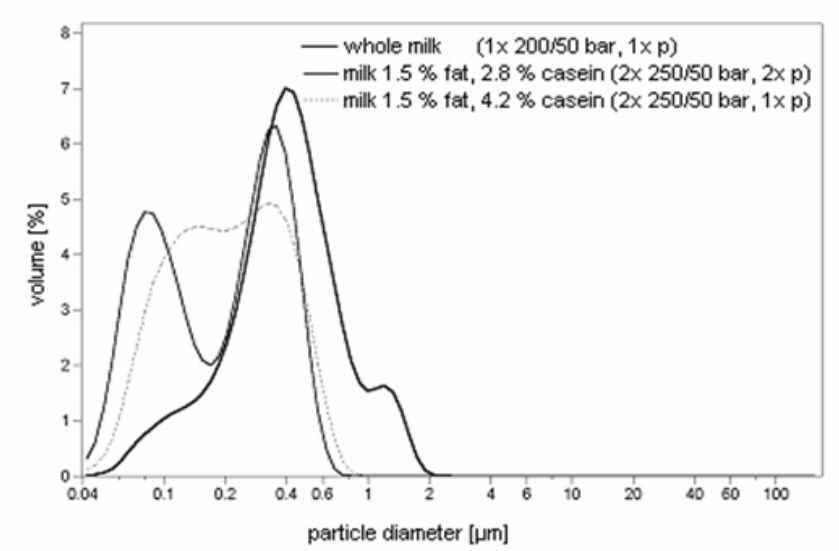

Figure 2. Volume-related particle size distribution of differently treated milk samples (p - pasteurised).

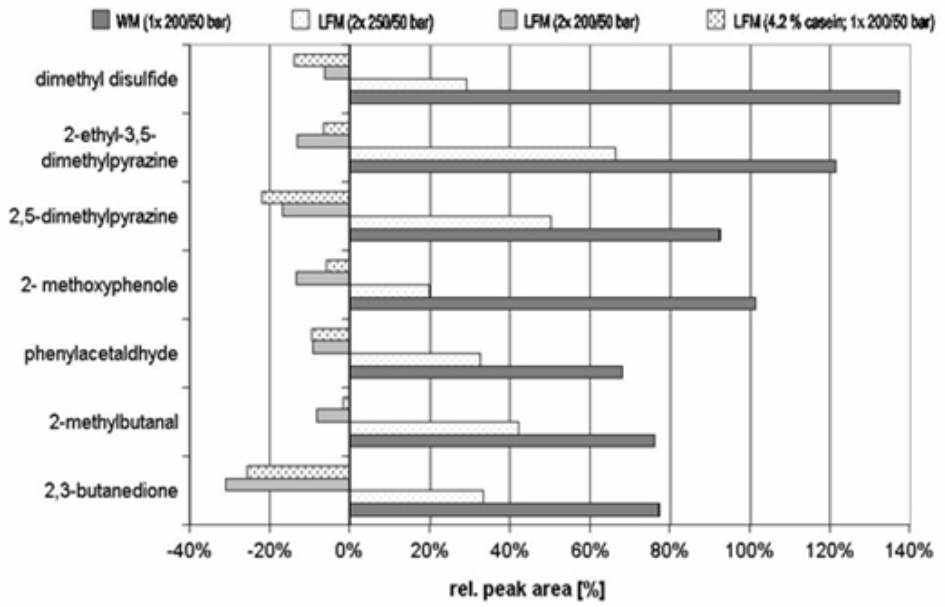

Figure 3. Static Headspace-GC/MSD peak areas (average of three measurements) of selected flavour compounds shown as percentage related to a white coffee beverage (once homogenised low-fat milk as additive; corresponds to 0%).

An intensity test was therefore performed, in order to identify the responsible descriptors. The odour and the milk-typical taste descriptors of the twice homogenised low-fat milk did practically not differ from the whole milk. The coffee-typical descriptors for taste were generally more perceived with the addition of the low-fat milk. For the twice homogenised low-fat milk with a raised casein content of 4.2%, the 'creamy' and 'milky' taste impressions were more intensively noticed, while the 'sour' taste descriptor was weaker in comparison to the whole milk. It has already been noticed in previous investigations that an addition of casein reduces the 'sour' taste. The addition of casein increased the viscosity, and thus the creamy taste impression is understandable, while this cannot affect the odour in the same degree (Figure 2). In Figure 3 results of some volatiles are presented. It can be observed that the effects of an addition of twice homogenised low-fat milk and an addition of whole milk were similar. In both cases, more volatiles were released from the beverages than with the addition of low-fat milk that was only homogenised once. This is an unexpected result, as the whole milk has a higher fat content than the low-fat milk, and therefore a greater retention of the flavour compounds was expected. It is known that fat acts as a solvent for lipophilic aroma compounds and reduces the volatility of these compounds (de Roos, 1997). We assumed that a distribution of the flavour compounds in the fat phase is not possible, because the fat globule membrane prevents the aroma-transfer. In this case the retardation or release characteristics of the volatiles are only based on reciprocal effects of membrane compounds, probably mainly with proteins. The treatment of the milk influences the composition of the fat globule membrane. During the homogenisation the total surface of the fat globules is increased and the material of the original fat globule membrane is not sufficient to cover the new fat globules. Because of the hydrophilic and hydrophobic regions of the casein micelles they can immediately cover the exposed new fat globules. If the milk is homogenised below 65 °C, only casein adsorbs on the fat globules. The whey proteins begin to denature above temperatures of 65 and/or 70 °C. Only the denatured state can attach to the fat globules. The new secondary membranes have local differences in thickness, which depends on the coincidental adsorption of the new membrane components (Walstra and Jenness, 1984). If the thermal treatment takes place after homogenisation, an association of the denatured whey proteins with the residue of the original membrane or with the casein is possible (Dalgleish

and Sharma, 1993). In particular, the β-lactoglobulin associates with the hydrophilic components of the casein (κ-casein). If the thermal treatment takes place before homogenisation, then 'whey protein/casein complexes' are formed in the milk serum before the formation of the secondary membrane starts. Therefore, it can be concluded that the secondary membrane is not identical in the two cases. Whether the ratio of casein and whey protein at the membrane differs is controversially discussed in the literature (Lee, S.J. and J.W. Sherbon (2002; Sharma and Dalgleish, 1994). These complex coherences could be a reason for the unexpected results of the investigations.

ACKNOWLEDGEMENTS

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Combined Effect of Water Activity and Temperature on Chemical Stability of Coffee Beverage

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SUMMARY

This work was addressed to study the chemical stability of coffee brew derivatives as a function of a_w and storage temperature. To this purpose, coffee brew was freeze-dried, equilibrated at increasing a_w and stored for up to 10 months at different temperatures from –30 to 60 °C. The chemical stability of the samples was assessed by measuring $[H_3O^+]$ formation rate (*k*). Independently on storage temperature, a maximum *k* value was observed at *circa* 0.8 a_w, suggesting mechanisms other than lactones' hydrolysis to contribute to the increase in acidity during coffee staling. In addition, the temperature dependence of *k* was well described by the Arrhenius equation.

INTRODUCTION

In the past decade, ready-to-drink coffee beverages have encountered increasing success in eastern countries where coffee drinking represents an almost new habit. On the other hand, in countries with a coffee drinking long tradition, the popularity of these beverages is still hindered because of their poor quality, mostly attributable to their very low chemical stability. Quality depletion is characterised by a change in the flavour profile and an increase in perceived sourness, which are accompanied by a pH decrease whose clear explanation is not put forward at present (Sivetz, 1963). The increase in quinic acid, mainly attributed to hydrolysis of esters and lactones previously formed during roasting, has been considered the main driving factor of the pH decrease (Maier et al., 1984; Nicoli et al., 1991; Dalla Rosa et al., 1990). However, the formation of quinic acid would account for only 25% of the overall acidity developed (van der Stegen and van Duijn, 1987). It has also been suggested that the pH decrease could be the consequence of complex reactions, probably related to non enzymatic browning pathways, involving carbohydrates and acids (Maier and Ochs, 1973).

Considering both mechanisms to be involved in beverage quality depletion during coffee brew storage, water content, and hence water activity (a_w), are expected to play a key role in determining the rate of pH decrease. In addition, the effect of a_w is likely to be strongly affected by storage temperature. However, due to uncertainty and complexity of the chemical reactions concerned, the effect of these factors on acidity development can be difficulty figured out. Undoubtedly, such information would be of great interest in order to find out processing and storage conditions allowing to obtain more chemically stable ready-to-drink coffee beverages.

MATERIALS AND METHODS

Ground dark-roasted coffee was purchased on local market. Coffee beverage was obtained by extraction of 100 g coffee powder with 1 L distilled water at 90 °C (Kenwood aromatica CM720, New lane, Havant, England). After extraction, coffee beverage was freeze-dried

(Mini Fast mod. 1700, Edwards Alto Vuoto Spa, Milano, Italy) and equilibrated at 25 °C in vacuum dessicators containing saturated salt solutions (Carlo Erba, Milano, Italy) having equilibrium relative humidity (ERH %) up to 100 % (Table 1). Equilibration times resulted to be less than 4 hours.

Table 1. Solid concentration of coffee samples equilibrated at increasing a_w.

a_w	Solid concentration (% w/w)
0.01	100.00
0.33	97.04
0.52	93.70
0.84	77.98
0.99	15.00
$0.99\overline{}$	1.80

Coffee samples with different a_w were hermetically sealed in glass vials and stored in the dark at –30, –18, –7, 0, 10, 20, 30, 45 and 60 °C for up to 10 months. At different times during storage, samples were removed from the incubators and rehydrated to a solid concentration of 1.80 % (w/w). Preliminary trials showed that freeze-drying and rehydration had no significant effect ($p > 0.05$) on the pH of the beverage.

Total solid content was determined according to AOAC.

Water activity (a_w) was determined by means of a dew-point measuring instrument (AQUA LAB, Decagon, Pullman, WA, USA) at 25 °C.

pH of coffee beverages was assessed by a Mettler Toledo 355 pH-meter (Lou Analyzer, Halstead, England). The changes in $[H_3O^+]$ followed the zeroth order kinetic and relevant rates (k) were calculated by linear regression of $[H_3O^+]$ as a function of storage time. The effect of temperature on k was evaluated by means of the Arrhenius equation**:**

$$k = k_o \cdot e^{-\frac{E_a}{RT}}$$

where k is the reaction rate constant; R is the molar gas constant (8.31 J/K/mol), T is the absolute temperature (K); E_a is the activation energy (J/mol) and k_o is the frequency factor.

The results reported in this work are the average of at least two determinations carried out on different samples and the coefficients of variation were less than 5% for total solid content, aw and pH and less than 10 % for k. Least squares linear regression analysis was performed by using Statistica for Windows (ver. 5.1, Statsoft Inc., Tulsa, USA, 1997).

RESULTS AND DISCUSSION

Coffee samples with different a_w were stored for increasing time in the temperature range from –30 to 60 °C and assessed for pH. pH data were thus used to calculate the changes in $[H_3O^+]$ during storage and the apparent rate constants of H_3O^+ formation (k). The changes in $[H_3O^+]$ and k values for coffee with $0.99\overline{}$ a_w, corresponding to 1.8 % solid concentration, are shown, as examples, in Figure 1.

Similarly, the values of k ($R^2 > 0.90$, $p < 10^{-2}$) relevant to coffee with increasing a_w and stored in the temperature range from 60 to –30 °C were calculated. Figure 2 shows the effect of a_w on k values of coffee samples stored at different temperatures.

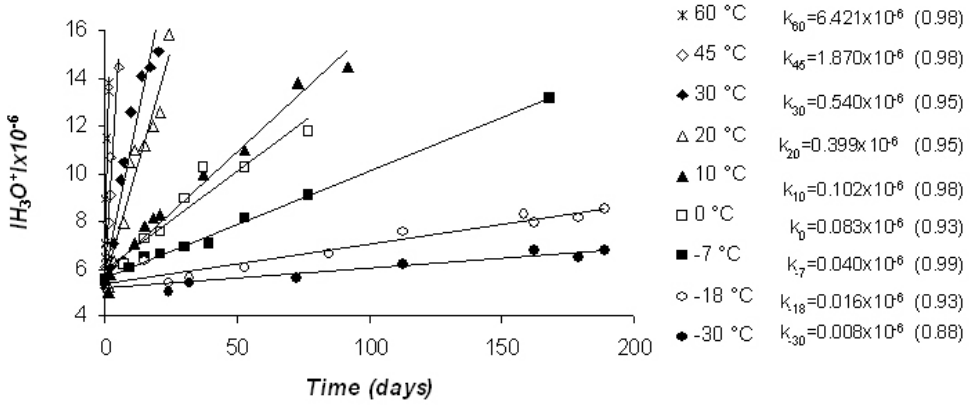

Figure 1. $[H_3O^+]$ of coffee with $0.9\overline{9}$ a_w as a function of storage time at different temperatures. The apparent zero-order rate constants of H_3O^+ formation (k, $[H_3O^+]$ day^{-1}) and the relevant determination coefficients (in brackets) are also shown.

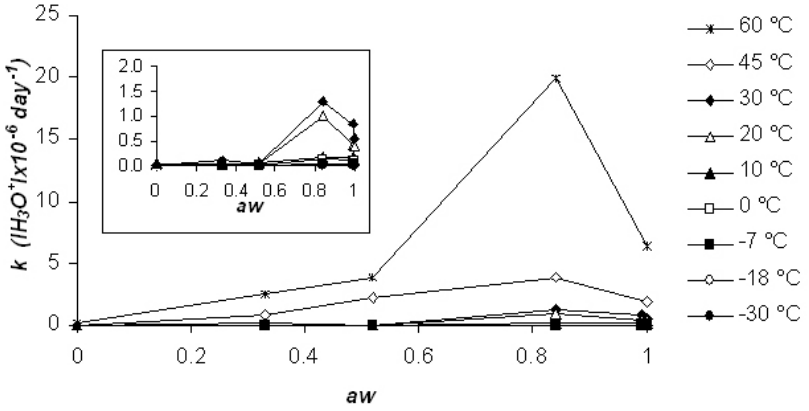

Figure 2. Apparent zero-order rate constants of H_3O^+ formation as a function of a_w of coffee samples stored at different temperatures. In the inset: detail of data in the k range from 0 to 2 $[H_3O^+]$ day^{-1}.

It can be observed that, at *circa* 0.8 a_w, corresponding to a solid content of 78 % (w/w), k presented a maximum value, whose intensity increased with temperature. This result clearly indicates that drying does not allow the stability of coffee to be obtained, unless very low a_w values are reached. In fact, drying to intermediate moisture levels promoted an unexpectedly fast quality depletion. These data seem to contradict the hypothesis that the decrease in pH during coffee beverage staling is solely attributable to hydrolysis of esters and lactones previously formed during roasting. In fact, in this case, water should be regarded as a reactant rather than as diluting media. Consequently, upon hydration, k should progressively enhance even beyond a_w 0.8, which was clearly not observed (Figure 2). It is noteworthy that the a_w dependence of the reaction rate tends to a bell-shape curve with a maximum at intermediate a_w values when food solids chemically react with each other in an aqueous system, as occurring during non enzymatic browning reactions (Duchworth, 1981). For this reason, the presence of a maximum rate at intermediate a_w suggests that coffee solids could undergo non enzymatic browning reactions following mechanisms which are the proceedings of those observed during roasting. In other words, browning reactions were not completed during

roasting but only temporarily blocked by the decreased mobility of the coffee matrix. In order to investigate the effect of temperature, *k* values were plotted according to the Arrhenius model (Figure 3).

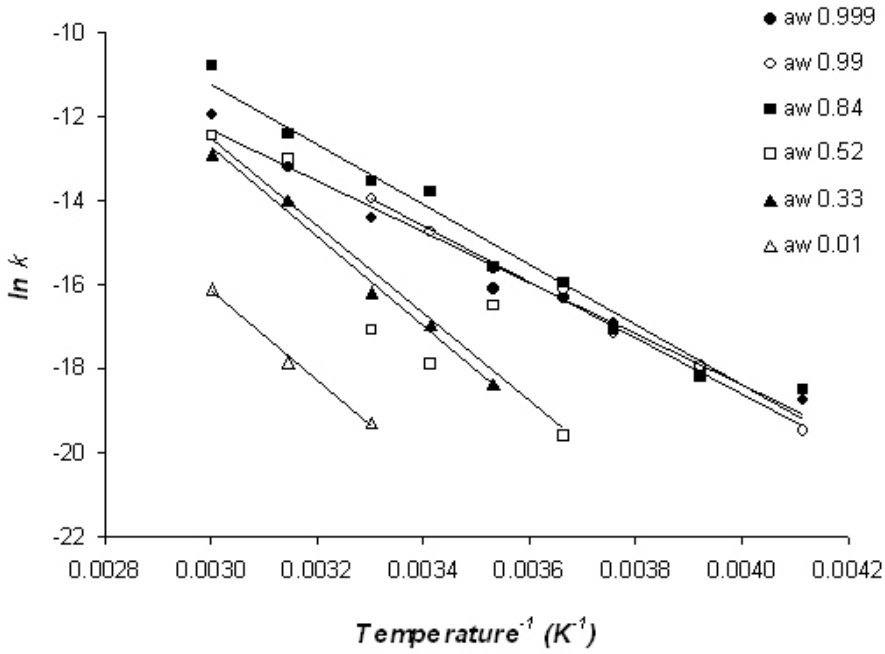

Figure 3. Apparent zero-order rate constants of H_3O^+ formation, expressed as ln *k* ($[H_3O^+]$ day^{-1}), as a function of storage temperature of coffee with different a_w. Lines were obtained by regression analysis according to equation 1.

An Arrhenius-type relation between *k* and temperature was clearly achieved in samples equilibrated at a_w higher that 0.52 in the entire temperature range considered. At lower a_w values, *k* was described by the Arrhenius model only in samples stored at temperature higher than 0-20 °C. Below this temperature, *k* resulted considerably lower (data not shown) indicating that, independently on storage temperature, the system was particularly stable from a chemical viewpoint.

Results obtained in this study clearly showed that the chemical stability of coffee brew derivatives is predictable by the Arrhenius equation but is strongly affected by a_w. In particular, beyond the critical 0.5 a_w value (94 % w/w), the quality depletion of coffee brew derivatives quickly proceeds reaching the highest rate in correspondence of intermediate moisture conditions (0.8 a_w, 78 % w/w), typical of most food ingredients which contain concentrated coffee brew.

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Influence of the Storage Temperature on the Colombian Coffee Brew Quality

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SUMMARY

The evolution of typical coffee compounds, such us caffeine and trigonelline, and hidroxycinnamic acids and their degradation products (i.e. 4-vinylguaiacol), and coffee brew sensory quality throughout the time as influenced by refrigeration temperature (4 ºC *vs* 25 ºC) has been studied. High losses of odour intensity and total volatiles during the first storage days were occurred. An acceptable odour quality (fresh and not rancid) was maintained until 7 and 15 storage days in 25 ºC and 4 ºC coffee brews, respectively. However, taste quality was maintained longer than odour. Traditionally, pH 4.8 was assumed as the limit of acceptability for coffee (Pangborn, 1982; Dalla Rosa et al., 1986). But, pH was maintained at 4.9 after 15 days at 4 °C, while sensory characteristics (odour and acid/sour taste) were unacceptable. Consequently, pH should not be the unique criterion of coffee brew acceptability. Caffeine and trigonelline concentrations were maintained along the time. An initial increase of 5-CQA followed by almost constant concentrations in both coffee brews was observed. There was a maximum peak of caffeic acid at 7 days and 20 days in coffee brews stored at 25 ºC and 4 ºC, respectively. Furthermore, higher 4-vinylguaiacol decrease in coffee brews stored at 25 ºC than in those at 4 ºC was observed. In conclusion, coffee brews stored at 4 ºC could have longer shelf-life than those at 25 ºC. Moreover, the storage temperature influenced on the evolution of some typical coffee compounds and their degradation products.

INTRODUCTION

One of the desires of coffee consumers is to combine good quality of coffee and easy preparation, for example, heating by microwave a cup of coffee prepared days ago. However, only few studies have been found about the chemical and sensory evolution of coffee brew. On the one hand, the relationship between pH and coffee quality is very well established (Dalla Rosa et al., 1990). But, on the other one, evolution studies of typical coffee compounds, such us caffeine and trigonelline, and hidroxycinnamic acids and their degradation products (i.e. 4-vinylguaiacol) have not been found. The aim of this work was to study the evolution of these compounds and coffee quality throughout the time as influenced by refrigeration temperature (4 ºC *vs* 25 ºC).

MATERIAL AND METHODS

Materials

Coffee brews were prepared by French press coffeemakers (90 g/L and 3 min. of time extraction), using Colombia Arabica roasted ground coffee. 135 mL of the freshly prepared coffee brews were aseptically poured into sterilized glass flasks (capacity 330 mL) and hermetically closed. The coffee brews were stored at 4 ºC and 25 ºC for 30 days. Pure

reference standards of caffeine, trigonelline, pentoxiphylline, 5-caffeoylquinic acid, caffeic acid, ferulic acid and 4-vinylguaiacol were purchased from Sigma-Aldrich (Aldrich, Saint Quentin Fallavier, France).

Caffeine and Trigonelline

Extract preparation, cleanup and HPLC analysis have already been described by Maeztu et al. (2001) HPLC analysis was achieved with an analytical HPLC unit (Hewlett-Packard 1100). A reversed-phase Hypersil-ODS (5_m particle size, 250 x 4.6 mm) column was used. The mobile phase was acetonitrile/water (15:85) in isocratic conditions at a constant flow rate of 2.0 mL min^{-1} at 25 ºC. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 280 nm.

5-caffeoilquinic acid (5-CQA)

1mL of coffee brew was diluted up to 100 mL with milliQ water. HPLC analysis was carried out with the equipment described above. Conditions of the gradient solvent system were the following: 12:88 acetonitrile/water adjusted to pH 3.0 with phosphoric acid solution, for 5 min, 7.5:92.5 acetonitrile/water (pH 3.0) for 5 min, 8:92 acetonitrile/water (pH 3.0) for 5 min, 25:75 acetonitrile/water for 5 min, at a flow rate of 1.6 mL/min, and finally, 12:88 acetonitrile/water (pH 3.0) for 5 min, at a flow rate of 1.1 mL/min, at 25 ºC. Wavelength of detection was 325 mn.

Hydroxycinnamic acids (caffeic acid and ferulic acid) and 4-vinylguaiacol

Extract preparation, cleanup and HPLC analysis were carried out according to the method of Álvarez-Vidaurre et al. (2005) with HPLC equipment described above.

pH

The measure was obtained with a Crison Basic 20 pH meter.

Volatile Profile

Volatile compounds profiles were obtained with the method described by Maeztu et al. (2001). SHGC was performed with an HP6890 gas chromatograph (Hewlett-Packard) equipped with a static headspace sampler (Hewlett-Packard model 7694).

Sensory analysis

A selected and trained 10-member panel was used. Odour intensity, freshness and rancidity odours, acidity and sourness were rated on 10-point scales from “none” (0) to “very high” (10). Each coffee brew sample was heated in a microwave over at 90 ± 2 ºC immediately before tasting. After the individual evaluation of each sample, results were discussed and established by panel consensus.

Statistical analysis

Analysis of Variance (ANOVA) was applied to the results along the time. T-Tukey was applied as the test *a posteriori* with a level of significance of 95%. t-Student analysis was applied to the results between the storage temperatures. Each parameter was determined by triplicate. All statistical analyses were carried out using SPSS v.11.0 software package.

RESULTS AND DISCUSSION

The evolution of the chromatographic total area volatiles, odour intensity, and fresh and rancid odours throughout storage at 4 and 25 ºC is shown in Figure 1. As expected, high losses of odour intensity and total volatiles during the first storage days were occurred. An acceptable odour quality (fresh and not rancid) was maintained until 7 and 15 storage days in 25 ºC and 4 ºC coffee brews, respectively. However, taste quality was maintained longer than odour, perceiving sour taste at 15 and 20 days in the same coffees when pH decreased fewer than 4.9 (Figure 2). Traditionally, pH 4.8 was assumed as the limit of acceptability for coffee [1,2]. But, it could be observed in Figure 2 that pH was maintained at 4.9 after 15 days at 4 ºC, while sensory characteristics (odour and acid/sour taste) were unacceptable. Consequently, pH should not be the unique criterion of coffee brew acceptability.

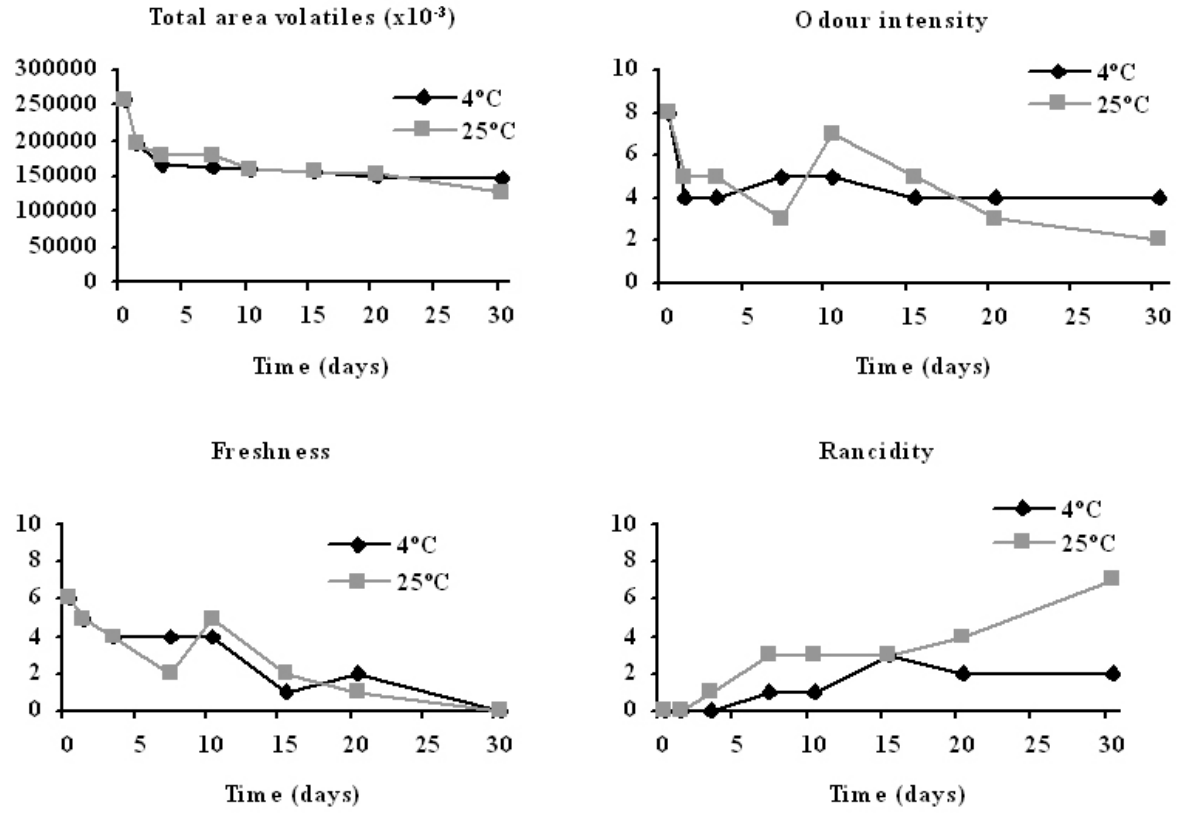

Figure 1. Evolution of total volatiles, odour intensity, fresh and rancid odours throughout storage at 4 and 25 °C.

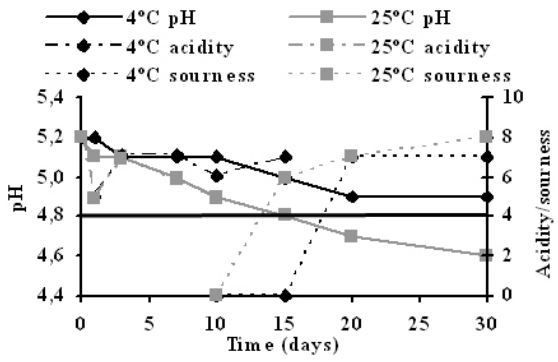

Figure 2. Evolution of pH, acidity and sourness of coffee brews throughout storage at 4 and 25 °C.

Table 1. Evolution of the chemical compounds of coffee brews throughout storage at 4 and 25ºC. Results are given as mean value ± standard deviation.

Time (days)	**0**	**1**	**3**	**7**	**10**	**15**	**20**	**30**
Caffeine (mg/mL)								
4ºC	1.08±0.01^{c}	0.94±0.04^{a}	0.97±0.03^{ab}	0.98±0.02^{ab}	0.93±0.01^{a}	1.10±0.01^{c}	0.94±0.01^{a}	1.02±0.03^{b}
25ºC	1.08±0.01^{d}	1.04±0.01^{c}	1.01±0.01^{ab}	1.03±0.00^{bc}	1.00±0.01^{a}	1.07±0.01^{d}	0.98±0.00^{a}	1.04±0.03^{e}
LS	ns	ns	ns	ns	***	ns	**	ns
Trigonelline (mg/mL)								
4ºC	0.14±0.01^{ab}	0.17±0.01^{c}	0.13±0.00^{a}	0.14±0.01^{ab}	0.15±0.02^{abc}	0.16±0.01^{bc}	0.14±0.00^{ab}	0.14±0.00^{ab}
25ºC	0.14±0.01^{a}	0.15.0.01^{a}	0.16±0.01^{a}	0.16±0.00^{a}	0.16±0.00^{a}	0.15±0.01^{a}	0.15±0.01^{a}	0.15±0.01^{a}
LS	ns	ns	*	ns	ns	ns	ns	ns
5-CQA (mg/mL)								
4ºC	0.58±0.00^{ab}	0.53±0.01^{a}	0.68±0.02^{cd}	0.67±0.00^{cd}	0.62±0.03^{bc}	0.64±0.04^{cd}	0.64±0.01^{cd}	0.69±0.05^{d}
25ºC	0.58±0.00^{a}	0.58±0.01^{a}	0.73±0.00^{cd}	0.70±0.00^{bc}	0.67±0.00^{b}	0.74±0.01^{cd}	0.72±0.01^{cd}	0.77±0.05^{d}
LS	ns	**	*	**	*	**	***	*
Caffeic acid (µg/mL)								
4ºC	1.32±0.05^{a}	2.66±0.00^{e}	2.14±0.08^{d}	1.75±0.01^{b}	2.13±0.08^{cd}	2.05±0.04^{c}	5.74±0.08^{f}	1.73±0.01^{b}
25ºC	1.32±0.05^{a}	2.62±0.02^{b}	3.45±0.03^{c}	4.77±0.19^{d}	2.47±0.01^{b}	2.62±0.03^{b}	3.39±0.04^{c}	2.61±0.12^{b}
LS	ns	*	***	***	***	***	***	***
Ferulic acid (µg/mL)								
4ºC	3.56±0.03^{d}	3.20±0.00^{b}	2.90±0.10^{a}	2.81±0.02^{a}	3.54±0.06^{d}	3.39±0.05^{c}	2.90±0.06^{a}	3.24±0.12^{b}
25ºC	3.56±0.03^{d}	3.38±0.10^{cd}	3.01±0.22^{b}	2.19±0.13^{a}	3.27±0.03^{c}	2.97±0.06^{b}	2.39±0.02^{a}	3.37±0.24^{cd}
LS	ns	**	**	***	***	***	***	**
4-Vinylguaiacol (µg/mL)								
4ºC	3.95±0.03^{h}	2.95±0.04^{g}	2.57±0.05^{f}	1.91±0.02^{d}	2.10±0.04^{e}	1.71±0.09^{c}	1.15±0.03^{b}	0.90±0.05^{a}
25ºC	3.95±0.03^{h}	2.70±0.01^{g}	1.97±0.10^{f}	0.91±0.02^{d}	1.14±0.03^{e}	0.58±0.01^{b}	0.66±0.02^{c}	0.36±0.00^{a}
LS	ns	**	***	***	***	***	***	***

*In each file, different superscripts indicate significant differences ($p < 0.05$) along the time. LS level of signification between two storage temperatures in each day: ns no significant ($p > 0.05$); *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$).*

The evolution of the chemical compounds of coffee brews throughout storage at 4 and 25 ºC is shown in Table 1. Caffeine and trigonelline concentrations were maintained along the time. An initial increase of 5-CQA followed by almost constant concentrations in both coffee brews was observed. There was a maximum peak of caffeic acid at 7 days and 20 days in coffee brews stored at 25ºC and 4ºC, respectively. Furthermore, higher 4-vinylguaiacol decrease in coffee brews stored at 25ºC than in those at 4ºC was observed.

CONCLUSIONS

In conclusion, coffee brews stored at 4 ºC could have longer shelf-life than those at 25 ºC. Moreover, the storage temperature influenced on the evolution of some typical coffee compounds and their degradation products. These results could be a good starting point to study any other storage conditions of coffee brews and to develop a coffee brew ready to be heated at microwave.

AKNOWLEDGEMENTS

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Green Coffee is ALIVE ! A Review on the Metabolic Processes taking Place in Coffee Beans during Processing and their Implication for Modern Coffee Research

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SUMMARY

Within the last few years, substantial progress has been made in the understanding of the metabolic processes occurring within the coffee seeds during the course of post harvest treatments. It was shown that seed germination is initiated during processing and, especially whilst drying, a stress metabolism is executed in the coffee beans and that the metabolic reactions involved strongly are determined by the mode of post harvest treatment and thereby influencing the coffee quality. These insights lead to a paradigm shift in coffee research: today, the green coffee beans are not longer considered just as an inanimate commodity, but rather as viable organisms, whose physiological capacity could be used for quality improvement. This however, requires the comprehensive knowledge of the related metabolic processes, especially that of their time courses and their successions. Using modern techniques of plant biochemistry and molecular biology, it became possible to analyze reliably the extent and amplitude of these processes. Based on competitive RT-PCR techniques, the time course of germination processes during the course of processing was analyzed by the expression pattern of the germination specific isocitrate lyase. In wet processed green coffees, maximal gene expression occurs about 2 two days after the onset of post harvest treatment, whereas the maximal activity in dry process coffees is abundant about one week after the start of processing. The progression of germination was also monitored by quantification of the cell cycle activity and cell division via Western blot analyses of ß-tubulin and by flow cytometry. As monitored for the gene expression, also the maximal cell division occurs in wet processed coffees already two day after the onset of processing, whereas the maximum in dry process ones is achieved about one week after the start of drying. A direct comparison of all time courses reveal that the processes analyzed do not run simultaneously, but that there are some minor shifts in the time courses. This is due to the fact that germination corresponds to an entire metabolic syndrome, where some events run in parallel, some delayed and others consecutively. Like germination, also a stress related metabolism proceeds in differentially processed green coffees seeds to a quite different extent. This is expressed by a massive accumulation of the stress metabolite γ-amino butyric acid (GABA) in dry processed beans, whereas in wet processed ones the GABA concentration is far lower; although also in the course of wet processing drought stress is induced when the parchment coffee is dried. The explanation for these differences in the extent of GABA accumulation is given by the quite different time frames for the related metabolism which can be deliberately altered by changing the drying conditions. As specified for germination, also stress metabolism corresponds to a metabolic syndrome. Hitherto, it is not known, if further complex metabolic events are also executed in green coffee during processing. Yet, we must be aware that numerous metabolic reactions are running in parallel or consecutively, altogether a complex and sophisticated intermixture of metabolic reactions, which only could be comprehended by using new and powerful methods like the new and promising approaches

of genomics and metabolomics. Up to now, the causal coherences between the various physiological events and the generation of flavour precursors and aroma relevant compounds are not elaborated. The clarification of this subject corresponds to one of the most intriguing and promising field of modern coffee research. The overall analyses of the substantial composition of green coffee in relation to physiological fundamentals, will provide powerful tools for the understanding and comprehension of influencing the coffee quality by modifying processing conditions.

INTRODUCTION

In the past, the quality differences between wet and dry processed coffees had been attributed exclusively to the facts that wet processing requires much more accuracy and diligence, and that only fully ripe cherries are used. However, when homogenous identical starting material is processed by the wet and dry method in parallel, also the typical flavour differences of wet and dry process coffees evolve (Selmar et al., 2002; 2004). This finding provides an unequivocal proof that the major distinctive characteristics of differentially processed green coffees are specifically generated during the course of processing. In other words: the overall substantial composition of the processed beans must have been generated differentially, conditional to the mode of processing.

Within the last few years, substantial progress has been made in the understanding of the metabolic processes occurring within the coffee seeds during the course of post harvest treatments. It was shown that seed germination is initiated during processing (Selmar et al., 2004) and, especially whilst drying, a stress metabolism is executed in the coffee beans (Bytof et al., 2005). The metabolic reactions involved are determined strongly by the mode of post harvest treatment and thereby influencing the coffee quality. These insights lead to a paradigm shift in coffee research: today, the green coffee beans are not longer considered just as an inanimate commodity, but rather as viable organisms, whose physiological capacity could be used for quality improvement. This however, requires the comprehensive knowledge of the related metabolic processes, especially that of their time courses and their sequences. Using modern techniques of plant biochemistry and molecular biology, it became possible to analyze reliably the extent and amplitude of these processes. In this paper an overview on the progression of metabolic reactions that run during the course of processing is presented and a substantial treatise of the complex metabolic situation in coffee beans during post harvest treatments is given.

EXPERIMENTAL

Experimental processings

The experimental processings were performed using both, fresh coffee cherries that have been shipped by air to our laboratory (Institute for Plant Biology, TU Braunschweig) from Brazil, Tanzania, and Mexico or, alternatively, fully ripe coffee cherries that had been harvested from *Coffea arabica* plants, grown in our green houses. Directly after harvesting, laboratory processing experiments were performed using identical material for both, dry and wet processing. The procedures applied correspond to the conditions at processing factories in the green coffee producing countries. For solid determination of the initial status (0 - unprocessed seeds), coffee seeds were shock frozen in liquid nitrogen promptly after detaching the fruits in the green house. In all cases, prior to processing, the fruits have been manually sorted very carefully. Only fully ripe red cherries (*Coffea arabica* L.) were used as “identical starting material” for both, dry and wet processing trials in parallel.

Wet processing

For wet processings, the fruits were manually pulped and the mucilaginous parchment beans were transferred into 5 L-Erlenmeyer-flasks adding an excess of fresh water. The coffee was fermented under the ambient conditions of the laboratory (21 °C) for 36 h. During the course of this approach, the water was exchanged three times. The resulting parchment coffee was dried in a laboratory drying oven at temperatures of 35-40 °C until the desired water content of 12 % (wet basis) was achieved. The beans were then manually hulled.

Dry processing

For dry processings, the mature coffee cherries were dried in a laboratory drying oven at temperatures of 35-40 °C until the desired water content of 12% (wet basis) was achieved. The beans were then manually de-husked.

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. For details see Knopp et al. (2006).

Quantification of free amino acids

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 (λ_{ex} = 334 nm; λ_{em} = 425 nm). For details see Bytof et al. (2005).

Expression studies

Based on known ICL sequences of various plants, redundant primers were created to generate a homologous probe for the ICL of *Coffea arabica*. A corresponding 480 bp-fragment was cloned into a bacterial vector (TOPO TA), transformed into *E. coli* (Cell DH 5α), amplified and used for both, as probe for Northern blots and for sequencing. Based on the sequence elucidated, specific ICL primers were created: ICL-forward (3´-gggattgggacctgcctagaacc-5`) and ICL-reversed (3´-agaggaaaggaaccatgaggttga-5`), which were used to produce a 424 bp-PCR product.

Extraction of RNA

Coffee seeds were shock frozen and ground in liquid nitrogen. 200 mg powder were extracted using 2 mL peqGold-RNA-pure solution (peqLab) according the corresponding instruction leaflet. In order to remove all carbohydrates, an additional purification step with peqGold OptiPure (peqLab) was performed. Then the DNA was removed by desoxyribonuclease-I treatment. The complete DNA-degradation was checked by PCR. The quality of the RNA was verified by electrophoretic analysis.

RT-and PCR-reaction

RT-reaction was performed with 100 ng total RNA, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (MBI Fermentas). Instead of oligo dT, a specific ICL-RT primer (3´-ccatgaggttgacacact-5`), had been used (0.25 µL of 10 µM). In order to quantify reliably

the transcript concentration, an internal RNA-standard that resulted in corresponding 1129 bp-PCR-product, was added to the RT-assay, which easily could be differentiated on the gel from the ICL-PCR-product (424 bp). For competitive RT-PCR assays, 1, 10, 25, and 50 pg of the internal standard have been added. For each sample, two independent sets of competitive RT-reactions have been performed. PCR was performed according standard procedure according to Selmar et al. (2006). PCR-products were stained with ethidium bromide after electrophoretic separation; quantification was performed using Scion Image computer program.

ß-Tubulin as a marker for cell division

Using a scalpel, embryos were extracted out of the coffee seeds and were homogenized in Modil-buffer (pH 6.8) according to de Castro et al. (1998), containing a protease-inhibitor cocktail (Complete Mini Protease Inhibitor, Roche ®). Proteins were separated by SDS-PAGE (10%) and blotted to a nitrocellulose membrane. ß-tubulin was detected using specific ß-tubulin antibodies (TUB 2.1, Sigma). The secondary antibody was an anti-mouse IgG, conjugated with alkaline phosphatase. Detection was performed using BCIP and NBT. Quantification was performed using Scion Image computer program.

Flow cytometry

Using a scalpel, embryos were extracted from the coffee seeds. 5 embryos were transferred into 200 µL of TBS buffer (pH 7.3), containing a protease-inhibitor cocktail (Complete Mini Protease Inhibitor, Roche ®) and chopped with a razor blade. To the embryo fragment suspension another 500 µL of TBS-buffer were added, containing 5 µL Rnase. After 10 min. incubation (RT) the suspension was filtered through 88 µm mesh nylon gaze. 5 µL aqueous propidium jodide (1 mg/mL) were added and incubated for 20 min (RT), before used for flow cytometric analyses. The flow cytometer was a DakoCytomation CYAN, the softwares used were Summit (v. 4.0) and ModFit LT(v. 3.1, Verity Software House).

RESULTS AND DISCUSSION

The approach to use the physiological capacity of the coffee beans for quality improvement requires a comprehensive knowledge of the proceeding metabolic processes, especially that of their time courses and their successions. As germination corresponds to an entire metabolic syndrome, which comprehends numerous reactions that either run in parallel, delayed and others consecutively, its extent was determined by the expression strength of germination specific enzymes as well as by the estimation of the magnitude of cell cycle activity and of cell division.

Germination of coffee seeds during the course of processing

Quantification of the expression strength of isocitrate lyase

The reliable quantification of the expression strength by RT-PCR techniques requires the application of an internal standard. With regard to the germination specific isocitrate lyase (ICL), this was achieved by elongation of a standard ICL probe to about 1129 bp. The results of the corresponding competitive RT-PCR experiments are mentioned in Figure 1. Maximal expression occurred two days after the onset of processing, when the seeds had been dried already for about twelve hours. Later, expression strength decreased, last but not least due to the fact that the seeds were dried down to a water content that prohibited increasingly all

metabolic reactions. In contrast, in dry process coffees, maximal expression of ICL was present at about one week after the start of drying.

Quantification of the cell cycle activity and cell division

The changes in the extent of cell division had been monitored via the occurrence of ß-tubulin. This small protein is an indispensable constituent of the microtubules, which in the course of cell division are necessary for the allocation of the chromosomes to the daughter cells. The abundance of ß-tubulin is a reliable marker for the intensity of cell division. The time course of cell division was similar to that of the expression of ICL: in wet processed coffee beans, maximal activity occurred about 2 days after the start of processing, whereas during the course of dry processing, maximal cell division took place about one week after the start of the drying (Figure 1). The amplitude of cell cycle activity was estimated by quantification of 4C-nuclei using flow cytometry. Indeed, also cell cycle activity was highest in wet processed seeds at the second day and in dry processed seeds at about one week after the start of drying, but the time courses and the amplitudes were not identical to those of the cell division. The direct comparison of all time courses reveals that the processes analyzed do not run simultaneously, but that there are some minor shifts in the time courses. This is due to the fact that germination corresponds to an entire metabolic syndrome, where some events run in parallel, some delayed and others consecutively.

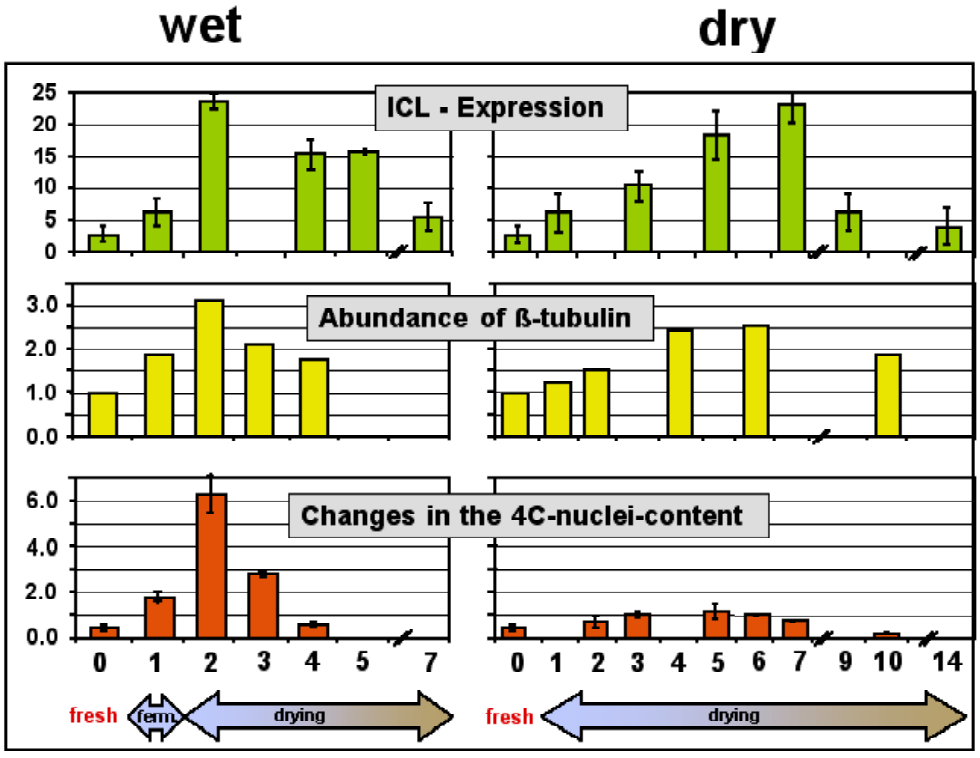

Figure 1. Quantification of germination related processes during wet and dry processing according to Bytof et al. (2006).

Up to now, the entire impacts and the overall consequences of the germination processes occurring during coffee processing are not yet known, or even understood. The elucidation of both, the causal relation-ship between metabolic events and the occurrence of certain aroma compounds and the comprehensive estimation of the complex germination related metabolism is a great challenge for coffee research in the future. Nevertheless, based on the data so far known, we can conclude: green coffee develops its processing specific characteristics as consequence of these metabolic events.

Stress related metabolism during the course of processing

In addition to germination, further metabolic processes occur in the coffee seeds during the course of processing. These processes are caused and related to the drying of the coffee beans. Bytof et al. (2005) showed that γ-amino butyric acid (GABA) is accumulated in dry process beans to a large extent, whereas the concentration of this stress metabolite is enhanced only slightly, although the wet processed beans are dried down to a final water content of 11%, too. These differences are due to distinctions in the time frame, determined by the induction of stress related metabolism, and the entire shutdown of all metabolic reaction due to water stress, respectively. Analysis of coffee leaves that have been exposed to drought stress revealed that the accumulation of GABA corresponds to a general and very fast stress response in coffee (Figure 2).

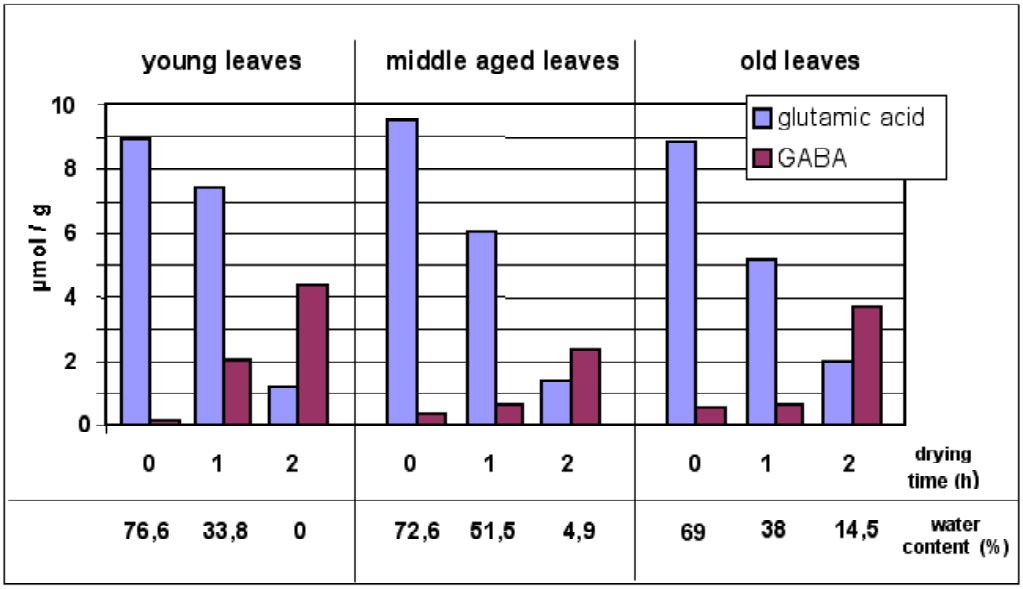

Figure 2. Accumulation of γ-amino butyric acid (GABA) in coffee leaves at various stages of drying.

Like germination, also the stress related metabolism corresponds to a metabolic syndrome, where some events run in parallel, some delayed and others consecutively, e.g. the accumulation of dehydrins, small protein that are thought to stabilize dehydrating cells. Also with regard to the stress related metabolism, we have to be aware that specific changes of the drying conditions applied will influence the metabolic processes within the coffee seeds and thereby affect coffee quality! In an actual research project, the complex metabolic processes related to the stress metabolism that run during the course of drying, will be examined by applying variation in the drying courses. The related differences in the progression of the water loss entail considerable distinctions with respect to the expected response of the coffee seed's metabolism (Figure 3). Furthermore, it has to be taken into consideration that – in the same manner as in the case of stress metabolism – the period germination related processes is governed by the gradual shutdown of metabolic processes due to water shortage. The research project is aimed to provide important information on the effects of drying conditions on both, the biochemical status of the drying coffees as well on the sensoric qualities of the related coffee beverages.

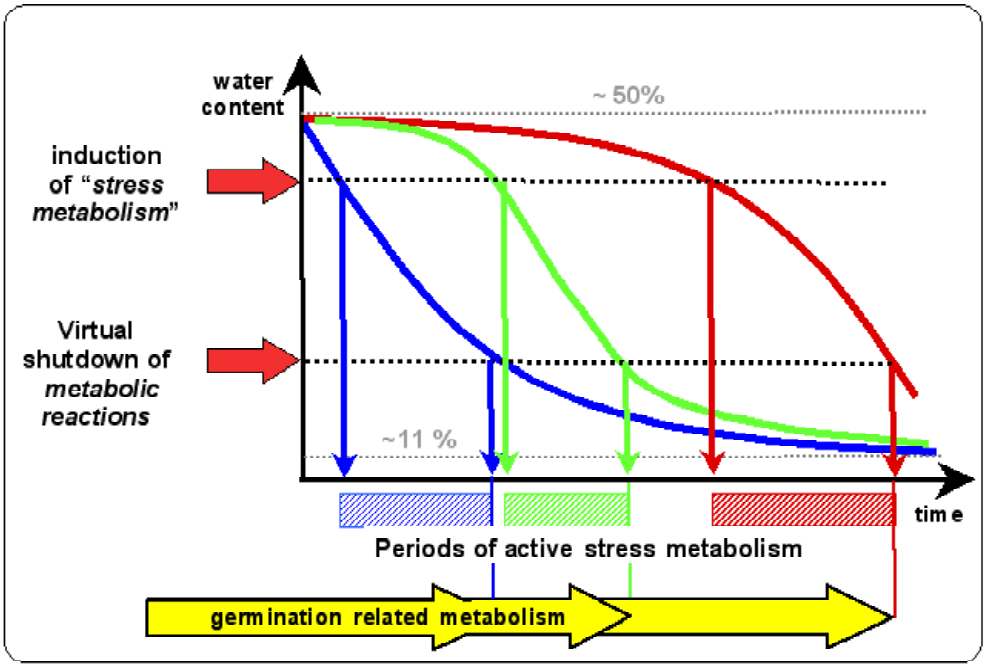

Figure 3. Model of various drying curves and their impact on the periods of an active metabolism within the seeds.

Further, prospective approaches and auspicious cogitations

We must be aware that in addition to germination and stress metabolism, further metabolic events might influence the physiological status of the processed coffee beans. Overall considerable amounts and extents of metabolic reactions are running in parallel or consecutively in the coffee seeds, resulting in a complex intermixture of a large variety of metabolic reactions that causes changes in the chemical composition and the physical properties of the coffee beans. In this context, the consequences of some enzymatic hydrolyses may be mentioned, e.g. activity changes of endomannanases in the seed, which affect as well the stability of the beans as the composition of soluble carbohydrates. Controlling the meta-events, (i.e. germination, stress) means effectuating the related enzymatic activities and in the consequence using the metabolic capacity of the coffee seed to deliberately form flavour profiles. Growth regulators, osmotica or other remedies applied in coffee post harvest treatment would be feasible means for such an approach. We executed also some preliminary experiments on the acceleration the germination metabolism in processed coffees seeds by the application of phytohormones, i.e. gibberillic acid, in Mexico. Unfortunately the related outcomes were not really clear and no significant effects on cup quality could be detected under the post harvest conditions chosen. However, unlike other authors (Amaral da Silva et al., 2005), we could not find any inhibiting effects of gibberillic acid on fresh coffee seeds.

Influence of the seed maturation

Another interesting aspect of post harvest treatment and coffee quality is fruit maturity. Red ripe and sound cherries produce the best cup quality and therefore in many regions they are carefully picked. However, in coffee growing regions, in which – mainly for logistic reasons – the stripping method or mechanical harvesters prevail, large parts of the lot may consist of overripe black and of unripe green coffee fruits. Unripe coffee fruits (*verdes*) produce beverages of inferior quality (Barel and Jacquet, 1994). In this context it should be noted that the concentration of asparagine in immature coffee seeds is far higher than in fully ripe ones;

beyond, in overripe seeds, the corresponding asparagine concentration is only about one fourth of the standard content (Figure 4). As asparagine is thought to be an important source for the generation of acrylamide during roasting (Stadler et al., 2002), the actua concentration of this amino acid has attracted special attention. In a commercial "*café verde*"-sample, the asparagine concentration revealed more than 2300 mg asparagine per kg green coffee, representing the major free amino acid and the fivefold content of asparagine in common green coffees. It cannot be excluded that also other substances could be affected analogously. Earlier works have shown that chlorogenic acid contents are also interconnected with coffee seed maturity (Clifford and Kazi, 1987).

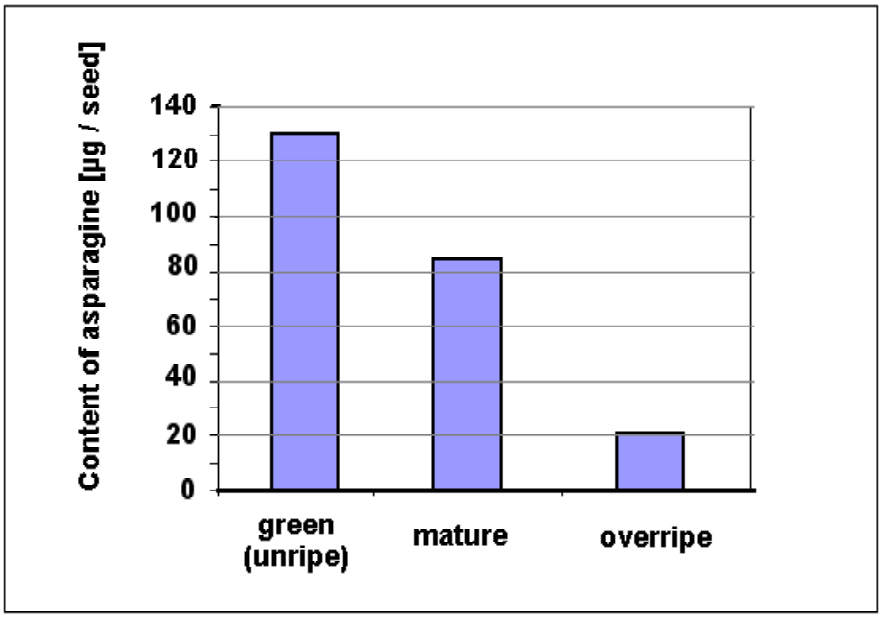

Figure 4. Accumulation of glutamine in coffee seeds of varying in their maturity.

Impact of the storage conditions on the viability of coffee seeds during storage

When green coffee is stored for more than one year, its quality decreases significantly. In addition to the well know generation of off-notes related to lipid oxidation, a general flatten of aroma is detectable, which is thought to be related to the loss of viability of the coffee beans (Sivetz and Desrosier, 1979). Up to know the reasons for this flattening are unknown. In this context, our findings on the impact of the parchment on the viability of green coffee become relevant. In accordance with the common conjecture that green coffee remains vital just for only several months after processing, our analyses revealed that after three months of storage, nearly 90% had lost their viability and after a half year, less than 5 are still vital. In contrast, the storage of coffee beans within the parchment resulted – under the same conditions – in a tremendously enhanced phase of viability: even after one year of storage, more than two third of the coffee beans were still alive. If indeed these differences in viability are the key in the understanding of the processes related to the aroma flattening during storage is not known yet, but a related research approach is very promising.

The* seed-centric viewpoint *to coffee post harvest treatment

In this overview only a few aspects of coffee seed physiology and its impact on coffee cup quality could be touched, last but not least due to the fact that we are only at the beginning of elucidating this special field of coffee science. We have to pay attention to the interrelations between the coffee bean submitted to post harvest treatment and its changing environment. In

addition to the multiplicity of events and reactions, we must take into consideration that all these processes are influence by external factors, e.g. temperature, irradiation, oxygen supply. A corresponding scheme is given as Figure 6.

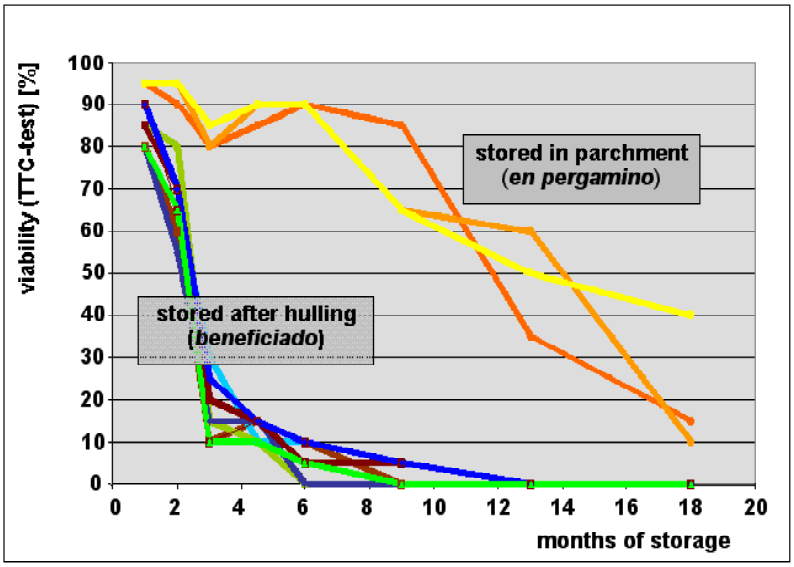

Figure 5. Changes in the viability of Brazil Arabica coffees, stored within the parchment and after hulling.

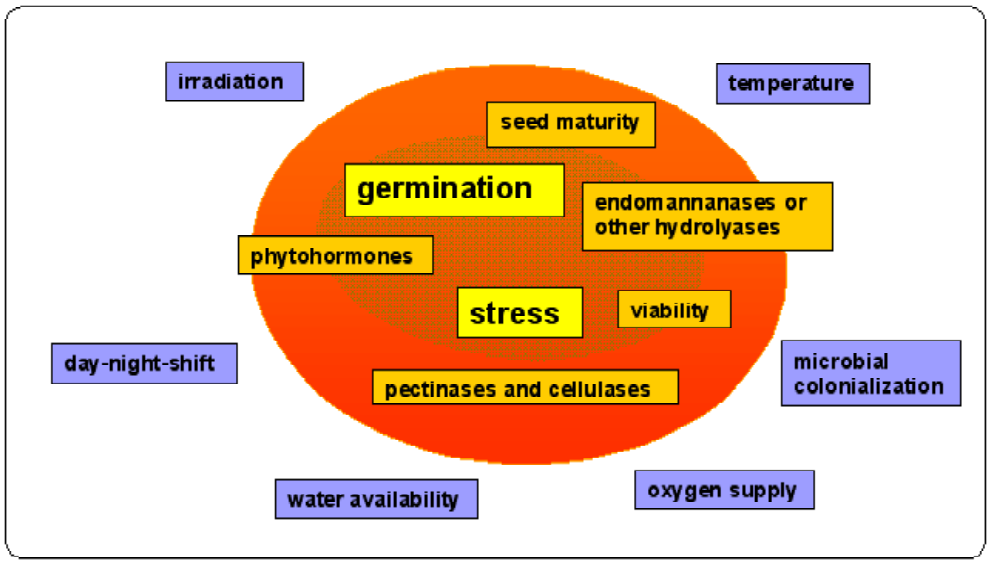

Figure 6. Further prospective approaches and auspicious cogitations and some internal factors affecting the beans´ metabolism.

Due to this complexity, the compilation of the reactions involved as well as their impact on the coffee quality requires integrative research efforts, combining the wide range of all modern research methods, including the promising genomics- and metabolomics-approaches. This also especially applies for any concept to modulate these events by deliberately changing the external factors to influence the coffee quality. However, with respect to combine or to link metabolic reactions or events, respectively with related influences on coffee quality, there are still some elementary approaches to be adapted.

CONCLUSION

Using modern methods of biochemistry and molecular biology, it was shown that the time course of the germination and stress related processes are significantly different in wet and dry processed seeds, demonstrating that these metabolic reactions strongly are influenced by the processing conditions. These differences in the time courses and the amplitudes of the metabolic events determine the distinction in the substantial composition of differentially processed green coffees, thereby affecting coffee quality, and, establishing the peculiarities of wet and dry process coffees. However, the entire impacts and the overall consequences of the metabolic processes occurring during coffee processing are not yet known, or even understood. Due to the high complexity, a comprehensive analysis of their impact on the coffee quality and their modification by external factors requires integrative research efforts, combining the wide range of all modern research methods, including the promising genomics and metabolomics-approaches. Moreover, the elucidation of the causal relation-ship between metabolic events and the occurrence of certain aroma compounds is a great challenge for coffee research in the future.

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Control of the Coffee Fermentation Process and Quality of Resulting Roasted Coffee: Studies in the Field Laboratory and on Small Farms in Nicaragua During the 2005-06 Harvest

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SUMMARY

This paper describes a field study conducted during the 2005-06 Nicaraguan coffee harvest investigating the relationship between scientific control of the coffee fermentation process and the quality of the resulting roasted coffee. In phase one of the study, small-scale, well-controlled laboratory fermentation was carried out on eleven batches of coffee at the farm La Canavalia in Matagalpa, Nicaragua. With otherwise identical treatment, fermentation of the small samples was halted by washing when the pH of the fermenting mass decreased to approximately 4.6, 4.3, or 3.9. After drying and roasting, these samples were individually evaluated by two certified cupping laboratories. The results indicate a weak positive correlation between pH at washing and subsequent roasted coffee quality. In phase two of this study, 100 small-holder coffee producers were asked to characterize their customary procedures using standard pH paper and then to "optimize" the fermentation times. Generally, this required somewhat shortening fermentation times, with termination at higher pH. The coffee from the "usual" and "optimized" procedures was dried, roasted, and evaluated by two cupping laboratories. The results indicated that the coffee producers were very successful in optimizing the fermentation process, but that the roasted coffee quality did not reflect these changes, possibly due to a general decrease in the quality of the crop at the end of the harvest.

RESUMEN

Este escrito describe un estudio en el campo de la cosecha cafetalera Nicaragüense llevado a cabo durante los años 2005 y 2006, en el cual se investiga la relación entre el control científico del progreso de fermentación del café y la calidad del café tostado resultante. La primera fase de estudio, en escala pequeña y en un laboratorio de fermentación muy bien controlado, se llevó a cabo en 11 lotes de café de la finca La Canavalia en Matagalpa, Nicaragua. Asimismo y con tratamiento idéntico, se interrumpió la fermentación de muestras pequeñas a través del lavado cuando el pH de la masa en fermentación decrecía aproximadamente a 4.6, 4.3, o 3.9. Después que estas muestras fueron secadas y tostadas, se evaluaron individualmente por dos laboratorios de catación debidamente certificados. Los resultados indican una débil correlación positiva entre el pH del café en el lavado y la calidad subsiguiente del café tostado.En la segunda fase de este estudio, se solicitó a 100 pequeños productores de café caracterizar sus procedimientos tradicionales usando papel pH estándar y luego optimizar el tiempo de fermentación. Generalmente, esto requirió de un tiempo corto de fermentación para finalizar con un alto valor del pH. Tanto el café "habitual" como el "optimizado" fueron sometidos a procedimientos de secado, tostado y evaluado por laboratorios de catación. Los resultados indicaron que el café de los productores fue muy

exitoso en la optimización del proceso de fermentación, pero la calidad del café no reflejó cambio, posiblemente debido a un decrecimiento en la calidad del café al final de la cosecha.

INTRODUCTION

While the global coffee crisis has somewhat eased since the lowest market prices were reached in 2001, the effects remain significant among the impoverished coffee producers in developing countries. In Nicaragua where 42% of rural labor is employed in coffee, over 120,000 jobs were lost during the hardest years of the coffee crisis, with continuing social and environmental consequences (ICO 2003). Nicaraguan small-holder coffee producers have responded by strengthening their cooperative organizations and seeking certification that can give access to specialty (organic and Fair Trade) markets (Bacon 2005). International development and relief organizations, such as Catholic Relief Services (CRS), have come to the aid of coffee producers by assisting in certification efforts, coffee quality improvement, and access to markets in developed countries (CRS/NI 2005, p10). The United States Agency for International Development (USAID 2003, p5) has contributed through projects designed to aid small-holder coffee producers in assessing and improving their coffee quality.

In this project, initiated in 2003, an international group of faculty and student chemists works in Nicaragua with coffee producer cooperatives and CRS to contribute scientific expertise with appropriate technology in order to put simple methods into the hands of producers for improvement of coffee quality, certification, and market access.

After a series of discussions with coffee producers and the staffs of CRS and USAID, it was decided to focus first on over-fermentation, a major concern of coffee producers. Processing methods had been known to be important for coffee quality (Wootton 1966; Puerta-Quintero 1999), and over-fermentation was generally considered detrimental to coffee quality (Lopez and others 1989). A field study conducted in 2004 on small-holder farms resulted in the characterization of chemical changes during fermentation and in particular the decrease in pH that is associated with the liquification of the coffee mucilage, allowing the coffee to be washed clean (Jackels and Jackels 2005). It was determined under a wide range of conditions on various farms that the batches of fermenting coffee could be washed clean when the pH fell from approximately 5.5 to 4.6. Upon receiving this finding, the coffee producers wanted to know if pH measurement could be used to improve and control fermentation on the farm, resulting in coffee quality improvement.

The primary goals of this study were: (1) to determine if a relationship exists between coffee quality, as evaluated in the cupping laboratory, and the pH when fermentation is terminated by washing; and (2) to determine the feasibility of producers themselves using pH measurements to improve coffee quality through a “fermentation optimization” method. These two questions were investigated simultaneously in December 2005 – March 2006. In phase one, small-scale, well-controlled laboratory fermentation was carried out on eleven batches of coffee processed on a Nicaraguan farm. With otherwise identical treatment, fermentation of the small samples was halted by washing when the pH of the fermenting mass decreased to approximately 4.6, 4.3, or 3.9. After drying and roasting, these samples were individually evaluated and rated by two certified cupping laboratories. In phase two, approximately 100 small-holder coffee producers were asked to characterize their customary procedures using standard pH paper and then to “optimize” the fermentation times. Generally, this required shortening fermentation times, with termination at higher pH. The coffee from the “usual” and modified procedures was dried, roasted, and evaluated by two cupping laboratories.

MATERIALS AND METHODS

Controlled Fermentation (Field) Experiments

Small-scale controlled fermentation (field) experiments were conducted at La Canavalia, the experimental and model farm of the Association for Agricultural Diversification and Development (ADDAC), located in Yasika Sur near the village of San Ramón, Matagalpa, Nicaragua. At 750 m altitude, the farm receives 200-240 cm of precipitation annually and has a temperature range of 20-26 °C. Typically, ripe coffee cherries (*Coffea arabica,* var. caturra) were harvested in the morning hours and were washed and selected by density, retaining only those that did not float. After being mechanically pulped in the wet mill building in late afternoon, they were placed in a cement tank with a drain (no water added) for natural fermentation, which typically required approximately 15 hours. For the field experiments, about 30 kg of freshly pulped coffee was divided among six fermentation buckets, which were constructed to mimic the process in the large tank. A five-gallon outer bucket served to collect the drain liquid, while a three-gallon inner bucket with a drain platform and holes contained the coffee (Figure 1). The apparatus was jacketed with high efficiency insulation and covered with mosquito net. The six buckets remained in a covered location where fermentation proceeded under ambient conditions.

Figure 1. Apparatus for controlled fermentation of coffee.

Each bucket of fermenting coffee was monitored by time, temperature, and pH. The pH readings were measured both semi-quantitatively (short range paper, EMD Chemicals, Inc. colorpHast™, two ranges, 4-7 and 2.5-4.5) and quantitatively (Reflectoquant, RQflex2™, Merck, Darmstadt, Germany, range 4-7). Sample preparation is described below in "Measurement of pH in Fermenting Coffee Batches". The fermentation process was terminated by washing the coffee when it reached the desired pH, denoted herein as pH_{term}. Washing consisted of transferring the coffee to a five gallon washing bucket that had several hundred small holes in the sides and bottom. The washing bucket was placed inside another five gallon bucket without holes. Approximately 3 gallons of clean water were added, and the coffee was stirred vigorously for approximately 5 minutes. Debris was skimmed, and the coffee was drained by pulling the inner bucket out of the water. The "dirty" water was discarded, and the washing process repeated five more times. The washed coffee was sorted and partially sun-dried in racks, before being transported to a commercial processing service (Sol Café) in the valley, where it was placed on a patio in the sun and dried to 10-12% moisture.

Each field experiment consisted of six buckets derived from a common batch of beans harvested on the day of the experiment. The fermentation was terminated so that approximately duplicate samples were created from coffee with pH_{term} 4.5-4.8 (Range 1), 4.1-4.4 (Range 2), and 3.6-4.0 (Range 3). Fermentation was always "complete" in Range 1, with Ranges 2 and 3 representing over-fermentation by 1.5 and 4 h (medians) respectively. Experiments were conducted over a three-week period, after which, the samples were roasted and their quality evaluated by cupping in two independent laboratories (see below).

Fermentation Optimization by Coffee Producers

Since it was not feasible to travel to each of the 100 project farms in order to train the coffee producers in the process of fermentation optimization, the technical staff of the cooperatives serving them was given hands-on training in the methods of the project, including pH measurements, and provided with kits of materials to deliver to each farm. The technicians trained the producers at the time of kit delivery and returned a few weeks later to answer any questions.

Each farm was asked to complete a questionnaire giving the following information: location, cooperative membership, altitude, coffee cultivation area, and traditional practice of wet processing, including batch size, time of initiating fermentation, and its usual duration. Each farm was provided a kit with the necessary materials: cups, sampling and stirring spoons, thermometer (digital), watch (digital), pH strips (EMD Chemicals, Inc. colorpHast™, two ranges, 4-7 and 2.5-4.5) and color charts, instructions and data sheets, pen, clipboard, and container. The instructions were for a three step process: 1) document the regularly practiced process (Step A), 2) make changes to the process (Step B), and 3) document the optimized process (Step C).

On the farm, coffee was typically picked in the morning, sorted and pulped in the early afternoon, and put in the fermentation tanks in late afternoon. In Step A of the procedure, the producer was asked to maintain the traditional schedule for three days, recording pH, temperature of coffee, and time of initiation of fermentation. The same data were to be recorded for the fermenting coffee early the next morning and again at the time of its washing. In Step B, the producer was asked to note the typical pH_{term} value at the time of washing (from Step A) and make changes in fermentation time if necessary. If pH_{term} was < 4.0, the time of fermentation during the next day was reduced by two hours. If the pH was between 4.0 and 4.2, the time of fermentation was reduced by one hour. If the pH was between 4.2 and 4.6, no change was made in fermentation time. In Step C, the same data were collected for a batch using the optimized fermentation process. The producers were asked to wash, sort and partially dry the parchment coffee from each batch, following their usual procedure. Samples of partially dried parchment coffee, about 1 kg from each of steps A and C, were collected from each farm, were dried to approximately 12% moisture in the sun using the usual procedure, and were sent to two laboratories for husking, roasting and cupping.

Measurement of pH in Fermenting Coffee Batches

The following instructions were provided to coffee producers along with a pictorial representation of each step. First, the date and time were noted on a data sheet provided. A reminder was given to start with clean, dry cups and spoons. The cups for coffee and water were marked with levels for filling. Approximately 50 mL volume of coffee (30 g) with its associated mucilage was taken from a hole about 10 cm deep in the mass of coffee and was mixed with 50 mL of fresh, pure water. The mixture was stirred for 15 seconds. Then the pH strip was dipped into the water and the color was immediately matched with the

manufacturer's chart to determine the pH. The data were recorded to the nearest tenth of a pH unit.

Quality Evaluation by Cupping Laboratories

All coffee samples, from both the field experiments and the producer optimization steps, were evaluated by roasting and cupping at certified cupping laboratories. The coffee was mechanically husked, brought to a medium roast in a small roaster, and then cupped in the Sol Café laboratory, a facility of CECOCAFEN, a second-tier cooperative well known in Nicaragua and internationally. The same roasted sample was then cupped in the laboratory of CECOSEMAC, a second-tier cooperative organized by Cáritas Matagalpa and directly serving the 100 coffee farms that participated in this project. In each cupping evaluation, the same procedure was followed. A 12 g sample of medium roasted coffee was finely ground and placed in a glass cup. The aroma of the ground coffee was sniffed and then the brew was made by adding freshly boiled water (Fuente Puro, heated in an aluminum kettle). The aroma of the crust and broken crust were sampled. Following crust removal with stainless steel spoons, the coffee was tasted by aspiration into the mouth and nose. Numerical scores were recorded for aroma, body, acidity, flavor, after-taste and balance. The total scores were tabulated on a 100 point scale where 90-100 is excellent, 80-90, very good; 70-80, commercial grade, and below 70, poor or damaged.

Statistics

Statistical analyses were carried out using SPSS version 14.0 for Windows™ (2005).

RESULTS

Comparison of Cupping Results for Equivalent Samples

A number of samples (both field and producer) were created under such similar conditions as to be considered "equivalent". A comparison of the results from a single laboratory for these "equivalent" sets gives an indication of the reproducibility of both the processes in the field and at the cupping laboratories. For the thirty-one such comparisons possible among the samples cupped at Sol Café the correlation coefficients are: $r_{Pearson} = 0.453$ ($p = 0.010$) and $\rho_{Spearman} = 0.564$ ($p = 0.001$). The twenty-six comparisons in the Cáritas laboratory yielded $r_{Pearson} = 0.436$ ($p = 0.026$) and $\rho_{Spearman} = 0.0.396$ ($p = 0.045$). Linear fits to these data sets account for only 15-30% (r^2)of the total variance.

Controlled Fermentation Experiments

Buckets were assigned to pH_{term} ranges (see above), with both instrumental and test-strip pH values being considered. In two batches, all six buckets were placed in Ranges 2 and 3 because fermentation had progressed beyond Range 1 at first measurement. After categorization of the 66 buckets from 11 batches, the three ranges contained 18, 25, and 23 samples, respectively. All samples were evaluated by Sol Café laboratory, and 59 of them were also evaluated at the Cáritas laboratory. After censoring scores below 70 ("damaged" coffee), there were 60 values from the Sol Café data and 50 from Cáritas. In the Sol Café data, nine of the eleven batches were represented in all three ranges, and the Cáritas data set had six such batches. Approximately 20% of the data points in these sets were single values rather than the average of "equivalent" buckets.

"Common knowledge" among producers is that over-fermentation degrades coffee quality. Since it has been shown that pH drops throughout the fermentation process, the working hypothesis of this study was that the quality of coffee as determined by cupping laboratories *decreases* as pH_{term} *decreases*. The null hypothesis is that coffee quality and pH_{term} are unrelated.

Average cupping scores for the three ranges could not be compared directly because of variation in coffee quality between single-day batches. The differences between batches would be expected to be larger than the differences between ranges within any batch, as was confirmed by ANOVA calculations. Accordingly, the data was analyzed using pair wise t-tests to compare data in Range 1 with data from the same batch in Ranges 2 and 3. In Table 1 are presented average cupping scores for the three ranges and the changes from Range 1 to Ranges 2 and 3. One-tail probabilities are appropriate here for the paired t-tests because the over-fermentation in going beyond Range 1 can only result in degradation of coffee quality. If, as in some instances, the evaluation rises, this change is assigned to random variation in the field and laboratory processing.

Table 1. Average cupping scores and changes for field experiments.

	Range 1[a]	Range 2[a]	Range 3[a]	Change (1→2)[b]	Change (1→3)[b]
Sol Café Results	80.26 (2.9; 9)	79.14 (3.1; 9)	78.76 (3.2; 9)	–1.1 (t = 1.41; p = 0.10)	–1.5 (t = 2.00; p = 0.04)
Cáritas Results	82.46 (4.0; 6)	82.38 (2.9; 6)	81.17 (3.7; 6)	–0.1 (t = 0.05; p = 0.48)	–1.3 (t = 0.81; p = 0.23)

[a]Reported as: mean (standard deviation; number of batches); [b]Reported as:change in mean (pair wise t-statistic; one-tail p-value.)

Although the changes reported in Table 1 are statistically significant ($p < 0.05$) in only one case, the overall set of negative changes is suggestive of a decrease in coffee quality with decreasing pH_{term} (over-fermentation). It is noted that the only case with a significant decrease in quality corresponded to the broadest pH range (1→3) and the more extensive of the two data sets (Sol Café).

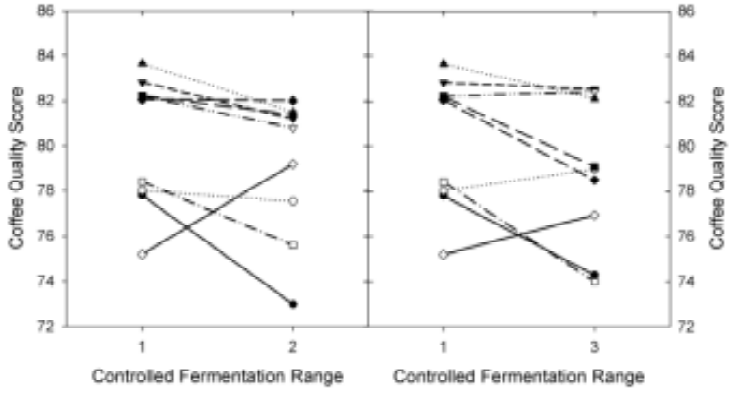

Figure 2. Coffee quality vs fermentation range.

In Figure 2 it is shown that the cupping score change (Sol Café) for individual batches increases in only one case from Range 1 to Range 2 and in only two cases from Range 1 to Range 3. Nonparametric analysis of this data using the Wilcoxon Signed Rank test indicated a marginally significant difference ($Z = –1.718$, $p_{1\text{-tail}} = 0.043$) for Range 1 to Range 2 and a marginally insignificant one ($Z = –1.599$, $p_{1\text{-tail}} = 0.055$) for Range 1- Range 3. The dominant

trend is clearly a decrease in cupping score with a decrease in pH_{term}, with the decreases between ranges being close to the p = 5% significance level in both parametric and nonparametric tests. It is suggestive that with more repetitions and larger data sets, this relationship would become more significant with decreased variance of the data and increased statistical power of the study.

Producer Data (fermentation optimization)

Seventy-seven producers returned data, of which sixty-nine had both fermentation times and pH_{term} values noted for each of Steps A and C. To determine if *on the average* the producers followed the protocol, comparison was made between fermentation times and pH_{term} values for Steps A and C. From Step A to Step C, the average fermentation time decreased from 18.0 h to 16.3 h (n = 69, t = 3.32, $p_{2\text{-tail}} = 0.0014$). From Step A to Step C, the average pH measured at the termination of fermentation increased from 3.97 to 4.28 (n = 69, t = –4.70, $p_{2\text{-tail}} = 1.3 \times 10^{-5}$). In going from Step A to Step C, the producers clearly shortened the fermentation time, resulting in higher pH_{term}. The two changes are significantly correlated, with $r_{Pearson} = -0.319$ ($p_{2\text{-tail}} = 0.008$) and $\rho_{Spearman} = -0.341$ ($p_{2\text{-tail}} = 0.004$).

The hypothesis to be tested is that the changes in process from Step A to C resulted in higher coffee quality. The average cupping scores (Sol Café) for 67 producers changed insignificantly from 81.7 to 81.8. After limiting the analysis to those producers who also reported valid pH measurements for both steps (N = 50), the average scores were unchanged (81.8). Further limiting analysis to those cases (N = 33) where the change in pH_{term} was greater than zero, in accord with the experimental design, the mean score changed from 82.0 to 81.8, an insignificant (p = 0.35) decline in quality. The correlation coefficients between change in quality and change in pH_{term} were negative, but statistically insignificant: $r_{Pearson} = -0.16$ (p = 0.37) and $\rho_{Spearman} = -0.23$ (p = 0.19). A subset (N = 43) of samples were also evaluated by Cáritas and similarly displayed only small and insignificant changes in quality.

DISCUSSION AND CONCLUSIONS

Previous work had shown that pH measurements could be used in the field to track the fermentation process of pulped coffee cherries (Jackels and Jackels 2005). Two further questions were addressed in the present study: 1) Does coffee quality as determined in cupping laboratories correlate with the pH of the fermentation mass at time of washing? and 2) Can producers themselves use pH test paper to effect change in their fermentation process and consequently in their coffee quality?

The first question was addressed by the controlled fermentation experiments carried out in our field laboratory. The results show a weak relationship in which a decrease in coffee quality accompanies a decrease in pH_{term}, corresponding to over-fermentation. This relationship is statistically significant only for the case of the largest pH difference (Range 1 to Range 3) considered and for evaluation at the more professional and experienced laboratory (Sol Café). This change is a decrease of 1.5 quality points (out of an average of 80) with a pH decrease of at least 0.5 units. The changes from Range 1 to Range 2, while even less significant, are still suggestive of this relationship.

Cupping laboratory data are semiquantitative in nature and inherently possess relatively large variance. It is expected that with increasing sample and cupping replication, the variances would decrease and that the correlations suggested here would become significant. Although the change in cupping score suggested by these results is modest, it would be important in the effort to improve and maintain coffee quality.

The question addressed in the producer study is complicated. First, it was necessary to determine whether or not pH could be measured and could be used to control fermentation time by producers with training from their cooperative technical staff*s*. Producers were clearly successful in raising the average pH_{term} of their fermentation process by shortening the fermentation time. Fermentation times decreased, and pH_{term} increased at a very significant level from Step A to Step C, with the two changes being significantly correlated. Our conclusion is that, on the average, the producers accomplished the desired changes in their fermentation processes.

There is no indication, however, of coffee quality improvement being effected by the process changes. In fact, the suggested correlation between the changes in pH_{term} and in coffee quality is an *inverse one.* Although the pH changes accomplished by the producers were smaller than those observed in the controlled field experiments, an additional uncontrolled factor is more likely dominant. The producer experiments were conducted during from December 20, 2005 through January 30, 2006. The 2005-06 coffee harvest in Matagalpa was earlier than expected and was approaching completion by January 1. It is well known to producers that the coffee quality declines markedly toward the end of the season. Since Step C typically occurred 2-3 weeks after Step A, Step C used coffee that may have been generally inferior to that in Step A. The experimental protocol assumed that the quality would be unchanged between steps, which was clearly not the case. This is very likely the underlying cause of the suggested decline in quality from Step A to C.

The overall conclusion is that under controlled conditions, the pH of washing shows a weak correlation with coffee quality, which is very likely to be strengthened with a statistically more powerful experimental design. The question of whether producers can use pH measurements on their farms to improve the quality of their coffee is unanswered. While the producers can clearly utilize the technology to control their processes, it is unknown if that control can result in practical improvement.

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Changing Chemical and Sensory Properties of Robusta Cultivar by Processing Method

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SUMMARY

Despite the importance of *C. canephora* in coffee commercialization around the world the beverage quality provided by the species is not so appreciated. It is well known, particularly from researches with *C. arabica*, that preparing method interferes in chemical composition and in cup quality of coffees. Most of these studies compares Arabica coffees processed by wet and dry method and their results suggest that changes induced by processing methods could be tried in an attempt to improve Robusta beverage quality. Therefore, the research herein reported deals with the effect of processing method on chemical and sensory properties of *C. canephora* var. Robusta. Dry, semi-dry and wet methods were investigated. Results showed that processing method affected the concentration of fructose, stachyose, galactose and also of acetic, malic and oxalic acids in green beans. Coffee processed by wet method exhibited the best beverage, which was less bitter, more aromatic, cleaner, sweeter and slightly more acid brew compared to the beverages from grains processed by dry and semi-dry methods. Grains prepared by semi-dry method provided beverage with characteristics between those promoted by wet and dry method.

INTRODUCTION

Although Robusta coffee is an alternative to coffee production in warm humid regions and its importance in the world market has increased in the last years, commercial value of Robusta green beans is about half of the Arabica. This difference is partially due to the Robusta cup quality. Contrary to Arabica beverages, there is no expectation for fruity aroma, or for sweetness in Robusta brews. These last have typical not so prized flavor, are less acid and, normally, bitter than Arabica coffee beverages. With these characteristics, Robusta coffee is mainly used in blends with Arabica in roasted and ground coffees for filter and espresso and in small scale as unique species in soluble coffees. Cultivars Conillon and Robusta are the most worldwide grown *C. canephora*. Although both are resistant to some biotic and abiotic factors, Robusta cultivar presents some advantages such as higher bean size and more vigorous plant. In spite of these advantages *C. canephora* var. Robusta does not provide better beverage than Conillon and some efforts in order to improve it could be undertaken. Modifications in preparing method seemed to be an attractive possibility to accomplish the intent. As known, most, if not all, of both cultivars are similarly prepared by dry method. A number of studies have been published describing the effect of processing method in sensory characteristics of coffee beverages. Few studies have been published on the effects in some physico-chemical properties of green beans, and most of them compares Arabica coffees prepared by dry and wet methods. A few reports describe experiments with Robusta varieties (Guyot et al., 1995; Leloup et al., 2004). Considering sensory characteristics in this context, coffees prepared by dry method are known to provide sweeter and more body brew, whereas

washed coffees provide beverages with higher acidity and aroma. Furthermore, few studies have evaluated chemical composition of grains and sensory properties of beverages from beans prepared by semi-dry method (Knoop et al., 2006). However, none of these is carried out with Robusta cultivars. In this work cup quality and chemical composition of green and roasted beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods were investigated.

MATERIAL AND METHODS

Plant Material and Sample Preparation

Coffee samples were harvested in 2001 by stripping a pool of trees of *C. canephora* var. Robusta grown at 22°54' S, 42°05' W and altitude 669 m. Triplicates of 10 kg of visually fully ripe and sound fruits were manually sorted and prepared by dry, semi-dry and wet methods. Grains prepared by these methods were herein called as natural, pulped natural and washed, respectively. Dry fermentation was employed in wet method. After sun dried up to around 11% moisture, coffee samples were hulled and sorted. Largest non-defective beans were employed on chemical and sensory analyses. Samples were composed of equal amounts of each replicate. Roasted beans used in chemical analyses were sub-samples of those used for sensory evaluation. Green and roasted beans were ground to less than 0.5 mm for chemical analyses.

Chemical Analyses

The concentration of acetic, lactic, malic, formic, oxalic and citric acids, sucrose, arabinose, galactose, glucose, fructose, maltose, and stachyose, as well as pH measurements and total titratable acidity were measured in green and roasted coffees. Carboxilic acids and sugars were extracted in hot water (Rogers et al., 1999). Dionex Column AS 11, electrochemical detector and ASRS supressor were used for carboxilic acid quantification. Separation was carried out isocratically until 4 min with 0.5 mM NaOH followed by linear gradient up to 25 mM NaOH in 30 minutes, using flux of 1 ml/min. Sugars were separated isocratically with 50 mM NaOH at flux 1ml/min in column Dionex CarboPac PA 1 and detected in pulsed amperometric detector. Absence of mannose and xilose were checked in the same column using water as eluent and 300 mM NaOH in post-column system. Total titratable acidity was measured by titration with 0.1 N NaOH to pH 8.2 (method 30.1.07-AOAC, 1998). Measurements of pH were performed in pH-meter.

Sensory Evaluation

Five designated experts tasted coffee brews prepared with 10 g of medium-light roasted and ground coffee and 100 ml of hot water. Sensory quality was assessed by aroma, astringency, body, cleanliness, acidity, sweetness, bitterness and overall quality. Scores ranged from 0 (worst quality or less intense perception) up to 10 (best quality or more intense perception).

Statistical Analysis

Data were analyzed by ANOVA and means compared using Tukey test at 95% of confidence.

RESULTS AND DISCUSSION

Regardless of processing method, sucrose was the main sugar in green beans of *C. canephora* var. Robusta. Sucrose concentration ranged from 4.59% (db), in pulped natural grains, up to 4.79% (db), in washed beans, with no statistical difference between these values (Table 1).

Table 1. Water soluble carbohydrate concentration in green beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods.

Processing Method	Arabinose (%db)	Galactose (%db)	Glucose (%db)	Sucrose (%db)	Fructose (%db)	Stachyose (%db)
Dry	0.06a	0.19b	0.11a	4.67a	0.18c	0.037b
Semi-Dry	0.06a	0.22a	0.12a	4.59a	0.26a	0.040a
Wet	0.05a	0.19b	0.13a	4.79a	0.22b	0.014c

Same letter indicates no difference between treatments ($p < 0.05$).

This behavior was similar to that observed in *C. arabica* var. Acaiá (Knoop et al., 2006), and also to the average result of six Robusta clones studied by Leloup (2004), although there was a clone which exhibited around 40% more sucrose in washed grains.

Experiments carried out in Instituto Agronômico (IAC) with Arabica and Robusta coffees allow to infer that probably there are interactions between variety and processing method on the grain composition. Thus, although there is a tendency for fructose be lower in washed grains (results not shown), opposite behavior was observed in *C. canephora* var. Robusta. In this variety fructose concentration was lower in natural grains. Higher fructose concentration in washed than in natural grains of *C. canephora var.* Robusta was quantified also by Guyot et al. (1995). It is possible that not only differences in analytical methodology, but also the way on which the fermentations were carried out in both studies might explain the differences found comparing to similar works with Arabica (Knoop et al., 2006). Therefore, dry fermentation would not favor passive leaching of sugars into the environment as could happen in underwater fermentation nor would favor alcoholic and lactic fermentation as discussed by In this context, endosperm *C. canephora* var. Robusta would be less aerated in dry processing.

There was no statistical difference between the concentrations of arabinose and glucose in grains prepared by the three methods, and stachyose was the free sugar present in lowest concentration in grains prepared by any process. Stachyose concentration was around 100 times lower than the concentration of sucrosein green beans and was not detected in roasted beans. Maltose was not detected in green and in roasted coffee

Sucrose was the most abundant sugar also in roasted coffee, although around 85% of the sugar in green beans had been transformed during roasting (Table 2). Among all sugars analyzed, arabinose concentration was almost the same in green and in roasted grains.

As reviewed by Balzer (2001), citric and malic were the main aliphatic acids in green beans of *C. canephora* var. Robusta (Table 3). There was no statistical difference in citric acid content in grains prepared by dry, semi-dry or wet method. However, preparing method influenced acetic and malic acid concentration in such a way that the highest acetic acid concentration occurred in natural grains and the lowest in washed grains. On the light of recent findings (Bytof et al., 2005), the highest concentration of acetic acid in natural grains could be a response to the stress caused by dehydration, or, less probably, due to the mucilage fermentation during the 23 days of drying process.

Table 2. Low molecular carbohydrate concentration in roasted beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods.

Processing Method	Arabinose (% db)	Galactose (%db)	Glucose (%db)	Sucrose (%db)	Fructose (%db)
Dry	0.05a	0.01a	0.04a	0.68b	0.03a
Semi-Dry	0.04a	0.01a	0.04a	0.70ab	0.03a
Wet	0.04a	0.01a	0.03a	0.76a	0.00b

Same letter indicates no difference between treatments ($p < 0.05$).

Table 3. Organic acid concentration in green beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods.

Processing Method	Lactic (%db)	Acetic (%db)	Formic (%db)	Malic (%db)	Oxalic (%db)	Citric (%db)	Total (%db)
Dry	0.11a	0.088a	0.031a	0.52a	0.042b	0.93a	1.62b
Semi-dry	0.12a	0.071ab	0.034a	0.47b	0.023c	0.96a	1.68a
Wet	0.12a	0.046b	0.031a	0.50a	0.076a	0.92a	1.71a

Same letter indicates no difference between treatments ($p < 0.05$).

Oxalic acid concentration was statistically higher in washed than in natural or natural pulped beans. Natural pulped grains are sometimes similar to natural, as for acetic acid, and sometimes similar to washed coffee, as for malic acid.

Lactic acid was not detected in roasted coffee. Roasting process increased acetic acid concentration by about 7 times in natural coffee, 9 times in natural pulped coffee and 15 times in washed coffee. The roasting process made the concentration of citric acid to be very similar to that of acetic acid. Formic and malic acid presented around half of that concentration. Oxalic acid was the acid present in lowest concentration in roasted coffee. Moreover, total acid concentration was higher in roasted beans, where it ranged from 1.97 to 2.13%, than in green beans where ranged from1.62 to 1.71%.

Table 4. Organic acid concentration in roasted beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods.

Processing Method	Lactic (%db)	Acetic (%db)	Formic (%db)	Malic (%db)	Oxalic (%db)	Citric (%db)	Total (%db)
Dry	0.00	0.67ab	0.36a	0.30b	0.07a	0.73a	2.13a
Semi-Dry	0.00	0.64b	0.31b	0.35a	0.06ab	0.60c	1.97c
Wet	0.00	0.69a	0.37a	0.30b	0.05b	0.66b	2.07b

Same letter indicates no difference between treatments ($p < 0.05$).

The lowest total titratable acidity in green beans occurred in natural pulped coffee (Table 5). There was no statistical difference between total titratable acidity in natural and washed coffees, although pH was significantly higher in natural beans. One reason for this observation could be acid composition of the grain and their pK values (Maier, 1987). Upon roasting, natural coffee showed the lowest acidity and washed coffee the highest one, however there was no difference in pH values of these coffees.

Total titratable acidity increases during roasting process, but it is not directly related to total acid concentration (Tables 3 and 4). So, although there was not statistical difference between the values in natural and washed green coffees, the total acid concentration was higher in

washed beans. Maybe this is due to the pH considered as the end point of titration. Here it was taken as 8.2 and at this pH not only aliphatic acid but also phenolic hidroxy groups begin to ionize (Woodman, 1985).

Table 5. Total titratable acidity and pH of roasted beans of *C. canephora* var. Robusta prepared by dry, semi-dry and wet methods.

Processing Method	Green Coffee		Roasted Coffee	
	pH	Total Titratable Acidity (meq/kg)	pH	Total Titratable Acidity (meq/kg)
Dry	6.08a	171.8a	5.19a	186.6c
Semi-Dry	6.02a	149.8b	5.22a	192.0b
Wet	5.88b	172.8a	5.20a	200.2a

Same letter indicates no difference between treatments (p< 0.05).

No perceptive astringency or off flavor was sensed in any beverage. The scores reached by the coffee beverages are shown in Table 6.

Table 6. Scores of beverages of *C. canephora* var Robusta prepared by different methods.

	Attribute						
Processing Method	Aroma	Cleanliness	Sweetness	Body	Acidity	Bitterness	Overall Quality
	points						
Dry	5.2 ± 0.4	6.5 ± 0.7	6.5 ± 0.7	6.3 ± 0.9	1.6 ± 0.0	5.0 ± 0.0	4.7 ± 0.6
Semi-Dry	5.8 ± 0.6	6.7 ± 0.6	6.7 ± 0.6	6.3 ± 0.9	1.7 ± 0.0	5.2 ± 0.4	5.7 ± 0.9
Wet	9.3 ± 0.4	8.7 ± 0.9	8.7 ± 0.9	7.5 ± 0.6	3.1 ± 0.4	0.8 ± 0.0	9.4 ± 0.3

Washed coffee beverage reached highest punctuation in all positive attributes. Therefore, it was better evaluated in aroma, cleanliness, sweetness, body and overall quality. Figure 1 shows graphically the same results.

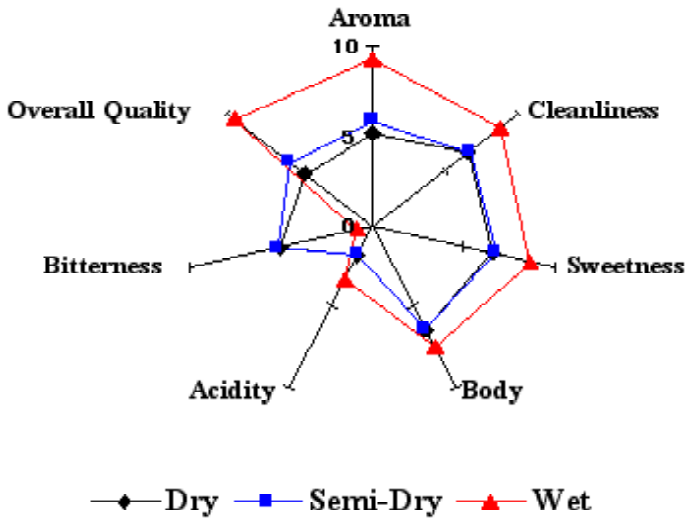

Figure 1. Cup quality of *C. canephora* var. Robusta beverages prepared by dry, semi-dry and wet methods.

Recently, Selmar et al. (2004) and Bytof et al. (2005) presented evidences that chemical and sensory characteristics of coffees processed by wet and dry methods are consequence of the degree of germination reached before dryness and also due to GABA accumulation in seeds. These findings could explain why healthy fruits of *C. arabica* cultivar at the same maturation degree could provide different beverages when prepared by different methods. Same behavior was also observed with *C. canephora* var. Robusta prepared by dry, wet and semi-dry methods. It is interesting to notice that contrary to expectation, even the body of washed coffee beverage was higher than of natural coffee. Maybe, it was also a consequence of dry instead of underwater fermentation carried out to remove mucilage. Although it was suggested before that the lower bitterness of washed coffee could be due to migration of some compounds from the grains to the aqueous environment, same thing was observed when dry fermentation was performed. Other typical characteristics, such as higher aroma and acidity, conferred to beverages by underwater fermentation were also perceived when dry fermentation was carried out.

Grains prepared by semi-dry method provide beverages which characteristics were intermediary between washed and natural grains. Concerning cup quality, however, the additional advantage of this method over dry method is that it implies in less risk of deleterious fermentation during drying process.

CONCLUSIONS

Results of experiments confirmed that fruits of a cultivar at the same maturation, origin and health stated may provide beverages with different quality when prepared by different methods. Therefore, coffee beverage of *C. canephora* var. Robusta was significantly improved through processing method. Washed beans prepared by dry fermentation provided less bitter, more aromatic, cleaner, sweeter and slightly more acid beverage compared to natural and pulped natural grains. Processing method did not affect sucrose concentration in grains but greatly interfered with organic acid balance. Citric acid concentration was quite similar in washed and natural grains, but acetic acid concentration was almost twice in natural grains.

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Importance of Water in the Wet Post-Harvest Process on the Quality of Mexican Coffee

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SUMMARY

Quality of Mexican coffee produced by four variants of the wet method were evaluated taking into account physical aspect of beans, aromatic content of green and roasted coffee and sensory character of the beverages. Two pulping machines and three types of mucilage removal of beans (natural fermentation in water, dry natural fermentation and mechanical removal of mucilage) were used to control how reducing water in the process induces changes in coffee quality. Three degrees of roasting were applied for better appreciation of difference based on aroma content. Physical aspect of green coffee beans was characterized using standard criteria of defects. Aroma of green and roasted coffees was analyzed by HS-SPME and flavor of beverages was evaluated by descriptive sensory analysis. Changes in the post-harvest process lead actually to physical, aromatic and sensory differences in coffees and beverages clearly distinguished by the principal components analysis (PCA) applied on the data obtained. The type of pulping involves differences on physical aspect of beans, the use of vertical drum equipment leads to beans with much more defects. The more important processing stage when considering the quality with the aromatic and sensory criteria was the mucilage removal method. Coffee produced by the traditional wet method using microbial mucilage removal was of better quality than coffee produced by the mechanical method recently introduced because it uses less water and is more ecological. Moreover mucilage removal in water gave coffees with better aromatic quality influence by fruity, floral and caramel notes, whereas dry mucilage removal gave more neutral coffees. These results confirm the importance of the microbial mucilage removal under water in the wet method for coffee quality.
Keywords: Coffee, Processing, Quality, Physical aspect, Aroma, SPME, Sensory analysis, PCA

RÉSUMÉ

La qualité du café mexicain issu de quatre variantes de la voie humide a été évaluée en prenant en compte l'aspect physique des grains, le caractère aromatique des grains verts et torréfiés ainsi que la qualité sensorielle des boissons. Pour cela, deux types de dépulpage et trois types de démucilagination (microbienne, sous eau et à sec et mécanique) ont été appliqués aux cerises afin de vérifier si la réduction de l'eau engendre des modifications de la qualité du café. De plus, trois degrés de torréfaction ont été appliqués aux échantillons de café vert afin de pouvoir mieux apprécier les différences aromatiques dues aux variantes de traitements. L'aspect des grains a été caractérisé par rapport à des critères standards de défaut. Les composés volatiles des grains verts et torréfiés ont été analysés par HS-SPME-GC

couplée à l'olfactométrie et à la spectrométrie de masse. La qualité de la boisson a été jugée par analyse sensorielle descriptive. Les modifications d'étape du traitement post-récolte engendrent effectivement des différences de qualité des échantillons qui sont discriminés lorsque l'on traite les données par analyse en composantes principales (ACP). Le type de dépulpage utilisé en traîne des différences d'aspect des grains, ceux issus d'un dépulpeur à tambour vertical montrant le plus de défauts. L'étape qui joue le rôle le plus important au niveau des différences entre échantillons du point de vue qualité aromatique et sensorielle est la démucilagination. Les cafés issus de la démucilagination microbienne sont de meilleure qualité aromatique que ceux issus de la voie écologique utilisant un dépulpeur et un démucilaginateur mécanique économe en eau. De plus, lorsque la démucilagination microbienne est réalisée sous eau elle permet d'obtenir des cafés plus typés caractérisés par des notes fruitée, florale et caramel alors que la démucilagination microbienne réalisée à sec donnent des cafés plus neutres. Ces résultats confirment l'importance des l'eau dans le procédé de traitement post-récolte du café notamment au niveau de l'étape de démucilagination pour l'obtention de cafés de qualité.
Mots clés : Café, Procédé, Qualité, Aspect physique, Arôme, SPME, Analyse sensorielle, ACP

INTRODUCTION

In Mexico, Arabica coffee is processed by the wet method, which involves mechanical pulping of ripe cherries (disc or drum pulper) to remove the skin and part of the mucilage, then removal of the remaining mucilage from the pulped cherries (microbially, enzymatically, chemically or mechanically), and lastly a drying stage (in the sun or artificially). This method uses large amounts of water (40 litres of water/kg of green coffee) and requires great care. It also results in substantial organic pollution in rivers (Bailly et al., 1992). However, it offers the advantage of giving a green coffee that looks better than coffee obtained by the dry method (Coste, 1958; Vincent, 1969; Pochet, 1983).

Coffee producers in the Veracruz region (Mexico) traditionally use the wet method, with different pulping and mucilage removal techniques. Since 1990, a new coffee preparation method has been used in that region to do away with microbial removal of mucilage from the beans and reduce the quantities of water used. This method, which is very often called the ecological wet process or wet unfermented process, uses mechanical mucilage removal. However, doing away with the microbial fermentation stage is likely to result in physico-chemical and sensory modifications to the coffee. The use of this so-called ecological system (Becolsub) showed that there was a good balance between the acid and bitter tastes of Arabica coffee from Colombia (Puerta-Quintero, 1999).

Most flavour compounds are produced when green coffee is roasted. Different degrees of roasting lead to distinct flavour profiles that can affect the sensory characteristics of beverages (Mayer et al., 1999; Yeretzian et al., 2002). The quality of roasted coffee will depend on the length and temperature of the operation (Obiero, 1998; Schenker et al., 2002). Green coffee quality largely depends on the existence of flavour precursors, themselves formed during fruit ripening then during post–harvest processing (Barel and Jacquet, 1994). The purpose of our work was to study how four wet post-harvest processing options affected first the quality of green coffee according to its aromatic potential and then the aromatic quality of roasted coffee and the sensory quality of beverage after 3 different roasting.

MATERIALS AND METHODS

Biological material

The *Coffea arabica* samples used in this study came from the Veracruz production zone (Mexico). During the 2001-2002 harvest, ripe, defect-free cherries were picked and divided into 40-kg batches of cherry coffee at three different periods (December 2001, January 2002 and February 2002)

Physical aspect of green beans

The number of total defects was determined by a visual examination of average samples of 300 g of green coffee from the post-harvest processes in accordance with ISO standards: undesirable beans, insect attacked beans, broken beans, white beans, shells (Defects ISO 1980; 1985; 1993).

Wet post-harvest processing treatments

Four wet post-harvest processing treatments were tested. They differed through the type of pulping and mucilage removal used. In treatments 1 and 2, pulping was carried out in water with a disc pulper, whilst in treatments 3 and 4 a vertical drum pulper (Penagos Hnos & CIA LTDA, Colombia) was used without water. The mucilage removal stage for treatments 1, 2 and 3 was carried out microbially (natural fermentation). Mucilage removal in treatment 1 was carried out in water in 0.5 m^3 polypropylene tanks. In treatments 2 and 3, microbial mucilage removal was carried out under dry conditions, in the same types of tanks as for treatment 1. The samples were washed as soon as the fermentation time had been judged sufficient; this was determined by assessing the breakdown of mucilage on the beans by touch (between 30 and 60 hours). Treatment 4 used a vertical mechanical mucilage remover (Penagos Hnos & CIA LTDA, Colombia). Once the mucilage had been removed, the beans were dried in the sun on metal trays, in layers around 2 cm thick until a moisture content of about 12%. The samples were frozen at –80 °C in plastic flasks pending their use.

Degree of roasting

Green coffee beans (100 g) with a moisture content of around 12% were roasted in a PROBAT® type RE1 laboratory roaster at constant air temperature (240 °C). Three degrees of roasting were performed by varying the length of the operation: light roast (6 min), medium roast (7 min) and dark roast (9 min). Two roasting indicators were used: luminance and weight loss. The luminance of the ground roasted coffees was measured on a Chroma Meter CR-210 tri-stimulus colorimeter (Minolta) and expressed on the Hunter scale (Clydesdale, 1978). Weight loss was expressed as a percentage of total weight.

Preparation of coffee samples

Green coffee samples (100 g) from each post-harvest treatment were frozen 12 h at –80 °C prior to grinding. Grinding was carried out in a Perten® grinder (Laboratory Mill type 3600) on a 500 micron setting. After grinding, the ground coffee samples were frozen at –80 °C in plastic flasks pending their use. Roasted coffee samples (≈ 80 g) from each roasting operation were degassed for 12 h at –20 °C prior to grinding; grinding was carried out in a Ditting® grinder on a < 400 micron setting. After grinding, the ground coffees were stored like the green coffee pending use.

Non-volatile compounds analysed by HPLC

Organic acids were quantified by the modified Van Der Stegen and Van Duijn method (1987) (Stegen and Duijn, 1987). An initial extraction on a Dowex 1x4 column (Fluka) with a water/methanol mixture (3/1, v/v) was used to recover the sugars, with the acids remaining fixed. A second extraction on an Amberlite XAD-4 column (Fluka) with 5 mM H_2SO_4 was used to recover organic acids whilst eliminating impurities. The solution obtained was filtered at 0.45 µm. The organic acids were separated by high performance liquid chromatography (Shimadzu LC-6A equipment) on a Biorad Aminex HPX-87H column (300 mm x 7.8 mm) and detected and quantified by a Shimadzu SPD-6A detector (UV Spectrophotometric Detector) at 210 nm. The sugar extracts were separated by HPLC on a PL Hi-Plex column (300 x 7.7 mm; particle size 8 µm) and detected and quantified by refractometry (Shimadzu RID-6A). The eluant used for the 2 analyses was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The concentrations of the different organic acids and sugars were deduced from calibration curves for each compound (SIGMA).

Free amino acids were quantified on an aliquot part of the aqueous extracts obtained from green coffee bean samples and after filtration on syringe-filters fitted with a 0.45 µm Millipore™ membrane. Free amino acids were analysed by HPLC after derivation and detection of the compounds formed under fluorescence. The OPA reagent consisted of a mixture of a 0.2 M borate buffer solution adjusted to pH 9.9 ± 0.05 and a methanol solution of OPA (ortho-phtaldialdehyde) at 38.2 mM supplemented with MPA (mercaptopropionic acid) (20/5/0,05, v/v). The concentrations of each acid were obtained by injecting standard solutions made from 22 amino acids (SIGMA). 100 µl of OPA reagent was added to 100 µl of amino acid solution (standard or sample), before injecting after a reaction time of 1 minute, at room temperature.

Amino acids were separated on an HPLC Varian™ 5000 Liquid Chromatograph combined with a Shimazu RF-530 fluorimeter (λ excitation = 340 nm; λ emission = 445 nm). The column used was an Adsorbosphere™ OPA HR Alltech™ measuring 150 mm in length and 4.6 mm in diameter, with a porosity of 5 µm. A binary elution gradient between 80 mM sodium acetate, pH 5.9, and methanol+ THF 97/7% (v/v) was applied with a flow rate of 1.5 ml/min.

Non-volatile compounds analysed by NIRS

The contents of various chemical compounds (caffeine, trigonelline, chlorogenic acids, fat, sucrose and moisture) in the coffee samples were determined by near infrared spectroscopy. Absorption measurements for the different samples were carried out on 3 g samples of green or roasted coffee powder using a NIR-System 6500 apparatus equipped with a spinning sample module and mini-cups measuring 3.8 cm in diameter. Spectra were acquired for wavelengths ranging from 400 nm to 2500 nm in 2 nm steps, following the 16/32/16 measuring sequence. On roasted coffee, only caffeine could be quantified by this method. The contents of all compounds were compared to CIRAD-CP's arabica green coffee spectral database.

Volatile compounds analysed by Headspace-SPME (HS-SPME) and gas chromatography

Ground coffee samples were brought to room temperature for 90 min prior to sampling for headspace analysis. A Carboxen/PDMS (CAR/PDMS) type 75 µm SPME fibre (Supelco Co., Bellefonte, PA, Ca.) was used to extract volatile constituents from the coffee headspace. One

gram of ground coffee was placed in a 2 ml hermetically sealed flask, which corresponded to a headspace of 1/3 of the sampling flask. The flasks were placed for 30 minutes in an oven thermostatically regulated at temperatures of 25, 40 or 60 °C, to reach sample headspace equilibrium. Then, volatile compounds were extracted by placing the SPME fibre in contact with the headspace for 5 to 15 minutes at the equilibrium temperature. For compound desorption, the fibre was placed in the GC injector heated to 250°C for 4 minutes. All the samples were taken in triplicate.

The chromatography analysis was performed with a Varian® 3300 chromatograph (Walnut Creek, CA, USA) equipped with a DB-WAX capillary column (J&W scientific) measuring 30 m * 0.32 mm i.d., with 0.25 µm phase coating. Injection was in splitless mode, at 250 °C with a Supelco specific SPME insert of 0.75 mm i.d. The vector gas (hydrogen) flow was 1.5 ml/min. The column temperature was programmed from 44 °C to 170 °C at 3 °C/min, followed by a rise from 170 °C to 250 °C at 8 °C/min. Detection was by a flame ionization detector (FID) at 300 °C.

The aroma characteristics of the volatile compounds from each coffee sample were analysed by combined FID-Olfactometry. A derivation with a deactivated column was used to bring half the effluents to an OD 01 sniffer system (SGE, Ringwood, Australia). The conditions used for olfactory perception were the same as those described above for HS-SPME-GC analysis. Detection was carried out independently by three judges and each detection was performed in triplicate on different days. For each odour stimulus panellists gave odour description and recorded the detection time.

The coffee SPME extracts were analysed on a GC-MS apparatus (HP-6890A GC connected up to an HP-5973N MS) with a DB-WAX capillary column (J&W scientific) measuring 30 m * 0.32 mm, with a 0.25 µm phase coating. Column temperature programming was identical to that described for GC-FID. Injection was in splitless mode for 4 min at 250 °C with a specific SPME insert. The mass range scanned was from 40 to 350 amu at a scanning rate of 2.89 scans/sec. The transfer line temperature was 260 °C. The vector gas (Helium) flow rate was 1.5 ml/min. The ionization method used was electronic impact with ionization energy of 70 eV. The volatile constituents of the headspace were identified by comparing their calculated relative retention indexes with those given in the literature, and their mass spectra with those in the database (Wiley Mass Spectral Data). The relative retention indexes were calculated from the retention times of the compounds and of the linear alkanes (Retention Index Standard, Sigma). The aromatic notes of the compounds perceived by olfactometry were also used as identification criteria by comparing them to references in the literature.

Descriptive analysis of beverage

The beverages were prepared from 50 ± 0.1 g of ground coffee (Ditting disc grinder) for 1 l of water with a low mineral content (Volvic®, France), pH 7,0 and a temperature of 95 ± 2 °C. The extractions were made by 5 min of infusion in a plunger coffee maker (French Press or plunger method). The cup temperature at the time of tasting was 55 ± 3 °C.

The beverages were tasted by a panel of 17 CIRAD experts (10 women and 7 men) aged 24 to 50 years and trained on samples of Arabica coffee from Mexico.

The panel assessed the cup quality of the coffee samples by the flavour profile method (Standard ISO 6564-1985) (AFNOR, 2002). Eleven sensory criteria were proposed to the panel: flavour intensity, flavour quality, body, acidity, bitterness, floral, fruity, sour, harsh, “green” taste, "fermented" taste. Scoring was on a scale of 0 to 5, where 0 corresponded to a

total absence of the criterion in the coffee and 5 to high presence of the criterion in the same coffee. Panel responses were entered on computer and analysed with ULISI software developed by INRA, France (Avelino et al., 2002). The analysis was carried out on the mean score attributed by the panel for each sample and each descriptor.

Statistical analysis of data

In order to distinguish between coffee samples from 4 post-harvesting processes a principal components analysis (PCA) was applied to the data by the Statistica software package (v6, Statsoft).

RESULTS

Physical aspect of green coffee beans

The results of the Figure 1 present a clear difference between samples according to the pulping method used. The number of green coffee defects was larger for post-harvest treatments using a vertical drum pulper without water compared to treatments 1 and 2, which included a phase of wet pulping. It was particularly with regard to undesirable beans that the post-harvest treatments displayed large and significant differences, followed by the existence of broken beans due to direct use of the drum pulper. Treatments 3 and 4 stood out from treatments 1 and 2 through a larger number of such undesirable beans.

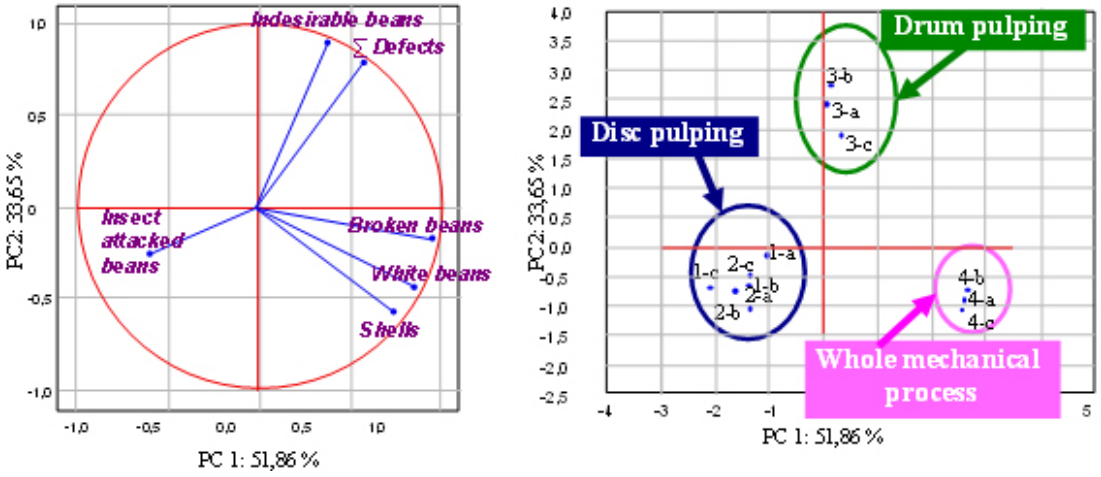

Figure 1. PCA on criteria of physical analysis of green coffee (trade characteristics) (a,b,c = three harvesting periods).

Sensory analysis of beverage

The panel did not succeed in identifying "fruity" and "floral" notes in a significant way. It was therefore decided only to keep nine of the eleven attributes. Figure 2 shows the PCA with the 9 sensory variables. The values given are means of the two tastings of beverages made with coffee from three harvesting lots (a,b,c) after four post-harvest processing variants (1, 2, 3, 4) and three degrees of roasting (L = Light, M = Medium, D = dark). Differences were found for each attribute between the three degrees of roasting. Flavour intensity and body increased in line with the degree of roasting. However, the "green" and "fermented" variables decreased as the degree of roasting increased (Aino and Motoyoshi, 2001; Schenker et al., 2001; Sanz et al., 2002). The Figure 2 shows that coffee beverages are grouped according to their roasting degrees. This is a well known behaviour of coffees sensory attributes. Dark roasting produces

beverages with bitter taste and high flavour intensity. While light roasting is characterized by acidity and fermented sensory attributes. Coffees of medium roasting were judged neutral.

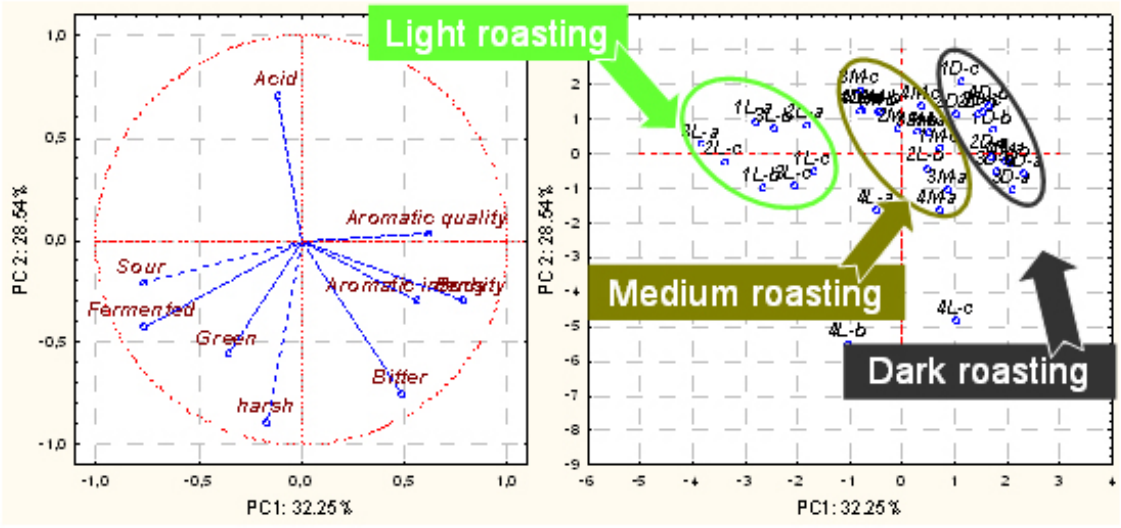

Figure 2. PCA on sensory attributes of coffee beverage by descriptive analysis of beverage tastings (means of 2 tastings) (L = Light, M = Medium, D = dark, a, b, c are the three harvesting lots).

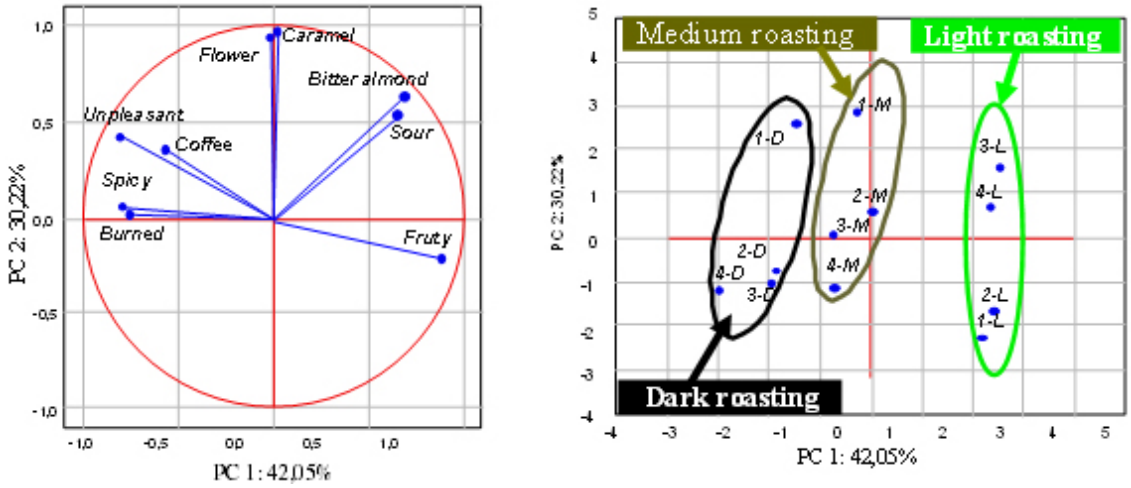

Figure 3. PCA on aromatic notes of roasted coffee volatiles given by HS-SPME-GCO.

Aroma of roasted coffees

We then characterized the coffee samples produced by the different post-harvest processing treatments, based on olfactory criteria, using the sniffing method. Olfactory characterization of coffees is recognized as being one of the most important criteria for distinguishing between the post-harvest processing treatments by which they were produced (Bytof et al., 2000). With the combined HS-SPME-GC-O we used, we detected 101 odorous compounds, 66 were identified and compounds with an olfactory impact are grouped in 9 different aromatic notes that describe coffee aroma in accordance with the data in the literature (Clarke, 1990; Furia and Bellanca, 1992; Cantergiani et al., 2001; Sanz et al., 2001): fruity, flowery, caramel, coffee, spicy, burnt, unpleasant, sour, bitter almond. The same roasting effect can be noticed in the aroma of roasted coffee (Figure 3). The aroma profile of light roasted coffees was characterized by fruity notes. When roasting degree is increased these typical aroma note are

lost and coffee, spicy, burnt notes were developed. Coffee from the medium roasting is the less characterized by aromatic notes.

Aroma of green and roasted coffees

Introducing aromatic notes characterizing green coffees in PCA data leads to a discrimination of samples according to the process used (Figure 4). It can be seen that there are three distinct groups formed as a result of the effect of water in the mucilage removal. Natural fermentation under water produced in the green coffee fruity notes and in roasted coffee caramel notes. The ecological method gave undesirable notes especially in green coffee beans such as vegetable, sour and bitter. Dry fermentation produces neutral coffee less characterized by aromatic notes. So, the type of the mucilage removal has an important effect on the aroma quality of green and roasted coffees, the natural fermentation under water being the best one.

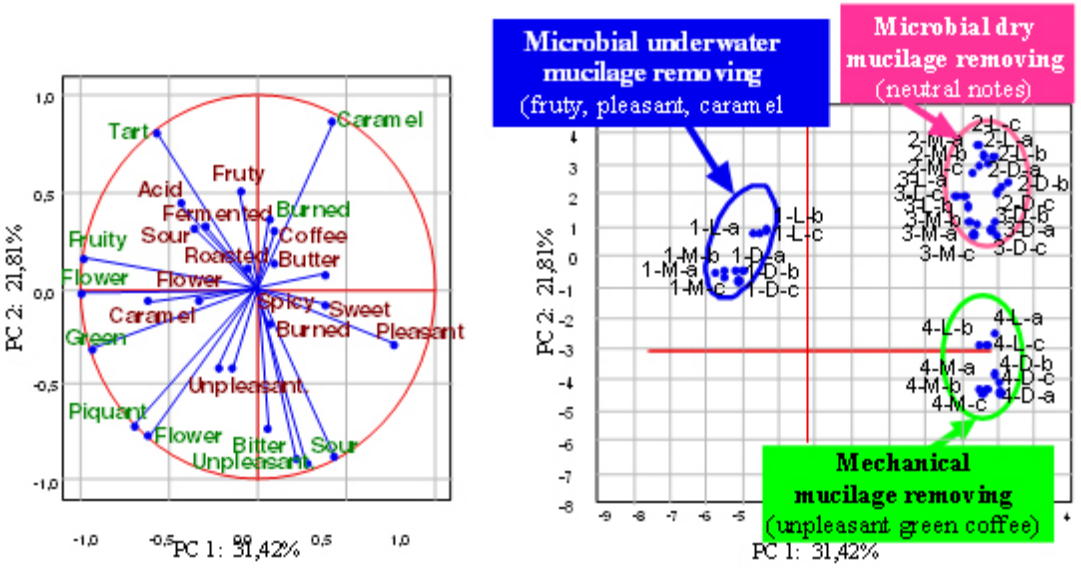

Figure 4. PCA on aromatic notes of green and roasted coffee volatiles given by HS-SPME-GCO.

Non-volatile compounds of green and roasted coffees

The Figure 5 present PCA made with non-volatile compounds of green and roasted coffees analysed by HPLC and NIRS. Organic acids were analysed in green and roasted coffee by HLC. These compounds were found in both types of coffee but their content changed: acetic, citric, formic, malic, lactic, oxalic, quinic and succinic. Amino acids and sugars recognized as principal precursors to aroma developed during roasting were analysed only in green coffee by HPLC. The simple sugars found were sucrose, glucose, fructose and arabinose. A lot of amino-acids were found in green coffee: alanin, arginin, asparagin, aspartic ac., glycin, glutamin, glutamic ac., histidin, isoleucin, leucin, lysin, methionin, phenylalanin threonin, tryptophan, tyrosin and valin. Caffein, trigonellin, chlorogenic acids, fat and sucrose also precursors of aroma and flavour of coffee were analysed by NIRS in green coffee.

Mucilage removal produced an important effect on the content of amino acids, organic acids and aroma precursors. This effect was manifested by the two groups. The microbiological mucilage removal method produced high content of certain amino acids which are important to coffee flavour both in green and roasted coffee. The mechanical method developed high content of organic acids such as quinic and succinic acid that are responsible of undesirable

sensory notes in roasted coffee. High contents of sugars are also found in green coffee from the mechanical method that could develop burnt notes after dark roasting.

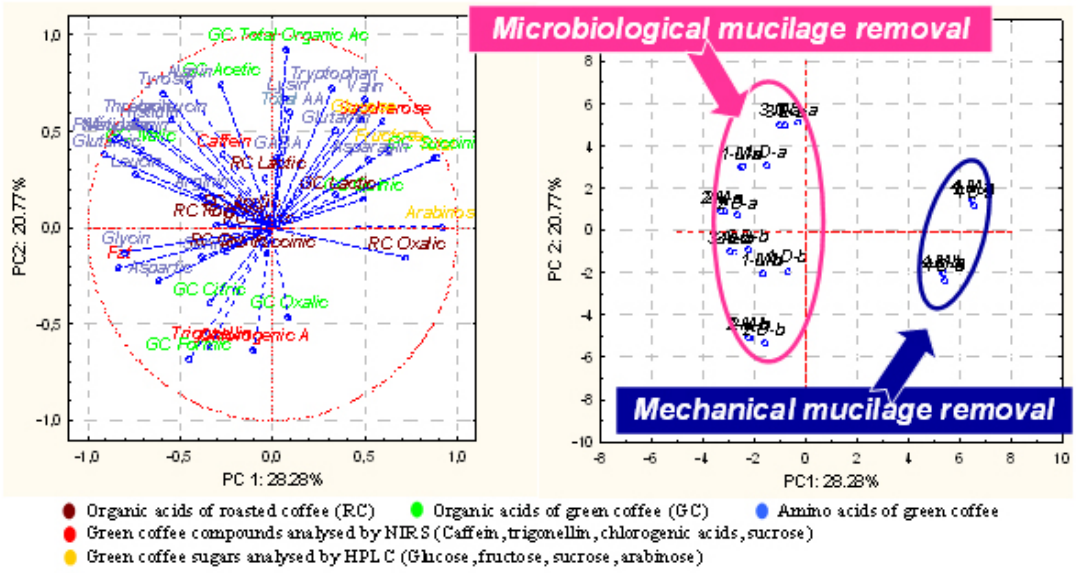

Figure 5. PCA on non-volatile compounds of green and roasted coffee analysed by HPLC and NIRS.

The whole PCA

All the compounds or attributes characterized by the different analytical methods used in this study were used as variable in a PCA for discriminate coffee samples of the 4 post-harvest processes (Figure 6). It can be seen the effect of water in the fermentation process on the aroma content of green and roasted coffees. The ecological method produced less aromatic precursors. The dry natural fermentation was characterized by undesirable sensory notes and fermented beverages.

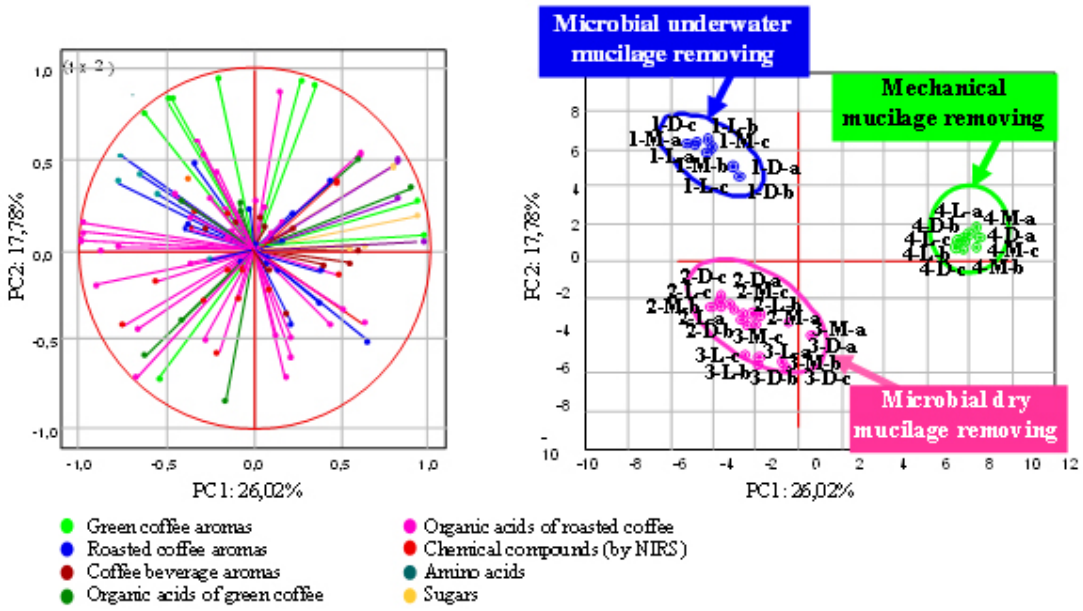

Figure 6. The whole PCA on all variables used to qualify coffee samples from the 4 process by the different analytical methods.

CONCLUSION

Each treatment studied has advantages and disadvantages. Choice of processing method depends on coffee grower project: water saving and ecological sensibility or speciality coffee production, with waste water treatment.

Pulping method has no effect on aromatic and sensory qualities of coffee. However, it impacts on the physical quality of green coffee which has a higher content of physical defects, when drum pulper is used.

Microbial mucilage removal under water gives green coffees of better aroma (fruity notes) and less physical defects than mechanical treatment, which saves water and gives better yield.

For these reasons, mucilage removal process is a key step for aromatic and sensorial qualities.

The post harvest process is very important for the quality of the final product. The choice of the method depends on the desired quality of the beverages.

In order to optimize the roasted process, the post harvest treatment of green coffee should be considered.

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A Critical Appraisal of Coffee Drying in Kenya

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SUMMARY

Coffee drying is the most critical phase of the coffee processing chain in. Normally, it also imposes serious challenges to the processors for being highly dependent on the solar energy, an input whose reliability can be impaired sometimes by prevalence of overcast, cloudy and rainy periods. This can cause prolonged drying even at critical stages of high moisture content, which is detrimental to the production of high quality coffee. Generally, the drying tables require vast areas out of the limited available land besides being labour intensive. These tables need regular repair and maintenance as well leading to frequent additional costs. Some other adverse aspects inherent to the drying process can however be checked by controlling the drying rate or exposing coffee to the sun at a certain phase of drying. These limitations among others have been stimulating the need for alternative coffee drying technologies. However, there has been very limited adoption of new innovations to at least supplement the drying tables particularly in the cooperative sector. As such, there is still need for further search for appropriate dryers. In view of this, the important outputs towards overcoming the critical constraints, which have been encountered with are discussed. Recommendations are also made on the way forward for the sake of sustaining the high quality of Kenya coffee particularly with respect to safety and health requirements.

INTRODUCTION

In Kenya, arabica coffee is mainly processed by the wet method with only very small unsuitable amounts, being committed to dry processing. The resultant parchment coffee is mainly sun dried because the final quality of such coffee is also highly appreciated despite its demand for labour and capital in substantial proportions (Wootton et al., 1968). However, this process also requires labour, capital and land in substantial proportions (Whitaker et al., 1984). Since it can also take up to 14 days on average to dry the coffee, the capacity of the drying tables must accommodate coffee harvested within 2 weeks. The drying process takes place in distinct stages identified by percent moisture content (MC%, wb) to including: Skin drying (55-45 %), White (45-33%), Soft black (32-22%), Medium black (21-16%), Hard black (15-12%) and, fully dry (10.5-11%) for parchment. The coffee cherry which is intended for dry process is simply dried from 65% to 12% MC (Kamau, 1980).

Initially, coffee drying must be rapid, and involves vigorous stirring and if necessary using heated air to remove the surface water and that between the parchment hull and the bean the shortest time possible within a day. The following white phase has to be slow and cool to avoid parchment cracking. Otherwise, subjecting wet and dry parchment to 38 $^{\circ}$C and 57 $^{\circ}$C can cause sourness in the liquor besides promoting uneven drying. During the soft black phase, exposure to the sun for an accumulated 50 sunshine hours induces some very attractive quality attributes to the coffee via ultra violet rays of the sun radiation. However, in a hot, dry weather, the rate of drying under fully exposed conditions can lead to a total drying period so short that the photosensitive reactions which appear to play a part in the development of quality do not have time to reach completion. Such coffee tends to be yellowish or brownish as well (Wootton et al., 1968). Mechanical drying is discouraged within this phase (Kamau,

1980). At 12% MC, the coffee can remain on the tables for long, if adverse weather prevails, supporting the practice of transferring such coffee from the tables to ventilated storage to pave way for relatively wetter coffee (Wootton, 1968).

Once coffee has been committed to the drying process, it must be ensured that it dries steadily as the drying status may require and devoid of reversing due to re-wetting. Hindered drying conditions should be overcome by provision of any suitable technology. Coffee should not be allowed to over-dry since valuable weight will needlessly be lost and coffee becomes pale yellow an attribute that is related to poor quality. Besides, over-dry coffee reabsorbs moisture thereby getting rewetted with a loss in quality. However, it is better to over dry slightly instead of under drying since a little moisture re-absorption has insignificant undesirable impact on coffee quality while under drying creates conditions conducive to mould growth. It is important therefore, that all the critical points during the drying process are controlled. How this has been done and the way forward in this regard is subject of this paper.

THE CRITICAL CONSTRAINTS AND THE CORRESPONDING REMEDIAL MEASURES

Microbial activities during Skin drying

It is evident that the fully wet state of freshly washed parchment provides conditions suitable for the development of microbial activity leading to onion flavour and other taints (Wotton et al., 1968). In order to avoid this hazard, it is essential to get wet coffee through the skin-dry stage within the shortest time possible in a day. This means a rapid rate of drying, maximum exposure to the sun and continuous stirring of the beans spread in a thin layer on the skin-drying tables until the surface water and that between the bean and the hull is removed. Coffee can be skin dried with warm air without heating beyond 35 °C. After skin drying, the parchment should be transferred to final drying table on the same day to prevent rewetting through dew formation on metal tray wire, which can occur reverse the gains made during the day.

Parchment cracking in the white stage

As soon as the coffee is skin-dry, the parchment begins to crack unless it is immediately shaded. This occurs if rapid and hot drying was adopted in this phase since the parchment hull would dry faster than the bean and raptures in an attempt to shrink over an otherwise swollen bean (Mburu, 1999; Wootton et al., 1968). Cracking can expose the bean to microbial attack, induce contamination from the ambient environment besides ageing and hence loss of coffee quality. The parchment should also be shaded from the intense sun between 10.30 am and 2.30 pm to ensure that it remains cool, but continuously ventilated via regular stirring. The minimum residence time in this phase is signified by change of colour from white to black accompanied by minimum parchment splitting. Intact parchment will not crack afterwards.

Sunlight requirement at the soft black stage

The beans at the soft black stage are elastic, translucent and allow the formation of the final colour of the bean. Sunlight (UV solar radiation) is therefore very essential because formation of the raw bean colours as well as the improvement of the chemical quality is photosensitive at this moisture level. Since drying is not very critical in this phase, an increased layer depth and stirring vigorously can be practiced if it gets too hot. However, for the remaining days to fully dry, coffee should be spread to a depth of 2.5 cm, more thinly in poor weather and

stirred frequently. It should also be kept under ventilated shade cover from 10.30am to 2.30 pm in hot weather (Mburu, 1999; Wootton et al., 1968).

Mixed drying

Mixed drying of coffee can contribute to uneven roast and light acidity in the coffee. Therefore, the relevant measures must be in place to check such an undesirable aspect. The design of the current coffee drying table for instance is meant to ensure that it remains absolutely flat, which is essential for thorough stirring and thus even drying of the coffee (Sonke, 1968). Other important features include a suitable width to ensure that one can stir the coffee to the middle from either side for even drying. These tables have a wooden framework on top of which are chicken and tension wires with adjustable J-bolts to keep them flat all the time. Scheduled repair and maintenance of these tables is therefore very crucial to prevent them from sagging and hence inducing mixed drying. Mixed drying is also avoided by drying coffee lots harvested at different times separately on the drying tables until they are fully dry. This applies also to dry processed cherry instead of allowing them to accumulate in heaps located sometimes in dump places pending attention at the end of the processing season.

Protection from adverse weather

Sun drying of coffee on the conventional tables can expose the coffee to extreme weather conditions. However, recommended suitable protection of the coffee from adverse weather is available. Covering materials like a “Nylex” P.V.C. or polythene films 1 m wide are for instance safely used for covering coffee at night and in dull/rainy weather during the day as alternatives to “sisal-craft” paper provided the coffee is wrapped in Hessian or sisal cloth to unsure that it is not in contact with the covering material; and the covering material is not passed underneath the coffee, which must be ventilated from below. An evepy reinforced P.V.C. covering can also be safely used over coffee drying tables provided the coffee is wrapped in hessian or sisal cloth. It is however, worth observing that covering coffee in the middle of the day from 10.30 am to 2.30 pm, every day even though the weather may be dull and cool merely serves to lengthen drying times and so increase pressure upon drying space with no compensating advantage.

In a hot and dry day, coffee should ideally be stored under a roof some distance above it, which can be very much cooler than its exposed counterparts. However, in practice, heaping the coffee longitudinally on the table coupled with vigorous stirring instead of covering is recommended to protect the coffee from intense heat from the sun (Wootton et al., 1968). A double-layer of hessian over a shallow layer of parchment coffee does not prevent the coffee attaining about the same temperature as fully exposed parchment.

Drying space limitations

The lack of adequate space for sun drying coffee can cause congestion in a primary coffee-processing factory thereby impairing the capacity of the factory to receive coffee in a timely manner as it ripens in the farms. The resulting delays in harvesting can have adverse effects in the processed coffee. Sun drying space has become increasingly limiting due technological advance in farming, hindered attributed to unreliable climate; prolonged skin drying periods during long rain spells or when the weather is very dull and cold; lack of enough well maintained drying tables, labour availability and management, some social implications and the absence of suitable equipment to supplement the drying tables. Shortage of tables can arise from limited factory land and deterioration of the drying tables caused by failure to maintain and repair them as required. Congestion can also occur where the cherry delivery to

a factory exceeds its design cherry intake capacity. Such adverse conditions can compromise the coffee quality perhaps by inducing mould formation. Since the main constraint arises from adverse weather, coffee drying trials were conducted within the global coffee project (Mburu, 2005). The results showed that the low frequency of initial OTA producers did not develop into significant OTA outcomes that could be ascribed to the treatments. These experiments also proved to regularly contain OTA-producing fungi. However, individual replicates were occasionally far removed from others. The quality of the coffee from all the treatments was poor (Class 5-8) possibly due to the prolonged drying (12-20 days), thick coffee layers (4-8 cm) among other factors.

Since fully dry parchment is normally at equilibrium with a relative humidity of 60% at 20 °C, coffee can dry to about 12% beyond which it can take as many as 8 days (Wooton et al., 1968) without attaining the fully dry status as long as the wrong RH% prevails. However, a suitable 9th day can see the coffee through to full dry within 2 hours only. At the same time, the drying rate might be too slow from the soft black stage during overcast condition due clouds. For these and other reasons, the very wet coffee can miss drying space on the tables because of congestion. These findings paved way for studies to consider the following options for sustaining an adequate capacity at the drying stage through scheduled repair, maintenance and expansion of drying tables, more adoption of coffee driers and ventilated conditioning/temporary storage bins as well as improving the operational and labour management.

Coffee drying tables

In Kenya, sun drying of coffee has been most preferred in comparison to mechanical drying and this has produced the best quality coffee. However, though congestion due to shortage of drying tables sometimes causes a reduction in the quality of the dried coffee, the remedy is not the building of more tables since many factories do not have or are unable to purchase the necessary land and/or do not have sufficient labour to operate extra tables. As such, this can become critical as plantings in the smallholder sector begin to expand the overall yields. The status of the existing tables must however, be confirmed to be in good order first and the necessary repairs if any done before considering expanding the drying space. Otherwise, doubling the coffee depth on the tables from the soft black to fully dry phase can also serve to over come congestion. The available factory land for the drying might also be the limiting factor for expansion. In such a case, it would be prudent to consider the services of a coffee dryer.

Conditioning bins

If the coffee is on the table for so many days without attaining fully dry status during adverse weather conditions, the factory would be experiencing serious congestion. This is just an example of the sort of frustrating circumstances, which justify the construction of ventilated conditioning bins to which it is advisable, therefore, to transfer nearly dry coffee for temporary storage in poor weather and so free drying tables for wet coffee. Such coffee must however, be returned to finish off at the tables when warm and dry conditions return or at the end of the harvesting season. In this regard, ventilated and manually stirred conditioning bins have been playing an important role in the estate and cooperative coffee sectors respectively. Further to that, ventilated bins not only eliminate heating, but also actual cooling is achieved. Finally, the total capacity of the bins should be sufficient for at least one week's production, or about twice as much as can be held on tables.

Soaking

Soaking of parchment after final grading can enhance the factory capacity by allowing further cherry intake even when congestion is prevailing. Since parchment can be soaked for a maximum of 7 days without deterioration in quality (Mburu, 1997), implementing such a practice would enhance the capacity of a factory to process more coffee particularly when drying is seriously affected by adverse weather. Soaking can actually play a pivotal role at the peak season within which 20% of the seasons harvest is expected within 2 weeks. The soaked coffee would then be dried when congestion eases.

Coffee driers

In view of the need to provide solutions to the constraints causing congestion in a coffee factory, the technical performance of alternative driers like ***PVC Tents*** or simple ***Plastic cover*** projected over the coffee (Kamau, 1982) for skin drying even when there is bad weather/rain and during the night; ***Drying trays*** (Anon., 1990) for wet parchment and which can be stacked on racks under a corrugated iron shed; ***Rotary drum*** and ***Batch*** driers (McCloy, 1960; Mburu and Mason, 1993); ***Sack driers*** (McCloy, 1959); ***Silo*** drying unit (Kamau, 1980); ***Low Continuous Column*** drier (Type BSR 133) (Kamau, 1980); ***Ventilated conditioning bins*** (McCloy, 1960); A ***new parchment Solar*** drying system (Trim et al., 1984) and Parabolico driers (Global Coffee Project, 2004) were evaluated. Other preliminary studies focused on driers for the small holder sector like the ***ITIPAT*** drier from Cote de Ivoire; ***cabinet solar*** drier from UCDA, Uganda); as well as ***concrete barbeques***. Although, some driers were capable of relieving tables when the weather could not permit parchment drying to fully dry or when the cherry intake was beyond the table capacity, loading in some of them was very laborious. These perhaps are some of the reasons why adoption of these driers has been very limited particularly in the cooperative sector despite accrued savings in labour (normally used in opening, covering tables and moving coffee) and the accelerated drying period by 7-11 days (Kamau, 1982) because of the ability of some of them to dry wet parchment even during the rain. All the same, mechanical drying has also been observed to reduce the drying period quite considerably and in some cases the ratio was 1:8. In general, therefore, few if any mechanical dryers have been installed in cooperative factories principally due to their high cost and technical sophistication but they have been employed in the estate sector for many years. However, the search for, new parchment drying systems for cooperative and smallholder factories is still in progress to supplement the sun drying of coffee in order to salvage coffee which could otherwise be ruined by lack of drying facilities during the peak period when weather is also not ideal for drying.

Specifications for coffee driers are as follows: The dyer must be indirectly fired and the fuel used specified. It should dry coffee from 55% (wb) to 33% (wb), and 23% to 10.5%); If not 55% (wb), then the highest moisture that it can safely handle should be indicated; The temperature of coffee beans should not exceed 35 °C at any time during the drying cycle. The total power requirement of the installation should be indicated in Kw; The average fuel consumption of the heat source per unit tone of coffee dried should be given for both moisture content ranges; The capacity of the dryer or batch capacity range should be indicated; The approximate drying time to dry from 55% to 33% and 23% to 10.5% should be indicated for the average ambient conditions of 25 °C dry-bulb temperature and 60% relative humidity; Installation requirements of the drier should be stated and drawings provided for this purpose; The minimum requirements of equipment necessary for loading and unloading the drier should be included in the supply; Possibility to offer an automatic moisture control for the dryer if required should be indicated and; It should also be indicated whether the dryer has been found to be satisfactory for drying wet processed Arabica coffee. These specifications

are necessary for screening the dryers before evaluation. At the end of evaluation, the dryer must be appraised economically and financially to enhance its adoption.

Efficiency in the human resources

The other constraining factor has been on efficiency of the factory operators to implement the drying procedure to the letter. This can also prevent the effective utilization of available labour. This shortcoming has been addressed through regular in-service and field training.

Moisture content of the coffee during drying

To ensure that coffee dries at the required rate throughout, the capability to measure the moisture content particularly from soft black to fully dry status is very necessary. This makes it easy to avoid over drying. Oral measurements by cracking beans with the teeth and accessing the ease of hulling parchment by rubbing a sample between the hands are the conventional means employed to determine whether the coffee has dried fully while use of moisture meters is very limited. However, weighing a known volume of coffee can be used to estimate its water content but its robustness must be proved and any shortcomings charted. One such specific issue relates to shrinkage during water loss. If the particles shrink to a greater degree than the volume of water removed, the packing changes and one could predict a systematic error due to the standard container accommodating increasing amounts of dry matter and a flattening in the relationship between weight loss and water loss as drying occurs. A second potential source of error would lie in any inherent differences in the density of the grains due to seasonal regional or physiological effects. A study to this effect was conducted on different parchment grades arising from density grading in a washing channel.

The relationship between weight and volume was clearly linear though with only a moderately good adherence to the best-fit line. As expected, grade 1 was denser than 2 and grade 2 was denser than lights. However, sometimes grade 2 from one source was denser than grade 1 from another source. If this were a real result and not one due to inaccurate extrapolation, establishing a weight, which deems Kenyan parchment coffee, even grade-by-grade, to be dry would be problematic. Further field-testing of weight of a constant volume of parchment during drying ultimately showed there was a consistent packing error and that there were significant differences depending on source and parchment grade.

Security

In the absence of effective security, the operators can transfer wet coffee to facilities not suited to drying though secure. Since that can have adverse effect on coffee quality, it warrants for provision of proper security of the drying coffee instead of taking such an aspect for granted.

DISCUSSION AND CONCLUSION

Drying coffee should never be covered with Hessian or sisal cloth alone. In hot dry weather, the finished coffee should be removed from the tables while cool. Over drying rather than under drying is preferable, but time should not be wasted deliberately over drying.

The foregoing analysis indicates that the conventional drying tables still remain the commonly used coffee drying technology in Kenya to date. This is much so in the cooperative and the smallholder sector unlike in the estates. However, new and appropriate technologies are still required to supplement the solar drying. This should be prioritized to ensure that the

cited constraints are decimated. In addition to that, the drying table capacity in all the coffee factories should be restored by expedient repair of the damaged ones. Otherwise, their necessary scheduled service and maintenance should be resorted to as required.

There are currently new innovations developed recently in the world towards this end. There is need therefore to sustain the evaluation activities to identify those which can be adopted into the Kenya coffee industry as they are or in a modified form.

Conditioning was introduced; it has been reported, to produce uniform drying though it has evidently become a tool to manage periods of high demand on the drying tables. The danger here is that very wet coffee can be transferred to manually conditioning bins where it will not dry further at all. This can easily happen in coffee factories with limited drying tables since most of the tables have deteriorated because of long lapse of low production, which led to minimal or no usage of such tables with hence no repair or maintenance. As a solution, the manual operated bins should be ventilated. This will require all the factories to electrified and electric fans installed for this purpose.

Finally, the know-how of the factory operators should be sustained through regular training. By so doing, the entire process as well as the existing and any new equipment will be operated efficiently for the improved quality, health and safety of Kenya coffee.

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Ultrastructural Analysis of Drying Damage in Coffee Endosperm Cells

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SUMMARY

Biological membranes are particularly sensitive to dehydration. The ability to keep the plasma membrane as a semipermeable barrier has been associated with the seed viability and grains quality. When the cellular organization is severely damaged the seed lose considerable amount of cellular solutes. Although it is well know the rate of drying is an important factor determining the level of plasma membrane damage, ultrastructural analysis of coffee seed endosperm submitted to different conditions of drying have not been carried out before. In the present work we have analyzed and compared the structural alterations in the cell wall, in the vesicles and plasma membrane integrities in coffee endosperm after drying at 40 °C and 60 ºC. Samples were prepared in ten replicates using histochemical tests with Sudan IV to light microscope and routine techniques to scanning and transmission electron microscopy. The results indicate any change in the cellular integrity of the plasma membrane and vesicles in seeds dried at 40 ºC. Histochemical analysis show higher amounts of putative oil body present into the endosperm cells after drying at 40 ºC. In contrast, seeds dried at 60 ºC display fused oil bodies giving rise to large droplets in the intercellular space, what leads to loss of cellular integrity. Both scanning and transmission microscopy show the plasma membrane collapse and coalescence of the vesicles when the coffee beans were dried at 60 °C. Thus, we might conclude that higher temperature is detrimental to the coffee endosperm cells integrity.

INTRODUCTION

The drying of the coffee is one of the most important coffee processing stages and consists in the removal of great part of the grains water. Several researches had been made with the objective to evaluate the drying system, the reduction of the energy consumption and the driers efficiency (Berbert et al., 1994; Freire, 1998; Guimarães et al., 1998). Recently, the importance of post-harvest treatments on coffee bean quality has received growing attention, and several studies describe the impact of wet and dry processing on the physiology and quality of coffee (Bytof et al., 2000). Nevertheless, ultrastructural analyses which occur upon drying are not well understood.

Studies have shown that, after desiccation, the plasma membrane is one of the first points of damage. Ultrastructural analysis of the endosperm tissues is essential to verifying these works. To respond to this deficiency, the objective of this work is to evaluate, through histochemical and ultrastructural analyses, the effect of different drying temperatures on the structures of coffee endosperm cells.

MATERIAL AND METHODS

The parchment coffee *(Coffea arabica* L.) was used in the present work**.** To decrease the interference of different primary matter in the results, only one variety (“Catucai”) was used

throughout the whole experiment. Harvesting was done manually, using the stripping system in four repetitions in time. The coffee was then separated according to the density of the fruit. The portion of ripe fruit was peeled and submitted to pre-drying on ground during one day. Than, the coffee was dried at 40 and 60 ºC in driers of fixed layers 0,13 m thick, using air flow of 20 $m^3 min^{-1} m^{-2}$. The driers (Figure 1) with fixed layers had a chamber measuring 0,61 x 0,61 x 0,61 m and a fan triggered by a 0,5 hp motor. The plenum chamber contained a group of 3.400 kw electrical circuits to heat the air and the drying chamber was composed of four removable sections that received the pre-dried samples. Each section received an average of 0,01 m^3 of coffee. After each drying the samples were cooled with ambient air. The final moisture content was determined using the oven method (Jensen, 1962). The samples were then stored in polythene bags until the moment of cleaning and the histochemical and ultrastructural analyses.

The defective grains were removed before the analyses were carried out so that they would not interfere in the results.

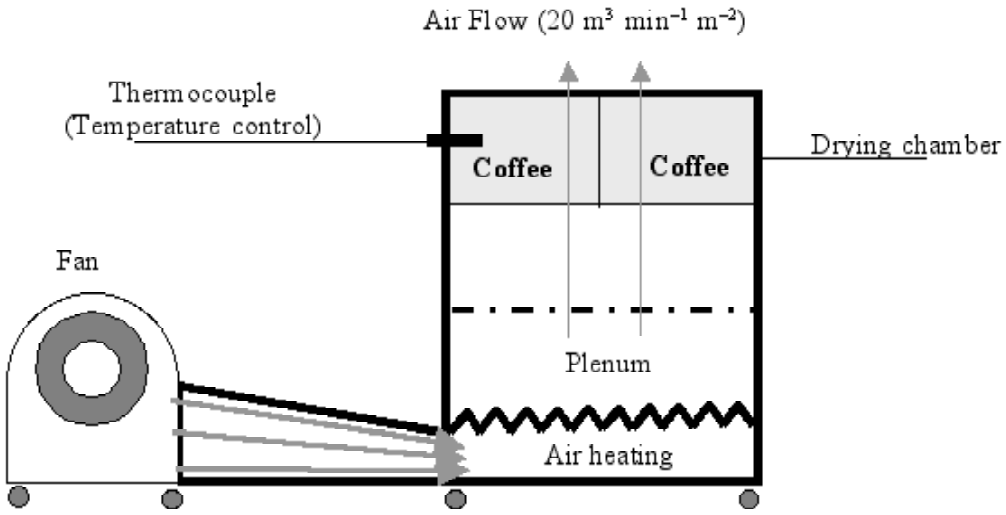

Figure 1. Scheme of the equipment used to dry the coffee samples.

Sampling

In this work samples composed of ten dry parchment coffee were used for each drying temperature, 40 and 60 ºC.

Histochemical and ultrastructural analyses

The sections destined for histochemical reactions, obtained by cutting with a hand microtome and kept in water drop**,** were treated for three minutes with the Sudan IV reagent in an ethanol solution at 80% to visualize the lipids (Jensen, 1962).

Preparation of the samples for SEM

The samples were cut lengthways and immersed in modified Karnovisky solution. They were then infiltrated with a crio-protectont, an aqueous solution consisting of 30% glicerol for 30 minutes and transversally sectioned in liquid nitrogen using a scalpel blade. The sections obtained were post fixed in a 1% aqueous solution of osmium tetroxide, dehydrated before critical point dried. The specimens were placed on aluminum support “stubs” and observed under a LEO EVO 40 XVP scanning electron microscope (Leo Electron Microscopy).

Preparation of the samples for TEM

The samples were cut lengthways and immersed in a fixative solution (Karnovisky modified). After that, they were then washed in cacodylate buffer 0.05 M, pH 7.2 (three times for 10 minutes), post-fixed in 1% aqueous osmium tetroxide solution for 1 hour, transferred to a 0,5% uranyl acetate solution for 12 hours at 4 ºC, dehydrated in a series of acetone solutions. The dehydrated tissue was gradually infiltrated with spur/acetone, 30% for 8 hours, 70% for 12 hours and 100% twice for 24 hours each. The specimens obtained were embed in pure spurr resin at 70 ºC for 48 hours. The blocks obtained were trimmed using razors thicker > 100 nm and ultra thin sections (<100nm). The sections were post contrasted in uranyl acetate followed by lead citrate for three minutes, and then examined with transmission electron microscopes (TEM) Zeiss Mod. EM-109.

RESULTS AND DISCUSSION

The results are presented in Figure 2. In the seeds dried at 40 °C (Figure 2A), it is possible to observe a greater concentration of oils in a globular shape inside the membrane. The oils were preserved in this shape due to the integrity of the vesicles. In the seeds dried at 60 ºC (Figure 2B). At 60 ºC, the oils are not as well defined as they are at 40 ºC, but spread inside the cells forming large droplets in the intercellular spaces, indicating a rupture of the membranes. These observations are essential to the preservation of the quality of coffee, as the rupture of the membranes can expose the oils to oxidation and rankness, which in turn lead to the formation of undesirable composts that alter the coffee's aroma and flavor.

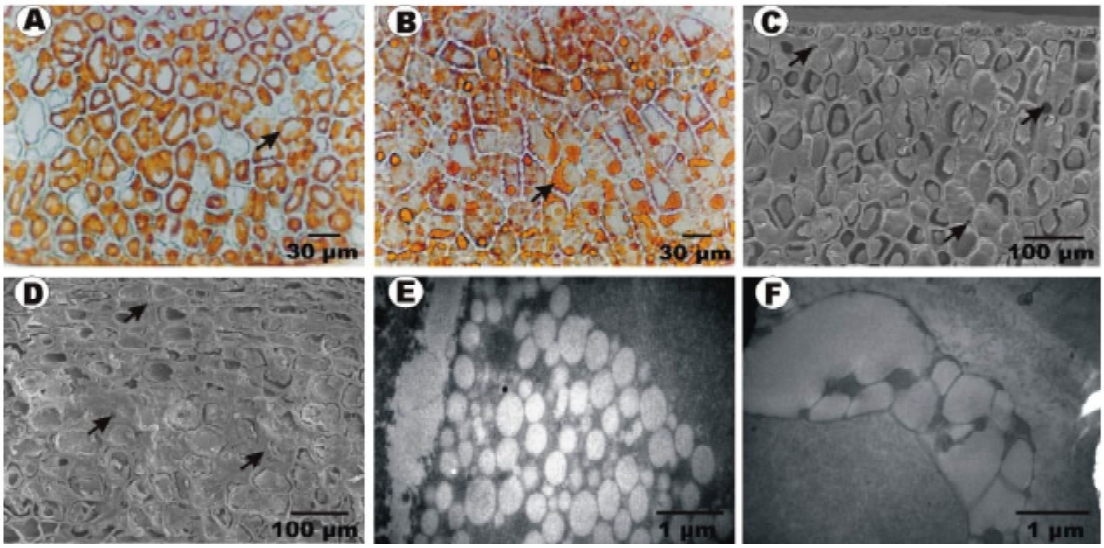

Figure 2. (A and B) Light micrographs of histochemical test of coffee endosperm showing (A) putative oil body inside cells (arrow) (B) large oils droplets in intercellular spaces and ruptured plasma membrane. (C to F) Electron micrographs of coffee endosperm showing (C) Scanning electron micrograph of endosperms cells after drying at 40 ºC. It is possible to see the cellular material inside the cell and intercellular space empty (arrows). (D) Scanning electron micrograph of endosperms cells after drying at 60 ºC. Observe that the endosperm cells were full of cellular material and the intercellular spaces were occluded (arrows), indicated rupture of the cells. (E) Transmission electron micrograph of endosperms cells after drying at 40 ºC. It is possible to see a lot of undamaged vesicles and in (F) Transmission electron micrograph of endosperms cells after drying at 60 ºC. It is possible to see the start of coalescence and collapse of vesicles.

The scanning electron microscopy results (SEM) can be observed in the Figures 2C and 2D. In the coffee seeds dried at 40 ºC (Figure 2C), the content of the cells remained intact and the intercellular spaces remained empty. At 60 ºC (Figure 2D), there was a complete rupture of the cellular membranes and a complete occlusion of the intercellular spaces. These results not only confirm the light micrograph observations but add some relevant information. As the SEM was carried out in dry seeds, any doubts as to the methodology used in the preparation of the fresh cuts, such as the possibility of the membrane ruptures occurring during the steeping process, are eliminated. The transmission electron microscopy analyses (TEM) of coffee seeds dried at 40 ºC is presented in the Figure 2E. Inside the endosperm cells, the vesicles remained undamaged. But in the coffee seeds dried at 60 °C (Figure 2F), coalescence and rupture of the vesicles can be observed.

CONCLUSIONS

The drying parchment coffee at 40 ºC preserves the integrity of the plasma membrane, keeping the putative oil bodies uniformly distributed in the cell. The drying of parchment coffee at 60 ºC leads to a coalescence and a rupture of the vesicles and of the membranes, causing occlusion of the intercellular spaces. Independent of the temperature, the pre-drying period does not interfere in the integrity of the cellular membrane of coffee seeds.

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Cooperative Unions and 'Sustainable' Coffee Initiatives in Ethiopia: Opportunities and Challenges

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SUMMARY

'Sustainable' Coffee initiatives have been flourishing in Ethiopia as a result of increasing demand for organic coffee by global organic coffee consumers and premium price received over the conventional coffee because of an environment friendly production system, socioeconomic concern for the smallholder coffee growers and waiver of market auction by the Ethiopian government which allowed the farmer cooperative unions to export their produce directly to their customers in Europe, Japan and the USA. Four Coffee Farmers Cooperative Unions (CFCU) have been established since 1999, namely, Oromia Coffee Farmers Cooperative Union (OCFCU), Sidama Coffee Farmers Cooperative Union (SCFCU), Yirgacheffe Coffee Farmers Cooperative Union (YCFCU) and Kafa Forest Coffee Farmers Cooperative Union(KFCFCU). Some member primary cooperatives of these unions have been certified organic and fair trade labelled as a result of which they are receiving floor price of 1.41 and 1.26 US$ per pound respectively. At a time when world coffee prices are at their lowest level in 30 years, having fallen by 50% in three years, member farmers of the cooperative unions are reported to be paid fair price for their produce which is invested in food, shelter, health care, education and improved management and conservation of coffee genetic resources. The general tendency for demand to out grow supply suggests the premium is not under an immediate threat and a recent FAO publication indicated that developing countries are likely to gain a relatively substantial market share for organic coffee in the coming years. In this paper, opportunities of sustainable coffee initiatives for and challenges to organized group of farmers in Ethiopia are discussed and a call for global assistance is made.

Key words: Cooperative union, sustainable, Coffee, organic, fair trade

INTRODUCTION

The concept of sustainability in the realm of specialty coffee includes aspects variously referred to as 'economic viability for farmers', 'environmental conservation' and 'social responsibility' (Daviron and Ponte, 2005). Some of these coffees are sold as certified coffee, such as organic, fair trade, bird friendly, Rain forest- Alliance certified, and Utz Kapeh.

The broad notion of sustainable coffee was developed within the North American specialty industry-although the first forms of sustainable certified coffee were developed in Europe by the fair trade movement (Daviron and Ponte, 2005). Organic coffee is produced with methods that aim at promoting a viable and sustainable agro-ecosystem; fair trade is an alternative

approach to conventional trade that aims to improve the livelihoods and well-being of small producers by improving their market access, strengthening their organizations, paying them a fair price with a fid minimum, and providing continuity in trading relationships' (Giovannucci and Koekoek, 2003); shade grown coffee is grown under forest cover, thus preserving biodiversity and providing an appropriate habitat for migratory birds; and the rainforest-alliance certification and the Utz Kapeh code of conduct attempt to combine some elements of the other three sustainable traditions (Daviron and Ponte, 2005).

The global volume of certified sustainable coffee was estimated to be around 272,000 bags for a retail value of US$ 490 million in 2000; if we include non-certified coffee sold as sustainable, the figures rise to 318,000 bags and US$65 million, around 1.2 present of the global coffee market. Estimated size of certified coffee markets (Organic, Fair trade, Utz Kapeh and Shade grown) in thousand 60kg bags for the 2003 were 440, 298, 233 and 11 in that order respectively (Daviron and Ponte, 2005). The total estimated volume (851,00 bags) is much higher that in previous estimations presented above and this is explained on the basis of much higher estimates on organic and the recent growth of a new sustainability initiative-Utz Kapeh. The sustainable coffee market is still a small niche-about 1 percent of the 85.7 million bags exported by International Coffee Organization (ICO) member countries in 2003 (Daviron and Ponte, 2005). Yet it is growing fairly rapidly and attracting increased interest in the industry. The share of one of the sustainable coffee initiatives (Organic) in Ethiopia does not exceed 0.1 percent (Mekuria et al., 2004) and there is very little information on the other forms of sustainable coffee initiatives (fair trade and Utz Kapeh).

At least four major premises can be cited for the emergence of sustainable coffee initiatives in Ethiopia (the recent fall in the market price of coffee, emergence of market based sustainable coffee initiatives, prevalence of an age-old cooperation, diverse and suitable coffee production system). The recent fall in the market price of coffee contributed to a socio-economic decline and affected an estimate of 125 million people. The decline has also resulted in returns that failed to cover production costs. The fall in farmer and government revenue in Ethiopia amounted to 42% within a year (Mekuria et al., 2004)

The emergence of organic coffee has been suggested as a viable option for impoverished farmers living in diverse ecosystems (Bray et al., 2002). Recognizing emerging market potentials of 'sustainable' coffee initiatives, four coffee farmers cooperative unions, established in the 1970s, were reorganized in Ethiopia and were granted permissions to bypass the local traders and the existing coffee auction system to sell directly to international buyers, eliminating the middlemen empowering farmers with far more control over coffee processing and distribution.

Despite the strong effort of organized producers to highly engage in sustainable coffee initiatives, however, the effort made so far to strengthen their effort, document their opportunities and share their challenges with policy makers and arabica coffee consumers is very little.

Therefore, article is prepared with the following objectives:

1. To provide information on the status of sustainable coffee initiatives in EthiopiaTo highlight opportunities and challenges of these coffee initiatives to organized group of farmers

COFFEE FARMERS COOPERATIVE UNIONS IN ETHIOPIA

Four Coffee Farmers Cooperative Unions, representing the major coffee growing regions in Ethiopia have been reorganized with the objective of improving farmers' income, maintaining the quality and improving the productivity of coffee and maintaining the sustainability of the coffee industry. A number of their primary cooperatives have been certified for organic, fair trade and responsible coffee production and sourcing (Table 1). Moreover, the current status of farm size, affiliated farmer numbers, estimated percentage of certified arabica coffee production and supply are indicated in Figures 1 and 2.

Table 1. Coffee farmers' cooperative unions in Ethiopia.

Name of Cooperative Union	No. of Primary cooperatives Certified as Organic (BCS Oko Grantie GMBH)	No. of Primary cooperatives Fair Trade labelled (FLO)	No. of Primary cooperatives double certified (Fair Trade and Organic)	No. of Primary cooperatives certified for responsible coffee production and sourcing (Utz Kapeh)
Oromya Coffee Farmers Cooperative Union (OCFCU)	36	--	6	----
Sidama Coffee Farmers Cooperative Union (SCFCU)	25	---	8	2
Yirgacheffe Coffee Farmers Cooperative Union (YCFCU)	12	2	3	----
Kafa Forest Coffee Farmers Cooperative Union (KFCFCU)	2	5	---	2
Total	75	7	17	4

Technical assistance by the unions has resulted in major expansions in organic coffee production, increases in small farmer and cooperative incomes and direct market linkages to the US, Japan and Europe. Members were paid of organic and fair trade premiums, in addition to their profit share, and this would supplement their meagre income during times of their financial hardship.

Fair trade coffee helped to provide living wages to farmers and up to three times as much income as the average coffee producer which would intern improve their quality of life and allow them to continue working in their farms. Member farmers of the cooperative unions reported that they have invested the dividend they have received in housing, back into their coffee, to start a new business or to pay for their children education.

Prior to the establishment of the unions, farmers used to receive between 27 and 54 percent of the coffee export price only (Weihe, 2005). Currently, Oromya Coffee Farmers Cooperative Union has increased the amount paid to members from export sales to about 70 percent through fair trade and organic certification and directly managing coffee sales. At present, members of primary cooperatives receive dividends based on the volume of coffee they sale. Farmers reported that dividends are used for short-term credit, health, food, and agricultural inputs. In general, these unions have played a vital role in improving the producer-buyer

linkage, directly exporting members coffee, providing goods and services, promoting high quality coffee production and processing, training and education, provision of saving and credit services for member farmers.

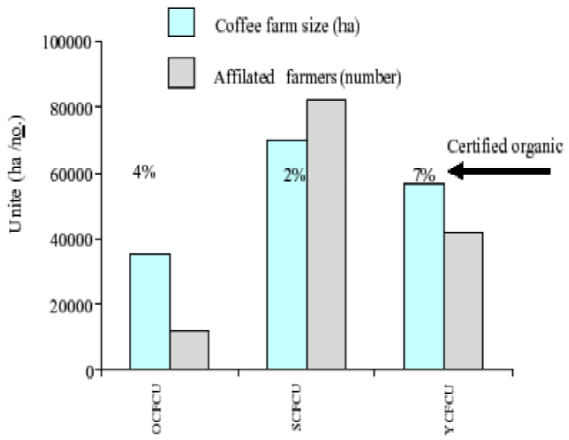

Figure 1. The current ststus of farm size, affiliated farmer numbers and an estimated percentage of certified organic Arabica coffee production by tne Oromia (OCFCU), Sidama (SCFCU) and Yirga Chefe (YCFCU) Coffee Farmers Co-operative Union's in Ethiopia (See Figure 2).

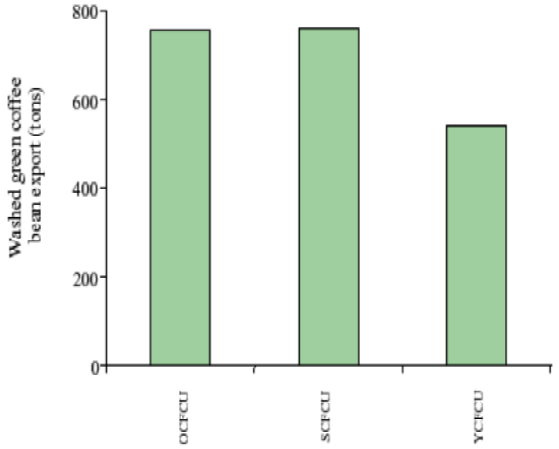

Figure 2. The average supply of green certified Arabica coffee to world market from the three farmers co-operative unions over the period of 200/2002 (unpublished data collected from each farmers cooparative unions).

Oromya Coffee farmers Cooperative Union (OCFCU)

The Oromya Coffee Farmers Cooperative Union (OCFCU) was founded in June 1999 and comprises 74 cooperatives with 22, 734 smallholder farmer members of which 36 are certified as organic by the German agency BCS Oko Grantie and six are double certified for organic and fair trade sales (Table 2). Moreover, the union is a member of the Specialty Coffee Association of America (SCAA) and Eastern Africa Fine Coffee Association (EAFCA). It produces the specialty coffees of Yirgacheffe, Limu, Harrar, Sidama, Jima and Lekemti on over 86,00 acres with annual production of 16,507 tons (Weihe, 2005) and obtains special permission to bypass the auction market and sell directly to its clients. It also offers its members with assistance in marketing and exporting, coffee processing, warehouse storage,

transportation, training on quality production and on coffee extension, coffee by-products, and savings and credit.

Table 2. List of primary cooperatives certified for Organic, Fair trade in Oromya.

Sr.No.	Name of cooperative	District	Zone	Organic	Fair trade
1	keleltu hasegola	Abaya	Borena	√	√
2	Jirme wachu	Gelana	Borena	√	
3	Kilenso mekonisa	Bule hora	Borena	√	
4	Garcha enshe	Krecha	Guji	√	
5	Guanga	Abaya	Borena	√	
6	Guji	Kerecha	Elaferda	√	
7	Dibisa	Bule Hora	Borena	√	
8	Negele gorbitu	Abaya	Borena	√	√
9	Haro	Mana	Jimma	√	√
10	Kenteri	Mana	Jimma	√	
11	Dawa	Mana	Jimma	√	
12	Choche guda	Goma	Jimma	√	√
13	Kaso dabu	Goma	Jimma	√	
14	Ilibu	Goma	Jimma	√	
15	Limu sedecha	Goma	Jimma	√	√
16	Babu	Limu kossa	Jimma	√	
17	Tulube	Metu	Ilubabora	√	
18	Dika gabe	Alledidu	Ilubabora	√	
19	Sineso	Dedesa	Ilubabora	√	
20	Homasiba	Gimbi	W/wollega	√	
21	Bule chala	Hara	W/wollega	√	
22	Figa kobora	Bojii	W/wollega	√	
23	Lalisa	Haru	W/wollega	√	
24	Ilili derartu	Bedeno	Ilili Derartu	√	√
25	Goromuti	Bedeno	Goromuti	√	
26	Riga damu	Bedeno	Riga damu	√	
27	Ababuna	Bedeno	Ababuna	√	
28	Biftu ganema	Bedeno	Biftu ganema	√	
29	Gemechis	Bedeno	Gemechis	√	
30	Warqa	Nensabo	Warqa	√	
31	Homa	Abaya	Borena	√	
32	Kilenso rasa	Bule Hora	Borena	√	
33	Chafe genata	Dedere	E/Haraghe	√	
34	Afata wanji	Mana	Jimma	√	
35	Kampi	Gechi	Ilubabora	√	
36	Koli filfili	Anfilo	Anfilo	√	

In its five years existence, the OCFCU has achieved remarkable success in facilitating smallholder farmers' direct engagement in international trade. The cooperative has built a closer link between farmers and international markets. Building local, national and international relationships with roasters and importers has paved the way to niche and specialty marketing opportunities for farmers, providing them with a living wage to improve their quality of life. The fact that several of its cooperatives are certified as fair trade and organic, member cooperatives earn 3-5 times more than they would receive through traditional marketing channels.

Table 3. List of primary cooperatives certified for Organic, Fair trade and responsible coffee Production and sourcing in Sidama.

	Woreda	Cooperative [Map Downloads Available]	Altitude	Organic	Fair Trade	UTZ Kapeh
1	Aleta Wendo	Chuko Lamala	--			
2		Dobena Wicho	600	√		
3		Dongora Kawado	1,786	√		
4		Gerbicho Lela	--			
5		Gidibona Sheicha	1,959	√		
6		Gure	--			
7		Halo Gelma	1,942	√	√	
8		Homacho Waeno (Haranjicho)	2,015	√	√	
9		Hondowana Borbodo (Habeja)	1,929	√		
10		Korkena Gunde	1,818			
11		Kosoricha	--			
12		Lela Honcho (Futahe)	1,948	√	√	
13		Titira	1,857			
14		Wottona Bultuma	--	√	√	
15	Bensa	Chabe	--			
16		Chire	--			
17		Hache	--	√		
18		Mokonisa	--			
19		Shanta Golba	--	√		
20	Dalle	Boa Bedegelo	1,890	√		
21		Bokasso	2,061	√		√
22		Fero (2nd Fero)	1,930	√	√	
23		Gane	1,810	√		
24		Goyida	1,801	√		
25		Hunkute (Mamana)	1,950	√		√
26		Keage	--			
27		Megara	--			
28		Shoye (Duba)	1,884	√	√	
29		Wayich (Wayicho)	1,837	√		
30		Wenanata	--			
31		Wicho	--	√		
32	Darra	Buna Buka (Tawaba)	1,949			
33		Nura Korate (Odola)	1,627			
34		Setamo	1,815	√		
35		Shilicho [	1,880	√	√	
36		Wata Dara	--			
37	Shebedino	Abela Galuko	--			
38		Fura	--			
39		Howoliso (Dobe Toga)	1,985	√		
40		Taramessa	--	√		
41		Telamo	1,888	√	√	

Source: http//www.sidacoop.com.

Sidama coffee farmers cooperative union (scfcu)

The Sidama Coffee Farmers Cooperative Union (SCFCU) was founded in July 2001 and comprises 42 cooperatives with 82,275 smallholder farmer members. Twenty-five cooperative members of the union, with a potential exporting capacity of over 7,000 tons of coffee per annum, are certified as organic by BCS Oko Grantie. Of the twenty five cooperatives eight primary cooperatives, having a potential production and supply of 6,875 tons per annum received fair trade certification and two primary cooperatives, with a potential production and supply of 360 tons of green beans per annum, are certified by the UTZ KAPEH foundation in the Netherlands for responsible coffee production and sourcing (Table 3). The average export price received by the union for the 2005 and 2006 sales are presented in Tables 4 and 5. In the 2005 cropping season, specialty, Utz Kapeh, double organic and fair trade certified, organic, fair trade and conventional coffee types are reported to account for 1, 4,8,10, 11 and 66 percent of the union's produce in that order respectively.

The union is granted special permission to by-pass the national auction market and sell directly to its clients. It is a member of the Specialty Coffee Association of America (SCAA), Eastern Africa Fine Coffee Association (EAFCA) and Addis Ababa Chamber of Commerce (AACC) and has received World Specialty Commodities Award, Paris 2005; Rosters Guild, Specialty Coffee Association of America Award, Seattle 2005; ECAFE Gold Cooperative Coffee Ethiopian Competition 2005, First place honour in washed and unwashed Sidama varietal class and International Quality Award, The Arch of Europe, Frankfurt, 2004.

Table 4. Average export price of certified coffees.

Types of product	2004/2005				
	Types of Markets				
	conventional	Organic	FLO	Double certified (Organic and fair trade)	UTZ
Washed	1.04	1.16 (11.54)	1.28(23.08)	1.41(35.58)	1.20(15.38)

Table 5. Average export price of certified coffees.

Types of product	2005/2006				
	Types of Markets				
	conventional	Organic	FLO	Double certified (Organic and fair trade)	UTZ
Washed	1.25	1.31(4.8)	1.33(6.4)	1.41(12.8)	1.30(4..0)

Yirgacheffe Coffee Farmers Cooperative Union (YCFCU)

The Yirgacheffe Coffee Farmers Cooperative Union (YCFCU) was founded in July 2000 and represents nearly 44,000 farmer members who are organized into 21 primary cooperatives in the Gedeo district of Southern Ethiopia. Of the 21 primary cooperatives, twelve are certified as organic. Of these twelve primary cooperatives certified for organic three are also double certified for fair trade and organic sales. Moreover, two are registered for fair trade sales (Table 6). The average export prices received for three years are presented in Tables 7, 8 and 9. The union was the first place winner for Ethiopia at the 2005 Taste of Harvest Coffee Cupping Competition, which was held in Levingstone, Zambia on February 28, 2005.

Table 6. List of primary cooperatives certified for Organic, Fair trade in Yirgacheffe.

Name of Cooperative	Type of Coffee Produced	Organic	Fair Trade	Volume Produced in Tons	Total Farm Size in Hectors	Number of Producers
Adado	Yirgacheffe*	√		2,060	3,296	2,424
Addis Ketema	Yirgacheffe*	√		1,430	2,289	1,683
Aramo	Yirgacheffe			2,498	3,997	2,939
Belekara	Yirgacheffe	√		826	1,322	972
Beloya	Yirgacheffe*	√		1,792	2,868	2,109
Dumerso	Yirgacheffe			1,584	2,535	1,864
Edido	Yirgacheffe	√		2,441	3,906	2,872
Hafursa	Yirgacheffe	√	√	886	1,418	1,043
Hama	Yirgacheffe	√	√	1,329	2,127	1,564
Haru	Yirgacheffe	√		1,389	2,223	1,635
Kello	Yirgacheffe*	√		1,182	1,892	1,391
Koke	Yirgacheffe	√		1,316	2,105	1,548
Konga	Yirgacheffe	√	√	1,862	2,979	2,191
Sigiga	Yirgacheffe*	√		3,083	4,933	3,627
Amaro Kele	Sidamo			2,870	4,800	1,729
Chichu	Sidamo			2,301	3,681	2,707
Dama	Sidamo			2,116	3,386	2,490
Finchwa	Sidamo		√	1,070	1,712	1,259
Hase Haru	Sidamo		√	1,573	2,517	1,851
Michele	Sidamo			1,749	2,799	2,058
Resa	Sidamo			1,749	2,799	2,058
Tumiticha	Sidamo			1,513	2,420	1,780
TOTAL	12	5	38,619	62,004	43,794	

Source: http//www.Yirgacheffe-coffee.com.

Table 7. Average export price received for certified coffees in the 2003/04 crop year.

Types of product	2003/04				
	Types of Markets				
	conventional	Organic	FLO	Double certified (Organic and fair trade)	UTZ
Washed	1.06	1.41	1.41	1.41	-

Table 8. Average FOB price received for certified coffees in the 2004/05 crop year.

Types of product	2004/05				
	Types of Markets				
	conventional	Organic	FLO	Double certified (Organic and fair trade)	UTZ
Washed	1.15	1.47	1.47	1.47	-

Table 9. Average FOB price received for certified coffees in the 2005/2006 crop year.

Types of product	2005/06				
	Types of Markets				
	conventional	Organic	FLO	Double certified (Organic and fair trade)	UTZ
Washed	1.18	1.51	1.51	1.51	

Kafa Forest Coffee Farmers Cooperative Union (KFCFCU)

The Kafa Forest Coffee Farmers Cooperative Union (KFCFCU) was established in 2004 by 15 primary cooperatives representing 4267 farmers. Currently, the number of primary cooperatives reached 26 embracing 6632 farmers. The union has made agreement with Bench Maji and Teppi Cooperative Unions to export their coffee and so far Five primary cooperatives are fair trade certified and two others are certified for organic and responsible coffee production and sourcing (Utz Kapeh).

OPPORTUNITIES

Supporting policy/Legislation

The Federal Democratic Republic of Ethiopia, House of peoples' representatives endorsed a bill (488/98) for the establishment of organic agriculture system in Ethiopia. This would contribute to further expansion of organic coffee production.

Genetic Diversity

The tremendous genetic diversity present in the Ethiopian arabica coffee gene pool offers an immense opportunity to breed for coffee types that are resistant or tolerant to pests and adaptable to diverse agro-ecologies. To date a number of CBD resistant cultivars are in production and the effort to develop resistant cultivars for major coffee growing regions is underway. This has curtailed the use of agrochemicals for the control of this disease.

Ease of conversion to organic

Around 75% of total coffee area in the world and more than 90% in Africa is cultivated by smallholder farmers (Scholer, 2000). Though not yet fully certified, more than 90% of the Ethiopian coffee production system is based on traditional practices under natural forest canopy and can be considered as bird friendly or shade grown organic coffee (Kufa and Shimber, 2001; Scholer, 2000). This makes conversion of uncertified coffee growers into organic very easy and is to the advantage of small-scale coffee growers as it is suggested that yield tends to be stable if conversion to organic departs from low input (often traditional) systems (Scialabba et al., 2002). Under conditions where numerous grower organizations and smallholder farmers do not use, or use a minimum of agro-chemicals, conversion seems a logical option especially when coffee prices are low (ITC, 2002). Nonetheless, care should be taken to ensure that the potential export production warrants the cost of conversion into organic production.

Prevalence of an age-old cooperation

Over forty years have elapsed since the modern farmers' cooperatives came into existence in Ethiopia. By being organized into cooperatives or companies, farmers are able to market their

own harvest in a cost effective and competitive manner, thus tapping into potential price incentives offered by the market (for quality, organic, fair trade). Consequently, producers learned about their customers how to improve quality at every processing stage and.

Availability of cheap labour

Organic coffee production have high labour requirement than that would be required for the use of agro chemicals particularly in weed control, but their production costs are lower (Sosa *et al.,* 2004). Therefore, given that organic coffee attracts premium prices, cost of production is lower and labour is cheap offers a better opportunity to small-scale coffee growers in Ethiopia to reap the benefits of producing it.

Demand versus supply

Different trade sources have varying views on the growth prospects of organic coffee sales. Organic coffee has been growing at a rate of 25% per year since 1993 (Griswold, 2000) and in 1996 it was reported to account for less than 2% of the $5 billion world market for specialty coffees, but was quickly increasing its meagre share (CEC, 2000).

At present the general tendency for demand to outgrow supply suggests that the premium received for organic products is not under immediate threat for most product categories and organic production is expected to offer premium prices and a profitable alternative to conventional production systems for many farmers (Scialabba et al., 2002). Similarly, the fact that the demand for organic coffee (western Europe, North America and Japan) exceeds the present supply, which is still less than 1% of annual world production (Van Der Vossen, 2004) opened possible markets for Ethiopian coffee cooperative unions, enabling them to enhance foreign exchange earnings and diversify their exports.

Impressive premium

Premiums reflect strong consumer demand with some consumers willing to pay higher premiums than others. Organic products tend to command impressive premiums in developed countries at retail level: on average between 10-50% and in some cases as high as 100-200% above conventional prices for the same commodity (Scialabba et al., 2002).

Premiums for organic coffee are difficult to estimate as they depend on the quality of the coffee and the market situation at a given moment. The potential producer premium (FOB) was 10-15% over the equivalent non-organic quality in 2002 (ITC, 2002). This compares with consumers generally accepting to pay retail prices of around 20% more for organic coffee that they do for conventional coffee. Another report by Burnett (1998) indicated that organic coffee is the fastest growing segment of the US $2.5 billion specialty market and certified organic coffee farmers earn 15-20% more than non-organic coffee farmers. Some exceptional coffees realize higher premiums but consumer interest tails off rapidly if premiums go beyond this unless the coffee's quality is absolutely outstanding (ITC, 2002). Guaranteed floor price of $ 1.41 and $1.26 (Table 10) for organic and fair trade coffee respectively, attracts coffee growers to engage in organic coffee production.

Table 10. Guaranteed minimum Fair trade and certified organic coffee prices in cents per pound.

Type of coffee	Fair Trade		Certified organic	
	Central America, Mexico, Africa, Asia	South America, Caribbean area	Central America, Mexico, Africa, Asia	South America, Caribbean area
Washed arabica	126	124	141	139
Unwashed arabica	120	120	135	135
Washed Robusta	110	110	125	125
Unwashed Robusta	106	106	121	121

Source: International Trade Center, 2002.

CHALLENGES

There seems to be both a general desire and movement to undertake efforts to advance sustainable coffee production initiatives and their related positive impacts. Nevertheless, it is important to recognize several significant challenges that will need to be addressed in the implementation of cooperative measures so that it can be sustainable. Despite the increasing demand and impressive premiums paid for Ethiopian sustainable coffee types, it appears that their production and supply by cooperative unions is largely constrained by:

Lack and/or limited access to credit

In general Farmers have had little access to credit to fund crop marketing activities. The fall in the price of coffee in the past few years have created a bad impression on lending institutions (Banks) in view of the fact that most of the cooperatives have had difficulty in paying back their loan on time making access to credit a problem.

High cost of inspection and certification

Certification is an expensive process and annual inspection may cost tens of thousands of dollars for coffee cooperatives (Rice and Ward, 1996). Foreign inspectors charge fees that is equivalent to those that prevail in the developed countries from which they come and these fees are as a general rule, very high, particularly when compared to socio economic condition of organic coffee producers in lesser developed countries (Sosa *et al*, 2004).Generally, due to its cost and reliance on external agents, certification is a fundamental problem for organic coffee growers. Therefore, this calls for the need to establish a national certifier and or internal control system to reduce costs associated with inspection and certification.

Little investment on research and extension

Despite the increasing expansion, certification, and export of organic coffee, the effort made so far to identify suitable organic materials, develop appropriate organic coffee cultivation methods, and disseminate various protocols developed elsewhere is very little.

Limited awareness of consumers

Despite the undoubted popularity of Ethiopian coffee, there is relatively little awareness of how coffee is cultivated in Ethiopia and the role that coffee cultivation plays in sustaining the environment. This can partly be attributed to limited effort of responsible authorities in Ethiopia in the promotion and advertisement of sustainable coffee types produced by organized group of farmers.

Lack of local and national certifiers

Ethiopian Coffee farmers' cooperative unions rely only on foreign inspectors and certifiers and local and or national certifiers have not yet been established. As a result cooperatives are charged a very high fee for inspection and certification which, in turn, has resulted in an increased cost of production.

CONCLUSION

The ever increasing social and environmental consequences of modern coffee production in terms of threat to farm workers, native wild life, local surface and ground water, diminishing local biodiversity and disruption of valuable habitat and disposal of millions of tons of coffee husks into nearby stream have stimulated interest in a return to more traditional and sustainable methods of producing this cash crop.

In view of this general scenario, the tremendous coffee genetic diversity and production system that Ethiopia is endowed with and little or no use of agrochemicals offers an immense opportunity to strengthen and further expand sustainable coffee initiatives and reap impressive premiums. However, it appears that lack and/or limited availability of credit, high cost of inspection and certification, little investment on research and extension, limited awareness by consumers and lack of local and national certifiers are major bottlenecks for the expansion of organic coffee production in Ethiopia.

Therefore, in light of the existing ecological concern in the world, socio-economic opportunities for and challenges encountered by small-scale coffee producers, coffee farmers must comply with the standards of ecological agriculture, vigorous marketing campaigns should be carried out, and credit facility should be available for the cooperatives. Moreover, there is a need for global assistance to help establish local and national certifiers and maximize exploitation of untapped potential of small-scale coffee producers in Ethiopia

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Information Management for Product Differentiation in Supply Chains: the Case of Speciality Coffee

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SUMMARY

Prices of most of agricultural commodities show a long-term trend to decline. Increasingly markets are signaling demand for differentiated products and in order to increase their incomes farmers and traders are looking to higher value options, including differentiated products. Product differentiation occurs when a product offering is perceived by the consumer to differ from its competition on any physical or non physical product characteristic including price. The differentiation can be based both on perceptual differences and also on actual product differences based on measurable characteristics. The information requirements for supply chain management of differentiated high value products are much more stringent than for traditional commodities requiring a two way information flow running from the primary producer to the end customer. The conceptual base for information systems to support supply chain management of differentiated products is described. CinfO, an internet based coffee information system, illustrates how these principles have been applied to the case of specialty coffees. CinfO provides farmers information on where and how to produce coffee with particular features, whilst at the same time providing traders with information on the availability of specialty coffees with particular traits. Furthermore, CinfO traces individual coffee batches indicating where and how the beans were produced, processed and distributed to the end consumer so as to facilitate identity preservation which is key to obtaining added value from differentiation.

INTRODUCTION

For many years the development workers talked of the subsistence farmer, staple foods and food security. We believe that at present there are very few truly subsistence farmers, and that this is not a new phenomenon. Most farmers, including the poorest, are in the business not only to feed and clothe themselves and their families, but also to make money or at least barter their goods. Markets, not production, increasingly drive agricultural development (World Bank, 2006). However, the price of most commodities shows a long-term trend to decline. A basket of various agricultural commodities that we have analyzed at different times show a general price decline of 1-3% per year. At the same time, in spite of major research efforts, yield potential of commodity crops is increasing at 0.2-2% per year (Evans and Fischer, 1999) and actual increases in crop yields are of the same order (ASA, 1999). Consequently, with rice as a partial exception, most of the major commodities are produced today on larger farmers than previously, and these farms now employ less people per unit area. At the same time some farmers and communities, particularly those with limited access to land, have switched to higher value differentiated crops or activities so as to increase their income per unit land area.

At the same time lack of reliable information at all stages along the supply chain is a severe limitation on the development of differentiated products, in spite of the existence of modern information technology. The development of differentiated high value crop products depends

on an overall organization framework, excellent communication and information exchange within that organizational framework, the ability to differentiate the product and to develop processes that ensure that the end customer obtains the desired product at an acceptable price in a timely manner. Specifically information is lacking on: how and where to produce and process products with specific characteristics; how to ensure that producers and processors are aware of consumer demands and preferences; how to raise the interest of processors if producers put forward a product with new characteristics; and coordination of activities and logistic along the supply chain. This paper first introduces briefly concepts related to product differentiation, then proposes a conceptual framework for information management systems in differentiated, high value crop supply chains and uses a case study to illustrate how these concepts can be turned into reality using an internet based information management system, CinfO, for speciality coffee.

CONCEPTUAL BACKGROUND

Supply Chain Management

Supply chain management is the integration of all key processes across the supply chain (Cooper et al., 1997) ranging from those involving end users to those that are original providers of products, services and information (Lambert et al., 1998). Supply chain management functions as a network of organizations that are involved, through upstream and downstream linkages, in the different processes and activities that produce value in the form of products and services in the hands of the ultimate customer (Christopher, 1992). Much SCM work has concentrated on markets where products are valuable and differentiated, rather than in commodity markets due to the importance of business-to-business relationships in the supply of differentiated products. Furthermore, SCM emphasizes the overall and long-term benefit of cooperation and information sharing by all members of the supply chain replacing the adversary relationships common in traditional commodity markets with a spirit of collaboration so that all benefit. In the light of these advantages SCM was chosen as an appropriate means to bring together the multiple actors in the supply chain to ensure that consumers obtain the differentiated products they desire, whilst at the same time using the network approach to make certain that primary producers and others along the supply chain reap just rewards for their efforts.

Product differentiation and Identity preservation

Increasingly markets are signaling demand for differentiated products. It is noteworthy that market segmentation, that implies heterogeneity in the demand function, can only be used as a strategy when accompanied by product differentiation (Dickson and Ginter 1987): if there is no product differentiation then demand functions will not differ and hence there is no heterogeneity of the demand function to be exploited by segmentation. Product differentiation occurs when a product offering is perceived by the consumer to differ from its competition on any physical or non physical product characteristic including price. The differentiation can be based both on perceptual differences obtained from usage, word of mouth, promotion campaigns and also from actual product differences based on product characteristics (Dickson and Ginter 1987, Clause 2003). “Credence attributes” or “process attributes” that relate to the process by which the products are produced (for example coffee produced in an ecologically sound and sustainable manner) are also value factors but can often only be measured through certified and auditable systems that accredit the process (Cause, 2003).

The specific characteristics of coffee quality can be (i) product inherent, (ii) personal and (iii) symbolic (Daviron and Ponte, 2005. The Coffee Paradox.). As is the case of wines there are

many traits that determine quality, but due to personal preferences there is no one particular coffee that has the best inherent quality. These inherent quality characters are subject to personal preferences: one person may prefer a sightly bitter coffee with a chocalatey flavour whereas others may prefer a milder coffee with a touch of nutty almond. It is precisely these different personal preferences that open up the possibilities for carving out niche markets for specialty diffentiated products that cater to the personal preferences of individual consumers. Symbolic quality characteristics are normally associated with a particular production area, production system or social context. The perceived, or symbolic, differentiation of a product in a similar manner to that of the inherent characteristics will often depend on personal preferences: the quality of the differentiated product depends on the personal individual preferences and desires of the end consumer.

Differentiation can be achieved through three different pathways: segregation, traceability and identity preservation (Smyth and Phillips, 2002) with traceability and segregation as means to preserve the identity of the product. However, it is identity preservation that aggregates value to the product in a sustainable manner (Smyth and Phillips, 2002). Furthermore for identity preservation the features that characterize that particular identity must be determined and described. The objective of identity preservation is to increase revenue by capturing the increased value associated with specific product traits. The consumer must be able to identify the value of the product: if there is no way of identifying the particular product traits the consumer will not pay extra for them (Smyth and Phillips, 2002; Goldsmith and Bender, 2004). An identity preserved production and marketing (IPPM) system is a “closed loop” channel that facilitates the production and delivery of a certain assured quality through the characterization of the overall production, processing and marketing procedures and the product itself. This characterization covers all aspects of the process from the initial variety to the label on the product that the end consumers purchases. As many of the product attributes are not visible or readily detectable in the product itself, systems are required to provide information to consumers about the provenance of a product.

CONCEPT OUTLINE OF INFORMATION SYSTEMS TO INNOVATE

To support supply chain management and product differentiation we envisage a modern information system that compiles valuable information and knowledge from multiple sources, has the power to interpret that information in a relevant manner, and thereby finally provide rural producers and their supply chain partners with answers pertinent to their questions on how to increase their incomes.

Value of information

The value of better information and an improved information system for SCM is that it provides a solid base for decision-making, and thereby enables the discovery of income generating opportunities or avoids costly errors. In statistical analysis type I and type II errors are well known. A type I error occurs when an alternative hypothesis is accepted due to a chance observation, whilst a type II error is when an alternative hypothesis is rejected even though it is the true state of nature. We have adapted these type I and II errors to producers’ situations. A type I error occurs when a farmer fails to act in a way which is of potential benefit, for example, by failing to change when she or he should have. Poor farmers have the reputation for making type I errors due to risk aversion (Antle, 1987; Kingwell, 1994).

Relevance of information

It is evident that effective development in rural communities is closely related to active participation of the members of the community and their ability to make informed decisions

(For example World Bank 2006). The growers and producers and their supply chain partners are essentially interested in what will function under their particular conditions, they are not interested in prescriptive generalized information that may or may not be pertinent for their particular circumstances. Modern information management systems, particularly Geographic Information Systems (GIS) provide the opportunity to target site-specific results from one specific site to other similar sites that may be geographically distant from the original area of development. Furthermore, if feedback from farmers can be linked into GIS systems, the immense local knowledge on what works and equally important what fails under particular conditions can be used as a guide to rural communities for technology for their specific conditions.

Provision of information

There are several reasons why specific interventions are needed to provide rural communities with improved and more effective knowledge systems. First, there is a plethora of available knowledge, and much of it is not relevant to the particular circumstances of the poor. Second, relevant knowledge may not be available to the poor: they may simply not have access to the knowledge, the knowledge may not have entered into easily reached information systems or the way it is presented and shared may make it unintelligible, suspect and not credible. Third, asymmetry in access to knowledge may disempower the growers and producers. Fourth, the social, economic and environmental conditions of rural communities are extremely diverse and hence the specific knowledge relevant to each community is distinct. Hence generalized prescriptive knowledge systems are not effective. Fifth, effective systems must allow people to query the knowledge systems and at the same time people must know how to formulate questions and seek answers and become confident in doing this. Finally, much potentially useful information is in the minds of an enormous number of people ranging, *inter alia*, from "campesinos" to local and international experts, technologists, social scientists and researchers.

THE CASE OF CINFO

Technical Specifications

CINFO is a prototype internet based information management system for high value agricultural products. It consists of a central database and an internet interface that provide general data management functions, user tailored information presentation, data input and export functions and simple analysis tools. It is based on a security framework that ensures data privacy through a multi-level security system. CINFO offers a highly flexible structure for managing the dynamic and sensitive content tailored to the users' demand. Access is only provided for to users for the specific information that he/she is allowed to see.

Content and functionality

The CINFO platform provides general information related to the coffee tracking project and specific information about coffee production in pilot regions. A series of set queries have been established that enable simple statistical analyses and an information presentation interface has been set up and adapted to specific user-groups. The specific information section provides each user-group with detailed information on management practices, coffee production and quality (including flavour) for specific farms. In addition historic data on the quality of coffee from specific farms is available. Farm and production data is stored in a relational data model (see graph), allowing consistent and storage and dynamic linkage of data. CINFO manages basic farm data including the description of the post harvest process,

management units (e.g. GPS coordinates), field data (e.g. varieties and shade system), harvest (coffee) lots (harvest date, lot quantity, certification), cupping quality data and physical quality data. Different information management modules enable and support product differentiation. The modules are grouped under three domains: inherent, symbolic and personal product information, with each module closely interlinked with the others. Inherent product quality data such as sensorial attributes (cupping data) and physical coffee quality (grain size) is captured for each lot of coffee produced on each participating farm. Separately managed fields or production units are processed apart. The cupping data allows different quality characteristics of lots to be identified and targeted to specific markets segments. Production practices and harvest date are tracked separately for each coffee-lot, as well as symbolic product attributes for preserving product identity through product certification and labeling (organic, fair trade etc.). Photographic images of the farm and the production area and environment as well as farm maps are available and can be linked to each particular batch of coffee so as to add personal symbolic value to the product. The coffee buyer (customers, roasters) can search the database, online, for his specific taste and flavour preferences, preferred growing region, environmental conditions and symbolic traits. The potential buyer also may review farm history data that includes quality characteristics and production volumes over time. The product quality and production data is also available to enable qualified feedback to producers so that they can adapt the production practices met market needs in terms of both quality and quantity. Thus CINFO facilitates two way information flow and network communication, and provides the actors in the supply chain with the means of obtaining his specific preferred product whilst at the same time providing the farmers with information that allows them to develop products that are demanded by specific high value segments of the market. The following sections illustrate in more detail some of the specific functions of CINFO.

Coffee quality module

The coffee quality module manages sensorial cupping data (e.g. flavor, acidity, sweetness, body) supporting several data input and storage formats (SCAA, CoE, and several CinfO proprietary formats) for this data. Key attributes of coffee appraised by all the common cupping schemes can be compared thus allowing simple searches and statistical analysis to be carried out. In addition physical quality data for each harvest lot (e.g. humidity, physical defects and screen size) are maintained in the data base. The quality module also displays key quality attributes like aroma, flavour, balance, defects, etc. of one or multiple samples for a selected farm. The graphic display facilitates rapid quality profile appraisal and evaluation of homogeneity between samples. A similar function presents important physical coffee data for coffee buyers and roasters (e.g. humidity and “factor de rendimiento” as well as physical coffee bean defects). Each physical and sensorial cupping dataset is linked to farm and production data, including certification, with GPS coordinates for spatial analysis and map presentation.

Image and farm map modules

The image module manages images of farmer, farm facilities, coffee fields, landscape, etc. The farm map module stores maps, aerial view images, farm sketches, etc. to show, how the farm is distributed and organized in terms of production units. The images enable the coffee consumer to get familiar with the environment where the preferred coffee is produced. These modules are accessed through specific, both a central and separate interfaces. The farm map interface enables the authorized CINFO user, to search for farm subsets, meeting certain product quality characteristics or geographical attributes. The resulting set (one or more farms) is displayed on an interactive map with several functions including zooming, panning

or loading different spatial information layers such as altitude models, county and cities borders and rivers. By clicking on a certain farm, basic farm information (farm name, municipality, GIS data), farm images, farm maps, cupping quality data, appear.

Quality control mechanisms for tracking and segregating

CINOF enables product identity preservation through a variety of technologies including an integrated product tracking system and product segregation functions. The coffee tracking system collects and compiles product data along the product supply chain from production units until the end-point product with unique tracking tags. Product data can be tracked over space and time. The coffee-lot module accepts the input of several coffee-lots per farm, thus a single farm can produce several batches with different production units, treatments and harvest methods. All data is linked to GIS data (geo-reference coordinates) that enable presentation of the data on digital (web) maps.

The coffee product track runs from the field where coffee is grown to consumers all over the world while the physical state of coffee changes from the green, fruit pulp-covered coffee bean to a wide variety of roasted, blended, ground and packed coffee of different flavours for different uses. The main product tracking nodes through which coffee runs are: production in the field, (on-farm) post harvest processing (fruit pulp peeling, fermentation, and washing, drying), further processing in coffee cooperatives (sorting, quality determination, peeling and others), change of ownership and transportation through exporters to importers to roasters (roasting, blending, grinding), and finally the marketing process with the end-point sale to a customer (general public or food industry). To link the final product to geographical origin and processing information a set of product tracking codes are applied. These codes are directly attached to the product and participants can obtain information about the product from the central data storage and monitoring system (CINFO database).

CINFO distinguishes three different code systems, the management unit code, the coffee lot code (also named cupping code) and the product code. The management unit code is used for linking all production and processing data as well as geographic information about the production zones to a specific environment or site in time, defined as the management unit. The coffee lot code links the management unit code with the harvest date and lot quantity enabling the tracking of quality data and other harvest dependent parameters. The product code is mainly for roaster and end customer use to identify the origin of the packed and ready-to-sell product. Due to the fact that many coffee roasters use a mixture of coffee from different regions and producers, this code is needed to link several coffee-lots with the help of a new code that appears on the coffee pack. The management unit code has to be a simple code, easy to read and process. Furthermore the code is unique system-wide and comes with a simple error detection mechanism. On registering a new field in the CINOF system, a new system-wide unique number is generated. Afterwards a checksum bit is added to this number and the whole expression is converted to a hexadecimal number. The hexadecimal format is shorter than purely numeric formats and the alphanumeric presentation is easier to read. The coffee-lot code (cupping code) follows a different scheme. It is designed to facilitate objective coffee quality determination through “blind cupping” and determination of physical characteristics. It exists of one number (between 1-9) followed by a character (A-Z). This number-character-combination is repeated depending on CINFO’s demand for codes. The prefix “C-“ is used to identify this code as coffee-lot code. There are two possible ways of creating the product code. The roaster can either select the corresponding coffee-lot codes for all coffees used for some blend and receives a product code from the CINFO system, or the roaster chooses a bar or other code unique for that particular batch and links it with the coffee-lot codes from the CINFO database. The code is presented in hexadecimal numbers. Electronic scan devices and RFID technology are not currently available for the CINFO pilot

project phase, however current hexadecimal codes are totally compatible with these technologies and have the advantage of incorporating an error detection system which is essential for use with scan devices.

Geographical analyses and data export

If specific product characteristics (e.g. coffee quality) in real time are linked to existing spatial models (altitude model, climate, political division) the combined data can be used to discover production niches for particular types of coffee. Since computing power and software technology for real time applications like the generation of dynamic web content is still limited, only basic models can be run directly on CINFO. For more sophisticated analyses or data interchange with other systems, data stored in the central CINFO database can be made available as download-file in different formats: csv-file (comma separated value, readable with any spreadsheet program), shape-file for GIS software, Google-Earth® format and a special file format for Expector – a software program for statistical analyses. For example, an intelligent feedback (two way dialogue) system provides the farmer with a quick overview of his productivity status in terms of yield and quality compared with others with similar conditions, thus increasing his knowledge about his potential opportunities for profitable differentiation. Complex automated data analysis processes produce easily understandable and powerful reports in different presentation.

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Importance of Tree Revenues and Incentives from the Programme "Coffee-Practices" of Starbucks for Coffee Farmers in Costa Rica and Guatemala

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SUMMARY

From 2003 to 2005, studies were undertaken in low altitude regions of Costa Rica and Guatemala on the economic importance for farmers' revenues of the sales of timber and fuel wood derived from trees in coffee agroforestry systems. In Costa Rica, they showed that sales of timber can account for 15 to 34% of the value of coffee revenues accumulated over a rotation period of 25 years while timber and fuel wood represented up to 52% and 25%, respectively, of the annual coffee revenues of medium size farms of low altitude in Guatemala. The commodity chains were found to be poorly organized with an absence of agreements and little cooperation among stakeholders so that farmers would benefit more by eliminating intermediaries and selling directly to wholesalers. In 2005, a study was undertaken in 51 farms from 3 cooperatives of high altitude zones (> 1200 m) of Costa Rica in order to analyse the financial profitability for farmers willing to participate in a pilot project of the "CAFE-Practices Programme" (CPP) of Starbucks. The financial analysis indicated that farmer's income was positive with a relation Benefit/Costs superior to 1, irrespective of the adoption of the CPP, for all farm types (from small farms of ~3 ha^{-1} relying exclusively on household labour to large farms of ~24 ha^{-1} with permanent labour). As the environmental requirements of the CPP were not demanding and evaluation criteria not precisely defined, their implementation did not represent a large extra cost for farmers. Therefore, results indicated that adoption of these CPP requirements improved only slightly the financial return of all farm types as coffee prices were already high in these high altitude regions renowned for their coffee quality. Consequently, this Starbucks initiative guaranteed good incomes to farmers in the medium term, encouraged them to take higher social responsibility towards their workers, but did not motivate them to fundamentally change their agricultural practices towards a more environmentally friendly coffee production.

INTRODUCTION

The coffee sector has a strong impact on the economy of Central America. Coffee has been the main agricultural crop and source of export earnings over the last 100 years. Currently, coffee production sustains approximately 300,000 farmers in this region. Consequently, the instability of coffee prices, and especially low prices, has led to a strong social crisis in Central America during the last 5 years with rural unemployment and poverty increasing alarmingly (Varangis et al., 2003).

Coffee plantations have also large-scale environmental impacts as they cover nearly 1,000,000 hectares of the Central American isthmus which is one of the world hotspots for biodiversity. These coffee plantations are often situated in fragile mountainous ecosystems,

many in watersheds that supply water to urban centres. Around 75% of coffee is cultivated under shade in this region (Beer et al., 1998). Therefore, these coffee agroforestry systems (AFS) could play a major role in providing connectivity within degraded and fragmented landscapes, help facilitate movement and maintain viability of wildlife populations such as migratory birds within the Mesoamerican Biological Corridor. Nonetheless, the last cycle of low coffee prices (1999-2004) has resulted in the elimination of up to 20% of coffee AFS in Central America and the exploitation of new forest land or tree fallows to grow food crops on slopes prone to soil erosion.

To reduce farmers' vulnerability to coffee price volatility while preserving natural resources, new marketing opportunities (premiums for quality coffee, organic and eco-friendly labels) have been identified as one of the strategies to improve the economic sustainability of coffee farms and the Central American coffee sector. Indeed, an increased environmental awareness has contributed to the emergence of new markets in developed countries for environmentally-friendly coffee during the last 10 years (Ponte, 2004). Although this still represents only 4% of coffee exports by Central America, the most advanced region with respect to these niche markets, major roasters such as Kraft, Tshibo, Nespresso and Starbucks are developing product lines based on environmentally produced coffee (Salazar, 2005). Diversification of revenues through the production and improved marketing of timber and fuel wood is another strategy that should contribute to improve the livelihoods of coffee farmers. The raw material sources for major furniture distributors in Europe are more and more limited to timber harvested in sustainable manners such as those recommended by "Smartwood" and Forest Stewardship Council.

The objectives of these studies were: (1) to assess the relative economic importance of tree revenues in coffee AFS and to identify ways to increase financial return to farmers; and (2) to analyse the financial profitability for farmers willing to participate in the "CAFE-Practices Programme" of Starbucks, one of the main coffee roasters engaged in pilot projects aimed at rewarding social and environmental responsibilities of coffee farmers in Central America.

MATERIALS AND METHODS

Economic importance of tree products to coffee farmers' revenues

From 2003 to 2005, three studies were undertaken in Costa Rica and Guatemala on the economic importance for farmers' revenues of the sales of timber and fuel wood derived from trees in coffee AFS. In the low altitude regions (< 800 m) of Costa Rica, one tree species generally predominates in coffee plantations and fuel wood is rarely used as households in urban and rural centres are usually equipped with electrical or gas stove. Therefore, case studies concentrated on coffee systems shaded with timber tree species planted at plantation establishment (*Eucalyptus deglupta, Terminalia amazonia, Cedrela odorata*) or from natural regeneration (*Cordia alliodora*). Volume of commercial timber was estimated for a rotation of 4 to 25 years depending on the timber species and use. The importance of timber revenues with respect to that of coffee was estimated with average coffee plantation duration of 25 years and coffee productivity of 23 bags (1050 kg) of green beans produced per hectare and per year. In 2004, this corresponded to a gross coffee revenues of 782\$ ha^{-1} yr^{-1} and a net revenues of 382\$ ha^{-1} yr^{-1} at the price of 34 \$ per bag (46 kg) and with production costs of 400\$ ha^{-1} yr^{-1}. The price of timber (standing tree or log) and costs of tree planting, thinning, transportation, sawing and drying were evaluated locally through interviews with the different stakeholders of the wood sector.

The study in Guatemala was undertaken in 2005 in the watershed of Ocosito where coffee is produced in the altitudinal range of 500 m to 1400 m. The potential revenues derived from timber and fuel wood in these coffee AF systems were quantified by assessing the volume and market price of standing timber trees with a diameter superior to 40 cm in 36 coffee farms. The actual economic contribution of timber, fuel wood and coffee was determined for the six farm typologies encountered in this watershed via quantification of the volume sold during the last 3 years and costs of production.

In both countries, main stakeholders of the local commodity chains of timber and fuel wood were identified through interviews and informal discussions, their interactions determined and the financial importance of tree products for these various stakeholders was assessed (from coffee farmers to local end users).

Analysis of costs/benefit of a pilot project promoting high quality, sustainable coffee in high altitude regions of Costa Rica

In 2005, 51 coffee farms from 3 cooperatives were surveyed in 3 zones of Costa Rica (Dota, San Marcos and San Luis) with altitude superior to 1200 m where the pilot programme "CAFE-Practices" (CPP) of Starbucks was started in 2004; this pilot programme aimed at improving the social, environmental and economic sustainability of producers and other stakeholders of the coffee commodity chain.

In order to analyse the financial profitability of this CPP, coffee farms were grouped according to their technical and biophysical characteristics via a multivariate analysis. Costs of production were evaluated through interviews of farmers and cooperative managers. Coffee price was assumed constant over a 3 year period for which Starbucks has contracts with cooperatives. This price was 120 $ per bag of green beans. After deducting the costs (processing and exportation) assumed by cooperatives and considering that only 70% of the coffee produced corresponded to the top quality commercialized within the CPP, coffee producers received a price of 88 $ per bag. In the absence of CPP, the price paid by cooperatives to producers was calculated as the average one of the last 10 years (for Dota: 90 $/bag, San Marcos: 80 $/bag and San Luis: 68 $/bag). For each farm type, coffee productivity was estimated from the last 3 production years.

RESULTS AND DISCUSSION

Economic importance of tree products to coffee farmers' revenues

In the sub-optimal regions (altitude < 800 m) of Costa Rica, the studies undertaken in 2003 and 2004, showed that sales of timber (*Eucalyptus deglupta, Cordia alliodora, Terminalia amazonia* and *Cedrela odorata*) can account for 15 to 34% of the value of coffee revenues accumulated over a period of 25 years (Table 1). Most coffee farmers sell standing trees to intermediaries in charge of harvest and commercialization to wholesale traders or end users (local furniture and construction sectors). It appears clearly that farmers could benefit more by hiring contractors to fell their trees and transport logs, but sell themselves timber directly to wholesale traders. By eliminating intermediaries, farmers' timber revenues could increase by up to 31% in the case of *Cedrela odorata*, 42% for *Cordia alliodora* and up to 51% for *Terminalia amazonia*.

Table 1. Tree density, timber production, duration of rotation, timber prices, actualised revenues and economic importance of timber in coffee agroforestry systems planted with *Cedrela odorata*, *Cordia alliodora*, *Terminalia amazonia* and *Eucalyptus deglupta* in low altitude regions (< 800 m) of Costa Rica.

Tree species	Tree Density (trees ha^{-1})	Timber Production (m^3 ha^{-1} yr^{-1})	Rotation (yr)	Timber Price ($ m^3)	Actualised Revenues[(1)] ($ ha^{-1} yr^{-1})	Timber Revenues (%)
Cedrela (St)[(2)]	75	3	25	117	350	23.4
Cedrela (Dsl)[(3)]	75	3	25	169	507	33.7
Cordia (St)	200	5	25	36	290	19.3
Cordia (Dsl)	200	5	25	85	500	33.3
Terminalia (St)	125	5	25	47	233	15.5
Terminalia (Dsl)	125	5	25	95	475	31.6
Eucalyptus (poles)	120	4	4	39	425	28.3
Eucalyptus (board)	120	8	8	25	345	23.0

[(1)]Based on inflation rate of 10% per year as registered in Costa Rica for the last 10 years; [(2)]St: sale of standing trees to intermediate; [(3)]Dsl: direct sale of logs by farmer to wholesaler.

Despite a lower market value of *Eucalyptus deglupta* (Table 1), this study showed that sale of poles or board accounted to 23 and 28% of accumulated coffee revenues, respectively. This is due to the fact that *Eucalyptus deglupta* grows faster than the other 3 tree species and hence 3 to 6 rotations can take place over 25 years.

These timber revenues, currently representing up to 33% of coffee revenues, are likely to increase in the future as timber price has strongly increased over the last 20 years due to an increasing demand in the region. Indeed, price for *Cedrela odorata* increased 6.3 fold and for *Cordia alliodora* 5.7 fold between 1985 and 2004 in Costa Rica.

The Guatemalan study showed contrasting results depending of farm type and location along the altitudinal range (500 m to 1400 m) in the Ocosito region. For the 36 farms surveyed, mean density was 235 trees ha^{-1} with a total 34 tree species encountered, 44% of timber species, 26% of fuel wood species (mainly *Inga* spp.), 15% of fruit trees (9% of citrus) and 6% of other perennial crops (cacao, macadamia and rubber).

Traditional, small-size coffee farms (Trad-small) of low altitude (< 800 m) with shade predominantly composed of non-timber species were the least profitable with low coffee productivity and 10% of their revenues derived from tree harvesting (Table 2). Farms located at higher altitude (Int-Alt) generated the highest income, mainly from coffee, due to slightly higher yield and especially higher price paid for quality. The sustainable exploitation of timber, originating from natural regeneration, and fuel wood generated up to 76% of the annual revenues of medium size farms of low altitude (Trad-med), planted with a high density of mixed shade tree species (leguminous and timber species). The results also showed that the present rate of timber extraction in these farms was generally sustainable as less was extracted

(1.1 m^3 ha^{-1} yr^{-1}) than the annual timber tree growth rate (up to 3 m^3 ha^{-1} yr^{-1}). However, the recent tendency is to increase this exploitation to compensate losses in coffee revenues of the last 5 years due to low prices and to respond to an increasing demand for these resources by the local commodity chains. Only in semi-intensive large farms, fuel wood was exploited at a higher rate (14 m^3 ha^{-1} yr^{-1}) than that of growth (10 m^3 ha^{-1} yr^{-1}). It was shown that for small organic farms, tree exploitation generated only 5% of the farm revenues including the valuation of fuel wood used for coffee drying and household consumption.

Table 2. Main characteristics (farm management and sizes, altitude, productivity) and importance of coffee, timber and fuel wood revenues for coffee systems in the Ocosito watershed of the Northern-Pacific region of Guatemala.

Farm Type	Farm Size (ha)	**Altitude** (m)	Tree Density (trees ha^{-1})	Production (kg ha^{-1})	Coffee Revenues (%)	Fuel wood Revenues (%)	Timber Revenues (%)
Trad-small	26	777	259	526	90	3	6
Trad-med	41	757	237	644	23	25	52
Trad-large	253	691	219	493	74	7	13
Int-large	123	790	204	874	99.6	0.4	0
Int-Alt	195	1179	205	736	87	8	3
Organic	2.2	1400	273	440	69	5	0

In Costa Rica and Guatemala, surveys indicated that the timber and fuel wood commodity chains were found to be poorly organized with an absence of agreements and little cooperation among stakeholders and hence with great potential for improvement. In both countries, it was observed that there were potentially important local markets, especially for high value timber, high market acceptance for new timber species, and high demand in Guatemala for fuel wood produced by *Inga* species.

Analysis of cost/benefits of the CPP pilot project of Starbucks in high altitude regions of Costa Rica

Four types of farms were defined according to their technical and biophysical characteristics via a multivariate analysis: 1) small size farms relying exclusively on familial labor (S-F: 2.7 ha^{-1}); 2) medium size farms with familial and temporary labor (M-FT: 5.6 ha^{-1}); 3) large size farms with temporary labor and, to a lesser extent, permanent and household labor (ML-TPF: 10 ha^{-1}); and 4) large size farms depending exclusively on temporary and permanent labor (L-TP: 24 ha^{-1}). Based on the last 3 production cycles, the mean farm productivity was evaluated at 1200 kg ha^{-1} for S-F, 1600 kg ha^{-1} for M-TF, 1150 kg ha^{-1} for ML-TPF and 1500 kg ha^{-1} for L-TP.

To be eligible to the CPP, farms had to go through a certification process following social and environmental criteria. The results indicated that small and medium farms received a grade of 62% and 64%, respectively, just above the minimum to be considered as “Preferred suppliers” (60%) within the CPP, but not high enough to be classified as “Strategic Suppliers” (80%) and hence received an extra premium of 5$ per bag. Medium-large size farms received an average grade of 71% while the situation was critical for large farms as they scored 41% well below the threshold of 60%.

For small and medium farms, social responsibility was adequate due to the fact that they rely mostly on familial and temporary workforce, respect local labor laws (minimum wage, no child labor) and many criteria did not apply in the absence of permanent workforce (vacation

pay, social benefits). Still, many requirements were not satisfied such as adequate protection while using agrochemicals and absence of first-aid-kit for medical emergency. For large farms (L-TP) relying on permanent and seasonal workforce, minimum requirements according to local labor laws were generally adequate, but many additional ones required by the CPP were not satisfactory such as periodic wage increases, incentives for education, labor contracts and improved social benefits for permanent workers, decent on-farm living conditions for seasonal workers, and training for use of agrochemicals for all workers.

Respect to environmental responsibility, the minimum of 2 m free of cultivation to protect water resources was the least respected in small, medium and medium-large farms. Other weak points were the poor monitoring of pests and diseases, the basis for an improved control relying on lower chemical applications, and the absence of anti-erosion management. For large farms (L-TP), the environmental evaluation was generally very poor due to the absence of shade trees, high use of herbicides and fertilizers, intensive and systematic preventive use of chemicals for control of pests and diseases, high level of erosion and, to a lesser extent, the absence of protected areas and refuge for wild life. Therefore, strong efforts have to be accomplished by large producers to comply with CPP environmental requirements.

To perform a financial analysis "with and without CPP" with the Benefit/Costs ratio as an indicator, costs of technical and social improvements to be made were assessed through economical surveys; for examples, the cost of implementing anti-erosion measures was estimated at 3 workdays per ha, the cost of a first aid-kit at around 20 $, and the cost of training of a permanent worker at around 250 $ per year. Although all the CPP requirements are important and hence should all be put into practice, it was assumed that the producers will opt for a gradual transition towards this goal by first complying with the ones that have the major incidence on their “grade”, but are also the most easily and financially achievable with respect to their specific farm conditions. This priority ranking of improvements is not a simple task and varies from farm to farm. This was further complicated by the fact that many evaluation criteria were vague such as “significant effort towards minimizing the use of agro-chemicals” or “minimum shade level to insure significant biological conservation”.

In all 3 cooperatives, farmers were volunteering to participate to the CPP and hence social and environmental conditions on farms were generally above average. Furthermore, social and environmental requirements of this CPP were not that highly demanding and consequently their implementation did not represent a large extra cost for coffee farmers (Table 3). It was estimated at a total of 250-430 $ per ha and hence around 100-190 $ per year for the 3 year transition period.

Table 3. Transition and maintenance costs for complying with the requirements of the CPP as “Strategic Partner” according to farm type.

Farm type	Costs of transition for CPP. “Strategic level” (in $ ha^{-1})				Constant costs for maintaining “Strategic level” (in $ ha^{-1})
	Year 1	Year 2	Year 3	Total	Year 4 and on
S-F (2.7 ha)	130	80	75	285	75
M-TF (5.6 ha)	100	70	65	235	65
ML–TP (10 ha)	110	75	75	260	75
L-TP (24 ha)	190	140	100	430	105

For the coffee cycle of 2004/05, financial analyses in the short term indicated that net farmer’s income was positive with a ratio Benefit/Costs superior to 1 for all farm types,

irrespective of adoption of the CPP. The results also indicated that adoption of these CPP requirements improved only slightly the financial profitability of farms as coffee prices were already high in these high altitude regions renowned for their coffee quality, with exception of San Luis where coffee price are historically lower (around 70 $ per bag for San Luis versus 80-90 $/bag for Dota and San Marcos). Consequently, this Starbucks initiative guaranteed good incomes to farmers in the medium term (up to 3 years as duration of the contract), but does not financially motivate them to fundamentally change their agricultural practices towards a more environmentally friendly coffee production. Still, the CPP appears to increase producers' awareness towards a higher social responsibility for their workers, especially living conditions for seasonal workers during coffee harvesting. Therefore, this pilot program could appear to be mostly a strategy for Starbucks to secure medium term access to high quality coffee produced in these high altitudes zones rather than one promoting farmers' adoption of sustainable coffee practices.

Table 4. Ratio Benefit/Costs for various farm types of 3 cooperatives with and without CPP and with and without premium for "Strategic Partner" status (5 $ per bag).

Farm type	Cooperative	Without CPP*	With CPP (60%) "No Premium"	With CPP (80%) "+ Premium "
S-F	San Luís	1,02	1,23	1,27
	San Marcos	1,59	1,65	1,69
	Dota	1,53	1,43	1,46
M-TF	San Luís	1,39	1,72	1,77
	San Marcos	1,85	1,94	2,00
	Dota	2,03	1,90	1,97
ML-TPF	San Luís	1,18	1,46	1,50
	San Marcos	1,26	1,32	1,36
	Dota	1,81	1,67	1,73
L-TP	San Luís	1,37	1,61	1,67
	San Marcos	1,70	1,69	1,78

CONCLUSIONS

These studies indicate that income diversification through the production of timber and fuel wood is an interesting option for producers in low altitude regions where coffee plantations generally are heavily shaded. As the demand for tree products is increasing in Central America, this tendency is likely to persist and improve in the near future. Tree harvest originating from coffee AFS has also positive, environmental impacts as farmers rely less on natural forest remnants for fuel wood household energy. Clearly, farmers and cooperatives entrepreneurial skills need to be enhanced so that farmers can benefit much more financially by harvesting trees by themselves and directly commercialising wood products rather than selling standing trees to intermediates. Through access to technical information and business contacts, farmers should also be able to improve their incomes via sales of eco-certified timber produced on their farms.

In Central America, the "CAFE-Practices" of Starbucks is aimed at high-altitude coffee zones (> 1200 m) as high quality coffee with distinct features (aroma, flavor, acidity) is a prerequisite for this company. This results in higher coffee prices than common international prices, but has also direct effects on farmers' management strategies and impacts on the environment. In the coffee producing zones of Dota, San Marcos and San Luis in Costa Rica, coffee management is generally very intensive with high inputs of fertilization, systematic and preventive use of fungicides for pest control, low diversity and density of shade trees and a

strong tendency of expanding coffee cultivation up to steep slopes; all these agricultural practices with the aim of maximizing productivity and economic return. The praiseworthy efforts of Starbucks would be better served by insuring high enough and longer term incentives that compensate farmers for a reduction in coffee productivity while adopting more environmentally-friendly coffee management practices.

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Detection and Enumeration of Mycofloral Populations Associated with Ethiopian Arabica Coffee Bean Contamination

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SUMMARY

Ethiopia is the country with the longest tradition of coffee production and consumption in the world that witnessed by unique Ethiopian 'coffee ceremony'. The country is also the oldest exporter of world's finest original coffee. Although most coffee is exported as sun dried (natural), the amount of high quality wet processed (washed) Arabica coffee has been tremendously increasing since the 1990s. There have been enormous concerns about mycotoxin especially ochratoxin A (OTA) contamination associated with severe mould development in coffee. Consequently, great deals of research and extension efforts are being underway globally on mould management. In this regard, Ethiopian coffee farmers are strongly advised to adopt good harvesting and drying practices to improve quality and reduce mould contamination. Nevertheless, except few flying publications, baseline information is either lacking or scanty hitherto about mould contamination of Ethiopian Arabica coffee. In an attempt to address and tackle this prominent issue, investigations were undertaken to detect and enumerate mould species associated with Arabica coffee, determine their distribution and relative importance in the country. Three sample components; viz. coffee cherries fallen and dried on the ground, cherries dried on the tree and wet processed parchment coffee, were structured in 3 locations namely, Gera, Jimma and Teppi (each with distinct agroecological zone) in major coffee belts of Ethiopia. The former two sample groups meant to represent bulk of the traditional sun dried coffee production while the latter constituted the wet processing method. Accordingly, about 300 cherries/parchments were randomly collected, dehulled and then plated following two standard procedures (agar plate and blotter methods) after surface disinfection. The experiment was repeated at least twice during the main harvesting seasons for two consecutive years. The isolated species identification was confirmed at BBA, Berlin, Germany. The result showed that seven major fungal species belonging to the genera *Aspergillus* (6) and *Penicillium* (1) were detected in the different coffee samples. The enumerated mycofloral populations turned to vary significantly across the sample components and geographic origins of coffee. *Aspergillus phoenicis* was abundantly isolated from coffee cherries dried on the tree and in wet processed parchment coffee from Gera and Jimma. While *A. parasiticus* was the dominant mould species (81.5%) detected in coffee samples fallen and dried on the ground at Teppi. *A. melleus* found in high proportion (87.5%) in fallen coffee beans, and in moderate percentage (45%) in dried coffee samples collected from the tree at Jimma. *Penicillium stoloniferum* was recorded most frequently in coffee samples collected at Gera followed by Teppi. The present findings revealed that the presence of different mould populations associated with Ethiopian coffee beans not reported elsewhere and interestingly, the most toxigenic species, *A. ochraceus* was not encountered.

INTRODUCTION

Ethiopia is the country with the longest tradition of coffee production and consumption in the world. Coffee drinking is well embedded in the daily life of almost all Ethiopians that is being witnessed by the performance of unique 'coffee ceremony'. This cultural heritage, nevertheless, significantly contributed to a sustainable production of the crop for centuries in Ethiopia. The economic value of C*offea arabica* genetic resources contained in Ethiopian highland forests was estimated to amount USD 1458 million and USD 420 million at a 5 and 10% respective discount rates (Hein and Gazweiler, 2005). Thus the crop is socially, commercially as well as genetically important to the country in particular and to the world in general. Ethiopian Arabica coffee is "empirically organic by default" owing to its ecological and traditional way of exploitation although not yet fully certified (Taye and Tesfaye, 2001; Daviron and Ponte, 2005). According to ICO report, Ethiopia is the second organic coffee exporter in 2005, exceeded by Peru (ICO, 2006). Besides there are famous localities such as Harar, Jimma, Limu, Nekemet, Gimbi, Sidamo and Yirgacheffe specifically known for premium coffee quality in world market.

The county is also the largest fine Arabica coffee producer in Africa and nearly 60-70% of which is sun dried or naturally processed coffee exported to Germany, Italy, France, the Netherlands, USA, and Japan. The proportion of washed coffee supply is booming since privatization in the 1990s. There have been enormous concerns about mycotoxin especially ochratoxin A (OTA) contamination associated with severe mould growth in coffee. Consequently, great deals of research and extension efforts are being underway globally on mould management (Daniel, 2000; Raghuramulu and Naidu, 2002). In this regard, Ethiopian coffee farmers are strongly advised to adopt good harvesting and drying practices to improve quality and reduce mould contamination. Analysis of thousands of coffee samples in different countries revealed that badly processed and handled coffees, irrespective of origin, coffee types and processing methods, possess OTA (Raghuramulu and Naidu, 2002). Daniel (2000), however, argued that OTA differ across geographical regions and processing methods, explaining high OTA risks in East Africa where Arabica is mostly processed by the dry method. Nevertheless, except such flying publications in some importing countries, baseline information is either lacking or scanty hitherto about mould development in Ethiopian Arabica coffee. This report presents the occurrence and distribution of mycofloral populations (mould species) associated with Ethiopian green coffee beans.

MATERIALS AND METHODS

Description of sample collection areas

Three locations; namely, Gera, Jimma and Teppi, distinctly differing in climatic conditions were selected to represent the major coffee producing regions of southwestern Ethiopia. Gera, located at higher altitudes above 1950 m, belonging to the humid tepid to cool highlands agro-ecological classification. The annual total precipitation is about 1880 mm fairly extended throughout the year; with mean minimum and maximum temperatures of 10.6 and 24.4 °C, respectively. Jimma, an area categorized as sub-humid tepid to cool mid-highland receiving about 1600 mm annual rainfall fairly distributed over 165 days. The maximum temperature varied between 23 °C in August to 28 in February and March. Teppi is, situated at 1200 m, enjoying warm humid conditions, receiving 1700 mm of total annual rainfall with average minimum and maximum temperatures of about 15.4 and 30.0 °C, respectively. In south west, where monomodal rainfall pattern is experienced, coffee flowering mostly occurs in either January or February and fruits mature in August and September that become ready for harvest in October through December.

Sample collection

Three sample groups were structured as coffee cherries fallen and dried on the ground (dry cherries on earth) and cherries dried on the tree both locally referred to as 'jenfel' that represented the bulk of traditional sun dried (natural) coffee production. About 300-400 fallen and dried cherries were independently collected from the ground and on the tree in the field (3-5 fields/ location). The same number of parchment coffee was sampled on the drying tables (3-5 tables/ location) from washing station in the abovementioned localities throughout the harvesting season for two consecutive years.

Isolation and identification of mycofloral spp.

Two isolation procedures- the standard agar plate and blotter methods were followed for isolation of the microorganisms. Two batches each consisted of 100-200 green coffee beans (10 beans/plate) were plated on moist filter paper and the remaining seed lots on Dichloran 18% Glycerol Agar (DG18) and potato dextrose agar (PDA) after surface disinfection. Disinfection was achieved by rinsing the seeds in 5% sodium hypochlorite ($NaHCO_3$) in plastic Petri dishes for 1 minutes followed by twice rinsing in distilled sterile water. After drying under running laminar airflow chamber, the beans were aseptically transferred into plates and all the plates were incubated at room temperature of 24 ± 1 °C. After 7-10 days, each plate was checked for microbial growth and the number of seeds with and without moulds was counted and recorded. Infected coffee bean specimens were examined under microscope, and then slides were prepared for detailed examination of morphological structures for identification (Pitt and Hocking, 1997; Thomas and Raper, 1965). Finally the number of infected coffee beans by each mycofloral species were counted and expressed as infection percentage of the total number of seeds per plate. The isolated species were confirmed by expert identification at BBA, Berlin, Germany.

RESULTS AND DISCUSSIONS

Six fungal species belonging to the genera *Aspergillus* and a *Penicillium* were identified in association with Arabica coffee bean contaminations in Ethiopia (Table 1 and Figure 1). The distribution and proportions of these mycofloral populations significantly varied across sample groups and coffee growing areas in the country. *A. phoenicis* was the highest mould species (65-75%) isolated in coffee cherries fallen on the ground and dried leftover on the tree at Gera and Jimma. *A. melleus* was the second highest mould population (50%) identified in the dried coffee samples collected on the ground and picked on the tree as compared to the washed parchment coffee in both methods of isolation (Tables 1 and 2). This species was prevalent in the intermediate coffee growing areas of Jimma as compared to high altitude areas of Gera and the low laying areas of Teppi. *A. parasiticus* was frequently infecting cherries fallen to the ground at the lower altitudes of Teppi (Table 3) but almost absent in all coffee samples either at Gera or Jimma (Figure 2). *A. auricomus* and *A. fuligmosus* were encountered in a few coffee samples. *Penicillium stoloniferum* was common in the samples originated from the fields with wet and humid high altitude regions (Tables 1 and 2). Amongst the samples, fallen cherries to the ground and cherries left on the tree showed more mould contamination than wet processed parchment coffee (Table 4).

A. ochraceous A. niger, A. carbonarius and *P. verrucosum* were the most abundantly isolated species from Arabica and Robusta coffees in different coffee growing countries (Naidu et al., 2005; Pittet et al., 2001; Ngubirano et al., 2001; Frank, 2001). Although a number of *Aspergillus* spp. are found to be associated with Arabica coffee beans in Ethiopia, those species known to form OTA such as *A. ochraceous* was not encountered in this study.

Instead, *A. melleus* and *A. auricomus* (few samples) which belong to the *A. ochraceus* group were recorded in coffee samples collected from Jimma-mid altitude areas and Teppi-low laying regions. Verga et al. (1996) reported that these two *Aspergillus* spp. produce ochratoxin A (OTA) in other crops. *A. parasiticus* was one of the mould species associated with "rio-taste" coffee beans (Vanos, 1987). Naidu et al. (2005) discussed that gleanings and tree dried fruits would be one of the sources for toxigenic mould in dry processed coffee, mixing of tree dried and gleaning during processing of coffee was found to increase the risk of toxigenic mould in the final product. Vargas et al. (2005) also 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%). The mean infection rates for cherries from the tree were very low, but higher in fruit from the ground, the drying yard and storage indicating infection by toxigenic species after removal from trees (Pittet et al., 2001).

Table 1. Proportions (%) of mycofloral species identified in Arabica coffee beans collected from Gera areas (> 1950 m) by agar plate and blotter methods.

Mould species	BBA Collection No.	Fallen cherries on ground		Cherries on tree		Parchment coffee		Mean
		Agar	Blotter	Agar	Blotter	Agar	Blotter	
Aspergillus auricomus	BBA72076	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. flavofurcatus	BBA72074	0.0	5.3	0.0	0.0	12.9	0.0	3.1
A. fuliginosus	BBA72078	0.0	0.0	0.0	0.0	0.8	0.0	0.2
A. melleus	BBA72077	0.0	10.0	0.6	14.2	12.6	20.0	9.6
A. parasiticus	BBA72075	1.1	11.4	0.0	21.4	10.0	20.0	10.2
A. phoenicis	BBA72073	72.1	97.3	85.6	94.5	55.8	46.0	75.2
Penicillum stoloniferum	BBA72062	58.9	29.4	1.1	22.5	64.2	77.4	42.3

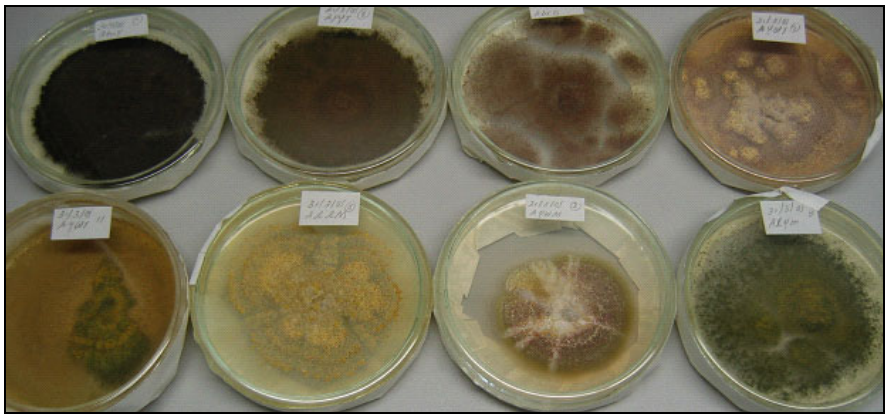

Figure 1. Colony appearances of some mycofloral mould species isolated from Arabica coffee in Ethiopia.

In line with other studies, mould contaminations are reported mostly in coffee beans harvested from the ground and those left on the tree in Ethiopia. It was noted during the study that cherries could have fallen off the tree by unusual torrential rain fall during harvesting seasons of October and November that caused most ripe cherries fall to the soil mud favoring fungal growth. The cloudy and rainy conditions also complicated some of the post-harvest activities such as prolonged the drying period and/or rewetting of dried cherries or parchments. In natural drying, freshly harvested cherries were spread on mat or raised

platforms, turned the cherries around for uniform drying that takes more than a week depending on the prevailing weather conditions. As family labor is the major source for picking and transporting the cherries which was inadequate owing to overlap of activities such as harvesting of cereals and larger size of coffee farms. The dried cherries were mainly stored in jute sacks or big baskets in the house. The storage period is mainly influenced by expectation of better prices of coffee; and the lower the current price the longer the period that might have effected mould development and quality deterioration. Among the insect pests, coffee berry borer damage was encountered in some coffee beans sampled from Teppi and Jimma. Esayas et al. (2004) reported occurrence of coffee berry borer (*Hypothenemus hampei*) under wide range of altitudes from 1200 to 1800 m inflicting as high as 60% infestation of dry leftover cherries on the trees and fallen to the ground.

Table 2. Proportions (%) of mycofloral species identified in Arabica coffee beans collected from Jimma areas (1750 m) by agar plate and blotter methods.

Microflora spp.	Fallen cherries on ground		Cherries on tree		Parchment coffee		Mean
	Agar	Blotter	Agar	Blotter	Agar	Blotter	
Aspergillus auricomus	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
A. flavofurcatus	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
A. fuliginosus	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
A. melleus	94.5	80.5	10.0	83.6	10.0	21.7	50.1 ± 0.1
A. parasiticus	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
A. phoenicis	71.0	60.0	99.5	99.0	43.5	21.7	65.8 ± 30.8
P. stoloniferum	13.3	0.0	0.0	0.0	10.0	3.9	4.5 ± 5.8
Fusarium spp.	5.7	1.5	6.4	0.9	10.0	0.8	4.2 ± 3.7

Table 3. Proportions (%) of mycofloral species identified in Arabica coffee beans collected from Teppi areas (< 1200 m) by agar plate and blotter methods.

Microflora spp.	Fallen cherries to the ground		Cherries on the tree		Parchment coffee		Mean
	Agar	Blotter	Agar	Blotter	Agar	Blotter	
Aspergillus auricomus	0.2	0.6	0.5	1.4	0.2	0.7	0.6 ± 0.4
A. flavofurcatus	15.0	0.0	0.0	0.0	0.0	0.0	2.5 ± 6.1
A. fuliginosus	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
A. melleus	14.7	0.0	12.5	15.0	10.0	24.4	12.8 ± 7.9
A. parasiticus	63.5	99.5	0.0	37.4	15.0	15.5	38.5 ± 27.2
A. phoenicis	0.0	0.0	10.0	20.0	12.0	26.2	11.4 ± 5.5
P. stoloniferum	0.0	0.0	18.2	41.6	18.5	64.5	23.8 ± 15.2

On the other hand, it was noted that Ethiopian coffee farmers are well advised by extension staffs to follow appropriate coffee handling practices (harvesting red ripe cherries only, use proper drying and storage materials) and even forced to sell soon after harvest. There were also coffee quality control task forces at district and lower levels that mandated to tour and ensure proper harvesting and drying activities performed by farmers. However such extension approach has its own shortcomings as small-scale coffee farmers are not encouraged by premium price payment to quality produce. Also provisions of inputs such as wire meshes, canvas, clean sacks, and simple moisture meter are limited. Further investigations involving

analysis of large number of coffee samples under different conditions for potential OTA production should be addressed.

Table 4. Proportions (%) of mycofloral species identified in various coffee samples collected from Gera, Jimma, and Teppi areas in southwestern Ethiopia.

Mycofloral species	Fallen cherries			Cherries on the tree			Parchment coffee		
	Gera	Jimma	Teppi	Gera	Jimma	Teppi	Gera	Jimma	Teppi
Aspergillus auricomus	0.0	0.0	0.4	0.0	0.0	0.95	0.0	0.0	0.45
A. flavofurcatus	2.7	0.0	7.5	0.0	0.0	0.0	12.5	0.0	0.0
A. fuliginosus	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
A. melleus	5.0	87.5	7.4	7.4	46.8	13.8	16.3	15.9	17.2
A. parasiticus	6.2	0.0	81.5	10.7	0.0	18.7	15.0	0.0	15.3
A. phoenicis	84.7	65.5	0.0	90.0	99.2	15.0	50.9	32.6	19.5
P. stoloniferum	44.2	6.7	0.0	11.8	0.0	29.9	70.8	6.9	41.5
Others	0.0	0.0	0.0	2.8	0.0	0.0	0.0	5.0	0.0
Mean	17.8	20.0	12.1	15.3	18.3	9.8	20.7	7.6	11.7

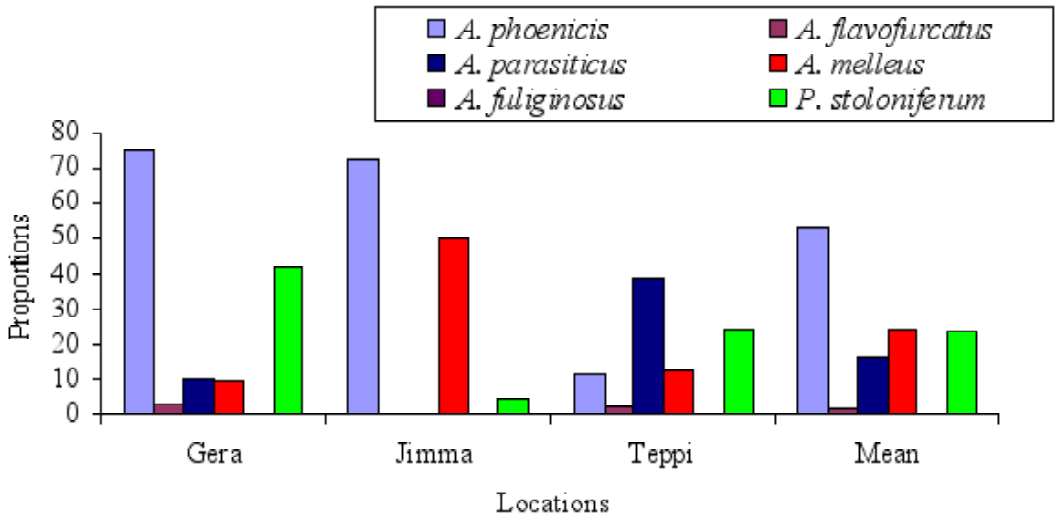

Figure 2. Distribution of mycofloral species associated with Arabica coffee beans in southwestern Ethiopia.

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Ochratoxin A Reduction in Coffee Beans (*Coffea arabica*) Using a Spouted Bed Roaster

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SUMMARY

Conflicting data are found in literature with respect to the influence of roasting, grinding and beverage preparation on the destruction of ochratoxin A (OTA). In the present study, *Coffea arabica* was contaminated with spores of toxigenic *Aspergillus ochraceus*. After an incubation period, a sample was analyzed for OTA. Contaminated coffee was roasted in a vertical spouted bed roaster at four different temperatures (180 °C, 200 °C, 220 °C and 240 °C) and three different time periods (5, 8 and 12 min). After roasting, coffee beans were ground and OTA was analyzed. An average of 247 μg/kg of OTA was produced in coffee contaminated with *A. ochraceus*. Coffee treated with spouted bed roasting significantly reduced the OTA level. At 180 °C for 5, 8 and 12 min, OTA reduction was 8% (226 μg/kg); 42% (142 μg/kg) and 53% (114 μg/kg) respectively. At 200 ºC for 5, 8 and 12 min, OTA reduction was 30% (173 μg/kg); 56% (108 μg/kg) and 66% (80 μg/kg) respectively. At 220 ºC for 5, 8 and 12 min, OTA reduction was 46% (131 μg/kg), 76% (58 μg/kg) and 87% (31 μg/kg). Finally, at 240 °C for 5, 8 and 12 min, OTA reduction was 76% (57 μg/kg), 94% (14 μg/kg) and 98% (2 μg/kg) respectively. The results of this experiment showed that hot air spouted bed roasting can be an efficient tool for eliminating OTA in coffee.

INTRODUCTION

Roasting is a heating process in which the raw coffee is submitted to a temperature of 180-250 °C for a period of 5 to 15 min. During roasting, chemical changes occur with aroma and dark color compound formation, and physical changes with water loss and production of carbonic gas and other volatiles (Clarke, 1987).

Conflicting data are found in the literature with respect to the influence of roasting, grinding and beverage preparation on the residual levels of ochratoxin A (OTA). Initial data suggested that almost the complete destruction of OTA takes place during the roasting process. However, occurrence of OTA in market samples of roasted coffee, as well as in coffee beverage, was reported after an improved detection method. Blanc et al. (1998) carried out experiments to investigate the behaviour of OTA during roasting and the production of soluble coffee. In this study, a small part of the OTA was eliminated during the initial cleaning process of the coffee, due to the discarding of defective and black beans, but the most significant reduction occurred during the roasting process. The ground roasted coffee contained only 16% of the original OTA present in the raw coffee. The cleaning of the coffee and thermal degradation were the most important factors in the elimination of OTA from the coffee.

Heilmann et al. (1999), studied the behaviour and OTA reduction in raw coffee beans roasted industrially, showing that the levels of OTA were significantly reduced, especially in coffee decaffeinated by solvent extraction.

The data in the literature show evidence that the roasting process is efficient in reducing OTA, but there is a lack of more conclusive research on the effects of the stages of roasting, grinding and beverage preparation on the stability of the toxin.

MATERIAL AND METHODS

Coffee Sample

A green *Coffea arabica* sample, previously dried and de-husked, was obtained directly from a farm (São Paulo State, Brazil).

Selection and preparation of the Aspergillus ochraceus spore suspensions

One isolate of *A. ochraceus* from coffee grown in Brazil was used to prepare the inoculum. The fungus was inoculated into malt extract agar (MEA) and incubated at 25 °C for 5 days. The growing culture was transferred into a test tube containing 30 mL of 0.1% peptone water plus 0.1% Tween 80, and the suspension agitated in a vortex for 1 minute. A spore concentration of 10^7 CFU/ml was obtained by dilution technique.

Inoculation of Aspergillus ochraceus spores into coffee

Sixteen PET plastic flasks of 2000 mL capacity were decontaminated with 70% alcohol and dried under UV light for 15 min. Each flask received 1 kg of raw coffee. Fifteen of these flasks with coffee were contaminated with 120 ml of the inoculum. The flasks were shaken manually. One flask was not inoculated for control purposes. The flask tops were loose and the flasks were incubated at 25 °C for 30 days. Each day the flasks were shaken manually and opened for aeration inside a cabinet flow. After 30 days, all the coffee contaminated with *A. ochraceus* from the flasks was placed in a big plastic bag and homogenized manually. Five samples were taken from this bag and analyzed for OTA.

Roasting of coffee

A vertical spouted bed roaster was heated by dry heat until the roasting temperature was achieved. An aliquot of 300 g of contaminated coffee was placed in this roaster at four different temperatures: 180, 200, 220 and 240 °C and three different time periods: 5, 8 and 12 min. Each treatment was carried out twice, and temperature variation reading was made by three type T thermocouples installed in the equipment, as shown in Figure 1. After the roasting period, the coffee was immediately removed from the spouted bed and placed in a dry air cooler until it reached room temperature. Roasted coffee was ground in a Probat Emmerich Stawert mill (Probat Emmerich Stawert Mühlenbau Typ K32/20 700 W 230 V 1 - 4,5A 60 Hz 3400 min-1 40 minKB Made in Germany) and sieved.

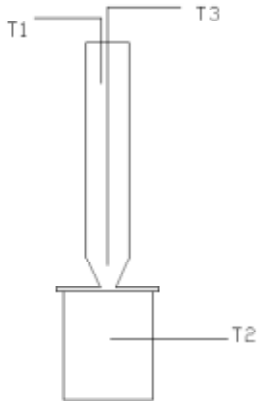

Figure 1. Schematics of the spouted bed roaster and Thermocouple positioning.

Ochratoxin A (OTA) analysis

Ochratoxin A from green and roasted coffee was analyzed according to Vargas et al. (2005). An aliquot of 25g of each sample was extracted with a 200 ml 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 methanol:acetronitrile:water:acetic acid (35:35:29:10). The flow rate was 0.8 mL/min. The equipment used was a Shimadzu LC-10VP system (Shimadzu Corporation, Japan) set at 332 nm excitation and 476 nm emission. The HPLC was equipped 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, Bellefonte, PA). Each analysis was carried out twice for each treatment and each repetition. The OTA detection method in raw coffee beans was validated at the Food Technology Institute. Samples with 3 repetitions were spiked with OTA standard (Sigma, USA) at three levels (4.8; 8.0 and 80.0 μg/kg). The OTA recovery percentages obtained were 86.5; 78 and 81%. The detection and quantification limits of the method were 0.2 and 0.6 μg/kg respectively.

RESULTS AND DISCUSSION

Ochratoxin A

The average of ochratoxin A produced by *A. ochraceus* in raw coffee after 30 days of incubation was 247.29 μg/kg .

Figure 2 shows the kinetics of ochratoxin A destruction at 4 different temperatures (180, 200, 220 and 240 ºC) and 3 times (5, 8 and 12 min.). At 180 ºC for 5, 8 and 12 min, OTA reduction was 8% (226 μg/kg); 42% (142 μg/kg) and 53% (114 μg/kg) respectively. At 200 ºC for 5, 8 and 12 min, OTA reduction was 30% (173 μg/kg); 56% (108 μg/kg) and 66% (80 μg/kg) respectively. At 220ºC for 5, 8 and 12 min, OTA reduction was 46% (131 μg/kg), 76% (58 μg/kg) and 87% (31 μg/kg). Finally, at 240 ºC for 5, 8 and 12 min, OTA reduction was 76% (57 μg/kg), 94% (14 μg/kg) and 98% (2 μg/kg) respectively. The results of this experiment showed that the roasting process can destroy a great part of OTA.

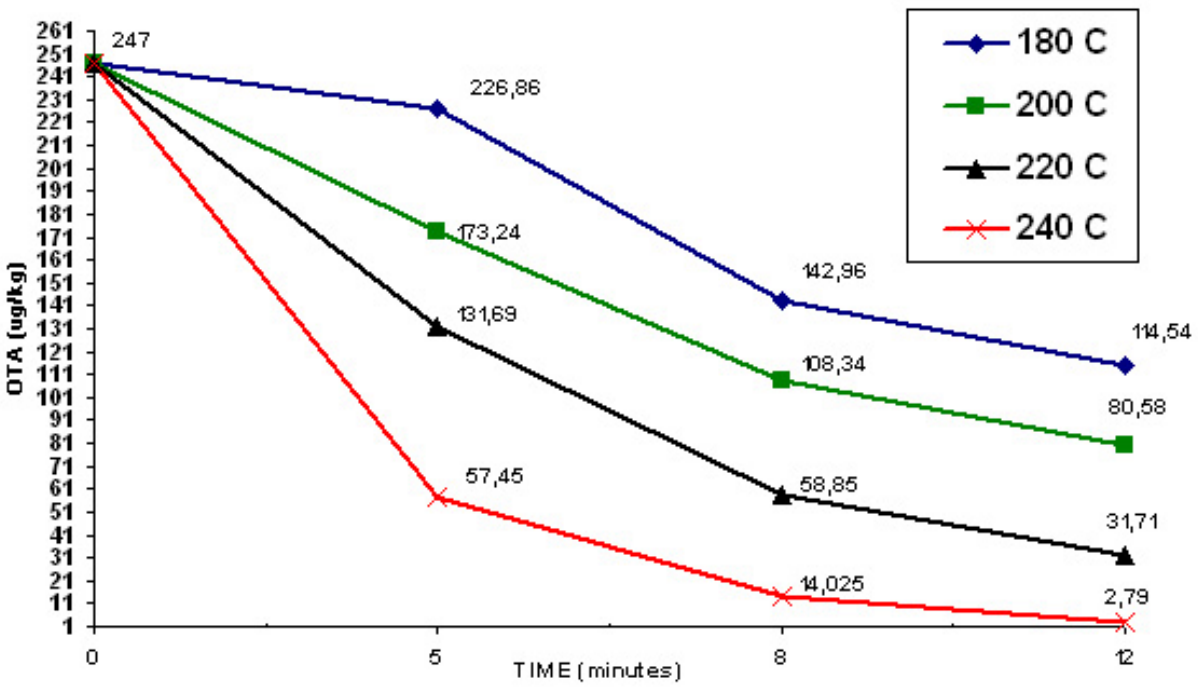

Figure 2. Kinetics of ochratoxin A destruction at four different temperatures.

Figure 3 shows a more representative outlook of the percentage of Ochratoxin A destruction in each heat treatment.

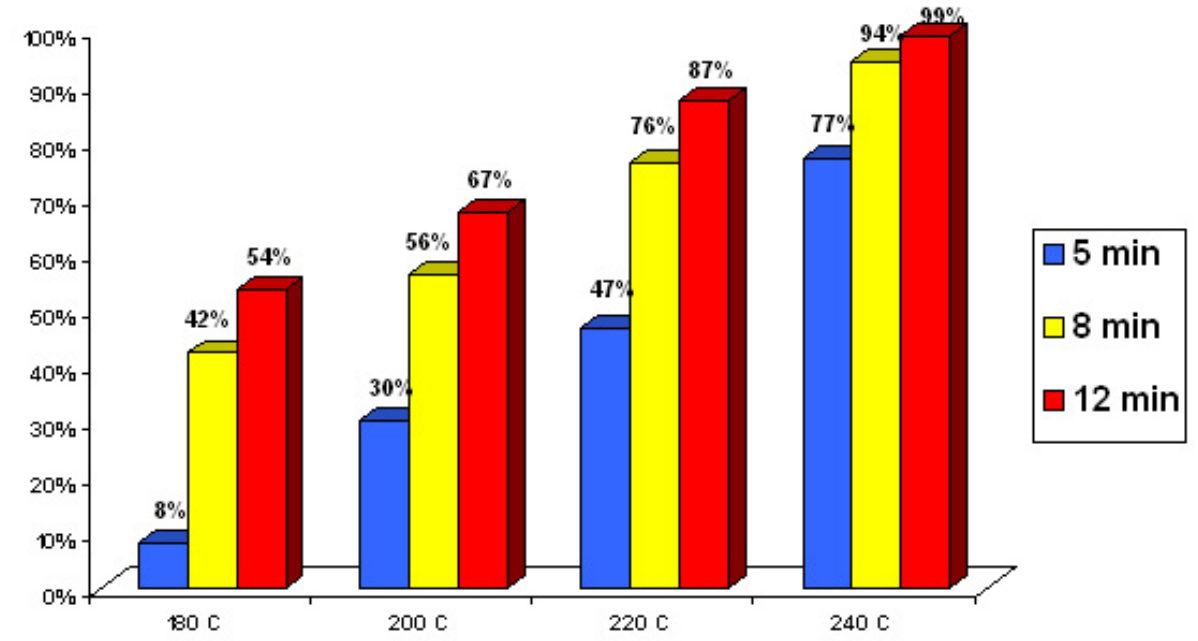

Figure 3. Percentage of Ochratoxin A destruction in four heat treatments.

Differences in OTA destruction using roasting process have been published. Tsubouchi et al. (1987) studied the effect of roasting on the stability of OTA, using an air heater, at roasting temperature of 200 °C. At this temperature, roasting for 20 minutes, Tsubouchi et al. (1987) were able to achieve only 12% of OTA destruction. On the other hand, Blanc et al. (1998) found that roast and ground coffee contains only 16% of the original OTA contamination.

Santos et al. (2005) found that roasting coffee at 210 °C could lead to a level of OTA degradation of about 87%, but variation of results were up to 134%. All these results are probably due to the roasting method used in their experiments.

Urbano et a1. (2001) studied the destruction of OTA in coffee during roasting. Using a contamination technique very similar to our study, they were able to achieve a high contamination level on green coffee (160.5 μg/kg), and roasting at 220 °C for 15 minutes led to a OTA destruction of 94%.
As the destruction of OTA showed a linear behaviour, with this results, we can assume that OTA destruction follows first order kinetics, allowing researchers to calculate D and Z values, and also activation energy levels. This data is already being analysed by our research team and a complete study in the kinetics of OTA destruction should be published soon.

CONCLUSIONS

The results of this research show that spouted bed roasting is a very efficient procedure for ochratoxin A reduction in coffee, depending on the time and temperature combination chosen.

As the spouted bed roaster is a very efficient heat exchanger, we can trust the results obtained because of the stability of the thermic profile shown in the process. Kinetic studies are very important, because they allow engineers and technicians to design a specific process with greater efficiency and avoiding energy and product losses.

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Routine Use of DNA Based Traceability from Field to Factory Gates for Nespresso Coffee Products

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SUMMARY

Coffea arabica represents approximately 62% of worldwide coffee production and this species is considered as the best for cup quality in comparison to *Coffea canephora*. This international commodity is used for specialty coffees for premium products such as Nespresso ®. DNA based traceability has been routinely used to ensure the authenticity of pure Bourbon Amarelo selected for new Nespresso ® limited edition based on a single Arabica variety. The aim of this quality control was to detect any unexpected adulteration of Bourbon Amarelo batches delivered to the factory. Thanks to previous analysis on the genetic diversity of a large collection of Arabica varieties and the establishment of a database of SSR, several specific DNA markers of the Bourbon Amarelo and possible adulterant varieties have been identified. A statistical protocol for bean sampling and analysis has been defined, allowing to evaluate a defined level of adulteration. This DNA system of traceability constitutes one of the first industrial applications to coffee. This technology can also be applied to the identification of the varieties used in roasted coffee products.

INTRODUCTION

Coffee is the second traded commodity on global markets after oil. But because of an over-emphasis on high volume sales, only a fraction of global coffee production represents harvests of outstanding quality, so called specialty or gourmet coffee. According to a World Bank survey, just 10 percent is devoted to specialty beans. Of this, no more than 10 to 20 percent meets Nespresso's expecting standards.

It is important to know the geographic and genetic origin of coffee for the purposes of fair international trade (Prodolliet, 1996). The origins used impart to the end 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 genetic and /or geographic origin of coffee is to support claims and to prevent any deliberate or accidental adulteration of a raw material of reputed origin by a cheaper coffee originating from another species or country.

A sensory analysis of a given coffee is usually sufficient to assess the species origin of coffee as the sensory and physical attributes of these two main species, Robusta and Arabica, are very different. However, the sensory properties are not only influenced by the coffee species but also, within the same species by the coffee variety itself. Consequently, the variety authenticity demonstration would then require DNA-based techniques and especially microsatellite (SSR) markers (Martellossi et al., 2005).

Arabica coffee is usually traded as a blend of different varieties but Nespresso have recently launch in 2006 a “Limited edition” called Bourbon amarelo prepared from a pure variety, growing in Brazil, and selected for the character of its vintage and its specific flavor. The main objective of the authenticity DNA based analysis was to develop a routine test in order to select the DNA markers allowing to guarantee the authenticity of the Bourbon amarelo variety and eventually to determine the level of adulteration.

MATERIAL AND METHODS

Material

A representative set of arabica varieties including 63 accessions was selected in a worldwide collection for the SSR analysis. This sampling of arabicas contains 10 Bourbon varieties from different origins in order to select the most relevant SSRs capable to differentiate among them.

Fourteen batch bean samplings (100 to 200 grams) of coffee containers from Brazil and a control from the single pure variety: Bourbon amarelo have been expertise in order to detect any adulteration using SSRs.

DNA extraction

For each sample a random set of 90 to 270 green coffee beans were ground under liquid nitrogen. The DNA extractions were performed using DNeasy 96 (QIAGEN) according to manufacturer recommendations.

The DNA extraction from roasted and ground coffee in Nespresso® capsule was performed according to Meyer and Jaccaud (1997).

SSR analysis

Seven microsatellites were selected according to their capacity to differentiate among closely related Arabica accessions (Table 1).

Table 1. List of the seven SSRs used for DNA diversity and traceability study.

SSR code	Linkage group	Range size amplicon (bp)
R338	E	220-240
R264	A	180-210
R268	G	125-150
A8814	Unknow	110-125
A8845	K	200-230
A8847	K	160-200
AY2449	C	270-300

PCR DNA amplification

The amplification is performed for each SSR marker with two specific primers and one of them is labelled with a fluochrome allowing the detection of the PCR fragment. Amplified DNA was separated on the sequencer ABprism 3100 and the experimental data were analysed by GENSCAN and GENOTYPER softwares (Applied Biosystems).

AND	:	2.5 µl
Primer A (labelled primer, 10µM)	:	1 µl
Primer B (20µM)	:	0.5 µl
AmpliTaq Gold PCR Master Mix	:	20 µl

DNA amplification is performed on a thermocycler PCR Applied Biosystems 9700:
10 min à 94 °C

30 sec à 94 °C
30 sec à 50 °C 35 cycles
1 min à 72 °C

7 min à 72 °C

Statistic analysis

Statistic analyses were performed using NCSS2001 software (Hintze, 2001). The establishment of the Arabica diversity dendrogram was made with Ward's minimum variance as clustering method and Euclidian distance from data obtained with the seven SSRs selected.

A specific statistic test was performed on each batch of green beans analysed in order to estimate (p = 0.95) that the true proportion of adulteration is below 10%. This test is depending on several factors as: the p threshold, the size of the batch, the number of beans analysed and the number of adulterated beans.

RESULTS AND DISCUSSION

The first step of the elaboration of this quality control test was to determine the genetic diversity of the main cultivated Arabica accessions in regards to Bourbon amarelo cultivar and some Ethiopian Arabica in order to select the most relevant SSRs showing the higher level of differentiation. The selection includes seven SSRs (Table 1) that allowed a description of the Arabica genetic diversity (Figure 1).

Two main groups are clearly characterized, represented by the Ethiopian accessions on one hand and main cultivated varieties on the other hand. Another interesting trait is that the number of accession duplicates is higher in the cultivated Arabica (20) than in Ethiopian group (1). This data illustrates the narrow genetic diversity among cultivated Arabicas in regards to the more diversified genetic pool from Ethiopia where is the centre of diversity for this species.

These results were confirmed by molecular analysis (Anthony et al., 2001; Steiger et al., 2002) illustrating the drastic reduction of genetic diversity in cultivated accessions due to limited dissemination from its primary centre of diversity. Nevertheless, the set of seven SSRs selected was good enough to differentiate the cultivated Arabica accessions as well as the different Bourbon cultivars. This allows us the establishment of a DNA test based on SSR markers to identify and trace Bourbon amarelo bean samples from Brazil.

Using the seven SSRs on the different coffee beans samples at the origin of the "Limited Edition Blend": Bourbon amarelo we obtain a reference database for which each specific SSR DNA profile of this variety is referenced (Figure 2). During the quality procedure analysis each bean sample was assessed with the seven SSRs and only four of them detected putative adulterated beans (Figure 3) showing a different DNA profile from the reference variety used.

Even if these differences are present in a low frequency and always below a statistical threshold of 10% we have tried to determine the genetic origin of these heterogeneity.

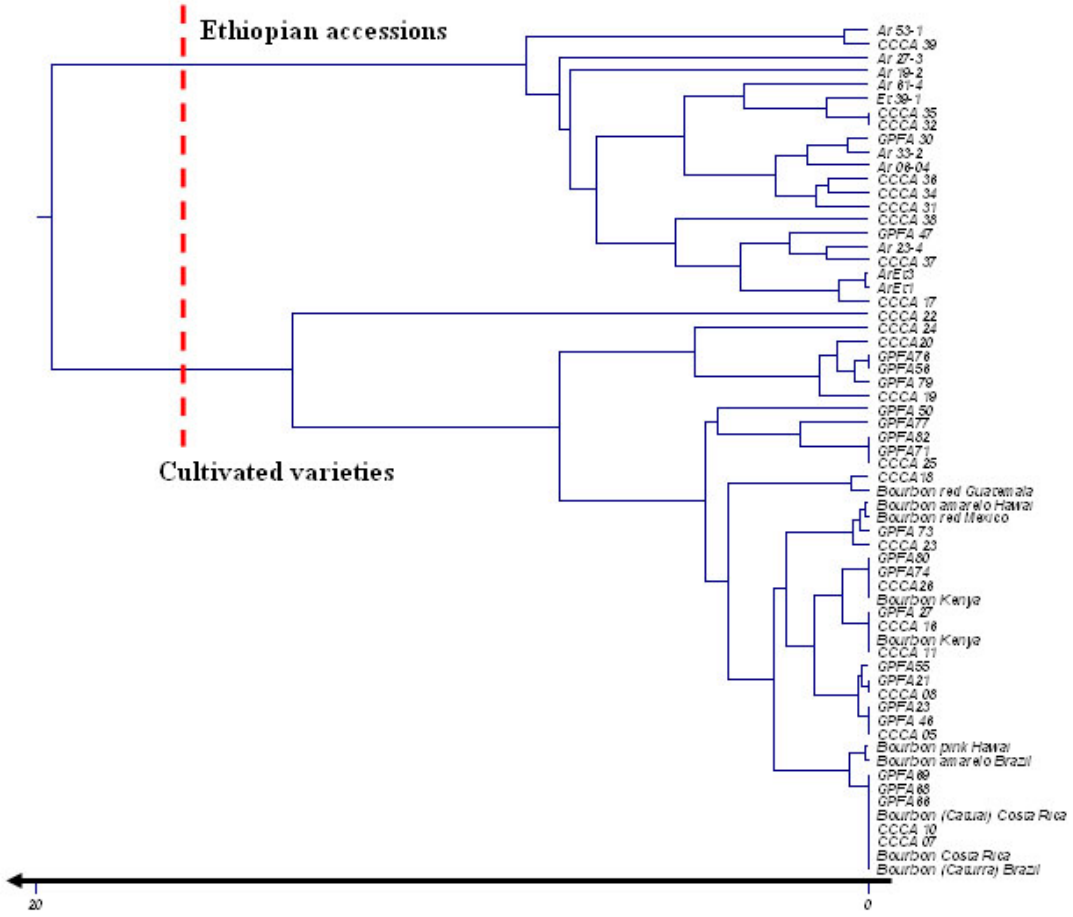

Figure 1. Dendrogram based on the use of seven SSRs with 63 Arabica accessions representative of the genetic diversity of this species. The dissimilarity scale at the bottom of the figure shows the genetic variability between the Arabica analysed.

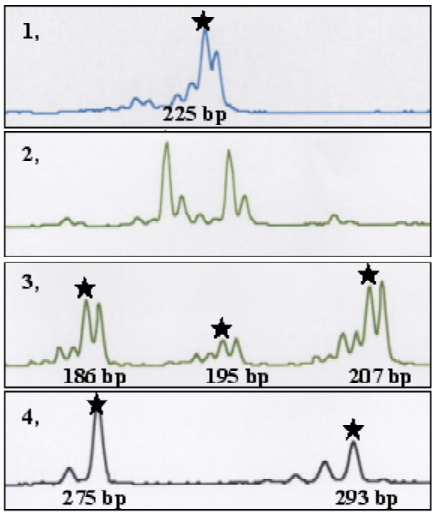

Figure 2. Examples of DNA fingerprinting using four SSRs on Bourbon amarelo arabica variety. 1 = SSR A8845, 2 = SSR R268, 3 = SSR R264, 4 = SSR AY2449. The size in base pair (bp) of each PCR product is given below each allele detected. The symbol ★ indicates the exact location of each allele.

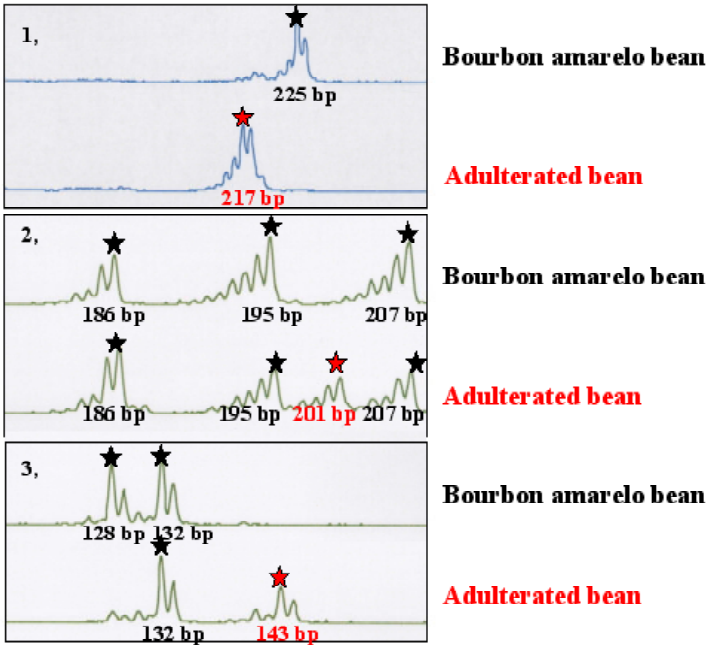

Figure 3. DNA fingerprinting using three SSRs showing the patterns on different bean samples. 1 = SSR A8845, 2 = SSR R264, 3 = SSR R268. The size in base pair (bp) of each PCR product is given below each allele detected. The symbol ★ indicates the location of each control (Bourbon amarelo) allele and the symbol ★ highlights the adulterating alleles.

Because of the tetraploid origin of Arabica generally the SSR analysis detects two homologous loci characterizing the two coffee diploid genomes at the origin of Arabica species. This species is largely autogamous explaining why a high frequency of the loci analysed are homozygous (Figures 4-1). The majority of the bean tissues (endosperm) have a hexaploid status due to the fusion of the antherozoid (2n) and the central cell nuclei (4n).

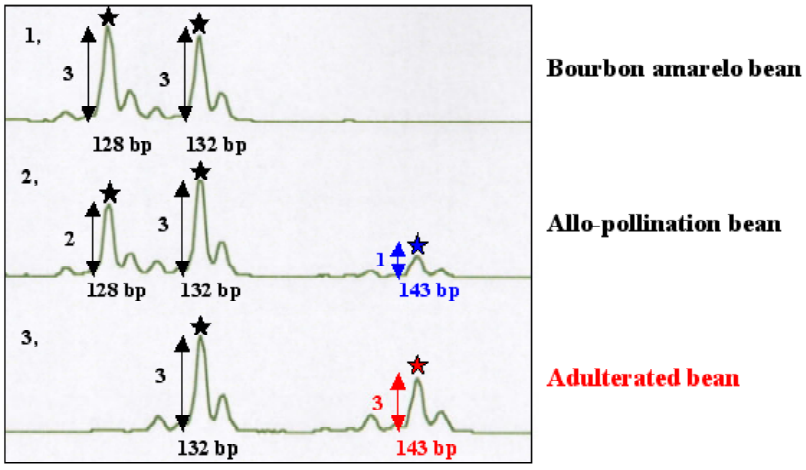

Figure 4. DNA characterization using SSR R268 on Arabica samples from 1, Control Bourbon amarelo (self-pollinated) bean, 2, allo-pollinated bean, 3, adulterated bean. The symbol ★ shows the alleles characterizing the Bourbon amarelo variety and the symbols ★ or ★ highlights the allo-pollination or adulteration status of the bean samples analysed, respectively.

With the use of a semi-quantitative assay using fluorescent labelling of the DNA amplification it is possible to quantify each allele in a given SSR DNA profile. Following these analyses two different types at the origin of the "no true to type pattern" (Figures 4-2, 3) can be detected:

- One bean SSR pattern displays an extra allele (143 bp) in comparison to the reference control (Figures 4-2). This allele represents a relative ratio of 1/3 and the other allele (128 bp), at the same locus, had a ratio of 2/3. The genetic characterization of such bean led to the conclusion that there is no adulteration but that bean analysed is the product of an allo-pollination between the Bourbon amarelo variety and another Arabica. In our database we detected only one Arabica variety carrying the allele of 143bp, this variety is an Icatu that is a usual Arabica growing in Brazil.
- The other SSR pattern (Figures 4-3) is characterized by the absence of the alleles of 128bp and the presence of the alleles of 143 bp. This genetic determination led to the identification of the bean adulteration coming from an Icatu or closely related to this arabica variety.

A total of 15 coffee batches including a control were analyzed. For each lot 90 to 270 beans were studied. The adulteration level was ranking from 0 to 3 beans per sample corresponding to an estimated statistical rate of adulteration from 0% to 6%. These results were confirmed by performing the SSR DNA fingerprinting (Figure 5) using the DNA extracted from the Nespresso capsules containing Bourbon amarelo product. Despite of the factory processing including roasting and grinding steps our SSR DNA analysis is producing DNA fingerprints. It was no possible to differentiate the SSR patterns obtained from this cultivated variety in regards to the end product demonstrating that the level of adulteration is low.

At the opposite, the SSR DNA fingerprinting can be used on the end product to determine the Arabica varieties used in a given blend. It could be also a powerful tool to detect and quantify the presence of Robusta, as adulterant, in 100% Arabica claimed products.

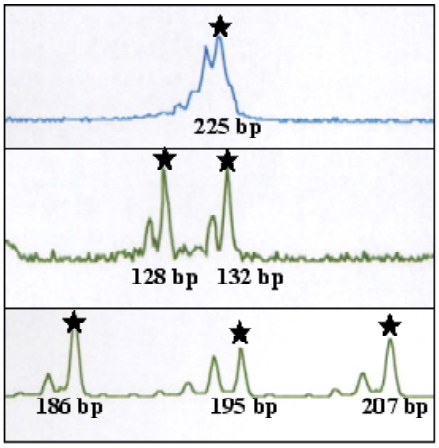

Figure 5. DNA fingerprinting pattern of a ground coffee from " Bourbon amarelo" capsule using three SSRs: 1 = SSR A8845, 2 = SSR R264, 3 = SSR R268. The size in base pair (bp) of each PCR product is given below each allele detected. The symbol ★ indicates the location of each control (Bourbon amarelo) allele.

ACKNOWLEDGMENTS

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The Status of Coffee Processing Capacity in Kenya

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SUMMARY

The capacities along the coffee processing chain were assessed in 24 primary coffee factories. The results were compared with those of a standard design. The processing capacities in most factories with 1, 2, 4 and 6 discs were less than the optimum designs but to different extents. The 3 disc pulper category however, had more incidences of excess capacity than the others and 1 factory in this category demonstrated satisfactory capacities in the consecutive critical stages. An inconsistent variation in the available capacity was also observed in consecutive stages thereby limiting the capacity of each factory to the status of the most inadequate stage. These findings can be used to upgrade the capacities to the correct standard design where found inadequate or to determine any additional cherry intake which can be accepted to a factory incase of increased coffee yields arising from intensified or expanded production.

INTRODUCTION

In the wet processing of coffee in Kenya, the just ripe coffee is selectively picked from every tree at preferably fortnightly intervals within a harvesting season of about 3 months. The trend of ripening (Figure 1) confirms that about 20% of the entire season's harvest is realized within 2 weeks or about 15% within one (1) week of the peak season. There is normally a serious tendency of congestion at the primary coffee factories during the peak season. This is mainly so where the factory design capacity does not measure to the cherry intake at the peak season. Previously, the factory's annual cherry intake capacity had been considered to suffice greatly if technically operated as required. However, due to persistence of congestion in most of the factories, a rapid survey was conducted in the main coffee growing region in the East of the Rift Valley to establish whether the existing capacity still remained adequate. This was thought important since coffee quality for instance, in relation to the recent concern of mould formation depends on the technical efficiency of a primary coffee factory.

MATERIALS AND METHODS

Coffee factories in the cooperative, medium and smallholder farm sectors were categorized with respect to the number of pulping discs per factory. Coffee factories from each category were then selected at random for the survey.

The survey was conducted by measuring the functional dimensions of the equipment used at the pulping, fermentation, drying and storage stages of processing. More information, which was pertinent to the survey, was obtained from the factory managers using a questionnaire designed for this purpose.

Using the recorded data, the capacities at each processing stage were calculated for each selected factory. The design capacities of each factory category were also determined following a procedure by Mburu (2004) and compared with the findings of the survey.

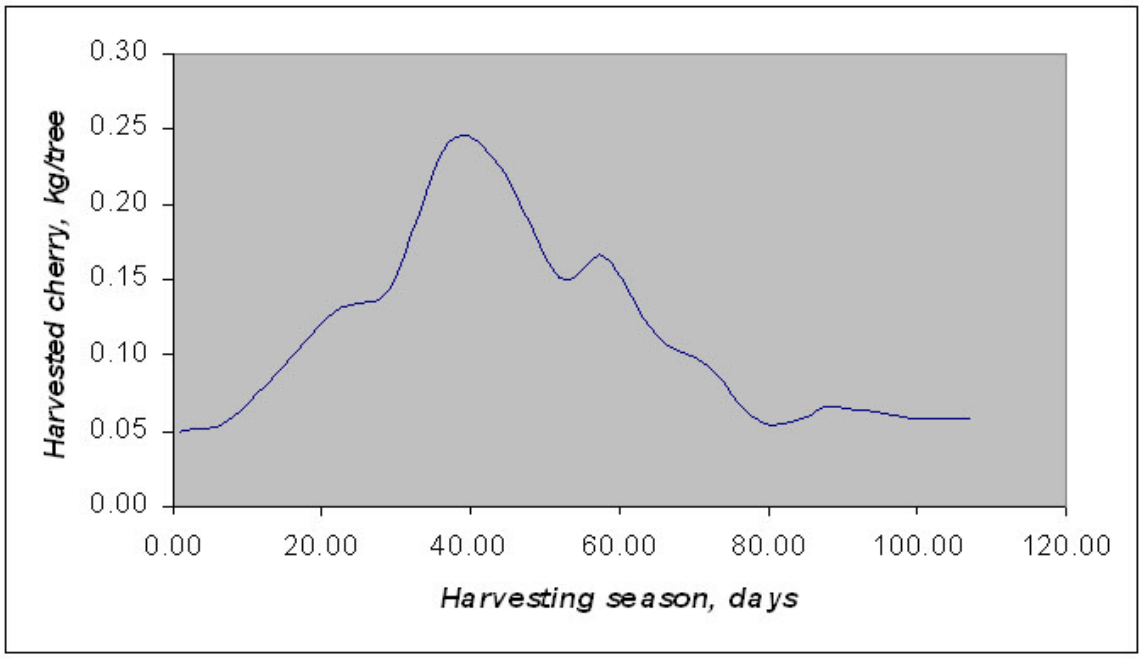

Figure 1. The ripening trend of coffee.

RESULTS AND DISCUSSION

- According to the results in Table 1 the potential cherry intake in some cases exceeded the design capacity and more so with the 3 disc pulpers commonly used in the co-operative sector.. The opposite was however, observed in the drum pulper factories. The actual annual cherry intake was always less than the design capacity except in one 3-disc factory processing 31,000 kg above the design capacity.

- The existing fermentation capacities exceeding the design capacities in 22% of the 1-2 disc factory categories. However, 100% of factories with more than 3 discs used more space than the design capacity. Under such circumstances the facilities could be seriously strained to cope with the coffee being availed for this process.

- The available skin drying space was less than required except for 60% of the 3 disc coffee factories which had more than the design capacity. This was an indication of a serious lack of space for skin drying. Perhaps these factories cope with the deficit by rapid skin drying of parchment throughout the day.

- The available final drying space was always inadequate except in the 3 and 4 coffee factories where 60% and 40% had excess capacity respectively. Inadequate final drying tables can induce a tendency of hastening to transfer the semi dry coffee to the conditioning bins at relatively high moisture content of between 20-35% (Mburu and Mick, 2005).

- About 33% of all the factories surveyed did not have conditioning bin facilities. Besides this, the conditioning bin capacities were always below the design level possibly signifying the factories less reliance on them as required except at times of adverse weather and a sudden peak crop.

Table 1. Design and available coffee factory capacities.

	Pulper	Annual Cherry intake,		Fermentation	Skin drying	Final drying	Conditioning	Storage	Waste disposal
Sn	Discs	Potential, kg	Current, kg	m3	m2	m2	m3	m2	m2
DC1	1	150,000		12.60	280.00	945.95	473-1892	36.00	
1	Drum	30,000	18,080	4.71	42.00	341.60	0.00	0.00	0.00
2		60,000	30,000	7.04	34.94	165.87	4.68	14.06	0.00
3		6,000	5,700	17.39	12.60	41.95	0.00	16.50	0.00
4		60,000	45,300	8.21	34.40	19.20	0.00	9.90	5.00
5		30,000	27,400	7.32	10.40	187.90	0.00	31.59	5.00
DC2	2	350,000		25.20	560.00	1891.89	946-3784	84.00	
1	2	84,000	30,000	5.53	33.80	181.30	0.00	29.00	54.60
2		400,000	316,169	12.76	77.12	648.84	0.00	99.00	36.58
3		280,000	70,000	0.00	15.55	0.00	0.00	0.00	0.00
4		400,000	109,393	31.82	25.60	773.17	0.00	84.96	16.76
DC3	3	800000		37.80	840.00	2837.84	1419-5676	192.00	
1	3	1,000,000	501,000	182.30	1081.90	6181.00	121.12	224.10	56.60
2		900,000	250,000	330.32	536.10	2607.10	254.33	213.60	123.00
3		1,900,000	600,000	131.50	960.06	5606.87	230.00	521.61	144.00
4		800,000	450,000	343.86	984.96	2679.27	216.00	169.01	182.00
5		1,500,000	831,000	207.68	393.90	6907.00	226.40	571.90	196.00
DC4	4	1,200,000		50.40	1120.00	3783.78	1892-7568	288.00	
1	4	700,000	326,757	81.17	780.10	4607.70	228.20	216.00	134.30
2		1,000,000	300,000	233.47	394.90	4073.20	204.80	191.26	168.00
3		900,000	710,000	81.49	251.40	3738.00	120.20	203.75	521.00
4		1,500,000	216,000	108.72	232.30	2258.00	384.60	283.65	169.00
5		1,200,000	476,833	116.79	207.56	1759.93	617.22	165.43	951.11
DC6	6	1600000		75.60	1680.00	5675.68	2838-11351	384.00	
1	6	1,500,000	1,010,674	272.31	1345.00	3070.00	160.00	1150.00	42.70
2		2,000,000	839,756	266.22	150.18	4829.00	280.00	259.11	145.70
3		1,500,000	570,643	100.52	373.00	1485.35	282.05	246.18	30.47
4		1,200,000	494121	214.61	495.45	2599.15	428.05	93.96	40.97
5		1,600,000	651830	274.20	655.00	3755.40	1068.00	84.71	86.54

- The smallholder sector had either limited or no storage facilities at all while one of the 2-disc factory category had excess storage and another no store at all. Factories without the recommended stores resorted to other undesignated buildings for this service. Although 80% of the 3-disc factories had excess storage space, only one 3 disc pulper factory had adequate capacity in all the critical consecutive stages of fermentation, skin drying, final drying and storage. All the 4 disc factories had inadequate storage space while 1 of the 6-disc factories had a 300% space above the design capacity.

- The most excessive capacity was realized in the fermentation (70.8%) followed by storage (25%), final drying (20.8%) and skin drying (12.5%) in that order. Finally, all the factory categories had some area reserved for waste disposal except half of the smallholder planters.

- Finally, all the factory categories had some area reserved for waste disposal except half of the smallholder planters. This was as expected since the smallholder sector was just evolving from the co-operative sector. Besides, personal observations have revealed that they are even lacking in more basic facilities than this including processing water. Although, their output of the processing effluents may be far less and more scattered away from the surface water sources than the other factory categories, they should still be provided with the relevant technical know how to develop their processing units to model mini factories.

CONCLUSION

1. There was no consistent trend in the capacity of processing facilities provided in comparison to the functional design requirements.

2. The processing capacity status should be identified and upgraded if need be in all the factories ountry wide to enhance the coffee quality.

3. Where the capacity was in excess, the need for additional annual cherry delivery to that factory from increased coffee yields via intensified or expanded production was also established. This could prevent the factory from incurring depreciation cost for underutilized equipment.

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Evaluation of Some Innovative Primary Processing Practices Observed in the North Rift Valley Region of Kenya

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SUMMARY

Over the last 10 years North Rift Valley has increasingly become important as far as the future of coffee production in Kenya is concerned. A survey conducted during 2004/2005 revealed processing practices and arrangements that appeared unique and divergent from practices elsewhere in Kenya. Fermentation stage is one area that farmers have attempted to overcome constraints by improvising and at times coming up with what would be described as innovative ideas. This research focused on the cup quality and presence of infective fungi in the coffee fermented using the improvised fermentation receptors alongside the conventional fermentation tank. Various fungi were isolated in the coffee under the study. Yeasts were the highest occurring fungi. No significant difference (at $p \leq 0.05$) was found in the aroma, body, acidity and the overall acceptance between the coffees fermented using the various improvised fermentation tanks and the conventional fermentation tank under the conditions of this study.

INTRODUCTION

Kenya produces mild arabica coffee that is of unique high quality. The desirable quality attributes of Kenya coffee are derived from inherent genetic characteristics of selected coffee varieties, climatic conditions and proper field and post harvest management. Coffee fermentation involves subjecting pulped coffee to appropriate condition so that the mucilaginous coating may be degraded and become separable from the parchment Apart from the concrete fermentation tanks widely used and studied, other materials that can serve as fermentation tanks exist (Sivetz and Desrosier, 1979). However materials made of iron and zinc are not recommended as they are easily corroded by the products of fermentation and may impart metallic flavour to the fermenting coffee (Wrigley, 1988). Concerns over food safety have become a major issue in the international trade. If coffee is not handled properly during processing moulds may grow and under certain conditions may affect the quality of the product (Wrigley, 1988) and or produce mycotoxins (Taniwaki et al., 1999). The aim of this investigation was to evaluate the cup quality and presence of infective fungi in the coffee fermented using some improvised fermentation receptors alongside the recommended concrete fermentation tank.

MATERIALS AND METHODS

Coffee processing

Cherry was pulped and drained off excess water using meshed screens. The mucilaginous parchment coffee was placed into aluminium milking cans and aluminium cooking pots, which were used to serve as fermentation tanks alongside the recommended concrete

fermentation tank. After fermentation the parchment was washed and then sun dried to moisture content of 10.5-11.0%.

Mycological examination of internal bean surfaces

In the laboratory total internal fungal population in the coffee beans were enumerated on DG18 medium after surface sterilization with 1% sodium hypochlorite. Fungal growth was recorded from 4 to14 days of incubation.

Coffee quality evaluation

Dry parchment samples were hulled and graded. The grades AA and AB were mixed and submitted for quality assessment.

RESULTS

The diversity of fungi in the internal surfaces of cherry (before any processing) and parchment fermented using different receptors are presented in Table 1. Yeasts were observed as the highest occurring fungi in the internal surfaces of cherry before it was subjected to any form of processing and after fermentation. No discernable trend was observed in the other moulds occurring in the samples assessed. There was a decrease in the percentage fungal levels on drying the coffee (Table 2). The sensory evaluation data is presented in Table 3. Variation in, aroma quality, body, acidity and the overall acceptance of the coffees fermented using the various improvised fermentation receptors and the conventional concrete fermentation tank was not significantly different at $p \leq 0.05$.

Table 1. Types of fungi isolated from the internal bean surfaces before drying expressed as proportions of the total fungal population.

	Runs	Yeast	*Aspergillus* spp.	*Penicillium* spp.	*Fusarium* spp.	*Cladosporium* spp.	*Alternaria* spp.	Mucor	NI
Fresh Cherry	Run 1	N.D	0.07	0.07	N.D	0.86	N.D	N.D	-
	Run 2	0.50	N.D	0.05	0.10	0.35	N.D	N.D	-
	Run 3	0.64	N.D	N.D	0.2	0.16	N.D	N.D	-
CFT	Run 1	0.42	N.D	0.08	0.17	0.25	N.D	N.D	0.08
	Run 2	0.71	N.D	N.D	0.25	0.00	N.D	0.04	-
	Run 3	0.66	N.D	0.06	0.19	0.03	0.06	N.D	-
ACP	Run 1	0.65	N.D	N.D	0.24	0.09	0.02	N.D	-
	Run 2	0.84	N.D	N.D	0.11	0.05	N.D	N.D	-
	Run 3	0.67	N.D	0.02	0.23	0.04	N.D	0.02	0.02
AMC	Run 1	0.78	N.D	N.D	0.16	0.06	N.D	N.D	N.D
	Run 2	0.95	N.D	N.D	0.04	0.02	N.D	N.D	-
	Run 3	0.96	N.D	N.D	0.02	N.D	N.D	N.D	0.02

ACP: Parchment fermented using Aluminium Cooking Pot; AMC: Parchment fermented using Aluminium Milking Can; CFT: Parchment fermented using Conventional fermentation tank; NI: Moulds not identified; N.D: Not detected.

Table 2. Internal fungal infections levels observed in the coffee fermented using different receptor before and after drying.

Fermentation receptors	Runs	%Fungal infection in coffee beans before drying	% Fungal infection in coffee beans after drying
CFT	Runs 1	0.19	0.00
	Runs 2	0.76	0.11
	Runs 3	0.33	0.00
AMC	Runs 1	0.81	0.00
	Runs 2	0.57	0.04
	Runs 3	0.76	0.11
ACP	Runs 1	0.52	0.04
	Runs 2	0.76	0.04
	Runs 3	0.67	0.07

ACP: Aluminium Cooking Pot; AMC: Aluminium Milking Can; CFT: Conventional fermentation tank; NI: Moulds not identified.

Table 3. Cup quality of coffee samples fermented using different receptors.

Quality parameters assessed	Runs	CFT	ACP	AMC	ANOVA
Aroma quality	1	3.33	3.00	3.11	NS
	2	3.22	2.89	2.89	NS
	3	3.11	2.78	3.00	NS
Body	1	3.44	2.89	3.44	NS
	2	3.44	3.11	3.00	NS
	3	3.56	3.00	3.22	NS
Acidity	1	2.89	2.56	3.11	NS
	2	3.00	2.89	2.89	NS
	3	2.89	2.67	2.89	NS
Preference	1	2.89	2.89	3.11	NS
	2	3.00	3.00	2.78	NS
	3	3.11	2.56	3.00	NS

ACP: Quality parameters of coffee fermented using Aluminium Cooking Pot; AMC: Quality parameters of coffee fermented using Aluminium Milking Can; CFT: Quality parameters of coffee fermented using Conventional fermentation tank; NI: Moulds not identified; NS: No significant variation in quality parameters from the treatments (at $p \leq 0.05$).

Aroma	**Body**	**Acidity**	**Preference**
5. Very strong	5. Very strong	5. Very strong	5. Excellent
4. Strong	4. Strong	4. Strong	4. Strong.
3. Moderately strong	3. Moderately strong	3. Moderately strong	3. Good
2. Moderately weak	2. Moderately weak	2. Moderately weak	2. Moderately weak
1. Weak	1. Weak	1. Weak	1. Very bad
0. None	0. None	0. None	0. Undrinkable

CONCLUSION

In Kenya coffee processing recommendations exist only on the usage of concrete fermentation tanks. The innovations by farmers when tested under controlled conditions, passed as good practices and stood out as possible opportunities that need to be further

understood. For the sake of small scale farmers constrained by lack of individualized or centralized fermentation facilities, research work should be put in place to validate some of the innovations set in place by farmers. However the whole question of coffee production expansion in the North Rift Valley region presents enormous challenges and opportunities for the coffee sector in Kenya.

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Arabica Coffee from El Salvador: Comparison of Drying Dynamics, Chemical Profile and Sensorial Properties between Samples Dried on the Patio and in a "Solar Drier"

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SUMMARY

An experimental campaign was undertaken in February 2005, to investigate the drying behaviour of Arabica coffee during the harvest season in El Salvador. Samples of *C. arabica* cv. Bourbon were harvested at peak maturation, processed by the "washed" method and dried in parallel on the patio and in a sheltered, greenhouse-type solar drier. Drying parameters (time, temperature, relative humidity, weight loss) were recorded for the whole duration of the experiment. The solar drier not only protected the coffee from rain and weather; moreover, it showed a slight but significative reduction in drying time in comparison to the patio, and guaranteed a more uniform product and a reduction in visual defects. Parchment was then removed, and samples were shipped to Trieste where sensorial and chemical characterization was performed. Sensorial evaluation was in agreement with the chemical analysis of aroma compounds, and underlined no relevant difference between patio- and solar-drying, thus confirming that the solar drier does not alter the quality of the product, and is a more efficient alternative to patio-drying in order to reduce processing time.

INTRODUCTION

In recent years, there has been increasing interest in the use of so-called "solar driers" to improve green coffee drying. These devices come in various shapes and size, from simple roofs protecting the coffee from rain, to complex structures with forced air-circulation and heating (Mulato, 2004; Berrueta-Soriano et al., 2003). The basic concept common to all types of solar driers is heat accumulation, obtained in a closed environment that captures solar radiation and generates a higher temperature and lower relative humidity. This system has two obvious advantages over the traditional patio: accelerated drying, and protection from rain.

In this experiment, we investigated the behaviour of a greenhouse-type solar drier during the 2005 harvest season in El Salvador.

MATERIALS AND METHODS

The campaign was carried out during February 2005 in Beneficio El Molino, situated on the Cordillera de Apaneca in El Salvador, at an altitude of 1300m above sea level. The drying process was never disrupted by rain.

Fruits of *C. arabica* cv. Bourbon were harvested at peak maturation and processed by the "washed" method. The wet parchment coffee was divided in two batches, that were dried in parallel on the patio and in the solar drier. Several experiments were made with growing thickness of the coffee layer, from 10 kg/m^2 to 40 kg/m^2 (the typical density used on the patios). In addition, a sample was dried according with the traditional Ethiopian procedure

(suspended bed of metal net, low density, covered at night and mid-day), which is a widely recognised “high quality” method.

The drying patio (Figure 1) was made of concrete tiles and was kept as clean as possible, to ensure the highest quality of the process.

Figure 1. Drying patio.

The solar drier (Figure 2) was built by eng. D. Malcangio (Italy), and was made of an external frame of steel and aluminium poles covered in UV-resistant plastic sheet. Inside, the coffe was laid on suspended beds of plastic net, favouring air circulation and cleanliness of the process.

Figure 2. Greenhouse-type solar drier.

The suspended table (Figure 3) was hand-made on location from wood poles, with a fine metal mesh supporting the coffe mass, that was covered in plastic sheets at night and mid-day, to ensure a more gradual drying.

The following table summarize the characteristics of the processed samples:

sample #	1	2	3	4	5	6
drying method	solar drier	solar drier	solar drier	patio	patio	raised bed
drying density	10 kg/m?	30 kg/m?	40 kg/m?	30 kg/m?	40 kg/m?	10 kg/m?
drying time	6 days	7 days	8 days	9 days	9 days	6 days

Samples were dried to a water content of 11 ± 1% (measured with a portable electronic moisture meter). Drying time varied from 6 to 9 days, depending on drying method and density (see table above).

Figure 3. Suspended-table drier.

Drying parameters (time, temperature, relative humidity, weight loss) were recorded for the whole duration of the experiment. Parchment was then removed , and samples were shipped to Trieste, where sensorial and chemical characterization were performed.

RESULTS AND DISCUSSION

Heat accumulation effect

During the whole campaign, the average ambient temperature was 23.5 °C, and the average ambient RH (Relative Humidity) was 58.3%. Inside the drier, average temperature was 26.4 °C, with an average RH of 50.5%.

The heat accumulation inside the solar drier caused an average rise in temperature of 2.9 °C, and a decrease in RH of 7.8%.

Coffee drying dynamics

In the following graphs, we compare the samples with the same density, keeping the suspended table as a reference sample. Drying is indicated as percentage weight loss.

As can be seen, the solar drier always presents the steepest curve in the initial phase of drying, indicating that it is the quickest method for elimination of the free and weakly-bound water (the most critical for fermentation problems). At every coffee density, the solar drier is quicker than the patio, and even at the maximum load of 40 kg/m^2 (equivalent to standard patio-drying), the solar drier is only 2 days slower than the suspended table, which works with only 10 kg/m^2.

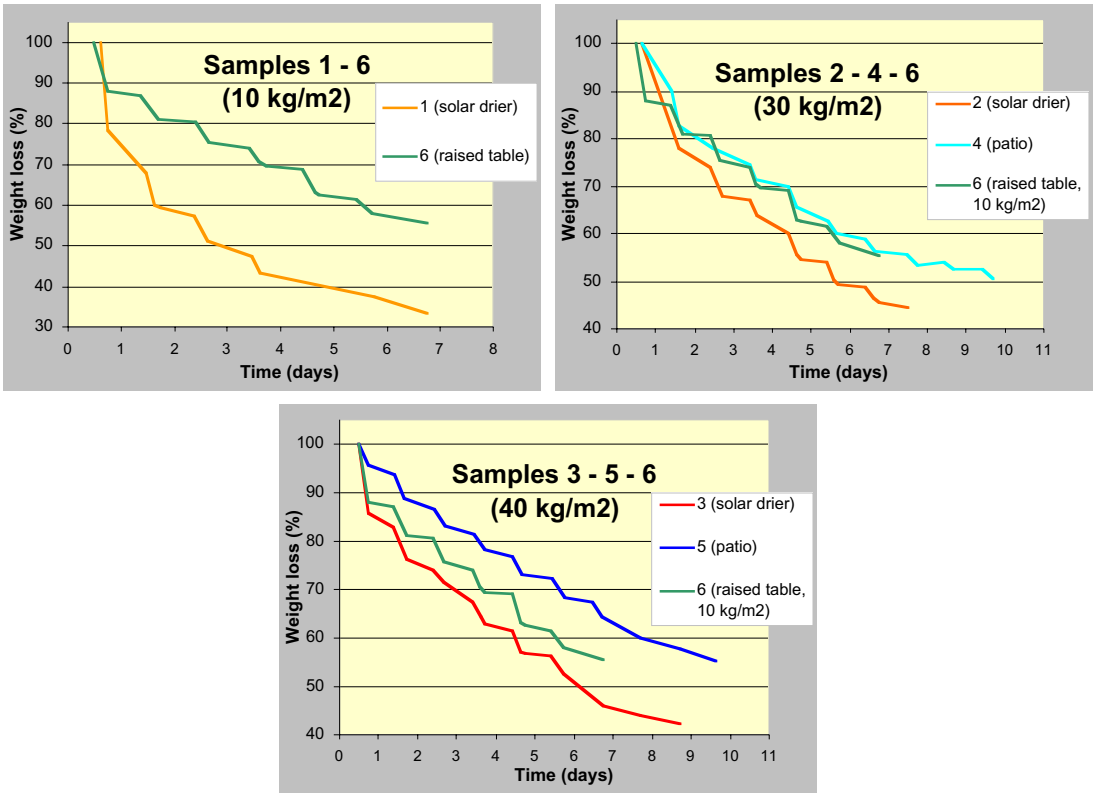

Figure 4.

Sensory analysis

Samples were evaluated by a panel of 6 trained judges in 3 successive sessions. Samples were presented anonymously and in random order. Data were collected and processed with FIZZ analysis software.

No significative difference was found between the samples: all of them were found to be of excellent quality and free of defects.

Chemical analysis

Aroma profile was obtained with extraction by SDE (Simultaneous Distillation Extraction) followed by GC-MS (Gas Chromatography-Mass Spectrometry) analysis.

Chemical analysis was in accordance with sensory evaluation, evidencing no defects and no relevant difference between the samples.

CONCLUSIONS

Coffee drying in a greenhouse-type solar drier presents many advantages:

- protection from atmospheric agents
- heat accumulation inside the drier, with a rise in temperature and decrease in relative humidity, resulting in accelerated drying
- maximum working load equivalent to patio-drying (40 kg/m^2)
- quality indistiguishable from the best possible “traditional” techniques

It must be noted that all these advantages apply not only with respect to patio-drying, but also compared to the suspended tables, which is a well-known high-quality technique. Quality of "solar dried" coffee was equivalent to coffee dried with the "raised bed" method, with the advantages of quicker and greater production, and independence from weather conditions.

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Water Content in Green Coffee: Comparison of Methodologies

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SUMMARY

The main objective of this study was to correlate methods for water content evaluation in green coffee based on oven drying to Karl Fischer titration (KFT). The methodologies evaluated were (i) ISO 6673 (16h drying at 105 °C) and (ii) the reference method employed by the Brazilian Agriculture Ministry (24 h drying at 105 °C) using both whole (BAMw) and ground (BAMg) coffee beans. Reference oven drying methodologies ISO 6673 and BAMw presented results statistically equivalent ($p > 0.05$) to those from KFT in the moisture content range that is of interest for green coffee commercialization (8-13% w/w). Both methodologies also correlated well (0.99 correlation coefficient) to KFT. However, for coffee samples that were submitted to drying procedures, significant differences between KFT and ISO 6673 and BAMw measurements were observed, with both oven drying methodologies providing higher moisture content values than KFT. Regarding the effect of grinding on method performance, lower average moisture contents were observed for all ground coffee samples (BAMg) compared to whole beans (BAMw). Nonetheless, significant differences ($p < 0.05$) only occurred for the samples that undergone drying (7-5% w/w moisture content range).

INTRODUCTION

Reference methods for water content determination in green coffee are based on oven drying. Such methods do not measure water content itself, but the mass loss under the heating conditions employed, including other volatile substances. The term moisture content is usually employed for this type of measurements, even though the term mass loss would be more accurate (Isengard, 2001).

A recent study by Reh et al. (2006) presented a comparison of ISO Standards for water content determination in green coffee and showed they were highly correlated to each other (R^2 values above 0.99). They also showed that ISO 6673 (1983) was appropriate for routine analysis since it was the standard that required the least input of labor (one step of drying, no grinding required) and was found to be independent of climate conditions and the presence of forced ventilation. The Brazilian reference method employed for water content determination in green coffee is based on oven drying at 105 °C for a 24 h period (Ministério de Agricultura e Reforma Agrária, 1992). This methodology was found to overestimate moisture content in comparison to ISO1447 (1978) by approximately 1% (Cabrera and Taniwaki, 2003).

Karl Fischer titration (KFT) is the most important chemical direct method for the determination of water content in food products, being based on the reaction of water with iodine in an alcoholic solution. Its major advantage with respect to weight loss techniques is the high selectivity for water coupled to the fact that it does not require sample heating (De Caro et al., 2001). In view of the above, the objective of this study was to compare Karl Fischer titration (KFT) to mass loss methods for water determination in green coffee.

METHODOLOGY

Arabica green coffee (Viçosa, MG, Brazil) previously classified by cup as soft was employed in all tests. The coffee was placed in shallow recipients and allowed to thermodynamically equilibrate for a 48 h period (Lot 0). Some coffee samples were submitted to drying and humidification procedures. Humidification consisted on spraying distilled water over the coffee beans. The water amounts employed were 1 g (Lot -1), 2 g (Lot -2) and 5 g (Lot -3) per 100 g coffee. Drying was performed in a convective oven with forced ventilation (model 400/3ND, Nova Ética, Brazil) at 200 °C for 30 (Lot 1), 60 (Lot 2) and 90 s (Lot 3). Samples were let to equilibrate for 24 h in sealed containers prior to the analyses.

The oven drying methodologies evaluated were (i) ISO 6673 (16 h drying at 105 ^{o}C) and (ii) the reference method employed by the Brazilian Agriculture Ministry (24h drying at 105 °C) using both whole (BAMw) and ground (BAMg) coffee beans. All oven drying methods employed 5 g coffee samples placed in a convective oven with forced ventilation (model 400/3ND, Nova Ética, Brazil). The reference water content was determined by Karl Fischer titration (KF 1000, Analyser, Brazil) using Karl Fischer reagent with pyridine (Merck) as titrating solution. External extraction procedure consisted of adding 10 mL methanol/chloroform solution (3:1) to each ground coffee sample (~1 g). The mixture was homogenized in an ultrasonic bath for 1 h and then vortex mixed for 10 min (2800 rpm). After phase separation, 0.2 mL of the supernatant phase were injected in the titration vessel. Two injections were performed for each sample.

Determinations were performed randomly in a total of five replicates per experiment. The experimental data were submitted to analysis of variance and the mean values were compared by the Duncan test at 5% probability.

RESULTS AND DISCUSSION

Average water content values are displayed on Table 1. In the moisture content range that is of interest for green coffee commercialization (8-13%), reference oven drying methodologies ISO 6673 and BAMw presented results statistically equivalent to those from KFT. These results indicate that the organic volatile substances contribution to mass loss was not significant for that moisture content range. However, for coffee samples that were submitted to drying, significant differences between KFT and ISO 6677 and BAMw measurements were observed. Both oven drying methodologies presented higher moisture content values than KFT. Such differences are attributed to the combination of the following factors: (i) loss of volatiles becoming more significant for lower moisture samples; and (ii) occurrence of moisture loss during sample grinding, since this procedure must be perfomed for KFT and is not required for both ISO 6673 and BAMw. The moisture loss during grinding can be confirmed by comparing results for KFT and BAMg for Lots 1, 2 and 3. Such results are equivalent, due to the fact that both methodologies employ sample grinding. The effect of sample grinding can also be assessed by comparing the results for methodologies BAMw and BAMg. Significant differences ($p < 0.05$) between these methodologies were observed for all samples that undergone drying. Therefore, these results indicate that loss of moisture during grinding becomes significant as sample moisture content decreases. The effect of sample grinding was also relevant for the sample with the highest moisture content (Lot -3). This was attributed to the fact that grinding is hindered by increases in moisture content as the sample becomes less brittle. Thus, sample overheating could occur. Such problem could be minimized by lowering sample temperature prior to the grinding step, using dry ice, for example.

Table 1. Moisture content (%w/w) for arabica green coffee.

Method Sample	KFT	ISO 6673	BAMw	BAMg
Lot -3	13.3 ± 0.2a	13.3 ± 0.5a	13.2 ± 0.4a	10.6 ± 0.1b
Lot -2	11.0 ± 0.4bc	10.9 ± 0.2c	11.5 ± 0.8ab	11.8 ± 0.1a
Lot -1	9.2 ± 0.3a	9.1 ± 0.1a	9.2 ± 0.3a	9.9 ± 0.1a
Lot 0	6.3 ± 0.6b	7.8 ± 0.1a	8.4 ± 0.1a	8.0 ± 0.1a
Lot 1	5.5 ± 0.4b	7.0 ± 0.1a	7.4 ± 0.1a	5.6 ± 0.3b
Lot 2	5.2 ± 0.1c	5.9 ± 0.1b	7.1 ± 0.1a	5.05±0.3c
Lot 3	4.2 ± 0.2 d	5.8 ± 0.1b	6.5 ± 0.1 a	4.7 ± 0.2c

Average value ± Standard Deviation. Values followed by the same letter in the same line do not differ significantly by the Duncan test at 5% probability.

Correlation coefficients are shown in Table 2. ISO 6673 presented the highest correlation to KFT. The reference method employed in Brazil for moisture determination in green coffee (BAMw) also correlated well to KFT. The lowest correlation coefficients were observed for BAMg, probably as a result of the sample grinding effect.

Table 2. Correlation coefficients among moisture content data.

	KFT	ISO 6673	BAMw	BAMg
KFT	1.0000			
ISO 6673	0.9893	1.0000		
BAMw	0.9859	0.9927	1.0000	
BAMg	0.9167	0.8969	0.9166	1.0000

CONCLUSIONS

Reference oven drying methodologies ISO 6673 and BAMw presented results equivalent to those from KFT in the moisture content range that is of interest for green coffee commercialization (8-13% w/w), thus being appropriate for water content determination in that range. For coffee samples that were submitted to drying procedures, water content values measured by both oven drying methodologies were higher than water content values obtained by KFT. These results indicate that loss of volatiles becomes significant as moisture content decreases. Furthermore, mass loss during grinding should also become a problem as moisture content decreases.

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Safety Control of Pesticides in Raw Coffee

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SUMMARY

Input of agrochemicals has long been marginal in coffee farming, where good agrarian practices and minimal application of traditional non-synthetic chemicals like copper salts and lime prevail. In any case, pesticide residues in food are nowadays a growing concern for consumers and regulatory bodies, and deserve stringent monitoring. Our company is tackling since 1993 the issue of possible contamination of raw coffee by pesticides, drawing off samples according to the ISO sampling method and analyzing for all-purposes pesticides with a corporate acceptance limit set at the quantitation threshold. Among over 7000 negative samples, only few positive cases have shown up with the discovery of traces of Lindane and Chloropyrifos. The possible contact pathways with the raw coffee beans seem to exclude the radicular/vascular route. No trace of contaminants has ever survived in the roasted product.

INTRODUCTION

Coffee, together with commodities like sugarcane, cocoa and bananas, has played a central role in the economic development of many tropical countries (Wrigley, 1988). Not unlike any other agricultural activities all over the world, the expansion of these export crops in the last century has left a distinct ecological footprint in terms of reduced biodiversity. Due to cost and handling problems, however, the input of agrochemicals (fertilizers and pesticides) has long been marginal in coffee farming, where good agricultural practices have been used for decades with success to control weeds and cryptogamia by minimal application of traditional non-synthetic chemicals like copper salts and lime (Cambrony, 1992; Matiello, 1997).

Pesticide residues in food have been a growing concern for consumers and regulatory bodies throughout the last decades (EPA, 2006). The issue of pesticide residue in food is quite controversial: pesticides are used because they have beneficial properties in terms of crop production and yield; by their use, farmers can maximize their efforts in the field thus minimizing the cost of the product to the consumer. Moreover, the use of pesticides has a positive benefit in terms of public health because fungi, insects, and non-crop plants (often poisonous) can contaminate the harvest with many natural toxins.

While both crop protection against pest attacks and the toxicological aspects of food safety have been much investigated, the balance point between them is still awaiting a definite positioning. The commodity coffee seems in general very little affected by the problem of pesticide residues (McCarthy et al., 1991; Jacobs and Yess, 1993).

Our company started back in 1993 to tackle the issue of possible contamination of raw coffee by pesticides, in compliance to specific Italian norms on insecticides and fungicides in green beans (O.M. 6/6/1985; O.M. 18/7/1990). Experimental activity included drawing off samples from several lots according to the ISO sampling method (ISO, 1982). As result of that pioneering activity, the samples analyzed for general-purposes pesticides popular in that

period did not bring up suspicions about the existence of any contamination problem in coffee.

To the aim of guaranteeing the upmost wholesomeness of our products, we decided in 1996 to adopt a corporate acceptance limit, provisionally set at the value of instrumental threshold of quantitation. All incoming lots of raw coffee (approximately 800 per year) were submitted to analysis since then.

Such approach is codified in our corporate certification of product conformity, as issued by the agency 'Qualité France', accredited by the 'Commission Nationale des Labels et des Certifications' of French Ministry of Agriculture and Fisheries. Since then, a systematic analytical control of several chemical and microbiological contaminants likely to be presents in raw coffees has been integrated in the existing procedure.

EXPERIMENTAL

All chemical analyses were executed by Chelab laboratories, a leading Italian company in the sector of chemical analysis and technical assistance for industry, agriculture and environment, certified according to UNI CEI EN 45001/90 by SINAL (SIstema Nazionale per l'Accreditamento Laboratori).

The contaminants data gathered from 1996 to date can be seen on Table 1.

Table 1. Overall analyses and contaminants-positive cases.

Year	Analyzed lots	Positive stocklots
1996	460	0
1997	421	0
1998	469	0
1999	757	0
2000	948	0
2001	662	0
2002	811	0
2003	838	5
2004	919	4
2005	978	0
TOTAL	7263	9

After twelve years of scrutiny and more than seven thousand lots analyzed, the first few positive cases have been found only recently (years 2003 and 2004) in lots from India. Table 2 shows the contaminants detected.

Some removal trials have been carried out to see whether these contaminants could be eliminated by double-stage ventilation: the result was somehow erratic, pointing at an overpowering effect of sampling incertitude over process (see Table 3). The same table shows also data on roasted coffee, analyzed in order to confirm the disappearance of pesticides from the finished product, where no trace of contaminants has ever shown up.

Table 2. Summary of positive cases.

Year	Lot	Pesticide	Value [mg/kg] in raw coffee	Note	Value [mg/kg] after roasting
2003	1	Lindane (gamma-HCH)	0.019 - 0,023 - 0,012	3 samplings	see Tab. 3
		Ethyl chloropyrifos	<0,01 -<0,01 - 0,054	3 samplings	see Tab. 3
	2	Lindane (gamma-HCH)	0.023 – 0.019 - 0.023	3 samplings	see Tab. 4
	3	Lindane (gamma-HCH)	0.01 – 0.014 – 0.015	3 samplings	see Tab. 4
	4	Lindane (gamma-HCH)	0.026 – <0.01 – 0.01	3 samplings	see Tab. 4
	5	Lindane (gamma-HCH)	0.013 – 0.011 – 0.01	3 samplings	see Tab. 4
2004	6	Methyl bromide	0.017	after fumigation*	**< 0,01**
	7	Methyl bromide	0.016	after fumigation*	**< 0,01**
	8	Ethyl chloropyrifos	0.230 - 0,020	2 samplings	**< 0,01**
	9	Ethyl chloropyrifos	0.017		**< 0,01**

**Since March 2005, methyl bromide fumigation in the container before shipment from India to Europe has been discontinued, in conformity with US EPA deliberation (F.R. Vol. 65, N. 229).*

Table 3. Contaminants in raw material vs. processed.

	Value [mg/kg]	Note	Value [mg/kg]	Note	Value [mg/kg]	Note
Raw original	0.019	1st sample	0.023	2nd sample	0.012	3rd sample
1 st air-cleaned	0.015	1st sample	0.018	2nd sample		
2 nd air-cleaned	0.021	1st sample	0.023	2nd sample		
Roasted original	< 0.01	1st sample	< 0.01	2nd sample		
Roasted 1 st clean	< 0.01	1st sample	< 0.01	2nd sample		
Roasted 2 nd clean	< 0.01	1st sample	< 0.01	2nd sample		
Industrial roast					< 0.01	3rd sample

Instance A: Lindane (gamma-HCH) analyzed according to UNI EN 12393/99 (detection threshold < 0,01 mg/kg).

	Value [mg/kg]	Note	Value [mg/kg]	Note	Value [mg/kg]	Note
Raw original	0.014	1st sample	0.017	2nd sample	0.029	3rd sample
1 st air-cleaned	< 0.01	1st sample	0.030	2nd sample		
2 nd air-cleaned	0.01	1st sample	0.017	2nd sample		
Roasted original	< 0.01	1st sample	< 0.01	2nd sample		
Roasted 1 st clean	< 0.01	1st sample	< 0.01	2nd sample		
Roasted 2 nd clean	< 0.01	1st sample	< 0.01	2nd sample		
Industrial roast					< 0.01	3rd sample

Instance B: Ethyl Chloropyrifos analyzed according to UNI EN 12393/99 (detection threshold < 0,01 mg/kg).

DISCUSSION AND CONCLUSIONS

Lindane (CAS 58-89-9) is generally administered on *Coffea arabica* plants by swabbing on the stem; this is to prevent the adult beetle of *Xylotrechus quadripes* and congeners from laying eggs on the stem itself from which at a later stage larvae bore through, resulting in wilting of the whole plant. As Lindane is very expensive, growers use to the same aim the other formulation Chloropyrifos (CAS 2921-88-2) (Raghuramulu, 2004).

Neither product is a systemic fungicide (Kidd et al., 1987), so they are not likely to be readily taken up by the plant and diffuse into the seeds. While residual Chloropyrifos in the soil is expected to half its concentration in 81 days (Kookana, 1995), common practice in India assumes that traces will not persist over 30 days: our green coffee beans as analyzed had been collected from the plant already since 4 months at least.

It is worth to highlight that both products are commonly used in warehouses to prevent various pests from attacking any edible material stored there (Ulmann, 1972). Since all positive coffee lots came from a single warehouse, where the application of the two mentioned pesticides could have been used during storage, it may be inferred that this might be the most likely pathway for our case of coffee bean contamination.

The amounts of pesticide residuals were not homogeneously distributed within the same raw coffee lot, even if the ISO method of sampling has been used: this suggests that only some bags had been contaminated. As time went by from July to October 2003, quite all the lots quarantined in the factory's warehouse have lost most contamination by natural way (see Tab. 4). What is more, roasting process did eliminate completely the presence of said contaminants, making the finished product free from specific risks.

Table 4. Evolution on time of contaminants [mg/kg].

	Late raw coffee	**Late raw coffee**	**Roasted coffee**	**Roasted coffee**
Lot	Lindane	Ethyl Chloropyrifos	Lindane	Ethyl Chloropyrifos
2	0.011	**< 0.01**	**< 0.01**	**< 0.01**
3	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
4	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
5	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**

In conclusion, our experience shows - in good agreement with previous surveys – that the presence of pesticide residues in raw coffee is infrequent. Storage seems effective to get rid of most of the contamination, while roasting eliminates it altogether.

WARNING

Even if encompassing data from more than 7000 samples, this large data base of ours cannot be seen as exhaustive. Nor is it representative of the general trade, because the coffee that underwent testing was the only one of our interest i.e. high quality grade, where particular care had been exerted on cultivation style, cherries' process, handling, storage, transport and sensibilization of farm staff.

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The Quality of Wet Processed Arabica Coffee as Influenced by Depth of Parching and Covering Period during Drying

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SUMMARY

Different drying depths and covering periods were investigated to determine the optimum depth and suitable exposure (covering) period that correlates with above average or acceptable cup quality characteristics of arabica coffee under Jimma condition. Coffee dried at depths 2, 3 and 4 cm attained the highest cup quality grades while the other depth (5 cm) was associated with lower quality standards. On the other hand, covering drying parchment coffee for a long time or exposure to strong sunshine for a long time in the day decreased the quality of arabica coffee. Hence, the uncovered (check) and the covering treatment from 10:00 to 16:30 attained the lowest cup quality grades. On the other hand, coffee beans dried at depths of 2, 3, and 4 cm and covered during 10:30-14:30, 10:30-16:30 and 11:00-15:30 were found to have better cup quality characteristics. It is therefore essential to avoid drying at a depth higher than 4 cm as it prolongs the drying period and hence lowers the cup quality standard. In addition, covering drying parchment coffee should be done only to avoid exposure to the strongest sunshine hours in the day. During drying parchment coffee, covering for a very short time or covering it for a very long period should be avoided as it can negatively affect the quality of the beverage.

INTRODUCTION

Coffee is one of the world's largest traded commodities and is produced in many countries, which are often heavily dependent on coffee export earnings. Due to the increasing competition in the world market, producing high quality coffee has become so crucial. Coffee with out physical and sensorial defects and with a good physical appearance is normally required. One principal quality-determining factor is the development of moisture content in the coffee bean during drying (Coffee growers' handbook, 1968).

Wet parchment coffee, at a stage immediately after fermentation, consists of approximately 50-60% moisture (www.ico.org/field_processing.asp). To reduce to an optimum 12.5% the parchment coffee is dried either in the sun, in a mechanical drier, or by a combination of the two. For sun drying the beans are laid out in a layer of 2 to 10 cm, and turned frequently to ensure even drying. Sun drying usually takes 8 to 10 days, depending upon ambient temperature and humidity.

Coffee produced following the wet method of processing is known to have superior quality characteristics and hence commands a higher market price. Each step in the wet method of processing is critical in maintaining the inherent quality of the coffee variety being processed. Drying is however the most important stage of processing affecting the quality characteristics of coffee. Almost all types of bean colors are developed during the drying process, which in turn determine the coffee quality standard or the value in green analysis (Coffee growers' handbook, 1968).

In addition, parchment coffee needs to be dried quickly (*after wet processing*) to stop microbiological activities which can develop severe cup defects as well as toxins which pose health risks for consumers (GTZ-PPP, 2002). Agronomic practices, research and development linkages, method of processing, lack of affordable credit, drying, storage, high cost of farm inputs, and transportation have generally been reported as the major factors affecting coffee quality in Africa (http://www.fao.org/DOCREP/003/X6939E/X6939e02.htm).

Due to the relatively higher price for wet processed coffee, the number of wet processing plants in southwestern Ethiopia has increased tremendously in recent years. Despite the increment in washed coffee production, no standard depth of drying parchment coffee is practiced in coffee washing stations in the country. Consequently, the coffee is heaped unevenly yielding inferior cup taste or quality. Higher heaps show delay in drying while sparsely spread once result in crack of parchment and thus an admixture of under dried and over dried beans.

Similarly, prolonged exposure of parchment coffee to very strong sunshine causes high loss due to cracking of parchment resulting in discolored beans (Personal observation at washing stations; ULG Consultants limited, May 1986). Determining the optimum condition for parchment drying was thus highly indispensable.

Therefore, this experiment was carried out to determine optimum drying depth that could avoid unnecessary congestion and parchment cracking problems, and to identify suitable covering period from excessive sun heat to minimize losses in quality.

MATERIALS AND METHODS

A split plot design with four replications, covering period as main plot and depth of parching as subplot treatments was used. The main plot (covering periods) included: uncovered (check), 10:30-14:30, 11:00-15:00, 10:30-16:30, and 10:00-16:30. The subplot (parchment depth) treatment comprised: 2cm, 3cm, 4 cm and 5 cm.

Six kilograms of fresh cherry were pulped by hand (disc pulper) and fermented under recommended conditions. The drying tables were constructed from wire mesh on firm wood posts to accommodate the design requirement. The beans were dried up to moisture content of 11%.

Sensorial evaluation of cup quality was accomplished by a panel of cup tasters. Scoring values used in coffee classification report and commercial descriptive standards were used to determine and categorize coffee quality. Analysis of variance was also employed where appropriate to determine treatment differences.

Cup quality evaluation consisted of raw (40%) and liquor (60%). Raw value was evaluated as *shape & make*, *color*, and *odor*. Liquor was also evaluated as acidity, body and flavor. Finally coffee quality data were collected and analyzed accordingly. The details of the sensorial evaluation procedure are given in *Annex1*.

RESULT AND DISCUSSION

The interaction between covering time and drying depth significantly ($p < 0.05$) affected the total time required to dry parchment coffee to an optimum moisture level (Table1). Parchment coffee dried at depth of 5 cm and covered during 10:00-16:30 had the longest drying time (10.8 days), while the uncovered parchment coffee at a depth of 2 cm dried more quickly

(5.98 days) (Table 1). The sensorial evaluation indicated that the both the uncovered and the longest covering (10:00-16:30) treatments had relatively lower cup quality values (Table 2).

The main effects i.e., covering period and depth of parching significantly ($P < 0.05$) affected drying time of parchment coffee (Table 1). Covering during 10:30-16:30 resulted in prolonged drying (9.47 days), but the uncovered parchment coffee dried in a relatively short time (6.56 days). Drying period was also significantly ($P < 0.05$) affected by depth of drying parchment coffee (Table 1).

The result of cup quality evaluation is given in table 2. Hence, parchment coffee uncovered during the day and the one covered during 10:00-16:30 had inferior cup quality than the other treatments. Therefore, the result indicated that covering parchment coffee for a very prolonged period or for a very short duration could cause quality deterioration. Avoiding both extremes of covering parchment coffee, i.e. extended and very short covering periods will be critically important to keep the inherent quality characteristics of coffee. Though no variation in the final quality class range was observed, parchment coffee dried at the highest drying depth (5 cm) gave the lowest value of cup quality, while the other drying depths (2, 3, and 4 cm) gave better cup quality values.

Table 1. Effect of *covering period* and *drying depth* on length of drying time of parchment coffee.

Covering period (CP)	Drying depth (cm)				CP means
	2	3	4	5	
Uncovered	5.98	6.2	6.88	7.18	6.56
10:30-14:30	6.77	7.68	8.98	9.73	8.29
11:00-15:30	6.9	7.8	8.83	9.74	8.32
10:30-16:30	8.19	8.82	9.63	10.47	9.28
10:00-16:30	8.04	8.83	10.21	10.8	9.47
DD means	7.18	7.87	8.91	9.58	

CV% = 1.84; $Lsd_{0.05}$ = 0.11.

Table 2. Mean values of cup quality evaluation for different drying depths and covering periods of parchment coffee.

Covering period (CP)	Drying depth (cm)				CP means
	2	3	4	5	
Uncovered	54.31	50.56	54.25	48.5	51.91
10:30-14:30	56.94	53.94	56.81	51	54.67
11:00-15:30	55.06	53.38	56.13	52.56	54.28
10:30-16:30	56.38	53.19	55.38	52	54.23
10:00-16:30	52.44	50.06	52.88	49.75	51.28
DD means	55.03	52.23	55.09	50.76	

This result agrees with previous reports that neither too quick drying at excessive temperatures nor too slow drying with low temperatures will bring about good quality standards (GTZ-PPP, 2002). When beans are dried too quickly, a hard shell on the out side is developed which prevents air to penetrate the bean and hinders drying; hence, beans will be over-dried at the outside while remaining moist in the inside which causes cracking of beans during hulling and sorting, and finally reduce its value in the quality evaluation. Too slow drying may also increase the risk of mould development (GTZ-PPP, 2002).

The data also revealed that there was a clear association between drying time (drying days) and drying depth and covering period (Figures 1 and 2). As drying depth increased, the drying time was apparently extended and finally lower values of cup quality were recorded. Similarly, as the duration of covering increased, extended drying time and lower values of cup quality were found. Moreover, the quickly dried (6.56 days) uncovered treatment was clearly associated with lower cup quality values (Figures 1 and 2).

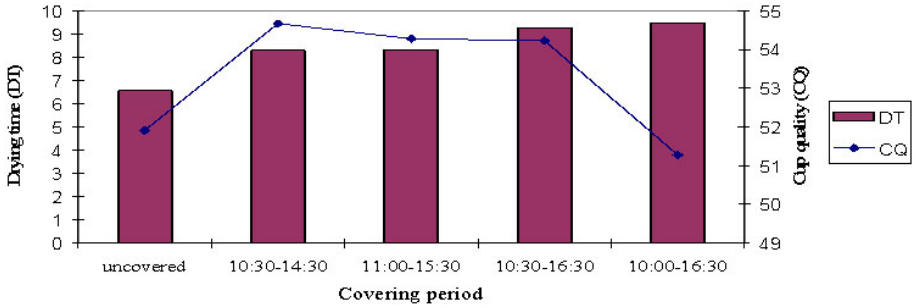

Figure 1. Effect of covering period on drying time and cup quality.

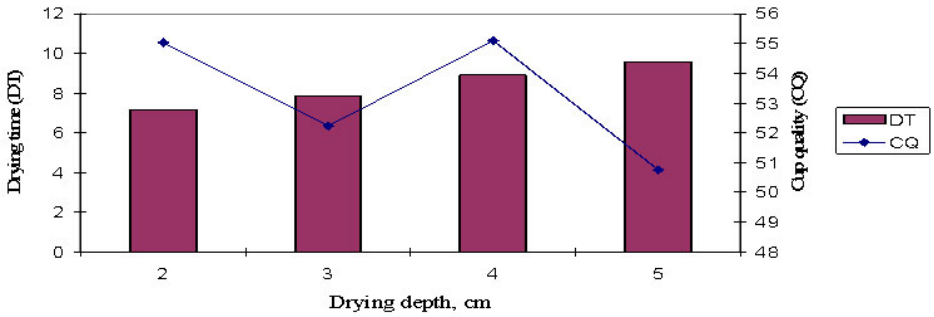

Figure 2. Effect of depth of parcking on drying time and cup quality.

CONCLUSION AND RECOMMENDATION

As drying depth increased, there was a linear increase in drying time and vice versa. On the other hand, based on the cup quality evaluation, all depths of parching resulted in cup quality values that fall with in similar class rang, but a relatively lower cup quality value was found at the highest drying depth. Therefore, a depth 2 to 4 cm is recommended to dry parchment coffee. The highest depth in this study (5 cm) was found to prolong the drying time and lower the general quality characteristics. Though there were no quality problems with the parchment coffee dried at the lower depth (2 cm), considering the larger area required to dry parchment coffee at such low depth, the higher depths are normally preferred.

The longest covering duration (10:00-16:30) during parchment coffee drying resulted in extended drying time (10.84 days) compared to the uncovered parchment coffee that dried much earlier (6.56 days); consequently, both treatments (*uncovered* and *covered* during 10:00-16:30) had the lowest mean values of cup quality evaluation. Therefore, covering should be practiced during the very strong sunshine hours to avoid excessive heating and care should also be taken not to cover for a long time in the day. Under Jimma condition, during the day (starting from 10:00 in the morning), drying parchment coffee should be covered from 4 to $5^{1}/_{2}$ hours.

Finally, the result of this experiment indicated the need for an effective management of temperature and moisture during the drying stage of wet processing of arabica coffee.

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Annex 1. Sensorial evaluation of arabica coffee quality (raw & liquor).

Raw Value (40 %)						Liquor Value (60 %)					
Shape & make	Pts	Color	Pts	Odor	Pts	Acidity	Pts	Body	Pts	Flavor	Pts
v. good	15	Bluish	15	Clean	10	Pointed	20	Full	20	v. good	20
Good	12	Grayish	12	Trace	8	Medium/p	15	Med./F	15	Good	15
Fair/ Aver.	8	Greenish	8	Light	5	Medium	10	Med.	10	Aver.	10
Mixed	5	Faded	5	Moderate	2	Light	5	Light	5	Fair	7
Small	2	Brownish	2	Strong	1	Lacking	2	thin	2		

Class Range: 1 = 81-100, 2 = 61-80, 3 = 41-60, 4 = 21-40 or 1-2 defective cups, 5 = < 20 or more than 2 defective cups (defective cups: foul, earthy, chemical).

The Influence of Shade During Fermentation Stage of Wet Processing on the Cup Quality of Arabica Coffee

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SUMMARY

A trial was conducted to test the effect of shade during fermentation on cup quality characteristics of arabica coffee. Three CBD resistant lines (741, 74110 and 74165) were wet processed following the normal procedure and fermented under *Corrugated iron sheet shade* and *no shade* treatments. Fermentation period was significantly ($P < 0.05$) affected by the interaction between shade and variety. Variety 74165 fermented under shade had the longest fermentation period (40 hrs), while variety 74110 fermented without shade had the shortest fermentation period (25 hrs). On the other hand, no clear association between fermentation period and cup quality was observed. However, better cup quality value was recorded for variety 74165, while variety 74110 had relatively lower value. Despite such numerical variations in the values of cup quality evaluation, all varieties belonged to the same quality class range (class three).

INTRODUCTION

Ethiopia is the second largest coffee producer in Africa and the coffee crop accounts for about 5% of the GDP, 12% of the total agricultural output, 15% of the employment and more than 60% of the foreign exchange earnings (Coste, 1992). Due to its remarkable economic significance to the country, the volume of washed coffee production has increased tremendously especially in recent years. Currently the international market for coffee is highly competitive and hence keeping the maximum possible quality standard has become a necessity.

Cup quality is a complex characteristic which depends on a series of factors such as the species or variety (genetic factors), environmental conditions (ecological factors), agronomical practices (cultivation factors), processing systems (post harvest factors), storage conditions, industrial processing, preparation of the beverage and taste of the consumer (Moreno, et al., 1995).

Coffee produced following the wet method of processing is known to have superior quality characteristics and hence commands a higher market price. Each step in the wet method of processing (Figure 1) is critical in maintaining the inherent quality of the coffee variety being processed.

Fermentation is one of the most important stages in the wet method of coffee processing. Fermentation stage or “mucilage removal” by means of a biochemical reaction or hydrolysis of the mucilage which covers the parchment beans is a reaction caused by enzymes (pectinases & pectase) which are naturally present in the coffee cherry (Wintgens, 2004). Mucilage is insoluble in water and clings to parchment too strongly to be removed by simple washing; but it can be removed by fermentation followed by washing or by strong friction in

machines called mucilage removers. Fermentation may be natural or accelerated by chemicals or enzymes. Natural fermentation may be dry (with out water) or under water.

The importance of fermentation process in regard to the development of coffee quality is undeniable but the duration of fermentation process is very variable. It depends on the principal elements involved, such as the volume of the mass of beans, the species or variety of coffee, the maturity of the fruits, water used, potential natural enzymes, the ambient temperature and altitude (Coste, 1992; Kenya coffee, 43:513).

The role played by microclimate on Ethiopian coffee fermentation procedure is quite inconsistent across locations and among heterogeneous coffee lines in the vast coffee growing areas of the country (ULG, 1986). The problem is thought to depend heavily on environmental factors and the inherent characteristics of coffee lines which show different quality potential for various reasons.

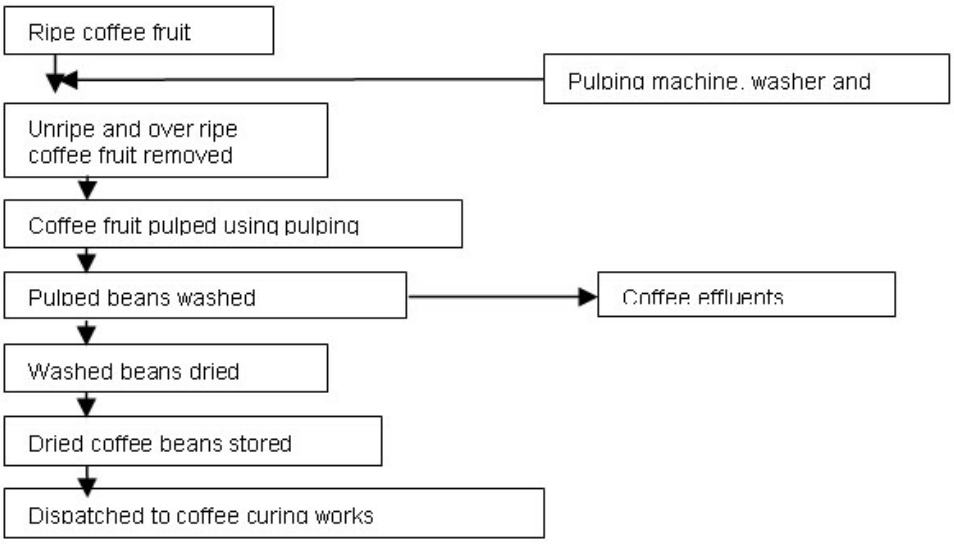

Figure 1. Schematic representation of "wet" coffee processing.

This experiment was therefore carried out to determine the effect of shade on fermenting coffee, and to evaluate the quality of some coffee cultivars under controlled microclimate.

MATERIALS AND METHODS

The experiment was carried out using split plot design with shade level as main plot and three CBD resistant coffee varieties (741, 74110 and 74165) as sub-plot treatments. Two sets of concrete tanks (25 cm x 30 cm x 40 cm) were constructed under shade and in the open. 6 kg red ripe cherry from each cultivar was pulped by hand (disc pulper) and subjected to ferment under normal conditions. The samples were then washed and dried to a moisture content of 11%.

Sensorial evaluation of cup quality was accomplished by a panel of liquorers. Scoring values used in coffee classification report and commercial descriptive standards were used to determine and categorize coffee quality. Analysis of variance was also employed where appropriate to determine treatment differences.

Cup quality evaluation consisted of raw (40%) and liquor (60%). Raw value was evaluated as shape & make, color, and odor. Liquor was also evaluated as acidity, body and flavor. Finally

coffee quality data were collected and analyzed accordingly. The details of the sensorial evaluation procedure are given in Annex 1.

RESULTS AND DISCUSSION

Fermentation period was significantly ($P < 0.05$) affected by the interaction between shade and variety (Table1). Variety 74165 fermented under shade had the longest fermentation period (40 hours), while variety 74110 fermented without shade had the shortest fermentation period (25 hours). From the data obtained, positive association between fermentation period and cup quality was observed (Figures 2 and 3). This might be due to uniformity of fermentation under shade than the unshaded treatment.

Under Jimma condition, mucilage normally takes between 24 to 40 hours to become completely removed from the bean (Woelore, 1993). It is generally believed that, within this range, the longer the coffee is left in fermentation tanks, the better the final quality, provided the development of taints is avoided (Wootton, 1963).

Though there wasn't any change in the final quality class as a result of the interactions or the main effects (Table 2), a positive correlation between fermentation period and cup quality was observed. Variety 74110 had the shortest fermentation period (25.5 hrs) and was associated with the lowest cup quality value compared to variety 74165 that had 34.96 hrs of fermentation period and the highest cup quality value (Tables 1 and 2).

On the other hand, strong varietal difference has been noticed for cup quality. Hence, better cup quality value was recorded for variety 74165, while variety 74110 had relatively lower value (Table 2). Despite such numerical variations in the values of cup quality evaluation, all varieties belonged to the same quality class range (*class three*).

The main effects were also statistically significant ($P < 0.05$) for fermentation period. Among the three varieties, variety 74165 had the longest fermentation period (34.94 hours), and variety 74110 had the shortest (25.5 hours). Fermentation under shade took 33.06 hours as compared to 28.25 hours for fermentation with out shade. This result agrees with previous reports that the variety of coffee and microclimate are among the factors that affect the duration of fermentation process (Coste, 1992; Kenya coffee, 43:513).

CONCLUSION AND RECOMMENDATION

Shade by variety interaction was highly significant for fermentation period. Hence variety 74165 fermented under shade treatment had the longest fermentation period (40 hrs). The same variety (74165) fermented with out shade had shorter period of fermentation (29.92 hrs). Despite the difference in the *fermentation period* of the variety, no noticeable variation in the final cup quality class was obtained. However, positive association between fermentation period and cup quality was observed (Figures 2 and 3). Shade during fermentation might help to create a uniform condition for fermentation process and hence improve the cup quality characteristics. Therefore, using shaded fermentation tanks might help to achieve uniform fermentation process and better quality coffee.

On the other hand, existence of varietal difference in cup quality has been noticed. Among the three varieties studied, 74165 had a relatively better cup quality than the other varieties. Therefore, apart from good processing practices, choosing varieties that are known for their quality attributes will be equally important for coffee producers.

In general, variety 74110 had the shortest fermentation period and relatively lower cup quality. Variety 74165 had the longest fermentation period and relatively higher cup quality value. Despite such differences, mean values of cup quality evaluation from all the treatments fall within similar quality class range.

Table 1. The interaction effect of shade and variety on fermentation period (in *hrs*).

Shade	Variety			*Shade means*
	V1 (741)	V2 (74110)	V3 (74165)	
Shaded	33.17	26.00	40.00	33.06
Unshaded	29.83	25.00	29.92	28.25
Variety means	31.50	25.5	34.94	

CV% = 8.37; $LSD_{0.05}$ = 2.112.

Table2. Mean values of cup quality evaluation for different varieties and shade treatments.

Shade	Variety			*Shade means*
	V1 (741)	V2 (74110)	V3 (74165)	
Shaded	53.75	46.50	55.42	50.22
Unshaded	52.75	43.50	53.42	49.89
Variety means	53.25	45.00	54.41	

Quality Class Range: 1 = 81-100, 2 = 61-80, 3 = 41-60, 4 = 21-40 or 1-2 defective cups, 5 = 20 or more than 2 defective cups (defective cups: foul, earthy, chemical).

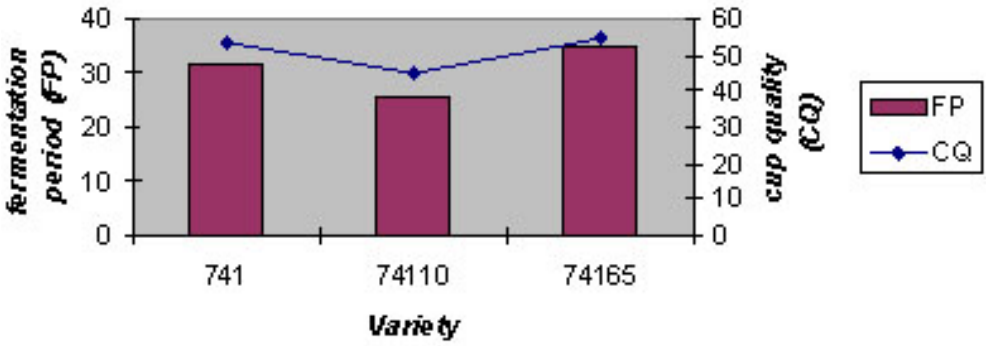

Figure 2. Effect variety on fermentation period and cup quality.

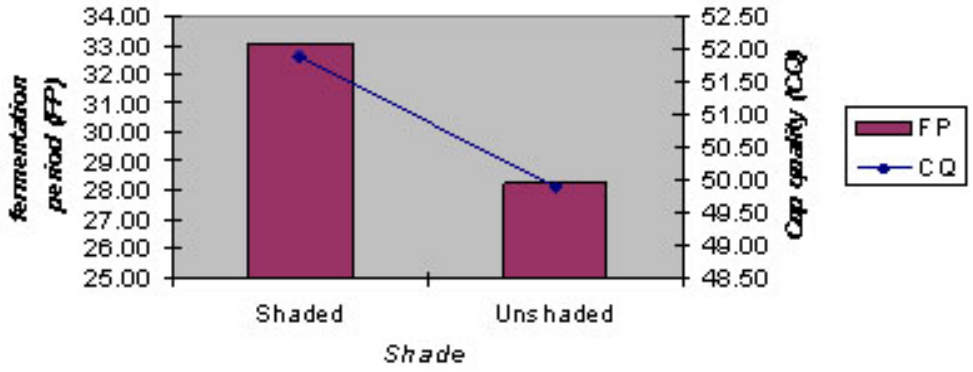

Figure 3. Effect of shade during fermentation on fermentation period and cup quality.

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Annex1. Sensorial evaluation of arabica coffee quality (raw & liquor).

Raw Value (40%)						Liquor Value (60%)					
Shape & make	Pts	Color	Pts	Odor	Pts	Acidity	Pts	Body	Pts	Flavor	Pts
v. good	15	Bluish	15	Clean	10	Pointed	20	Full	20	v. good	20
Good	12	Grayish	12	Trace	8	Medium/p	15	Med./F	15	Good	15
Fair/ Aver.	8	Greenish	8	Light	5	Medium	10	Med.	10	Aver.	10
Mixed	5	Faded	5	Moderate	2	Light	5	Light	5	Fair	7
Small	2	Brownish	2	Strong	1	Lacking	2	thin	2		

Class Range: 1 = 81-100, 2 = 61-80, 3 = 41-60, 4 = 21-40 or 1-2 defective cups, 5 = 20 or more than 2 defective cups (defective cups: foul, earthy, chemical).

Effect of Sorting and Soaking on Coffee Robusta during Delayed Post-harvest Treatment in Côte d'Ivoire – Preliminary Results

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SUMMARY

The coffee trade on the international market requires, in addition to the criteria such as water content, new norms of qualities such as the mycotoxin level, in particular the ochratoxine A (OTA) that presents a risk for the human and animal health. Its high level consumption can have serious consequences on vital organs as the kidneys and the liver. OTA is a chemical substance from obtained from some moulds metabolism such as *Aspergillus ochraceus*. Regularly consumed at a weak level (chronic poisoning), ochratoxine A can be source of cancer. Tolerated level in coffee have been fixed to a maximum of 10 µg/kg of green coffee and 4 µg/kg of roasted coffee. Delayed treatment of coffee cherries after harvest is a current practice in Cote d'Ivoire. Coffee cherries are kept in jute bags or in heap several days before drying. This practice constitutes an important source of OTA contamination. In order to reduce the level of contamination, research works have been carried out in the objective to study the delayed treatment impact on OTA contamination. Interactions between different factors of post harvest treatment have been studied. Results showed that either the applied treatment, OTA level in green coffee remains superior to the tolerated levels. However coffee sorted by floating and kept by immersion, would reduce the OTA contamination in green coffee.

Key words: coffee, OTA, moulds, *Aspergillus ochraceus,* Cote d'Ivoire

INTRODUCTION

The marketing of the agricultural produce lets appear nowadays, of new constraints related to the sanitation quality. Regarding African countries, agricultural produce of export are dominated by coffee for some, coffee and the cocoa for others, palm oil, peanuts, cotton, rubber trees, banana, pineapple…

Coffee samples taken in Europe and coming from West and East Africa (OIC, 1996), revealed rates of contamination in ochratoxin A respectively of 9% and of 18%. For some countries of the same areas, the rate of contamination would be up to 25%. These high rate of contamination affect the quality of the marketable coffee and would represent significant losses of currencies. For health reason and in order to protect consumers, European Union proposed a limit of 8 and 10 µg/kg in the raw coffee and 3 to 4 µg in the torrefied coffee. The application of these standards would generate in the case of Côte d'Ivoire, the refusal of 54.000 tons of raw coffee, that is to say a loss of more than 20 billion francs CFA. The coffee rejections coming from Côte d'Ivoire were already recorded in Italy and Spain.

Coffee constitutes the base of Côte d'Ivoire economy. Coffee rejections will not be beneficial for this country if the proposed rates fixed by the European Union were adopted. In the same time, certain batches of coffee coming from the various producer countries show rates of OTA higher than 10 ppb.

The scientific committee of the human consumption, in his report of September 17, 1998, estimated necessary, taking into account the harmful effects of the mycotoxins on human health, to take care on the contents these toxins and in particular the content of OTA in the coffee and certain foodstuffs. According to this report, it would be advisable to reduce as much as possible the content of OTA in the coffee to 5 ng/kg P.c/day.

Several different post harvest treatments are used around the world. The dried and the wet processes are wildly used. However, for several reasons, these schemes are not followed by all producers. There are some traditional techniques used by farmers in order to save time during drying.

Keeping fresh harvested coffee cherries in sacks or heaps for several days before the proper drying, is a common practice of coffee (Robusta) producers in Côte d'Ivoire. This is in part due to a lack of drying spaces or available transportation, and in part is the belief that delayed processing can reduce drying times and give better coffee quality.

Research activities carried out according to Franck (2001), Samson and Pitt (1985), at the CNRA Research Station in Bingerville, in order to evaluate the effects of delayed processing, the type of the dryers, the stirring and the load of coffee cherries to be dried, on coffee quality, showed that delay between harvesting of coffee cherries and the beginning of drying increases the risk of OTA contamination in green coffee. Delayed processing may also have negative impact on the coffee quality in term of increasing moisture content and hence microbial growth and OTA production.

According to Juarez de Sousa e Silva (Hygienic coffee processing

Professor of Agricultural Engineering Federal University of Viçosa juarez@ufv.br), keeping fresh harvested coffee cherries under clean' and renewed water instead of in sacks or on heap, reduce the risk of moulds infection and OTA contamination.

This research work was carried out in order to test the hypothesis that keeping fresh harvested coffee cherries under water, reduce the risk of moulds infection and OTA contamination

MATERIALS AND METHODS

Fresh harvested coffee cherries (robusta) from National Agronomic Resaerch Center of oe d'Ivoire (CNRA) were immersed in pans previously filled with water. By hydraulic separation, in function of the density, the perfect cherries were separated from the coffee that floats. In order to study the impact on OTA level in coffee, six samples were obtained and were analyzed.

1. Unsorted coffee cherries in 4 days delayed processing
2. Sorted coffee cherries in 4 days delayed processing
3. Sorted fresh coffee cherries 0 days delayed processing
4. Unsorted fresh cherries 0 days delayed processing
5. Immersed, unsorted cherries 4 days delayed processing
6. Immersed sorted cherries 4 days delayed processing

Water was renewed every 24 hours. Cherries were dried on bamboo table at a load of 25 kg/m^2 After OTA extraction and purification, OTA levels were carried out by HPLC (SHIMADZU)

Reagents

- Standard: OTA, 100 ng/ml (R. BIOPHARM)
- Column: Immun0-affinity column (R. BIOPHARM)
- Buffer: PBS (R. BIOPHARM)

Method performance

- Linearity: 1ng / ml to 40 ng / ml
- Repeatability: Coefficient variation (CV) for 10 ng/ml and 25 ng/ml concentrations were respectively 0,71 % and 0,65 %
- Precision: 95,22 %
- Detection limit.: 0,1 ng/ml
- Stationary phase: column Spherisorb C 18 S5 ODS 2,5um (25 cm _4,6 mm) with precolumn
- Mobile phase: acetonitrile-water-acid glacial acetic acid (55: 43: 2)
- Flow: 1 ml / mn in isocratic
- Fluorescence detector: excitation 333 nm; emission 460 nm
- Injection volume: 20 µl
- Analysis time: 12 minutes

OTA QUANTIFICATON (ng/g):

C = Ss x Zstd / Sstd x V/ m ou (v) x Vext / Va x V1 / V2
C: OTA concentration (µg / kg or µg / l in samples
S: OTA pic surface in samples
S STD: OTA pic surface in standard solution
Z STD: OTA concentration (µg/l) in standard solution
V: volume of solvant (3 ml)
M: Analysed sample weight (g)
V: Analysed sample volume (ml)
Vext: Extraction solvent volume (ml)
VA: volume (ml) after extraction
V1: Sample volume (ml) for purification
V2: diluted sample volume (ml) and purified in the column

PRELIMINARY RESULTS

The OTA level in the unsorted coffee cherries in 4 days delayed processing is 2,15 ng/g whereas it is 0,45 ng / g in the sorted coffee cherries in 4 days delayed processing.

OTA level in sorted fresh coffee cherries in 0 days delayed processing is inferior to the detection limit which is 0,1 ng/ml OTA level is approximately 0,215 ng/g. in the unsorted fresh cherries 0 days delayed processing compared to the immersed unsorted and sorted samples which present an OTA contamination les than 0,1 ng/g. after 4 days delayed processing.

COMMENTS

Coffee cherries present a variety of active microflora comprising yeasts (Saccharomycopsis, Candida and Pichia…genus), moulds such as *Fusarium*, *Penicillium*, *Aspergillus (*Niger, *Carbonarius, Ochraceus*…), saprophyte bacteria comprising Gram-Positives bacteria such as

Bacillus, Cellulomomas and Gram-Negative bacteria such as Pseudomonas and Enterobacter detected on coffee cherries, in the mesocarp, inside the beans, in earth, air**,** tree steams…

Interactions between biological factors, chemical factors and also the post-harvest quality handling (fungi, substrate, Aw, dryers, load, stirring, storage, transport, …) participate into OTA contamination. Previous research activities have revealed that the source of OTA contamination is still unknown (GCP/INT/…). However, keeping coffee cherries in immersion reduces all these interactions and contributes to prevent OTA contamination.

OTA level is low in the sorted coffee cherries in delayed processing and in fresh harvested coffee cherries dried immediately after harvesting. No OTA was found in the immersed coffee cherries at a detection limit of 0,1 ng / ml. The weak rate of OTA in the immersed samples could be due to the slowing of metabolic reactions from the microorganisms. Sorted or unsorted, OTA is not detected in the coffee cherries. However, it is known that defects beans are niches of fungi and also sources of OTA contamination. Sorting and soaking should reduce the risk of mold growth and OTA contamination. The constraint of this method is water disponibility in rural areas

CONCLUSION

Coffee sorting and soaking could have positive effects on coffee quality. The risk of OTA contamination by sorting and soaking is less than in the common delayed practice. Further studies will be carried out in order to find out the real impact of the immersion technique on coffee OTA contamination during delayed processing. Also, a comprehensive research work should be done to detect the real point of infection in order to cease or to reduce the possibility of OTA contamination in coffee cherries.

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Comparative Fungal Profile in Wet Primary Processing Methods of *Coffea canephora* Pierre in Nigeria

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SUMMARY

In line with the recent focus at preventing mycotoxin contamination of commodity crops in the export market, an experiment was initiated at the Headquarters of the Cocoa Research Institute of Nigeria (CRIN) to determine fungal profile in wet processing methods of robusta coffee. From the five methods evaluated eight fungi species were isolated, three of these: *Aspergillus niger* Van Tieghen, *Aspergillus ochraceous* Wilhelm and *Fusarium* sp. Link have been previously implicated as mycotoxigenic fungi. *A. niger* was the most frequently isolated (0.73) followed by *Fusarium* sp. (0.11) and *A. ochraceous* with the least value of 0.02 from all the evaluated methods. *A. ochraceous* was isolated from only two wet-processing methods. The unfermented-washed method had the least overall fungal isolates compared with other methods.
Key words: Coffee, fungal profile, wet processing method, mycotoxigenic fungi

INTRODUCTION

Coffee is an important export crop traded globally. In Nigeria, Coffee is cultivated extensively in more than 20 states (Williams, 1989; Filani, 1989). The presence of mycotoxins produced by some fungi in coffee is undesirable because it may be used as a trade barrier. This could be as a result of primary processing method among other things (Studer-Rohr et al., 1994). Farmers in Nigeria prefer dry primary to wet-processing method because it is cheap, simple and does not require any formal training whatsoever (Adenikiju et al., 1989), however the quality produced is considerably low compared to the wet method. Nonetheless there is variation in wet processing methods which could consequently affect the quality of the coffee (Rene, 1989). This study therefore determined the fungi profile in five wet processing methods of robusta coffee in order to ascertain the presence or otherwise of mycotoxigenic fungi.

MATERIALS AND METHODS

This study was conducted at the headquarters of the Cocoa Research Institute of Nigeria (CRIN) Ibadan, between November-December, 2005.

Matured, ripened berries of robusta coffee were selected from the lot. The process was varied to give rise to five treatments viz:

1. Unfermented- unwashed: Selected coffee berries were de-pulped, without washing dried in the sun for 8 days to obtain 10% moisture content.
2. Unfermented- washed: The process was the same as in (Adenikiju et al., 1989) above except that the samples were washed after de-pulping before sun dried.

3. Basket fermented: Selected berries were de-pulped, the beans were than put in the basket, covered with polythene sheet to ferment for 24 hours, then washed and sun dried.
4. Under-water unwashed: Selected berries were de-pulped, the parchment beans were put inside (soaked in) water for 24 hours and later sun dried.
5. Under-water washed: The process was the same as in (Essien, 2000) above except that the parchment beans were washed after soaking for 24 hours and then sun dried.

DETERMINATION OF FUNGAL PROFILE

To determine fungal profile, a modified method of (Oyeniran, 1977) was used. A sample of ten beans per treatment was macerated in a blender with 90ml of sterile watery (0.2%) agar using a high-speed homogeniser. Series of dilution were then prepared and 1ml each was used to inoculate 10 ml, cooled (45 °C) molten Czapecks agar inside 9 cm Petri dishes. Each treatment was replicated thrice and incubated for 3 days. The colonies of fungi were recorded and identified while their frequency of occurrence were determined.

RESULTS AND DISCUSSION

As shown in Table 1, eight different fungal species were isolated, three of which: *Aspergillus niger*, *A. ochraceous* and *Fusarium* sp. are mycotoxigenic fungi (CABI, 2001; Bankole, 1994; Essien, 2000; Studer-Rohr et al., 1994; Taniwaki et al., 1999). *A. niger* and *Fusarium* sp. were frequently isolated from all the samples while *A. ochraceous* was isolated only from unfermented-unwashed samples. Analysis of variance further revealed that *A.niger* was prevalent in all the samples (0.73), however the least value (0.58)(Table 2) was obtained in unfermented washed method

Table 1. Occurrence of mycoflora in different wet processing methods.

	Fungi isolates*							
Processing methods	An	Af	Ao	Fs	Ms	Pi	Ts	Lt
Unfermented-unwashed	+**	–***	+	+	+	+	–	–
Unfermented-washed	+	–	–	+	–	–	+	+
Basket-fermented	+	–	–	+	–	–	–	–
Underwater-washed	+	–	–	+	–	–	–	–
Underwater-unwashed	+	+	–	+	–	–	–	+

An = Aspergillus niger, Af= Apergillus fumigatus, Ao= Aspergillus ochraceous, Fs = Fusarium sp., Ms = Mucor sp., Pi = Phytophthora infestans, Ts = Trichoderma sp., Lt = Lasiodiplodia theobromae; ** + = Present; * – = Absent*

This study has revealed that proliferation of mycotoxigenic fungi can be minimized through unfermented- washed wet-processing of coffee berries as only 0.58 of *A..niger*, 0 of *A. Fumigatus*, 0 of *A. ochraceous* and 0.02 of *Fusarium* sp (Table 2).

Table 2. Frequency of occurrence of mycoflora in different wet processing methods.

Fungal isolates	Unfermented unwashed	Unfermented washed	Basket fermented	Under water unwashed	Under water washed	Means
Aspergillus niger	0.67	0.58	0.89	0.67	0.82	0.73a
A. fumigatus	0	0	0	0.14	0	0.03c
A. ochraceuos	0.08	0	0	0	0	0.02c
Fusarium sp.	0.11	0.02	0.08	0.14	0.17	0.11b
Mucos sp.	0.04	0	0	0	0	0.01c
Phytophthora infestans	0.06	0	0	0	0	0.01c
Trichoderma sp.	0	0.28	0	0	0	0.06
Lasiodiplodia theobromae	0	0.15	0	0.06	0	0.04c

Means followed by the same alphabets are not significantly different at P < 0.05 by Duncan's Multiple Range Test (DMRT).

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Recent Developments in Soluble Coffee and Coffee Based Beverages Technology

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SUMMARY

Soluble coffee has been an important and profitable product for years. Even so in mature markets consumers are shifting more and more towards coffee based beverages (like cappuccini or café au lait). Due to the variability in appearance (spray dried, freeze dried, shiny flakes etc.) and the importance of yield when extracting, soluble coffee is much more technology driven than roasted coffee. When it comes to coffee based beverages additional physico-chemical aspects play a role. For example, the composition of an otherwise perfect dairy or non-dairy creamer may not be a good match for some soluble coffees regarding dissolution kinetics and local acidity, resulting in a rather unpleasant flocculating product. For those who are not insiders to soluble coffee production this presentation first introduces to the technological background of soluble coffee production. In addition to conventional technology, recently published patents on technology and new coffee and coffee based beverages are summarised. In the second part of the presentation some examples are given about how business drivers influence new developments in technology and why some of these developments have not been successful on the markets.

INTRODUCTION

Soluble coffee has been an important and profitable product for years. In mature markets consumers are shifting more and more towards coffee based beverages (like cappuccini or café au lait) and liquid (canned or dispensed) coffee products.

Soluble coffee is much more technology driven than roasted coffee due to the variability in appearance (spray dried, freeze dried, shiny flakes etc.) and the importance of yield when extracting roasted coffee beans. When it comes to coffee based beverages additional physico-chemical aspects play a role. For example, the composition of an otherwise perfect dairy or non-dairy creamer may not be a good match for some soluble coffees regarding dissolution kinetics and local acidity. A wrong combination can therefore result in a rather unpleasant flocculating product.

Publications on new soluble coffee technology from research institutes are rare. More information can be gathered from patents or equipment manufacturers. Both sources need a thorough check for valid information. Research in itself is already an investment and implementing new technology into factories even more. Therefore a connection between changes in the coffee market and the overall economic situation and research activities should be detectable.

High green coffee beans prices should foster development of better extraction technologies to maximize profit from increased extraction yield. In the same way the relation between Arabica and Robusta bean prices should for unfavourable Arabica prices increase the demand for technologies that diminish differences in taste and aroma. One of these technologies is

steam treatment of Robusta beans to reduce typical Robusta taste (U.S. Pat. No. 5,019,413). Figure 1 shows, how the ratio of Arabica to Robusta prices influence the return on invested capital for an installation requiring two Million $ US in investment.

This is an example of a nearly perfect timing. The patent was granted in 1991, from 1995 on the price for Robusta was falling, while Arabica green coffee beans continued to rise, and stayed high in price till 1999. At the same time interest rates (Bank of England) dropped from nearly 15 % in 1990 to below 6 % in 1993.

RECENT DEVELOPMENTS

Conditions were still favourable for an additional hydrolization step to increase yield during extraction (U.S. Pat. No. 5,897,903 filed in 1997) with low cost of capital for an investment. The improved aroma recovery to keep or even improve aroma levels to compensate for the higher yield (U.S. Pat. No. 6,592,922 filed in 2002) follow suit.

A lot of developments in texturization were finalized during the period from 1991 to 1999. They were ranging from extrusion of SD coffee (U.S. Pat. No. 5,474,792 (1992) and prior patents), and one step processing from conventional evaporator (about 50 % TC) to powder by means of thin film evaporator (U.S. Pat. No. 5,035,908 (1991)) to processing of green beans without roasting (U.S. Pat. No. 5,972,409 (1999)) therefore creating the roasting aroma during the final texturization step. All these processes are meant to substitute a costly process (e.g. freeze drying) by something less expensive (e.g. spray drying followed by extrusion). By the time the patents were filed coffee beans were expensive, while cost of capital was dropping to extremely low rates. Apart from the uncertainties of consumers accepting changes in final product textures the financial pressure of investing into a new process, with all its intrinsic difficulties, ceased.

While activities on texturization came more or less (WO2004034798) to a halt, patents on coffee based beverages are frequent.

A typical problem that occurs in reconstitution of mixtures from coffee and milk is a flocculation of milk proteins. This is due to the local acidity around a soluble coffee grain when dissolving. The flocculation is depending on the water temperature as well and increases with temperature. When coffee and milk are not dry mixed but wet mixed before spray drying the flocculation is even more severe. One way to hide this effect is to pre-flocculate proteins before spray drying in a way that very fine aggregates are created that are close to being invisible in the final product (US Pat. No. 5,620,733 (1997)).

An other way to get around this problem is to use non dairy creamer or whitener based on an oil/water emulsion for the whitening effect of milk as described in US Pat. No. 6,277,429 (2001).

Apart from reconstitution of powders re-diluting canned coffee or coffee concentrate is a way to produce ready-to-drink coffee beverages. For dispensing usually the freshly extracted concentrated coffee is rapidly frozen and transported from the factory to the vending machine in a frozen state and kept refrigerated in the vending machine to maintain the rich aroma and taste of coffee.

New developments are room temperature storage and provision of customized varieties and strengths of fresh-brewed coffee on demand (US Pat. No. 6,808,731 (2004)).

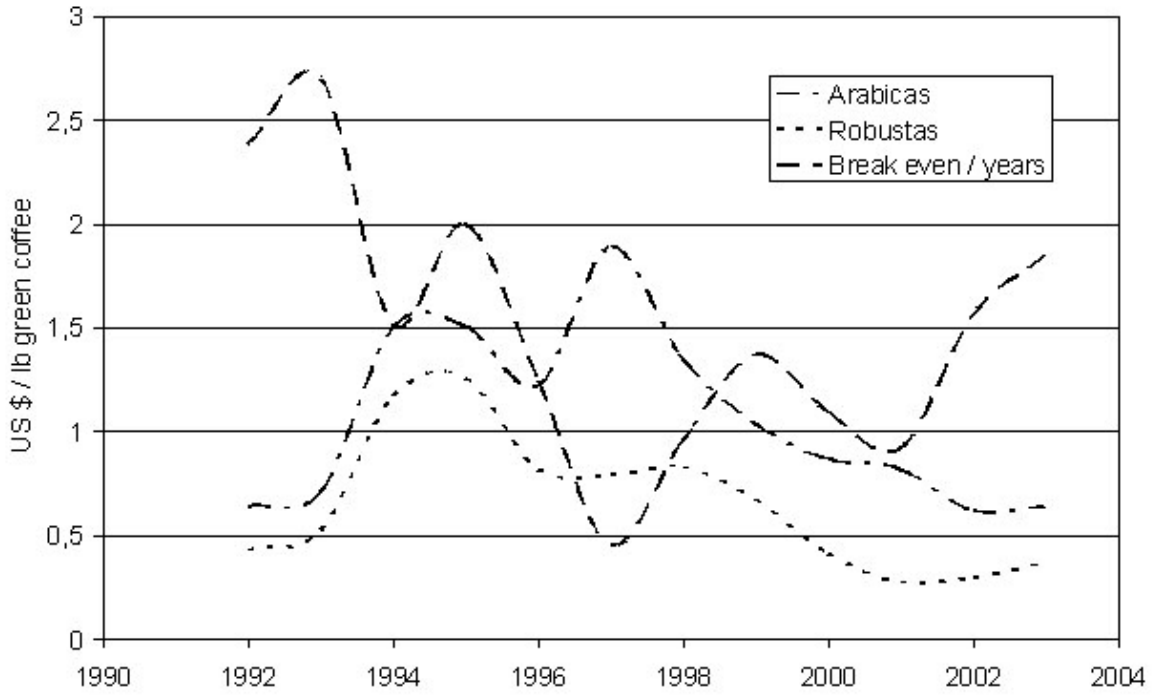

Figure 1. Ratio of Arabica to Robusta prices between 1992 and 2003 and its influence on return on investment or time for break-even based on a two Million $ US investment and a capacity of 10000 tons/year (cost of capital not included).

A new controlled-release technology was developed using novel encapsulated buoyant water-immiscible volatile carrier liquids (VCLs) to strengthen beverage preparation aroma. This method can be used to capture natural coffee aroma frosts in VCLs to further enhance the quality of high-impact coffee aromas (US Pat. No. 6699518 (2004); EP1 353566 (2005)).

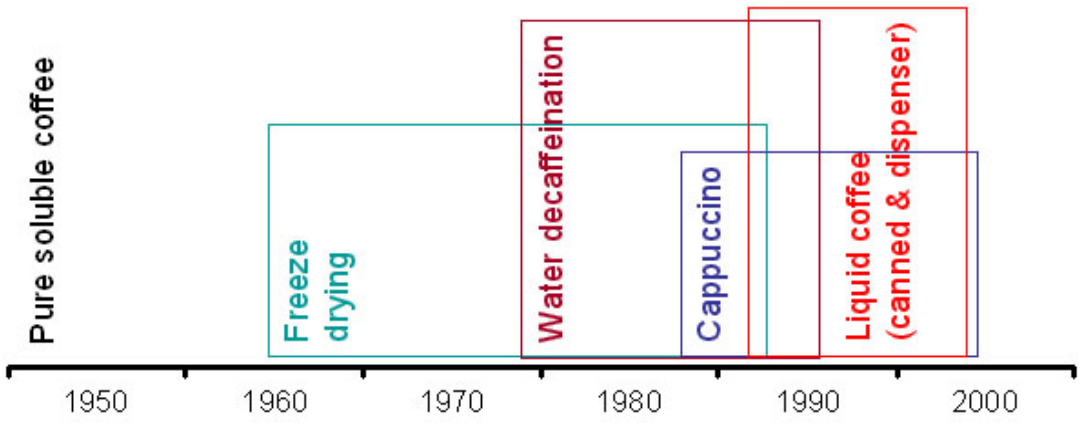

Figure 2. Period of intensive patent filing for selected topics.

The filing of patents indicates the time span of major activities in a certain area. Figure 2 gives four examples for patents in the coffee and coffee based beverage area. While activities on freeze drying and de-caffeination have come to a rest, patents on Cappuccino-style beverages as well as on liquid coffee continue to be filed. Even though these trends have continued over more than ten years, a new trend is not visible.

A renewed interest in a former field of activity is rarely seen clearly due to use of expired patents.

In general patents in soluble coffee and related beverages have moved from process and technology driven patents towards product or product class specific activities, often strongly related to taste.

OUTLOOK

Roasting green coffee, a process that moved to shorter roasting times for a long time, has been limited lately by the exploration of acrylamid formation during short time/high temperature roasting. In the area of coffee extraction and aroma recovery work will continue until the theoretical limit is achieved, while for texturization the limits are set by marketing. A recently published comparison (Stiftung Warentest 7/2006) shows that there is room for improvement on Cappuccino foam, but that there are clearly differences in quality already. Here the question is rather if the consumer is willing to pay for investment in new installations and for high quality raw materials.

Modeling Exothermic Heat Generation during the Roasting of Coffee

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SUMMARY

Exothermic heat generation during coffee roasting was modeled as a single, first-order reaction with a rate coefficient governed by the Arrhenius equation. With best-fit values for parameters, E and A_q, heat-generation rates calculated with the resulting equation, Eq [2], agreed fairly well with rates measured by DSC by Raemy (1981). Improved agreement with Raemy's rate data was obtained by similarly modeling four parallel reactions, i.e. by using Eq [6]. Results calculated with Eq [6] for typical temperature versus time behavior for roasting bean show that the exothermic reactions producing the most heat are far from complete at the end of normal roasting; but others with higher E and/or A_q essentially go to completion. Raemy's DSC test method prevented evaporative cooling from partially cancelling exothermic heating, but probably also created an abnormal reaction environment. DSC tests carried out with suitably predried coffee beans should provide a more normal environment and provide data permitting better modeling.

RÉSUMÉ

J'ai modélisé le chauffage exothermique pendant la torréfaction du café comme une réaction chimique de ordre 1 régie par l'équation d'Arrhénius. Avec le modèle, Eq [2] de cette étude, et les valeurs les meilleures des paramètres E et A_q, j'ai obtenu un rapport assez bon entre les vitesses de chauffage calculées et les vitesses des données DSC de Raemy (1981). J'ai obtenu un meilleur rapport avec Eq [6], un modèle semblable pour quatre réactions faites parellèment. A la fin des torréfactions, les réactions avec des niveaux élevés de E et A_q sont assez complètes. La fin des autres réactions n'est que partielle, mais celles-ci produisent le plus de chaleur. La méthode d'essais DSC de Raemy a empêché l'évaporation de l'eau des grains et la nullification partielle du chauffage exothermique par la refroidissement à cause de cette évaporation. Mais probablement sans cette évaporation le milieu de réaction est anormal. Donc, si on voudrait modéliser avec succès le chauffage exothermique pendant la torréfaction du café, it faut faire usage des données des essais DSC executées de nouveau dan des conditions qui fournissent un milieu semblable au milieu actuel de réaction exothermique de la torréfaction.

INTRODUCTION

I started this work to provide subroutines for computer programs (Schwartzberg, 2002; 2004) used to predict coffee bean temperature, T, versus time, t, behavior during roasting. In these subroutines, I calculated rates of exothermic heat generation by assuming such heating could be modeled as a single, first-order reaction with a rate coefficient, k, governed by the Arrhenius Equation. I further assumed that the initial amount of reactant was proportional to Q_T, the total amount of heat the reaction can produce, and that the amount of reactant remaining at time t was proportional to $(Q_T - Q_R)$, where Q_R is the amount of heat generated

up to t. Thus, $(Q_T - Q_R)/Q_T$ is the fraction of reactant remaining at t. From the Arrhenius equation, k expressed in kW/kg is

$$k = A_q \exp(-E/T) \quad [1]$$

where $E = H_A/R$, H_A is the enthalpy of activation for the reaction, R is the perfect gas law constant, T is the bean temperature in degrees K and A_q is the Arrhenius equation prefactor (in sec^{-1}) times a heat generation factor. Thus A_q has the dimensions (kW/kg coffee); and dQ_R/dt, the specific rate of heat generation, is

$$dQ_R/dt = k\,[(Q_T - Q_R)/Q_T] = A_q \exp(-E/T)[(Q_T - Q_R)/Q_T] \quad [2]$$

Though many reactions occur during roasting, I thought (hoped) that use of Eq [2] might describe those causing exothermic heating accurately enough for engineering purposes. To use Eq [2], I had to obtain suitable values for A_q, E and Q_T.

Raemy (1981) used differential scanning calorimetry (DSC) to measure exothermic heating in green, Mexican Arabica coffee beans when dT/dt = 1 K/min. He measured Q_T and dQ_R/dt versus T while applying 20-25 bar of pressure by Argon to suppress evaporation of water from the beans. Thus, unlike some other thermal analysis data for coffee (Baltes, 1977; Eggers and von Blittersdorff, 2005), evaporative cooling did not partially cancel exothermic heating. His Figure 3 is a plot of dQ_R/dt versus T for the exotherm. I calculated dQ_R/dt and Q_R versus T values from his Figure 3, and used those values to determine E and A_q.

PROCEDURE

I enlarged Raemy's Figure 3; drew a base line connecting the 140 °C and 230 °C ends of the exotherm and vertical lines from the base line to the dQ_R/dt curve at 5°C intervals starting at 140 °C. Then I measured L_m, the lengths of these lines, with a precision ruler. Based on the trapezoid rule

$$Q_T = 0.5K(\_T)/(dT/dt)\Sigma(L_m + L_{m-1}) \quad [3]$$

with Σ summed from m = 2 to m = 19. L_1 is L_m at m = 1, i.e. at T = 140 °C and L_{19} is L_m at T = 230 °C. (dT/dt) = 1 °C/min = 0.01667 °C/sec, the temperature rise rate used in Raemy's test. (ΔT) = 5°C is the T interval between m and $(m - 1)$. $K \equiv (dQ_R/dt)_m/L_m$. Thus $(dQ_R/dt)_m = KL_m$. Raemy's Q_T ranged from 250 to 375 kJ/kg, a rather large range. I used Q_T = (250 + 375)/2 = 312.5 kJ/kg. I substituted Q_T and the measured L_m in Eq (3) and solved it to obtain K. I then obtained a set of $(dQ_R/dt)_m$ values from corresponding KL_m. $(Q_R)_m$ values for T_m = 140 °C to 230 °C were obtained by replacing Q_T in Eq [3] with $(Q_R)_m$ and using an upper summation limits, m, < 18. This approach was used because Figure 3 had a dQ_R/dt axis with no scale. Use of $(dQ_R/dt)_m$ and $(Q_R)_m$ versus T data and Q_T provided as printout from a DSC system would be preferable.

BORCHARDT AND DANIELS METHOD

Reaction rate coefficient values k_m at selected T, i.e. T_m, were determined by the Borchardt and Daniels method (Borchardt and Daniels, 1957; E2041-03). Based on Eq [2]

$$k_m = (dQ_R/dt)_m\, Q_T/[Q_T - (Q_R)_m] \quad [4]$$

ASTM E 2041-03 indicates that $(dQ_R/dt)_m$ and $(Q_R)_m$ for $0.1 < (Q_R)_m/Q_T < 0.9$ should be used

when computing k_m. This minimizes errors caused by variation in base-line placement and inaccurately determined low values of $(dQ_R/dt)_m$. Thus, data for $7 \geq m \geq 16$, i.e. T between 170 °C (443.2 K) and 215 °C (488.2 K) were used to determine k_m.

From Eq [1]

$$\ln(k_m) = \ln(A_q) - E/T \qquad [5]$$

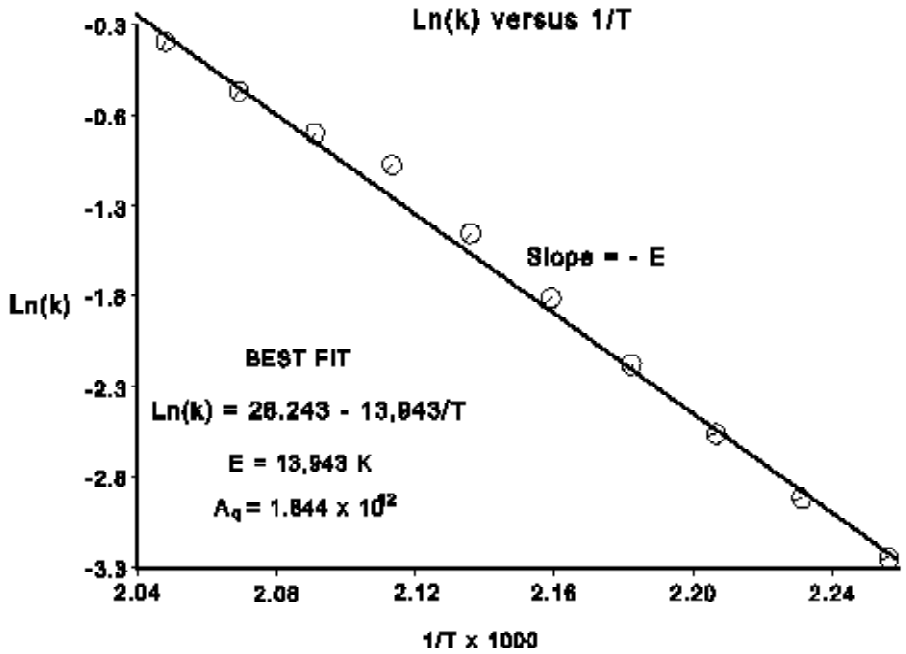

Figure 1. Ln(k) versus 1/T, with T in degrees K. The k were obtained from Raemy's data using the Borchardt and Daniels method.

When k_m obtained from Eq [4] are used, a plot of $\ln(k_m)$ versus $1/T_m$ (with T_m in degrees K) should be a straight line if the Arrhenius equation applies and if the heat generating reaction is first-order. Despite some deviating points, the plot in Figure 1 is straight. Thus, the heat-generating reaction apparently is first order and governed by the Arrhenius equation. From linear regression, E = 13,943 K and $A_q = 1.844 \times 10^{12}$ kW/kg.

COMPUTED RESULTS

These E and A_q and Q_T = 312.5 kJ/kg were substituted in Eq [2], which was then integrated by a computer program to obtain predicted dQ_R/dt versus T and the root mean-square error (RMSE) based on differences between the predicted and experimental dQ_R/dt over the entire T range. The program was also used in an "automated search mode" to find best-fit E and A_q by systematically varying E and A_q till a RMSE minima was found. This occurred for E = 14,400 K and $A_q = 5.54 \times 10^{12}$ kW/kg. The resulting RMSE, 5.5×10^{-3} kW/kg, is slightly lower than the RMSE, 6.1×10^{-3} kW/kg, obtained from use of the Borchardt and Daniels method. Use of reaction orders other than 1, i.e. use of $[(Q_T - Q_R)/Q_T]^n$, with $n \neq 1$ in place of $[(Q_T - Q_R)/Q_T]$ in Eq [2], produced larger RMSE, even for n = 1.001 or 0.999. A dQ_R/dt versus T curve obtained with the E and A_q found by the search method is also plotted in Figure 2.

Though the RMSE obtained with the predicted curves are fairly low, the predicted data deviates from the experimental data in a systematic way rather than randomly.

MULTIPLE REACTION MODEL

Raemy carried out a DSC test where dT/dt = 0.5 K/min instead of 1.0 K/min, and obtained three (dQ/dt) peaks (two of them poorly resolved). Thus a realistic model should involve at least three parallel reactions.

Eq [6] provides a model describing heat production from J independent reactions

$$(dQ/dt)_{\text{total}} = \Sigma(dQ_R/dt)_j = \Sigma(A_q)_j \exp(-E_j/T)[\{(Q_T)_j - (Q_R)_j\}/(Q_T)_j] \qquad [6]$$

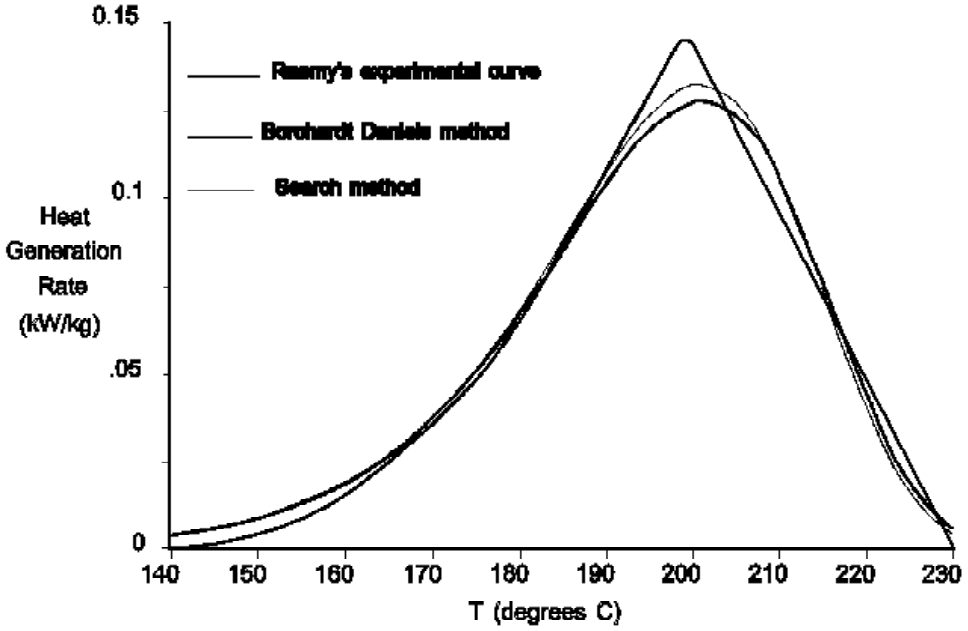

Figure 2. Experimental and predicted heat evolution rates versus T (in ^{o}C). Predicted rates were calculated with Eq (2) using parameters found either by the Borchardt and Daniels method or by the search method.

Values for the $(dQ_R/dt)_j$ are obtained by use of $(A_q)_j$, E_j and $(Q_T)_j$ in Eq [2]. Four parallel reactions were used to obtain a $(dQ/dt)_{\text{total}}$ versus T curve that peaked as sharply as Raemy's Figure 3. For four reactions, j goes from 1 to J = 4 in each summation in Eq [6]; and twelve parameter values: four $(A_q)_j$, four E_j and four $(Q_T)_j$, are needed. Raemy's DSC curves do not provide enough data to find true best-fit values for that many parameters. Nevertheless, parameter values were found that, when used in Eq [6], provided computed $(dQ/dt)_{\text{total}}$ versus T values that fitted Raemy's curve very well, i.e. the RMSE = $1.7\text{x}10^{-3}$ kW/kg. With twelve adjustable parameters, one should be able to fit a curve quite well.

$$\text{RMSE} = \{\Sigma[(dQ/dt)_{\text{exp}} - (dQ/dt)_{\text{total}}]^2/19\}^{1/2} \qquad [7]$$

Differences between $(dQ/dt)_{\text{exp}}$ and $(dQ/dt)_{\text{total}}$ for use in Eq [7] were computed at 5 ^{o}C intervals, i.e. at 19 values of T between 140 °C and 230 °C.

The parameter values used are listed in Table 1. They were found by use of a computer program that converted $(dQ/dt)_{\text{exp}}$ and T values obtained from Raemy's DSC curve into pixel co-ordinates that were used to plot $(dQ/dt)_{\text{exp}}$ versus T on a PC screen. $(dQ_R/dt)_j$ versus T values computed with Eq [2] for each reaction and $(dQ/dt)_{\text{total}}$ versus T found with Eq (6) were

similarly plotted on the screen, so that curves could be rapidly compared. An RMSE found by use of Eq [7] was also displayed on the screen.

DSC curve peaks shift toward higher T when dT/dt is increased. Thus for dT/dt = 1.0 K/min, the maxima in Raemy's 0.5 K/min DSC curve s shift to roughly 175 °C, 199 °C and 215 °C, but the first and last peak become hidden. Initial E_j values were selected that provided needed degrees of peak sharpness. To provide overall peak sharpnes matching that shown in Raemy's Figure 3, reaction 4 was caused to peak near 199 °C, and the E_4 used was quite large. $(A_q)_j$ and $(Q_T)_j$ were chosen to provide appropriate peak height. Initial $(A_q)_j$ values that provided peaks at the cited T were found by use of Eq [8]

$$(A_q)_j = [(dQ_R/dt)_j]_{max}/\{[1 - (Q_R)_j/(Q_T)_j]_{max} \exp[-E_j/(T_{max})_j]\} \quad [8]$$

Subscript "max" appended to a variable indicates that the value used for that variable is the one occurring when $(dQ_R/dt)_j$ is at its maximum.

Initial E_1 $(A_q)_1$ and $(Q_T)_1$ were selected to cause $(T_{max})_1$ to occur at 198.6 °C, and to provide $(dQ_R/dt)_1$ that were lower than Raemy's $(dQ/dt)_{exp}$ over as large a T range as possible. The remaining initial E_j and $(Q_T)_j$ were selected to reduce $(dQ/dt)_{exp} - (dQ/dt)_1$ gaps in each curve region where $(dQ_R/dt)_j$ for one of the remaining reactions reached its peak. Then, the parameters were adjusted to reduce further $(dQ/dt)_{exp} - (dQ/dt)_{tot}$ gaps seen on the PC screen. The parameter values were then fine-tuned to minimize the RMSE. This required roughly four hours of computation. Computation took less than 1 second for the Borchardt and Daniels method for a single reaction and 20 to 40 seconds for the search method for a single reaction.

Table 1. Parameter values used to calculate $(dQ/dt)_{total}$ with Eq [6] and $(dQ_R/dt)_j$ with Eq [2].

j	E_j	$(A_q)_j$	$(Q_T)_j$
	K	kW/kg	kJ/kg
1	16,200	1.84×10^{14}	220.8
2	20,700	2.86×10^{16}	25.8
3	35,100	7.51×10^{29}	32.8
4	38,700	4.35×10^{34}	33.9

RMSE only slightly higher than that obtained with the listed parameter values were obtained with markedly different parameter values. The computer program was used to compute and plot a $(dQ/dt)_{total}$ versus T curve for the listed parameter values when dT/dt = 0.5 K/min. The curve obtained did not match Raemy's 0.5 K/min curve well. A discernible $(dQ/dt)_{total}$ peak appeared at 215 °C; and $(dQ_R/dt)_3$ peaked at 175 °C, but did not cause $(dQ/dt)_{total}$ to peak at or near that temperature. The listed parameter values are not suitable for Raemy's 0.5 K/min curve, nor are they unique. I have not yet been able to find parameter values that work well at both 0.5 K/min and 1.0 K/min.

ROASTING

Eqs [2] and [6] and the program used to obtain data for Figure 3 were also used to compute $(dQ/dt)_{total}$, $(Q_R)_{total}$ and the $(dQ_R/dt)_j$ and $(Q_R)_j$ for typical T versus t behavior for roasting. During roasting, reactions with high E_j and $(A_q)_j$ essentially go to completion; but the reactions producing most of the exothermic heating have markedly smaller E_j and/or $(A_q)_j$, and are less than 50% complete at the end of roasting. Thus $(Q_R)_{total}/Q_T < 0.55$ at the end of

roasting. Like roast color and dry matter loss or total weight loss, $(Q_R)_{total}/Q_T$ may serve as an index for the degree of completion of a roast.

$(Q_R)_j/(Q_T)_j$ for a particular reaction similarly may serve as a measure for the extent of that reaction. Determining $(Q_R)_j/(Q_T)_j$ would be useful if flavor and aroma generation correlate with a particular exothermic reaction. Better E_j, $(A_q)_j$ and $(Q_T)_j$ values have to be obtained to compute $(Q_R)_j/(Q_T)_j$ and $(Q_R)_{total}/Q_T$ accurately. Measurement of dQ_R/dt at quasi-isothermal conditions at several T can be used to identify individual exothermic reactions or reaction clusters more clearly and to determine values for parameters characterizing these reactions.

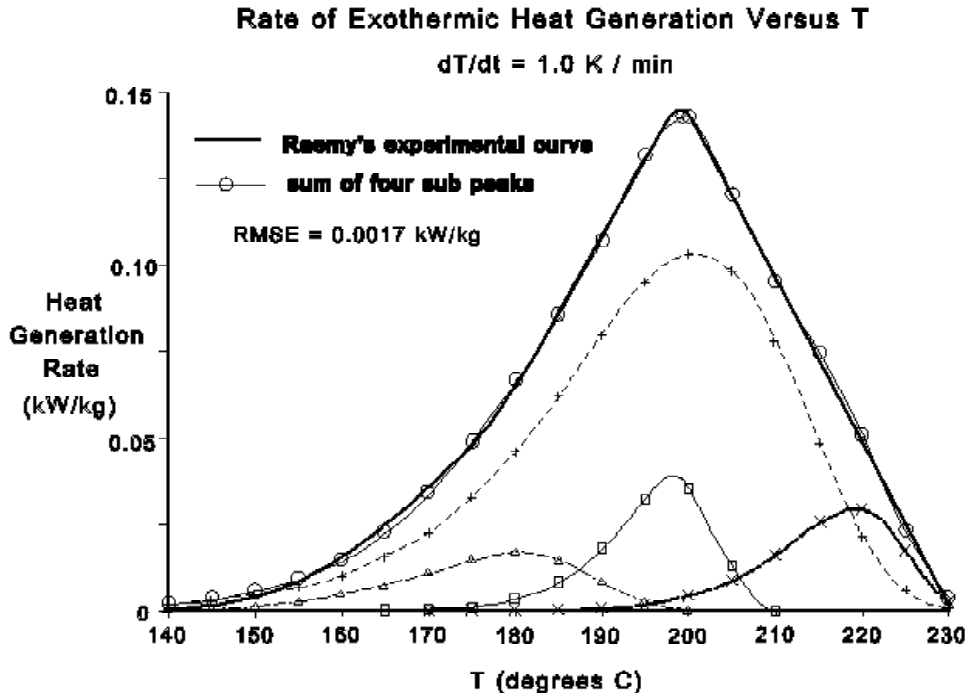

Figure 3. Experimental heat evolution rate versus T (in ^oC) and corresponding heat evolution rates computed by means of Eq [6] with parameters found by a search method.

REACTANT MOBILITY

Low reactant mobility can reduce dQ_R/dt in ways not predicted by Eq [2]. Reactants probably are less mobile before chlorogenic acid melts (at roughly 150^{o}C) or sucrose melts (at 171 °C) or they melt together at a lower T. Failure to account for low reactant mobility may have caused my predicted $(dQ/dt)_{total}$ to be higher than $(dQ/dt)_{exp}$ at $T <$ 160 °C. But, if the exotherm baseline is shifted slightly downward at its 140^{o}C end, $(dQ/dt)_{total}$ and $(dQ/dt)_{exp}$ agree quite well at $T <$ 160 °C. T passes through the 140 °C – 160 °C range very rapidly during roasting. Thus reduced reactant mobility probably does not depress exothermic heat generation significantly during roasting.

EFFECTS OF EVAPORATION SUPPRESSION

Roasting reactions produce water (Geiger et al., 2005), much of which normally evaporates. If that evaporation is suppressed, measured Q_T will be higher than actual Q_T for normal roasting. Eq [9] can be used to calculate X_{eq}, the equilibrium, dry-basis water content of coffee beans heated to temperature T in a closed system whose free volume is V

$$X_{eq} = X_o - V/(Mv) \quad [9]$$

M is the dry mass of beans, X_o is their initial dry-basis water content and v is the specific volume of water vapor at T and P_w, the equilibrium partial pressure of water at X and T. Because of slow mass-transfer, X, the actual dry-basis water content of beans during DSC testing, will be greater than X_{eq}. Nevertheless, changes causing $(X_o - X_{eq})$ to increase also cause $(X_o - X)$ to increase. Evaporative cooling is proportional to $(X_o - X)$. Thus more evporative cooling will occur and X will be smaller when $V/(Mv)$ is large.

Raemy's Figure 2 (Raemy, 1981) shows that his DSC cells were connected to a much larger, unheated, buffer chamber filled with Argon at 20-25 bar. Therefore V was large; and appreciable evaporation almost certainly occurred., The heat removed by consequent evaporative cooling has to be added to his measured Q_T to obtain true Q_T. Thus true Q_T for his tests are probably somewhat larger than the measured values he reported.

V/M changes when sample weight changes. Thus measured Q_T were affected by changes in the weight of samples used for testing. Sample weight variation probably contributed to the large variation in measured Q_T observed by Raemy.

$v \approx RT/(18P_w)$. At constant X, T/P_w is roughly 6.6 times higher at 413.2 K (140 °C) than at 503.2 K (230 ^{o}C). Thus V/Mv, $(X_o - X)$ and evaporative cooling tend to increase progressively as T increases. This could be the reason why Raemy's $(dQ/dt)_{exp}$ values decreased almost linearly rather than tailing off gradually as T approached 230 °C.

SEALED CELLS

Raemy and Lambelet (1982) carried out DSC tests with the same batch of coffee beans tested by Raemy, but with sealed cells providing a much smaller V. X_o = 0.081 kg water/kg dry coffee and, again, dT/dt = 1 K/min. The coffee bean DSC curve depicted in their Figure 5, contains features not found in Raemy's Figure 3. These features include: 1) a partially emergent peak around 175 °C; 2) a clear added peak around 215 °C; 3) a 10 °C to 20 °C upward curve shift with respect to T; and 4) a shallow shoulder at $T >$ 250 °C. The upper Q_T limit they found was 420 kJ/kg, which is somewhat higher than the 375 kJ/kg upper Q_T limit found earlier by Raemy. Were these changes due to reduced evaporation of water caused by use of a lower V? Reduced evaporation would cause X and water activity, a_w, to be higher, thereby changing the reaction environment and possibly changing reaction behavior.

Plots by Baltes (1977) depict exotherms for coffee beans obtained by DTA with sealed cells in one case and open cells in another. The two exotherms differ in shape in ways that do not appear to be caused wholly by evaporative cooling in the test with open cells. Baltes' exotherms peak at much higher T than the peaks previously discussed. Baltes probably he used much higher dT/dt, perhaps 10 or 20 K/min. Therefore his exotherms may overlap those for pyrolysis of hemicellulose and cellulose.

UNSUPPRESSED EVAPORATION

Eggers and von Blittersdorff (2005) used reaction calorimetry to measure Q_T and dQ_R/dt versus T for exothermic heating in coffee beans without suppressing evaporation. Their dQ_R/dt versus T curve for dT/dt = 1 K/min has a different shape than Raemy's. That shape difference does not appear to be caused wholly by overlap of the exotherm and the evaporation endotherm. For green beans, their Figures 1, 3 shows Q_T = 117.6 kJ/kg and that 184 kJ/kg of evaporative cooling occurred before the apparent start of the exotherm. The

latent heat of evaporation of water is roughly 2,300 kJ/kg around 200 °C. The heat of desorption of water from low moisture content foods is roughly 1.2 times water's latent of evaporation. Thus at least roughly 184/(2300 x 1.2) = 0.0667 kg water/kg dry beans evaporated before the apparent start of the exotherm.

X_o range from 0.0888 to 0.1364 for beans with as-is water contents in the usual range, 8% to 12%. For such beans, 0.0221 to 0.0697 kg water/kg dry beans at most could evaporate during the exotherm. Thus the cooling produced at most could only cancel 184 x (0.0221/0.0667) = 61.3 kJ/kg to 184 x (0.0697/0.0667) = 192.6 kJ/kg of exothermic heating, and their real Q_T probably lie between 117.6 + 61.3 ≈ 179 kJ/kg and 117.6 + 192.6 ≈ 310 kJ/kg. The average of these values, 245 kJ/kg, is slightly lower than 250 kJ/kg, the lowest Q_T reported by Raemy (1981) and Raemy and Lambelet (1982).

CONCLUSIONS

Based on comparisons of the exothermic heating curves and data of Raemy (1981), Raemy and Lambelet (1982) and Eggers and von Blittersdorff (2005), it appears that: 1) repressing moisture loss prior to and during exothermic heating in coffee beans provides a reaction environment different from that existing in beans during exothermic heating in roasting; 2) the nature of exothermic reactions probably changes somewhat in response to such environment change; and 3) exothermic heating may decrease as bean moisture content decreases. To model exothermic heating during roasting: one must: 1) account for water loss during roasting; and 2) obtain DSC data that show how residual water content affects exothermic roasting reactions. Such data probably can be obtained from DSC tests carried out with sealed cells containing coffee beans predried to provide different X_o. These tests may also provide information about how residual water content affects solubles creation by hydrolysis during roasting. Eqs [2] and [6] and methods used in this work probably will prove useful for analyzing data from these tests.

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Study of Physical Properties of Coffee Beans during Roasting. Application of Hyperspectral Image Analysis

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SUMMARY

During the roasting process, coffee beans change their physical properties, with the increase of volume, loss of strength and toughness and the progressive increase of browning. Variations in the relative reflectance of coffee beans at different time of roasting (3, 6, 8, 9, 10 minutes) were evaluated with the aim to define the optimal roasting degree by means a physical determination. Simultaneously, the variations in the bean physical properties, such as density, expansion index, textural change, colour index, weight loss and moisture, were also monitored. Results shown that the reflectance seems to be different for each roast degree, such as the intensity. The peak of reflectance becomes larger the longer the roasting time. The typical brittleness of roasted coffee beans seems to be related to both the water loss and the change of tissue structure determined by the pyrolysis phenomena and decrease of density. Besides, roasting process caused a decrease of moisture content with a reduction of deformability and strength.

RÉSUMÉ

Pendant le procès de torréfaction, les grains de café changent leurs propriétés physiques par augmentation de volume, perte de dureté et progressive brunissement. Les variations de refléctance relative des grains de café en temps différent de traitement (3 6, 8, 9, 10 minutes), ont été évalués pour établir un critère physique pour la détermination du degré de torréfaction. Les variations des propriétés physiques des grains de café, comme densité, expansion, structure, index de couleur, perte de poids et d'humidité, ont simultanément, été étudiées. Les résultats ont montré que la refléctance semble être différent pour chaque degré de torréfaction, comme aussi l'intensité. Le pic de refléctance devient plus grand avec l'augmentation du temps de torréfaction. La fragilité typique des grains de café torréfié semble être due à la perte d'eau et au changement de structure du tissu déterminé des phénomènes de pyrolyse et de diminution de densité.

INTRODUCTION

Roasting is one of the most important step in coffee processing because of marked chemical, physical, structural and sensorial changes. During this process coffee beans are subjected to high temperature for different times that vary greatly according to the type of roaster, the process conditions, the origin, variety and characteristics of coffee beans (size and shape) and the degree of roasting required for the final product (light, medium or dark). Coffee roasting is a process carried out in different ways throughout the world. In the case of Italian-style roasting, it is usually performed in a very intense way, in order to obtain “dark” or “very dark” coffee, the bean temperature ranges between 220 and 240 °C (Lerici and Nicoli, 1990; Severini et al., 1992). These process conditions determine a strong acceleration of changes

that occur in the last steps of roasting: an excess or defect of few seconds plays a significant role on the quality characteristics of coffee brew. However, these changes result to be insignificant for the "light" (Dalla Rosa and Lerici, 1996) or "medium" degrees of roasting . The perfect control of the roasting time is particularly important to reach a fully developed aroma and a homogeneous colour of the coffee beans, by the required chemical reactions. The degree of roast can be evaluated by the colour of beans, the loss of mass, the flavour developed and the chemical changes of several compounds (Lerici et al., 1980a; Pittia et al., 1996). Roasting control is often a manual operation assigned to a trained inspector and only in advanced plants, it can programmed tanks to the automatism based on optical and/or thermal sensors. Visual inspection is then impossible in "high yield" or fast roasting because of the short-time that characterise this type of roasting. Imaging techniques have been developed as an inspection tool for quality and assessment of a variety of agricultural food products. Imaging is generally non-destructive, reliable and rapid, depending on the specific technique used. Spectroscopic methods provide detailed fingerprints of the biological sample to be analysed using physical characteristics of the interaction between electromagnetic radiation and the sample material, such as reflectance, transmittance, absorbance, etc. The analytic spectral region include the near-infrared region, which has been successfully used for food quality and food safety analysis during the past two decades. The hyper-spectral imaging technique combines analysis of both spatial and spectral characteristics of a sample. A discrete number of wavelengths are selected using filters in the machine vision system to reconstruct sample images before processing and analysis (Mehl et al., 2004). Hyper-spectral imaging, an emerging technology developed in recent years, can be used as a tool to determine the roasting degrees of the coffee beans. The purpose of this research was to study the main physical changes of Arabica and Robusta coffee samples roasted from light to dark level, using the hyper-spectral imaging technique besides the conventional methods.

MATERIALS AND METHODS

Samples of Arabica (*Coffea arabica*) and Robusta (*Coffea canephora,* var. Robusta) green beans provided by *Essse Caffè S.p.A.* (Bologna, Italy) were used for different treatment times. Three hundred grams of green coffee beans were roasted in a laboratory roaster of 500 g capacity (mod. EXPO 500/E, STA plants s.r.l., Bologna, Italy); at different times during the process (3, 6, 8, 9, 10 minutes), sample of coffee beans were removed in order to observe the effects of different degrees of roasting on coffee physical changes. The roasting process was carried out in duplicate for each samples.

Analytical determinations

- -moisture (%): by weight on ground coffee samples after heating in oven according to AOAC (1980) methods;
- -total weight loss (%): by weight of coffee samples before and after roasting;
- -density: using a suitable picnometer according to Lerici et al. (1980) methodology;
- -color analysis: using a tristimulus colorimeter (Chromameter-2-Reflectance, Minolta, Japan) equipped with a CR-300 measuring head. Standard C.I.E. conditions with illuminant "C" were used. Chromaticity was calculated as L*, a*, b*. The instruments was calibrated on a white tile (L* = 95.3; a* = + 1.0; b* = + 0,8) before each series of measurement;
- -water activity: with a point of dew hygrometer Aqualab CX-2 (Decagon Devices Inc., Pullman, WA). Calibration was performed using saturated solutions of known a_w;
- -textural analysis: using INSTRON 3343 (Instron Ltd., High Wycombe, UK), equipped with an compression kit (two metal parallel plates). For the measurements, 20 beans of

each sample were used. Compression force (50%) was applied at a rate of 2 mm/s until failure occured.

- -carbon dioxide: determinated by head space gas chromatographic analysis according to Massini et al. (1990) methods, using a Dani gas chromatograph model 3200 equipped with a termal conductivity detector and a recorder. A glass column (2mx2mm) filled with Porapak Q (80-100 mesh) was used.
- hyper-spectral image analysis was carried out using a scanner, equipped whit a Spectrometer (Specim V10, Specim Ltd., Finland), having a bandwidth from 400 to 1000 nm and resolution of 5 nm. The Spectrometer were electronically calibrated in order to set 0% and 100% of reflectance after each measuring, by means of a white tile having known reflectance. 121 images, from 400 to 1000 nm, were acquired and elaborated by an algorithm coded in MATLAB 7.1. (The Mathwork inc, USA) in order to determine the relative reflectance and morphological parameters of the grains.

Data processing

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

A preliminary study based on PCA has been applied for a better understanding of the discriminating efficiency of the selected descriptors and also a visualization of the samples trend. Two plots (loadings and scores) are often used to observe the structure present in the multivariate data matrix. Both scores of variables and coffee bean samples obtained from covariance analysis of autoscaled data matrix are represented in the space of two principal components (PCs). Figure 1 shows the plots corresponding to the variables (a_w, density, chroma, carbon dioxide, total weight loss, dry matter, luminosity, volume, breaking strength and deformability) and the sample scores (Arabica and Robusta green and roasted coffee beans), explaining the 91.1% of total variance. The first PC, (PC_1) explains 82.86% and the second PC (PC_2) explains 8.25% of total information.

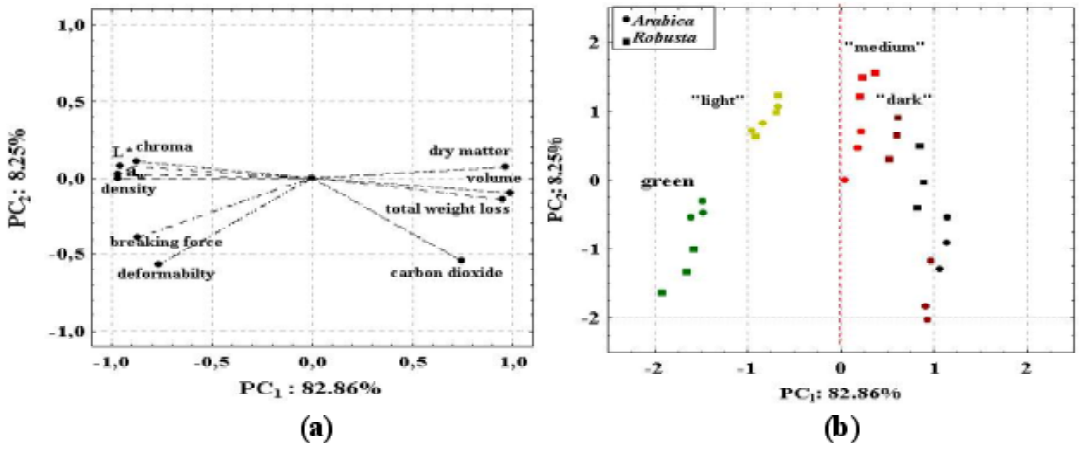


Figure 1. Principal component projection of coffee bean samples at different roasting degrees. (a) Loading plot for coffee variables (b) Score plot of green and roasted coffee samples.

A detailed examination of the variable loadings of PC_1 (Table 1) shows that all descriptors selected for analysis (with factorial weight over 0.70) had more influence on PC_1,

discriminating coffee beans only in terms of roasting degree and not in terms of variety. It can be observed that green and "light" roasted samples (3 minutes of heat treatment) are located at negative values of PC_1, whereas "medium" (6 minutes of heat treatment) and "dark" roasted beans (8, 9, 10 minutes of heat treatment) appear at positive values of this PC. Water activity, luminosity and density highly contributed on green and "light" roasted beans, whereas volume, total weight loss and dry matter on "medium" and "dark" roasted samples. In particular, the most contribution on green beans was caused by breaking force and deformability descriptors, which discriminated green from roasted coffee beans because of their marked toughness.

Table 1. Loadings of each variables for PC_1 and PC_2.

Loadings	PC_1	PC_2
Water activity	-0.970945	-0.001895
Total weight loss	0.950380	-0.143965
Dry matter	0.965741	0.072149
Luminosity	-0.959868	0.080155
chroma	-0.877258	0.108025
Breaking force	-0.868951	-0.387429
Deformability	-0.764672	-0.568576
Carbon dioxide	0.746242	-0.544661
Density	-0.970648	0.028214
Volume	0.988982	-0.101709

Besides, luminosity and chroma variables distinguished the "light" roasted beans from all other samples. In the first 3 minutes of process, in fact, the loss of beans cuticle caused an increase of their brightness. "Medium" roasted beans differing from other samples for the contribution of dry matter variable because of the major water loss that occur during the first 6 minutes of heat treatment. Finally, volume, carbon dioxide and total weight loss variables allowed to discriminate the "dark" roasted beans from other samples. These descriptors, in fact, greatly influenced the last minutes of roasting process. It was possible to observe that Arabica samples subjected to the heat treatment for 8 and 9 minutes differed from the same roasted of Robusta because of the high contribution of carbon dioxide variable on these samples: the high content of sugar in Arabica variety, could determine a more intense pyrolysis and carbon dioxide release. In Figure 2, changes in breaking force and water activity of Arabica and Robusta beans, as a function of the roasting process time are shown. The very high breaking force values of the Arabica and Robusta green coffee beans could be, partially, attributed to the presence of some structural polysaccharides, especially those derived from the polymerization of mannan (Trugo, 1985). In all cases, the increase of the roasting time caused a break force decrease, which reached similar and very low values at the end of the process; so to indicate a progressive reduction in beans strength. Breaking force showed a progressive and significant decrease ($P < 0.5$) as the water activity becomes lower. The lowest values of breaking force are obtained by the samples of both coffee varieties, which presented similar low values of water activity. Also we can observe that the highest breaking force values are showed by Robusta beans, but not significant differences ($P > 0.5$) there are between Arabica and Robusta varieties. These results according to those reported by Pittia et al., (2001) could be, probably, affected by the irregularity of the size and shape of the beans and by structural variability inside each group of coffee bean samples which determined a coefficient of variation over to 25%. The values of deformability index followed the same trend of breaking force (data not shown) to indicate a great change in texural of roasted samples due to heat treatment.

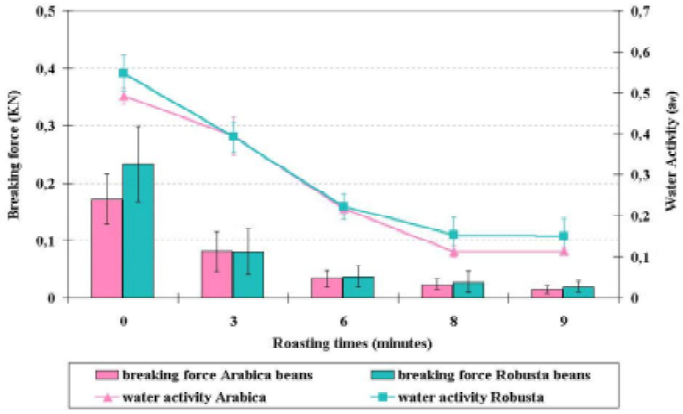


Figure 2. Breaking force and water activity of Arabica and Robusta coffee beans as a function of roasting time.

In particular, a low toughness together with a low deformability are a typical index of a brittle and fragile texture (Borges and Peleg). In Figure 3, changes in roasting loss (moisture and total weight loss) and carbon dioxide content of Arabica and Robusta coffee beans during roasting treatment, as a function of the heating time are shown. The roasting loss is either measured by the dry mass loss (the weight loss based on dry green beans) or by the total weight loss (moisture plus organic matter loss) (Eggers, 2006). As expected the moisture loss of both coffee varieties increased during heat treatment. At the same time an increase in total weight loss was observed.

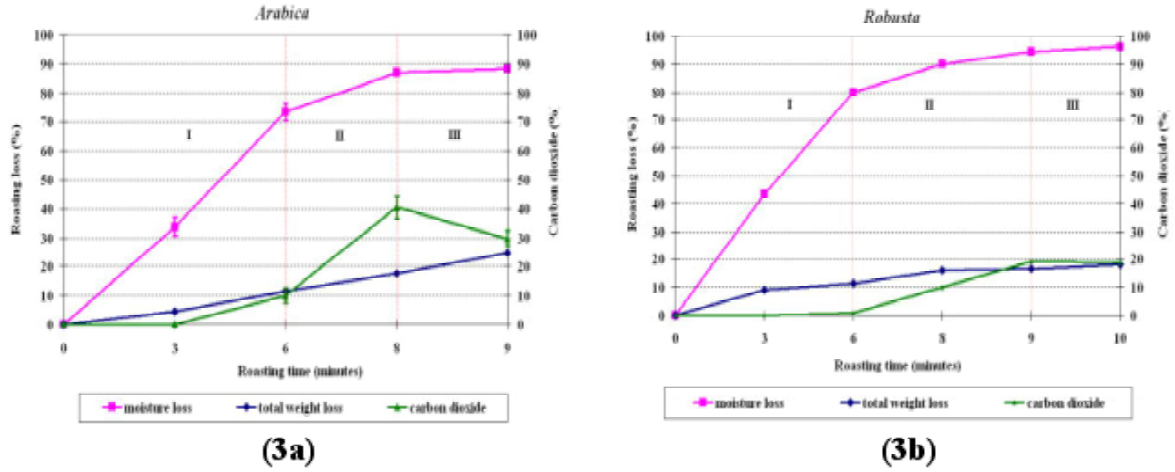
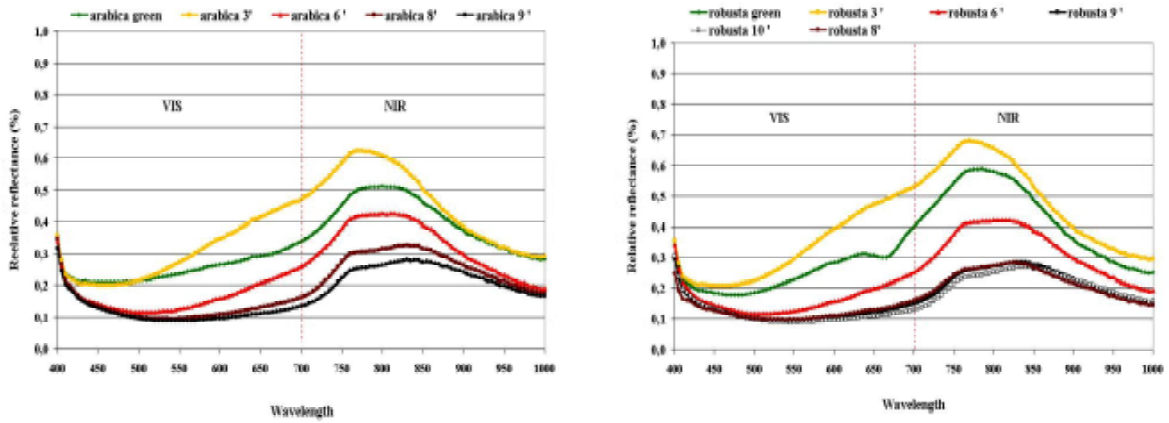


Figure 3. Roasting loss (moisture and total weight loss) and carbon dioxide content of Arabica (3a) and Robusta (3b) coffee beans as a function of roasting time.

Roasting can be considered as a succession of different stages. During the first 6 minutes (first stage) of processing the release of water vapour from the green coffee was the main effects, in fact, coffee bean loses over 70% of initial moisture content. The second stage of process is characterized by sugar pyrolysis and a slow release of carbon dioxide and other volatile compounds (Dutra et al., 2001). Water vapour and volatile substances which develop inside the bean caused an increase in the internal pressure with consequent expansion. Moreover, weight reduction due to release of water, CO_2 and other volatile components determine a decrease of bean density (data not shown). This second stage, ends when the coffee loses its elasticity as a consequence of the significant sugar content. Due to the elasticity loss, the increase in the internal pressure causes cracks on the bean surface. Finally, in the third stage

the progressive carbonization of the roasted bean causes a further increase in weight loss and a lowered retention capacity as shown by the release of CO_2. Figure 4 shows the trends of relative reflectance corresponding to Arabica and Robusta coffee beans at every roasting degree, as a function of different wavelengths (between 400 and 1000 nm), are shown.



(4a) (4b)

Figure 4. Relative reflectance of Arabica (4a) and Robusta (4b) coffee beans at every roasting degree, as a function of different wavelengths.

From an initial observation of both coffee variety spectrums, it was possible to note that the reflectance peaks obtained in the near infrared region, between 750 and 850 nm, allowed to distinguish the different roasting degrees. In particular, the intensity of reflectance peak decreases during roasting time, becoming wider and wider. This reduction indicate a great absorption in this spectrum region, from roasted coffee beans. In fact, the lowest reflectance peaks in the visible spectrum (between 400 and 700 nm) corresponding to the "dark" roasted samples, point out that coffee beans colour verges on black. A comparison between Arabica and Robusta at the same roasting degree showed differences in the intensity of reflectance peaks. In particular, in the first stage of roasting (3 minutes) Robusta reflectance peaks showed a higher intensity than those of Arabica, because of its more light colour. No differences in reflectance values between Arabica and Robusta are shown by "medium" roasted beans, whereas in the last minutes of process Arabica reflectance peaks appear more intense then Robusta because of the progressive exudation of oil towards the surface of bean which becomes more shining. Considering colour index a* and b* obtained by hyper-spectral image analysis (Figure 5a), it's interesting to note that the colour of the coffee moved from green zone, of the raw coffee beans, to the red zone of "medium" roasted samples, then returned to the green zone but in a lower yellow band (Luciane et al., 2001). In addition colour index values were reduced to colour functions such as arctg b*/a* and $(a^{*2}+b^{*2})^{1/2}$, and these functions of colour were represented in a polar graph (Figure 5b). When polar coordinates (Hue angle and Chroma) were used, coffee beans at every level of roasting, were located in a well defined colour zone. This is the typical behaviour of non enzymatic browning in vegetable matrices. This is shown by the progressive and marked decrease of the hue angle degree and chroma values of samples. During the first 3 minutes of roasting it was observed an increase of chroma values because of two physical changes that occur in this step of process: water vapour at the coffee surface and loss of bean cuticle, which determine a great brightness of it.

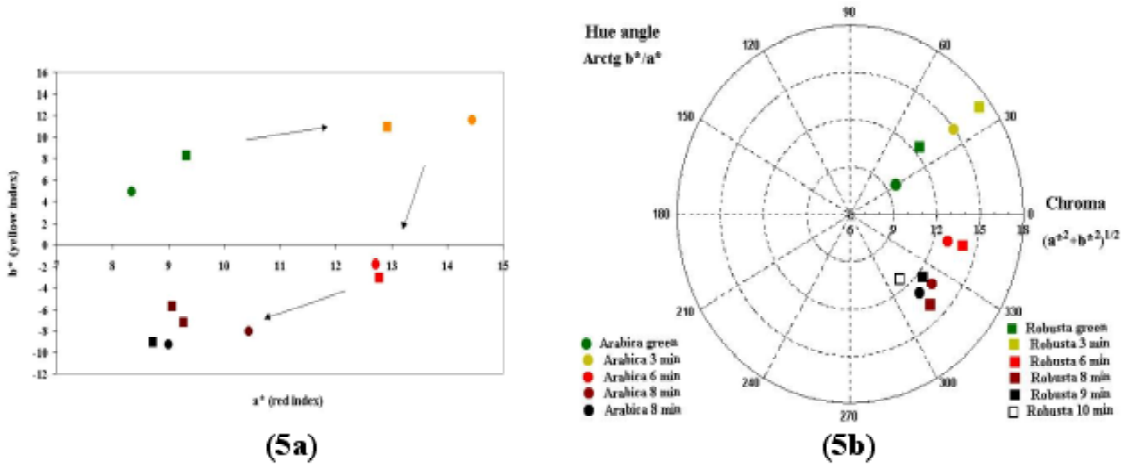


Figure 5. Changes of colour coordinates (a* and b*) (5a) and colour polar coordinates hue angle and chroma, (5b) of Arabica and Robusta coffee beans as a function of roasting time.

Morphological characteristics of Arabica and Robusta beans for each roasting time are shown in Table 2. At 8 minutes, the geometric indexes, such as area, perimeter, equivalent, diameter, minor (x) and major (y) axis increased, due to release of water, CO_2 and volatile compounds in the gas phase. At 9 minutes of roasting these indexes decreased, but at the same time volume increased of 100% (data not shown), to indicate that in this last minutes of process the bean is subjected to a contraction of x and y axis because of z axis extension. This trend is confirmed by eccentricity index, which indicate that in the first minutes of roasting process, the bean surface has an elliptic shape, but then it becomes more and more spherical.

Table 2. Morphological analysis of Arabica and Robusta beans as a function of roasting time. Different letters indicate significant differences with a confidence level of 95%.

Samples	Area (pixel)	Perimeter (pixel)	Major Axis (pixel)	Minor Axis (pixel)	Equivalent diameter (pixel)	Eccentricity (pixel)
Arabica green	580.07 a	89.87 a	30.90 a	24.08 a	27.13 a	0.60 a
Arabica 3’	712.29 b	99.60 b	33.66 b	27.09 b	30.03 b	0.57 b
Arabica 6’	806.75 c	105.43 c	35.50 c	28.94 c	31.94 c	0.56 b
Arabica 8’	985.85 d	117.47 d	40.26 d	31.31 d	35.34 d	0.60 a
Arabica 9’	854.54 d	109.36 c	38.13 c	28.64 c	32.93 c	0.65 c
Robusta green	502.86 a	83.61 a	29.75 a	21.59 a	25.24 a	0.67 a
Robusta 3’	580.18 b	89.66 b	31.32 b	23.60 b	27.08 b	0.64 b
Robusta 6’	648.03 c	94.60 c	32.88 c	25.12 c	28.63 c	0.63 b
Robusta 8’	768.73 e	103.16 d	36.01 e	27.20 e	31.19 e	0.64 b
Robusta 9’	723.48 d	100.36 d	35.30 d	26.06 d	30.20 d	0.65 c
Robusta 10’	756.30 e	102.72 d	35.96 e	26.82 e	30.94 e	0.65 c

CONCLUSIONS

This work demonstrated that hyper-spectral image analysis may be usefully applied as a tool to determine the optimal roasting degree of coffee beans. In comparison to other techniques of analysis as the tristimulus colorimeter and NIR spectroscopy hyper-spectral image technique introduces the advantage to be more precise because the physical characteristics obtained from this analysis are the result of the whole sample analysed and not of some points.

Besides, this analysis is contemporarily conducted on a big number of samples and requires a minimal preparation of this. In addition to identify pure samples of each variety, future work will explore whether it's possible to detect blends of Arabica and Robusta, and this may offer a new, fast method for the detection of adulteration.

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Acrylamide in Roast Coffee – Investigations of Influencing Factors

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SUMMARY

After the first publication in 2002 on the presence of acrylamide in food products an initiative within the coffee industry has started to consider a joint investigation on the formation and presence of acrylamide in coffee. This initiative resulted in a major research programme which was executed from early 2003 to mid 2004 with participations of more than 20 companies representing the European coffee industry. The objective of the study was to understand the presence of acrylamide in coffee covering the influence of green coffee types, processing conditions and brewing and to identify mitigation opportunities. The results showed that coffee in various aspects is different to most other affected food categories. During the roasting process the acrylamide levels are determined by formation and reduction mechanisms. Formation reactions are dominating in the beginning of the roasting process and lead to increasing levels at this stage. The reduction mechanisms begin to dominate already at very light roast degrees and result in decreasing levels of acrylamide during the following phases of roasting. This results in final levels in the finished products which are only a fraction of the observed maximum interim levels. Although differences between Arabica and Robusta in acrylamide formation potential have been found and the effects of varying the roast degree and roast time have been identified the conclusion is that only very limited options are available to reduce the acrylamide level in existing products. The same parameters also have very distinct effects on the sensorial characteristics and therefore potential adjustments to reduce the acrylamide levels would have a significant impact on the quality and the consumer acceptability of the final product. Furthermore, it was shown that acrylamide in packed roast coffee products is not stable. The acrylamide levels are decreasing significantly during the shelf life period when products are stored at ambient or higher temperatures. This should be taken into account when comparing monitoring data of market samples. Further investigations are necessary in order to understand the mechanism behind.

INTRODUCTION

Since Swedish researchers reported in April 2002 about the occurrence of acrylamide in heated foods (Swedish National Food Administration), extensive efforts were undertaken by the different stakeholders towards mechanistic elucidation and prevention. The presence of acrylamide was confirmed in a whole range of foods and drinks, which were heated above 120 °C during their processing or preparation. Acrylamide was detected in fried potatoes, bread, cookies, potato and cereal based snacks, coffee and other products. The formation of acrylamide appeared to be dependent on the composition of the food and the imposed thermal conditions. The mechanism of acrylamide formation was reported to involve reducing sugars

and amino acids, predominantly asparagine (Stadler et al., 2002; Mottram et al., 2002; Zyzak et al., 2003; Murkovic, 2004).

Already in 2002 the European coffee industry initiated a comprehensive study to understand the levels of acrylamide in coffee products which was executed from early 2003 to mid 2004 with participation of more than 20 companies representing the European coffee industry. The aim was to investigate the influences of raw material and processing parameters on the formation of acrylamide and the effects of storage and brewing to identify potential mitigation options. The results of the coffee industry study have been published (Lantz et al., 2006) and have also been integrated into the efforts of the European Food Industry under the umbrella of the European Food and Drink Federation (CIAA) to summarize the efforts of food manufacturers and their associations of different sectors to identify potential intervention steps to reduce acrylamide levels in food. These activities of the industry wide cooperation has lead to the development of the CIAA Acrylamide Toolbox document (European Food and Drink Federation) with the intention to provide brief descriptions of the evaluated intervention steps to manufactures as basis for an individual (plant and product specific) assessments with regard to the effect on acrylamide formation and on sensorial or other consumer relevant properties of the product.

MATERIALS AND METHODS

Green coffees: The roasting experiments were executed with the three major types of green coffee: Arabica coffee (*Coffea arabica*) from Brazil (dry processed) and from Colombia (wet processed) and Robusta coffee (*Coffea canephora robusta*) from Vietnam (dry processed). These three cover the major types of internationally traded green coffee (dry respectively wet processed Arabica and dry processed Robusta). The three countries of origin were in recent years the three largest exporters of green coffee and supplied together more than half of the international export.

In an additional check on representativity of the results obtained with these three coffees, a number of random samples of green coffee were taken and roasted, 5 Robusta's from respectively Cameroon (2x), Cote d'Ivoire, Indonesia and Uganda and 15 Arabica's from respectively Brazil (2x), Colombia (2x), Costa Rica, Ethiopia (2x), Guatemala, Honduras, India, Kenya, Mexico, Peru, Papua New Guinea and El Salvador.

Roasting equipment: The roasting experiments were all executed in the following three roasters: Roaster A (Probat RT 3SY/Emmerich/Germany), a fluidized bed roaster with mechanical supported movement of the coffee beans, with heat transfer predominantly by convection and a batch size of 2 kg green coffee; Roaster B (Neuhaus Neotec RFB6/Reinbek/Germany), a rotating fluidized bed roaster with heat transfer by convection and a batch size of 2 kg green coffee and Roaster C (Probat PRG500/Emmerich/Germany), a drum roaster with heat transfer mainly by conductivity and a batch size of 0.5 kg green coffee. The process conditions in the roasting experiments were varied beyond the normal commercial range, i.e. from under-roasted till over-roasted. The roast degree, as measured by light reflectance using a Lange colorimeter, was varied from very light roast, 100-110 LRU (Lange reflectance units) corresponding to 4-6 % dry-weight-loss, till very dark roast, 40-50 LRU and 9-11% dry-weight-loss. The roast time was varied from very fast roast (1.5 min) till very slow roast (16 min). Before the execution of the storage trials the samples of roasted coffee were analyzed for acrylamide within one week or stored at –18 °C until analysis.

To make sure that representative results were obtained with these three roasters, in total 51 roasted coffee samples were drawn from different types of commercial scale roasting equipment operated by 17 different European partners in this study.

The storage trials for roasted coffee were done with vacuum-packed roast and ground coffee produced two weeks before and stored at 4°C until onset of the storage study, that was carried out at four different temperatures: –18 °C, +4 °C, ambient temperature and at +37 °C. Samples taken during the standardized storage conditions were analyzed for acrylamide on the next day.

Brewing experiments were carried out using several brewing machines widely used in Europe. The tested brewing strengths ranged from 46 up to 146 g. coffee/l. water.

Analyses: The degree of roast was measured both by light reflectance (LRU = Lange Reflectance Units measured with colorimeter of Hach-Lange/Duesseldorf/Germany) and also by measurement of the dry-weight-loss. The acrylamide analyses were performed by the lab of Eurofins Analytic GmbH, Hamburg using liquid chromatography tandem mass spectrometry using deuterium-labeled acrylamide as an internal standard. For coffee with a content of 282 µg/kg acrylamide the method is reported to have a standard deviation of 25.9 µg/kg (= 9%) (Hoenicke et al., 2004). The analysis of asparagine and reducing sugars was performed by the Institute of Plant Biology, Technical University Braunschweig using for asparagine the OPA method (Roth, 1971) and a DIONEX® BIO-LC HPAEC-PAD for the (reducing) sugars.

RESULTS AND DISCUSSION

The general characteristics and in particular the sensorial characteristics of roasted coffee largely depend on the blend (Arabica/Robusta), on the amount of heat effectively transferred into the coffee beans during roasting, and on the roasting time. Therefore these parameters need to be fixed in narrow ranges to consistently achieve the target flavour profile of an existing product. Arabica and Robusta coffee are botanically different species and they are distinctly different in sensorial properties. The amount of transferred heat is largely dependent on the temperature of the hot air used to roast, on the applied coffee/air ratio and on the achieved heat transfer rate. A larger amount of transferred heat results in a darker roast. The roasting experiments were designed to cover the three main factors, Arabica/Robusta, roast-time and degree of roast.

Roasting of the 23 different green coffees in roaster B during 2.5 minutes to a medium roast (80 LRU) revealed a difference between the two botanical different species, Arabica and Robusta coffee, with Robusta's producing on average a higher acrylamide level (see Table 1). No difference appeared for the two different ways of processing (dry versus wet processing) Arabica coffee cherries to green coffee beans (data not shown).

Table 1. Effect of Arabica versus Robusta coffees on acrylamide levels (roasted in 2.5 minutes to a medium roast).

	Number of samples	Acrylamide mean (µg/Kg)	Standard Deviation	Variation (%)
Robusta's	6	378	32	8
Arabica's	17	251	45	18

The 20 additional green coffees (5 Robusta's and 15 Arabica's) were also analyzed for aspartic acid, glutamic acid, asparagine, glucose, fructose and sucrose. A weak positive correlation ($R^2 = 0.56$) for the asparagine level in green coffees and the acrylamide in the corresponding roasted coffee is apparent from Figure 1. This applies to the Arabica coffees as well as to the combined Arabica plus Robusta coffees. Taking the 5 Robusta's samples separately, a correlation is not evident. Also, glucose levels in the green coffees did not show a clear correlation with the acrylamide in the roasted coffees (data not shown). It is very well possible that correlations between green coffee components and acrylamide formation in the beginning of the roasting process are obscured by the reduction of acrylamide in the second part of the roasting.

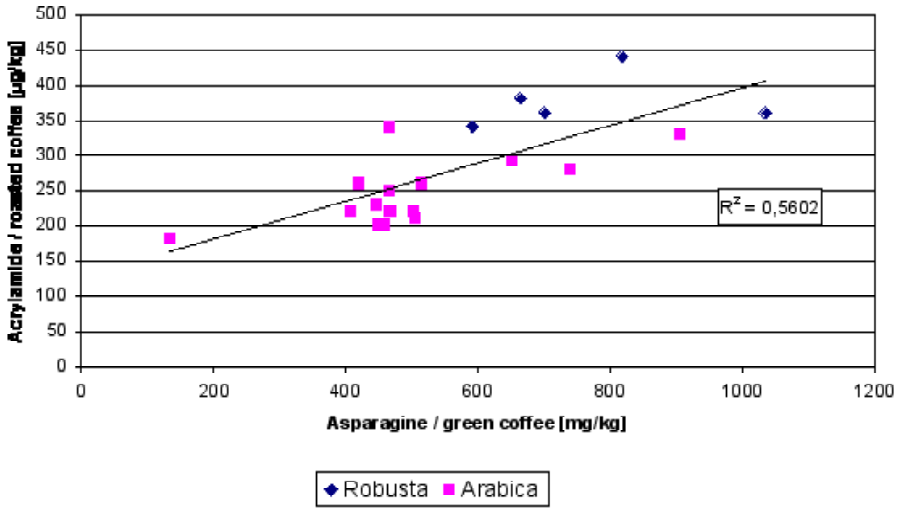

Figure 1. Asparagine levels in green coffees versus acrylamide level in corresponding roasted coffee (medium roast time and medium degree of roast).

In contrast to potatoes and potato chips, where a rather stringent correlation is evident between the precursors and acrylamide content in the final product, there is for coffee an only rather weak positive correlation observed between asparagine in green and acrylamide in roasted coffee.

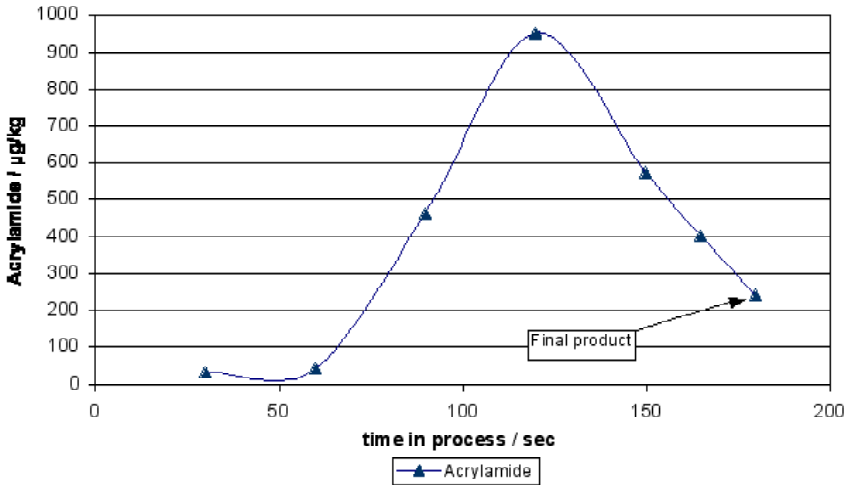

Figure 2. Acrylamide levels of partially roasted coffees, by prematurely stopping the process of roasting (Colombia coffee up to medium roast in 3 minutes).

To study the acrylamide formation during the roasting process, partial roasting was applied by prematurely stopping the roasting process. It confirmed that both, formation and reduction of acrylamide occurred at roasting. Figure 2 shows a typical curve for the formation and reduction of acrylamide during roasting up to medium roast. During the roasting process the acrylamide levels are determined by formation and parallel reduction mechanisms. Formation reactions are dominating in the early phases of the roasting process and lead to increasing levels at this stage. The reduction mechanisms begin to dominate already at very light roast degrees and result in decreasing levels of acrylamide during the following phases of roasting. This results in final levels in the finished products which are only a fraction of the observed maximum interim levels.

Basically similar patterns of acrylamide formation and reduction were observed in other studies (Senyuva and Gökmen, 2005; Taeymans et al., 2004; Taeymans et al., 2005). Heating experiments with ground green coffee in a sealed headspace vial in a laboratory oven, without forced cooling afterwards indicated that the acrylamide content went through a maximum of about 300 µg/Kg (Senyuva and Gökmen, 2005). Taeymans et al. (2004), reported, that peak levels of around 2000 µg/Kg had been observed early in the process of roasting coffee beans. Differences in observed absolute maximum values might be partly attributable to the type of green coffee and the way of roasting. However, differences in the ways of cooling of the (partially) roasted coffees might be of major importance. Not applying a forced cooling or varying the cooling time/ cooling efficiency might result in the process to be "frozen" in a quite different state with impact of the analysed acrylamide of the sample. At least such would make the observed variation in early roasting acrylamide peak values plausible.

The degree of roast is usually measured by reflectance of light. In the present study it is expressed as reflectance units measured in a Lange colorimeter.

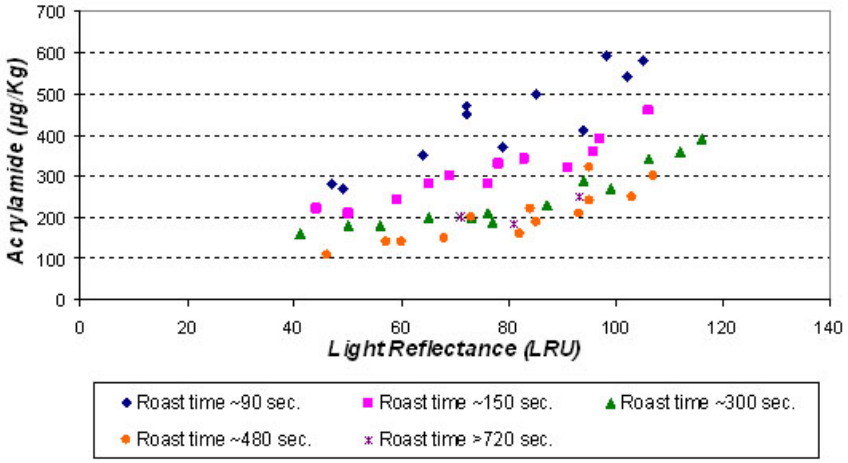

Figure 3. Effect of the degree of roast and roast time on acrylamide in Colombia coffee as measured over the full experimental range, from under- till over-roasted coffee

The extreme values in reflectance reached in the full experimental range, were on the light "roasted" side 116 LRU and on the dark side 41 LRU. In practice however the roasted coffees in Europe range in their roast degree from 55 to 105 LRU with a gradient from north Europe with typically lighter roasted coffees to south Europe with darker coffees. The colour bandwidth of roasting in a specific country is usually about 10 LRU, whereas the sensorial bandwidth of an individual brand of roasted coffee is not more than 5 LRU. Differences larger than that reflect products with clearly different sensorial characteristics. The effect of the degree of roast is shown in Figure 3, over the full experimental range from under- to over-roasted (116 till 41 LRU).

Granby et al. (2004) and Senyuva et al. (2005) reported market samples of roasted coffee respectively the corresponding brews to show lower levels of acrylamide for the darker roasted coffees. This was also seen the present study encompassing 51 samples from commercial roasting of Arabica respectively Robusta coffee across Europe, supplied by the study partners, showing the effect of Arabica versus Robusta coffee and that darker roasts on average had lower levels of acrylamide (see also Table 1 and Figure 3).

The effect of roast time as observed in the roasting experiments is shown in Figure 3 as well. Again with regard to roast time, the experimental range goes beyond the commercially practiced range. European commercial scale roasting practices roast times from 2 till 15 minutes. Shorter roast times tend to induce higher levels of water extractable solids, which make a stronger tasting brew (Clarke and Vitzthum, 2201). This means that customers will use less coffee per cup. At very short roast times (< 2 $^{1}/_{2}$ minutes) the acrylamide reduction is still progressing and the level is still going down.

Bagdonaite et al. (2004) reported about two different series of experiments. In one series they used a lab-scale roaster (batch size 80 g.) and observed that Robusta coffees produced higher acrylamide levels at roasting than Arabica's did. In their second series they used pre-heated glass dishes in a lab-oven. Their results indicated that longer roasting resulted in lower acrylamide levels. For both series there were no measurements of the degree of roast reported. The lacking of info on the degree of roast makes it impossible to draw conclusions from their results for coffee for actual consumption.

A decrease of the acrylamide level during storage has been reported in several publications (e.g. Delatour et al., 2004 and Andrzejewski et al., 2004, reported a reduction of 30 to 65% after a 6-7 month storage at ambient temperature and Hoenicke et al. (2005) reported a reduction of 30% after a 3 month storage at 10-12 °C). In the present study the acrylamide reduction in vacuum-packed roast and ground coffee was measured over 12 months at 4 different temperatures (Figure 4). The reduction after a 6-7 month storage at ambient temperature of 50-60% is consistent with data reported in the publications.

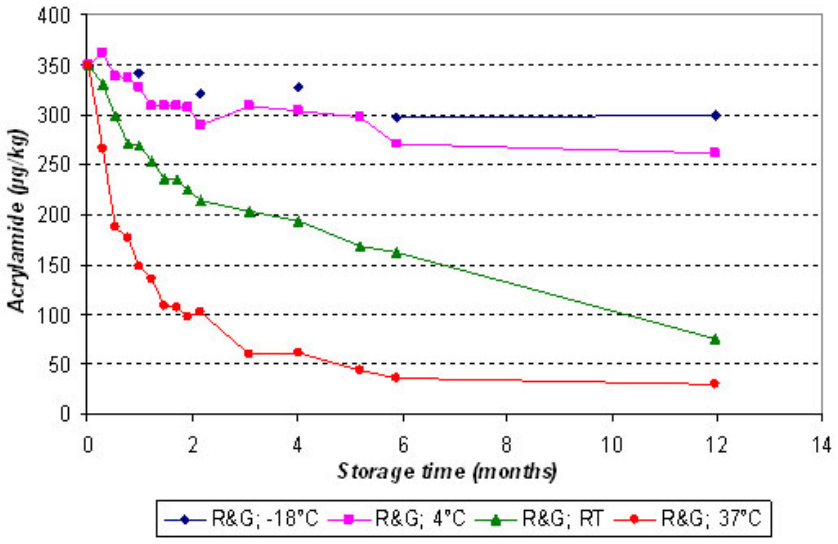

Figure 4. Reduction of acrylamide in vacuum-packed roast & ground coffee during storage

A sample of a roasted coffee (whole beans) stored at room temperature showed a decreasing curve parallel to the room temperature curve for roast and ground coffee in Figure 4 indicating that the reduction mechanism is not depending on the particle size.

A cross-section of generally available brewing systems was used to test the extraction of acrylamide from the roast and ground coffee into the brew. The results are presented in Table 2.

Table 2. Extraction of acrylamide into coffee brew.

Brewing system	Coffee/water (g/l)	Extraction efficiency (%)
Horeca pour-over system (5 l. capacity)	46	104%
Household drip-filter (1 l. capacity)	49	102%
Plunger pot	49	99%
Fresh-brew filter coffee (vending machine)	62	102%
Espresso machine (manual equipment)	146	75%

At-home brewing strengths range from about 30 g/l upward. The tested brewing methods showed complete extraction of acrylamide into the brew up to the double of this brewing strength. Only for espresso (146 g/l) the extraction of acrylamide was incomplete. The lower extraction efficacy of espresso brewing might be due to the combination of the higher coffee to water ratio plus the much shorter extraction time as used in espresso brewing.

No reduction of acrylamide was observed at holding the coffee beverage from the household drip-filter coffee in a thermos-jar for a period of $1^1/_2$ hour (data not shown).

CONCLUDING REMARKS

Different to most other affected food products in coffee the acrylamide level went through a peak level during processing. Both formation and reduction of acrylamide occurred during the roast process. The remaining levels in the fully roasted product for brewing of coffee were only a small fraction of the intermediately observed peak levels.

The main factors in affecting the acrylamide level in roasted coffee appeared to be: The Arabica/Robusta ratio in the blend, with Robusta giving higher levels; time and degree of roast, with both shorter and lighter roasting at the edges of the normal roasting range giving the higher levels; conditions and time of storage after roasting, with clear reduction by ambient storage during a few months. Acrylamide, present in the roast and ground coffee at the moment of brewing, was largely extracted into the brew. This extracted acrylamide was stable in the beverage within the normal time for consumption. The Arabica/Robusta ratio in the blend, the time of roast and the degree of roast have very distinct effects on the sensorial characteristics of the final product. Therefore, within the accepted boundaries of product specific taste profiles, only relatively small effects on the acrylamide level are expected to be achievable.

These results leave a number of quite relevant and interesting questions for further research. The European coffee sector already initiated a study into the mechanism and kinetics of the acrylamide reduction reaction. Part of the variation observed in commercial samples might be actually due to differences in freshness of the coffee. Another subject for investigation is the co-variance of other desired/undesired coffee components with any changing of the roast conditions.

Present study researched the chain from green coffee till the coffee beverage for the factors affecting the acrylamide level. Taking the results together, it can be concluded that within the sensorial range, as accepted by the consumers, only relatively small reduction of acrylamide levels appears achievable

ACKNOWLEDGEMENTS

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Impact of Roasting Conditions on Acrylamide Formation in Coffee

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SUMMARY

The impact of steam treatment applied prior to or during coffee roasting on acrylamide content was investigated. As results, steam treated and roasted samples contain more acrylamide content (at constant colour) than conventionally roasted coffee. This is due to the shorter time needed to roast those samples, allowing for less efficient acrylamide degradation. On the other hand, steam-roasted samples need longer roasting time to reach the target colour and thus contain less acrylamide. At fixed colour and roasting time, conventional roasting leads to similar content in acrylamide than both tested processes. Sensorially, however, both tested processes lead to important changes, compared to conventional roasting.

RÉSUMÉ

L'impact d'un traitement à la vapeur avant ou pendant la torréfaction du café sur la teneur en acrylamide a été étudié. Il apparaît que tous les échantillons traités à la vapeur puis torréfiés contiennent plus d'acrylamide que du café torréfié conventionnellement (à couleur constante). En effet, le temps de torréfaction de ces échantillons est plus court, ce qui permet une dégradation moins efficace de l'acrylamide. Par contre, les échantillons torréfiés sous vapeur ont besoin de plus de temps pour être torréfiés. Ainsi, leur teneur en acrylamide est réduite. A couleur et durée de torréfaction constantes, une torréfaction conventionnelle conduit à des teneurs similaires en acrylamide que les deux procédés testés. Par contre, sensoriellement, les deux procédés testés induisent à des changements importants comparés à une torréfaction conventionnelle.

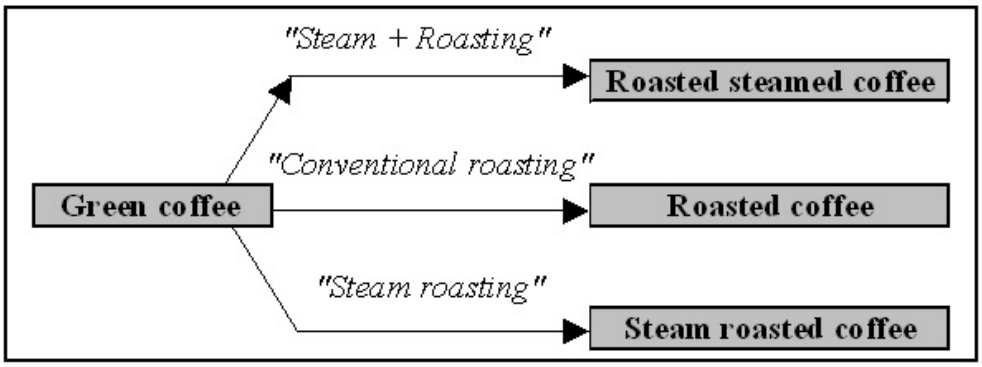
INTRODUCTION

In 2002, the Swedish National Food Administration published data on high concentrations of acrylamide, a neurotoxic and potentially carcinogenic compound, in heated carbohydrate-rich foods (Swedish National Food Administration, 2002). The levels were considered alarming, as the acrylamide containing foodstuffs included daily-consumed products such as crisp bread, roast potatoes, French fries, and roasted coffee.

Roasted coffee, which contains between 150-500 μg/kg acrylamide, was considered as an important source of acrylamide (Andrzejewski et al., 2004). Up to now, no legislation on acrylamide exists. However, Germany has recently introduced a minimization concept ("signal value"), setting the recommended maximal value for roasted coffee at 370 μg/kg (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2004). In California, a working plan considers that a variety of cooked foods, including roasted coffee, may require carcinogen warnings. Therefore, many efforts in the scientific community of coffee have focused on the understanding and mitigation of acrylamide generation upon roasting (Bagdonaite and Murkovic, 2004). Under conventional coffee roasting, acrylamide is formed

by Maillard-type reactions at temperature above 110 °C and starts degrading above 130 °C. Asparagine and reducing sugars are the key precursors, asparagine being the limiting factor.

Recently, TNO (Netherlandse Organisatie Voor Toegepastnatuurwe Tenschappelijk Onderzoek) patented a steam roasting process (WO 2004/066751) claiming to significantly decrease acrylamide levels in various foods (e.g. cacao spices). Two different steam treatments (i.e. steam treatment + roasting and steam roasting) were applied to coffee and compared to conventional roasting. The impacts of roasting temperature and both steam treatments were assessed on acrylamide content and on sensory properties.



EXPERIMENTAL

Green coffees

- A commercial green *Robusta* (Vietnam) was used for the "Steam+Roasting" trials.
- A commercial green *Arabica* (Colombia) was used for the "Steam Roasting" trials.

Roasting

Three roasting processes were applied:

- *"Conventional roasting"* (i.e. reference) was performed in a fluidized bed roaster (Neuhaus Neotec).
- *"Steam+Roasting"* The steam treatment of green coffee was performed in an autoclave. Kinetics were carried out in the temperature range 120-170 °C. The wet coffee was immediately dried and roasted in a fluidized bed roaster (Neuhaus Neotec) at fixed temperature (i.e. 215 °C) and colour (i.e. CTN 90).
- *"Steam roasting"* Steam roasting trials were performed in a rotating roaster equipped with steam injection. A full experimental design was performed. Temperature varied between 200 and 240 °C and water activity between 0.04 and 0.16. For all the roasting trials, time was adapted to reach CTN 90.

Analytical methods

- Roasting colour was determined using a Nehaus Colour test II
- Acrylamide was analyzed using a LC-MS/MS and d_3-acrylamide (Cambridge Isotope Laboratory) as internal standard (Delatour et al., 2004).
- Sensory evaluation was performed by comparative profiling using the conventionally roasted coffee as a reference. The panel was composed of 6 trained assessors familiar with the roasted coffee tasting. The preparation consisted in brewing grounded roasted coffee (concentration: 4%) for 2 min. Sixteen attributes were used to describe the products.

RESULTS & DISCUSSION

"Conventional roasting"

Coffee was roasted in a fluidized bed roasted at three different temperatures (i.e. 210-230 °C). Time was adapted to reach a fixed colour (i.e. CTN 90). Acrylamide was analyzed after roasting (Figure 1). As results, acrylamide depends on roasting time (at fixed colour). Longer roasting time leads to lower acrylamide content, due to more efficient acrylamide degradation.

Roasting T. °C	Roasting time min	Acrylamide ppb
230	4.5	176
220	6.7	145
210	10.0	127

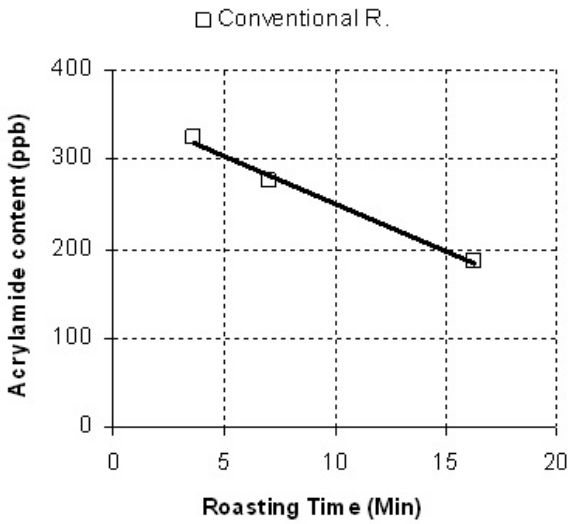


Figure 1. Impact of roasting time on acrylamide content in roasted coffee during a conventional roasting (Roasting in a Neotec roaster at CTN 90).

"Steam+Roasting"

Regarding steam treatment + roasting, steam treatments of various severities (i.e. T = 120-170 °C, t = 30-120 min) were applied. Acrylamide was analyzed after roasting at fixed colour (Figure 2)**.** As results, the steam treated coffee reaches the target colour faster than the conventionally roasted coffee at the same temperature. The more severe the steam treatment, the faster the roasting is. This is probably due to the colour that already starts to develop during steam treatment.

Regarding acrylamide, steam treatment at higher temperature leads to higher acrylamide content after roasting. Indeed, the acrylamide content mainly depends on the roasting time (at fixed colour), independently of the steam treatment applied prior to roasting. As observed for the conventional roasting, longer roasting time allows for more efficient acrylamide degradation. The conventionally roasted coffee also behaves in the same manner leading to a similar content in acrylamide compared to steam treatment and roasting, at fixed colour and roasting time.

Sensorially, the differences are directly related to the steam treatment intensity (Figure 3). Low steam treatments lead to coffees that are relatively close to the conventionally roasted coffee. On the other hand, strong steam treatments lead to sweeter, more acid, less bitter, less *Robusta* notes.

	Steam treatment °C / min	Roasting time min	Acrylamide ppb
Steam + Roasting	120 / 30	12.2	247
	120 / 38	12.3	247
	120 / 50	12.5	214
	120 / 67	14.0	230
	120 / 90	13.4	237
	140 / 22	10.0	227
	140 / 36	10.3	247
	140 / 55	9.1	222
	140 / 82	7.6	248
	140 / 120	4.9	307
	170 / 10	3.7	306
	170 / 13	3.4	323
	170 / 17	3.2	335
	170 / 22	2.4	300
Conven-tional roasting	-	16.3	185
	-	7.0	276
	-	3.6	324

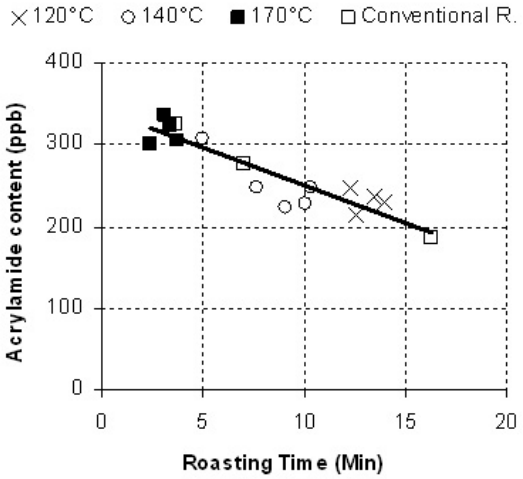


Figure 2. Impact of steam treatment + roasting on acrylamide content in roasted coffee (Steam treatment performed in autoclave, roasting in a Neotec roaster at CTN 90).

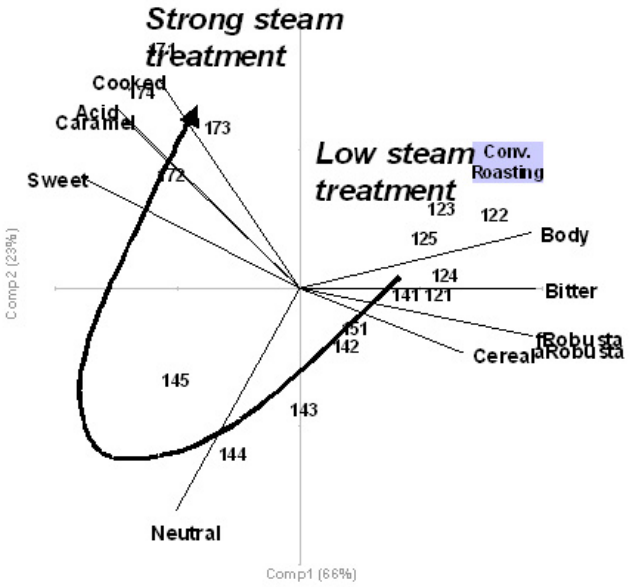


Figure 3. Impact of steam treatment and roasting on sensory properties.

"Steam Roasting"

Regarding steam roasting, roasting trials using different temperatures (i.e. 220-240 °C) and different water activities (i.e. 0.04-0.16) were performed. They were compared to a conventionally roasted coffee (Figure 4). As results, steam roasting leads to longer roasting time than conventional roasting (at fixed temperature), possibly due to the use of another roaster type and/or to the steam addition. Water activity had no impact on acrylamide as shown in Figure 5. On the other hand, the roasting temperature has again an important impact, lower roasting temperature leading to lower acrylamide content. As for the steam treatment + roasting and conventional roasting, this could be explained by longer time needed to reach the target colour, allowing more efficient acrylamide degradation. Again, conventional roasting

behaves in the same manner leading to a similar content in acrylamide than steam roasting at fixed colour and roasting time.

	Water activity / Roasting T. (°C)	Roasting time min	Acrylamide ppb
Steam roasting	0.04 / 200	18.0	88
	0.08 / 200	18.0	100
	0.16 / 200	18.0	95
	0.04 / 220	11.0	125
	0.08 / 220	11.0	113
	0.16 / 220	14	111
	0.04 / 240	8.0	126
	0.08 / 240	8.6	149
	0.16 / 240	8.1	163
Conventional roasting	- / 230	4.5	176
	- / 220	6.7	145
	- / 210	10.0	127

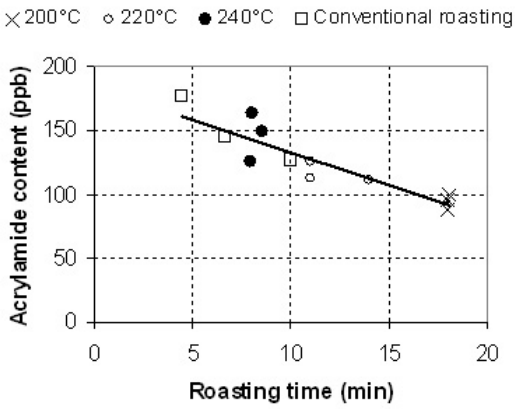


Figure 4. Impact of steam roasting on acrylamide content in roasted coffee (Steam treatment performed in a drum roaster, roasting in a Neotec roaster at CTN 90).

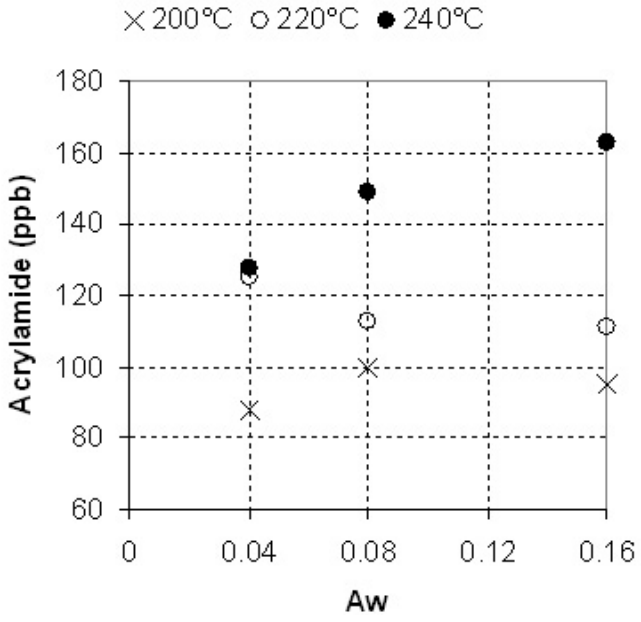


Figure 5. Impact of water activity during steam roasting on acrylamide content in roasted coffee (Steam pre-treatment performed in autoclave, roasting in a Neotec roaster).

Sensorially, all samples were found to be very different from the conventionally roasted coffee. Some differences were systematic, the steam-roasted samples showing less coffee, body and roasty notes, but more bitterness. Some attributes were dependent on water activity. Higher water activity leads to coffees that were found more acid, less roasty and bitter as shown in Figure 6.

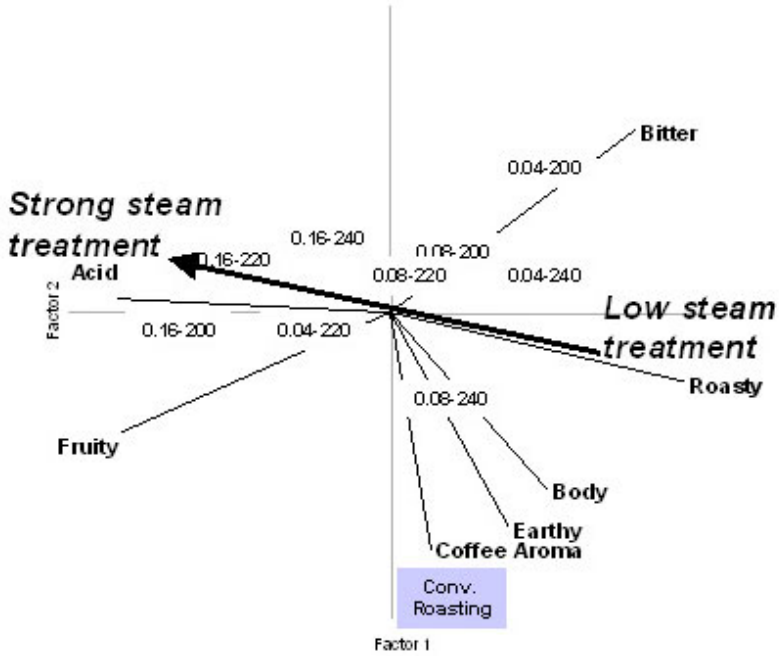


Figure 6. Impact of steam roasting on sensory properties.

CONCLUSIONS

In conclusions, both steam treatment and roasting and steam roasting do not impact the acrylamide content. The most prevailing roasting parameter is the roasting time. A longer roasting time allows a more efficient the acrylamide degradation and thus leads to lower final acrylamide content. Sensorially, both steam treatments lead to considerable changes compared to conventionally roasted coffee. In both treatments, more severe steam treatment leads to more acid, less roasty and bitter coffees.

ACKNOWLEDGEMENT

We thank TNO for the coffee roasting and for helpful discussions.

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Method of Preparing Novel Coffee Aromatizing Compositions

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SUMMARY

Increasing the intensity of aroma released during reconstitution of instant coffee powders may provide some consumers with a more fulfilling sensory experience. A new controlled-release technology was developed by the authors using novel encapsulated buoyant water-immiscible volatile carrier liquids (VCLs) to strengthen beverage preparation aroma (Zeller et al., 2003a, b). At the 20th ASIC we demonstrated the ability to create synthetic coffee aromas compounded in VCLs that accelerate aroma release and increase peak intensity relative to aromatized conventional carrier liquids (Zeller et al., 2004a). At this meeting we will describe an effective patented method to fix natural coffee aroma frosts in VCLs to further enhance the quality and authenticity of these novel high-impact coffee aromas (Zeller et al., 2004b).

MATERIALS

Food-grade d-limonene was sourced from Firmenich and food-grade 2-ethylfuran was sourced from Sigma-Aldrich. Each VCL was deodorized prior to aromatization by passing through a silica gel column to remove polar impurities. Coffee oil expelled from spent grounds and coffee aroma frosts produced from vent gases were obtained from Kraft manufacturing plants. Percolation vent gas frost was produced by cryogenic condensation of coffee volatiles released during hot water extraction of fresh roast coffee. Grinder vent gas frost was produced by cryogenic condensation of coffee volatiles released during grinding of fresh roast coffee. Frosts were predominantly solidified coffee-derived carbon dioxide (CO_2) and generally contained about 5-15%, by weight, water ice in addition to relatively small amounts of condensed coffee volatiles.

METHODS

VCL Aromatization

The following process was used to separately aromatize VCLs with coffee aroma frosts. VCLs were removed from a conventional freezer and placed in a pre-chilled jacketed stainless steel vessel fitted with a vented lid. Frost was added directly to VCLs in small amounts over 2-4 hours while temperature was gradually increased from about –75 °C to a maximum of about –20 °C effective to slowly sublime CO_2 from the frost and dissolve aroma components in the VCLs. VCLs remained liquid throughout the process due to their very low freezing points. After the final portion of frost was added to the vessel and sublimation of CO_2 was complete, aromatized VCLs were filtered or decanted to remove water ice present throughout the process. Aromatized VCLs were stored in a freezer in sealed glass vials. Frost-to-VCL weight ratio ranged between 10:1 and 20:1 to permit attainment of high aroma fixation levels. Additional details can be found in US 6,699,518.

Oil Aromatization

The following process was used to aromatize coffee oil with the same frosts used to aromatize VCLs. Oil was placed in the same stainless steel vessel and frost directly added while temperature was increased and held between 15-25 °C effective to sublime CO_2 from the mixture and dissolve aroma components in the oil. The process was conducted at a relatively high temperature over a longer period of time to prevent the oil from freezing and permit equilibration of volatiles in the oil. The aromatized oil was decanted from liquid water present during the process. Aromatized oils were stored in a refrigerator in sealed glass vials. Frost-to-oil weight ratio was set at 1.6:1 to provide sufficient oil to adequately dissolve aromas and permit attainment of normal fixation levels. Additional details can be found in US 6,699,518.

Encapsulation

The following process was used to separately encapsulate aromatized VCLs in instant coffee granules. A 50% coffee solution was prepared by dissolving 37.5 g Kenco® freeze-dried coffee in 37.5 g hot water. The solution was chilled to 3 °C and aerated for one minute at 10,000 rpm using an immersion mixer to disperse air bubbles in the solution. Aromatized VCLs were removed from the freezer and 7.0g added to the aerated coffee solution. The aeration process was repeated to disperse the aromatized VCLs in the coffee solution. The solution was dripped into liquid nitrogen using a syringe fitted with 24-guage needle. The frozen particles were dropped onto a 1500 g bed of vacuum-dried milled freeze-dried coffee (100 μm average particle size and 1% moisture) and desiccated in this powder for 48 hours in a closed container. The powder was sieved to recover the dry granules containing encapsulated aromatized VCLs which were stored in sealed vials at room temperature.

Aromatized Carrier Analysis

The following process was used to compare relative amounts of coffee aroma transferred to carriers under different aromatization conditions. A fixed weight (1.0g) of each aromatized carrier was sealed in a 22 mL glass vial and equilibrated at 60 °C for 30 minutes. A fixed volume (0.25 mL) of headspace was removed from the vial with an automated sampler and injected into a gas chromatograph equipped with a capillary column and flame ionization detector. Aroma counts were obtained by subtracting from the total any counts contributed by VCLs and then rounding to the nearest hundred counts. Aroma counts were compared to appropriate references to permit estimation of the amount of aroma recovered from frost during carrier aromatization. The approximate moisture content of aromatized carriers was obtained using Karl-Fischer analysis.

Coffee Granule Analysis

The following method was used to assess the intensity and quality and of preparation aromas provided by coffee granules containing aromatized VCLs. Granules were dry-blended with Kenco® freeze-dried coffee at fixed levels ranging between 3-10% by weight and placed in an empty beaker. Each blend was reconstituted using near-boiling water and the aroma released into the air above the hot beverage immediately evaluated by members of a trained sensory panel. The apparent particle density of aromatized coffee granules was approximated by dry-blending with an excess of ignited sand having much smaller particle size and measuring displacement volume in a graduated cylinder versus a reference without granules. The liquid density of aromatized VCLs was approximated by measuring weight and volume in a graduated cylinder.

RESULTS

Details of aromatization of d-limonene and coffee oil with percolation vent gas frost are summarized in the table. By comparison, aromatization of 2-ethylfuran with grinder gas vent frost at a 20:1 frost:carrier ratio produced an aroma content of 1000 counts with aroma recovery and moisture content comparable to aromatization of d-limonene with percolation vent gas frost.

Table 1.

Carrier	Frost:Carrier Ratio (w/w)	Fixation Time (hours)	Aroma Content (counts)	Aroma Recovery (%)	Moisture Content (%)
d-Limonene	10:1	2	2100	90	0.1
d-Limonene	12:1	4	2300	90	0.1
Coffee Oil	1.6:1	8	300	80	1.5

Aromatized VCLs had approximately 0.9 g/cc liquid densities compared to 1.0 g/cc for water. Coffee granules containing encapsulated aromatized VCLs had apparent particle densities of approximately 0.8 g/cc. Relatively low density and aerated structure caused coffee granules to float and rapidly dissolve on the surface of reconstituted beverages to essentially instantaneously release the encapsulated aromatized VCLs. Relatively low density, low water solubility, and high volatility of aromatized VCLs caused them to rapidly evaporate from the beverage surface to provide an aroma burst without leaving behind an oil slick. The panelists agreed beverages formulated with coffee granules containing aromatized VCLs produced preparation aroma intensity and quality superior to reference beverages prepared without granules. The former were generally described as providing intense preparation aroma having fresh, rich, roast coffee character.

CONCLUSIONS

The intensity and quality of instant coffee preparation aroma can be greatly improved by use of floating coffee granules containing encapsulated VCLs aromatized with natural coffee frosts. The low freezing point and viscosity of VCLs enable rapid and efficient aromatization at temperatures below the melting point of water ice to provide high aroma contents at low moisture levels. The low density and aerated structure of coffee granules in combination with the high volatility and low water solubility of aromatized VCLs encapsulated therein provides rapid controlled-release and evaporation from the surface of hot instant coffee beverages without creation of residual oil slicks.

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Production of Biodiesel from Defective Coffee Beans

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SUMMARY

The present study aimed at an evaluation of the production of biodiesel from the oil of defective coffee beans. Alcohol-to-oil molar ratios, time and temperature were the reaction parameters studied. Sodium methoxide was used as alkaline catalyst. Direct transesterifications of triglycerides from oil extracted from defective coffee beans were performed with both methanol and ethanol. Gas chromatography was employed for biodiesel analysis. The highest values of ester yields were 74% for coffee oil, for 1h transesterification with methanol at 25 °C. Also, the evaluated physical properties of the coffee oil biodiesel (density, viscosity and heat value) were within the limits specified by European standards. Further studies regarding the identification of the factors affecting conversion are needed in order to optimize the production of biodiesel using the oil from defective coffee beans. Preliminary tests involving the removal of unsaponifiable matter prior to transesterification has been demonstrated to be a suitable processing step for improving the ester yield.

INTRODUCTION

Brazil is the largest coffee producer in the world. However, approximately 20% of its production consists of defective beans, which upon roasting decrease beverage quality. Regardless of growth, harvest and processing conditions, some defects are intrinsic in nature and will always be present. The ones that affect beverage quality the most are black, brown and immature beans. These defective beans are physically separated from the non defective beans prior to commercialization in international markets. However, since to coffee producers they represent an investment in growing, harvesting, and handling, these defective beans are put in the internal market in Brazil, where the roasting industry uses them in blends with non defective beans. Thus, the quality of the roasted coffee consumed in Brazil is depreciated (Oliveira et al., 2006). In view of this situation, several studies are currently under development in order to find an alternative use for defective coffee beans. One of the alternatives being considered is biodiesel production.

Biodiesel is a name applied to fuels manufactured by the esterification of renewable oils, fats and fatty acids. Biodiesel can be employed as a fuel for unmodified diesel engines (Graboski and McCormick, 1998). This type of fuel has been commercially produced in Europe since the early 1990's (Barnwal and Sharma, 2005). Research studies indicate that, when used as a diesel fuel substitute, biodiesel can replace diesel fuel without causing harmful effects to an unmodified diesel engine, while simultaneously reducing harmful exhaust emissions. Also, biodiesel is completely miscible with petroleum diesel fuel and can be employed as a blend. However, biodiesel is still not economically feasible in comparison to petroleum diesel. Grain (soybean, rapeseed, sunflower seed) production costs are responsible for approximately 70% of total fuel production costs. Such drawback could be minimized by the use of a "less valuable" product, such as the oil of rejected defective beans, with the advantage of allowing coffee producers to produce and use their own fuel. Therefore, the present study aimed at an evaluation of the production of biodiesel from the oil of defective coffee beans.

METHODOLOGY

Materials

The coffee beans used in the present work, both healthy and defective, were acquired from Santo Antonio Estate Coffee, an association of coffee producers in Minas Gerais State, Brazil. The reagents employed were all of analytical grade.

Oil extraction

Coffee beans were ground in a Rotatec grinder (Brazil) prior to oil extraction. The oils of the ground healthy and defective coffee beans were obtained by solvent (hexane) extraction in an industrial Soxhlet apparatus (Sociedade Fabbe Ltda, Brazil), with an extraction capacity of 25 kg of beans per batch. The oils were extracted in batches of 16 hours of duration. After extraction, the solvent was removed in a rotary evaporator (Fisatom, mod. 5502, Brazil) at 70 °C, until no traces of hexane were detected by gas chromatography.

Transesterification reactions

The transesterification reactions were performed in a 500 mL cylindrical three-necked reactor, mechanically stirred (600 rpm) and heated by a hot water jacket. The temperature of the water in the jacket was controlled by a thermostatic water bath and it was set to keep the reacting medium at the desired temperature (55 °C when using methanol and 60 °C for ethanol). The reactions were carried out with eighty grams of coffee oils from defective and healthy beans. Sodium methoxide 1% (based on the mass of oil) was used as an alkaline catalyst for oil of healthy coffe beans. For the defective coffee beans oil, the amount of catalyst used was calculated as the minimum necessary for the transesterification of the triglyceride fraction plus that for the neutralization of the titrated free fatty acids (Pregnolatto and Pregnolatto, 1985). Methanol and ethanol were employed in a 6:1 alcohol-to-oil molar ratio. The reactions were carried out at ambient temperature (25 °C) and at 55 °C and 60 °C for methanol and ethanol, respectively. After completion of reaction, the pour-off time was 24 hours, a time long enough to allow for the complete separation of an upper ester layer and a lower glycerol layer. The upper layer was analyzed by gas chromatography to determine the yield of fatty acids alkyl esters. The parameters used in the transesterification tests are presented in Table 1.

Gas Chromatography

The samples taken after transesterification of the oils were analyzed by gas chromatography. 25 μL of the samples were dried in a nitrogen stream for the removal of the excess solvent and subsequently weighed and diluted in 1mL methanol. The fatty acids methyl and ethyl esters were injected in a Varian 3380 gas chromatograph, equipped with a flame ionization detector. The analytic conditions employed were: initial temperature of 200 °C, followed by a temperature increase at a rate of 10 °C per minute up to 240 °C. The duration of the analysis was 10 minutes. A Carbowax 20M column was employed. Peak identification was carried out by comparison of the retention times with those for the respective fatty acid methyl esters standards (FAMEs), which were prepared by esterification of fatty acids standards with BF3 in methanol solution.

Table 1. Parameters employed in the transesterification reactions.

Oil	Temperature (°C)	Reaction time (hours)
Coffee healthy	25	1.0
Coffee healthy	25	2.0
Coffee healthy	55	0.5
Coffee healthy	55	1.0
Coffee defective	25	1.0
Coffee defective	25	2.0
Coffee defective	55	0.5
Coffee defective	55	1.0

RESULTS AND DISCUSSION

The yield of coffee beans oil ranged from 10 to 12% on a dry weight basis. The titrated acidities of the oils of healthy and defective coffee beans were 2.62 ± 0.29 and 10.04 ± 0.03% (w/w), respectively. Notice that the contribution of the defective beans to the acidity of the oil is fairly high, since these beans are partially comprised of fermented beans (black and brown). The triglyceride composition of the coffee oils, from both healthy and defective beans, were determined by a thermogravimetric procedure adapted from that proposed by Goodrum and Geller (2002). Coffee oil from healthy beans was determined to be comprised of 81% (w/w) triglycerides and the oil from defective beans presented a 76% (w/w) triglyceride fraction, both values falling within the range previously published in the literature (Oliveira et al., 2006; Speer and Kölling-Speer, 2001). The oils were subjected to transesterification reactions without any refinement, i.e., with a 19 and 24% fraction of unsaponifiable matter for the oils of healthy and defective beans, respectively. The moisture content of the oils were determined as 0.28 and 0.11 kg_{water}/kg_{oil} for the oils of healthy and defective beans, respectively. The fatty acid composition of the coffee oils were determined in a previous study (Oliveira et al., 2006) and the major constituents are linoleic and palmitic acids (44 and 34%, respectively), followed by oleic (9%) and stearic (7%) acids.

The characteristics observed for the transesterification reactions with oils from healthy coffee beans are summarized as follows. The oil samples reacted with methanol presented good separation of phases during pour-off time, with negligible amounts of saponified and gelatinous matter being observed in the products. When using ethanol, a partial separation of phases was observed only for the reaction carried out at room temperature for two hours. The other reaction conditions did not lead to separation of phases during pour-off time. All the samples reacted with ethanol presented significant amounts of gelatinous matter in the reaction products. In order to evaluate whether ester conversion occurred or not, the reactions products that did not separate into two phases were diluted in alcohol and submitted to an acid treatment for neutralization of unreacted catalyst and to facilitate separation of phases. The non-soluble gel was separated by centrifugation, and the upper layer was removed and resubmitted to an evaporation procedure for alcohol removal. After solvent removal, a new separation of phases occurred and formation of gel was not observed. The ester layers were analyzed by GC. The chromatogram for the products obtained in the reaction with ethanol at 55 °C, for 1 hour, is presented in Figure 1. Peaks related to both ethyl and methyl esters are clearly noticeable. The methyl esters peaks are due to the presence of methanol in the solution in which the catalyst is commercially available (30% CH3ONa in methanol). All the other chromatograms presented similar ester profiles.

The average ester conversion results for the reactions with healthy coffee oil are presented in Table 2. Standard deviation values ranged from 0.4 to 1.7 percentage points. The conversions

obtained for the coffee oil were lower than those for the refined soybean oil. These lower conversions may be attributed to the fact that part of the catalyst was consumed for the production of soap, since there was no addition of catalyst to compensate for the higher free fatty acid content of the healthy coffee oil (2.6%). Also, the presence of unsaponifiable matter (19%) might have hindered the interactions between the reactants (triglycerides and alcohol). Also, the presence of emulsion in the products increases the solubility of alkyl esters in the glycerol-rich phase (Vicente et al., 2004), which was separated by centrifugation prior to the collection of samples for the analysis, and part of the produced ester might have been removed in that phase. When methanol was employed, there were no significant differences in ester conversion with an increase in reaction time. The reactions with ethanol presented lower conversions than those with methanol. Also, regarding the reactions with ethanol, an increase in reaction time promoted higher ester conversions when the reaction was carried out at room temperature, and did not affect the conversion for the reaction at 60 °C. Although ethanol is expected to lead to higher conversions in shorter reaction times, due to its higher miscibility with the oil, it is also known to hinder phase separation. Thus, it makes the determination of the true ester yields difficult, as the reaction products should undergo further processing (acid treatment or addition of glycerine) to allow for good separation of phases.

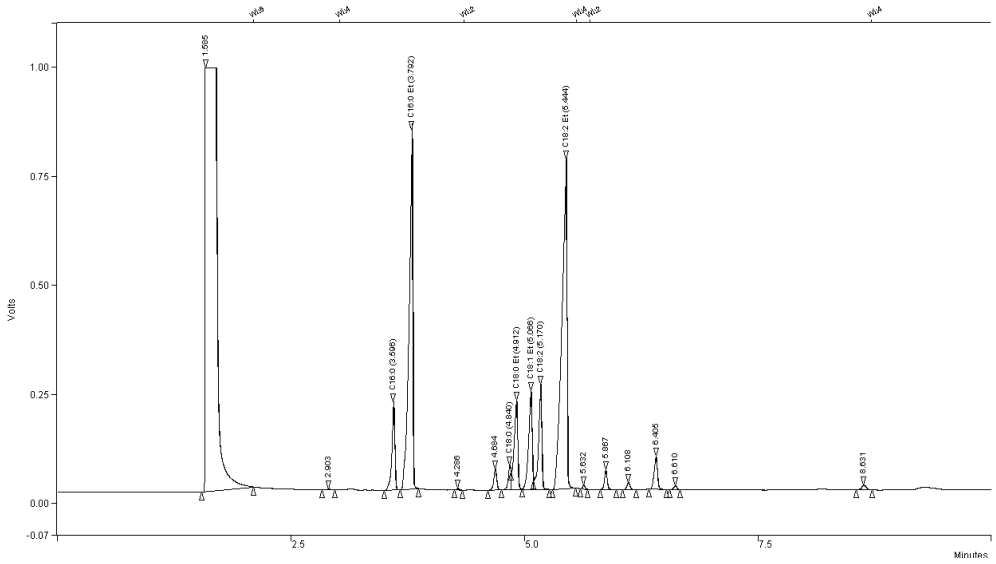

Figure 1. Chromatogram for biodiesel from coffee oil (healthy beans) after 1 h transesterification with ethanol at 55 °C.

Table 2. Ester yields for the reactions with oil from healthy coffee beans.

Reaction conditions	Ester yield (%)
MetOH 25 ºC 1 h	70
MetOH 25 ºC 2 h	68
MetOH 55 ºC 0,5 h	62
MetOH 55 ºC 1 h	60
EtOH 25 ºC 1 h	35
EtOH 25 ºC 2 h	45
EtOH 60 ºC 0,5 h	64
EtOH 60 ºC 1 h	64

The oil of defective coffee beans was subjected to transesterification reactions employing the same conditions as for the oil of healthy beans with the exception that the amount of catalyst was recalculated to account for its higher free fatty acid content (10%). Saponification of the

free fatty acids was observed instantaneously as the mixture of alcohol and catalyst was added to the reactor, causing an increase in the viscosity of the reactants mixture. As the alkyl esters began to form, the viscosity of the reacting medium decreased again. After completion of the reaction, the product was set to rest, not separating into phases and acquiring a paste-like texture as it cooled down. After alcohol removal (evaporation), the mixture acquired a solid-like texture. It was then submitted to an acidified alcohol treatment as suggested in the literature (Encinar et al., 2005) and centrifuged afterwards. Following centrifugation, good separation of phases was attained. The ester layer was sampled and analyzed by GC. The chromatogram for the reaction with methanol at room temperature is presented in Figure 2. All the other chromatograms presented similar ester profiles.

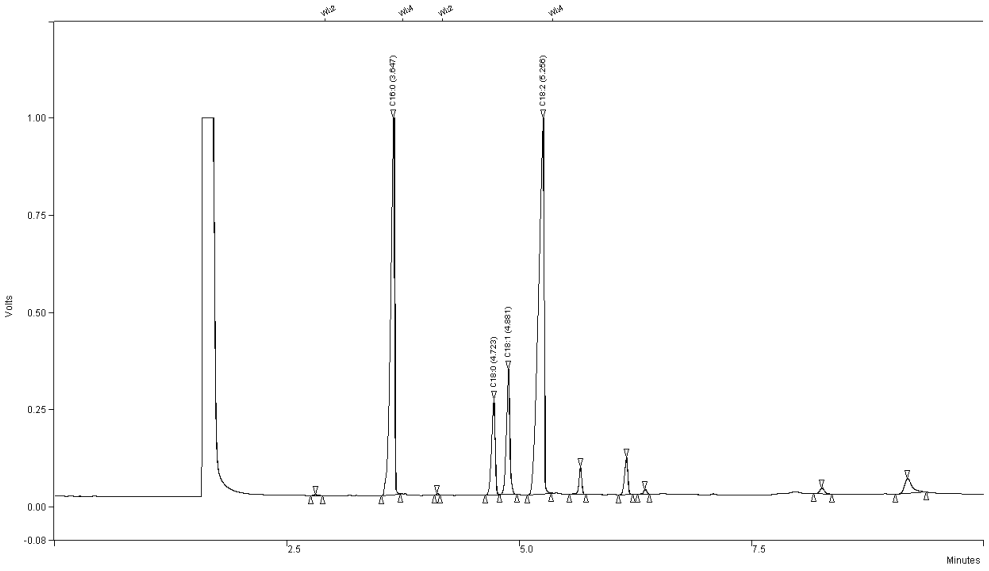

Figure 2. Chromatogram for biodiesel from coffee oil (defective beans) after 1h transesterification with methanol at 25 °C.

The average ester yields for all the reactions carried out with defective beans oil are presented in Table 3. Standard deviation values ranged from 0.5 to 1.8 percentage points. The ester yields in this case were higher than those obtained for the oil of healthy coffee beans. This can be attributed to the fact that the amount of catalyst used for the oil of defective beans was increased to compensate for a higher amount of free fatty acids. A higher amount of catalyst will allow for reactions with free fatty acids to produce soap and assure that the remaining amount of catalyst is enough to promote transesterification of the entire triglyceride fraction. Again, when compared to the results for the refined soybean oil, the ester yields were lower. Recall that the oil of defective coffee beans presented a 24% fraction of unsaponifiable matter that might have hindered interactions between reactants, thus contributing to the lower yields. The unsaponifiable fraction is responsible for imparting a higher viscosity to the oil. In this study, the viscosity of coffee oils was determined to be about four times higher than the viscosity of the refined soybean oil (170 mPa.s against 43 mPa.s).

An increase in reaction time did not significantly affect conversions to esters, except for the reactions with ethanol at 60 °C. A major difference in ester yields was observed for the reactions with ethanol at 60 °C, when an increase of 30 minutes in reaction time caused an increase of 16 percentage points in ester yield. In the case of ethanol, it is known from literature that the transesterification reaction is kinetically controlled, since the reactants (ethanol and oil) are completely miscible (no mass transfer resistance) and, also, the reaction rates are more dependent on temperature than when using methanol. Thus, at higher

temperatures (60 °C), this dependence on temperature is evidenced and it is further supported by the fact that an increase in time did not favor the ester yield at 25 °C.

Table 3. Ester yields for the reactions with oil from defective coffee beans.

Reaction conditions	Ester yield (%)
MetOH 25 °C 1 h	74
MetOH 25 °C 2 h	71
MetOH 55 °C 0,5 h	68
MetOH 55 °C 1 h	71
EtOH 25 °C 1 h	72
EtOH 25 °C 2 h	73
EtOH 60 °C 0,5 h	55
EtOH 60 °C 1 h	71

Physical properties for the biodiesel produced from coffee oils are presented in Table 4, together with European specifications for commercial biodiesel and Diesel No. 2. All the physical properties for the coffee oil biodiesel were within the limits specified by European standards. Regarding the amount of free glycerol in the ester-rich phase, after one acidic methanol washing step (Tomasevic and Siler-Marinkovic, 2003) and a few water washing steps, it was determined to be in the range of 123 to 141 mg/kg. The method employed for the free glycerol determination was that proposed by Bondioli and Bella (Bondioli and Bella, 2005).

Table 4. European biodiesel[a] and Diesel No. 2[b] specifications.

Property	Unit	Lower Limit (Knothe et al., 2005)	Upper Limit (Knothe et al., 2005)	Tobacco seed oil biodiesel (Usta, 2004)	Coffee oil fatty acid methyl esters[a]		Soybean oil Biodiesel[a]	Diesel No. 2 (Usta, 2004)
					defective	Healthy		
Density	Kg/m^3	860	900	886.8	894.1	892.5	876.7	841.5
Viscosity	mm^2/s	3.5**	5**	3.5**; 6*	4.9**;8.9*	3.1**;3.4*	5.7*	2.9**
HHV[b]	kJ/kg	35000	-	39811	38414	38498	39100	44631

**Value at 2 5ºC; **Value at 40 ºC; [a]This study, reactions with methanol at 25ºC; [b]HVV=High Heating Value.*

Preliminary tests with removal of unsaponifiable matter prior to transesterification of oil of healthy beans, at 25 °C, for 1h, were conducted and results demonstrated that increases in esters yields of the order of 21 percentage points could be achieved, when compared to the situation when the oil was transesterified with 19% of unsaponifiable matter present.

CONCLUSIONS

Oils extracted from healthy and defective coffee beans were successfully converted to biodiesel by transesterification with both methanol and ethanol in the presence of sodium methoxide as an alkaline catalyst. The yields for the reactions with the oil of healthy coffee beans were lower than those for the oil of defective beans, indicating the need for correction of the amount of catalyst to be used due to the content of free fatty acids of the oil. Further studies regarding the identification of the factors affecting conversion are needed in order to optimize the production of biodiesel using the oil from defective coffee beans. Removal of

unsaponifiable matter prior to transesterification has demonstrated to be a suitable processing step for improving the ester yield.

ACKNOWLEDGEMENTS

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Improving Industrial Measurement of the Temperature of Roasting Coffee Beans

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SUMMARY

Coffee roasting reactions depend on roasting bean temperature versus time, t, history. Thermometric lag and bean heat-transfer resistance, cause measured bean temperatures, T_A, to differ significantly from bean mean temperatures, T_M. Eqs [5] and [9] in this paper can be used to compute T_M versus t histories from T_A versus t data.

RÉSUMÉ

Les réactions de la torréfaction du café dépendent de l'évolution temporelle de la température du café pendant la torréfaction. Le retard thermometrique et la résistance thermique des grains de café causent les divergences marquées entre T_A, les températures mesurées des grains, et T_M, les températures moyennes des grains. On peut utiliser les données de l'évolution des T_A vis-à-vis des temps, t, et les Eqs [5] et [9] de cette étude pour calculer l'évolution temporelle de T_M.

INTRODUCTION

Coffee beans heat differently in different locales in a roaster and at different points on their surface, but heating effects tend to even out soon because roasters mix beans well. Bean temperatures are measured by a sturdy probe, a sheathed thermocouple or thermistor, immersed in a dense flow of beans. A properly positioned probe responds to the mean bean-surface temperature, T_S, and not to roaster gas temperature, T_G. The measured temperature, T_A, often is recorded versus time, t. The probe diameter, D, may be 6.4 mm or in some large roasters even 9.5 mm. Thus thermometric lag is large. In small roasters, D are smaller, and lag is smaller, but still significant.

Roasters are preheated before beans are loaded. Therefore T_A initially is much higher than the corresponding bean mean temperature, T_M. Entering beans cool the probe. So T_A decreases rapidly at first. T_A still drops for a while after loading ends, then passes through a minima, T_{min}, at $t = t_{min}$ and rises. Typical t_{min} range from 40 to 60 sec for a 15 kg drum roaster, to 70 to 90 sec for a 240 kg drum roaster, to 100 sec or more for a large rotating bowl roaster where a 9.5 mm diameter thermocouple was used.

THERMOMETRIC LAG

Thermometric lag in coffee roasters is governed by Eq [1]

$$T_S = T_A + K(dT_A/dt) \qquad [1]$$

where K is the thermometric lag coefficient. Eq (1) shows $T_S = T_A = T_{min}$ when $dT_A/dt = 0$, i.e. at t_{min}. $T_S < T_A$ for $t < t_{min}$; but $T_S > T_A$ at $t > t_{min}$.

In flowing air or water, K are more than linearly proportional to D, depend mainly on external heat-transfer resistance rather than in-probe resistance, and depend on air or water flow rate. Listed K for probes in coffee bean streams were not available. K of 40 sec and 68 sec respectively were obtained for probe-roaster combinations examined in this work. If K = 40 sec, T_S will be 28 °C higher than T_A when dT_A/dt = 0.7 °C/sec

TEMPERATURE DIFFERENCES WITHIN BEANS

$T_S > T_M$ and $T_M > T_o$, the bean's center temperature. Based on Duhamel's theorem, T_S, dT_S/dt and bean thermal properties determine how T_M and T_o behave. Thus, if Eq [1] applies, T_M and T_o as well as T_S depend on T_A and can be computed from T_A versus t data; and roaster control systems that reliably control T_A versus t behavior, similarly control internal bean temperature behavior and the outcomes of flavor-production by roasting reactions.

To estimate T_M and T_o by use of Duhamel's theorem, I modeled beans as spheres having the same thermal properties and surface/volume ratio; and showed that shortly after bean loading is complete that

$$(T_S - T_M) = F_M[a^2/\gamma](dT_S/dt) \quad [2]$$

and

$$(T_S - T_o) = F_o[a^2/\gamma](dT_S/dt) \quad [3]$$

γ is the thermal diffusivity of beans and a is the radius of the equivalent sphere. Eqs [2] and (3) apply at $t > t_L$ + 10 to 15 sec, where t_L is the bean loading time. Equations for $(T_S - T_M)$ and $(T_S - T_o)$ are similar, with F_M used in one and F_o in the other. Therefore only equations for $(T_S - T_M)$ will be shown hereafter.

F_M and F_o depend on the shape of the T_S versus t curve. If T_S rises at a constant rate, F_M = 0.0667 and F_o = 0.1667. F_M and F_o are larger when, as often occurs in roasters, T_S rises at a progressively decreasing rate. As shown later, F_M was 0.092 and F_o was 0.213 for T_S versus t behavior in a typical drum roaster. If γ = 1.35 x 10^{-7} m^2/sec, as reported for Brazilian Arabicas (Eggers and v. Blittersdorff, 2005) and a = 2.78 mm, $[a^2/\gamma]$ = 57 sec. If the cited F_M and F_o apply and K = 40 sec and, as in my earlier example, dT_A/dt = 0.7 °C/sec, T_M will be 3.7 °C lower than T_S, but 24.5 °C higher than T_A; and T_o 8.5 °C will be lower than T_S but 19.5 °C higher than T_A. In general, T_M is much closer to T_S than to T_o at t where Eqs [2] and [3] apply. Thus fine probes implanted at the center of beans do not sense T_M well. Thermometric lag affects computed T_M more strongly than in-bean heat transfer resistance whenever K > 10 sec; and much more strongly when K > 20 sec.

Exothermic heating or evaporative cooling were neglected in computing T_M and T_o values. However, if Q, the rate/unit mass of exothermic heating or evaporative cooling, is spatially uniform in beans

$$(T_S - T_M) = F_M[a^2/\gamma](dT_S/dt - Q/C) \quad [4]$$

where C is the beans' heat capacity. Q is positive for exothermic heating and negative for evaporative cooling. Combining Eqs [1] and [4]

$$T_M = T_A + K(dT_A/dt) - F_M[a^2/\gamma][dT_A/dt + K(d^2T_A/dt^2) - Q/C] \quad [5]$$

T_S versus t curve shape and F_M and F_o values are affected by the Biot number, B. $B = h_G\, a/k$, where h_G is the gas-to-bean heat-transfer coefficient and k is the thermal conductivity in beans. To determine how B affects F_M and F_o for T_S versus t curves for typical roasts, I examined how ($T_S - T_M$) and ($T_S - T_o$) vary in spheres (i.e. modeled beans) convectively heated by gas at constant temperature T_G when Q again is spatially uniform. For t long enough for T_S, T_M and T_o to be governed by the first term in the infinite series solution for the partial differential equation governing such heating, it can be shown that 0

$$F_M = (3B/q^2 - 1)/q^2 \qquad [6]$$

and

$$F_o = [(q/\sin(q) - 1)/q^2] \qquad [7]$$

where q is the first root of $q[\cot(q)] + B - 1 = 0$. Derivation of Eqs [2] to [7] will be provided elsewhere. $B < 3$ for beans in most roasters. If $B < 3$, $q^2 \approx 3B/[1 + 0.235B]$ with little error; $F_M = .0783(1 + 0.235B)$; and $F_o = (1 + 0.235B)(q/\sin(q) - 1)/3B$

Eqs [2] to [7] were derived for cases where T_S is uniform. T_S is not uniform on a roasting bean's surface. Non-uniform T_S develop for objects whose geometry is not wholly symmetric. When the conditions used to develop Eqs [6] and [7] apply, it can be shown for several such geometries (e.g. short cylinders and rectangular blocks) that both ($T_S - T_M$) and ($T_S - T_o$) will be proportional to ($dT_S/dt - Q/C$) where T_S is the mean surface temperature. Thus, both ($T_S - T_M$) and ($T_S - T_o$) may also be proportional to ($dT_S/dt - Q/C$) in the same sense for hemiellipsoidal coffee beans.

When Eqs (6) applies, it can be shown that

$$(T_S - T_M) = F_M\, q^2\, (T_G - T_S)_{lm} = (3B/q^2 - 1)(T_G - T_S)_{lm} \qquad [8]$$

where $(T_G - T_S)_{lm} = (T_{G1} - T_{G2})/\ln [(T_{G1} - T_S)/(T_{G2} - T_S)]$ and T_{G1} and T_{G2} are the respective temperatures of gas entering and leaving the roasting chamber.

Combining Eqs [1] and [8]

$$T_M = T_A + K(dT_A/dt) - F_M\, q^2\, [T_A + K(dT_A/dt) - T_G]_{lm} \qquad [9]$$

Unlike Eq [5], Eq [9] does not involve use of Q/C or determination of d^2T_A/dt^2. Eqs [4] to [9] are based on Q being uniform in beans, and are inaccurate when Q is markedly non-uniform. Eqs [2] to [9] apply for $t > t_L$ + 10 to 15 sec for most roasters.

TESTING EQ [1]

To test whether Eq [1] applies for a thermocouple surrounded by flowing beans in roaster, I: 1) heated a small load of beans to roughly 140 °C in a Probat sample roaster; 2) let the roaster drum continue to rotate with heat and air flow shut off until bean temperatures equalized and T_S stopped changing; 3) rapidly inserted a 9.5 mm D thermocouple; into the tumbling pile of beans; and 4) measured T_A versus t for some time thereafter.

Figure 1 shows the test set-up and a plot of log($T_S - T_A$) versus t obtained using T_S *and the* T_A versus t values measured. The plot is a straight line, indicating use of Eq [1] was valid for this test. Thus Eq [1] probably applies generally for thermocouples exposed to coffee beans flows

in roasters. Points deviated from the straight line because manual measurements of T_A and t were imperfectly synchronized. K = the plot slope divided by –2.303 and was 235 sec. for the test conditions used,

DATA PROCESSING

T_A versus t data are noisy and must be smoothed to obtain T_A and dT_A/dt suitable for use in Eqs [2] to [9]. Polynomials based on t and best-fit coefficients fitted sections of T_A versus t curves well and smoothly, but did not fit entire curves. Therefore overlapping sections were used and best-fit polynomials were generated for each section. Polynomial coefficients were interpolated in overlap zones to preventT_A, dT_A/dt and d^2T_A/dt^2.from jumping at junctions between sections.

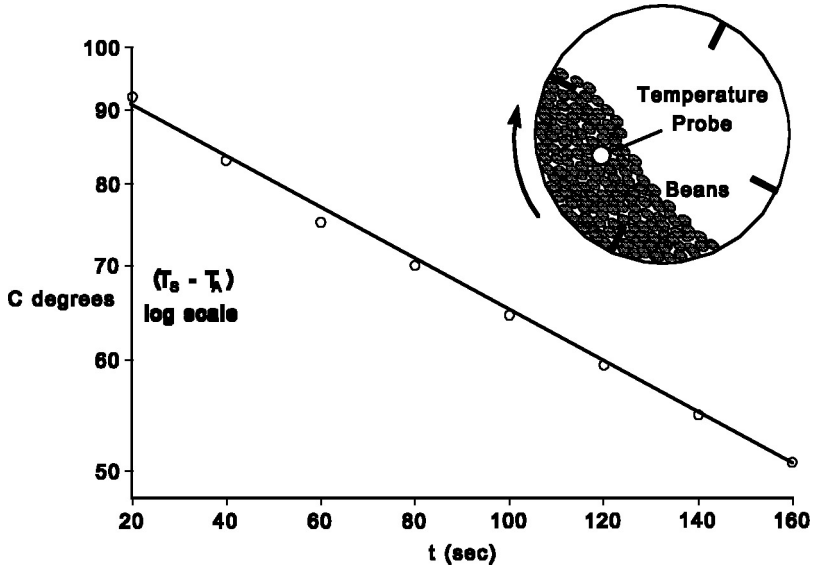

Figure 1.

DETERMINING K AND F_M

Using the polynomial that correlated T_A for a 9.5 mm D thermocouple in a RZ4000 roaster in the $t < t_{min}$ range, I calculated dT_A/dt and T_A values at several t between t_L + 10 sec and t_{min} – 10 sec. Estimated T_S for the same t were read from T_S versus t curve drawn between T_1, the initial bean temperature and $t = t_L/2$ and $T_{\min}$ and t_{min} . $K = (dT_A/dt)/(T_S - T_A)$. K varied little at the selected t. The averaged K = 68 sec. In the sample roaster, K was 235 sec for virtually the same thermocouple. The two K differ because bean flow rates differ markedly in these two roasters.

Based on S and V for hemiellipsoids, a = 2.78 mm for beans 10.3 mm long, 7.25 mm wide and 3.98 mm high. Using the Nusselt number correlation for spheres, I estimated h_G for beans whose a = 2.78 mm while roasting in a 240 kg batch drum roaster.. Based on that h_G and k based on k correlations for wood: B = 1.5. Only part of the beans in roasters, receive heat from gas at a time. The rest tumble and mix in piles and temporarily pick up little heat. Thus B effectively is lower than just calculated. If beans contact hot gas only half the time in the roaster, B = 0.75, q^2 = 1.913 and $F_M \approx 0.092$ and F_o = 0.213, values used earlier in this paper. As roasting proceeds, beans expand, k decreases and a and B increase. Gas mass-flow rates and h_G also decrease as roasting progresses. Apposing cell walls exchange heat radiantly and paired evaporation and condensation of water occur on those walls. Thus k is markedly larger at T_M than at room temperature. Thus, during roasting, B increases less than projected from

changes in a and k measured at room temperature for green, partly roasted and fully roasted coffee beans.

DISCUSSION

For a given roaster and bean temperature probe, specific T_A versus t records provide specific T_M versus t histories for T_M and t where most roasting reactions occur. Identical T_M versus t histories should be used to prepare identical product in different types of roasters. Eqs [5] to [9] in this paper can be used to achieve that goal.

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Eggers, R. and v. Blittersdorff, M., (2005) Temperature Field During Roasting and Cooling of Coffee Beans, Eurotherm Seminar 77, Parma, Italy

Heat Transfer Effects in Roast and Quench Processing of Coffee Beans

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INTRODUCTION

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. Complementary to the paper presented by Eggers et al. (2004) the focus is set on results of thermal analyses, calculations of heat transfer intensities based on both theoretical and experimental approaches, and mass transfer phenomena during roasting and quenching. They became part of a mathematical model 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. The experimental procedure of roasting and quenching was described in the former paper.

HEAT SOURCES AND SINKS

Thermal analyses were performed in order to investigate endothermic as well as exothermic changes that influence both energy conversion, and material properties of the coffee beans. An endothermic effect, assigned to mainly water evaporation, is sharply delimitable between 30 °C and 180 °C. The evaporation enthalpy amounts to 130 J/g to 150 J/g, almost independent of the heating rate, so that the kinetics of the evaporation can be determined by partially integrating the endothermic peaks, as depicted for green coffee grounds in Figures 1 a,b. Thus, a basis is given for calculating moisture evaporation during any heating process like isothermal roasting, Figure 1 c. In thermal analyses and roasting of whole coffee beans physical and chemical changes are shifted to higher temperatures (endothermic peak at about 140 °C to 160 °C) due to mass transfer resistances, internal pressure, and higher temperature changes, respectively. This fact is generally accounted for when calculating temperature profiles during roasting (Eggers et al., 2004; Schwartzberg, 2002). Exothermic reactions, though, starting at about 150 °C, cannot be quantified neither for coffee grounds nor for coffee beans owing to the lack of a base line in DSC-thermo-grams (Raemy et al., 1982), and their course being dependent on the analysis method (Eggers 2005). An indirect approach to attaining the enthalpy and kinetics of exothermic reactions during the roasting process makes use of heat of combustion measurements of green, par-tially, and fully roasted coffee beans. Fischer (2005) ana-lysed coffee beans that had been taken from a fluidised bed roasting process at 242 °C gas temperatures at different times. Naturally, the dry basis heat of combustion rises with roasting degree. When related to green bean weight, though, one can deduce from a falling heat of combustion an exothermic release of energy with roasting. Still, the determination of conversion-related, formal kinetics requires further systematical analyses of differently roasted coffee beans.

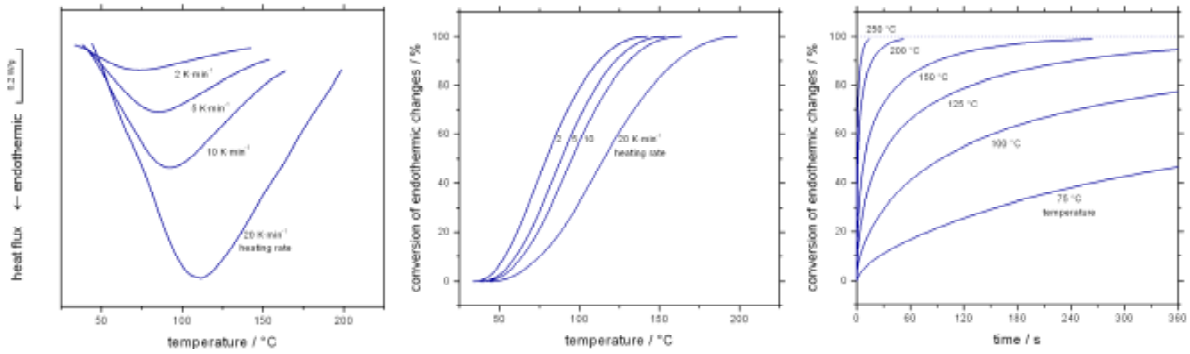

Figure 1. Kinetics of endothermic evaporation derived from DSC-Roasting of green coffee grounds. a) DSC-thermograms, b) conversion at constant heating rate, c) conversion during isothermic roasting.

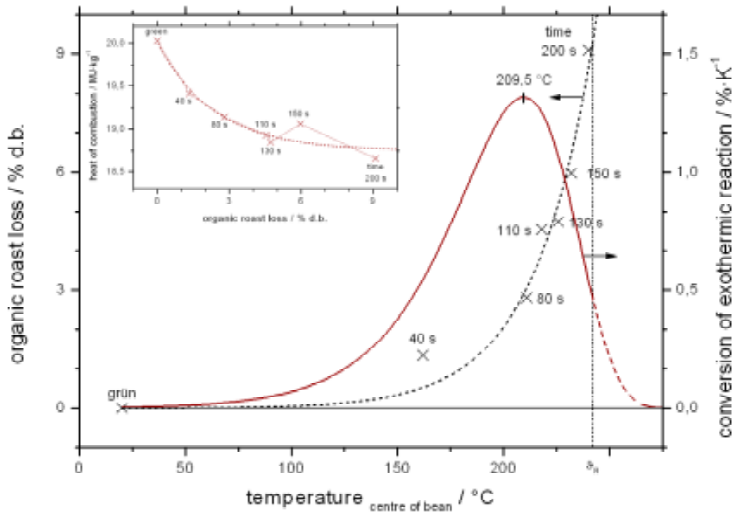

Figure 2. Kinetics of exothermic reactions derived from combustion calorimetry of coffee beans after fluidised bed roasting at 242 °C.

EXTERNAL HEAT TRANSFER

Heat transfer intensities were calculated using equations for the dimensionless Nusselt number and estimated from the experimental deter-mination of the transient temperature field inside the coffee beans. During convec-tive roasting, the heat transfer coefficient is decreasing, having reached a maximum value of about 250 $W \cdot m^{-2} \cdot K^{-1}$ according to the Nusselt equation for the volume equivalent sphere in a fluidised bed (Gnielinski 1978). It is slightly lower during convective air cooling. Water addition accelerates cooling down to the saturation temperature of the applied water. The fastest cooling is achieved by a spray quenching process and by applying cold water, which has the highest quenching power.

Spontaneous evaporation and highest cooling rates im-mediately after water addition are realised, though, only at water temperatures above 65 °C. Depending on the bean surface temperature, thus roasting conditions, and water temperature, the evaporation characteristics are similar to those found for compact sur-faces (Nukiyama 1966). Re-gions of convective, nucle-ate, and film boiling are qualitatively distinguishable by their different heat transfer intensities, Figure 4. Moreover, the Leidenfrost phenomenon of a closed vapour film on the surface can be seen in the temperature curves in Figure 3, that show a steep temperature

decrease only after nucleate boiling set in. Then the heat transfer coefficient is higher by 2 orders of magnitude when compared to air cooling. Still, the absolute quantity of heat transfer during water cooling remains uncertain, since evaporation and condensation effects inside the porous body were not considered, and the surface temperature used for heat transfer calculations is not accessible directly during water quenching.

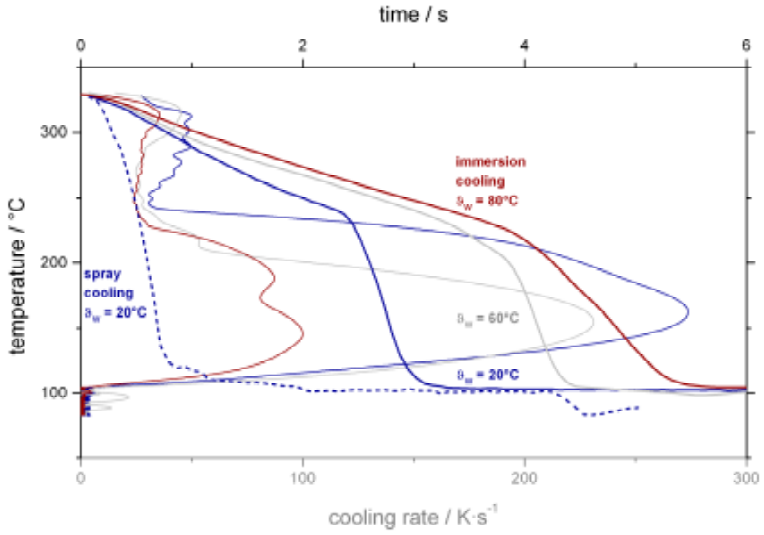

Figure 3. Cooling kinetics during spray and immersion quenching of coffee beans.

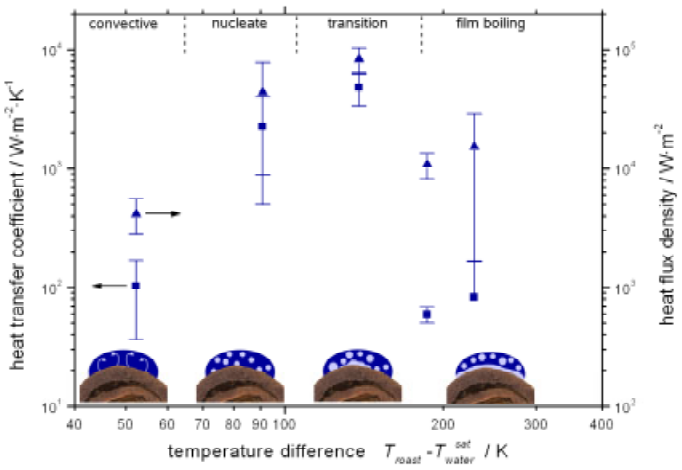

Figure 4. Heat transfer during immersion quenching of coffee beans in water at 80 °C.

COFFEE BEAN QUALITY AND FURTHER PROCESSING

The abovementioned heat transfer effects represent some of the processes and parameters, that, being interdependent and furthermore related to accompanying mass transfer phenomena, exert an influence on coffee bean quality and further processing. In this respect, especially the mass transfer during water quenching can be altered in different ways by the appropriate temperature of the quenching medium.

While water quenching ge-nerally enables remoistening of the coffee beans, the ap-plication of cold water leads to an accumulation of water at the beans' surface. Since for grinding a homogeneous pro-duct is advantageous a resting time is required, until the coffee beans can

be further processed. The contact angles of water droplets impinged on the beans' surface, Figure 5, hint at the dependency of surface conditions both on quenching parameters and the subsequent resting time. In this respect, hot-water cooled coffee beans, having a dry surface shortly after quenching, behave more similar to air cooled coffee, being earlier equilibrated. Leading to almost the same final moisture, remoistening of coffee beans is facilitated by transport of mainly water vapour through hot water quenching.

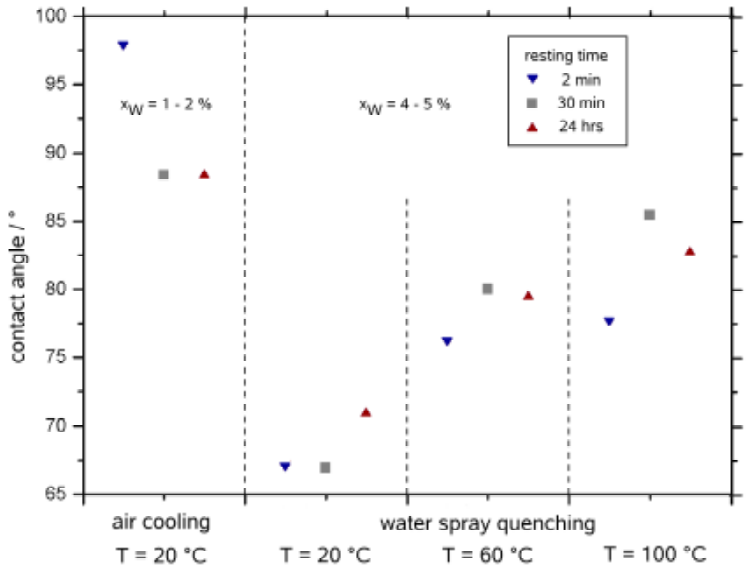

Figure 5. Contact angle of water droplets impinged on differently quenched coffee beans.

Moreover, the extraction kine-tics are different at first contact with hot water, as shown in Figure 6. Thus, the composition of both the cof-fee beans and the exhaust gas might be influenced by the temperature of the applied water. The higher the tempe-rature, the more homogeneous the coffee beans, and the less solid material is extracted during quenching.

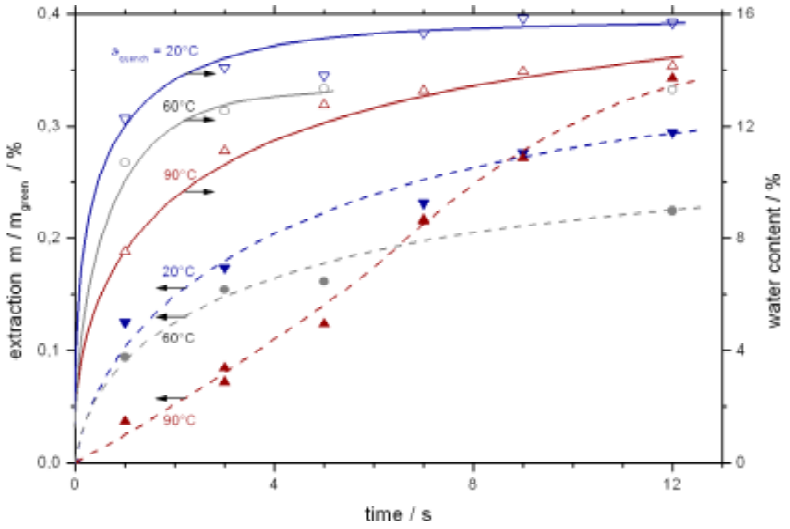

Figure 6. Extraction of soluble material into quenching water and remoistening of coffee beans during immersion quenching.

MODELLING OF ROASTING AND QUENCHING PROCESSES

The above-mentioned heat and mass transfer phenomena were incorporated into a model for calculation of the transient temperature distribution inside the coffee bean, namely as appropriate boundary conditions and heat sources and sinks when solving the partial differential equation of heat conduction including temperature dependent material properties. Basics and first results of this approach to calculation of the processes have been presented formerly (Eggers et al., 2004).

Being aware of the ongoing heat and mass transfer phenomena, mathematically describing same, and knowing their influence on product quality and further processing allows for optimization of industrially performed roasting and quenching processes.

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Coffee Husks as Biosorbents for Heavy Metals

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SUMMARY

The objective of this work was to propose an alternative use for coffee husks. In this study, dry coffee husks were successfully used as biosorbents for the removal of copper from aqueous solutions. After chemical treatment, the organic leaching from the husks was reduced to acceptable levels and the biosorption capacity was maintained. Parameters such as the adsorption equilibrium time and the effect of adsorbate initial concentration on the removal of copper from solution were studied. The maximum adsorption capacity of copper was found to be in the order of 12 mg/g at pH 4.0, for an initial concentration of 50 mg/L at room temperature. The experimental data demonstrated coffee husks to be a suitable candidate for use as biosorbent in the removal of heavy metals of industrial wastewaters.

INTRODUCTION

The processing of coffee generates expressive amounts of agricultural waste. Coffee husks, comprised of outer skin, pulp and parchment, are probably the major residues from the handling and processing of coffee. For every ton of coffee beans produced, approximately one ton of husks are generated during dry processing, whereas for wet and semi-wet processing this residue amounts to more than two tons (Saenger et al., 2001). To our best knowledge, there are no profitable uses for this type of residue and its disposal constitutes a major problem. Although combustion of agricultural wastes is usually considered as an alternative source of energy, it has already been demonstrated that major problems are prone to happen, such as agglomeration, fouling and excessive emissions, due to the low melting point of the ash of burnt coffee husks and the expressive amount of volatile organic matter present in the husks (Saenger et al., 2001). Furthermore, since sustainable development should be prioritized, the development of techniques for giving additional value and reusing this type of residue should be sought.

The production of adsorbents (activated carbons) or the direct use as biosorbents are two of the potential alternative uses for agricultural wastes that have been extensively studied in the last decade (Castro et al., 2000; Rajeshwarisivaraj et al., 2001; Mohan and Singh, 2001; Baquero et al., 2003; Mohanty et al., 2005; Carmona et al., 2005; Nasernejad et al., 2005). Bansal et al. (1988) reviewed several criteria to be considered when selecting a potential precursor for the manufacture of activated carbon and Pollard et al. (1992) pointed out that few materials would satisfy all the requirements, and that selection is often made on the basis of raw material availability. Furthermore, Evans et al. (1999) stated that the manufacture of activated carbon should balance economic viability with performance, with the precursor materials being readily available and a minimum of resources being used in the process of conversion. Based on these criteria, the possibility of using raw agricultural waste that is readily available in expressive amounts as biosorbents for the removal of heavy metals from industrial wastewaters seems rather appropriate. Additionally, the heavy metals can be easily recovered from the biosorbents for reuse. Therefore, coffee husks are potential candidates for use as biosorbents, since this type of residue satisfy the mentioned criteria.

In view of the aforementioned, the objective of this study was to investigate the feasibility of using dry coffee husks as natural biosorbents for the removal of heavy metals from industrial wastewaters.

MATERIALS AND METHODS

Samples

Dry coffee husks were acquired from Santo Antonio Estate Coffee, a coffee producers association located at Santo Antônio do Amparo, Minas Gerais State, Brazil. The husks were obtained from a dry processed coffee after de-hulling.

Biosorbent preparation

Coffee husks were dried overnight at 105 °C in a convection oven, ground and sieved into fractions, with the fractions between 14 and 16 mesh (Tyler series) being separated for use as biosorbents. The separated fraction was successively washed with distilled water to eliminate soluble components and coloring agents.

Preparation of metal ion solutions

The stock solution of Cu^{2+} was prepared by dissolving its sulfate salt in distilled water. The test solutions were prepared by diluting a 1 g/L stock solution. The initial copper ion concentration ranged from 50 to 100 mg/L and the pH of each solution was adjusted to 4 prior to the addition of the biosorbent.

Biosorption studies

Batch experiments of biosorption were performed in 250 mL Erlenmeyer flasks at room temperature, with the flasks being agitated on a shaker for periods of time that ranged from 1 to 72 hours. In all sets of experiments, 1 g of coffee husks were thoroughly mixed in a 100 mL solution of copper ions.

Analysis of metal ions

After the specified time period, 5 mL aliquots were taken from the Erlenmeyer flask and the concentration of the copper ions was determined by atomic absorption spectrophotometry. The amount of metal adsorbed was determined by taking the difference between the initial ion concentration and the concentration of the solution at the time of sampling. All determinations were performed in a total of three replicates per experiment.

Sorption isotherms

For each initial concentration, sorption isotherms were built with the calculated adsorbed quantity based on the average residual ion concentrations of the sampled solution, measured at periods of 1, 2, 3, 4, 24, 48, 56 and 72 hours of batch biosorption tests.

RESULTS AND DISCUSSION

The sorption kinetics of Cu(II) for coffee husks are presented in Figure 1, for initial ion concentrations ranging from 50 to 100 mg/L. It can be readily observed that sorption occurred at faster rates in the first 4 hours for all initial ion concentrations. For a sorption period of 48

hours, the amount of adsorbed metal ion was in the range of 80 to 90% for all the initial ion concentrations. Equilibrium was attained after a 24 hours sorption period for all initial ion concentrations, with the highest initial concentrations leading to lower maximum adsorption capacities at equilibrium. The maximum adsorption capacity of copper was found to be in the order of 50 mg/g at pH 4.0, for an initial concentration of 50 mg/L at room temperature.

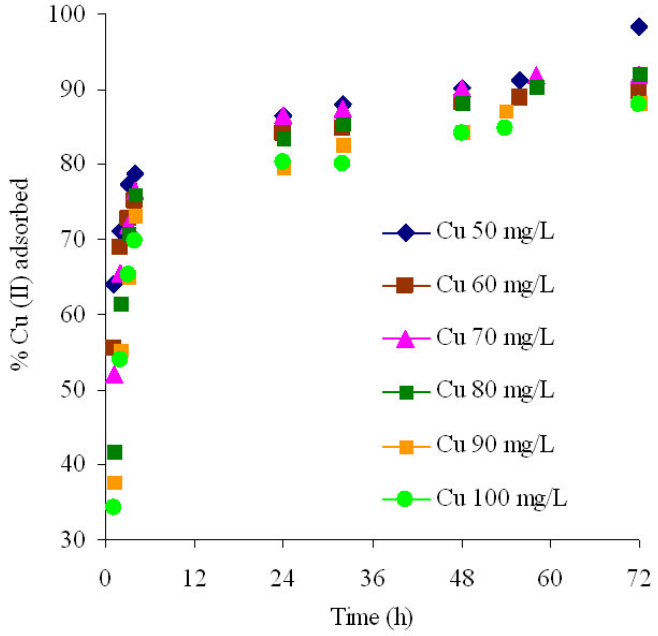

Figure 1. Sorption kinetics of Cu (II) for coffee husks.

CONCLUSIONS

A study of the feasibility of using dry coffee husks as biosorbents for the removal of copper ions from aqueous solutions was successfully carried out. Coffee husks presented better sorption performance for low concentrations of copper. The experimental data demonstrated coffee husks to be a suitable candidate for use as biosorbents in the removal of heavy metals of industrial wastewaters.

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Investigations by S.E.M. and Helium Pycnometry of the Effect of Water on the Physical Properties of Roast and Ground Coffee

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SUMMARY

S.E.M. (Scanning Electron Microscopy) and helium pycnometry were used to investigate the physical changes during wetting of roast and ground coffee. The SEM showed to be an indirect way to see where liquid water is present inside the coffee particles. On the other hand, the plasticization of coffee cell walls by water induced an increase in He permeability and an increase in the particles mean volume diameter. For roast and ground coffee containing 50% w/w of water, liquid water was present in cells only at the periphery of the particles as evidenced by the S.E.M. technique, while the He pycnometry showed a permeability up to the core of the particles. Diffusivity of water (5-6 x 10^{-11} m^2s^{-1}) was determined from the swelling kinetics of the particles.

RÉSUMÉ

L'utilisation de la M.E.B. (Microscopie Electronique à Balayage) et de la pycnométrie à l'hélium ont permit d'étudier les changements physiques du café torréfié moulu pendant son mouillage. La M.E.B. s'est avérée être une méthode indirecte pour détecter la présence d'eau liquide à l'intérieure des particules de café. D'autre part, la plastification par l'eau des parois cellulaires du café a provoqué une augmentation de la perméabilité à l'hélium et une augmentation du diamètre moyen des particules. Pour du café torréfié moulu contenant 50% d'eau (% par rapport à la masse finale), l'eau liquide n'était présente que dans les cellules à la périphérie des particules d'après les résultats de M.E.B., alors que les résultats de pycnométrie à l'hélium ont montré une perméabilité jusqu'au cœur des particules. La diffusivité de l'eau (5-6 x 10^{-11} m^2s^{-1}) fut déterminée d'après les cinétiques de gonflement des particules.

INTRODUCTION

Extraction is a key operation in the manufacture of instant coffee, which allows soluble and volatile compounds to be recovered. To ensure a "100% coffee" claim for instant coffee, liquid water is the only solvent allowed to improve compound mass transfer. In order to optimise the process of coffee making, a particular understanding of water distribution is required. However, only a few studies were devoted to the study of water distribution and its effects on physical properties of coffee (Spiro et al., 1989; Spiro and Selwood, 1984; 1990; Kino and Takagi, 1995).

The objectives of this work were to determine the state of water and its role on the physical changes during wetting of roast and ground (R&G) coffee, using Scanning Electron Microscopy (SEM) and helium pycnometry.

MATERIALS AND METHODS

Coffee samples

Colombian Arabica coffee was roasted (Neuhaus Neotec, Germany) and ground (Ditting grinder, Switzerland). R&G coffee was wetted according to a standardized procedure: distilled water was added manually and the sample was stirred for 30 seconds. This procedure provides homogeneous wetting of particles (RSD < 5%) as shown by measuring the water content of particles in different parts of the samples.

Scanning Electron Microscopy (SEM)

Coffee particles were mixed with Tissu –tek™ 45 83-OCT and a portion of the mixture deposited on a cryo-specimen holder. Sample freezing was done in nitrogen slush using the ALTO freezing station followed by a transfer under vacuum into the Gatan ALTO2500 cryo-system, with the cold stage at approx –170 °C. The sample was then freeze-fractured by whacking it with a razor blade to reveal its internal structure and inserted in the microscope chamber, under high vacuum (3-4 x 10^{-6} Torr). The fractured specimens were lightly etched on the cryo-stage in the microscope (heating to –100 °C) and then stabilized at –115 °C. Visualization of samples was done without any coating in a Quanta 200 FEG (The Netherlands FEI Company) operated at 15 kv in HighVac mode.

Helium pycnometry

Closed porosity was determined from volumetric measurements by helium pycnometry. The device used was the AccuPyc 1330 from Micromeritics™ (France) with a filling pressure of 19.6 psi (1.35 bar) and a working temperature < 35 °C. For each sample, the relative standard deviation between three replicates was < 5%.

The specific volume [cm^3/g] of R&G coffee was calculated knowing the volume (measured by the pycnometer) and the weight of the sample, and is defined as follows:

$$\upsilon_{He} = \frac{V_{particles}}{m_{particles}} = \frac{V_{solids} + V_{closed\ pores}}{m_{particles}}$$

where; $V_{particles}$ [cm^3]: total volume of the R&G particles measured by the He pycnometer; m [g]: mass of the R&G sample; V_{solids} [cm^3]: volume of the coffee polymers constituting the matrix ≈ volume measured for ~100 μm dry coffee particles; $V_{closed\ pores}$ [cm^3]: volume not accessible by the helium, assumed to be 0 for ~100 μm dry coffee particles.

For wetted particles, the specific volume is calculated after subtraction of water specific volume.

Closed porosity [cm^3/g] was deducted from values of specific volume:

$$\varepsilon_{closed\ pores} = \upsilon_{He} - \upsilon_{solids}$$

Particles sizing

The mean volume diameter D[4,3] of the wet coffee particles was measured by use of sizing laser beams (Mastersizer from Malvern Instruments, UK; with butanol). Measurements were repeated at least three times. Particles swelling was predicted using the model of diffusion in a sphere (Crank, 1975).

$$\frac{D[4,3]_t}{D[4,3]_\infty - D[4,3]_0} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left[\frac{-D_{app}\,n^2\,\pi^2\,t}{r^2}\right]$$

where: $D[4,3]_t$, $D[4,3]_\infty$, and $D[4,3]_0$: particle mean volume diameter at time t, after infinite time, and at time t=0, respectively [m]; n: number of composite spheres; D_{app}: apparent diffusion coefficient [m^2/s]; r: radius of the sphere [m], determined for each data point from the calculation of D[4,3], and iteratively used in the equation.

RESULTS

Several authors studied the porosity of dry R&G coffee by SEM (e.g. Schenker, 2000) or He pycnometry (Shimoni and Labuza, 2000; Anderson et al., 2003). To our knowledge, these techniques have not yet been used with wetted coffee particles.

S.E.M.

Coffee immerged in water showed cryo-concentrated soluble components inside the cells, not visible in dry coffee (see Figure 1 a and b). This shows water penetration inside the cell voids. At higher magnification, it is possible to see the structure of the cryo-concentrated materials and the gas vacuoles remaining inside the cells (Figure 1 c). The soluble structures are more or less porous depending on the shape of the water crystals formed.

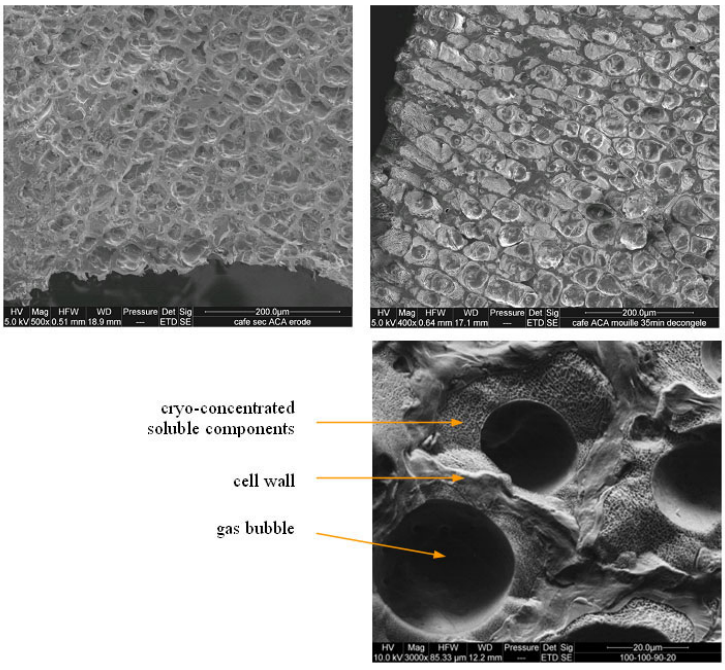

Figure 1. SEM micrographs of a dry coffee particle (a) and a particle immerged in water at ambient temperature for 30 min (b and c) – CTN 60.

For coffee samples with 50% w/w of water content and stabilized at 90 °C for 20 min (Figure 2), liquid water was evidenced only at the periphery of the particles (D[4,3] ~1.0 mm).

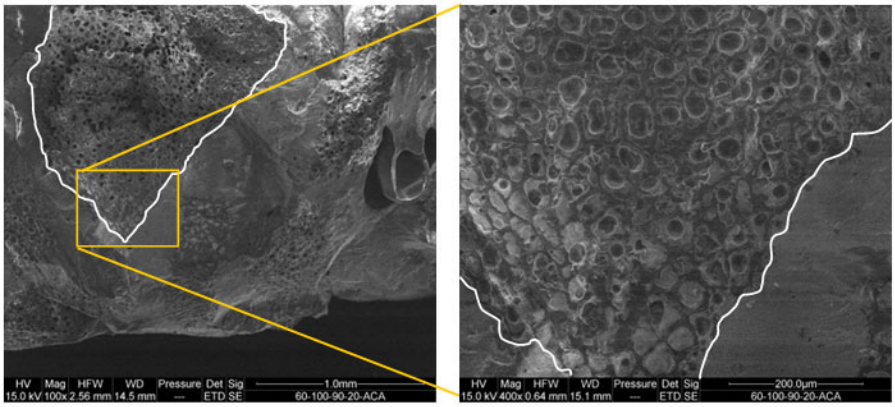

Figure 2. SEM micrographs of coffee particles (CTN 60) after wetting with liquid water at 90 °C and stabilized at this temperature for 20 minutes – Water content 50% w/w.

Helium pycnometry

Closed porosity was higher for larger particles (~0.45 and 0.20 cm^3/g for 1100 and 400 μm dry particles respectively, for dark roast) and for darker roast, as expected.

The closed porosity decreased with increasing water content, showing plasticization of coffee polymers by water (Figure 4). For all studied samples, above ~30% of water content, the closed porosity was lower than 0.1 cm^3/g, showing permeability of helium up to the core of the particles (400 to 1100 μm).

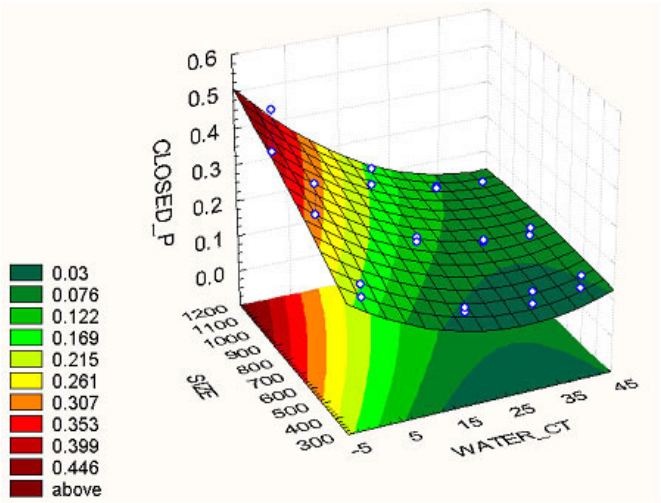

Figure 3. Closed porosity as a function of coffee particles size and water content (each couple of points represents data for CTN 60 and CTN 100; the mapping is made on the intermediate value: CTN 80).

Particles sizing

Water absorption kinetics as determined by particles sizing, are reported in Figure 4. They show that the D[4,3] of coffee particles with a water content of 50% w/w (wetting ratio 100%) increased by up to ~30% at 10-15 minutes following wetting (for ~750 and 1050 μm

initial particles sizes). Calculated water diffusion coefficients (D_{app}) were ~$5.2*10^{-11}$ and $6.1* 10^{-11}$ m^2/s for the lower and the larger particles sizes, respectively.

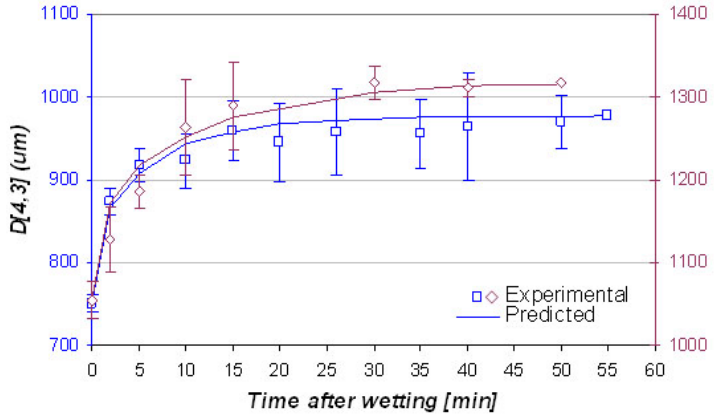

Figure 4. Mean volume diameter of coffee particles after wetting at 90 °C with a water content of 50% w/w – CTN 80.

The time scale of water absorption by coffee particles after wetting compares well with the time needed for most of the soluble compounds to be released (Voilley and Simatos, 1980).

CONCLUSIONS

The three techniques used in this study are complementary. The presence of liquid water inside roast and ground coffee was evidenced by S.E.M.

Helium pycnometry and particle size analysis showed the plasticization of coffee polymers by water and the dynamics of water absorption.

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Valorisation of Spent Coffee Grounds by Conversion into Activated Carbon - Dynamic Behaviour During Adsorption of Textiles Dyes

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SUMMARY

The food industry and households are generating of coins products that it would be necessary to valorise and to reinject in the economic circuit in so much that, for raw material of new utilizations. To shortcoming this sturdy, we tried to valorise the loss of coffee (marc de café) and to transform it in carbon actived and to test it for the removal of dyes from wastewater of the textile industry, by the process of absorption in a column, in fixed bed, in dynamic system. The dynamic behavior of our actived carbon valorized (in presence of one textiles dye ,the pink direct has been studied, and this by the establishment of all Hydrodynamic parameters, and an operative zone optimized for the working of our absorption column has been appraised; showing real capacities of adsorption of our activated carbon

RÉSUMÉ

L'industrie alimentaire et les ménages sont générateurs de sous produits qu'il faudrait valoriser et réinjecter dans le circuit économique en tant que matière première, pour de nouvelles utilisations. A travers cette étude; nous avons essayé de valoriser le déchet de café «marc de café» en le transformant en charbon actif et après l'avoir caractérisé nous l'avons testé pour la décoloration des eaux de rejets de l'industrie textile, par le procédé de l'adsorption dans une colonne, en lit fixe; en système dynamique. Le comportement dynamique de notre charbon actif valorisé en présence d444e colorants textile [le rose direct] a été étudié. et ceci par L'établissement de tous les paramètres hydrodynamiques ; et une zone opératoire optimisée pour le fonctionnement de notre colonne d'adsorption a été estimée. montrant des capacités réelles d'adsorption de notre charbon actif
Valorisé.

INTRODUCTION

The era of the deposit and the hiding is completed, the future must force us to search a valorization of matter or an energy valorization of waste and making it possible to reinject them in circuits of production and why not the creation of new technologies. It is within this framework that our present study is whose objective is: to eliminate the textile dyes rejected into the rivers by the use of an activated carbon obtained by the valorization of under foodstuff, in fact, the loss of coffee (marc de café) rejected in very great quantities in Algeria; the statistics given by the Algerian office of the statistics; in 2001, indeed the coffee consumption in Algeria east around 2,75 kg per year and per person. This loss of coffee will be transformed into carbon activated and used like adsorbent for the elimination of the dyes (pink direct), and to study the behaviour of our carbon activated developed in dynamic system and finally to optimize our process of adsorption

THEORETICAL APPROACH OF ADSORPTION IN DYNAMIC SYSTEM

The process of adsorption on fixed bed activated carbon in grain requires to be able to predict the breakthrough curves of the filter according to the operating parameters of the system. This curve translates the profile of concentration, of one pollutant to eliminate out of the column according to time. The dimensioning and the improvement of the performances of the column and it is what justifies our study by the use the activated carbon of the loss of coffee (ACLC) the process is based on the determination of the used Bed Zone, (UBZ). This concept was studied by Mikaels (1952) and used for the exchanging resins of heavy ions, then generalized by Pansini (1996) .The method is very simple, effective and highly reliable for the practice and makes it possible to establish correlations for the systems of adsorption (Newcombe, 2000). However, the operating conditions should well be fixed:

1. A uniform fixed bed
2. A volume throughput and a uniform concentration of the liquid phase
3. Not of phase shift
4. A negligible adsorption energy
5. No interaction between the adsorbed molecules

Generally the operations of adsorption are uninterrupted in fixed-bed or in mobile bed.

The dynamic of adsorption can be visualized in term of zone of adsorption the used Bed Zone, (UBZ) which moves along the column by leaving behind it a zone of saturation or of balance such as the Figure 1 shows it. the transfer is once completed the UBZ reached the bottom of the column.

The exchange capacity of a fixed bed measures the effectiveness of adsorption or elimination of an adsorbent and indicates which is the fraction of solid which takes part in the phenomenon of adsorption, one defines it as being the quantity of adsorbate (dye) really eliminated compared to the potential capacity from elimination from the adsorbent (loss of coffee) inside the UBZ.

EXPERIMENTAL PART

Preparation of the Activated carbon of loss of Coffee (ACLC)

The marc of the coffee collection will be transformed into activated carbon by using the operations of activation per chemical process (Benrachedi and Messaoud-Boureghda, 2001) using the phosphoric acid (3N)- and a carbonization at 500-700 °C during two hours. The Specific area of the ACLC: 1248 m^2/gr.

DYNAMIC STUDY

Preparation of the colouring solution

To conclude our study, we used an direct acid dye (pink direct), dissolved in water distilled with a concentration of 50 mg/l .le pH of the solution equal to 5, 5.

Preparation of the adsorption column (reactor)

A granulated carbon Activated of loss of coffee, once weighed according to the height (H1 = 0.5 cm, H2 = 10 cm; H3 = 15 cm) is placed in the column (1.5 cm in interior diameter); The column is fed by a peristaltic pump with variable flow (Q1 = 0.55 l/h; Q2 = 0.99 l/h, Q3 =

1.12 l/h; Q4=1.31 l/h; Q5 = 1.57 l/h) ; Once the rate of feed and the height of the column fixed, we start the peristaltic pump, and the solution of dye is recovered in a graduated flask. Recovered volumes are analyzed. The residuals concentrations of the dye are raised and the breakthrough curves will be drawn the concentration of the dye is determined by molecular adsorption spectrophotometer. The initial concentration ($\mathbf{C_P}$) is fixed 5% of C0 = 2, 5 Mg/l and saturation concentration (**Cs**) = 95% of C0 = 45 mg /l.

RESULTS AND DISCUSSION

Establishment of the various experimental parameters

Once the breakthrough curves specific to each couple of flow and height are established, one raises times of breakthrough (**tp**) and saturation (**ts**) by direct graphic reading. These last parameters enable us to evaluate volumes of breakthrough (**Vp**) and of saturation (**Vs**) for each couple of flow and height and one the of the zone height of transfer of matter ($\mathbf{H_{UBZ}}$) calculates thereafter. the rate of travel of the transfer zone of matter ($\mathbf{U_{UBZ}}$) is evaluated; the determination of the quantities adsorbed at the breakthrough (**Xp**) and saturation (**Xs**) are graphically given one establishes the zone of optimization, one using report/ratio ($\mathbf{H_{UBZ}}$ /**H**) the results are summarized in the table n° 1.

Breakthrough curves obtained for the pink dye the according the flow rates and the heights

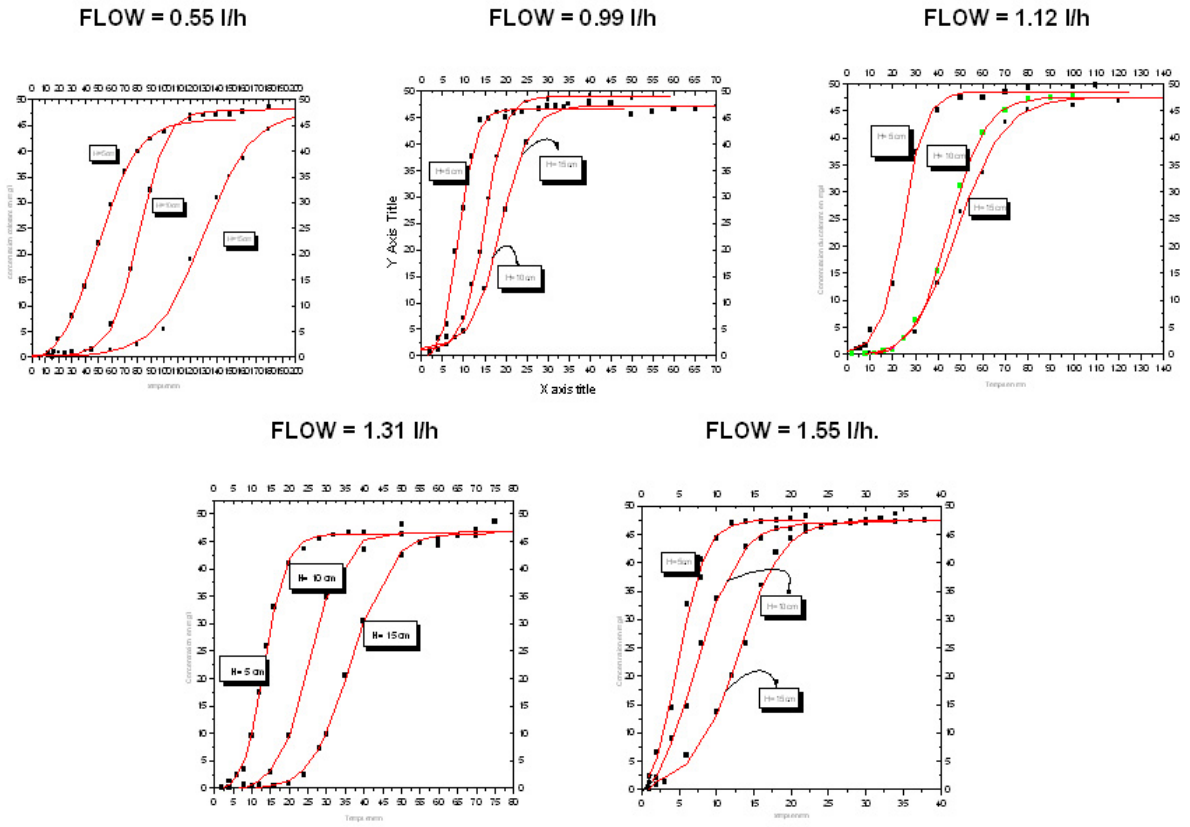

Figure 1.

Table 1. Able of the differents experimental parameters values.

Débits	Hauteur	Tp	Ts	Vp	Vs	H_{ZTM}	U_{ZTM}	H_Z/H	Xp	Xs
L/h	cm	mn	Mn	I	L	cm	m/mn		mg/g	mg/g
Q=0,55	H=5cm	16.50	04.70	0.15	0.96	2.50	1.20	0.50	1.06	2.75
	H=10cm	46.35	111.60	0.42	1.02	5.40	4.90	0.54	1.07	2.94
	H=15cm	82.41	177.80	0.75	1.63	8.42	5.20	0.56	1.09	3.04
Q=0.99	H=5cm	9.88	39.40	0.16	0.65	3.09	5.94	0.62	0.92	1.66
	H=10cm	23.47	70.30	0.38	1.16	6.43	8.06	0.64	0.93	1.80
	H=15cm	22.40	79.40	0.36	1.31	9.92	9.14	0.66	0.94	1.95
Q=1.12	H=5cm	5.68	23.58	0.10	0.44	3.39	11.30	0.67	0.86	1.07
	H=10cm	12.10	41.80	0.22	0.78	6.77	13.26	0.67	0.88	1.28
	H=15cm	20.50	55.40	0.38	1.03	10.19	17.43	0.75	0.90	1.41
Q=1.31	H=5cm	3.21	14.42	0.07	0.31	3.78	20.17	0.75	0.82	0.77
	H=10cm	5.30	21.52	0.11	0.47	10.99	22.10	0.69	0.85	0.82
	H=15cm	5.41	31.60	0.12	0.69	10.94	27.90	0.90	0.85	0.96
Q=1.51	H=5cm	1.98	11.20	0.04	0.28	5.83	37.02	1.16	0.79	0.28
	H=10cm	2.31	15.49	0.05	0.39	11.85	38.98	1.18	0.80	0.38
	H=15cm	3.60	20.66	0.08	0.52	12.65	41.04	1.22	0.82	0.53

Evolution of the height used bed zone

It informs about the quantitative effectiveness of adsorption, weaker is this better height is the speed of exchange. It remains the principal parameter of the dynamics of exchange. This height of UBZ, increases with the increase in the volume throughput, and undergoes the same evolution for an increase height of the column of ACLC. Indeed, by increasing these parameters, one increases the residence time of the colouring molecules in the column, and this by the establishment of new preferential sites of adsorption, is because of the steric obstruction, being flow it is noted that the height of the UBZ sensitive to the hydrodynamic conditions and that the evolution the height of the UBZ the dye according to the volume throughput shows a certain stability for the low flows then increases quickly for the high flows.

Dye quantity adsorbed at the point of opening per unit of developed activated carbon mass

The experimental results showed that the quantity of dye adsorbed at the breakthrough is very interesting for our dye. The adsorbed quantities increase with the height and decrease with the increase in the volume throughput what is confirmed by the results found by Hanini for Mexican zucchini (1996e) and Naamane (Pansini, 1996). And there a better adsorption of the dyes is low flow for heights of zones of rather weak transfer of matter; this assumption was checked by Chern (1990); Walker (2000) and Panswad (2000).

We note that the adsorption of the pink direct is better at the point of saturation; this observation can be due to the properties of the ACLC because the direct pink dye (chemical structure) has a coplanar configuration (Madani, 1996) it is adsorbed horizontally and causes an adsorption of cover, therefore of superposition (Nicolas and Kroppa, 1996).

Determination of the optimum zone of operation

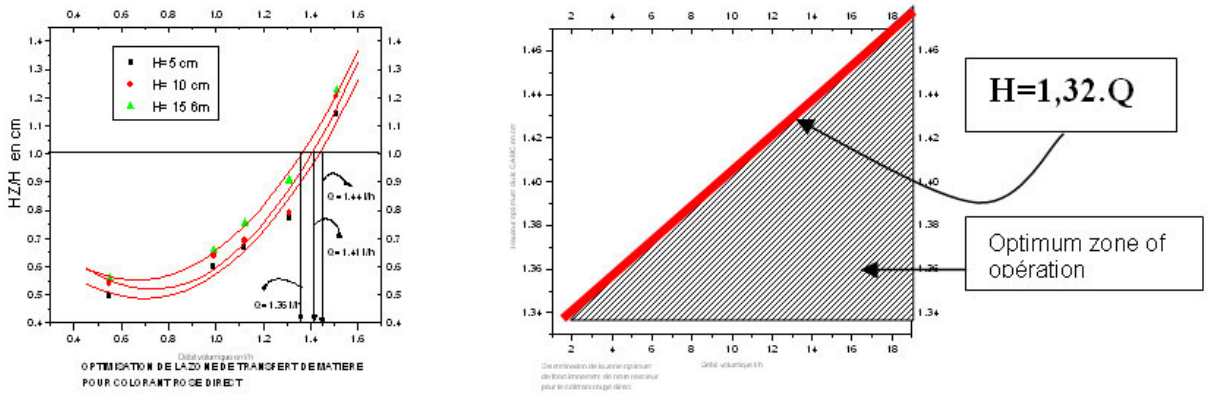

Figure 2.

It passes imperatively by the basic idea which consists in saying that the optimum zone of operation of a column must be determined by knowledge the aptitude of the column of adsorption, with employment for which it is intended. For that one is obliged to estimate the ratio (height of the UBZ on the height of the fixed bed (H) of ACLC) it must be smallest possible and in the worst case equal to the unit, On the basis of the principle that the activated carbon bed work under optimum conditions limit when the height report/ratio of the UBZ on the height of the column is equal A 1. then from the graphs and taking the horizontal one with the x axis for y = 1, we deduce from the couples from values H and Q which enable us to plot the curves in the limiting conditions of operation. For the zone of operation of each dye, it must be located at the corresponding line lower part H = Q X. From there one establishes the operational zone for the operation of the column.

CONCLUSION

Our work has as a principal objective the description of adsorbent qualities of a by-product which one sought A develops We sought has to include/understand his behaviour during the adsorption of the dyes in dynamic system, like one considered the influence of the hydrodynamic parameters and one been able to establish an optimization of the control of our unit according to the flows of percolation and height of the activated carbon bed in the column and especially the specific area of CALC that confirm us that the adsorption of the dyes is done according to nature and of properties of each dye (standards). The activated carbon obtained from the loss of coffee present of some qualities if it is used in fixed-bed and the percolation must be done has low flow

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Coffee Functional Genomics in Brazil

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SUMMARY

The Brazilian Coffee Genome Program was designed with the objective of making available modern genomic resources to the coffee scientific community. The initial phase funded by the CBP&D-Café (Brazilian Consortium of Coffee Research and Development), EMBRAPA and FAPESP, consisted on single-pass sequencing of 214,964 randomly picked clones from 37 cDNA libraries. The libraries were constructed with plant material of *Coffea arabica*, *Coffea canephora* and *Coffea racemosa*, submitted to abiotic and biotic stresses and/or from specific stages of cells and plant development that after trimming resulted in 130.792, 12,381 and 10,566 sequences for each species, respectively. The ESTs clustered into 19,031 clusters and 13,928 singletons resulting in a Unigene close to 33,000 different sequences. Blast analysis of these sequences revealed that about one third had no significant matches to sequences in the NCBI database. Annotated sequencing results have been stored in two online databases at http://www.lge.ibi.unicamp.br and http://www.cenargen.embrapa.br. Current research on functional genomics is being carried out by several groups aiming at identifying genes involved in tolerance to biotic (leaf rust, nematods, coffee leaf miner, etc) and abiotic (drought, salinity and cold) stresses, and genes involved in flowering, fruit development and quality. Transcription profiling using cDNA arrays associated with protein profiling by mass spectrometry analysis of tryptic peptides are techniques currently being used in these functional studies. In addition, several groups are concentrated on identifying SNPs and other molecular markers, aiming at establishing a breeding program based on marker-assisted selection. Resources developed in this research program will provide genetic and genomic tools that may hold the key to the sustainability, competitiveness and future viability of the Brazilian coffee industry.

INTRODUCTION

Coffee is an important agricultural commodity in the world and is produced in more than 60 countries. It generates a turnover of US$10-12 billion per year and ranks second on international trade exchanges, representing a significant source of income to several developing countries in Africa, Asia and Latin America. Brazil, Vietnam and Colombia are responsible for about 50% of the world-coffee production, and Brazil alone responds for more than one third of the global coffee production and exports. This fact ranks coffee amongst the most important commodities in the Brazilian trade balance.

Coffee has long been bred to improve important agronomical characteristics as flowering, yield, bean size, cup quality, caffeine content and disease and drought resistance. Despite solid efforts and considerable progress, genetic improvement in coffee breeding using conventional approaches has been slow due to many factors such as the narrow genetic basis

of cultivated coffee, lack of genetic markers and efficient screening tools, as well as the long time for generation advancement.

The recent development of applied technologies in biology is leading to an enormous production of information in the area of plant genomics, through the sequencing of different organisms. Large-scale sequencing of cDNAs to produce Expressed Sequence Tags (ESTs) and comparing the resulting sequences to public databases has become the method of choice for the rapid and cost-effective generation of data on the coding capacity of genomes and to potentially identify new genes.

The Brazilian Coffee Genome Program was designed to develop and deploy useful tools for gene discovery and functional genetic analysis in coffee and related species and to help the advancement of knowledge on the structure and evolution of the coffee genome. The generated coffee EST database from *C. arabica*, *C. canephora* and *C. racemosa* resulted in the identification of more than 30 thousand different unigenes and will facilitate genetic studies on coffee. This basic information provides a very valuable resource for studies on the biology and physiology of coffee plants that will considerably enhance the isolation and characterization of important agronomical genes for genetic improvement of Coffea.

MATERIALS AND METHODS

Generation of ESTs

The Coffee Genome Project for large-scale sequencing of ESTs was formulated in 2002 through a cooperative agreement signed between the Brazilian Coffee Research and Development Consortium (CBP&D-Café), a national consortium of 40 public Universities and Research Institutes, the Brazilian Enterprise for Agricultural Research (Embrapa), the São Paulo State Research Support Foundation (FAPESP) and the Permanent Forum for University-Company Relations (UNIEMP).

cDNA Libraries and Sequencing

The Instituto Agronômico de Campinas (IAC), which possesses a significant germplasm collection of *Coffea* species, supplied the material for the construction of cDNA libraries covering a wide range of tissues, developmental stages, and plant material submitted to biotic and abiotic stress conditions. All cDNA libraries constructed by the AEG group used plant material from *C. arabica* cv. Mundo Novo, while those constructed at Embrapa Recursos Genéticos e Biotecnologia were made from tissues and organs from *C. arabica* cv. Catuaí . Also, cDNA libraries were made from tissues of *C. canephora* and *C. racemosa* lines belonging to the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER) and IAC's collection, respectively. The details of the sequencing and the construction of cDNA libraries were described by Vieira et al. (2006).

Database analysis

For functional annotation of ESTs and categorization of contigs, the masked (http://www.phrap.com/) and trimmed sequences (Telles and da Silva, 2001) were compared with the protein sequences stored at NCBI databases (National Center for Biotechnology Information), particularly to the NR-(Non-redundant) database (http://www.ncbi.nlm.nih.gov/ blast/html/ blastcgihelp.shtml#databases). In addition, tools for electronic analysis of gene expression based on the number of reads in a given contig have also been developed by the bioinformatic's group of Embrapa.

Protein Profiling by Mass Spectrometry: Protein extracts were trypsin digested and separated by Reverse-phase HPLC using a C18 column at a constant flow rate of 1 mL/min using a gradient spanning from 0 to 100% of acetonitrile:TFA (99.9:0.1) in 40 minutes. Molecular masses (MS) of the major detected peptides were determined by MALDI-TOF/MS using the UltraFlex II controlled by the FlexControl 2.4.30.0 software. The MS/MS spectra were carried out in the positive mode precursor ion fragmentation (LIFT/CID) at a laser frequency of 50 Hz with external calibration, using the Peptide Calibration Standard-Starter Kit 4. Resulting data were analyzed using FlexAnalysis. Obtained peptide sequences were identified by tBlastn analysis against the Brazilian Coffee EST Database.

RESULTS

Coffee EST libraries

The description of the coffee EST libraries and the number of valid reads produced are presented on Table 1.

Table 1. Description of the coffee EST libraries.

Library code	Tissue/Developmental stage	Number of valid reads
AR1, LP1	Plantlets and leaves treated with araquidonic acid	5664
BP1	Suspension cells treated with acibenzolar-S-methyl	12379
CB1	Suspension cells treated with acibenzolar-S-methyl and brassinoesteroids	10311
CL2	Hypocotyls treated with acibenzolar-S-methyl	11615
CS1	Suspension cells treated with NaCl	10803
EA1, IA1, IA2	Embryogenic calli	9191
EB1	Zygotic embryo (immature fruits)	192
EC1	Embryogenic calli from *Coffea canephora*	8050
EM1, SI3	Germinating seeds (whole seeds and zygotic embryos)	9201
FB1, FB2, FB4	Flower buds in different developmental stages	23036
FR1, FR2	Flower buds + pinhead fruits + fruits at different stages	14779
FR4	Fruits (*Coffea racemosa*)	7967
FV2	Fruits, stages 1,2 and 3 (*Coffea racemosa*)	7195
CA1, IC1, PC1	Non embryogenic calli with and without 2,4 D	12135
LV4, LV5	Young leaves from orthotropic branch	15067
LV8, LV9	Mature leaves from plagiotropic branches	11864
NS1	Roots infected with nematodes	569
PA1	Primary embryogenic calli	2483
RM1	Leaves infected with leaf miner and coffee leaf rust	5567
RT3	Roots	560
RT5	Roots with acibenzolar-S-methyl	2311
RT8	Suspension cells with stressed with aluminum	9119
RX1	Ramos infectados com *Xylella spp*.	9563
SH1	Leaves from water deficit stresses plants (*Coffea canephora*)	7368
SH2	Water deficit stresses field plants (pool of tissues)	6824
SS1	Well-watered field plants (pool of tissues)	960

BlastX analysis

The results of a BlastX analysis of the generated contigs after clustering and assembly using the CAP3 program (Huang and Madan, 1999) are presented in Figure 1. The consensus sequences were analyzed for their similarity with known genes, present in the non-redundant protein database at NCBI, considering 10^{-5} for the *e-value* as a threshold for identity.

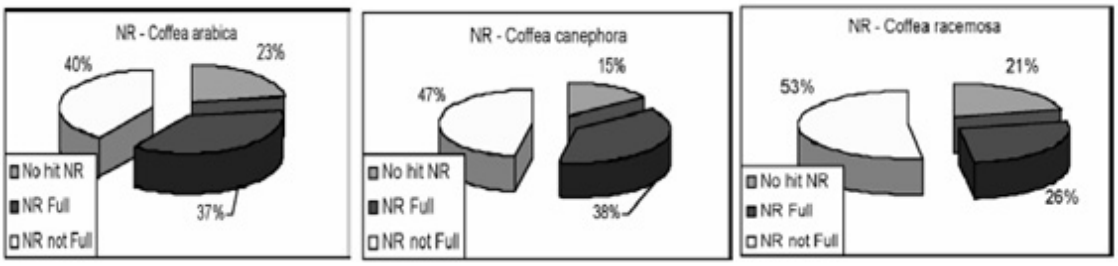

Figure 1. Distribution of the unigenes (contigs plus singletons) according to their comparison against non-redundant protein database (NR at NCBI) by BlastX analysis considering a threshold of 10^{-5} *e-value*. No hit NR: no similar sequence has been found at NR; NR full: coffee sequence with a significantly similar sequence deposited at NR and may encompass the complete ORF.

Protein Profiling by Mass Spectrometry

The identification and characterization of tryptic peptides from the 11S globulin of *C. arabica* beans is presented in Figure 2.

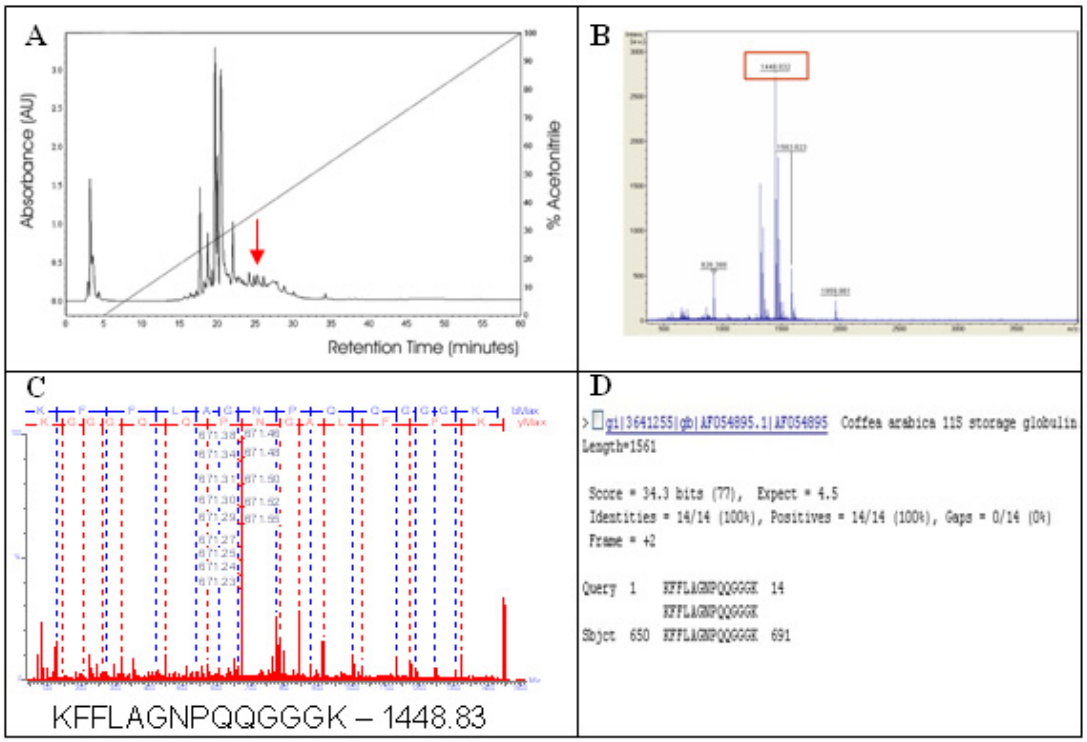

Figure 2. Protein profiling of coffee beans by Mass Spectrometry. (A)RP-HPLC fractionation of tryptic peptides. (B) Molecular masses of the major detected peptides from fraction 15 determined by MALDI-TOF/MS. (C) The MS/MS spectra of the ion 1448.83. (D) BlastP analysis against NR database at NCBI.

Sequence and electronic expression data of contigs matching previously described protein sequences of 11S globulins from *C. arabica* and *C. canephora* (Rogers et al., 1999 and (Chenwei et al., 2005).) retrieved from the Brazilian Coffee EST Database.are shown in Figure 3. As expected (Lashermes et al., 1999), results indicate that one group of sequences from *C. arabica* matches the 11S protein sequences from *C. canephora* and another group does not. Most interestingly, the pattern of electronic expression of these two distinct groups of sequences are distinct.

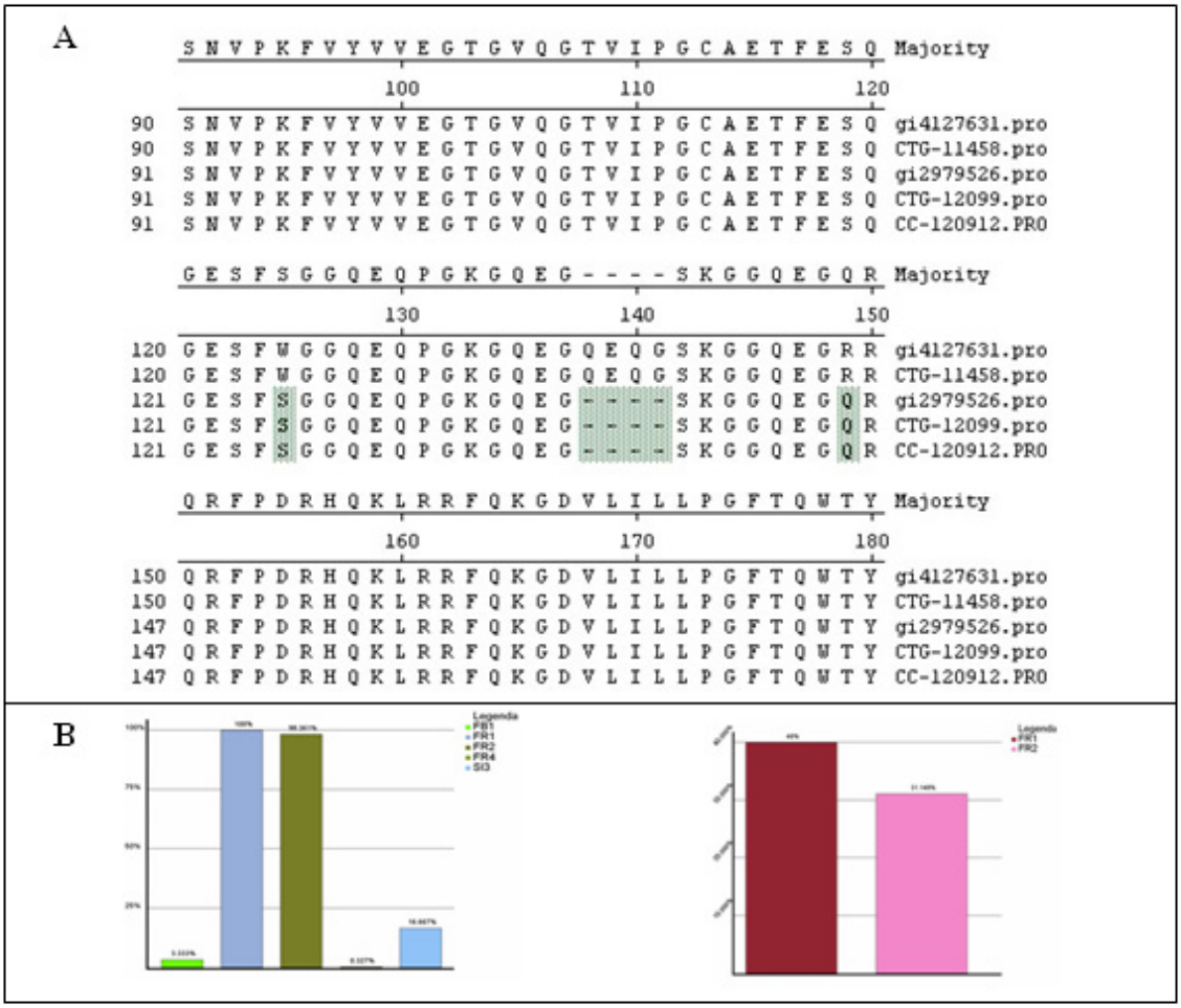

Figure 3. Sequence and electronic-expression data of 11S globulins available in the Brazilian Coffee EST Database. (A) Clustal alignment of partial protein sequences of 11S globulins. GI4127631 and GI2979526 are protein sequences of 11S globulins from *C. arabica*, previously characterized (Rogers et al., 1999); CC-120912 is a protein sequence of *C. canephora*, recently described (Chenwei et al., 2005). CTG-11458 and CTG-12099 are matching contigs present in the Brazilian Coffee EST Database. (B) Differential pattern of electronic expression of CTG-12099 (left) and CTG-11458 (right), based on occurrence of sequence reads in the different EST libraries.

DISCUSSION AND CONCLUSION

As in any EST project, unwanted sequences are produced like ribosomal sequences, poly-A fragments, low quality and short sequences, and slippage that needed to be remove to avoid the introduction of irrelevant information into the ESTs database. From the total of 214,964 reads produced by 26 laboratories, an overall sequencing efficiency of 70% was obtained. The

trimming was carried out with reads from *C. arabica*, *C. canephora* and *C. racemosa* that resulted in 130,792, 12,381 and 10,566 sequences (respectively).The quality of the submitted sequences is an important information to validate a database and the majority of the ESTs analyzed at the end of the sequencing stage of the Coffee Genome Project had length above 500 bp (Phred quality > = 20).

Regarding the cDNA libraries from *C. arabica*, *C. canephora* and *C. racemosa*, the sequences were analyzed for their similarity with known genes by BlastX against NR, considering 10^{-5} for the *e-value* as threshold for identity. It is interesting to note that there is a very similar partition between known and unknown sequences (No hit NR) for the three species. Moreover, among the sequences that generated hits, a significant number comprehends full cDNAs (full lengh – NR full). Close to 11,000 contigs from *C. arabica* (29% of the dataset) lacked significant similarity to any sequence in the Gene Ontology Database (GO).

Proteomics as used to identify proteins in complex mixtures is only effective when a sequenced and annotated genome is available or a large Unigene set becomes established (Rounsley et al., 1996). Proteomics is complementary to the ESTs because it focuses on gene products. Proteomic studies consist of profiling the protein expression found in samples derived from different cultivars, tissue types, cultivation or post-harvest conditions in order to understand what proteins may be responsible for a trait of commercial significance, such as resistance to pathogens, stress tolerance and quality . Mass spectrometry methods such as described here, integrated with the information available in the Coffee EST Database may provide a powerful tool to a better understanding of the complex molecular network working in all kinds of interactions of a coffee plant and its environment. Furthermore, these integrated analyses will certainly speed the gain of basic knowledge up about the molecular factors affecting coffee quality.

The Brazilian Coffee Functional Genomics Program briefly presented here brings new perspectives and approaches to carry out biological research on coffee. Genome related databases, as the one made available by the Coffee Genome Project, have become an invaluable asset for the scientific community to move onto the use of a number of these new technologies. Ultimately, through the use of the coffee ESTs dataset, genetic markers can be found for breeding programs, coffee genes can be cataloged in association with their location on the genome, the study of gene function and how activity of the gene products fits into complex metabolic pathways can be facilitated, and the regulation of the genes in response to different developmental and environmental stimuli can be examined holistically. In addition, applied research aiming at the development of new cultivars to address the various constraints related to the coffee production chain will certainly benefit from these newly available technologies.

ACKNOWLEDGMENTS

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Development of a Bioinformatics Platform at the Colombia National Coffee Research Center

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SUMMARY

We have implemented a web-based Bioinformatics platform that functions as a genomics information resource for coffee and other organisms studied at the Colombia National Coffee Research Center – CENICAFE. The Bioinformatics platform includes a Laboratory Integrated Management System (LIMS), the implementation of wEMBOSS, home-developed perl tools for data analysis, InterproScan for annotation of sequence domains, and the implementation of wBLAST and wNetBLAST among other tools available. The main backbone of the system is an adaptation of the SOL Genomics Network (SGN) databases developed at Cornell University for ESTs, molecular markers and BAC sequences storage and analysis (http://sgn.cornell.edu). The system is based on the postgresQL relational database, the use of perl scripts for the manipulation of data, the Apache Web server with the mod–perl integrated perl interpreter, and the servers run the Debian distribution of the GNU/Linux operating system. Although SGN has mainly developed as a plant genomics oriented resource, the Cenicafe platform has implemented several new tools and databases for the analysis of other organisms sequence data such as fungi and insects. The Cenicafe databases contain to date 32.000 coffee EST sequences from 22 libraries organized in 9.257 *C. arabica* and 1.239 *C. liberica* unigenes, 6.000 *Beauveria bassiana* EST sequences organized in 2.404 unigenes, and 4.000 *Hypothenemus hampei* (coffee berry borer) EST sequences organized in 885 unigenes, besides the more than 100.000 Solanaceae unigene sequences annotated at SGN. The sequences are annotated based on Solanaceae, Arabidopsis, Swissprot and Genbank sequence comparisons using BLAST homology searches, aminoacids are predicted using ESTScan, the domains are annotated using InterproScan and Gene families are annotated using a perl script developed at SGN. The system will implement in the near future a database of coffee genetics resources developed at Cenicafe, a proteomics platform, and a Microarray database. We will also be incorporating other components to the platform specially for the visualization of genetic maps from the Gmod project (Gbrowse), the SGN system, TIGR, and other open source projects.

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 July 2006, roughly 3.000 DNA sequences from the species *C. arabica* had been deposited in the GeneBank database. Only recently, a large Expressed Sequenced Tag data set from the species *C. canephora* developed jointly by Nestlé and Cornell University scientists were deposited in public databases (Lin et al., 2005).

Genomic research is a field that continuously faces the problem of storing, indexing and retrieving large amounts of data; fortunately for bioinformaticians there is a trend in the field

to rely to a greater extent on standard methods for the analysis of this data. It is possible nowadays to share Bioinformatics resources between different research groups and the integrity of the data is not jeopardized in anyway (Teufel et al., 2006).

ESTs are being produced for a number of plants as a rapid method for gene discovery. For instance rice has more than 1 million EST sequences and there are 12 plant species, most of them grasses, with at least 200.000 EST sequences in dbEST (release 082506, August 2006). The ultimate aim in most projects is to catalogue all the expressed genes in a particular genome.

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 the development of a Bioinformatics platform for the storage, comprehensive analysis and easy retrieval of molecular data generated in Colombia from the Coffee Genome Initiative taking place in Cenicafe.

DEVELOPMENT OF THE PLATFORM

The coffee genomics project started in 2003 in Colombia with the financing of the Ministry of Agriculture and the National Coffee Growers Federation. The main outcomes expected from this research include the development of molecular tools and markers for coffee, construction of a *C. arabica* genetic map, identification of agronomic important genes, and the development of a Bioinformatics platform to store and analyse the data generated in the project. A major part of the molecular tools been developed involve; the generation of a large set of ESTs; construction, fingerprinting and sequencing of a *C. arabica* BAC library; and the detection of microsatellite, COS and SNP markers. We have also incorporated as part of our genomics research the development of tools to study the genomes of *Hypothenemus hampei* and *Beauveria basiana*.

From the start, the Bioinformatics platform has been a major component of the genomics research at Cenicafe. Our efforts have concentrated in the development of relational databases, tools for data analysis, and web-based user-friendly interfaces to access data, based on open source technology. We use commercial software just in very special scenarios of our analysis. We have engaged in close collaborations with research groups that adopt this kind of approach in their Bioinformatics developments and consequently our main partners in this area are the Solanaceae Genomics Network based in the Department of Plant Breeding at Cornell University and The Institute for Genomic Research – TIGR in Rockville, Maryland.

Computer resources

We have a cluster that consists of a master server (IBM x346. Disk space RAID5: 1,2 TB, RAM: 5 GB, CPU: 2 x 3,6GHz Intel Xeon) and 7 server nodes for data processing (Opteron e-325, 64 bits). For the functioning of the cluster, we use NFS (Network File System) and samba to share directories.

Software resources

The master server runs the Apache webserver, most machines run Debian GNU/Linux as operating system, and generally we develop programs in perl for processing data, and perl-cgi and PHP for Web development. Our first Database Management System was constructed using the MySQL relational database and we are currently migrating to the PostgreSQL database system. We implemented the applications MPI-BLAST and ClustalW-mpi to run on the cluster. By running these processes on the cluster we have calculated up to 6 times reduction in processing of data.

The Bioinformatics group efforts are concentrated in two major areas, service and production (development). The service routines include the analysis of sequence and other types of data produced by Cenicafe genomics scientists. The development activities include the setting up and administration of Bioinformatics servers, construction of structured databases, development of web-based interfaces for the display of data and the writing of scripts in perl and other languages for the manipulation of data.

IMPLEMENTATION OF SOFTWARE

The Bioinformatics system is accessible through a web-based interface from which all databases and tools are available (Figure 1). The system is built-in a Laboratory Integrated Management System that incorporates a project administration resource, coffee and other organisms databases (SGN Schema), the wEMBOSS suite of tools (Rice et al 2000) and local implementations of BLAST (Altschul et al., 1990) to run particular types of analysis (wBLAST, BLASTXtract and wNetBLAST among others).

DATA ANALYSIS

While the Sol Genomics Network has mainly developed as a plant genomics oriented resource, the Cenicafe platform has implemented several new tools and databases for the analysis of other organisms molecular data such as fungi and insects. The core of the system is to date the SGN database schema, but our platform has incorporated several additional modules to annotate fungi and insects, given that as mentioned above, genomics data is also been produced from *H. hampei* (the coffee berry borer) and *B. bassiana* (biological control agent).

ESTs are analyzed based on an adaptation of the SGN pipeline (Mueller et al., 2005). In synthesis, chromatograms are called with phred (Ewing et al., 1998), assemblies are performed with CAP3 (Huang and Madan 1999), full length EST sequences are computed by TargetIdentifier (Min et al. 2005), aminoacid prediction is accomplished by ESTScan (Iseli et al., 1999), and functional annotation of sequences is performed with several databases among them GenBank, and more specialized databases like Solanaceae and Arabidopsis for plants, Sacharomyces and Magnaporthe for fungi, and Drosophila and Tribolium for insects. Additional functional annotation of Gene Ontology terms (Ashburner et al., 2000) is performed in house with InterProScan (Mulder et al., 2003; Zdobnov and Apweiler 2001). Several steps of the process include scripts written in perl at SGN and Cenicafe.

Other Bioinformatics analysis include the discovery of SSR markers, development of specific PCR primers, prediction of SNPs and homology comparisons between large sets of sequences. SNP prediction is accomplished through the comparison of several homologous sequences and their visualization is performed with the software CodonCode Aligner (CodonCode Corporation, Dedham, MA).

Table 1 illustrates the number of ESTs analyzed sequences deposited in Cenicafe databases. These numbers are continuously increasing and there will shortly be an update of the databases to incorporate a number of new coffee ESTs and BAC-end sequences. It is possible to retrieve the data from the databases in very specific ways according to the scientists needs; an example of a tissue-specific expression analysis of *C. arabica* transcripts is shown in Table 2.

Cenicafe databases also include more than 1000 coffee microsatellite sequences used for the construction of coffee genetic maps and diversity studies and data gathered from COS and SNP markers studies.

FINAL REMARKS AND FUTURE PROSPECTS

The system will implement in the near future a database of coffee genetics resources developed at Cenicafe, a proteomics platform, and a Microarray database. We will also be incorporating other components to the platform specially for the visualization of genetic maps from the Gmod project (Gbrowse "Generic Genomic Browser", Stein et al 2002), the SGN system, TIGR, and other open source projects. A BAC relational database is in the process of construction and it will include over 60.000 BAC-end sequences been generated at Arizona University.

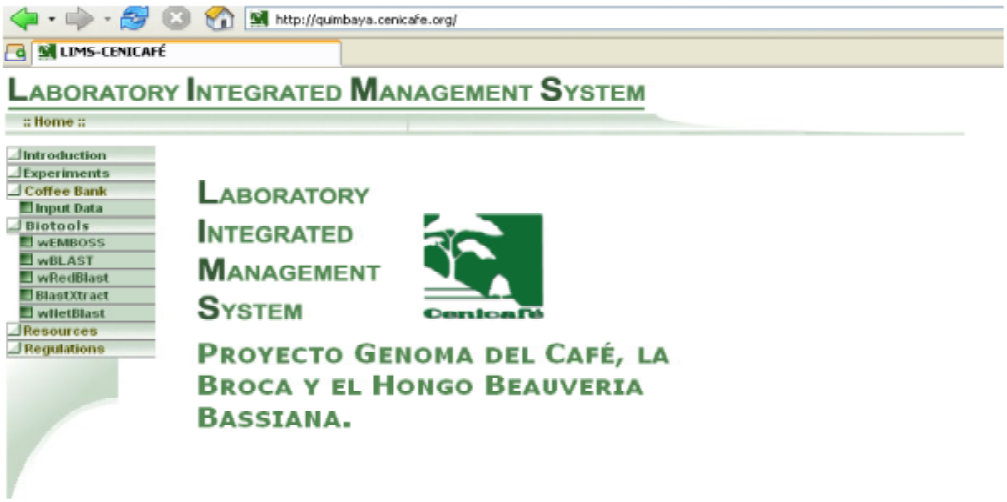

Figure 1. Web interface of the Cenicafe LIMS system which integrates access to databases and analysis tools.

Table 1. Number of EST sequences deposited in Cenicafe databases.

	LIBRARIES	CHROMATOGRAMS	UNIGENES
COFFEE			
Coffea arabica	17	35.000	9.257
Coffea liberica	4	3.613	1.239
Coffea canephora	5	47.000	13.750
Coffea spp.	1	497	210
FUNGI			
Beauveria bassiana	8	5.300	2.404
INSECTS			
Hypothenemus hampei	2	3.563	885

We are in the process of mirroring the Solanaceae Genomics Network site (http://sgn.cornell.edu/) that will be accessible from (http://sgn.cenicafe.org/). The projected web interface of the mirror site can be viewed in Figure 2. We will have to develop ways of defining complex interactions, functional annotation and integration of proteomics, microarrary and other data that will emerge from the project. Several new ways of integrating these data are emerging (Rhee et al., 2006).

Table 2. BLAST homology searches of *C. arabica* Unigene sequences, number of ESTs that compose each unigene and representation in 3 tissue-specific libraries.

Unigene No.	No. Members	FR	L	FL	BLAST	ORGANISM
sgn|U269499	244	133	110	1	No hits	
sgn|U269496	201	101	76	24	Metallothionein	C. arabica
sgn|U269332	192	25	113	54	acidic endochitinase	At
SGN-U269331	168	21	108	39	acidic endochitinase	At
SGN-U268736	143	26	100	17	lipid transfer protein	At
SGN-U268469	138	40	29	69	DNA replication	SV40
SGN-U269498	122	86	36	0	No hits	
SGN-U268200	98	72	19	7	metallothionein	Citrus unshiu
SGN-U269495	80	40	30	10	Metallothionein	C. arabica
SGN-U268647	68	16	52	0	chlorophyll a /b binding protein	At
SGN-U269497	64	48	16	0	No hits	

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Construction of an Interspecific Genetic Linkage Map from a *Coffea liberica* x *C. eugenioides* F1 Population

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SUMMARY

Coffee is globally one of the most important export crops and is a prominent part of the economy in more than 50 countries in Latin America, Africa and Asia. In Colombia for more than a century it has been the leading export commodity. In spite of that, genetic research on coffee has been sparse and has been focused mainly on the two major cultivated species, *Coffea arabica* L. and *C. canephora* P., leaving unexplored the genetic potential in other species in the genus *Coffea*. In this work we describe the construction of an interspecific genetic linkage map of coffee, using a mapping population obtained at CENICAFE and consisting in 91 plants from the F_1 of de diploid species *Coffea liberica* x *C. eugenioides* cross. A total of 327 molecular genetic markers developed at CENICAFE were evaluate, of which 116 exhibited segregation patterns that allowed linkage analysis using JoinMap 3.0 genetic software. The results indicated that 76 loci incorporate into 12 major linkage groups. Another 40 loci produced minor linkage groups. The segregation analysis show a clear tendency for segregation distortion at a high proportion of loci (39%). The knowledge derived from this study will have applications in plant breeding through marker assisted selection programs, linkage disequilibrium analysis and the identification of quantitative traits, among others.
Key words: coffee, genetic map, genetic linkage, genomics.

INTRODUCTION

Coffee belongs to the Coffea Genus, Rubiaceae family, whose presence is restricted mainly to tropical and subtropical areas. This crop plays an important role in the commercial activity of more than 50 countries of Africa, Asia and America. The genus includes around 100 species, in its majority diploides with 2n = 22, with the exception of *Coffea arabica* with 2n = 44 considered an alotetraploid (Bridson and Verdcourt, 1988).

The size of the coffee genome based on fluocitometry indicate that *C. liberica* contains 1.68 pg of DNA and *C. eugenioides* 1.39 pg, being the rank for all the diploides species of coffee of 0, 95 pg for *C. racemosa* to 1.78 pg for *C. humilis* (Cros et al., 1995). The same study reveals that *C. arabica* contains 2.61 pg of DNA by nucleus, which is compatible with its tetraploide state. According to this, the species of *Coffea* in general can be considered with a low level of nuclear DNA content.

Coffee is a perennial crop and therefore breeding programs for its improvements face among problems the long generation time, which is around five years, which mean that the improvement process takes long time and high costs. In addition, the cultivars have a low

degree of genetic variability given their origin (Haarer, 1956) and its autogamous condition. Because of that the scientific work to improve coffee must take into account the use of wild materials, applying modern technologies of molecular biology, in combination with classic breeding methods and the appropriate schemes of introgresion. Within this context, one important objective is to get knowledge of the genome of the plant and then the possibility of manipulating accurately the genes of interest, shortening the time and the costs of the program. For this purpose it is fundamental to have a genetic linkage map that facilitates the selection of the most desirable genotypes based on selection of quantitative traits of interest such as, resistance to diseases, quality and production, among others. In this work we constructed a genetic linkage map using an F1 population obtained of the crossover between *C. liberica* x *C. eugenioides*. These two species are diploides of fundamentally wild habitat, with 2n = 2x = 22 chromosomes. *C. eugenioides* is considered one of the progenitors of *C. arabica*, *C. liberica* maintains a narrow genetic distance with *C. canephora* this makes to this population a very interesting one, non only due the quantitative detection of loci that regulate characteristics of agronomic interest but also because of the availability of the population that is on the fields.

Besides, *C. eugenioides* has lower caffeine content in the grain, around 0.2%, compared with *C. liberica* that reaches 1% (Barre et al., 1997). Also he has reported that the grain of *C. liberica* has a high content of clorogenic acids which is related to cup quality (Ky et al., 1999). *C. liberica* radicular system is vigorous and of fast regeneration, in contrast to *C. arabica*. This characteristic makes of *C. liberica* a tolerant species to the attack of nemátodos (Calles, 1993).

MATERIALS AND METHODS

DNA extraction

The DNA extraction was made from young leaves of each plant using 10 gr of fresh tissue. The used method of extraction is the same one described by Ky et al. (2000).

Plant material

The mapping population includes the parents, and 91 hybrid plants F_1 2 years old, planted in the experimental station Naranjal of CENICAFE, Chinchiná, Colombia. This population was obtained from the cross between *C. liberica* x *C. eugenioides*, two diploides wild species with 2x = 2n = 22.

Molecular markers

A total of 327 molecular markers were evaluated in the parents of the population. These include: 235 SSRs generated by CENICAFE in association with Cornell University (Cristancho, Greene and Gaitan, McCouch laboratory, Cornell University, Ithaca, NY), 72 SSRs from IRD, France and 20 EST from Cenicafe were evaluated in this population.

Detection of the polymorphism

The markers were evaluated by reactions of amplification with the following profile: Step 1. Preheating to 94 °C for 5 minutes. Step 2. Denaturation 94 °C for 1 minute. Step 3. Association to 55 °C for 1 minute. Step 4. Polymerization to 72 °C for 1 minute. Step 5. To repeat from step 2 34 cycles. Step 6. Final extension to 72 °C for 10 minutes. Step 7. The

amplification products were run on 4% poliacrilamida gels and for staining silver nitrate was used.

Linkage analysis

For estimations of recombination frequencies, distances estimation, and linkage groups the program JoinMap 3.0 was used. It allows to construct joint maps from both parents or individual maps for each one (Van Ooijen and Voorrips, 2001). For tests of hypothesis of linkage versus independence, the statistical LOD Score was used (Z) introduced by Haldane and Smith in 1947 and Morton in 1955 for studies of human genetics (Nuez and Carrillo, 2000). The term is an abbreviation of the English expression "Log of the odds" and statistically is define as the logarithm of the proportion of the probabilities of linkage and independence, that is to say, $L(r=\hat{r})$ with respect to L(r = 0.5), where r is the recombination frequency.

RESULTS AND DISCUSSION

Detection of polymorphic and segregant markers.

The level of polymorphism was evaluated using the parentals and 6 individuals from the population. In total 327 markers were evaluated and 116 of them showed segregation patterns that allow linkage analysis.

Segregation analysis.

This analysis was based on the seven segregation models that exist for a seudo-cross population like this one. From them, five models fitted to the markers already mapped. The models include loci that segregate for one of the parents lm x ll or nn x np (cases 1 and 2 for the father and the mother, respectively), when both parents are heterozygotes but have the same alleles hk x hk (case 3) and when both parents are heterozygotes but with different alelos, ab x cd or ef x eg (case 4). Case 6 with both parents heterozygotes but the mother includes ona null allele (ab x a0) and it is is codified as case 1 (l m x ll). Models five and seven were not observed for any of the markers in this population.

On model one are included all loci for which *C. eugenioides* segregates two alleles and *C. liberica* is homozygote. A total of 21 loci with these characteristics were found. They correspond to 18,1% of the total number of segregant loci. .

On model 2 60 markers were classified which correspond to the 51,7% of tota segregant loci. On this case *C. eugenioides* is homozygote and *C. liberica* has two alleles.

For model three, two markers were found that correspond to 0,02% of the total number of segregant loci. On this case both parents present the same two alleles.

On model 4 which is the most informative model of segregation, each parent has two different alleles. With this segregation type 29 loci were identified. They correspond to 25,0% of the total analyzed loci. There were not loci with model 5 of segregation.

For model 6, four loci were found. This model is similar to case one. The difference is that the homozygote has a null allele .

Model 7 which is the inverse of model 6 was not represented oin any loci of this map.

From the 116 markers with information for linkage analysis, 39% (45) showed distortion segregation.

Linkage analysis.

A genetic map was constructed in a family of 91 descending plants of the interspecific cross of *C.liberica* x *C. eugenioides*. This preliminary map contains 76 microsatellite markers distributed in a distance of 378 cM. With values of LOD over 3.0 with exception of the linkage group 12, with a LOD of 2.0. (Figure 1). These markers constitute a standard initial set of reference to be used as anchor markers for comparative mapping and to saturate the coffee genome consensus map.

Is interesting the fact that the microsatellites were distributed at random on the genome as observed in Figure 1. The construction of this map is in a preliminary stage, and 40 markers have not yet been mapped on any of the linkage groups. Five segregation models fitted to the markers already mapped.

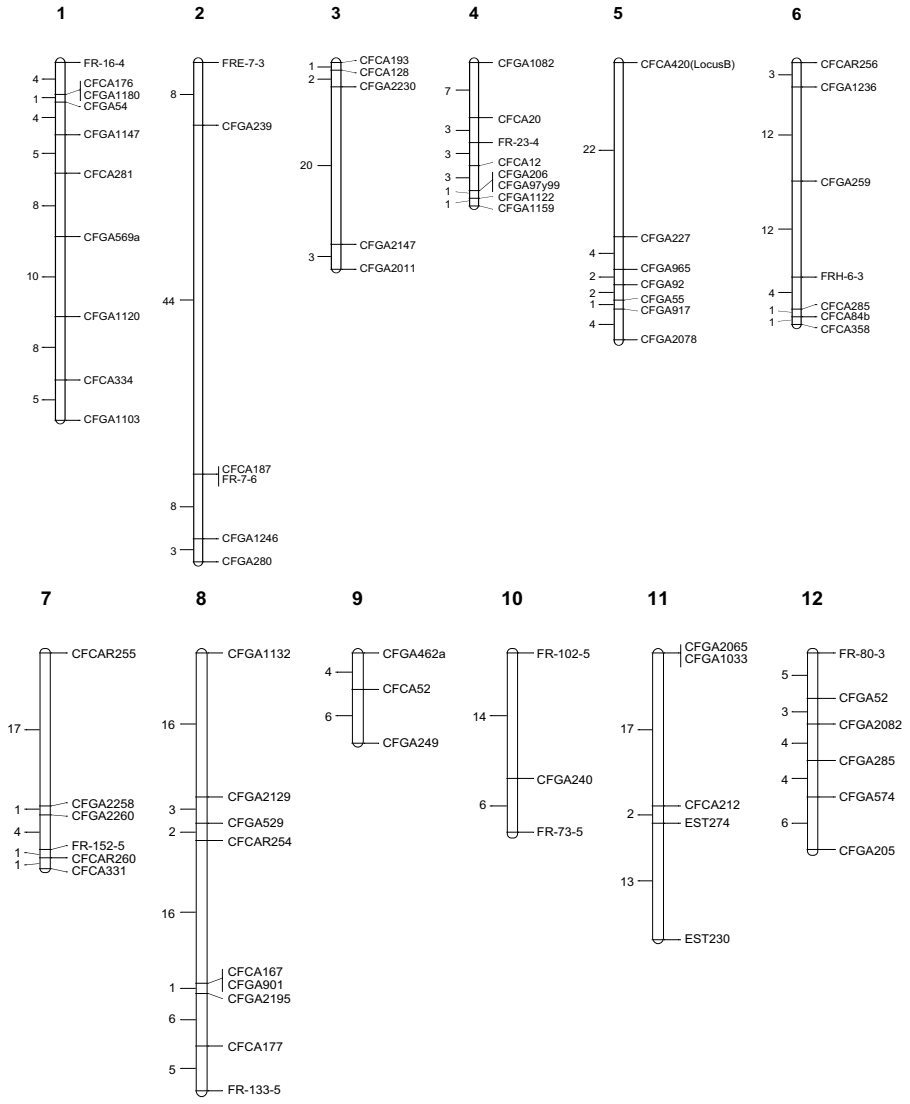

Figure 1. Genetic linkage map of *C. liberica* x *C. eugenioides* constructed on 91 interspecific hybrids. 74microsatellite markers and 2 EST were grouped, using the Kosambi function and the program JoinMap 2.0. 12 linkage groups were obtained with a total length of 378 cM.

A remarkable aspect of the study is the number of loci that present distortion of the mendelian proportions of segregation. Several works have reported widely distortions of segregation in plants, in mapping populations of different species (19, 20, 37, 46, 64). In general, the works in this field argue diverse causes to explain this phenomenon. Some of the most mentioned are: the abortion of zygotes, the selective exclusion of a particular gametic genotype at the moment of the fertilization due to mutual incompatibility and the zygotic selection, among others (35). In agreement with these hypotheses and considering that the population of our map is an interspecific one, it is very probable that the distortion of segregation in this population is related to processes of gametic or zygotic selection, although the described experiments can not give answer to this hypothesis and then it is required to do specific experiments to solve it.

Currently, studies are being developed to increase the number and type of microsatellite markers in this population, as well as the exploration of the possibility of including markers associated to expression regions, called functional markers.

Another interesting finding in this study is the high proportion of loci with null alelos (32% of 114 microsatellites and 2 ESTs). To a genotype in a particular locus null alleles are assigned when the expected amplification products, according to the segregation model, are not detected for a particular combination plant-marker, after several repetitions. This can be due to the origin of the microsatellite sequences from Caturra variety (*C. arabica*) and to possible mutations in the course of the evolution, that prevent the detection of some alelles in other species of *Coffea* such as the ones used as parens for the mapping population.

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Transgenic Roots for Functional Genomics of Coffee Resistance Genes to Root-Knot Nematodes

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SUMMARY

The possibility of rapid validation and functional analysis of nematode resistance genes is a major objective for coffee. We developed an *Agrobacterium rhizogenes*-mediated transformation protocol for *Coffea arabica* enabling efficient and rapid regeneration of transformed roots from hypocotyls of zygotic embryos, and subsequent production of composite plants (transformed roots induced on non-transformed shoots). Embryos from two *C. arabica* varieties, resistant or susceptible to *Meloidogyne exigua*, were transformed with *A. rhizogenes* A4RS strain armed with binary vectors possessing *gus* or *gfp* reporters genes. Transformed roots with *A. rhizogenes* were regenerated at the inoculum site for 90% of infected embryos. These roots did not exhibit 'hairy' disturbed phenotype. Putative transformed roots could be identified and further selected by histochemical GUS assay and fluorescence microscopy without using herbicide or antibiotic selection. Transgene incorporation was confirmed by PCR. Co-transformation frequencies [i.e. co-integration of both T-DNA from "root inducing" Ri plasmid and binary plasmid] ranged from 40-80%. Composite plants, bearing a single co-transformed ramified root were obtained 12 weeks after transformation (all non co-transformed or weakly ramified roots were eliminated). Nematode infection in nursery conditions of 16 weeks-old composite plants from the Caturra susceptible variety resulted in the development of numerous big sized-galls, while very few small galls were observed in the IAPAR-59 resistant variety. No difference was found in the number of extracted nematodes between the transformed and non-transformed roots ($P < 0.95$) for both varieties, demonstrating that transformed roots retained the resistance/sensibility phenotype of varieties from which they are derived. These results suggest that composite plants constitute a powerful tool for studying genes involved in nematode resistance and root development.

INTRODUCTION

For Latin American coffee growing regions, phytopathogenic nematodes, particularly root-knot nematodes, are frequently found on coffee tree roots. They cause spectacular drops in yields, and decay that often leads to early death. In coffee plantations, nematodes are usually controlled with nematicides, which are among the most toxic molecules used in agriculture. Moreover, the effectiveness of those products is limited as nematodes live part of their life cycle in the soil and are sheltered from the pesticides. One alternative consists in developing integrated pest management (IPM) and using resistant coffee tree varieties. Those two approaches are an ecological and sustainable alternative to often irrational use of phytosanitary products, which are a danger to human health and his environment.

In coffee trees, several *C. arabica* lines derived from the interspecific Timor hybrid (wild *C. arabica* x *C. canephora*) have displayed resistance to the *M. exigua* nematode and the IRD-CIRAD team confirmed that resistance to that nematode came from the *C. canephora* parent (Bertrand et al., 2001). Recently, Noir et al. (2003) identified molecular markers associated

with the resistance to *M. exigua*. A simply inherited major gene, called the *Mex-1* locus, was identified. *Mex-1* gene may have incomplete dominant expression because most of an F2 population derived from a cross between resistant and susceptible genotypes showed a gall index that was higher than the mean value of the resistant parent.

Until date, functional validation of cloned resistant genes to nematodes in different plants has been done preferably using *A. tumefaciens*-mediated transformation. Principal reasons for this are: i) disposition of reliable protocols for efficient obtention of transformation events, ii) higher availability of vectors and iii) the fact that larger size of T-DNA can be transfer into the plant genome compared with direct transformation methods. However, an important inconvenient of transformation protocols using *A. tumefaciens*, is that regeneration of whole plants for nematode tests from initially transformed callus still constitutes a time consuming and tedious process, e.g. with actual regeneration procedures approximately 1 year is required before the plant with the introduced sequences can be evaluated.

On other hand, resistant genes to nematodes can be rapidly and effectively validated by producing *in vitro* transformed roots expressing candidate resistance genes obtained after infection by *A. rhizogenes* (Remeeus et al., 1998). *A. rhizogenes* is a soil pathogenic bacteria which has the property to regenerate roots genetically transformed at the site of inoculation in plants and is also able to transfer T-DNA from binary vectors, allowing the regeneration of transformed roots expression foreign genes (Zupan et al., 2000).

Coffee genetic engineering emerged during the last decade as a potential tool to achieve objectives from two different research strategies: i) study the introgression impact (functional *in vivo* validation) of agronomically interesting genes that would be introduce later by traditional breeding programs, ii) serve as a tool to introduce desirable traits into commercial genotypes (i.e. protection against insects to which no source of resistance has been identified naturally in *Coffea* sp.).

However, the procedures of coffee transformation reported in the literature before this study were complex, tedious and inefficient in order to be used for the functional analysis of putative genes (e.g. 0.3% transformation efficiency reported by Leroy et al., 1997, 3% by Kumar et al., 2006). In that context, we developed an efficient *Agrobacterium rhizogenes*-mediated protocol that enables efficient and rapid regeneration of transformed roots and composite plants exhibiting a suitable phenotype to be acclimatized within 16 weeks following the transformation.

MATERIALS AND METHODS

Plant material, binary vectors and transformation procedures

The *C. arabica* var. Caturra (susceptible) and Iapar-59 (resistant) to *M. exigua* was used in this study. Two months-old germinated embryos were transformed with three vectors pBIN19 35S-*uidA*, pBIN19 35S-*gfp* and pCAMBIA2300 35S-*gfp* carried by A4RS strain of *Agrobacterium rhizogenes* using the protocol described by Alpizar et al. (2006a).

Selection of co-transformed roots and production of composite plants

Decontamination of *A. rhizogenes* following co-culture period was done by subculturing embryos every 4-weeks in MS germination medium containing decreasing cefotaxime concentrations (500, 200, 100 $\mu g ml^{-1}$). Co-transformation efficiency was calculated after 12 weeks as the percentage of inoculated embryos that regenerated at least one GUS or GFP-positive root at the wound site. Selection of GUS co-transformed roots was done during the third subculture by dipping one root tip of well-ramified roots in Xgluc staining solution (Jefferson et al., 1987), whereas screening of GFP co-transformed roots was done using a stereomicroscope with fluorescence, through a set of filters GFP2 [λ excitation: 480 ± 40 nm and λ excitation stop: 510 nm].

Analysis of transformed root morphological variability

Another major technical challenge facing the functional validation of resistance genes to nematodes by *A. rhizogenes*-mediated transformation is the production of transgenic roots exhibiting desirable and stable phenotypes (the most similar as possible to non-transformed roots). For the assessment of root morphological variability, root images of branched root fragments from both composite plant and non-transformed plants (controls) were taken prior to nematode multiplication assessment. The images were acquired by a scanner and were analyzed using the software procedures of WhinRHIZO V5.0 (Instrument Regent, Quebec, Canada). For each root clone, the variables acquired were: number of lateral roots per cm of mother root to evaluate root branching, the total root length (cm) at the end of 3 weeks of subculture, and the percentage of fine roots (%) with a diameter less than 0.5 mm.

Nematode infection on composite plants

Composite plants were transferred into 200 cm^3 plastic pots filled with a sterilized culturing substrate composed soil and a mixture of pure silica sand and water-absorbent synthetic polymer proposed by Reversat et al. (1999) placed in a growth chamber under 14 h photoperiod (50 $\mu mol\ m^{-2}\ s^{-1}$ light intensity) at 26 °C and 60% of relative humidity. Once the composite plants reached the three pair of leaves stage, they were inoculated with *M. exigua* individuals (J2 larvae + eggs) collected from inoculated susceptible *C. arabica* plants grown in a greenhouse. 10 composite plants and normal plants of each variety were inoculated, with three replicates. The inoculum dose was 300 individuals (eggs + J2) applied to the collar of each plant. Four months after inoculation, nematodes were extracted following the protocol of Hussey and Baker (1973). Number of nematode individuals per gram of fresh root was determined under stereo-microscope in three replicates.

Statistical analysis

The data were compared by ANOVA followed by a comparison of the means using a Neuman-Keuls multiple range test. Values followed by different letters are significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

Regeneration and selection of co-transformed roots

By using the armed A4RS with pBIN19-p35S-*uidA*, pBIN19 35S-*gfp* and pCAMBIA2300 35S-*gfp* binary vectors it was possible to measure the co-transfer frequency of the Ri TDNA and of the T-DNA of the binary vector containing the *uidA* and *gfp* reporter gene following 14 days of co-cultivation at 20 °C. The three constructions responded in a similar way to agroinfection (Figure 1). Between 66.5 and 92.5% of infected embryos regenerated at least one root at the wound site and between 38.5 and 75.9% generated at least one GUS or GFP-positive root. The average number of co-transformed roots per inoculation site was higher for pBIN19 35S-*gfp*. Then, after the GUS staining and GFP visual tests performed on a root tip of each root system, allowed to preserve one GUS or GFP-positive root on the stem of each plant. Other roots were eliminated.

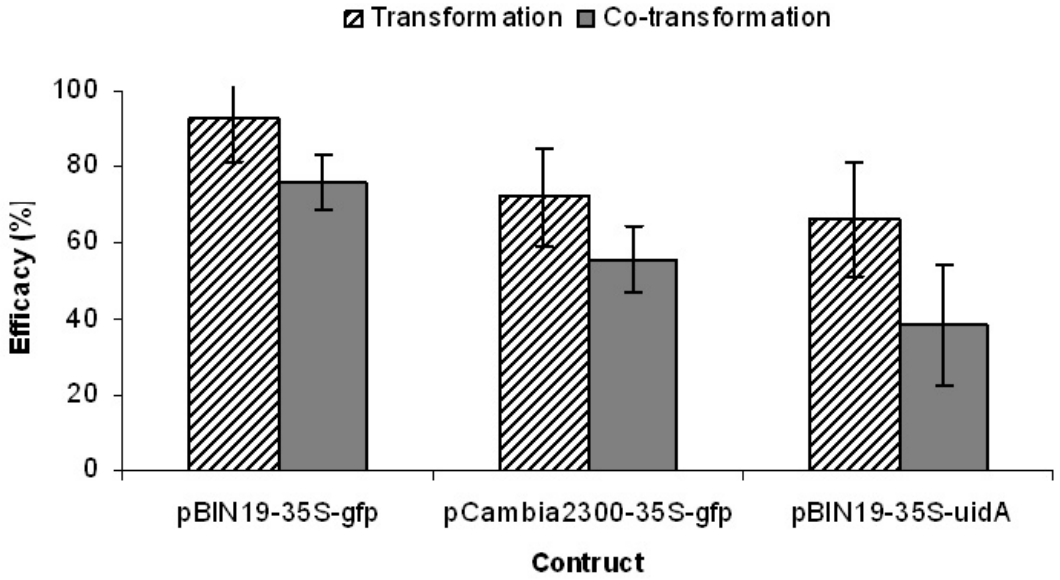

Figure 1. Comparison of transformation efficiency and co-transformation frequency of binary vectors pBIN19-35S-*uidA*, pBIN19-35S-*gfp* and pCAMBIA2300-35S-*gfp* in *C. arabica* var. Caturra. Each value represents the mean of four replicates on 20 embryos each. *Transformation*: frequency of inoculated embryos that gave rise to at least one transformed root. *Co-transformation*: frequency of inoculated embryos that gave rise to at least one *uidA* or *gfp*-positive transformed root.

One of the main problems using *A. rhizogenes*-mediated transformation is the difficulty to eliminate the agrobacteria after co-cultivation period. It is well known that this difficulty is particularly strong with the A4 strain (Kumar et al., 2006). Conventionally, decreasing concentrations of the bacteriostatic cefotaxime in culture medium is used. However, with this protocol, a significant percentage of transgenic roots still contain the bacteria. The previous poses a significantly problem since infected root clones cannot be used in functional analysis studies because of the impossibility to screen through molecular analysis (i.e. PCR test) whether the presence of the reporter gene or transgene belongs to the transformed plant or to bacteria. The assessment of the efficiency of the decontamination is conventionally realized through PCR analysis checking for the presence of the *virD* gene from the Ri plasmid of the bacteria in a region outside the T-DNA. By consequence, the hairy roots bearing this gene are

supposed to be contaminated by the agrobacteria. At the beginning of our experiments, using cefotaxime treatment, the efficiency of decontamination achieved was 60% (evaluation assessed on 62 root clones). We developed a new decontamination treatment using a mix of cefotaxime and PPM® (Plant Cell Technology, WA, USA) which is known to have an affect on wide spectrum of pathogens (bacteria and fungi). The application of this treatment resulted in a significantly increase of the decontamination efficiency; only 11.3% of clones exhibited presence of *virD* gene.

Evidence of limited phenotypic variability among coffee hairy roots

The existence of morphological variability between transformed and non-transformed roots on the one hand, and between hairy root clones on the other, was revealed using two variables: the percentage of branched fine roots and the total root length. Both variables were confirmed to be stable and to provide immediate application on the routine measurement through image acquisition and analysis of hairy roots to screen and discard aberrant phenotypes. Most of the coffee hairy root clones were morphologically similar. The results of the image analysis of morphological parameters confirmed that approximately 90% of transformed coffee roots are morphologically similar to non-transformed roots and enabled the use of *A. rhizogenes*-mediated transformation systems for functional genomics of root genes.

Nematode infection on composite plants

The average number of nematodes extracted from transformed roots of the susceptible Caturra and Iapar-59 varieties (3001 ± 2687 and 218 ± 281 nem. g. root^{-1}) compared with non-transformed roots (3258 ± 2200 and 135 ± 90 nem. g. root^{-1}) was not significantly different according to ANOVA test (Figure 2). These results prove that *M. exigua* nematodes multiply normally on transformed roots and that their transgenic status does not represent a problem to develop studies on the interaction between coffee/*M. exigua*. The results above also showed that nematode reproduction levels varied largely between different transformed roots as well as for control plants. Nematode multiplication variability has been reported in *A. rhizogenes* mediated-transformed roots of sugar beet (Cai et al., 2003) and tomato (Plovie et al., 2003) and similarly, in coffee resistance tests to *M. exigua* carried in nursery conditions on normal non-transgenic seedlings under natural tropical conditions of substrate, temperature and humidity (F. Anthony, pers. comm.). We hypothesized three causes that may induce this variability, the first two ones are specific to the bioassay on composite plants: i) the fragility and small size of the plant material induce development delay and losses during the acclimatization to *ex vitro* conditions, ii) problems related to the horticultural management: inconsistencies in substrate composition, limited root biomass, timing and localization of nematode inoculum, iii) a variability related specifically to the living material (i.e. maintenance of nematodes virulence).

The use of soil with a mixture of pure silica sand and water-absorbent synthetic polymer substrate (3:1 ratio) led to a considerable reduction of the mortality rate of "composite plants", plants with non-transformed aerial part bearing a transformed root (from 50 to less than 20%) as the time of acclimatization (from 12 to 7 months). Dozens of composite plants of both Caturra and Iapar-59 varieties have been inoculated at the six leaf pair stage in these improved conditions; at this stage the root system from these composite plants exhibits an exponential growing phase and the formation of nematode galls is observed three weeks after the nematode inoculation. In other hand, the gall size analysis described by Alpizar et al.

(2006b) is proposed as a complementary method for screening the resistant root phenotype from transformed plants. This visual evaluation can be done rapidly and would provide complementary qualitative data to increase the accuracy of the determination of the resistant/susceptible character. According to the experience accumulated from several inoculations in controlled conditions, *M. exigua* maintained in tomato or pepper often exhibited lost of virulence to multiply in either transgenic or non-transgenic coffee roots. Nematode maintenance in those plant species is often used since multiplication rate is higher than coffee. Therefore, an important improvement for future bioassays is to maintain the nematode inoculum in susceptible coffee plants at least 4 months before carrying out the experiment.

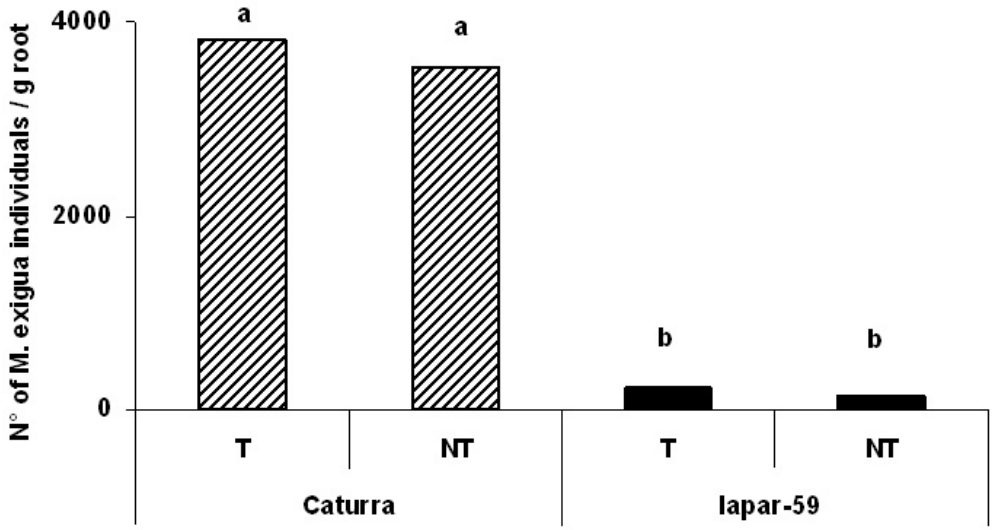

Figure 2. Numbers of *M. exigua* individuals extracted from on *A. rhizogenes*-transformed vs. normal roots in susceptible (Caturra) and resistant (IAPAR-59) varieties. Numbers of nematode individuals per gram of fresh root were determined 4 months after *M. exigua* inoculation. Each value represents the mean of three replicates from 10 composite or normal plants for each variety. For each variety, values with different letters are significantly different at $P \leq 0.05$ (Neuman-Keuls test).

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Utilization of Chromosome Painting as a Complementary Tool for Introgression Analysis and Chromosome Identification in Coffee

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SUMMARY

The term "chromosome painting" widely implies painting of differential chromosome segments with sequence specific probes based on the technique of *in situ* molecular hybridization. Development of fluorescence *in situ* hybridization (FISH), further enhanced sensitivity and versatility of *in situ* hybridization procedures. Despite recent development and application of FISH in plant genome analysis, this technology remains unfamiliar to most coffee scientists. Here we report through of different examples, the potential of FISH technique as a tool for genome analysis in coffee. We investigated the presence of alien chromatin in interspecific hybrids between *C. arabica x C. canephora* as well as in one *C. arabica* line introgressed from *C. liberica*. Further, we demonstrated that it is possible to identify one specific chromosome on the whole genome, despite the morphological similarity between coffee chromosomes. Overall, our results illustrate how molecular cytogenetics approach would provide complementary information for genetic mapping studies based on molecular markers.

INTRODUCTION

Together with marker-based genetic linkage mapping, fluorescence *in situ* hybridization (FISH) represents some of the most significant advances in molecular techniques for studying hybridization and introgression in plants. Main advantage of FISH procedure is that it allows to detect the extend of introgression across the entire genomes in a single hybridization experiment thanks to the *in situ* labeling of homologous chromosomes or chromosome regions on basis of divergent dispersed repeats. *In situ* hybridization could be carried out using total genomic DNA (GISH), chromosome derived DNA probes or large genomic insert clones, like bacterial artificial chromosomes (BAC-FISH). In plants, the FISH analysis has facilitated the identification of individual chromosomes and parental genomes in hybrids, the physical mapping of genes on chromosomes, the integration of genetic and physical maps with marker tagged BAC clones, and the analysis of genomic distribution of mobile genetic elements and other repetitive DNA sequences.

While GISH has provided valuable information for characterization of genomes and chromosomes in hybrid polyploids, hybrid plants and recombinant breeding lines, FISH using specific-probes remains difficult to apply in most of plant species (Schubert et al., 2001; Raina and Rani, 2001). Therefore, the aim of this report was to investigate whether total genomic DNA from the two diploid relatives *C. canephora* and *C. liberica*, could be used as a probe to identify the presence of alien chromatin in introgressed genotypes of *C. arabica* by genomic *in situ* hybridization (GISH). Triploid and tetraploid interspecific hybrids derived from crosses between *C. arabica x C. canephora* were examined by GISH in order to differentiate alien genomes as well as inter-genomic relationships into the chromosome set of

the hybrids. GISH analysis was also used to detect chromosomes carrying the introgressed *C. liberica* fragments in a recombinant line of *C. arabica* (S.288 line).

MATERIAL AND METHODS

Plant material

Root tip meristems were obtained from clonal propagated plants of triploid (*3x* = 33, E^aC^aC) and tetraploid (*4x* = 44, E^aC^aCC) interspecific hybrids between *C. arabica x C. canephora* as well as from seedling plants of the S288 line a *C. arabica* gentype introgressed from *C. liberica*,. Both cloned and seedling plants were growth in pots under greenhouse appropriate conditions. Triploid F1 plants were generated by crossing the tetraploid *C. arabica* (accession ET 30) as female parent with the diploid *C. canephora* (IF 181) accession. Tetraploid hybrids resulted from controlled crossing between *C. arabica* (used as female parent) with a colchicine-duplicated diploid *C. canephora* plant (IF 181 accession). The S 288 line is derived from a selfed offspring of S.26, a natural hybrid between *C. arabica* and *C. liberica* (Vishveshwara, 1974). This line has been reported to carry two different resistance factors from *C. liberica* (i.e. *SH3* and *SH5*) against the coffee orange rust, *Hemileia vastatrix*.

Chromosome preparation

Chromosome preparation was carried out as described previously by Lashermes et al. (1999). For mitotic metaphase chromosome spreads preparation approximately 0,5 cm-long actively growing root tips were collected. In order to arrest mitotic division at the metaphase stage, root tips were treated in the dark with a saturated α-bromonaphthalene solution. Root tip meristems were treated in an enzyme mixture at 37 °C. Chromosomes from digested cells were fixed on slides and then air dried. Selected slides containing optimal chromosome spreads were stored at 4 °C until utilization.

Probe isolation and labelling

Probe labeling, GISH and BAC-FISH analyses were conducted according to methods described by Jiang et al., (1995) and Benabdelmouna et al., (2001) with some modifications. Genomic probes for GISH were generated from total genomic DNA isolated from young leaves of *C. arabica*, *C. canephora* and *C. liberica* species. For BAC-FISH experiments, different BAC clones were previously identified in high-density colony filters from a BAC library of *C. arabica* by using a RFLP chromosome-specific probe (gA67A) corresponding to the linkage group 1 (i.e. chromosome 1) of the *C. canephora* genome (Noir et al., 2004). Only one BAC clone (i.e. 81-13H), belonging to the E^a sub-genome of *C. arabica* and exhibiting any or very low unspecific hybridization signals, was selected for further BAC-FISH identification of chromosome 1.

FISH analysis

Double hybridization experiments of GISH were carried out using simultaneously the genomic DNA from either *C. arabica* and *C. canephora* or *C. arabica* and *C. liberica*, as probe. In order to determine chromosome localization of *C. liberica* introgressed fragments present in the S.288 line, both genomic DNA (from *C. liberica*) and a BAC chromosome-specific clone (i.e. 81-13H) were used as probes in a combined GISH/BAC-FISH analysis. Whenever two probes were used together, each one was labeled with different color (red or green).

RESULTS AND DISCUSSION

GISH detection of alien chromosomes in *C. arabica* x *C. canephora* interspecific hybrids

GISH analysis of triploid and tetraploid hybrid genomes resulted in a weak differentiation between parental chromosomes. Differentiation between *C. arabica* ($E^aE^aC^aC^a$) and *C. canephora* (CC) chromosomes resulted very difficult because an elevated cross hybridization between genomic probes. Although it was not possible to differentiate between the C and C^a chromosomes in the hybrid genomes, we observed a weak discrimination between the E^a chromosomes (issued from the parental Eugenioides sub-genome of *C. arabica*) and the "Canephora derived-chromosomes", including C and C^a (Figure 1a).

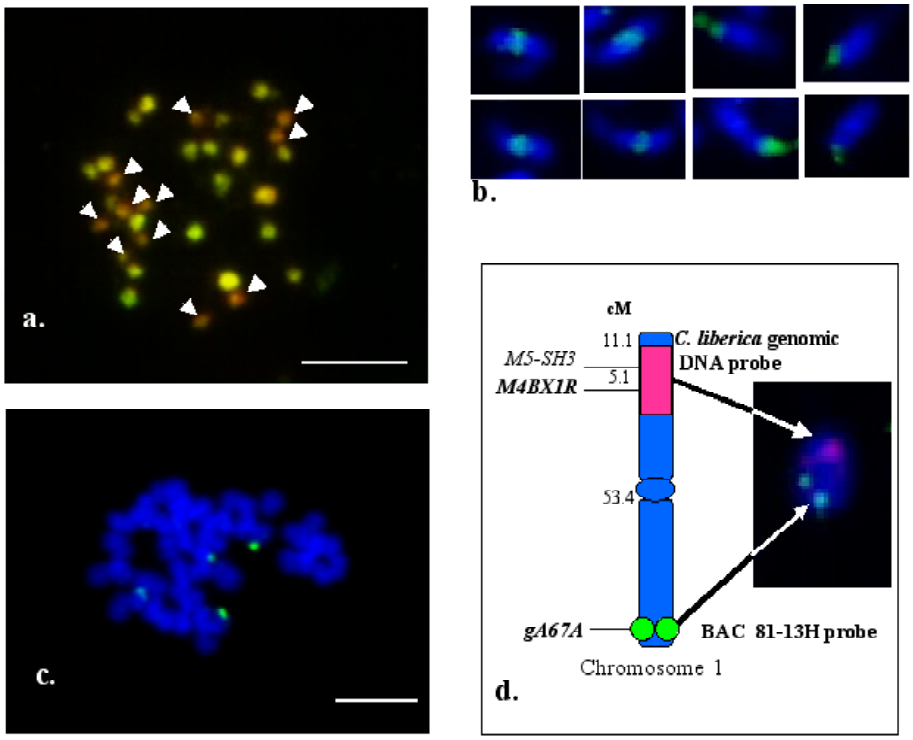

Figure 1. (a), GISH in chromosome preparations from a triploid interspecific hybrid between *C. arabica* and *C. canephora* after double hybridization using total genomic DNA from each parent. Red chromosomes correspond to the E^a chromosomes from *C. arabica* (indicated by arrows). (b), Individual mitotic metaphase chromosomes of the introgressed S.288 line of *C. arabica*, carrying different intregressed fragments from *C. liberica* (green signals). (c), FISH localization of the fourth homologous set of chromosomes corresponding to the linkage group 1 on metaphase preparations of the introgressed line S.288. Chromosome identification was carried out using the chromosome-specific BAC clone 81-13H as probe (labeled in green). (d), Link between physical and cytogenetic information regarding introgressed fragment from *C. liberica* in the chromosome 1. The scheme shows the expected chromosome positions of the introgressed fragment involving the SH3 factor for rust resistance, and the chromosome-specific BAC clone 81-13H. In b, c and d, chromosomes were counterstained with DAPI, in bleu. Scale bar = 5 µm.

Difficult genome discrimination in interspecific hybrids through GISH analysis, confirms the close genetic affinity between the two parental species *C. arabica* and *C. canephora*, as well as between *C. canephora* (C genome) and the constitutive *C. eugenioides* sub-genome (E^a) present into *C. arabic*a as demonstrated previously by molecular analyses (Herrera et al.,

2002). According to GISH analysis, the observed genomic resemblance between the modern *C. canephora* genome (C) and the ancestral *C. canephora* parental species of *C. arabica* (C^a) appears rather considerable, at least in that regarding frequency and distribution of dispersed repetitive sequences. This low divergence between the C^a sub-genome of *C. arabica* and the C genome of the actual *C. canephora* species also suggest limited genome reorganization during the evolution of the tetraploid archetype and the present amphidiploid *C. arabica*.

Detection and chromosomal location of *C. liberica* chromatin introgressed into the *C. arabica* genome

GISH analysis on mitotic chromosomes of the introgressed S288 line allowed clear localization of *C. liberica* introgressed fragments. *In situ* hybridization with total genomic DNA from *C. liberica* as probe, consistently showed the presence of eight fluorescent signals corresponding to fourth introgressed fragments (Figure 1b). Apparently the introgressed DNA fragments were distributed on fourth different homologous chromosomes (i.e. two signals for each homologous set of chromosomes).

On the other hand, and in order to identify the homeologous set of chromosome 1 on the S.288 genome, we used a chromosome-specific BAC clone as probe. Therefore, after BAC-FISH hybridization we observed fourth signals (Figure 1c), corresponding to the expected fourth homoeologous set of chromosomes belonging to the linkage group 1 in the *C. canephora* map described by Lashermes et al. (2001). As inferred by previous genetic information, not also the introgressed fragments but the chromosome-specific BAC 81-13H, were observed at the expected physical position on the chromosome 1 (Figure 1d).

Finally, in order to identify the individual chromosomes from linkage group 1 and carrying the introgressed fragments from *C. liberica*, a combined GISH/BAC-FISH analysis was carried out. As result, two individual chromosomes were detected carrying both, the BAC signals (in green) and the genomic signals (in red) corresponding to the introgressed fragments from *C. liberica* (Figure 1d).

The S288 line is a *C. liberica* introgressed genotype derived from an spontaneous interspecific hybrid (Prakash et al., 2002). Using a molecular marker approach Prakash et al., (2004) identified three genetically independent *C. liberica* introgressed fragments in the S288 genome. Authors estimated that alien introgression represented a half chromosome equivalent of the *C. liberica* species distributed over at least three chromosomes in the S288 line. Our results, using GISH consistently showed the presence in this line of eight fluorescent signals corresponding to four introgressed fragments distributed on four different homologous chromosomes (i.e. two signals for each homologous set of chromosomes). It is conceivable that using *in situ* hybridization we detected additional small fragments from alien DNA, which were not detected through molecular marker analysis. Current development of additional linked markers will provide insights to elucidate this discrepancy.

CONCLUSIONS

Overall results illustrate the successful utilization of molecular cytogenetics methods for coffee genome studies, providing additional insights into genome organization and evolution. This report offers an starting point for different future applications of FISH techniques in coffee, and in particularly in *C. arabica* breeding. For instance, our results demonstrated that GISH analysis appears as a useful tool for detection of alien chromosomes or chromosome fragments in introgressed genotypes derived from diploid species displaying substantial genomic differentiation with *C. arabica*. On the other hand, the positional mapping of large

DNA fragments (like BAC clones) carrying interesting genes, could be expected by FISH hybridization on either mitotic metaphase or meiotic prophase (i.e. pachytene) chromosomes. Thanks to the adaptation of FISH to coffee, some forthcoming applications of this technique can be envisaged like: (i) the differentiation of parental chromosomes in hybrids from wide crosses, (ii) localization of alien fragments in introgressed material (e.g. BCn), (iii) identification of specific chromosomes (i.e. karyotyping), and (iv) analysis of distribution of repeated sequences along the genome.

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Phylogenetic Origins and Expression Analysis of a Duplicated *WRKY* Gene in the Polyploid Species *Coffea arabica*

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SUMMARY

C. arabica is an allotetraploid species resulting from a recent hybridization between two wild diploids *Coffea* species. The merger of two genomes in a common nucleus may be accompanied by considerable genomic reorganization. In particular, gene redundancy may lead to gene silencing or to the functional divergence of duplicated genes. We recently identified a *C. arabica* gene (*CaWRKY1*) encoding a transcription factor of the WRKY superfamily associated with plant defense responses to pathogens. We describe here the isolation in the *C. arabica* genome of two *CaWRKY1* gene copies (*CaWRKY1a* and *CaWRKY1b*) that probably originate from each ancestral subgenome of *C. arabica*. *CaWRKY1a* and *CaWRKY1b* shared only 94.7% nucleotide identity and 98.5% aminoacids identity over the whole gene sequence but a strict conservation of the DNA-binding region (WRKY domain) was retained. To assess the phylogenetic relationships between *CaWRKY1a* and *CaWRKY1b*, a comparative sequence analysis of the *WRKY1* gene was conducted in 6 related diploid *Coffea* species, including the putative *C. arabica* ancestral progenitors, *C. canephora* and *C. eugenioides*. PCR cloning, sequencing and phylogenetic analysis of the *Coffea WRKY1* genes showed that *CaWRKY1a* grouped together with the *C. canephora* and *C. congensis WRKY1* genes whereas *CaWRKY1b* was genetically closer to the *C. eugenioides WRKY1* gene. To verify whether *CaWRKY1a* and *CaWRKY1b* were both functional in the *C. arabica* genome, gene expression studies were conducted using real-time quantitative reverse-transcription Polymerase Chain Reaction (qRT-PCR). Monitoring of the *CaWRKY1a* and *CaWRKY1b* mRNA levels during plant infection with the rust fungus *Hemileia vastatrix* and wounding or salicylic acid treatments showed that both genes were simultaneously and equally expressed. Overall, results evidenced that *CaWRKY1a* and *CaWRKY1b* are homoeologous sequences in the polyploid *C. arabica* genome, and are probably descents of the *C. canephora* and *C. eugenioides WRKY1* genes. Both genes contributed to the transcriptomic expression of plant defense responses to pathogens, suggesting that no functional divergence had occurred between the duplicated genes in the *C. arabica* genome.

INTRODUCTION

The WRKY transcription factors belong to a major group of DNA – binding proteins in plants, and function as transcriptional activators as well as repressors in a number of developmental and physiological processes (Eulgem et al., 2000; Robatzek and Somssich, 2002; Zhang et al., 2005). In particular, WRKY transcription factors can regulate plant responses to biotic and abiotic stresses (reviewed *in* Ülker and Somssich, 2004). Expressed Sequence Tags (ESTs) with homology to *WRKY* transcription factors genes were recently isolated in the coffee plant *Coffea arabica* (Fernandez et al., 2004). One of them, *CaWRKY1*, showed altered expression patterns upon infection with the rust fungus *Hemileia vastatrix* (Berkeley & Broome) as well as in response to wounding and salicylic acid (SA) treatments (Fernandez et al., 2004; Ganesh et al., 2006). In allopolyploid species such as *C. arabica*,

studying gene expression is complicated by gene duplication and therefore by the problem of distinguishing transcript pools derived from each homoeologous genome. Altered gene expression, including silencing and up- or downregulation of one of the duplicated genes, may be common in allopolyploids and may lead to subfonctionalization within the ancestral gene pair (Adams et al., 2003; Adams and Wendel 2005; Udall et al., 2006). To explore the regulation of the duplicated *CaWRKY1* genes in coffee, we cloned the two *C. arabica* homoeologous copies and assessed their expression patterns by real-time quantitative reverse-transcription Polymerase Chain Reaction (qRT-PCR) during coffee defense responses.

MATERIALS AND METHODS

Rust, wounding and salicylic acid (SA) treatment

Coffea arabica var. Caturra were challenged with *H. vastatrix* isolates eliciting an incompatible interaction (race VI) or a compatible interaction (race II) as described in Fernandez et al. (2004). Biological samples originated from at least three independant experiments conducted in the greenhouse at different periods of the year. For abiotic treatments, Caturra leaves were treated as described in Ganesh et al. (2006). Wounding was performed by applying an average of 7 transversal cuttings per half-leaf using cissors. SA treatment was performed by infiltrating leaves with a 0.5 mM solution of SA using a needless syringe.

Full length cDNA cloning by RACE experiments

Specific oligonucleotides (DSS16-5R and DSS16-3R) were designed from the DSS16 sequence (Genbank accession CF589188) for RACE experiments (Figure 1). 3'RACE and 5'RACE experiments were conducted by combining Omniscript RT kit (Qiagen) and SMART PCR cDNA synthesis kit (Clontech). The resulting PCR product was cloned into pGEM-T easy vector (Promega, France) and sequenced (Genome Express, France). Full length cDNA (FL cDNA) clones were obtained by PCR amplification using two oligonucleotides (CATW5 and CATW3) designed from 5'- and 3'RACE sequences. The resulting 2-kb PCR product was cloned and sequenced.

Cloning of genomic CaWRKY1 sequences

CaWRKY1 sequences were obtained by PCR experiments on *C. arabica* var. Caturra DNA using CATW5 and CATW3 primers. The PCR products (around 2.5 kb) were cloned and sequenced.

Quantitative gene expression analysis

RNA extraction and qRT-PCR were performed as described in Ganesh et al. (2006). For absolute quantification of cDNA molecules, the threshold cycle (Ct) values of the triplicate PCRs were averaged and the copy number of each cDNA was estimated from calibration curves data obtained on calibrated amounts (10^2, 10^3, 10^4 and 10^5 copies) of purified plasmids bearing the cloned gene tested. The *CaWRKY1a* and *CaWRKY1b* gene copy numbers were normalized to the *CaUbiquitin* gene chosen as internal reference of gene expression. Linear regression analysis was used to calculate the correlation coefficient between the *CaWRKY1a* and *1b* gene copy number obtained in each experiment, and over all experiments.

Cloning of WRKY1 sequences in related *Coffea* spp.

Fresh leaves of *C. arabica* var. Caturra, *C. canephora*, *C. eugenioides*, *C. congensis*, *C. liberica*, *C. racemosa* and *C. humilis* were used for genomic DNA extraction (DNEasy Plant minikit, Qiagen, France). Partial genomic *WRKY1* sequences were obtained by PCR experiments on DNA of each *Coffea* spp. using CATW5 and DSS16-n5R primers. The resulting 2-kb PCR products were cloned and sequenced.

Bioinformatic analysis of CaWRKY1 sequences :

Homology to sequences present in international databases were searched using Basic local alignment search tools (BLASTN and BLASTX) (Altschul et al., 1997). Sequences were aligned using ClustalW version 1.8 (Thompson et al., 1994). Search for specific protein domains were performed on Pfam database website (http://www.sanger.ac.uk/Software/ Pfam).

Phylogenetic analysis of CaWRKY1 sequences

Parsimony analyses with unweighted, unordered characters were conducted with PAUP 4.0b10 (Swofford, 1998) in Macintosh environment. Bootstrap analysis was performed using 1000 replicates and unrooted consensus trees were constructed.

RESULTS

Cloning of two *C. arabica CaWRKY1* genes

CaWRKY1 was originally isolated as a differentially expressed sequence fragment (DSS16) (Fernandez et al., 2004). Specific oligonucleotides designed from the DSS16 sequence were used for 3' and 5' RACE cloning experiments and two distinct FL - cDNA sequences named *CaWRKY1a* and *CaWRKY1b* were identified. Clone *CaWRKY1a* (1974 bp) and clone *CaWRKY1b* (1960 bp) contained an open reading frame (ORF) encoding a predicted polypeptide of 573 aminoacids (aa) and 572 aa, respectively (Figure 1). The two sequences shared 97.5% nucleotidic (nt) identities and 98.3 % aa identities. *CaWRKY1a* and *CaWRKY1b* nt sequences varied by insertions / deletions (Indels) and single nucleotide polymorphism (SNP) in their 5' - untranslated region (5'UTR), as well as in the ORFs leading to a total of 10 aa changes in the predicted polypeptide sequences (Figure 1). Two distinct *CaWRKY1* genomic sequences sharing 96.4% of nucleotide similarity were obtained from *C. arabica* DNA. The two genes exhibited a similar structure and consisted of six exons and five introns (Figure 2).

***CaWRKY1a* and *CaWRKY1b* putatively encode transcription factors of the WRKY plant proteins superfamily**

Homologies searches run with the nucleotide and amino acid sequences of the FL – cDNA clones revealed a highly significant similarity to proteins belonging to the *A. thaliana* WRKY transcription factors subgroup IIb (Eulgem et al., 2000). Both CaWRKY1a and CaWRKY1b deduced proteins matched the same *A. thaliana* WRKY factor (At1g62300). The CaWRKY1a and CaWRKY1b aa sequences were characterised by the presence of a single WRKY domain containing the core motif WRKYGQK together with a C2H2 – type zinc finger motif in the C – terminal region (Figure 1). In addition, a potential leucine – zipper –motif (LZ) and a nuclear localization signal (NLS) were identified (Eulgem et al., 2000).

```
CaWRKY1a   MDKGWGVTVDNPDKIGFFGNKPVFGFNLSPRLNPSKGSLSMFPATEFLANQNRREDSHAA  60
CaWRKY1b   MDKGWGVTVDNSDKIGFFGNKPVFGFNLSPRLNPSKGSLSMFPAAEFLANQNRREDSHAA  60

                     NLS                                                
CaWRKY1a   ASSDGEKRVVVGEVDFFSDKKKANDILIKKEDCHGEDKMKTNMDVVNTGLQLVIANTGSD 120
CaWRKY1b   ASSDGEKRVVVGEVDFFSDKKKANDILIKKEDCHGEDKMKTNMDVVNTGLQLVIANTGSD 120

                                    LZ domain                          
CaWRKY1a   QSTVDDGVSSDIEDKRAKLELAQLQVELERMNAENRRLREMLSQVSNNYTALQMHLMTLM 180
CaWRKY1b   QSTVDDGVSSDIEDKRAKLELAQLQVELEGMNAENRRLREMLSQVSNNYTALQMHLMTLT 180

CaWRKY1a   HQQQQNAKPQTTQDHEIGERKSEENKPENGGVVVPRQFLDLGPSGTAEMDEPTNSSSEER 240
CaWRKY1b   HQQQQNAKPQTTQDHEIGERKSEENKPENGGVVVPRQFLDLGPSGTAEMDEPTNSSSEER 240

CaWRKY1a   TLSGSPRNNMELSRNKGVGREESPESQGWAPNKVAKLNAPSKTVDHAQAEATMRKARVSV 300
CaWRKY1b   TLSGSPHNNMELSRNKGVGREESPESQGWAPNKVAKLNASSKTVDHAQAEATMRKARVSV 300

                                    WRKY domain                        
CaWRKY1a   RARSEAPMITDGCQWRKYGQKMAKGNPCPRAYYRCTMAVGCPVRKQVQRCAEDRTVLITT 360
CaWRKY1b   RARSEAPMITDGCQWRKYGQKMAKGNPCPRAYYRCTMAVGCPVRKQVQRCAEDRTVLITT 360
                                        *       *                      

CaWRKY1a   YEGTHNHPLPPAAMAMASTTSAAANMLLSGSMSSADGLMNPNFLARTILPCSSNMATISA 420
CaWRKY1b   YEGTHNHPLPPAAMAMASTTSAAANMLLSGSMSSADGLMNPNFLARTILPCSSNMATISA 420
             * *                                                          

CaWRKY1a   SAPFPTVTLDLTQTPNPLQFQRQPSTPFQLPFGTPPQNFPPVANPQMHQVFGQALYNQSK 480
CaWRKY1b   SAPFPTVTLDLTQTPNPLQFQRQPSTPFQLPFGTPPQNFPPVANPQMHQVFGQALYNQSK 480

CaWRKY1a   FSGLQVSQDIEAAAAAAAQMQNQGQHPQVQQGQHQPSFADTLSAATAAITADPNFTAALA 540
CaWRKY1b   FSGLQVSQDIEAAG-AAAQMQNQGQHPQVQQGQHHPSFADTLSAATAAITADPNFTAALA 539

CaWRKY1a   AAISSIIGGSHPSSSNNSNPTTSTPPTANFPGN                             573
CaWRKY1b   AAISSIIGGSHPSSSNNSNPTTSTPPTANFPGN                             572
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Figure 1. Alignment of *CaWRKY1a* and *CaWRKY1b* deduced amino-acid sequences. The open boxes indicate the domains corresponding to WRKY factors from group IIb (NLS: nuclear localization signal. LZ: leucine zipper, WRKY domain). Arrowheads represent intron positions. The stars represent the zinc finger motif. Non-conserved aa are shown as grey boxes.

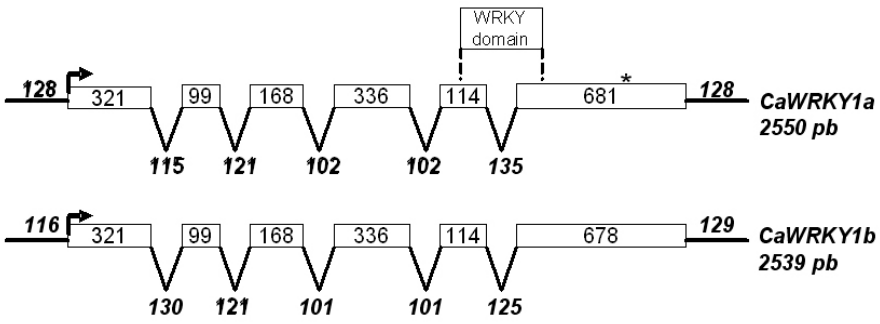

Figure 2. *CaWRKY1a* and *CaWRKY1b* genomic structures. Exons are indicated by boxes and introns are shown as broken lines. Arrows indicate the translational initiation sites and the star the aa insertion. The sequences corresponding to the WRKY domain are interrupted by an intron.

CaWRKY1a and *CaWRKY1b* expression in plant defense

To investigate the respective involvement of *CaWRKY1a* and *1b* genes into coffee defense responses, the mRNA levels of each gene were monitored by qRT-PCR analysis during plant infection with the coffee rust pathogen. Time-course experiments were conducted using the Caturra variety challenged with *H. vastatrix* isolates either eliciting an incompatible interaction (resistance) or a compatible interaction (susceptibility). 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. Absolute quantification of the *CaWRKY1a* and *CaWRKY1b* mRNA levels in coffee leaves allowed to assess the copy number of each gene in each sample. The relative changes in gene expression showed that both genes were induced by rust infection (Figure 3). Between 12 and 16 hours post-inoculation, depending on time-course experiments, a marked induction of the *CaWRKY1* genes by fungal infection was evidenced. Statistically significant differences ($P < 0,05$) in the relative expression of the *CaWRKY1* genes were found between the compatible and incompatible interactions.

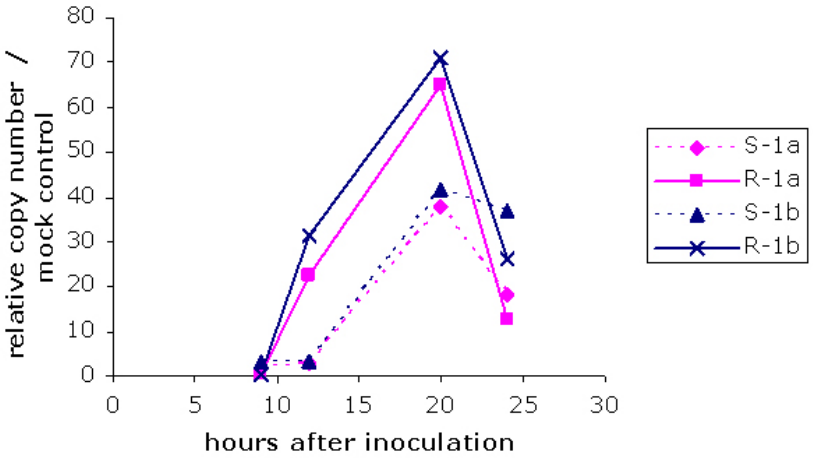

Figure 3. Example of *CaWRKY1a* and *CaWRKY1b* expression patterns obtained in resistant (R) and susceptible (S) coffee leaves after rust inoculation. The *CaWRKY1a* and *CaWRKY1b* copy number in each sample was related to that obtained in control plants at each time point.

CaWRKY1a and *CaWRKY1b* were also activated by wounding or SA – treatment (Figure 4). A statistically significant correlation (Pearson $R = 0.92$, $p < 0.001$) between the two genes expression was obtained across 44 data points measured (including rust, SA and wounding responses). These results clearly evidenced that both genes were simultaneously and equally expressed in response to biotic as well as to abiotic treatments.

Phylogenetic analysis of the coffee WRKY1 gene in the *Coffea* genus

To assess whether *CaWRKY1a* and *CaWRKY1b* were homoeologous-, allelic- or paralogous-sequences, we conducted a genetic diversity analysis of the coffee *WRKY1* gene in the *Coffea* genus. Homologous sequences of the *CaWRKY1* gene were obtained from *C. canephora*, *C. eugenioides*, *C. congensis*, *C. liberica*, *C. racemosa* and *C. humilis* genomic DNAs. Alignement of the partial sequences (1540-bp in total) using ClustalW showed that *WRKY1* sequences essentially differed by numerous SNPs as well as distinct INDEL events in the 5'UTR and the first two intronic sequences. *WRKY1* nucleotidic sequences similarity ranged from 94.4 to 99.3% between *Coffea* species (data not shown). Highest similarity was observed

between *CaWRKY1a* and *canephora WRKY1* sequences (99.3%) and between *CaWRKY1b* and *eugenioides WRKY1* sequences (98.7%). Alignement of the deduced aminoacids sequences (324 aa in total) showed a strict conservation of CaWRKY1a with the *congensis* WRKY1 sequence and of CaWRKY1b with the *eugenioides* WRKY1 sequence.

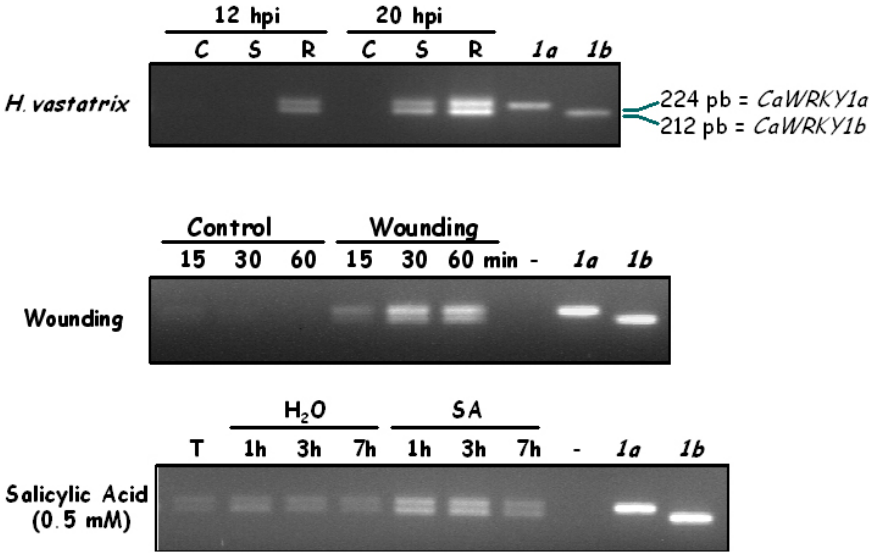

Figure 4. *CaWRKY1a* and *CaWRKY1b* expression patterns obtained after rust inoculation [control (C), resistant (R) and susceptible (S) coffee leaves], wounding or salicylic acid treatment.

Because INDELs may severely bias phylogenetic inferences derived from molecular sequences, the DNA sequences of each species – representative clone were cleared of INDELs for phylogenetic analyses.

Parsimony analysis conducted on the resulting DNA sequences (1481 characters) produced a unique most parsimonious tree that clearly separated the two *C. arabica WRKY* sequences into distinct phylogenetic clades (Figure 5). *CaWRKY1a* grouped with the *canephora* and *congensis* sequences while *CaWRKY1b* was closely related to the *eugenioides* sequence.

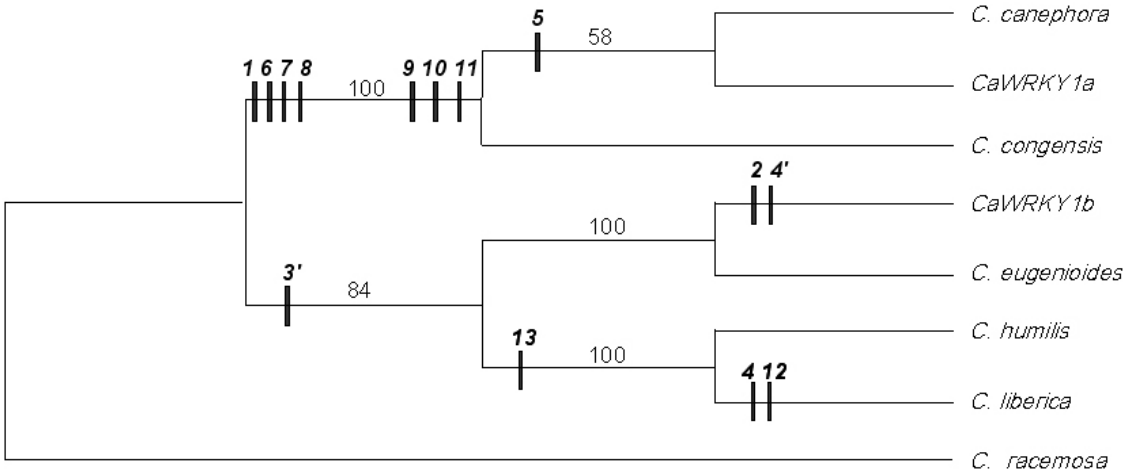

Figure 5. Phylogenetic tree (maximum parsimony) of *WRKY1* genes in *Coffea* species. Numbers above branches are bootstrap values (100 replicates). Vertical bars (INDELs mutations) were manually assigned to each branch but were not taken into account for the phylogenetic analysis.

DISCUSSION

Molecular data obtained so far from the coffee genome suggested that *C. arabica* formed recently and that low divergence occurred between the two constitutive genomes of *C. arabica* and those of its progenitor species (Raina et al., 1998; Lashermes et al., 1999). A higher level of polymorphism is thus expected between the *C. arabica* homoeologous sequences than among them and the ancestor sequences. In this study, a genetic study was conducted on the *WRKY1* gene cloned in six diploid *Coffea* species and in *C. arabica*. *Coffea* species were chosen based on several DNA analyses (Raina et al., 1998; Lashermes et al., 1999) that suggested that *C. arabica* is an amphidiploid formed by hybridisation between two wild diploid species closely related to *C. congensis, C. canephora* and *C. eugenioides*. A higher similarity was observed between *CaWRKY1a* and *canephora WRKY1* sequences (99.3%) and between *CaWRKY1b* and *eugenioides WRKY1* sequences (98.7%) than between *CaWRKY1a* and *CaWRKY1b* genes (97.5%). Alignement of the deduced aminoacids sequences showed a strict conservation of CaWRKY1a with the *congensis* WRKY1 sequence and of CaWRKY1b with the *eugenioides* WRKY1 sequence. In contrast, the putative CaWRKY1a and CaWRKY1b proteins shared only 98.3% aa identities. The close phylogenetic relationships observed between the two *C. arabica WRKY* sequences and other diploid *Coffea* species (Figure 5) strongly suggests that *CaWRKY1a* and *CaWRKY1b* are homoeologous rather than paralogous sequences in the tetraploid *C. arabica* genome.

Expression analysis showed that both *CaWRKY1* homoeologous genes contributed to the transcriptomic expression of coffee defense responses to pathogens, and were activated by abiotic treatments. These results evidenced that the duplicated genes were functional in the *C. arabica* genome. Similar amount of *CaWRKY1a* and *CaWRKY1b* transcript levels were found in *C. arabica* leaves under all treatments, suggesting that the homoeologous genes undergo the same transcriptional control. It is likely that the two inherited *WRKY1* gene copies were functionally conserved after the polyploidization event that gave rise to the *C. arabica* species.

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Soluble Sugars, Enzymatic Activities and Gene Expression during Development of Coffee Fruits Submitted to Shade Condition

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SUMMARY

Effects of shade (50% light) condition on development and sugar metabolism was analyzed in fruits of *Coffea arabica* L. var. IAPAR 59. Fresh weight measurements of separated tissues (pericarp, perisperm and endosperm) showed that the increased size observed in beans of shaded plants was correlated with high perisperm development. Reducing sugar contents in the pericarp and endosperm of fruits from shaded plants appeared higher than in the same tissues of control ("full-sun") plants. On the contrary, sucrose content was slightly lower in shade than in control beans. At the enzymatic level, sucrose synthase activities detected in the latest stages (197-231 DAF) of perisperm development from shaded plants were higher than from of control, confirming the importance of this tissue in coffee fruit development. At the molecular level, the *CaSUS1* and *CaSUS2* gene expression was also analyzed in the endosperm from control and shaded fruits. Expression level of *CaSUS2* was higher in mature beans (260 DAF) of shaded plants than in control plants (231 DAF). These results showed that light condition affect sucrose metabolism of coffee beans.

INTRODUCTION

In coffee, many factors are known to influence "coffee cup quality", like the species or varieties cultivated, the plant age, harvest period and the post-harvest processes applied to obtain dried beans (Leroy et al., 2006). Shade either provided naturally or artificially was also reported to give high coffee quality probably by lowering tree stress, favoring slow ripening of cherries and adequate bean filling (Vaast et al., 2006). Near infrared reflectance spectrophotometry (NIRS) analyses of coffee beans showed positive correlations between fat matter content and altitude and shaded conditions (Decazy et al., 2003). In addition, inverse correlation was often observed between sucrose and caffeine, trigonelline and chlorogenic acids levels (Vaast et al., 2006). In a previous study, we analyzed sucrose metabolism during fruit development of *Coffea arabica* L. var. IAPAR 59 at the biochemical and molecular levels (Geromel et al., in press). It was shown that sucrose synthase (SUS: EC 2.4.1.13) had the highest activities during the last stage of endosperm and pericarp development that paralleled the accumulation of sucrose in these tissues. Pulse-chase experiments with ^{14}C-fructose and ^{14}C-sucrose also indicated the existence of intensive exchange of sugars between fruit compartments occurring mainly through simultaneous biosynthetic and catabolic processes of sucrose (Geromel et al., in press). Because sucrose is important precursor of

coffee flavor and aroma (Homma, 2001; Grosch, 2001), the objectives of the present work were to study the effects of shade on sugar metabolism.

MATERIALS AND METHODS

Plant material

Fruits were harvested from plants of *Coffea arabica* L. var. IAPAR 59 cultivated in field conditions (Agronomic Institute of Paraná State, Londrina-Paraná, Brazil). Plants were cultivated under control ("full-sun", FS: photosynthetic photon flux density of 2250 $\mu Em^{-2} s^{-1}$ at noon in summer season) or shaded (SH) conditions. In the latter case, light was artificially reduced using a net allowing 50% of the photosynthetic photon flux density.

Coffee sampling and processing

Cherries (100 gr) were randomly collected every four weeks from the flowering (09-2003) up to the complete maturation (05-2004) from 10 individual field-grown plants For sugar quantification and enzymatic analyses, cherries were immediately frozen in liquid nitrogen and stored at –80 °C.

Sugar content and enzymatic activities

Sugar contents and enzymatic activities were determined as described before (Geromel et al., in press).

Gene expression

RNA extraction, Northern-blot analysis and probe labeling were as described before (Geromel et al., in press).

RESULTS

Characteristics of fruit growth

In our field conditions, fruits from plants of *C. arabica* L. var. IAPAR 59 grown under FS and SH condition completed their maturation in around 231 DAF ± 30 and 260 DAF ± 30 respectively. Tissue fresh weights (FW) were determined separately for perisperm and endosperm at regular stages of coffee cherry development. Perisperm expansion was rapid after 60 DAF, with higher FW in SH compared to FS condition at 87 DAF (Figure 1A). The endosperm only became easily detachable from the perisperm at 120 DAF and reached FW of 0.47 ± 0.03 gr and 0.51 ± 0.07 gr at the mature stage of FS and SH conditions, respectively at the 231 DAF and 260 DAF (Figure 1B).

Reducing sugar and sucrose contents during coffee cherry development

Reducing sugar (RS: mainly glucose and fructose) contents were measured in separated tissues of fruits grown in FS and SH conditions (Figure 2A-B). The main difference between FS and SH conditions concerned the maintenance of higher hexose content ("sink") in the perisperm of SH fruits (Figure 2A and B). In the pericarp, RS were low up to 175 DAF and increased rapidly after this time to reach 404 mg g^{-1} DW and 326 mg g^{-1} DW respectively in SH and FS conditions. Sucrose was also accumulated during latest stages of endosperm

development, reaching 72.5 and 61.2 mg g^{-1} DW, respectively at 231 DAF in FS and 260 DAF in SH conditions (data not shown).

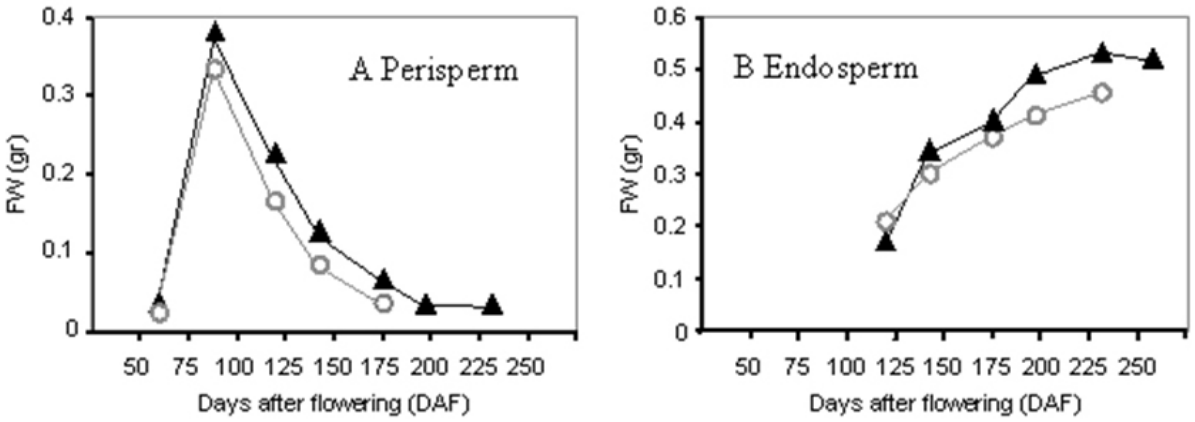

Figure 1. Evolution of tissue fresh weights during ripening of *C. arabica* L. var. IAPAR 59 fruits grown under "full sun" (open circle) and shade (closed triangle) conditions. Fresh weights are given in grams for perisperm (A) and endosperm (B) separated tissues.

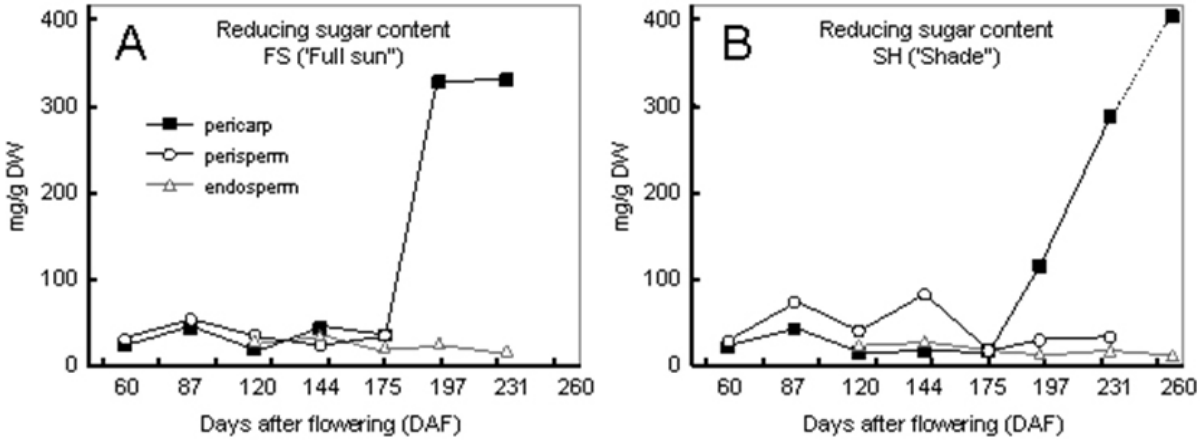

Figure 2. Evolution of reducing sugar contents in separated tissues from fruits of *C. arabica* L. var. IAPAR 59 grown under FS (A) or SH (B) conditions. Data are given mg.g^{-1} of dry weight (DW) in pericarp (black square), perisperm (open circle) and endosperm (open triangle) tissues.

Enzymatic activities during coffee fruit development

Sucrose synthase (SUS) activity was monitored in pericarp, perisperm and endosperm tissues separated from FS and SH cherries (Figure 3A-C). In the pericarp, SUS activities showed similar patterns in FS and SH conditions, with a continuous increase from 60 to 197 DAF where a peak was detected (Figure 3A). A regular increase of SUS activity was observed in the perisperm of FS cherries, particularly between 120 and 175 DAF (Figure 3B). SUS activity in SH perisperm was higher than in FS, maximal at 231 DAF and declined at the mature stage (260 DAF). In FS endosperm, SUS activities reached a peak at 175 DAF and decreased towards the harvest (Figure 3C). The profile differed in SH endosperm where maximal SUS activity was observed at the mature stage (260 DAF).

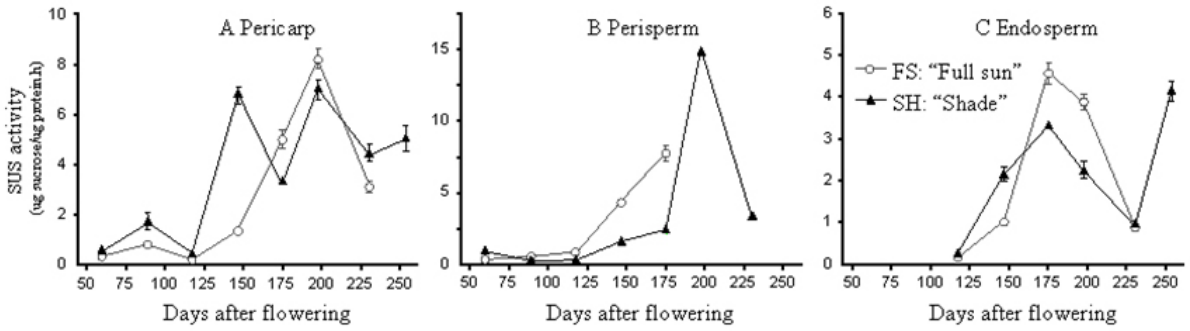

Figure 3. Profiles of SUS (sucrose synthase) activity during coffee fruit development. Activity was assayed in the sense of sucrose synthesis (µg sucrose. hr^{-1} μg^{-1} protein) in pericarp (A), perisperm (B) and endosperm (C) tissues separated from fruits grown in full-sun (FS: open circle) or shade (SH: black triangle).

Expression of SUS-encoding genes

CaSUS1 and *CaSUS2* gene expression was monitored during the maturation of endosperm that developed under FS and SH conditions. SUS expression profiles in FS endosperm for the maturation period 2003-2004 reported here where similar to those obtained in endosperm of fruits harvested from plants growing at FS condition in 2002-2003 (Geromel et al., in press). However, SH plants showed an early expression of *CaSUS1* in the endosperm, as well an increase on expression of *CaSUS2* during the late stages of development, compared with expression patterns observed in endosperm of FS plants.

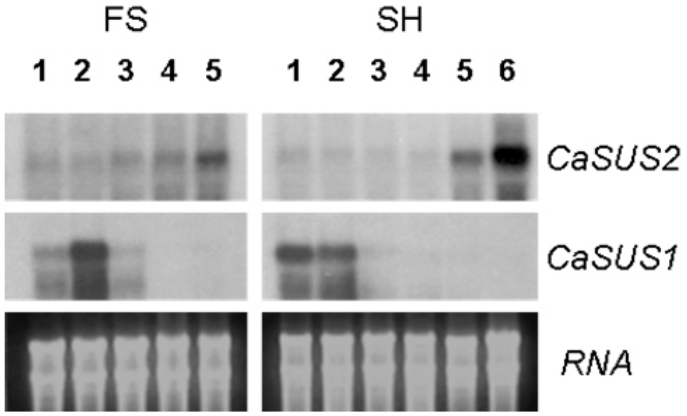

Figure 4. Expression of *CaSUS1* and *CaSUS2* genes in developing endosperm from fruits grown in full-sun (FS) or shade (SH). Total RNA (15 µg) isolated from endosperm at regular developmental stages (lane 1, 120 DAF; lane 2, 144 DAF, lane 3, 175 DAF; lane 4, 197 DAF; lane 5, 231 DAF and lane 6, 260 DAF) was separated in a formaldehyde-agarose gel and transferred onto a nylon membrane. Probes used correspond to *CaSUS1* and *CaSUS2* cDNA sequences (Geromel et al., in press). RNAs stained by ethidium bromide (bottom) were used to monitor the equal loading of samples.

CONCLUSION AND DISCUSSION

In coffee, the first tissue to develop soon after the fecundation is the perisperm and its volume defines the locule space that will be further occupied by the endosperm (bean) (De Castro and Marraccini, 2006). Here, we showed that the one month delay of the endosperm (bean)

development observed in SH condition was a consequence of the longest persistence of the perisperm tissue. In addition, high development of the perisperm tissue during early developmental stages in shade condition could also explain the increase in size of SH beans (Geromel et al., in preparation). We also reported higher reducing sugar content in pericarp of SH than in FS condition, a situation that was also observed in mature (dried) coffee beans (Geromel et al., in preparation). As reported in other works (Vaast et al., 2006; Decazy et al., 2003), lower sucrose content was observed in SH than in FS mature beans. Even limited, these data reinforce the idea that intensive sugar exchanges exist between the pericarp and endosperm (Geromel, in press) and that the slow down ripening process of shaded berries favors a complete filling of coffee beans (Vaast et al., 2006).

The decrease on light intensity affected SUS activities in coffee fruits. For example, SUS activity in the latest stages of perisperm development was significantly higher in SH than in FS condition. Even reduced to a thin membrane (silver skin) surrounding the endosperm at this time (De Castro and Marraccini, 2006), these observations confirmed the important function of this tissue in bean development process (Geromel, in press; Rogers et al., 1999). In a previous work, the CaSUS2 isoform of SUS was proposed to be the main enzyme responsible of sucrose accumulation because its expression coincided with the peak of SUS enzymatic activity and sucrose accumulation observed during the latest stages of endosperm development (Geromel, in press). On the other hand, the CaSUS1 isoform of SUS was proposed to function as a sucrose-degrading enzyme since its expression (by Northern-blot) and detection (by Western-blot) was never accompanied by sucrose accumulation (Geromel, in press). The situation appeared quite different here where SUS activities appeared lower than those measured in 2002-2003 and did not showed a continuous increase during latest stages of endosperm development. Since no acid invertase activity exists in endosperm (Geromel, in press; Geromel et al., in preparation), higher sucrose-degrading activity in SH than in FS mature endosperm could explain high RS and low sucrose contents in the former. At the molecular level, *CaSUS1* expression detected in young stages (120-175 DAF) of SH and FS endosperm correlates quite well with SUS activity in this tissue. High *CaSUS2* expression in SH endosperm at 260 DAF could also explain the SUS peak observed at the same time in this tissue. However, *CaSUS2* expression in FS endosperm at 231 DAF was not followed by SUS activity. This could be a consequence of the biannual bearing pattern commonly reported in sun-grown coffee plants (Vaast et al., 2006) since FS-grown plants analyzed here effectively presented high and low productivity in 2002-2003 and 2003-2004 respectively (data not shown). Another explanation could be that plants suffered of water limitation for several weeks in 2003-2004, mainly during endosperm expansion and storage phase (Geromel et al., in preparation). Finally, the existence of post-translation modification of SUS protein (Winter and Huber, 2000) could also explain the asynchrony between the transcription of SUS-encoding genes and SUS activities measured in the endosperm.

Altogether, these results showed that environmental (i.e. lighting) conditions modified sugar metabolism in developing coffee beans both at gene expression and enzymatic levels. Rapid degradation and (re)synthesis of sucrose between pericarp, perisperm and endosperm tissues as well as transfers of C-compounds between these tissues also explain the lack of clear relationships between sugar contents and enzymatic activities (Geromel et al., in press). Howeve

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Nucleotide Diversity of Genes Involved in Sucrose Metabolism. Towards the Identification of Candidates Genes Controlling Sucrose Variability in *Coffea* sp.

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SUMMARY

Quality and drought stress tolerance are two important targets for *Coffea* species cultivation. Currently, efficient genetic improvement of these traits is still hampered by the lack of early and cheap predictors. In this context, identification of molecular tools linked to these traits would significantly improve breeding efficiency. Based on the available literature, different metabolisms involved in the variability of both drought tolerance and coffee quality can be proposed. Based on this information, a study was initiated in *Coffea* species, aiming at estimating nucleotide diversity of four sucrose metabolism enzymes (Sucrose Synthase, Cell Wall Invertase, acid Vacuolar Invertase and Sucrose Phosphate Synthase). The two mains objectives of this work were i) to assess the level of variability of these genes within the whole area of distribution of *Coffea canephora,* and within 15 related *Coffea* species representing the four groups of diversity of this genus, and ii) to identify polymorphisms useful for mapping and association genetic studies. Almost 200 polymorphisms (SNP, INDELS, SSR) were identified through sequencing of *Coffea canephora* genotypes. In addition, analysis of the variability of these genes between different *Coffea* species allowed the identification of 300 additional polymorphic sites. Parallel in-silico analysis of EST resources confirmed the interest of this approach towards the identification of polymorphisms in *Coffea* sp. Identification of nucleotide polymorphisms will not only provide useful markers for traditional genetic studies (genetic mapping, population genetics, association studie) but also provide criteria to infer the evolutionary history of the analysed genes. Such information will be particularly relevant to select the best candidate genes to test in future association studies.

INTRODUCTION

Coffee is one of the world's heavily traded commodities. However, little is known about the genomic control of cup quality and abiotic stresses tolerance in *Coffea sp* which are two key components of the sustainability of the coffee market. Currently, rapid genetic improvement of these traits is still hampered by the lack of early and cheap predictors, phenotypic ones being cost and time consuming to use. In this context, identification of molecular tools linked to these traits would significantly improve breeding efficiency. Among the different metabolisms involved in fruit quality development and drought tolerance, sucrose metabolism

is particularly relevant. Several studies underlined the importance of enzymes/genes of this biosynthesis pathway in drought stress response (Andersen et al., 2002; Hazen et al., 2005; Pelah et al., 1997). At the coffee quality level, sucrose has been pointed out as an important precursor because its degradation during roasting leads to allyphatic acids, hydroxymethyl furfural and furans that contribute to flavours and aromas (Grosch, 2001; Homma, 2001). In addition, part of the preference for *C. arabica* compared to *C. canephora* coffee, has been frequently attributed to sucrose content differences between these species (Guyot et al., 1996; Casal et al., 2000; Ky et al., 2001).

Identification of the genomic regions and genes controlling the variability of traits of agronomic importance is a long and difficult task. Identification of QTLs in mapping pedigrees, despite its importance, only provides partial information on the genetic control of these traits, the underlying genes and the responsible polymorphisms remaining unknown. Such lack of information often hampers the application of marker assisted breeding in conventional breeding schemes.

In this context the aim of this study was to evaluate the nucleotide diversity of genes encoding 4 enzymes of the sucrose biosynthesis pathway (Cell Wall Invertase (CWI), acid Vacuolar Invertase (VI), SUcrose Synthase (SUS) and Sucrose Phosphate Synthase (SPS)). This analysis was performed using two strategies: i) direct sequencing of *C canephora* and *C.* spp genotypes and ii) in-silico analysis of the EST resources available. In addition, analysis of the landscape of diversity of these genes from an evolutionary point of view provides information useful to identify the best candidate gene/ sites to use in future mapping and association experiments.

MATERIAL AND METHODS

Polymorphism discovery by direct sequencing

The primers used to amplify the targeted genes were designed based on the sequences available in The Brazilian Coffee Genome Project (Vieira et al., 2006; http://www.lge.ibi.unicamp.br/cafe/). After a first polymorphism discovery step in *C. canephora* based on 7 genotypes belonging to the different genetic groups (Congolese SG1, Congolese SG2, Congolese B, Congolese C, Guineans, Uganda wild and Uganda N'Ganda, (Cubry et al., 2006)) a larger sample size (35 to 70 genotypes belonging to the different genetic groups) was analysed for the most interesting genes (SUS1, SUS2 and SPS). *C. canephora* genotypes belonging to the Congolese and Guineans groups were provided by the CNRA (Centre National de Recherche Agronomique) of Ivory Coast Republic whereas the Ugandan genotypes (Wild and N'Ganda) were obtained from the NARO-CORI of Uganda. All the genotypes used for the interspecific analyses (14 species) came from the IRD (Institut de Recherche pour le Développement) collection (Montpellier, France).

In-silico polymorphism discovery

In parallel with the polymorphism discovery based on the direct sequencing strategy, a search of the polymorphisms available in the EST resources generated by the Brazilian Coffee Genome Project and the Nestlé/ Cornell project (Lin et al., 2005; http://harvest.ucr.edu/) was performed using exclusively the contigs containing at least four sequences. Only polymorphisms for which the rare allele was present at least twice were considered.

RESULTS AND DISCUSSION

Sucrose metabolism genes: Mining of the Brazilian Coffee Genome Project

As a starting point of this project, data from the Brazilian Coffee Genome Project were analysed with a particular emphasis on the genes encoding the four main enzymes of sucrose metabolism. Between 6 (for SPS) and 237 ESTs (for SUSY) were identified (Table 1). Bioinformatic analyses of these sequences allowed the identification of 7 putative genes for the CWI and two for the other genes (2 SUS, 2 SPS and 2 VI). Based on this information, primers were defined to amplify 3 CWI genes, 1 VI gene and the 4 putative genes encoding SUS and SPS proteins.

Table 1. Sucrose biosynthesis pathway: Mining of the Brazilian Coffee Genome Project.

Gene	Number of ESTs	Number of singletons	Number of contigs	Number of contigs > 4 seq	Deduced gene number[a]
Cell Wall Invertase (CWI)	22	3	6	2	7 (2)
Vacuolar invertase (VI)	9	1	1	1	2 (1)
Sucrose Phosphate Synthase (SPS)	6	0	2	0	2 (0)
Sucrose Synthase (SUS)	237	18	9	9	2 (2)

[a]*Number of genes expected based on the BLASTX results obtained using the contigs and singleton. Between parentheses: the number of genes for which contigs, with at least 4 sequences, are available.*

Assessment of *Coffea canephora* nucleotide diversity by direct sequencing

All the fragments were sequenced in at least one genotype belonging to the different genetic groups (7 genotypes). The SUS and SPS genes were then analysed using a larger sample size (35 to 70 genotypes). Sizes (in bp) of the explored regions and polymorphic sites discovered are indicated in the Table 2.

Table 2. Polymorphism discovery in *C. canephora.*

Gene	Regions explored (bp)					Polymorphic sites			
	5'UTR	Coding	3'UTR	Introns	Total	Ntot	SNP	INDELS	SSR
CWI_1	0	191	158	0	349	2	2	0	0
CWI_3	0	529	0	0	529	3	3	0	0
CWI_5	0	276	60	195	531	12	11	0	1
VI_1	0	732	0	314	1046	13	13	0	0
SUS_1	542	2183	273	1159	4157	36	31	0	5
SUS_2	0	2375	570	1865	4810	97	79	11	7
SPS_C1	0	286	377	0	663	17	17	0	0
SPS_C2	0	664	0	535	1199	1	1	0	0
Total	542	7236	1438	4068	13284	181	157	11	13

A total of 13 kb was explored leading to the identification of 181 polymorphisms including 157 Single Nucleotide Polymorphisms (SNP), 11 INsertions/DELetionS (INDELS) and 13 microsatellites (SSR). On average 1.2 SNP was detected every 100bp. When considering all the sequenced fragments, most of the SNP were located in untranslated regions (69%) and one third of the polymorphisms detected in the coding regions led to Non Synonymous

mutations (33 Synonymous [S] vs 16 Non Synonymous [NS]) (Table 3). In the context of association studies aiming at identifying the genes/sites controlling the genetic variability of agronomic traits, this class of polymorphism will be the most interesting to use as they are likely to induce modification of enzyme's activity/affinity.

Table 3. Landscape of nucleotide diversity (SNP only) in *C. canephora.*

Gene	Ntot	Intron	5'UTR	3'UTR	ExsonS	ExsonNS
CWI_1	2	–	–	2	0	0
CWI_3	3	–	–	–	2	1
CWI_5	11	6	–	1	2	2
VI_1	13	5	–	–	4	4
SUS_1	31	8	10	9	4	0
SUS_2	79	46	–	8	17	8
SPS_C1	17	–	–	12	4	1
SPS_C2	1	1	–	–	0	0
Total	157	66	10	32	33	16

In a second step, according to the results obtained through physiological and genomic studies (Geromel et al., 2006; Marraccini et al., unpubished) it has been chosen to focus our efforts on the Sucrose Synthase and Sucrose Phosphate Synthase genes, which are the ones that seem to regulate sucrose accumulation in the coffee bean.

For SUS1 and SUS2, almost the full-length genomic sequences were analysed (4.1 and 4.8 kb respectively). For both, much more Synonymous (respectively 4 and 17) than Non Synonymous polymorphisms (respectively 0 and 8) were detected, suggesting that these genes are under strong evolutionary constraints due to their key role in plant development.

For SUS1, SUS2 and SPS_C1, examination of at least 5 genotypes per genetic group allowed the analysis of the population genetic structure based on nucleotide polymorphism information. Genetic differentiations (Fst) ranging from 0.46 (SPS) to 0.54 (SUS1 and SUS2) were observed. These values correspond to the one's obtained with SSR (0.380; Cubry et al., 2006). If this analysis did not reveal, in this particular case, a discrepancy between candidate genes and neutral markers (SSR), this strategy can be extremely useful to identify genes of agronomic interest (these genes usually presenting diverging patterns of evolution compared to neutral genomic regions (Pot et al., 2005)).

Nucleotide diversity of SUS1, SUS2 and SPS in *Coffea* sp

For SUS1, SUS2 and SPS_C1, in addition to *C. canephora*, 14 species were analysed. These species belong to the 4 groups of diversity of *Coffea* and are characterized by different amounts of sucrose and present variable levels of tolerance to drought stress. For the 3 genes analysed, 311 polymorphic sites corresponding to 265 SNP, 28 INDELS and 18 SSR were detected (Table 4).

Table 4. Polymorphism discovery in *Coffea* species.

Gene	Ntot	SNP	Indels	Microsat
SUS_1	142	121	9	12
SUS_2	107	90	11	6
SPS_C1	62	54	8	0
Total	311	265	28	18

Consistently with the results reported at the *C. canephora* level, most of the SNP were located in untranslated regions (76 %), and within the coding regions the Synonymous mutations were predominant (53 S vs 10 NS) (Table 5). These results suggest that at both *C. canephora* and *C.* spp levels these genes are under high selective constraints confirming their key role in plant development.

Table 5. Landscape of nucleotide diversity (SNP only) in *Coffea species.*

Gene	Ntot	Intron	5'UTR	3'UTR	ExsonS	ExsonNS
SUS_1	121	88	–	–	26	7
SUS_2	90	47	–	23	19	1
SPS_C1	54	–	–	44	8	2
Total	265	135	0	67	53	10

In addition, this study allowed the confirmation of the origin of *C. arabica*. Lashermes et al. (1997) and Cros et al. (1998) using respectively ITS from nuclear DNA and chloroplastic sequences proposed that the two parental species of *C. arabica* could be *C. canephora* and *C. eugenioides*. The results obtained at the nucleotide level for SUS1, SUS2 and SPS_C1 confirmed this hypothesis revealing for these three genes close relationships between the haplotypes of *C. arabica* and the ones of these two species (Figure 1). It is also interesting to note that when considering the 26 sites heterozygotes in *C. arabica*, in 92 % of these cases (24), the two alleles were present in *C. canephora* and/or *C. eugenioides*. And within these 24 sites, 19 present fixed differences between the two species.

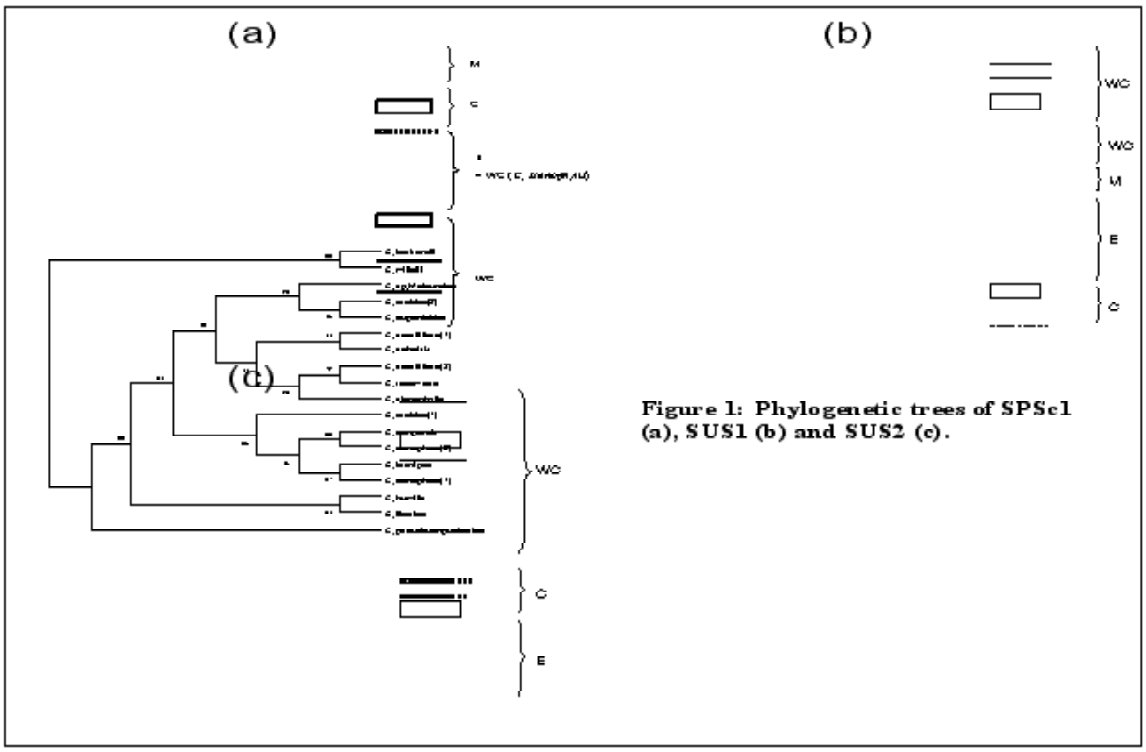

Figure 1: Phylogenetic trees of SPSc1 (a), SUS1 (b) and SUS2 (c).

Figure 1. Phylogenetic trees of SPS_C1 (a), SUS_1 (b) and SUS_2(c).

In-silico polymorphism detection

SNP have recently become the marker of choice in genetic analyses due to their abundance and stability compared to SSR and INDELS. Although the most classical way to identify SNP is, as presented earlier, direct sequencing of amplicons, an alternative method takes advantage

of the redundancy of gene sequences generated in EST sequencing programmes. Such programmes have been developed for coffee, generating a total of 261 964 sequences (47000 from the Cornel/Nestle project and 214 964 from the Brazilian Coffee Genome Project) offering the opportunity to initiate an in-silico polymorphism discovery for the coffee species.

Table 6. Polymorphic sites detected in the Brazilian Coffee Genome Project.

Gene	Lengh	Number sequences[a]	Number of polymorphic sites[b]	Number of SNP	Number of Indels	Number of SSR	Number of Non Synonymous mutations[c]
CWI_1	1432	4 (0)	0	0	0	0	0
CWI_5	1748	6 (0)	1	1	0	0	1
VI_1	1723	8 (0)	20	20	0	0	8
SUS_2	1804	13 (0)	0	0	0	0	0
SUS_2	257	5 (0)	0	0	0	0	0
SUS_1	2985	200 (8)	50 (10)	43	4	3	4 (0)

[a]Between parentheses, the number of sequence of Coffea racemosa; [b]Between parentheses, the number of polymorphic sites corresponding to fixed differences between Coffea arabica and Coffea racemosa; [c]Between parentheses, the number of synonymous mutations corresponding to fixed differences between Coffea arabica and Coffea racemosa.

Analysis of Brazilian Coffee Genome Project allowed the identification of 64 polymorphic sites (Table 6). These polymorphisms were mainly detected in VI_1 (acid Vacuolar Invertase I) and SUS1 (Sucrose Synthase I). Out of these 64 polymorphic sites, 13 lead to NS modifications. According to the availability of *C. arabica* (which is of allotetraploid origin) and *C racemosa* sequences in the Brazilian Coffee Genome Project, comparison of the polymorphisms discovered through the direct sequencing methodology at the interspecific level and the ones discovered In-Silico was possible. Based on this comparison it appeared that 50 % (34/66) of the polymorphic sites detected by traditional sequencing and potentially present in the Brazilian Coffee Genome Project (polymorphisms located in the coding sequence and in regions available in the Brazilian Coffee Genome Project) were detected.

At the *C. canephora* level, 34 % (12/35) of the polymorphic sites detected by traditional sequencing and potentially present in the Brazilian Coffee Genome Project were detected. This result suggests that a significant part of the variability present in *C. canephora* is still present in *C. arabica*. In addition, this result underlines the interest of the analysis of *C. arabica* EST resources not only in the frame of project concerning exclusively *C. arabica* but also *C. canephora*.

Regarding the Nestle/Cornell EST resource, only SUS1 was analysed (70 sequences). Indeed for the other genes, the minimum number of sequences per contig (i.e 4) was not reached. For this gene, 25 polymorphisms were detected. Out of these, 10 had been detected by traditional sequencing (4 of them were initially considered as false positive in the traditional sequencing strategy according to their localization in low quality sequences). Out of the 10 "validated" polymorphic sites detected by the traditional sequencing strategy in the same region in *C. canephora*, 6 were detected in the Nestle/Cornell EST database (60%). When compared to the sequencing approach, 15 additional polymorphisms were found in the Nestlé/Cornell dataset. Several reasons can be proposed to explain the detection of additional polymorphic sites in-silico: i) low density of sequencing in the region analysed (1 genotype per group), ii) small sample size at the within group level…

The results obtained through the "In-Sillico polymorphism discovery" strategy revealed its importance and complementarity with the strategy of direct sequencing. This strategy could easily provide the Coffee community with a large set of polymorphic markers useful for various purposes: genetic mapping, association studies, analysis of geographic origin, certification of varieties…

CONCLUSION AND PERSPECTIVES

Analysis of nucleotide diversity of sucrose biosynthesis genes allowed the identification of several polymorphisms at the intra and interspecific levels. In addition to their interest for traditional genetic studies like genetic mapping, population genetic analysis, association studies… their pattern of diversity will also provide criteria to infer the evolutionary history of the analysed genes. Such information will be particularly relevant to select the best candidate genes to test in future association studies.

The results obtained through the In-Silico polymorphism discovery strategy confirmed the importance of this approach towards the identification of markers useful for the coffee community. A whole genome scan could be rapidly initiated using the EST resources currently available (Brazilian Coffee Genome Project and Nestlé/Cornell project); such approach which presents an extremely low cost compared to traditional sequencing efforts would provide in a short term a large set of polymorphism relevant towards the identification of molecular markers useful for marker assisted breeding of coffee sp.

ACKNOWLEDGEMENTS

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Targeted Transcriptome Profiling during Seed Development in *C. arabica* cv. Laurina

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SUMMARY

Due to its economic importance, *Coffea* is receiving increasing attention with respect to genomic research. As a first approach on coffee seed development, we carried out a preliminary targeted transcriptomic study using carefully selected cDNAs. On the basis of EST libraries generated from mRNAs of *C. canephora* and *C. arabica* fruits and leaves we focused on cDNAs encoding enzymes potentially involved in seed development and/or in biosynthesis of precursors playing a role in beverage quality. We selected 266 candidate genes that were PCR-amplified and spotted onto a nylon membrane. So obtained cDNA arrays were used to screen the transcriptional status of the endosperm of *C. arabica* cv. Laurina grown in La Réunion Island, at seven different development stages (sampled between 60 and 250 days after flowering). Clustering analysis was performed using gene expression data and was used to highlight observed phenological changes. Analysis of variance sorted seventy five cDNAs differentially expressed during seed development. We classified these genes into five distinct groups of expression pattern through multivariate analysis.

INTRODUCTION

Coffee flavour is the product of a complex chain of chemical transformations. The green bean only has a faint smell, but it contains all the precursors able to generate coffee aroma during roasting. One of the main focuses of research on coffee quality is to establish relationships between the chemical composition of green coffee beans and beverage quality, and particularly, to identify aroma precursors involved in flavour development. Those precursors are synthesized and/or transported in the endosperm during seed development and maturation and their levels may vary according to genetic and environmental factors. Characterization of gene expression profiles during phenological changes occurring in seed development could lead to identify key quality-related genes, a main prerequisite for the development of efficient and rapid quality breeding strategies.

The development and maturation of the fruits of *C. arabica* cv. Laurina require 210-250 days after flowering (DAF) and are similar to those of *C. Arabica* cv. Bourbon. As summarized by De Castro and Marraccini (2006) and Eira et al. (2006), different developmental stages can be described. First, initial seed expansion (0-90 DAF) is due to the rapid growth of the transient perisperm until it fulfills the entire volume of the locule. After this period, the developing endosperm progressively replaces the perisperm inside the locule, almost entirely at 120-150 DAF. The remaining perisperm looks like a thin pellicle surrounding the endosperm. Finally,

the ripening phase takes place 200 DAF when seed maturation is associated with pericarp softening and pigmentation.

Large-scale partial sequencing of anonymous cDNAs to produce Expressed Sequence Tags (ESTs) and comparison of the resulting sequences with public databases have become a method of choice for a rapid and cost-effective identification of new genes. On this basis, molecular knowledge of coffee is rapidly increasing through the recent release of several *Coffea* EST databases. First, HarvEST Coffea database (http://harvest.ucr.edu/; Lin et al., 2005) is based on sequences from approximately 47,000 cDNA clones of *C. canephora*, issued from different laboratories. Second, the Brazilian coffee genome project also released 130,000 ESTs from *C. arabica* (http://www.lge.ibi.unicamp.br/cafe/; Vieira et al., 2006). In addition, an EST database was simultaneously developed in our laboratory, containing 10,420 sequences derived from *C. canephora* fruit and leaf (GenBank accession numbers EE191792-EE200565).

These resources are now freely available to pursue transcriptome analyses which may lead to the identification of quality-related genes involved in determining the final chemical composition of seeds. For this purpose, we selected and amplified 266 cDNA clones potentially involved in seed development which were further arrayed on nylon filters. These filters were then hybridized with probes deriving from seven developmental stages, from aqueous perisperm to ripen endosperm. The main objectives of this work were: (i) to characterize phenological stages on the basis of gene expression patterns; (ii) to identify genes which are expressed in specific developmental stages (iii) to classify genes with similar expression patterns into functional clusters.

MATERIAL AND METHODS

Experimental site and plant material

Developping seeds were harvested from plants of *Coffea arabica* cv 'Laurina' (a natural mutant of cv. 'Bourbon') grown in the field (21.16°S, 55.33°E, 1015 m a.s.l, Grand Tampon, Réunion Island, France) on an Andosol soil, with an average temperature of 18.5°C and a mean annual rainfall of 1300 mm. Coffee trees were planted in 2002 without shade and were in their second (2005) production cycle (about 2 m high). Plant spacing was 2 m between rows and 1 m within rows.

For collection purposes, fruits were tagged after flowering and harvested at one of the seven following development stages: Stage 1 (0-60 DAF, days after flowering): seed mainly formed by aqueous perisperm, Stage 2 (60-90 DAF): aqueous perisperm surrounding a small liquid endosperm, Stage 3 (90-120 DAF): aqueous endosperm tissue growing and absorbing the perisperm, Stage 4 (120-150 DAF): soft milky endosperm, Stage 5 (150-210 DAF): hard white endosperm , Stage 6 (210-240 DAF): ripening cherry fruits with pericarp turning to yellow, Stage 7 (> 240 DAF): mature cherry fruits. To ensure developmental synchrony of fruits harvested from each stage, they were visually inspected externally and internally via cross section using a scalpel. After this cross section, the seed was separated from the pericarp and locules and immediately frozen in liquid nitrogen. For each stage, three pools of seeds were collected at ten days interval each. Each such pool was analyzed in duplicate for RNA extraction, labelling and hybridization.

cDNA array building

The coffee EST databases used in this project were developed in our laboratory. They contained 10,420 sequences derived from *C. canephora* fruit and leaf cDNA libraries (GenBank accession numbers EE191792-EE200565), as well as around 2,000 sequences of *C. arabica* (cv. Bourbon and Laurina) issued from SSH subtractive libraries (Lecolier et al. unpublished results). Selected candidate genes were PCR-amplified, purified (adjusted to 300 ng/µl) and randomly spotted in duplicate on Hybond N^+ membranes at a 10 clones.cm^{-2} density using a FLEXYS arrayer and a 384-pin tool (Genomic Solutions) at the Montpellier Languedoc-Roussillon Genopole genotyping platform (http://www.genopole-montpellier-lr.org). For each spot, around 15 ng of each amplimer was spotted (four hits). After denaturation, neutralization, and rinsing, cDNA were crosslinked on filters via UV treatment (70 mJ.cm^{-2}).

RNA extraction, cDNA synthesis, labelling and membrane hybridization

After total RNA extraction (according to Azevedo et al., 2003), samples were treated with RNase-free DNase I and passed through RNeasy MinElute Cleanup columns (Qiagen) to clean and concentrate the sample. RNA integrity and purity was assessed by agarose gel electrophoresis and spectrophotometry ($A_{260}/A_{280} > 1.9$). Complex probes, synthesized from 10 µg denaturated total RNA using anchored oligo-dT (VT_{18}), were prepared in 50µl by simultaneous reverse transcription and labelling for 2 hours at 37 °C in the presence of LabelStar reverse transcriptase (Qiagen), RNase inhibitor, 50 µCi α-^{32}P-dCTP, 6 µM dCTP and 0.5 mM each dATP, dTTP and dGTP. Membranes were prehybridized 15h at 65 °C in 40 ml of the hybridization mix (5x SSC, 5X Denhardt's, 0.6% (w/v) SDS) with 40µg/ml sheared denaturated salmon sperm DNA. Complex probes were denaturated and added to the mix for 20h of hybridization. Membranes were washed twice in 1X SSC, 0.1% SDS for 20 min at 65 °C, then once in 0.1X SSC, 0.1% SDS for 15 min at 65 °C and finally exposed to phosphor screens. The screens were scanned in a phosphor plate system (Cyclone, Perkin Elmer). Hybridization signatures were quantified with the Arrayvision analyser software (GE Healthcare) by integrating all spot pixel intensities. A local background value was determined in the neighbourhood of each spot. Practically, twelve expression values per gene were available for each developmental stage.

Statistical analysis

Data were transformed (using Statistica 7.1 software) to get the required conditions for analysis of variance, i.e. to obtain a Gaussian (normal) distribution of residuals, independent of tested factors and showing constant variance. The data transformation was empirically determined and corresponded to $\log_{10}$[crude signal+2]. So transformed local backgrounds were then subtracted from the transformed data giving a corrected expression value (net signal Y) for each gene under study ($Y = \log_{10}$[crude signal+2] - $\log_{10}$[background+2]). The absence of any uncontrolled effect (such as differences between hybridizations) was further tested. Indeed, a two-factor ANOVA model with fixed factors (gene and hybridization) was used on crude and net signal dataset and unambiguously showed the efficacy of transformation. After this transformation, "gene" effects were the main significant factors, "hybridization" factor can be considered as negligible. All the following analyses were performed on this transformed dataset (net signal Y).

Concerning gene expression analysis, three different statistical methods were used.

First, a clustering analysis (Ward's criteria, Euclidian distance) was performed on transformed gene expression data set in order to highlight similarities between developmental stages.

Secondly, principal component analysis (PCA) was used to identify factors structuring gene expression variability. In order to get gene expression profiles independent from mean intensity, transformed data were standardized by dividing them by the mean expression value for each gene. Weak gene expression signals close to background signals were discarded by arbitrarily setting a threshold value of 0.02. A first analysis with orthogonal rotation by normalized varimax was carried out and factors interpretation led to define new explicative synthetic variables which were added to the other variables in the final analysis.

Thirdly, a clustering analysis (Ward's criteria, Euclidian distance) was applied to factorial scores obtained from PCA analysis in order to pinpoint clusters and similarities gene expression profiles. Finally, statistical significance of variations in gene expression was verified by analyses of variance (one-way ANOVA with fixed effect) on transformed expression values using developmental stages as criteria of classification.

RESULTS

Discontinuous structure of phenological stages during seed development

Targeted cDNA arrays were used to compare gene expression profiles among seven phenological stages during coffee seed development. A first clustering analysis was performed on gene expression dataset to differentiate stages in terms of gene expression (Figure 1). A discontinuous structure was found and, at the highest level, two clusters Ca and Cb were found. Ca included stages 6 and 7, corresponding to maturation of the endosperm occurring during ripening stages, while Cb included stages 1 to 5. At a lower level, Cb can be split into Cb-1 and Cb-2, corresponding respectively to early stages of seed development when perisperm represents the major part of the seed (stages 1 and 2) and later stages when endosperm is developing (stages 3, 4 and 5). It is worth noting that stage 5 (unripe fruits with fully developed endosperm) is closer in terms of gene expression to early stages 1 and 2 (when perisperm is the main constituent of the seed) than to late stages 6 and 7 corresponding to ripe fruits (stages 6 and 7).

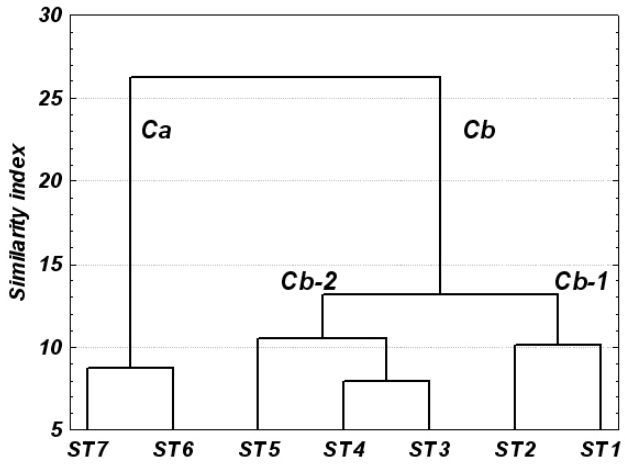

Figure 1. Clustering analysis (Euclidean distance, Ward's criterion), performed on gene expression data set, showing similarities between normalized gene expression values at seven development stages during coffee seed development.

Factors structuring gene expression diversity

Structuration of the gene expression variability was carried out by a PCA analysis. Two principal components explained 71.7% of the variance (Figure 2). The first factor represents the effect of ripening on gene expression as it separates stages 6 and 7 from others. The second could be associated with endosperm development as it opposes stages 4 and 5 (endosperm fully developed) to early stages when the perisperm dominates. This interpretation is reinforced by results obtained with synthetic variables (Figure 2). Indeed, 99% of variations in the synthetic variable SVa are explained by factor 1, while 80% of variations in the synthetic variable SVb are explained by factor 2 (data not shown).

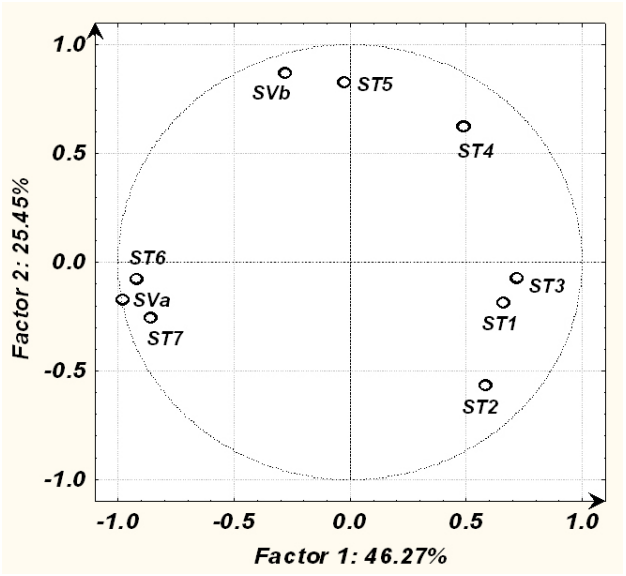

Figure 2. Principal components of the variability of normalized gene expression profiles amongst the seven seed developmental stages. The two first factors explain 71.7% of variance. SVa ([Stages 6+7]/[Stages 1+2+3+4+5+6+7]) and SVb ([Stages 4+5]/[Stages 1+2+3+4+5]) are synthetic variables which are tentatively explicative.

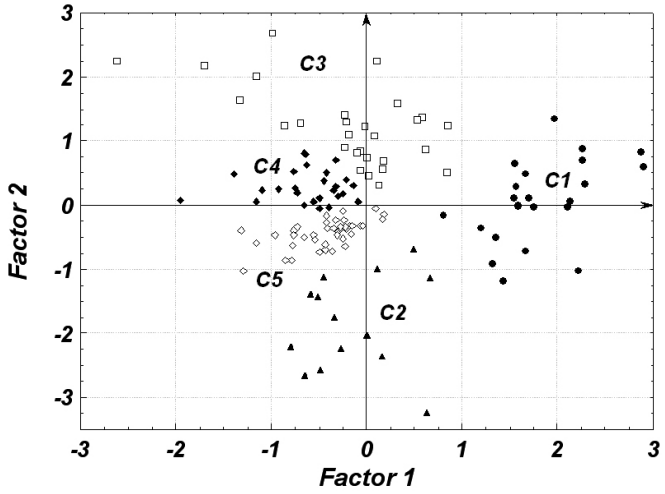

Figure 3. PCA of the distribution of 131 normalized gene expression profiles at seven development stages. Genes are labelled according to similarities shown by clustering analysis: Class 1 (●), C2 (□), C3 (▲), C4(◆) and C5 (◇). Clustering analysis (Euclidean distance, Ward's criterion) was performed on the two first factorial scores and defined five classes of genes sharing similar expression profiles.

Identification of clusters of co-expressed genes

Factorial scores of the PCA were then used to determine similarities among gene expression profiles through a clustering analysis. This second clustering analysis was performed on the two first factorial scores (dendogramm not shown). Five major clusters, named C1 to C5, were observed and plotted on PCA graph (Figure 3). Finally, the mean profile of each cluster was plotted in order to visualize expression patterns of co-expressed genes (Figure 4). Due to high variability observed for stage 1, data are not presented.

Cluster 1 comprises a set of 25 transcripts which strongly accumulate during the ripening phase (stages 6 and 7). During this phase, our data show the induction of cell-wall-related genes like annexin, expansin, cellulase, arabinosidase, pectate lyase, xylosidase and laccase, representing the largest functional class of genes of C1. Cluster 2 is formed by 25 genes expressed at a high level in early stages of development (stages 1, 2 and 3) but repressed in mid-stages 4 and 5. In this cluster, the expression level of some genes is also enhanced in late stages 6 and 7. This cluster contains several transcripts encoding plasma transporters such as aquaporin, hexose carrier and ABC transporter. Cluster 3 is composed by a small set of 14 genes which are highly expressed during intermediate stages 3, 4 and 5 (endosperm growth and maturation corresponding to the storage phase). Those genes are involved in lipid storage (oleosin, caleosin, steroleosin), protein storage (11S and 7S globulin) and secondary metabolism (secretory peroxidase and 4-cinnamate hydroxylase). Cluster 4 is represented by 28 genes whose expression is progressively repressed during seed development reaching their lowest level during stages 6 and 7. Amongst these genes, several are involved in photosynthesis and carbon metabolism. Finally, cluster 5 comprises a set of 38 genes with relatively stable expression during the first five stages. Most of them are non-developmentally regulated transcripts and may be considered as representative control genes (e.g. spermidine synthase, actin, histone 2B and omega 6 fatty acid desaturase).

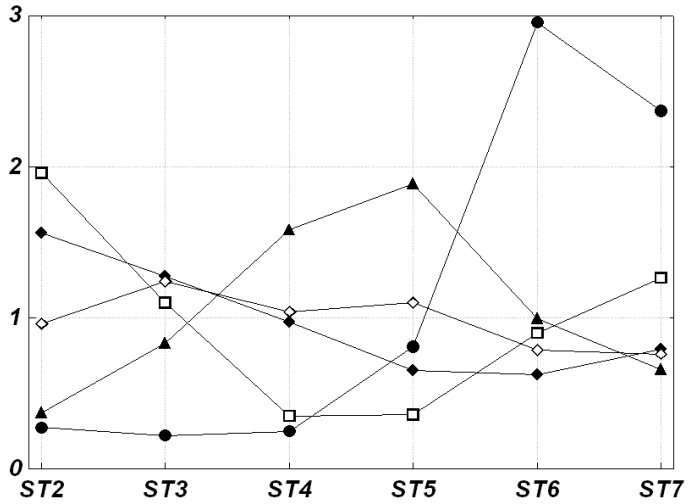

Figure 4. Mean expression profile of clusters: C1 (●), C2 (□), C3 (▲), C4(◆) and C5(◇). The Y-axis represents expression value as normalized ratio (mean expression value of the stage/ mean expression value for all stages).

CONCLUSION

The cDNA array approach was successfully used for the first time in studying coffee seed development and ripening. Although our array contained only 266 genes, it was large enough to allow typology analyses and dissect seed development stages at a transcriptome level. Our

results show a discontinuous structure of phenological stages between seed development (stages 1 to 5) and seed maturation (stages 6 and 7). At a lower level, a continuum is observed during seed development between early stages 1-2 (when perisperm represents the major constituent of the seed) and late stages 4-5 (when endosperm is fully developed). Phenological changes are associated with variations in expression of functionally related genes. Cluster C1 gathers genes linked to seed maturation while Cluster C2 comprises genes associated with perisperm division and elongation. Finally, Cluster C3 is associated with endosperm development and storage phase.

Important candidate genes will be further studied by real-time PCR to confirm their expression profile. Expression data from this study will also be connected to the accumulation of some selected metabolites along the same developmental stages. However, it is worth noting that our results are already in complete accordance with recent studies of the expression of specific genes during fruit development such as genes from the oleosin family (Simkin et al., 2006), dehydrin family (Hinniger et al., 2006), 11S storage protein (Rogers et al., 1999) or ACC oxidase (Pereira et al., 2005). Our results illustrate the advantages of using small boutique arrays. Microarray techniques at genome-scale level require the availability of knowledge on a high number of cDNA sequences and, even so, these techniques are still long, costly and tedious to initiate. Our relatively cheap, small-scale approach could be used to screen a large number of environmental/developmental situations and could be helpful in identifying *ad hoc* comparisons for more detailed analyses using oligonucleotide microarrays.

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Caffeoylquinic Acids Distribution in *Coffea canephora* Plantlets

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SUMMARY

Caffeoylquinic acids (CQAs) are cinnamate conjugates derived from the phenylpropanoid pathway. One of them, i.e. the chlorogenic acid (5-*O*-caffeoylquinic acid, 5-CQA), appears as an intermediate in the lignin pathway. CQAs, and particularly 5-CQA, are accumulated in coffee beans, where they can form vacuolar complexes with caffeine. *Coffea canephora* beans are known to have high CQAs content, but little is known about the content and diversity of these compounds in other plant parts. We attempted to gain new insight into the *C. canephora* CQAs metabolism by assessing the CQAs content and *in situ* localization in 4-month old plantlets. HPLC analyses revealed that the content was higher in juvenile organs. The most abundant cinnamate conjugate was 5-CQA, but dicaffeoylquinic acids and feruloylquinic acids were also present. Using specific reagents, histochemical analysis showed that caffeoylquinic acids (mono- and diesters) were closely associated with chloroplasts in very young leaves, suggesting a protective role against light damage. During leaf aging, they were found to be associated with vascular tissues, indicating that they are mobilized via phloem transport and confirming their involvement in lignification processes. Compound biosynthesis and accumulation are discussed with regard to the differential expression of the last genes involved in their biosynthesis. In accordance with the hypothesis of a complex formation with caffeine, the alkaloid distribution corresponds to that observed for caffeoylquinic acids.

RÉSUMÉ

Les acides caféoylquiniques (CQAs) sont des conjugués de cinnamate, dérivés de la voie des phénylpropanoïdes. L'un d'entre eux, l'acide chlorogénique (acide 5-O-caffeoylquinique, 5-CQA), est un intermédiaire dans la voie des lignines. Des CQAs, et en particulier le 5-CQA, sont accumulés dans les grains de café, où ils peuvent former des complexes vacuolaires avec la caféine ou d'autres alcaloïdes. Les grains verts de *Coffea canephora* sont connus pour avoir une teneur élevée en CQAs, mais il existe peu de données sur la teneur et la diversité de ces composés dans d'autres parties de la plante. Par HPLC, nous avons évalué la teneur en CQAs dans des plantules de *C. canephora* âgées de quatre mois et montré que le contenu était plus élevé dans les organes juvéniles. L'ester le plus abondant est le 5-CQA, mais les acides dicaféoyl- et les acides féruloylquiniques sont également présents. En utilisant les réactifs spécifiques, l'analyse histochimique a montré que les acides caféoylquiniques (mono- et diesters) sont étroitement associés aux chloroplastes des feuilles très jeunes, suggérant un rôle protecteur de ces composés contre les rayonnements UV. Au cours du vieillissement des feuilles, ces composés sont fortement accumulés dans certaines zones des tissus conducteurs, indiquant leur mobilisation par transport dans le phloème et confirmant leur participation au

processus de lignification. Leur biosynthèse et accumulation sont discutées en tenant compte du niveau d'expression des gènes impliqués dans les dernières étapes de la biosynthèse. Une distribution semblable a été observée pour les alcaloïdes.

INTRODUCTION

Esters formed between hydroxycinnamates and quinic acid represent a major family of plant phenolics. Chlorogenic acid (5-caffeoylquinic acid, 5-CQA) is the most widespread of all the esters formed between caffeic and quinic acid (CQA) (Molgaard and Ravn, 1988). It is commonly considered to be an intermediate in the lignin pathway. This route involves an esterification between coumaroyl-CoA or caffeoyl-CoA and quinate, catalyzed by two different hydroxycinnamoylquinate transferases (HQT and HCT), and an hydroxylation, catalysed by a CYP98A, an enzyme from the cytochrome P450 family (Schoch et al., 2001). Some plant families, including *Solanaceae*, *Asteraceae* and *Rubiaceae*, also produce diesters, principally dicaffeoylquinic acids. CQA are potent antioxidants that are synthesised in response to oxidative stresses (Grace et al., 1998), and act particularly against lipid peroxidation (Rice-Evans et al., 1997). This antioxidant activity may also be beneficial for human health, e.g. limiting atherosclerosis and carcinogenesis (Jin et al., 2005), or inhibiting HIV-1 replication (Zhu et al., 1999). CQA are involved in a broad range of stress responses, but mechanisms underlying their biosynthesis and protective action *in vivo* are still unclear. In coffee trees, CQA accumulate in beans. This is particularly marked in *Coffea canephora* green beans where their content can exceed 10% of dry bean weight and 5-CQA alone represents about 68% of the total hydroxycinnamoyl quinic acid content (Anthony et al., 1993; Ky et al., 2001; Campa et al., 2005). CQA content is well documented in coffee beans as these compounds are involved in the bitterness of the coffee beverage due to their degradation into phenolics during roasting (Leloup et al., 1995). Extensive biochemical analyses have shown that beans of some *Coffea* species contain high levels of 5-CQA, but also dicaffeoyl- and feruloylquinic acids (diCQA and FQA). They are also thought to be involved in the vacuolar sequestration of caffeine in seeds due to their ability to form complexes with this alkaloid (Mösli Waldhauser and Baumann, 1996). Nevertheless, little is known about their biosynthesis in coffee trees or their presence in other parts of the plant. The present study investigated the biosynthetic pathways leading to 5-CQA biosynthesis but also the biochemical composition and histochemical localization of caffeoylquinic acids in *C. canephora* plantlets.

MATERIAL AND METHODS

Seedlings of *Coffea canephora* Pierre were cultivated in tropical greenhouses (natural daylight, 25 °C night, 28 °C day, 80% humidity) at the IRD research centre in Montpellier (France). Nodes were ranked from node 1, for the youngest (juvenile leaves), to node 4, for the oldest (mature leaves). Five 4-month old seedlings were harvested during spring, at midday, and bulk samples were made with shoot tips, leaves and petioles from different nodes (node 1 to node 4), apical stems (from apex to node 1), medium stems (from node 1 to node 2), basal stems (from node 2 to node 4), cotyledon leaves, hypocotyls and roots. The bulk samples were divided in two batches and immediately frozen in liquid nitrogen. One batch was directly used for RT-PCR experiments and the other was lyophilised before extraction for analyses of hydroxycinnamoyl ester content according to the methods previously described (Ky et al., 2001; Bertrand et al., 2003).

For gene isolation, *C. canephora* cDNA libraries from young leaves and fruits at different development stages were screened. Then, specific primers were designed from the cDNA sequence for PCR amplification of full-length genes. For RT-PCR analysis, total RNA from

the different plant parts were isolated. cDNA was synthesised, and then each cDNA was amplified by PCR using specific primers for each gene.

For histochemical examination, cross-sections were prepared and observed as already described (Mondolot et al., 2006). With UV light, Neu's reagent (Neu, 1957), a standard reagent for phenolic compounds, allows to detect caffeoylquinic acids by a greenish-white fluorescence while feruloylquinic derivatives are bright blue. Under visible light, Dragendorff's reagent modified by Schute (Merck, 1968) colours in reddish-brown alkaloids and associated compounds such as caffeine, theobromine and trigonelline.

RESULTS

Hydroxycinnamoylquinic acid content

The major hydroxycinnamoyl quinic esters accumulated in the different organs of 4-month old *C. canephora* plantlets are the 5-CQA and the 3,5-DiCQA. Young organs and cotyledonary leaves are particularly rich in caffeoylquinic acids (Table 1).

Table 1. Evaluation of the content in the major hydroxycinnamoyl esters present in the different organs of 4-month-old seedlings of *C. canephora*. Values are expressed in percentage of the dry mass (% DW). For the same class of compounds, values followed by the same letter indicate no significant between-organ difference at $p \leq 0.05$ according to one-way ANOVA.

Organ		5-CQA	3,5-DiCQA	5-FQA
Shoot tip		4.90 ^{c}	7.73 ^{b}	0.10 ^{cd}
Stem	Apical	5.78 ^{b}	9.23 ^{a}	0.10 ^{cd}
	Medium	4.80 ^{c}	2.01 ^{c}	0.19 ^{b}
	Basal	1.59 ^{f}	0.50 ^{de}	0.05 ^{de}
Hypocotyl		1.04 ^{f}	0.35 ^{de}	0.04 ^{de}
Petiole *Node 1*	Young	4.16 ^{c}	1.52 ^{cd}	0.18 ^{b}
Node 2	Medium	3.27^{d}	1.09 ^{cde}	0.13 ^{bc}
Node 3	Old	2.24 ^{ef}	0.51^{d}	0.08 ^{cde}
Leaf *Node 1*	Young	2.59 ^{de}	0.97 ^{de}	0.08 ^{cde}
Node 2	Medium	2.53 ^{de}	0.55 ^{de}	0.08 ^{cde}
Node 3	Old	2.92 ^{de}	0.40 ^{de}	0.09 ^{cd}
Cotyledon		7.63 ^{a}	1.51 ^{cd}	0.47 ^{a}
Root		1.09 ^{f}	0.16 ^{d}	0.02 ^{e}

As already observed in coffee green beans, 5-CQA is the most accumulated compound except in very young organs (shoot tip or apical stem) where 3,5-DiCQA content is largely higher than that of the 5-CQA. Total CQA content decreased from the top to the bottom of the plant.

Caffeoylquinic acid biosynthesis in C. canephora

By screening leaf and fruit *C. canephora* cDNA libraries, we isolated two genes encoding hydrycinnamoylquinate transferases, one of them encoding a HQT, the other one a HCT, and two genes encoding CYP98A, CYP98A35 and 36. By heterologous expression in yeasts, only CYP98A35 seemed to be able to catalyse the hydroxylation of the coumaroylquinate. Figure 1

describes the proposed pathways for 5-CQA biosynthesis in *C. canephora* according to these findings.

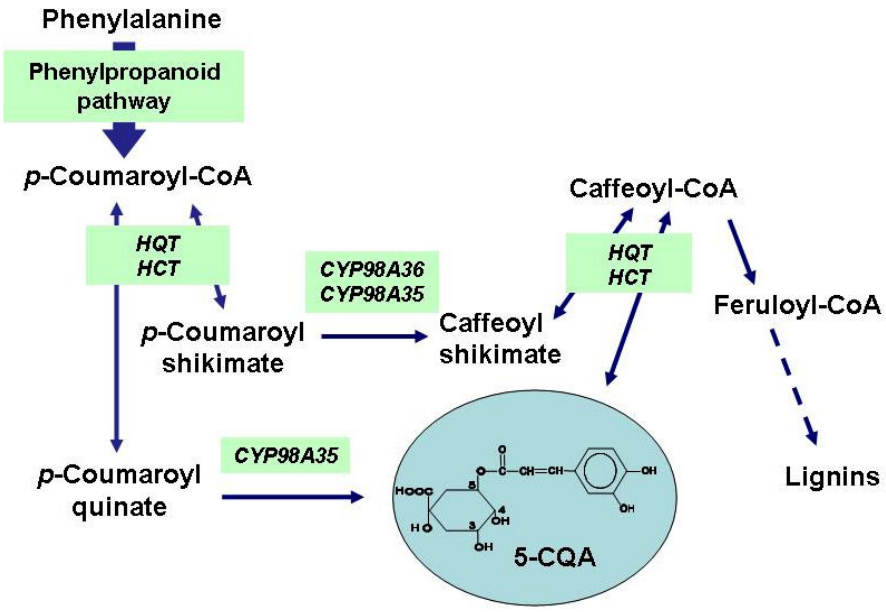

Figure 1. Proposed pathways for 5-CQA biosynthesis in *Coffea canephora*.

Expression levels of these genes were evaluated by RT-PCR analysis. Different patterns were observed according to the plant organ (Figure 2). A high expression of *CYP98A35* and *HQT* genes was observed in the apical part of the plant, where CQAs accumulated, indicating that CQA biosynthesis and accumulation take place in these organs. In the roots, the three genes, *CYP98A35*, *HQT* and *HCT*, were highly expressed. As CQA were not accumulated in these organs, their biosynthesis may constitute a transitory step in that of lignin.

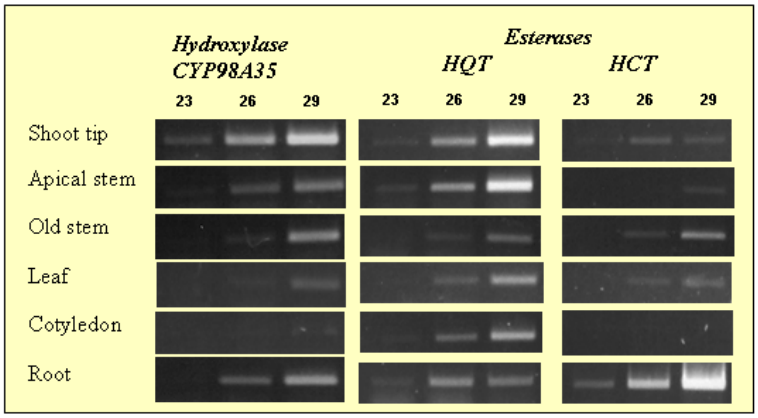

Figure 2. Expression levels of the last genes involved in caffeoylquinic acid biosynthesis in different organs of 4-month old *C. canephora* plants. mRNA accumulation level was determined by semi-quantitative RT-PCR after 23,26 or 29 cycles of amplification.

Histolocalization of caffeoylquinic acids

A specific greenish-white fluorescence is emitted by caffeoylquinic acids under UV light after treatment with Neu's reagent. In juvenile leaf blades, this fluorescence was observed in all chlorenchyma cells from palisadic and spongy parenchyma (mesophyll), indicating a high

concentration of CQAs (Figure 3A). This specific fluorescence appeared in the area where chloroplasts are concentrated highlighting the close association between CQAs and chloroplasts in chlorenchyma cells.

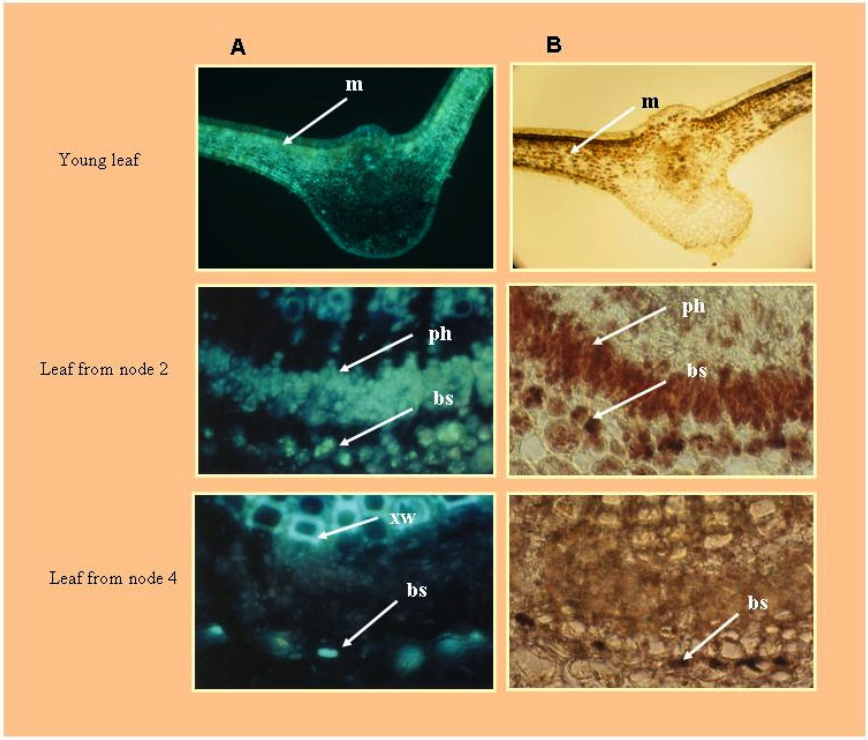

Figure 3. Histochemical localization of CQA (A, Neu's reagent, UV light x 100) and alkaloids (B, Dragendorff's reagent, visible light, x 100) in *C. canephora* leaves. Bs, bundle sheath, m, mesoplyll, ph, phloem, xw, xylem wall.

In maturing leaves, from node 2, the highest intensity was observed in chlorenchymatous bundle sheath cells as well as in phloem cells. In leaves from node 4, this specific fluorescence was particularly intense in the cell walls of newly formed xylem vessels and in the chlorenchymatous bundle sheath cells. In similar cross-sections, Dragendorff's reagent was used to localize alkaloidic compounds (Figure 3B). A brownish colour appeared in the same leaf tissues as the fluorescence, except in the xylem vessels.

DISCUSSION

This study showed that chlorogenic acid (5-CQA), the most widespread caffeoylquinic acid, and also 3,5-DiCQA, a diester of quinic acid, were both present at high concentration in the apical part of young *C. canephora* plants. In immature leaf blades, CQAs were specifically localized in chlorenchyma cells, where they appeared to be biosynthesized and associated with chloroplasts. This close link between CQAs and chloroplasts in young leaves suggests that, in the newly formed organs whose tissue architecture is not complete, CQAs could play a special role by protecting chloroplasts against light damage. As suggested by Alibert et al. (1976), another explanation for this chloroplastic localization may be that CQAs synthesis takes place in chloroplasts. Complementary biochemical and enzymatic analyses on isolated chloroplasts are needed to clarify this point. In developing leaves, CQAs were localized in phloem cells where they could be biosynthesized or transported throughout the plant. In this case, they would be synthesized in the upper part of the plant and transported by the phloem sap to other plant organs where they would be required in the lignification process. Their

impregnation in xylem cell walls of mature leaves argues in favour of the assumption that CQAs contribute to cell wall building (Aerts and Baumann, 1994; Schoch et al., 2001).

Our study also revealed that CQAs and alkaloids accumulate in the same area. This is fully in line with the hypothesis of a complex formation between chlorogenic acid and caffeine, a purine alkaloid (Spencer et al., 1988; Mösli Waldhauser and Baumann, 1996). In *C. arabica*, purine alkaloid biosynthesis appeared to occur in very young leaf tissues (Zheng and Ashihara, 2004). It is possible that CQAs and alkaloid synthesis take place in young tissues of coffee leaves where they act as protective agents. Their transport to mature organs could occur via the phloem vessels, especially when they are required for response to biotic or abiotic stresses.

It would be of interest to gain further insights into the biosynthetic pathway leading to CQAs biosynthesis in *Coffea* species through *in situ* hybridization and protein immunolocalization.

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Functional Validation of *Coffea PAL* Genes Using Genetic Engineering

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SUMMARY

Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the phenylpropanoid pathway and plays a critical role in the biosynthesis of phenolic compounds such as anthocyanins, flavonoids, coumarins, chlorogenic acids or lignins. PAL enzymes are encoded by a small multigene family. Screening *Coffea canephora* EST libraries, three encoding PAL genes have been isolated. In this paper, their functional validation is considered using a sense or antisense approach in two model plants differing by their ability to accumulate chlorogenic acids (CGAs), compounds known to play a critical role by adding bitterness and astringency to the final coffee beverage quality. In view of this, understanding and identifying the role of each candidate paralogue in CGA biosynthesis is an important step towards the improvement of organoleptic properties. Sense and antisense constructs, comprising individual *Coffea* PAL genes (PAL1, PAL2, PAL3) were prepared in the binary vector pCAMBIA1305.1. All six constructs were introduced into *Arabidopsis* by floral dip method and in tobacco plants by co-cultivation with *Agrobacterium tumefaciens* LBA 4404 or GV 3101 strains. The preliminary analysis of the transformed plants are presented.

RÉSUMÉ

La phénylalanine ammonia-lyase (PAL) catalyse la première étape de la voie de biosynthèse des phénylpropanoïdes (PPP) et joue un rôle critique dans la biosynthèse des composés phénoliques tels que les anthocyanines, les flavonoïdes, les coumarines, les acides chlorogéniques ou les lignines. Les PAL sont codées par une petite famille multigénique. Par criblage de banques EST de *Coffea canephora*, trois gènes codant des PAL ont été isolés. Leur validation fonctionnelle est envisagée en utilisant l'approche transformation génétique (sens ou antisens) de deux plantes modèles différant par leur capacité à accumuler les acides chlorogéniques (CGAs), composés connus pour jouer un rôle critique en ajoutant l'amertume et l'astringence à la qualité finale du café boisson. L'identification du rôle de chaque paralogue de PAL dans la biosynthèse des CGAs est une étape importante vers l'amélioration des propriétés organoleptiques du café boisson. Des constructions géniques sens et antisens, avec les différents gènes PAL de *C. canephora* (PAL1, PAL2, PAL3) ont été réalisées dans le vecteur binaire pCAMBIA1305.1. Chacune des six constructions a été introduite dans *Arabidopsis* par la méthode d'immersion florale et dans *Nicotiana* par co-culture avec les souches d'*Agrobacterium tumefaciens* LBA 4404 ou GV 3101. L'analyse préliminaire des plantes transformées est présentée dans ce travail.

INTRODUCTION

The first enzyme of the phenylpropanoid pathway (PPP) is the phenylalanine ammonia lyase (PAL, EC 4.3.1.5). PPP is involved in the biosynthesis of phenolic compounds such as anthocyanins, flavonoids, coumarins, chlorogenic acids or lignins. It has been shown that in plants PAL enzymes are encoded by a small multigene family and that *PAL* paralogues differ by both their sequence and the regulation of their expression (Cramer et al., 1989; Kao et al., 2002). This regulation, acting at the entry point of the PPP, may lead to various changes in the subsequent steps of the pathway, tending plant metabolism towards specific routes and biosynthesis (Hahlbrock and Scheel, 1989). For example, the over-expression of one bean paralogue (*PAL2*) in tobacco has lead to increase 5-CQA level in leaves, one of the major chlorogenic acid (CGA) accumulated in plants (Howles et al., 1996). This family of hydroxycinnamoyl quinic acid esters plays an important role in coffee plants. These compounds, and especially the 5- caffeoyl quinic acid (5-CQA), represent the second most important metabolites in coffee next to caffeine. In coffee drink, the CGA content plays a critical role in the cup quality by adding bitterness and astringency to the beverage (Clifford, 1999; Leloup et al., 1995). Three PAL cDNAs have been recently isolated from *Coffea canephora* EST libraries (Mahesh, 2006). Their expression in sense and antisense orientation in model plants will be helpful to understand their specific role in the orientation of the PPP through biosynthesis of one or another specific phenolic compound. In the present study we report the construct preparation for genes of interest from *C. canephora* in both orientations and their expression analysis in model plant systems.

MATERIALS AND METHODS

Agrobacterium tumefaciens strains GV 3101 or LBA 4404 were used to transform independently *Arabidopsis thaliana* by floral dip method and *Nicotiana tabacum* SR-1 variety by the co-cultivation method with the six different constructs carrying the candidate cDNAs in either sense or antisense orientation. pCAMBIA 1305.1 (after deleting the *GUS* gene) carrying hygromycin resistance (*hptII* gene) or pCAMBIA 2301 with kanamycin resistance (*nptII* gene) under the control of the CaMV 35S promoter were used as binary vectors (Figure 1).

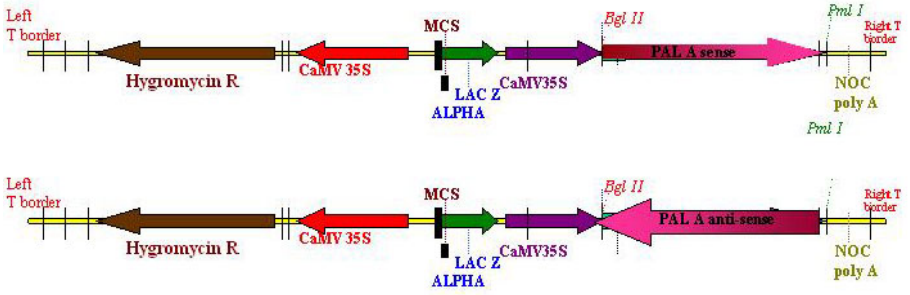

Figure 1. Construct design in sense and antisense orientation. pCAMBIA 1305.1 back-bone with candidate gene after removal of the *GUS* gene. The three PAL cDNA were cloned on the same scheme.

For *Arabidopsis thaliana*, six different lots of flowering plants were immersed in the bacterial preparations. The T0 seeds were then harvested, pre-treated to decontaminate and then inoculated onto water agar medium with hygromycin (15 mg/l). Selected transformants of *Arabidopsis* were transplanted to micropots for their further growth under controlled conditions to get the T1 plants and subsequently the T2 generation that could be used for the expression studies of gene of interest and also for molecular and biochemical analysis.

For *Nicotiana tabacum* SR-1 variety, MS medium with 1.0 mg/l BAP and 0.1 mg/l NAA was used during co-cultivation (with acetosyringone) and selection stages. The putative transformants were checked for the presence of *hptII* or *nptII* genes respectively for hygromycin selection and kanamycin selection. The transformed plants of *Nicotiana tabacum* were rooted on MS medium containing 2.0 mg/l IBA with respective antibiotic for selection and cefotaxim for preventing *Agrobacterium.*

RESULTS AND DISCUSSION

Best yield of selected embryos were obtained using hygromycin as selection agent (Figure 2).

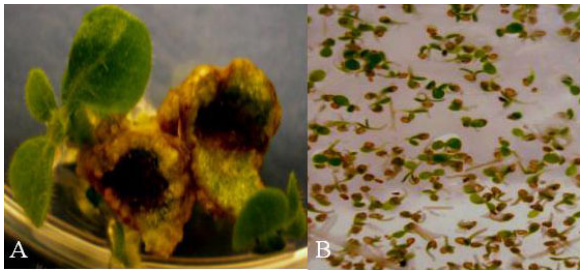

Figure 2. A) Direct shoot formation from cut leaf explants of *Nicotiana* after co-cultivation with *A. tumefaciens* GV 3101 with PAL 1 construct on hygromycin selection medium. B) Germination of *Arabidopsis* T1 seeds (transformed with PAL 1) on hygromycin selection medium.

PCR experiments, using the *hptII*and *nptII* specific primers provided molecular evidence of the transgenic nature of plants resistant to hygromycin or kanamycin for both *Nicotiana* and *Arabiodopsis*.

The expected sizes of the amplified fragments i.e. 708 bp from the kanamycin resistance gene (*nptII*) and 479 bp from the hygromycin phosphotransferase gene (*hptII*) were obtained in all samples using as template the total DNA extracted from plants growing on selection media (Figure 3).

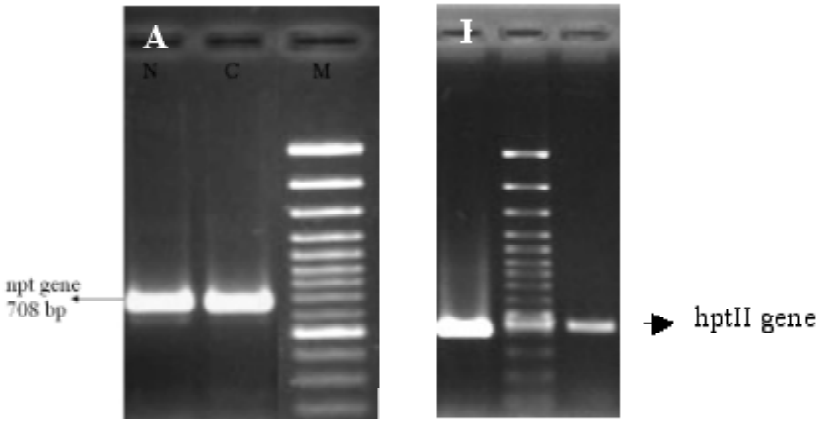

Figure 3. Confirmation of transgenic nature of control transformants of *Nicotiana* and *Arabidopsis* by PCR using *nptII* gene primers for kanamycin. Confirmation of transgenic nature of transformants (with *Coffea* PAL1 insert) of *Nicotiana* and *Arabidopsis thaliana* by PCR using *hptII*gene primers for hygromycin (407bp).

The *Arabidopsis* transformed plants are now under controlled conditions at the T1 generation. They will be used to produce T2 plants, which will serve for further molecular and biochemical studies. Some contamination problems on the tobacco plants obliged us to restart the transformation. Differences on plant growth and major metabolite of interest (chlorogenic acid) in model plants will be checked in plants transformed by each PAL paralog either in sense (overexpression) or antisense (suppression) orientation.

ACKNOWLEDGEMENT

The author GP is thankful to Department of Science & Technology, Govt. of India, for awarding Boyscast Fellowship. This work was financed by IRD and the Indo-French Center for the Promotion of Advanced Research (IFCPAR).

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Cold Tolerance Evaluation in *Coffea* sp. Impact on the Photosynthetic Apparatus and the Control of Oxidative Stress Assessed by Gene Expression Analysis

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SUMMARY

Among *Coffea* sp., some differential ability to cold acclimation has been observed. During previous work of our group, cold acclimation in *Coffea* sp. was associated to the maintenance of membrane function and the strengthening of control mechanisms against oxidative stress (Campos et al., 2003; Ramalho et al., 2003). In order to understand the molecular impacts and the mechanisms that allow plants to cope with chilling exposure, the expression of 24 genes related to oxidative stress control and photosynthesis (Fernandez et al., 2004) has been studied by means of semi-quantitative RT-PCR. From the 24 genes, 18 seem to be related to cold-response, 10 of which might be involved in cold tolerance mechanisms. Here we present preliminary data concerning the expression analysis of three genes, *caCP24*, *cacytf*, and *cadhar*, by means of qRT-PCR.

INTRODUCTION

Low temperature chilling is one of the major stresses that limit plant productivity and the geographical distribution of many important crops. Low temperature stress is of particular importance for coffee production, since the two economically most important coffee species (*Coffea arabica* and *C. canephora*) are sensitive to low non-freezing temperatures (Ramalho et al., 2003; DaMatta et al., 1997). Low positive temperatures are known to depress growth, photosynthetic performance and yield (Ramalho et al., 2003; DaMatta et al., 1997; Bauer et al., 1985; DaMatta and Ramalho, 2006). Among the cell structures, chloroplasts are quickly and deeply affected (Kratsch and Wise, 2000) and in chilling-sensitive plants, such as coffee, net photosynthesis ceases almost completely at 5-10 °C (Larcher, 1981). Under such conditions, the triggering and control of oxidative stress is of utmost importance, since the use of photochemical energy is greatly diminished. The aim of the present work was to analyze the expression of coffee genes related to oxidative stress and photosynthetic response to low non-freezing temperatures.

MATERIALS AND METHODS

Plant material and growth conditions

The experiments were carried out as described in Campos et al. (2003) with minor modifications, using 1.5 years old plants from genotypes *C. canephora* cv. Apoatã (IAC 2258), *C. arabica* cv. Catuaí (IAC 81), Icatu (IAC 2944 – *C. canephora* x *C. arabica*) and *C. dewevrei*. The plants were transferred into a growth chamber (700EDTU, ARALAB, Portugal) and submitted successively to: 1) a gradual temperature decrease (0.5 ºC/day) from 25/20 ºC to 13/8 ºC (day/night), over 24 days, 2) a 3 day chilling cycle (13/4 ºC), where the plants were subjected to 4 ºC during the night and in the first 4h of the morning (thus, concomitantly with light), followed by a rise to 13 ºC applied throughout the rest of the diurnal period, 3) a rewarming period of 6 days at 25/20 ºC, in order to allow recovery. Photoperiod was set to 12 h, RH to 65-70% and PPFD to *ca*. 750 μmol m^{-2} s^{-1}. Determinations were made in the 2 top pairs of mature leaves from each branch in 6-8 plants per cultivar.

Real-time quantitative PCR assays

cDNA sequences were provided by Dr. Diana Fernandez, IRD, Montpellier. The homologies of the studied genes are summarized in Table 1. Based on the cDNA sequences, specific primers were designed in order to perform the expression studies.

Individual PCR reactions were carried out with the SYBR Green method in 20 μl final volume according to the manufacturer's instructions (qPCR Core kit for SYBR® Green I (Eurogentec). The *Coffea arabica* ubiquitin gene (*caubi*, accession n. AF297089) was used as internal control. Real-time PCR was conducted in the iQ™5 system (Bio-Rad) using the program: 95 ºC – 3 min; 95 ºC – 30 s, 60 ºC – 30s, 72 ºC – 30s (35 cycles) and 55 ºC – 10 min. The analysis of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In order to validate our method, amplifications were performed on diluted samples, using primers for the reference gene (*caubi*) and for the target genes (*caCP24*, *cacytf* and *cadhar*). After validation of the method, results of each sample were expressed in N-fold changes in treated target genes copies, normalized to *caubi* relative to the copy number of the untreated (control = 20/25 ºC) target gene, according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{target} - Ct_{Caubi})_{treatment} - (Ct_{target} - Ct_{Caubi})_{control}$ (Livak and Schmittgen, 2001).

Table 1. Genes and homologies.

Gene	Homology
caCP24	Chlorophyll a/b-binding protein CP24
cacytf	Cytochrome *f*
cadhar	Dehydroascorbate reductase

RESULTS AND DISCUSSION

As it can be seen in Fig. 1, only in Icatu a sharp accumulation of *caCP24*, *cacytf* and *cadhar* transcripts was observed at the middle of the acclimation period. At the end of that period (13/8 ºC) and after chilling exposure (3x13/4 ºC), the amount of transcripts decreased in all genotypes, but the levels were always higher in Icatu (except for *cacytf* at 13/8 ºC). In general, upon recovery (Rec 25/20 ºC) the transcriptional activity of all genes was enhanced in all

genotypes, particularly in Icatu, with the exception of *cadhar* were a decrease was observed (despite the presentation of the highest *cadhar* expression amongst genotypes). The results seem to be in agreement with the data reported in Ramalho et al. (2003; 2006), where it was shown that, among the studied genotypes, Icatu had higher photosynthetic tolerance to low temperatures, which could be attributable to the reinforcement of the electron transport chain and antioxidative system components.

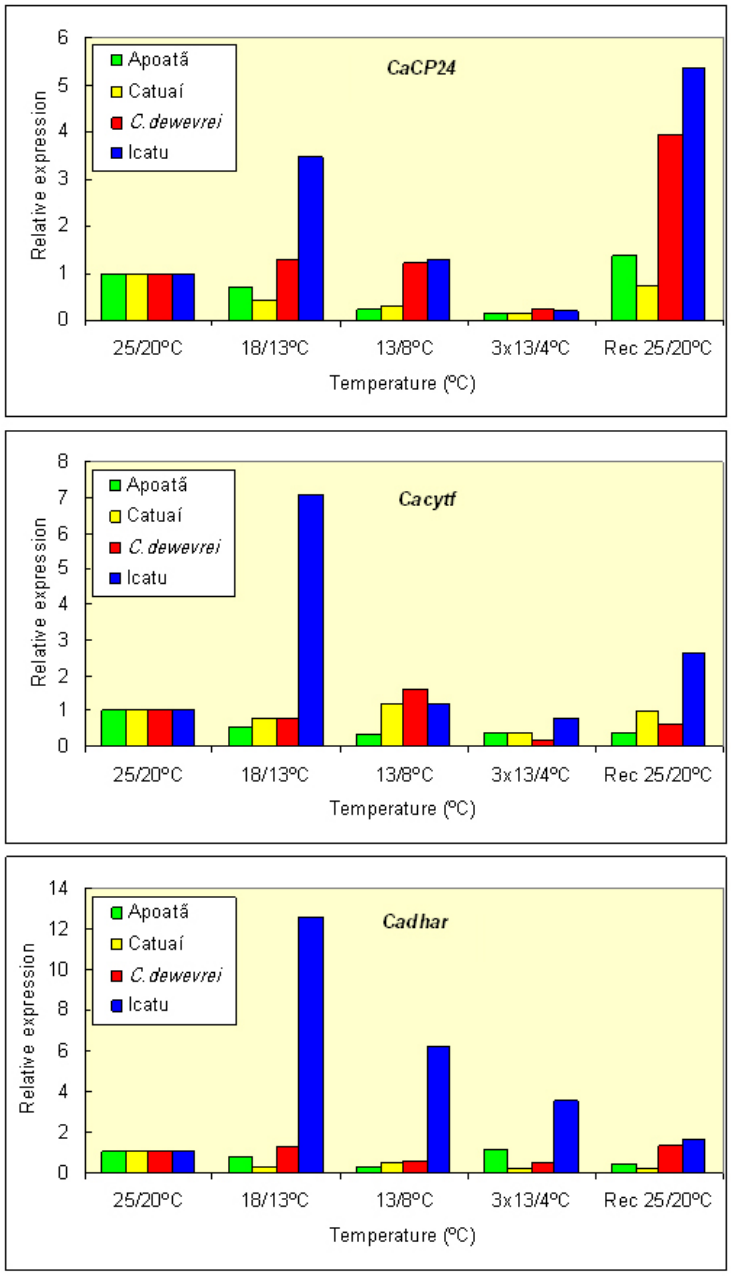

Figure 1. Relative expression level of *caCP24*, *cacytf* and *cadhar* monitored by real-time PCR. 25/20 °C – control; 18/13 °C – middle of the acclimation period; 13/8 °C – end of the acclimation period; Rec 25/20 °C – end of the recovery period. Each of the gene under study was normalised against *caubiquitin* gene.

The early produced transcripts (and, eventually, proteins) might have "prepared" the plants, allowing them to endure the chilling conditions and is in agreement with the higher photosynthetic functional levels at PSII (related to *caCP24*), PSI (related to *cacytf*) and higher ROS protection (related to *cadhar* expression) observed on this genotype (Campos et al.,

2003; Ramalho et al., 2003; Ramalho et al., 2006). Surprisingly, Catuaí, that is considered moderately tolerant to cold (Campos et al., 2003; Ramalho et al., 2003) has shown a pattern of transcript accumulation very similar to that observed in *C. devewrei* and Apoatã (considered as susceptible genotypes), suggesting that post-transcriptional regulation or other mechanisms are underlying cold tolerance in this genotype. Further experiments are in progress in order to increase the knowledge of cold tolerance mechanisms in coffee.

ACKNOWLEDGEMENTS

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Sucrose Metabolism during Fruit Development of *Coffea racemosa*

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SUMMARY

Sucrose metabolism was studied during the of fruit maturation of *Coffea racemosa*. Cherries were harvested regularly after anthesis up to the complete maturation occurring at around 70 days after flowering (DAF). This development was followed by measuring individual weight of pericarp, perisperm and endosperm tissues at regular (every 2 weeks) time after flowering. Along the development, the pericarp was the main tissue, representing around 70-80% of fresh weight of cherries. After 10 DAF, the perisperm decreased rapidly and was replaced by the growing endosperm. Sucrose content appeared relatively stable during endosperm development, representing around 8% of fruit dry weight at the mature stage. Acid invertase and sucrose synthase activities were also monitored in the endosperm. No invertase activity was observed in developing endosperm of *C. racemosa*, confirming the limited role of this enzyme in sucrose metabolism of coffee fruits. On the other hand, sucrose synthase showed highest activities at around 40 DAF, coinciding with endosperm expansion phase. The sucrose synthase activity also paralleled sucrose accumulation observed at the same stage in this tissue.

INTRODUCTION

Sucrose metabolism was studied during fruit maturation of *Coffea racemosa* fruits since the flowering to mature fruit cycle of this species is around three times more rapid than in *C. arabica.* For that, sucrose metabolism was investigated in separated tissues (pericarp, perisperm and endosperm) of *C. racemosa* fruits under development.

MATERIALS AND METHODS

*Plant material***:** Fruits of *C. racemosa* were collected from a unique plant from the germoplasm collection at IAPAR (Instituto Agronômico do Paraná, Londrina-PR Brazil). Harvests were performed every two weeks, from flowering (27/10/2004) towards full ripening (03/01/2005). Collected fruits were rapidly frozen in liquid N_2 and analyzed for sugar content and enzymatic activities as described previously (Geromel et al., 2006).

RESULTS

Characteristics of C. racemosa *fruit growth*: In field conditions, fruits of *C. racemosa* completed their development in around 70 days (Figure 1). Perisperm tissue disappeared

rapidly between 12 and 40 days after flowering (DAF), concomitant to the rapid growing of the endosperm occurring between 26 and 40 DAF (Figure 2A). Along the fruit development, the pericarp was the main tissue, representing around 70-80% of total fresh weight (FW).

Figure 1. Fruit development of *C. racemosa*: flowers at anthesis stage (1), green fruit at around 40 DAF (2) and fruit at the mature stage, at around 70 DAF (3). Photos were kindly provided by Dr. Oliveiro Guerreiro Filho, Agronomic Institute of Campinas (IAC), Campinas – SP, Brazil.

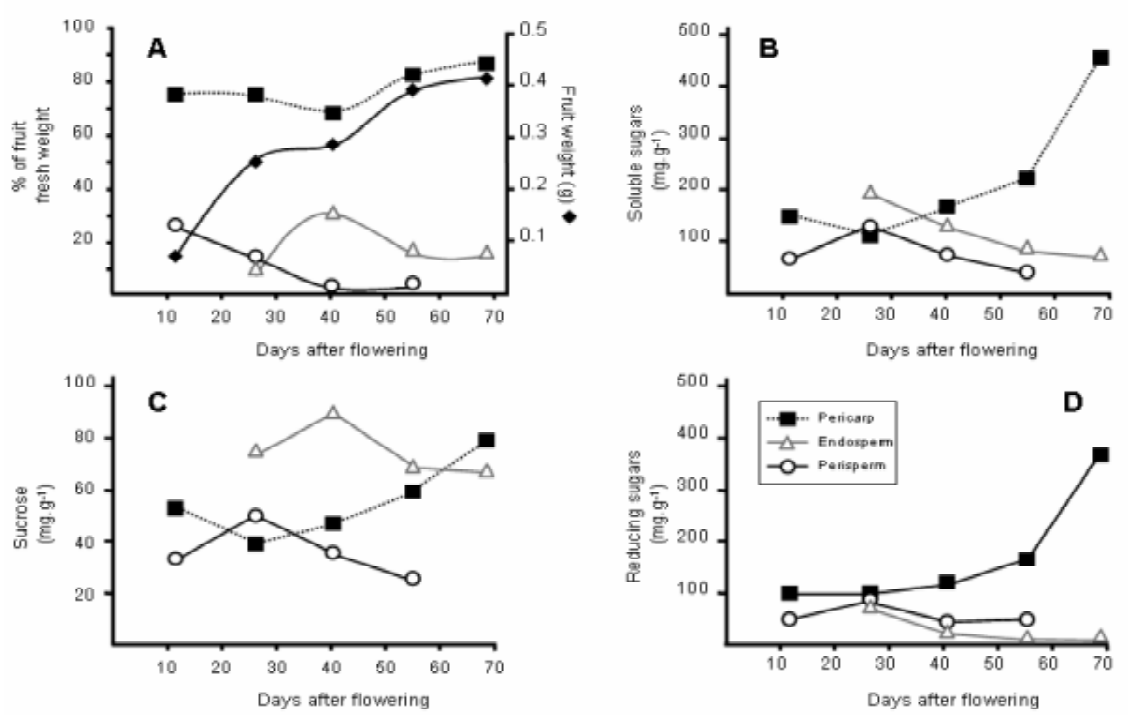

Figure 2. Weight of tissues and sugar contents during *C. racemosa* fruit ripening. (A) Evolution of cherry fresh weight-FW (◆) and pericarp (■), perisperm (○) and endosperm (Δ) tissues expressed in percentage of cherry FW. Contents of total soluble sugars (B), sucrose (C) and reducing sugars (D) in isolated tissues were expressed as mg.g^{-1} dry weight.

Evolution of sugar contents during C. racemosa *cherry development*: Total soluble sugar, as well as sucrose and reducing sugar (mainly glucose and fructose) contents were measured in separated tissues (pericarp, perisperm and endosperm) of developing fruits of *C. racemosa* (Figure 2B-D). Sugars (soluble, reducing) and sucrose accumulated gradually during pericarp development, particularly during the two weeks before the maturation. Sucrose contents

always appeared quite high during endosperm development (Figure 2C), reaching a maximal value (90 mg g^{-1} DW) at 40 DAF and 68 mg g^{-1} DW at the mature stage.

Enzymatic activities in developing endosperm of C. racemosa: Low levels of acid invertase were observed in the endosperm (Figure 3) throughout the maturation. However, sucrose synthase (SUS) activity showed a peak at 40 DAF and decreased after up to the mature stage.

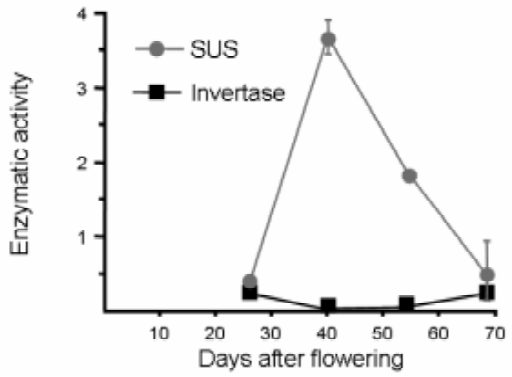

Figure 3. Activity profiles of sucrose synthase (SUS) and invertase in endosperm of *C. racemosa* fruits under development. Invertase activity (■) was expressed as µg reducing sugars. hr^{-1} $µg^{-1}$ protein. SUS activity (●) was assayed in the sense of sucrose synthesis and expressed as µg sucrose. hr^{-1} $µg^{-1}$ protein. Time scale is given in days after flowering.

DISCUSSION AND CONCLUSION

In *C. arabica* and *C. canephora*, the first tissue to develop soon after the fecundation is the perisperm followed by the endosperm (De Castro and Marraccini, 2006). Even reduced to a few cell layers ("silver skin") surrounding the endosperm in the latest stages of coffee fruit development, recent works showed that the perisperm retains gene expression (Geromel et al., 2006) and enzymatic activities (Geromel et al., in these proceeding) confirming the important contribution of this tissue for endosperm development. Recent studies also indicate that the maximal volume occupied by the perisperm in earlier stage of fruit development predetermines the final size of mature beans (Geromel et al., in these proceeding; Rogers et al., 1999). The results presented here, showing that maximal endosperm FW at 40 DAF also corresponded to perisperm FW at 12 DAF, also corroborates such a control. In addition, sucrose content in the endosperm appeared quite high and stable during the development, a situation that differed from that previously reported in *C. arabica* (Geromel et al., 2006). Negligible acid invertase activity was observed in the endosperm, therefore confirming the limited role of this enzyme regarding sucrose metabolism in this tissue (Geromel et al., 2006; Geromel et al., in these proceeding). SUS activity in endosperm showed a peak at 40 DAF that overlapped the time of intensive endosperm development which also coincided with the peak of sucrose accumulation in this tissue. Expression analysis of SUS-encoding genes is underway and should permit to better define the biological functions of SUS1 and SUS2 isoforms of SUS (Geromel et al., 2006) during coffee fruit development.

ACKNOWLEDGMENTS

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Transcription Factors Associated with Flowering and Fruit Ripening in Coffee Plants

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SUMMARY

The completion of the Brazilian coffee genome project in addition to new developed technologies, such as transcriptomics, can be used to identify and to investigate genes associated with key events during coffee plant development. One of the main problems in coffee plant is the unsynchronized flowering causing an uneven fruit ripening, leading to more difficult harvesting, and reducing the quality of the final product. Transcription factors play important roles in various developmental processes controlling the expression of important genes. To understand the mechanisms involved during flowering and fruit ripening, it is necessary to analyze the cellular behavior at various stages of such processes which can unravel the molecular genetic mechanisms underlying them. These mechanisms are complex network of factors interacting during flowering and fruit ripening controlling these two processes in an activation or suppression way. Thus, this work aims to study the transcription factors associated with coffee flowering and fruit ripening. Therefore, various stages during flower development and fruit ripening will be used for RNA extraction. RNAs will be converted to radioactively labeled cDNAs and hybridized against a macroarray made with genes from the Brazilian Coffee EST project.

INTRODUCTION

One of the main problems in coffee plant is the unsynchronized flowering which can cause an uneven flowering, leading to unequal fruit set. This makes more difficult harvesting, consequently, lower quality of the final product. Transcription factors are proteins that bind to DNA sequence being able to control their transcription as activators or suppressors. Therefore, they play important roles in various developmental processes controlling the expression of several genes. To understand the mechanisms involved during fruit flowering and fruit ripening, it is necessary to analyze the cellular behavior at various stages of such processes. This work aims to study the transcription factors associated with coffee flowering and fruit ripening, trying to set light on these complex traits in order to unravel the molecular genetic mechanisms underlying them.

EXPERIMENTAL SET UP

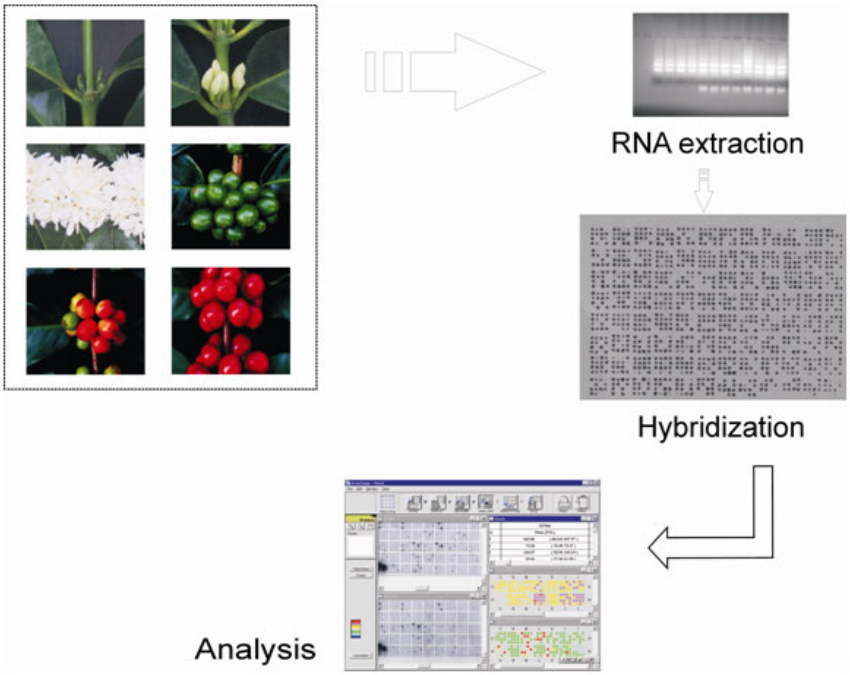

Figure 1. Experimental setup. RNA of different stages of coffee flowering and ripening are extracted and cDNAs are synthetized to be hybridized against the macroarray membrane. Membranes are spotted with cDNAs from several coffee libraries of the Brazilian Coffee EST project. Images are read using the FLA3000 scanner (FUJIFILM) and data collected and analysed using the Fujifilm ArrayGauge™ software.

FOLLOWING UP

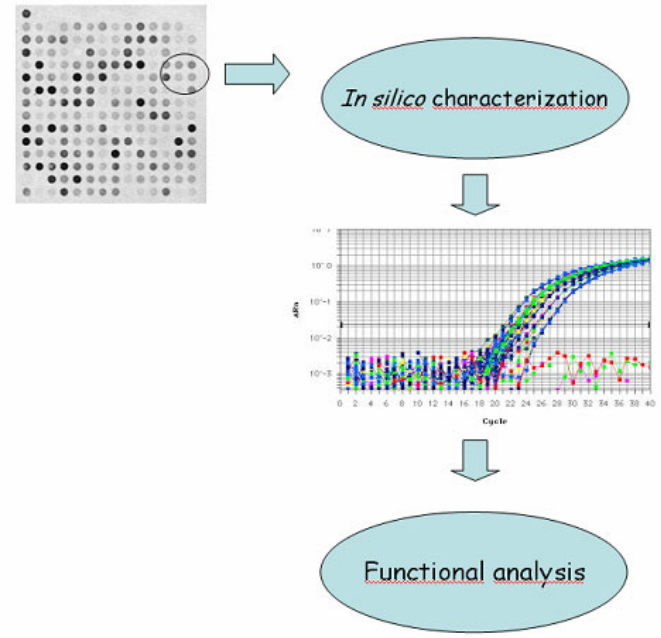

Figure 2. Schematic representation of further analysis of the selected genes. Criteria for selecting genes will be based on the expression level (at least 2-fold up- or down-regulation at P ≤ 0,05) and on *in silico* characterization.

Based on their expression profile, genes will be selected upon the several stages and validated using Quantitative Reverse Transcription PCR (qRT-PCR). Candidate genes will be used for further functional analysis Comparative analysis will be performed using available databases for picking up those whose expression are either down or upregulated during flowering and ripening. A selection of the most interesting genes will be done, taking in account previous knowledge of key genes in these processes.

HETEROLOGOUS EXPRESSION

Candidate genes will be further characterized by heterologus expression using the *Arabidopsis thaliana* model plant. Arabidopsis plants will be transformed by the floral dip-based method (Clough and Bent, 1998). Phenotypic analysis will be carried out in these plants with respect mainly to flowering and ripening stages

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Sequence Analysis from Leaves, Flowers and Fruits of *Coffea arabica* var. Caturra*

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*Research co-sponsored by the Colombian Ministry of Agriculture and The National Science Foundation Plant Genome Research Program

SUMMARY

Coffee is one of the main agricultural products in Colombia and increased agronomic and genetic knowledge is necessary to establish effective strategies for its improvement. Currently, molecular studies are in progress to identify genes of agronomic importance. In this study, we report on cDNA libraries from three different tissues (leaf, flower, and fruit) from *C. arabica* var. Caturra which were developed to provide data on genes expressed in leaf, flower, and fruit tissues. In total, 32,961 sequences were generated and following cleaning, clustering and assembly, collapsed into 10,799 unique sequences. Putative homologs for the *C. arabica* sequences could be found in Arabidopsis and rice, finished dicot and monocot genomes. Furthermore, putative homologues could be identified in publicly available EST collections of *Coffea canephora* as well as in the Solanaceae species, *Lycopersicon esculentum* and *Solanum tuberosum.* Annotation of the sequences revealed a diversity of genes including similarity to proteins involved in metabolism, protein synthesis, and cellular defense. This distribution agrees with previous studies made in tomato, where transcripts were mainly involved in metabolic processes of transcription and protein synthesis. These categories were also found in Arabidopsis and can represent a conserved set of metabolic functions in plants.
Key words: *Coffea arabica*, cDNA libraries, ESTs, sequence similarity, putative function., genomics

INTRODUCTION

Coffee is an important international agricultural, species, providing large resources for the economies of many developing countries. Despite its economic importance, coffee has received little attention with respect to molecular genetics and genomic research. Such information is important in order to modify or introduce traits of interest in the species through breeding programs.

Coffea arabica is the only tetraploid species (2n = 4x = 44) in the *Coffea* genus and is generally self-incompatible. According to recent reports, *C. arabica* is an allopolyploid formed by hybridization between two closely related diploid species (2n = 22), *Coffea eugeniodes* and *Coffea canephora* (Lashermes et al., 1999). *C. arabica* is characterized by low genetic diversity which has been attributed to its allotetraploid origin, reproductive biology, and evolutionary process of this species.

The recent development of applied technologies in biology is leading to an enormous production of information in the area of plant genomics through the sequencing of different organisms. Large-scale sequencing of cDNAs to produce Expressed Sequence Tags (ESTs), was initially used for the model species *Arabidopsis thaliana* (Höfte et al., 1993) and rice (Yamamoto and Sasaky, 1997), that together with maize, wheat, tomato and soybean ESTs projects represent more than 50% of the total entries in dbEST (GenBank http:// www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) although ESTs sequences from a number of other species including coffee have been deposited in dbEST. As of July 2006, 47,999 gene sequences from coffee had been deposited in the dbEST, mostly from *C. canephora*. ESTs provide not only a rapid and cost-effective generation of data on gene expression and regulation, but also a practical tool to potentially identify new genes, to identify polymorphic markers useful for construction of genetic maps and to quantify expression data for digital expression analysis. As a result of advances in computational molecular biology and biostatistics, it is possible analyze large-scale EST datasets efficiently and exhaustively (Ronning et al., 2003; Ogihara et al., 2003).

Recently, Lin et al. (2005) reported on 47,000 cDNA sequences of *C. canephora,* their putative function, the expression profiles in different tissues, and a comparative analysis with *Arabidopsis* and Solanaceae species, particularly tomato. In this study, we report the isolation and characterization of ESTs from tissue-specific cDNA libraries of *C. arabica.*.

MATERIALS AND METHODS

Molecular methods

Total RNA was extracted from pre-anthesis flowers (CACF), 22 week old fruit (CACB), and young leaves (CAFL) of *C. arabica* var. Caturra using RNAgents total RNA isolation system (Promega, Madison, WI). mRNA was obtained by magnetic separation of poly $(A)^+$ mRNA using PolyAtract mRNA isolation system (Promega, Madison, WI), and its integrity was examined using agarose gel electrophoresis. 24-200 ng mRNA was reversely transcripted to single-stranded cDNA by PowerScript Reverse Transcriptase at 42°C for 1 hr. First-strand cDNA was synthesized with a *Sfi* I-Oligo (dt) adapter-primer (CDSIII/3' primer). The resulting single strand cDNA was amplified by PCR using CDSIII/3' primer and 5' primer (5' PCR primer) following parameters 24 cycles 95°C for 15 s, 68°C for 6 min.

The double strand cDNA synthesis and library constructions were carried out mainly according to the manual of Creator Smart cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). After second-strand synthesis, cDNA was digested by Sfi I enzyme. Digested cDNAs were size-fractionated with Chroma Spin-400 columns and ligated in to the pDNR-LIB vector predigested by Sfi IA/Sfi IB. Ligations were used to transformation of recombinant plasmid into DH10B electrocompetent cells *E. coli*. Recombination efficiency was screened by performing PCR directly on colonies and by digestion of miniprep DNA with Sfi enzyme to excise inserts.

Bacteria containing coffee cDNAs were cultured in 384-well pates and cDNAs inserts sequenced by Rexagen (Seattle, WA). Sequencing reactions were performed on ABI 3700 sequencers using the T7 primer which anneals at the 5' end of cDNA.

Bioinformatic methods

The vector-derived sequences, low quality sequences, and ambiguous sequences were removed from each EST using the Lucy program (Chou and Holmes 2001). After trimming,

the sequences were screened to remove adapters, vector, and *E. coli* contamination using Seqclean. Contigs were assembled from redundant reads using the CAP3 program (Huang and Madan 1999) using 95% identity, overlap of 50 and overhang of 20; only sequences equal or longer than 100 bases were included in the assemblies. Blastn and tblastx based on an E value cutoff of 1e-5 were performed against Arabidopsis, rice, and Uniprot reference databases to annotate the coffee sequences. The predicted coffee peptides were subjected to InterProScan annotation, which integrates the most common used protein signature databases and gene ontology annotation. We also performed a pairwise comparison between tissues to identify shared and tissue-specific sequences.

RESULTS AND DISCUSSION

Quality of cDNA libraries

The number of ESTs generated from each library is summarized in Table 1. The number of good sequences per library was on average 67%. The length before and after trimming was on average 791 and 364bp, respectively.

Table 1. Number and length of sequences for each library.

	Fruit	Flowers	Leaves
No. trace files	9531	11406	12024
No. good sequences	6284	8707	8875
Seq %	66%	76%	59%
Average Length of sequences:			
Raw	794	821	758
After Lucy trimming	314	376	331
After seqclean	329	377	400

In Table 2, the assembly results are shown. In total, there are 10,799 non-redundant sequences (contigs plus singleton ESTs). These sequences were analyzed using Blastx against rice, Arabidopsis and Uniprot databases.

Comparative analyses with the *C. arabica* sequences

In Table 3, the number of homologues found in the Arabidopsis, rice, and the Uniprot databases are shown. The ability to readily detect putative homologs within our *C. arabica* sequences is consistent with a high degree of conservation in highly to moderately expressed genes which have conserved function throughout plants. The flower and leaf libraries have more putative homologues than the fruit library which may be suggestive of more diverged genes required for fruit development or genes which are novel to coffee.

Examination of the putative homologues in Arabidopsis, rice and Uniprot revealed a diversity of genes within our sequences. The main categories include proteins involved in metabolism, protein synthesis, and cellular defense similar to previous studies in tomato and Arabidopsis

Comparison of our *C. arabica* sequences (contigs and singleton ESTs) with assembled ESTs from *C. canephora* revealed a putative homolog for 62% of the *C. arabica* sequences using a relaxed cutoff criterion of E-value < 1e-5. Using a more stringent cutoff of > 95% identity over 10% length of the *C. arabica* sequence, a putative homolog could be found in *C. canephora* for 45% of the *C. arabica* sequences. With respect to the Solanaceae, a putative

homologue could be found in assembled ESTs of *Lycopersicon esculentum* and *Solanum tuberosum* for 58 and 59% of the *C. arabica* sequences, respectively.

Table 2. CAP3 assembly results.

	Fruit	Flowers	Leaves	Combined
No. input sequences	6284	8707	8875	23866
Total No. contigs and Singletons	3637	4911	4797	10799
No. of contigs	761	1132	1127	3181
No. of singletons	2876	3779	3670	7618
Avg_contig length	509	598	571	419
Total ESTs clustered	3408	4928	5205	16248
Avg. EST/contig	4,5	4,4	4,7	5,1

Table 3. Number of putative coffee homologues in Uniprot, Arabidopsis, and rice.

	Total No. Sequences	Uniprot	%	Arab	%	Rice	%
Fruit	3637	1659	46%	1648	45%	1556	43%
Flower	4911	2948	60%	2888	79%	2735	75%
Leaves	4797	3398	71%	3294	69%	3147	66%
Combined	10799	6450	60%	6295	58%	5968	55%

Pairwise comparison of the *C.arabica* sequences was performed to identify those sequences which are tissue-specific. Table 4 shows that a significant proportion of sequences are tissue-specific which is consistent with previous reports which show that a majority of transcripts are expressed in more than one tissue.

Table 4. Pairwise comparison of *C.arabica* ESTs

	Database		
	Fruit	Flower	Leaves
Fruit	-	1240	1237
Flower	1242	-	1254
Leaves	1290	1295	-

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Characterisation of Three Ethylene Receptor Genes in *Coffea canephora* and their Expression in Model Plants

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SUMMARY

The full-length cDNAs corresponding to genes encoding three ethylene receptors: CcETR1, CcETR2 and CcEIN4 were isolated from *Coffea canephora* cDNA libraries (fruit and leaves). The corresponding genomic sequence of each gene was characterized from at least one *C. canephora* BAC clone. Three full length CcETR1 cDNAs were isolated they differed only by the length of their 3'UTR, with three putative polyadenilation sites. The 5'UTR contains a short ORF, interrupted by one intron, and capable to encode a 35 amino acids polypeptide, which has no similitude with known proteins. The CcETR1 ORF itself is 2,192 bp long, it contains five introns. The fifth intron is sometimes alternatively spliced leading to a polypeptide that lacks the receiver domain. *CcETR2* and *CcEIN4* are less complex. Their ORF is 2289 and 2298 bp long respectively and both have only one intron. In order to study the relationship between ethylene perception, fruit ripening time and caffeine content in some *Coffea* species, *CcETR1* segregation has been analysed in a population issued from a back cross between *C. dewevrei* and *C. pseudozanguebariae*. Some quantitative traits were found to be influenced by this gene: presence of PSE allele in a plant induces a 27 days reduction of the fructification cycle, 24.7% decrease in the green beans caffeine content and a 11.5% lowering of the seed weight. Over-expression of these genes in *Arabidopsis thaliana,* is discussed.

RÉSUMÉ

Les ADNc pleine longueur correspondant aux gènes codant trois récepteurs d'éthylène: CcETR1, CcETR2 et CcEIN4 ont été isolés à partir de banques d'ADNc de *Coffea canephora* (fruits et feuilles). La séquence génomique de chaque gène a été caractérisée à partir d'au moins un clone BAC de *C. canephora*. Trois ADNc de CcETR1 ont été isolés; ils ne se différencient que par la longueur de leur 3' UTR, indiquant la possibilité de trois sites alternatifs de polyadenilation. Le 5' UTR contient une ORF, intérrompue par un intron et capable de coder un polypeptide de 35 acides aminés qui n'a aucune similitude avec des protéines connues. L'ORF de CcETR1 couvre 2.192 pdb avec 5 introns. Le cinquième intron est quelques fois alternativement épissé donnant un polypeptide dont le domaine récepteur manque. CcETR2 et CcEIN4 sont moins complexes. Ils ont une ORF de 2289 et 2298 pdb respectivement chacune interrompue par un seul intron. Afin d'étudier la relation entre la perception de l'éthylène; le temps de maturation du fruit et le contenu en caféine chez quelques espèces de *Coffea*, la ségrégation de *CcETR1* a été analysée dans une population issue d'un backcross entre: *C. dewevrei* et *C. pseudozanguebariae*. La forme allèlique de *CcETR1* a un effet sur la durée du cycle de fructification, sur la teneur en caféine des grains et sur le poids de 100 graines. La surexpression de ces gènes dans *Arabidopsis thaliana* est discutée.

INTRODUCTION

The phytohormone ethylene plays a central role in physiological and developmental processes, such as germination, growth, flower initiation, leaf and flower senescence, organ abscission and fruit ripening (Abeles et al., 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 induces transcription of a wide range of genes involved in wound signalling and defence.

A family of five receptors mediates ethylene perception in Arabidopsis: ETR1, ERS1, ETR2, ERS2, and EIN4 (reviewed in Guo and Ecker, 2004)). 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 contains additionally a putative signal sequence in the aminoterminal region that could target the proteins to the secretory pathway. They have a degenerated hystidine kinase domain. Two of the receptors (ERS1 and ERS2) lack a receiver domain at the C terminus.

Species from the *Coffea* genus show great differences in fruit ripening time. In *C. pseudozanguebariae* (PSE), a wild East African species, the fruit ripens 9 weeks after anthesis, while in other species this process lasts much longer, generally about 35 to 37 weeks (Hamon et al., 1984). Caffeine content of PSE green beans is nil, while in the two major cultivated species, *C. arabica* (ARA) and *C. canephora* (CAN) it varies from 1.2% to 2.8% (dmb) respectively (Anthony et al., 1993).

Co-location of an ethylene receptor encoding gene and a QTL related to the fruit quality has been established for melon (Perin et al., 2002). In *Coffea*, a major gene related to the fructification time was mapped on an interspecific ((PSE x DEW) x DEW) genetic map (Akaffou et al., 2003) and this gene had an effect on the caffeine content of green coffee beans.

Up to date, little information is available on coffee fruits response to ethylene despite the fact that coffee trees are of the climacteric type (Pereira et al., 2005). Previously, we had characterised the *CcERF2* gene. This gene encodes a transcription factor localised at the end of ethylene signal cascade and it might be related with fruit ripening (Bustamante et al., 2005). Now, we present the isolation and characterization of the first three genes encoding ethylene receptors in coffee plants (*CcETR1, CcEIN4* and *CcETR2*).

MATERIAL AND METHODS

Identification and characterisation of the ethylene receptor genes was carried out on CAN, PSE and DEW. Trees were grown under tropical conditions in a green house at the IRD centre in Montpellier, France. A cross between PSE (female) x DEW (male) was made at IRD Agricultural Station (Man, Côte d'Ivoire). Sixty two backcross offspring on DEW (BCDEW) were obtained by open pollination of thirteen F1 hybrids using DEW as pollen source. Leaf samples were collected and total DNA was extracted. cDNA libraries from young leaves and fruits at different development stages of CAN were screened. Then, specific primers were designed from the cDNA sequence for PCR amplification of full-length genes. Amplification was done for three species that differ in fruit ripening time and caffeine content, i.e. DEW, CAN and PSE. Total RNA from three different fruit ripening stages were isolated from CAN fruits. cDNA was synthesised, and then each cDNA was amplified by PCR using specific primers for each receptor gene. RT-PCR products were verified by restriction. Full length

coding sequences of *CcETR1*, *CcETR2* and *CcEIN4* were inserted into pCAMBIA1300 or pCAMBIA1305.1 plasmids, (CaMV 35S promoter). The resulting plasmids were introduced into Arabidopsis Columbia wild type by the floral dip procedure.

RESULTS AND DISCUSSION

Table 1 shows a general characterisation of three ethylene receptors genes in CAN. *CcETR1* shows some aspects that could indicate a strong regulation of its expression.

Table 1. General characterisation of 3 ethylene receptors genes in *C. canephora.*

	CcETR1 cDNA	CcEIN4 cDNA	CcETR2 cDNA
Long	2,649 bp. 2,683 bp. 3,162 bp.	2,906 bp.	2,985 pb.
ORF	2,223 bp.	2,298 bp.	2,289 pb.
Putative Protein	740 aa, 82.48 kDa	765 aa, 85.63 kDa	762 aa, 85.46 kDa
Identity	• 87.1% to ETR1 of Petunia x hybrida	• 74.4% to LeETR5 of Solanum lycopersicum • 35.3% to CcETR1	• 71.5% to LeETR4 of Solanum lycopersicum • 37.7% to CcETR1 • 59.7% to CcEIN4
GENOMIC SEQUENCE			
Introns in coding region	5	1	1
Intron size	1,240; 125; 95; 163; 1,148 bp.	2,045 bp.	652 bp.
Upstream Open Reading Frame (uORFs)	Yes, 35 aa	No	No
Intron in 5'UTR	Yes, 978 bp.	No	No

1. Three independently isolated full length cDNA clones had the same coding sequence and an identical 5'UTR, but they differed by the size of their 3'UTR suggesting that these three clones only differed by alternative polyadenilation sites.
2. The 5'UTR comprised a short putative open reading frame (uORF). This uORF is interrupted by one intron, which is conserved in some *Coffea* species.
3. RT-PCR analyses made on total RNA purified at different fruit development stages in CAN and PSE show that the primary transcript issued from *CcETR1* transcription had different figures of intron retention suggesting the presence of different alternative splicing sites.

CcETR2 increases its expression at the climacteric stage in ripening fruits. *CcEIN4* increases slowly its expression with fruit development. However, *CcETR1* is identically expressed in all stages of fruit development in CAN and PSE.

Interestingly, over-expression of *CcETR1* or *CcEIN4* in Arabidopsis etiolated plants grown on a medium without ethylene precursor (ACC) nor inhibitor of ethylene synthesis (AVG) yield a negative gravitropic growth in seedlings.

Segregation analysis of the *CcETR1* alleles in a backcross progeny of an interspecific cross (PSE x DEW) x DEW, showed an effect of the present alleles on some agronomical traits

such as: fructification time, time of endosperm maturation and weight of 100 seeds (P100) (Table 2). Presence of the PSE allele (P) in the genome of a plant, by opposition to a homozygote DEW-DEW situation (DD), induces a significant reduction of the fructification cycle by 27 days. This reduction in fructification time is explained at 77% by the reduction of the endosperm maturation period. On average, maturation of the endosperm in a heterozygote DP occurs 21 days prior to the homozygote DD. A DP heterozygote also shows a reduction of the caffeine content in its grains by 24.7%.

Table 2. Effects of *ETR1* alleles on fructification cycle, endosperm maturation, 100 seeds weight, and caffeine content in the fruits of a PSE-DEW progeny. DD: DEW homozygote for *ETR1*. PD *ETR1* heterozygote. %PSE: percentage of decrease in the heterozygote progenies.

Trait	DD	DP	%PSE	F	p
Duration in days of the fructification cycle (DCF)	279,813	252,391	9,8%	8,88	< 0,0051
Duration in days of the endosperm maturation (DN)	128,125	107,087	16,4%	8,48	< 0,0060
100 seeds Weight in g (P100).	10,247	9,064	11,5%	5,49	< 0,0215
Caffeine content	0,653	0,492	24,7%	8,76	< 0,0048

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Use of Leaf-Disk Technique for Gene Expression Analysis of the Coffee Responses to *Hemileia vastatrix* Infection

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SUMMARY

The most acknowledged method for coffee leaf-rust resistance evaluation uses leaf disks inoculated with *Hemileia vastatrix* and kept in moisture chambers. Besides an efficient control of inoculation conditions, this technique allows a simultaneous evaluation of innumerous plants, with diverse fungal race/coffee genotype combinations, and using low uredospore quantities. The objective of this study was to evaluate the suitability of this technique for the functional gene analysis of coffee responses to leaf-rust infection. A comparison of gene expression in the presence or absence of the pathogen was performed on intact leaves and on leaf disks. To avoid non-specific gene expression due to leaf injury, the leaf disks were prepared 24h and 48h before inoculation and kept moist. *Coffea arabica* plant samples of the resistant Obatã and the susceptible Ouro Verde cultivars were challenged with *H. vastatrix* race II and were collected 24 h after inoculation. Semi-quantitative reverse transcription (RT)-PCR and real time quantitative PCR were used to evaluate expression of several coffee genes. Genes known to be constitutively expressed such as the Glyceraldehyde 3-phosphate deshydrogenase gene or the Ubiquitine gene were used, as well as genes involved in disease resistance responses. Results demonstrated that overall there are differences in the gene expression patterns observed in leaves and disks, either prepared 24h or 48h before inoculation. The genes *PAD3* and *PR1b* showed induction in leaves and in the 48h-disks of Obatã upon rust fungus inoculation, and gene suppression in the 24h-disks treatment. The genes *WRKYs* were activated in leaves and suppressed in disks in the same cultivar. Opposite patterns of WRKY expression were detected in disks of Ouro Verde. Our results showed that most of the defense-related genes studied displayed altered patterns of gene expression compared to intact leaves. These results suggest that the leaf-disk technique cannot be successfully used for transcriptomic analysis of coffee-rust interactions.

INTRODUCTION

The orange rust (Hemileia vastatrix Berk. and Br.) is the most important fungal disease of coffee (*Coffea arabica*) in Brazil, and, depending on the defoliation intensity, yield loss, may account for 30% losses in coffee production (Kushalappa and Eskes, 1989).

The Instituto Agronômico de Campinas (IAC), São Paulo, Brazil, develops since the 70's, a genetic breeding program aiming at the development of resistant varieties to the rust fungus. In this program were generated the main resistant coffee cultivars of Brazil, as Icatu Vermelho, Icatu Amarelo, Icatu Precose, Tupi and Obatã.

Despite the high degree of resistance showed for some of these varieties, the fast development of new races of the fungus become the selection of resistant plants a non-stop work.

The most acknowledged method for coffee leaf-rust resistance evaluation uses leaf discs inoculated with *Hemileia vastatrix* and kept in moisture chambers (Eskes and Toma-Braghini, 1981). Besides an efficient control of inoculation conditions, this technique allows a simultaneous evaluation of innumerous plants, with diverse fungal race/coffee genotype combinations, and using low uredospore quantities. The objective of this study was to evaluate the suitability of this technique for the functional gene analysis of coffee responses to leaf-rust infection.

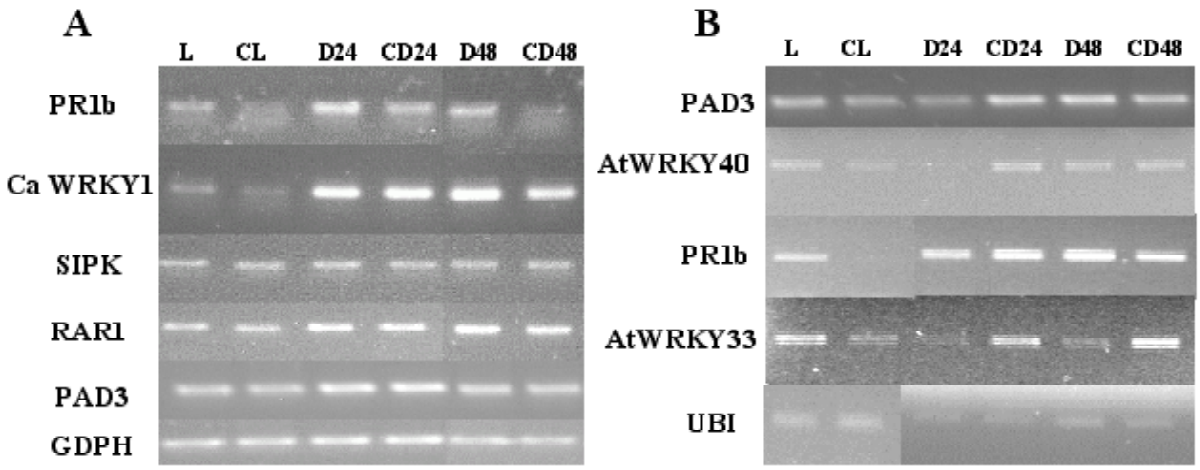
MATERIAL AND METHODS

A comparison of gene expression in the presence or absence of the pathogen was performed on intact leaves and on leaf disks. To avoid non-specific gene expression due to leaf injury, the leaf disks were prepared 24 h and 48 h before inoculation and kept moist. *Coffea arabica* plant samples of the resistant Obatã and the susceptible Ouro Verde cultivars were challenged with *H. vastatrix* race II and were collected 24 h after inoculation. Four eight-month-old coffee plants of each cultivar were kept under a regime of 16h light and 8h dark at 23 ± 2 °C during the assays. Two leaves per plant were inoculated *in situ* and the two opposites leaves were detached for the confection of the disks (1.8 cm diam.), with a cork borer. Each treatment was composed of eight disks. The leaves and disks were inoculated with droplets of 0.025ml spore suspension, with 1 mg spore/ml of distilated water.

Semi-quantitative and quantitative "real time" reverse transcription (RT)-PCR (Ganesh et al., 2006), was used to evaluate expression of several coffee genes previously isolated (Fernandez et al., 2004; Lecouls et al., 2006). Genes known to be constitutively expressed such as the *Glyceraldehyde 3-phosphate deshydrogenase* (*GDPH*) gene or the *Ubiquitine* (*UBI*) gene were used, as well as genes involved in disease resistance responses. The genes chosen for the analyses were: the WRKY transcription factors (*CaWRKY1*, *AtWRKY33*, *AtWRKY40*), bZIP transcription factor (*RAR1*), acid and basic pathogenesis-related gene (*PR1a* and *PR1b*), the salicylic acid induced protein kinase (*SIPK*), non-expressor of PR1 (*NPR1*) and the Cytochrome P450 (*PAD3*).

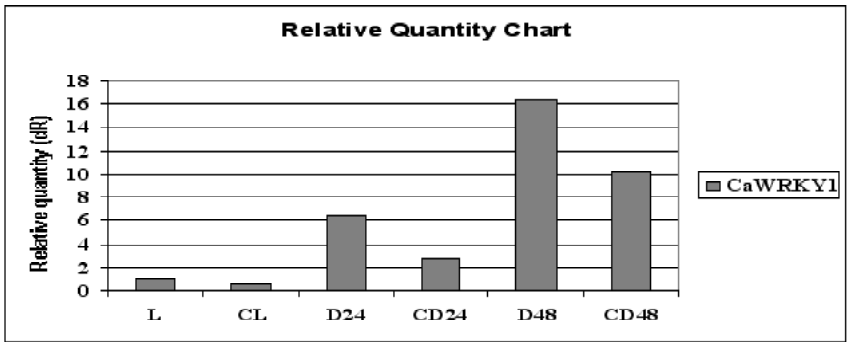
RESULTS

In the semi quantitative RT-PCR analyses, the susceptible cultivar Ouro Verde showed similar patterns of expression between leaves and disks (Figure 1A). The *CaWRKY1* and the *PR1b* were induced in inoculated samples as compared to control samples. Gene expression was slighly higher in inoculated disks than in inoculated leaves, but not in the control disks compared to the control leaves. The inoculation with *H. vastatrix* did not induce the genes *SIPK*, *RAR1* and *PAD3* in these treatments**.** However, in the resistant cultivar Obatã, there was a difference in the gene expression patterns observed in leaves and discs, either prepared 24 h or 48 h before inoculation (Figure 1B). The genes *PAD3* and *PR1b* showed induction in leaves and in the 48h-disks upon rust fungus inoculation. In the 24 h-disks, gene suppression occurred. The genes *WRKYs* were activated in leaves and suppressed in disks. The genes *SIPK* and *RAR1* were not induced and showed similar patterns of expression between leaves and disks in Obatã (data not shown).



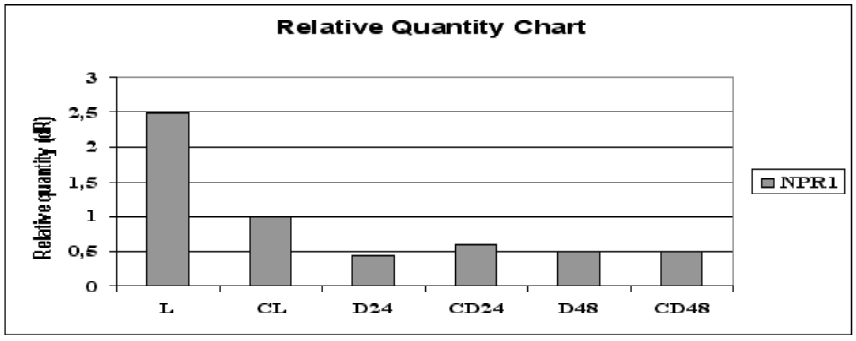
L = inoculated leaves; CL = non inoculated leaves; D24 = 24h-discs inoculated; CD24 = 24h-discs non inoculated; D48 = 48h-discs inoculated; D48 = 48h-discs non inoculated.

Figure 1. Expression of several genes in leaves and leaf discs of two *Coffea arabica* varieties, inoculated with race II of *Hemileia vastatrix*. (A) susceptible variety of coffee Ouro Verde; (B) resistant variety of coffee Obatã.



L = inoculated leaves; CL = non inoculated leaves; D24 = 24h-discs inoculated; CD24 = 24h-discs non inoculated; D48 = 48h-discs inoculated; D48 = 48h-discs non inoculated.

Figure 2. Relative expression of the gene *CaWRKY1* in leaves and leaf disks of the resistant cultivar Obatã of *Coffea arabica*, 24 hours upon orange rust fungus inoculation.



L = inoculated leaves; CL = non inoculated leaves; D24 = discs with 24h inoculated; CD24 = discs with 24h non inoculated; D48 = discs with 48h inoculated; D48 = discs with 48h non inoculated.

Figure 3. Relative expression of the gene *NPR1* in leaves and leaf disks of the resistant cultivar Obatã of *Coffea arabica*, 24 hours upon orange rust fungus inoculation.

In the Real Time RT-PCR analyses, the acid pathogenesis-related gene (*PR1a*), showed similar patterns of expression in leaves and disks treatments (data not shown). In the same way, the gene CaWRKY1 was suppressed in leaves and disks after inoculation in the resistant cultivar Obatã (Figure 2). The *NPR1* gene was quite suppressed in the discs treatments of the Obatã (Figure 3).

DISCUSSION

Our results showed that the stress provoked by the leaf disk cuttings altered the expression of many genes. When challenged with the rust fungus *H. vastatrix*, most of the defense-related genes studied displayed altered patterns of gene expression in coffee leaf discs compared to intact leaves.

Several genes involved in plant pathogen defence mechanisms are also activated by wounding (Cheong et al., 2002). The WRKYs transcription factors are a family of genes activated by many biotic and abiotic stresses. There are strong evidences that the WRKY genes are involved in defence genes regulation, acting upstream genes like PR1 and NPR1 (Yu et al., 2001; Liu et al., 2005). Increased expression of NPR1 and PR1 are related to enhanced levels of resistance (Liu et al., 2005). Here we found that the expression of the WRKYs is hardly affected by the disk lesion and NPR1 was quite suppressed by the disk treatment.

These results suggest that the leaf-disc technique cannot be successfully used for transcriptomic analysis of coffee-rust interactions.

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Phylogenetic Analysis of the *WRKY* Transcription Factors Gene Superfamily in Coffee Plants

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SUMMARY

WRKY family proteins are transcription factors involved in the regulation of development and plant defense response pathways. The *Arabidopsis thaliana* WRKY superfamily is made of 75 members. Common to these proteins is a DNA-binding region of approximately 60 amino acids in length which comprises the absolutely conserved sequence motif WRKY adjacent to a novel zinc-finger motif. A comparative phylogenetic analysis of the *WRKY* gene family in coffee and *A. thaliana* was conducted to assess the diversity of this family in coffee and to identify homologous coffee genes with putative function in defense responses to pathogens. Bioinformatic analysis of around 200 000 coffee Expressed Sequence Tags (ESTs) identified 313 ESTs with BLAST homologies to WRKY proteins. Almost 30 different putative WRKY genes were obtained, but only 25 unigenes encoding a protein with a WRKY domain were identified. Alignement of the WRKY domain sequences of the 25 coffee unigenes together with those of 72 *A. thaliana WRKY* genes showed a high conservation of the WRKY motif and the zinc-finger motif in the coffee WRKY domain. The 25 coffee WRKY members were distributed among the 3 main *A. thaliana* WRKY subgroups, with group I members displaying two WRKY domains, as expected. Conservation of the intron position within the WRKY domain sequence was evidenced when cloning the genomic sequence of one *WRKY* coffee gene (*CaWRKY1*). Clustering of the coffee *WRKY* genes based on the EST distribution in cDNA libraries made from tissues under several physiological conditions allowed to identify genes associated with development or with plant defense responses. To assess the involvement of *WRKY* genes in the coffee defense response pathways, gene expression patterns are being tested in coffee plants under several defense-related conditions.

INTRODUCTION

WRKY proteins are plant transcription factors encoded by a multigene family comprising over 74 genes in *Arabidopsis thaliana* (Eulgem et al., 2000 ; Dong et al., 2003) and more than 80 in rice (*Oriza sativa*) (Xie et al., 2005). WRKY proteins are characterized by the presence of one or two DNA - binding domains which comprise the conserved WRKYGQK core motif (Eulgem et al., 2000). Transcriptional regulation of a number of genes involved in several physiological processes may be driven by WRKY transcription factors (Eulgem et al., 1999; Ülker and Somssich, 2004). So far, recent studies have shown that WRKY proteins probably have regulatory functions in seed development, sugar signalisation and plant defence responses to pathogens (for review Ülker and Somssich, 2004). Indeed, pathogen infection, wounding or treatment with salicylic acid (SA) have been shown to induce rapid expression

of several *WRKY* genes from a number of plants (Dong et al., 2003; Ryu et al., 2006). In coffee (*Coffea arabica*), the *CaWRKY1* gene displayed altered expression patterns in response to biotic and abiotic treatments (Fernandez et al., 2004; Ganesh et al., 2006). Identification of regulatory genes involved in several physiological mechanisms such as disease resistance or seed development would offer new tools for improving coffee (*C. arabica*) varieties for important agronomic traits. The aim of this study was to identify *WRKY* genes in the coffee genome by data mining large sets of Expressed Sequence Tags (ESTs) and to predict their involvement in different physiological processes based on their expression patterns.

RESULTS

Identification of coffee *WRKY* genes

Table 1. List of coffee unigenes encoding a putative WRKY transcription factor.

Coffee clone	Origin	AtWRKY best BlastX	*A. thaliana* group	Expression group
CaWRKY-C5	*C. arabica*	33	I	C
CaWRKY-FR2-5E8	*C. arabica*	33	I	A
CaWRKY-126831	*C. Canephora*	33	I	Pericarp
CaWRKY-C10	*C. arabica*	33	I	A
CaWRKY-23-A03	*C. arabica*	44	I	Rust-induced
CaWRKY-119460	*C. Canephora*	40	IIa	Early-stage cherry
CaWRKY-C14	*C. arabica*	40	IIa	C
CaWRKY-130063	*C. arabica*	40	IIa	Early-stage cherry
CaWRKY-C23	*C. arabica*	40	IIa	A
CaWRKY1	*C. arabica*	460	IIb	Rust-induced
CaWRKY-C2	*C. arabica*	31	IIb	C
CaWRKY-C4	*C. arabica*	57	IIc	B
CaWRKY-C18	*C. arabica*	75	IIc	C
CaWRKY-C22	*C. arabica*	21	IId	C
CaWRKY-FR2-82A10	*C. arabica*	74	IId	A
CaWRKY-130733	*C. Canephora*	21	IId	Early-stage cherry
CaWRKY-125957	*C. Canephora*	15	IId	Pericarp
CaWRKY-C25	*C. arabica*	7	IId	C
CaWRKY-CB1-73G5	*C. arabica*	11	IId	B
CaWRKY-C24	*C. arabica*	27	IIe	A
CaWRKY-EA1-7B7	*C. arabica*	14	IIe	A
CaWRKY-125811	*C. Canephora*	69	IIe	Leaf
CaWRKY-C12	*C. arabica*	53	III	A
CaWRKY-C13	*C. arabica*	53	III	A
CaWRKY-C21	*C. arabica*	70	III	B
CaWRKY-C28	*C. arabica*	54	III	B

Coffee *WRKY* genes were retrieved from ESTs databases by keyword searches of annotated unigenes as well as by multiple BLAST searches using the WRKY domain sequence. The databases searched included (i) the Brazilian Coffee Genome Project ESTs database (http://www.lge.ibi.unicamp.br) which comprises more than 30 000 unigenes isolated from 27

cDNA libraries made from coffee (mostly *C. arabica*) tissues under several physiological conditions (Vieira et al., 2006), (ii) the *C. canephora* ESTs database developed from 5 cDNA libraries made from coffee leaves and seeds at a range of developmental stages (http://www.sgn.cornell.edu) and comprising more than 13 000 unigenes (Lin et al., 2005) and (iii) the IRD *C. arabica* EST database made of 1900 unigenes from defence-specific subtractive cDNA libraries (Fernandez et al., 2004; Lecouls et al., 2006).

We identified 313 ESTs with BLAST homologies to WRKY proteins. Search for the specific DNA-binding protein domain (WRKYGQK sequence followed by a C2H2- or C2HC-type of zinc finger motif) (Eulgem et al., 2000) was manually performed on the coffee unigene sequences. Almost 30 different putative *WRKY* genes were obtained, but only 25 unigenes encoding a protein with one or two WRKY domains were identified (Table 1). The remaining unigene sequences either did not cover the WRKY domain or ended within the domain, thus impairing further analyses.

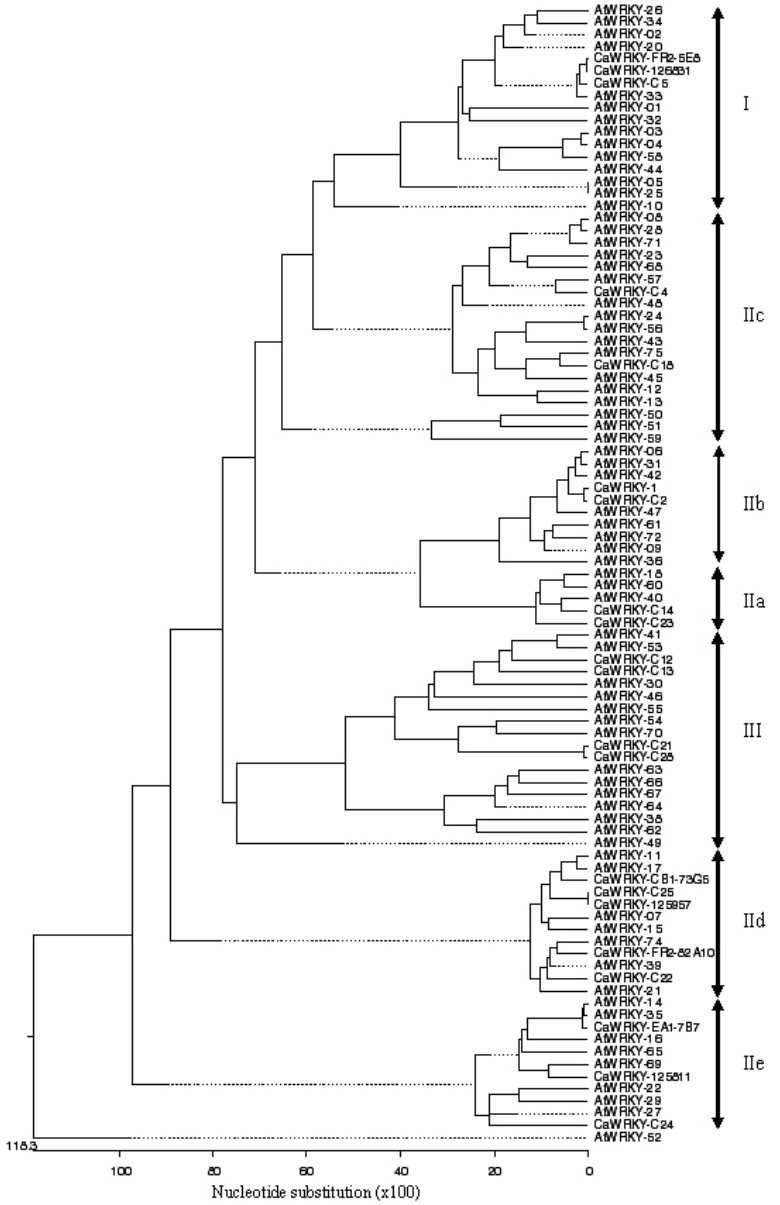

Figure 1. Dendrogram showing phylogenetic relationships between coffee and *A. thaliana* WRKY domains. Numbers on the right are the phylogenetic groups assigned to *A. thaliana* WRKY proteins (Eulgem et al., 2000).

Classification of *WRKY* genes on the basis of the WRKY domain sequences

BLAST homology to *A. thaliana* WRKY sequences were searched in GenBank database. The C-terminal WRKY domain sequences (68 amino acid residues) of 72 *A. thaliana WRKY* genes and the 25 coffee unigenes were aligned and a phylogenetic tree was constructed using the Lasergene software package (DNAStar, Inc., USA). Coffee genes were classified into the 3 main *A. thaliana WRKY* genes groups (Eulgem et al., 2000) (Figure 1 and Table 1). A high conservation of the WRKY motif and the zinc-finger motif was observed between the two plants. Group 3 *WRKY* coffee genes had a C2HC-type zinc-finger motif (C-$(X)_7$-C-$(X)_{23}$-H-*X*-C) whereas all other coffee WRKY genes had a C2H2-type (C-$(X)_n$-C-$(X)_p$-H-X-H).

Alignement of the *CaWRKY1* genomic and cDNA sequences (Petitot et al., 2006) showed the presence of an intron within the WRKY domain. The intron position (after the first Q residue of the zinc-finger domain) was highly conserved with that of *A. thaliana WRKY* genes (Eulgem et al., 2000).

Hierarchical classification of ESTs into expression groups

To identify coffee *WRKY* genes putatively associated with important physiological mechanisms such as development or plant defense responses, we analyzed the distribution of 17 *C. arabica WRKY* unigenes into the 27 cDNA libraries of the Brazilian coffee genes database. The presence/absence of WRKY ESTs in each cDNA library was recorded as a (0;1) matrix and used to construct a distance matrix (Simple-matching index) and a dendrogram with the UPGMA algorithm (Sneath and Sokal, 1973) contained in the software package TREECON, version 1.3b (Van de Peer and De Wachter, 1994). Coffee unigenes could be separated into 3 main groups based on their library distribution (Figure 2). The first cluster (expression group A) grouped unigenes only present in cDNA libraries involved in plant development (different fruit stages, embryogenic calli and lines), the second cluster (expression group B) contained ESTs from a cDNA library made from acibenzolar-S-methyl and brassinosteroide-induced tissues. The remaining unigenes (expression group C) were each largely distributed over 4-10 cDNA libraries and could not be assigned to a particular physiological trait. Future work will aim at identifying coffee *WRKY* genes involvement in agronomically important traits.

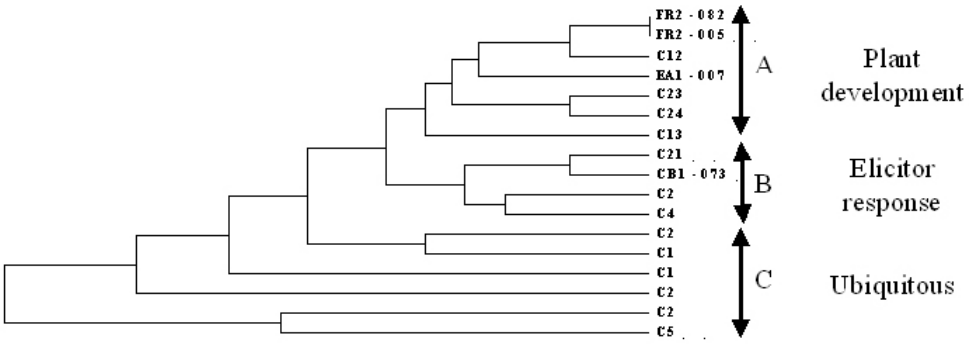

Figure 2. Dendrogram showing relationships among *C. arabica WRKY* unigenes based on their expression data.

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Early Expressed Genes in the Coffee Resistance Response to Root-Knot Nematodes (*Meloidogyne* sp.) Infection

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SUMMARY

To understand physiological and molecular mechanisms underlying the resistance response of coffee to the root-knot nematode *M. exigua*, we undertook a genomic approach based on the construction of substractive (SSH) cDNA libraries enriched in genes induced during the early stages of HR. Two different libraries were generated from root tips of resistant vs. susceptible and resistant vs. control coffee varieties 2-4 days (pooled time-points) after inoculation with *M. exigua.* A total of 1180 non-redundant ESTs were obtained and these sequences appeared to be specific of each library since only 41 (4%) were common to both. Functional annotation of the unigene set showed that 30% of the ESTs encoded putative homologues of known defence-related proteins involved in disease resistance (resistance gene analogs, NPR1, HIN1), programmed cell death (cathepsin, cystein proteinase, 26S proteasome, Bax inhibitor), cell wall strengthening (peroxidase, HRGP, xyloglucan transglycosylase), signalling (ABC transporters, calcium dependent-kinase, WRKY and AP2 transcription factors), antimicrobial activity (PR10, beta-1,3-glucanase) and the production of anti-microbial coumpounds (phytoalexin precursors, snakin, cytochrome P450). In addition, 14% of the annoted ESTs encoded proteins of unknown function and 35% shared no significant similarity to plant protein database entries. Ongoing studies focus on differential screening of the cDNA libraries and on expression analyses of genes during an infection time-course of resistant and susceptible coffee varieties with *M. exigua* using real-time quantitative RT-PCR in order to identify candidates genes activated in HR exhibiting plants.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* sp.) are major pests damaging coffee culture (*Coffea arabica*) in Latin America. Resistance to *Meloidogyne* carried by the related diploïd species *C. canephora* has been introgressed into *C. arabica* genome by traditionnal breeding programs. Resistance to *M. exigua* is conferred by the single gene *Mex*-1 (Noir et al., 2003) and resistant coffee trees exhibit a typical phenotype of hypersensitive response (HR) when challenged by nematodes (Anthony et al., 2005). The HR occurs 5 days post-inoculation (dpi) and is characterized by the formation of necrotic lesions in the region of pathogen attack resulting from a programmed cell death directly responsible for the pathogen confinement and growth limitation. The HR is associated with activation of defence mechanisms in the dying area as well as in the surrounding tissues such as changes in protein phosphorylation, generation of reactive oxygen species (the oxidative burst), modification of ion fluxes, cell wall reinforcement by deposition of lignin and callose, lipid peroxidation, synthesis of antimicrobial molecules (phytoalexines), production of signalling hormones, and activation of pathogenesis-related (PR) genes (Greenberg, 2004; Morel, 1997; Heath, 2000).

To understand the cellular and molecular mechanisms involved in coffee defence reactions to root-knot nematodes, a genomic approach based on the establishment of a catalogue of genes

implicated in resistance have been carried out. It consisted in the construction of subtractive cDNA libraries (SSH technology, Diatchenko et al., 1999) followed by systematic ESTs sequencing.

MATERIAL AND METHODS

Plant material and nematode inoculation

Two *C. arabica* varieties, Caturra and Iapar59, respectively susceptible (compatible interaction) and resistant (incompatible interaction) to *M. exigua*, were used in this study. Ten plants of each variety were inoculated with 800 nematodes juveniles freshly hatched. Two and 4 days after the nematode challenge, root tips were collected and immediately frozen. Mock inoculation was considered as the non-inoculated control.

RNA extraction and SSH libraries contruction

Root tips total RNA was extracted using the Rneasy Plant kit (Qiagen, France) with some modifications. Construction of the SSH libraries was performed with the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA) as described by Fernandez et al., 2004. Systematic sequencing of 1500 clones was made by MWG (Germany).

Bioinformatic analyses

The bioinformatic analysis was achieved using "ESTdb", an automated annotation and clustering pipeline developed in the IRD laboratory (Fernandez et al., 2004). Sequence homologies were searched using BlastN and BlastX (Altschul et al., 1990) and sequences with significant matches (E-value < 10^{-3}) were assessed to a fonctionnal categories based on the Expressed Gene Anatomy database classification scheme (White and Kerlavage, 1996).

RESULTS

Two cDNA subtractive libraries specifically enriched in genes expressed during *C. arabica* incompatible interaction with *M. exigua* were built. These libraries were generated from root tips of the resistant *vs.* susceptible varieties (library #1, named "nematode-SSH 2/4dpi") and resistant *vs.* control (library #2, named "nematode-SSHincompatible 2/4dpi"), 2-4 days after inoculation (pooled times). A total of 1731 randomly chosen clones were partially sequenced resulting in 1400 exploitable ESTs. Clustering analysis using ESTdb allowed to defined a set of 1180 non-redundant ESTs consisting in 1049 singletons and 131clusters.

Inter and intra-library comparisons showed that most of the ESTs are specific of each library since only 41 sequences (4%) are common to both (Figure 1). Furthermore, comparison with the *C. arabica* ESTs obtained in response to the orange rust fungus (Fernandez et al., 2004) showed that only 8 sequences are shared by the two interactions. In addition, only 56% were already represented in the public databases.

Homology searches using BlastX revealed that 51% of the annotated ESTs shared similarity with plant genes of known function while 14% encoded proteins of unknown function. Besides, 35% showed no significant homology with any protein present in the databases. Functional annotation of the unigene set was performed according to the categories defined for *A. thaliana* (Figure 2). The 1180 ESTs were distributed in 10 categories, the global repartition being similar from one library to the other.

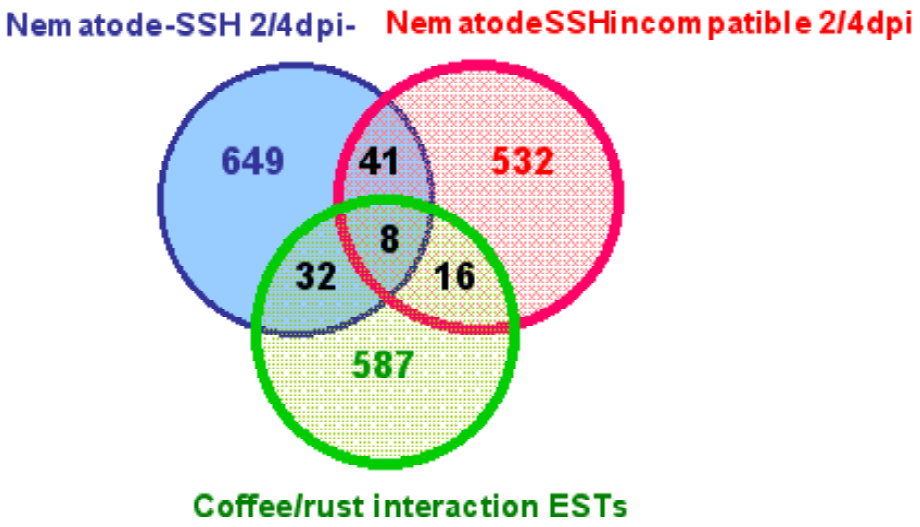

Figure 1. ESTs distribution over the different libraries.

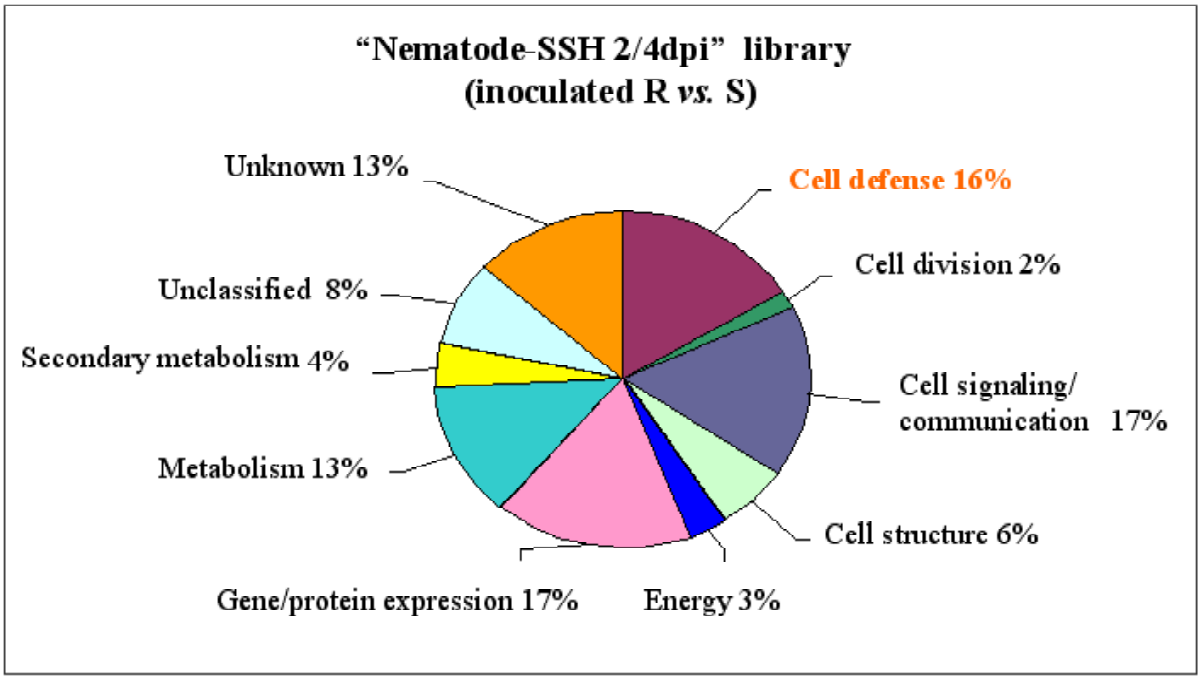

Figure 2. EST classification according to putative biological function. Exemple of the library "Nematode-SSH 2/4dpi".

The best represented categories concerned the modulation of the genic and protein expression (18.5%), the cell communication and signalling (17%) and the defence responses (16%). These 3 merged categories included a majority of genes encoding putative proteins homologues known to be involved in plant/pathogen interactions as well as in the mechanisms, specific or general, of biotic and abiotic stress responses. In particular, are represented genes encoding resistance effectors (R gene analogues, NIM-1, HIN-1, EDR1), genes involved in the programmed cell death (cathepsin, cystein proteinase, Bax inhibitor), in the reinforcement of cell walls (peroxidase, HRGP, N-rich protein), or in cell signalling (ABC transporters, calcium dependent-kinase) as well as defence genes (PR1, PR10, beta-1,3-glucanase), genes responsible for the production of anti-microbial compounds (phytoalexin precursors, snakin, cytochrome P450) and transcription factors (WRKY, SCARECROW and AP2).

CONCLUSIONS

In this study, a catalog of genes potentially involved in the resistance of *C. arabica* to *M. exigua* was generated. Expression analyses on an infection time-course using real-time qantitative RT-PCR (qPCR) are in progress and will drive candidates' choice among the directory previously established.

So far, our results are the first reported on the coffee physiological response to a ground plant parasite They should allow the identification of key components of coffee resistance to nematodes and supply useful data for a better understanding of the defence mechanisms in a tropical woody plant. Furthermore, they provide other possibilities to develop new management strategies for a coffee durable resistance to pest and diseases.

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Genes Associated with Coffee (*Coffea arabica*) Seed Germination

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SUMMARY

The recent development of Brazilian coffee genome project together with new technologies developed, such as high throughput sequencing analyses and transcriptomics can be used to investigate and identify genes associated with coffee seed germination and subsequently establishment of the coffee seedling. Coffee seed germination is slow and shows wide variation in timing of emergence. Thus, the overall objective of this work was to identify and study the genes expression profile during coffee seed germination. Therefore, RNAs extracted from embryo, micropylar and lateral endosperm during coffee seed germination will be converted to radioactively labeled cDNAs and hybridized against a macroarray performed with genes from the coffee genome project (Coffee ESTs).

INTRODUCTION

The coffee (*Coffea arabica*) fruit is a drupe containing two seeds. The coffee seed is comprised of an endosperm and embryo. The coffee embryo is surrounded by endosperm tissue (Krug and Carvalho, 1939; Mendes, 1941). The endosperm cell walls are composed by mannans with 2% of galactose (Wolfrom et al., 1961). Coffee embryo is small with 3 to 4 mm long composed of an axis and two cotyledons (da Silva et al., 2004).

Seed germination "begins with the water uptake by the seed (imbibition) and ends with the elongation of the embryonic axis, usually the radicle" (Bewley and Black, 1994). Germination in coffee seed is the net result of embryo growth inside the endosperm prior radicle protrusion and endosperm weakening (da Silva et al., 2004; 2005). Increases in pressure potential and cell wall extensibility are responsible for embryo growth inside the coffee endosperm prior to radicle protrusion (da Silva et al., 2004). Endo-β-mannanase (E.C.3.2.1.78) and β-mannosidase (EC 3.2.1.25), are the main enzymes involved in weakening of the endosperm prior radicle protrusion in coffee seed (da Silva et al., 2004; da Silva, 2002).

Although this has already been established, the studies about the molecular physiology of the coffee seed during germination considering the endosperm and the embryo are still scanty. However, the recent development of Brazilian coffee genome project together with new technologies developed, such as high throughput sequencing analyses and transcriptomics can be used to investigate and identify genes associated with coffee seed germination. Therefore, the overall objective of this work is to identify and study the gene expression profile during coffee seed germination.

MATERIALS AND METHODS

Seed material

Seeds from *Coffea arabica* L., cultivar Rubi, were harvested in 2006 in Lavras, MG, Brazil, from different plants, depulped mechanically, fermented, dried to 12% moisture content (fresh weight basis), and stored at 10 °C.

Germination conditions

The seed coat was removed by hand and seeds were surface sterilised in 1% of sodium hypochlorite for 2 minutes. Seeds were then rinsed in water and placed in Petri dishes on filter paper with 10 ml of the water. During imbibition seeds were kept at 30 ± 1 °C in the dark (da Silva et al. 2004). The germination percentage, as radicle protrusion, was recorded daily.

Imbibition curve

Intact seeds coffee seeds had the surface sterilized in 1% of sodium hypochlorite during 2 minutes were rinsed and imbibed in demineralized water and placed at the temperature of 30 °C in the dark. The fresh weight was monitored daily by weighting the seeds during imbibition.

RNA isolation

Total RNA will be extracted using the modified hot borate method (Wan and Wilkins, 1994). Endosperm cap, endosperm lateral and embryo will be dissected from one hundred coffee seeds after 5 days of imbibition. The seed parts will be first frozen in liquid nitrogen and ground to a powder. The powder will be suspended in 700 µl hot (80 °C) borate buffer (0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1% SDS and 1% sodium deoxycholate, pH 9,0) with 1.1 mg DTT, 14 mg PVP, 14 mg ascorbic acid and 1 mg proteinase K per sample.

RESULTS AND FUTURE WORK

The water uptake during imbibition followed the common triphasic pattern. Most of the seeds reached phase II of germination at 3 days of imbibition. Germination as radicle protrusion, started at day 5 in water-imbibed seeds and after 10 days of the beginning of imbibition, 50% of the seeds showed radicle protrusion. As future work, RNA will be extracted from endosperm micropylar, endosperm lateral and embryo, converted to radioactively labeled cDNAs and hybridized against a macro-array performed with genes from the coffee genome project (Coffee ESTs).

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Galactinol Synthase Gene Expression in *Coffea Arabica* L. under Water Stress

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SUMMARY

Galactinol synthase (GolS) catalyzes the formation of galactinol, which will produce raffinose oligosaccharides (RFOs). Expression of GolS during water deficit stress has been reported, probable due to the function of RFOs as osmoprotectant. Three galactinol synthase isoforms (*CaGolS1, 2* and *3*) were identified from the Coffee Genome Brazilian Project database (http://www.lge.ibi.unicamp.br/cafe/). The isoforms have similar length to others *GolS* genes. Northern blot analysis showed an increased in *CaGolS1* transcripts in coffee plants under water shortage when compared to plants in well-watered conditions. Transcripts of *CaGolS1* were specifically found on leaves and not in other tissues.

INTRODUCTION

Galactinol synthase (GolS) is the key enzyme in the biosynthesis of oligosaccharides from raffinose (RFO) family (Keller and Pharr, 1996). It plays a regulatory role in carbon partioning, acts directly in many processes of plant physiological development and in plant responses to abiotic stresses. Galactinol is formed from UDP-galactose and *myo*-inositol by the action of galactinol synthase (EC 2.4.1.123; GolS), which belongs to glycosil transferase 8 family (Campbell et al., 1997). Increased transcription of *Galactinol synthase* gene during water deficit has been reported. In C*ucumis melo*, it was observed that GolS activates the RFO metabolism in plants submitted to drought stresses. It was also demonstrated that *CmGolS1* transcription occurs in mature leaves and seeds during plant development, while *CmGolS2* trasncription was observed only in mature leaves (Volk et al., 2003). In *Arabidopsis thaliana*, from a family of seven *GolS* genes, three of them were stress responsive. *AtGolS1* and *2* were induced by drought and high-salinity stress, while *AtGolS3* was induced by low temperatures. These results showed that GolS plays a key role in the accumulation of galactinol and raffinose under abiotic stress conditions, conferring drought-stress tolerance to plants, since galactinol and raffinose may function as osmoprotectants (Taji et al., 2002). In *Ajuga reptans,* a model plant used to study the regulation of RFO metabolism, two distinct *GolS*, *ArGolS1* and *ArGolS2* were identified (Sprenger and Keller, 2000). The objective of this study was to increase the knowledge on the transcription expression of *GolS* genes in coffee plants under drought stress.

MATERIALS AND METHODS

Three galactinol synthase isoforms (*CaGolS1, 2* and 3) were identified from the Coffee Genome Brazilian Project database (http://www.lge.ibi.unicamp.br/cafe/). The isoforms

presented integrity and similarity to *GolS* gene, which allowed the primer design. The contigs formed by different tissues of coffee stressed plants were analyzed using bioinformatic programs such as BlastP and BlastX. Also, it was used the TargetP 1.1 Server program to detect signal peptides, and the program PSORT – *Prediction of Protein Localization Sites* version 6.4 for prediction and localization of proteins. Six month-old plants of *Coffea arabica* cv. IAPAR-59, cultivated in 1L pots in greenhouse, were submitted to a 5-day period without water, being evaluated by total water potential, osmotic potential and photosynthetic rates. Total RNA from different tissues was extracted based on protocol (Chang et al., 1993) for Northern blot analysis to detect the *Galactinol synthase* transcripts in leaves submitted to drought stress, as well as in different tissues under well-watered conditions (Figure 1).

RESULTS AND DISCUSSION

From 91 sequences found into the Brazilian Coffee Genome Project database (http://www.lge.ibi.unicamp.br/cafe/) three full-length galactinol synthase isoforms were identified (Table 1). CaGolS1, CaGolS2 and CaGolS3 isoforms have 1005, 1026 and 1017 bases pairs (bp), coding for proteins of 314, 341 and 338 amino-acids (aa), respectively. All the three isoforms present the glycosyl transferase domain pfam01501.

Table 1. Eletronic Northern of *Coffea arabica GolS* contigs.

Gene	Total ESTs	Leaves	Callus	Root	Cells suspension	Branch	Walter stress	Fruits	Plantlets
CaGolS1	49	32	X	1	5	1	10	X	X
CaGolS2	15	4	1	X	2	1	3	3	1
CaGolS3	6	2	X	X	X	X	4	X	X

When submitted to water deficit, the plants showed no symptom during the first two days without watering. A progressive decreased in water and osmotic potential was observed from the third day until the end of the treatment (Figure 1). Photosynthesis rates were stable until the second day of stress, falling quickly after that. Lost of turgor from the leaves after the second day was also observed.

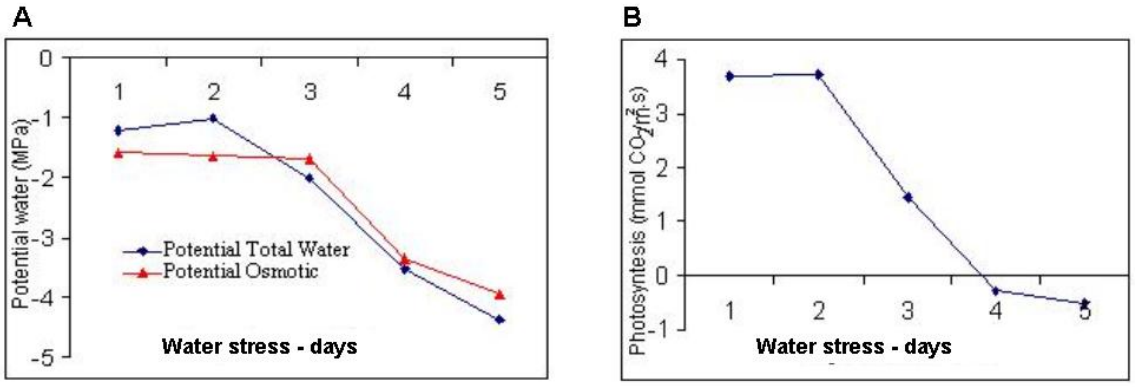

Figure 1. A - Total water and osmotic potential during water stress. B –Photosynthesis rate during water stress.

From the third day under water shortage (leaf water potential –2,02 MPa), coffee plants presented increased *CaGolS1* expression compared to plants in well-watered conditions (–1,2 MPa) (Figure 2). Similarly to found in *Cucumis melo* (Volk et al., 2002) and *Arabidopsis thaliana* (Taji et al., 2002; Liu et al., 1998), the *GolS* gene in *C. arabica* is involved in the

plant response to water deficit.Northern blot analyses showed that transcription of *CaGolS1* was tissue specific for mature and young leaves, and were not observed in roots, branches, floral buds, red fruit pulp and ripe seeds of *Coffea arabica* cv. IAPAR-59 grown under field conditions (Figure 2).

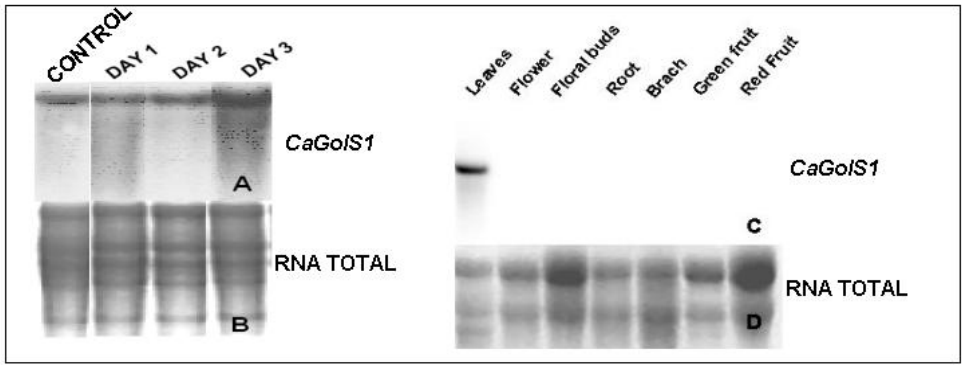

Figura 2. Northern blot analysis of total *C. arabica* RNA hybridized with CaGolS1. A) RNA from leaves of plants growing under different water deficit stress. B) RNA from different coffee tissue as described. Total RNA loading control is represented under the blots.

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In Silico and *in Vitro* Analysis of the Isoprenoid Pathway in Coffee

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SUMMARY

The most important lipids in coffee, the diterpenes khaweol and cafestol, are originated from the isoprenoid pathway. Despite their diversity in functions and structures, all isoprenoids derive from the common-five carbon building unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In higher plants, there are two independent pathways located in the cytosol (mevalonic acid or MVA pathway) and in the plastids (methylerythritol phosphate – MEP – or non mevalonic pathway). Throughout the data mining of the Brazilian Coffee Genome Project we studied the genes that code for the enzymes 3-hydroxy-3-methyglutaryl-CoA reductase (HMGR) and mevalonate diphosphate decarboxylase (MPDC) for the MVA pathway and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and isopentenyl diphosphate/dimethylallyl diphosphate synthase (IDS) for the MEP pathway.

INTRODUCTION

Coffee is one of the most important world agricultural commodities. Although the cup quality is one of the most important aspects for consumption, very little is known about several components, which can contribute for quality, including lipids. The most important lipids in coffee, the diterpenes khaweol ($C_{20}H_{26}O_3$), and cafestol ($C_{20}H_{28}O_3$), are originated from the isoprenoid pathway. Despite their diversity in functions and structures, all isoprenoids derive from the common-five carbon building unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Rodriguez and Boronat, 2002). In higher plants, two independent pathways located in separate intracellular compartments are involved in the biosynthesis of IPP and DMAPP (Figure 1). In the cytosol, IPP is derived from the mevalonic acid (MVA) pathway (Qureshi and Porter, 1981; Newman and Chappell, 1999), that starts from the condensation of acetyl-CoA, whereas in plastids, IPP is formed from pyruvate and glyceraldehyde 3-phosphate through the methylerythritol phosphate (MEP or non-mevalonate) pathway (Esenreich et al., 1998; Rohmer, 1999). The key enzyme of the cytoplasmic MVA pathway is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) (EC 1.1.1.34). *HMGR* in plants is reported to be encoded by two genes (Bach et al., 1991;Weissenborn et al., 1995). These *HMGR* isoforms are differentially expressed, depending on physiological conditions (Weissenborn et al., 1995; Stermer et al., 1994). The enzyme mevalonate diphosphate decarboxylase (MPDC) is responsable for the formation of IPP.

For the MEP pathway, the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is currently considered as the first specific step for biosynthesis of isoprenoid in plastids. The last enzyme on the MEP pathway is the isopentenyl diphosphate/dimethylallyl diphosphate synthase (IDS), which can form either IPP or DMAPP. With the aim to understand the process of formation of the isoprenoids in *Coffea* we have started the characterization of the genes involved on the MVA and MEP pathway.

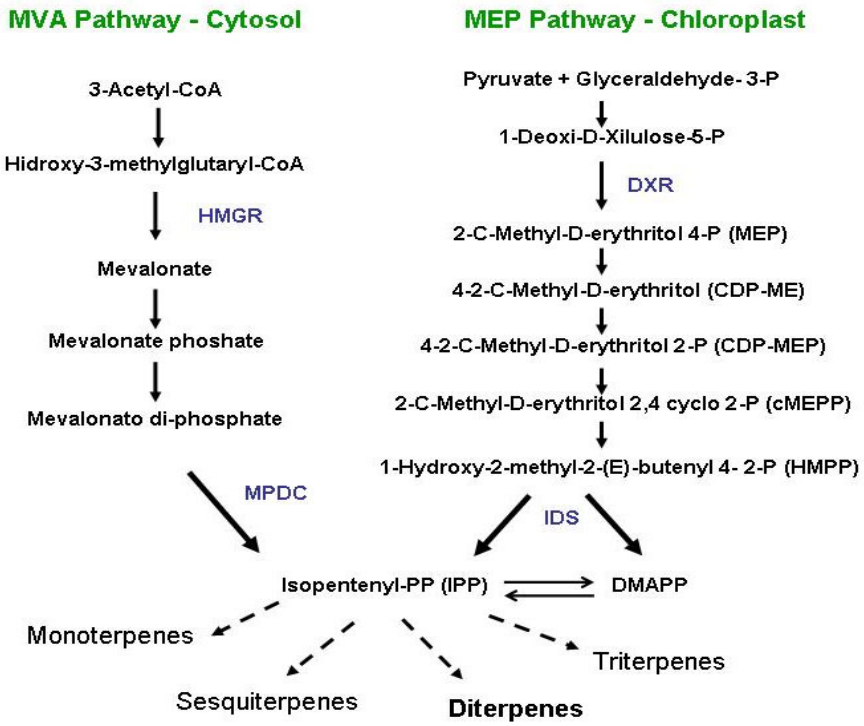

Figure 1. Diagram of the isoprenoid pathway in the cytosol and in the plastids in plants.

MATERIAL AND METHODS

A key word search for ESTs from genes of the MAV and MEP pathway was conducted on the database of the Brazilian Coffee Genome Project (Vieira et al., 2006) (http:/www.lge.lbi.unicamp.br/cafe/). The sequences were clusterized for contig formation using the Sequencher 4.5 software. Singlets and contigs were analyzed using the basic local alignment search tool (BLAST) in the tblastx mode at NCBI database. The consensus contig were translated using the Open Reading Frame Finder available at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The proteins sequences were aligned with homologous sequences from other organisms using Clustal W at http://clustalw.genome.ad.jp/.

Total RNA was isolated from pulp, perisperm and endosperm from fruits of *Coffea arabica* cv. IAPAR 59 at different stages of maturation (Chang et al., 1993). One µg of total RNA was used to produce cDNA with Thermoscript^{TM} oligo DT System (Invitrogen). To check the complementation of the HMGR contigs, different primer combinations were used on PCR reaction of the cDNA. For Northern Blot analysis 10 ug of total RNA was transferred to nylon membranes and hybridized using UltraHyb solution as previously described (Pereira et al., 2005).

RESULTS AND DISCUSSION

The keyword search for the *DXR* (MEP pathway) resulted in twenty two ESTs and formed only one full length contig. Electronic Northern showed higher expression in libraries of germinating seeds (Table 1). Search for *IDS* ESTs retrieve 47 sequences, which also formed only one full length contig. The sequences were originated mainly from hypocotyls induced with acylbenzolar-S-methyl, a SAR inducer. It is interesting to observe that several of ESTs coming for the MEP pathway were obtained from cell tissue culture libraries or stress induced libraries. On the other hand, the number of ESTs from the two genes from the MVA pathway (HMGR and MPDC) on those libraries was very low (Table 1).

Table 1. Eletronic Northern of genes from the MVA and MEP pathway.

Gene	ESTs	Fruits and flowers buds	Leaf	Tissue culture cells	Walter stress	Hypocotyl/ acilbenzolar	Germinating seeds
CAHMGR1	11	8	2	1			
CAHMGR2	2			2			
MPDC	7	2		4		1	
DXR	22	4	3	6	3	1	5
IDS	47	9	9	14	4	10	1

For the MVA pathway, 13 ESTs were found for HMGR that originated three incomplete contigs named: 6479, 6175 and 0003. For MPDC only 7 ESTs were found forming one full lenght contig.

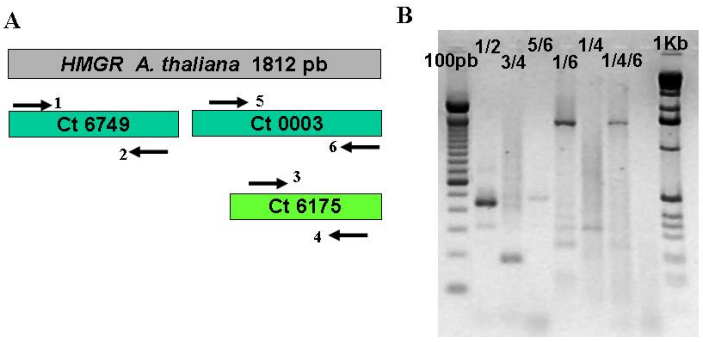

Figure 2. Characterization of *HMGR* isoforms by PCR. A) Contigs obtained and their position related to a consensus *A. thaliana* sequence. Number on the arrows indicated the primer position for fragment amplification. B) Partial PCR amplification of coffee *HMGR*. Numbers in the columns indicated the used primer combination. Expected sized for the fragments: 1/2: 6749/6749C = 450 pb; 3/4: HMGR 0/0C = 186 pb; 5/6: HMGR 6175/6175C = 483 pb; 1/4: 6749/0C= 1556pb; 1/6: 6749/6175C=1694pb; 1/4/6: 6749/0C/6175C = 1556pb.

For HGMR, the translated sequence of the contig 6749 presented high similarity with the N-terminal region of a consensus *Arabidopsis thaliana* HMGR protein. Contigs 6175 and 0003 presented high similarity with the C-terminal region of the same protein (Figure 2A) As the presence of two isoforms were expected, primers based on those contigs were used to check whether they could belong to the same contig 6749 or represent another *HMGR* isoform. When we used primers from sequences of contig 6749 and 0003, it was possible to amplify a fragment corresponding to the expected size of the full-length cDNA, indicating that they are

the same isoform (Figure 2B, primers 1 and 6), and was called *CaHGMR1*. The combination of primer 6749 and 6175 did not amplify the expected fragment size, suggesting that 6175 is a different isoform, called *CaHMGR2*.

The number of isoforms of the four genes datamined was the same reported for *Arabidopsis thaliana*, with the exception for the *MPDC* that has two isoforms in *A. thaliana* and only one in the Brazilian Coffee Genome Project *C. arabica* ESTs database. A keyword search from *MPDC* in *C. canephora* ESTs (LLIn et al., 2005) using the Harvest platform (http://harvest.ucr.edu/) also retrieved only one contig that was mainly expressed during the late stages of fruit maturation.

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Coffea Expansin Gene Family and Expansin Expression during Fruit Maturation

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SUMMARY

Expansins (EXP) are plant cell-wall loosing proteins involved in cell enlargement and developmental process such as organogenesis, seeds germination, cell wall dissolution and fruit ripening. Two families of EXP are known, α-expansin (EXPA), involved in the control of cell wall extension, and β-expansins (EXPB), the major allergens of grass pollen that also have cell wall-loosening activity. With the objective to study the role of EXP in coffee fruit maturation we selected EXP homologous sequences on the Brazilian Coffee Genome Project database. Full-length contigs were classified according the EXP family. Northern blots of pulp from fruits at the latest stages of maturation (22-32 weeks after flowering) showed increased transcription of one contig (*CaEXP1*). This higher transcription corresponds with a climacteric burst, during the expansion of pulp, with a decline in "cherry" fruits. The transcription of *CaEXP1* was also observed in roots, shoots, flower and flower buds, but not in leaves.

INTRODUCTION

Fruit softening is associated with cell wall disassembly mediated by the action of a complex of enzymes and proteins (Dotto et al., 2006). Expansins are a group of extracellular enzymes that directly modify mechanical properties of plant cell walls inducing cell wall extension and stress relaxation at acid pH condition (Li et al., 2002; McQueen-Manson et al., 1992). They act by causing a reversible disruption of hydrogen bonds between cellulose microfibrile and matrix polysaccharides, particularly xyloglucan, resulting in an irreversible elongation of plant cell walls. Localized expression of expansins is associated with the meristems and growth zones of the root and stems, as well as the formation of leaf primordia on shoot apical meristems (Reinhardt et al., 1998)**,** expansins activity occurs in fruit softening (Brummell et al., 1999; Rose et al., 1997; Kalamaki et al., 2003; Yoo et al., 2003; Harrison et al., 2001; Obenland et al. 2003), abscission (Belfield et al., 2005), xylem formation (Gray-Mitsumune et al., 2004), seed germination (Chen and Bradford, 2000), penetration of pollen tubes through the stigma and style (Cosgrove et al., 1997; Pezzotti et al., 2002), formation of mycorrhizal associations with symbiotic fungi in root tissues (Balestrini et al., 2005), growth of parasitic plants (O'Malley and Lynn, 2000). Expansins represent a superfamily of plant proteins that are made of four families designated α-expansin (EXPA), β-expansin (EXPB), expansin-like α (EXLA) and expansin-like β (EXLB) (Kende et al., 2004). Members of the EXPA and EXPB families are known to have wall-loosening activity (Cho HT, Kende, 1997; Cosgrove et al., 1997; McQueen-Manson et al., 1992), whereas the other two families have been identified only from sequence homology, without protein function analysis (Lee et al., 2001; Li et al., 1992).

MATERIALS AND METHODS

In Silico Analysis

Contigs and singlets related with expansins were selected in the ESTs database of the Brazilian Coffee Genome Project (http://www.lge.ibi.unicamp.br/cafe/) through keyword search. After local Blast, all the sequences and contigs were clustering again using the Sequencher – Gene Codes program. Sequences were also analyzed by BlastX, BlastP at NCBI. Deduced amino-acid sequences were obtained using ORF finder at NCBI and aligned using Sequencher. ScanProsite (http://www.expasy.org) was used to verify the two domains specific for mature exp protein and to indicates an N-glycosilation linkage. The pollen allergen domain was verified for each using BlastP. The peptide signal region was observed using SignalP (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree was constructs (Mega 3.1 version), to verify the similarity degree within the coffee sequences and to determine their exp family (EXPA, EXPB, EXP-like-A and EXP-Like-B) according to *Arabidopsis thaliana* and *Oriza sativa* sequences from NCBI.

Total RNA was isolated from different tissues and pulp from fruits at different stages of maturation of *Coffea arabica* cv. IAPAR 59 according to Chang et al. (1993). One µg of total RNA was used to produce cDNA with Thermoscript^{TM} oligo DT System (Invitrogen) to amplify a Coffea exp gene. For Northern Blot analysis 10 ug of total RNA was transferred to nylon membranes and hybridized using UltraHyb solution.

RESULTS AND DISCUSSION

In Silico Analysis

Based on Brazilian Coffee Genome Project database 162 sequences from *EXP* were identified. After two rounds of clusterization, the sequences were saturated and aligned using the software Sequencher, forming 28 contigs. Contigs with ESTs from fruit libraries were listed in the Electronic Northern (Table 1).
The deduced amino-acid sequences were analyzed for the presence of expansin domains. A phylogenetic tree with heterologous EXP of different families showed that EXPA represents the major family in coffee genome. Contig 6 and 16 were classified as EXLB and contig 8 as an EXLA (Figure 1).

Table 1. Expansin coffee sequences including ESTs from fruit libraries.

Expansis	ESTs	Fruits and flowers	Leaves	Cells suspension	Flowers bud	Callus
CaEXPA1	28	4	5	13	1	2
CaEXPA2	11	1	X	1	4	3
CaEXPA3	8	2	4	X	1	1
CaEXPA4	8	3	X	1	3	3
CaEXPA5	51	2	3	3	X	2
CaEXPA6	4	1	1	X	X	X

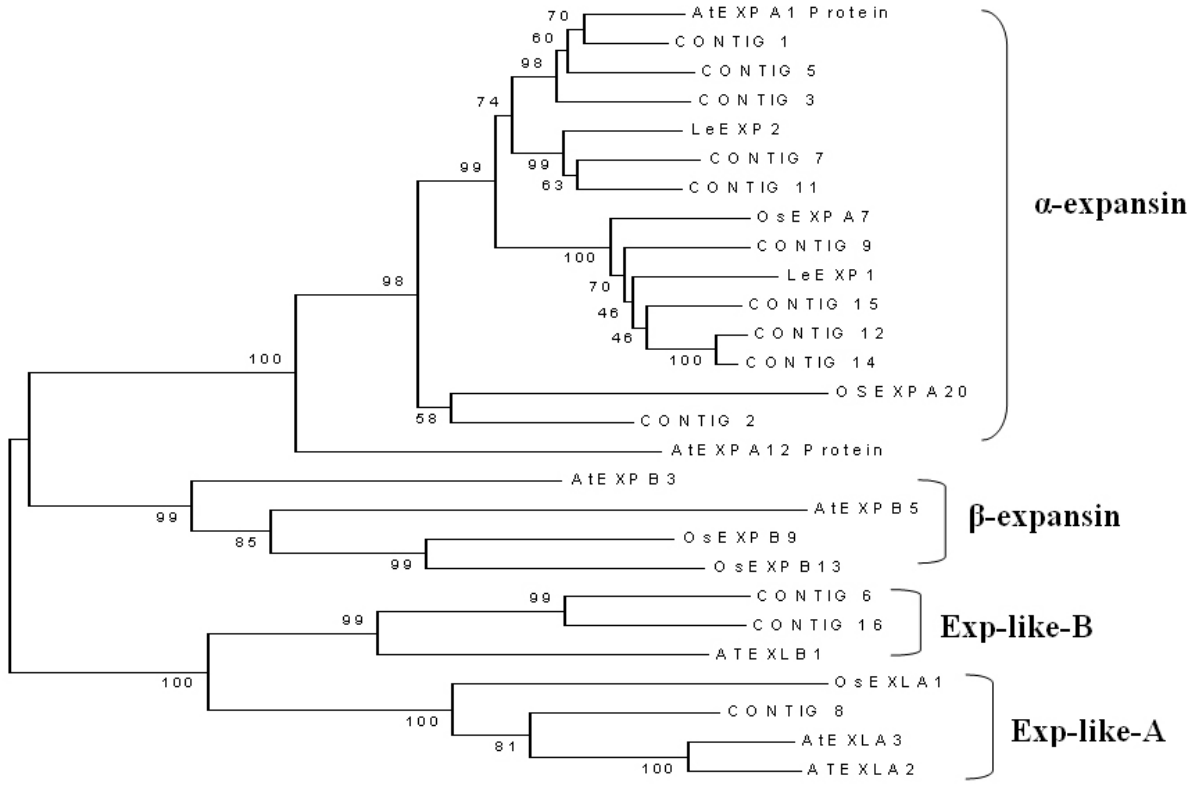

Figure 1. Phylogenetic tree of the expansin superfamily, including sequences (contigs) of *Coffea arabica* from the Brazilian Coffee Genome Project, *Arabidopsis thaliana* (At), *Oriza sativa* (Os) and *Lycopersicon esculentum* (Le) from the NCBI. The tree was constructed with MEGA 3.1 using neighbor joining and bootstrap values.

Gene Expression Analysis

The transcripts of coffee EXP, using *CaEXPA1* (Contig07) as a probe, were observed in different tissues (Figure 2A) and in the pulp at the latest stages of fruit maturation (Figure 2B). *CaEXP1* transcripts were detected mainly in flower buds, roots and during the stages of fruit pulp. Lower transcription was detected in flower and young shoots, but no transcripts were observed in leaves.

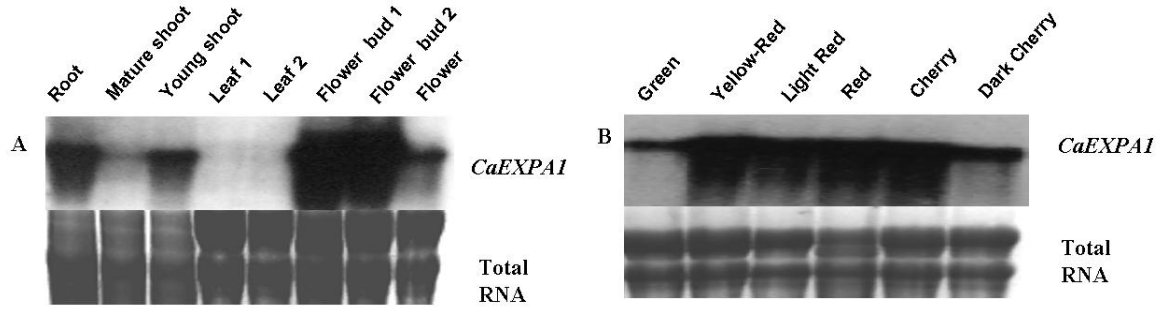

Figure 2. Northern Blot analysis of *C. arabica* total RNA hybridized with *CaEXPA1*. A) RNA from different coffee tissues. Leaf and flower bud samples were in duplicates. B) RNA from fruit pulp from different ripening stages. The total RNA used for the blot is showed below each sample in A and B.

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Isolation and First Characterization of Two O-Methyltransferase Genes Involved in Phenylpropanoid Pathway in *Coffea canephora*

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SUMMARY

The phenylpropanoid pathway plays an important role in plant secondary metabolism as it leads to the synthesis of a great variety of phenolic compounds, such as flavonoids, coumarins, or lignin. This pathway plays a particular role in coffee, especially in *Coffea canephora* where high levels of cinnamic esters were found in green beans. These compounds are involved in the determination of the coffee cup quality. Several O-methyltransferases intervene in this pathway and participate to the lignin biosynthesis. Two full length cDNA clones corresponding to genes encoding S Adenosyl Methionine (SAM) dependent O-Methyltranferases were isolated from *C. canephora* cDNA libraries. The first cDNA contained an open reading frame encoding a 350 amino acid protein. A blastx search showed that this protein belongs to the Methyltransferase II family and more precisely, that it was 85 % identical to the Caffeic acid O-Methyltransferase (COMT) from *Catharanthus roseus*. The second cDNA's ORF encoded a 247 amino acid peptide belonging to the Methyltransferase III family. This protein presented 89 % identity with the Caffeoyl CoA O-Methyltransferase (CCoAOMT) from *Betula platyphylla*. These results showed that the *Coffea canephora* genome contains at least one gene for each of these two types of structurally and functionally different OMTs involved in the phenylpropanoid pathway by performing the methylation of caffeic acid or caffeoyl-CoA Interestingly, in two EST *C. canephora* libraries, from young leaves and fruits, representing over 5 000 unigenes, only 1 CCoAOMT cluster was found but not a single EST for COMT.

RÉSUMÉ

La voie des phénylpropanoïdes joue un rôle important dans le métabolisme secondaire des plantes conduisant à la synthèse d'une grande variété de composés phénoliques, tels que des flavonoïdes, des coumarines, ou de la lignine. Cette voie joue un rôle particulier chez les caféiers, particulièrement chez *Coffea canephora* où des niveaux élevés d'esters d'acide cinnamique ont été trouvés dans les grains verts. Ces composés sont impliqués dans la détermination de la qualité de tasse du café boisson. Plusieurs O-methyltransferases interviennent dans cette voie et participent à la biosynthèse de lignine. Deux ADNc pleine longueur correspondants aux gènes codant pour des O-Methyltranferases-S Adenosyl Méthionine (SAM) dépendantes ont été isolées dans banques d'ADNc de *C. canephora.* Le premier ADNc possède un cadre ouvert de lecture codant une protéine de 350 acides aminés. Une recherche par Blastx a montré que cette protéine appartenait à la famille des Méthyltransférases II et qu'elle était à 85% identique à la «Caffeic acid O-Methyltransferase» (COMT) de *Catharanthus roseus*. Le deuxième ADNc code pour un peptide de 247 acides aminés appartenant à la famille des Méthyltransférases III. Cette protéine a présenté 89%

d'identité avec la «Caffeoyl CoA O-Methyltransferase» (CCoAOMT) de *Betulla platyphylla*. Ces résultats ont montré que le génome de *Coffea canephora* contient au moins un gène de chacune des deux OMTs différemment impliquées dans la voie des phénylpropanoïdes, en méthylant soit l'acide caféique soit le caffeoyl-CoA. Il est intéressant de noter que dans les 2 banques EST de *C. canephora* réalisées à partir de jeunes feuilles et de fruits, représentants plus de 5 000 unigènes, seulement 1 EST a été trouvé pour la CCoAOMT et aucun pour la COMT.

INTRODUCTION

S-Adenosyl L-methionine (SAM) dependent O-methyltransferases (OMTs) are involved in phenylpropanoid pathway. These enzymes catalyse the transfer of a methyl group on hydroxycinnamates, leading to the synthesis of ferulate, sinapate or feruloyl-CoA. In plants, they are implicated in lignin synthesis, response to pathogen and secondary metabolism synthesis. A high content of chlorogenic acids (11% DW), hydroxycinnamoylquinic acids including feruloyl esters, has been found in *Coffea canephora* seeds. This accumulation led us look for genes encoding for OMTs and expressed in fruits or leaves of *C. canephora*.

RESULTS

Two coding sequences, corresponding to 2 different SAM-OMTs were isolated by screening *Coffea canephora* leaf and fruit cDNA libraries.

The first sequence was identified as a Caffeoyl CoA O-methyltransferase (CCoAOMT), which catalyses the methylation of caffeoyl CoA to form feruloyl CoA. The cDNA was 744 bp long. The deduced peptide (247 amino acids) shared the domains E, F, G and H, characteristic of plant OMT I. (Figure 1)

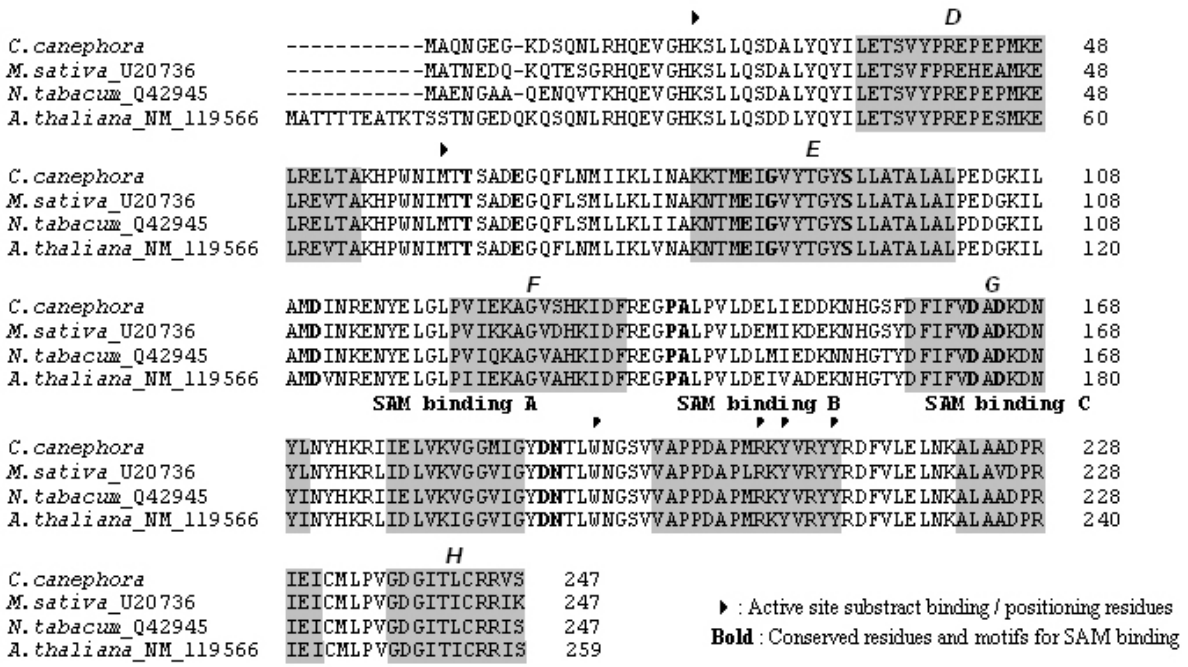

Figure 1. Sequence alignment of 4 CCoAOMTs. Alignment was performed using ClustalW algorithm.

The second sequence (1153 nucleotides) was identified as a Caffeic acid O-methyltransferase (COMT), which catalyses the conversion of caffeic acid to ferulic acid. The deduced peptide sequence (350 amino acids) was analysed by alignment with other COMTs. It shared the domains I, J, K, L that characterize the plant OMTs II. (Figure 2)

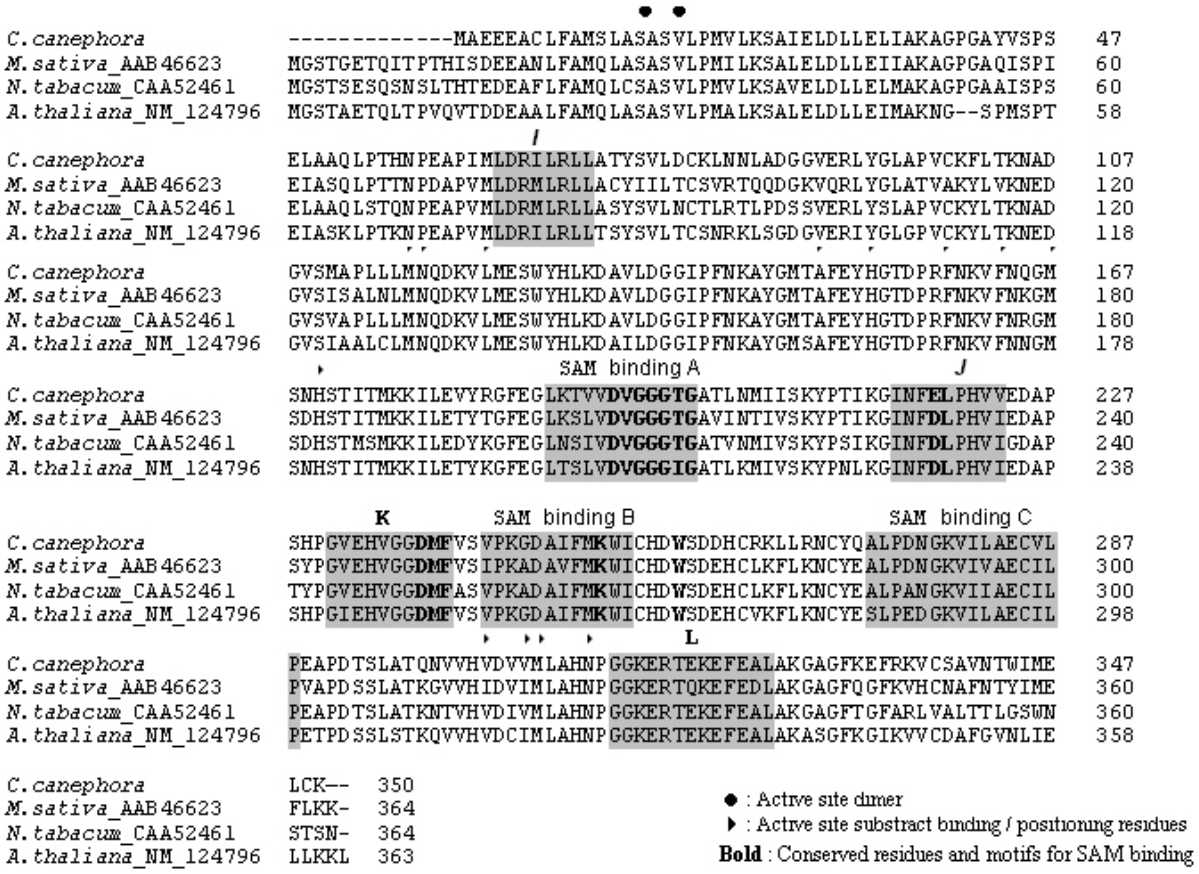

Figure 2. Sequence alignment of 4 COMTs. Alignment was performed using ClustalW algorithm.

On both sequences, alignment with *Medicago sativa* OMTs allowed to identify amino acids implicated in substrate and cofactor binding. (Figures 1 and 2).

A putative transit peptide (20 amino acids) was only identified in the COMT sequence. The addressing of this peptide to the secretory path suggests that phenylpropanoid compound methylation occurs in different cell compartments.

Interestingly, in two EST *C. canephora* libraries, from young leaves and fruits, representing over 5 000 unigenes, only 1 CCoAOMT cluster was found but not a single EST for COMT.

Alignment of the two OMTs of *C. canephora* was performed with homologues from species where the two kinds of peptide were simultaneously found. (Figures 3 and 4).

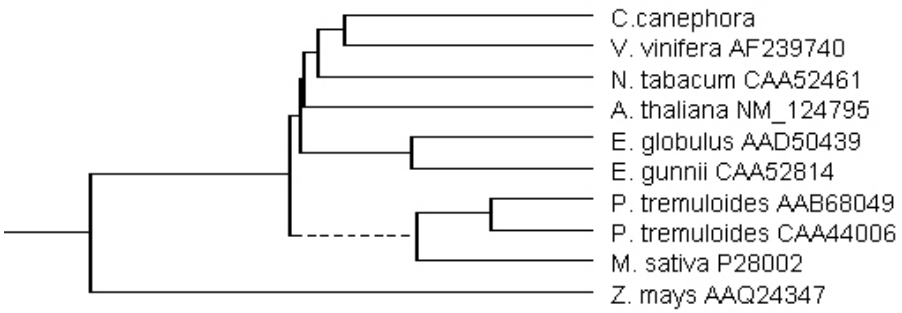

Figure 3. Phylogenic tree of COMT proteins. Multialignment was performed using ClustalW algorithm.

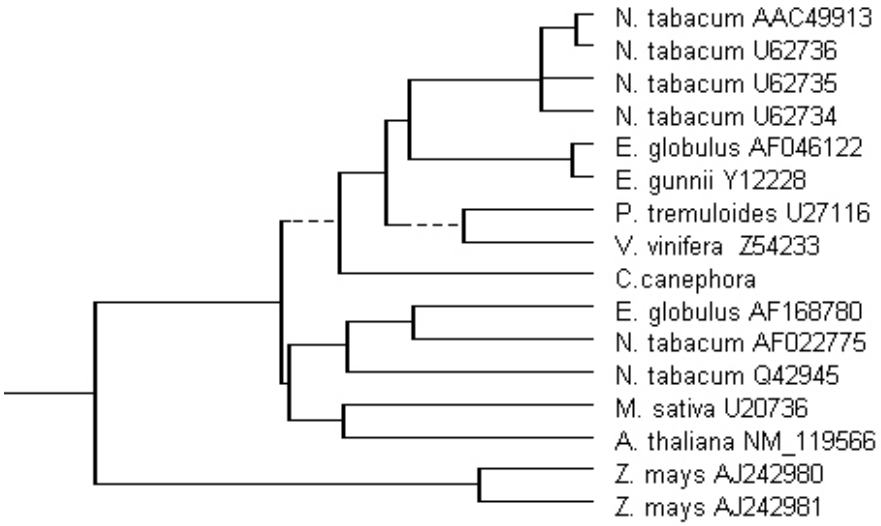

Figure 4. Phylogenic tree of CCoAOMT proteins. Multialignment was performed using ClustalW algorithm.

The two *C. canephora* OMTs gather together with dicotyledonous OMTS, suggesting the implication of these OMTs in lignin synthesis and response to pathogens.

CONCLUSION

This preliminary work is ongoing with the identification of homologous OMT genes and the functional characterization of the encoded proteins in coffee plants. The recent isolation of a second CCoAOMT sequence led us to understand the implication of each isozyme in chlorogenic acids biosynthesis.

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Nickel Elicits a Fast Antioxidant Response in *Coffea arabica* Cells

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SUMMARY

The antioxidant responses of *C. arabica* cell suspension cultures to nickel were investigated. Growth was stimulated at 0.05 mM $NiCl_2$, but reduced at 0.5 mM $NiCl_2$. It was detected an increase in lipid peroxidation at 0.5 mM $NiCl_2$. Catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidase (GOPX) and superoxide dismutase (SOD) activities were increased, particularly at earlier $NiCl_2$ exposure times and the activities were higher at 0.5 mM $NiCl_2$ for most of exposure times tested. Non-denaturing PAGE revealed one CAT isoenzyme, nine SOD isoenzymes and four GR isoenzymes. The SOD isoenzymes were differentially affected by $NiCl_2$ treatment and one GR isoenzyme was increased by $NiCl_2$. $NiCl_2$ at 0.05 mM did not induce lipid peroxidation and the main response appeared related with the induction of SOD, CAT, GOPX and APX activities and throught the induction of GR to ensure the availability of reduced glutathione.

INTRODUCTION

In higher concentrations Ni is a toxic pollutant for plants. Molecular O_2 is relatively unreactive in its ground state, but the production of active oxygen species (AOS) such as $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$ and O_2^1, is an unavoidable consequence of aerobic metabolism. Under normal growth conditions the production of AOS in cells occurs at a low rate, however, adverse environmental factors that disrupt the cellular homeostasis enhance the production of AOS, leading to oxidative stress. Ni is another agent that may cause oxidative stress in plants.

Efficient destruction of AOS requires the action of several antioxidant enzymes such as SOD, CAT, APX, GR and GOPX. SODs are responsible for the dismutation of $O_2^{\bullet -}$ generating H_2O_2 and O_2. CAT, APX, GOPX are enzymes that catalyse the conversion of H_2O_2 to water and O_2.

So far very little is known about the antioxidant response of coffee to stresses. However, sewage sludge has been adopted by coffee farmers as an agricultural practice. Therefore, we studied the effect of $NiCl_2$ on coffee cell metabolism, with special attention to the physiological parameters related to cell growth, lipid peroxidation and the enzymatic antioxidant system.

MATERIAL AND METHODS

Cell suspensions and treatments

Friable callus were obtained from leaves of *C. arabica* variety Catuaí Vermelho (Neuenschwander and Baumann, 1992) and used to produce suspension cultures (100 rpm, dark, 25 °C) being transferred to new medium every week. Seven-day-old cells were

transferred t media containing $NiCl_2$ at 0.05 and 0.5 mM and the cells were grown for 288 h being harvested at distinct periods during the growth cycle.

Analyses

Ni was analysed by energy disperse X-Ray fluorescence. Lipid peroxidation was determined by TBARS method. Enzymes were extracted with 100 mM KP buffer, pH 7.5, 1 mM EDTA, 3 mM DTT and 5% PVPP. After centrifugation the supernatant was used for CAT, GR, APX, GOPX and SOD analyses. CAT, GR (Azevedo et al., 1998), APX (Neuenschwander and Baumann, 1992) and GOPX (Medici et al., 2004) were assayed by spectrophotometric methods. Additionally CAT, GR and SOD were assayed by activity staining on non denaturing PAGE. SOD (Azevedo et al., 1998), CAT (Woodbury et al., 1971) and GR (Nakano and Asada, 1981) were stained with already established protocols.

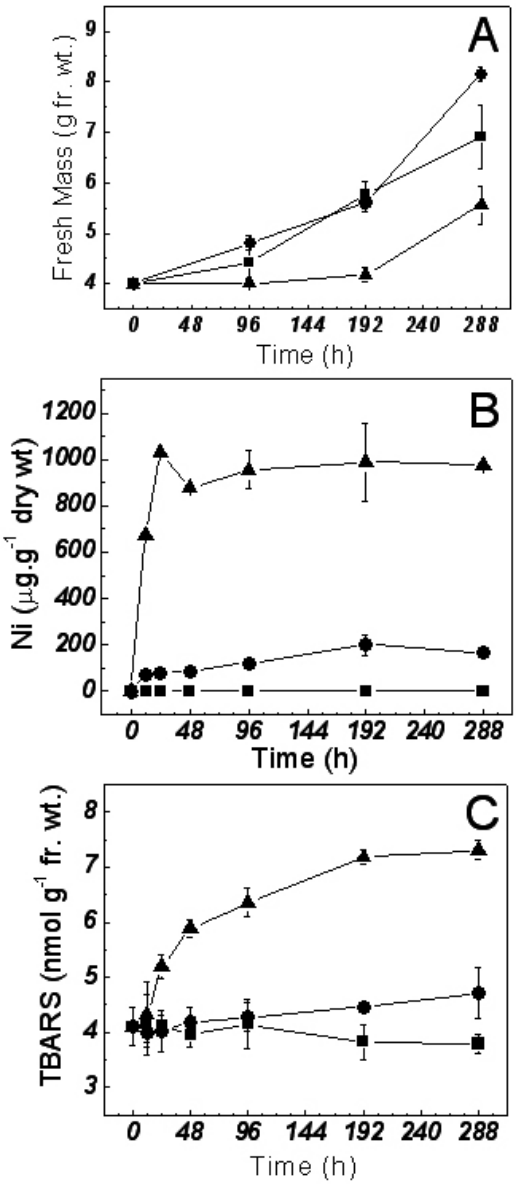

Figure 1. Cell growth (g FW) (A); Ni accumulation (μg.g^{-1} DW) (B) and TBARS content (nmol.g^{-1} FW) (C) in coffee cells grown for a 288 h period in three concentrations of NiCl2. Control zero $NiCl_2$ (■), 0.05 mM $NiCl_2$ (●) and 0.5 mM $NiCl_2$ (▲).

RESULTS

Cell growth, Ni accumulation and induction of oxidative stress

It was observed an inhibition with 0.5 mM $NiCl_2$ and a stimulation of growth with 0.05 mM $NiCl_2$ after 288 h of growth (Figure 1A). Ni entered the cells very rapidly (Figure 1B). Depending on the $NiCl_2$ concentration a faster lipid peroxidation was observed (Figure 1C).

SOD activity

Several SOD isoenzymes were observed on PAGE (Figure 2A) and they were classified according to their metal co-factor based on their inhibitory pattern to hydrogen peroxide and cyanide (data not shown). Band V (Mn-SOD) exhibited a general increase in activity following $NiCl_2$ exposure, but band VI (Mn-SOD) was inhibited. The four minor Mn-SODs isoenzymes (bands I, II, III and IV) were not detected (12 h) or were faint (24 h) with 0.5 mM $NiCl_2$. The Fe-SOD isoenzymes VII, VIII and IX were not affected by $NiCl_2$.

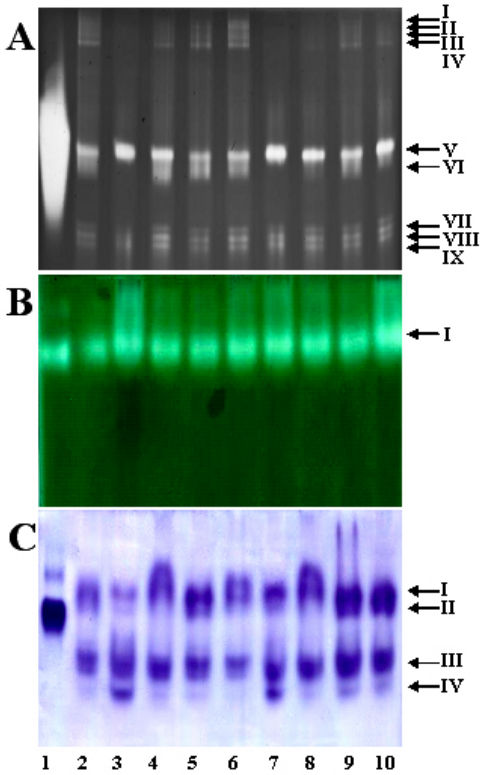

Figure 2. Activity staining for SOD (A), CAT (B) and GR (C) in coffee cells. Lane 1, bovine SOD, bovine liver CAT and *Saccharomyces cerevisiae* standards for (A), (B) and (C), respectively; lane 2, control (zero $NiCl_2$) after 96 h; lane 3, 12 h; lane 4, 24 h; lane 5, 192 h and lane 6, 288 h of growth in 0.05 mM $NiCl_2$; lane 7, 12 h, lane 8, 24 h, lane 9, 192 h and lane 10, 288 h of growth in 0.5 mM $NiCl_2$.

CAT activity

CAT activity staining (Figure 2B) revealed one isoenzyme, which varied similarly to the pattern observed by the spectrophotometer assay (Figure 3A), with a sharp increase in the beginning of treatment with a further increase after 288 h.

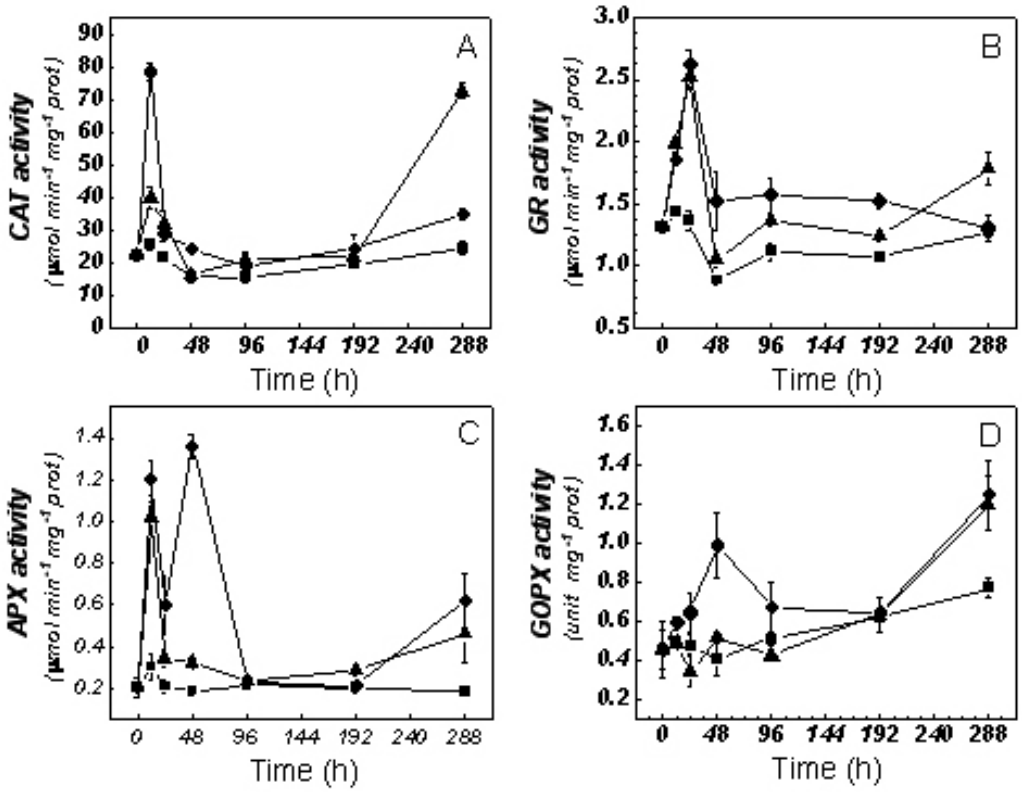

Figure 3. Specific activity of CAT (µmol.min^{-1}.mg^{-1} prot) (A); GR (µmol.min^{-1}.mg^{-1} prot) (B); APX (µmol.min^{-1}.mg^{-1} protein) (C) and GOPX (u.mg^{-1} prot) (D) in coffee cells grown for a 288 h period in three concentrations of $NiCl_2$. control zero $NiCl_2$ (■), 0.05 mM $NiCl_2$ (●) and 0.5 mM $NiCl_2$ (▲).

GR activity

GR staining revealed at least four bands (I, II, III and IV), that were differently affected by $NiCl_2$, particularly band IV (Figure 2C). Total GR activity increased sharply during the first 24h of growth in the presence of $NiCl_2$, but was reduced to lower activity levels after that but still remaining higher than the control.

APX activity

Total activity of APX increased and decreased alternately during the experiments (Figure 3C). In cells subjected to 0.5 mM $NiCl_2$ also exhibited an increase, but to a lower extent than those subjected to the 0.05 mM $NiCl_2$ concentration.

GOPX activity

Two peaks of GOPX activity (96 and 288 h) were observed with 0.05 mM $NiCl_2$. On the other hand, with 0.5 mM $NiCl_2$ activity increased after 288h (Figure 3D).

DISCUSSION

Plant cells incorporate Ni proportionaly to the Ni concentration in the medium and/or the incubation time. In plants, Ni toxicity can cause inhibition of shoot and/or root growth and shoots (Baccouch et al., 2001). Ni is also considered an essential element and it may be a

cause of coffee cell growth induction by 0.05 mM $NiCl_2$ as observed in other plants (Samantaray et al., 2001).

The coffee cell cultures exhibited a brownish colouration mainly at 0.5 mM $NiCl_2$, which might be an indication of lipid peroxidation as shown by the large enhancement in the TBARS. Lipid peroxidation can be initiated by AOS. One mechanism AOS may be generated *in vivo* is the Fenton reaction, which involves the reduction of H_2O_2 by a transition-metal ion, such as Ni, to form the $^{\bullet}OH$ radical, one of the most potent oxidants. Heavy metals can induce oxidative stress indirectly by producing disturbances in chloroplasts, peroxisomes and mitochondria. Since the coffee cells were grown in dark, the oxidative stress may have been due mitochondrial alteration and/or by Fenton type reactions.

The enzyme SOD is unique in that its activity can affect the cell $O_2^{\bullet-}$ and H_2O_2, concentrations. The Ni-dependent enhancement of SOD activity in response to Ni treatment observed in coffee cells has also been reported in several other plants (Rao and Sresty, 2000). We have been able to identify and classify up to nine distinct SOD isoenzymes in coffee cells. The two major SOD bands, V and VI (Mn-SODs) were detectable in native PAGE gels and band V seemed to be largely responsible for the global and rapid increase in SOD activity, suggesting a major mechanism to avoid the Ni stress. Mn-SOD is located in the mitochondria and peroxisomes, and therefore should prevail in coffee cells. Three minor bands of Fe-SOD isoenzymes did not respond to the Ni treatment. Fe-SOD has been associated with chloroplasts (Vitória et al., 2001).

The rapid elevation in CAT activity at 0.05 mM $NiCl_2$ may be associated with the elevation of H_2O_2 level by the higher SOD activity. CAT activity has been associated with Ni toxicity in other plants (Baccouch et al., 2001). Many plants contain multiple CAT isoenzymes, however, only one major CAT isoenzyme was detected here.

GR activity increased rapidly after $NiCl_2$ treatment. Other studies showed that GR activity increases as part of the defence mechanism against Ni stress (Daza et al., 1993). Such results suggest that GR responds to Ni stress by maintaining glutathione in the reduced form, prior to the formation of a stable Ni-GSH complex (Rao and Sresty, 2000) and/or the activation of the ascorbate-glutathione cycle for the removal of H_2O_2 (Asada, 1999). A fourth GR band (IV) in coffee cells was more significantly affected by Ni mainly after 12 h exposure. Furthermore, all GR isoenzyme bands in Ni-treated cells exhibited changes in activity. In plants most of the GR activity is located in chloroplasts, so, it appears that in coffee suspension cells cytosolic GR isoenzymes are likely to be predominant due to the dark growth.

Plants also contain plastidic and cytosolic isoenzymes of APX. In the coffee cells, the activity of APX increased rapidly upon $NiCl_2$ treatment although the activity trends were slightly different between the two $NiCl_2$ concentrations used. APX activity has been shown to increase under Ni treatment in other plants (Baccouch et al., 2001).

GOPX exhibited a general activity elevation in response to Ni treatment, mainly at 0.05 mM $NiCl_2$, confirming previous findings reported for other plants (Rao and Sresty, 2000).

It appears that 0.05 mM $NiCl_2$ might have induced the antioxidant defences by the rapid increase of the activity of all of the major antioxidant enzymes tested (SOD, CAT, GR, APX and GR) protecting against the deleterious effects of AOS, which might explain the lack of increase in lipid peroxidation observed.

Coffee cells subjected to 0.5 mM $NiCl_2$ exhibited oxidation damage based on visual evaluation and the increase in TBARS content, leading to inhibition of cell growth. This effect may be due a reduction in the Ni-tolerance mechanisms that include the insuficient increase in the activity of the antioxidant enzymes that were in general lower when compared to the activities detected at 0.05 mM $NiCl_2$ treatment. Thus, with the elevation of Ni concentration, a higher enhancement in the activity of antioxidant enzymes would be necessary to alleviate the Ni-induced oxidative stress to maintain the Ni-tolerance in coffee cells at 0.5 mM $NiCl_2$.

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Allantoin Has a Limited Role as Nitrogen Source in Cultured Coffee Cells

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SUMMARY

In coffee plants allantoin and allantoic acid are catabolites of caffeine degradation. Using coffee cell suspension as a model we investigated the contribution of ALN as a source of N in coffee. ALN was incorporated in the liquid medium and after 20 d of cultivation, cell mass, NO_3, NH_4, amino acids, soluble proteins, ALN and caffeine were determined in the cells. The activity of glutamine synthetase was also studied. The results showed that despite being taken up by cells ALN does not contribute significantly as a source of N in coffee cells. Compared with mineral N sources, cells grown with ALN-N accumulated much less mass. The inclusion of ALN in the medium caused significant alterations in the content of some N compounds indicating a stress condition.

INTRODUCTION

The ureides allantoin (ALN) and allantoic acid (ALA) are the main N transport compounds found in the xylem sap of many nodulated tropical legumes and it may reach as much as 95% in *Vigna* (Pate, 1973). Contrary to legumes, little is known about the physiological role of ureides in non-nodulating plant species. Some reports suggest the ureides play a role in the N transport and economy of N in algae (Piedras et al., 1998; Desimone et al., 2002) and *Arabidopsis*. ALN and ALA are formed from xanthine degradation as follows: xanthine → uric acid → ALA → ALA.

ALA and ALN are also metabolites of caffeine degradation in coffee, tea and other caffeine-containing plants, since xanthine is formed after three consecutive demethylation steps of the alkaloid (Mazzafera, 2004). *Coffea arabica* and *Coffea dewevrei* seeds were found to contain 0.9 $mg.g^{-1}$ of ureides which is a little higher than the level found in soybean seeds ~0.3 $mg.g^{-1}$ (Vitória and Mazzafera, 1999). Caffeine and ureides are present in the xylem sap of *C. Arabica*, suggesting that they might play some role in the N transport in this species (Mazzafera and Gonçalves, 1999).

Here we investigated the role ALN as a source of N in coffee, using coffee cell suspensions as an experimental model.

MATERIAL AND METHODS

Cell suspensions and treatments

Friable callus were obtained from leaves (Neuenschwander and Baumann, 1992) and used to produce suspension cultures (100 rpm, dark, 25°C) being transferred to new medium every week. Three weeks before the beginning of treatments, the cells were adapted in medium without N. Cells adapted without N were transferred to medium where the N of the medium

was changed by substituting mineral N with the corresponding amount of N from ALN. Three treatments were used: 0% N-ALN (100% mineral), 25% N-ALN (75% mineral N + 25% N from ALN) and 100% N-ALN. After 20 d the cells were harvested by filtration and the fresh mass determined.

Analyses

Freeze-dried cells were extracted and analysed for amino acids and caffeine by HPLC, and for proteins, ALN, ALA, NO_3^- and NH_4^+ by colorimetry. Activity of glutamine synthetase (GS) was also assayed. Cells were fed with [2-^{14}C]caffeine and after 3 days caffeine was extracted and the radioactivity in the alkaloid used to calculate the degradation rate.

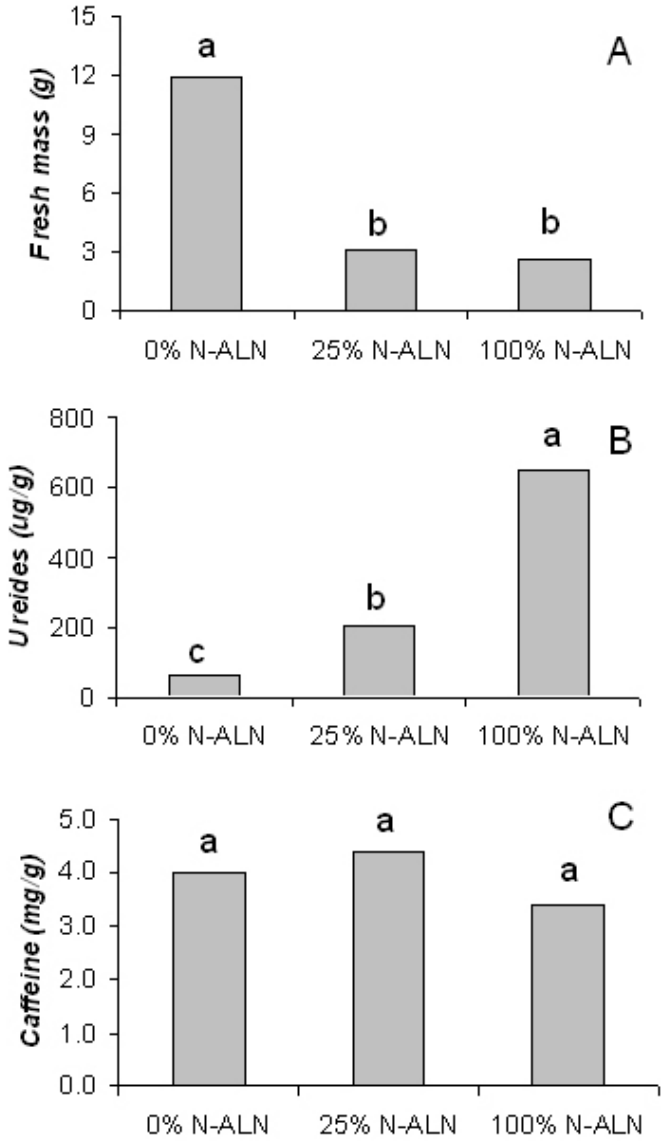

Figure 1. Fresh mass (A), and ureide (B) and caffeine (C) in coffee cells cultivated in 0%, 25% and 100% N-ALN media. Initial cell mass was 2 g.

RESULTS AND DISCUSSION

After 20 d in liquid medium, when ALN was the exclusive source of N, the cells doubled their initial (2 g) fresh mass (Figure 1A) but, this growth rate was much lower than that

observed with cells growing exclusively on mineral N (0% N-ALN). Curiously, cells in the 25%N-ALN medium did not show an intermediary growth rate relative to the other two treatments. This suggests that ALN might have been toxic to the cells. Nevertheless, the ureide content in the cells increased with ALN in the medium (Figure 1B).

By feeding coffee cells suspensions with [2-^{14}C]caffeine it was possible to estimate that the caffeine degradation rate was 93 $\pm$ 5 $\mu g.g^{-1}$ cells.day^{-1}. Caffeine was also measured in coffee cells cultivated for 20 d in 0%, 25% and 100% N-ALN media (Figure 1C) and although some variation was observed the values did not differ significantly among treatments. Therefore, during the experimental period it seems that caffeine synthesis was maintained.

NH_4^+ content of cells in the 0% N-ALN medium was more than twice that found in the media containing ALN (Figure 2A). NO_3^- was also lower in the media containing ALN but in this case the 100% N-ALN showed the lowest content (Figure 2B). It is noteworthy that even with cells that were acclimated for 3 weeks in media deprived of N they still contained NO_3^-, probably in view of an internal pool in the vacuole.

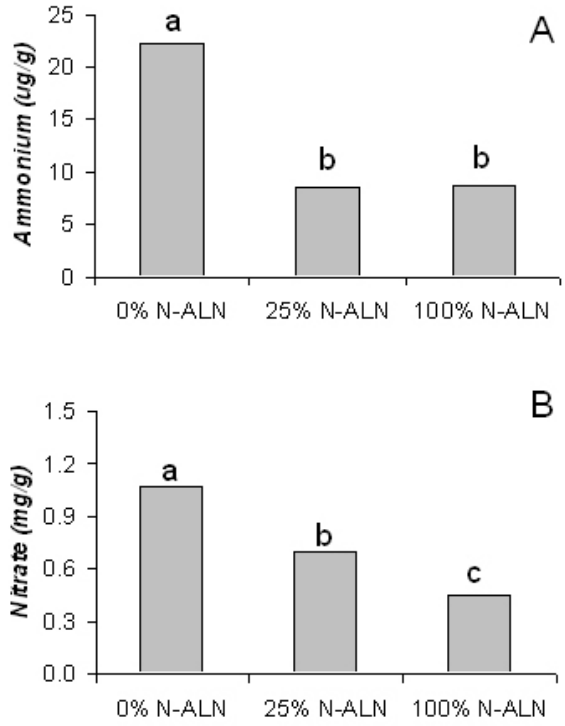

Figure 2. NH_4 (A) and NO_3 (B) in coffee cells cultivated in 0%, 25% and 100% N-ALN media.

Considering the initial composition of the media, by the end of the experiment, the NH_4^+ content in the 0% and 25% N-ALN media had dropped to 1.33 and 0.51 $\mu g.mL^{-1}$, respectively (data not shown). The NO_3^- content of the media also decreased, reaching values of to 171 and 37 $\mu g.mL^{-1}$, respectively. Calculating the percent of NH_4^+ and NO_3^- remaining in the media, the former fell to levels lower than 0.5% while the later to levels varying between 2 and 7%. Therefore, compared with ALN remaining in the media it is clear that cells given ALN did not take up the same amount of N as the cells on mineral N. Nevertheless, the presence of ALN did not reduce the uptake of mineral N, apparently.

As observed here for coffee cells, deficient growth was observed with *Arabidopsis* growing exclusively on ALN as N source, although the plants completed their life cycle producing

seeds (Desimone et al., 2002). Therefore, the low growth observed with coffee cells on ALN appears to have been a consequence of N depletion because of deficient uptake of this ureide. However, it is not possible to rule out that N depletion was due to deficient catabolism of ALN inside the cells.

The specific activity of GS was highest in cells growing on 25% ALN and lowest in cells receiving only mineral N (Figure 3A). Soluble protein content was contrary to the GS activity (Figure 3B).

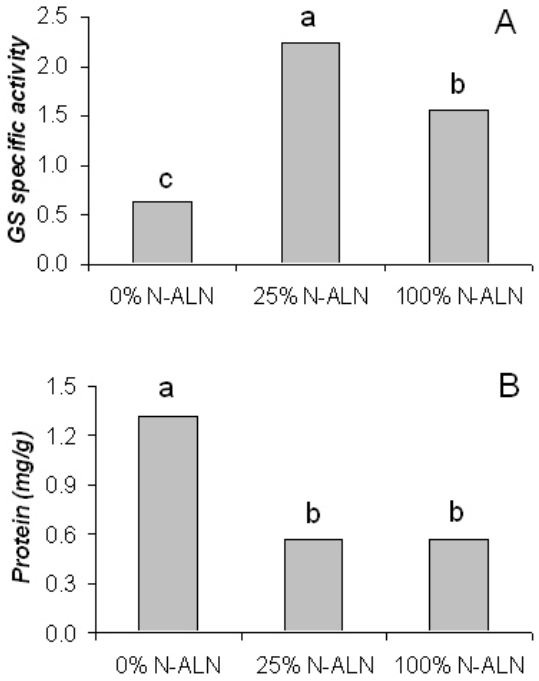

Figure 3. Glutamine synthetase activity (A) and soluble protein contents (B) in coffee cells cultivated in 0%, 25% and 100% N-ALN media.

Cells on 0% N-ALN had more amino acids (Figure 4A). Together with the protein data, amino acid contents indicate that despite ALN being taken up by the cells, N-ALN was not apparently incorporated in amino-N compounds. In addition to a variation in content, the amino acid profile was also changed by ALN nutrition. Cells on 0% N-ALN medium contained relatively more asparagines and glutamine (Figure 4B). When ALN was added to the medium there was a large relative increase of aspartic acid and glutamic acid, with a corresponding decrease of asparagine and glutamine.

The presence of ALN obviously has a profound influence on the N metabolism of the cultured coffee cells. It would appear to lead to a N deficiency condition. On a fresh weight basis, less protein and much less free amino acids was found in the cells grown in the presence of ALN, indicative of a lower N:C ratio. Lower N:C ratios result from N-deficiency and appear to have a negative influence on asparagine synthetase activity (Lam et al., 1995) which would explain the higher aspartic acid/asparagine ratios found in the free amino acids of the cells. By analogy, the increase in glutamic acid/glutamine ratios under the same conditions might result from decreased GS activity. However, GS activity was not lower in the cells growing on ALN. Nevertheless, GS activity is known to be less affected by N stress than other enzymes of N assimilation (Atkins et al., 1984). Consequently, the higher glutamic acid/glutamine ratios may simply be a reflection of the strongly reduced assimilation of N due to the N deficient conditions, due to a reduced throughput of N in the GS reaction.

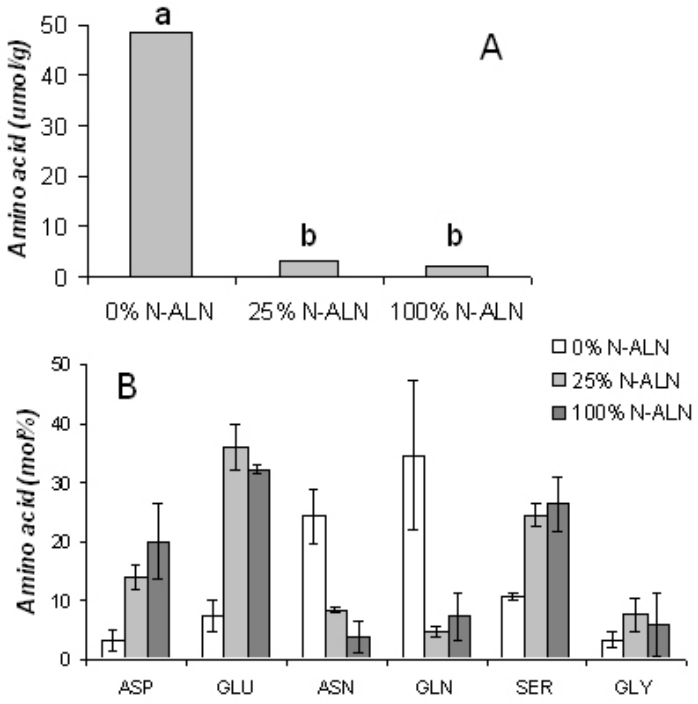

Figure 4. Amino acid contents (A) and amino acid composition (B) in coffee cells cultivated in 0%, 25% and 100% N-ALN media. ASP = aspartic acid, ASN = asparagines, GLU = glutamic acid, GLN = glutamine, SER = serine, GLY = glycine.

Compared with mineral N, coffee cells were not efficient in metabolize of ALN and therefore there might have been a shortage of N for cell growth, explaining the diminished mass accumulation and the low contents of N compounds. Like coffee seeds [10], coffee cells in culture contained larger relative amounts of serine which increase with ALN supply (Figure 4B). GS activity is higher in plants submitted to stresses that cause an increase of photorespiration such as drought (Wingler et al., 1999). The ratio GLY/SER has been suggested as an indicator of increased photorespiration (Diaz et al., 2005). Here we observed that this ratio was 0.32, 0.31 and 0.22 in the 0, 25 and 100% N-ALN treatments, respectively. But this decrease in the ratio was mainly caused by an increase of serine (see Figure 4B). Since these cells were maintained in the dark, it would appear that the rise in serine was stress-related involving N starvation provoked by ALN, since the ureide does not seem to be satisfactorily metabolized. Serine might arise by glycolytic activity generating 3-phophoglyceric acid, the substrate of serine biosynthesis (Coruzzi G., Last et al., 2000).

As N-containing compounds, it has been suggested that alkaloids might play a role as N-reserve molecules but this relationship was not established for caffeine in coffee (Mazzafera, 1999). ALN is formed during caffeine degradation and one might expect the metabolic recovery of the NH_4^+ from the ureide breakdown. Our results do not exclude this possibility but show that it would not be of physiological importance in terms of plant N-economy.

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