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Coffee and Cancer: A Benefit-Risk Evaluation of the Experimental and Epidemiological Evidence

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SUMMARY

Animal carcinogens found in heated foods and beverages have been a significant health concern since the 1970's, when trace levels of these compounds were discovered in many foods. Many of these compounds have been studied in the U.S. National Toxicology Program's (NTP) carcinogen bioassays in rats and mice as well as in the European Union's HEATOX Project, including acrylamide, furan and 4-methylimidazole (4-MEI), all of which have been found in roasted coffee as a result of the Maillard Browning Reaction (MBR). Perhaps the most prominent animal carcinogen in coffee is acrylamide, but there is now extensive epidemiologic evidence indicating that it is not a human dietary carcinogen. Furan, a rodent liver carcinogen, is also well-known constituent in coffee, and coffee is known to be the primary dietary source of furan. But paradoxically, coffee consumption actually reduces the risk of human liver cancer, the target organ for furan. And 4-MEI has been shown to induce only mouse lung tumors in chronic testing, while in the same NTP bioassay it reduced the incidence of tumors in several organs of the rat.

Currently there is little human epidemiologic evidence linking these trace level animal carcinogens in coffee with the risk of human disease, including cancer. The health benefits of a food such as coffee, including the effects of many health-protective, naturally occurring compounds (e.g., chlorogenic acids) and those produced by heat in the MBR (e.g., melanoidins), are often neglected by public health and regulatory authorities when assessing the overall safety of a food. In fact, coffee consumption has actually been shown to reduce the risk of several human cancers while not increasing cancer risk in other organs systems, in spite of the fact that coffee contains trace levels of many animal carcinogens. While it is obviously important to evaluate the toxicological risks of individual heat-induced carcinogens in foods including coffee, it is equally important to fully evaluate the safety of whole foods containing these carcinogens using a combination of modern clinical, toxicological, nutritional and epidemiological techniques using a benefit-risk evaluation of the whole food. Such an evaluation gives us the new concept of the "Coffee-Cancer Paradox".

INTRODUCTION

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 safe 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 focused on investigating the possible health benefits of coffee consumption. As evidenced by the conclusions of many recent expert reviews, a growing literature appears to be concluding that moderate coffee consumption may be associated with reduced risk of type 2 diabetes, coronary heart disease, Alzheimer's disease, Parkinson's disease and even some cancers, in addition to the well-established database on the benefits of human coffee and caffeine consumption in improving both physical and mental performance (Higdon and Frei, 2006).

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 (the chlorogenic acid derivatives) (Hoelzl et al., 2010) and heat-produced antioxidants (various Maillard Reaction Products, including volatile heterocyclic compounds and brown melanoidin polymers) (Moreira et al., 2012; Somoza, 2005). 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 such as glutathione-S-transferase, which has been shown to detoxify acrylamide in the human body. Therefore, while trace parts-per-billion levels of many animal carcinogens can be found in brewed coffee, there are also many compounds now identified in coffee that may reduce the risk of cancer.

What we do or don't eat or drink is oftentimes linked to an increased risk of human cancer, where we have witnessed the "carcinogen-of-the-month" syndrome. However, a great deal of this information has been preliminary in nature and based largely on extremely high-dose animal cancer studies and small epidemiologic studies. But once a larger body of studies has been accumulated, especially human studies as it has for coffee over the past decade, the absence of any real human cancer concern over coffee has been demonstrated, and there is now strong evidence that coffee consumption is reducing the risk of several forms of human cancer. This paper will address key animal and human studies on coffee and its components and the need to take a "Holistic Approach" [a "risk vs. benefit" approach] in determining the health effects of coffee in today's diet, especially in relation to the possible protective effect of coffee in human cancer.

HUMAN EPIDEMIOLOGIC EVIDENCE

Coffee consumption is a major and frequent dietary exposure in diverse cultures around the globe, but its safety related to human cancer causation has been questioned and studied for decades. A substantial body of epidemiologic evidence (over 500 studies) relating coffee consumption to cancer of various organ sites has been accumulated to date. Numerous, organ-specific studies using meta-analysis, as well as comprehensive reviews, have been undertaken more recently, and have concluded that there is not only no increased cancer risk for numerous organs but also a reduced risk for several other forms of cancer.

With the emergence of many more studies over the past decade, Arab (2010) comprehensively reviewed and summarized the findings of meta-analyses and of more than

500 studies on site-specific human cancers among coffee consumers. This reviewer concluded that the evidence largely points to an overall lack of effect across all cancer sites (breast, pancreatic, kidney, ovarian, prostate, gastric cancer): "For most cancer sites, there is a significant amount of evidence showing no detrimental effect of consumption of up to 6 cups of coffee/day in relation to cancer occurrence. In fact, some of the evidence…suggests that coffee might prevent some cancers." Arab concluded that there is evidence of a strong and consistent preventive effect in hepatocellular and endometrial cancer and, possibly, breast cancer, and that the association between colorectal cancer appears to be borderline protective. She noted that the risk of bladder cancer appears to be very weak when associated with heavy coffee consumption in some study populations, but that this effect may be an indication of confounding by smoking.

In a review of methylxanthines and health, Beaudoin and Graham (2011) concluded that the research did not find a strong association between long-term coffee consumption and the risk of colorectal, breast, lung and bladder cancers. These reviewers found that the strongest beneficial association between coffee and cancer is for liver cancer and that this protective, dose-dependent association is evident even in low consumers (one cup of coffee daily). In another recent major review and meta-analysis of 59 epidemiological studies evaluating the risk of cancer associated with one cup/day increments of coffee consumption, the authors found that an increase in consumption of one cup of coffee per day was associated with a 3% reduced risk of cancers (Yu et al. 2011). In subgroup analyses, the authors also noted that coffee drinking is associated with reduced risk of bladder, breast, buccal and pharyngeal, colorectal, endometrial, esophageal, hepatocellular, leukemic, pancreatic and prostate cancers. Findings from this meta-analysis suggest that coffee consumption may reduce total cancer incidence and it also has an inverse association with some types of cancers.

Another large-scale cohort study in Japan that followed 97,753 Japanese men and women aged 40-79 for 16 years found no association between coffee consumption and total cancer mortality among men, whereas a weak inverse association was found among women (Tamakoshi et al., 2011). And the European Investigation into Cancer and Nutrition (EPIC) prospective cohort study that investigated the association between coffee consumption and the risk of chronic diseases (including cancer) in the 42,659 participants that were followed over the 8.9 year period found no association between caffeinated or decaffeinated coffee consumption and total cancer risk (Floegel et al. 2012).

As noted above, there has been some concern that coffee consumption may be associated with a weak increase in risk of bladder cancer in some earlier studies. Zhou et al. (2012), however, recently evaluated 23 case-control studies with 7,690 cases and 13,507 controls, as well as 5 cohort studies with 700 cases and 229,099 participants. They concluded that although data from case-control studies suggested that coffee was a risk factor for bladder cancer, there was no conclusive evidence on this association because of inconsistencies between case-control and cohort studies.

ANIMAL CARCINOGENS IN COFFEE

Coffee contains trace levels of over two dozen identified animal carcinogens, including acrylamide, furan, caffeic acid, various aldehydes, polycyclic aromatic hydrocarbons, ochratoxin A and others. While a few of these chemicals are naturally occurring in green coffee beans, many of the others are chemicals formed at trace levels during the roasting of coffee by means of the Maillard Browning Reaction (MBR) (Somoza, 2005). Carcinogens from heated foods, produced by the MBR between carbohydrates and amino acids and proteins, have been a health concern since the 1970s. Following acrylamide's discovery in

foods, the European Commission undertook a 40-month project to research and evaluate many heat-generated food toxicants and issued a very comprehensive report on this effort (HEATOX Project, 2007). But an important toxicological consideration that has received little attention to date is that many MBR products are beneficial to health because many demonstrate anti-oxidative, anti-mutagenic and anti-carcinogenic properties, and some MBR products even induce the formation of carcinogen-detoxifying enzymes such as glutathione-S-transferase.

For roasted coffee overall, however, the International Agency for Research on Cancer (IARC, 1991) concluded that animal studies of long-term coffee consumption (brewed and dried instant coffee powder) have demonstrated no increased risk of cancer. But it is important here to discuss two animal carcinogens in coffee, acrylamide and furan, each of which has gained widespread attention and concern over the past decade.

Acrylamide

Acrylamide was discovered in a wide array of carbohydrate-rich Maillard browned foods by Swedish researchers and was first reported in 2002 (see Lineback et al., 2012 for a comprehensive review). Coffee was one of many browned products shown to contain acrylamide (typical levels in brewed coffee are 8-13 parts per billion). As noted in Figure 1, much has been learned about acrylamide's toxicology and carcinogenicity, including the recent reporting of the NTP two-year chronic carcinogenicity bioassay of acrylamide in rats and mice (Beland et al., 2013). The updated NTP animal tumor results should now lead the FAO/WHO Joint Expert Committee on Food Additives (JECFA) to reevaluate the human risk of acrylamide. JECFA should consider dismissing consideration of the NTP's benign tumors in the rat mammary gland and mouse Harderian gland as not biologically relevant to human risk assessment.

- Human occupational neurotoxin, genotoxic / mutagenic in cell cultures.
- Known rat carcinogen, classified as a "probable human carcinogen".
- Metabolized to glycidamide (an epoxide), an animal carcinogen.
- Acrylamide & glycidamide can bind to DNA/amino acids/proteins.
  - DNA adducts → carcinogenic potential.
  - Blood hemoglobin adducts → biomarker of exposure.
  - Dietary proteins may reduce acrylamide uptake in humans.
  - Protective enzymes can detoxify acrylamide and glycidamide.
- NTP Acrylamide Bioassay (July 2012 Report).

"Clear Evidence of Carcinogenicity" for male and female rats and mice.

Figure 1. Acrylamide Snapshot: Chemistry and Toxicology.

While these were the most sensitive tumor endpoints, they are not malignant tumors and are not tumor types relevant to human risk. Therefore, JECFA and others (FDA, European Union, Health Canada) should reevaluate acrylamide's potential for human risk based on the lower incidences of more relevant NTP malignant rat and mouse tumor endpoints, since acrylamide is too important and too widespread a contaminant in the human diet to have its risk determined by biologically irrelevant rodent tumor endpoints and with no consideration of acrylamide's lack of increased risk in humans.

There are two recent comprehensive reviews of dietary acrylamide's human cancer epidemiology. The first concluded that "Available studies consistently suggest a lack of an increased risk of most types of cancer from exposure to acrylamide" (Pelucchi et al., 2011). And more recently Lipworth et al. (2012) concluded that "…epidemiologic studies of dietary acrylamide intake have failed to demonstrate an increased risk of cancer." The authors further concluded that "…continued epidemiologic investigation of acrylamide and cancer risk appears to be a misguided research priority".

Most of the major countries of the world have advised consumers to follow the dietary recommendations for a balanced diet issued by their food regulatory and public health agencies. The data available to date have been insufficient to warrant any recommendation for a significant change in dietary recommendations because of acrylamide's occurrence in foods. Current epidemiologic and toxicologic evidence are insufficient to indicate that the amounts of acrylamide consumed in the normal diet are likely to result in adverse human health effects, particularly cancer.

So, does acrylamide in foods, including coffee, pose a real risk to human health? We conclude that, in spite of acrylamide's known animal carcinogenicity, the human cancer epidemiology database is reassuring and supports the conclusion that there is little if any increased cancer risk in humans. Furthermore, acrylamide's potential dietary risk should be assessed in light of the known health-protective, beneficial components of many acrylamide-containing foods.

Furan

Furan is a simple heterocyclic flavor compound produced by heating many foods and beverages, and it has been known for decades to occur at trace ppb levels in many heat-processed foods (e.g., coffee and other browned foods). Its formation, exposure, toxicity, carcinogenicity and aspects of its risk assessment have recently been reviewed (Moro et al., 2012). Furan is a known rat and mouse liver carcinogen and has been classified by IARC as "possibly carcinogenic to humans." Brewed coffee has been found to be responsible for about 70% of total furan exposure in the human diet. It has been reported at levels up to about 200 ppb in some roasted, but Guenther et al. (2010) demonstrated that furan is reduced significantly during roasting, grinding, storage, brewing and drinking, and that actual levels in roasted coffees are probably closer to 10-35 ppb.

Furan, however, is certainly the best example of the "Coffee-Cancer Paradox" (discussed below). While furan has been shown to produce only liver tumors in rats and mice, and although coffee is the top dietary contributor to furan intake, the human cancer epidemiology described above shows that coffee consumption actually protects against human liver cancer in spite of the presence of this animal liver carcinogen.

BENEFIT-RISK EVALUATION – THE "HOLISTIC APPROACH"

Considerations of comparing health risks and benefits are critical in determining whether the consumption of a particular food or beverage should be considered safe.

Consequently, benefit-risk evaluation is absolutely essential to assess the safety of foods containing heat-produced carcinogens. Regulators and public health authorities have been much too focused in the past decades on simply evaluating the risk of individual chemicals in a food or beverage one by one. Thus, we believe that the correct approach going forward is to

evaluate the safety of the whole food by comparing its risks vs. benefits using the "Holistic Approach".

Various "benefit-risk" evaluations have recently been undertaken around the world. The U.S. FDA's 2009 "Draft Risk and Benefit Assessment of Fish" evaluated the risks of methyl mercury contamination vs. health-protective omega-3 fatty acids. The European Food Safety Authority has provided detailed guidance on human health risk-benefit assessment of foods (EFSA, 2010). Some additional expert guidance was also provided by the *Risk-Benefit Analysis for Foods* (BRAFO) project, an effort funded by the European Commission and coordinated by the International Life Sciences Institute/Europe to develop a framework that allows a quantitative comparison of human health risks and benefits of foods and food compounds, using a common scale of measurement (Hoekstra et al. 2012). A very ambitious evaluation of acrylamide's benefit-risk considerations was contained in a comprehensive expert report that was commissioned by the International Life Sciences Institute/Europe, an effort by 12 collaborating institutes, universities and food companies (Seal et al., 2008). This study reported on risk-benefit considerations of mitigation measures on the acrylamide content of foods, including case studies on potatoes, cereals and coffee. Key issues addressed were the impact of pre-harvest, post-harvest and processing conditions on acrylamide formation, consideration of the nutritional value and beneficial health impact of consuming these commodities and the calculated impact of mitigation using probabilistic risk-benefit modeling to demonstrate the principle of this approach.

When both the benefits and risks of a heat-processed food are assessed, instead of just a single focus on one carcinogen in this food, the beneficial health effects of certain whole foods may be shown to outweigh the health effects of trace levels of animal carcinogens and other toxicants in these foods. We believe that coffee is one of the best examples of such a food, where the health benefits of its constituents do outweigh the risks. Coffee's benefit-risk evaluation, therefore, provides us with some key principles that should be taken into account when evaluating the safety of a whole food:

- we must press global health and regulatory authorities to use improved toxicology, epidemiology and risk assessment methods on individual carcinogens tested at very high doses in animals.
- we must undertake more research and evaluation on both the qualitative and quantitative assessment of the benefits of whole foods.
- we must consider the health benefits of protective compounds both naturally occurring and those produced by heating.
- we must assess the safety and benefits of the whole food, not just individual food carcinogens and toxicants one by one.

THE "COFFEE-CANCER PARADOX"

Coffee contains about 2,000 identified compounds (hundreds are flavors and aromas), including trace levels of many animal carcinogens noted above. But global health and regulatory authorities now agree that coffee consumption is not causing any increased risk of human cancer, and in fact, epidemiological studies show significant risk reductions for several forms of human cancer in spite of the trace level presence of numerous animal carcinogens. How can this paradox be explained? We believe that the presence of many health-protective compounds in roasted coffee may be responsible for the observed cancer risk reductions, including naturally occurring antioxidants (the chlorogenic acids), heat-formed antioxidants (the brown melanoidin polymers), inducers of detoxification enzymes such as glutathione-S-transferase and numerous as yet identified beneficial compounds. So herein lies the "Paradox"

– although coffee contains numerous animal carcinogens, the consumption of the whole food product most likely reduces human cancer risk. This conclusion can serve as the basis for using a similar "Holistic Approach" in conducting benefit-risk evaluations on a wider range of other heat-processed food products beyond coffee.

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Caffeine and Age-Related Cognitive Decline

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SUMMARY

Caffeine consumption has long been associated with enhancement of mood and cognitive functions, notably learning, memory and speed of information processing.

We examine the epidemiological evidence for a causal relationship between caffeine consumption and cognitive deterioration in the elderly. In a population study of 641 elderly persons we examined cognitive functioning, caffeine consumption, magnetic resonance imaging volumetrics and other factors known to affect cognitive performance. Our findings demonstrate the association between caffeine consumption and lower cognitive change over time to be statistically significant for women only, taking into account multiple confounders, to be dose-dependent and temporally related (caffeine consumption precedes cognitive change). Mean log transformed white matter lesion/cranial volume ratios were found to be significantly lower in women consuming more than 3 units of caffeine per day after adjustment for age. This observation is coherent with biological assumptions that caffeine through adenosine is linked to amyloid accumulation and subsequently white matter lesion formation. We also explore the possibility that this association may be modulated by diabetes risk factors.

INTRODUCTION

Ageing of the population has led to an increase in both the prevalence of ageing-related memory loss and dementia. In the absence of specific treatments for either of these conditions, epidemiological research has directed its efforts towards the identification of protective factors which may at least slow down the process of memory loss and delay the onset of dementia. Within this context caffeine has recently become a subject of interest. While it has long been known that caffeine has a positive effect on vigilance, attention, mood and arousal, more recent evidence suggests that it may also be neuro-protective. While having multiple biological effects, including increased cortical activity, the non-selective antagonism of adenosine receptors, particularly A1 and A2A receptors, is the only known central pharmacological effect that occurs in the dose-range of voluntary caffeine intake.

Blockade of adenosine A2A receptors may attenuate damage caused by Aβ, the toxic peptide that accumulates in the brain of patients with Alzheimer's disease (AD). Long-term caffeine administration protects AD transgenic mice against cognitive impairment while limiting brain Aβ levels and increasing brain adenosine levels. This finding suggests a potential role for caffeine in at least slowing the process of neuro-degeneration. A small case-control study found lower caffeine intake during the preceding 20 years in AD patients compared to controls, and a prospective study found regular consumption of coffee but not tea to be associated with a reduced risk of AD at 5 year follow-up.

A recurrent difficulty with clinical and population studies of coffee consumption and cognitive functioning is the elimination of confounding variables; that is, factors associated

with coffee consumption which may in themselves explain the association, for example coffee drinkers may be less disabled and more sociable, and these factors are both associated with memory performance. In order to examine the complex question of caffeine intake and cognitive functioning we looked at data from a population study of 9077 dementia-free community-dwelling persons aged 65 years and over from three French cities: Montpellier, Dijon and Bordeaux.

METHODS

Questions relating to caffeine consumption were included in a standardized interview administered by either psychologists or research nurses. It was assumed that one cup of coffee contained 100 mg of caffeine and tea 50 mg (14), the total average consumption per day being calculated per subject in caffeine units (one unit=100mg).

The cognitive examination consisted of a test of visuo-spatial recall, the Benton Visual Retention Test, and a test of verbal recall and fluency, the Isaacs Set Test. Scores on the Set Test were the number of words produced within 30 seconds. In the 3 centers, clinicians diagnosed prevalent cases of dementia.

Information was also collected on demographic characteristics, education level, mobility and confinement to home and neighborhood, height, weight, alcohol consumption, tobacco use, past history of respiratory disorders, cancer, hypertension, hypercholesterolemia, diabetes, stroke, angina pectoris, myocardial infarction, cardiac and vascular surgery, dementia vulnerability genes, and presence of depressive symptomatology.

On the basis of previous animal and human studies cited above, indicating that blockade of adenosine A_{2A} receptors limits brain Aβ levels, we assumed high caffeine consumers to have lower levels of plasma Aβ. While our study did not include direct measures of either plasma or brain amyloid levels, volumetric measures of microvascular brain injury (WML) were obtained by structural magnetic resonance imaging (MRI) in the Montpellier subjects only; WML having been previously shown to be related to plasma Aβ in both mild cognitive disorder and dementia. In this case we assume that higher rates of caffeine consumption will be associated with lower total WML volume.

RESULTS AND DISCUSSION

Our findings showed that caffeine consumption of over 300 mg per day reduces the rate of decline over time on a verbal fluency task, sensitive to dysfunction in most cortical areas. This protective effect was found to be true for women only. Over the study period caffeine consumption did not, however, reduce the risk for dementia. The advantage of large epidemiological data is that it may take into account multiple biological, environmental, and clinical confounding factors which may have obscured the true cause of this association. We reported that these results persisted even when all known potential confounding factors (age, education, gender, depressive symptoms, hypertension, cardiovascular disease, anti-cholinergic medication, smoking, and alcohol use) were taken into account.

We furthermore found from MRI imaging that higher caffeine consumption was associated with lower WML volume in women only. Furthermore, as with the cognitive testing, this relationship is dose-dependent. There are numerous biological hypotheses linking caffeine intake and WML, for example adenosine associated decreases in blood brain barrier permeability may reduce the amount of amyloid passing into the brain and WMLs being linked to both clinical and silent stroke, changes in extracellular adenosine during ischemia

may provide a neuroprotective response. The reasons for gender differences remain unclear. They could involve differences in caffeine metabolism or in sensitivity to the pharmacologic effect as well as hormonal factors which could not be evaluated in the present study but warrant further examination.

Our work thus suggests that coffee consumption may slow cognitive decline and WML accumulation in women. Further studies are required, however, to determine whether the active component is indeed caffeine or another constitutent of coffee.

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Table 1. Description of base-line population.

	Men (N=2820) %	Women (N=4197) %	Chi2 p
Caffeine consumption per day			
[0-1] unit	27.4	24.6	
]1-2] units	32.4	31.5	0.0006
]2-3] units	27.0	27.5	
> 3 units	13.2	16.4	
Age			
65-69	26.0	25.1	
70-74	35.0	32.8	0.03
75-80	24.8	28.0	
80+	14.2	14.1	
Education			
5 years	21.7	24.8	
9 years	30.1	40.4	< 0.0001
12 years	19.7	21.0	
12 +	28.5	13.8	
CESD ≥ 16	13.8	28.1	< 0.0001
Cardiovascular disease[1]	15.3	4.4	< 0.0001
BMI			
Normal	38.0	53.7	< 0.0001
Overweight	49.4	33.0	
Obese	12.6	13.3	
Diabetes2	12.8	6.7	< 0.0001
Cancer3	1.8	1.2	0.02
Hypertension4	64.2	57.4	< 0.0001
LLA (statin or fibrate)	29.7	32.1	0.03
Hypercholesterolemia5	78.7	60.2	< 0.0001
Smoking			
Never	30.0	81.2	< 0.0001
Former	61.9	15.1	
Current	8.1	3.7	
HRT			
Current		15.0	
Former		16.7	
Never		68.3	
Anticholinergic drugs	4.1	9.3	< 0.0001
Mobility			
Confined home	1.2	1.8	< 0.0001
Confined neighborhood	2.0	4.4	
Not confined	96.8	93.8	
Alcohol			
0	8.0	27.1	
1-12 g/day	31.8	54.0	< 0.0001
13-36 g/day	41.2	17.3	
> 36g/day	19.0	1.6	
Apo E4	20.7	19.2	0.12
MMSE < 26¶	11.8	15.5	< 0.0001
Isaacstest < 40¶	20.2	20.0	0.82
Benton test < 11¶	23.6	30.8	< 0.0001

[1]History of stroke, myocardial infarction, angina pectoris, or arteritis; [2]Diabetes defined as glucose ≥7.2 mmol/l or treated; [3]Cancer over the past two years; [4]Systolic blood pressure ≥ 160 or diastolic blood pressure ≥ 95 mm Hg or intake of antihypertensive drugs; [5]Total cholesterol level ≥6.20 mmol/l; % of subjects with lowest cognitive performances at base-line (lowest quintile); Abbreviations: BMI = body mass index; LLA = lipid-lowering agent; HRT = hormone replacement therapy.

Table 2. Polytomous logistic regression: age, sex and centre-adjusted OR of taking caffeine (cross-sectional at base-line).

	]1-2] units OR (CI)	**]2-3] units OR (CI)**	**> 3 units OR (CI)**	**Wald test p value**
Education				
5 years	1	1	1	
9 years	1.18 (1.01;1.39)	1.58 (1.33;1.87)	1.59 (1.28;1.97)	< 0.0001
12 years	1.07 (0.89;1.28)	1.52 (1.25;1.85)	1.78 (1.40;2.25)	
12 +	1.29 (1.06;1.56)	1.88 (1.53;2.30)	2.66 (2.09;3.38)	
CESD ≥ 16	0.77 (0.66;0.90)	0.78 (0.66;0.91)	0.75 (0.62;0.90)	0.001
Cardiovascular disease	0.81 (0.64;1.02)	1.14 (0.91;1.43)	1.12 (0.85;1.48)	0.02
Hypertension	1.03 (0.90;1.17)	0.96 (0.83;1.09)	0.78 (0.66;0.91)	0.003
Smoking				
Never	1	1	1	< 0.0001
Former	1.26 (1.08;1.48)	1.44 (1.23;1.70)	2.26 (1.87;2.74)	
Current	1.66 (1.22;2.28)	1.66 (1.20;2.30)	3.70 (2.63;5.21)	
HRT (n=4197)				
Current	1.02 (0.79;1.32)	1.24 (0.96;1.61)	1.46 (1.10;1.94)	0.03
Former	1.07 (0.86;1.34)	1.07 (0.85;1.35)	0.91 (0.69;1.19)	
Never	1	1	1	
Anticholinergic drugs	0.77 (0.61;0.97)	0.70 (0.55;0.90)	0.81 (0.60;1.08)	0.03
Alcohol				
0	1	1	1	
1-12 g/day	1.38 (1.17;1.63)	2.06 (1.72;2.46)	1.45 (1.18;1.77)	< 0.0001
13-36 g/day	1.60 (1.32;1.94)	2.24 (1.82;2.76)	1.60 (1.26;2.03)	
> 36g/day	1.83 (1.39;2.40)	2.51 (1.88;3.35)	1.39 (0.98;1.98)	

The polytomous logistic regression models the odds of being in one class of consumption versus being in the "≤ 1 unit" class (reference category).

Table 3. Age, education, base-line cognitive performances, and centre-adjusted OR of cognitive decline according to base-line caffeine intake (longitudinal).

	Men N=2820			Women N=4197		
	OR (CI)	**p**	**p trend**	**OR (CI)**	**p**	**p trend**
ΔIsaac ≤ -6						
]1-2] units	0.92 (0.73;1.17)	0.50	0.19	0.91 (0.75;1.10)	0.33	0.0003
]2-3] units	1.08 (0.85;1.38)	0.51		0.82 (0.67;1.00)	0.05	
> 3 units	1.18 (0.87;1.59)	0.29		0.66 (0.52;0.83)	0.0005	
ΔBenton ≤ -2						
]1-2] units	0.99 (0.80;1.24)	0.96	0.94	0.95 (0.79;1.14)	0.58	0.21
]2-3] units	1.11 (0.88;1.40)	0.36		0.99 (0.82;1.20)	0.92	
> 3 units	0.92 (0.69;1.23)	0.57		0.83 (0.66;1.04)	0.10	
ΔMMSE ≤ -2						
]1-2] units	1.02 (0.81;1.28)	0.87	0.40	0.97 (0.81;1.17)	0.78	0.24
]2-3] units	1.00 (0.79;1.27)	0.99		0.89 (0.73;1.08)	0.23	
> 3 units	1.19 (0.89;1.59)	0.25		0.91 (0.73;1.14)	0.42	

Decrease of at least two points from the base-line for MMSE and Benton test or of at least 6 points for the Isaacs test.

Table 4. Multivariate OR of a cognitive decline (longitudinal).

	Women N=4197			
	ΔIsaacs ≤ -6		ΔBenton ≤ -2	
	OR (CI)	p	OR (CI)	p
Age (yrs)	1.06 (1.05;1.08)	< 0.0001	1.07 (1.05;1.09)	< 0.0001
Education				
5 years	1		1	
9 years	0.81 (0.67;0.98)	0.03	0.73 (0.61;0.88)	0.0009
12 years	0.52 (0.42;0.66)	< 0.0001	0.45 (0.36;0.56)	< 0.0001
12 +	0.57 (0.44;0.74)	< 0.0001	0.46 (0.36;0.58)	< 0.0001
Centre				
Montpellier	1	1	1	1
Bordeaux	1.02 (0.81;1.29)	0.84	1.55 (1.24;1.93)	0.0001
Dijon	1.17 (0.98;1.41)	0.09	1.95 (1.63;2.33)	< 0.0001
Baseline cognitive test	1.08 (1.07;1.09)	< 0.0001	1.78 (1.70;1.86)	< 0.0001
CESD ≥ 16	1.17 (0.99;1.37)	0.07	1.20 (1.02;1.41)	0.03
Cardiovascular disease	1.25 (0.89;1.75)	0.21	1.38 (0.98;1.96)	0.07
BMI				
Normal	1		1	
Overweight	0.96 (0.81;1.13)	0.59	1.14 (0.98;1.34)	0.08
Obese	0.98 (0.78;1.22)	0.83	1.14 (0.92;1.43)	0.24
Diabetes1	1.19 (0.89;1.58)	0.23	1.17 (0.88;1.55)	0.30
Hypercholesterolemia	1.12 (0.96;1.29)	0.15	0.86 (0.75;1.00)	0.04
HRT				
Current	0.88 (0.70;1.10)	0.25	1.04 (0.84;1.28)	0.73
Former	0.95 (0.78;1.17)	0.64	0.94 (0.78;1.14)	0.54
Never	1		1	
Anticholinergic drugs	1.40 (1.10;1.78)	0.007	1.08 (0.85;1.38)	0.53
Mobility				
Confined home	1.77 (1.07;2.95)	0.03	1.30 (0.77;2.20)	0.32
Confined neighborhood	1.20 (0.85;1.71)	0.30	1.51 (1.06;2.16)	0.02
Not confined	1		1	
Alcohol				
0	1		1	
1-12 g/day	0.87 (0.73;1.03)	0.11	1.09 (0.92;1.28)	0.34
13-36 g/day	0.88 (0.70;1.10)	0.26	1.01 (0.81;1.25)	0.96
> 36g/day	1.17 (0.65;2.11)	0.59	1.20 (0.66;2.16)	0.55
Caffeine				
[0-1] unit	1		1	
]1-2] units	0.94 (0.77;1.13)	0.49	0.96 (0.79;1.16)	0.64
]2-3] units	0.85 (0.70;1.04)	0.12	1.00 (0.83;1.22)	0.97
> 3 units	0.67 (0.53;0.85)	0.001	0.82 (0.65;1.03)	0.09

Table 5. Association between caffeine consumption at baseline and Isaac score over time, random-effect models.

	Men N=2820			Women N=4197		
	β estimate (SE)	**p**	**p trend**	**β estimate (SE)**	**p**	**p trend**
Univariate[1]						
Time (years)	4.31 (0.58)	<		3.49 (0.46)	<	
Caffeine		0.0001			0.0001	
]1-2] units	-0.25 (0.46)			0.14 (0.39)		
]2-3] units	-0.35 (0.49)	0.59	0.19	0.56 (0.40)	0.72	0.004
> 3 units	-0.82 (0.60)	0.48		1.31 (0.47)	0.16	
Caffeine x		0.17			0.005	
time	0.06 (0.10)			0.07 (0.08)		
]1-2] units	-0.01 (0.11)	0.60	0.85	0.14 (0.09)	0.40	0.002
]2-3] units	0.06 (0.13)	0.91		0.31 (0.10)	0.10	
> 3 units		0.63			0.002	
Multivariate[2]						
Time (years)	4.33 (0.58)	<0.0001		3.47 (0.46)	<0.0001	
Caffeine						
]1-2] units	-0.37 (0.46)	0.43		-0.05 (0.39)	0.88	
]2-3] units	-0.34 (0.48)	0.48	0.21	0.25 (0.40)	0.53	0.02
> 3 units	-0.82 (0.60)	0.17		1.10 (0.46)	0.02	
Caffeine x						
time	0.06 (0.10)	0.59		0.07 (0.09)	0.41	
]1-2] units	-0.01 (0.11)	0.92	0.85	0.14 (0.09)	0.10	0.002
]2-3] units	0.06 (0.13)	0.64		0.31 (0.10)	0.002	
> 3 units						

[1]Adjusted for age, age x time, educational level, centre; [2]Adjusted for age, age x time, educational level, centre, CESD, cardiovascular disease, BMI, diabetes, hypercholesterolemia, anticholinergic drugs, mobility, alcohol, HRT (for women only) and smoking (for men only).

Does Long-Term Coffee Intake Reduce Type 2 Diabetes Mellitus Risk?

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SUMMARY

This review reports the evidence for a relation between long-term coffee intake and risk of type 2 diabetes mellitus. Several epidemiological studies have evaluated this association and studies revealed a lower risk of type 2 diabetes mellitus with frequent coffee intake. Moderate coffee intake (≥4 cups of coffee/d of 150mL or ≥400mg of caffeine/d) has generally been associated with improvement of risk factors for type 2 diabetes mellitus. Therefore, this review highlights the current evidence for the intricate relationship between constituents' coffee on reduction DM2 risk.

INTRODUCTION

Type 2 diabetes mellitus (DM2) is characterized by insulin resistance and/or abnormal insulin secretion, resulting in a decrease in whole-body glucose disposal. Individuals with chronic hyperglycemia, insulin resistance, and/or DM2 are at greater risk for hypertension, dyslipidemia, and cardiovascular disease.

Although genetic factors may play a role in the etiology of DM2, there is now convincing evidence that DM2 is strongly associated with modifiable factors, such as diet. Interestingly, among the several factors present in diet, coffee, one of the most widely consumed non-alcoholic beverages in Western society, is highlighted as a potent dietary-component associated with reduced risk of several chronic diseases, including DM2 and its complications. Coffee is a complex mixture of more than a thousand substances, including caffeine (primary source), phenolic compounds (chlorogenic acid and quinides), minerals and vitamins (magnesium, potassium, manganese, chromium, niacin), and fibers and several of these coffee constituents have a possible role in glucose metabolism.

The present review provides an overview of the role of long-term coffee intake on the risks of glucose tolerance, insulin sensitivity, and DM2.

COFFEE INTAKE AND TYPE 2 DIABETES MELLITUS

The association between the coffee intake and the risk of developing DM2 has been examined by several researches. Data from a prospective study indicated an inverse association between coffee consumption and the risk of DM2 in men independently of race, age or serum concentration of magnesium. Individuals who drank at least seven cups of coffee daily had 50% lower risk to develop DM2 than those who drank two cups or fewer per day. However, this study has not differenced the intake of caffeinated and decaffeinated coffee and didn't evaluate other sources of caffeine.

Salazar-Martinez et al. evaluated the intake of coffee and caffeine from any sources and found an association between coffee intake and the risk of DM2. Besides, this association was found to be more prominent in women than in men and a protective effect of caffeine intake against DM2 was also revealed.

In the Nurses' Health Study II, the researchers observed, after adjustment for several variables, a lower risk of DM2 in women who consumed any dose of coffee when compared to those who did not have this habit. This association was similar in both caffeinated 0.87 (CI: 0.83-0.91), decaffeinated 0.81 (CI: 0.73-0.90) and filtered coffee 0.86 (CI: 0.82-0.90), suggesting that moderate, either caffeinated, decaffeinated or filtered, coffee consumption decreases (13-19%) the risk of DM2 in young and middle-aged women.

The 11-year prospective Iowa Women's Health Study, carried out with postmenopausal woman verified that the intake of both types of coffee, caffeinated and decaffeinated, was inversely associated to the risk of DM2. In accordance to this, the Nurses´ Health Study I (1989-1990) revealed a 16% lower concentration of C-peptide in individuals who ingested at least 4 cups of caffeinated or decaffeinated coffee per day, indicating that the chronic consumption of caffeinated/decaffeinated coffee might reduce insulin secretion since it decreases C-peptide secretion, a marker of insulin secretion and reducing insulin secretion is consistent with increased insulin sensitivity. The results from these studies indicate that coffee constituents other than caffeine might have a protective role against DM2.

Additionally, an epidemiological study indicated that coffee processing seems to have an effect in the risk of DM2 and pointed an advantage of the filtered coffee over the boiled one (without filtering) in reducing the risk of DM2. Since the lipidic substances from coffee grains, namely cafestol and kahweol, are removed in filtered coffee, it is reasonable to suggest that these substances might act indirectly by increasing the risk of DM2. Moreover, another epidemiological study observed that the protective effect of coffee intake depended on the doses and a prospective study reported that both current and former (~20 ago) coffee consumers had, respectively, 62% and 64% reduction in the risk of DM2.

As verified, not all studies have observed an inverse association between coffee consumption and the risk of DM2. In fact, a Finnish cohort study didn’t report this association. In addition, a study in Pima Indians, a population with high prevalence of DM2, didn’t find different incidence of DM2 among coffee consumers and who those who never drink coffee. Nevertheless, a systematic review elaborated from nine cohort studies supports the inverse association between coffee consumption and the risk of DM2. The individuals who ingested 4-6 cups per day and those with higher intake (more than 6 cups of coffee per day) had 28% and 35% lower risks of DM2 when compared to the lowest ingestion category (less than 2 cups or none daily).

Collectively, numerous epidemiological studies, systematic reviews and meta-analysis of prospective studies observed reduction of DM2 risk occurs independently of caffeinated or decaffeinated coffee intake, with largest inverse association for consumption of approximately 4 servings per day.

MECHANISMS UNDERLYING THE PROTECTIVE EFFECTS OF COFFEE INTAKE ON DM2

Up to the moment, several mechanisms of action as well as the precise coffee constituent responsible for the association between coffee intake and DM2 have been proposed.

The hypothesis that coffee consumption lowers the risk of DM2 involves several possible mechanisms as its likely effects on obesity and insulin sensitivity, which are important risk factors for DM2. In accordance to this, Tagliabue et al. proposed that coffee consumption might stimulate thermogenesis. Some studies showed that caffeine intake is inversely associated with body weight gain and satiety. Lopez-Garcia et al., in his latest research of a 12-year follow-up assessing men and women showed that individuals who consumed coffee lost more weight than those who did not.

Besides, a randomized, placebo-controlled and double-blind study with overweight and moderately obese men and women noticed that the intake of a high-caffeine diet (~524 mg/d) reduces body weight, fat mass and waist circumference, and increases the satiety, when compared to a low-caffeine diet (~151 mg/d). Accordingly, Kovacs et al. observed that high caffeine consumption (511 mg/d) led to higher satiety than low caffeine intake (149 mg/d).

Additionally, coffee influences the secretion of gastrointestinal peptides such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), lowering glucose absorption in the small intestine, and activating central anorexigenic peptides (POMC/CART) as well as inhibiting orexigenic peptides (AgRP/NPY). In accordance to this, McCarty reports a higher GLP-1 production after the intake of drinks containing chlorogenic acid, such as coffee. Another suggested mechanism is the direct stimulation of pancreatic beta cells by caffeine and theophylline.

The beneficial effects of coffee's constituents other than caffeine on insulin sensitivity should be considered. Coffee is a major source of the polyphenol chlorogenic acid in the human diet and may affect glucose metabolism by different mechanisms: increasing insulin sensitivity; inhibiting glucose absorption; inhibiting or retarding the action of α-glucosidase; inhibiting glucose transporters at the intestinal stage; reducing or inhibiting glucose-6-phosphatase hydrolysis at the hepatic stage, what may reduce plasma glucose output, leading to reduced plasma glucose concentration; and activate AMP-activated protein kinase (AMPK) in muscle. Moreover, this chlorogenic acid neutralizes the deleterious effects of free fatty acids over the function of beta cells in insulin-resistant overweight individuals, reducing the risk of DM2. However, it is important to take into account potential confounding by other foods sources of chlorogenic acid, such as apples.

Furthermore, it has been suggested that the inhibition of iron absorption by polyphenol compounds present in coffee might be one of the mechanisms underlying the protective effects of coffee intake on glucose metabolism as evidences points that higher body iron stores are associated with an increased risk for DM2. In line with this, the induction of iron deficiency in impaired glucose tolerant subjects has improved insulin sensitivity.

Each cup (237mL) of regular instant coffee has nearly 7mg of magnesium, a micronutrient involved in glucose homeostasis. Preliminary data evidenced an association between low dietary magnesium intake and insulin resistance. Accordingly, low plasma magnesium concentrations were found in the Pima Indians, probably due to their high degree of insulin resistance.

Collectively, the studies indicates that the coffee intake, caffeinated or decaffeinated, can reduce the risk of DM2, being moderate coffee intake (≥4 cups of coffee/d of 150mL or ≥400mg of caffeine/d). Moreover, several substances other than caffeine, e.g. chlorogenic acid and magnesium, have been suggested as responsible for the protective effect of coffee in the risk of DM2. Although habitual moderate coffee intake seems to be safe and reduce the

risk of DM2, referenced researchers in the theme state that it is early to recommend an increase in coffee consumption as a public health strategy for preventing diseases.

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Effect of Blanching and Air Drying Temperature on Antioxidant Capacity and Fiber Content of Coffee Pulp for Human consumption

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SUMMARY

Blanching and drying effect on the antioxidant capacity, fiber, total polyphenols, anthocyanins, chlorogenic acid and caffeine content was evaluated in coffee pulp, as well as its potential as an ingredient for human consumption. Pulp from organic coffee was submitted to six different treatments that combined acidification, blanching and drying. These samples were analyzed for antioxidant capacity, and results showed that blanched samples had higher antioxidant capacity than the unblanched pulp and that drying temperature had no significant effect on the antioxidant capacity. Total polyphenol content was not affected by treatment. Total anthocyanins were higher for the treated samples than untreated ones. Chlorogenic acid and caffeine contents were higher in acidified blanched and dry samples. Fiber content did not change with any of the treatment.

INTRODUCTION

Coffee wet processing removes the exocarp and most of the mesocarp, resulting in the coffee pulp. Coffee pulp represents about 263 million kilograms per year in Costa Rica mostly used to make organic fertilizer through composting or vermiculture processes. However, reduced space in some coffee mills to do composting or high costs to transport husk to processing facilities cause serious environmental problem.

For a long time, secondary processes have been proposed for coffee by-products, in order to obtain different products, such as: animal feed, organic fertilizer, pectin source, biogas, ethanol, particleboards, mushroom substrate, organic acids, with no references indicating use of coffee pulp in human foods.

Coffee pulp contains significant amounts of polyphenols, tannins and caffeine (1,3,7-trimethylxanthine), representing an opportunity to obtain new functional ingredients to use as food ingredients with high health benefits potential. Among coffee pulp polyphenols are low molecular weight phenols, chlorogenic acid, tannins, anthocyanins and proanthocyanidins.

Anthocyanins provide the coffee cherry red pigment being cyanadin-3-rutinoside the major anthocyanin. High phenolic compounds content, antioxidant activity and glucosidase inhibitor of coffee fruit peels indicate that they impart health benefits.

In the wet process, fresh coffee husks are removed prior to drying and their red color is rapidly degraded by the action of enzymes (peroxidases and polyphenoloxidases (PPO)) liberated by the damaged cells of the outer skin and pulp or by other oxidizing agents, such as oxygen. Pulp color changes from a deep red when recently separated from coffee beans to a

dark-brown color. Therefore, pretreatment before processing is necessary to reduce phytochemicals changes to obtain a stable product.

Food industries has achieved browning control by PPO inhibitors, including ascorbic and citric acids, that have been successful inhibiting the PPO in potatoes, apples, pears, grapes, plums and lettuce.

A physical way to inactivate enzymes is blanching, usually combined with drying and freezing. Blanching helps to maintain stability of the remaining phenolics during apple drying process. Food dehydration is one of the most effective methods to ensure adequate preservation of products. However, drying impact on a nutrient can be important, especially when the process conditions are not controlled to minimize negative effects. High temperatures applied during drying may cause degradation and change bioactivity of thermally sensitive compounds, such as flavonoids. This degradation may be reduced by pretreatments before the dehydration process, such as blanching or the addition of chemical additives, to inhibit or reduce the thermal degradation of polyphenolics.

Fiber is another important component in coffee husk and it represents 21% of the total weight. The main advantage of dietary fiber from agro by-products, when compared with cereals, is its higher proportion of soluble dietary fibre.

The aim of the present study was to measure the effects of blanching and drying of coffee pulp on its antioxidant capacity, fiber, total polyphenols, anthocyanins, chlorogenic acid and caffeine content and its potential as a dried ingredient for human consumption.

MATERIALS AND METHODS

Arabica organic coffee pulp was collected immediately after crushing from Tarbaca region in San Jose, Costa Rica. Coffee was pulped by a small pulper machine that was previously sanitized. Ripe coffee cherries were washed very well with water before pulping.

Coffee pulp was immediately sprayed with a 5% citric acid solution stirring pulp constantly, for about 2 minutes, and working with 1 k batch at a time. All samples were packed in metallized bags and transported at low temperature to the University laboratories to be processed. Two control samples were also obtained. About 500 g of coffee pulp without acid treatment and another 500 g of acidified coffee pulp were frozen in liquid nitrogen and kept frozen until analysis.

Blanching was applied to half of the acidified coffee pulp and the other half was not blanched. Pulp batches were place in a small steam-jacketed kettle at 95 °C for 5 min.

The blanched and the unblanched coffee pulp batches were evenly distributed in single layers (20 mm depth) on wire trays, which were then placed in a cabinet dryer with an air cross-flow (2 zone Excalibur commercial dehydrator). Two different drying temperatures were used, 55 °C and 70 °C, for 5 h. Experimental design rendered six different treatments, four combinations of blanched and unblanched samples at two drying temperatures and two control samples without blanching and drying. Control samples were kept at -80 °C. All treatments were done by triplicate. These samples were analysed for antioxidant capacity using oxigen rate antioxidant capacity method, ORAC (dry matter basis).

Samples with high antioxidant capacity were analysed for total polyphenols caffeine, total anthocyanins, and chlorogenic acid.

Total fiber was measured in the highest antioxidant capacity sample and in the two control samples, in dry matter basis. Method 991.43 from AOAC was used to quantify fiber.
The results were analysed by analysis of variance (ANOVA), when F was significant means were compared by contrast test at $p<0.05$ using XLSTAT (2010).

Finally, product concept study was carried out to identify potential uses of treated coffee pulp. Four potential products (biscuits, fizzy drinks, condiment and tea) with added dried coffee pulp were evaluated. Fifty consumers were interviewed. Two products by consumer were presented and they filled up a questionnaire.

RESULTS AND DISCUSSION

There were significant differences among the six treatments ($p=0.002$). Contrast analysis allowed comparison of specific treatments. Only two contrasts were significant. Differences between acidified coffee pulp without treatment and acidified non blanched and dried at 55 °C and at 70 °C husks ($p=0.002$) were found, and differences between acidified non blanched husks that were dried at both temperatures and acidified blanched and dried at both temperatures husks ($p<0.0001$) were found.

As seen in Figure 1 blanched samples had higher antioxidant activity (487 and 492 umolTE /g dry sample at 70 °C and 55 °C respectively) than dry samples without blanching (165 and 146 umolTE /g dry sample at 70 °C and 55 °C respectively). Therefore, blanching had a positive effect on preservation of antioxidant compounds.

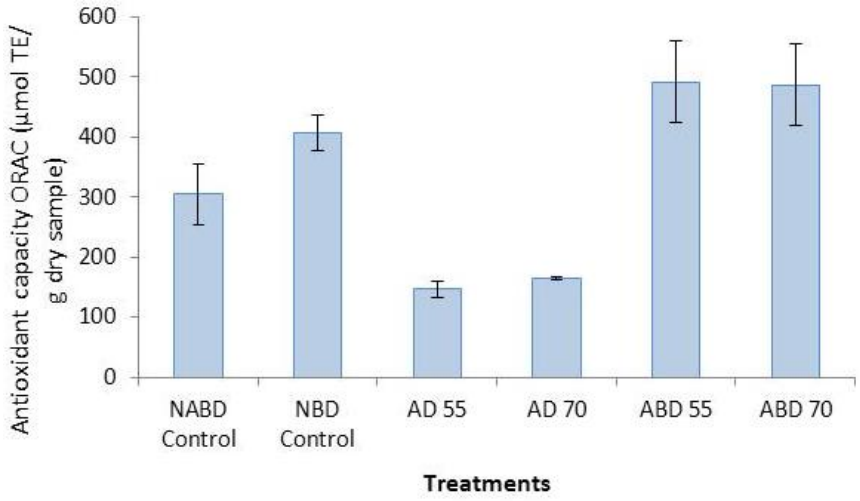

Figure 1. Citric acid, blanching and drying effect on antioxidant capacity (dry matter basis) in coffee pulp. (NABD Control: coffee pulp without acidification, blanching and drying, NBD Control: coffee pulp with acidification without blanching and drying, AD 55: coffee pulp acidified without blanching and dried at 55 °C, AD 70: coffee pulp acidified without blanching and dried at 70 °C, ABD 55coffee pulp acidified blanched and dried at 55 °C and ABD 70 coffee pulp acidified blanched and dried at 70 °C).

Blanching is a unit operation that seeks enzyme activity reduction. Blanched coffee pulp lost less compounds with antioxidant activity due to PPO inactivation. Similar results were obtained by Rossi et al. who found that PPO inactivation, through blueberry fruit steam blanching prior juice extraction, significantly increased recovery of anthocyanins and

phenolic compounds, and therefore gave a significant increase in the antioxidant activity of the juices. Also other studies showed a decrease in the content of total polyphenol for unblanched carrot peel dehydration and for apple pomace when same dehydration temperatures were compared.

Acidified non blanched and dried at both temperatures husks resulted in lower antioxidant content than acidified coffee pulp with neither blanching nor drying (165 umolTE / umolTE 146 g / g dry sample at 70 ° C and 55 ° C respectively). Antioxidant compounds may be lost by enzymatic action in unblanched samples exposed to hot air since the decrease was larger than in acidified untreated husk that was not exposed to hot air. Other studies reported an antioxidant activity decrease due to PPO action. Irreversible oxidative processes during drying can reduce the antioxidant activity of red pepper and pre-treatments like blanching or additives like SO_2 and $CaCl_2$ can improve the retention of vitamin C and antioxidant capacity.

The fact that there were no significant differences between two drying temperatures (55 °C and 70 °C) applied to acidified and blanched husk as well between the same temperatures applied to acidified unblanched husks indicated that temperature during drying process did not have an effect on coffee pulp antioxidant capacity. Some reports indicate that dehydration at high temperatures (i.e. 80 and 90 °C) shows higher antioxidant activity rather than at low temperatures (i.e. 50, 60 and70 °C). This behaviour could be related to the drying process, at low temperatures, which implies long drying times which may promote a decrease of antioxidant capacity. Besides, in the present study both temperatures were applied during the same time.

Several fruit antioxidant capacities from literature can be compared with these coffee pulp values. Strawberries, plums and grapes antioxidant capacity is about 154 ± 8, 79 ± 2 and 36 ± 1 umol TE /g dry sample respectively and they were lower than blanched and dried coffee husks (70 °C or 55 °C) antioxidant activity in dry basis. These fruits are considered antioxidant sources; therefore, dried coffee husk can be considered an antioxidant source also.

Treatments with higher antioxidant capacity were: non treated coffee pulp that was frozen, acidified frozen coffee pulp and acidified blanched and dried at 50 ºC and at 70 ºC. Total polyphenols, caffeine, chlorogenic acid and anthocyanins were evaluated in these four tretaments.

ANOVA for coffee pulp total polyphenol content shows non-significant differences ($p > 0.05$) among four treatments. Total polyphenols were not affected by treatments applied to coffee pulp (freezing, acidification, blanching and drying). Samples received different treatments; therefore, different mechanisms participated preserving their polyphenols. Coffee pulp PPO was probably inactivated by blanching and citric acid addition, avoiding browning. On the other hand, samples without neither acid nor blanching that were kept at -80 ºC probably formed an amorphous solid state or glass within the frozen food that reduced diffusion-controlled reactions, in such a way that the enzymatic degradation could not occur.

Total polyphenol contents have been reported and they can be compared with coffee husk polyphenols. Ulloa et al. reported a total polyphenol content of 20 mg gallic acid /g coffee pulp that was dried at 60 °C for 24 hours in an oven. This value was lower than total polyphenols, on a dry matter basis, of coffee pulp dried at 70 °C and at 55 °C (32 and 26 expressed as mg gallic acid /g dry sample, respectively). Also dry blanched acidified coffee pulp polyphenol content was higher than blackberry, raspberry and strawberry juices, indicating that acidified, scalded and dried coffee husk could be attractive to consumers for healthy products.

A different trend was observed for anthocyanins. Anthocyanin content, expressed as cyanidin 3-rutinoside, is shown in Figure 2.

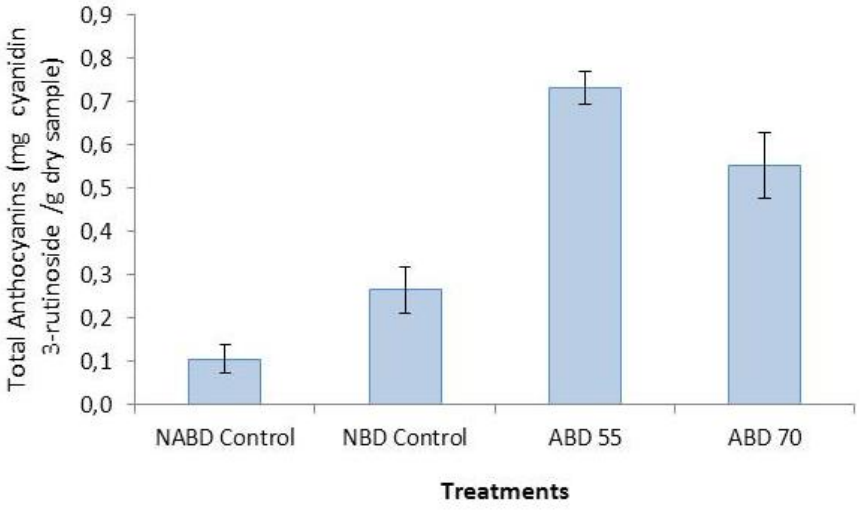

Figure 2. Total anthocyanins, in dry basis, for acidified blanched coffee that was dried at either 55 ºC or 70 ºC and non-acidified and acidified coffee pulp without blanching and drying. (NABD Control: coffee pulp without acidification, blanching and drying, NBD Control: coffee pulp with acidification without blanching and drying, AD 55: coffee pulp acidified without blanching and dried at 55 °C, AD 70: coffee pulp acidified without blanching and dried at 70 °C, ABD 55coffee pulp acidified blanched and dried at 55 °C and ABD 70 coffee pulp acidified blanched and dried at 70 °C).

There were significant differences among treatments ($p = 0.001$) according with the ANOVA. Contrast tests found significant differences between no treated samples (non-acidified and acidified without blanching and drying) and acidified, blanched and dried at 55 ºC and 70 ºC samples ($p = 0.0001$). Coffee pulp without blanching and drying had the lowest anthocyanin values (0.11 and 0.27 expressed as mg of cyanidin-3-rutinoside /g dry samples).

When contrasting acidified without blanching and drying sample (control sample) with acidified, blanched and dried (at 55 °C and 70 °C) coffee pulps a significant difference was also found ($p = 0.0009$), with a higher anthocyanin values obtained for the acidified, blanched and dried at 55 °C and 70 °C samples. PPO in samples without blanching was active and it displays great affinity for o-diphenols. Anthocyanins were reduced by enzymatic action, since they can be degraded by polyphenoloxidase. Similar results were obtained by Rossi et al., who found that PPO inactivation, through a steam blanching blueberry fruit prior to the juice extraction, significantly increased the recovery of anthocyanins.

There were not significant differences between acidified and no acidified coffee pulp without blanching and drying ($p > 0.05$). Acid treatment by itself was not enough to retain anthocyanins. It was expected that acidified samples would retain more antioxidants since red color was preserved with citric acid. Citric acid and phosphate ions are known to act as chelating agents upon the Cu-containing PPO. Copper complexation, at the active site of the enzyme, results in enzyme inhibition, since copper is essential for PPO activity.

Significant differences ($p = 0.048$) were found in total anthocyanin content between acidified blanched and dried at 55 °C sample and acidified, blanched and dried at 70 °C coffee pulp. Acidified blanched and dried at 55 °C coffee pulp total anthocyanins values were higher than coffee pulp acidified blanched and dried at 70 °C. Heat treatments cause destruction of

anthocyanins especially at high temperatures, and, therefore, high temperatures and high oxygen concentrations involved in air-drying process lead to rapid anthocyanin degradation.

When comparing anthocyanin content for acidified blanched and dehydrated at 55 or 70 °C coffee pulp (0.73 and 0.55 expressed as mg of cyanidin-3-rutinoside /g dry sample, respectively) with other fruits such as blackberry (1.83 mg /g of cyanidin-3 glucoside), strawberry (0.10 to 0.80 mg /g of cyanidin-3 glucoside), blueberry (0.20 to 3.60 mg /g of cyanidin-3 glucoside) and raspberry (0.20 to 2.20 mg /g of cyanidin-3 glucoside), coffee pulp content was within range reported for these fruits which are considered high anthocyanin sources. Therefore, dry coffee husk regardless of the drying temperature (55 or 70 °C), has an anthocyanin concentration that could be interesting to produce healthy products. Coffee pulp contains significant amounts of chlorogenic acid. ANOVA did not show significant differences ($p > 0.05$) among treatments. Application of citric acid, blanching and drying at 55 or 70 °C contributed to preserve chlorogenic acid. It must be pointed out that chlorogenic acid can act as an intermediary in PPO enzymatic degradation of the anthocyanins and therefore be decreased in samples were PPO is active. Control samples kept frozen at -80 °C and acidified blanched samples did not suffer PPO action.

Moon et al. reported values between 61.15 to 86.42 mg chlorogenic acid /g dry sample in fresh coffee beans. Clifford & Ramirez reported, for Arabica sundried coffee pulp, between 10.1 to 11.6 mg chlorogenic acid /g dry sample depending on the variety. These results are similar than those of the present study when acidification, blanching and drying at 55 °C or at 70 °C were used in coffee pulps (12 mg of chlorogenic acid / g dry sample) and, therefore, they could be a suitable raw material for the development of products that provide benefits to the consumer.

Coffee pulp caffeine values were not significant different ($p > 0.05$) among treatments. Caffeine content was between 15 and 16 mg/g dry sample and it was lower than brewed tea and coffee caffeine content and higher than colas.

No significant differences were found among fiber content in different coffee pulp samples ($p> 0.05$). Fiber content was between 35% - 38% in dry matter basis. Blanching did not reduce fiber by leaching. Several studies reported a 20% in dry matter basis of fiber in fresh coffee pulp. Therefore, dried coffee pulp can be a good fiber source.

The consumer concept study showed that coffee pulp was considered an innovative food that can be an ingredient for tea infusions, condiments, cookies, bread and more. The idea that consumers can have an ingredient with high antioxidant activity and fiber content was very attractive.

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Coffee Polyphenols Protect Human Plasma from Postprandial Carbonyl Modifications

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SUMMARY

The antioxidant capability of coffee polyphenols to inhibit red-meat lipid peroxidation in stomach medium and absorption of malondialdehyde (MDA) in human was studied. Roasted-ground coffee polyphenols were found to inhibit lipid peroxidation in stomach medium and be 2-5 fold more efficient antioxidant than those of instant coffee. Human plasma from 10 volunteers after a meal of red-meat cutlets (250gr) revealed a rapid accumulation of MDA. Accumulation of MDA in human plasma modified LDL known to trigger atherogenesis. Roasted-ground coffee (200ml), consumed by 10 volunteers during a meal of red-meat cutlets, inhibited postprandial plasma MDA absorption by 80% and 50%, 2h and 4h respectively. The results obtained *in-vitro* simulated stomach model on MDA accumulation were predictive for the amount of MDA absorbed into circulating human plasma, *in-vivo*. Timing the consumption of coffee during the meals may transform it to a very active functional food.

INTRODUCTION

Coffee consumption found to be potentially beneficial for health and decrease the risk of coronary heart disease, type-2 diabetes, and possible other diseases. Atherogenesis may results at least partly from processes that occur after ingestion of high-fat foods that contain advanced lipid oxidation end-products (ALEs), some of which are cytotoxic and genotoxic compounds. We demonstrated that the stomach could act as a bioreactor and be an excellent medium for enhancing lipid oxidation and co-oxidation of vitamins and other dietary constituents. Red-meat is oxidized in the stomach and this process contributed to absorption and accumulation of cytotoxic ALEs, such as malondialdehyde, into rat and human blood system. It was assumed that the gastrointestinal and especially the stomach are the main location of polyphenols as antioxidants in human. Most recently we have demonstrated for the first time (to the best of our knowledge) that absorption and accumulation in human plasma of MDA, from a dietary source, increases in parallel the modification of low-density lipoprotein (LDL) to MDA-LDL. Data supported the involvement of modified LDL in atherogenesis, removal of blood circulating modified-LDL in mice results in complete prevention of atherosclerosis progression. Phenolic compounds and phenolic-melanoidins are the main groups of antioxidants found in coffee brews. Chlorogenic acids are the most abundant polyphenols in green coffee, during coffee roasting a significant part of the polyphenols and especially chlorogenic acid are oxidized and polymerized into high molecular weight coffee melanoidins. The high molecular lipophylic antioxidants activity was on average 30-fold higher in roasted coffee than in green coffee. We studied the antioxidant capability of coffee to inhibit food lipid peroxidation in stomach medium and the effect of consuming coffee during a meal of red-meat on accumulation of MDA in human plasma.

MATERIALS AND METHODS

All coffee brands and fresh turkey red-meat were purchased at commercial stores in Israel. Simulated gastric fluid (SGF) was freshly prepared accordingly to the U.S. Pharmacopeia (U.S Pharmacopeia Inc. Rockville, MD, 2000).Coffee beverages brands: A-"Turkish" roasted-ground coffee (Elite); AG- the same "Turkish" roasted-ground coffee (Elite) (98%) + 2% powder of freeze-dried green coffee extract; B- Instant spray dried soluble coffee (Elite); C - Instant freeze dried soluble coffee bland with green coffee beans extract (Nestle); D- Instant freeze dried soluble coffee "Tester's Choice" (Nestle). All coffee brands were purchased in Israeli grocery stores and are commercially available. Roasted-ground coffee (5g) was brought up to boiling in 200ml of water and the upper phase was taken for analysis. Instant soluble coffee, spray-dried and freeze-dried (2.5g) were dissolved in 200 ml of boiled water and the supernatant was taken for analysis.

Preparation of green coffee freeze-dried powder (GCEP)

Green coffee extract as a freeze-dried powder (GCEP) was prepared by freeze drying of Robusta green coffee beans, powdering by a grinding machine, solubilized the powder by boiling water, and freeze-dried the extract into a powder. The powder was further extracted by 80% ethanol, the ethanol was evaporated under vacuum and the concentrate was freeze dried into a powder.

Determination of total coffee polyphenols

The polyphenol contents of the coffee beverages were determined with Folin-Ciocalteau reagent and calculated as chlorogenic acid equivalent.

Determination of coffee polyphenols by HPLC method

The coffee beverage after filtration was analyzed by a HPLC gradient system using two high precision pumps (Varian ProStar, Walnut Creek, CA, USA); RP18 column (Licrosphere, 5 μm; 254 × 4.6 mm, Merck, Darmstad), autosampler (Varian ProStar model 410) and UV detector (Varian ProStar 325 Dual Wavelength UV-Vis Detector), operating at 325 nm. Quantification of all other CGA was performed using the area of 5-CQA standard calibration curve, combined with molar extinction coefficients.

Determination of lipid peroxidation in SGF

Red meat, lipid peroxidation was determined by hydroperoxide and MDA accumulation as described by Kanner et. al.

Subjects

A randomized crossover study was conducted with 10 healthy subjects. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Hebrew University-Hadassah Institutional Committee for Human Clinical Trials and the approval of the Israel Ministry of Health Committee for Human Trials. Written informed consent was obtained from all subjects/patients. Subjects were excluded if they had any metabolic disorders, were taking dietary supplements, were smokers (1 cigarette/day), were heavy exercisers (4-30 min of aerobic exercise/wk), or were drinkers (~5 U of alcohol/wk).

The volunteers comprised four men and six women with normal blood lipid and glucose levels, with a mean (±SD) age of 28.5±3.1 years. Each volunteer consumed, in a random order, three different test meals on three different occasions separated by 1 wk. The subjects were asked to fast 12 hours before the test meal and to avoid eating meat or fish products for 2 days before the day of the experiment.

Test meals

Turkey red-meat was minced in a MG-600 food processor (Kenwood, Japan), cooked as cutlets on an electric grill for 6 min until well done, divided into 100g portions, and frozen at –80°C pending the experiment. Three test meals consisted of A, control, composed of red meat cutlets (250 g) and a glass of water (200 ml); B, composed of red meat cutlets (250 g) and a glass of "Turkish"A roasted coffee (5g/200 ml, containing 550 mg of polyphenols); C, composed of red meat cutlets (250g) and a glass of "Turkish" AG roasted coffee (5g/200 ml containing 620mg polyphenols). All meals were prepared in advance and kept at –80°C pending use (within 4 wk).

Determination of plasma malondialdehyde

Malondialdehyde was extracted from plasma as described previously. The supernatant was reacted with TBA and filtered through a 0.2 µm membrane. A 25µl sample was injected into an SCL-10A VP HPLC (Shimadzu, Japan), separated with a Lichrocart column, model RP-18, 125–4S (Merck, Darmstadt, Germany), and detected with an RF-10AXL HPLC fluorescence detector (Shimadzu, Japan) set at 532 nm excitation and 553 nm emissions. The mobile phase consisted of a 35:65 (v/v) mixture of methanol and 0.05 mol/L potassium phosphate buffers, pH 7, at a flow of 1 ml/min. MDA standard solution was used to generate a standard curve and to spike plasma samples for determination of the recovery.

Statistical analysis

The results were subjected to repeated-measures analysis of variance (ANOVA) as a three-period crossover design with in Stat version 3.01, (Graph Pad Inc.) software, followed by application of the Student–Newman–Keuls test.

RESULTS AND DISCUSSION

The results showed that a cup of 200ml coffee beverage from brands A, B, C and D contain polyphenol amounts of 550, 511, 595 and 401 mg (chlorogenic acid equivalent) respectively.The HPLC separation shows that the soluble polyphenols in dry green coffee are mostly chlorogenic acids). Using chlorogenic acid (5-CQA) as standard for separation and calculation, the freeze dried powder of green beans (GCEP) contain 80% polyphenols by which ~50% of them are chlorogenic acids, in agreement with those reported in literature,. The inhibition of red meat lipid peroxidation (determined by TBARS) by Turkish roasted-ground coffee (A) in SGF is presented in Figure 1A. The inhibition of red meat lipid peroxidation by coffee products is presented in Figure 1B. The most active antioxidant beverage was found to be the roasted-ground coffee A following B, C and D. The inhibition IC_{50} of beverages A, B, C and D for 100g meat lipid peroxidation is 21, 32, 39 and 94ml respectively. However, most of the people eat ~200g meat, and we would like to inhibit meat lipid peroxidation by 100%. The calculation of IC_{100}/200g meat shows that for a total inhibition of meat lipid peroxidation it is a need from beverages A, B, C and D of 168, 256, 312 and 752 ml, respectively. We determined the effect of two products of roasted-ground coffee A and roasted-ground coffee AG (A enriched by 2% GCEP) on meat lipid peroxidation

in stomach medium. Notably, coffee A and coffee AG contain 110 and 123 mg polyphenols /1g DM, respectively. The results obtained in Figure 1C led to a paradox that increasing the concentration of polyphenols increased the IC_{50} by 2.5 fold, and coffee amount, for IC_{100}/200 g meat, from 168ml to 375ml. We confirmed the antioxidant decrease of coffee AG by generation of hydroperoxides and accumulation of MDA.

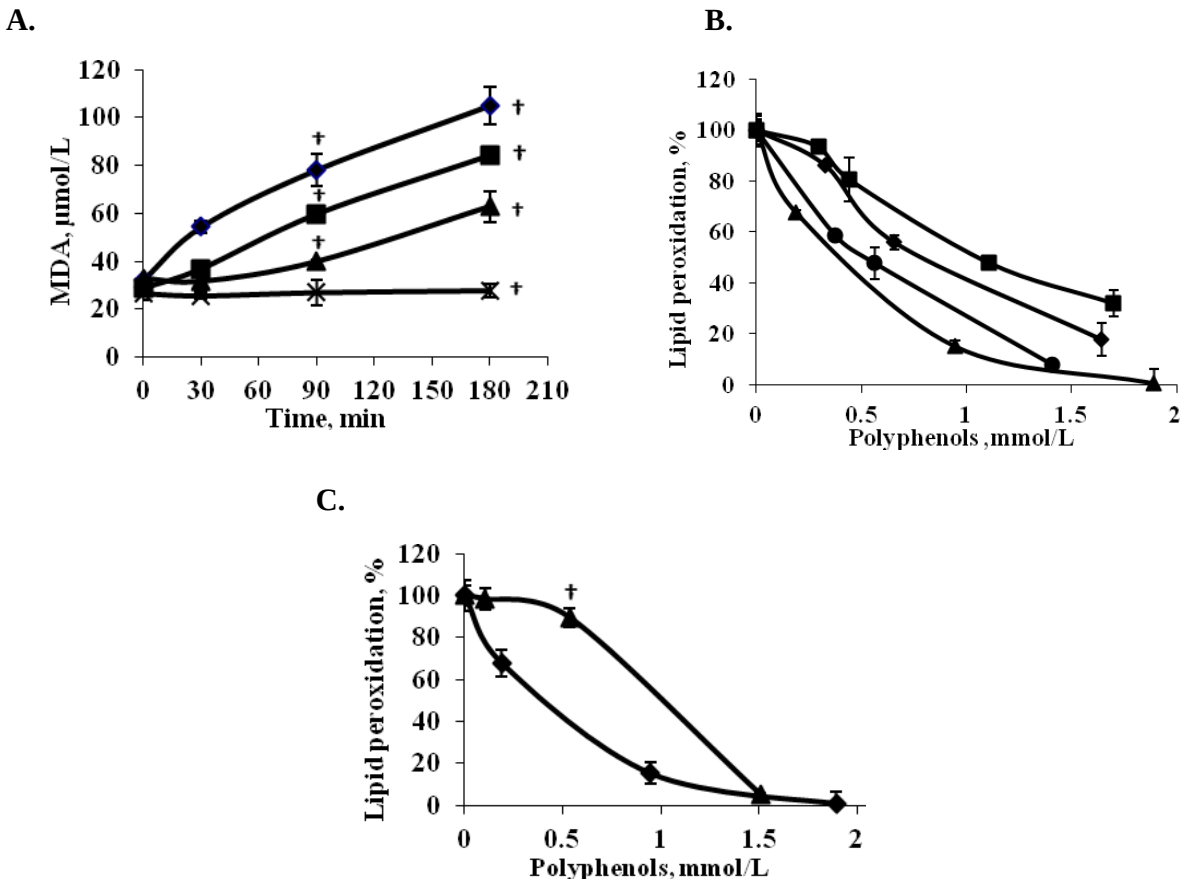

Figure 1. Coffee antioxidant activity in SGF.
A) Inhibition of red-meat lipid peroxidation by roasted-ground coffee A.
Control, no coffee (♦); 193.6µmol/L coffee polyphenols (■); 968 µmol/L coffee polyphenols (▲); 1936 µmol/L coffee polyphenols(x).
B) Inhibition of red-meat lipid peroxidation (t 90min=100%) by coffee commercial brands. Turkish coffee roasted-ground A (▲); Coffee instant B (●); Coffee instant C (♦); Coffee instant D (■).
C) Inhibition of red meat lipid peroxidation (t 90min=100%) by coffee A and AG.
Turkish coffee roasted-ground A (♦);Turkish coffee roasted-ground AG (▲).
Data are presented as mean ± SD for three parallels for each prepared sample.

Ten healthy volunteers consumed the meals on different days after an overnight fast. All the volunteers consumed the same red-meat cutlets, drunken water or coffee beverages during the meal bringing interaction of red-meat and coffee antioxidants for the first into the stomach lumen.

After treatment A, postprandial levels of plasma MDA of volunteers increase by ~110nmol/L after 4h and remained high after 6h. Drinking coffee A reduced significantly the absorption of MDA. Consumption of cutlets and drinking of coffee AG inhibited less MDA absorption, (Figure 2A). Changes in area under the curve (AUC) of plasma MDA concentration of 10

volunteers after treatments A, B and C are presented in Figure 2B. The average changes in area under the curve show a significant increase in MDA concentration after ingestion of cutlets of red meat without coffee and a significant inhibition and reduction in MDA concentration by drinking during the meal roasted-ground coffee A and AG Figure 2C.

MDA is the most abundant active carbonyl generated from lipid peroxidation in-vitro in food, its concentration in red-meat could reach 300 µmol / Kg, in-vivo it is also generated after an oxidative stress. The MDA generated in the stomach seems to be critical for its transfer and absorption into the blood system. Its absorption in the gastrointestinal tract is through N-E-(2-propenal) lysine adducts, a more electrophilic active conjugated α-β-carbonyl, which modify proteins through a schiff-base with basic amino-acids. Evaluation of MDA-lysine bioavailability in rats showed incorporation in the small intestine and plasma. Modification of LDL by MDA was found to be dependent on the increase of plasma MDA level following a red-meat meal. Modified-LDL is one of the main initiators of atherogenesis and removal of circulating modified-LDL results in complete prevention of atherosclerosis progression.

Roasted-ground coffee has an inhibitory effect (IC50) on muscle food lipid peroxidation of 2 to 5 fold higher than instant coffee per cup. We conclude that during production of the instant coffee only the hydrophilic, more soluble and small polyphenols molecules are extracted. Those molecules act less efficiently as antioxidants. Thus, the amount and ration between the low molecular and high molecular weight polyphenols in coffee beverages affect the antioxidant tone very significantly and could explain in part the difference results obtained between the roasted-coffee brand A and coffee AG which contain more phenolic acids. Lipid peroxidation in red-meat is mostly catalysed by an iron-redox cycle.The hydrophilic phenolic acids in coffee AG seems to enhance especially pro-oxidative iron redox-cycle catalysis and less scavenging of lipid free radicals and by this to decrease the overall antioxidant effect of the system.

We determined the effect of two products of roasted-ground coffee A and roasted-ground coffee AG (A enriched by 2% GCEP) on meat lipid peroxidation in stomach medium. Notably, coffee A and coffee AG contain 110 and 123 mg polyphenols /1g DM, respectively. The results obtained in Figure 1C led to a paradox that increasing the concentration of polyphenols increased the IC50 by 2.5 fold, and coffee amount, for IC100/200 g meat, from 168ml to 375ml. We confirmed the antioxidant decrease of coffee AG by generation of hydroperoxides and accumulation of MDA.

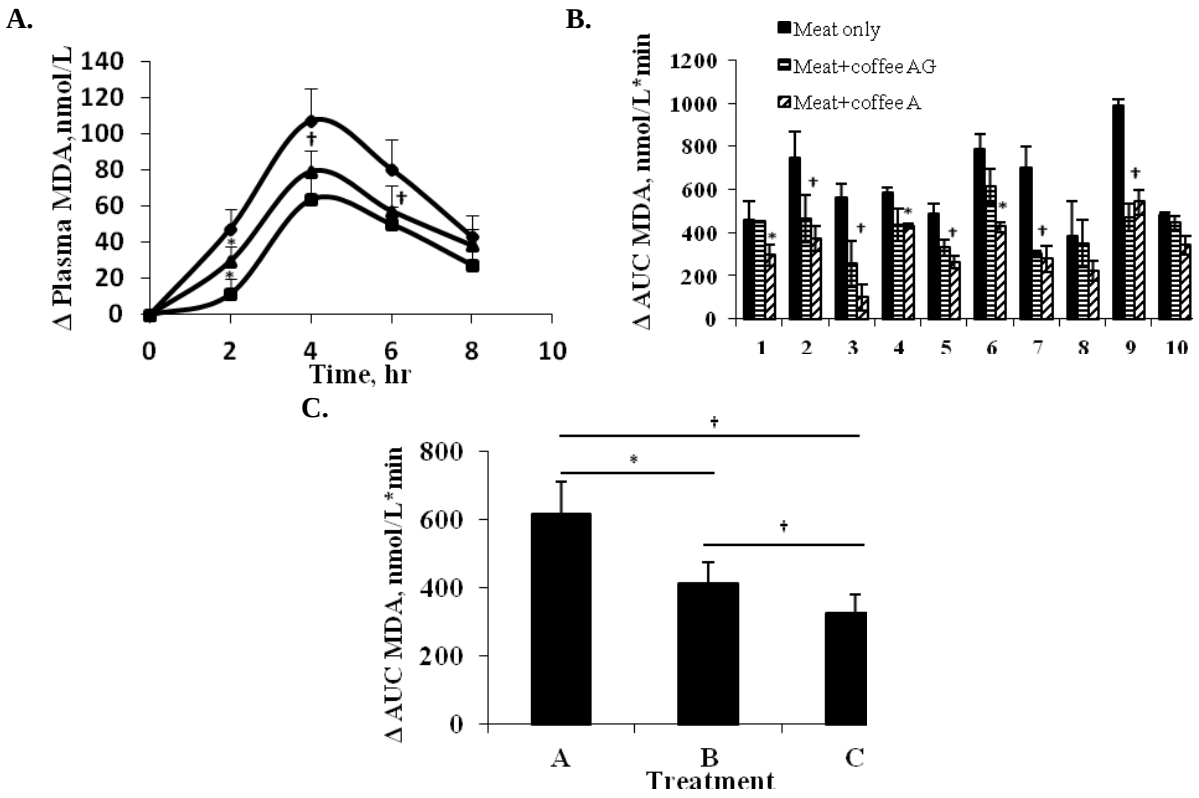

Figure 2. Inhibition of MDA accumulation in human plasma by coffee beverages. A.

- **A. Postprandial plasma MDA accumulation (average) in volunteers after test meal of red meat (250g) and (200 ml) different beverages.**
- **B. Area under the curve (AUC) of plasma MDA vs. 8h of 10 volunteers.**
- **C. (Black), test meal and water;(Horizontal), test meal and Turkish coffee roasted-ground A; (Diagonal), test meal and Turkish coffee roasted-ground AG.**
- **D. Average absorption of MDA in plasma volunteers after 8h. Treatments described in B.**
- **E. Data are presented as mean±SE.† Very significant p<0.006, *Significant p<0.05.**

Our results revealed a rapid accumulation of MDA in plasma volunteers after a meal of red meat cutlets. Consuming of roasted-ground coffee AG inhibited less the absorption of MDA in plasma in all volunteers. These results correlate with the *in-vitro* study of simulated stomach model system, showed a better inhibition of lipid peroxidation by coffee A than AG.

We found from human clinical trials, one from coffee and three from red-wine that the results from *in-vivo* carbonyl modification of human plasma and *in-vitro* red-meat lipid oxidation in SGF are in high correlation and very predictive. The results seem to be of great importance for further investigation of the involvement of dietary polyphenols and other antioxidants on human health. Recently several studies adopted our model systems.

Our study shows that coffee the most popular beverage in the world, supplying the most significant portion of daily intake of dietary antioxidants, effectively control lipid peroxidation in stomach medium and prevent postprandial absorption and plasma MDA modification. We hypothesize that by timing better the consumption of coffee during the meals we could transform coffee into a real active functional food (24).

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Development of Acrylamide-Free "Ready-to-Drink" Coffee by *Aspergillus oryzae*

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SUMMARY

In 2009, Wakaizumi et al. reported that *Aspergillus oryzae*, called "National fungus", a filamentous fungus or a mold, used to saccharify starch from rice into sugars in the sake brewing industry can reduce acrylamide by acrylamide-degrading enzymes (amidase: EC 3.5.1.4) present in their mycelia. The main purpose of this study was to confirm the acrylamide-reducing effect of *A. oryzae* during the production of ready-to-drink coffee on a laboratory scale and to evaluate the properties of this treated coffee liquid. For the acrylamide removal test, YPD medium was used and added cellulose fiber to immobilize the mycelia of *A. oryzae*. After incubation, immobilized mycelia were washed with water. The immobilized mycelia were then added to the coffee brew and incubated at 35°C. After that, the coffee was collected to evaluate its quality. This strain degraded nearly all acrylamide in a 10 ppm-acrylamide aqueous solution at 6 h, and this effect was also observed in a sample of commercially extracted coffee. Caffeine, chlorogenic acid, and organic acid (citric, quinic, malic, glycolic, lactic, acetic, and formic acids) contents in the treated coffee liquid tended to decrease compared with that in the control coffee liquid. Flavor analysis by GC-MS showed that the concentration of 1-propanol, ethyl acetate, 2-methyl-1-butanol, isobutyl alcohol, and isoamyl alcohol, which are constituent elements in fruits, flower, wine, and sake, were markedly increased in the treated coffee liquid compared with that in the control. Moreover, the results of sensory tests by trained panels showed that the treated coffee had a fruity, sweet floral flavor. We hope that these data will help make "acrylamide-free ready-to-drink coffee" a practical reality.

INTRODUCTION

Acrylamide is a suspected carcinogen that is formed when sugar and the amino acid asparagine are heated together, and is classified as a probable human carcinogen (group 2A) by the International Agency for Research on Cancer (IARC). Since the release of a report by Sweden's National Food Administration in April 2002, consumers have had something else to think about when choosing what to eat. Acrylamide is formed through reactions between free amino acids and reactive carbonyls (e.g., reducing sugars) via the Maillard reaction typically occurring at temperatures above 120^{o}C. Although the relevance to human health of dietary exposure to acryl amide is unclear, regulatory agencies such as the World Health Organization (WHO) continue to encourage food manufacturers to take measures to reduce acrylamide levels in processed foods. With regard to coffee, the acrylamide content is low compared to potato chips and French fries. But people consume a lot of coffee in a day.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) uses the Margin of Exposure (MOE) approach to evaluate the risks of acrylamide to humans. MOE is the quotient of the lowest dose of a substance causing a health issue divided by the estimated intake of that substance by the general human population. According to the JECFA's calculations, MOE values for acrylamide carcinogenicity in humans are 300 for high consumers and 75 for average consumers. These values were estimated by research on mammary tumors in rats. Generally, MOE values less than 10,000 indicate that a substance is "probably carcinogenic to humans." JECFA therefore concluded that the presence of relatively high levels of acrylamide in food is of human health concern.

On the other hand, there is a concept called the "Holistic approach." While brewed coffee does contain trace levels of acrylamide, there is no epidemiological evidence linking consumption of those levels with a risk of cancer in humans. Moreover, the health benefits of coffee (such as anticancer and antidiabetic effects) are often overlooked by public health and regulatory authorities when assessing the overall safety of a food product. We definitely need an assessment of the overall safety and benefits of the whole food product, and not just focus on individual food carcinogens in isolation. However, some evidence has been found of an association between dietary acrylamide and endometrial and ovarian cancers. In 2012, Bongers et al. found that acrylamide may increase the risk of multiple myeloma and follicular lymphoma in men. Little is currently known about adverse effects of very high intakes of acrylamide in humans. Further studies are needed to determine the effects of trace amounts of dietary acrylamide on cancer risk in humans.

In 2009, Japanese research groups reported that *Aspergillus oryzae* (called koji in Japanese; it is a kind of mold) might be useful for reducing acrylamide levels in roasted tea. Some scientists reported that there are enzymatic, agronomic, and other approaches for lowering the acrylamide content in coffee, but there are no viable strategies for minimizing the acrylamide content without adversely affecting the sensory qualities. For this reason, we thought it was worth trying a microbiological approach for reducing acrylamide levels in coffee. The objectives of this investigation were to control acrylamide removal by *A. oryzae*, and to explore the feasibility of producing ready-to-drink coffee. *A. oryzae* is on the FDA's Generally Recognized as Safe (GRAS) list.

MATERIALS AND METHODS

A. oryzae strain

Previously, we developed self-cloned *A. oryzae* strain that was used for this work (It carries 4 amidase transgenes in its genome). *A. oryzae* produces an amidase, an enzyme that converts acrylamide into acrylic acid. The acrylic acid is metabolized into various intermediate compounds before being incorporated into the TCA cycle and ultimately converted to H_2O and CO_2. Amidase is an inducible enzyme, but this self-cloned strain does not need pre-cultivation to activate the amidase genes. Figure 1A shows relative amidase gene expression for the parent and self-cloned strains, as determined by real-time PCR. The rate of amidase transcription in the self-cloned strain was 21,000 times higher than that in the parent strain. Figure 1B shows specific activities of amidase in the parent and self-cloned strains. Amidase activity was 42 times higher in the self-cloned strain than in the parent strain. The important point is that acrylic acid is also a carcinogen, so acrylamide removal by degradation with amidase is not a useful process.

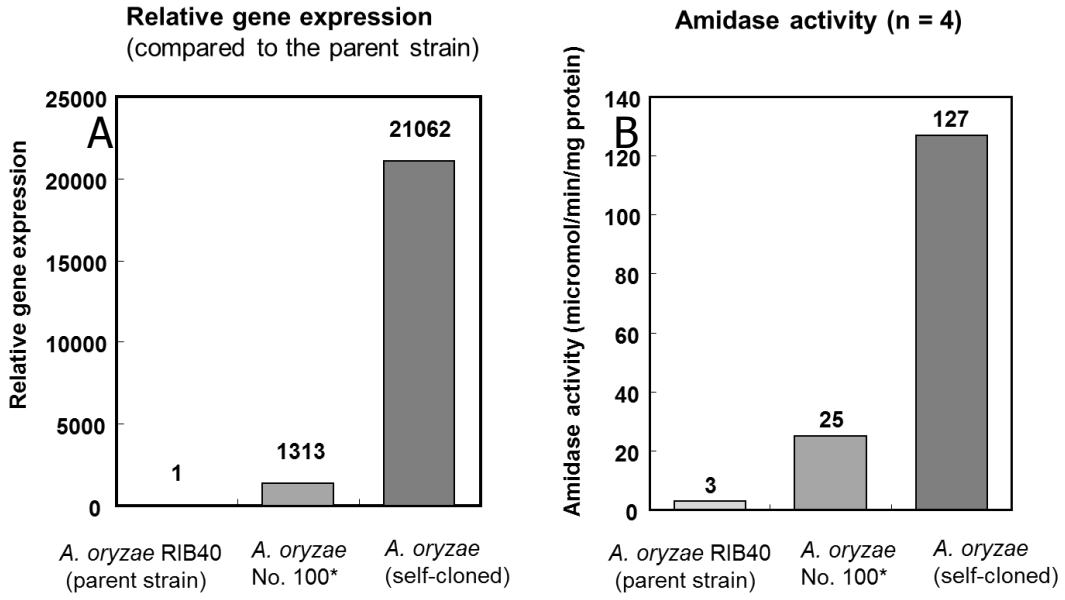

Figure 1. Evaluation of self-cloned *A.oryzae*. *A.oryzae* No.100 (Already reported strain: Acrylamide degradation characteristic of *Aspergillus oryzae* mycelia. *Seibutsu-Kogaku Kaishi*. 2009. 87, 490–495).

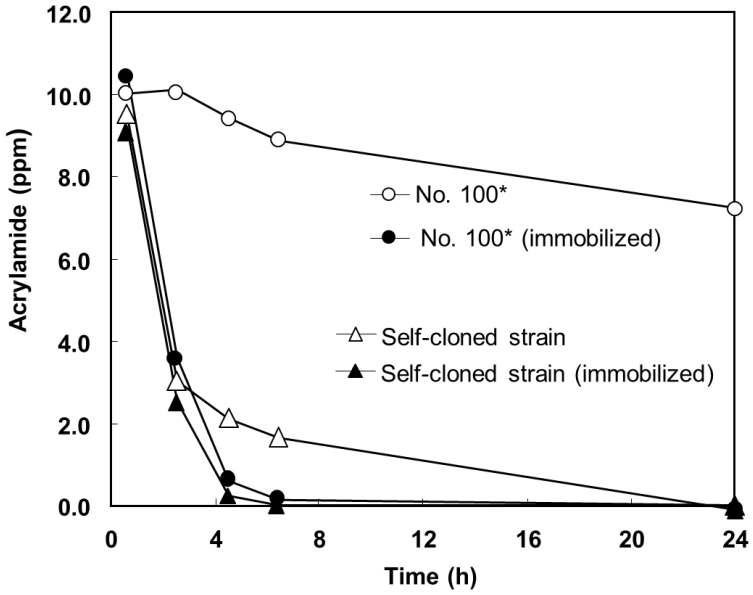

Figure 2. Change in acrylamide content (10 ppm aqueous solution).

Subsequently we evaluated the abilities of *A. oryzae* strains to reduce the concentration of acrylamide in solution. As shown in Figure 2, 150 mg of *A. oryzae* No. 100, a well-known strain, lowered the acrylamide concentration by almost 10 ppm in about 6 hours. The self-cloned strain was also able to reduce acrylamide levels, but this strain does not need pre-incubation to induce amidase. Acrylamide removal by both strains was much faster when the mycelia were immobilized on cellulose fiber as compared with using the free mycelia.

For the acrylamide removal test, YPD medium was used and added cellulose fiber to immobilize the mycelia. Growth was carried out in a shaking incubator at 100 rpm for 3 days at 30°C. After incubation, immobilized mycelia were washed with water. The immobilized

mycelia were then added to the coffee brew and incubated at 35°C. After that, the coffee was collected to evaluate its quality (Figure 3).

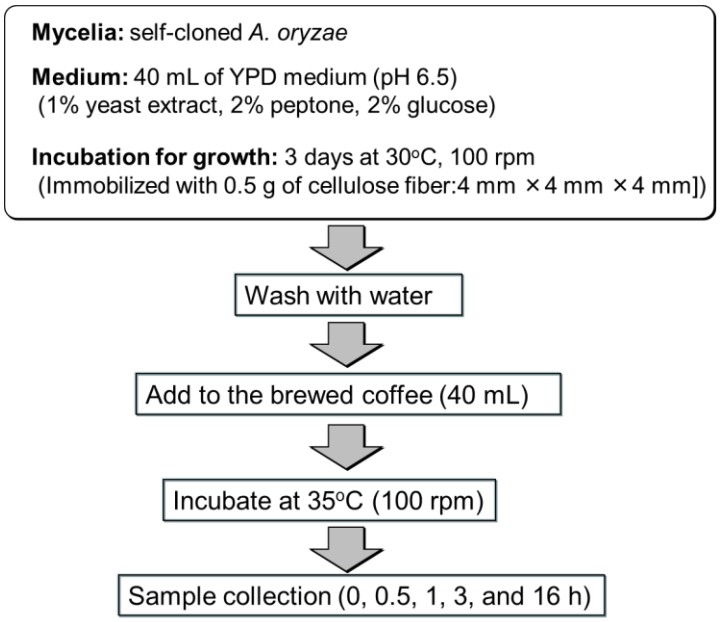

Figure 3. The protocol for the acrylamide removal test.

Coffee samples

Roasted coffee beans (*Coffea Arabica*; from Brazil) were ground (L value was 17.7). The coffee was brewed with hot water at a 17:1 ratio of water to ground coffee. The brewed coffee contained 2% soluble solids.

Evaluation of the treated coffee

Acrylamide content

The acrylamide content was analysed by GC/MASS QP2010 (Shimadzu corp., Kyoto, Japan), where conditions were as follows: GC condition: needle temperature, 120°C; Carrier Gas,110kPa; Column, ZB1 (0.32mm i.d. thickness, 3.0 micrometer); column temperature, 40°C (5min)-5°C / min -60°C-15°C / min-250°C (3min); Helium pressure, 80kPa; inserting temperature, 250°C; split ratio, 0; split flow, 20.4ml/min. Mass-spectrometer condition: interface temperature, 300°C; SIM sampling ratio, 0.2sec. A solid phase extraction procedure was applied for clean-up and sample pre-concentration before acrylamide measurement.

Aroma compounds

Fifty kinds of aroma compounds were measured with a GC/MS QP2010 (Shimadzu corp., Kyoto, Japan) with HS-sampler HS-40EX (Perkin Elmer Japan Co, Ltd., Yokohama, Japan). GC-MS conditions were the same as described above. Headspace sampler condition: ample temperature, 60°C heating time 30min; Transfer temperature, 180°C.

Polyphenol content

Chlorogenic acids (mono-caffeoylquinic acids, feruloylquinic acids, and di-caffeoylquinic acids) were analysed by the method of Matsui et al.

Organic acids content

Eight kinds of organic acids contents were measured by HPLC. The collected sample was subjected to GL-7480 system (GL Sciences Inc., Tokyo, Japan), where the conditions were as follows: Column, Shodex RSPack KC-811(30cm x 8mm i.d. x 4); Column temperature, 60^{o}C; Mobile phase, 3mM $HClO_4$ / H2O; flow rate, 1ml/min; labeling solution, 15mM Na_2HPO_4, 2mM NaOH, and 0.2mM BTB; detector: UV 445nm.

The sensory test

The sensory test was carried out by 11 trained panelists (7 men and 4 women; average age: 30.5 years). The selected evaluation terms were "flower-like," "fruit-like," "caramel-like," "acidity," "bitterness," "astringency," "body," and "after taste," as have been used in some previous studies. The evaluation scales used a 9-level rating system, from -4 to +4, with the ratings provided by the panelists. The panelists frequently commented on the treated coffee over the course of the sensory test. The statistical analyses were performed with SPSS 16.0J for Windows (SPSS Inc., Chicago,IL, USA), and the level of the significance was set at under 5%.

RESULTS AND DISCUSSION

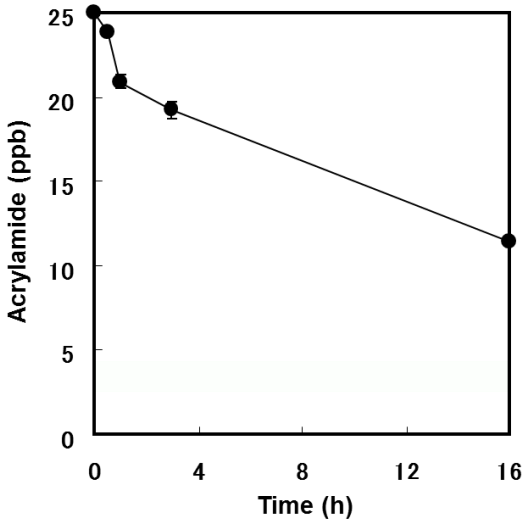

Figure 4. Change in acrylamide content in the coffee brew.

Figure 4 shows the changes in acrylamide levels in the coffee. Acrylamide was reduced to 54% after 16 hours. As described before, this strain lowered acrylamide by almost 10 ppm aqueous in about 6 hours in a preliminary test without the coffee components.

One difference is that the coffee contained much less acrylamide: in ppb instead of ppm. The results depended on the quantity of mycelia added and the incubation time. We repeated the test using a thinner coffee brew (soluble solids: 1.2%), and the acrylamide content was found to have reduced to 84%. Further studies are needed to optimize the process by, for example, increasing the quantity of mycelia added to accelerate the removal because it is not practical to incubate coffee for 16 hours.

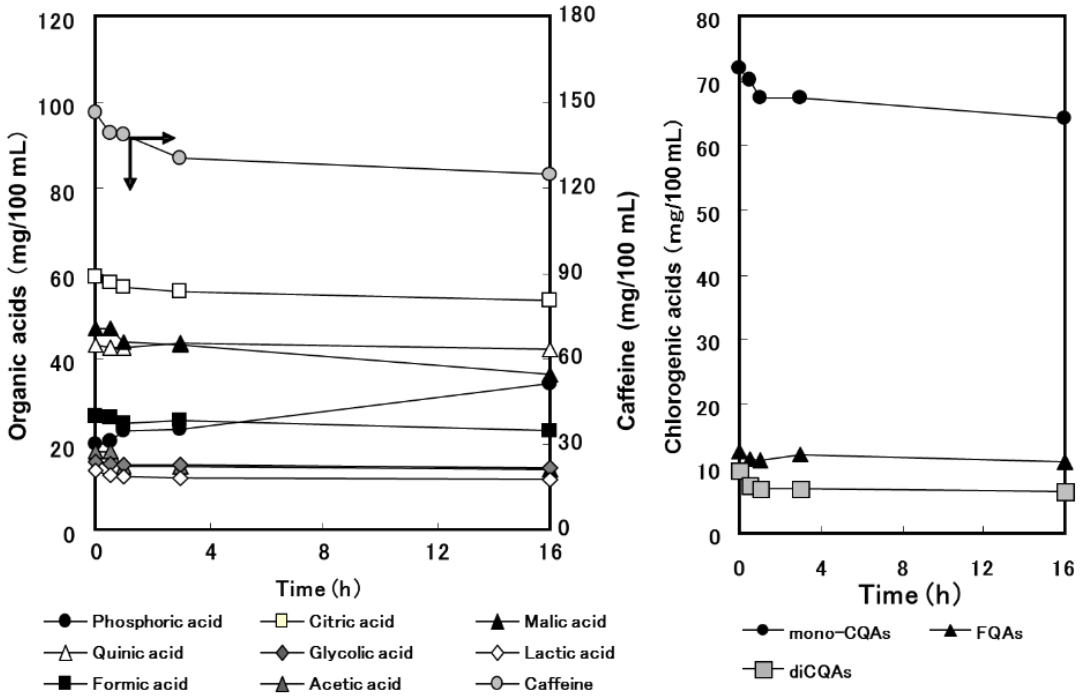

Figure 5. Changes in the levels of caffeine, organic acids, and chlorogenic acids in the coffee brew.

Figure 5 shows the changes in the levels of caffeine, organic acids, and chlorogenic acids in the coffee brew. Caffeine and mono-caffeoylquinic acids (mono-CQAs) decreased by about 15% after 16 hours. Generally, organic acid content in the coffee is closely associated with its quality. These decreased by only 6% after 16 hours. Fifty aroma compounds analysed that were the most enhanced following self-cloned *A. oryzae* treatment are listed in Table 1. The concentrations of 1-propanol, ethyl acetate, and 2-methyl-1-butanol in the coffee were 15.5, 9, and 8.7 times higher than the untreated control. These alcohols and esters give coffee "alcoholic," "fruit-like" and "sweet" flavor characteristics. These flavors are possibly a result of the metabolites released into the coffee from the mycelia.

Table 1. Aromatic components enhanced by self-cloned *A. oryzae* treatment.

Volatile compounds	Sensory features	Enhanced ratio after 16-h treatment
1-Propanol	Alcohol-like, sweet	15.5
Ethyl acetate	Fruits-like	9.0
2-Methyl-1-butanol	Fruits-like (Grape), pungency	8.7
Isobutyl alcohol	Sweet	7.3
Isoamyl alcohol	Fruit-like (Banana), wine-like	6.2
Ethanol	Alcohol-like	2.7
2-Pentanon	Fruit-like, ether-like	2.2
Pyridine	Roasted	1.2

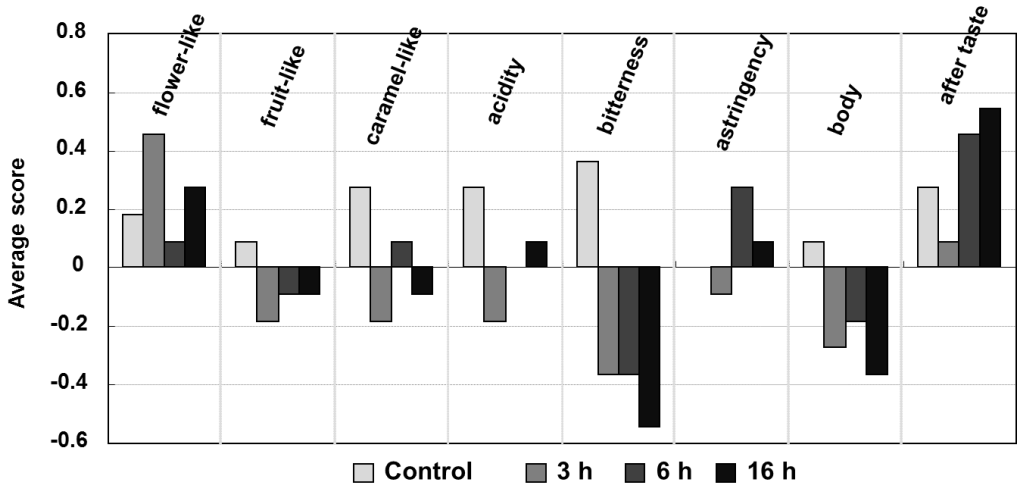

Figure 6. The results of the sensory test.

Figure 6 shows the results of the sensory test. No significant differences have observed over the course of the sensory test. However, the score for "flower-like" flavor increased; on the other hand, the score for "bitterness" and "body" flavors decreased with increased treatment time. These results suggest that the panelists evaluated the treated coffee as smooth and mild flavors. The panelists commented that the coffee has a characteristic flavor, one that is associated with Sake or alcohol. These results suggest that the sensory score and comments are related to increased levels of alcohols and esters in the treated coffee. It is suggested that treatment conditions (time, temperature, amount of mycelia, etc.) can be manipulated to achieve the optimal product and process. These results provide us with significant insights into how to develop acrylamide-free ready-to-drink coffee.

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Effects of Oxidative Component-Reduced Coffee Containing Higher Levels of Chlorogenic Acids on Blood Pressure, Body Fat, and Energy Metabolism in Humans

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SUMMARY

Hydroxyhydroquinone, pyrogallol, and hydroquinone, all of which generate reactive oxygen species, are produced as oxidative components (OCs) by green coffee beans during the roasting process. These OCs are thought to inhibit the reported beneficial effects of chlorogenic acids (CGA) in coffee. Therefore, we developed an oxidative component-reduced (OC-R) coffee by removing most of the roasting process-derived OCs while maintaining a large amount of CGA. The effects on blood pressure, body fat, and energy metabolism were then analyzed in clinical trials in humans.

Study A.

The antihypertensive effect of OC-R coffee containing CGA was evaluated in high-normotensive and mildly hypertensive adults, using a double-blind, randomized controlled study design. The subjects drank one can of Active or Placebo coffee daily for a period of 12 weeks. Systolic blood pressure (SBP) was significantly lower in the Active group (n=49) than in the Placebo group (n=51) throughout the intervention period.

Study B.

The effects of coffee with or without OCs on blood pressure were evaluated. The protocol and method were the same as for Study A, except the Control coffee (Roasted Coffee) contained OC and CGA. The results were similar to those in Study A. These results suggest that the intake of OC-R coffee improves blood pressure.

Study C.

The preventive effect of OC-R coffee against body fat accumulation was evaluated in obese adults using the same Active and Placebo coffee as used in Study A. Body weight was significantly lower in the Active group (n=53) than in the Placebo group (n=56).

Study D.

To investigate the mechanism underlying the anti-obesity effect, energy metabolism after ingestion of OC-R coffee was evaluated in a double-blind cross-over study. Postprandial oxygen consumption and fat utilization were significantly enhanced in the Active group compared with the Placebo group.

INTRODUCTION

Green coffee bean extract, chlorogenic acids (CGA), and ferulic acid, the CGA metabolite, have antihypertensive effects in animal studies. Among various antagonists and blockers, intravenous injection of a nitric oxide (NO) synthase inhibitor inhibits the effect of ferulic acid, suggesting that the mechanism underlying the antihypertensive effect of CGA is associated with NO-mediated vasodilation by vascular endothelial cells. A previous study in humans reported that ingestion of a drink containing 140mg of CGA significantly decreased SBP and increased the vasodilation response as an index of vascular endothelial function.

On the other hand, however, coffee consumption is also associated with a slight increase in blood pressure. We recently demonstrated in an animal study that hydroxyhydroquinone (HHQ), an OC produced by roasting green coffee beans, inhibits the antihypertensive effects of CGA in a dose-dependent manner.

Therefore, we developed an OC-R coffee by removing most of the roasting process-derived OC by the absorption process while maintaining a large amount of CGA. The effects on blood pressure, body fat, and energy metabolism in humans were then analyzed in clinical trials.

METHODS

Study A

The purpose of this study was to evaluate the antihypertensive effect of OC-R coffee containing CGA in high-normotensive and mildly hypertensive adult men and women. Commonly consumed commercially available roasted coffee products contain CGA and OCs, such as HHQ. Two test beverages (Active and Placebo) were used in this study (Table 1). Before ingesting the test beverage, subjects were randomly assigned to one of two groups (Active and Placebo group). During the trial period, the subjects were given the following instructions: drink one can of Active or Placebo beverage daily for 12 weeks; continue usual dietary habits.

Study B

The purpose of this study was to evaluate the effect of coffee with or without OCs on blood pressure in high-normotensive and mildly hypertensive adult men and women. Two test beverages (Active and Control) were used in this study (Table 1). The Control beverage was a common coffee product containing OC and CGA. The protocol and method were the same as for Study A, except the Control beverage was used instead of the Placebo beverage.

Study C

The purpose of this study was to evaluate the anti-obesity effect of OC-R coffee containing CGA in overweight or obese adult men and women. Two test beverages (Active and Placebo) were used in this study (Table 1). The protocol and method were almost the same as for Study A.

Study D

The purpose of this study was to evaluate the effect on energy metabolism to gain insight into the mechanism underlying the anti-obesity effect of CGA with a double-blind, randomized controlled cross-over design. Two test beverages (Active and Placebo) were used in this study

(Table 1). After a 1-week intervention period, energy metabolism was evaluated by indirect calorimetry after fasting and up to 3.5 h postprandially.

Table 1. Composition of test beverages.

	Active beverage	Placebo beverage	Control beverage
Chlorogenic acids (mg)	297 - 359	0 - 2	299
Hydroxyhydroquinone (mg)	0.05	0.02	1.69
Caffeine (mg)	77 - 82	78 - 81	75
Energy (kJ)	24 - 29.3	22 - 29.3	37.7

The test beverages were prepared as a canned Ready-to-Drink beverage (184mL or 185g).

RESULTS AND DISCUSSION

Study A

Throughout the 12-week intervention period, SBP was significantly lower in the Active group than in the Placebo group (Group effect, p=0.044, Table 2). A clear, although non-significant difference in DBP, was also observed between groups (Group effect, p=0.059). Changes in the forearm arterial blood flow as an index of vascular endothelial function were improved in the Active group after the 12-week intervention (data not shown). No clinically significant side effects were noted in a safety evaluation (data not shown).

Table 2. Changes in blood pressure in high-normotensive and mildly hypertensive subjects in Study A.

		Active	Placebo	Estimated difference (95%CI)
SBP (mmHg)	Baseline	140.4 ± 1.3	141.1 ± 1.2	
	4wk	135.5 ± 1.5	137.8 ± 1.6	-1.9, (-5.5: 1.7)
	8wk	135.6 ± 1.5	138.0 ± 1.8	-1.9, (-5.9: 2.1)
	10wk	135.9 ± 1.6	140.7 ± 1.4	-4.4, (-8.3: -0.6)
	12wk	135.6 ± 1.5	139.6 ± 1.5	-3.6, (-7.6: 0.3)
Group	*p*	*0.044*		
DBP (mmHg)	Baseline	86.7 ± 0.8	87.2 ± 0.8	
	4wk	84.9 ± 1.0	85.9 ± 0.9	-0.8, (-3.1: 1.5)
	8wk	85.2 ± 1.0	86.2 ± 0.9	-0.8, (-3.2: 1.7)
	10wk	83.4 ± 1.0	86.5 ± 1.0	-2.9, (-5.7: -0.2)
	12wk	84.0 ± 1.0	86.2 ± 1.0	-1.9, (-4.6: 0.7)
Group	*p*	*0.059*		

mean±SEM. n; Active = 49, Placebo = 51.
Estimated difference between Active and Placebo groups at each week and 95% confidence interval by a linear mixed-model.

Study B

Throughout the 12-week intervention period, the Active group had a significantly lower SBP than the Control group (Group effect, p=0.031, Table 3). No clinically problematic effects in individual subjects were noted in the safety evaluation (data not shown).

Table 3. Changes in blood pressure in high-normotensive and mildly hypertensive subjects in Study B.

		Active	Control	Estimated difference (95%CI)
SBP (mmHg)	Baseline	139.8 ± 1.2	140.6 ± 1.0	
	4wk	135.2 ± 1.4	140.0 ± 1.4	-4.2, (-7.2: -1.3)
	8wk	136.0 ± 1.4	138.4 ± 1.5	-1.8, (-5.2: 1.6)
	10wk	137.5 ± 1.5	140.8 ± 1.3	-2.6, (-6.2: 0.9)
	12wk	139.0 ± 1.5	143.0 ± 1.6	-3.4, (-7.3: 0.5)
Group	*p*	*0.031*		
DBP (mmHg)	Baseline	88.2 ± 0.8	88.2 ± 0.6	
	4wk	85.3 ± 0.9	86.7 ± 0.9	-1.3, (-3.4: 0.7)
	8wk	86.3 ± 0.8	87.6 ± 1.0	-1.2, (-3.3: 1.0)
	10wk	87.0 ± 1.0	88.9 ± 1.0	-1.8, (-4.3: 0.7)
	12wk	88.4 ± 0.9	89.8 ± 1.0	-1.4, (-3.8: 1.1)
Group	*p*	*0.092*		

mean±SEM. n; Active = 51, Control = 47.
Estimated difference between Active and Control groups at each week and 95% confidence interval by a linear mixed-model.

These studies demonstrated that ingestion of OC-R coffee for 12 weeks significantly decreased SBP in the Active group compared with the Placebo and Control groups.

These findings suggest that CGA in coffee has antihypertensive effects and OCs in coffee inhibit the beneficial effects of CGA. Therefore, reducing the OC content in coffee can improve blood pressure.

Suzuki et al. reported that ingestion of HHQ-free coffee improved vascular endothelium-dependent vasodilation concomitant with an increase in NO bioavailability and a decrease in NADPH-dependent superoxide anion (O_2^-) production. These findings suggest that CGA inhibits the production of excess reactive oxygen species and improves NO bioavailability, thereby improving vascular endothelial function, and this may be one of the mechanisms underlying the blood pressure-lowering effects.

Study C

Body weights of the Active (n=53) and Placebo (n=56) group were 75.8±1.4 kg and 75.6±1.6 kg (mean±SEM) at baseline, respectively. After the 12-week consumption, body weight was significantly decreased in the Active group compared with the Placebo group (Group-by-time interaction, $p<0.05$, data not shown). Other visceral fat areas and waist circumference were significantly lower in the Active group than in the Placebo group (data not shown). No adverse events attributable to the ingestion of CGA were noted.

Study D

Seven healthy men randomly consumed either the Active or Placebo beverage in a crossover design. After a 1-week intake period of the test beverages, postprandial oxygen consumption and fat utilization in sedentary conditions were significantly enhanced in the Active group compared with the Placebo group (data not shown). These effects on energy metabolism may partially underlie the anti-obesity effects observed in Study C. These findings suggest that

daily consumption of OC-R coffee could contribute to the prevention of fat accumulation by enhancing fat utilization as energy.

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Health and Coffee Update 2012

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SUMMARY

Coffee consumption contributes to the control of Type 2 diabetes mellitus (metabolic syndrome), Parkinson's disease, liver disease and perhaps depression, suicide risk, dementia, and migraine. Moderate coffee intake, below 300mg per day in adults, or 3mg/kg in children, will not increase the risk for stroke, arrhythmia, hypertension, cardiovascular disease, cancer, infection, complications of pregnancy, calcium imbalance, bone disease, or kidney stones. Finally, coffee may be associated with a variety of adverse but relatively inconsequential side effects such as sleeplessness, heart palpitations and urinary frequency.

INTRODUCTION

Quality over quantity is an emerging market consideration for heavy coffee drinkers. From the roaster-retailer's standpoint, these changes in public opinion are increasing the demand for specialty coffee. Specifically, they are focusing the public's attention on brewing techniques, caffeine content, and quality. From the public's perspective, coffee in moderation is regarded as generally beneficial and, with minor qualifications, seldom harmful.

MATERIALS AND METHODS

The author's material consisted of his experience as a practicing physician, clinical research collaborator and coffee roaster-retailer. The methods consisted of an extensive review and focused summary of relevant technologies and findings.

RESULTS AND DISCUSSION

The major results in this review came from large western European databases. The studies were consistent and reproducible.

Modern advances in cell biology have provided laboratory confirmation of data based epidemiologic studies. High performance liquid chromatography has been used to identify the molecular composition of coffee. Mass spectroscopy has been employed to define the chemical structure of each molecule of interest.

Recent biomedical discoveries have come from our ability to selectively "knock out" genes coding for specific molecules one gene at a time. Hence, every human disease including neurodegenerative and metabolic disorders has a corresponding mouse model. Coffee related health issues are no exception.

Four agents found in coffee affect health: caffeine, diterpenes, chlorogenic acids, and niacin. Caffeine in the brain acts by a variety of chemical and electrical mechanisms. Relevant effects include control of Parkinson's disease, cognition, performance, and perhaps migraine. In the

liver, acute caffeine administration impairs glucose tolerance, but increases insulin sensitivity, and promotes energy metabolism. Chronically, coffee administration improves glucose tolerance. Caffeine is also a diuretic and a selective smooth muscle relaxant. Whereas, there is nothing to unsubstantiate the use of caffeinated coffee in moderation, there remains a significant and growing demand for quality, whole bean, decaffeinated coffee.

The diterpenes, cafestol and kahweol, are associated with increased enzymes which may contribute to increases in cholesterol. They are absent in coffee brewed with filtration. The adverse effect on cholesterol is marginal but sufficient to contribute to the increasing demand for filtration brewing in preference to boiled or steam extraction techniques.

Chlorogenic acid and cinnamic acid are polyphenol antioxidants. Resveratrol, another antioxidant, is of intense interest to the lay public even though there are few reports validating its clinical relevance.

Finally, one of five micronutrients may be important Niacin in conventional coffee usage may modify lipids and thus contribute to the control of atherosclerosis. It also corrects pellagra. The other four, magnesium, potassium, zinc, and vitamin E, are only 0.1-5% of recommended daily requirements.

Caffeine and caffeine-related methylxanthenes inhibit HIV-1 replication. No epidemiologic data have been reported.

Low acid coffees are hitting the markets as a coffee source that provides symptom relief for consumers with dyspepsia, diarrhea, bloating, pain, and irregular defecation. Coffee increases gastrointestinal motility but lifestyle studies suggest that the dyspepsia's are the consequence of food intolerance rather than the cause.

Advocates of organic coffee cite the use of potentially carcinogenic pesticides in standard, non-organically grown coffee. However, one German study tested pesticide residues in raw and roasted coffee and their degradation during the roasting process. The residues were reported to be reduced to insignificant amounts during the roasting process.

Epidemiologic data do not support dietary acrylamide intake as an important public health factor for cancer risk. Genetically modified food products are subject to intense market pressure without underlying scientific validation.

In a recent frequently cited study, regular coffee consumption was not associated with increased mortality rate in either men or women.

From the health standpoint, coffee consumption contributes to the control of type 2 diabetes mellitus (metabolic syndrome), Parkinson's disease, liver disease, bone disease, and perhaps depression and suicide risk, dementia, and migraine,. Moderate coffee intake, below 300 mg per day in adults, or 3 mg/kg in children, will not increase the risk for stroke, arrhythmia, hypertension, cancer, complications of pregnancy, kidney stones, as well as HIV, acrylamides, genetics, or mortality. Finally, coffee in excess of three cups per day may be associated with a variety of adverse side effects such as sleeplessness, heart palpitations and urinary frequency.

From the public's perspective, coffee in moderation is regarded as generally beneficial and, with minor qualifications, seldom harmful. As a result, the demand for specialty coffee is increasing.

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Does Coffee Prevent Prostate Cancer?

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SUMMARY

Heretofore, coffee has not been shown to decrease the risk of prostate cancer. With the advent of an understanding of the large cohort of low risk, nonlethal disease, there is a possibility that coffee with or without caffeine may prevent prostate cancer as reflected by the reduction of high risk, lethal disease in the coffee drinking cohort. Confirmatory population studies should be pursued. Standard murine models for high risk prostate cancer are available and should also be utilized to address this issue.

INTRODUCTION

Prostate cancer preoccupies coffee drinkers. Approximately, one in 9 coffee drinkers has prostate cancer. For many, their prostate cancer might never have been discovered without screening. For others, their prostate cancer is lethal. Preventive measures include control of weight and cholesterol. Benefit from selenium and vitamin E has not been substantiated. Active surveillance rather than conventional definitive therapy is now currently recommended for low risk individuals because their disease is likely to be nonlethal. It is only the minority of high risk individuals who warrant aggressive prophylaxis and treatment. High risk individuals have poorly differentiated histology, advancing tumor size and/or spread, an escalating prostate-specific antigen and/or a risky genome.

Coffee's antioxidants have been credited with playing a preventative role with regard to some major health problems such as diabetes type II, dementias and Parkinson's disease but not cancer. Recent developments in our understanding of prostate cancer justify a review of whether coffee may prevent this disease.

Prostate cancer has similarities with other major cancers such as those arising from breast and bowel. However, in other ways, it is unique. Fifty percent of men aged eighty and older have been found at autopsy to have prostate cancer yet the vast majority died of other causes. Moreover, prior to screening the vast majority of these cancers remained undiscovered. Men died with the disease rather than of the disease.

In the laboratory, antioxidants have been shown to be anti-proliferative. Two switch points are glutathione S-transferase pi (GST pi) and hypoxia-inducible factor-1 alpha (HIF-1 alpha). A possible mechanism of action for one of coffee's antioxidants, resveratrol, is induction of DNA damage response and cell death. It is not mutagenic. It has been proposed as a chemotherapeutic agent. The antioxidant activities of coffee are similar regardless of brewing technique or caffeination. The United States Task Force on screening recently cited two epidemiologic studies as evidence that screening for prostate cancer might not save lives.

In another recent study, using the Harvard Health Professionals Follow-up Study database, a subset of high risk prostate cancer target men with symptomatic disease and disease specific

mortality. The influence of anti-inflammatory drugs and antioxidants on prostate cancer has been extensively measured but the influence of tumor risk was not addressed. Results from clinical trials have been mixed. For example, a prospective study of selenium and vitamin E was halted before completion because the controls did better than the recipients of the agents.

MATERIALS AND METHODS

The Harvard Health Professionals Follow-up Study involved 51,529 male health professionals at baseline from 1986 to 2008. It included biennial health outcomes reports. Five food frequency questionnaires were completed during the course of the study, including intake of decaffeinated and regular coffee.

Most importantly those men reporting prostate cancer were separated into low and high risk groups. The groups were developed on the basis of advanced and/or lethal cancer versus non-advanced cancer. Criteria for assignment included extent of disease (stage) and microscopic appearance (Gleason score). Extent of disease was based upon the World Health Organization TNM classification and microscopic appearances were judged by Gleason scoring of glandular morphology.

A subsequent study from Glasgow, Scotland concentrated on the microscopic appearance (Gleason score) in a prospective cohort of 6,017 men. Hazard ratios were determined for Gleason specific prostate cancer by coffee consumption categories.

RESULTS AND DISCUSSION

In the Harvard study, the strongest associations were for lethal and advanced prostate cancer. The age related incidence rates of lethal prostate cancer for heavy coffee drinkers and non-coffee drinkers were 34 and 79 per 100,000 person-years, respectively. (P=.03 for lethal cancer and P=.004 for advanced cancer). Results were similar for drinkers of caffeinated or decaffeinated coffee.

In the Glasgow collaborative study, the hazard ratio reduced significantly from no coffee to 3 or more cups/day among those with high Gleason score. P value for trend was 0.03. No other significant differences were identified.

In a parallel study of tea drinking men, the Gleason group failed to find an association of tea intake with Gleason score.

The United States Preventive Services Task Force report on prostate cancer screening has underlined the large proportion of prostate cancer that is low risk, nonlethal and probably would not have been identified in the absence of prostate cancer screening. It is this subset that probably masked previous attempts to identify a positive role for coffee in reducing the risk of clinical, high risk and lethal disease.

The lower incidence was also observed in decaffeinated coffee drinkers; it was not observed in tea drinkers. Polyphenolic antioxidants have not been shown to alter the risk of prostate cancer. However, further studies are indicated to investigate the likelihood of these antioxidants playing a role in controlling the small subset of high risk prostate cancer patients.

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Kinetic Analysis on the Inhibition of Porcine Pancreas A-Amylase by Chlorogenic Acids from Green Coffee Beans and Cinnamic Acid Derivetives

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SUMMARY

Coffee is a popular beverage throughout the world and is a major source of dietary polyphenols, particularly chlorogenic acids (CGAs). In this study, the inhibitory effects of nine types of CGAs [3-caffeoylquinic acid (3-CQA), 4-CQA, 5-CQA, 3-feruloylquinic acid (3-FQA), 4-FQA, 5-FQA, 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-diCQA and 4,5-diCQA] from green coffee beans and eight cinnamate derivatives [cinnamic acid (CiA), *m*-methoxycinnamic acid (*m*-MCiA), *p*-methoxycinnamic acid (*p*-MCiA), ferulic acid (FA), isoferulic acid (IFA), *p*-coumaric acid (*p*-CoA), *m*-coumaric acid (*m*-CoA), and dihydrocaffeic acid (DHCA)] against porcine pancreas α-amylase isozyme I (PPA-I) were evaluated and the inhibition modes were modeled for each compound by kinetic analysis using Hanes–Woolf plots. All CGAs used in this study had inhibitory effects on PPA-I. 3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,5-diCQA, CA, DHCA, FA, IFA, *m*-CoA, *p*-CoA, *m*-CiA, *p*-CiA, and CiA exhibited mixed-type inhibition with $K_i > K_i'$, suggesting that these inhibitors bind to the ES complex more strongly than the free enzyme (E). In contrast, 3,4-diCQA and 4,5-diCQA showed mixed-type inhibition with $K_i < K_i'$, suggesting that these inhibitors bind to E stronger than to ES. More recently, it was reported that decaffeinated green coffee beans extract contains a high concentration of CGAs (ca. 40%) and significantly decreased postprandial blood glucose levels when administered to rats with soluble starch. One interpretation of these results holds that the biological activities of CGAs relates to their ability to inhibit pancreatic α-amylase.

INTRODUCTION

Coffee is one of the most frequently consumed drinks in the world and is a major source of polyphenols, chlorogenic acids (CGAs) in particular, in the human diet. CGAs comprise a family of esters that include derivatives of hydroxycinnamic acid [most commonly caffeic acid (CA), ferulic acid (FA) or *p*-coumaric acid (*p*-CoA) and quinic acid (QA). CGAs comprise three main subgroups: caffeoylquinic acids (CQAs), feruolylquinic acids (FQAs), and dicaffeoylquinic acids (diCQAs). CGAs have a variety of biological activities, such as antioxidant activity, tyrosinase inhibitory activity, and pancreas lipase inhibitory activity.

α-Amylase inhibitors can effectively prevent of diabetes and obesity by limiting elevation of plasma blood glucose levels because of delayed postprandial carbohydrate digestion and absorption.

We previously reported the inhibitory effect of 5-CQA and its components, CA and QA, on the PPA isozymes, PPA-I and PPA-II, using *p*-nitrophenyl-α-D-maltoside (G_2-*p*NP) as a

substrate at pH 6.9 and 30°C. The inhibition potencies of the respective inhibitors against both isozymes were almost identical and can be ranked in order of potency, 5-CQA > CA > QA. The inhibition modes of 5-CQA and CA on PPA-I and PPA-II were investigated by kinetic analysis, and it was found that 5-CQA and CA showed mixed-type inhibition with $K_i > K_i'$ (the inhibitor constants for E and the ES complex, respectively), against both PPA-I and PPA-II. The bindings of PPA-I or PPA-II with 5-CQA or CA were both found to be all exothermic and enthalpy-driven by thermodynamic analyses. For PPA inhibition of 5-CQA and its components (CA and QA), no difference was observed in the inhibition of the PPA-I and PPA-II isozymes. Although the inhibition of 5-CQA against PPA has been reported previously, the effects of other CGAs contained in green coffee beans remain to be elucidated.

In this study, based on our previous report, the inhibitory effects of eight types of CGAs (3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) and eight cinnamate derivatives (CiA, *m*-MCiA, *p*-MCiA, FA, IFA, *p*-CoA, *m*-CoA, and DHCA) against PPA-I were tested and structure-function relationships were evaluated for each compound.

MATERIALS AND METHODS

Inhibition of PPA-I by CGAs using G_2-*p*NP as substrate

Inhibitory activities of each compound against PPA-I were examined spectrophotometrically under the same conditions used for the PPA activity measurement with G_2-*p*NP as substrate at pH 6.9 and 30°C.

Determination of the mode of PPA-I inhibition by 5-CQA, 5-FQA, 3,5-diCQA, and 4, 5-diCQA

The inhibition mode of 5-CQA, 5-FQA, 3,5-diCQA, and 4,5-diCQA was investigated by kinetic analysis using Hanes–Woolf plots. Inhibitor constants Ki and Ki' that correspond to the dissociation constant Kd of the EI complex into the I plus E and the Kd of the ESI complex into I plus ES were determined.

RESULTS AND DISCUSSION

Inhibition of PPA-I activity of CGAs and cinnamate derivatives

Table 1 shows IC_{50} and the inhibitor constants (K_i and K_i') of CGAs and cinnamate derivatives. The inhibitory activities of nine types of CGAs and their components, CA, FA, and QA, against the PPA-I-catalyzed hydrolysis of G_2-*p*NP were determined by comparing the IC_{50} values and were found to be, in order of potency, 3,4-diCQA = 4,5-diCQA > 3,5-diCQA > 5-CQA > 4-CQA > 3-CQA > CA > 5-FQA > 4-FQA > 3-FQA > FA >> QA. 3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,5-diCQA CA, DHCA, FA, IFA, *m*-CoA, *p*-CoA, *m*-CiA, *p*-CiA, and CiA showed mixed-type inhibition with $K_i > K_i'$, suggesting that the inhibitors bind to the ES complex stronger than to the free enzyme E. In contrast, 3,4-diCQA and 4,5-diCQA showed mixed-type inhibition with $K_i < K_i'$, suggesting that these inhibitors bind to E stronger than to ES. More recently, it was reported that extract of decaffeinated green coffee beans contains a high concentration of CGAs (ca. 40%) significantly decreased postprandial blood glucose levels when administered with soluble starch in rats. These results suggest the possibility that the biological activity of CGAs will be related to their inhibition of pancreatic α-amylase.

Table 1. IC_{50} and Inhibitor constants, K_i and K_i', of nine chlorog. acid from green coffee beans and cinnamate derivates[a].

Inhibitor	IC_{50} (mM)	K_i (mM)	K_i' (mM)
3-CQA	0.23 ± 0.03	0.61 ± 0.16	0.13 ± 0.01
4-CQA	0.12 ± 0.01	0.24 ± 0.03	0.10 ± 0.01
5-CQA	0.08 ± 0.02	0.23 ± 0.02	0.05 ± 0.01
3-FQA	2.55 ± 0.12	6.14 ± 0.49	1.74 ± 0.05
4-FQA	2.02 ± 0.09	4.34 ± 0.57	1.40 ± 0.39
5-FQA	1.09 ± 0.03	1.95 ± 0.11	0.99 ± 0.03
3,4-diCQA	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
3,5-diCQA	0.03 ± 0.00	0.10 ± 0.00	0.01 ± 0.00
4,5-diCQA	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
CA	0.40 ± 0.03	1.12 ± 0.14	0.27 ± 0.04
DHCA	1.94 ± 0.10	10.21 ± 1.91	1.61 ± 0.02
FA	5.45 ± 0.12	11.17 ± 0.91	4.87 ± 0.37
IFA	4.27 ± 0.17	7.16 ± 0.58	4.11 ± 0.08
m-CoA	4.51 ± 0.20	8.12 ± 1.12	3.31 ± 0.03
p-CoA	4.86 ± 0.27	7.82 ± 0.25	3.88 ± 0.14
m-MCiA	> 4.5[b]	39.46 ± 3.29	4.96 ± 0.46
p-MCiA	> 4.5[b]	45.83 ± 7.41	3.63 ± 0.32
CiA	> 6.0[b]	45.99 ± 10.08	8.69 ± 0.84
QA	26.5 ± 1.8	NA[c]	NA[c]

[a] A mean of triplicate analysis ± SD.

[b] Narita & Inouye [5].

[c] Not determined correctly because of the low solubility of the ir

[d] NA, not analyzed.

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Effect of Coffee Decaffeination on the Lipid Peroxidation in Hyperlipidemic rats

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SUMMARY

This study aimed to evaluate the effect of coffee decaffeination, in arabica and robusta species, on the lipid peroxidation in hyperlipidemic rats liver. Filter coffee was used, from arabica (A), decaffeinated arabica (DA), robusta (R), and decaffeinated robusta (DR) coffees. Rats (n=30) were assigned into six groups, with a negative (C-) and positive control (C+), and one group for each coffee brew type. Rats were fed *ad libitum* on a hypercholesterolemic diet (0.5% cholesterol and 0.25% cholic acid), except for C- (normal diet), receiving 7.2 mL/kg/day of brew by oral gavage, for 42 days. MDA was estimated by quantification of the thiobarbituric acid reactive species (TBARS) by HPLC on the liver homogenates. The concentration of MDA in the groups treated with the coffee brews was significantly lower than the values in the C- group. As regards to the effect of coffee decaffeination, no significant differences were observed, as well as between species. These results suggest that the ingestion of coffee brews might protect the liver cells from oxidative stress damage independently of coffee species and decaffeination process.

INTRODUCTION

Coffee is one of the most widely consumed beverages in the world. Regular coffee drinking has been associated with the prevention of several important diseases, including cardiovascular disorders, obesity, diabetes, neurodegenerative disorders, liver diseases such as cirrhosis and a reduced risk of developing hepatocellular carcinoma. The studies show that these health benefits are mostly due to the presence of bioactive compounds in coffee brews, particularly those with antioxidative properties. Among the bioactive compounds investigated, the focus has been driven to its phenolic acids, such as chlorogenic and caffeic acid, as well as to caffeine and its metabolites, both with recognized antioxidative properties.

Regarding caffeine, many studies have been conducted on its physiological effects in coffee brews, caffeinated waters or energetic drinks. Still, a general consensus on its positive and negative impacts in human health is yet to be achieved.

The decaffeinated coffee market has been expanding increasingly in the last years. Although there are several studies about the chemical composition and antioxidant activity of whole coffee, few studies have evaluated the influence of decaffeination on lipid peroxidation. This study aimed to evaluate the effect of coffee decaffeination, in arabica and robusta species, on the liver lipid peroxidation in hyperlipidemic rats.

MATERIALS AND METHODS

The coffee samples were analyzed before and after decaffeination in a local industry with dichloromethane. The samples were roasted (roaster model Probatino - Leogap, Brazil, 1 kg capacity) to a medium roasting degree as usual in Brazil. The roasted beans were ground (electric grinder Pinhalense, ML-1, Brazil) at a particle size of 20 mesh, packed in polyethylene/aluminum packs, sealed and stored at -20 ° C until use.

The beverages were prepared according to the method of Nicoli et al. (1997) with few modifications. Briefly, 100 ml of deionized water at 90 °C were dripped into 10g of coffee powder in a commercial filter paper. The beverages were prepared daily.

The rats (n=30) were maintained at 22±2 °C, 55±10% humidity, with a 12-h light-dark cycle. The animals were divided into six groups, all on a hypercholesterolemic diet (0.5% cholesterol and 0.25% cholic acid) *ad libitum*, except for the negative control (C-) feed with a normal diet. The hypercholesterolemic rats included a positive control (C +) and a group for each type of coffee brew: arabica (A), decaffeinated arabica (DA), robusta (R) and decaffeinated robusta (DR). The coffee brews were administered daily by gavage (7.2 mL. kg^{-1}) for 42 days, while the control groups (C- and C+) receiving water by the same administration method. At the end of the experiment the animals were sacrificed and evaluated for malonic dialdehyde (MDA) by quantification of the thiobarbituric acid reactive species (TBARS) by HPLC on the liver homogenates. Protein was estimated by the Bradford assay. The University Ethical Committee for Animal Research (Nintec/PRP-UFLA-MG/Brazil) approved the protocols used in this study (protocol 046/2009).

RESULTS AND DISCUSSION

The results for MDA quantified in the liver homogenates, standardized for protein, are shown in Figure 1.

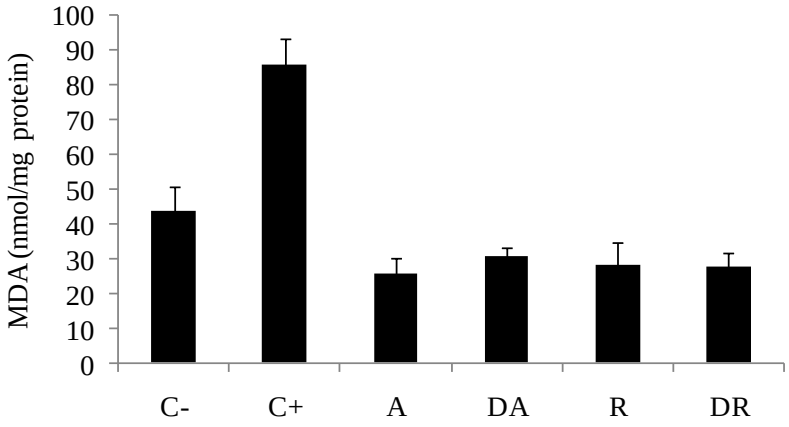

Figure 1. MDA levels in rats liver (nmol/mg protein; A = arabica; R = robusta; D = decaffeinated) (Different letters indicate significant differences by the Scott-Knott test at 5% probability).

The C + group showed significantly higher levels of MDA than the C- group, indicating that the hypercholesterolemic diet was effective in the induction of liver lipid peroxidation. All groups treated with coffee brews showed a significant reduction in hepatic MDA content when compared to the C+ group. The concentration of MDA in the groups treated with the coffee brews was also significantly lower than the values in the C- group, suggesting that the antioxidant compounds in coffee are able to reduce lipid peroxidation, not only in

hypercholesterolemic rats but also in non-hypercholesterolemic ones. As regards to the contribution of caffeine, no significant differences were observed between whole and decaffeinated brews, as well as between coffee species.

The chlorogenic acids present in coffee brew are known to have antioxidant activity in animal tissues. The reduction of TBARS levels observed in the animals liver in our study may be, at least partially, attributed to the presence of these compounds in the coffee brews administered to rats. Other authors also suggest that the Maillard reaction products formed during coffee roasting have antioxidant activity, including melanoidins, and may have contributed to the results obtained in the present work.

These observations suggest clearly that the ingestion of coffee brews might protect the liver cells from oxidative stress damage, being this observation independent of coffee species and caffeine amounts.

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Consumer Acceptance Limit for Iron and Zinc Concentrations in Brews Obtained from Fortified Ground Roasted Coffee

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SUMMARY

In the present study, we evaluated the consumer acceptance limit for iron (Fe) and zinc (Zn) concentrations in brews obtained from fortified ground roasted coffee. Fe bisglycine chelate and Zn bisglycine chelate were chosen for fortification of ground coffee (80% *C.arabica*, 20% *C.canephora*, medium-light roast, #65 SCAA, USA, fine grid) after sensory tests previously carried out. Although our goal was to offer 30% of the daily dietary recommended intake (DRI) for adults in a 50mL cup of brew, six coffee samples containing increasing amounts of minerals were evaluated (0, 30%, 50%, 80%, 100% and 150% of Fe and Zn Brazilian DRI per 50mL). Fe and Zn analyses in the ground coffee and in the brews were carried out by inductively coupled plasma atomic emission spectrometry (ICP OES). Recovery in the brew of Fe and Zn added to ground coffee was, on average, 95.3% and 48.5%, respectively. Acceptance test was used to determine acceptance limit, followed by Preference Mapping to analyse data. Results showed that, on average, fortifications up to 50% Fe and Zn DRI per 50mL were well accepted by consumers. Considering the efficiency of extraction, at this dose, consumers were receiving about 6.7 mg Fe (48% DRI) and 3.6 mg Zn (24% DRI). Although further sensory tests should be performed, roasted and ground coffee appears to be a suitable food vehicle for the fortification with Fe and Zn.

INTRODUCTION

An adequate intake of nutrients contributes to the normal growth, development and wellbeing of the human organism, protecting individuals against the risk of diseases caused by nutritional deficiencies. However, micronutrients malnutrition is still observed worldwide, especially in developing countries. Of major concern is the prevalence of iron (Fe) and zinc (Zn) deficiency. Literature data indicate that the intake of these minerals in Brazil is below recommended intakes (RDI), despite the fortification of wheat flour as well as other commercial food products. The fortification of foods with nutrients is an accepted practice used by food processors since the mid-twentieth century aimed to enhance the nutritional value and prevent or correct deficiencies of one or more nutrients. For a program of food fortification to be effective, it is essential to take into account the dietary habits and nutritional needs of the target population. The food vehicle chosen must be accessible and widely consumed by the population and the fortification compounds should be added in the most bioavailable forms. However, the micronutrients added should not significantly change the original sensory characteristics of the food. Coffee is the most traded food product in the world and the most consumed beverage after water. Due to the high content of phenolic compounds and other bioactive compounds, coffee has been considered beneficial to human health. Furthermore, a previous study showed that the addition of Fe and Zn to instant coffee

presented satisfactory bioavailability. Therefore, the use of coffee as a vehicle for fortification appears to be promising and may have social and public health relevance. Considering the need to maintain the original sensory characteristics of the fortified food product, and taking into account that such characteristics affect consumer product liking, in the present study we evaluated the consumer acceptance limit for Fe and Zn concentrations in brews obtained from fortified ground roasted coffee.

MATERIALS AND METHODS

A popular blend comprised by 80% of good quality and 20% of defective (PVA) *Coffea arabica* beans (COCAPEC, MG, Brazil) was used. Beans were roasted to medium-light colour degree (# 65 Agtrom-SCAA) and ground in a discs grinder (Gourmet M-50, LEOGAP, Curitiba, PR, Brazil fine grid, # 1). This color degree was chosen because beans at such color degree contain high amount of chlorogenic acids and bioactive derivatives, as well as a considerable amount of niacin. The forms of micronutrients chosen for coffee fortification were Fe bisglycine chelate and Zn bisglycine chelate. These forms have previously presented good solubility, bioavailability, and sensory quality, which are prerequisites for food fortification.

In order to establish the concentrations of Fe and Zn used for coffee fortification, the following aspects were considered: the daily consumption of roast and ground coffee of at least 1-4 cups of coffee (5g of coffee in 50mL of water per cup), the levels of Fe and Zn in fortified foods reported in the literature, the current Brazilian legislation for wheat flour and cornmeal fortification with Fe, and results from a previous study, which assessed the bioavailability of Fe and Zn added to instant coffee. Although our goal was to fortify coffee with 30% of Brazilian DRI for Fe and Zn, five concentrations of added minerals were tested, as described below.

For minerals analysis, a sample of 10.0 mL brew or 1g of ground coffee was digested with concentrated nitric acid. Analyses of Fe and Zn in the ground coffee and in the brews were carried out by inductively coupled plasma atomic emission spectrometry (ICP OES), on a Perkin Elmer Optima 4300DV. All determinations were performed in the simultaneous mode of operation, and the conditions used were: generator power, 1500 W; auxiliary Air flow, 0.2 L/min; coolant flow, 15 L/min; nebulizer flow, 0.45 L/min; pump speed, 1.50 mL/min.

Seventy one regular coffee consumers were invited to take part in the study and the only condition for participation was to consume at least one cup of coffee per day. Six coffee samples including a control sample and increasing amounts of added minerals were evaluated. Samples provided 0 (control), 30%, 50%, 80%, 100% and 150% of Fe and Zn DRI, according to ANVISA, Brazil, per 50mL of brew.

Brews at 10% (w/v) were prepared in electric coffee maker (Britânia® - NCB27, Brazil), and the preparation time was 1m and 50s. Brews were served to participants in 50mL disposable Styrofoam thermal cups coded with three digit numbers at 68 ± 2 °C, in individual computerized sensory booths, following a balanced presentation order. Participants were allowed to choose to sweeten (using sugar or artificial sweetener) or not their brews, according to their habits. When sugar or sweetener was used, they were told to add the same amount to all samples. To evaluate the brews acceptability, a 9-point hedonic scale was used, ranging from "extremely dislike" to "extremely like".

Statistical analyzes were performed using the program XLSTAT ® Version 2010.3.01 (Addinsoft, USA). Acceptance data were also treated by ANOVA followed by Fischer (LSD),

test for comparison of means, as well as Cluster Analysis and Preference Mapping. Mean results were considered significant when $p < 0.5$.

RESULTS AND DISCUSSION

The percentage of minerals extracted from fortified coffees in the electric coffee maker was, on average, 95.3% and 48.5% for Fe and Zn, respectively. The mean acceptance scores for the coffee beverages are presented in Figure 1. Despite the low acceptance means for all samples (including the unfortified coffee, i.e. 0%), possibly caused by the roasting degree to which the participants were not used to, on average, no difference in acceptance was found among the brews providing minerals at concentrations equivalent to 0%, 30%, and 50% of Brazilian DRI (ANVISA), the latter corresponding theoretically to 7.0 mg of Fe and 7.5 mg of Zn in a cup. Considering the efficiency of mineral extraction in the brew, actual amounts provided were 6.7 mg Fe and 3.6 mg Zn per cup in the latter sample. These results indicate that participants did not observe differences in terms of liking between the control (unfortified coffee) and coffee fortified up to about 50% DRI of Fe fortification and 25% DRI of Zn fortification, which is close to our goal of fortification at 30% of the Brazilian DRI (ANVISA). These results also suggest that further sensory tests should be performed to evaluate the sensory acceptance for each mineral separately.

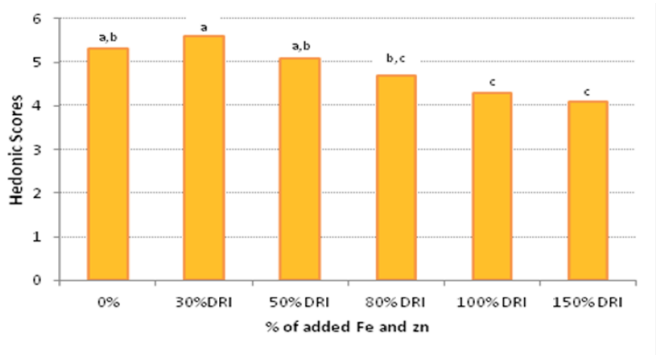

Figure 1. Mean preference scores for coffee beverages obtained from ground roast coffees fortified with different levels of minerals. Different letters above bars indicate significant difference by ANOVA.

As consumers do not exhibit the same behaviour, and a mean is not representative of all participants, Cluster Analysis and Preference Mapping were carried out. Figure 2 presents the first two dimensions of internal preference mapping generated from the hedonic responses associated with acceptance of six samples of coffee beverage, containing concentrations from 0% to 150% of DRI Brazilian recommendation for Fe and Zn.

Three distinct groups of consumers with different acceptance scores were identified (Figure 2 and Table 1). These segments are still being characterized considering socio-demographic characteristics, and coffee consumption habits. The mean acceptance scores of each segment are represented in Table 1. The largest segment (Segment 1, n=30) liked all samples, and attributed relatively high scores to 0%, 30%, 50% and 80% levels of fortification (mean scores between 6.10 and 7.03). Segment 2 (n=24) gave low scores to all products, including the unfortified coffee, and decreased their scores as the percentage of minerals increased. Consumers in segment 3 (n=17) did not like the unfortified beverage, and preferred samples fortified with 30%, 80%, 100% and 150% of RDI percentage. The overall low scores given by segments 2 and 3 were probably due to the fact that they were not used to drink good quality coffee beverages or because they were not used to drink medium-light roasted coffee.

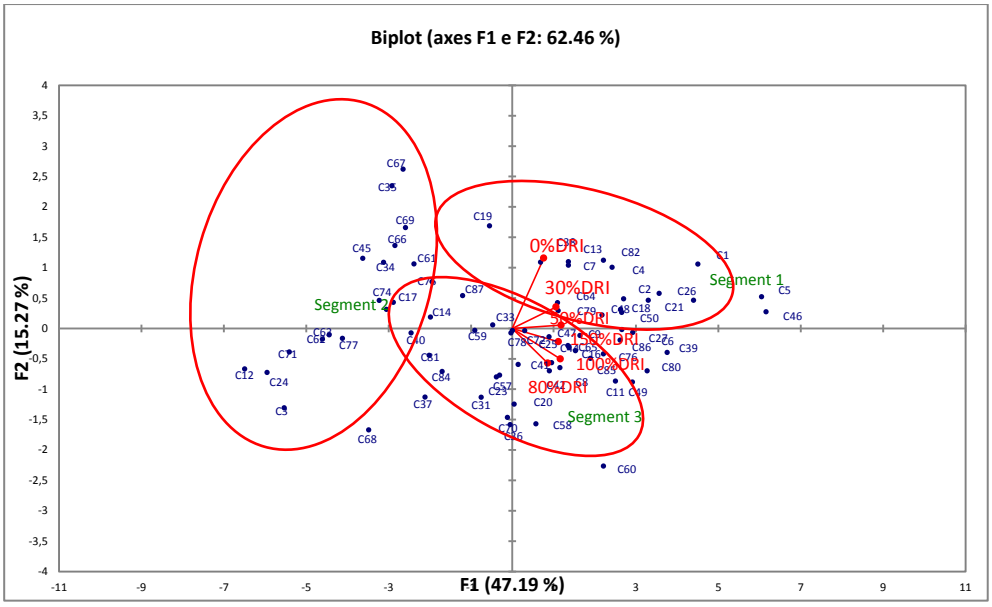

Figure 2. Internal preference mapping obtained for beverages prepared from ground roast coffees fortified with different levels of Fe and Zn.

Table 1. Mean preference[1] scores for consumer segments for brews from ground roast coffee fortified with Fe and Zn.

Samples	(n = 71)	Segment 1 (n = 30)	Segment 2 (n = 24)	Segment 3 (n = 17)
	100%	***42.25%***	***33.80%***	***2395%***
Unfortified (Control)	5.3 ^{a,b} ± 2.4	7.0^{a} ± 1.3	4.6 ^{a} ± 2,5	3.0 ^{b} ± 1.6
30% DRI	5.6 ^{a} ± 2.3	6.5 ^{a,b} ± 1.9	3.7 ^{a,b} ± 2.4	6.6 ^{a} ± 1.3
50% DRI	5.1 ^{a,b} ± 2.4	7.0 ^{a} ± 1.9	3.0 ^{b,c} ± 1.6	4.8 ^{a,b} ± 2,4
80% DRI	4.7 ^{b,c} ± 2.4	6.1 ^{a,b} ± 1.6	2.7 ^{b,c} ± 1.8	5.0 ^{a,b} ± 2.6
100% DRI	4.3^{c} ± 2.4	5.6 ^{b} ± 1.9	2.1 ^{c} ± 1.6	5.2 ^{a} ± 2.1
150% DRI	4.1 ^{c} ± 2.7	5.4 ^{b} ± 2.3	1.7 ^{c} ± 1.3	5.0 ^{a,b} ± 2.7

Mean ± SD. Means in the same row followed by different letters indicate difference between test samples by Fischer (LSD) (p<0,05). [1]Evaluated in 9-point hedonic scales, varying from 1: disliked extremely to 9: liked extremely.

Considering the adequate bioavailability of added Fe and Zn to the coffee matrix and the relatively good recovery of these minerals during brewing, especially regarding Fe, sensory data from the present study indicate that roast ground coffee is a suitable food vehicle for fortification with Fe and Zn. Further sensory tests are being performed.

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Effect of *Coffea Canephora* Aqueous Extract on *Ex Vivo* Oral Biofilms: A Case Study

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SUMMARY

In the present manuscript, the *ex vivo* antimicrobial effect of brewed coffee was tested on oral biofilms. For this, unsweetened and sweetened (10% sucrose) brewed light-roasted *Coffea canephora* were applied on biofilms formed by non-stimulated saliva from three volunteers. After contact with unsweetened and sweetened brews, the average microorganisms count in the biofilms reduced by 15.2% and 12.4%, respectively, with no statistical difference among them. Although no difference was observed among the microbial counts in oral biofilms treated with both coffee extracts, with and without sucrose, it is important to emphasize that the amount of sugar added to coffee vary considerably, according to people's habits, which could influence the coffee properties against the oral biofilm reduction.

INTRODUCTION

Among natural products that have demonstrated antibacterial properties, coffee is the most popular in terms of consumption. This opens a promising avenue of applications, since it is relatively safe and has a taste and odor largely appreciated.

Among many existing coffee species, the two most commercialized are *Coffea arabica and Coffea canephora* . In a recent study, Antonio et al. reported a better performance of *C. canephora* extracts, comparing to *C. Arabica,* in relation to inhibition of biofilm formation by *Streptococcus mutans* - a cariogenic bacterium, probably because of its higher content of polyphenols and caffeine. In addition, the same authors demonstrated *in vitro* that *C. canephora* beans also hindered the de-remineralization process of deciduous tooth fragments in the presence of an *ex vivo* mixed biofilm.

Besides all the evidences involving the antibacterial activity of coffee against *S. mutans*, studies clarifying the role of coffee on the reduction of microorganisms count within the biofilm are still needed. In addition, in all parts of the world, coffee is largely consumed with sugar. However, there is no evidence that sweetened coffee extracts would also exert antibacterial activity. Therefore, the objective of this study was to investigate the effect of unsweetened and sweetened *C canephora* extracts on the microorganisms count in mixed *ex vivo* artificial biofilms.

MATERIALS AND METHODS

Coffea canephora extract

Regular *Coffea canephora* cv. Conillon beans were roasted as in Antonio et al. to produce a light roasting degree coffee. An unsweetened (CE) and sweetened (SCE) aqueous coffee extract at 20% was obtained by a coffee brewing procedure, percolating 100 mL of pre-boiling (95°C) Milli-Q purified water through 20 g of ground roast coffee. The sweetened coffee extract was prepared just like the first extract and sweetened with 10% sucrose.

Volunteers and saliva samples collection

The study was approved by the Local Ethics Committee (IESC – UFRJ). Young adult volunteers (n = 3) were in good general and oral health, not making use of medication and had all natural teeth. Subjects were instructed not to consume food or beverages except for water during 1 hour before saliva collection. Moreover, none of them consumed coffee habitually. Non-stimulated saliva samples were collected after the volunteers spit into a graduated collection sterilized tube. Saliva produced in the first 30 sec was discarded and then, it was collected for exactly 5 min. Their mean DMFT (4.6) and mean whole saliva flow rate (0.83mL/min) were registered. The saliva samples, stored in melting ice, were immediately transferred to the microbiology laboratory for biofilm assay.

Biofilm assay

Twenty microliters of non-stimulated saliva collected from volunteers (n = 3) were placed on 0.22 mm membrane disks (diameter of 0.13mm) (Millipore). Each membrane was placed over plates containing BHI-S (Brain Heart Infusion medium, from Difco, Sparks, USA; supplemented with hemin, menadione (Inlab, Maranhão, Brazil) and yeast extract (Oxoid, Hampshire, England). The system was incubated anaerobically for 48h at 37^{o}C. After the biofilm growth, disks were collected and placed inside a glass flask, for 30 min, containing 2 mL of the following substances: (i) coffee aqueous extract (CE, 20mg/mL); (ii) coffee aqueous extract (20mg/mL) with sucrose at 10% (SCE); (iii) chlorhexidine (0.05%) – positive control; (iv) Milli-Q water with sucrose at 10%; and (iv) Milli-Q water – negative control. This procedure was performed in duplicate. Then, the disks were briefly washed in sterile saline to rinse out the added substances and the biofilm was extracted by vortexing the disks in 1 mL of sterile saline during 2 min. To enumerate viable cells, serial dilutions (10^{-1} to 10^{-8}) were performed and 50 mL of each dilution were streaked out on BHI-S agar (short plate) in triplicate. Plates were incubated anaerobically for 48 h at 37^{o}C and then, CFU/mL was counted. The activity of the coffee extracts on microbial biofilms was estimated by comparing the microbial population collected from the biofilms placed in the controls and the microbial population collected from the biofilms placed in coffee extracts.

Sucrose end-point for mixed oral biofilm growth

Mixed biofilms were formed from human saliva in artificial membranes. The biofilm/membranes were transferred to test tubes (n = 7), which contained saline solution (control) and sucrose (1%, 3%, 5%, 7%, 10%, and 20%). After the same treatment with the protocol described above for the antimicrobial assay with artificial biofilm, diluted samples (10^{-1} to 10^{-8}) were inoculated onto a BHI-S agar plate and incubated anaerobically for 48 h at 37^{o}C. The number of CFU was calculated. The experiment was performed in duplicate.

Statistical analysis

The difference between the applied treatments was observed through Kruskal-Wallis test followed by Mann Whitney for comparisons of substances two-by-two. Differences between means were considered significant when values of $p<0.05$.

RESULTS AND DISCUSSION

A reduction in biofilm microbial count was observed in the systems incubated with both extracts of *C. canephora* (SCE and CE), with no statistical difference among them as shown in Figure 1. The positive control was the most effective agent against the microorganisms from biofilm, reducing their count by about 32.1% ($p<0.05$), whereas SCE and CE were able to reduce the CFU counts by 15.2% and 12.4%, respectively, with no statistical difference among them (Table 1). A drop in biofilm microorganisms count was also observed after treatment with Milli-Q water with sucrose at 10% ($p <0.05$) when compared to the treatment with negative control (Figure 1). According to the literature, the exposure to sugar can be considered a determinant factor in dental caries disease, especially if it occurs at a high frequency (e.g. more than 6 times/day). The difference between the results from the biofilm treatment with water and sweetened water, which demonstrated a drop of CFU counts from biofilm after treatment with the last one is in contrast with our hypothesis according to which high levels of sucrose should have raised the *ex vivo* biofilm, since Vale *et al.* affirmed that sucrose is able to significantly increase bacteria counts in dental biofilm.

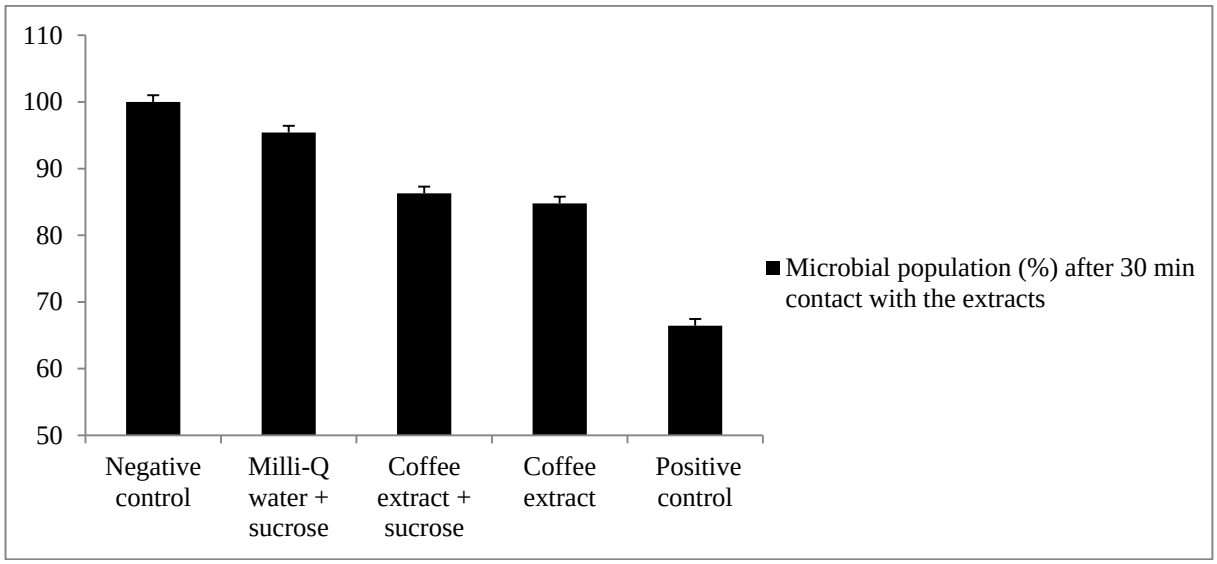

Figure 1. *In vitro* microbial biofilm susceptibility assays for *C. canephora* aqueous extracts (with and without sucrose addition), chlohrexidine at 0.05% --positive control and Milli-Q water with sucrose at 10%. The percentage of viable microorganisms in the biofilm was estimated by comparing the CFU/mL of the biofilm placed in Milli-Q water – negative control (100% viable) and the CFU/mL from the biofilms placed in all other referred substances after 30 min of incubation.

Table 1. Microbiological analysis of artificial oral biofilms after treatment with: negative control (NC); unsweetened (CE) and sweetened (SCE) *C. canephora* extract; positive control (PC) and sweetened Milli-Q water (SMQ).

Volunteer	microbial count from biofilm (n $\times 10^6$) ± SD ($\times 10^6$) after treatment with				
	NC	***CE***	***SCE***	***PC***	***SMQ***
1	15.08 ± 6.81^a	1.90 ± 0.65^b	2.62 ± 0.70^b	0.03 ± 0.00^c	6.23 ± 0.64^d
2	1.43 ± 0.30^a	0.14 ± 0.06^b	0.21 ± 0.17^b	0.03 ± 0.01^c	0.62 ± 0.15^d
3	2.56 ± 0.91^a	0.24 ± 0.10^b	0.51 ± 0.14^b	0.02 ± 0.00^c	0.87 ± 0.14^d

Note: In rows, means values followed by distinct letters differ among them at a significant level of 5%, considering the results independently, with respect to the individual microbiological characteristics of the volunteers.

In order to investigate the role of sucrose in the bacterium cell metabolism, we performed an additional experiment incubating membranes with the same mixed biofilm in sucrose solutions from 1% to 20%. Sucrose concentrations from 5% up to 20% were capable of reducing the number of bacteria colonies from biofilm compared to control (Figure 2). According to Lemos et al. although the ability to survive for long periods of carbohydrate starvation is considered crucial for oral bacteria, sudden exposure to an excessive amount of sugar in cells can result in an accumulation of toxic levels of glycolytic intermediates, resulting in bacteria death. The authors believe that in the present *in vitro* conditions, the high levels of sucrose provoked the drop of bacteria osmotic tolerance. Recently we tested a green (unroasted) *C. canephora* extract against *S. mutans* and observed that it did not inhibit the bacteria growth. This was attributed to the high sucrose concentration in the green coffee, which promoted the growth of the bacteria. However, sucrose concentration of the extract used in the referred study was lower than 5%. In this context, the results of the present work do not contradict the previous data, since we only observed the influence of sucrose on the reducing growth of bacteria in concentrations higher than 5%.

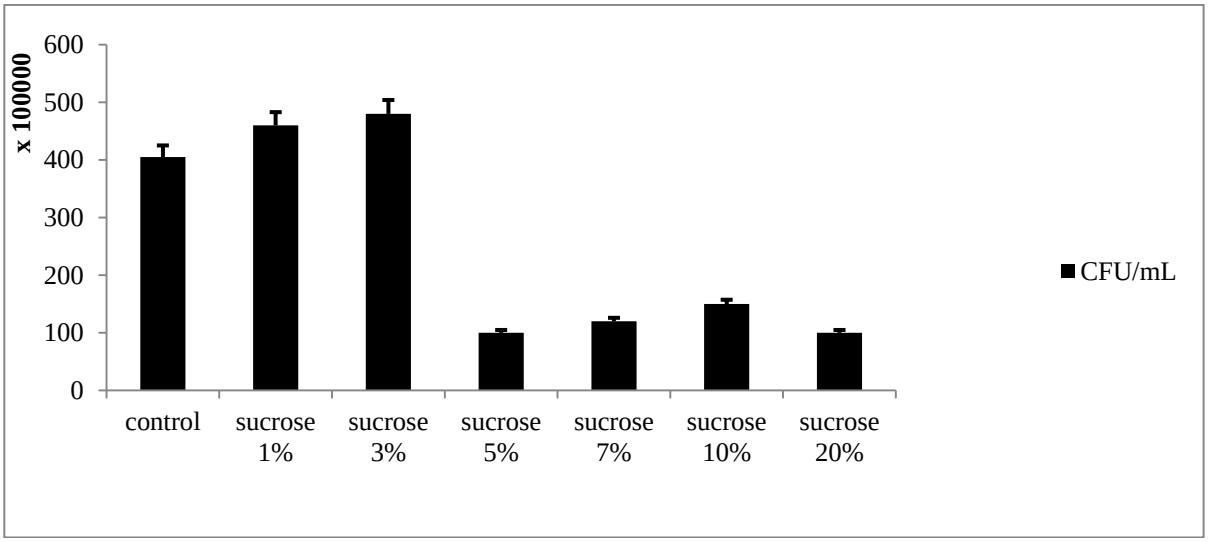

Figure 2. Number of colonies forming units (CFU) using biofilm treated with increasing concentrations of sucrose and control.

The present results show that the *C. canephora* extract reduced the microbial count in *ex vivo* oral biofilms by around 15%. Considering that coffee is a natural ingredient that may be obtained in large quantities and at low cost it may be highly beneficial as an anticariogenic ingredient, mainly because no difference was observed among coffee extract with and without

sucrose. However, as much as we have tried to mimic a real situation, these results were obtained from preliminary screening in *in vitro* circumstances. In addition, it is important to emphasize that the amount of sugar added to coffee vary considerably, according to people's habits, which could influence the coffee properties against the oral biofilm reduction. Thus, further *ex vivo/in vivo* studies should be performed with the aim of investigating the influence of the sweetened coffee extract against oral biofilm.

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ß-Sitosterol – A Parameter to Study the Coffee Proportion in Coffee Drinks

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SUMMARY

The popularity of coffee-drinks-to-go has been increasing over the last few years. These commercial milk-based drinks contain 70% of milk and 30% of other ingredients, including coffee. The aim of our study was to calculate the coffee proportion in these coffee drinks. Within the sterol fraction, which is only little influenced by the roasting process, ß-sitosterol proved to be a possible indicator. Model solutions containing different proportions of milk and coffee were analysed, and some parts of the obtained results were presented. It could be demonstrated that in the case of a constant coffee proportion, similar ß-sitosterol content was analyzed when the milk proportion is increased to 80%. Moreover, by increasing the coffee proportion, the ß-sitosterol content increased linearly even at a defined milk percentage of 80%. However, due to the fact that the content of ß-sitosterol in both coffee beverages and instant coffees strongly depends on the production process, the assessment of the coffee proportion is only possible if the used coffee preparation is available.

INTRODUCTION

In recent years, coffee drinks have become more and more popular as a quick and uncomplicated solution for an occasional consumption of coffee. They are available in the refrigerated sections of most supermarkets. They belong to the milk-based drinks and consist of at least 70% to 80% milk and, at most 20% to 30% of other ingredients such as coffee, sugar, rheological additives, cocoa, flavourings, and sometimes caffeine. According to the declaration on the packaging, the coffee proportion is a solid or liquid coffee extract, or a coffee brew, and ranges between 0.8 to 19% of the beverage.

To study the coffee proportion in commercial coffee drinks it is necessary to find a suitable component as a characteristic for coffee. It is reasonable to investigate a water-soluble and a lipid-soluble substance in regard to the differing extraction processes. Hence, the question is whether it is possible to assess the coffee proportion in coffee drinks.

The substances selected must accomplish several requirements:

- Adequate content in green, roasted, and instant coffee.
- Low natural variability.
- Stability during the roasting process.
- Adequate passage into the coffee beverage.
- Absence in the other ingredients.

The suitability of trigonelline as a water-soluble substance as well as the suitability of ß-sitosterol as a lipid-soluble substance was tested. The results for trigonelline are presented

in Poster PC 410 (Trigonelline and HMF — Parameters to Study the Coffee Proportion in Coffee Drinks).

RESULTS AND DISCUSSION

Due to its sufficiently low natural limits of variation and its stability during the roasting process the sterol fraction was chosen in order to assess the percentage of the coffee proportion in commercial coffee drinks. In the sterol fraction, ß-sitosterol is the main component with an approximate but nearly constant ratio of 50%. Therefore, it presents itself as a good component for quantification.

A method for analyzing ß-sitosterol was established using model drinks consisting of milk and, due to its use in commercial coffee drinks, of instant coffee. Thereby, 100 mL model drinks were prepared consisting of 10% to 80% low-fat milk and 2 g of an instant coffee with 1.1 mg/100 g ß-sitosterol. The high cholesterol level of the milk was responsible for some analytical problems during the development of the method. As a result, when increasing the milk proportion up to 80% at a constant coffee content the cholesterol content increased whereas the analyzed level of ß-sitosterol remained stable (Figure 1).

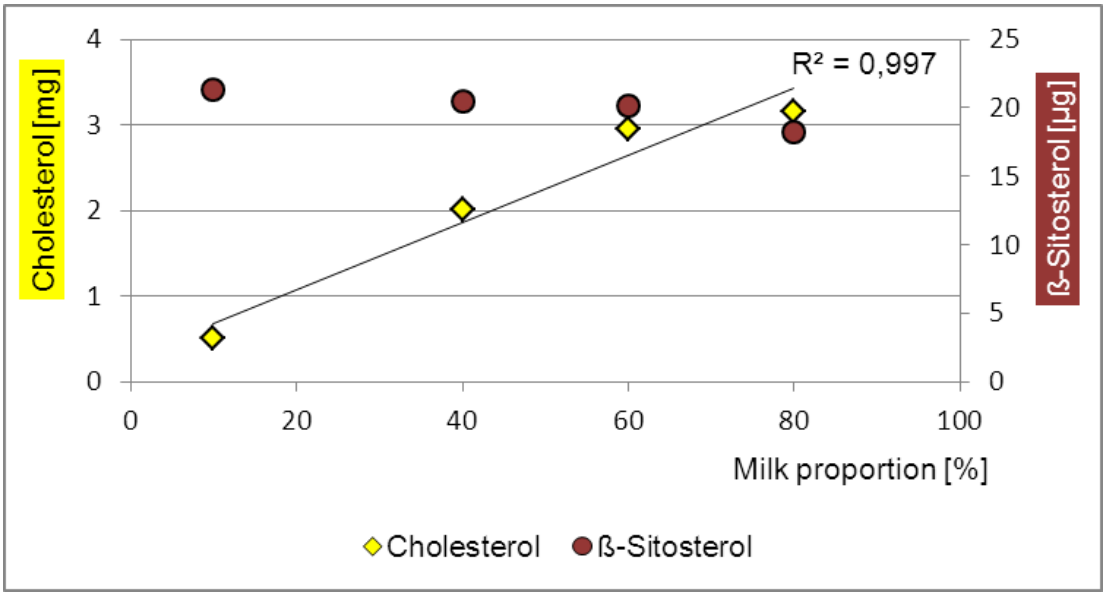

Figure 1. Contents of cholesterol and ß-sitosterol of model drinks with increasing milk proportion.

Figure 2 shows the results when increasing the portions of coffee at a constant milk proportion of 80%. There is a linear relation between the coffee proportion and the ß-sitosterol content. For these coffee drinks, 0.5 to 6 g of an instant coffee with a ß-sitosterol level of 0.6 mg/100 g was used.

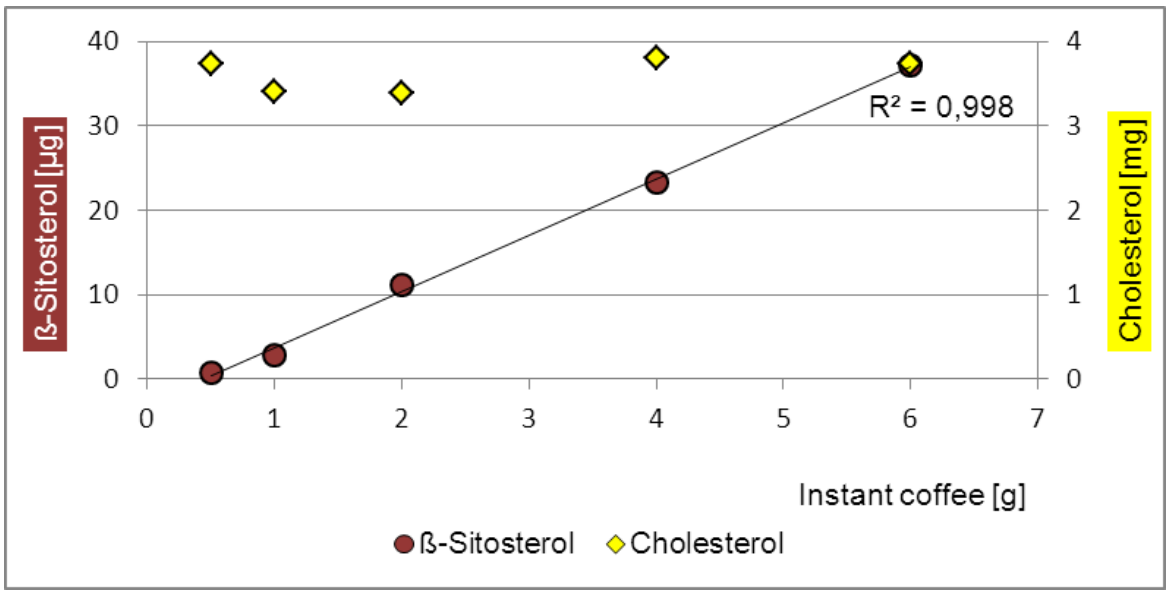

Figure 2. Contents of ß-sitosterol and cholesterol of model drinks with increasing coffee proportion.

It is important to note that although the ß-sitosterol content in roasted coffees is nearly constant, the passage into the coffee water extract depends very strongly on the production conditions. For example, the transfer of the lipids into the beverage ranged from 0.3% for a filtered coffee to 20% for a Scandinavian type coffee. In different instant coffees, the ß-sitosterol content also varies between 0.6 mg/100 g to 2.1 mg/100 g (n = 7).

Based on the results of the model drinks, it is possible to assess the coffee proportion in coffee drinks via the determination of ß-sitosterol if the coffee preparation is known or available.

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On-line Analysis of the Coffee Roasting Process with PTR-ToF-MS: Changes in Flavor Formation for Different Coffee Varieties

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SUMMARY

Roasting is the process where volatile organic compounds (VOCs), responsible for the aroma of a cup of coffee, are generated. This strongly depends on the type of coffee being roasted, as well as on the time-temperature-profile of the roasting process. Proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS) allows examining directly and in real-time the formation of the VOCs during the roasting process itself, and in particular of the VOCs responsible for the flavor. In this study, different roasting profiles to a medium roast degree were monitored for five different coffee origins: Arabica from Columbia, Antigua (Guatemala), Djimmah (Ethiopia), Yirga Cheffe (Ethiopia) and Robusta Malangsari (Indonesia). Clear differences in the VOC formation can be seen between Robusta Malangsari, Arabica from Middle America and Ethiopia; only minor differences are observable between Arabica from Columbia and Guatemala. Off-line analysis of the respective coffee brews revealed the differences in the cup, including HS SPME GC/MS for aroma analysis.

INTRODUCTION

The quality of a cup of coffee depends on a large number of parameters. Starting with the climate (sun-exposition, altitude, rain), the soil and irrigation, the species and variety of the green bean, their post-harvest processing, roasting and grinding, and the way they are brewed are all influencing the aroma and taste of a cup of coffee. In this study, the focus is on a particular but critical factor, namely the impact on the flavor of coffee of different time-temperature roasting profiles applied on different varieties of the green bean. It is known that the characteristic aroma compounds of a cup of coffee are generated through various physical changes and chemical reaction mechanisms during the roasting process: During the initial endothermic phase of roasting, the green beans are drying, reducing the water content from about 8-12% to a few percent. Further heating of the beans lets the exothermic pyrolysis reactions start. This can be perceived during roasting as a popping sound, called the first crack (at about 175-185°C). If one is keeping on roasting to a very dark roast degree, at higher temperatures (above 200°C) the second crack can be heard. At the end of the roasting process the beans are removed from the roaster and are cooled down quickly (quenched), either by spraying water on the beans or by air. Mainly between the first and the second crack, the typical coffee aroma compounds are formed in chemical reactions. Several pyrazines, e.g., are produced in Maillard reactions, whereas pyridines are obtained mainly by degradation of

trigonellines. Thermal decomposition of ferulic acid leads, among others, to the key coffee aroma compound 4-vinyl-guaiacol. Besides aroma compounds, melanoidines are also formed, giving roasted coffee its characteristic brownish color. Several chemical reaction mechanisms are still not well understood, but some are critically depending on moisture content, temperature and the formation of intermediates in complex multistep and competing processes. One can, therefore, expect that the final flavor does not only depend on parameters measured at the beginning and the end of the roasting, but that the path connecting these also matters. In other words, the specific time-temperature roasting profile is a relevant parameter for the cup quality, as stated by Yeretzian et al. in 2002 "Flavor quality … is a path dependent quality"[1,2]. This reflects the everyday-experience of roasters.

The pathway of formation of the flavor of coffee can be studied on-line by proton-transfer-reaction time-of-flight mass spectrometry (PTR-ToF-MS)[3]. This dynamic method allows the detection of VOCs in very small concentrations at a high mass resolution of more than 5000 m/Δm (FWHM) for benzene, for example. In the soft ionization process, the VOCs are protonated via transfer of H^+ from H_3O^+ to the analyte, leading to almost no fragmentation of the ions. The VOCs, often at very low concentrations in the air, can be measured without any preconcentration, as the proton affinities of the main constituents of air (N_2, O_2 and CO_2) are lower than that of H_2O, and hence they are not protonated. For the on-line analysis of the formation of these VOCs during the roasting of coffee, the PTR-ToF-MS is coupled to the off-gas of the coffee roaster (
Figure 2).

Several studies have so far been performed on the on-line analysis of coffee roasting focusing in general on only one type of coffee roasted along different roasting profiles, either with PTR-quadrupol-MS [1,2] (having a much lower mass resolution than the time-of-flight mass spectrometer), via laser ionization [4-8], or with PTR-ToF-MS [9,10]. Here, the influence of different time-temperature roasting profiles on the formation of VOCs during the roasting of different types of coffee is presented based on on-line analysis with PTR-ToF-MS.

MATERIALS AND METHODS

Roasting Profiles

Coffee from Columbia (*coffea arabica*, Probat-Werke, Germany), Guatemala (Antigua La Ceiba, *coffea arabica*, Rast Kaffee AG, Switzerland), Ethiopia (Yirga Cheffe and Djimmah, *coffea arabica*, Rast Kaffee AG, Switzerland) and Indonesia (Malangsari, *coffea caneophora var. robusta*, Rast Kaffee AG, Switzerland) was roasted on a Probatino roaster (PROBAT, 2008, heating gas: propane, PanGAs, Winterthur). For all trials, 1 kg batches of green beans were roasted. The different profiles were achieved by varying the heating intensity of the Probatino, the roast degree was measured by color (Colorette, 3b, Probat-Werke, Germany). The roasted beans were quenched with air and filled in bags (Wicovalve PET/ALU/PE 12-8-75 80x50x280 mm silver) à 250 g and stored at -22°C until further analysis.

The roasting profiles were chosen to cover (i) a medium time-temperature roasting profile optimal for each type of coffee and (ii) a long time-temperature roasting profile and are shown as a time-temperature plot in
Figure 1. The details of the respective roast batches are given in Table 1.

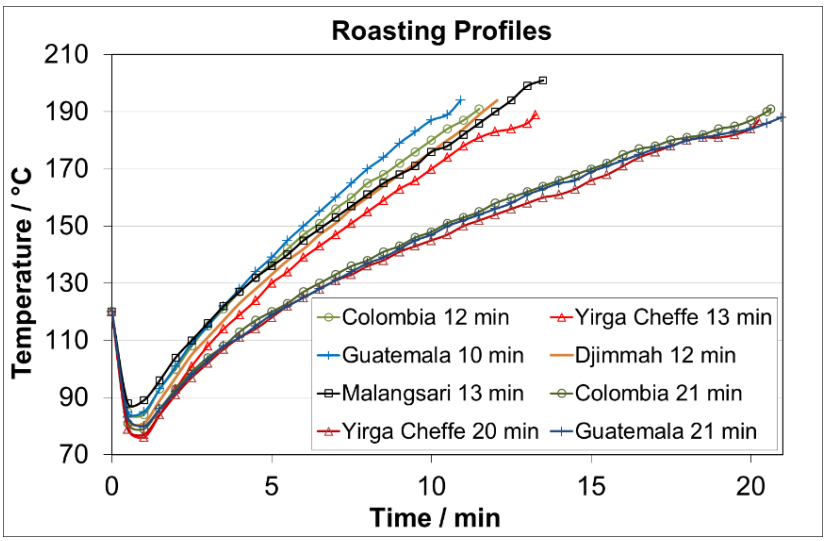

Figure 1. Time-temperature roasting profiles. Plotted is the temperature insight the roasting drum in degree centigrade versus the roasting time in minutes.

Table 1. Experimental results for the respective roast batches for the following time-temperature roasting profiles to a medium roast degree: Colombia 12 min, 21 min; Guatemala 10 min, 21 min; Yirga Cheffe 13 min, 20 min; Malangsari 13 min; Djimmah 12 min. Given are the weight loss after roasting in percent, the gain in volume in percent, the roasting time in minutes, the end temperature of the roasting process in °C, and the roasting degree in Pt (Colorette 3b).

	Colombia					**Guatemala**					
	12 min			**21 min**		**10 min**			**21 min**		
weight loss / %	-14	-13	-14	-13	-13	-13	-13	-13	-13	-12	-1
gain in volume / %	71	75	71	64	64	68	68	71	64	61	57
roasting time / min	11.46	11.29	11.43	21.25	20.38	10.55	9.37	9.25	20.57	22.27	21.13
end temperature	194	193	193	188	191	194	193	196	188	187	187
roast degree / Pt	103	107	102	103	100	102	112	103	102	106	109
	Yirga Cheffe								**Malangsari**	**Djimmah**	
	13 min				**20 min**				**13 min**	**12 min**	
weight loss / %	-13	-13	-13	-13	-13	-14	-13	-13	-15	-15	-14	-14
gain in volume / %	54	54	54	61	43	43	43	43	62	65	71	71
roasting time / min	12.43	13.26	13.42	13.17	20.24	19.5	20.46	19.35	12.51	13.14	13.02	12.06
end temperature	192	189	188	190	187	187	185	186	202	201	193	194
roast degree / Pt	98	102	107	103	123	96	100	99	98	102	109	103

On-line monitoring

The VOCs and hence the aroma profile of the coffee beans were monitored on-line during roasting with PTR-ToF-MS (PTR-ToF-MS 8000, Ionicon Analytik GmbH, Austria). The experimental setup is shown schematically in
Figure 2. The roasting gas from the Probatino is withdrawn from the exhaust gas outlet with a vacuum membrane pump (Typ N86 KN.18, KNF Neuberger AG, Switzerland) through stainless steel tubes (deactivated, BGB Analytik AG, Switzerland). To prevent condensation of the VOCs, the roasting gas is diluted with activated carbon-filtered compressed air, and the

stainless steel tubes are heated to 70°C. A constant flow of 100 ml/min of the diluted roasting gas is transferred to the PTR-ToF-MS. The measurement started slightly before the green beans were filled in the Probatino, and was continued a short time after the beans had been removed from the roasting drum.

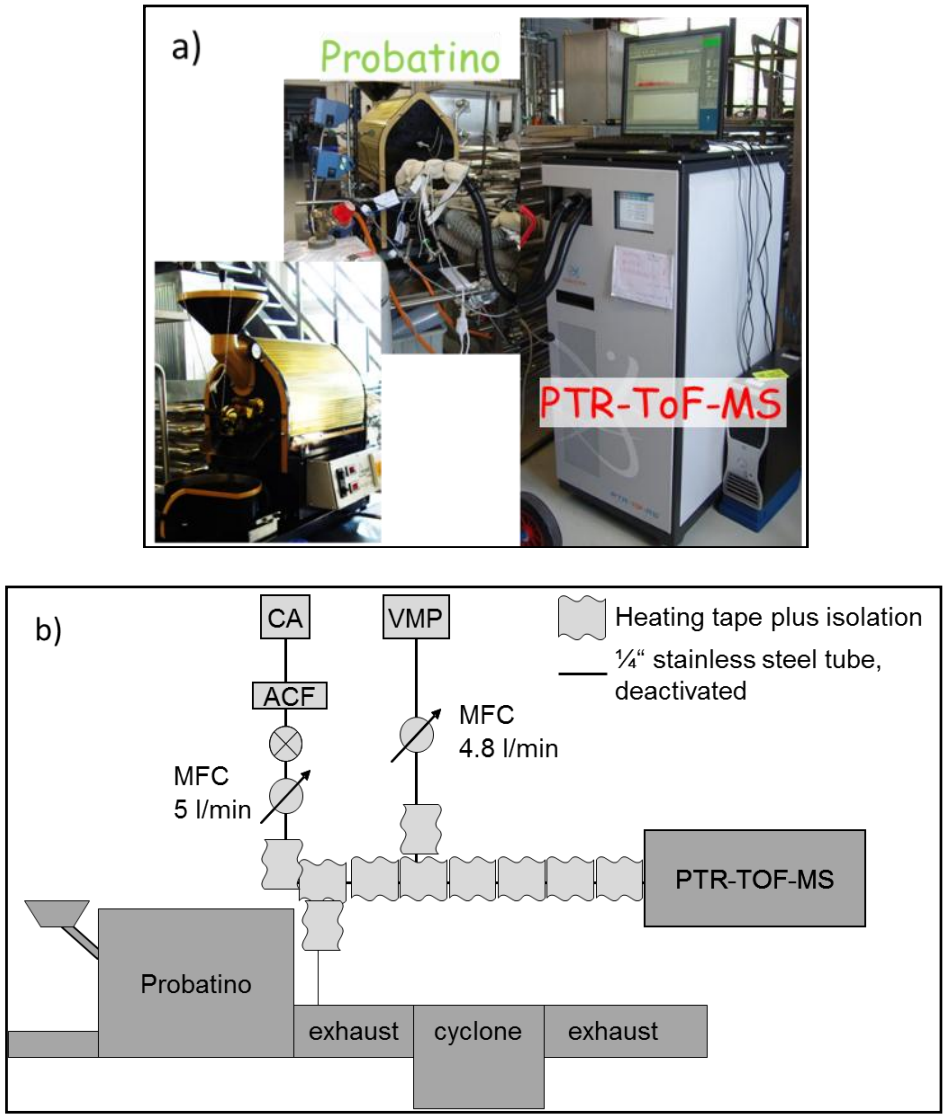

Figure 2. a) Left: Front view of the Probatino, middle: back view of the Probatino coupled to the PTR-ToF-MS (right). b) Schematics of the experimental setup. CA: compressed air; ACF: active carbon filter; VMP: vacuum membrane pump; MFC: mass flow controller.

Off-Line Analysis

Coffee brew: Coffee brew was prepared with 12 g roast and ground coffee (espresso grinder KED 640, Ditting, Switzerland, grinding degree 8) per 200 millilitre water (Evian, France) at 90°C, the extraction time was 4 min. The brew was filtered with a ceramic filter (Bayreuth coffee machine, Erste Bayreuther Porzellanfabrik Walküre Siegmund Paul Meyer GmbH, Germany) before further analysis.

HS SPME GC/MS: 10 ml of coffee extract were analyzed immediately after preparation with headspace solid phase micro extraction gas chromatography / mass spectrometry (HS SPME GC/MS) with a Polydimethylsiloxane/ Divinylbenzen (PDMS/DVB) SPME fiber (65 µm film

thickness, Supelco, Sigma-Aldrich Chemie GmbH, Switzerland) on a DB-WAX column (30 m x 250 µm x 0.25 µm, Agilent Technologies, Switzerland). *SPME parameters* (Gerstel, Switzerland): Incubation: 4 min at 50 °C, agitating at 250 rpm; Extraction time: 7 min at 50°C; Desorption time: 5 min at 240°C; GC/MS parameters (7890/5975N, Agilent Technologies, Switzerland): 35 °C for 1 min; then 4 °C/min to 100 °C for 10 min; then 30 °C/min to 130 °C for 8 min; then 6 °C/min to 220 °C for 5 min; splitless mode; flow 1 mL/min; EI source 70 eV, 230°C; detector 150°C. For data analysis, the software MSD Chemstation (Version G1701 EA E.02.00.493, Agilent Technologies, Switzerland) and the database NIST08 was used. Chemical identification was performed via the respective mass spectrum and retention time. 61 molecules contributing to the aroma of coffee are chosen for evaluating the headspace of the respective coffee brew extraction methods, their HS SPME GC/MS intensities have been summed up for the total headspace intensity.

RESULTS AND DISCUSSION

In this study, the temporal evolution of different VOCs during the roasting process was monitored on-line for five different types of coffee with PTR-ToF-MS. Typical mass spectra of the composition of the VOCs in the exhaust gas of the roaster are shown in

Figure 3, one at the very beginning of the roasting process, at 10 s (

Figure 3a), and one at the maximum of the roasting process, just before the beans have been removed from the roasting drum, here at 705 s (

Figure 3b). A comparison of the two mass spectra illustrates the huge amount of VOCs generated during roasting. Interesting at this point is how these VOCs are formed in real-time, that means how their intensity changes during the roasting time. Focusing firstly on one time-temperature roasting profile, one type of coffee, and one VOC,

Figure 3c illustrates the time-intensity profile of $C_5H_5O^+$ as an example of the formation pathway during a roasting profile of a 12 min roasting of Colombian coffee. This molecule was not formed up to a roasting time of about 8 min, then the intensity reached a slight plateau after the first crack at about 10 ½ min (184°C), and at about 11.3 min (190°C) the intensity rose again until the end of the roasting process. There are several molecules evolving the same way as $C_5H_5O^+$, like $C_5H_9O_2^+$ or $C_4H_5O^+$ and others, in the case of Colombian coffee. Others, however, were generated in a different formation pathway. This is shown in Figure 4 for the VOCs $CH_3O_2^+$, $C_6H_9O_4^+$, CH_5O^+ for the same 12 min time-temperature roasting profile of Colombian coffee to a medium roast degree of Pt 103.

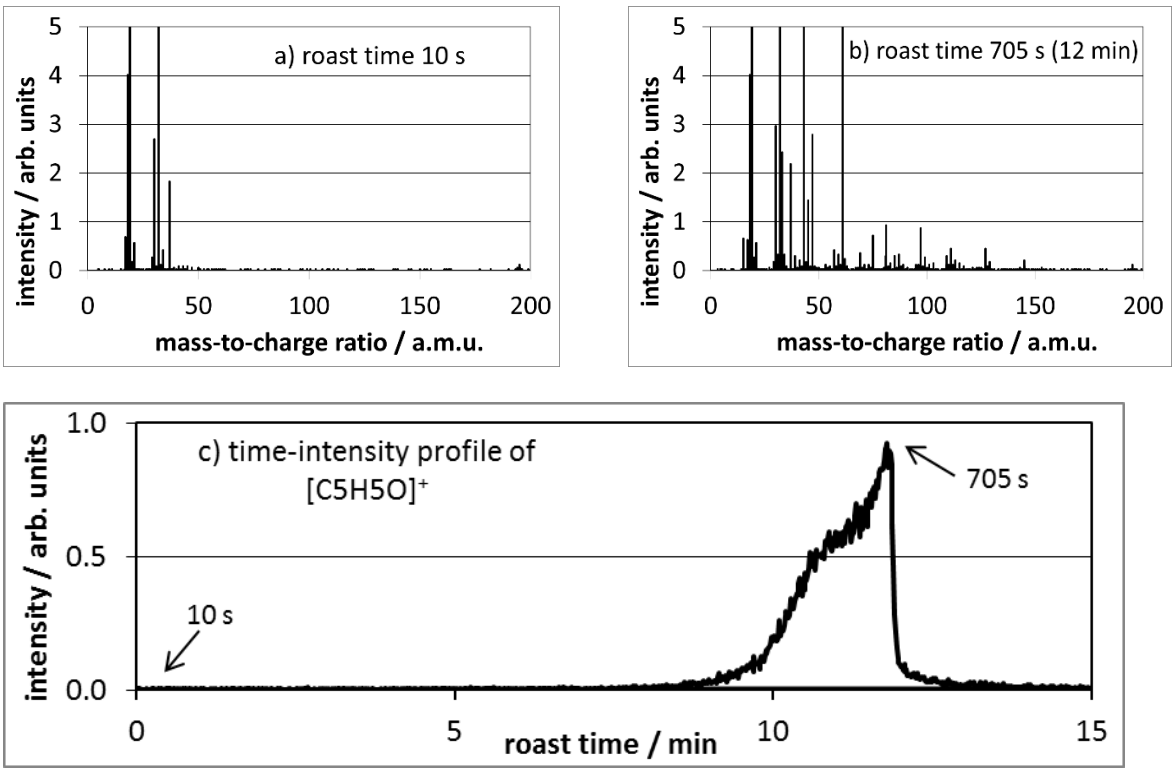

Figure 3. a) Mass spectrum at the very beginning of the roasting process at a roasting time of 10 s, b) mass spectrum at the maximum of the roasting process, after a roasting time of 705 s (12 min) and c) time-intensity profile of the VOC $C_5H_5O^+$.

The results in

Figure 3 and
Figure 4 refer to one coffee variety roasted along one specific time-temperature roasting profile.. It is well known, however, that the aroma of coffee changes when changing the time-temperature roasting profile. The analysis of the roasting process with PTR-ToF-MS can give insight in how these changes in the aroma are happening.

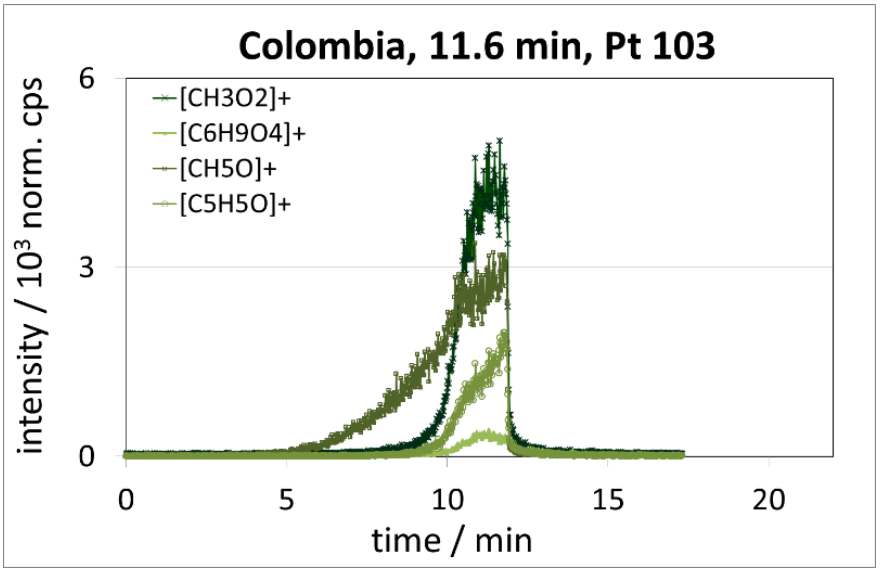

Figure 4. Time-intensity profiles of four different VOCs ($CH_3O_2^+$, $C_6H_9O_4^+$, CH_5O^+, $C_5H_5O^+$) during the time-temperature roasting profile of 11.6 min of Colombian coffee.

In Figure 5, e.g., the change in the formation pathway of CH3O2+, CH5O+ and C5H5O+ is shown when prolonging the time-temperature roasting profile from twelve to 21 minutes. In the case of the formation of CH3O2+, this leads to a second plateau in the early part of the time-intensity profile shortly after 10 minutes roasting time. Instead of a quite continuous formation of this VOC during the roasting in a 12 min profile, the formation is kind of split in two parts in a 21 min roasting profile (keeping in mind that both are leading to the same roast degree of Pt 103).

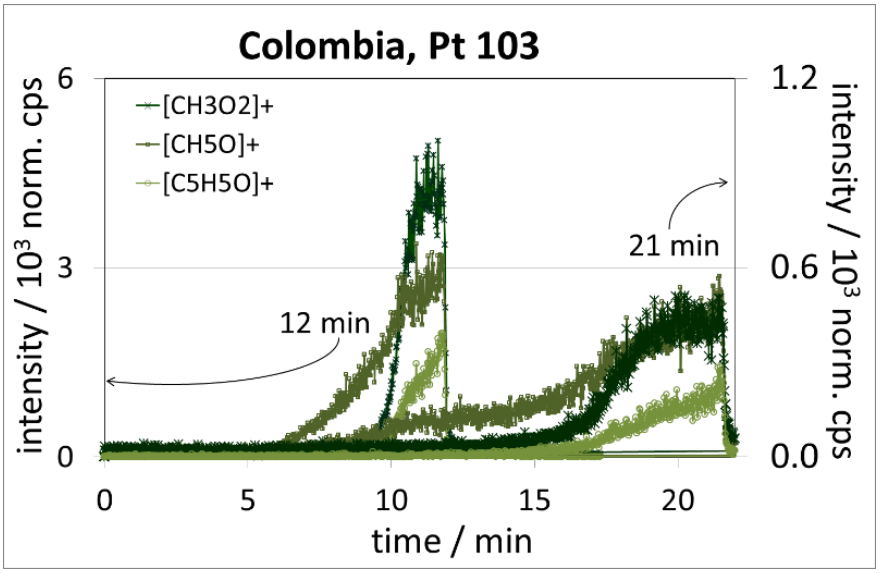

Figure 5. Time-intensity profiles of three different VOCs ($CH_3O_2^+$, CH_5O^+, $C_5H_5O^+$) during the time-temperature roasting profiles of 11.6 min and 21 min of Colombian coffee.

The analysis of different time-temperature roasting profiles of one coffee has shown that within one roasting profile, different VOCs are formed differently, and that changing the roasting profile leads to a change in the formation pathway of the VOCs. The question is, how the time-intensity profiles of the VOCs change when changing the type of coffee. For this, five different coffees were roasted along different time-temperature roasting profiles. The results are given in Figure 6a for one roasting profile and in Figure 6b for two roasting profiles, 12-13 minutes and 21 minutes. Whereas $C_5H_5O^+$ was formed along a more or less continuous time-intensity profile with a slight plateau in the case of the 12 min roasting of Colombian coffee, this VOC was formed, in the case of a 13 min roasting profile of Yirga Cheffe, continuously up to the first crack where the intensity reached a distinct plateau until the end of the roasting process. In the case of Malangsari coffee, the difference was even more pronounced: the formation of $C_5H_5O^+$ started at around the same time as in the case of Yirga Cheffe, but then the intensity rose continuously until the end of the roasting process, without any plateau in between. Comparing the time-intensity profile between Colombian and Guatemalan coffee, there were almost no differences in the formation pathway. As in the case of the roasting of Colombian coffee, the formation pathway changed when changing the time-temperature roasting profile, as given in Figure 6b.

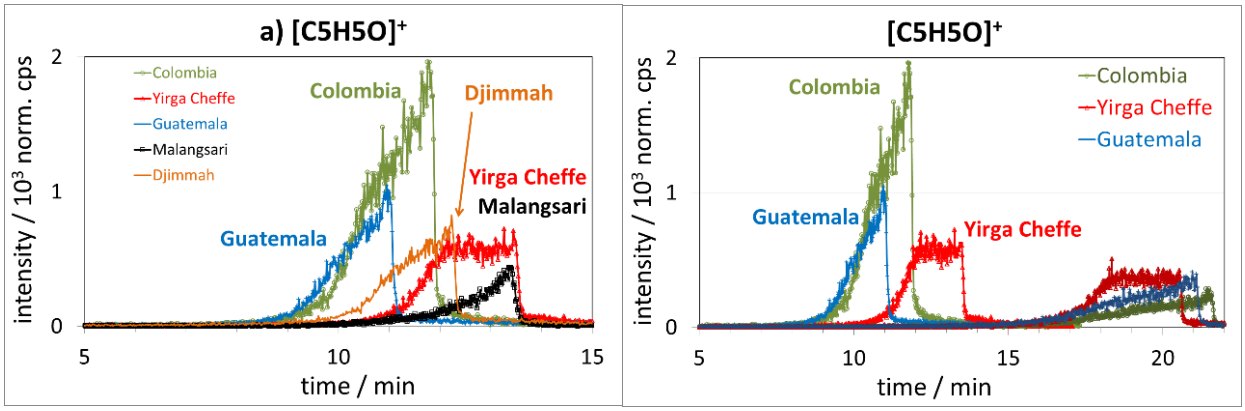

Figure 6. Time-intensity profiles of one VOC ($C_5H_5O^+$) during the time-temperature roasting profile of a) about 12 min (higher heating gas temperature, shorter roast time, medium roast degree) and b) about 12 min and 21 min (lower heating gas temperature, longer roast time, medium roast degree) for the different coffees Colombia, Yirga Cheffe, Djimmah (only 12 min), Guatemala and Malangsari (only 13 min).

This study revealed that during roasting of coffee, (i) different VOCs were formed differently within one time-temperature roasting profile, and (ii) that this formation pathway was altered by changing the roasting profile, and (iii) that the VOCs are formed differently when roasting different types of coffee. In addition, offline analysis of the roasted coffee was performed to get information about how these different formation pathways of the VOCs are reflected in the cup of coffee. In Figure 7, the total headspace intensity, corresponding approximately to the aroma intensity above a cup of coffee, is given for the roasting profile of 12-13 min (a) and 21 min (b) for the different coffees. Here, again, differences between different types of coffee are revealed: Malangsari had a significantly higher total headspace intensity in the 12 min roasting profile than Yirga Cheffe, Colombia and Guatemala, the latter two having almost the same total headspace intensity. Yirga Cheffe had the significantly lowest total headspace intensity. Going on to the prolonged roasting profile of 21 min, the total headspace intensity decreased slightly. Yirga Cheffe had the lowest total headspace intensity, again, and Colombia and Guatemala had similar values. Hence, prolonging the roasting profile from about 12 min to 21 min led to a decrease in aroma intensity above the cup of coffee, whereas the relative intensities between different types of coffee remained more or less the same.

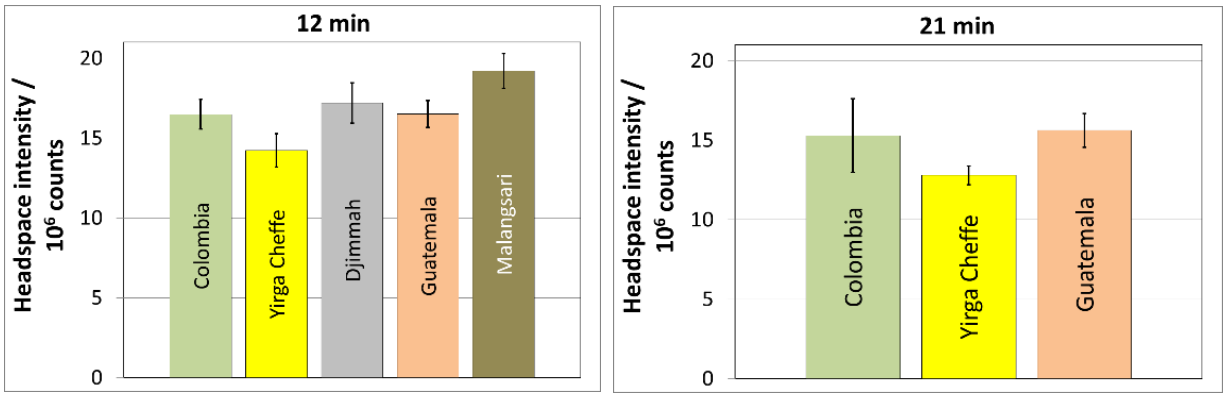

Figure 7. Total headspace intensity of 61 molecules analyzed with HS SPME GC/MS for a) the roasting profile of about 12 min and b) for the roasting profile of about 21 min for the following coffees: Colombia, Yirga Cheffe, Djimmah (only 13 min), Guatemala and Malangsari (only 13 min).

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Potential of Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFTS) for Detection of Adulteration in Roasted Coffees

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SUMMARY

The objective of this work was to evaluate the potential of Diffuse Reflectance Fourier Transform Infrared spectroscopy (DRIFTS) for simultaneous discrimination between roasted coffee and the following adulterants: spent coffee grounds, coffee husks, roasted corn and roasted barley. Coffee beans and adulterants were submitted to batch roasting in an oven at temperatures ranging from 200 to 270^{o}C. Roasting conditions were established based on color measurements in comparison to commercially available roasted coffee samples. Multivariate statistical analysis was performed in order to verify the possibility of discrimination between coffee and adulterants. Principal Components Analysis provided separation of the samples into four groups: coffee, coffee husks, spent coffee grounds and corn/barley. Classification models were developed based on Linear Discriminant Analysis and recognition and prediction abilities of these models were 100%, with the samples being separated into six groups: pure coffee, adulterated coffee, spent coffee grounds, coffee husks, corn and barley. Such results confirm that DRIFTS presents potential for the development of an analytical methodology for detection of adulteration in roasted and ground coffee.

INTRODUCTION

Ground roasted coffee is quite vulnerable to adulteration because it presents physical characteristics (particle size, texture and color) that are easily reproduced by roasting and grinding a variety of biological materials. Thus, a wide variety of spurious materials, such as twigs, coffee berry skin and parchment, spent coffee, roasted barley, maize, cocoa, soybean and others, have been reported in the literature to be commonly used in fraudulent admixtures with coffee and a few analytical methodologies have been developed for their detection in roasted and ground coffee. Some studies have established suitable parameters and markers for detection of adulterants in ground roasted coffee and instant or soluble coffee, but most of the developed methodologies are based on chromatographic methods. Although effective, such methodologies are time demanding, expensive and involve a considerable amount of manual work, and thus are not appropriate for routine analysis. Furthermore, they do not allow for simultaneous discrimination of two or more adulterants in a given sample.

The need for new and rapid analytical methods in the field of food adulteration has prompted extensive research on spectroscopic methods, including Fourier Transform Infrared Spectroscopy (FTIR). Spectroscopic methods are either based on transmittance or reflectance readings, with reflectance-based methods being more commonly employed as routine methodologies for food analysis, since they require none or very little sample pre-treatment. FTIR reflectance methods can be divided into Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFTS) and Attenuated Total Reflectance Fourier Transform Infrared

Spectroscopy (ATR-FTIR). While ATR collects information from the sample surface, DRIFTS provides information from the entire sample, being a combination of internal and external reflection. In general ATR-FTIR is more appropriate for analysis of liquid samples, while DRIFTS is commonly employed for analysis of solids. A few studies have employed DRIFTS for coffee analysis, and the specific applications were discrimination between Arabica and Robusta varieties, detection of glucose, starch or chicory as adulterants of freeze-dried instant coffees, separation between decaffeinated and regular roasted coffees and discrimination between high and low quality coffees before and after roasting. Given the need for establishing a reliable and fast methodology for dectection of adulteration in coffee, the objective of this work was to evaluate the potential of DRIFTS for simultaneous discrimination between roasted coffee and commonly employed adulterants such as spent coffee grounds, roasted corn, roasted barley and roasted coffee husks.

MATERIALS AND METHODS

Green Arabica coffee, barley and corn samples were acquired from local markets. Coffee husks were provided by Minas Gerais State Coffee Industry Union (Sindicato da Indústria de Café do Estado de Minas Gerais, Brazil). Spent coffee grounds were provided by a local soluble coffee industry (Café Brasília).

Coffee beans, coffee husks and corn samples (30 g) were submitted to roasting in a convection oven (Model 4201D Nova Ética, São Paulo, Brazil). After roasting, the samples were ground (D < 0.5 mm) and submitted to color evaluation. Color measurements were performed using a tristimulus L*a*b* colorimeter (HunterLab Colorflex 45/0 Spectrophotometer, Hunter Laboratories, VA, USA) with standard illumination D65 and colorimetric normal observer angle of 10^{o}. Previous studies have shown that roasting degree will be dependent on the type of sample and on the roasting temperature [1]. Preliminary tests showed that it would take higher temperatures (over 240 °C or 250 °C) to promote significant color changes so corn or barley could be considered roasted to degrees comparable to those for coffee. Roasting of coffee husks, on the other hand, required milder temperatures. Therefore, roasting conditions were established for each specific type of sample. Roasting degrees were defined according to luminosity (L*) measurements similar to commercially available coffee samples (19.0 < L*< 25.0), corresponding to light (23.5 < L*< 25.0), medium (21.0 < L*< 23.5) and dark (19.0 < L*< 21.0) roasts. The corresponding roasting times and temperatures are displayed in Table 1.

Spectra were collected from a Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan) with a DLATGS (Deuterated Triglycine Sulfate Doped with L-Alanine) detector at room temperature (20 ± 0.5 °C). Diffuse reflectance (DR) measurements were performed in diffuse reflection mode with a Shimadzu sampling accessory (DRS8000A). Each roasted and ground sample (D < 0.15 mm) was mixed with KBr (10g/100g) and then 23 mg of this mixture was placed inside the sample port. All spectra were recorded within a range of 4000–400 cm^{-1} with 4 cm^{-1} resolution and 20 scans, and submitted to background (pure KBr) subtraction. The spectra were also truncated to 2500 data points in the range of 3200-700 cm^{-1}, in order to eliminate noise readings present in the upper and lower ends of the spectra. The following data spectra pretreatment techniques were tested: (0) no additional processing (raw data), (1) mean centering, (2) normalization, (3) baseline correction employing two (3200 and 700 cm^{-1}) or three (3200, 2000 and 700 cm^{-1}) points, (4) first derivatives and (5) second derivatives.

Table 1. Roasting parameters and conditions.

Roasting temperature	Luminosity values (Roasting time)		
Coffee			
	Light Roast	**Medium Roast**	**Dark Roast**
200°C	24.28±0.02 (40 min)	21.48±0.08 (70 min)	19.62±0.37 (90 min)
220°C	23.18±0.12 (20 min)	21.51±0.01 (22 min)	19.96±0.13 (25 min)
240°C	25.17±0.04 (11 min)	22.01±0.33 (13 min)	19.89±0.08 (15 min)
Coffee husks			
	Light Roast	**Medium Roast**	**Dark Roast**
200°C	22.22±0.05 (20 min)	21.66±0.15 (30 min)	20.16±0.12 (50 min)
220°C	23.00±0.06 (10 min)	20.41±0.30 (13 min)	19.88±0.13 (15 min)
240°C	25.16±0.04 (6 min)	21.34±0.17 (7 min)	20.47±0.06 (9 min)
Corn			
	Light Roast	**Medium Roast**	**Dark Roast**
240°C	24.45±0.21 (30 min)	22.01±0.33 (35 min)	19.89±0.08 (40 min)
250°C	24.63±0.26 (15 min)	22.17±0.08 (17 min)	19.33±0.07 (19 min)
260°C	22.25±0.06 (11 min)	21.10±0.16 (12 min)	19.26±0.10 (13 min)
Barley			
	Light Roast	**Medium Roast**	**Dark Roast**
250°C	24.07± 0.13 (30 min)	22.59±0.39 (35 min)	21.16±0.21 (40 min)
260°C	24.31± 0.45 (15 min)	22.06± 0.56 (17 min)	21.72±0.3 (19 min)
270°C	25.44± 0.12(11 min)	-	20.37 ± 0.33 (13 min)

Using the DR spectra (raw or normalized) and its derivatives as chemical descriptors, pattern recognition (PR) methods (PCA and LDA) were applied in order to establish whether adulterants could be discriminated from roasted coffee samples. LDA model variable selection was based on the data that presented higher influence on group separation (high loading values) from the PCA analysis. The statistical package XLSTAT Sensory 2010 (Addinsoft, New York) was employed for all the chemometric calculations.

RESULTS AND DISCUSSION

Average normalized spectra obtained for roasted coffee, spent coffee grounds, roasted coffee husks, roasted corn and roasted barley are shown in Figure 1. Two sharp bands at 2916 and 2840 cm-1 can be clearly seen in the spectrum corresponding to roasted coffee. Such bands have been previously reported present in spectra of roasted Arabica and Robusta coffee samples and also of crude coffee samples, with the band at 2829 cm−1 attributed to stretching of C–H bonds of methyl (–CH3) groups, and employed as a reference for quantitative analysis of caffeine in soft drinks. Similar bands can be viewed in the spectra obtained for roasted coffee husks, roasted corn and roasted barley. Coffee husks have been reported to present similar levels of caffeine (~1% dry basis) in comparison to coffee beans, whereas corn and barley do not contain any caffeine. However, FTIR studies on corn and corn flour have also reported two bands at 2927-2925 and 2855 cm-1, respectively attributed to asymmetric and symmetric C-H stretching in lipids. Although the samples in those studies were not submitted to roasting, the lipids content is not expected to vary during roasting of corn (or barley for that matter), as it is known to occur with coffee, and the peaks assignment to C–H stretching in lipids might still be valid. Therefore, such bands may be affected by both caffeine and lipids levels in the case of coffee, and are most likely primarily associated to caffeine in the case of

coffee husks and only to lipids in the cases of roasted corn, roasted barley and spent coffee. It is noteworthy to point out that the majority of the caffeine present in coffee is extracted during soluble coffee production, whereas the lipid fraction is partially extracted, hence, leading to spent coffee grounds virtually devoid of caffeine but still containing some lipids.

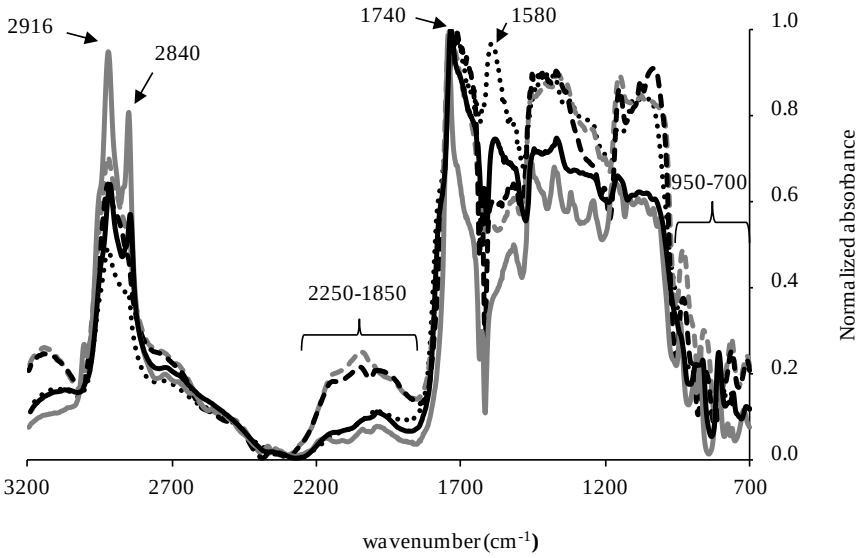

Figure 1. Average normalized diffuse reflectance spectra obtained for roasted coffee (———), roasted coffee husks (.....), roasted corn (– –), roasted barley (– –) and spent coffee grounds (—).

Sharp bands at 1743-1740 cm-1 are evident in the coffee, corn and spent coffee spectra. Such bands have been reported in FTIR studies of roasted coffee and are attributed to carbonyl (C=O) vibration in esters (triglycerides). Such literature reports and the fact that these bands are rather weak in the spectra obtained for roasted coffee husks and barley (low lipid content) are strong indications that it can be associated to lipid concentration. Several bands can be viewed in all the spectra in the range of 1700 to 700 cm-1. Many substances that naturally occur in coffee are reported to present absorbance bands in this range, the 'double bond region' as classified in accordance with the spectra segmentation presented by Stuart. For example, Ribeiro et al. performed DRIFTS analysis of roasted coffees and observed lower absorbance of decaffeinated samples in the range of 1700 to 1600 cm-1. The band at 1659-1655 cm-1 has been consistently used as a chemical descriptor of caffeine in FTIR spectroscopic detection and quantification of caffeine in coffee extract samples. Another substance that can be associated to peaks in this range is trigonelline, a pyridine that has been reported to present several bands in the range of 1650-1400 cm-1, and is present in both crude and roasted coffee. Some of the bands in this range may be attributed to axial deformation of C=C and C=N bonds in the aromatic ring of trigonelline. Furthermore, sharp bands can be observed at 1585-1575 cm-1 for the spectra of coffee and coffee husks and they may be both attributed to the presence of non-degraded trigonelline and nicotinic acid (one of trigonelline major degradation products upon roasting). The spectrum for spent coffee does not present a pronounced band in this region and this can be attributed to the fact that, during production of soluble coffee, trigonelline and nicotinic acid are extracted for they are both quite soluble in water. No reports were found on the presence of these types of compounds in both corn and barley, thus, corroborating the assignment of the peaks at 1585-1575 cm-1 to trigonelline and its degradation products. The wavenumber range of 1400 to 900 cm-1 is characterized by vibrations of several types of bonds such as C–H, C–O, C–N and P–O. Chlorogenic acids, a family of esters formed between quinic acid and one to four residues of caffeic, p-coumaric

and ferulic acids, present strong absorption in the region of 1450 to 1000 cm-1. Carbohydrates also exhibit several absorption bands in the 1500 to 700 cm-1 region, so it is expected that this class of compounds will contribute to many of the observed bands.

Using the DR spectra as chemical descriptors, pattern recognition (PR) methods (principal components analysis – PCA and linear discriminant analysis - LDA) were applied in order to establish whether the adulterants could be discriminated from roasted coffee. Data matrices were assembled so that each row corresponded to a sample and each column represented the spectra datum at a given wavenumber, after processing as previously described. The spectra pretreatment steps that provided a satisfactory level of simultaneous discrimination between roasted coffee and all adulterants simultaneously were the following: no additional treatment of raw data, normalization with three point baseline correction and first derivatives. The corresponding scatter plots obtained after PCA analysis are displayed in Figure 2.

Sample grouping can be observed in all the plots displayed in Figure 2, even though separation is more evident for the spectra submitted to normalization and first derivatives (Figs. 2b and 2c). Roasted barley and roasted corn were not effectively separated, regardless of the spectra pretreatment employed. Evaluation of the loadings plots obtained after PCA analysis of the normalized spectra indicated that the spectral ranges that presented the highest influence on PC1 values in association with coffee and spent coffee grounds (negative PC1) were the following: 3018-3000, 2970-2900, 2875-2830, 2422-2355, 1753-1741, 883-870 and 822-800 cm-1. The highest influence on PC1 values in association with roasted corn and barley (positive PC1) was observed in the following ranges: 3200-3090, 2270-1820, 1732-1674, 1480-1270, 1164-974, 934-904, 862-830 and 796-700 cm-1.

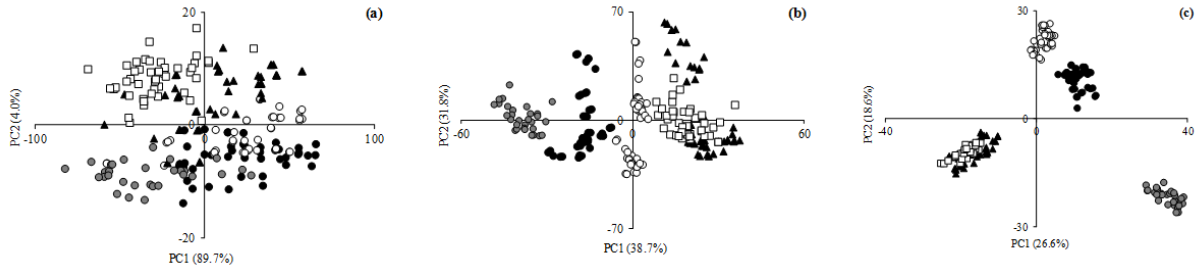

Figure 2. PCA scores scatter plot (PC1 vs. PC2) based on diffuse reflectance spectra (3100 - 700 cm-¹) of roasted coffee in comparison to adulterants after the following pretreatment steps: (a) no treatment; (b) normalization and baseline correction; (c) first derivatives (● coffee; ○ coffee husks; ● spent coffee grounds; □ barley; ♦ corn).

The satisfactory results obtained from the principal components analysis indicate that the data could provide enough information to develop classification models for roasted coffee and each specific roasted contaminant. Therefore, linear discriminant analysis (LDA) was employed in order to obtain classification models for pure coffee, its adulterants and adulterated coffee (total adulteration levels ranging from 66 to 1% of one or more adulterants). Model validation was performed using 30% of the samples as the evaluation set. Recognition ability was calculated as the percentage of members of the calibration set that were correctly classified, and prediction ability was calculated as the percentage of members of the evaluation set that were correctly classified. The corresponding score plots obtained for the first three discriminant functions of the developed models are shown in Figure 3. A clear separation of all groups (pure coffee, adulterated coffee, coffee husks, spent coffee grounds, corn and barley) can be observed for all models, that presented 100% recognition and prediction abilities. Altough some overlapping between corn and barley is observed, given

that the main goal is to discriminate pure and adulterated coffee, an evaluation of plots shown in Figure 3 shows that, for the model based on first derivatives, the third discriminant function alone provides separation between pure and adulterated.

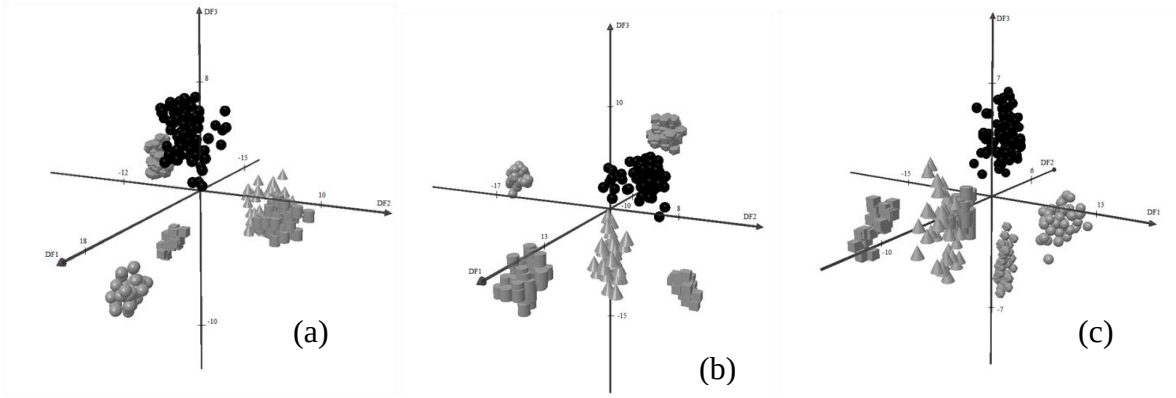

Figure 3. Scores of the discriminant functions provided by the LDA models of diffuse reflectance spectra (3100 - 700 cm^{-1}) after the following pretreatment steps: (a) no treatment; (b) normalization and baseline correction; (c) first derivatives (pure coffee; adulterated coffee; coffee husks; spent coffee grounds; barley; corn).

CONCLUSIONS

The potential of DRIFTS for simultaneous discrimination between roasted coffee and common adulterants was evaluated. PCA provided separation of the samples into four groups: coffee, coffee husks, spent coffee grounds and corn/barley. Classification models were developed based on Linear Discriminant Analysis and recognition and prediction abilities of these models were 100%, with the samples being separated into six groups: pure coffee, adulterated coffee, spent coffee grounds, coffee husks, corn and barley. Such results confirm the potential of DRIFTS for detection of adulteration in roasted and ground coffee.

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Oleosomes in Defective Arabica Beans

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SUMMARY

Coffee defects can be mainly identified by observing the colour of silverskin and/or that of the bean endosperm. Black beans result from dead beans or beans that fall naturally on the ground; sour beans are often associated with 'over-fermentation' during wet processing. According to the standard ISO 10470:2004, black beans, immature and sour beans are defects included in the category of beans with irregular appearance, known to affect beverage quality. Defective beans, however, can represent a source of valuable chemicals or raw materials and then the cell content of these beans is an interesting topic to be studied. Coffee oil extraction from defective beans has been suggested as possible process to obtain chemicals and energy through biodiesel conversion. The objective of this work was to characterize the oil bodies of defective beans and to carry out a comparison with normal beans both dry and wet processed.

Transversal sections of 1-2 mm of brazilian black, sour, immature and normal beans were pre-fixed in glutaraldehyde in phosphate buffer. Portions of these sections, that represent only the external endosperm, are subsequently fixed in the same solution. After a post-fixation in osmium tetroxide, samples were dehydrated with increasing acetone series and included in Spurr's resin (Spurr, 1969). Then, they were cut to obtain ultrathin sections, treated with standard double contrasting stain and examined by TEM. The oil fraction was isolated by a standard Soxhlet extraction.

Other seeds, fixed in formaldehyde solution for almost 3 weeks, were cut with a cryostate to obtain frozen section, stained with some histochemical techniques and examined by a standard optical microscope.

The morphological aspect of immature seeds was similar to normal seeds, with small oil bodies at the cell periphery. Black beans were different from the others, with their very hard cell walls and the presence of mold. Oil bodies were inside the more internal endosperm cells, sometimes completely fused. In sour beans, there are bigger oil drops and an unknown protein matrix, probably a consequence of the fermentation process. Oil bodies diameter and the content oil of defective beans lie in the range of normal arabica seeds, even if there are slight differences among the distribution of oil bodies diameter classes.

INTRODUCTION

Defects is a term used in commercial practice to describe the quality factor of the amounts of defective beans present and of extraneous matter. Producing countries have their own criteria for the classification of coffee but the ISO (International Organization for Standardization) has produced in the past Standard ISO 10470:2004, a defect reference chart for green coffee. According to ISO, black, immature and sour beans are defects that belong to the category of beans with irregular visual appearance, known to affect the beverage quality. Black beans

result from dead beans or beans that fall naturally on the ground, related to the fermented or stinker flavour; immature beans are unripe seeds responsible for the astringent or metallic flavour and sour beans are often associated with 'over-fermentation' during wet processing, that produce fermented flavour.

Approximately 20% (0,5 million tons per year) of the Brazilian coffee production consists of low quality defective beans, which are commercialized in the internal market in Brazil, being used by the roasting industry in blends with non defective ones. The extraction of oil would be an alternative to the use of lower quality coffee. Coffee oil extraction from defective beans has been suggested as possible process to obtain chemicals, used in cosmetic formulations or in food and pharmaceutical applications and energy through biodiesel conversion. An interesting alternative is the use of defective coffee press cakes, a residue from coffee oil biodiesel production, that was evaluated as an adsorbent for removal of basic dyes from aqueous solutions or a good source to obtain polyphenols and chlorogenic acids, known for their anti-oxidant activity.

Lipids in coffee are in the form of oleosomes or oil bodies functioning as an energy reserve for the germination. Oil bodies are isolated in the cytoplasm of cells and form masses close to the cell walls; they originate from the endoplasmic reticulum and consist of tryacylglicerols surrounded by a phospholipids monolayer. Proteins, called oleosins, are partially submerged in this monolayer, preventing the fusion between oil bodies. In a previous work, we discover slight differences about oil content among arabica samples from different geographical origin related with the size of oleosomes.

The objective of this work is to deepen the aspect of content cells and, in particular, of coffee oil in some brazilian defective samples (black, sour and immature), in comparison with normal beans with the same origin.

MATERIALS AND METHODS

Transversal sections of five seeds per type (normal Arabica both dry and wet processed, black, immature and sour arabica) were pre-fixed in a glutaraldehyde solution. Small portions (1-2 mm) of these sections that represent only the external endosperm were fixed overnight in the same solution. After a post-fixation in Osmium tetroxide, samples were dehydrated with an increasing acetone series and embedded in Spurr's resin. Samples were cut with an ultramicrotome (Leica Ultracut) to obtain thin and ultrathin sections, treated respectively with a Toluidin Blue O solution and the standard double contrasting stain. Sections were respectively examined with an optical microscope Leica Leitz DMRXE and with an electron microscope Philips EM 208 at 100 kV. Measures of oil bodies diameter were obtained using the program TESI Imaging µImage, at the same magnification (5,6 Kx). Other ten seeds per defective beans type were fixed in a formaldehyde solution and cut with a cryostate 2800 Reichert Jung. Frozen sections (10-12 mm) were stained with some histochemical techniques (TBO, PAS, UV-schiff) to highlight the content cells. The oil content was determined by gravimetry after oil isolation by means Soxhlet extraction.

RESULTS AND CONCLUSIONS

Oil bodies in seeds are normally spherical, generally pressed by other cell components, at the periphery of the cell, inside the cytoplasm. Arabica samples wet processed, more common in the global market, preserve this typical aspect of oil bodies (0.3-2.3 µm minimum and maximum diameter, oil content 12-17% dry weight) with endosperm cells rich in lipids (Fig.2b). Some cells are characterize by the presence of glyoxysomes (Figure 2d), that

mediate the lipids degradation inside the cells. Samples dry processed (Figure 2e, f) have cells that have suffered for the drying: they are partially empty or with an evident separation of plasma membrane from the cell wall. Oil bodies are apparently smaller but they preserve their global shape, thanks to the presence of oleosins on the membrane.

Black samples (0.4-1.3 µm minimum and maximum diameter, 13.5% oil content) are dead beans that have cells attached by mold and yeast: the consequence is that cells are partially destroyed at the epidermal and subepidermal layers; the endosperm is stiffer and the cell content seems to be different from the normal bean: vacuoles are contracted and merged together, as highlighted with TBO and UV-schiff stains (Figure 3e, f). Although oil bodies remain in their spherical form, when not fused (Figure 3c) and they are more variable in size diameter, generally bigger than the others (Figure 1).

The endosperm cells of immature beans (0.2-1 µm minimum and maximum diameter, 12.2% oil content) are younger, with thinner cell walls and bigger protein vacuoles, observed in yellow with a UV-Schiff stain (Figure 4f); the curve of distribution of oil bodies diameter classes is shifted to the left, towards smaller diameter than those of a ripe seed (Figure 1; 4c, d).
Sour beans (14.9% content oil) are apparently not so different from a normal bean, but at the microscopic level, cell content has an aspect completely different, with unknown spongy vacuoles stain in blue-violet and oil bodies (in grey, fig. 5 a, b) completely fused to form oil drops (> 2.5 µm in diameter) or large vacuoles (Figure 5 c, d), maybe a consequence of enzymatic activities during the fermentation. Oil contents in defective beans lies in the range of normal arabica seeds and apparently it makes no remarkable differences among the oil bodies size diameter, even if they show a slight different distribution in classes.

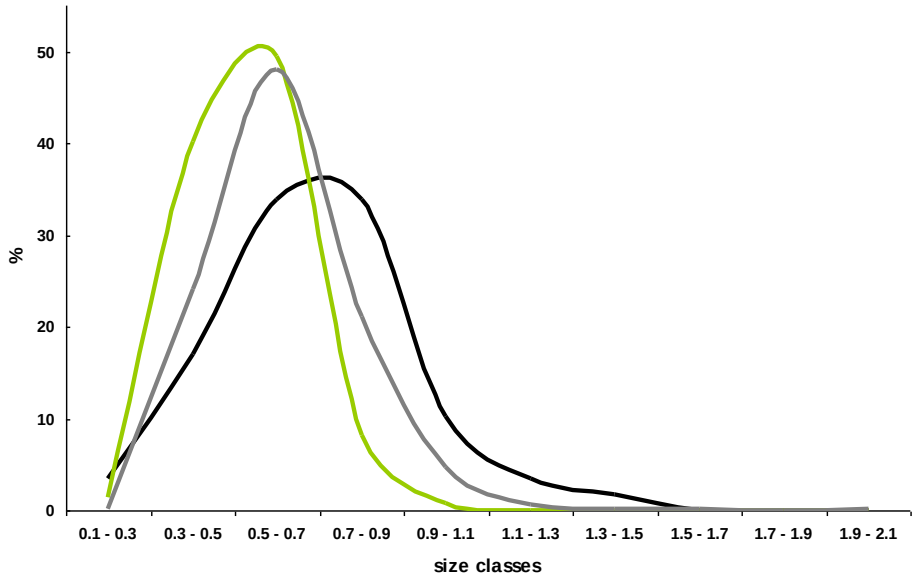

Figure 1. Distribution of oil bodies diameter classes (µm) of non defective (grey), black (black) and immature (green) samples in relation with their percentage per each class.

According to previous study, there are no significant differences in the amounts of total fatty acids between defects and non defective beans. However the observations made on the endosperm cells show remarkable morphological differences of the oily component, especially in sour samples, as a consequence of a strong fermentation. These differences may reflect the variation of minor lipid components (unsaponifiable fraction) not yet studied as far as we know.

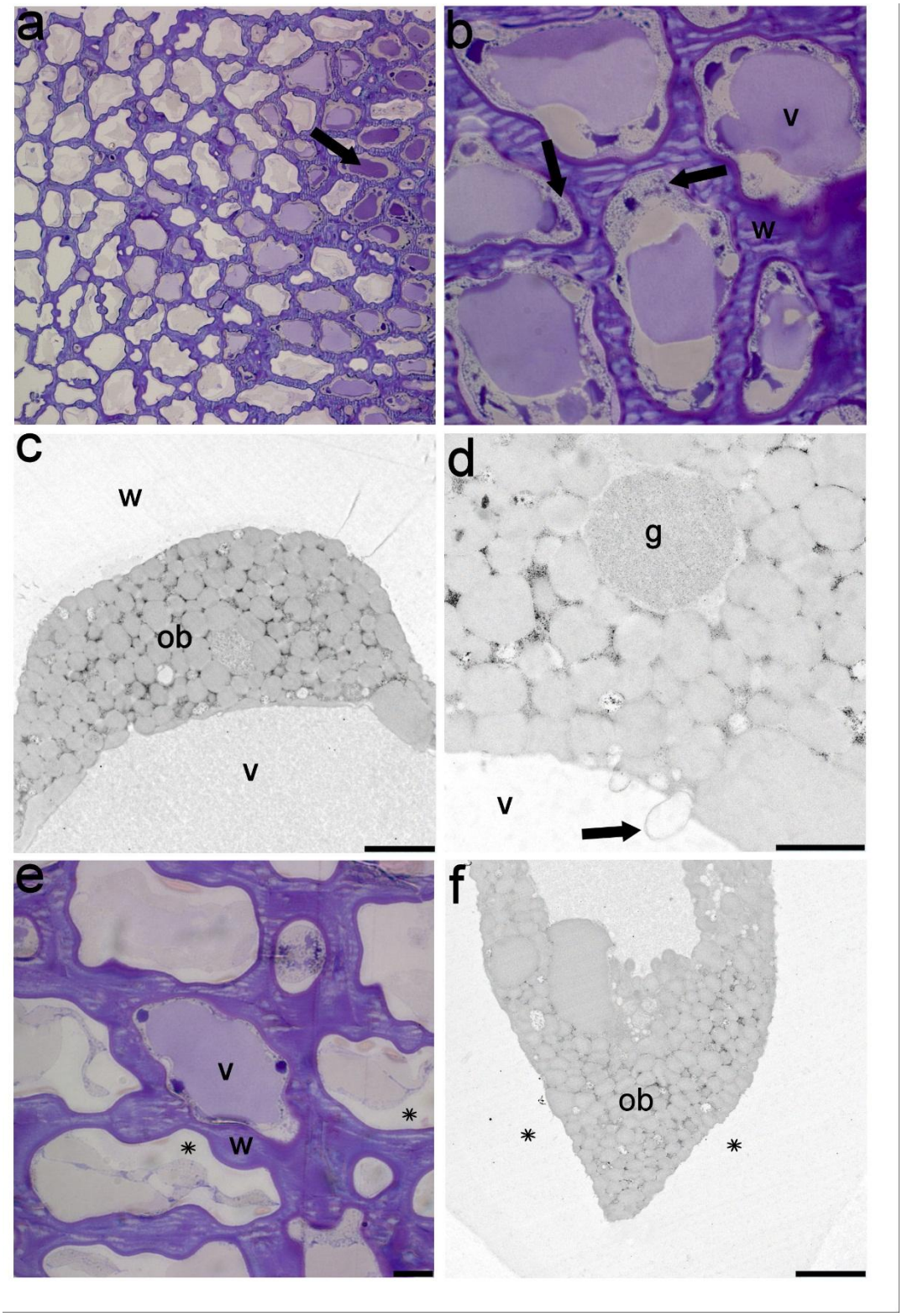

Figure 2. Non-Defective Beans; a, b: transversal sections of endosperm in a sample wet processed (TBO, bars: 50 and 10 µm), portion of external endosperm rich on oil bodies (arrows); c, d: ultrathin sections of an endosperm cell (TEM, bars: 2 and 1 µm), presence of lipid vesicles in degradation (arrow); e, f: thin and ultrathin section in a sample dry processed. w, cell wall; ob, oil bodies; v, vacuole; g: glyoxysome; *: empty zones.

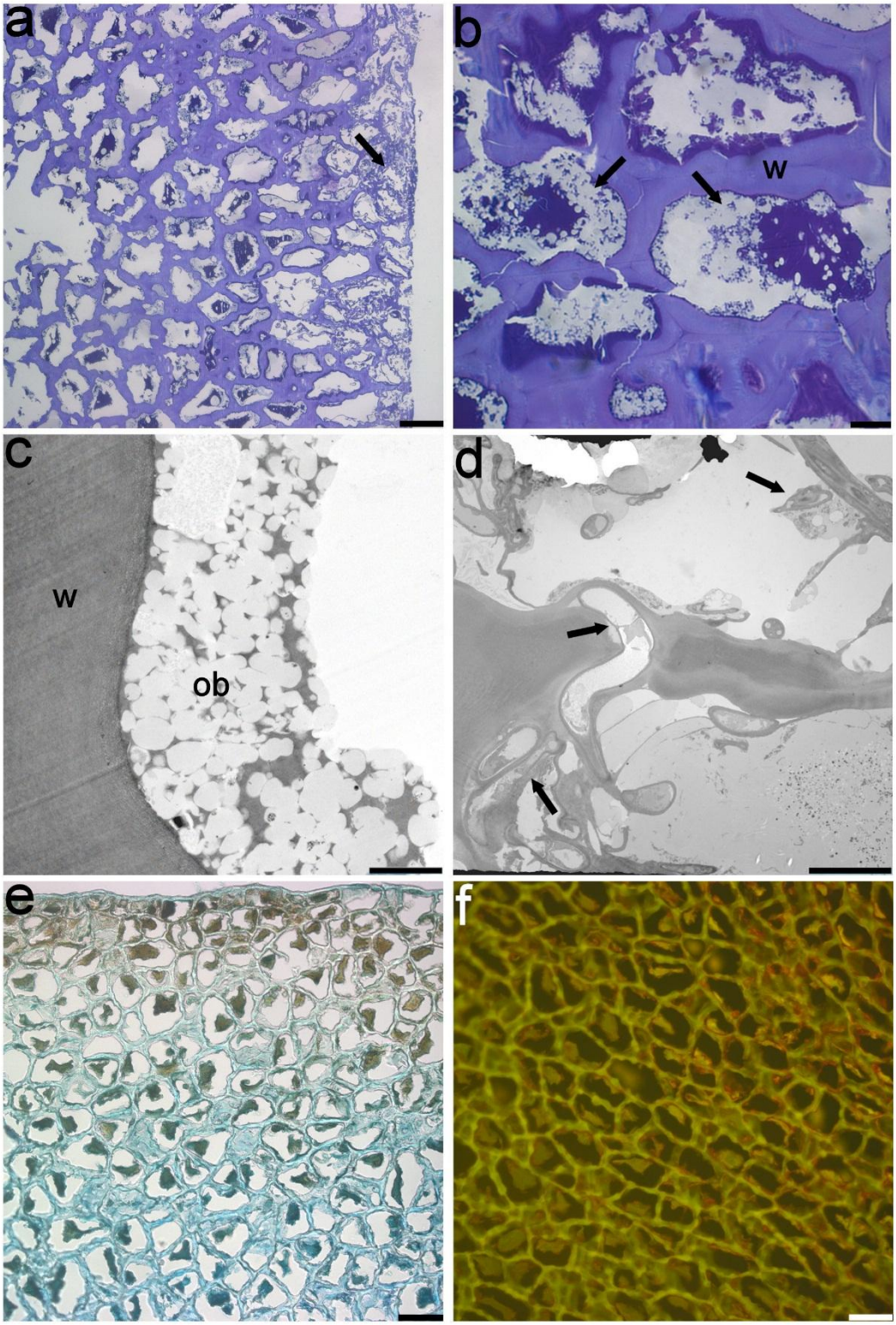

Figure 3. Black Beans; a, b: thin sections of endosperm (TBO, e, bars: 50 and 10 µm), a: bacterial and mold contamination (arrow); b: oil bodies and fused bodies (arrows); c, d: ultrathin sections of an endosperm cell (TEM, bars: 2 and 10 µm), presence of hyphae that cross the cell wall (arrows). E, f: transversal section of seed stain in TBO and UV-schiff (bars: 50 µm). w, cell wall; ob, oil bodies.

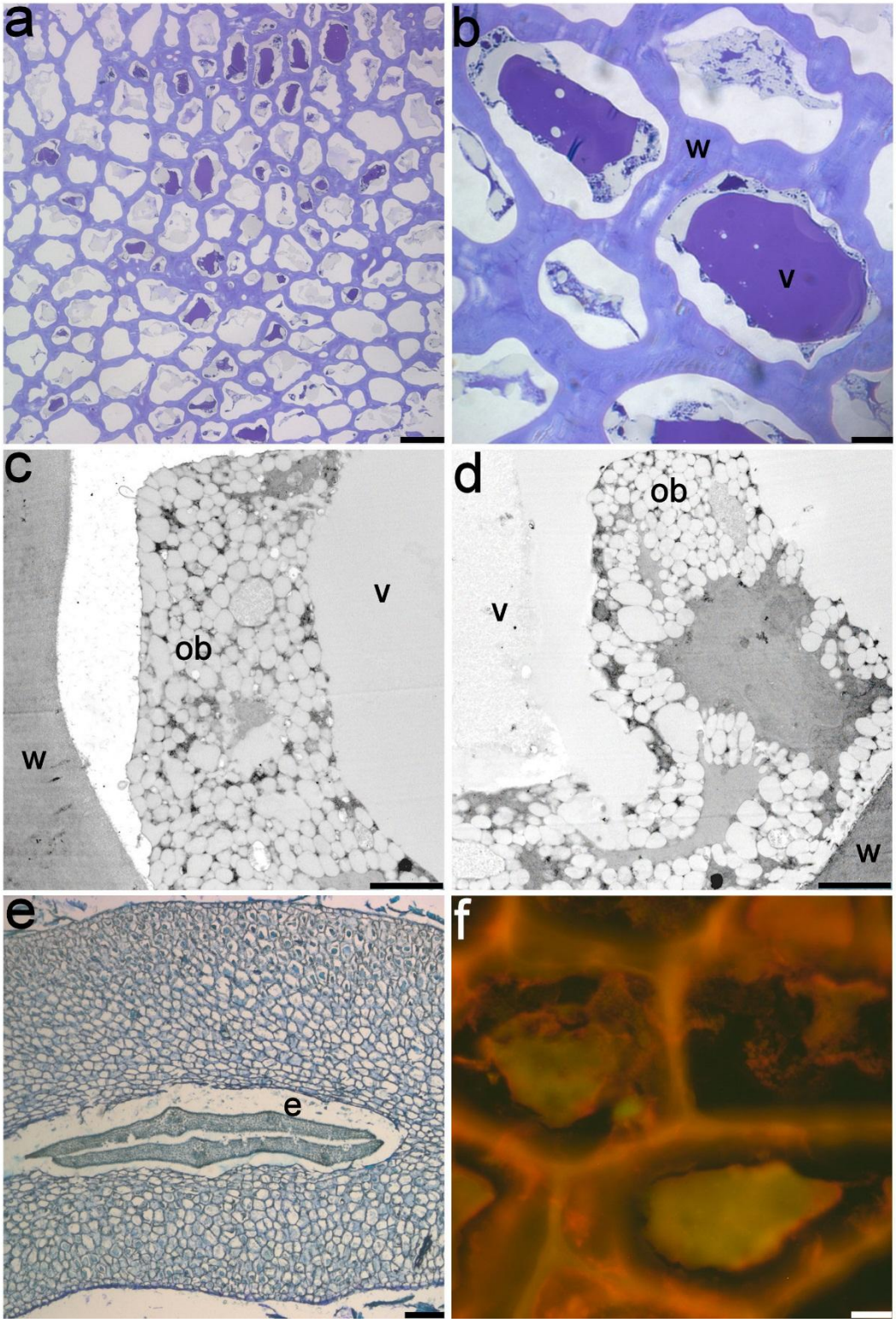

Figure 4. Immature Beans; a, b: thin sections of endosperm (TBO, e, bars: 50 and 10 µm); c, d: ultrathin sections of an endosperm cell (TEM, bars: 2 µm), presence of smaller oil bodies (ob); e, f: transversal section of seed stained in TBO (e) and UV-schiff (f) (bars: 200 and 10 µm). w, cell wall; ob, oil bodies; v, vacuole; e, embryo.

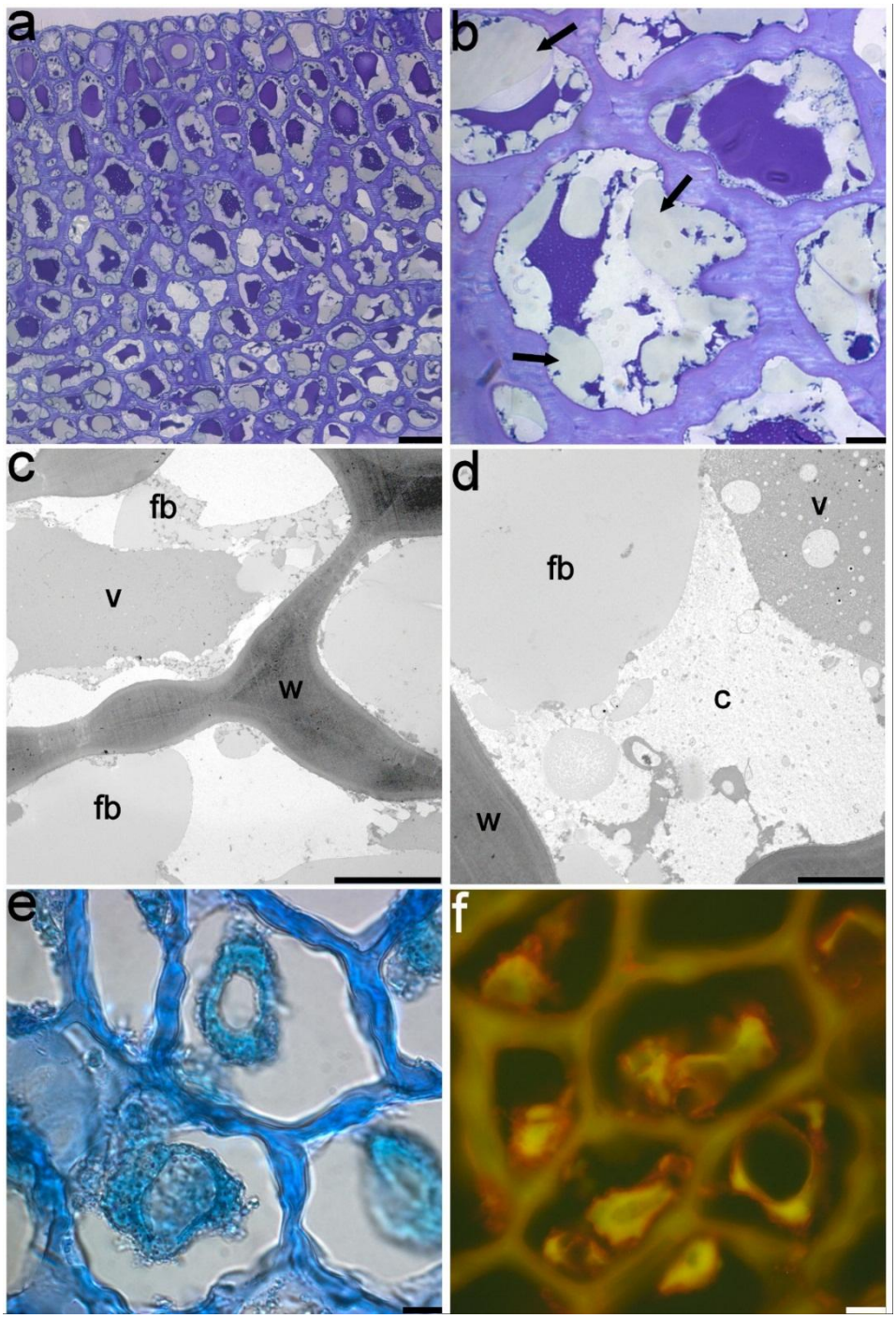

Figure 5. SOUR BEANS; a, b: thin sections of endosperm (TBO, e, bars: 50 and 10 µm); c, d: ultrathin sections of an endosperm cell (TEM, bars: 10 and 5 µm), presence of oil drops derived from fused oil bodies (fb); e, f: transversal section of cells stained in TBO (e) and UV-schiff (f) (bars: 10 µm). w, cell wall; fb, fused oil bodies; v, spongy vacuole; c, cytoplasm.

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Identification of Phenolic and Carotenoid Compounds in Coffee (*Coffea Arabica*) Pulp, Peels and Mucilage by HPLC Electrospray Ionization Mass Spectrometry

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SUMMARY

Coffee pulp, peels, and mucilage are by-products accruing from wet coffee processing. Like coffee seeds, these fruit tissues contain substantial amounts of polyphenols and other bioactives. In the present study, polyphenols and carotenoids were identified separately in different tissues (pulp and peels) of coffee cherries from five coffee varieties planted in Costa Rica, and in the mucilage obtained after commercial coffee cherry depulping. Peels and pulp of each fruit were manually separated and pooled. After extraction, polyphenols and carotenoids were characterized by high-performance liquid chromatography (HPLC) coupled to mass spectrometry. Most polyphenols detected were chlorogenic acids (CGAs), such as isomeric caffeoylquinic acid (CQA), feruloylquinic acids (FQA), and p-coumaroylquinic acids. Differences in the concentration of several phenolics were found among coffee varieties and by-products (pulp and peels). Other phenolic compounds, such as epicatechin and proanthocyanidin B, were also detected. Besides typical accessory carotenoids of chloroplasts, like violaxanthin, neoxanthin, lutein, β-carotene, α-carotene and various lutein esters were also identified.

INTRODUCTION

Phenolic compounds are ubiquitous constituents of higher plants. While their occurrence at high levels as well as their qualitative composition in coffee seeds has been investigated in detail, knowledge about their presence in coffee by-products (peels, pulp, and mucilage) is scarce. Representing more than 50% of the coffee fruits, coffee processing generates a significant amount of these by-products, which are considered as "waste" material by the coffee processors. Identification and detailed knowledge on the amounts of these valuable bioactives might foster the utilization of coffee by-products for nutritional and pharmaceutical purposes.

In contrast to dry coffee processing, the wet procedure usually allows the recovery of non-degraded peels and pulp. As previously reviewed in detail, a mucilage fraction may also be obtained when remnants of pulp and mucilage are separated from the coffee beans by mechanical scrubbing instead of degradation by controlled fermentation. These coffee processing by-products were previously shown to contain several isomers of CQA and di-CQA, epicatechin and FQAs, being considered as potent antioxidants. Furthermore, various

anthocyanins, such as cyanidin-3-rutinoside and cyanidin-3-glucoside, have more recently been described.

The chemical composition of coffee beans varies depending on the genotype, which also seems to affect the composition of other fruit parts. To the best of our knowledge, investigations on compositional pattern of coffee by-products from different genotypes cultivated on the same site are lacking, except for one report by Clifford and Ramírez-Martínez. Therefore, a characterization of polyphenols and carotenoids in peels, pulp and mucilage of five Costa Rican coffee (*Coffee arabica*) varieties was performed in this study.

MATERIALS AND METHODS

Plant material and sample preparation

Ripe *Coffea arabica* L. cherries of the varieties Caturra Rojo, Caturra Amarillo, Anaranjado, Catuaí and Arábica were manually picked from a farm located in Tres Ríos, Cartago, Costa Rica. While Caturra Rojo, Catuaí and Arábica had red peel color, the exocarp was yellow and orange colored in Caturra Amarillo and Anaranjado, respectively. After removing the seeds, peels and pulp were manually separated from each other. Coffee mucilage was obtained from commercial depulping (aqua-pulping), consisting of a blend of different coffee varieties. Each sample was immediately frozen in liquid nitrogen and subsequently freeze dried. Standards were obtained from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). All further reagents or solvents were purchased from VWR International GmbH Darmstadt, Germany), at least of analytical or HPLC grade. Deionized water was used throughout.

Polyphenol extraction and analysis

The protocol used for polyphenol extraction and analysis was modified from Kammerer *et al*., using 0.25 g of pulp, 0.5 g of peels or 5 g of mucilage. Polyphenols were extracted twice from each sample with MeOH/0.1% HCl, and the combined supernatants were evaporated to dryness *in vacuo* at 30°C. The residue was made up to 1 ml, 2 ml and 5 ml of deionized water (pH 3) for pulp, peels and mucilage, respectively. Subsequently, samples were membrane filtered (0.45 µm) into amber vials, and stored at -20°C until analysis. For HPLC analysis, an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, G1322A degasser, G1312A binary gradient pump, G1329/G1330A thermo autosampler, G1316A column oven and G1315A diode-array detector. The separation was conducted with a Phenomenex (Torrance, CA, USA) C18Hydro-Synergi (150 x 3.0 mm i.d., 4 µm particle size) with a C18 ODS guard column (4.0 x 2.0 mm i.d.) operated at 25°C. For the analysis of phenolic acids, the mobile phase consisted of a mixture of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and MeOH (10:90, v/v; eluent B). A mixture of 5% (v/v) formic acid (eluent A) and MeOH/water/formic acid (80/10/10, v/v/v, eluent B) was used for analysis of flavonols. Total run time was 60 and 70 min for phenolic acids and flavonols, respectively, both at a flow rate of 0.4 ml/min. The injection volume was 10 µl. CGAs were monitored at 280 nm and 320 nm. For anthocyanin analysis, the gradient used ramped from 10 to 14% B (5 min), 14 to 23% B (25 min), 23% to 32% B (15 min), 32% to 62% B (15 min), 62 to 100% B (5 min), 100% B isocratic (5 min), 100 to 10% B (5 min), 10% B isocratic (5 min). Total run time was 80 min. The injection volume was 4 µl. Monitoring was performed at 520 nm and a flow rate of 0.4 ml/min was used. Compounds were quantified by integration of the peak areas at the respective wavelength using a calibration curve of the corresponding standard compound: 5-caffeoylquinic acid (Sigma Aldrich Chemic, Taufkirchen, Germany), proanthocyanidin B1 (Extrasynthese, Lyon, France), ferulic acid and p-coumaoylquinic acid (Roth Chemie, Karlsruhe, Germany) and

cyanidin-3-O-glucoside (Polyphenols, Sandnes, Norway). LC/MS analyses were performed with the HPLC system coupled online to a Bruker 3000+ ion trap mass spectrometer (Bruker Daltronic, Bremen, Germany) operating in a negative mode for phenols and in positive mode for anthocyanins and an ESI source, as detailed by Kammerer *et al*.

Carotenoid extraction and analysis

Carotenoid extraction and HPLC-PDA-MS^n analysis was performed as described by Schweiggert *et al*. For selected samples, saponification of carotenoid esters was performed as follows: Carotenoid extracts were evaporated to dryness *in vacuo* at 25°C, re-dissolved in 50 mL of petroleum ether, and 50 ml of methanolic 10% (m/v) KOH were added. The solutions were maintained in agitation at 300 rpm overnight under nitrogen atmosphere. After saponification, the organic phase was separated, washed twice with deionized water, evaporated to dryness, and prepared for HPLC analysis as reported by Schweiggert *et al*. Identification of carotenoids was performed by comparing their UV-vis spectra and mass spectral behavior with data published previously.

Statistics

Determination of significant differences between means was carried out using one-way analysis of variance, followed by Tukey's test for mean comparison, using the statistical program Statistica 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Polyphenols (non flavonoids)

Predominant compounds identified were CQAs accompanied by CGAs (hydroxycinnamic acids) and FQAs. Three isomeric CQAs (3, 4, 5-CQA) and three di-CQAs (3,4, 3,5, and 4.5-di-CQA) were detected. One isomer of the FQA (5-FQA) and *p*-coumaroylquinic acid (5-*p*-coumaroylquinic acid) and one *O*-dimethoxycinnamoylquinic acid (5-*O*-dimethoxycinnamoylquinic acid) isomer were also identified. The location of these compounds in coffee by-products was found to be different, for example, in the peels and pulp three isomeric CQAs (3, 4, 5-CQA) were detected, while only two isomers were found in the mucilage (4 and 5-CQA). FQA was only detected in the pulp and mucilage (Table 1). Ramirez-Martinez, Clifford and Ramirez-Martinez and Ramirez-Coronel *et al*.also reported the occurrence of some of these compounds in the coffee pulp, which in their case were equivalent to our peels and pulp fractions because in previous works they were not separated from each other. Due to different pigmentation of peel and pulp, they were analyzed separately, the latter usually being much less pigmented. So far, there are no reports of the characterization of these compounds in both coffee tissues.

Table 1. CGAs pattern of different coffee by-products analyzed.

Compound	Coffee by-products		
	Pulp	**Peels**	**Mucilage**
3-CQA	X	X	
4-CQA	X	X	X
5-CQA	X	X	X
3,4-di-CQA	X		
3,5-di-CQA	X	X	X
4,5-di-CQA	X	X	
5-FQA	X		X
5-p-coumaroylquinic acid		X	
5-O-dimethoxycinnamoylquinic acid			X

In the case of the pulp and the peels, different concentrations of these compounds were also observed among varieties (Table 2). Pulp of the Arabica variety presented the highest values for three of the CGAs evaluated (3-CQA, 3,4-di-CQA, 4,5-di-CQA). Remarkably, the 5-CQA isomer was predominantly found in the pulp of the five varieties, while 3,5-di-CQA isomer was prevailing in the peels (Table 2) and the mucilage (data not shown). Clifford and Ramirez-Martinez reported similar concentrations of these compounds in the pulp of Caturra Rojo except for 5-CQA where higher concentrations (up to 9200 mg/kg) were reported. This could be related to distinct growing conditions, which have been associated with differences in the chemical composition of coffee fruits parts.

Polyphenols (flavonoids)

Among the flavonoids identified the flavanol epicatechin, two anthocyanins (cyanidin-3-glucoside and cyanidin-3-O-p-coumaroylglucoside) and four condensed tannins (proanthocyanidin B1 and B4, procyanidin dimer, proanthocyanidin dimer and proanthocyanidin trimer) were found. Most of them were extracted from the peels, and only proanthocyanidin B was detected in the pulp (Table 3). Both anthocyanins were found in the pink mucilage. Epicatechin has already been reported in the pulp of eight coffee cultivars by Ramirez-Martinez, while the proanthocyanidins have previously been reported by Ramirez-Coronel *et al*. in the pulp of *Coffea arabica.*

The concentration of condensed tannins was very low in the pulp whereas a procyanidin dimer showed the highest values in the peels (Table 3). These compounds were not detected in the mucilage. Condensed tannins are considered as anti-nutrients for ruminants, and this is especially important when feed use of coffee by-products is intended.

Table 2. Concentrations of the CGAs in the pulp and peels of the five coffee varieties evaluated.

CGAs	Concentration (mg/kg DM)				
	Coffee variety				
	Anaranjado	Arábica	Catuaí	Caturra Amarillo	Caturra Rojo
PULP					
3-CQA	242.2b	259.9a	220.8d	237.2bc	227.5cd
4-CQA	408.5a	434.9a	305.7b	419.9a	328.7b
5-CQA	1329.6a	1206.1a	1061.4a	1255.6a	1255.6a
?-CQA Rt=22	1998.9ab	2220.7a	1738.2c	2201.6a	1941.5bc
?-CQA Rt=28	182.7b	185.8b	180.2b	182.8b	218.9a
3,5-di-CQA	506.3ab	575.5a	450.5bc	569.3a	372.4c
3,4-di-CQA	420.1b	514.6a	353.9c	382.7bc	288.8d
4,5-di-O-CQA	280.1b	326.3a	266.1b	281.4b	235.3c
PEELS					
3-CQA	133.2a	136.9a	65.3b	156.0a	71.1b
?-CQA Rt=24,2	4526.1bc	5693.4ab	2745.6c	8029.8a	4242.6bc
4-CQA	177.7b	182.9b	87.8c	248.3a	97.1c
5-CQA	830.3ab	828.4ab	568.7b	899.5a	772.1ab
5-p-coumaroylquinic acid	79.7a	74.9ab	50.8b	89.2a	69.0ab
3,5-di-CQA	1537.3a	1510.0a	962.5a	922.2a	992.1a
4,5-di-CQA	367.2b	401.8ab	289.8c	440.9a	289.5c

Table 3. Concentrations of the flavonoids in the pulp and the peels of the five coffee varieties evaluated.

Flavonoids	Concentration (mg/kg DM)				
	Coffee variety				
	Anaranjado	Arábica	Catuaí	Caturra Amarillo	Caturra Rojo
PULP					
Proantocyanidin B	36.3bc	41.9b	39.2bc	30.5c	62.0a
Cyanidin-3-glucoside	514.1a	220.4a	469.7a	534.0a	293.6a
PEELS					
Epicatechin	1367.5a	1362.7a	846.1a	815.9a	1312.5a
Proantocyanidin B4	141.3b	51.1cd	32.1d	238.4a	80.6c
Procyanidin dimer	1692.4a	1795.1a	746.7b	1971.4a	1259.1ab
Proantocyanidin B1	78.3a	90.0a	51.3a	104.6a	88.1a
Cyanidin-3-O-glucoside	4.9b	22.8a	15.9a	ND	16.8a
Cyanidin-3-O-p-coumaroylglucoside	10.2b	97.7a	62.9a	ND	75.4a
MUCILAGE					
Cyanidin-3-O-glucoside	3.8b				
Cyanidin-3-O-p-coumaroylglucoside	8.9a				

Anthocyanins (cyanidin-3-glucoside and cyanidin-3-O-p-coumaroylglucoside) were not present in Caturra Amarillo, and their concentration in the Anaranjado variety was much lower than in the red-colored genotyes, as can be expected according to the peels colors. Anthocyanins were previously reported in the pulp of red coffee varieties by Patra and Oliveira and by Esquivel et al.

Carotenoids

While several carotenoids were found in the peels of the yellow (Caturra Amarillo) and orange (Anaranjado) colored genotypes, carotenoids were neither detected in the pulp of most varieties (except from Caturra Amarillo) nor in the mucilage of all samples analyzed. Carotenoids and chlorophylls identified in different coffee peels are presented in Table 4.

Table 4. Carotenoids and chlorophylls identified in the peels of three coffee varieties and in the pulp of cv. Caturra Amarillo, respectively.

Compound identity	Rt (min)	UV/VIS absorption maxima (nm)	$[M+H]^+$ *m/z*	Coffee variety[1]			
				Caturra Rojo (peels)	Anaranjado (peels)	Caturra Amarillo (pulp)	Caturra Amarillo (peels)
Violaxanthin	10.6	414/440/466	601	-	+	-	+
Neoxanthin	11.3	412/436/464	601	-	+	-	+
Chlorophyll b	22.1	466/650	906	+	+	+	+
Lutein -	23.8	420/444/472	569	+	+	+	+
apo-8´-carotenal (Istd)	26.5	468	464	+	+	+	+
Chlorophyll a	29.5	432/666	892	+	+	-	+
unidentified	37.5	420/440/470	537	-	+	-	+
α-carotene	43.6	422/446/474	537	-	+	+	+
β-carotene	47.2	424/450/478	537	+	+	+	+
Carotenoid esters	>48			-	+	+	+

[1]*presence (+) or absence (-) of the compound.*

Chromatographic separation of unsaponified carotenoids from Caturra Rojo, Anaranjado and Caturra Amarillo is illustrated in Figure 1. As indicated by the accompanying chlorophylls a and b, mostly carotenoids characteristic of green tissues were identified in the peels. As described in detail by Britton, with very few exceptions, the chloroplasts of green tissues of higher plants contain the same set of major accessory carotenoids, usually comprising β-carotene, lutein, violaxanthin, and neoxanthin. Due to the qualitative composition of the carotenoids from the peels, our study supports the hypothesis of their chloroplastidal origin. However, besides typical chloroplast pigments, various carotenoid esters were identified after saponification (not shown), revealing the presence of mostly lutein esters, supposedly acting as accessory pigments in photosynthesis. When comparing peel and pulp carotenoids, an apparent similarity of carotenoid composition could be confirmed, as can be seen from Figure 1, although the pulp of Caturra Amarillo only contained minor amounts of lutein esters. The other pulp samples analyzed were devoid of carotenoids, or their levels were too low to be detected by our analyses.

Besides considerable antioxidant properties, particularly β-carotene and, to a lesser extend regarding its conversion, α-carotene, are vitamin A precursors, thus playing an important role in human nutrition.
Lutein was identified as a major carotenoid in coffee peels, demonstrating their potential as a source of this valuable constituent. Lutein is found in very high concentrations in the macula lutea, which is the most sensitive region of the retina of humans and other primates. These carotenoids are believed to be important for the prevention of age-related macular degeneration, being a cause of impaired vision and blindness in elderly people. Furthermore, frequent lutein intake has been associated with improved cognitive functions like verbal fluency in the elderly, although further study was claimed by the respective researchers.

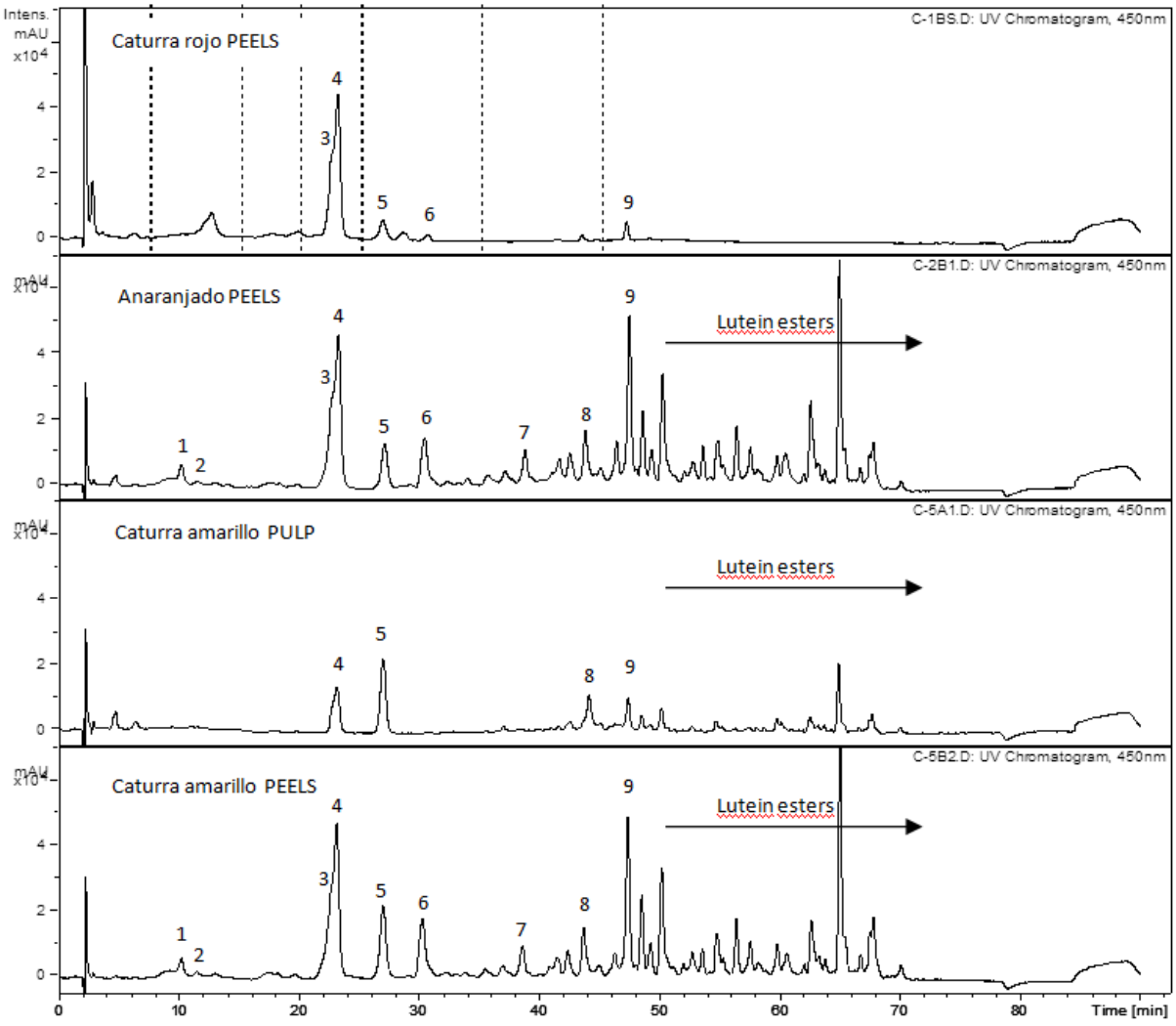

Figure 1. HPLC separation of unsaponified carotenoids from peels of Caturra Rojo, Anaranjado, and peels and pulp of, Caturra Amarillo respectively. 1) Violaxanthin. 2) Neoxanthin. 3) Chorophyll b (co-elution). 4) Lutein. 5) Istd. 6) Chlorophyll a. 7) Unidentified. 8) α-carotene. 9) β-carotene.

Coffee by-products (mainly peels) showed different pattern and quantity of pigments depending of their color. In the case of red colored cherries, presence of anthocyanins was obvious, while in yellow and orange fruits typically carotenoids were to be expected. Considering red-colored fruits, carotenoids might have been degraded upon ripening or even

lacking, while in orange fruits the presence of yellow and red pigments belonging to different groups contributed to their distinct color.

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Characterization of Animal Preference to Arabica Coffee Varieties and Cup Taste Profile on Domesticated "Luwak" (*Paradoxorus Hermaphroditus*)

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SUMMARY

"Luwak" coffee was known as a very unique coffee, because the coffee was passing trough inside disgestion of a wild civet cat (*Paradoxorus hermaphroditus*). During in the civet cat disgestion, a unique fermentation was occured eventually, that's producing any unique flavors substances. Production of wild "Luwak" coffee was very limited quantity. To get more "Luwak" coffee, a voluntar to domestication of the civet cat had been tried. Domestication of civet cat as coffee processing was a new method. There are 3 (three) types of civet cat hutch, those are "ranch", individual aviary, and individual cage. Individual cage method was easier to handle. Those civet cats were fencing only during harvesting season, after that's released to wild nature. Ration of a civet cat during in the hutch is 300 grams of fruits (banana, papaya, etc.) in the morning, 1 - 1,2 kg of fresh coffee cherry in the afternoon. Every week, 250 grams of fresh chiken meat is served. Fresh water always be available in the hutch. All the ration must be served in fresh condition, and the hutch must be cleaned everyday. A trial of civet cat preference on Arabica varieties had been conducted. The most prefered variety by "Luwak" is a new promising Composite variety developed by ICCRI, followed by Andungsari 2K, S795 and Kartika. A "luwak" can drop 300 - 400 grams of wet parchment a day or equivalent to 100 - 150 g green coffee. Average yield every civet cat is around 0,12 Kg a day similar to 14 Kg a season. Wild Luwak coffee contained black been, partly black beans, holed been, whereas the domesticated luwak coffee almost no physical defects. Wild Luwak coffee has strong Fragrance, Aroma, Flavor and Aftertaste, medium Acidity, medium body, and a good ballance. But, sometimes accompanied by *earthy* of flavour. Domesticated luwak coffee performed very strong fragrance, Aroma, Flavor and Aftertaste, medium acidity, heavy body, and very good balance. Cafeine content of luwak coffee is not significantly different with wet processing coffee.

INTRODUCTION

The genus *Paradoxurus* (Mammalia, Carnivora, Viverridae) includes three species: the golden palm civet, *Paradoxurus zeylonensis*, endemic to Sri Lanka; the brown palm civet, *Paradoxurus jerdoni*, endemic to the Indian Western Ghats; and the common palm civet, *Paradoxurus hermaphroditus*, which occurs across South and Southeast Asia. The common palm civet (*P. hermaphroditus*), which is widely distributed from Pakistan to the Lesser Sunda Islands. These species are largely frugivorous, although their diet includes small prey such as insects, earthworms, molluscs and small vertebrates. They are solitary, nocturnal and mainly arboreal, and play an important role as seed dispersers. Furthermore, common palm civets are the source of 'Kopi Luwak'. "Kopi Luwak" was known as one of a very unique coffees, because the coffee was passing trough inside disgestion of a wild civet cat (*Paradoxorus hermaphroditus*). Production of wild "Luwak" coffee was very limited

quantity. To get more “Luwak” coffee, a voluntar to domestication of the civet cat had been tried. There are 3 (three) types of civet cat hutch, those are “ranch”, individual aviary, and individual cage. Individual cage method was easier to handle. Those civet cats were fencing only during harvesting season, after that’s released to wild nature. Ration of a civet cat during in the hutch is 300 grams of fruits in the morning, 1,0 - 1,2 kg of fresh coffee cherry in the afternoon. Every week, 250 grams of fresh chiken meat is served. Fresh water always be available in the hutch. All the ration must be served in fresh condition, and the hutch must be cleaned everyday.

A number of scientific references on Kopi Luwak had been published, which reported on various physicochemical properties of “Kopi Luwak”. There are some major physical differences between them that include color differences, where Kopi Luwak was found to be higher in red color hue and was overall darker than control beans. Scanning electron microscopy revealed that all Palm Civet beans possessed surface micro-pitting caused by the action of gastric juices and digestive enzymes during digestion. Large deformation mechanical rheology testing revealed that Luwak coffee beans were harder and more brittle than their controls indicating that digestive juices were entering into the beans and modifying the micro-structural properties. Also after roasting, it was noted that there were significant differences in the flavor profile of the Kopi Luwak vs. the controls when analyzed by an electronic nose for volatile aroma compounds. The Indonesian Ulema Council has decided that "Kopi Luwak" permitted to consumed by moslem peoples.

MATERIALS AND METHODS

A trial of civet cat preference on Arabica varieties had been conducted. In 2010, 4 varieties had been tried, those are Andungsari 2K (BP416A), Komposit, Kartika, and S 795. In 2012, 7 varieties had been tried, those are Andungsari 1, Andungsari 2K (BP416A), BP 430A, BP 542 A, Komposit, Kartika, and S 795. Fresh coffee cherrie were served with paired 2 varieties. Every variety was 1 kg of fresh cherry, and so every civet cat get 2 kg everyday in the evening. After overnight, the unconsumed cherries were weighed. On the other hand, the fresh “Luwak” droping was colected, water soaked over night, washed, and dried on bamboo rack. The dried parchment of “Kopi Luwak” was hulled to get green coffee. The green coffee samples were graded to get the first quality according to Indonesian National Standard (SNI). Cupping evaluations were carried out by using medium roasting and grinding degree. Sensory components observed were fragrance and aroma, flavor, body (mouth feel), acidity, sweetness, balance, after taste, and preference (overall) by making 1 – 10 score.

RESULTS AND DISCUSSION

Level of consumption of luwak animal and production of dried parchments bean as mentioned in Table 1.

Table 1. Cherries comsumption and wet coffee parchment production of caged luwak.

	Coffee cherry comsumption (grams /day)	**Production of wet coffee parchment (grams /day)**
Average	860	381,5
Standard Deviation	139	101,8
Minimum	0	0
Maximum	1944	725,0

Number of caged Luwak were 4 – 74, 126 days.

Table 2. Luwak preference to some Arabica coffee varieties (2010)[1].

Pair number	Varieties	Cherries consumption (Gr / Luwak/ day)	% of Cherries consumed by luwak	Score[2]	Total cherries consumed (Gr/ Luwak/ day)	% of Total cherries consumed by luwak
1	Andungsari 2K	514	51.40	0	665.00	33.25
	Komposit	821	82.10	1		
2	S795	281	28.10	0	1072.00	53.60
	Komposit	647	64.70	1		
3	S795	539	53.90	0	894.00	44.70
	Andungsari 2K	567	56.70	1		
4	S795	474	47.40	1	1196.00	59.80
	Kartika 1	330	33.00	0		
5	Kartika 1	204	20.40	0	1080.00	54.00
	Composite	716	71.60	1		
6	Andungsari 2K	506	50.60	1	1149.00	57.45
	Kartika 1	345	34.50	0		

[1] Number of Luwak were 20. A "Luwak" was served 1000 gram of coffee chery.[2] Scoring: 1 – for more consumed, 0 – for less consumed.

A "luwak" consumed 860 grams of coffee cherry/day in average (0 – 1944 grams coffee cherry/day). Production of wet parchment was 300 - 400 gr a day or equivalent to 100 - 150 gr of green coffee. Average yield every civet cat is around 0,12 kg a day similar to 14 kg a season.

Table 3. Luwak preference to some Arabica coffee varieties (2012).

Pair nr.	Varieties	Average of cherries consumed (gr / Luwak/ day)	Standard of deviation	% of cherries consumed by luwak	Score[2]	Total cherries Consumed (gr / Luwak/ day)	% of total cherries consumed by luwak
1	- Komposit	550	215	55	1	1.035	52
	- Andungsari 2K	486	201	49	0		
2	- Composite	412	282	41	0	958	48
	- BP 542 A	546	203	55	1		
3	- Composite	296	223	30	0	845	42
	- S 795	549	282	55	1		
	- S 795	506	261	51	0		
4	- Composite	523	183	52	1	929	46
	- Kartika 1	406	171	41	0		
5	- Composite	374	256	37	0	948	47
	- BP 430 A	574	269	57	1		
6	- Composite	351	227	35	0	744	37
	- Andungsari 1	392	233	39	1		
7	- Andungsari 2K	541	222	54	1	1079	54
	- BP 542 A	538	231	54	0		
8	- Andungsari 2K	546	294	55	1	820	41
	- S 795	275	246	27	0		
9	- Andungsari 2K	542	124	54	1	948	47
	- Kartika	405	147	41	0		
10	- Andungsari 2K	438	239	44	1	762	38
	- BP 430 A	323	243	32	0		
11	- Andungsari 2K	518	297	52	1	765	38
	- Andungsari 1	247	232	25	0		
12	- BP 542 A	511	218	51	1	1017	51
	- S 795	506	261	51	0		
13	- BP 542 A	403	215	40	1	711	36
	- Kartika	309	198	31	0		
14	- BP 542 A	352	289	35	0	863	43
	- BP 430 A	511	324	51	1		
15	- BP 542 A	381	315	38	1	555	28
	- Andungsari 1	174	130	17	0		
16	- S 795	274	183	27	0	822	41
	- Kartika	548	227	55	1		
17	- S 795	625	151	62	1	1076	54
	- BP 430 A	451	157	45	0		
18	- S 795	359	177	36	0	850	42
	- Andungsari 1	491	184	49	1		
19	- Kartika	579	217	58	1	899	45
	- BP 430 A	320	228	32	0		

20	- Kartika	371	268	37	0	799	40
	- Andungsari 1	428	237	43	1		
21	- BP 430 A	355	213	36	1	637	32
	- Andungsari 1	282	225	28	0		

[1] Number of Luwak were 20. A "Luwak" was served 1000 gram of coffee cherries. [2] Scoring: 1 – for more consumed, 0 – for less consumed.

Experiment in 2010, the most prefered variety by "Luwak" was a new promising variety of Composite variety developed by ICCRI, followed by Andungsari 2K, S795 and Kartika. But in 2012, the most prefered variety by "Luwak" is Andungsari 2K, followed by BP 542 A. BP 430 A, BP 542 A, Kartika, Komposit, and S 795 are is not significantly difference on the consumed coffee cherries (Table 4).

Table 4. Average coffee cherry consumed and total score of luwak preference on some Arabica varieties.

Nr.	Arabica Varieties	2012		2010	
		Total score of luwak preference[1]	Average chery Consumed (gram / luwak/ day)	Total score of luwak preference[1]	Average chery Consumed (gram / luwak/ day)
1	Andungsari 1	3	336	Untested	Untested
2	Andungsari 2K (BP 416 A)	5	512	2	529
3	BP 430	3	423	Untested	Untested
4	BP 542	4	455	Untested	Untested
5	Kartika	2	436	0	293
6	Komposit	2	418	3	728
7	S 795	2	431	1	431

[1] Higher score is more preference.

In 2010, the most prefference of varieties pair were S795 and Kartika (1196 grams cherry/Luwak/day), followed by Andungsari 2K (BP 416 A) and Kartika pair (1149 grams cherry/Luwak/day), and Komposit and Kartika pair (1080 grams cherry/Luwak/day). Kartika variety may increase prefference of Luwak.

In 2012, the most prefference of varieties pair were Andungsari 2K (BP 416 A) and BP 542A pair (1079 grams cherry/Luwak/day), BP 430A and S 795 pair (1076 grams cherry/Luwak/dayi), and BP 542A and S 795 pair (1017 grams cherry/Luwak/day). Andungsari 1 had total preference score 3, but least chery consumed (336 grams cherry/Luwak/day). Some continued experiments must be done to get more comprehensive data.

Green Coffee Quality

Wild Luwak coffee contained black been, partly black beans, holed been, whereas the domesticated luwak coffee almost no physical defects (Table 5.). This case showed that's wild Luwak consume not only healthy coffee cherry, but also pest attacked cherry if the cherry still in "red and fresh". On the other hand, caged Luwak consume only healthy coffee cherry. The coffee cherry had been picked and selected by worker before serving to Luwak. The good

coffee cherry was served more quantity than Luwak bodily needs, so that's Luwak may select the best coffee chery only, until satisfied. The unconsumed coffee cherry must be processed as usually wet processing method.

Table 5. Number of deffect bean in "Luwak" green coffee[1].

Deffect	Unit	Arabica/ Wild Luwak		Arabica Caged Luwak		Robusta/ Wild Luwak	
		Washing	Soaking and Washing	Natural	Soaking and Washing	Lampung - Sumatra	Other - Sumatera
Black Bean	Bean	12	1	7	0	0	0
Partly Black Bean	Bean	15	0	9	0	5	21
Broken Black Bean	Bean	3	1	3	0	0	0
Brown Bean (Sour Bean)	Bean	10	11	26	0.5	3	0
One holed bean	Bean	3	0	1	2	96	39
Several holed bean	Bean	1	0	1	0	17	13
Several stained bean	Bean	0	0	2	0	0	0

[1] *Sample weight is 300 grams.*

Cup taste profile of Luwak Coffee

Wild Luwak coffee has strong Fragrance, Aroma, Flavor and Aftertaste, medium Acidity, medium body, and a good ballance. But, sometimes accompanied by *earthy* of flavour. Caged luwak coffee performed very strong fragrance, Aroma, Flavor and Aftertaste, medium acidity, heavy body, and very good balance. The best flavour of luwak coffee may be produced if the feces of luwak is sorted, soaked all night long, washed with clean water, and sundried. Washing wihtout soaking, the luwak coffee still contain somewaht earthy and moldy. Unwashed luwak coffee certain to be earthy and moldy (Table 6.).

Table 6. Cup taste profile of Arabica luwak coffee processed by some processing method[1].

Taste Atribute	Wash	Soaking and Wash	Natural
Quality of Aroma	7.25	7	7.33
Intensity of Aroma	7.25	7.5	7.67
Quality of flavor	7.25	7	7.17
Intensity of flavor	7.5	7.5	7.33
Body	7	8	8
Acidity	5	4.5	5.17
Quality of Aftertaste	7	6.5	7.17
Intensity of Aftertaste	7	7	7.33
Bitterness	4	5	4.33
Preference	6.75	7.5	6.83
Flavour defect			
Earthy	2.5	0	4.5
Moldy	0	0	2.5

[1] *Notation for intensity*

0 = Nil	*3 – 4 = Moderately weak*	*7 – 8 = Strong*
1 – 2 = Weak	*5 – 6 = Moderately strong*	*9-10 = Very strong*

Notation for quality and preference

0 = Inconsumable	*3 – 4 = Bad*	*7 – 8 = Very good*
1 – 2 = Very Bad	*5 – 6 = Good*	*9 - 10 = Excellent.*

Wild luwak coffee commonly has strong Fragrance, Aroma, Flavor and strong Aftertaste, medium Acidity, medium body, and good ballance, but occasionally it is tainted by earthy and moldy off-flavour. Arabica caged luwak coffee has flavour profile close to Full Wash Processed coffee with stronger body (Table 7). Robusta wild luwak coffee has a good flavour but tainted by earthy and moldy off-flavor (Table 8). Wild luwak coffee mostly has earthy and moldy off-flavor, becouse the "feces" had been grown by mold when collected.

Table 7. Flavor profile of some Arabica luwak coffee compared with Arabica Full Wash Processed[1].

Flavor Atribute	Wild Luwak Argopuro Mountain	Caged Luwak Argopuro Mountain	Arabica Full Wash Processed Argopuro Mountain	Caged Luwak Ijen Mountain	Arabica Full Wash Processed Argopuro Mountain
Fragrance and Aroma	6.98	7.65	7.44	7.1	7.82
Flavor	7.17	7.71	7.5	7.44	7.61
After Taste	7.17	7.65	7.48	7.53	7.6
Acidity	6.83	7.25	7.02	6.38	6.62
Body	7.4	7.31	7.13	7.1	7.25
Balance	6.94	7.58	7.27	7.47	7.49
Uniformity	10	10	10	9.63	10
Clean Cup	6.98	7.65	7.44	9.38	10
Sweetness	7.43	7.04	7.69	7.71	7.21
Overall	7.04	7.69	7.38	7.23	7.51

1 Note : See Table 6.

Flavor profile of Liberica luwak coffee is impresionable with the origin and variety. The specific characters are very low bitterness, medium sweetness, but very strong green and grassy. For someone had been familiar with Robusta, Liberica coffee is strong aroma but very low body. For someone had been familiar with Arabica, Liberica coffee is too low acidity and tainted with winy and fruity.

On "The 2nd Indonesia Specialty Coffee Auction" in 2012, some Indonesian Luwak Coffees had been evaluated by 25 International Cuppers according to Standard of Specialty Coffee Association of America (SCAA) [4] for bidding. The total scores are very widely spread from 82.20 until 86.04, and the special characters are very widely differences. Up to now there is no specific flavour characters to authenticate on the originality of those luwak coffee yet. Originality of luwak coffee may be provable only by a good documentation, not by cupping test method.

Table 8. Flavor profile of Robusta luwak coffee compared with Robusta Full Wash Processed[1].

Flavor Atribute	Robusta Wild Luwak	Wash Robusta	Liberica luwak coffee
Quality of Aroma	6.33	6.5	7.19
Intensity of Aroma	7.83	7	7.19
Quality of Flavor	7	6.5	7.68
Intensity of Flavor	783	6.5	7.68
Body	7.42	6.5	7.44
Quality of Aftertaste	6.33	6.5	7.55
Intensity of Aftertaste	7.67	6.5	7.55
Sweetness	Not scored	Not scored	8.85
Bitterness	5.92	4.5	6.27
Astringency	3.5	3.5	Not scored
Green	0	1	Very Green
Grassy	1.5	1.5	Very Grassy
Harsh	0.67	0	Somewhat harsh
Earthy	4.33	0	Somewhat earthy
Preference	6.83	6.5	7.74
Comments	Strong Earthy	Clean	Low body, low acidity winy, fruity.

[1] *Note : See Table 6.*

Cafein Content

Cafein content of some Arabica luwak coffee processed by some processing methods are not significantly different, also if compared with Arabica full wash processed (Table 9.)

Table 9. Cafein content of some Arabica luwak coffee compared with Arabica full wash processed.

Luwak Coffee Processing	Cafein content of green coffee (% of dry matter)	Cafein content of medium roasted bean (% of dry matter)
Washing only	1,39	1,51
Soaking and Washing	1,37	1,42
Natural	1,40	1,40
Full Wash Arabica (control)	1,38	1,51

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Identification of the Fungal Microflora of Coffee Beans from Different Origins and Evaluation of Different Decontamination Concepts

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SUMMARY

The aim of this work was to identify the fungal microbiota naturally occurring on commercial green coffee beans, belonging to both *Coffea arabica* and *Coffea robusta* varieties, and to evaluate different approaches for the disinfection of the bean surface. To this end, endogenous fungi were isolated using standard microbiological techniques and identified by morphological analysis and DNA sequencing. The fungal isolates belonged to *Aspergillus* and *Penicillium* spp. Different chemical, physical and biological methods were employed to control and/or inhibit the fungal growth on the beans. Overall, the use of chemical methods and application of lactic acid bacteria (LAB) were shown to be the most successful approaches.

INTRODUCTION

Green coffee beans host indigenous microbial microflora whose composition and load vary depending on geographical origin, farming practices and storage. In particular, the fungal microbiota may lead to the presence of spores that represent a concern from health, economical and agricultural perspectives, especially if the farming practices are not tightly controlled. In 2010, the total global coffee production amounted to 8,230,000 tonnes. However, the Food and Agriculture Organization (FAO) has estimated that 25% of the world's crops are affected by mycotoxins each year, with annual losses of around 1.3 billion metric tonnes of foods and food products, representing between $5 – 17 billion in the US alone (~25% of food production worldwide lost post-harvest). The price of fungal crop spoilage globally seriously effects coffee producing farmers and reduces coffee quality.

The European Food Safety Authority (EFSA) and FAO have stringent fungal toxin limits for foods. According to the EFSA, roasted coffee beans must not exceed 5 μg/kg for ochratoxin A (OTA); a commonly detected mycotoxin from ochratoxigenic *Aspergillus* species. However, certain countries have adopted FAO recommendations and in Bulgaria, Cuba, Greece, Hungary, Indonesia, Italy, and Singapore, levels ranging from undetectable to 15 μg/kg of OTA are acceptable. In light of the economical and health implications, the issue of fungal spoilage needs to be addressed. However, industry, government and consumer preferences demand minimal processing and reduced use of chemicals or environmentally harsh treatments of foods and beverages, particularly in developed and Western countries. Thus, natural, 'green' environmental and consumer friendly approaches are preferred. The present work aims to evaluate different strategies for the on decontamination of coffee borne fungal microorganisms.

MATERIALS AND METHODS

Commercially available green coffee beans were sourced from Ecuador, Brazil, Ethiopia, India, and Thailand. The coffee microflora was isolated by standard aseptic microbiological methods; 10g of green coffee (with the parchment intact) were homogenised in sterile stomacher bags (Seward Ltd, West Sussex, UK) containing 90mL Ringer's physiological solution (Sigma, Arklow, Ireland) in a Stomacher® 400 circulator (Seward Ltd) for 2 min at maximum power. The liquid component of the mixture was then serially diluted and plated directly onto agar media. Media (Oxoid, Thermo Fisher Scientific Inc, Hampshire, UK) used to isolate fungal strains included; Malt Extract, Potato Dextrose, Rose Bengal, Sabouraud Dextrose, and Yeast Extract-Phosphate agars, with and without 10 µg/mL erythromycin (Sigma) and 30 µg/mL chloramphenicol (Sigma) to inhibit bacterial growth.

Fungal isolates were purified by at least three contaminant-free sub-cultured generations for each isolate. Strains were identified by morphological analyses of the mycelial biomass and light microscopy analyses of the lactophenol cotton blue enhanced spores and mycelia. Molecular identification of the fungal isolates was performed by PCR using the universal primers ITS1 (tccgtaggtgaacctgcgg) and ITS4 (tcctccgcttattgatatgc).

The green coffee beans were subject to decontamination approaches of a chemical, physical and/or biological nature. Chemical decontamination was performed by soaking the coffee beans in various disinfection liquids (Table 1) for 1 to 20 min, with continuous shaking (200 rpm) followed by rinsing twice in sterile tap water. The physical decontaminations comprised high pressure (HP), microwave treatment, anaerobic incubation, UV, soaking at low pH (pH) and heat treatment under the specifications listed in Table 1. The efficacy of each decontamination approach was evaluated by determining residual fungal spore counts on the coffee beans after the treatment. The spore counts were measured by using serial dilution for counts on agar media and/or using a Thoma chamber counting cell. Furthermore, the effects of each treatment on the viability of the coffee beans was assessed using the 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) method where viable seed embryos convert colourless TTC to red formazan after incubation.

The biological decontamination methods investigated are listed in Table 1. They comprised soaking the green coffee beans in different solutions.

Furthermore, *in vitro* antagonistic tests were performed using the strain *Lactobacillus amylovorous* DSM 19280 LAB, from the University College Cork microbe collection, or its by-product as antifungal agents against the growth of the coffee fungal isolates. Briefly, the strain was cultured anaerobically, at 37°C in MRS broth overnight and subcultured for a further 16 hours. The cultured cells were either separated by centrifugation and the biomass (LAB) or the cell-free supernatant (LAB-CFS) were collected. The LAB plate test was done on MRS agar with three 5µL LAB spots (10^7 CFU/mL from an anaerobic overnight MRS broth culture at 30°C) placed equidistantly from the centre of the plate. These LAB spots were incubated anaerobically for a further 16 h at 30°C with subsequent application of cooled 0.7% agar containing 10^8 fungal spores. The plates were then incubated aerobically at 25°C for 7 days. The LAB-CFS test was done by incorporating 50% CFS-agar (freeze dried and aseptically reconstituted to 5 X concentration in 0.7% bacteriological agar) into MRS agar, with subsequent application of cooled 0.7% agar containing 10^8 fungal spores. The plates were then incubated aerobically at 25°C for 7 days. The efficacy of the *in vitro* approach was evaluated based on visual determination of fungal growth and measurement of fungal free halos surrounding LAB spots in the LAB plate tests, or measuring % plate covered by fungal mycelia in the LAB-CFS plate tests.

Table 1. List of decontamination methods used for green coffee beans.

Chemical:	**NaOCl**	**Sulfitox**	**H_2O_2**	**Pyne**	**Ecosol**	**Quatrol T**
Conditions:	A concentration of 1–5% for 1–20 min soaking time was used for disinfectants					
Physical:	**HP[1]**	**Microwave**	**Anaerobic**	**UV**	**pH[2]**	**Heat**
Conditions:	25-50°C, 100-200 mPa, 10 sec-10 min	800W oven, 10-60 sec, 0-6 h pre-soak	7 days, 0-6 h pre-soak	Laminar flow, 1-60 min	pH 3-10, 1–20 min soaking	30-80°C, in hot water
Biological:	**Acetic acid**	**Citric acid**	**Lactic acid**	**Propionic acid**	**LAB (*in vitro)***	**LAB-CFS (*in vitro)***
Conditions:	A 1 – 5% concentration was used for each organic acid, with a soaking time of 0-24 h				*Lactobacillus amylovorous* DSM19280	

[1]HP, high pressure, [2] pH, soaking at different acidic and basic pHs (3 to 10).

RESULTS AND DISCUSSION

On receipt of the green coffee beans, the species, origin, moisture content, fungal microbial load and other parameters were recorded (Table 2). The coffee bean microflora varied greatly depending primarily on geographical origin.

Harvest	Species	Origin and description	Moisture content[1] (%)	Viability[2] (%)	Spore count[3] (CFU/g)	Fungal species isolated[4]
1 – Feb 2011	*Coffea canephora* var *robusta*	Region A, parchment coffee	10.7 ± 0.8	61.0 ± 8.4	2.4 x10^6	– *Aspergillus niger* – *Penicillium griseofulvum* – *Meyerozyma guilliermondii* – *Aspergillus fumigatus*
2 – July 2011	*C. canephora* var *robusta*	Region B, parchment coffee	11.2 ± 0.7	94.0 ± 6.5	1.1 x 10^6	– *Aspergillus tubingensis* – *Mucor indicus* – *Rhizopus oryzae* – *Fusarium solani*
3 – Jan 2012	*C. canephora* var *robusta*	Region A, parchment coffee	13.5 ± 1.6	94.0 ± 6.5	1.1 x 10^6	– *Penicillium griseofulvum* – *Aspergillus niger* – *Aspergillus flavus* – *Aspergillus nomius* – *Aspergillus tubingensis* – *Penicillium citrinum* – *Aspergillus oryzae*
4 – Feb 2012	*C. canephora* var *robusta*	Region C, parchment coffee, wet processed	13.2 ± 2.0	19.3 ± 8.1	1. 6 x 10^5	– *Aspergillus clavatus* – *Aspergillus fumigatus* – *Debaryomyces hansenii* – *Meyerozyma guilliermondii*

[1]According to Pesquisa Agropecuaria Brasileira; [2]TTC test was done on 25 beans at least separate 3 times for each of the reported results; [3]Determined in duplicate by plate counts using antibiotic supplemented PDA and RBA and using a Thoma chamber count cell; [4]By morphological studies and PCR according to Fungi and Food Spoilage and A Guide to Methods and Application.

Toxigenic fungi isolated from the green coffee samples primarily included representatives from the *Aspergillus, Penicilium* and *Fusarium* genera. Regardless of geographical origin or post-harvest processing of the green coffee beans, approximately 10^6 cfu/g fungal spores were detected. In this study, chemical disinfectants, physical methods, and biological decontamination using natural organic acids (under optimal conditions) were employed to reduce the fungal load or inhibit growth. The results are reported in Table 3.

Table 3. The most efficient parameters for chemical decontamination of all batches of green coffee beans, reported as an average.

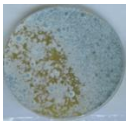
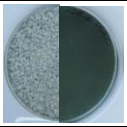
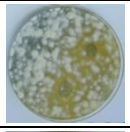
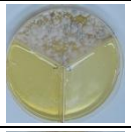
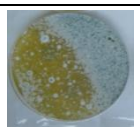
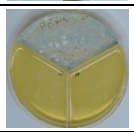
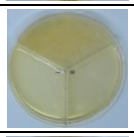
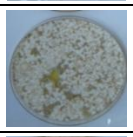
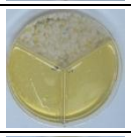
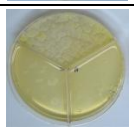
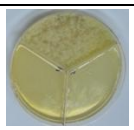
Decontamination approach	Process conditions	pH	Reduction in fungal counts (↓ LOG cfu/g)	% Δ Viability[1] (TTC test)
Chemical decontamination				
NaOCl	1.0 %	8.6	1.32	–17
Sulfitox	1.0 %	3.7	1.49	+23
H_2O_2	3.0 %	5.5	0.85	+33
Pyne disinfectant	1:10 %	ND	2.07	–9
Ecasol	2 ppm	ND	0.89	0
Quatrol T	1.3 %	9.2	1.60	+192
Physical decontamination				
High pressure machine	100mPa, 2 min	ND	5.00	–79
Microwave (800W oven)	Medium, 30 s	ND	6.04	–100
Anaerobic	7 days	ND	6.04	–11
UV (laminar flow hood)	1 h	ND	1.59	–50
pH	21 days[2]	10.0	1.93	+21
Heat (water bath)	70°C, soaking	7.0	6.04	–100
Biological decontamination (organic acids)				
Acetic acid	2 %, 15 min soak	2.8	1.50	+7
Citric acid	2 %, 15 min soak	2.2	1.53	–36
Lactic acid	1 %, 15 min soak	2.9	0.09	–78
Propionic acid	1 %, 15 min soak	7	1.68	–73

[1]As a percentage of control, [2]The beans were left to imbibe the liquid for 21 days and not soaked; ND not determined; PDA potato dextrose agar; RBA rose Bengal agar

From the data in Table 3, it is clear that physical decontamination such as microwave, anaerobic, heat, and HP treatments reduce the fungal load most efficiently. However, this is at expense of coffee viability which was reduced by 11 to 100%. Chemical and organic acid biological decontaminations generally reduced the fungal cell load by approximately 1 LOG cfu/g and affected the coffee bean viability to varying extents. Anaerobic treatment seems to be the most efficient method tested, however this approach resulted in undesirable smell after 7 days of incubation.

Since LAB have been previously shown to efficiently inhibit the growth of fungal spores from different matrices, a further biological approach to green coffee decontamination consisted of challenging the coffee bean fungal isolates using a LAB cell culture or in antifungal compound containing LAB-CFS (a by-product of LAB biomass production). These treatments allowed complete inhibition of fungal growth (Table 4).

Table 4. *In vitro* 7 day trials using the Lactic Acid Bacteria (LAB) *Lactobacillus amylovorous* or cell-free supernatant (CFS) as viable fungal antagonists for green coffee beans.

Fungal species	LAB plate test[1]	LAB-CFS plate test[2]	Fungal species	LAB plate test[1]	LAB-CFS plat test[2]
Penicillium griseofulvum			*Aspergillus flavus*		
Penicillium griseofulvum			*Aspergillus nomius*		
Aspergillus tubingensis			*Aspergillus tubingensis*		
Aspergillus niger			*Aspergillus tubingensis*		
Aspergillus tubingensis			*Rhizopus oryzae*		

[1]Top left and bottom spots are Lactobacillus amylovorous DSM19280, Covered right side spot is MRS control; [2]Top section is PDA only, left and right section is CFS-containing PDA in duplicate.

When using LAB as antagonists of the coffee-derived fungal species, incorporation of the CFS into the agar media was frequently more efficient at inhibiting contaminant growth than direct use of the LAB cells. As illustrated in Table 4, there is retarded or no growth of fungal species when using *L. amylovorous* LAB or LAB-CFS as antagonists, respectively. In general, the anti-fungal LAB-CFS was very efficient at inhibiting or preventing fungal growth to the end of the trial, up to 7 days, when using the coffee isolated strains. Further analyses using a wider array of LAB strains would be of particular interest against mycotoxigenic and phytotoxigenic fungal isolates. Additionally, future research should focus on analysis of the anti-fungal compounds present in the antagonistic LAB strains. To summarise, LAB and LAB-CFS perform well as fungal antagonists *in vitro* and could potentially replace chemical fungicides.

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Efficient Digestion of the Coffee Bean Cell Wall Using a Selected Food-Processing Cellulase

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SUMMARY

Screening of effective food processing-cellulase for digestion of cell walls of coffee beans was carried out, and the cellulase from *Trichoderma reesei* was selected. The digestion of the cell walls of green and roasted coffee beans was carried out by sequential procedures of autoclaving with 0.1 M NaOH, and cellulase re-digestion. The cell walls were easily broken into micro pieces. The enzyme solution were eluted with the size exclusion chromatography of Sephacryl S-200, then two active fractions were found, The key enzymes of the fractions were investigated by SDS-PAGE and LC-MS/MS. In the supernatant of the digestion of the alkali treated coffee beans, the high molecular galactan along with low levels of arabinose, digested oligo-mannose, and mono sugar of glucose and arabinose from the arabinogalactan protein were found by size exclusion chromatography of TOSO-HW50. In the precipitated micro particles, rhamnose and galacturonic acid were newly found as ratio of 1:1.86. The microscopy observations staining with calcofluor white for the cellulose of the digested cell wall were investigated. The cellulose was completely digested off, and the blue white-fluorescence was not stained in the residual cell-wall segments and the collapsed particles. The key enzymes of the selected cellulase are studying and identifying for the efficient digestion.

INTRODUCTION

Raw crops and their materials should be understood for the more usage to make more good products, processing, and also application of their residues. For these purposes, enzymes are often used for the tools. For coffee beans cell-wall, many researchers have been reported and cleared the compositions of the cell wall. Although huge amounts of coffee beans are processed annually and their residues are valuable resource, there is little known about the enzymatic digestion of the cell wall of coffee beans. Generally, mannanase would be described as a efficient enzyme to digest the cell wall of coffee beans, but the reports are hardly to find. Plants are principally made of carbohydrates, and proteins; therefore, effective digestion is possible with some enzymes and chemical or physiological treatments. However, fibers, cell walls, and tissue contents of coffee beans have a unique composition or complex such as galactomannan and arabinogalactan protein (AGP), and they have been sometimes strongly resistant to general enzymes.

Our study aims to achieve efficient enzymatic digestion of coffee beans, and various trials and screenings of food-processing enzymes have been carried out. In our results, a food-processing cellulase from *Trichoderma reesei* was found to be an efficient enzyme for digesting the coffee bean cell-wall, and the cell wall was drastically collapsed and digested.

In this presentation, we would like to present the digestion components and characteristics of this efficient enzymatic digestion of coffee bean cell-walls, and also investigate the characteristics of the key enzymes or the mechanism.

MATERIALS AND METHODS

Coffee beans

Coffee beans (*Coffee arabica*), the roasted coffee beans, and their cracked and milled powders were gifts from UCC Ueshima Coffee Co., Ltd., Kobe, Japan. The defatted powdered samples were prepared with a 10-fold volume of hexane extraction at room temperature for 24 h. The sample was then washed with hexane and dried at room temperature.

Enzymes

Cellulase (for food-processing cellulase from *Trichoderma reesei*, 5000 units/g) was a gift from Godo Shusei Co., Ltd. (Tokyo, Japan). Pectinase (Pectinex Ultra SP, from *Aspergillus aculeatus*; 26000 units/ mL) and Cellclast 1.5L FG from *A. aculeatus* were gifts from Novozyme Japan. Cellulase XL531 from *Aspergillus niger* was a gift from Nagase chemtex, Japan. Cellulocine GM5, AC40, and HC100, cellulases from *Aspergillus* sp. and TP25 and T2 from *Trichoderma viride* were gifts from HBI, Inc., Japan. Other cellulases were gifted from several companies, the screened cellulase were 23 kinds. All other reagents were of reagent grade.

Screening of Enzymes

The roasted residues was autoclaved with 0.1 M NaOH at 121 ºC for 60 min. The treated residues were washed with water and 0.1 M acetate buffer (pH 5.0) and placed in a 96-well microplate; a 1 or 5% enzyme solution (0.1 M acetate buffer, pH 5.0) was mixed at 40 ºC overnight, and the collapse or any visual change was observed.

Digestion of the cell walls of sliced green coffee beans

The green coffee beans were autoclaved with water at 121 ºC for 10 min. The cooled beans were sliced, and the sliced sections were autoclaved with 0.1 N NaOH at 121 ºC for 10 min. The milled powdered coffee beans or the sections were incubated in an eppen-tube with 1% the selected cellulase from *Trichoderma reesei* at 40 ºC for 24-72 hr.

Estimation of sugar and protein

The amount of uronic acid was measured according to the 3-phenyl phenol method. The total sugar was estimated by using the phenol-sulfuric acid method. The reducing sugar was estimated according to the Nelson-Somogyi method. Protein was estimated by using the Bradford method. Each amount was calculated using a colorimetric standard curve with D-galacturonic acid, D-glucose, and serum albumin as the standards.

Neutral sugar analysis

Analysis of the neutral sugars was done using the alditol-acetate method. The composition of the neutral sugar was analyzed by a GC system with a capillary column of DB- 225 (J&W Co., 0.25 mm × 30 m) connected to a Yanaco G-2800 (Yanaco Co., Ltd., Kyoto, Japan). The standard solution was adjusted with a 1% solution of each of the seven kinds of following

sugars: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose.

Staining

Calcofluor white were used for the staining reagents of the coffee residue. Calcofluor white, BactiDrop, was obtained from Remel, Lenexa, KS.

SDS-PAGE and LC-MS/MS

SDS-PAGE was done according to the Laemmli method. The electrophoresis equipment used was an AE-9631M/P with an attached concentration slope gel (5-20 and 10- 20%) (manufactured by ATTO, Tokyo, Japan). The standard molecular marker was the Kaleidoscope Prestained Standards from Bio-Rad. The buffer system was Tris-glycine or Tris-tricine, pH 7. The each protein band was digested by trypsin (trypsin gold, Promega), and LC-MS analysis was carried out by Hitachi Nano Frontier LD.

Light and fluorescent microscopic observation

The microscopic observations and photos were done using an Olympus BH-21 (Olympus Optical Co., Ltd., Tokyo, Japan) light microscope and a digital DP-II microscope photographic device. Fluorescent microscopic observations and photos were done using an Nikon Eclipse E-600 (Nikon Co., Ltd., Tokyo, Japan) fluorescent microscope and a digital Hamamastu ORCA- ER C4742-95 camera (Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan).

RESULTS AND DISCUSSION

Screening of cellulases

Twenty three-kinds of cellulases as food- processing enzymes (1%, 0.1 M acetate buffer, pH 5.0) were mixed with the alkali-treated coffee residue (0.1 M NaOH, 121 C for 60 min) at 40 ºC overnight. The diluted alkali treatment was effective for pre-treatment of coffee cell-wall. In the cell walls without the body complex or residual coffee brew by the diluted alkali, the autoclaving with water (121 C for 10 min) was sufficiently effective for the digestion of the outer cell walls. The most of the tested cellulases were not effective to digest, however, only the cellulase of *Trichoderma reesei* (GODO-TCF) caused the collapse of residues. The cells were dispersed, and the cell walls were partially digested. The decomposition of the cell walls was recognized as the autoclaving treatment and the cellulase effectively digested the cell walls of the coffee beans (Figure 1).

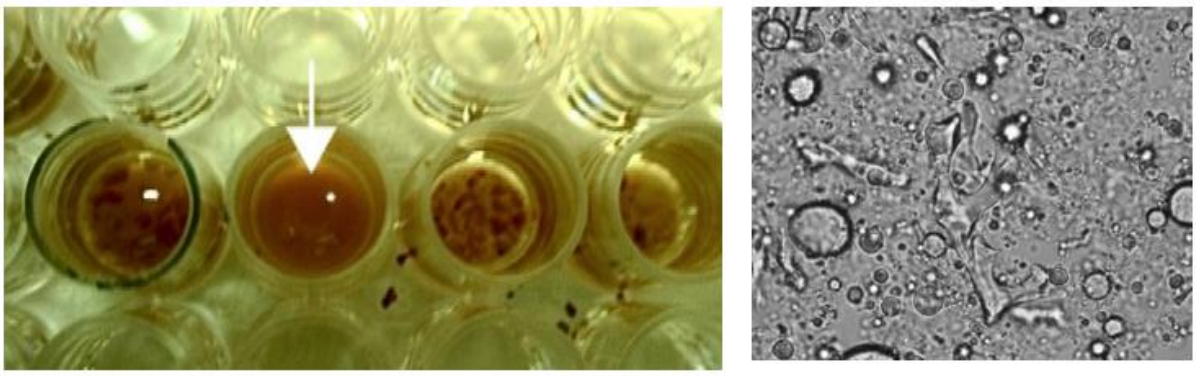

Figure 1. A food processing enzyme was selected as a efficient enzyme to digest coffee bean cell-wall (Left). The photo of microscopy of the digested and collapsed coffee bean cell-wall by the selected enzyme (Right).

Investigation for the key enzymes of the selected cellulase

The key enzymes of the cellulases was investigated by the size exclusion chromatography of Sephacryl S-200 HR. The eluted fractions were reacted with micro milled coffee beans in 96 hole plates. Two active fractions were found as shown in Figure 2. A fraction was in high molecular fraction in fraction No.29, and another one was low molecular fraction of Fraction No.39. The Fraction No. 39 was only to digest and to occur strong collapse the cell wall, and CMC and mannan were well digested. Mannan from brewed coffee was well digested in fractions from No.30 to No.40. The fraction No. 29 was synergetic for the fraction No.39, the digestion activity was strengthened as 6-fold.

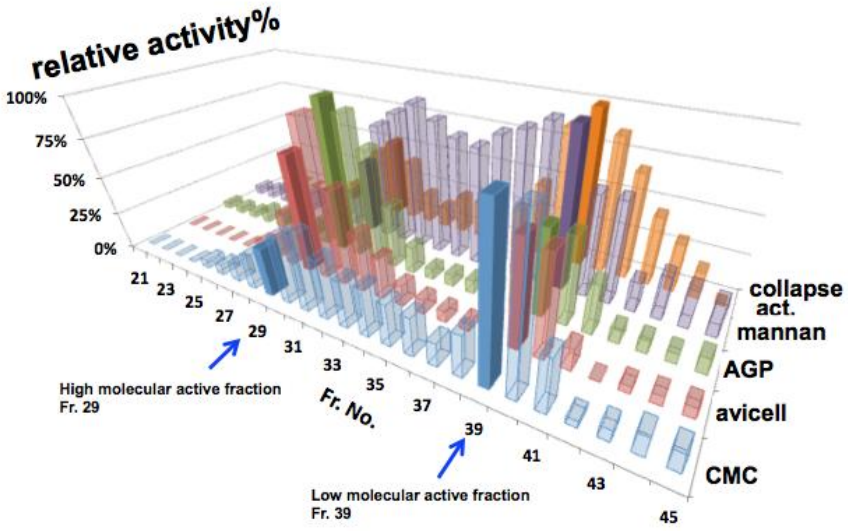

Figure 2. A size exclusion chromatography of Sephacryl S-200 HR of the slected cellulase from *Tricoderma reesei* of GODO TCF. The graph shows the overlayed activities of the collapse, digestion of mannan, AGP, avicell (micro crystalline od celulose) and CMC of the each fraction. The two fractions of Fr. 29 and Fr.39 were found as characteristic active fractions.

SDS-PAGE and LC-MS/MS analysis of enzymes of the active fraction

SDS-PAGE and the LC-MS/MS analysis of the two active fractions were carried out, and the gragments were searched in the CAZy data-base. α-*N*-Arabinofurasidase was detected in the former fraction, and xylanase II, endo glucanaseVII, endo-xylanase of *Tricoderma reesei* were found in the latter fraction, but mannase was not found as a main enzyme.

Analysis of the digestion of cell wall of coffee beans by the eluted fractions

Micro milled coffee beans were alkaline treated and digested for 2 days by the highest active fraction, the soup sugar analysis was carried out. The reaction mixture was centrifuged, and the precipitated the micro broken fragments. In the micro broken fragments, rhamnose and galacturonic acid were newly identified, and the molar ration was 1:1.86. The results mean the rhamnogalacturonan existence. The inner pectinic part would be exposed by digesting the outer part. A sugar analysis-profile of size exclusion-chromatography of the supernatant of digestion with the enzyme of the active fraction No.39 was shown in Figure 3. Y-axis is sugar content, and X-axis is the fraction numbers. Polymerization numbers of sugars are expressed as from Left to right, 7-8, 4-5, 3, and monosugar. High molecular weight of galactan was found. Three, and 4 or 5 oligo mannans and monosugar of Man were also detected, this result mean that mannan would be digested to oligo mannoses. Mono sugar of Glc and Ara were also found. The total Gal and Ara ratio was 2:1. These results showed the most of Ara of AGP was well released. Mannanase were not found by the LC-MS analysis, but this result show the small amount of mannanase should be also essential to collapse. Mono sugar of Glc was also characteristic.

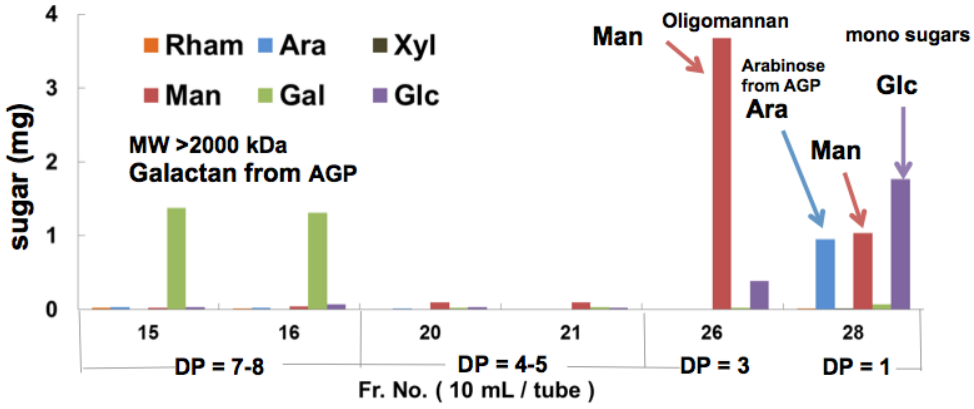

Figure 3. The sugar analysis of the digestion of cell wall from coffee beans are shown. The high molecular weight of AGP wihout Ara, oligo mannose and mono sugar of Ara, Man, and Glc were characteristic digestion.

Cellulase from *Trichoderma reesei* digestion of the sliced section of green coffee beans

The selected cellulase (1%, from *Trichoderma* reesei of GODO-TCF) digestion was carried out for the sliced green coffee beans treated by autoclaving with water (121 C for 10 min). Cellulose was stained by calco white flur, the segment was seen by fluorescent microscopy. In the before digestion segment, cellulose was clearly detectable as blue white fluorescent part. In the after enzyme reacted segment, the blue white part was not found. The result and the previous sugar analysis means that the cellulose was completely digested and formed mono

sugar of Glc. Therefore, Fraction No.39 had the activity of complete and strong digestion for the cellulose. These digestions would occur to easy collapse of the coffee cell wall.

AGP releasing and cellulose complete digestion would important to digest coffee cell wall. In the AGP digestion, Ara was mostly released, but the residual galactan was not digested to low moleculars, but the galactan would be mostly and still remained.

It is said that AGP would be deeply existed in the galactomannan, and the agglutinate the cell wall. Strong releasing of the Ara from the AGP is possible, and would be relaxing the agglutinated cell wall-components. This relaxing lead next strong cellulose digestion and mannan, and then the easy collapse of the cell wall would be possible.

Mannanase was not detactable in SDS-PAGE and LC-MS analysis, but small amounts of mannanase would be exist, and made to form oligo mannan.

We are now studying and investigate the high active fraction to clear the key enzymes, the each enzyme is now separately purifying and isolating, and investigate the action to the cell wall by microscopy. And, we are also studying on the function of the enzymes and the least desirable combination or why are the key enzymes, why is easy to digest by using this cellulase? Some trials on the digestions of the key components of AGP, and its pre-glycosylated protein or coffee bean hulls are also investigated for good coffee production.

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Impact of Crema Quantity and Appearance on Actual Coffee Perception

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SUMMARY

A set of six espresso coffees with different foam characteristics and similar above cup and in-mouth flavour sensory profiles was produced by combination of two varying parameters, the extraction pressure and the filtration of the coffee beverage. The coffees were subsequently evaluated in a comparative manner by a set of analytical (headspace, nose-space) and sensory (Temporal Dominance of Sensations) techniques. The presence of espresso crema in its standard quantity was demonstrated to be associated to the optimum release of pleasant high volatiles, both in the above cup headspace and in-mouth. On the other hand, the TDS study demonstrated that increasing amount of crema was associated to increasing roasted dominance along coffee consumption.

INTRODUCTION

For consumers, the smooth, dense, hazelnut brown foam (the so called “crema”) on top of a freshly brewed espresso is highly appreciated as part of the coffee experience. Crema is formed during the extraction of the coffee as a result of pressure created in the coffee bed and gas present in the roast and ground. The gas phase of the crema consists of water vapour from the percolation process and carbon dioxide due to the Maillard reaction during the roasting process of coffee beans, as well as aroma molecules that are released from the roast and ground coffee during the extraction.

The crema plays a very important and integral role in the coffee tasting experience. A visually appealing smooth and dense crema indicates a good extraction of the coffee. In previous work we have shown that the crema acts as a vehicule to release aromas above the cup and will therefore impact the smell of the coffee. Finally, it is well known that the crema brings smoothness and body to the espresso coffee. In the current study we aimed to understand how the crema quantity impacts the in mouth sensory perception, the coffee headspace and the in-mouth aroma release.

MATERIALS AND METHODS

Commercial Nespresso blend was used. All capsules came from the same batch. To modify the foam properties, series of six coffees with foam were produced by playing with the extraction pressure (Normal pressure = 12-16 bars; High pressure = 20 bars), and/or the filtration of coffee after extraction. For filtration two fabric types were used: Petex 07-64/45 (based on polyethylene terephthalate, 64 mM pore size, 45% open area) and Fluortex 02-70/22 (based on polyvinylidene fluoride, 70 mM pore size, 22% open area). A third type of filter, Petex 07-33/21 (based on polyethylene terephthalate, 33 mM pore size, 21% open area)

was used for totally removing the crema. This produced a reference coffee without foam. Coffees were prepared using standard or modified Nespresso machines. Coffee sample preparation was done by filling the machine with Acqua Panna mineral water (Sanpellegrino S.p.A., Milan, Italy), placing the capsule inside and setting the operating pressure. A transparent coffee cup, either with or without filter according to the coffee prototype, was placed below the coffee nozzle exit and on top of a Mettler type PM6 balance (Mettler Instrument AG, Zurich, Switzerland). Coffee preparation was carried until an approximate volume of 40 mL (i.e. exactly 40 g, as measured with the balance) were extracted into the cup. A sensory profiling was carried out on the entire set of 7 espresso coffees using 12 trained panellists. The profiling confirmed that neither the odour nor the in-mouth attributes were significantly affected by the preparation parameters (i.e. pressure and filtration).

Caliper method

Foam volume was determined using a digital caliper (Garant IP65-CI50, Hoffmann Group, Munich, Germany). A detailed description is given in *J. Agric. Food Chem.*, 2011.

Crema volume decay

Crema volume decay was followed dynamically inside the cup by using computer-assisted macroscopic imaging and image analysis. The method consisted in placing the freshly prepared coffee cup inside a dark chamber directly in front of an Infinity 2 digital microscope camera (Lumenera, Ontario, Canada) connected to a zoom lens 18-108/2.5 (Computar, California, USA) and illuminated using a light ring TL-E Circular 22W/33-640 (Phillips, The Netherlands). The camera, focused at the middle of the cup at the crema/liquid interface, was used to take a time image sequence. Images of the draining coffee crema were taken every 15 seconds during 5 minutes (first image was taken 15 seconds after coffee was prepared, time required to transfer the cup and initiate image acquisition). Image storage and following analysis were done using in-house developed software (Colibri). Quantitative analysis of the volume decay was performed by selecting the analysis region over the image and calculating then the crema area in every picture of the time sequence. Data was normalized by equating the area at t = 0 s to 100 %. All samples were analyzed in triplicate.

PTR-MS (head space and nose-space)

Details on The Proton Transfer Reaction Mass Spectrometry (PTR-MS) instrumental parameters as well as the assignment of the major volatile contributors to PTR-MS ion traces are described in detail in *Food & Function*, 2012 and *Anal. Chem*., 2005.

Headspace aroma was measured by PTR-MS as described by *Anal. Chem*., 2008. A double-jacketed, water-heated sample cell (glass vial) was placed inside an oven at a temperature of 100 °C with active air circulation. A water bath at a temperature of 50 °C was connected to the sample cell to keep the sample at constant temperature. To be as close as possible to a real consumer situation, the coffees (40 g) were directly prepared in a small size espresso cup. Then the cup was placed inside the water-heated cell at 50 °C. The sample cell was connected to the fix-installed top cover of the cell by a clamp and sealed by a Silicone O-ring. The coffee headspace was purged at 300 mL min-1 and diluted with synthetic air at 3000 mL min-1 prior to entering into the PTRMS. The above the cup aroma release was measured 90 s after preparation of the coffee beverage (time after the last drop of coffee has fallen into the cup) and followed during 6 minutes.

Nose space aroma was measured on 8 panelists that also participated in the TDS study. Each panellist had an individual nose-piece, tailor-made in order to smoothly and comfortably fit into the nostrils. The nose-piece was fixed on laboratory eyeglasses. Nose-space air was sampled via two 6 mm OD glass-tubings fitted into the nostrils and connected to the main part of the nosepiece with a ¼ inch OD Teflon tube. This Teflon tube connector allowed proper adjustments in distance and height of the glass-tubings, to be placed just at the nostril outlet of the panellist. The air from both tubes was combined and directed to a PTR-MS apparatus through a flexible and heated tubing. A small fraction of the nose-space air was sampled and introduced into the drift-tube of the PTR-MS. The tasting protocol was aligned with that of the TDS study, i.e. each coffee was consumed in 7 sips. At every sip, only the data of the first breath cycle was taken into account, as it was by far the richest in terms of aroma concentration. Furthermore the other cycles had about the same proportions of m/z, so there was no big advantage of considering them. Each coffee was evaluated in four replicates.

A detailed description of the analysis of the PTRMS (head space and nose space) data can be found in Barron 2012.

Temporal Dominance of Sensations (TDS) study

A panel of 16 trained coffee tasters was used for this study. The panellists were trained first on the attributes to describe the products, then on the TDS method. The TDS was performed on 7 sips, starting 90 seconds after coffee preparation. This number of sips was standardised for all panellists and allowed the consumption of the whole cup. For each sip, the panellists had to choose the dominant attribute of in-mouth perception among a list of eleven attributes: carbony, roasted, cereal, fruity, sweet, bitter, acidic, liquid, thick, gritty and silky. The panellists had 30 seconds to perform this evaluation between two sips. The very same analysis approach as for the nose space data was conducted on the TDS data, but the variable taken into account was the dominance rate over the panel.

RESULTS AND DISCUSSION

Sample space

A caliper method was used to validate the variation in crema volume between the different samples. The crema stability was measured using macroscopic imaging. It can be observed from
Figure 1A that the crema volume for the coffee extracted at high pressure (HP) is larger than the reference coffee (NP) and that the filtered coffees had a smaller crema quantity (FC/FCH). It is shown in Figure 1B that the crema stability was lower for the coffees extracted at high pressure vs the reference coffees.

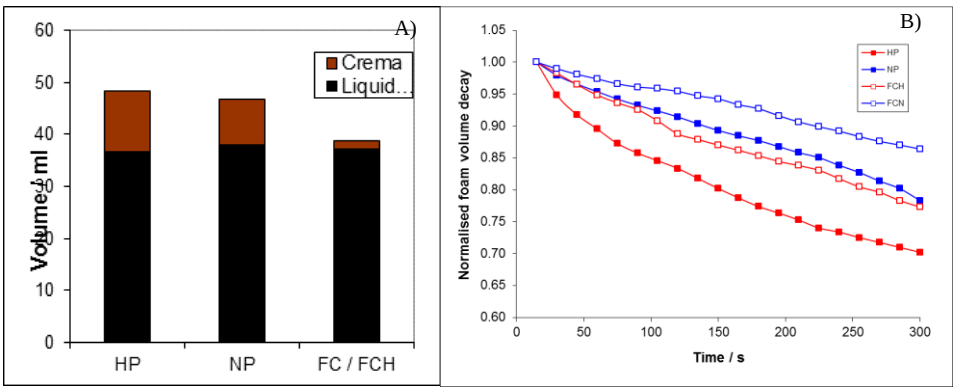

Figure 1. A) Crema volume at time 0 and B) crema volume decay over time (right) for espresso extracted at normal pressure (NP), with high pressure (HP) and with filtered coffee extracted at normal (FC) and high pressure (FCH).

In the next step the influence of crema on coffee headspace aroma release was studied. The two ANOVAs in
Figure 2A demonstrate that the impact of pressure and filtration on crema volume and stability did not impact the same m/z ion traces. The masses most impacted by pressure (increased crema quantity vs normal pressure) were in majority tracers of low volatiles. When pressure was increased the amount of low volaties was decreased. The masses most impacted by filtration (decreased crema quantity vs normal extraction) were the tracers of high volatile ones. These findings were confirmed in a PCA analysis. The projection was based on the coordinates of the observations on the two principal components. The coordinates of the projected variables corresponded to the correlation between the coordinates of the observations on the principle components and the respective pressure and foam data recorded for each sample. The masses pointing to the left (
Figure 2B), i.e. in the opposite direction compared to pressure were negatively correlated to pressure, so they decreased if pressure increased. These ion traces were in their vast majority tracers of low volatiles (
Figure 2B; circled in red). The masses negatively correlated with filtration, i.e. pointing to the top of the graph, increased when decreasing filtration (i.e. the foam amount was higher). These ion traces were all tracers of high volatiles (
Figure 2B; circled in green). The masses positively correlated with filtration, i.e. pointing to the bottom right of the graph, increased when increasing filtration (i.e. the foam amount was lower). These were in majority tracers of low volatiles (
Figure 2B; circled in red). It was noteworthy that the unfiltered coffees (NP and HP) released the highest amount of high volatiles in the headspace. Among them, 2-, and 3-methylbutanal (m/z 41 and 58), methanethiol (m/z 49), dimethylsulfide (m/z 63), and 2-methylpropanal (m/z 73) which have been previously demonstrated to contribute to the pleasant aroma freshness of roasted coffee.

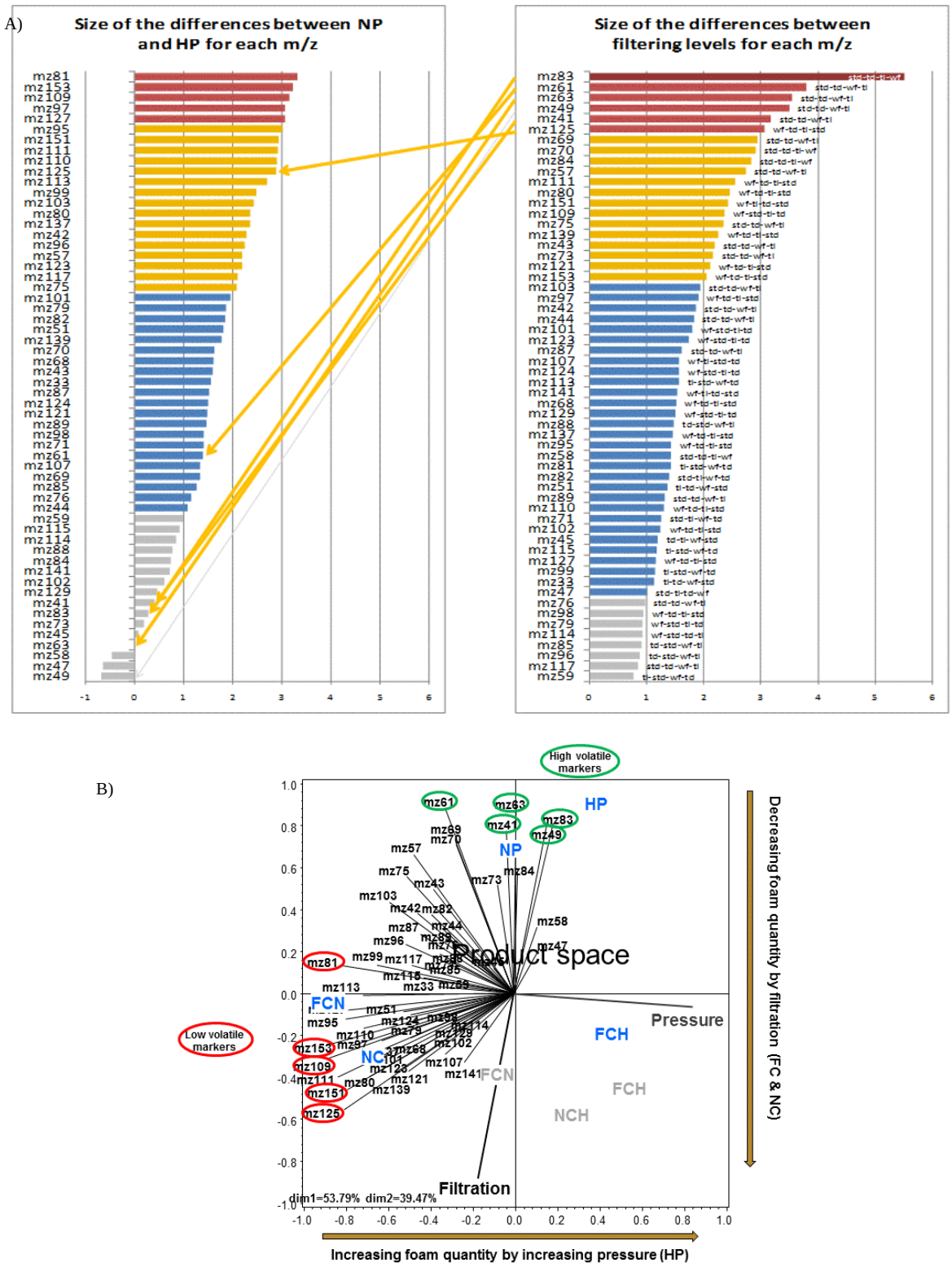

Figure 2. Statistical correlations between above the cup aroma release and the parameters of espresso coffee preparation. A) ANOVA analysis. The classification of ion traces was made according to their resolution. Red: >3. Yellow: between 2 and 3. Blue: between 1 and 2. Grey: <1. The arrows represent the correspondence of the m/z values between the two ANOVAs. B) PCA analysis. The X axis is mainly related to the influence of pressure (increasing pressure from right to left). The Y axis is mainly related to the influence of filtration (increasing filtration effect from top to bottom). The ions traces circled in green are tracers of high volatiles, while those circled in red are tracers of low volatiles.

Next the coffee aroma release was investigated in vivo in a method called nose-space analysis. The sensitivity and the dynamic capability of PTR-MS allow the fast analysis of the aroma present in the exhaled air in a dynamic way, along the consumption of an entire cup of coffee. Here the technique has been applied to the comparative analysis of the in vivo aroma release of our espresso coffee set. The effects of pressure and filtration have been separately assessed. To be aligned with the protocol of the TDS study (see below), the coffees were consumed in 7 sips. In
Figure 3 is shown the relative release compared to other products and sips. It can be concluded that there is an impact of filtration. While all filtered samples have a total amount of aromas which is lower than the non-filtered samples, the increased pressure does not impact the presence of aromas in the nose-space.

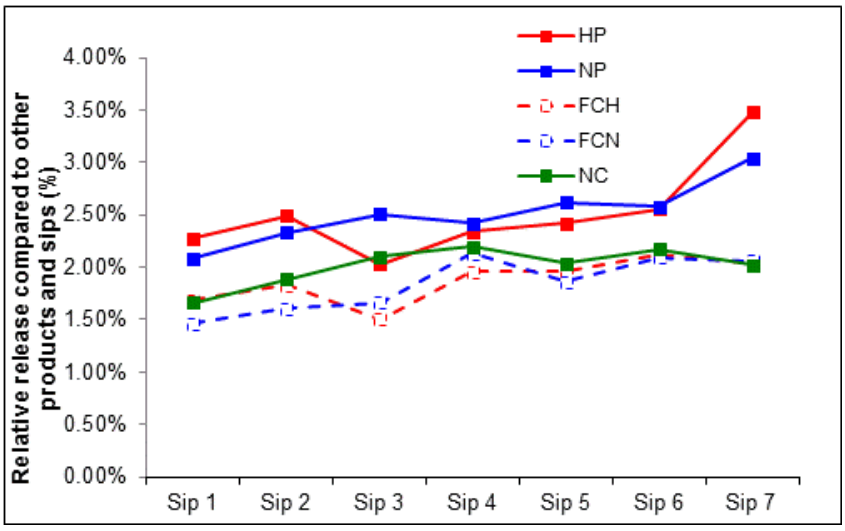

Figure 3. Impact of the amount of foam modulated by filtration on the in-mouth release of selected high and low volatile ion traces. The Y axis is the relative release of the ion traces under the nose space peak for each ion trace at the first breath cycle of each sip, compared to the other products analysed.

Next step was the sensory dynamic evaluation of coffee consumption. For this purpose we selected the Temporal Dominance of Sensations (TDS). In TDS, the variable measured by the panellists is not the intensity of each sensory attribute, but the dominance. An attribute is considered as dominant when it triggers the most attention from the panellists. In this study, the dominance was followed during the consumption of the coffee cup. To be aligned with the protocol of the nose-space, the coffees were consumed in 7 sips. TDS proved to be very efficient in evidencing differences among our panel of espresso coffees. The dominant in-mouth sensation at the first sip and the evolution of the dominance during consumption varied depending on the considered espresso prototype. The main impact was observed for the roasted note (
Figure 4) as well as for the carbony note (not shown). When the amount of foam was increased by increasing pressure (HP), the roasted dominance was increased and remained the highest along the entire coffee consumption. In contrast, when no crema was present (NC), the roasted attribute was not dominant any more, whereas bitterness was dominant from sip 2 to sip 7.

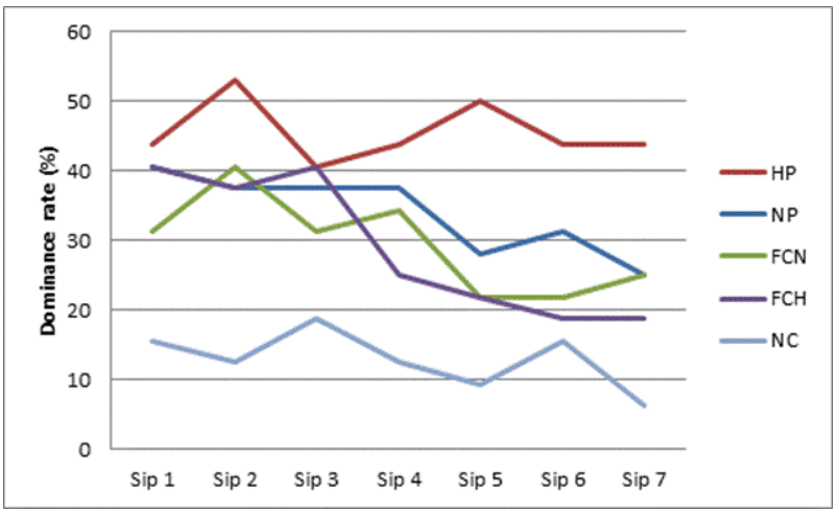

Figure 4. The effect of crema volume on the dominance of the roasted note in TDS profiling for espresso extracted at normal pressure (NP), with high pressure (HP) and with filtered coffee extracted at normal (FC) and high pressure (FCH) and with no crema (NC).

CONCLUSIONS

In this study, the aroma release of espresso coffees with crema of different physical characteristics were studied. It was confirmed that the above and in cup sensory profile of the coffees was similar for the coffees with different cremas. The cremas were however different both in quantity and in stability. Some general trends could be deduced from the experimental work which show that even for coffees with similar sensory profiles, differences can be observed both analytically and by sensorial methods.

Reducing crema quantity through filtration lead to reduced aromas above the cup as measured by PTR-MS. The nose-space study could confirm this as a reduced amount of aromas were analysed, especially high volatiles. Finally, the TDS study showed a reduced dominance of the roasted note, which may be related to the reduced amount of high volatiles as was observed both in the analytical above the cup and nose space study. It could be hypothised that the reduced amount of high volatiles as caused by filtration, could be related to a reduced crema quantity as well as a lower crema instability vs the corresponding non-filtered coffees.

When increasing the amount of crema by increasing the pressure PTR-MS data showed an increase in the release of more high volatiles in the head space. Furthermore, among these high volatiles released in the headspace, a good number were contributors to the pleasant freshness note of roasted coffee. The presence of crema (no difference observed for HP and NP) also favoured the in-mouth aroma release of high volatiles along coffee consumption. The recently developed TDS sensory technique proved once again here its efficiency in distinguishing coffees having different crema properties. The presence of foam was shown to be associated with the dominance of the roasted attribute.

Overall, the presence of crema favoured the release of pleasant high volatiles and removing part of it by filtration was clearly shown to be detrimental. On the other hand, there was no real added value to significantly increase the crema amount in espresso coffee by increasing the extraction pressure.

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Milk Modifies Sensory Properties and Reveals Unexpected Notes

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SUMMARY

More than half of coffee beverages worldwide are drunk in combination with milk. The in-cup result can range from a dash of milk to a latte macchiato, beverage dominated by milk. The choice of beverage depends both on culture as well as on personal preferences.

The choice of coffee to be used for the preparation of the milk based beverage is of very high importance. General assumptions are to use strong coffees in order to allow the perception of the coffee aromas in the presence of milk.

In the current study we aimed at understanding how a coffee range with a wide aromatic variety will express its aromatic notes in the presence of milk, and more specifically, how the quantity of milk impacts the result in cup. Thirty assessors were invited to four sensory sessions, each one dedicated to a specific beverage i.e. black coffee, coffee with a dash of milk, cappuccino and latte macchiato. The sensory procedure was the same for each session and consisted in sorting tasks followed by description of the groups using a common list of attributes.

The comparison of mappings obtained with different milk quantities shows that the perceptual space is modified. However, this modification is not due to a simple masking of coffee notes in presence of milk but to a real modification of the coffee sensory properties. Thus, some of the sensory dimensions of the black coffees remains, some seem to be masked and new ones seem to be revealed as biscuits or caramel notes.

It can be concluded that not only intensively roasted coffees should be recommended and that distinctive coffees allow the preparation of differentiated milk based beverages.

INTRODUCTION

There are various reasons to add milk to a coffee beverage and the usage depends on personal preferences but also on culture and habits. Some people add milk to reduce the bitterness and strength of the black coffee. Milk can also be added to create a recipe where the pleasurable moment is the focus. The most common way of adding milk to coffee is through a dash of milk, also called ‘cortado’ in Spain or ‘noisette’ in France. The most common recipe with frothed milk is the cappuccino. The consumption of coffee and foamed milk recipes is often considered more modern, stemming from the coffee shop culture.

While milk quantity and coffee strength are well known to play a role in the sensorial profile of the cup, less is known about the aromatic variety of the coffee. Until now the recommendations for milk based recipe preparation has been limited to the choice of the milk and the milk fat quantity. To the coffee itself little attention was given.

In the current study we aimed at understanding how coffees with different aromatic specificities express themselves in the presence of different quantities of milk.

MATERIALS AND METHODS

Products

The thirteen beverages included in one set were prepared from 12 commercial nespresso espresso varieties, one variety being repeated. All the beverages were prepared using a commercial Nespresso Latissima +. Aqua Panna water and Cremo UHT milk (2.5% fat) were used for preparation depending on the recipes. Black coffees were extracted at 40 ml directly into double-wall stainless steel espresso cups. Espressos with a dash of milk were prepared in the same cups by adding 7ml of cold milk to a 40 ml espresso. Cappuccinos were prepared in porcelain cups by adding 40 ml espresso to 70 g foamed milk. This resulted in the addition of 50 g of liquid milk to the espresso. Latte macchiatos were prepared in glass cups by adding espresso coffee to 120 g of foamed milk. This resulted in the addition of 90 g of liquid milk to the espresso.

Assessors and sensory procedure

Thirty assessors participated in four sensory sessions, each one dedicated to a specific beverage i.e. black coffee, coffee with a dash of milk, cappuccino and latte macchiato. The sensory procedure was the same for each session. The assessors received a set of thirteen beverages presented according to a Williams' latin square. They were asked to sort the beverages according to the simililarity of their base beverage flavour. They were asked to gather beverages with a similar flavour and to separate beverages with a different flavour. They were free to make as many groups as they want and to put as many beverages as they want in each group. After sorting beverages, the assessors were asked to describe each group using a predefinite list of 41 attributes. This list of attributes was established after a free description session conducted with a small group of coffee experts. The assessors were free to add vocabulary if needed.

Data analysis

The results from the sorting study were consolidated into a similarity matrix prepared by summing overall the number of times assessors put a particular pair of beverages in the same group. The greater the number in a cell of the matrix the greater is the presumed similarity between beverages. Sorting data were analysed by cluster hierarchical analysis applying Ward criterion. A similarity level has been chosen arbitrary to compare results from the different sorting tasks.

Vocabulary results collected for the four beverages were consolidated in a co-occurrence matrix summing the number of times a word has been cited for a given beverage. Vocabulary data were analysed by principal component analysis. Flashes have been drawn on the PCA map to indicate increasing milk quantities and help to better understand the impact of milk addition on the beverage description.

RESULTS AND DISCUSSION

Figure 1 shows dendrograms, stemming from the hierarchical cluster analysis, for the four types of beverages. In these representations, the higher the distance is, the lower the sensory similarity is. The cluster analysis which was performed on the 4 dissimilarities matrix shows

that the number and the structure of the groups varies according to the milk quantity. This indicates that there is an interaction between coffee and milk leading to a change in the structure of the sensory space.

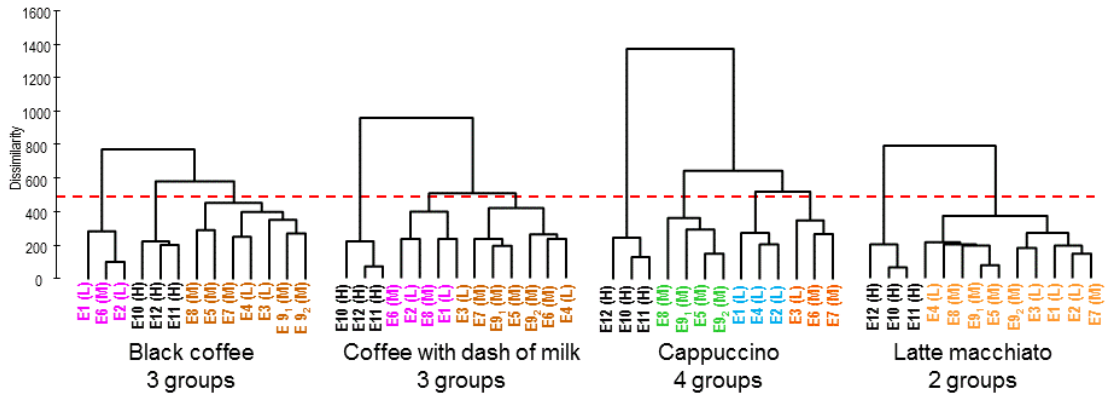

Figure 1. Dendrograms obtained by hierarchical cluster analysis for the four different beverages. From left to right: black coffee, dash of milk, cappuccino and latte macchiato. Coffee strength of the black coffee is indicated as low (L), medium (M) and high (H).

Three different groups have been defined from the black coffee sorting task, which are mainly defined by aromatic specificity. The first group consists of the very roasted coffees, the second group of the fruity coffees, and the third group of the cereal coffees. For small milk quantities the groups are not significantly changed vs the black cups. Coffees seem again to be gathered according to their aromatic families (very roasted, fruity, cereal). For larger milk quantities coffee, strength starts playing a more dominant role. In the case of latte macchiatos, only 2 groups separate the coffees where the strong coffees form one group and all other coffees are grouped together. Cappuccinos show a very interesting behavior with the highest number of groups explained by an interplay between sensory properties associated with both coffee and milk (i.e. coffee aromatic notes, coffee strength and the flavor of milk).

Placing all the products on a PCA (
Figure 2) which has been built using the sensorial vocabulary from the sorting study shows the evolvement of the coffee descriptions as a function of milk addition. The direction of the flash indicates increasing milk quantities. Even if each coffee keeps its own character, some trends can be identified.

The position of strong coffees (E12, E11, E10) will hardly change with milk. Theses coffees keep their character with a high body, strong roasted notes and bitterness.

Acidity as well as fruity notes which are associated with delicate coffees will gradually be masked when milk is added. On the opposite, milk reveals unexpected notes such as biscuit and caramel. Milk also brings sweetness as well as a smooth and silky texture.

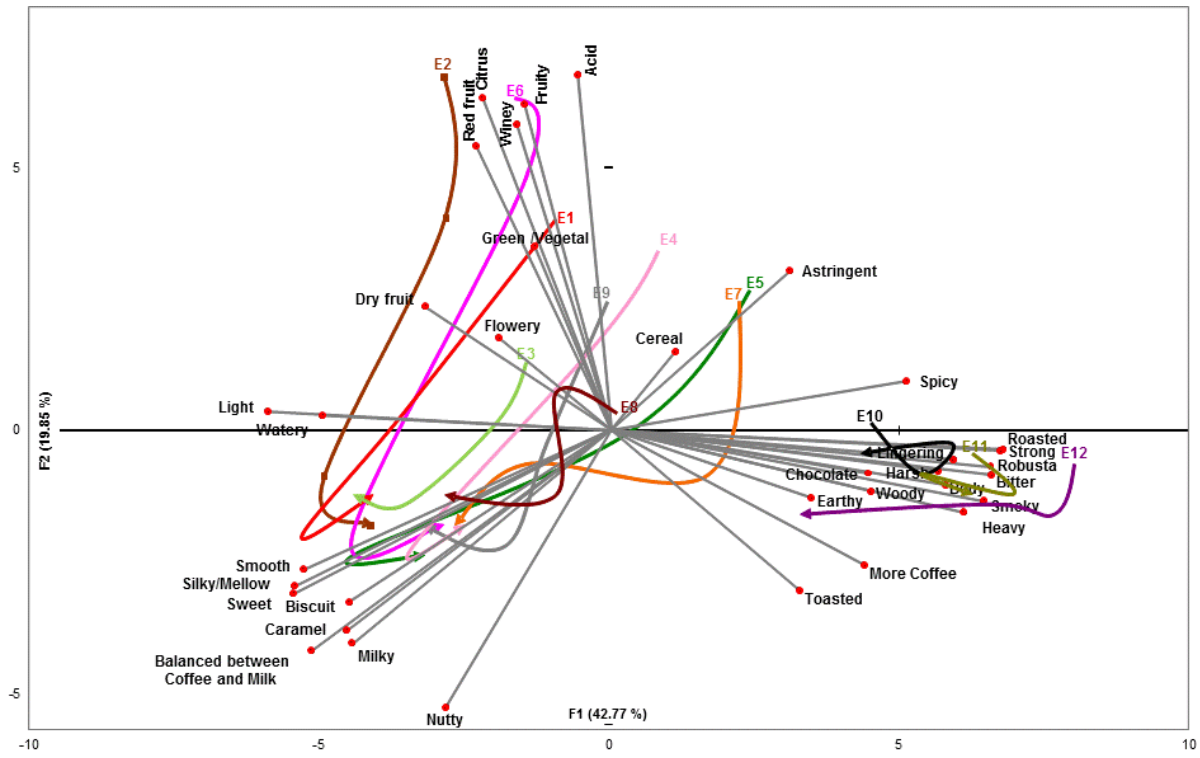

Figure 2. PCA in which the vocabulary is projected as well as the modification of each individual coffee when increasing milk quantities are added.

In conclusion, the coffee aromatic attributes, coffee strength as well as milk quantity have been shown to have a significant impact on the final in-cup result of a coffee and milk based recipe. Some aromatic notes and taste aspects will be enhanced, some level off and new ones will be revealed. While for small milk quantities the result is dominated by aromatic groups, for large milk quantities coffee strength will dominate. Cappuccinos show a more complex behavior as an interplay of aromas and strength will take place. From this study we can conclude that all twelve Nespresso coffees are of interest to prepare milk based recipes, each with their own distinctive character. The aromatic families are however modified, not only through a masking of aromatic notes but through a real modification of sensory properties.

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^{1}H-NMR Profiling of Green and Roasted Coffee Extracts: Classification of Colombian Coffees

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SUMMARY

The design of new products is crux for the competitiveness of Colombian coffee commercialization and thereby for the sustainability of half a million of coffee producers. As important is the ability to protect genuine Colombian coffee products from being adulterated with products of other origins. To achieve those aims several methods are currently being used, ranging from cupping to Near Infrared (NIRS) spectroscopy.

Here we present results obtained using Nuclear Magnetic Resonance (NMR) to profile and classify Colombian coffees.

INTRODUCTION

In order to ensure a constant income for coffee growers and a better product for the coffee consumers all over the world, the quality of the production has to be guaranteed if not increased requiring constant technical and technological developments. This quest for a better quality is sustainable only if it can translate into new products sold at a proper value and if the origin of this product, a region, a single farm, etc can be protected against frauds. Thus for decades, mechanisms are being developed to distinguish coffees.

Mainly two coffee species represent the vast majority of the global market, *C. Arabica L.* y *C. canephora Pierre*. The superior quality of the former is widely accepted and methods have been developed to discriminate them, initially looking for biomarkers, i.e., molecules that allow to differentiate both species. Methods based on the analysis of biomarkers, GC-MS and HPLC, include a separation step during which the different compounds are isolated and a detection step for the identification and quantification of each pure compound. Faster and cheaper methods have been developed that enable the analysis of the whole sample without the need for separation, NIRS and RAMAN have been demonstrated effective at determining the species of coffee beans and to monitor their origin. One drawback of NIRS is that the spectra cannot be directly interpreted in terms of the composition of the sample. This relation between intensity measured in the spectra and concentration of compounds in the sample is only possible after a model is build that implies determining the composition of a large sample sets.

Conversely, Nuclear Magnetic Resonance (NMR) is a powerful tool that allows to directly observing nuclear spins from different environment and form different molecules. It is thus possible to assign a signal in the spectrum with an atom of a molecule as illustrated in Figure 1, where several signals could be assign to caffeine for example allowing for its direct quantification. Indeed, in 1999, proton NMR experiments showed useful to study the composition of espresso coffees and a large number of the principal constituents were identified from the spectra. Recently it has been shown that discrimination of *C. arabica* vs.

C. robusta species was possible by direct integration of the one caffeine signal in NMR spectra. More recently, it has been shown that NMR spectra could be used to classify the origin of coffees from America, Africa and Asia and to even study the process of roasting by HR-MAS.

MATERIALS AND METHODS

Sample preparation

Samples were prepared readily by extraction in methanol at room temperature. 0.1 g (green) or 0.2 g (roasted) of milled coffee beans (45 μm) was extracted into 1.0 mL of non-deuterated solvent by vortexing 2 minutes. After two consecutive centrifugations at 8k rpm during 10 minutes, the supernatant was transferred into the NMR tube and 90 μL of the corresponding deuterated sample was added for the lock.

NMR experiments

^{1}H-NMR spectra were acquired with a Bruker 400 MHz Bruker spectrometer equipped with a triple gradient automatic indirect probe and a topshim board. A standard pre-saturation approach was chosen to achieve the suppression of solvent signals by applying a 25 Hz rf field during 4s. A special care was taken to calibrate the spectrometer before each run. Therefore, reference samples were used to precisely determine both the temperature and the shims, while a set of experiments were used to optimize the parameters for solvent suppression for each sample. After Fourier transform, apodization and baseline correction, the resulting 128k complex points vector was stored. Tuning, matching, shimming and calibrating the pre-saturation field frequency were performed automatically without any human supervision.

RESULTS AND DISCUSSION

Sample preparation

Several protocols were compared for the preparation of the samples and evaluated based on three criteria, the reproducibility, the cost and simplicity and the amount of signal present. For example, spectra obtained by extraction in aqueous buffer, methanol, chloroform and dimethylsulfoxide were compared. The differences can be appreciated in Figure 1 that led to select methanol for further experiments. Indeed, the amount of signal present in the 7-9 ppm advocates for the use of methanol and aqueous solutions. With the exception of quinic acid, all of the compounds identified by Tavares et al. have signal in that area that is much less crowded than the 4-2 ppm region were sugar signal dominate.

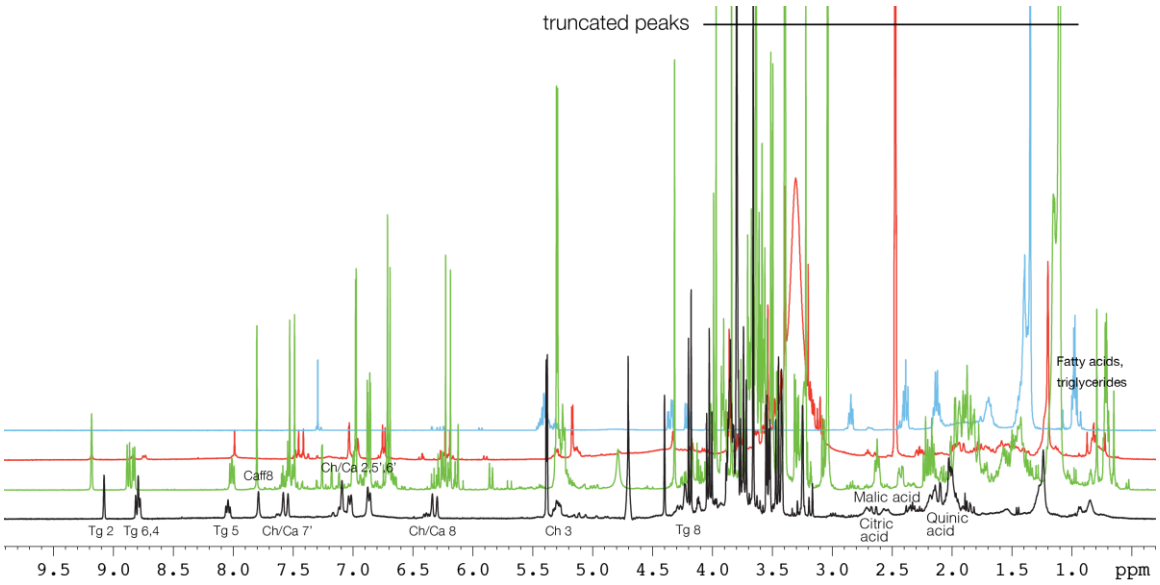

Figure 1. Spectra of green coffee in aqueous buffer (black), in methanol (green), in DMSO (red) and in chloroform (blue). The labels correspond to the assignment of main compounds found in aqueous buffer solution.

Test of reproducibility, achieved by repeated sample preparation, pointed out that the quality of the spectral baseline strongly depends on the concentration of particles in suspension. These latter were not completely removed by filtration on 45 μm membranes, thus leading to poor reproducibility. This result can be explained by the effect of small particles on the field homogeneity and consequently on the efficiency of the pre-saturation field. Conversely, the two consecutive centrifugations proposed in the previous section allowed to obtain clear solutions resulting in a highly reproducible spectrum.

Spectra of samples obtained by extraction at 80ºC were acquired and the intensity of the signal intensities in the region of interest were compared with spectra obtained for sample extracted at room temperature. Although a gain in intensity was achieved by increasing the temperature, this gain didn't compensate for the increasing complexity of sample preparation and for the potentially negative effects of temperature extraction on reproducibility.

Data pre-processing

Once acquired the spectra were apodized, Fourier transformed and phase corrected. This procedure is performed automatically and no manual interaction is ever permitted. A battery of statistical tests is performed on each original spectrum, including the determination of signal to noise ratio, position and intensity for several targeted peaks to estimate the quality of the experiment. At this point, outliers were eliminated from the analysis. Low quality shims, obtained automatically, and badly calibrated pre-saturation rf field were found the common causes for rejecting spectra, but not more than 10% of experiments were ignored.

Analysis and classification

A set of 176 coffees samples from different origins and different species (Colombia: 65, Suramerica: 26, Centroamerica: 37, Asia: 28, Africa: 20) prepared and analysed as described earlier was used to construct an expert system aiming at discriminating Colombian coffees from beans from other origins. The chosen method consists in consecutives binary

classifications using partial least square discriminant analysis (PLS-DA). Therefore, the opensource caret package for R software was used. The number of spectra selected for the construction of the model, the training set, was chosen to represent 80% of the total amount of spectra for each class, i.e., department. The remaining spectra, referred to as test set, were used to evaluate the predictive power of the model. This procedure was repeated 1000 times, each one with a different randomly selected training set. The resulting average confusion matrix was used to compute the sensitivity and specificity parameters reported in Figure 2.

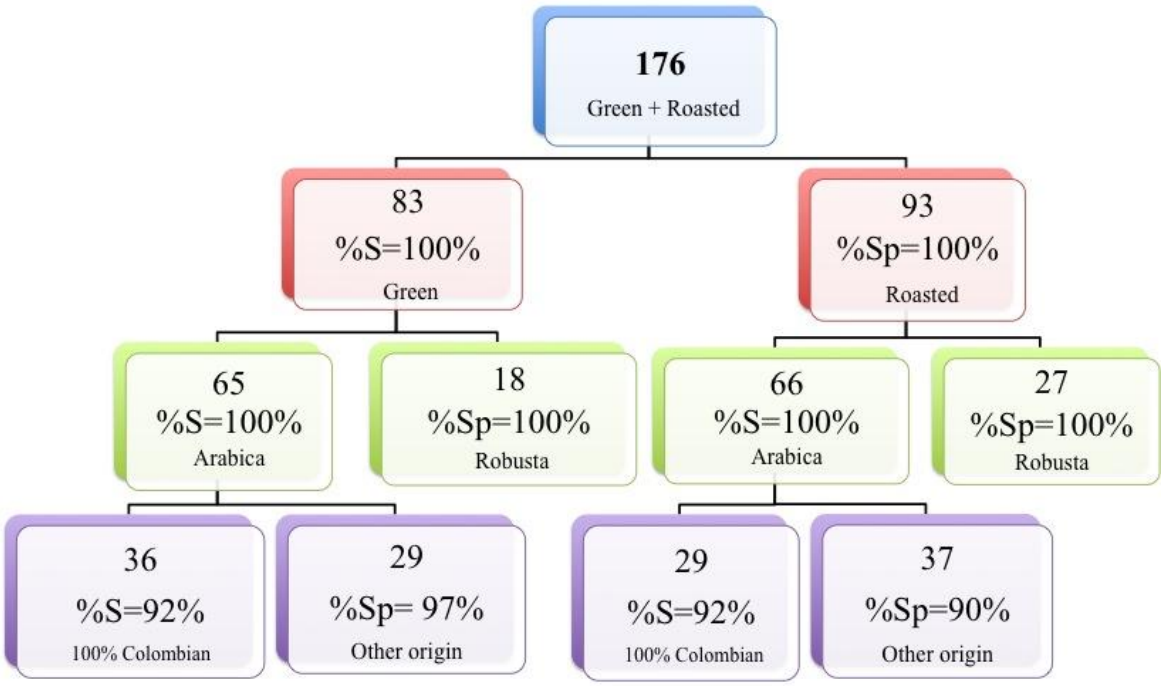

Figure 2. Classification of coffee samples. The sensitivity is calculated using S = TP / (TP + FN), while the specificity is obtained using the following relation Sp = TN / (TN + FP) and where TP, FN, TN, FP stand for true positive, false negative, true negative, and false positive.

The sequence of classification is organized as follows: first the presentation of the coffee bean is determined; green vs. roasted, second its species is determined. Finally, each *C. arabica* sample is categorized either as "100% Colombian" or as "Other origin". As expected, the presentation and species are readily determined and the classification of the coffees according to their origin is very reliable. Closer examination of misclassified origins shows that the confusion arises with coffees from Central America, Guatemala and Dominican Republic, which were mistaken with true Colombian coffees. The difference in specificity achieved for the two presentations is readily explained, since different coffee samples were analysed.

At this point is worth noting that no statistical pre-processing has been performed to the spectra, except a size reduction from 128k points to 2k points. No selection of particular signals or spectral regions has been performed, thus discrimination is completely untargeted. This means that further work can to be done to examinate the discriminant areas of the spectra, determine biomarkers involved in the discrimination and compare them with the information already available from the literature.

A set of 68 samples *C. arabica* coffees from five different Colombian departments (Cauca, Tolima, Huila, Magdalena y Norte de Santander) has been used to evaluate the discrimination power of our approach. In this case, the question that should be answered is: from which of thes e five departments does this coffee originate? The model was obtained using the same statistical tool as described for the previous analysis. The results are exposed in Table 1 and 2.

Table 1. Summary of the statistic achieved after 1000 repetitions of the classification have been performed. The diagonal elements represent the true positive, while the remaining elements of the same column represents false negative. The remaining elements of the same row represents false positive.

	Reference				
Prediction	**Cauca**	**Huila**	**Tolima**	**Norte de Santander**	**Magdalena**
Cauca	3170	418	415	0	9
Huila	806	3371	100	0	50
Tolima	15	205	2283	0	185
Norte de Santander	4	4	53	1000	20
Magdalena	5	2	149	0	1736

The results exposed in Table 1 can be recast in terms of sensitivity, also referred to as true positive rate (TPR), and specificity that can be expressed as 1 minus the false positive rate (FPR). Table 2 shows that classification is possible but the reliability drops when compared with the results obtained in the previous experiment. This result could be anticipated by looking carefully at the spectra. Indeed, it is possible to discriminate spectra belonging to different species by eyes, while the differences observed between spectra of coffee from different department of Colombia are much finer.

Table 2. Sensitivity and specificity achieved when classifying coffees from five different Colombian departments.

Department	**Population**	**%Sensibility**	**%Specificity**
Cauca	18	79.2	91.6
Huila	20	84.3	90.4
Tolima	15	76.1	96.3
Norte de Santander	5	100	96.4
Magdalena	10	86.8	98.7

Further work – including cutting edge statistical data analysis – and a higher number of spectra – obtained for samples representing additional variables, e.g. coffee processing, etc. – are required to increase the reliability of our expert system to separate coffee from different departments. However we would like to stress that both efforts might not suffice and that a plateau value is expected for both sensibility and specificity, since departments don't necessarily represent meaningful divisions in term of the agro-climatical parameters that are expected to translate into distinguishable coffees.

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Free Amino Acids Determination in Green Coffee Beans by GC-MS

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SUMMARY

Amino acids in green coffee are of extreme importance as they act as precursors for aroma formation during the roasting process. Some chemical classes of essential contributors to the roasted coffee aroma, such as pyrazines and pyridines, are only slightly represented in green coffee, while others are not present in the raw material as thiophenes, oxazoles and thiazols, being mainly formed through the Maillard browning by thermal reactions between amino acids and reducing sugars. Therefore, to better understand how the composition of the raw material can influence the final quality of the roasted coffee with respect to the aroma formation, the determination of the free amino acidic content in green coffee is essential. Moreover, as far as amino acidic profile is concerned, data on literature are scarce and often contrasting. This largely stimulated the present investigation aimed at characterizing the free amino acidic profile of several coffee samples from different species (*C. arabica*, *C. canephora and C. liberica*) and geographical origins. In order to be determined in a complex matrix such as green coffee, the free amino acids were first extracted with hot water, purified by solid phase extraction, derivatised and then analysed by GC-MS. The derivatization step, which consists in the alkylation of amino groups and the simultaneously esterification of carboxylic groups of amino acids, is crucial and it is required to turn amino acids into more volatile components that are consequently detectable by a gas-cromatographic technique. Using this procedure it was possible to detect more than 20 amino acids simultaneously in a single run in full scan mode. Generally, Arabica and Robusta show similar free amino acid profile. For both species main amino acids are glutamic acid, aspartic acid and asparagine. Liberica show a different profile, being similar the contribution of aspartic and glutamic acid. The content of γ-aminobutyric acid (GABA) in Robusta and Liberica is remarkably higher than that in Arabica. In the present work differences in free amino acids concentrations among different species and geographical origins will be discussed.

INTRODUCTION

Free amino acids in green coffee are of extreme importance, since they act as precursors of several compounds in roasted coffee, such as many aroma volatiles, colored substances and undesirable molecules like acrylamide. Free amino acid profiles have been studied in coffee by several authors during the past decades. The published results vary widely, depending on several parameters with species/varieties one of the most explored. The high potential of free amino acids as coffee species' discriminators has been underlined. Generally Arabica and Robusta have the same major and minor amino acids, and from a quantitative point of view Robusta shows a higher content of amino acids than Arabica. However, in comparing the two main coffee species, reported data show remarkable differences as far as the quantitative distribution of the individual free amino acids is concerned. In particular, in some cases

glutamic acid, asparagine and aspartic acid are indicated to be present as main amino acids in both Arabica and Robusta, with glutamic acid the main one for Arabica and asparagine the main one for Robusta. However, other studies indicate glutamic, aspartic and γ-amino-*n*-butyric (GABA) acids as main amino acids with glutamic the main one for both Arabica and Robusta some other indicate as the main free amino acids in green coffee alanine, asparagine and phenylalanine with alanine having the highest concentration for both Arabica and Robusta. As far as we know, *Coffea liberica* has not yet investigated as far as the free amino acid profile is concerned.

As regard to the great variations in amino acid concentrations determined within the main coffee species, this may be ascribed to various analytical and experimental set up including clean-up, extraction and detection methods, in addition to botanical, geographical and post-harvest processing reasons.

Analyses of amino acids can be carried out by GC or HPLC techniques. In both cases amino acids need to be derivatised. Concerning GC methods, this step is necessary in order to obtain volatile compounds whereas for HPLC, amino acids need to be derivatised to be detected by UV, fluorimetry or electrochemical techniques. Chosing the proper analytical method is crucial in selecting the range of amino acids which can be determined. In facts, depending on the analytical method, some amino acids can be overlooked or some others can be determined without resolution.

In the present work, free amino acidic profile of several green coffee samples from different species *(C. arabica, C. canephora and C. liberica)* and geographical origins has been determined by GC-MS after hot water extraction, SPE and derivatisation. The derivatisation step we used in present work consists in the alkylation of amino groups and the simultaneously esterification of carboxylic groups and by using this procedure it is possible to detect more than 20 amino acids simultaneously in a single run in full scan mode. In agreement with previous studies, free amino acid profile seems to be a promising marker for green coffee inter-species discrimination purposes.

MATERIALS AND METHODS

20 green coffee samples of different botanical species were studied: 7 *Coffea canephora var. robusta* from Cameroun, Ivory Coast, Brazil, Burundi, Uganda, Vietnam and Indonesia; 11 *Coffea arabica* (wet-processed) from Ruanda, Kenya, Brazil, China, Mexico, Ethiopia, India, Uganda, Guatemala and Burundi; 2 *Coffea liberica* (dry-processed) from Indonesia and India. Amino acids standards were all from Phenomenex (Torrance, CA, USA), with the only exception of GABA, from Sigma (St.Louis, MO, USA). Whole green coffee beans (30g) were extracted with 180 g of Milli-Q water at 80°C for 5 hours, under both constant stirring and reflux conditions. The extract, after filtration, was immediately frozen and kept to -20°C up to analysis. Average extraction yield ranging from 22.0 to 23.0 % w/w was determined independently on coffee botanical species.

100 µl of extract sample in the presence of 200 nmol of norvaline as internal standard were cleaned up through an ion exchange resin in order to reject the proteins. The amino acids were released from the resin through n-propanol used as an eluting medium. The samples were then derivatized through propyl chloroformate, which allowed the alkylation of the amino and the esterification of the carboxylic group of each amino acid simultaneously. The sample preparation was in accordance with the procedures described by Phenomenex in the usage of EZ-faast® kit for amino acids determination.

Compounds were eluted by a He gas flow of 1 ml/min in split mode (split 1:15) and separated using a 10 m ZB-AAA capillary column (film thickness 0,25 µm). The oven temperature, was initially set at 120°C, increased to 190°C at 20°C/min., then to 200°C at a rate of 30°C/min., to 205°C at 5°c/min., and then at the final temperature of 270°C at 30°C/min. hold for 1 minute. Mass spectrometer was set to electron impact mode (MS-EI) generated at 70 eV and mass spectra were collected in full scan mode, collecting ions from 39 to 500 m/z.

RESULTS AND DISCUSSION

In Figure 1 typical GC profile of major amino acids in Liberica, Arabica and Robusta is shown.

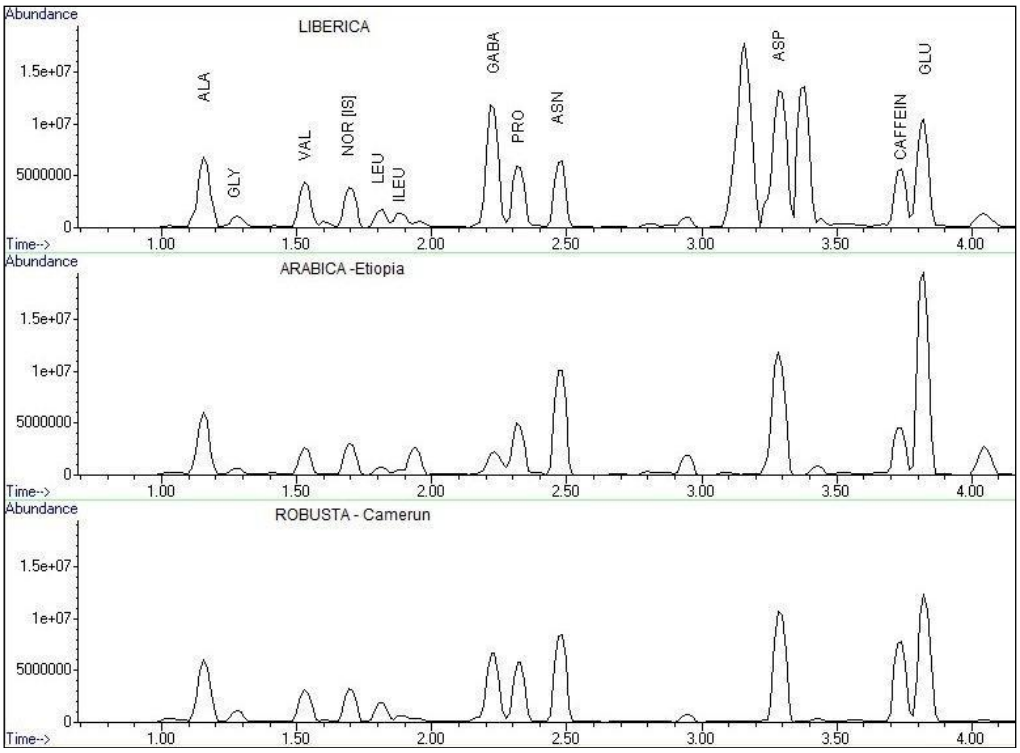

Figure 1. Typical GC profile for major free amino acids in Liberica, Arabica and Robusta respectively.

Arabica and Robusta show similar free amino acids profile. For both species main amino acids are glutamic acid (GLU), followed by asparagine (ASN) and aspartic acid (ASP), (with proline PRO as well in the case of Robustas) in full agreement with previous studies. Glutamic acid is more concentrated in Arabicas representing about 40% of total free amino acid concentration whereas asparagine, proline and in less extent aspartic acid are more abundant in Robustas.

Table 1. Average amino acid content (mg/kg of coffee) in Arabica, Robusta and Liberica green coffee samples.

	Arabica (n=12)	Robusta (n=7)	Liberica (n=2)
ALA	179	358	270
GLY	21	64	45
VAL	62	148	163
PRO	259	464	293
ILE	15	36	51
LEU	17	47	35
ASP	344	368	729
GLU	1554	1005	825
MET	29	89	116
PHE	88	177	112
HIS	16	29	40
LYS	31	111	50
TYR	30	120	62
TRP	41	154	43
B-ALA	-	3	10
GABA	305	633	448
ASN	357	612	329
SER	155	155	91
THR	47	83	58
Total	3550	4432	3763

However, there are significant differences depending on geographical origins; among Arabica samples, glutamic acid is more abundant in India and Ethiopia samples (around 1900 mg/Kg) and less in Brazil and China samples (around 1300 mg/Kg).

Liberica samples show a slightly different profile, being quantitatively similar the contributions of both aspartic and glutamic acid, and being γ-amino butyric acid (GABA) the third main amino acid. In comparing GABA concentration, after Liberica, Robusta is significantly richer than Arabica, and this may be the consequence of post-harvest processing of the examined samples.

In Table 1 the average concentration of amino acids is reported in mg/Kg of coffee. With the only exception of glutamic acid, free amino acids concentration is higher in Robustas than in Arabicas; in particular, asparagine and alanine were significantly higher. In a previous study proline has been found the only amino acid higher in Arabica but the present data do not confirm this finding. Minor amino acids for the three species are represented by glycine (GLY), leucine (LEU), iso-leucine (ILE), hystidine (HIS), and threonine (THR). The average total free amino acidic content determined in this study was 3550 mg/Kg (from 3007 to 3950 mg/Kg), 4432 mg/Kg (from 3405 to 5599 mg/Kg) and 3763 mg/Kg for Arabica, Robusta and Liberica, respectively. The higher concentration of total free amino acidic content in Robustas than in Arabicas is in good agreement with the literature, as already reported by several

authors. To our knowledge it is the first time total free amino acidic content of Liberica coffee samples is reported.

By resorting to PCA analysis, the free amino acid content of the whole set of samples has been processed in the attempt to cluster the coffee species (see Figure 2).

In the multivariate analysis, in addition to the concentration of free amino acids, both geographical origin and coffee species, were included in the data set. Figure 2, reporting the two principal components, clearly shows 3 distinctive groups: Arabica samples, characterized by higher concentration of glutamic acid, Liberica samples, clustered mainly by serine and aspartic acid, and Robusta samples, characterized by higher levels of GABA, asparagine and most of the other amino acids experimentally determined.

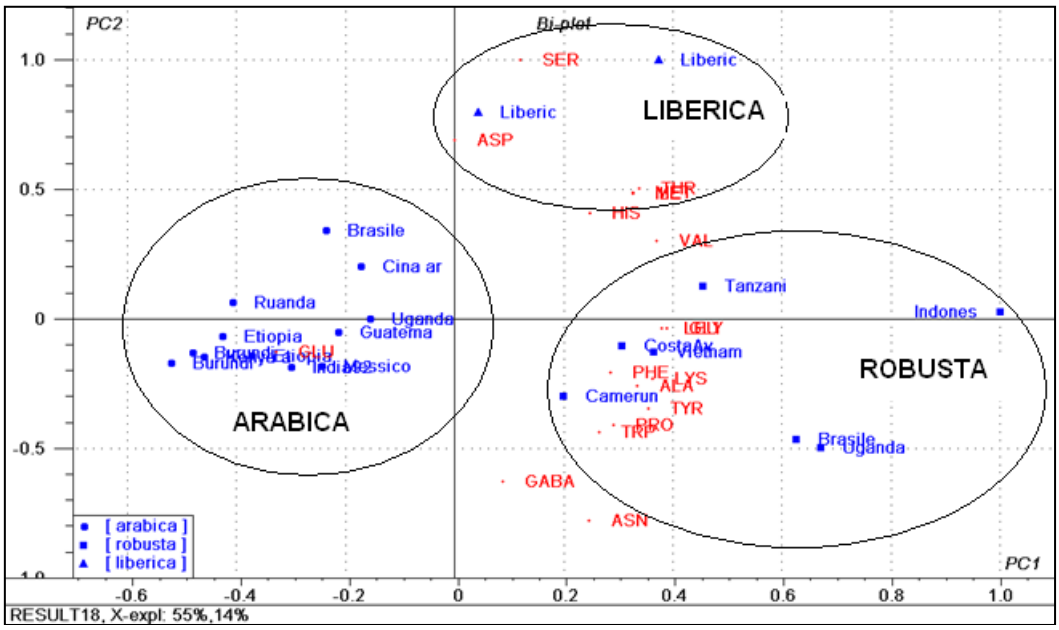

Figure 2. Discrimination of green coffee beans on the basis of free amino acids content.

CONCLUSIONS

Analysis of free amino acids in hot water extracts of whole green coffee beans was successfully performed. By comparing present quantitative data with literature ones it comes out that under the chosen experimental extraction conditions, free amino acids are recovered with good yield. Statistical processing of the complete data set put in evidence an excellent clustering of Arabica, Robusta and Liberica samples. Glutamic acid, aspartic acid, asparagine and GABA are particularly useful for inter-species discrimination purposes. Intra-specie variability linked to geographical origin has been also observed.

Free amino acids profile appears to be a useful and promising discrimination marker of coffee botanical origin, however, further studies including both other coffee species and higher number of samples, have to be performed, to validate a possible method.

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Physiological Markers for Quality Coffee Beverage

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SUMMARY

Physiological tests, such as germination, 2, 3, 5-triphenyltetrazolium chloride (TTC), electrical conductivity (CE) and potassium leaching (LK) are used in the seed industry to assess seed quality after processing and drying. The quality of coffee beverage is also affected by the post-harvest processes, since they may affect the chemical composition of the flavor and aroma precursors of coffee brew. Thus, physiological and biochemical fast, accurate, economical tests and easy standardization can constitute viable alternatives for assessing the quality of coffee beverage. However, studies about the using of physiological and biochemical to evaluate the quality of drinking coffee are rare. Thus the objective of the present study was to evaluate the potential physiological and biochemical analyses to assess quality of coffee beverage. The research was conducted at the Federal University of Lavras, Brazil. Fruits of *Coffea arabica* L., red ripe stage, were submitted to three types of processing, natural (dry), depulped and demucilated (wet), and dried to 11% moisture wb, by three methods, shade, sun and mechanical dryer at 40°C. Nine bean treatments were obtained from the combination of these factors and a commercial coffee bean sample was added, for a total of 10. One additional treatment was used, corresponding to coffee of lower sensory quality. The water content was determined and germination tests, EC, LK, TTC in the embryos, modified TTC in the endosperms were performed on the coffee beans. Determination of enzyme activity was assessed by electrophoresis technique. Coffee beans with high scores in sensory analysis also had high germination and viability percentages and low electrical conductivity values. There were high correlations between the sensory quality results of coffee beans and the physiological or biochemical tests results, with statistically significant levels less than 1% for radicle protrusion, TTC and CE. Furthermore, evaluating the coffee bean endosperms by TTC makes it possible to verify varieties of sensory quality in coffee beans.

INTRODUCTION

Coffee agribusiness sustainability in the international scenario depends more and more on the production of specialty coffees with better quality, and coffee quality assessment is currently based on sensory analyses that are in turn based on subjective data that are difficult to standardize. Thus it is highly desirable to identify objective, precise and easy standardization tests to assess quality and classify coffees for commercialization.

Seed quality is normally assessed after processing and drying by physiological and biochemical tests in the seed industry, such as the germination, tetrazolium, electrical conductivity potassium leaking tests, and enzyme profiles.

The germination test determines the maximum germination capacity of a seed sample and seed viability can be determined by the tetrazolium test, a biochemical test based on the activities of the dehydronenase enzymes reducing the 2, 3, 5-triphenyltetrazolium chloride or tetrazolium bromide in the live seed tissues by the reaction of hydrogen ions that are transferred to the referred salt. Salt reduction occurs only in the live cells so triphenyl formazan is formed, a red and indiffusible compound that confers red color to the live tissues of the embryo. The modified tetrazolium test used on coffee bean endosperms has demonstrated the potential of the test to monitor the quality of coffees during storage.

The electrical conductivity test is a simple, fast, cheap and efficient method that consists of quantifying the electrolytes lixiviated from the seed in the imbibition water and low vigor seeds release a large quantity of electrolytes in solution, resulting in a high electrical conductivity value or in high concentrations of certain ions, mainly potassium.

The quality of the coffee beverage can also be affected by operations during processing and drying, because these steps can affect the chemical composition of the flavor and aroma precursors of coffee drink. Results from recent research has have shown the association between reduced coffee bean sensory quality with physiological and biochemical alterations, such as oxidative stress, lipid hydrolysis, amino acid and carbohydrate levels etc. Thus research to assess the potential of physiological and biochemical analyses, associated to image analyses, to assess coffee beverage is a novel approach.

MATERIALS AND METHODS

Red ripe *Coffea arabica* L./Catuaí Amarelo 62 cultivar cherries were harvested and submitted to three types of processing, natural, depulped (mechanically de-pulped and the mucilage removed by fermentation in water at 20^{o}C), and demucilated (mucilage mechanically removed). The natural coffee (dry processing) and parchment grains (wet processing) were manually de-hulled and dried to 11% moisture (wb), by three methods, dry in shade, sun dried and mechanical dryer at 40°C. Nine bean samples were obtained from the combination of these two factors and a commercial coffee bean sample was added, for a total of 10. The bean water content was determined according to the standard international method ISO 6673. The sensory analysis was carried out by two trained panel qualified as Certified Judges of Specialty Coffees (SCAA Certified Cupping Judges), using the methodology proposed by Specialty Coffee Association of America (SCCA) to assess specialty coffees.

The beans were then submitted to physiological and biochemical assessment by the germination, tetrazolium, electrical conductivity and potassium leaching tests.

The germination test was carried out in roles of germination paper, moistened with a quantity of water 2.5 times the paper weight and 30°C constant temperature. The percentages of normal seedlings and strong normal seedlings were determined at 30 days, considering those with a hypocotyl at least 3 cm length; radicle protrusion percentage at 15 days, considering those radicle with at least 2 mm length; the percentage of plants with unfolded cotyledon leaves was calculated at 45 days.

The tetrazolium test was carried out with four replications on 25 embryos extracted after imbibition in water for 16 hours that were kept in a 0.5% tetrazolium solution, in the absence of light at 30°C for three hours. The embryos were classified as viable and unviable according to the damage location, extension and intensity.

The modified electrical conductivity test was carried out with four replications on 50 beans weighed with 0.001 g precision and immersed in 75 ml dionized water, and the electrical conductivity was measured in the imbibition solution after five hours at 25°C, with the results expressed in µS.cm-1.g-1 beans. The quantity of lixiviated potassium in the imbibition solution was determined in ppm, using a Digimed NK-2002 flame photometer.

The modified tetrazolium test was also carried out with adaptations. After imbibition in water for 36 hours at 30°C, four replications of 25 cross cut endosperms were imbibed in 0.1% tetrazolium solution in the absence light at 30°C for 24 hours. After staining, the sectioned endosperms were distributed with the flat surfaces facing upwards on 5 mm glass plates with a white background to take pictures with 300 dpi, in an inverted scanner. The TIFF format images were processed by the Matlab r2009b software, with image processing toolbox and SDC Morphology Toolbox 1.6. First isolation was performed using the blue band, a spectrum that permitted the greatest contrast between the bean and the image background. The automatic threshold was used for isolation proposed by Otsu and the bean was detected in the image using the blue band and the Otsu thresholds. To correct distinction imperfections in the picture, the mathematic morphology tool was used. The measurements were taken after defining the areas corresponding to the beans in the images, called the TZ index, according to the intensity of the band pattern in red, blue and green of each seed, determined by the equation number 1.

$$TZI = area\ (th,i) / area\ (segmentation,i)$$ Equation 1

Where: TZI= Tetrazolium indez; area (th, i) corresponding to the area (number of image pixels) in the region of the bean; and (segmentation, i) is the total area of each seed image i, after a threshold th. For each image i, five threshold values were used, namely: th1=0.2 th; th2=0.4 th; th3=0.6 th; th4=0.8 th; th5=th(i). Where: th(i) is the Otsu threshold of image i. Figure 1 illustrates the Otsu th3 of image (i).

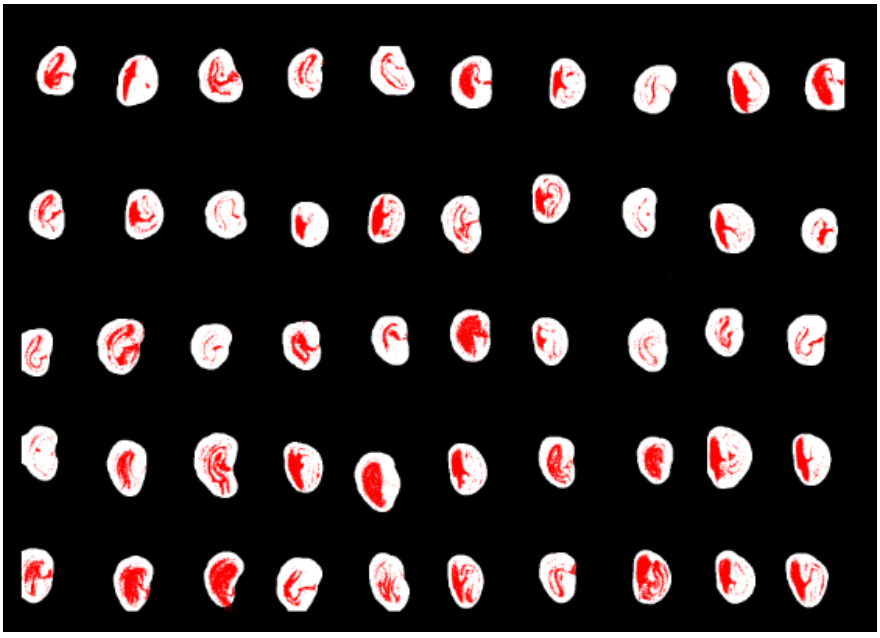

Figure 1. Illustration of the Otsu with the segmented image overlaid on the image of threshold 3, where the area in red represents the area index (*th,i*) and the total area of the seeds corresponds to the area (*segmentation,i*). Embrapa/UFLA, Lavras, MG, Brazil, 2012.

The results of the sensory analysis, physiological tests, and image analyses were submitted to ANOVA, analyses of correlation and the averages were compared by the Scott-Knott test at 5% probability, using the SISVAR software.

The sensory and physiological results were further submitted to an analysis of simple correlation, applying the multidimensional scaling technique, considering a dissimilarity matrix represented by $\Delta=[\delta ij]$, where each element indicated the similarity between the *i-th* and *j-th* variable, contextualized in the present study in two categories, the physiological and the sensory. The multidimensional reduction process was used based on the Euclidian distance matrix $D=[dij]$ formed from all the variables and the matrix was determined that best approximated the original dissimilarities matrix, that is, $D\approx\Delta$. It is emphasized that this approximation can be obtained not only for different distances. This problem therefore raised the need to verify, by means of a statistical criterion, which situation favors a better approximation to the original dissimilarity matrix. Thus the *Stress* function was used to measure the difference between the original dissimilarity matrix and the calculated distance matrix. The result of this function indicated that the smaller the value, the better the fit of the reproduced distance matrix to the observed matrix distance.

RESULTS AND DISCUSSION

Table 1 shows the results of the physiological and sensory analyses, where these tests show that the quality of the coffee beans treatments can be differentiated and the treatments can be classified by this quality. The results demonstrated that there was high correlation between the results of the physiological and sensory analyses with high coefficients of correlation (Figure 2) and minimum significance levels of 5%. These results indicated that coffee beans with high sensory quality also had physiological quality. Other recent studies have shown this correlation, corroborating the results obtained in the present research. The results of the sensory analysis presented a 0.94 correlation index with the results of the tetrazolium test and -0.87 with those of the electrical conductivity test. As the embryo viability percentage increased there was an increase in the values of the total scores of the sensory analysis. Inversely, the electrical conductivity test relates to the coffee beverage quality, so that increase in electrical conductivity implied inferior quality coffee.

Table 1. Results of coffee bean physiological assessment and sensory analysis. Embrapa/UFLA, Lavras, MG, Brazil, 2012.

Treatments[2]	Tetrazolium		Germination		Sensory Analysis		Electrical Conductivity
	(%)		(%)		(Total)		($\mu S.cm^{-1}.g^{-1}$)
T1	83.0 b		28.0 d		75,75 c		26.16 d
T2	79.0 b		42.0 c		83,75 a		31.89 e
T3	79.0 b		40.0 c		79,00 b		24.67 d
T4	100.0 a		86.0 a		82,25 a		5.86 a
T5	97.0 a		81.0 a		80,25 a		6.56 a
T6	95.0 a		74.0 b		81,00 a		8.77 b
T7	93.0 a		67.0 b		81,50 a		13.80 c
T8	96.0 a		86.0 a		81,75 a		13.08 c
T9	94.0 a		75.0 b		83,00 a		15.01 c
T10	6.0 c		0.0 e		56,00 d		74.06 e
CV (%)	6.59		12.26		1.45		12.49

Means followed by different letters differ statistically by the Scott-Knott test at 5% probability. [2]*T1: Natural/Sun; T2: Natural/Shade; T3: Natural/Drier; T4: Fermented/Sun; T5: Fermented/Shade; T6: Fermented/Drier; T7: Demucilated/Sun; T8: Demucilated/Shade; T9: Demucilated/Drier; T10: Additional treatment.*

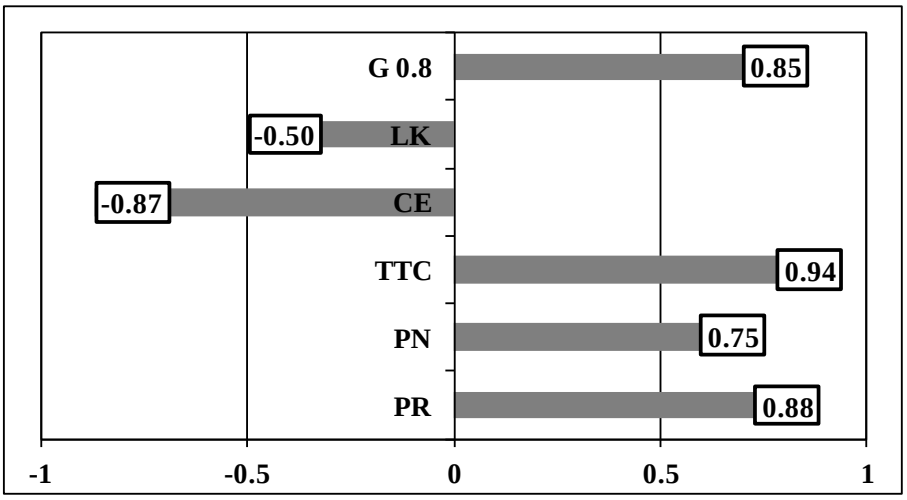

Figure 2. Correlation coefficients (r^2) between the physiological and sensory analyses of *Coffea arabica* L. beans. PR=radical protrusion; PN=normal seedlings; CE=electrical conductivity; TTC=tetrazolium; LK=potassium leaching; and G 0,8=TZI for the green band, threshold 4. Embrapa/UFLA, Lavras, MG, Brazil, 2012.

The electrical conductivity test is based on the integrity of the bean cell membranes and the high results showed the disorganization or rupture of the cell membranes, which results in the leaking of cell ions and consequently oxidative or catalytic reactions with undesirable and harmful products to the sensory quality of the coffee beverage. Higher electrical conductivity values were observed in poorer quality coffee beverages, as has been reported in other studies.

Results from image analyses by the Matlab software demonstrate a greater response to the color spectrum of the red band in the endosperm in coffee beans. For the red band, threshold 5

enabled a greater differentiation of the treatments. However, for the bands of the green and blue spectrum threshold 3 showed greater differentiation of the treatments (Figure 3). The additional treatment (T10) that included the beans intended for consumption, presented the worst physiological quality, indicated by the tetrazolium and germination tests and the poorest beverage quality, according to the sensory analysis (Table 2). This treatment also presented the lowest response to the light spectrum assessed, showing the embryos and the endosperms were unviable, which affected the sensory quality of the coffee.

The treatments processed naturally and dried in the sun and drier (T1 and T3) presented a relative reduction in viability compared to the other treatments, according to the tetrazolium test on the embryo. But the same treatments presented high response to the color spectrum assessed. There are two possible explanations for this performance. The embryos are the most delicate part of the seed, which show signs of viability loss faster when compared to the endosperms, which are more resistant reserve tissue. It is expected therefore that during coffee bean processing and drying the embryo may be the first tissue to lose viability, while the endosperm cells remain alive.

Another factor that justifies the intense staining on the endosperm of the coffee bean treatments processed naturally, dried in the sun in and the dryer may have been because of the intense respiratory activity present in this tissue.

The sensory analyses differentiate the treatments, but the physiological tests and the image analysis classified the coffees in more groups.

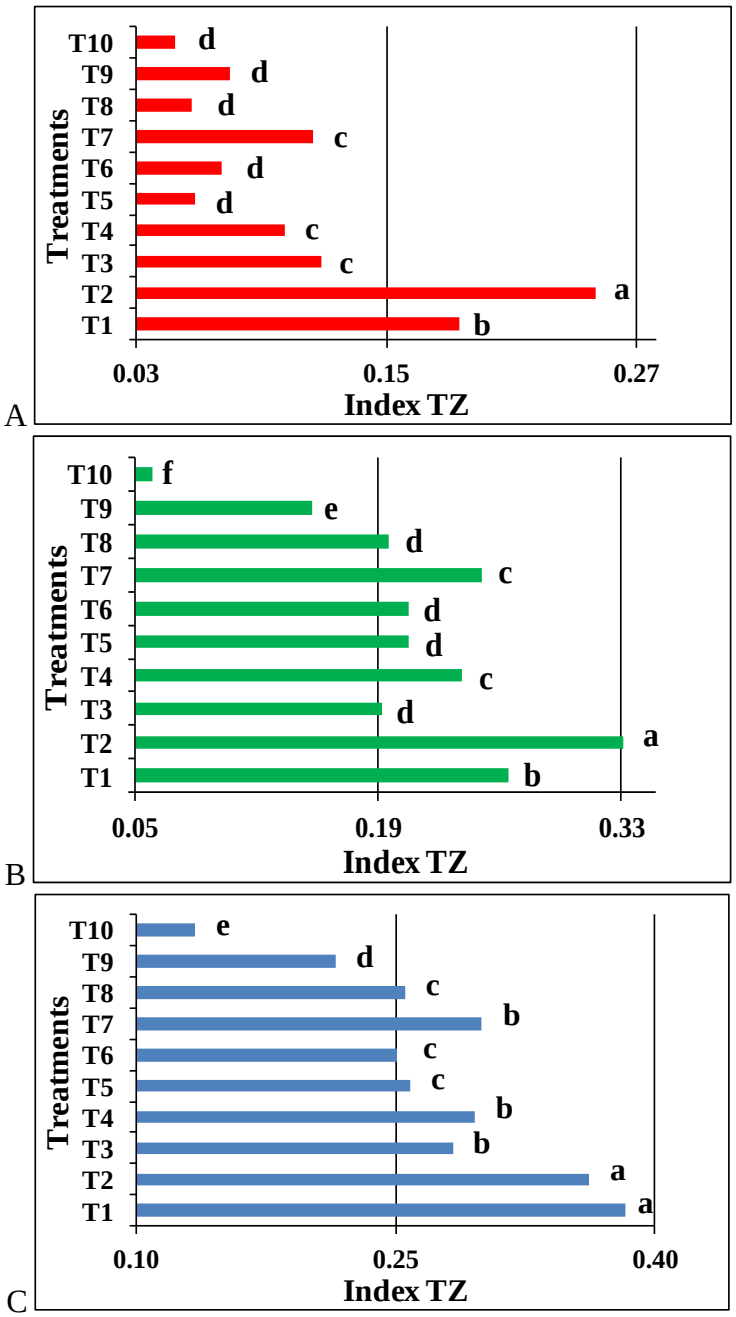

Figure 3. TZ indexes for the red band at threshold 5, and at threshold 3 for the green and blue band. Lowercase letters differ the treatments statistically by the Scott-Knott test at 5% probability. * T1: Natural/Sun; T2: Natural/Shade; T3: Natural/Drier; T4: Depulped/Sun; T5: Depulped/Shade; T6: Fermented/Drier; T7: Wet/Sun; T8: Wet/Shade; T9: Wet/Drier; T10: Additional treatment.

Regarding the multi-dimensional scaling technique, the results shown in Figure 4 correspond to the bi-plots constructed from variables related to sensory and physiological quality that permit identification of treatments groups that can be considered similar. Assessing the responses to the treatments showed that treatments T1-T9 can be considered similar regarding the sensory variable with the physiological variable percentage of normal seedlings at 30 days (N30d). It also showed that the T10 treatment presented heterogeneous responses of the others. It is emphasized that other clusters formed by other variables identified other similar groups.

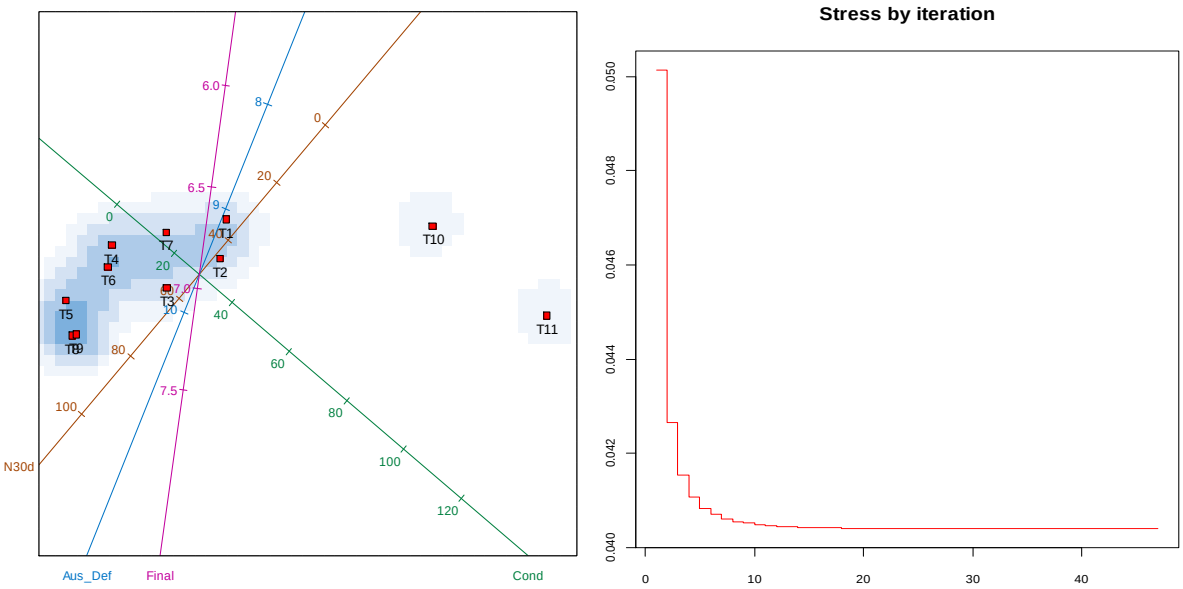

Figure 4. Bipbatches for sensory and physiological variables (left). Values obtained for the STRESS function in each interaction to validate the selected variables used (right).

It is important to emphasize those different combinations of the sensory and physiological variables can be used to assess cluster formation among the treatments. However, it is necessary to assess the quality of fit to ensure that the selected variables are suitable for dimensional reduction, in the sense that the difference is minimal between the similarity matrix formed with all the variables and the matrix formed only with the chosen variables. Using the Stress function, the values obtained for this function in each numerical interaction can be observed from the graphic analysis (Figure 4).

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Detection of Barley and Corn as Adulterants in Commercial Coffees Using Real-Time PCR.

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SUMMARY

It is well known that coffee is one of the main food products commercialized in the world. During processing, coffee can be intentionally adulterated with cheaper materials, including grains and cereals, such as barley and corn. Many techniques have been developed in order to establish suitable parameters and markers for detection of adulteration of ground roasted coffee and instant coffee. However, these methods may present low sensitivity and specificity. Although the recombinant DNA technology has shown to be a promising tool to determine the authenticity of processed foods, it has not been used to detect coffee adulteration. The objective of this study was to use a real-time Polymerase Chain Reaction (PCR) based method for detection of barley and corn in commercial coffees obtained in South American markets. The method was sensitive and specific to quantify down to 8.1pg and 0.3pg of barley and corn DNA, respectively. Ten control samples were evaluated and did not present barley or corn. The eight samples classified as gourmets or superior also did not present barley or corn in their composition. However, barley was detected in all twelve traditional (cheaper) coffee samples evaluated. Corn was only detected in one traditional sample, in addition to barley. The Real-Time PCR method showed to be suitable for detection of low amounts of food adulterants in roasted coffee.

INTRODUCTION

Coffee is one of the most popular drinks across the world. As such, it has been a target of fraudulent admixtures with a diversity of cheaper materials, roasted barley, corn, soybean and others. This practice leads to unfair competition, disrupting local and global economies, with defrauders gaining over reputable competitors. In addition, this practice is considered to be crime under the normative instruction of worldwide Surveillance Agencies. Many techniques have been developed in order to establish suitable parameters and markers for adulterations of ground roasted coffee and instant coffee. While visual inspection is traditionally used to detect the addition of cheap substitutes to green coffee, conventional methods for identification of adulterants using techniques of pattern recognition of colors and features photographs and digital image processing by computer are used for roasted coffee. However, these methods are subjective and consequently rely on the experience of the analyst, being subject to human error. Most of the analytical techniques were based on determination of chemical components by liquid or gas chromatography coupled to multivariate statistical

analysis. Recently, volatile markers have been identified for low quality coffee beans. Techniques that do not rely on the determination of specific chemical tracers but rather on strictly physical measurements were also proposed for the detection of adulterants in coffee. An example is the use of photo-acoustic spectroscopy, infra-red spectroscopy, scanning electron microscopy, and thermal lens spectrometry. Nonetheless, the aforementioned techniques do not allow for the identification of individual constituents or constituents in admixtures of several adulterants. The use of recombinant DNA techniques has been used for quality control (authenticity) of processed foods as well as to detect and quantify genetically modified organisms. The real-time or quantitative Polymerase Chain Reaction (PCR) technique allows for monitoring of the amplification reaction in real time (cycle to cycle), in a closed system without outside interference in the progress of the reaction. In a fluorescent system, a fluorescent signal is detected proportionally to the increase in the amount of the target amplified product. This fluorescence is emitted by fluorophor compounds, which may be probes linked to or intercalated in double stranded DNA fragments SYBR Green. In brief, SYBR Green binds the minor groove of dsDNA, enhancing greatly the fluorescence. The fractional cycle in which the increase in the fluorescence generated by the accumulation exceeds 10 standard deviations of the mean baseline fluorescence, with a selected range of cycles, is referred to as the threshold cycle.

Considering the need for sensitive and specific methods for detection of food adulterants, the objective of this study was to use a real-time PCR based method for detection of barley and corn in South American commercial coffees.

MATERIALS AND METHODS

Samples

Fresh samples of barley (*Hordeum vulgare*), corn (*Zea mays*) and coffee were used as specific target and rice (*Oryza sativa*), wheat (*Triticum aestivum*) and soy (*Glycine max*), were used as non-specific targets. All grains were purchased at a local market and were not genetically modified. Ten green coffee samples (four *C. arabica* and six *C. canephora*) were obtained directly from producers in São Paulo, Espírito Santo and Minas Gerais, Brazil, and were used as control samples. Coffee was roasted in a fluidized bed roaster (I-roast, USA) at 230°C to give dark colour degree (# 35 Agtrom-SCAA); barley and corn were roasted in a microwave oven to reach the same color as coffee. Samples were ground in a mill (IKA A11basic to pass a 500µm sieve).

Twenty one commercial ground roast and instant coffee samples were purchased in different stores located in South American countries. From these samples, 9 were classified as gourmet or superior and 12 were traditional (cheaper). To build the five point curve for adulterants quantification, a blend containing 80% of arabica roasted beans and 20% of robusta roasted beans was used and 0,5%; 1%; 2%; 5% and 10% of barley and corn were added to the coffee blend.

DNA Extraction

Roasted samples were submitted to DNA extraction with DNeasy kit/ buffer CTAB. DNA concentrations were determined in all *in natura* and roasted samples by spectrophotometer (Shimadzu UV-1800 Japan) at 260nm.

Primers design

DNA sequences corresponding to the endogenous genes for coffee, barley and corn were surveyed from Genbank (accession number NC008590.1, M60837.1, EF044213.1, respectively). Sequences were submitted to the program Basic Local Alignment Search Tool (BLAST) to analyze the similarity among other species. The primer pairs were designed using Genefisher software setting up the size amplicon of 100 pb. Primers were synthesized by Eurofins MWG Operon and their amplification was confirmed using *in silico* PCR runs at BIOINFX (http://www.bioinfx.net/).

PCR parameters

PCR runs were achieved using the SDS-ABIPRISM 7000 (Applied Biosystems). The reaction mixture contained 1 x Power SYBR Green Master Mix (Applied Biosystems) 240nM primers and 50ng DNA in 25µl final volume. Thermal conditions were as follows: 10 min at 95º C, 45 cycles of 15 s at 95 º C and 1 min at annealing temperature (Tm) of each primer pair (Table 1).

In order to estimate the adulterants percentage in commercial coffee samples, the equation obtained after a standard curve construction for each adulterant, was used:

$$y = ax+b$$

where:

- x= log [% of intentionally added adulterant]
- y= Mean Ct of intentionally adulterated coffee samples

Table 1. Primer parameters for barley, corn and coffee markers.

Food	Primers	Sequence	Tm (ºC)	Efficiency (%)	R^2	LOD (pg)	LOQ (pg)
Barley	Cevada 3	F: CCGGACCAGAACTTCTTG R: CCTGAAGCACGATTTCTG	60	104	0,99	5,0	8,1
Corn	Zeina 2	F:CAGGCTCCAACAAGCAATG R:GCAACTGTTGTGCCCTGATG	62	95	0,99	0,1	0,3
Coffee	Café 1	F:TTCCGAAGTCCTGGAGAG R:CGGAGGATATCTCAATCG	60	88	0,99	0,7	2,4

F/R- forward/ reverse primers. Tm - annealing temperature. LOD and LOQ - limit of detection and limit of quantification. R^2- Correlation coefficient.

RESULTS AND DISCUSSION

The melting curves from specific (A, C and E) and non-specific (B, D and F) targets are shown in Figure 1. The melting curve for the specific targets demonstrated that these primer pairs were specific for barley, coffee and corn detection. The nonspecific amplification peaks were attenuated by increasing annealing temperature.

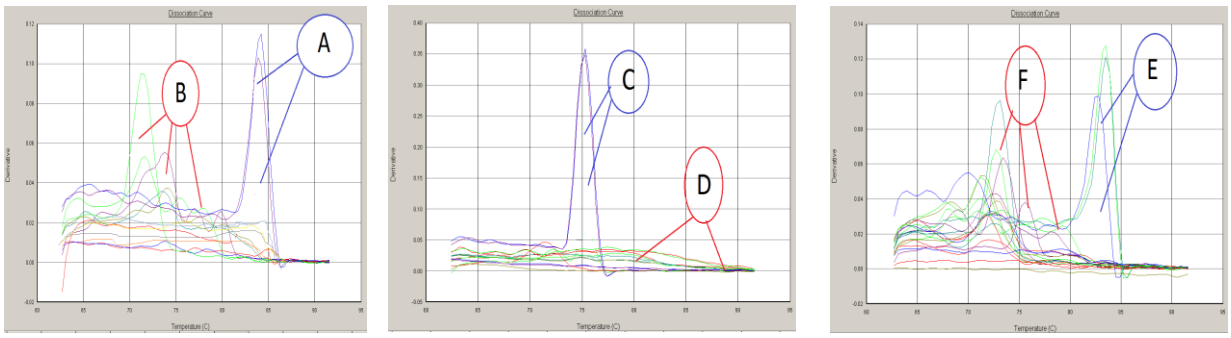

Figure1. Primer Cevada3 (AB), Café1 (CD) and Zeina2 (EF). The letters A, C and E shows amplifications specific and letters B, D and F demonstrates nonspecific amplifications like primer-dimer. Each primer pair was tested with genomic DNA of target and non-target (rice, wheat, soy, corn, barley, coffee *arabica* / *canephora*).

The method was sensitive and specific to identify levels down to 5,0pg and 0,1pg of barley and corn DNA, respectively, and quantify levels down to 8.1pg and 0.3pg of barley and corn DNA, respectively.

Figure 2 shows the laboratory adulteration standard curves and the equations, for percentage estimation, where the correlation coefficients (r^2) were 0.98 and 0.95 for barley and corn, respectively. Because recovered DNA is expected to be highly degraded, short amplicons are preferable for successful detection (Table1). Various factors may contribute to hydrolysis of DNA in food such as prolonged heat treatment, by action of nucleases (enzymatic hydrolysis), and depurination at low pH. The degree of PCR inhibition is to a great extent dependent on the food type. For example, heat treatment continuously degrades DNA resulting in a strongly reduced average fragment length what could explain the values of r^2. Improving homogenisation may also improve r^2 values.

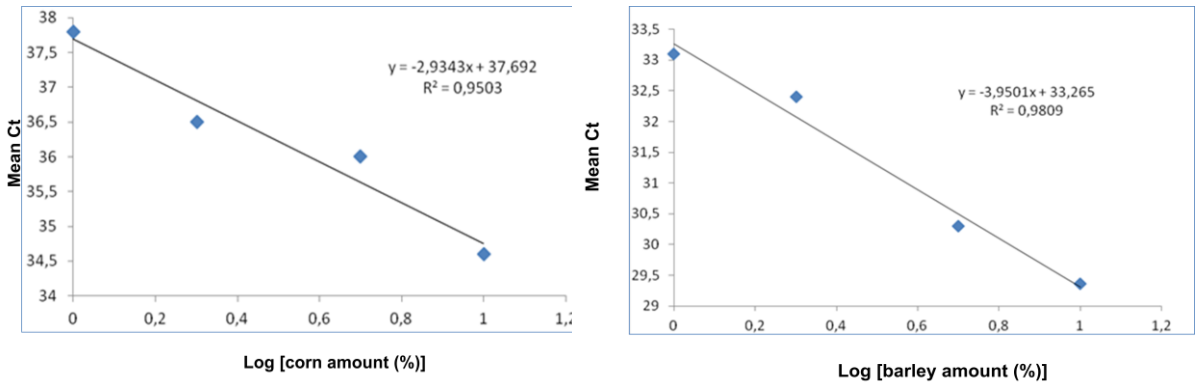

Figura2. Detection of the foods intentionally adulterated with 0.5%, 1%, 2%, 5% and 10% of corn and barley.

Regarding identification and quantification of adulterants in control and commercial coffee samples, as expected, barley and corn were not detected in any of the 10 evaluated control samples nor in any of the gourmets or superior commercial samples (Table 2). On the other hand, barley was detected in all 12 traditional (cheapest) evaluated coffee samples, and very low amount of corn (bellow the quantification limit) was present in only one sample, in addition to barley (Table 2, sample C). The percentage of barley in coffee samples was estimated based on curves built with data obtained from coffee adulteration at the laboratory (Figure 2). The percentage of barley found in commercial coffees varied from 1.0% to 4.8%, including instant coffee samples.

According to the Brazilian Surveillance Agency (ANVISA), commercial coffees containing 1% of strange materials are considered "dirty" and may be commercialized but not exported. Percentages of strange materials or adulterants above 1.3% are considered as resulting from criminal practice. Our results indicate that more than half (58%) of the traditional coffee samples evaluated, including instant coffee, contained percentages above the allowed limit. However, there is no limit specified for instant coffee in Brazilian regulation agencies. The regulations from other South American countries were not yet investigated and cannot be discussed here.

Still in Table 2, instant coffees (samples D[1], G[1], H[1]) showed higher amounts of barley comparing to ground roast coffes. In the process for producing instant coffee, the beans are ground and subjected to extraction under pressure at high temperatures which promotes, in effect, extraction of water soluble solids. The extract is then dehydrated yielding instant coffee. Given that DNA is considerably soluble in water, this would explain the higher percentage in instant coffee.

Table2. Detection of barley in South American Traditional, Gourmet and Superior commercial coffees.

	Samples	Mean Ct	Deteccion	Quantification [% adulterant]
Traditional	A	33,0±0,7	D	1,2
	B	31,9±1,3	D	2,2
	C	31,7±0,3	D	2,5
	D[1]	30,6±0,3	D	4,8
	E	33,6±0,7	D	NQ
	F	32,3±1,2	D	1,8
	G[1]	31,8±0,8	D	2,3
	H[1]	30,4±0,2	D	5,2
	I	32,8±0,3	D	1,3
	J	33,3±0,1	D	1,0
	K	32,6±0,1	D	1,5
	L	32,4±0,3	D	1,7
Gourmet and Superior	M	-	ND	-
	N	-	ND	-
	O	-	ND	-
	P	-	ND	-
	Q	-	ND	-
	R	-	ND	-
	S	-	ND	-
	T	-	ND	-

[1]*Instant coffee; Ct- Cycle threshold; D- detected; ND- Not detected; NQ- Amount bellow the limit of quantification.*

In conclusion, Real-Time PCR showed to be an effective tool for detection of adulterants in ground roast and instant coffee. Among the evaluated samples, those with the lowest prices were adulterated, especially with barley, in percentages varying between 1% and 4.8%. The method presented here is being validated in order to accurately estimate the percentage of adulterants so that it can be used for government agencies regulation. For this purpose, it is also necessary to develop and validate certified reference material or DNA-based calibrants for adulterants detection and quantification.

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Quality "In Cup" of Espresso Coffee: Data from over Ten Years of Investigation

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SUMMARY

In this work the results of a wide research concerning the "Quality in cup" of espresso coffee directly prepared in coffee shops (688) of six different geographic area of Italy (Central and Northern) performed during a period of over 10 years were discussed. In this way different process variables such as type of roasting plant, type of grinding-batcher plant, type of extraction machine (manual, automatic, semi-automatic), blend, water temperature and pressure, weight of powder and its compression inside the filter, were analysed before coffee extraction. Furthermore, the quality in cup was evaluated by measuring the most important indexes such as the consistency and the height of cream, pH, °Bx and volume of brew. Several experiment were performed in laboratory scale with the aim to evaluate the effects of possible uncontrolled variables during the coffee brew preparation in coffee shops.

Over than 9000 data were then statistically elaborated by Anova analysis, Mann-Whitney test and Levene test. The Principal component analysis (PCA) of extraction process data and of quality parameters of espresso coffee was used in order to discriminate the quality characteristics of coffee brews in relation to the different geographic areas and to discriminate the significant differences among the years of the investigation (from 2000 to 2011). From results, the type of extraction plant and of grinding-batcher plant as well as a good training of bar operators emerged as the most affecting variables the quality of espresso coffee.

INTRODUCTION

Among the several methods which is possible to use to prepare coffee brew, the so called "espresso" is the most important in Italy. However, due to the largely appreciated sensorial properties of "espresso" coffee, this method of extraction has been adopted in several places in the world. As known, the quality of espresso coffee is the result of a wide number of chemical and physic-chemical characteristics which interact among them affecting the final "quality in cup". For instance, by a physical-chemical point of view, "espresso" coffee was defined as *a polyphasic systems composed from a foam layer of small bubbles on top of an emulsion of microscopic oil droplets in an aqueous solution along with dispersed gas bubbles and fine coffee particles*. Also, a wide chemical complexity is responsible of sensorial attributes such as perceived acidity, mouth feel, bitterness and aftertaste which are greatly affected by internal and external variables. However, all above quality aspects of "espresso" coffee are greatly influenced by internal (raw material) and external (process conditions) variables. Among the process variables roasting process, coffee grind degree, water

temperature, extraction time, weight of powder, water pressure as well as pressure by which the powder is compressed in the filter have a crucial importance. As example, the pressure used to compress coffee powder greatly affects the water pathways during extraction phenomena hence, they are responsible of the type and the amount of chemical compounds inside the brew. In this paper the results of a wide research have been reported focused on the analysis of the effects of type of blends, type of extraction plant and the most important extraction parameters on some quality indexes of "espresso" coffee prepared in six geographic area of Italy from 2000 to 2011.

MATERIALS AND METHODS

Espresso coffee preparation

The investigation was performed in six geographic area of the Northern and Central Italy in a period ranged between 2000 and 2011. A total of 688 coffee shops were visited taking into account the three extraction machines (manual, M; semi-automatic, SA; automatic, A) and two types of blend and (M and BS) of roasted coffee.

The trials performed in university and company laboratories took into account two types of roasting plant (A and B), two types of blend with 60/40 and 70/30 ratio percentage of Arabica and Robusta respectively (S and M).

Chemical and physical analysis

Chemical and physical analysis were performed before coffee preparation and the following variable were analysed: water temperature (°C) and water pressure (atm) by tools supplied by ESSSE Caffè, weight of powder (g), extraction time (s), extraction rate (s^{-1}) and hardness of water (°F), by a kit supplied by the company. The principal characteristics of "espresso" brew were evaluated by measuring the following indexes: pH by a pH-meter (AMEL 334 –B, Milano, Italy); volume of coffee brew and cream eight with a graduate cylinder; soluble solid (°Bx) by a digital refractometer previously calibrated with distillate water; extraction rate (mL/s) by relating the measured volume of coffee brew with the extraction time; consistency of the cream by using the method reported from Severini et al.

The raw material was previously analysed to verify the homogeneity of its characteristics in terms of colour (by a Chromameter-2 Reflectance, Minolta, Japan, equipped with a CR 100 measuring head) and density of beans using a suitable picnometer according to the methodology described by Lerici et al. (1980) and powder (by a graduated cylinder): data are reported in table 1 as means, standard deviation (SD) and coefficient of variation (CV%).

Table 1. Means, standard deviations and Coefficient of variation of roasted coffee.

	L* (beans)	***a* (beans)***	***L* (powder)***	***a* (powder)***	***Density (beans)***	***Density (powder)***
Mean	20,39	5,33	22,44	9,25	0,57	0,41
SD	1,44	0,65	1,28	0,74	0,01	0,02
CV%	7,06	12,25	5,7	8,03	2,73	6,76

Statistical analysis

Significant differences (at p-level < 0.05) between means coffee "in cup" quality parameters were explored by using analysis of variance (ANOVA). Also the Mann-Whitney test was used in the case in which significant differences were observed between variance means at the Levene test. Also a principal component analysis (PCA) was performed to highlight possible difference between coffee beverage samples (coming from different geographic area and analysed during ten years) on the basis of quality parameters. All statistical analyses were performed by using Statistica ver. 7.0 (Statsfot Inc.).

RESULTS AND DISCUSSION

The total amount of data was firstly elaborated to realize which of them would be the most variable (figure 1). From results temperature of machine, hardness of water, extraction time, volume of brew (and consequently solids) and cream consistency resulted the most variable characteristics; whereas pressure of machine, powder amount, extraction rate, pH and cream height were the least variable. These were the first indications.

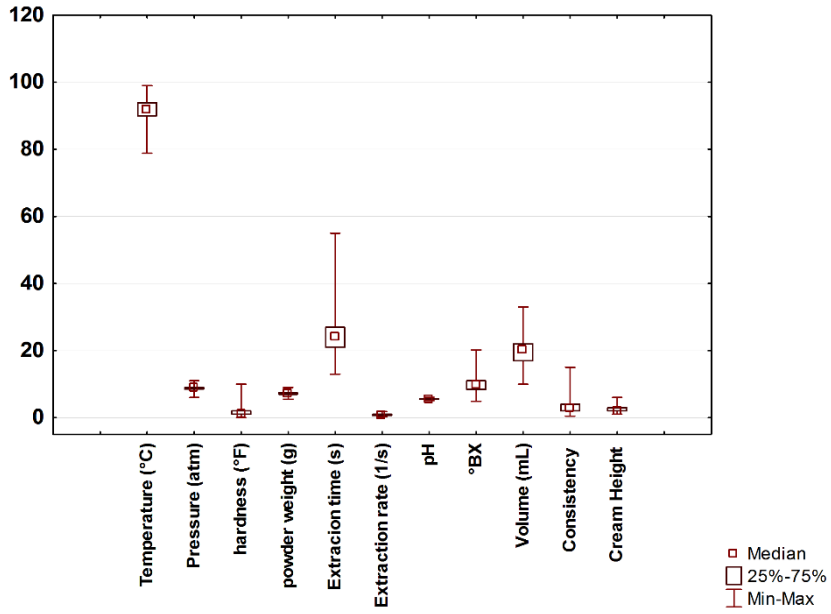

Figure 1. Variability of all the data collected in coffee shops.

The results of analysis of variance are shown in table 2, that reported means, standard deviations and Anova letters for total data concerning the brew characteristics. From ANOVA the differences based on type of extraction machine and blends (in red in the table) resulted non significant; so it wasn't used for PCAnalysis.

Table 2. Analysis of Variance (ANOVA) of means of all the data (in red data not significantly different).

	pH			°Brix			Volume (ml)			Cream consistency (s)			Cream height (ml)		
2000	5,62	0,16	a,b	9,55	1,63	a,c,d	20,98	3,85	a,c,d	2,65	1,83	a,c	2,02	0,61	a
2001	5,68	0,25	b,e	8,07	1,69	b	24,15	4,17	b	2,81	2,53	a,b,c	2,15	0,67	a,b
2002	5,66	0,08	b,e	9,80	2,60	a,c,f	20,15	3,38	c,e,f	3,34	2,33	c,d,e,f,h	2,21	0,59	a,b
2003	5,59	0,17	a,c	8,75	1,73	b,d,h	22,38	3,85	b,d	2,64	1,45	a,d	2,24	0,80	a,c
2004	5,56	0,09	c,d	9,73	2,32	a	19,71	3,94	a,c,e,f	3,71	2,08	e	2,11	0,75	a
2005	5,69	0,09	e	10,25	2,40	a,c,f	18,89	3,58	e,f	2,51	1,63	a	2,13	0,79	a
2006	5,53	0,09	d,f,g	9,87	2,12	a,c,e	19,32	3,76	e,f	2,80	1,51	a,f	2,48	0,71	b,c,d
2007	5,56	0,05	c,g	10,56	2,34	f	19,05	3,86	e,f	3,38	1,79	b,e,g	2,62	0,86	d,e
2008	5,52	0,05	f	9,62	1,95	a,c,g	20,20	3,99	a,c,e,f	2,76	1,78	a,g,h	2,57	0,83	c,e,f
2009	5,48	0,08	h	10,38	1,91	e,f	19,06	3,50	f	3,68	1,43	e	2,59	0,86	d,f,g
2010	5,37	0,13	i	9,52	2,13	a,c,d	20,19	3,92	a,c,e,f	3,52	2,27	b,e,i	2,57	0,72	d,f,g
2011	5,43	0,17	l	9,90	1,72	a,c,f	19,49	3,10	f	3,00	1,45	a,c,g,i	2,47	0,66	b,c,e,g
E	5,68	0,11	a	10,55	2,58	a	18,59	3,42	a	3,31	1,77	a	2,34	0,91	a
L	5,44	0,15	b	8,58	1,76	b	21,57	4,05	b	3,27	2,18	a,b	2,40	0,78	a
R	5,52	0,12	c	10,39	2,13	a	19,09	3,73	a,c	3,18	1,85	a,c	2,36	0,74	a
T	5,55	0,15	c	9,44	1,91	c	20,75	4,14	b,d	2,77	1,69	b,c,d	2,41	0,81	a
UM	5,60	0,11	d	9,80	1,88	c	19,87	3,44	c,d	2,99	1,72	a,d	2,34	0,76	a
V	5,52	0,16	c	9,61	2,05	c	20,47	3,91	d	3,30	1,91	a	2,41	0,77	a
M	5,53	0,15	a	10,22	2,26	a	19,25	4,09	a	3,05	1,85	a	2,23	0,80	a
A	5,52	0,13	a	9,71	2,01	b	20,15	3,70	b	3,15	1,78	a	2,45	0,76	b
S	5,58	0,14	b	10,11	2,40	a,b	19,16	3,82	a	3,09	1,81	a	2,29	0,83	a
M	5,54	0,14	a	9,97	2,15	a	19,76	3,82	a	3,16	1,88	a	2,33	0,75	a
BS	5,56	0,15	b	9,76	2,18	a	19,93	3,93	a	3,01	1,76	a	2,40	0,79	a

The score plots of PCA analysis of geographical areas in the plane defined by the first two principal components on the basis of espresso coffee samples quality parameters were reported in figure 2. A good separation between espresso coffee coming from the different visited geographic area was observed. In particular the best results were obtained, as shown, between Lombardia (L) and Emilia (E) (figure 2a), Lombardia (L) and Umbria (UM) (figure 2b) and Emilia (E) and Romagna (R) (figure 2c).

The score plots of PCA analysis were reported in figure 3. As shown, the best results were obtained between espresso coffee analysed in the 2000-2001 years (A-B) and 2010-2011 (M-N).

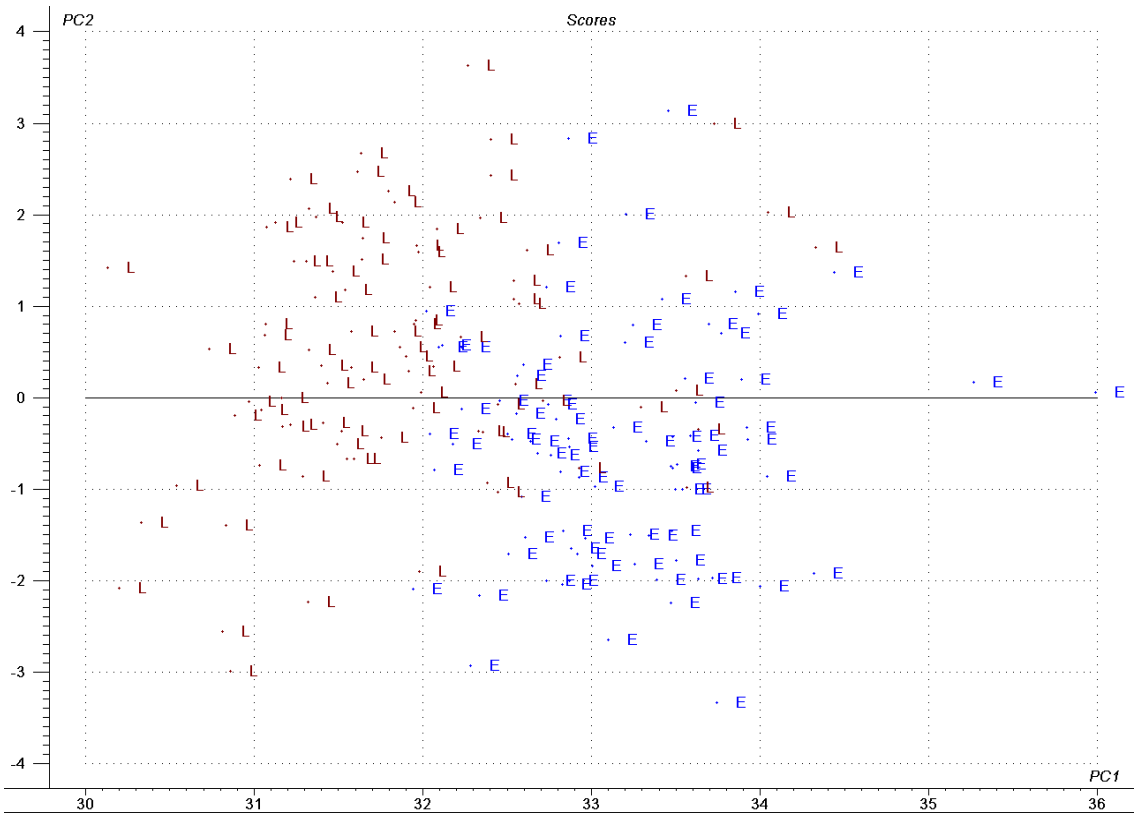

Figure 2a. Emilia (E) and Lombardia (L) discrimination.

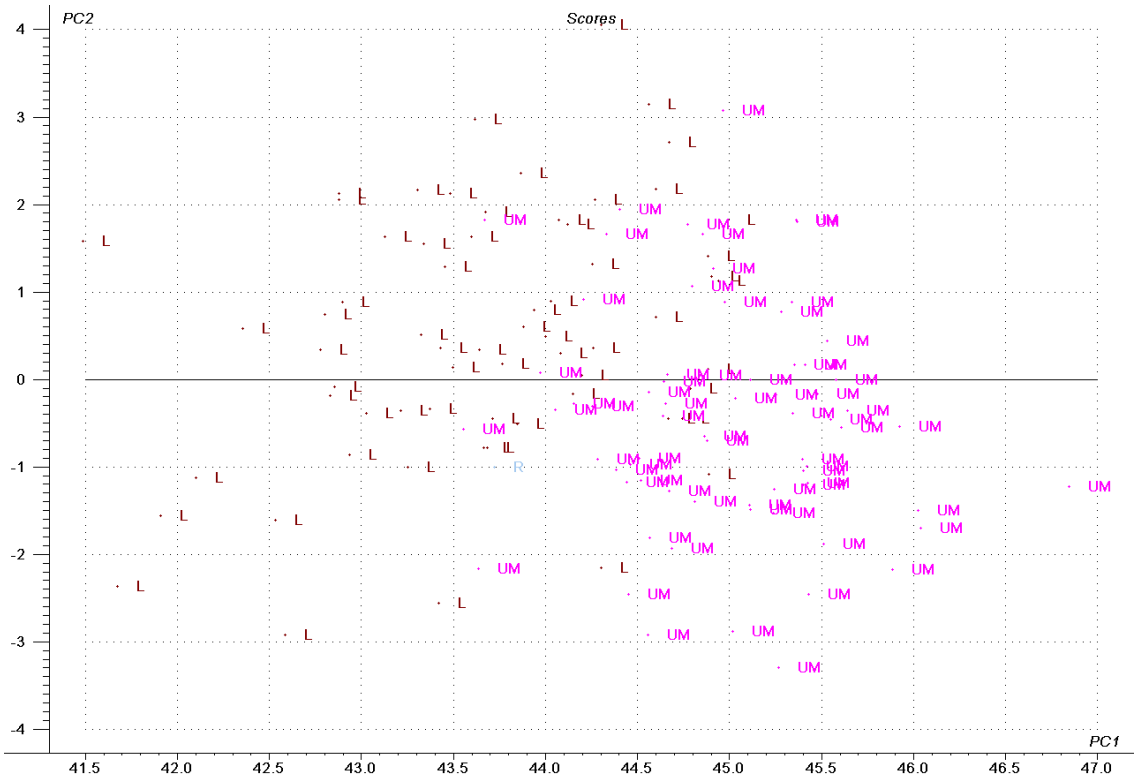

Figure 2b. Lombardia (L) and Umbria (UM) discrimination.

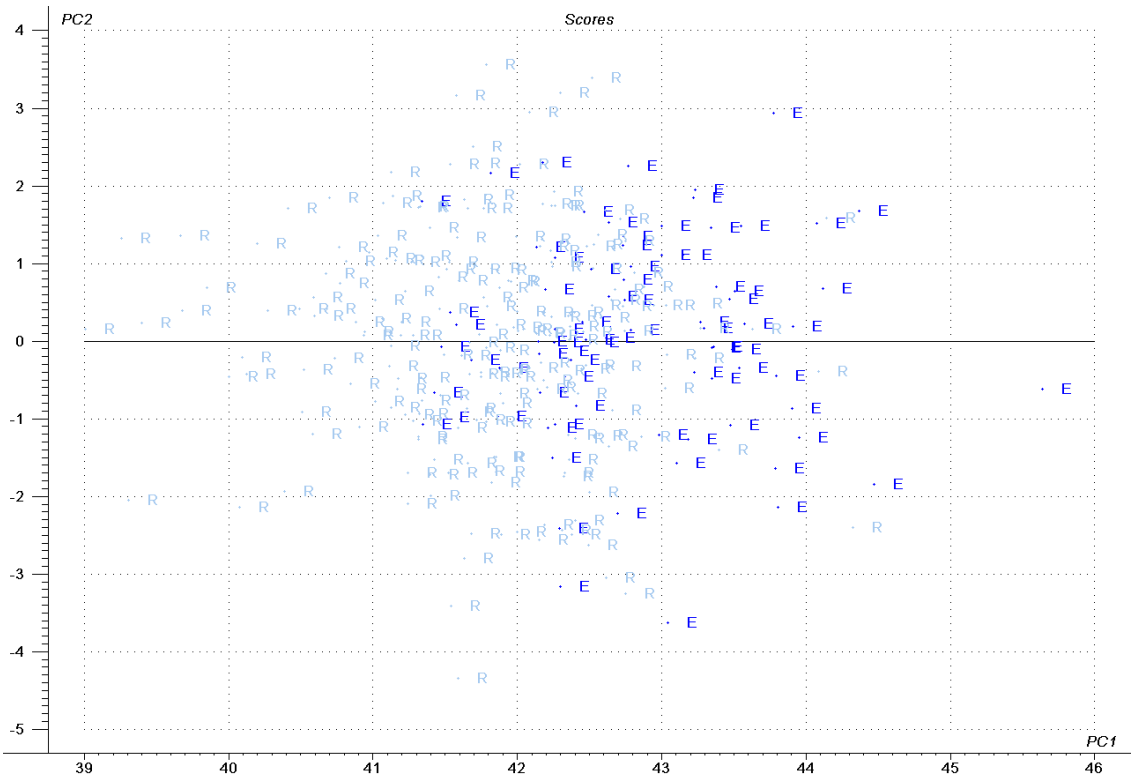

Figure 2c. Emilia (E) and Romagna (R) discrimination.

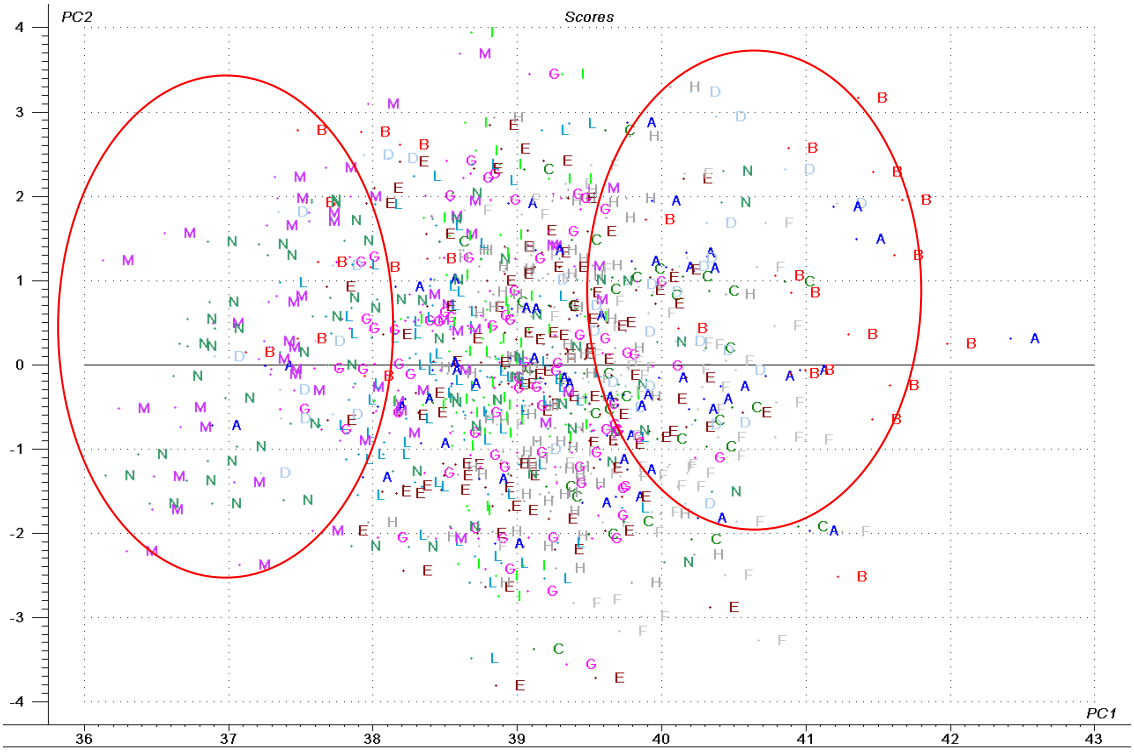

Figure 3. PCA score plot of years investigations in the plane defined by the first two principal components on the basis of espresso coffee samples quality parameters. A-B corresponded to 2000-2001 years; M-N corresponded to 2010-2011 years.

From results it seems that only the geographic area can affect the "espresso" coffee quality, that is substantially a cultural aspect. Moreover, an improvement of coffee quality has been detected throughout 12 years, which means that the training of bar operators carried out by ESSSE Caffè and certification of coffee shops has led to good results. Nevertheless the variability among samples was so great that, by the "on field" investigation, it was impossible to appreciate which variables really affected the quality of coffee.

Starting from these results several experiments were performed in both university and company laboratories with the aim to evaluate the effects of possible uncontrolled variables during the coffee brew preparation in coffee shops. In particular the effectiveness of two types of roasting plant, three types of grinding-batcher plant and three types of extraction plant on quality of "espresso" coffee was tested.

Only the figure concerning the sensorial profiles of brews obtained from coffee beans roasted by the two different roasting plants is shown (figure 4) from which it appears evident that the profiles are overlapping. So no significant differences emerged by using different plants, of course considering conditions for an Italian style roasting.

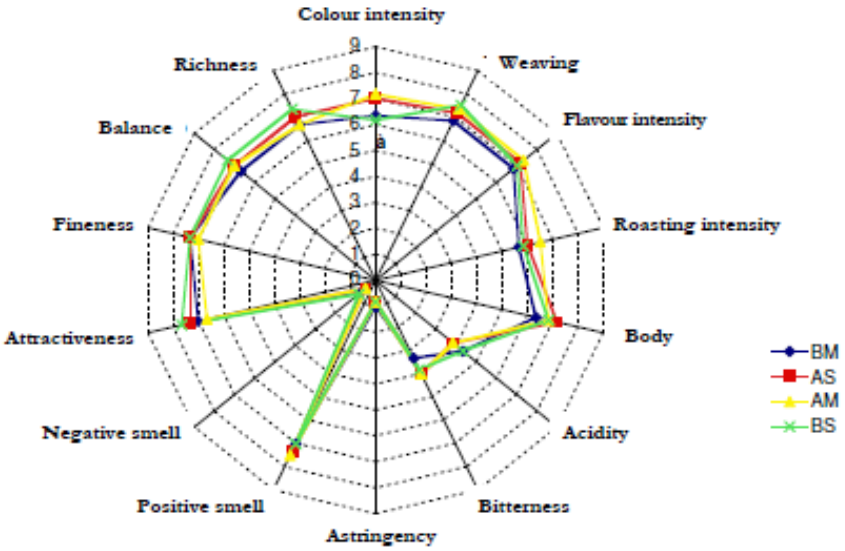

Figure 4. sensorial profiles of coffee brew obtained from beans of two blends (S and M) roasted by two different roasting plants (A and B).

More interesting was the assessment of effectiveness of grinding-batcher plants and extraction plants on coffee characteristics. In fact, using standardized conditions such as: blend (A, B or C), extraction time (25s), powder amount (7g), pressure on powder (12,5N/cm^2), pressure of machine (9atm ±0,5), temperature of machine (90 °C ±2), we obtained a great difference in terms of volume of espresso, ranged between 16 and 21 ml, with consequently differences of solid content, depending on the type of extraction plant, and in terms of temperature of brew with values ranged between 69 and 76 °C depending on both type of extraction plant and grinding-batcher plant. As well known, both volume and temperature of "espresso" are of prime importance for a correct and pleasant tasting. In particular, a correct volume of brew (about 20-25ml) contains a sufficient amount of extracted solids, caffeine, volatile and aromatic substances.

In conclusion, if it is true that the operators affect the coffee characteristics by hand making, in the same way it is true that machines decide coffee quality. Perhaps the standardization of each brand of "espresso" could be obtained by improving either the training activity for bar operators or perfecting the machine performances, with a particular carefulness about extraction machines and grinding-batchers.

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Multiple Role of Polyphenol Chemistry in Coffee Associated with Quality Attributes

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SUMMARY

Polyphenols such as chlorogenic acids (CGA, O-caffeoylquinic acids) and related compounds are the main components of the phenolic fraction of green coffee beans, reaching levels up to 14 % on dry matter basis. The main groups of CGA found in green coffee beans include caffeoylquinic acids, dicaffeoylquinic acids, feruloylquinic acids, p-coumaroylquinic acids and mixed diesters of caffeic and ferulic acids with quinic acid. During coffee processing, CGA may be isomerized, hydrolyzed or degraded into low molecular weight compounds. The high temperatures of roasting also produce transformation of part of CGA into quinolactones and melanoidins. Thus, complex polyphenol chemistry occurs during thermal treatment of coffee.

Flavour and aroma compounds derive from multiple chemical reactions, including the Maillard reaction, caramelization, polyphenol degradation, polymerization, lipid oxidation and pyrolysis. Polyphenols lead on the one hand to the generation of guaiacols and cresols that contribute to coffee flavour. On the other hand, they are precursors of phenolic compounds that can trap thiols and thus induce aroma degradation, which can be a particular problem when coffee is stored as a canned or bottled beverage or as a liquid coffee concentrate. Coffee aroma staling is mainly due to the decrease of the coffee-like smelling compound 2-furfurylthiol (FFT) trapped by the polyphenol degradation products di- and trihydroxybenzenes, and in particular hydroxyhydroquinone. This oxidative coupling reaction may also affect other odour-active thiols such as methanethiol that are present in freshly brewed coffee.

Coffee polyphenol derivatives are also significant contributors to bitterness, e.g. chlorogenic acid lactones and hydroxylated phenylindanes. Bean roasting either can break these phenolic acids down to form di- and trihydroxybenzenes such as hydroxyhydroquinone or can epimerize and dehydrate the acids to give various lactones that may provide a pleasant, coffee-like bitter taste quality in light- to medium-roast coffee.

Finally, coffee polyphenols have also a number of beneficial health properties related to their antioxidant activity. Nevertheless the exact chemical nature of antioxidant polyphenols in roasted coffee remains somehow unclear as the antioxidant activity can only partly be explained by well-known compounds such as the natural CGAs. Overall, it seems coffee polyphenols and their chemistry will likely influence cup quality by multiple effects. However, controlling the fate of polyphenols remains a challenging task due to their manifold implication in chemical reactions.

INTRODUCTION

Coffee is one of the most popular beverages in the world and is consumed in almost all countries over the world. It is mainly consumed because of its pleasurable aroma and taste

and for stimulation due to the high caffeine content. Beside this the image of coffee has changed from a rather negative to a more positive one in the past years. There is more and more evidence that a moderate coffee consumption (4-5 cups per day) has a positive health impact. These positive effects are, beside caffeine, mainly attributed to the presence of polyphenols in coffee. The term polyphenols is a general term that refers to several different chemical classes that are widely distributed in the plant kingdom. Prominent members are isoflavones in soy, catechins in tea, procyanidins in cacao, anthocyanidins in red fruits or ellagitannins in strawberries. Also phenolic acids like benzoic or cinnamic acids are commonly accepted as being part of the polyphenol family although they are lacking the "poly" aspect. The major polyphenols in coffee are the chlorogenic acids. Beside caffeine and trigonelline they are the main constituents of green coffee beans. Chlorogenic acids are composed of a cinnamic acid derivative such as caffeic acid or ferulic acid that is esterified with a quinic acid moiety. The most abundant chlorogenic acids in coffee are the 3, 4, and 5-caffeoyl quinic acids followed by the corresponding derivatives with ferulic acid, as well as the dicaffeoyl quinic acids (figure 1). These nine CGAs can make up to 90 % of the total polyphenol content in green coffee. Nevertheless in the past years several other compounds of the chlorogenic acid family have been identified containing e.g. coumaric acid, dimethoxycinnamic acid or sinapic acid as well as a lot of possible isomers of the di-CGA family.

Chlorogenic acids are mainly considered for their antioxidant properties but they play a much more important role for the quality of coffee and coffee beverages as multifunctional compounds. Some of the aspects of the multi-fold role of coffee polyphenols will be highlighted in this review.

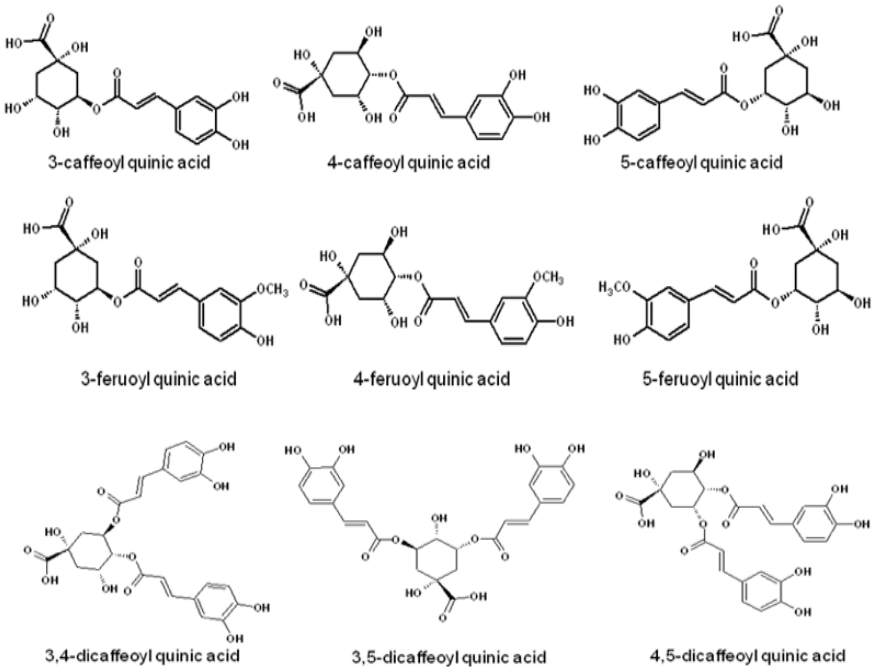


Figure 1. Chemical structures of the nine major chlorogenic acids in green coffee.

RESULTS

Coffee phenols as quality markers for green coffee

The CGA content differs significantly between Arabica and Robusta species with Robusta being richer in chlorogenic acid content. Therefore the CGA content has been frequently used for the classification of coffee samples in combination with other important constituents like caffeine and trigonelline. Different methodologies have been used for this approach such as NIR or LC/MS. Such approaches do not only allow the differentiation between pure Arabica and Robusta samples (figure 2) but also allow the set-up of prediction-models to calculate the blend composition of unknown samples. Like this e.g. adulteration of Arabica coffee with Robusta coffee can be determined up to a certain level dependend of the amount of Robusta added. The importance of CGA in such predictive tools is further highlighted when it comes to decaffeinated samples for which caffeine cannot be used as a marker.

More detailed classifications based on the chlorogenic acid content have been tested to evaluate green coffee quality e.g. differentiation of the cultivar or determination of the origin. While in general these approaches have been shown to work as well their limitations have also been clearly seen. E.g. the crop to crop variety of one cultivar has been found to be higher in some cases than the variation between different cultivars making the results only valid for a specific year of harvesting. Concerning the origin a differentiation between the zone (Africa, Asia, and America) appears possible in some cases but can fail due to limited differences between samples or to the crop to crop variations mentioned before.

Also the post-harvest treatment impacts the chlorogenic acid content as recently shown. Wet processed coffees were found to contain higher amounts of chlorogenic acids. Once again this can be used to evaluate the quality of a green coffee, but on the other hand allows also coffee producers to apply the right practises in order to preserve chlorogenic acids from loss upon post-harvest treatment.

In summary CGA content is one important marker to assess green coffee quality.

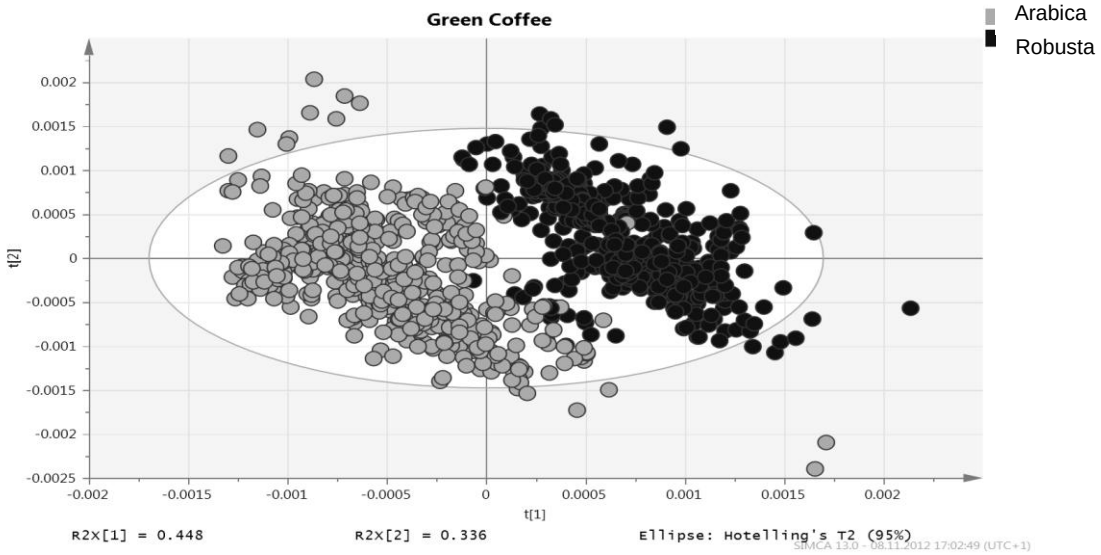


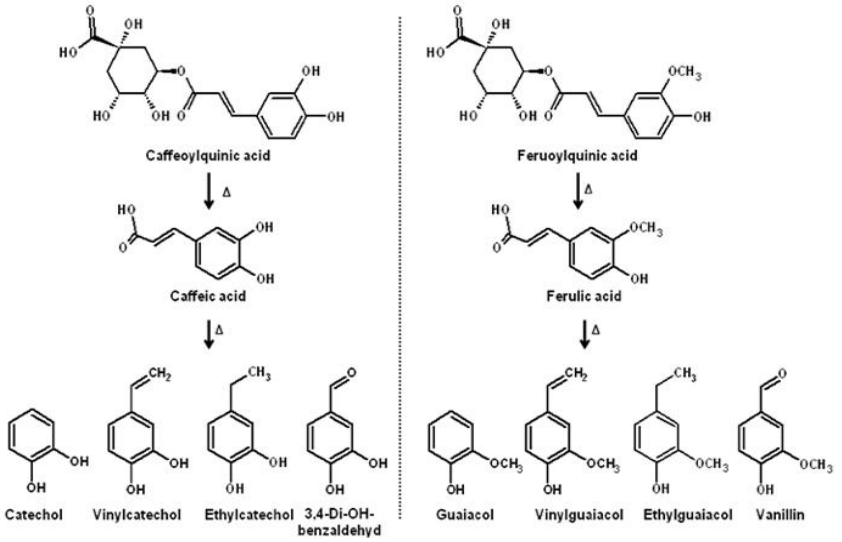
Figure 2. Differentiation of Arabica and Robusta coffees by NIR measurement including CGA.

Coffee phenols and their role in coffee aroma

Beside the quality of the green coffee roasting is a key step for the coffee quality as it is crucial for the development of the well-appreciated fresh coffee aroma. On the same hand it is well known that roasting has a strong impact on the degradation of chlorogenic acids. Depending on the roasting degree the loss of native chlorogenic acids can easily rise up to 90%. As at the same time the nice coffee aroma is generated it is very likely that chlorogenic acids are somehow involved in the aroma formation.

The thermal treatment of chlorogenic acids leads to a cleavage of the ester in first instance. This cleavage gives rise to a quinic acid moiety and the cinnamic part, either in form of caffeic acid or ferulic acid depending on the initial CGA. Both caffeic and ferulic acid are further degraded upon heat treatment. Decarboxylation leads to vinylcatechol, respectively vinylguaiacol. The vinyl moiety can undergo further reactions like oxidation to form 3,4-dihydroxybenzaldehyd or vanillin, hydration to form the corresponding ethyl derivatives, or can even be cleaved to form catechol or guaiacol, respectively. In particular the products formed from ferulic acid breakdown have been identified as being impact compounds of coffee aroma based on their dose-over-threshold factor which is the ratio of the concentration found in coffee and the perception threshold. If this value is above 1, meaning the concentration is above the perception threshold, this compound likely has an impact on the overall aroma of coffee. As seen in figure 3 all breakdown products of ferulic acid have been found at concentrations above their perception thresholds both in Arabica and Robusta samples.

Thus it can be clearly stated that chlorogenic acids play a crucial role in the aroma formation of coffee upon roasting.



Compound	Dose-over threshold Arabica	Robusta
(E)-beta-damescenone	260000	270000
furfurylthiol	110000	170000
MMBF	37000	33000
MBT	27300	27700
3-isobutyl-2-methoxypyrazi	16600	2400
homofuraneol	15000	12000
furaneol	11000	5700
2,3-butandione	3390	3190
4-vinylguaiacol	3200	8900
guaiacol	1700	11000
2,3-pentandione	1320	660
methional	1200	500
vanillin	192	644
2-ethyl-3,5-dimethylpyrazin	165	470
2,3-diethyl-5-methylpyrazin	95	310
sotolon	74	32
4-ethylguaiacol	32	362
abhexon	21	11

Figure 3. Aroma formation upon thermal breakdown of chlorogenic acids and the most important aroma compounds identified in Arabica and Robusta coffee (Semmelroch, 1995).

One well-known issue for coffee is the so-called aroma-staling which can be a particular problem when coffee is stored as a canned or bottled beverage or as a liquid coffee concentrate. Coffee aroma staling is mainly due to the decrease of the coffee-like smelling compound 2-furfurylthiol (FFT). Also here chlorogenic roles are heavily involved. It was

shown that FFT can effectively be trapped by di- and trihydroxybenzenes like catechol or in particular HHQ. These compounds are once again formed upon thermal degradation of chlorogenic acid via quinic acid and caffeic acid as intermediates as already shown in figure 3. The hydroxybenzenes are very sensitive to oxidation to form the corresponding quinones which can easily be attacked by nucleophils. Such a nucleophil is e.g. FFT. This leads to the formation of FFT adducts to the hydroxybenzenes. This pathway was first established in model reaction but the products formed were also detected in real coffee brews confirming the loss of FFT by trapping to benzenes. HHQ was found to be the most effective trapping agent for FFT making this compound very important in the prevention of aroma staling.

So finally the breakdown of chlorogenic acids is important for aroma formation while it plays also a crucial role in the stability of coffee aroma showing the importance of understanding chemical pathways in order to better control positive and negative effects of such reactions.

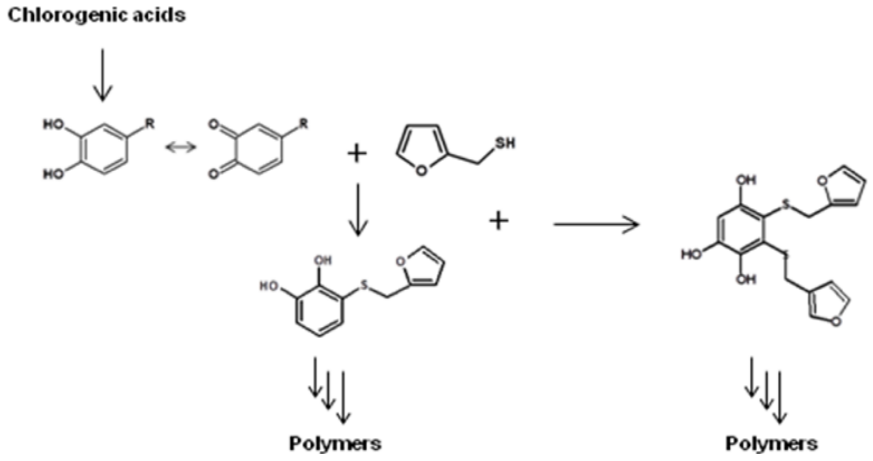


Figure 4. Trapping of FFT by oxidative coupling to HHQ (Mueller, 2006).

Coffee phenols and their role in coffee taste

Beside the coffee aroma also bitterness is one of the major preference drivers. For many consumers a pleasant and balanced bitterness is important which requires strong knowledge on the formation of bitter compounds. Also here CGA have been identified as playing an important role.

A well-known reaction of chlorogenic acids upon roasting is the formation of the corresponding lactones. The heat treatment easily leads to cyclization under loss of a water molecule to form different isomers of quinolactones. This lactone formation has been studied intensely and by applying the so-called taste dilution analysis these lactones have been identified as important bitter compounds in coffee. In particular for light to medium roasted coffees these lactones play a crucial role as they themselves undergo further degradation with higher roasting degree. Their detection thresholds have been found to range from 5-60 mg/L making these lactones quite active. As chlorogenic acids are highly abundant in green coffee beans such concentrations are commonly found in roasted coffee even though lactones are not formed exclusively.

Another class of bitter compounds identified in coffee brew derives from chlorogenic breakdown as well. These so-called phenylindanes are formed via coupling of vinylcatechol which is formed upon cleavage of CGA and decarboxlation of the resulting caffeic acid. The

bitter thresholds have been found being similar to those of the lactones but concentrations of these compounds in coffee brew are rather lower.

A third class of bitter compounds is formed in a similar way as the FTT trapping reported above. In the case of these recently reported bitter compounds the nucleophiles derive from sugar degradation e.g. furfurylalcohol which reacts with the quinone to form the corresponding adducts. The following scheme illustrates the different pathways of bitter compound formation deriving from CGA breakdown.

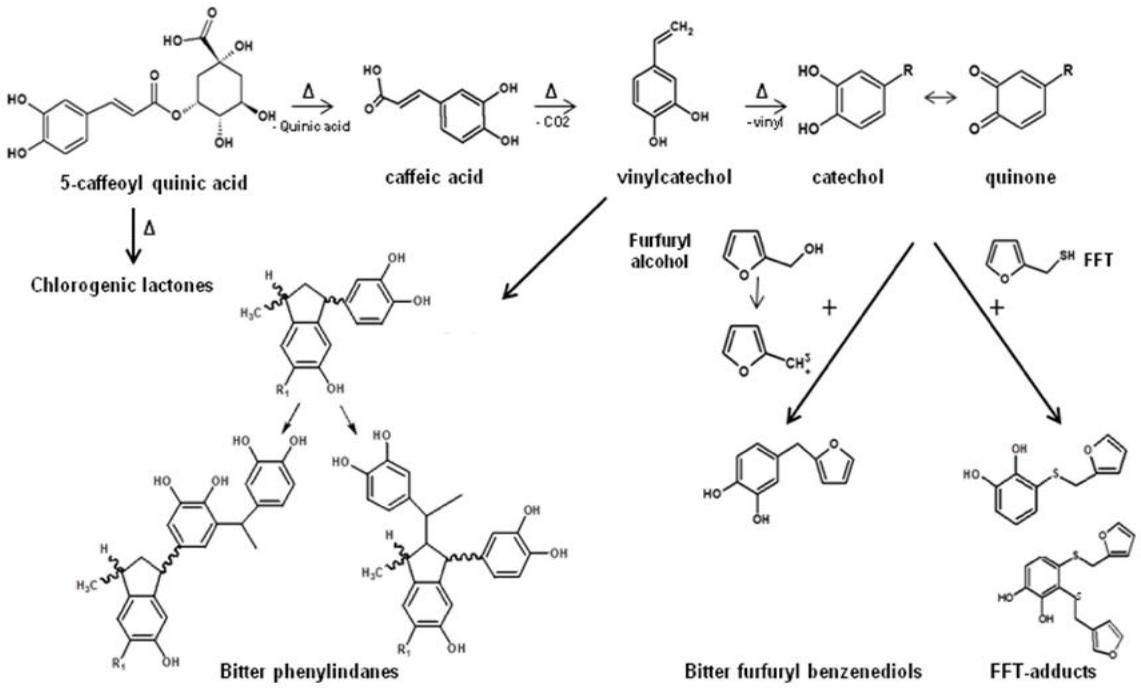


Figure 5. Formation of bitter compounds in coffee from chlorogenic acids.

Coffee phenols and their role for health

The chlorogenic acids as the major group of polyphenols in coffee have been considered as important antioxidants since a while. Several studies show a positive impact of a moderate coffee consumption on different health aspects such as cognitive decline or Type II diabetes which is often ascribed to the presence of coffee antioxidants. Also positive effects on different cancer cell lines have been described that are assigned to the presence of chlorogenic acids.

While in green coffee most of the antioxidant activity can be explained by the nine major chlorogenic acids only, the situation in roasted coffee is more complex. Despite of the rapid degradation of native chlorogenic acids upon roasting the antioxidant activity of coffee remains almost constant. By evaluating the response of the individual chlorogenic acids and their quantitative analysis it can be calculated how much activity derives from the nine major CGA. Proportionally the contribution of those nine CGA decreases with increasing roasting degree (figure 6).

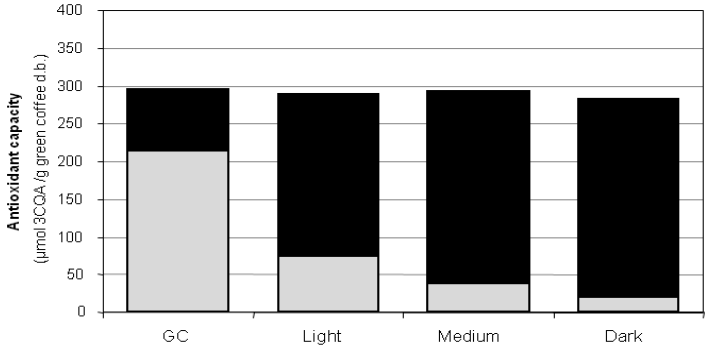


Figure 6. Antixidant capacity of different coffees and the contribution of CGA to it (grey part).

The antioxidant activity of roasted coffee has been investigated intensely and different explanations have been established like the incorporation of chlorogenic acids into melanoidins or the antioxidant activity of Maillard reaction products themselves. Also the interference of AOX assays by other compounds is regularly discussed.

As active principle for the antioxidant activity the vicinal diol plays the most important role based on the capability to interact with electrons and to form stable radicals.

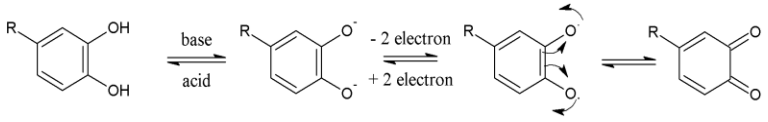


Figure 7. The role of the vicinal diol in antioxidant activity.

The residue R plays a less crucial role although further radical stabilization e.g. by the unsaturated bond in caffeic acid or steric hindrance like for dicaffeoyl quinic acids need to be taken into consideration.

Having a look at the reactions that occur to chlorogenic acids upon roasting (figure 5) it appears that the phenolic moiety remains intact and unchanged, no matter if it is lactone formation, dimerization or degradation to catechol derivatives. Even more complex reactions like trapping of nucleophils or indane formation as described before all take place while maintaining the vicinal diol structure entirely intact. Therefore at least part of the antioxidant activity of roasted coffee is likely caused by all the different compounds formed upon CGA transformation. The complexity of the different reaction pathways of which probably only a part has been identified yet as well as the tremendous challenge of analyzing all the different compounds in coffee samples explains why the understanding of the antioxidant activity of roasted coffee is still limited and requires further investigation. The elucidation of the complex chemistry of coffee polyphenols remains an important and challenging task but is crucial to better control quality attributes like aroma and taste, but also to better understand possible health effects of coffee.

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A new Approach for the Prediction of Caffeine Content in Raw Coffee by Near Infrared spectroscopy

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SUMMARY

In this work, a universal chemometric model based on diffuse reflectance near infrared spectroscopy (NIRS) and high pressure liquid chromatography (HPLC) data is proposed to predict caffeine content in raw coffees. However, the exploitation of the results of these analyses, in most situations, requires the prior confrontation between spectral data and results of the same samples obtained by the analytical reference methodology, creating models. So, the construction of a robust calibration model requires that both analytical methods are employed simultaneously to a large numbers of samples, which consists of a limiting factor for the widespread use of spectroscopy. Because of that, there are four main innovations in the construction of this multivariate model: (*i*) differences between artificial coffee matrices in caffeine and trigonelline concentrations were obtained; (*ii*) the use of different coffee species for the calibration steps, (*iii*) the selection of specific variables (wavelengths) for caffeine through the spectra of the pure compounds and the standard addition and (*iv*) low number of HPLC analyses. Partial least squares regression (PLSR) was used as a multivariate method to construct the model, and more than eighty different samples were used as external validation data sets. The prediction model for caffeine concentration, using 7 latent variables, presented 0.082 of root mean square error of prediction (RMSEP) and 0.98 of correlation coefficient (r_{cv}).

INTRODUCTION

Currently, the rapid growth of automation and technology has brought modern analytical methods for identification and quantification of the main compounds present in coffee beans and other agronomical products. Among them, HPLC, GC, NMR H^1 and electrochemical methods represent the most important technologies. Unfortunately, most of these techniques have disadvantages like a need for complex samples treatments (clean up). Moreover they are often time consuming, costly and destructive. To overcome these limitations, attention has been paid to the development of spectroscopic analysis coupled with chemometric tools.

However, to determine the content of a chemical compound present in coffee beans by spectroscopy, the relationship between the information of both, infrared spectra and reference methods, *e.g*. HPLC, must be established.

The aim of this work was the construction and validation of a universal multivariate model to estimate caffeine contents in raw coffees using near infrared diffuse reflectance. In the present work, the improvement of the methodology was verified by the use of different coffee species and artificial matrices. This approach allowed improving the range of caffeine content in the bean and a reduction of the number of analyses by the reference method.

MATERIALS AND METHODS

In order to identify the wavelengths related to calibrate caffeine in green coffee beans, spectra of pure caffeine (minimum 99 % purity), trigonelline (99 %), sucrose (99.5 %), 5-CGA (5-caffeoylquinic acid) (95 %), protein (casein 90%) and carbohydrate (cellulose high purity) were performed. The reagents were supplied by Sigma-Aldrich (Munich). The lipid fraction was extracted from crude coffee beans by mechanical pressing.

In the present work, ten green coffee varieties from the IAC germoplasm bank were analyzed using diffuse reflectance near infrared spectroscopy (NIRS) and HPLC in order to determine their caffeine contents (Table 1). The samples from *C. arabica* and *C. canephora* species were selected based on the different contents of this methylxanthine that ranged from 0.07 to 3.52% (w/w). One artificial coffee "matrix" with 0.4% of caffeine was obtained by extracting caffeine from a coffee sample with methanol/water (70/30). This matrix was considered as a "variety" (sample 10).

Table 1. Coffee samples from IAC germoplam bank used for the construction of the models.

Samples	Species	Varieties
1	*Coffea arabica*	Yellow Bourbon
2	*Coffea arabica*	Material with low caffeine content [6]
3	*Coffea arabica*	IAC 3996 - Ep 416 - Cova 1152 - Planta B
4	*Coffea canephora*	*Conillon* collection 66 - cova 3
5	*Coffea canephora*	Apoatã collection 3599 - Plant 9
6	*Coffea canephora*	Apoatã collection 3597 - Plant 1
7	*Coffea canephora*	Bukobensis collection 3
8	*Coffea canephora*	Ep 133 - 350 - 1653 - Cova 4
9	*Coffea canephora*	Ep 189 - Cova 128
10	*Coffea canephora*	*Conillon* collection 66 - cova 3 caffeine extracted

Diffuse reflectance spectra of green grounded coffee and of trigonelline, caffeine, lipids, cellulose, casein, 5-CGA and sucrose respectively, were obtained using a near-infrared NIRSystems 6500 spectrophotometer (Foss NIRSystems, Raamsdonksveer, Netherlands) equipped with a reflectance detector and sample transport module. Each spectrum was profiled with 256 scans in the 1100 to 2500 nm range and a resolution of 2 nm. In this work, three different aliquots of the sample were used and the spectrum of each aliquot was recorded.

All the 363 Near Infrared spectra were obtained by the mixture of proportions of different species and varieties and also through the addition of pure caffeine. Each spectrum was profiled with 256 scans from 1100 up to 2500 nm at 2 nm resolution respectively. All analyses were made in triplicate.

For the HPLC analyses, caffeine was extracted in methanol: water (70:30) at 60° C for 1 hour and quantified by high performance liquid chromatography (HPLC). A Shimatzu chromatograph used was equipped with UV-Vis detector and C-18 column Shim-pack CLC-ODS (M) of 4.6 x 250 mm and 5 mm in particle diameter, with pre-column of 4 x 1 mm. The elution was isocratic with mobile phase consisting of methanol: acetic acid: water (30:0,5:69,5, v: v: v) at a flow rate of 1 ml / min at 22° C. The wavelength reading of the samples was 272 nm. The concentration of caffeine was determined by the ratio between the

peak area of caffeine in the sample and the respective standards of known concentrations. The samples were analyzed in triplicate.

The original spectroscopic profiles were organized into a matrix format **X** (*I*x*J*), where each replicate was considered as one sample. Data analysis was carried out using Matlab 6.5 software (The MathWorks, Co., Natick, MA, USA) with the PLS_Toolbox computational package (Eigenvector Research, Inc. – PLS_Toolbox version 3.02.). In the present study, two pre-treatments were applied to the original data matrix: Savitzky-Golay smoothing with a window size of 7 points and first derivative function. Variable selection was carried out by the ordered predictors selection (OPS) method and visual inspection. The partial least squares method (PLS) was used as the regression method for modelling.

RESULTS AND DISCUSSION

The regions of the spectrum defined as important for the prediction of the caffeine content (selected variables) were visually selected comparing the spectra of the samples with the spectra of the pure caffeine and others pure compounds found in coffee and with the OPS algorithm. From the initial 700 variables (NIR spectrum), 128 were selected to construct the caffeine prediction model. These variables are grouped in 16 regions indicate in Figure 1.

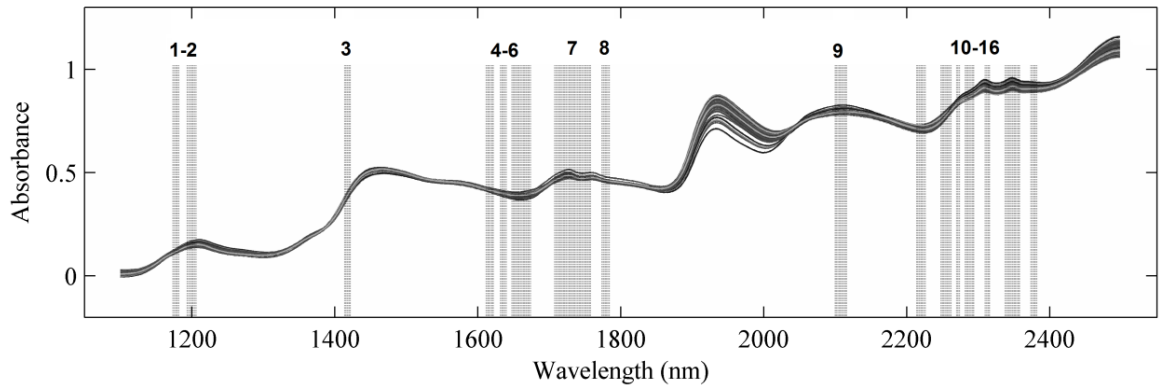

Figure 1. Regions of the infrared spectra selected for the regression model of caffeine.

Seven latent variables were used for the PLS model. The values of the statistical parameters RMSECV (root mean square error of cross-validation), r_{cv} (correlation coefficient of cross validation) and RMSEP (root mean square error of prediction) were 0.135 ± 0.01, 0.98 ± 0.00 and 0.082, respectively. The multivariate regression model for the determination of caffeine was built, tested and validated with 81 external samples (Figure 2).

The relative mean error between the external analysis by NIRS and HPLC for caffeine contents in raw coffees was 8.2%, for concentrations ranging between 0.8% and 3.0%. The reliability of the spectral bands (wavelengths) selected, suggests the potential of the PLS model to be used as a universal model for raw coffee analyses. It could be used by any research group working with multivariate models, using other coffee samples (species and varieties), different equipment brands, milling degree or any other variable.

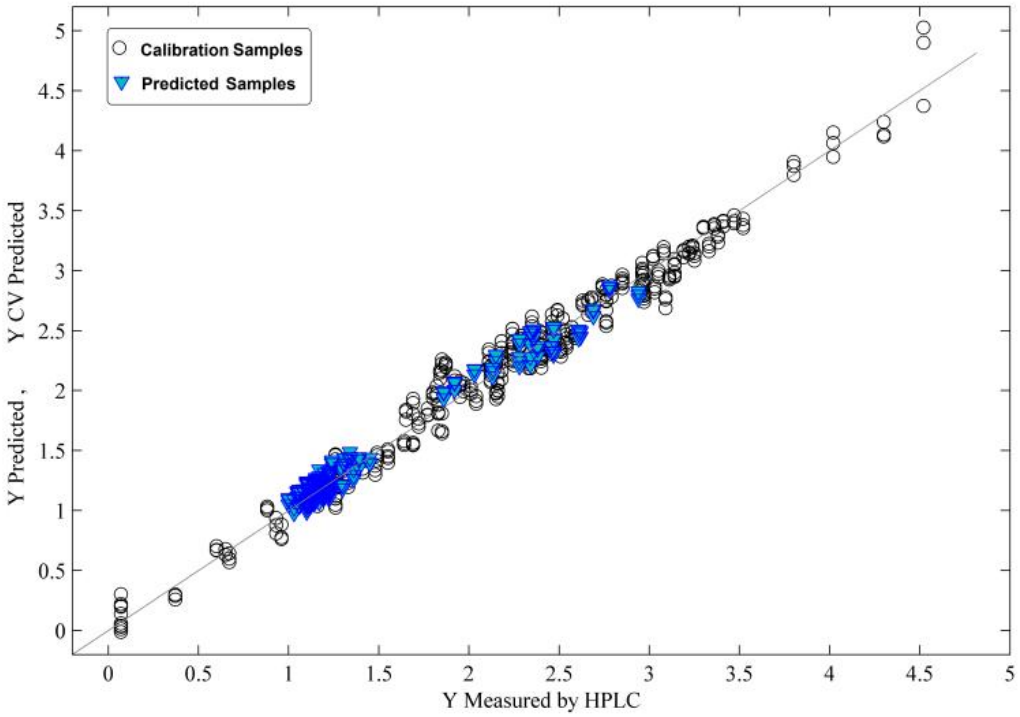

Figure 2. Plots of measured versus predicted samples in calibration (○) and prediction (▼) sets.

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Composition and Antioxidant Activity of Roasted Arabica Coffee from Different Cultivars Growing in the Same Edapho-Climatic Conditions

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SUMMARY

Coffee beans composition is affected by genetic characteristics, edapho-climatic conditions, post-harvest and roasting process and the final preparation of coffee brews. Hydrosoluble (nicotinic and chlorogenic acids, trigonelline and caffeine) and liposoluble (cafestol and kahweol) compounds and melanoidins stand out for their contribution to health mainly as antioxidants. The objective of this work was to study the composition and the antioxidant activity (AA) of roasted coffees of traditional cultivars (Catuaí and Icatu) and artificial crosses. The edapho-climatic conditions and roasting process were standardized, so the differences in composition and AA could be mainly attributed to the genetic characteristics of each cultivar. Hydrosoluble and liposoluble compounds were analysed by RP-HPLC, and melanoidins by spectrophotometry. Inhibition of linoleic acid autoxidation (formation of conjugated dienes), radical scavenging activity by ABTS (TEAC) and total phenolics (TP) were determined to estimate the antioxidant activity (AA). Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to data. PC1 (33% of the variance) was positively correlated to kahweol, melanoidins, TEAC and dienes values, and negatively correlated to cafestol and trigonelline contents. PC2 (22%) was negatively correlated to 5-CQA and TP and positively correlated to nicotinic acid and caffeine. HCA indicated three main groups of coffee varieties. Traditional cultivars showed high levels of cafestol, 5-CQA, trigonelline and caffeine, and lower contents of kahweol and melanoidins, nicotinic acid and AA. IPR100, 101 and 105 (Catuaí Sh2, Sh3 genetic background–GB) and IPR106 (Catuaí-GB) showed high levels of nicotinic acid, caffeine, melanoidins and AA (Dienes). Cultivars Sarchimor derived (IPR97, 104 and 107), IPR102 (Icatu X Catuaí) and IPR108 (Sarchimor X Icatu X Catuaí) were discriminated by the high levels of 5-CQA, trigonelline, kahweol, and high AA (TP and TEAC). In general, coffees originated by artificial crosses showed higher AA compared to traditional cultivars.

INTRODUCTION

The composition of coffee beans is affected by genetic characteristics, edapho-climatic conditions, post-harvest and roasting process and the final preparation of coffee brews (KY et al., 2001; LEROY et al., 2006). The breeding has the objective to transfer resistance genes from robusta coffee to arabica via an interspecific hybrid, the Timor Hybrid (BERTRAND et al., 2008). Besides conferring resistance to pests and diseases and an improvement of

agronomic characteristics, these crosses may also affect the composition, bioactivity and sensory quality of the coffees. Coffee consumption has beneficial effects to health with emphasis on its nutritional and functional importance related to its potential as an antioxidant (BERTRAND et al., 2008, BUTT; SULTAN, 2010). Hydrosoluble (nicotinic and chlorogenic acids, trigonelline, caffeine and melanoidins) and liposoluble (cafestol and kahweol) compounds stands out for their contribution to health mainly as antioxidants (SÁNCHEZ-GONZÁLEZ; JIMÉNEZ – ESCRIG; SAURA – CALIXTO, 2005; HIGDON; FREI, 2006). The objective of this study was to evaluate the composition and the antioxidant activity (AA) of roasted coffees of traditional cultivars (Catuaí and Icatu) and modern crosses. The edapho-climatic conditions and roasting process were standardized, so the differences of composition and AA could be mainly attributed to genetic characteristics of each cultivar.

MATERIALS AND METHODS

The coffees (eleven samples) were harvested at the Agricultural Technologic Park of Coop COCARI, Mandaguari, Paraná, Brazil, from May to July 2009 at latitude 23°32'52" (South), altitude of 650 m and average annual temperatures of 22 to 23°C. The traditional cultivars (Catuaí and Icatu) and modern crosses (IPRs 97, 100, 101, 102, 104, 105, 106, 107 and 108) (SERA et al., 2007a) were collected considering the time of harvesting according to the maturation of each cultivar. Post-harvesting conditions were standardized for all cultivars. Cherry fruits were manually selected, washed and sun-dried in patio. The traditional coffees and modern crosses were subjected to medium roasting process (8 to 11 minutes at 200-210°C, L* around 28) and grounded (0.5 mm particles) in the disk mill (PERTEN 3600, Sweden).

The contents of liposoluble (cafestol and kahweol) and hydrosoluble (nicotinic acid, trigonelline, 5-CQA and caffeine) compounds were determined by RP-HPLC (DIAS et al., 2010; ALVES et al., 2006) and melanoidins were estimated by spectrophotometry at 420 nm (LOPEZ-GALILEA; DE PENA; CID, 2007). To determine the antioxidant activity (AA) three methods were used: the inhibition of linoleic acid autoxidation which measures the formation of conjugated dienes at 234 nm (MAU et al., 2005); the determination of the radical scavenging activity by ABTS (TEAC) (SÁNCHEZ- GONZÁLEZ; JIMÉNEZ – ESCRIG; SAURA – CALIXTO, 2005) and by measuring the redox potential of the total phenolics (TP) (AOAC, 1990). Principal Components Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were applied by XLStat software (ADDINSOFT, 2007). Unweighted pair-group average method, as the linkage rule and Euclidean distance (similarities) were used to generate the dendrograms.

RESULTS AND DISCUSSION

PCA (Figure 1) and HCA (Figure 2, Table 1) were used for the simultaneous evaluation of the composition and antioxidant activity characteristics of the coffees. Correlations between variables and characterization of the different cultivars could also be observed. The first component (PC1) accounted for 33% of variance and was positively correlated to kahweol, melanoidins, TEAC and dienes values, and negatively correlated to cafestol and trigonelline contents (Equation 1). The PC2 (22%) was positively correlated to 5-CQA and TP and negatively correlated to nicotinic acid and caffeine (Equation 2). The HCA indicated three main groups of coffees (Figure 2) and the average value for compounds content and AA for each group (Table 1). It could be observed that there was genetic similarity among the coffees in each group.

$$PC1 = - 0,70 \text{ cafestol } +0,81 \text{ kahweol} – 0,74 \text{ trigonelline } +0,77 \text{ melanoidins } + +0,67 \text{ TEAC } + 0,54 \text{ dienes} \quad (1)$$

$$PC2 = - 0,58 \text{ nicotinic } + 0,74 \text{ 5-ACQ} – 0,53 \text{ caffeine } + 0, 68 \text{ TP} \quad (2)$$

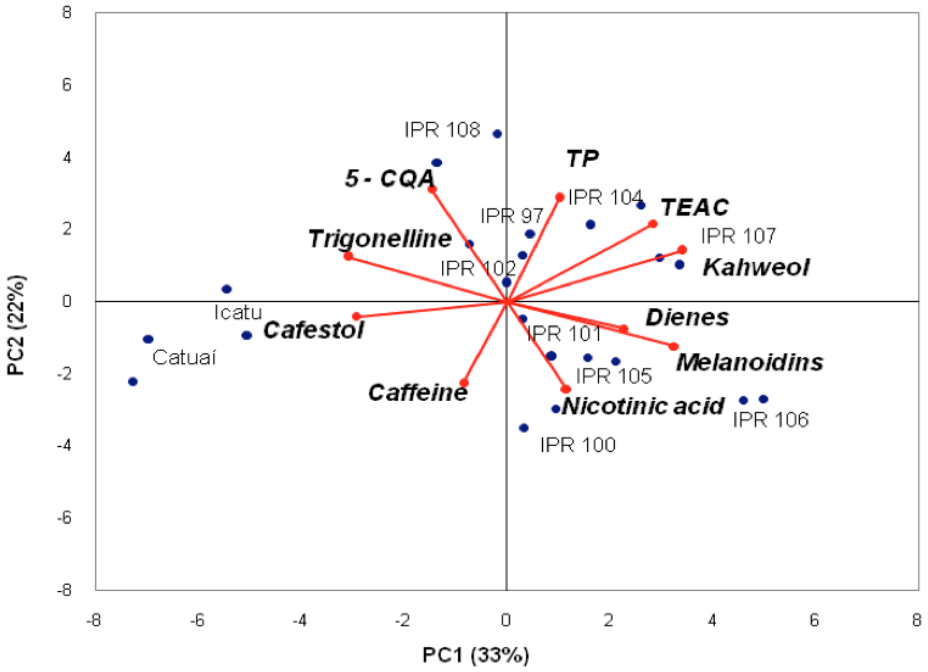

Figure 1. PCA Biplot considering composition and antioxidant activity of the arabica roasted coffee cultivars.

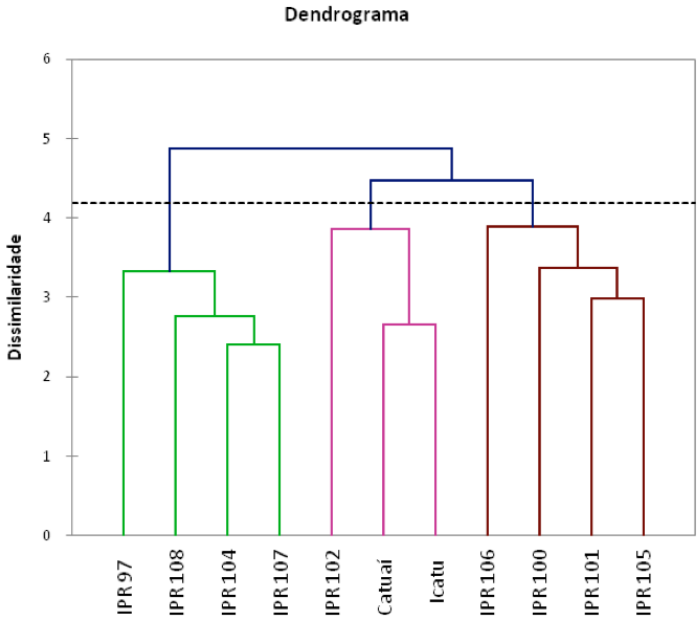

Figure 2. Dendogram considering composition and antioxidant activity of roasted coffee cultivars.

Table 1. Average value of compounds content and antioxidant activity for each group formed by HCA.

Group	Cafestol	Kahweol	Nicotinic	Trigonellie	5 CQA	Caffeine	Melanoidins	TEAC	TP	Dienes
1	1476	1132	22	1125	1476	875	0,58	620	4754	84
2	805	724	25	973	1308	1107	0,42	560	4636	83
3	596	1068	28	863	799	1101	0,70	584	4341	85

Composition expressed in mg 100 g^{-1}, melanoidins in UA at 420 nm, TEAC in µmol eq g^{-1}, TP mg 100 g^{-1} and Dienes in %.

The first group was formed by cultivars Sarchimor derived (Vila Sarchi and Timor Hybrid) such as IPR 97, 104 and 107, and IPR 108 (Sarchimor X Icatu X Catuaí) (Figure 2). These cultivars were allocated at the top of the plan and were discriminated by the high levels of 5-CQA, trigonelline and kahweol, and high AA (TP and TEAC) (Figure 1 and Table 1). The cultivars that presented genetic background associated to robusta coffee were discriminated by compounds related to environmental adaptation as 5-CQA and TP in which the high levels indicate poor adaptation. The lack of adaptation was also indicated by sensory analysis since IPR 97 was described as presenting green taste, green flavor, high acidity, low turbidity and brightness. The IPRs 102, 104, 107 and 108 cultivars showed intermediate sensory characteristics between IPR 97 and coffee with genetic background Catuaí (Kitzberger et al., 2011).

The second group was formed by traditional cultivars (Catuaí and Icatu) and IPR 102 (Icatu and Catuaí derived) (Figure 2). These coffee cultivars showed high levels of cafestol, 5-CQA, trigonelline and caffeine and low content of kahweol, compounds that were determinated by genetic and environmental factors (KY et al., 2001; MONTAVON; MAURON; DURUZ, 2003), and lower AA (Dienes, TEAC and TP) (Figure 1 and Table 1).

The cultivars IPR 100, 101 and 105 (Catuaí Sh2, Sh3 genetic background–GB) and IPR 106 (Catuaí-GB) formed the third group (Figure 2). These coffees showed high levels of nicotinic acid and melanoidins (compounds produced in the roasting process), high contents of caffeine, and higher AA (Dienes). These cultivars showed an opposite behaviour compared to the Catuaí that originated them and did not present genetic association with robusta genes. SH2 genes conferred resistance to some races of H. vastatrix and were identified in pure arabica coffees from Ethiopia. SH3 gene is probably derived from Coffea liberica (SERA et al., 2007b). For these IPR cultivars, Kitzberger et al. (2011) described sensory attributes such as coffee color, turbidity, coffee aroma, chocolate aroma, sweet taste, bitter taste and body positively correlated with coffee cup quality.

In general, coffees originated by artificial crosses showed higher AA compared to traditional cultivars.

CONCLUSIONS

Coffees from the modern crosses (IPRs 97, 100, 101, 102, 104, 105, 106, 107, 108) showed higher antioxidant activity and different composition from the traditional coffees studied (Catuai and Icatu). It could be also observed that coffees with similar genetic background had similar behaviour considering its composition and antioxidant activity.

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Kitzberger, C. S. G., Scholz, M. B. S., Silva, J. B. G. D., Benassi, M. T. Discriminação sensorial de cafés resultantes de cruzamentos entre *Coffea Arabica*, Villa Sarchi e

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Green Coffee Beans Composition, and Roasted Beans and Coffee Brews Characteristics: Diversity among Arabica Cultivars

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SUMMARY

Several genetic crosses result in different green beans composition which, coupled with cultural practices, climatic and post-harvest conditions, contribute to define the roasted coffee and beverages characteristics. The relationship between the composition of green beans and the characteristics of roasted beans and coffee brews for traditional cultivars (Bourbon, Catuaí and Icatu) and artificial crosses (Iapar 59, IPRs 97, 98, 99, 100, 101, 102 103, 104, 105, 106, 107 and 108) were studied. All coffees were grown in the same edapho-climatic conditions and had standardized processing conditions. Contents of cafestol, kahweol, trigonelline, 5-CQA, caffeine, total phenolics (TP), sugars (total and reducing), proteins and lipids and density (DensG) were determined in green beans. Density (DensR) and expansion were measured in roasted beans. Titratable acidity and pH were determined in the coffee brews. Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to data. Catuaí, IPRs 100, 101, 103, 105 and 106 (Catuaí x Icatu or Catuaí Sh_2Sh_3) were discriminated from others (Sarchimor crosses, Bourbon and Icatu) by the high values for nitrogen compounds (trigonelline and caffeine), expansion and pH and lower values of 5-CQA, TP, lipids, reducing and total sugar, DensG (compounds related to maturation) and DensR (PC1, 30% of variance). Catuaí, Icatu and Bourbon and IPR 98 were discriminated by lower kahweol and higher cafestol values (PC2, 18%). Therefore, some crosses had features in common with the traditional cultivars that originated them in the green, roasted beans and brews characteristics. The differences between traditional and breeding cultivars were mainly due to the diterpenes suggesting the importance of genetic effect on these compounds content.

INTRODUCTION

Composition of coffee green beans depends on the genetic characteristics, cultural practices, climatic and post-harvest conditions (JÖET et al., 2010) and contributes to define the characteristics of roasted coffees and coffee brews. Amongst the factors affecting the composition of coffee, genetic variability has been highlighted for direct contribution on the diversity considering the acidity, sugar, lipid and caffeine contents (SCHOLZ et al., 2000) and sensory quality (MEDINA FILHO, 2007). It is also known that parameters such as altitude and temperature affected the composition in a different way (cell wall carbohydrates, chlorogenic acids, lipids and caffeine) depending on the variety (JÖET et al., 2010). The breeding programs have focused their efforts to increase the resistance of pests and diseases by the transfer of genes from *Coffea canephora* to *C. arabica* via an interspecific hybrid, the Timor Hybrid (SCHOLZ et al., 2000). Those modern crosses have substantial amount of *C.*

canephora genetic material that influenced the green and roasted coffee composition. Therefore, the relationship between composition of the green beans, the characteristics of roasted beans and coffee brews for traditional cultivars and modern crosses grown in the same edapho-climatic and processing conditions were studied.

MATERIALS AND METHODS

The traditional cultivars (Bourbon, Catuaí and Icatu) and modern crosses (Iapar 59 and IPRs 97, 98, 99, 100, 101, 102 103, 104, 105, 106, 107 and 108) (SERA et al., 2007) were harvested at the Agricultural Technologic Park of Coop COCARI, Mandaguari, Paraná, Brazil, from May to July 2009. Although the time of harvesting was according to the maturation of each cultivar, harvesting and post-harvesting conditions were the same for all cultivars. The coffees were grown at latitude (S) 23°32'52", altitude of 650 m and average annual temperatures of 22 to 23°C.

The green coffee beans were frozen (-18°C) and immediately grounded prior to analysis using liquid nitrogen to prevent oxidation of the matrix compounds. The contents of liposoluble diterpens (cafestol and kahweol) and hydrosoluble compounds (trigonelline and 5-CQA) were determined by RP-HPLC (DIAS et al., 2010; ALVES et al., 2006). Caffeine (at 273 nm) and total phenolics content (at 760 nm) (AOAC, 1990), sucrose and reducing sugars (at 535 nm) (SOUTHGATE, 1976) were analyzed by spectrophotometry. Protein content was obtained by MicroKjeldahl and lipid content by Soxhlet extraction with petroleum ether (AOAC, 1990). The density of the green beans was also analyzed (DensG, mg g^{-1}) (BUENAVENTURA-SERRANO; CASTAÑO-CASTRILLÓN, 2002).

To obtain the roasted beans, green beans were subjected to medium roasting process (8 to 11 minutes at 200-210°C, L* around 28) and grounded (0.5 mm particles) in the disk mill (PERTEN 3600, Sweden). The roasted coffee beans were also characterized by density (DensR, mg g^{-1}), expansion and lightness (L*).

The beverages were prepared as described by Kitzberger et al. (2010). The pH was determinate by potenciometry and the titratable acidity was measured with NaOH 0.1N for 20 mL of beverage (AOAC, 1990).

Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were applied by XLStat, software (ADDINSOFT, 2007). Unweighted pair-group average method and Euclidean distance were used to generate the dendrograms in the HCA method.

RESULTS AND DISCUSSION

PCA and HCA were used to the simultaneous evaluation of the composition of green beans and the characteristics of roasted beans and coffee brews for different arabica coffee cultivars (Figure 1, Figure 2 and Table 1). The first-two components explained 48% of the variability of data: PC1 accounted for 30% of variance and PC2 for 18%.

PC1 was correlated to trigonelline, pH and expansion (positively) and 5-CQA, acidity, density (DensG and DensR) and reducing sugars. Cultivars with genetic background “Sarchimor” (Villa Sarchi and Timor Hybrid) and Icatu x Catuaí (IPR 102) and Sarchimor x Catuaí x Icatu (IPR 108) were separated in PC1 by compounds related to maturation and genetic aspects.

PC2 was mainly associated with diterpenes (positively for kahweol and negatively for cafestol) and negatively for total sugars. Cultivars Catuaí, Icatu and Bourbon and the cross

IPR 98, located at the bottom of the plan, were discriminated for their lower kahweol, higher cafestol, indicating that the diterpenes content should be determined by genetics crosses.

Protein and lipids contents presented less variation among cultivars (Figure 1, Table 1)

Three main groups of coffees could be observed by the HCA (Figure 2) and the average value for compounds content and other characteristics for each group were in Table 1.

PC1= – 0,56 5-CQA – 0,83 RS + 0,92 pH – 0,66 acidity – 0,83 DensR+ 0,72 trigonelline - 0,55 DensG + 0,61 Exp
Equation 1

PC2= - 0,71 cafestol + 0,79 kahweol -0,50 TS
Equation 2

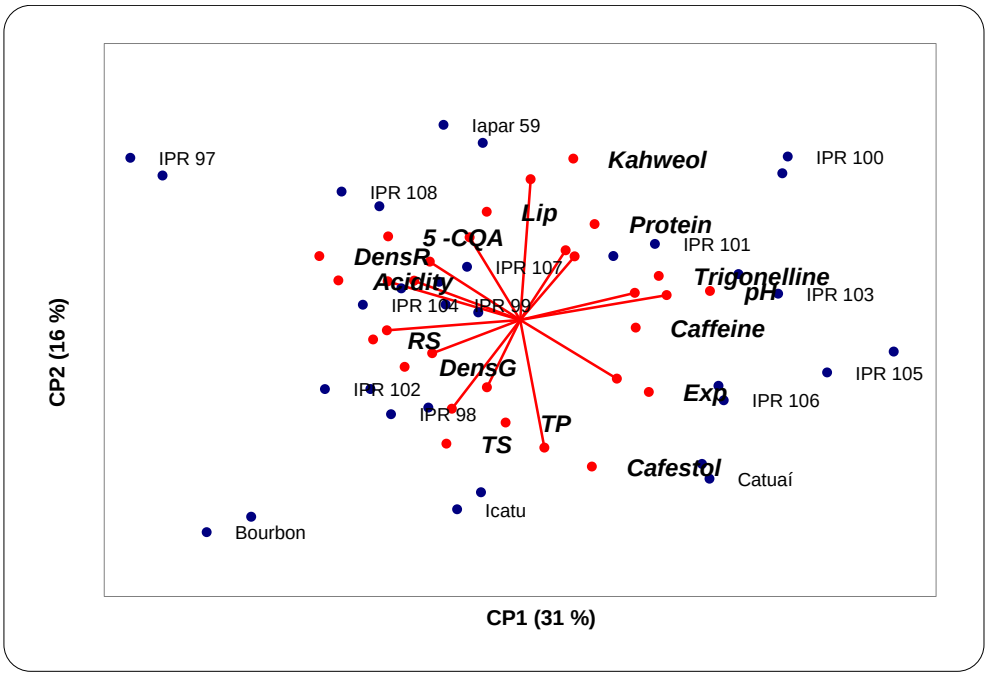

Figure 1. PCA Biplot of the coffee cultivars considering green beans composition and characteristics of roasted beans and coffee brews.

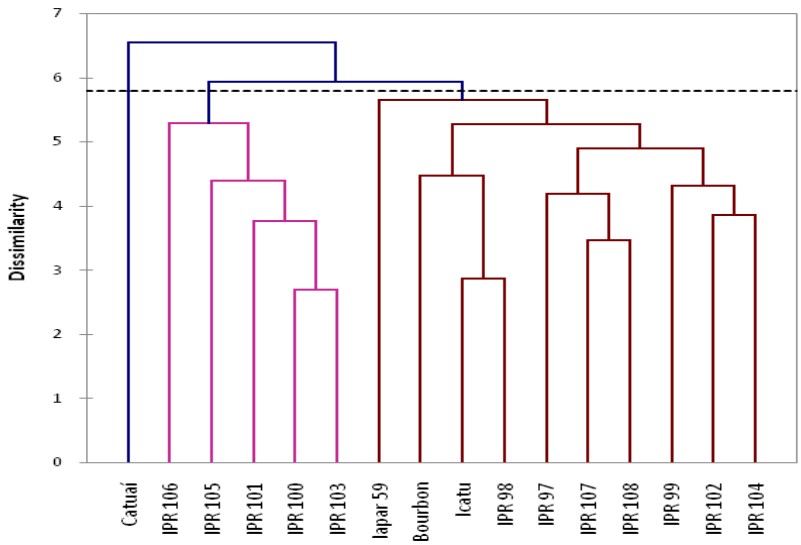

Figure 2. Dendogram of the coffee cultivars considering green beans composition and characteristics of roasted beans and coffee brews.

Table 1. Average values for green beans composition and characteristics of roasted beans and coffee brews for each group formed by HCA.

Group	Pro	Lip	Caf	Kahw	Caffei	Trig	5 CQA	TP	RS	SUC	DensR	DensG	Exp	L*	Acidity	pH
1	18	13	604	371	1385	996	3569	4551	200	7897	0,34	0,56	27	31	2,75	5,19
2	17	13	370	869	1459	1175	3931	4765	242	7509	0,32	0,62	38	27	2,73	5,31
3	17	13	402	771	1407	1021	4277	4956	452	7612	0,39	0,65	28	28	2,96	5,09

Composition expressed in mg 100 g of green coffee^{-1}, densR and densG mL g^{-1}, protein and lipids %.

The cultivars Catuaí, IPRs 100, 101 and 105 derived from a cross of Catuaí *Sh$_2$ Sh$_3$*, IPR 103 (Catuaí X Icatu) and 106 (Icatu) were allocated in the right side of the plan and were discriminated from others (Sarchimor crosses, Bourbon and Icatu) (Figure 1). The first group was formed by only Catuaí and was mainly characterized by the high value of cafestol (Table 1, Figure 2). A second group was formed by Catuaí *Sh$_2$ Sh$_3$* (IPR 100, 101 and 105), Icatu x Catuaí (IPR 103) and Icatu derived (IPR 106) (Figure 2, Table 1). Those coffees present higher values for kahweol, nitrogen compounds (trigonelline, caffeine and protein), expansion (Exp) and pH (Table 1, Figure 1). Caffeine, trigonelline and chlorogenic acids are genetically controlled while sucrose and proteins are influenced by environmental conditions and/or the cultural practices (KY et al., 2001; MONTAVON; MAURON; DURUZ, 2003; VAAST, 2006; GEROMEL et al., 2006). It was observed that modern crosses (IPR 100, 101, 102, 103, 105 and 106) had different characteristics from those cultivars that originated them (Catuaí and Icatu) (Figure 1 and Figure 2).

The Bourbon, Icatu, Sarchimor (Iapar 59, IPR 97, 98, 99 and 104), Icatu x Catuaí (IPR 102), Sarchimor x Mundo Novo (IPR 107) and Sarchimor x Icatu x Catuaí (IPR 108) cultivars formed the third group allocated on the left side of the plain (Figure 2). Those coffees presented higher values of 5-CQA, reducing sugar, total phenolics, density (DensR and DensG) and acidity (Table 1, Figure 1). Bertrand et al. (2008) reported that the chlorogenic

acids could be used to discriminate arabica coffee cultivars considering their genetics aspects and geographic origins. Chlorogenic acids, sucrose and TP concentrations were associated at the maturation stage of coffee beans and the high values of these compounds appointing to incomplete maturation at harvest (VAAST, 2006). In sensory analysis, Bourbon brew (growing in the region of Mandaguari) was characterized as being less body and for having lower color intensity and bitter taste comparing to Catuaí, Icatu, Iapar 59, IPR 98, 99 and 103 (KITZBERGER et al., 2010) and IPR 97 discriminated from others cultivars due the green coffee aroma and taste, high acidity, low turbidity and brightness (Kitzberger et al., 2011).

CONCLUSIONS

Considering the green beans, roasted coffee and beverage composition, some crosses had similar behavior to the traditional cultivars that originated them. The crosses Sarchimor, IPR 102, 107 and 108, Bourbon, Icatu and Catuaí were characterized by the presence of compounds that suggest a poor adaptation of edapho-climatic growing conditions.

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Diversity between *Coffea* Arabica Cultivars: Organic Acids Composition

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SUMMARY

The content of organic acids in coffees is affected by factors such as variety and growing conditions, roasting process (temperature and time) and brewing conditions. Quinic, malic, citric, acetic, and lactic and clorogenic (5-ACQ) acids were the most important compounds of this class in green and roasted coffees. The content of organic acids for traditional cultivars (Bourbon, Catuaí and Icatu) and modern crosses (Iapar 59, IPRs 97, 98, 99, 100, 101, 102 103, 104, 105, 106, 107 and 108) were studied in order to correlate the acids profile and the genetic background of the coffees. All coffees grown in the same edapho-climatic conditions and processing operations were standardized. Green beans, roasted coffees and coffee brews were evaluated; the organic acids were quantified by RP-HPLC. The green beans cultivars from a cross of Catuaí Sh_2 Sh_3 (IPRs 100, 101 and 105), IPR 102 (Catuaí x Icatu), 106 (Icatu), 107 (SarchimorxMN) and traditional cultivars were separated from Sarchimor crosses and IPR 103 (characterized by high contents of quinic and citric acids and lower of 5-CQA). Catuaí Sh_2 Sh_3 (IPR 100, 101, 105, 103), IPR 106, Sarchimor (IPR 98, 99 and Iapar 59) and Catuaí were discriminated by the high content of quinic acid and pH value observed in roasted coffee and coffee brews. Others cultivars presented high levels of malic, 5-CQA, lactic, citric and high titratable acidity. Beans of the cultivars Catuaí Sh_2 Sh_3 derived were characterized by its complete maturation, an opposite behavior to the others crosses.

INTRODUCTION

Flavor and aroma are important attributes of coffee brews and contributed for sensory quality. Organic acids content, mainly the free form, influences the acidity of the coffee brews (VERARDO et al., 2002). Coffee species and varieties, conditions of growing, stage of maturation, roasting and brewing processes and storage time of the coffee beverage are among the factors that influence the organic acids profile in coffee brews (VERARDO et al., 2002; ROGERS et al., 1999). Genetic variability is responsible for diversity in the composition of coffee (SCHOLZ et al., 2000) and influence the acidity, and content of sugar, fat and caffeine, and hence the sensory quality (MEDINA FILHO, 2007). The acetic and lactic acids seemed to be generated during roasting process from carbohydrate precursors (sucrose) and their contents are negligible in green coffee (BALZER, 2001; GINZ et al., 2001).

Organic acids play an important role in plant development, because they assist chelation and neutralization of the toxicity of aluminum (PING; RONG, 2006). Those compounds promote

rapid adaptation of cellular metabolism and they also activate and attach potential nutrients around the plant roots (PING; RONG, 2006). Breeding programs usually focus their efforts on the transference of genes from *Coffea canephora* to *C. arabica* in order to increase the resistance of pests and diseases. However, these crosses also modify the composition of coffees. Thus for new coffee crosses it is important to evaluate the effect of the genetic background on the composition. The objective of the research was to evaluate the content of organic acids of several new crosses (IAPAR 59, and IPRs 97, 98, 99, 100, 102, 102, 103, 104, 105, 106, 107, 108) comparing to traditional ones (Red Bourbon, Red Catuai, Yellow Icatu).

MATERIAL AND METHODS

Coffees of different genetic background were studied (Table 1). The coffees were harvested at the Agricultural Technologic Park of Cooperative COCARI, Mandaguari, Paraná, Brazil, from May to July 2009. Harvesting and post-harvesting conditions were standardized for all cultivars. The time of harvesting was variable, according to the maturation stage for each cultivar. Coffees were grown at latitude (S) 23°32'52", altitude of 650 m and average annual temperatures of 22 to 23°C. Defective beans were removed and green coffee beans were frozen (-18°C). Immediately prior to analysis, they were grounded (0.5 mm particles) in the disk mill (PERTEN 3600, Sweden) using liquid nitrogen to prevent oxidation of compounds. Green coffee beans were subjected to medium roasting process (8 to 11 minutes at 200-210°C, L* around 28) for preparing the brews. The beverage was prepared with 70 g L-1 and it was filtered through filter paper.

Table 1. Cultivars and their genetic background.

Cultivars	Genetic background
Traditional	
Red Bourbon	Pure arabica
Red Catuaí	Yellow Caturra (simple mutation of Red Bourbon) x Mundo Novo (hybridization between Red Bourbon and Sumatra)
Yellow Icatu	Red Icatu (hybrid of robusta and arabica) x Mundo Novo x Yellow Bourbon
Modern crosses	
Iapar 59, IPR 97, 98, 99, 104	Timor Hybrid and Villa Sarchi (Sarchimor)
IPR 100, 101, 105	Derived from a cross of Catuaí $Sh_2 Sh_3$
IPR 102	Icatu x Catuaí
IPR 103	Red Catuaí IAC 99 and Yellow IAC 66 x Icatu
IPR 106	Icatu
IPR 107	Sarchimor x Mundo Novo
IPR 108	Sarchimor x Icatu x Catuaí

SERA et al., 2007; Santos, 2011; Fazuoli, 2009; EIRA et al., 2007; SERA et al., 2005; ALTEIA et al., 2001; ITO et al., 2008.

Quinic, malic, lactic, acetic and citric acids were extracted and quantified based on the method described by Rodrigues et al. (2007) with modifications (Figure 1). The HPLC analysis was performed using an ACE 5 C18 column (250 mm x 4.6 mm id, 5 mm) (Advanced Chromatography Technologies, Aberdeen) with detection at 210 nm. Isocratic elution of 0.005 N H_2SO_4 solution at pH 2.5 was carried out with a gradient of flow: 0.7 ml min^{-1} for 0-2 min; 0.4 ml min^{-1} for 2-15 min; and 0.7 ml min^{-1} for 15-20 min. Oven temperature of 30 °C and temperature of the sample tray of 5 °C were applied. Green and roasted coffees were evaluated and identification of acids was done by comparison with standards and spiking. The concentration ranges for calibration curves and the

chromatographic parameters and data recovery are shown in Table 2. The recovery tests were carried out in duplicate. The acids standards were added to the samples (0.500 g) before analysis, in an amount of approximately 50% of the initial content.

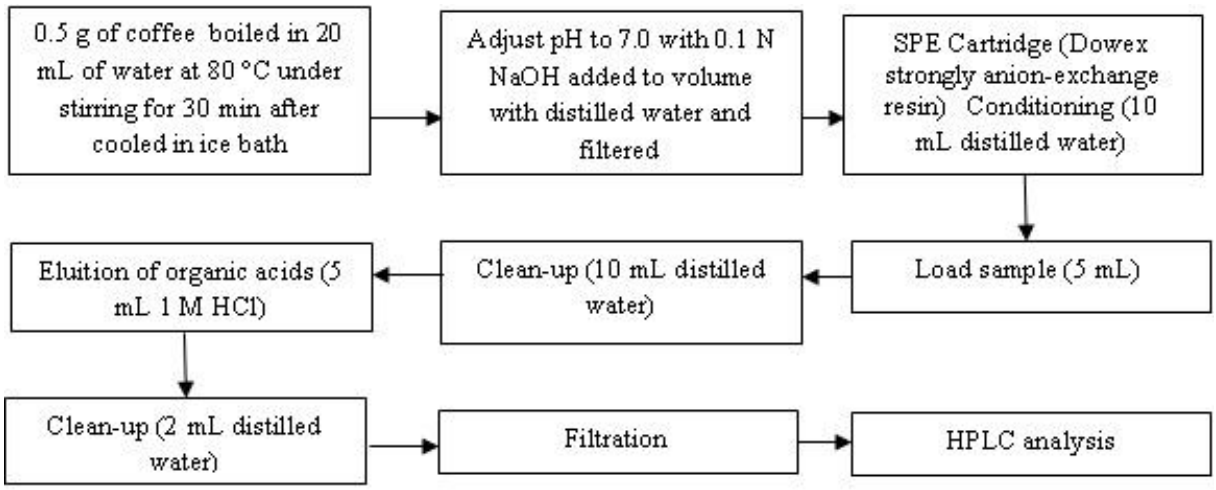

Figure 1. Flow-chat of the procedure for the determination of organic acids.

Table 2. Chromatographic parameters, concentration ranges and recovery data for organic acids.

Compounds	RT (min)	Linearity range (mg $100g^{-1}$)	Intercept	Slope	R^2(n=3)	LOD ($mg100g^{-1}$)	LOQ ($mg100g^{-1}$)	Recoveries (%)
Quinic	5.54	125-2000	43448	403.2	0.997	0.04	0.13	84
Malic	6.05	50-800	-18369	629	0.98	0.10	0.30	77.5
Citric	8.66	125-2000	10350	916.5	0.995	0.03	0.10	88
Latic	6.86	25-400	-935.4	280.8	0.998	0.08	0.23	79
Acetic	7.16	20-300	-1065	348	0.996	0.01	0.02	60

Chlorogenic acid (5-CQA) was quantified, by HPLC, in green and roasted coffees, as described by ALVES et al. (2006).

Coffee brews were characterized by pH and titratable acidity. pH was determined in 10 mL of coffee brew after reaching 25°C in a digital pH meter (Metrohm, model 744). Titrable acidity was determined in 10 mL of coffee brew titrated with 0.1 N NaOH to pH 8.2. The result was expressed in 0.1 mL of NaOH to 100 mL of brew.

In order to analyze the data, Principal Components Analysis (PCA) was applied by XLStat software (ADDINSOFT, 2007).

RESULTS AND DISCUSSION

In green coffees, the contents of the acids varied between 0.35 to 0.55% for quinic acid, 0.30 to 0.64% for malic acid, 0.93 to 1.31% for citric acid, and 4.17 to 5.35% for 5-CQA. These values were comparable to the reported by Steiman (2003) for coffee beans from different origins and crosses: 0.57% for quinic acid, 0.41% for malic acid, 1.37% for citric acid and 3.21 to 6.97% for 5-ACQ.

The organic acids content in coffee beans is highly associated with the level of maturation (Rogers et al. 1999). Concentration of quinic acid and malic decrease as the maturation has taken place (Rogers et al. 1999). High contents of 5-CQA were also usually related to immature beans (VAAST, 2006). Citric acid has an opposite behavior, presenting lower values in the initial stage of development of beans with an increase of 1.28 and 1.58% during the maturation (Rogers et al., 1999). Variations of citric, quinic and malic acids contents associated to genetic background have already been reported for other climacteric fruits (peaches) (SOUTY et al., 1999).

Principal Component Analysis was used for simultaneous characterization and comparison of organic acids profile in green and roasted coffee and some brews characteristics for different coffee cultivars (Figure 2).

The first two components of PCA explained 76% of the variability in the green coffee beans. PC1 accounted for 50% of variance and PC2 explained 26% (Figure 2a). The cultivars derived from a cross of Catuaí Sh2 Sh3 (IPRs 100, 101 and 105), IPR 102 (Catuaí x Icatu), 106 (Icatu), 107 (SarchimorxMN) and tradicional cultivars, allocated in the right side of the plan, presented high contents of quinic and citric acids and lower contents of 5-CQA and were discriminated by PC1 from others (Sarchimor crosses, and IPR 103). Malic acid was mainly correlated with PC2 and discriminated Iapar 59, IPRs 97, 98 and 99, Catuaí, Bourbon and Icatu (located in the bottom side) from other coffees.

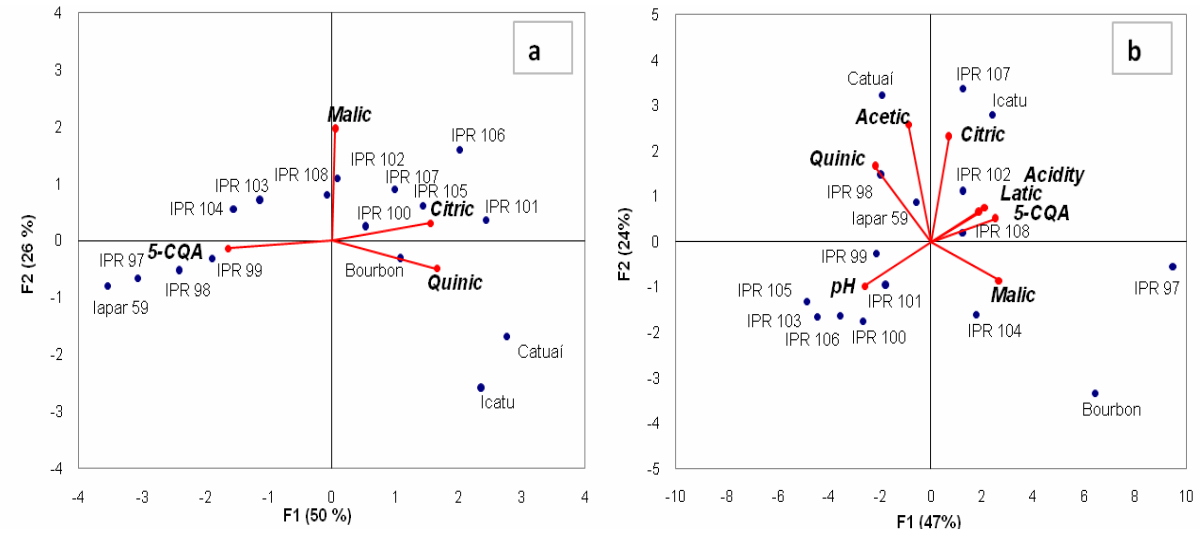

Figure 2. PCA Biplot of the coffee cultivars considering acids profile for green coffees (a) and roasted coffees and coffee brews characteristics (b).

Comparing the acids profile in green and roasted beans coffees, it was observed an increase in quinic acid contents (148 to 288%) and a decrease for 5-CQA (18 to 38%), citric acid (42 to 71%) and malic acid (10 to 132%). This behavior was also reported by Ginz et al. (2000). The acid profile for roasted coffees and the brew characteristics allowed the discrimination observed in Figure 2b. Catuaí Sh2 Sh3 (IPR 100, 101, 105, 103), IPR 106, Sarchimor (IPR 98, 99 and Iapar 59) and Catuaí were located in the right side of the plan. These cultivars showed high contents of quinic acid and pH values. The other cultivars (left side) presented high levels of malic, 5- CQA, lactic and citric acids and titratable acidity.

Interestingly, Bourbon and IPR 97 were discriminated by the high content of malic acid (0.6%) (Figure 2b). High levels of this acid is associated with the use of immature beans (ROGERS et al, 1999) and could eventually inhibit the sensory perception of sweetness if

present in high contents in fruits (SOUTY et al., 1999). Cultivars that present high pH brews values (located in the right side of the plan) showed lower levels of citric, lactic, 5-CQA and malic acids. The contribution of acids to pH brews also affects sensory properties due to the dissociation of various organic acid salts and the liberation of highly volatile free acids (Balzer, 2001). Kitzberger et al. (2010 and 2011) described that coffees with lower levels of malic and citric acids showed positive sensory characteristics and pointed that the presence of sensory attributes correspondent to immature coffees in brews of Bourbon and IPR 97 cultivars.

The changes in the acids profile after the roasting process has led to a different separation of cultivars comparing to green coffees (Figure 2). Cultivars of the same genetic background (Catuaí Sh_2Sh_3) remained close in the two PCAs. Sarchimor derivatives, more affected by the degradation of 5-CQA and formation of quinic acid, presented different behavior comparing the distribution in PCA for green beans and roasted coffees.

These results suggested that genetic background of each cultivar influenced the organic acids profiles in the final coffee brews. The differences attributed to the adaptation of each cultivar to growing conditions and the different behavior during roasting process, were probably reflected in different sensory profiles.

CONCLUSIONS

Considering the green and roasted beans coffees and brew composition, some crosses showed similar behavior to the traditional cultivars. The crosses Catuaí Sh2Sh3 derived were characterized by high contents of quinic and citric acids and lower content of 5-CQA suggesting a complete maturation in the conditions of the study. Sarchimor, IPR 102, 107 and 108, Bourbon, Icatu and Catuaí were characterized by the high levels of quinic, citric and 5-CQA that suggested a poor adaptation of edapho-climatic growing conditions.

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Physicochemical Composition of Green and Roasted Beans and Beverage of Coffee to Same Geographical Growing Origin

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INTRODUCTION

The association between coffee quality with post-harvest techniques (natural coffee or pulped coffee), cropping system (organic, irrigated) or the geographical origin is ways of valuation of coffee in a highly competitive market.

The growing conditions determine the composition of green beans and after roasted results in sensory attributes giving the peculiar characteristics of coffees from different locations. The many compounds of the coffee beans the carbohydrates, proteins, lipids, chlorogenic acids, soluble tannins, caffeine and trigonelline are among those main responsible for the formation of the characteristics of coffee beverages (FARAH et al., 2006). The concentrations of these compounds depend on the environmental factors, variety, maturation stage and post-harvest processing that are submitted to the coffee (KNOOP et al., 2005).

The Northeast Region - Paraná has great potential and climatic conditions of temperature and altitude suitable for obtaining high quality coffee (ACENPP, 2009). The objective of this study was to determine the physical aspects of green and roasted coffee beans and the physicochemical characteristics of the coffee beverages of Northeast Region for give subsidies in the process of geographical indication for this region.

MATERIALS AND METHODS

In the harvest of 2007-2008, in 16 municipalities of this region the farmers have been harvested, dried and dehulled samples of coffee. They were collected thirty-two natural cherry coffee (CN) which were dried without removing the hull and 21 pulped cherry coffees (CD), which were dried after removing the hull and without the removal of the mucilage.

Physical-chemical analysis of green beans

After removal of defective beans, coffee was frozen with liquid nitrogen (-196 °C) and was ground in a disk mill PERTEN 3600 (0.5 mm mesh). Moisture was determined 105°C until the constant weight, and proteins, lipids and caffein in green coffee beans ground were evaluated by the respective methods (AOAC, 1990). Total tannins were determined with Folin Ciocalteau reagent and gallic acid was used as standard (AOAC, 1990). Reducing sugars and total sugars were determined by the method of Somogyi and Nelson and chlorogenic acids were quantified by periodate reagent (CLIFFORD & WIGHT, 1976).

Physical-chemical properties of roasted beans

About 200g of coffee were roasted in a roaster (Rod Bel-São Paulo) and the temperature was maintained between 200-230 °C, for 8-10 minutes, depending on the initial moisture content of each sample. The end point of roasting was controlled by weight loss of green beans (NEBESNY & BUDRYN, 2006) and by visual color of roasted coffee (BAGGENSTOSS et al., 2008). The density and the expansion of roasted coffee bean were determined according to cited somewhere (FRANCA et al., 2005).

The beverage was prepared with 70g L^{-1} and it was filtered through Mellita filter paper. The beverage pH was determined in 10 mL of liquor after reaching 25°C in a digital pH meter (Metrohm, model 744). Titrable acidity was determined in 10 mL the titrated with 0.1 N NaOH to pH 8.2 and the result was expressed in 0.1 mL of NaOH to 100 ml of beverage. The lightness (L^1) and chromatic components a^1 and b^1 in the roasted and ground coffee were determined in portable Konica Minolta colorimeter CR 410.

The physical and chemical analyses of green and roasted beans were performed in duplicate. The analysis of variance, Tukey test and multivariate Principal Component Analysis was conducted using XLSTAT statistical program (ADDINSOFT, 2008).

RESULTS AND DISCUSSION

The coffee from the Northeast Region presented composition, beans size and density compatible with proper formation of beans (Table 1), suggesting favorable environmental conditions to promote greater accumulation of compounds that have a direct effect on beans formation. Concentrations of total sugars were higher (6,0 g 100 g^{-1}) than those founded in coffee in the final stage of maturation (GEROMEL, et al., 2006) and lower (from 5.1 to 9.4 g 100 g^{-1}) than those founded in coffees from different origins (CASAL et al., 2000). Reducing sugars (mainly glucose) were founded in lower levels in range from 0.10 to 0.69 g 100 g^{-1}average of 0.23, depending on the post-harvest processes they have undergone (KNOOP et al., 2005) and the stage maturation (GEROMEL, et al., 2006).

Table 1. Mean, minimum and maximum values (g 100 g-1) of the main constituents of green and roasted coffees.

	Constituents	Minimum	Maximum	Mean	Standard deviation
Green coffee	Beans size (BS)	28,92	100,00	75,88	18,51
	Green density[1] (GD)	0,62	0,68	0,66	0,01
	Reducing sugars (RS)	0,10	0,69	0,23	0,14
	Total sugars (TS)	3,88	9,20	7,47	0,99
	Total tannins (TT)	3,76	5,27	4,42	0,33
	Chlorogenic acids (CGA)	3,84	8,50	5,80	1,12
	Caffein (CAF)	0,95	1,65	1,29	0,13
	Proteins (Pro)	12,33	17,00	15,17	1,12
	Lipids (Lip)	13,42	18,91	15,37	1,10
Roasted coffee	Weight loss (WL)	12,17	17,17	13,81	1,17
	Expansion of roasted coffee (EXP)	22,87	84,58	53,90	13,63
	Roasted density[1] (RD)	0,31	0,46	0,37	0,04
	L[1]	25,31	29,55	27,10	1,08
	a[1]	8,69	10,40	9,43	0,42
	b[1]	9,83	15,13	12,31	1,45
	pH	4,82	5,44	5,08	0,13
	Titrable acidity (Actit)	9,83	15,61	13,24	1,27

[1]g mL^{-1}

The total chlorogenic acids and tannins, associated with astringency in wine and mate tea were in lower concentration in mature grains of coffee (FARAH et al., 2005). These compounds were founded in lower concentration in this coffee region, suggesting fully ripe grains (Table 1). Caffeine had values similar to those founded in coffees from different origins and commercial arabica coffees from Brazil, whose values ranged from 0.96 to 1.23 g 100 g^{-1} (FARAH et al., 2006). The concentration of lipids, mainly related to environmental conditions, was higher in the coffees of the region that the values founded in coffees growing in full sun and shade (11.5 to 13.1 g 100 g^{-1}, respectively) according to others authors (VAAST et al., 2006).

The roasted beans density presented above those founded in Brazilian coffees and volume expansion values between 22 to 84 g 100 g-1 (DUTRA et al., 2001), indicating great variability in the grain structure formation (VAAST et al., 2006). The pH ranged from 4.82 to 5.08 (Table 1) and the literature reported the ideal range (5.08 to 5.22) for consumer acceptance (MANZOCCO & LAGAZIO 2009).

Principal components analysis (PCA) of the matrix formed by the physicochemical variables (green and roasted beans and beverage) and the coffee samples demonstrated that the first three components explain 43,17% of the variance in the samples. The greater variance observed (26.01%) is associated with the physicochemical properties of roasted beans and beverages, as demonstrated by correlations of these variables with the F1, which were the main characteristics to discriminate the coffees of the Northeast Region. Weight loss and density of the roasted beans with the concentration of proteins and sugars contributed to the formation of F2 (17.15%).

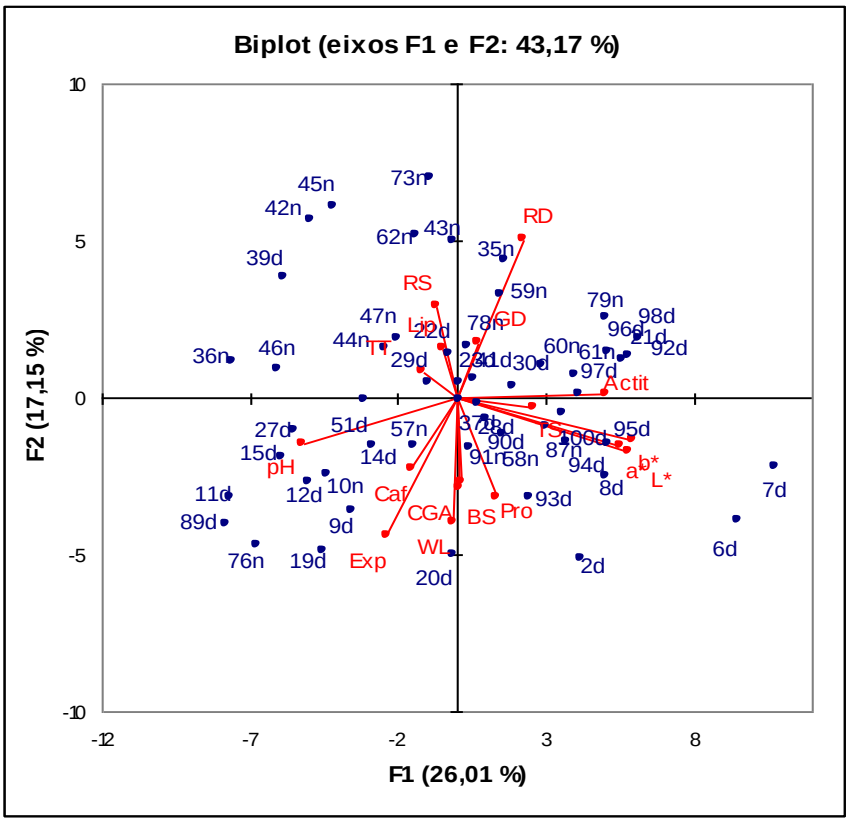

Figure 1. PCA biplot of coffee from Northeast Region-Paraná.

The process of post-harvest (CN or CD) had minor influence on the composition and characteristics of roasted beans and beverage in the coffees from Northeast Region and it was not possible to separate groups of each process. Similarly, the municipality of origin of these samples not presented separation of municipalities according to variables that formed the components in PCA.

CONCLUSIONS

The composition and physicochemical characteristics of green and roasted coffees and beverages in Northeast Region allow us to infer that the agronomic practices adopted by producers together the environmental conditions of the region results in coffees with special and particular characteristics and if the procedures for standardization were maintained, these coffees may be marketed as typical from this region.

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Sensorial Profile of Coffees Produced in Northeast Region of Parana-Brazil

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INTRODUCTION

Coffee is among the most widely consumed beverages worldwide and its consumption is associated with its aroma and flavor (NEBESNY & BUDRYN, 2006) and the presence of more than thousands compounds makes a coffee product sensory complex in terms these attributes (CZERNY et al., 1999).

The evaluation of sensory quality coffees requires a trained team or trained assessors, as professionals (experts) that use descriptors previously established to evaluate the coffee. In these evaluations are mentioned positive sensory attributes, for example, acid, fruity, citrus, caramel, chocolate and other of negative character as bitter, wood, astringent, green and fermented (NEBESNY & BUDRYN, 2006).

Another approach to the evaluation of coffee is the descriptive sensory analysis, where the score for the attributes will be later correlated with the quality of coffee. In this case, the assessor measures the set of qualitatively or quantitatively characteristics of each coffee in function both the sensory techniques and different levels of training as the consensus among team members.

Free Choice Profiling a descriptive sensory analyse has been used to evaluate other foods. This technique is based on the principle that people perceive the same characteristics but use different ways for expressing them (DIJKSTERHUIS & GOWER, 1991).

The tasters develop their individual evaluation forms, with the descriptors that seem sufficient and able to adequately describe the product. The training for use of consensual attributes and scale are eliminated in this type of evaluation, which reduces the time and cost of analysis (THAMKE, 2009). The data obtained were submitted to Generalized Procrustes Analysis (GPA) that was used to adjust the use of different parts of the scale and the different terms employed by tasters to describe and measure the same sensory characteristics (DIJKSTERHUIS & GOWER, 1991).

In Paraná-Brazil, the Northeast Region presents the climatic conditions of temperature and altitude suitable for obtaining high quality coffees (ACENPP, 2009), which, together with socio-cultural aspects of cities around the coffee, make the area favorable to commercialization of coffee with to a geographical indication. However, for such action is necessary an adequate characterization of the sensory profile of these coffees, in these cases it is desirable for a simple and efficient sensory methodology. The objective of this study was to describe the sensory profile of the coffees of Northeast Region by applying the technique of the Free Choice Profile.

MATERIALS AND METHODS

Thirty-two natural cherry coffees (CN) dried without removing the hull and 21 pulped cherry coffees (CD), dried after removing the hull and without the removal of the mucilage were analyzed. Approximately 800g were collected into a batch of at least 10 bags of coffee which were prepared by farmers of the Northeast Region in 2007-2008 seasons. For each samples 200 g of green beans without defects (immature grains, fermented and/or sour) was roasted in a roaster (Rod-Bel, São Paulo). The roasting temperature was kept between 210-220°C, during the time to achieve weight loss of 13-14%. Coffee beverage was prepared from 70 g of roasted and ground coffee per liter of water at 96-98°C. The filtered beverage were kept in thermo flasks and served in polystyrene cups (50 mL) coded with three digits for sensory evaluation. Assessors were recruited among employees of the Agronomic Institute of Paraná-IAPAR and professional tasters from Coffee Trade Center of Londrina – PR, according to the availability of time and interest and who had passed in the preliminary test of recognition of odors.

The attributes were selected by grid method where two samples with large differences were presented to assessors in order to identify the similarities and differences. Three sessions were carried out to make the list of attributes, using different pairs of contrasting sensory quality coffee. After choosing and defining attributes together with the leader, the chart and glossary of each taster. To evaluate the coffees the chart was prepared with a scale of 10 cm anchored in terms of intensity at the edges and also marking the center point was employed (VILLANUEVA et al., 2005). Samples of coffees (53) of the Northeast Region were presented sequentially in 13 sessions, with four samples in each session and a session with two samples. The results of each panelist were arranged in a matrix in which the samples and the attributes correspond to the rows and columns, respectively. These data were analyzed by Generalized Procrustes Analysis (GPA) using the statistical software XLSTAT (ADDINSOFT, 2008).

RESULTS AND DISCUSSION

To describe the sensory quality of coffees produced in Northeast Region, the panel employer different terms to characterize the appearance, aroma, flavor and texture of the coffee beverages. These attributes were similar to those for coffee in sachets (NARAIN et al., 2003) and samples formed from mixtures of arabica and robusta coffees (MOURA et al., 2007).

The tasters have used between ten and seventeen attributes and the number of attributes observed is comparable to that found by tasters to describe coffee genotypes (KITZBERGER et al., 2011). In relation to appearance of coffee all the team members determined the color as an important attribute for the coffees. The turbidity and brightness were indicated as important features of appearance by two seven p.m. assessors, respectively. The aroma was evaluated by a smaller number of tasters. Among them, thirteen tasters determined the coffee aroma, sweet, fermented and green and six others evaluated the aroma of caramel.

The flavor of coffee was evaluated by a greater diversity of attributes. The sour and bitter tastes were quantified by all assessors and the green and fermented flavors and astringency were cited by 13, 12 and 9 tasters, respectively. The attributes sweet, coffee, citrus, caramel, chocolate, good residual and bad residual tastes were mentioned with less frequently.

The texture of the coffee beverage was evaluated as body and washed for eight seven tasters respectively, and two tasters added viscous.

In the analysis of the interpretation of the Free Choice Profile dimensions in terms of attributes explains the different characteristics of coffees. The importance of an attribute is proportional to the frequency of mentions and the correlation coefficient: the higher these values, the greater the importance of these attributes to explain that dimension.

The attributes most frequently cited were those who had higher values of correlations with the dimension 1 (D1). In this dimension it was found negative correlations for coffee color, turbidity, aroma and taste of coffee. Opposed to these, the same dimension, the attributes are acid taste, aroma and green taste and fermented flavor, correlated positively with D1. The second dimension (D2) combines the attributes associated with brightness and body. The attributes of sweet aroma and taste, bitter taste were associated to D1 and D2, with different coefficient of correlations between these dimensions. Because of the difficulty to assess, quantify and express the attributes associated with the texture were not very significant in the analysis of the Free Choice Profile. The high quality coffees were associated with attributes similar to those used by tasters to describe the coffees (NARAIN et al., 2003).

The proximity of the samples with the descriptive vectors responsible for their characterization allows us to interpret the similarities and differences and understand the terminology used to describe these samples (Figure 1). Most of the coffee samples of Northeast Region (central region of Figure 1) showed intense coffee aroma, sweet and caramel taste and lower intensity of body. Other samples located in the lower right quadrant, (samples 60n, 61n and 47n) have a higher intensity of aroma and taste green and fermented taste and aroma probably due to the presence of immature grains and moisture above 12.5%.

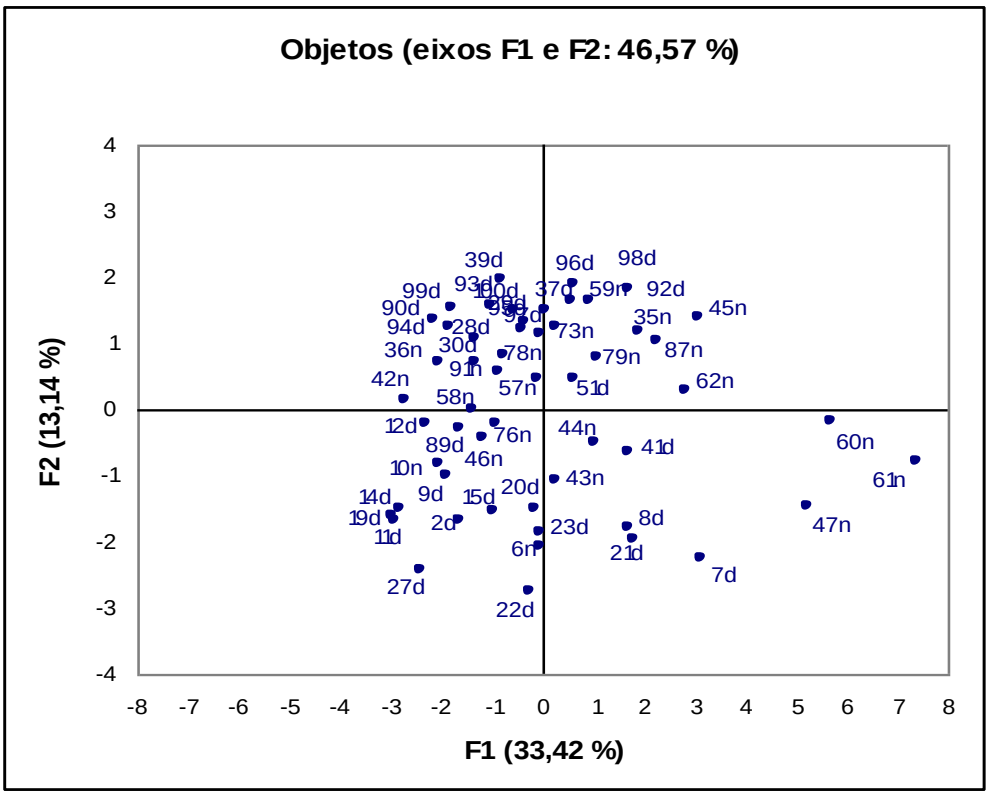

Figure 1. Configuration consensual from coffee samples CN and CD from Northeast Region. Label: numbers with the letter "d" refers to CD samples and "n" CN samples.

The coffees with greater aromatic intensity (lower left quadrant) have higher turbidity and are darker and lesser bright (opaque). This association between higher solids and higher intensity of attributes was observed for others authors (NEBESNY & BUDRYN, 2006).

Due to drying process (CN or CD) were expected coffees of different qualities (SELMAR et al., 2008) in this region. In most coffee samples CD was associated with the attributes of acceptability due to the presence of fully ripened beans. However, some coffee samples CD (7d, 8d, 21d, 41d, 92d, 96d and 98d) showed immature characteristics of coffee (green aroma and flavor fermented) probably by partial separation of immature and ripe coffees during removal of the hull.

Among the CN coffee beverages could be found with characteristics similar to coffee quality CD, but most samples had immature attributes of coffee. Such evaluations suggest that the raw material has a greater effect on sensory quality than the drying process. The distribution of the samples analyzed considering the municipalities found that only two sites (Tomazima and Carlópolis) predominate in the left quadrant. Probably harvest and post-harvest conditions for these samples were very similar, resulting in similar quality coffees. The other municipalities are distributed among all quadrants, with no evidence of association of quality at the production local.

CONCLUSIONS

Attributes of coffee aroma and flavor of coffee, sweet, acid and bitter tastes were described by most tasters were representatives to profile of these coffees in the region. It's possible to observe that CN and CD have similar sensory quality beverages without presented a clear distinction between these processes as expected. Coffees from different localities studied presented similar sensory characteristics without a clear separation between them. In this particular case the lack of separation is positive. Thus, through the technique of Free Choice Profile was possible to identify the key attributes of coffee beverages from Northeast Region, indicating the attributes responsible for quality.

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Trigonelline and Caffeine in Espresso and Fresh Brew Coffee—Impact of Grind Size, Water Temperature, and Coffee/Water Amount

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SUMMARY

Trigonelline and caffeine, two water-soluble substances, are constituents of the coffee brew. They should induce antibacterial and anti-adhesive effects in combination with other constituents of the coffee. The extraction behavior of trigonelline and caffeine in Espresso and Fresh Brew Coffee was studied as a function of the grinding grade, the water temperature and the coffee/water amount.

The MAAS SL 1000 vending machine was used to prepare the different coffee brews as previously reported for the lipo-soluble substances cafestol and carboxylic acid-5-hydroxytryptamides.

The influence of the brewing parameters depends on the brewing method used. The trigonelline and caffeine contents in the Espresso were mainly impacted by the amount of both coffee and water whereas in the Fresh Brew Coffee the contents were affected strongly only by the water volume.

No influence was detected for the water temperature. In contrast, the grinding grade showed a great impact. For Espresso a maximum extraction yield was achieved with 8.5 g coffee powder and 90 mL water volume.

INTRODUCTION

Coffee brews induce antibacterial and anti-adhesive effects on Streptococcus mutans, the main pathogenic organism for dental caries in humans. Caffeine and trigonelline are responsible for this effect, the same as nicotinic acid and chlorogenic acids. Andueza et al. studied trigonelline and caffeine as physico-chemical parameters for the quality of Espresso. They reported the influence of the coffee/water ratio, the water pressure, the grinding grade, and the water temperature.

Recently, our working group investigated the influence of the particle size and the coffee/water amount on substances of the coffee oil, namely cafestol, and carboxylic acid-5-hydroxytryptamides in Espresso and Fresh Brew Coffee. The aim of this study was to analyze the behavior of the water-soluble substances trigonelline and caffeine under the same conditions as those used for the lipo-soluble substances (Figure 1).

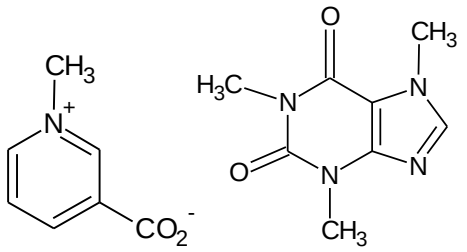

Figure 1. Trigonelline (left) and caffeine (right).

MATERIALS AND METHODS

For preparing the coffee brews, the MAAS SL 1000 vending machine equipped with an Espresso and a Fresh Brew unit was used. In addition to the grinding grade the variable preparation parameters were the coffee amount, the water temperature, and the water volume. Table 1 offers an overview of the preparation parameters.

Table 1. Preparation parameters

Parameter	Espresso	Fresh Brew
Temperature [°C]	80/95/110	
Coffee [g]	6.5/7.5/8.5	8/9/10
Water [mL]	40/65/90	70/125/180

The Espressos were directly collected in a 100 mL volumetric flask, and the Fresh Brew Coffees in a 250 mL flask, respectively. The brews were diluted and subsequently measured by RP-HPLC equipped with DAD. For the separation a Synergi Hydro column was used with a water/methanol gradient. Trigonelline was quantified at 264 nm and caffeine at 272 nm.

A Box-Benkten design was used to evaluate the main effects, the interaction effects, and the quadratic effects. This cubic design was selected since it requires fewer runs in the case of three or four variables than an on-factor-at-a-time-experiment.

The Box-Benkten design is characterized by a set of points lying at the midpoint of each edge of a multidimensional cube and the center point replicates (n=3). The coefficient for the determination (R^2) of all the models was above 0.94, indicating that the models adequately represented the actual relationship between the parameters chosen. It must be noted that the results of the statistic design are only valid within the ranges of the experimental limits (Table 1).

RESULTS AND DISCUSSION

The following experiments were carried out with a commercial Arabica coffee containing 8.8 g/kg trigonelline and 11.6 g/kg caffeine. The grinding conditions were the same as described by Zahm et al. For the Espresso, it is quite obvious that the finer the particle size the higher is the amount both of trigonelline and caffeine in the brew. Solely, the trigonelline content in the finely-ground coffee increased only a bit more in comparison to the medium-ground coffee. This is true for the Fresh Brew Coffee as well. Here, however, the extraction yield of trigonelline and caffeine was similar to that of the medium-ground coffee (Figure 2).

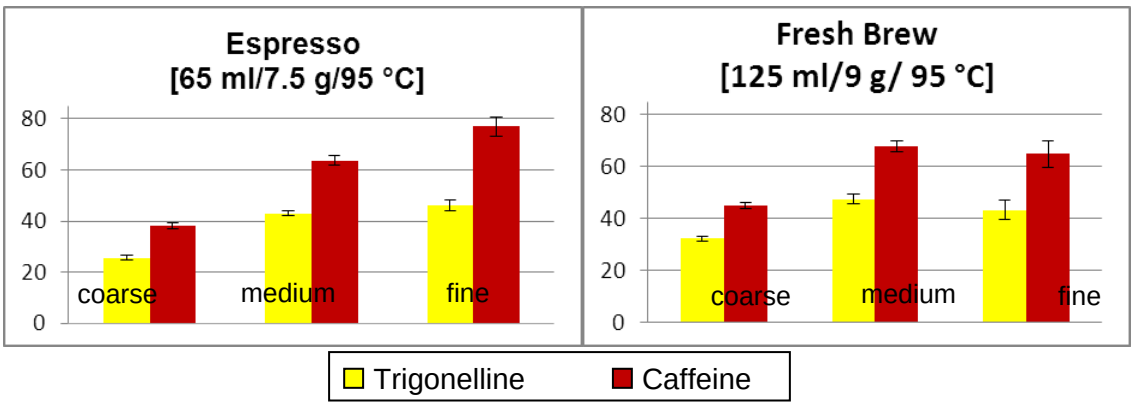

Figure 2. Impact of the grinding grade on trigonelline and caffeine in Espresso and Fresh Brew Coffee.

A typical chromatogram of a coffee brew at 264 nm is shown in Figure 3.

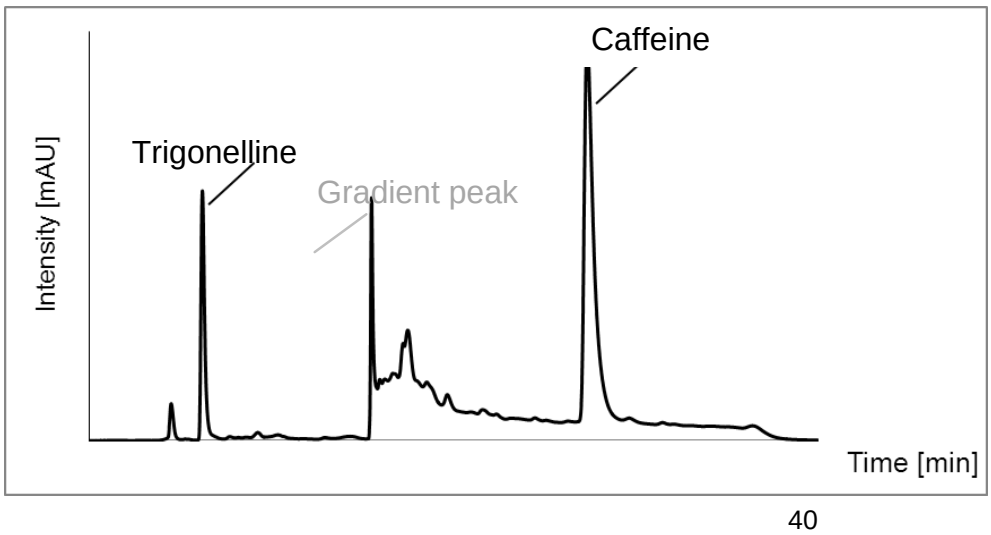

Figure 3. Chromatogram of an Espresso (λ=264 nm).

The influence of the water temperature was studied with an Espresso (40 mL; 7.5 g) and a Fresh Brew Coffee (70 mL; 9 g). Figure 4 makes evident that the water temperature affected neither the trigonelline nor the caffeine content.

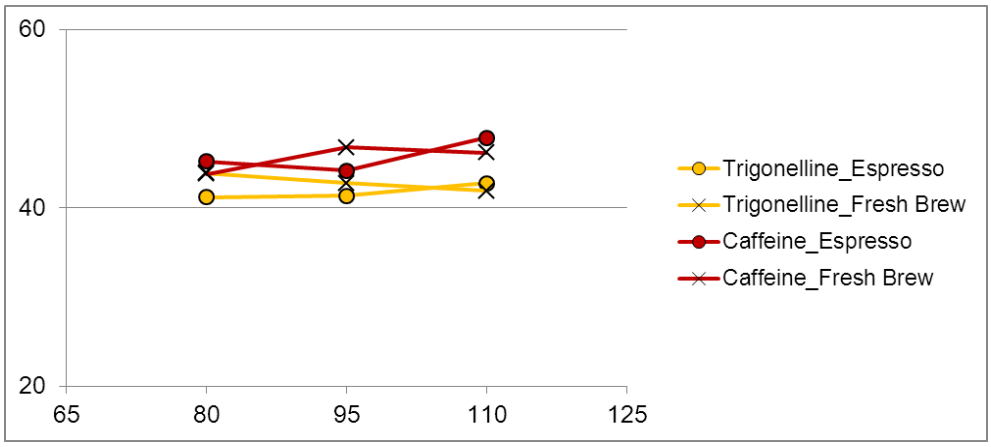

Figure 4. Impact of water temperature on trigonelline and caffeine level in Espresso (o) and Fresh Brew Coffee (x).

Figure 5 shows the 3D response surface plot of Espresso for trigonelline and caffeine. Both the trigonelline and also the caffeine content increased linearly with the increase of the coffee amount and the water volume. The values reached a maximum, using 8.5 g coffee powder and 90 mL water. In conclusion, for Espresso, the most important influencing factors on the trigonelline and caffeine contents were the coffee and the water amount.

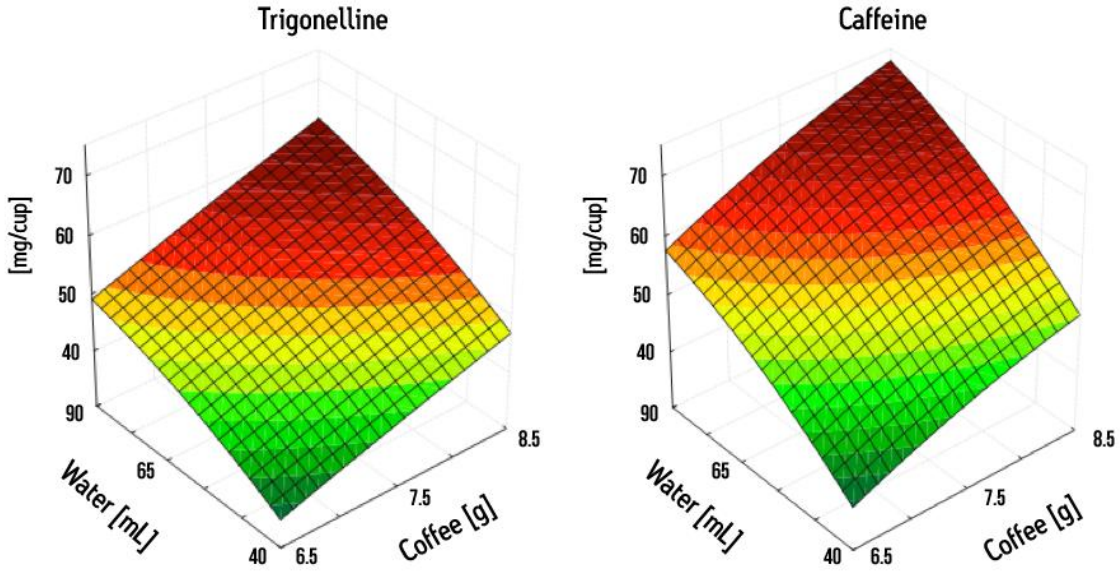

Figure 5. 3D response surface plot of Espresso with medium-ground coffee at 95 °C.

However, the composition of the Fresh Brew Coffee was impacted mainly only by the water quantity. This way the caffeine amount was influenced more strongly than the trigonelline content. For a constant water volume, the trigonelline and caffeine contents in the cup were only marginally affected by increasing the amount of coffee powder (Figure 6).

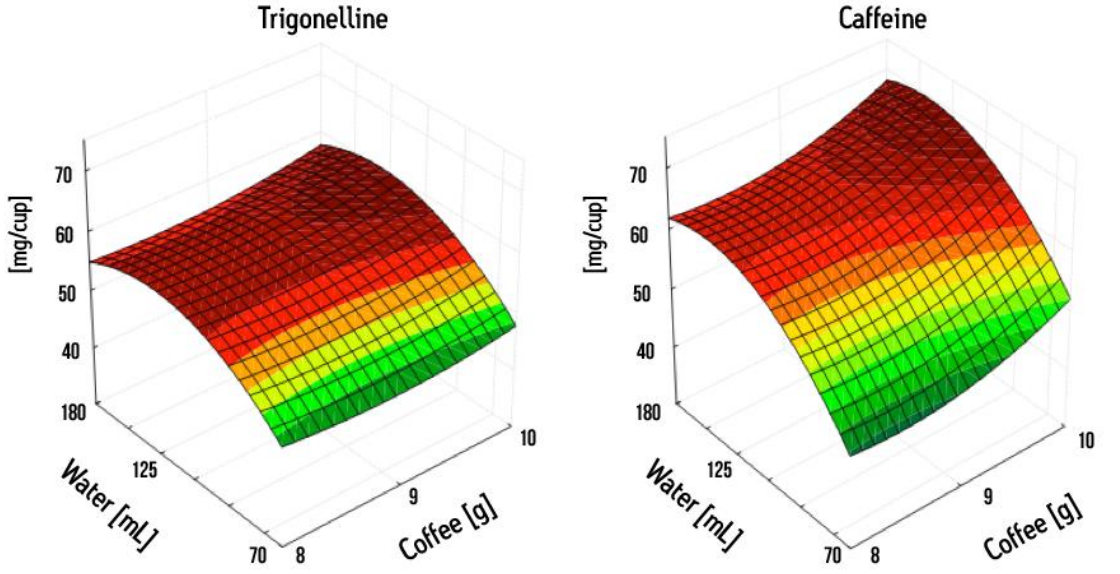

Figure 6. 3D response surface plot of Fresh Brew Coffee with medium-ground coffee at 95 °C.

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Steryl Glucosides in the Coffee Plant

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SUMMARY

Phytosterols as a part of the coffee oil have a lot of benefical properties. Up to now, only steryl esters and free sterols were reported for coffee. In other plants such as flaxseed, soybeans, and pumpkin seeds, steryl glucosides were identified as a third sterol component. Therefore, the aim of this study was to identify and to quantify the steryl glucosides in coffee, in addition to the steryl esters and the free sterols. At first, a method was developed and validated for analyzing the three sterol components, simultaneously.

The steryl glucosides were described for coffee for the first time. The main steryl glucosides in coffee were ß-sitosterol-, stigmasterol-, and campesterol glucoside. In Robusta coffee, Δ^5-avenasterol glucoside was identified as well. The contents in the green coffee beans ranged between 8% and 12% of the total sterols. In the roots of the three coffee varieties examined, the contents were in the same magnitude. The levels in the leaves were nearly twice as much as in the beans. Due to the small sample size, further research needs to be carried out in the future.

INTRODUCTION

Over the past years, phytosterols have achieved more importance due to their beneficial properties. In some of the scientific literature it was reported that the phytosterols reduce the cholesterol levels and have anticarcinogenic effects. Furthermore, an immune modulating effect was described.

In coffee, the total sterol content varies between 1.5% to 2.4% of the lipid fraction; with 5.4%, an even higher content was reported by Nagasampagi. These values represent only the free and esterified sterols. The sterol ß-sitosterol (Figure 1) was identified as the most important sterol in coffee with a content of about 50% of the sterol fraction.

However, in other plants, steryl glucosides were identified as a third group. They were analyzed, for instance, in wheat, flaxseed, soybeans, and pumpkin seeds with 9% to 37% of total sterols.

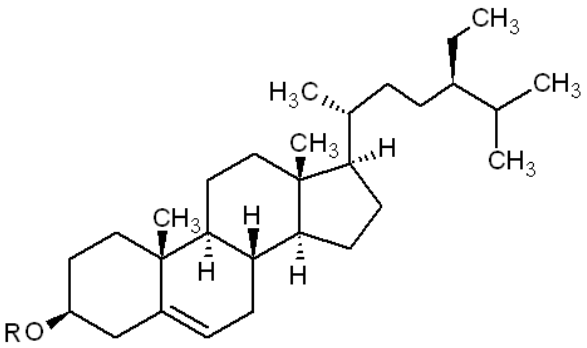

Figure 1. ß-sitosterol (R=H; R=fatty acid; R=glucoside).

MATERIALS AND METHODS

Green coffee of the species *Coffea arabica* and *Coffea canephora var. robusta* and the leaves and the roots of two *Coffea arabica* cultivars (*Caturra/San Ramon*) and of one *Coffea canephora* were exemplarily investigated. Figure 2 shows the analysis scheme.

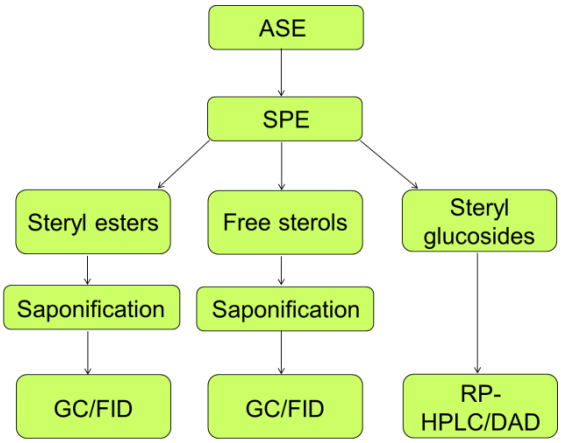

Figure 2. Analysis scheme.

By means of accelerated solvent extraction (ASE), the green coffee and the plant material were extracted with tert-butyl methyl ether (t-BME) and ethanol/water. An aliquot of the extract was separated on a silica gel 60 SPE-column and diluted with a mixture of n-hexane and ethyl acetate of a different polarity and with n-hexane/ethanol. Three sterol fractions were obtained, the first contained the steryl esters, the second the free sterols and, the third fraction the steryl glucosides. The steryl esters and the free sterols were analysed after saponification by GC/FID and quantified with ß-sitosterol. Two internal standards were used: cholesteryloleate for the steryl ester fraction and 5α-cholestan-3β-ol for the free sterols. The sterols that were quantified in the coffee samples with GC were: campesterol, stigmasterol, clerosterol, ß-sitosterol, Δ^5-avenasterol together with stigmastanol, Δ^7-stigmastenol, and Δ^7-avenasterol.

The separation of the steryl glucosides were accomplished by RP-HPLC on a C8-column equipped with diode array detector. The quantification was carried out either with ß-sitosterol or with stigmasterol, depending on the number of double bounds in the molecule. The recovery of the steryl glucosides were appreciated with a steryl glucoside standard (Matreya)

and ranged between 83% and 96%. The steryl glucosides quantified with HPLC were ß-sitosterol-, campesterol-, and stigmasterol glucoside.

The total sterol content of the coffee beans includes the free sterols, steryl esters and the steryl glucosides.

RESULTS AND DISCUSSION

The total sterol content of the analyzed Arabica green coffee was 1335 µg/g on average. This value was somewhat lower than that of Robusta with 1589 µg/g. These contents are in accordance with literature data.

Steryl glucosides were reported for coffee for the first time; between 8% and 12% of the total sterols were presented in the analyzed green coffees. The steryl esters were the main fraction in all green coffees with 45% to 61%, followed by the free sterols (Figure 2).

Figure 2. Percentage distributions of the sterol compounds in green coffee.

A typical GC chromatogram of a steryl ester fraction after saponification obtained from an Arabica coffee is shown in Figure 3 (left); on the right, an HPLC chromatogram at 195 nm of the subsequent steryl glucoside fraction is presented.

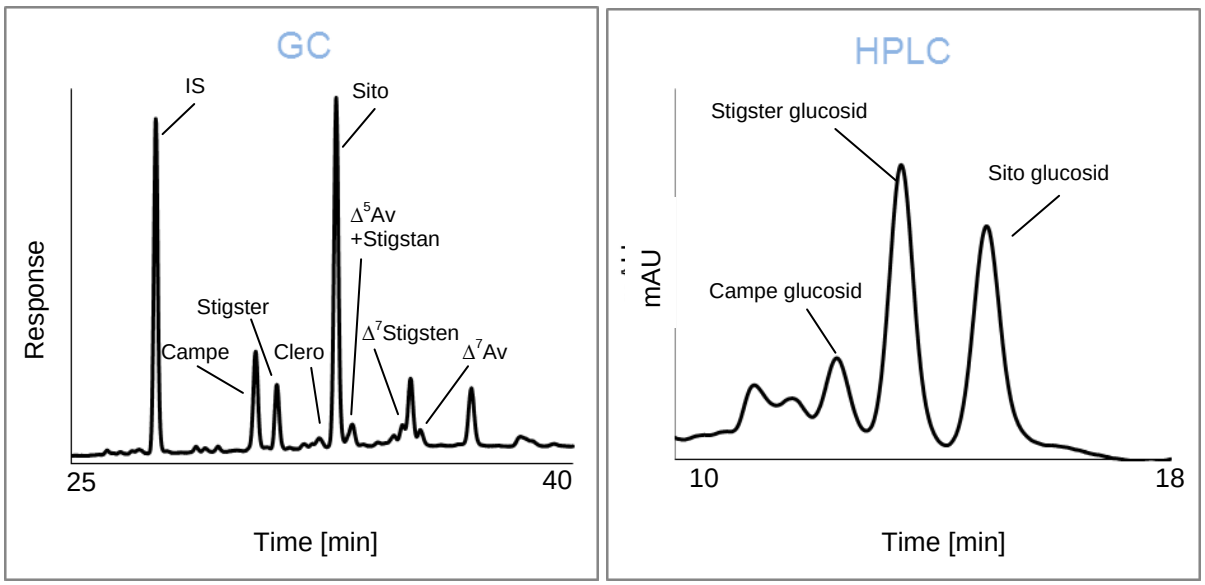

Figure 3. Section of a GC chromatogram of the steryl esters (left) and section of a HPLC chromatogram of the steryl glucosides (right) in Arabica green coffee (IS: Internal standard; Sito: ß-sitosterol; Campe: campesterol; Stigster: stigmasterol; Clero: clerosterol; Δ^5Av: Δ^5-avenasterol; Stigstan: stigmastanol; Δ^7Stigsten: Δ^7-stigmastenol; Δ^7Av: Δ^7-avenasterol).

The main component of the steryl glucoside fraction was ß-sitosterol, just as in the other fractions. The distributions of the other sterols were approximately equal in all groups. The content of the steryl glucosides in green coffee varied between 123 µg/g and 157 µg/g (Figure 4). Δ5-avenasterol, a characteristic sterol of Robusta coffee, could be identified as the glucoside by LC/MS in Robusta coffee, only.

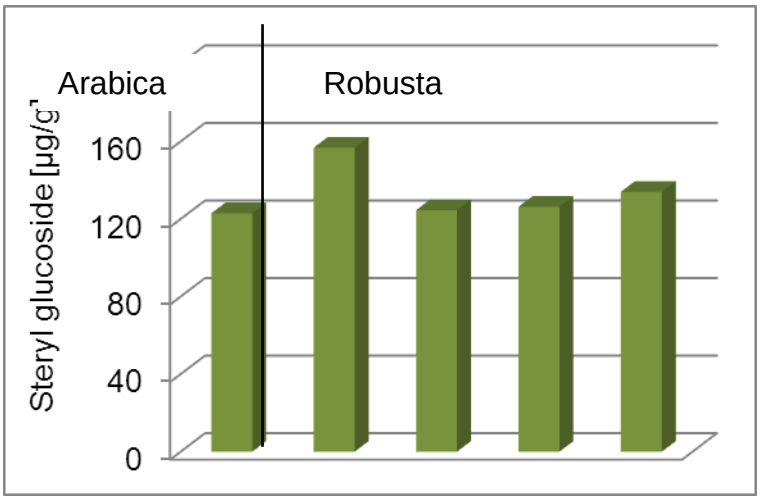

Figure 4. Steryl glucoside content in green coffee.

In the leaves of the two Arabica varieties, the steryl glucoside contents were 17% and 26% of the total sterols. The roots contained 7% and 6%, respectively. The contents of the Robusta were somewhat higher: in the leaves, 24% and in the roots 12% were analyzed (Figure 5).

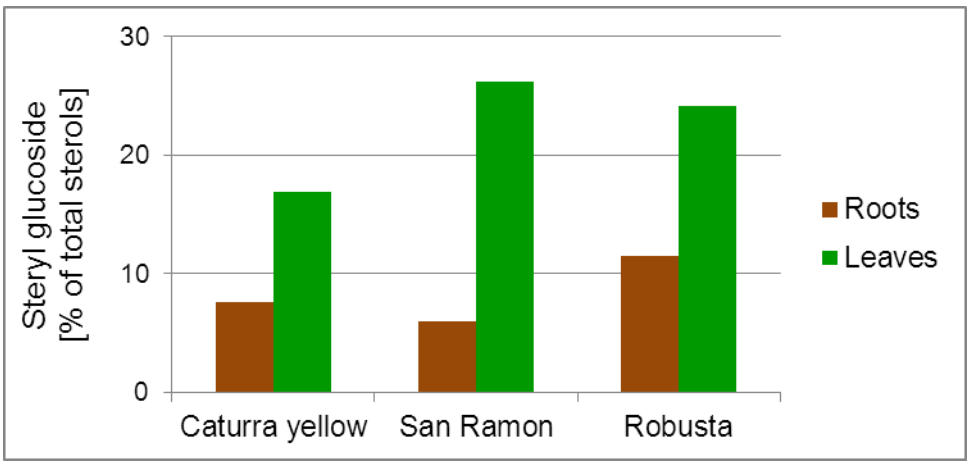

Figure 5. Percentage of the steryl glucosides on total sterols in roots and leaves.

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Trigonelline and HMF
Parameters to Study the Coffee Proportion in Coffee Drinks

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SUMMARY

Commercial coffee drinks belong to the milk-based drinks and comprised at least 70% milk and 30% of other ingredients such as a coffee preparation, sugar, rheological additives, cocoa, flavorings, and sometimes caffeine. The coffee proportion as a quality-determining compound constitutes between 0.8% and 19% of the coffee drink with mostly instant coffee or coffee extract applied for the coffee drinks per declaration. The question is whether it is possible to assess the coffee proportion in these coffee drinks. For this, as a water-soluble substance, trigonelline was studied. With a 100% carry-over from an instant coffee into the brew this appeared possible, but the high variability of the trigonelline content in different commercial instant coffees spoke against it.

The contents of trigonelline in coffee drinks were determined as being between 3.5 and 11 mg/100 mL drink. In addition, HMF was analyzed with 0.6 and 4.7 mg/100 mL drink. High HMF contents indicate the application of instant coffee. In conclusion, an assessment of the coffee proportion is feasible if the used coffee proportion is on hand.

INTRODUCTION

The popularity of ready-to-drink coffee has increased during the past years as a quick and uncomplicated solution to an occasional consumption of coffee. These chilled coffee drinks belong to the milk-based drinks and consist of at least 70% milk and, at most, 30% of other ingredients such as a coffee preparation, sugar, rheological additives, cocoa, flavorings, and sometimes caffeine. According to the declaration of the commercial coffee drinks, the coffee proportion is a solid or a liquid coffee extract or a coffee brew ranging from 0.8 to 19% of the whole drink. Nevertheless, the coffee proportion is the most valuable part of the drink.

To study the coffee proportion in commercial coffee drinks it is necessary to find a suitable component as a characteristic for coffee. It is reasonable to investigate a water-soluble and a lipid-soluble substance in regard to the differing procession of the extraction. Hence, the question is: “It is possible to assess the coffee proportion in coffee drinks?”

The substances must accomplish several requirements for the assessment of the coffee proportion:

- Adequate content in green, roasted, and instant coffee.
- Low natural variability.
- Low impact during the roasting process.
- Adequate passage into the coffee beverage.
- Absence in other ingredients.

In the majority of the coffee drinks, instant coffee or coffee extract are the ingredients of the product. This article deals with the utilizability of the water-soluble substance trigonelline as an indicator substance for the assessment of the coffee proportion.

MATERIALS AND METHODS

The commercial instant coffees were diluted with water and then directly measured with RP-HPLC/DAD. For the coffee drinks, an aliquot was taken and diluted with the same volume of methanol for precipitating the milk proteins. After filtration, RP-HPLC/DAD was carried out. The HPLC conditions were: column Synergi Hydro, water/methanol gradient, discrete wavelengths of 264 nm and 272 nm.

RESULTS AND DISCUSSION

In the following, some requirements mentioned above were to be considered for trigonelline.

Adequate passage into the coffee beverage

It is important, that the coffee amount and the trigonelline content in the coffee brews are linear for drawing conclusions to the coffee proportion concerned. As expected, the trigonelline in the instant coffee powder was 100% soluble in the beverage. Hence, there exists a linear relationship between the coffee amount and the trigonelline content (Figure 1).

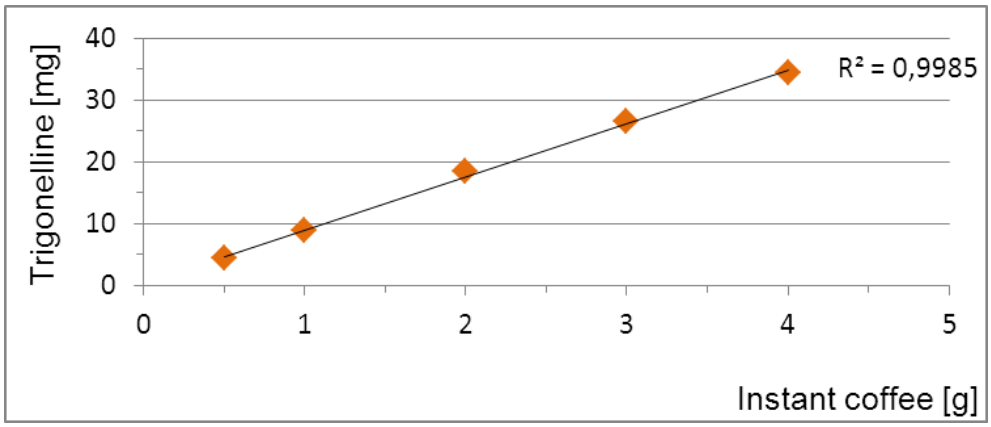

Figure 1. Linear relation between coffee amount and trigonelline in instant coffee.

Contents in commercial instant coffees

Five commercial instant coffees were analyzed in duplicates. The contents of trigonelline showed great differences. They varied between 9 g/kg to 17 g/kg (Figure 2). The literature also reported trigonelline contents between 3 g/kg and 26 g/kg. These variations may be caused by the different extraction conditions of the instant coffees. Furthermore, trigonelline is strongly influenced by the roasting process. The amount could be diminished to about 50%.

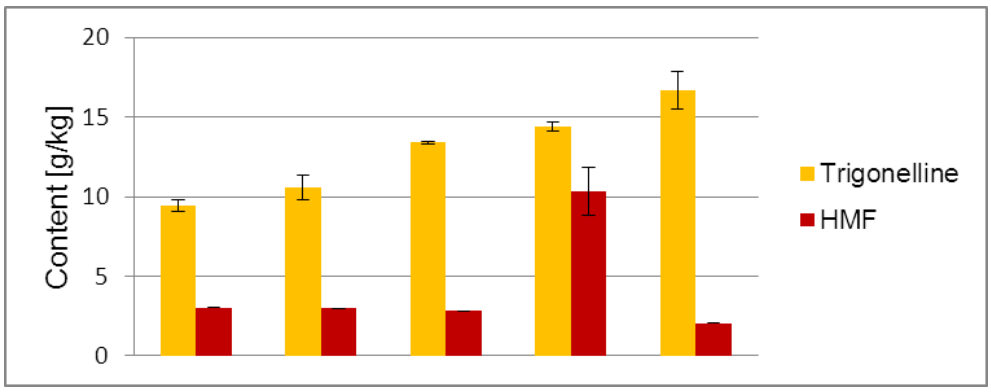

Figure 2. Trigonelline and HMF in different commercial instant coffees.

In addition to trigonelline, the contents of HMF (Hydroxymethylfurfural) were studied. The amounts are higher in instant coffees than in roasted coffees. In the analyzed commercial instant coffees, the levels ranged between 2 g/kg and 10 g/kg (Figure 2).

Figure 3 shows a typical chromatogram of a commercial coffee drink and an instant coffee. The analyses of the coffee drinks required a further clean-up-step. Therefore, methanol was added to precipitate the milk proteins.

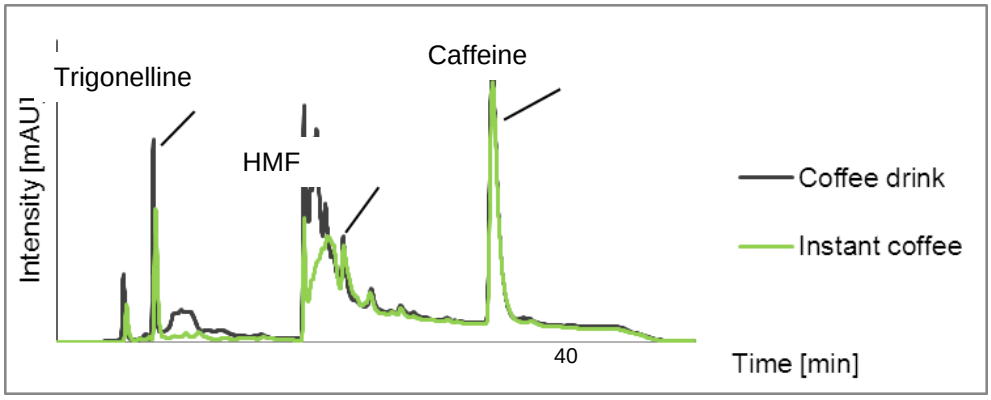

Figure 3. Chromatograms of a coffee drink and an instant coffee (λ=264 nm).

The contents of trigonelline in commercial coffee drinks varied between 3.5 and 11 mg/100 mL drink. The coffee drink with the highest declared coffee proportion showed the highest trigonelline content. The HMF levels were between 0.6 and 4.7 mg/100 mL drink. The highest amounts of HMF were analyzed in coffee drinks with the declaration “instant coffee”.

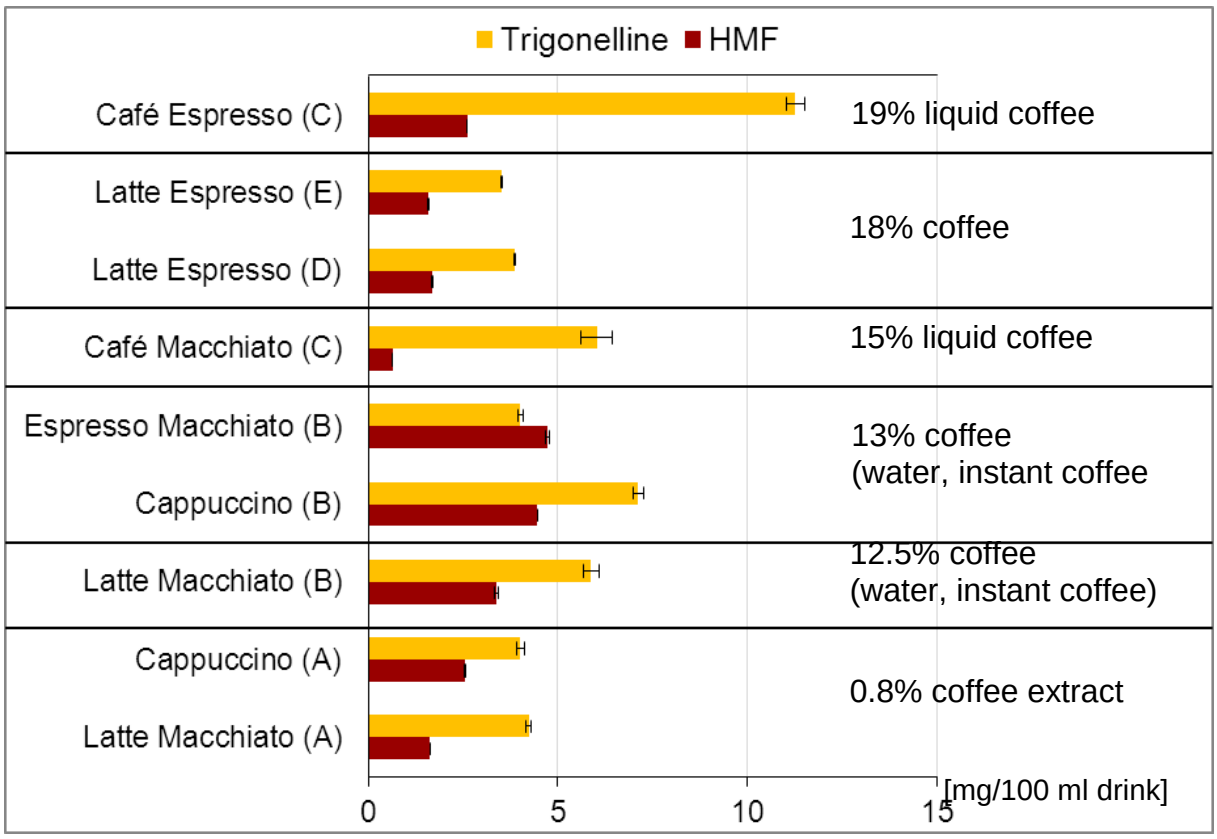

Figure 4. Trigonelline and HMF in commercial coffee drinks (analysis in duplicates; same letter means same producer).

Due to the high variability of the trigonelline content of instant coffees, it must be noted that the assessment of the coffee proportion in drinks via trigonelline is only possible when the coffee preparation used is available. High HMF contents indicate the application of instant coffee.

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Content of Carboxylic Acid-5-hydroxytryptamides (C-5-HT) in Decaffeinated Roasted Coffee: Influence of Decaffeination Process

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SUMMARY

The thin waxy layer covering the green coffee bean is mainly constituted by the so called carboxylic acid-5-hydroxytryptamides (more correctly $^{\beta}$N-alkanoyl-5-hydroxytryptamides, C-5-HT). These compounds stimulated several studies because they have been suggested as candidate stomach irritants with ulcerogenic effects on the gastric mucosa. It is well known that C-5-HT are partially thermally decomposed by roasting and by steam treatment. It is also known that a decrease in C-5-HT content can be observed by decaffeinating the green coffee beans, being dichloromethane (DCM) process more efficient than the supercritical CO_2 one.

In the present paper we report on the influence of the decaffeination process on the content of total C-5-HT in roasted coffee beans. Same lot of green Arabica blend has been decaffeinated by: water, dichloromethane, ethyl acetate and supercritical CO_2 processes and the total C-5-HT content has been determined on the roasted products. On the basis of the experimental results, the possibility to use the total amount of C-5-HT as a marker of decaffeination process has been explored by analyzing a number of decaffeinated roasted coffees purchased on the EU market.

INTRODUCTION

Carboxylic acid-5-hydroxytryptamides (C-5-HT) are the main constituents of the waxy layer which covers the green coffee bean. These coffee compounds are the amides of serotonin (5-hydroxytryptamine) and fatty acids with different chain lengths and grade of saturation. The barrier to hydration/de-hydration together with antioxidant properties may disclose their possible role in the protection of the green coffee bean. Coffee processing like steam-treatment, decaffeination, roasting or processes acting on the surface layer, remarkably reduce the content of C-5-HT originally present in green coffee beans. Roasting, for instance, induces the thermal degradation of C-5-HT to indole and quinoline derivatives via serotonin intermediate. The reduction of C-5-HT content in green coffee processed through steam-treatment or dewaxed coffee, has been related to less stomach irritation perceived by certain susceptible individuals after coffee consumption. This observation largely stimulated research aimed at individuating the coffee compounds inducing gastric acid secretion after coffee ingestion. Although the nature of these coffee constituents is not yet completely defined, it seems that the stomach irritating potential of C-5-HT can play a role in the undesirable physiological effects experienced by sensitive subjects. Beverage preparation is also very important in determining the amount of C-5-HT available for possible discomfort effects.

Whereas boiled coffees have amounts of up to 14 mg/L, *espresso* and paper filtered coffees have a total of C-5-HT contents close to or lower than 1mg/L.

Decaffeination process is known to reduce the total C-5-HT content, however the type of process seems to lead to different extent of the reduction. In particular, in comparing dichloromethane (DCM) and carbon dioxide, CO_2 decaffeination processes, the greatest effect in reduction has been observed for DCM decaffeination (decrease of 85%). These findings triggered our interest in determining the content of C-5-HT in products decaffeinated also according to ethyl acetate (EA) and water processes (W). By selecting an Arabica blend, the four different decaffeination processes have been performed and the obtained products, after roasting under different conditions, analyzed for total C-5-HT content. In order to explore the possibility to use total C-5-HT as analytical marker of decaffeination process, 37 samples of roasted decaffeinated commercial products have been analyzed. The total C-5-HT content may represent a promising analytical indicator of decaffeination process independently on roasting degree, blend composition and particle size distribution.

MATERIALS AND METHODS

Green coffee beans (Arabica blend) were decaffeinated according to DMC, CO_2, EA and W processes to achieve caffeine content according to Italian law (no > 0.10%). Decaffeinated green coffee beans were roasted to obtain medium roasting degree by using a lab roaster (Petroncini). Roasting degree was determined by color measurement (Probat Colorette 3B) with dark corresponding to < 70 and light to > 95 according to internal standards. Roasted and ground decaffeinated commercial products (n = 37) were purchased on the EU market. Products are named with an alphanumeric code. Coffee samples (ground and sieved when necessary) were prepared and analyzed for total C-5-HT according to Laganà *et al.* with minor modifications. Quantification (± 5% absolute error) was performed by reversed-phase HPLC with isocratic elution and fluorimetric detection (λex 280 nm, λem 340 nm) using docosanoic acid tryptamide (C22-5-HT) as external standard (Sigma). A 1100 HPLC with FLD fluorescence detector (Agilent) was used. EA was determined by GC-MS (Shimadzu QP2010) according to an internal method.

RESULTS AND DISCUSSION

In Table 1 the total content of C-5-HT determined on the same Arabica blend at a medium roasting degree (color 75-80) processed by the four well known decaffeination methods is reported. Whereas the total content of C-5-HT of CO_2, EA, and DCM processed coffees is within the range expected for decaffeinated roasted coffees, water processed decaffeinated coffee shows a very high value close to that typical of Arabica regular roasted coffee. In agreement with previous data, DCM process is particularly efficient in reducing the total C-5-HT content. In order to better investigate the performance of decaffeination processes in the total C-5-HT reduction, 8 commercial products with decaffeination process declared on the label have been analyzed.

Table 1. Total C-5-HT content in medium roasted 100% Arabica blend decaffeinated according different processes.

Decaffeination Process	Total C-5-HT (mg/Kg)
Water (W)	760
Carbon dioxide (CO_2)	453
Ethyl Acetate (EA)	181
Dichloromethane (DCM)	135

In Table 2, the total C-5-HT content of the commercial products is reported. The experimental data show that, independently on color, blend composition, and particle size distribution, the W process leads to a limited reduction of total C-5-HT content.

Table 2. [1]As declared on the label, A=Arabica, R=Robusta; nr = not reported.

Product	Decaffeination process[1]	Roasting degree[1]	Color	Blend[1]	Total C-5-HT (mg/Kg)
A1	CO_2	medium	71	nr	369
A2	CO_2	light	83	nr	448
A3	CO_2	dark	75	100% A	403
E1	CO_2	nr	83	nr	486
A4	CO_2	medium	92	60% A 40 %R	592
A5	CO_2	dark	82	30% A 70% R	484
O1	Water	nr	72	nr	1096
A6	Water	nr	61	100% A	954

The investigation has been extended to 29 commercial products (unknown decaffeination process) in order to assess a possible clustering. Table 3 reports on this new set of experimental data. As shown in the Table 5 samples characterized by a low total C-5-HT content (< 200 mg/Kg) reveal the presence of EA in amount significantly higher than that of all the other examined samples. This finding strongly suggests that the samples I1, R1, O3, O4 and U1 have been decaffeinated by EA process.

In Figure 1, the total C-5-HT content has been reported as a function of color measurement. By putting in evidence the samples processed by known decaffeination method, it is possible to individuate three areas where the samples decaffeinated by unspecified methods are clustered. In particular area A (total C-5-HT > 700 mg/Kg) and area B (250 mg/Kg < total C-5-HT < 700 mg/Kg) where water and CO_2 decaffeinated products are present, respectively and area C (total C-5-HT < 200 mg/Kg) where organic solvent decaffeinated products are shown. For the latter, the total C-5-HT content cannot permit the discrimination between DCM and EA processes. However, when EA determination is combined to C-5-HT analyses, it is possible to establish almost unambigously the type of organic solvent used for decaffeination.

In the case of W and CO_2 processes, Figure 1 shows a weak, albeit clear, trend of total C-5-HT content as function of roasting degree with the expected lower values for darker products.

Table 3. [1]As declared on the label, A=Arabica, R=Robusta; nr = not reported.

Product	Roasting degree[1]	Color	Blend[1]	Total C-5-HT (mg/Kg)	EA
I1	nr	84	nr	143	+
E2	nr	77	nr	119	
C1	nr	106	100% A	1062	
C2	nr	66	100% A	969	
R1	nr	90	nr	149	+
E3	nr	58	nr	166	
P1	dark	59	nr	350	
E4	medium	84	60% A 40% R	451	
O2	nr	80	nr	551	
E5	nr	85	nr	497	
C3	nr	92	100% A	1105	
E6	nr	56	A/R	800	
I2	nr	80	85% A 15% R	677	
R2	nr	92	nr	680	
O3	nr	81	nr	120	+
O4	nr	91	nr	80	+
O5	nr	80	nr	440	
T1	dark	44	100% A	159	
E7	nr	91	nr	206	
L1	nr	79	nr	125	
U1	nr	66	nr	116	+
A7	nr	71	nr	92	
E8	nr	65	nr	79	
E9	nr	53	nr	137	
A8	nr	90	nr	123	
E10	nr	48	A/R	785	
E11	nr	48	A/R	729	
E12	nr	58	nr	750	
L2	medium	76	100% A	129	

This trend is not evident for the DCM and EA processes. As far as the efficiency of the decaffeination process in total C-5-HT reduction is concerned, the present investigation indicates that both DCM and EA processes are the best performers. In view of the apolar character of the coffee waxes it is conceivable that the W process is the least effective in decreasing the total content of C-5-HT.

The present work suggests that the total C-5-HT content may represent a promising analytical indicator of decaffeination process of roasted and ground commercial products independently on roasting degree, blend composition and particle size distribution. Further studies are necessary to better understand how the different W and CO_2 process procedures affect the C-5-HT content in roasted decaffeinated coffee.

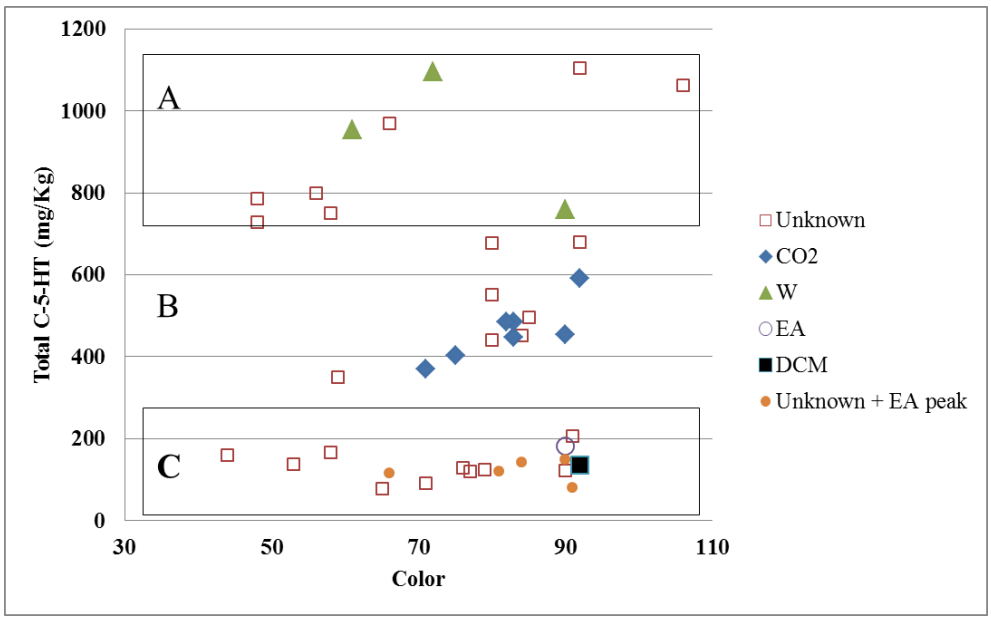

Figure 1. Total C-5-HT content as a function of color. A, B, C are described in the text.

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Can we Measure the Freshness of Roasted Coffee?

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SUMMARY

Seven different ratios of volatiles organic compounds were tested as chemical freshness indices using headspace gaschromatography with mass spectrometric detection (HS GC/MS) for (i) whole beans in four different types of packaging and (ii) six capsule systems from the Swiss market. Three indices are presented here: dimethyl disulfide/methanethiol, 2-butanone/2-methylfuran and 2,3-butanedione/ methanethiol. The first reflects the intrinsic instability of the coffee, but is also affected by the barrier properties of the packaging material and possibly by factors prior to packaging. The two other indices depend mainly on the barrier properties of the packaging and allows for a differentiation between packaging with and without aluminum barrier. Besides the evolution of the freshness over time, the indices also provide insight into capsule-to-capsule consistency.

INTRODUCTION

With the growing demand for specialty coffee and the rising consumer awareness of quality in the cup, it is becoming increasingly important to establish objective and quantitative measures of coffee quality. But quality is a complex concept and difficult to measure *(1)*. Nowadays *tasting* by a trained panel is a prevalent technique. Yet sensory evaluation is aimed at describing the sensory profile of coffee, without judgment. *Cupping* is a technique developed to evaluate green coffee quality, and focuses on defects. Yet, since one of the characteristics of specialty coffee is that it is expected to be free of defects, tasting for defects will not allow differentiating effectively amongst highest quality specialty coffees. *Hedonic evaluation* finally aims at assessing preferences. But equating preferences with quality and defining quality by what pleases and sells best does not allow deriving an absolute definition of coffee quality.

The focus of this project has been to develop quantitative analytical measures for positive coffee quality attributes, aimed at specialty coffees. Among others, this includes making the concept of freshness measurable. Indeed, we believe that freshness is one of a few central attributes that is closely connected to high quality coffee.

Here we report on the development and application of chemical freshness indices. Immediately upon formation, the flavor of a freshly roasted coffee starts to change *(2-6)*, leading to a loss of freshness. This includes loss of aroma by evaporation as well as chemical reactions (e.g. oxidation or other chemical reactions). Concurrently, the concentration of other compounds in the headspace (HS) increase. Hence the loss of freshness can best be described as a gradual change of the relative concentrations of aroma compounds as well as an overall decrease of intensity. A second quality concept that will be briefly addressed is consistency.

MATERIALS AND METHODS

Chemical indicators of freshness were explored for (i) roasted whole beans (coffea arabica, La Ceiba, Antigua, Guatemala) in four different packaging materials and (ii) six capsule systems from the Swiss market, labeled K1 to K6 (see Table 1). The packaging materials of the whole beans were: paper bag, plastic composite film (paper50/PE23), plastic composite film with a thin aluminum layer (paper50/PE25/ALU7/PE35), and plastic composite film with a thick aluminum layer (PET12MY/ALU8/PE90MY). Each package contained 250 g of roasted whole beans and was stored at room temperature. The following seven freshness indices were measured via HS analysis: dimethyldisulfide/methanethiol, 2-butanone/2-methylfuran, 2,3-butanedione/2-methylfuran, propanal/2-methylfuran, 2,3-butanedione/methanethiol, 2-butanone/methanethiol and methanol/2-methylfuran. Changes of the indices were monitored as ratios of Headspace (HS) concentrations of the selected VOCs. Three ratios are discussed below (**Errore. L'origine riferimento non è stata trovata.**-4): dimethyl disulfide/methanethiol (DMDS/MeSH), 2-butanone/2-methylfuran and 2,3-butanedione/ methanethiol.

In the case of the single serve capsule systems, capsules were opened and 4 g of the roasted and ground (R&G) powder was immediately filled in HS vials under nitrogen atmosphere. In the case of whole beans, the packages were opened, the beans ground and 4 g of the R&G powder was immediately filled in HS vials under N_2-atmosphere. All measurements were performed five-fold and values are expressed by their mean value and their 95 % confidence interval. HS GC-MS parameters are: *sample incubation time*: 20 min at 70 °C, *HS*: 2.5 ml HS syringe, 70 °C, injection volume 1 mL, (MPS2, Gerstel, Switzerland), *GC/MS*: (7890/5975N, Agilent Technologies, Switzerland) injector temperature 250 °C, split 30:1, DB-WAX (30 m x 250 µm x 0.25 µm), helium flow: 1 ml/min, oven: 20 °C for 6 min, then subsequent temperature increase ramps of 10 °C/min up to 70 °C, 5 °C/min up to 170 °C and finally 40 °C/min up to 220 °C.

Table 1. Packaging materials of capsule systems. Capsules consist of a body (mold) and cover foil that closes the capsule and is being perforated for extraction.

Code	Packaging Material		
	Body	***Cover***	***Secondary packaging***
K1	PP/EVOH/PP	PP/EVOH/PP	No
K2	PP/EVOH/PP	PP/EVOH/PP	No
K3	PP/EVOH/PP	PP/EVOH/PP	Aluminum
K4	98% Aluminum	Aluminum foil	No
K5	PP/EVOH/PP	Alu / PP/EVOH/PP	Aluminum (16 capsules)
K6	PP/EVOH/PP	Aluminum foil	No

K1 and K2 were the only systems without aluminum barrier, neither in the body nor the cover. K3 and K5 had secondary aluminum packaging. K4 was the only systems with a thick aluminum body and an aluminum cover. Finally the system K6 had a PP/EVOH/PP body and an aluminum foil as cover.

RESULTS AND DISCUSSION

DMDS/MeSH

This ratio reflects the degradation of MeSH leading to the formation of DMDS (the educt MeSH reacts to the product DMDS), and it is expected to be independent of the blend and the

roast degree. MeSH is typical for freshly roasted coffee, while fresh coffee should have very little DMDS. Hence we expect a small DMDS/MeSH ratio for freshly roasted coffee, which rises with decreasing freshness. For the whole beans in 250 g packages (Figure1), the paper bags show the fastest increase of DMDS/MeSH, which then flattens after a few weeks. The flattening may be attributed to one of the following reasons (or a combination thereof): (i) Most of the MeSH (educt) has reacted and the reaction ceases, (ii) DMDS reacts e.g. to form dimethyl-trisulfide, (iii) MeSH is lost by evaporation. At the other end of the spectrum, the plastic composite film with thick aluminum layer shows initially very little change but starts to rise after week 15 to achieve a value after 24 weeks that is no different than for the other types of packaging. We are currently extending the analysis up to one year storage to better assess the interaction and relative importance of the three processes cited above. Preliminary results indicate that at short times the DMDS/MeSH ratio is affected by both, the reaction of MeSH to DMDS, as well as the evaporation of MeSH.

Figure2 shows the evolution of the DMDS/MeSH ratio for different capsule systems. All capsules show a gradual increase, although at different rates. The system with a full aluminum body and aluminum foil (K4) has the lowest starting value and also remains low throughout one year of storage, while the only other capsule with an aluminum cover is performing second best (K6). In contrast, the two systems without aluminum barrier (K1, K2) show the fastest decrease in freshness. The two other systems (K3, K5) are intermediate with respect to the rate of change during storage and both are characterized by capsules with a secondary aluminum packaging. Another interesting aspect is the starting value for the ratio, for each of the packaging materials. Since the different 250 g coffee bags have all been filled from the same green coffee and roasting batch, values are initially identical. Capsules, in contrast, start at different values. We attribute this to differences in the blends and/or upstream processing.

2-butanone/2-methylfuran

This ratio differentiates well for the whole beans, between packaging with and without aluminum barrier (Figure3). The presence of aluminum as part of the barrier of the packaging prevents this ratio to change, whereas packaging without aluminum leads to a gradual and steady increase of this ratio. Hence we interpret an increase of this value as a loss of freshness.

2,3-butanedione/ methanethiol

This ratio differentiates well for capsule systems with and without aluminum barrier (Figure4). The two capsule systems K1 and K2 without aluminum barrier show the strongest changes during storage.

Consistency – a second attribute of quality

The uncertainty from the five repetitions shows large differences among the capsule systems. For instance, all five measured capsules for K4 showed nearly identical values for the ratios, which we attribute to high consistency in the upstream and packaging processes.

In conclusion, we have discussed two quantitative analytical measures to assess the quality of specialty coffee. These are freshness and consistency. Both were discussed in relation to roasted bean in 250 g packaging and single serve capsule systems. The data suggest that the evolution of DMDS/MeSH is driven both by oxidation of MeSH to DMDS and evaporation of MeSH. Furthermore, butanone/2-methylfuran (for whole bean packaging) and 2,3-butanedione/methanethiol (for capsule systems) are good indicator for the barrier properties of

the packaging. We are currently extending the storage time to one year and will report on the complete study including all seven freshness indices.

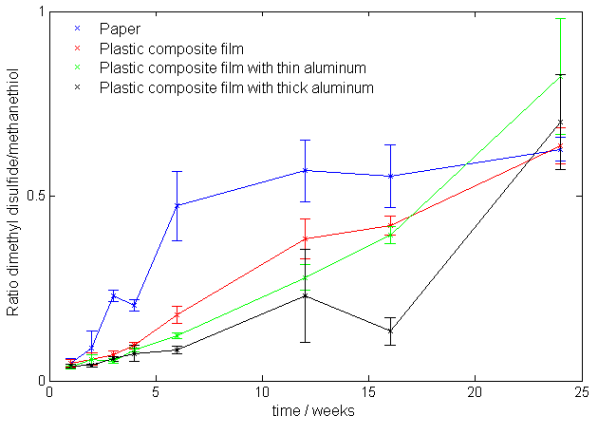

Figure 1. Whole beans. DMDS/MeSH ratio of roasted coffee beans in different packagings.

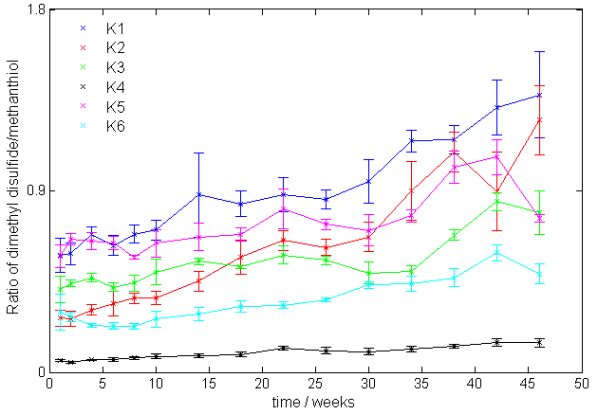

Figure 2. R&G. DMDS/MeSH ratio in different single-serve capsule systems.

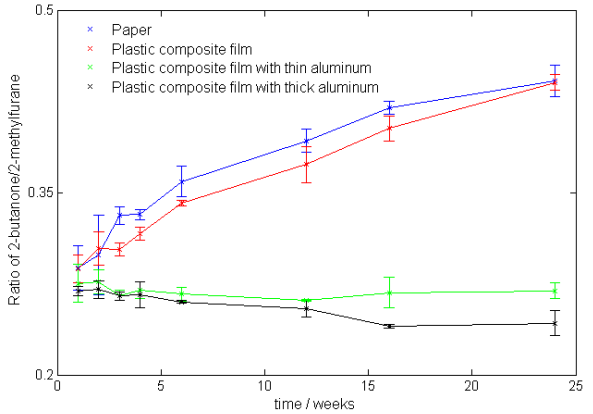

Figure 3. 2-butanone/2-methylfuran ratio for whole beans in different packaging materials.

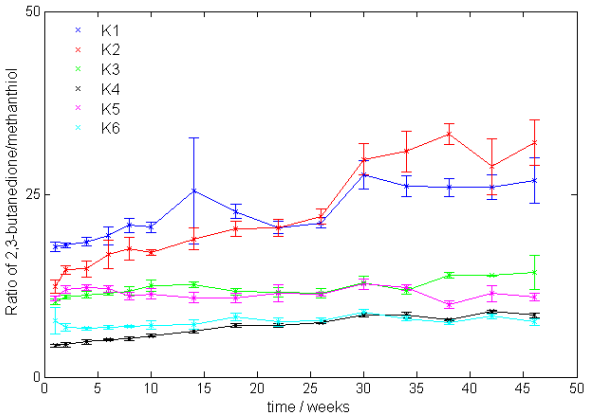

Figure 4. 2,3-butanedione/ MeSH ratio for capsules in different capsule systems.

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Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FITR) for Discrimination between Roasted Coffee and Adulterants

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SUMMARY

The objective of the present study was to develop an analytical methodology for the verification of coffee adulteration with roasted corn and coffee husks. The methodology was based on ATR-FTIR analysis of several samples of coffee beans, corn and coffee husks, after roasting and grinding. Principal Components Analysis provided separation of the samples into three groups: coffee, coffee husks and corn. Classification models were developed based on Linear Discriminant Analysis, with the adulterantes being satifactorily separated (100% correct classifications) from pure coffee. Such results confirm that ATR-FTIR is an appropriate analytical methodology for detection of adulteration in roasted and ground coffee.

INTRODUCTION

Ground roasted coffee has been the target of fraudulent admixtures with a diversity of cheaper materials (twigs, coffee husks, spent coffee grounds, roasted barley corn and others) because it presents physical characteristics (particle size, texture and color) that can be easily reproduced by roasting and grinding a wide variety of biological materials. Recent studies have shown that FTIR-based methods have been successfully applied in the food industry, in association with food quality evaluation, with a few applications focusing on roasted coffee. Among the commonly employed FTIR sampling techniques, attenuated total reflectance (ATR) can be employed for either solid or liquid samples, while Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFTS) is the technique usually employed for solid samples. ATR-FTIR has been shown to be appropriate for the analysis of roasted coffees. The specific applications were detection of glucose, starch or chicory as adulterants of freeze-dried instant coffees, determination of caffeine content, evaluation of roasting conditions and geographical discrimination. Thus, it was the aim of this study to evaluate the potential of ATR-FTIR for discrimination between roasted coffee and common adulterants such as roasted corn and coffee husks.

MATERIALS AND METHODS

Green arabica coffee beans and corn (acquired from local markets) and coffee husks (provided by Minas Gerais State Coffee Industry Union) samples (30 g) were submitted to roasting in a convection oven at 200, 220, 240, 250 and 260^{o}C. After roasting, the samples were ground (0.39 mm < D < 0.5 mm) and submitted to color evaluation. Color measurements were performed using a tristimulus colorimeter (HunterLab Colorflex 45/0 Spectrophotometer, Hunter Laboratories, VA, USA) with standard illumination D_{65} and colorimetric normal observer angle of 10^{o}. Measurements were based on the CIE $L^*a^*b^*$ three dimensional cartesian (xyz) color space represented by: Luminosity (L^*), ranging from 0

(black) to 100 (white) – z axis; parameter a^*, representing the green–red color component – x axis; and parameter b^*, representing the blue–yellow component -y axis. In order to attain different levels of roasting that could be representative of commercially available coffee, for each sample and temperature the roasting times were selected based on L* values measured for commercially available roasted coffee samples, corresponding to light (23.5 < L*< 25.0), medium (21.0 < L*< 23.5) and dark (19.0 < L*< 21.0) roasts. The corresponding roasting times and temperatures are shown in Table 1.

Table 1. Roasting parameters and conditions.

Roasting temperature	**Luminosity values (Roasting time)**		
Coffee			
	Light Roast	*Medium Roast*	*Dark Roast*
200°C	24.28±0.02 (40 min)	21.48±0.08 (70 min)	19.62±0.37 (90 min)
220°C	23.18±0.12 (20 min)	21.51±0.01 (22 min)	19.96±0.13 (25 min)
240°C	25.17±0.04 (11 min)	22.01±0.33 (13 min)	19.89±0.08 (15 min)
Coffee husks			
	Light Roast	*Medium Roast*	*Dark Roast*
200°C	22.22±0.05 (20 min)	21.66±0.15 (30 min)	20.16±0.12 (50 min)
220°C	23.00±0.06 (10 min)	20.41±0.30 (13 min)	19.88±0.13 (15 min)
240°C	25.16±0.04 (6 min)	21.34±0.17 (7 min)	20.47±0.06 (9 min)
Corn			
	Light Roast	*Medium Roast*	*Dark Roast*
240°C	24.45±0.21 (30 min)	22.01±0.33 (35 min)	19.89±0.08 (40 min)
250°C	24.63±0.26 (15 min)	22.17±0.08 (17 min)	19.33±0.07 (19 min)
260°C	22.25±0.06 (11 min)	21.10±0.16 (12 min)	19.26±0.10 (13 min)

Spectra were collected from a Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan) with a DLATGS (Deuterated Triglycine Sulfate Doped with L-Alanine) detector at room temperature (20 ± 0.5 °C).). For the attenuated reflectance measurements (ATR-FTIR), a horizontal ATR sampling accessory (ATR-8200HA) equipped with ZnSe cell was employed. Each roasted and ground sample (D < 0.15 mm) was mixed was placed inside the sample port. All spectra were recorded within a range of 4000–400 cm^{-1} with 4 cm^{-1} resolution and 20 scans, and truncated to 2500 data points in the range of 3200-700 cm^{-1}, in order to eliminate noise readings present in the upper and lower ends of the spectra. The following data spectra pretreatment techniques were tested: (0) no additional processing (raw data), (1) mean centering, (2) normalization, (3) baseline correction employing two (3200 and 700 cm^{-1}) or three (3200, 2000 and 700 cm^{-1}) points, (4) first derivatives and (5) second derivatives.

Using the ATR spectra (raw or normalized) and its derivatives as chemical descriptors, pattern recognition (PR) methods (PCA and LDA) were applied in order to establish whether samples adulterated with coffee husks and roasted corn could be discriminated from roasted

coffee samples. LDA model variable selection was based on the data that presented higher influence on group separation (high loading values) from the PCA analysis. The statistical package XLSTAT Sensory 2010 (Addinsoft, New York) was employed for all the chemometric calculations.

RESULTS AND DISCUSSION

Regarding PCA, the spectra pretreatments that provided a satisfactory level of group separation were the following: (0) no additional treatment of raw data, (2) normalization and (3) baseline correction employing two (3200 and 700 cm^{-1}) or three (3200, 2000 and 700 cm^{-1}) points. The corresponding scatter plots are displayed in Figure 1. Roasted coffee, roasted coffee husks and roasted corn can be identified as separated groups. Roasted coffee is clearly separated from the others, with some group overlapping between corn and coffee husks for the plots based on raw spectra (Figure 1a).

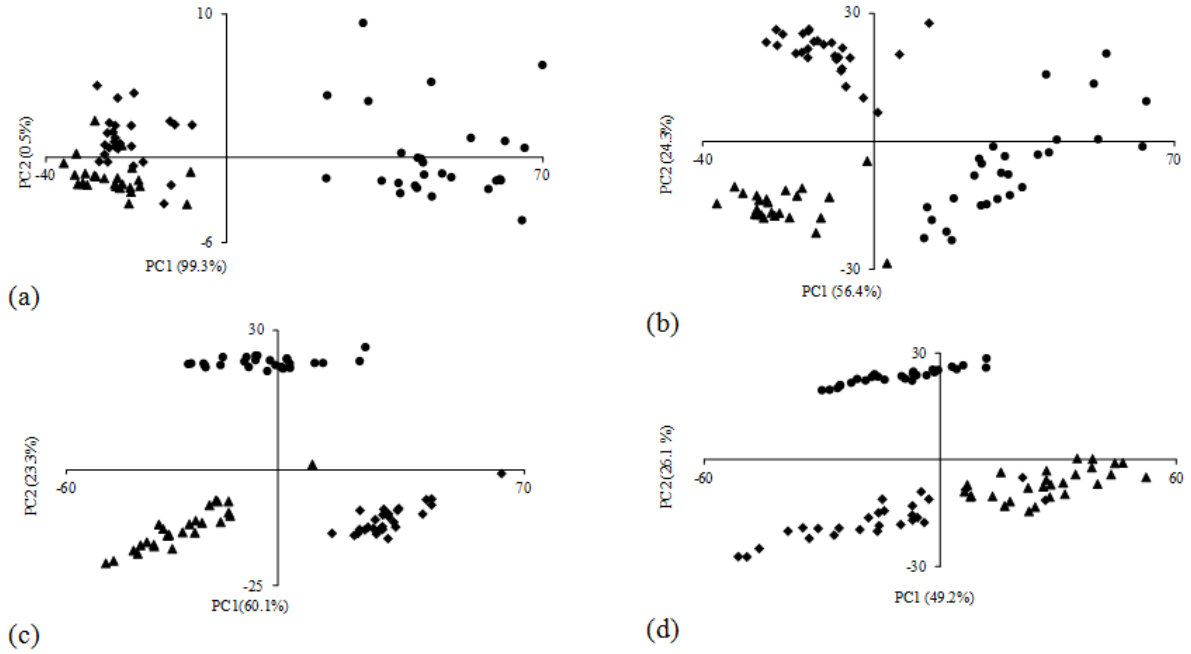

Figure 1. PCA scores scatter plot (PC1 vs. PC2) based on ATR spectra of roasted coffee in comparison to roasted corn and coffee husks after the following pretreatment steps: (a) no treatment; (b) normalization; (c) two and (d) three point baseline correction (● coffee;♦ coffee husks;▲corn).

The satisfactory group separation results obtained from the PCA indicate that the data should provide enough information to develop classification models for coffee and adulterants. Thus, linear discriminant analysis (LDA) was employed in order to obtain classification models. The score plots obtained for the discriminant functions are shown in Figure 2. A clear separation between roasted coffee, coffee husks and corn, can be observed for the developed models, that provided 100% recognition and prediction.

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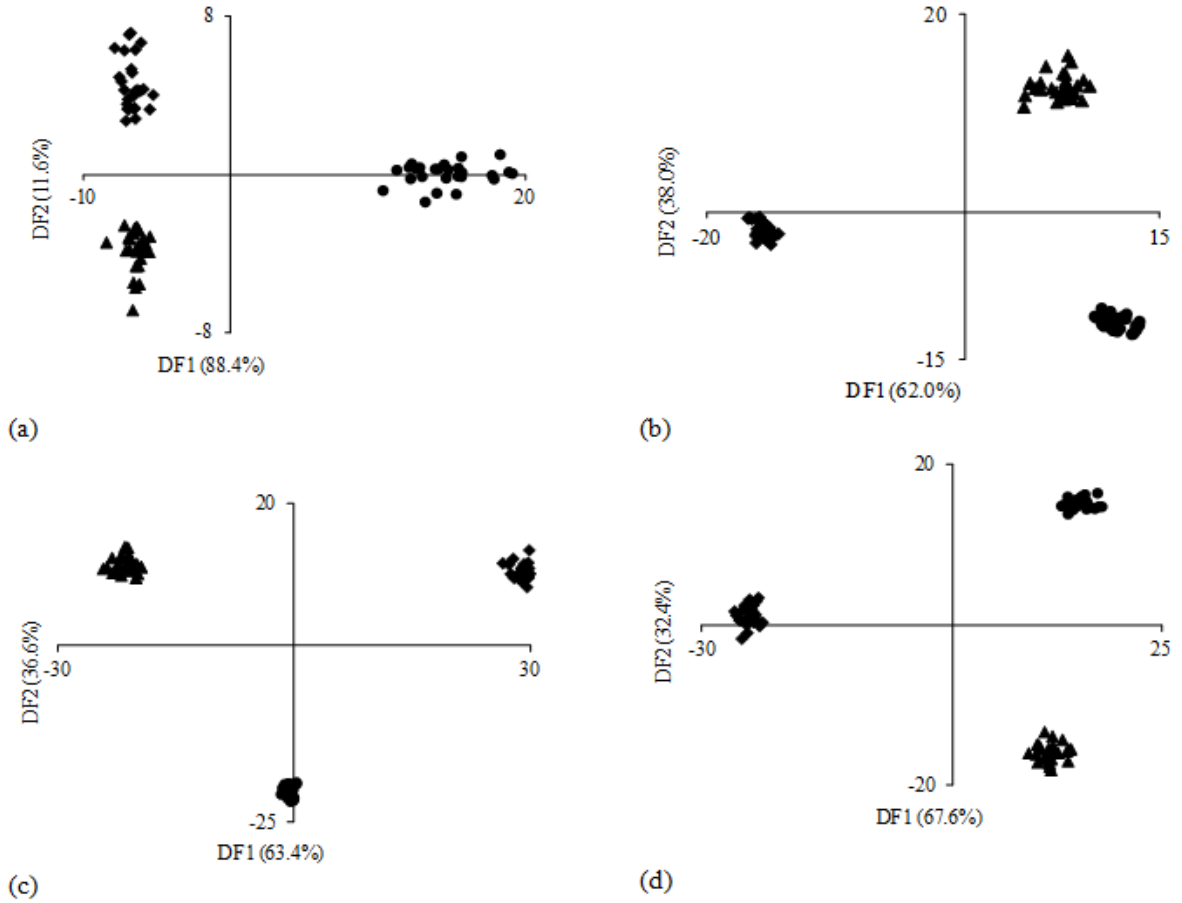

Figure 2. Scores of the discriminant functions provided by the LDA models of ATR spectra (3100 - 700 cm-1) after the following pretreatment steps: after the following pretreatment steps: (a) no treatment; (b) normalization; (c) two and (d) three point baseline correction (● coffee; ◆coffee husks; ▲corn).

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Detection of Coffee Husks and Roasted Corn in Admixtures with Roasted Coffees By Fourier Transform Infrared Spectroscopy (FTIR)

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SUMMARY

The objective of the present study was to develop an analytical methodology for the verification of coffee adulteration with roasted corn and coffee husks. The methodology was based on FTIR analysis of several samples of coffee beans, corn and coffee husks, after roasting and grinding. Samples of coffee were adulterated with varying proportions of corn and coffee husks. Principal Components Analysis provided separation of the samples into three groups: coffee, coffee husks and corn. Classification models were developed based on Linear Discriminant Analysis, with the adulterated samples being satifactorily separated (100% correct classifications) from pure coffee. Such results confirm that FTIR is an appropriate analytical methodology for detection of adulteration in roasted and ground coffee.

INTRODUCTION

Not only is Brazil the largest coffee producer and exporter, but also it is one of the largest consumers in the world. Because of the way coffee is consumed, i.e., an infusion of ground roasted beans, the product of consumption is quite vulnerable to adulteration with other roasted grains and beans, such as corn and barley. Also, coffee by-products such as spent coffee grounds and coffee husks are commonly used for adulteration of ground roasted coffee since, after roasting, they become difficult to be detected by visual inspection and common routine analytical methods. Recent studies have shown that FTIR-based methods have been successfully applied in the food industry, in association with food quality evaluation, with Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFTS) being the technique usually employed for solid samples. DRIFTS has been proven to be appropriate for the analysis of roasted coffees, providing satisfactory discrimination between Arabica and Robusta varieties, between regular and decaffeinated coffees, and between high (non-defective) and low quality (defective) coffees. Thus, it was the aim of this study to evaluate the potential of this technique for discrimination between roasted coffee and common adulterants such as roasted corn and coffee husks.

MATERIALS AND METHODS

Green arabica coffee beans and corn (acquired from local markets) and coffee husks (provided by Minas Gerais State Coffee Industry Union) samples (30 g) were submitted to roasting in a convection oven at 200, 220, 240, 250 and 260oC. After roasting, the samples were ground (0.39 mm < D < 0.5 mm) and submitted to color evaluation. Color measurements were performed using a tristimulus colorimeter (HunterLab Colorflex 45/0 Spectrophotometer, Hunter Laboratories, VA, USA) with standard illumination D65 and colorimetric normal observer angle of 10o. Measurements were based on the CIE L*a*b*

three dimensional cartesian (xyz) color space represented by: Luminosity (L*), ranging from 0 (black) to 100 (white) – z axis; parameter a*, representing the green–red color component – x axis; and parameter b*, representing the blue–yellow component -y axis. In order to attain different levels of roasting that could be representative of commercially available coffee, for each sample and temperature the roasting times were selected based on L* values measured for commercially available roasted coffee samples, corresponding to light (23.5 < L*< 25.0), medium (21.0 < L*< 23.5) and dark (19.0 < L*< 21.0) roasts. The corresponding roasting times and temperatures are shown in Table 1.

Table 1. Roasting parameters and conditions.

Roasting temperature	**Luminosity values (Roasting time)**		
	Coffee		
	Light Roast	**Medium Roast**	**Dark Roast**
200°C	24.28±0.02 (40 min)	21.48±0.08 (70 min)	19.62±0.37 (90 min)
220°C	23.18±0.12 (20 min)	21.51±0.01 (22 min)	19.96±0.13 (25 min)
240°C	25.17±0.04 (11 min)	22.01±0.33 (13 min)	19.89±0.08 (15 min)
	Coffee husks		
	Light Roast	**Medium Roast**	**Dark Roast**
200°C	22.22±0.05 (20 min)	21.66±0.15 (30 min)	20.16±0.12 (50 min)
220°C	23.00±0.06 (10 min)	20.41±0.30 (13 min)	19.88±0.13 (15 min)
240°C	25.16±0.04 (6 min)	21.34±0.17 (7 min)	20.47±0.06 (9 min)
	Corn		
	Light Roast	**Medium Roast**	**Dark Roast**
240°C	24.45±0.21 (30 min)	22.01±0.33 (35 min)	19.89±0.08 (40 min)
250°C	24.63±0.26 (15 min)	22.17±0.08 (17 min)	19.33±0.07 (19 min)
260°C	22.25±0.06 (11 min)	21.10±0.16 (12 min)	19.26±0.10 (13 min)

Spectra were collected from a Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan) with a DLATGS (Deuterated Triglycine Sulfate Doped with L-Alanine) detector at room temperature (20 ± 0.5 °C). Diffuse reflectance (DR) measurements were performed in diffuse reflection mode with a Shimadzu sampling accessory (DRS8000A). Each roasted and ground sample (D < 0.15 mm) was mixed with KBr (10g/100g) and then 23 mg of this mixture was placed inside the sample port. All spectra were recorded within a range of 4000–400 cm^{-1} with 4 cm^{-1} resolution and 20 scans, and submitted to background (pure KBr) subtraction. The spectra were also truncated to 2500 data points in the range of 3200-700 cm^{-1}, in order to eliminate noise readings present in the upper and lower ends of the spectra. The following data spectra pretreatment techniques were tested: no additional processing (raw data), mean centering, normalization, baseline correction employing two (3200 and 700 cm^{-1}) or three (3200, 2000 and 700 cm^{-1}) points, first and second derivatives.

Using the DR spectra (raw or normalized) and its derivatives as chemical descriptors, pattern recognition (PR) methods (PCA and LDA) were applied in order to establish whether samples adulterated with coffee husks and roasted corn could be discriminated from roasted coffee samples. LDA model variable selection was based on the data that presented higher influence on group separation (high loading values) from the PCA analysis. The statistical package XLSTAT Sensory 2010 (Addinsoft, New York) was employed for all the chemometric calculations.

RESULTS AND DISCUSSION

Regarding PCA, the spectra pretreatment steps that provided a satisfactory level of group separation between coffee and both adulterants were were the following: no additional treatment of raw data, normalization with three point baseline correction and first derivatives. The corresponding scatter plots are displayed in Figure 1. Roasted coffee, roasted coffee husks and roasted corn can be identified as separated groups. Roasted corn is clearly separated from the others, whereas some group overlapping occurs between coffee and coffee husks for the spectra-based plots (Figure 1a and 1b). First derivatives provided complete separation of the three groups (Figure 1c).

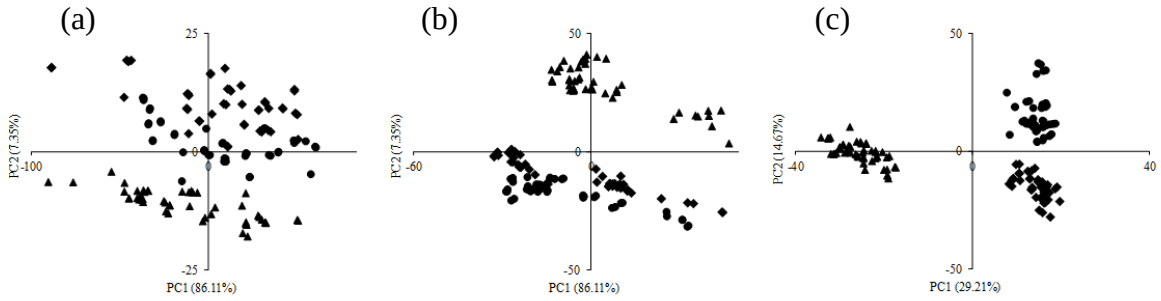

Figure 1. PCA scores scatter plot (PC1 vs. PC2) based on diffuse reflectance spectra of roasted coffee in comparison to roasted corn and coffee husks after the following pretreatment steps: (a) no treatment; (b) normalization and baseline correction; (c) first derivatives (♦ coffee; ▲coffee husks; ●corn).

Based on these results, the analysis was repeated to verify if the technique would also allow discrimination between pure and adulterated coffee (adulteration levels ranging from 50 to 10% of one or both adulterants). The corresponding PCA scatter plots are displayed in Figure 2. Pure and adulterated coffees are clearly separated into two groups, with some overlapping for plots based on normalized spectra (Figure 2b).

The satisfactory group separation results obtained from the PCA indicate that the data should provide enough information to develop classification models for pure and adulterated coffee. Thus, linear discriminant analysis (LDA) was employed in order to obtain classification models. The score plots obtained for the discriminant functions are shown in Figure 3. A clear separation between pure and adulterated coffee samples, can be observed for the spectra-based models (Figures 3a and 3b), with both models providing 100% recognition and prediction. The model based on first derivatives (Figure 3c) presented poorer performance, with some overlapping between pure and adulterated samples. Values for recognition and prediction abilities were 91 and 87%, respectively.

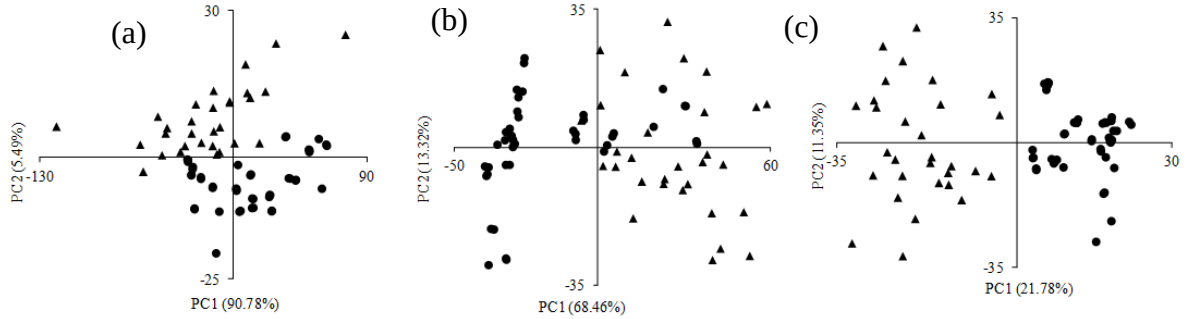

Figure 2. PCA scores scatter plot (PC1 vs. PC2) based on diffuse reflectance spectra of pure and adulterated roasted after the following pretreatment steps: (a) no treatment; (b) normalization and baseline correction; (c) first derivatives (▲ pure coffee;● adulterated coffee).

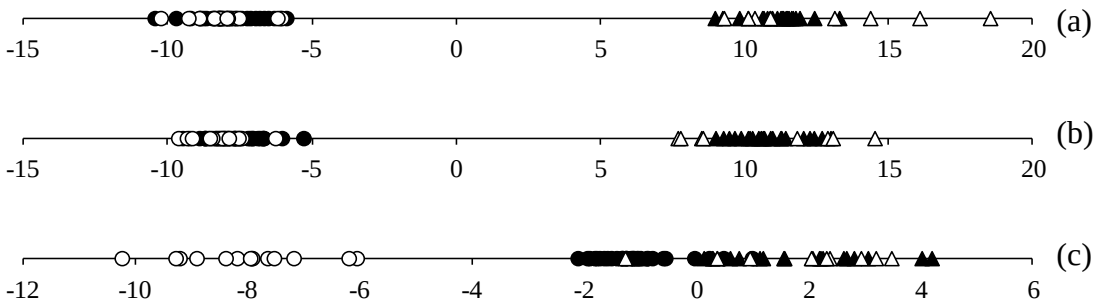

Figure 3: Scores of the discriminant functions provided by the LDA models of diffuse reflectance spectra (3100 - 700 cm^{-1}) after the following pretreatment steps: (a) no treatment; (b) normalization and baseline correction; (c) first derivatives (▲ pure coffee; ● pure coffee/validation;△ adulterated coffee; ○ adulterated coffee/validation).

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Quantification of Roasted and Ground Defective Coffees in Admixtures With Non-Defective Coffees by Atr-Ftir and Chemometrics

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SUMMARY

Mathematical models based on chemometric analysis of Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) spectra of defective coffees in admixtures with non-defective coffees were developed. 1st and 2nd derivative transformations were applied to raw data in order to remove unimportant baseline signal from samples and reduce instrument effects. Partial Least Squares (PLS) was used to construct the models aiming to predict the percentage of defective coffee in the coffee blends. Besides the chemical similarity between defective and non-defective coffees, high degrees of accuracy were achieved.

INTRODUCTION

Defective coffee represent about 20% of the total coffee produced in Brazil and similar amounts can be expected in other areas around the world. The negative effect that such beans have on coffee quality can be associated to specific problems that occur during harvesting and post-harvest processing operations. Black beans result from dead beans within the coffee cherries or from beans that fall naturally on the ground by action of rain or over-ripening. The presence of these beans in a roasted batch usually imparts a heavy flavor to the beverage. Sour beans can be associated with 'overfermentation' during wet processing and with improper drying or picking of overripe cherries, imparting sour and oniony tastes to the beverage. Immature beans come from immature fruits, and contribute to the astringency of the beverage.

Research interest on defective and low quality coffee beans has intensified over the past years, given the increasing awareness on the negative aspects they impart to the quality of the beverage. In the present study, ATR-FTIR and PLS regression were used to quantify defective coffees in blends with non-defective coffees.

MATERIALS AND METHODS

Arabica green coffee samples were acquired from a Coffee Roasting Company located in Minas Gerais (MG) State, Brazil (Café Fino Grão, Contagem, MG). Beans were manually sorted (by a professional trained and certified for green coffee classification) into five lots: non-defective, immature, black and dark sour. Coffee samples (25 g) were taken from each lot and submitted to roasting in a convection oven (Model 4201D Nova Ética, São Paulo, Brazil) at 235 °C until achieving a medium degree of roasting (21 < Luminosity* <23.5). The determination of the roasting degree was based on color evaluation of the ground coffee using a tristimulus colorimeter (HunterLab Colorflex 45/0 Spectrophotometer, Hunter Laboratories, VA, USA) with standard illumination D_{65} and colorimetric normal observer angle of 10^{o}. Previous results on ATR-FTIR analysis of defective and non-defective coffees indicated that

the influence of the roasting degree is not or less significant than the influence of the quality of the beans. Considering the large number of samples to be analyzed, samples roasted at only one roasting condition were used in this work. Thereafter samples were sieved (0.15 < particle diameter < 0.25 mm).

Black, immature and dark sour coffees were mixed with non-defective coffee, with % defects ranging from 3 to 30 % in steps of 3% (10 blends for each of the three defects). Blends were disposed in Falcon tubes and shaken for one minute in a tube shaker (Fisatom, Brazil).

A Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan) with a DLATGS (Deuterated Triglycine Sulphate Doped with L-Alanine) detector was used in the measurements that were all performed in a dry atmosphere at room temperature (20 ± 0.5 °C). A horizontal ATR sampling accessory (ATR-8200HA) equipped with ZnSe cell was employed. Measurements were performed using approximately 2 g of ground coffee sample. The empty recipient was used to obtain the background spectrum. The approximate total time required for sample preparation was 5 min. All spectra were recorded within a range of 3100–800 cm^{-1} with a 4 cm^{-1} resolution and 20 scans and submitted to background subtraction. Each coffee blend was analyzed in five replicates. The pure non-defective coffee was also analyzed in six replicates, obtaining a total of 56 readings for each defect.

Prior to statistical analysis, derivative transformations were applied to raw data in order to remove unimportant baseline signal from samples and reduce instrument effects. The Savitzky-Golay algorithm was used to simultaneously smooth the data as it takes the derivative.

The software Matlab (The MathWorks, Co., Natick, MA) using the statistical package PLS_Toolbox (Eigenvector Research, Inc.) was employed for the chemometric calculations. PLS was applied to determine the concentration of defective coffee mixed with non-defective coffee. The calibration set used to build the models included 40 samples randomly selected, and the 16 remaining samples were used at the validation set. The models were chosen based on the optimal number of factors, the minimum value of the predicted residual sum of squares (PRESS), and the maximum value of the correlation coefficient (R^2). The performance of the models developed was estimated by Random subset cross-validation, in which training and validation data are randomly split, and for each split predictive accuracy is assessed and then averaged. The predictability of the resulting models was evaluated based on the standard error of prediction (SEP) and the R^2 value.

RESULTS AND DISCUSSION

The scatter plots of actual and calculated values for percentage of defective in non-defective coffee blends for both calibration and validation models are shown in Figure 1. The models were constructed based on 1st and 2nd derivative spectra. A visual inspection of the scatter plots suggests that a higher predictability is achieved when 1st derivative is applied to dark sour and immature coffee blends spectra, and 2nd derivative is applied to black coffee blend spectra.

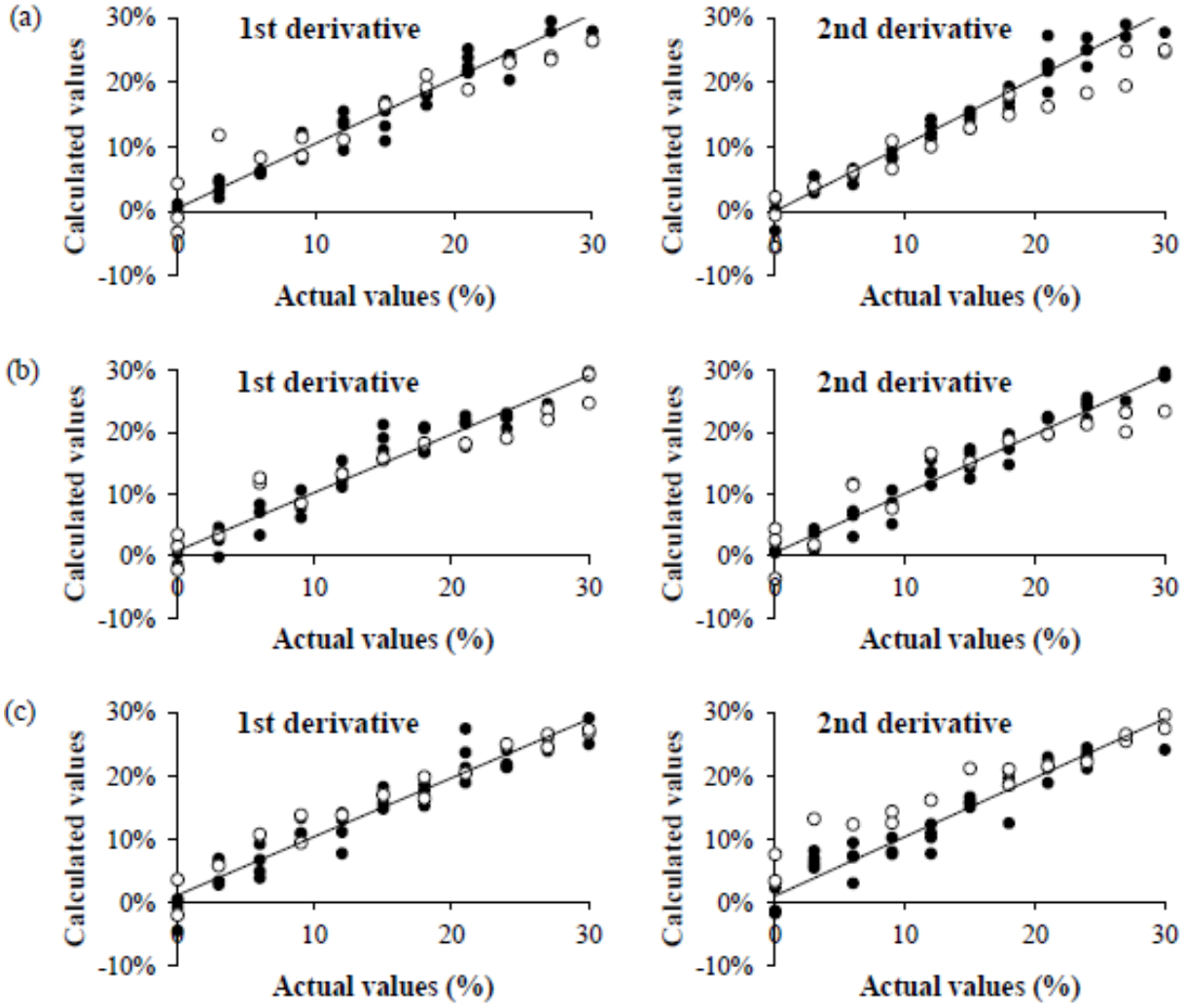

Figure 1. Scatter plots of actual and calculated values for percentage of (a) dark sour, (b) black, and (c) immature coffee in blends with non-defective coffees. ● calibration and ○ validation.

Table 1 summarizes and confirms the prediction results. For the models constructed for black coffees, 1st derivative treatment provided higher degree of accuracy with lower SEP and higher R^2. Although the SEP was slightly lower in the 1st derivative treatment for dark sour blends, a substantially higher R^2 was obtained in the 2nd derivative treatment model. However, it must be considered that a larger factor, or latent variable number, was used. In general, models with fewer factors are less likely to exhibit over fitting and tend to be more stable and have better generalization ability. With respect to the immature coffee blends, 1st derivative treatment allowed for the highest degree of accuracy.

Table 1. Calibration and validation parameters for the prediction models.

Data treatment	*Factors*	*Calibration*			*Validation*	
		RMSEC	*R²*	*RMSECV*	*RMSEP*	*R²*
Black						
1st derivative	8	0.022	0.938	0.055	0.035	0.917\
2nd derivative	8	0.018	0.96	0.056	0.039	0.883
Dark sour						
1st derivative	5	0.018	0.96	0.031	0.033	0.919
2nd derivative	6	0.015	0.975	0.039	0.038	0.931
Immature						
1st derivative	7	0.026	0.928	0.046	0.025	0.949
2nd derivative	6	0.024	0.934	0.053	0.045	0.936

CONCLUSIONS

The feasibility of employing ATR-FTIR as a method for quantification of defective in blends with non-defective coffee samples was evaluated and successfully demonstrated. Based on 1st and 2nd derivative spectra, the PLS models developed were found to be suitable predictors. The obtained results reinforce that the nondestructive evaluation, ease of operation, and fast determination of ATR-FTIR technique not only allow the discrimination of coffees from different specie or origin, but also the assessment of coffee quality.

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Potential of Atr-Ftir for Discrimination Between Defective and Non-Defective Roasted Coffees

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SUMMARY

The objective of this work was to evaluate the feasibility of employing Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) for discrimination between defective and non-defective ground and brewed coffees. Principal Component Analysis (PCA) of the ATR spectra (after derivative transformations) provided separation of the samples into two groups: (a) non-defective and light sour, and (b) black, dark sour and immature beans. It was found that except for light sour beans, it is possible to dicriminate between defective and non-defective coffees by ATR-FTIR, using either solid or liquid samples.

INTRODUCTION

The determination of food quality and authenticity are major issues in the food industry. Most instrumental techniques required for assessing this quality are time demanding, expensive and involve a considerable amount of manual work. Therefore, there is a need for new and rapid analytical methods, as infrared spectroscopy, which is fast, reliable and non-destructive. This technique, coupled with chemometric data analysis techniques, has been successfully applied for food quality evaluation, and for authentication of species and geographical origin of coffees and coffee quality evaluation prior and after roasting. In view of the aforementioned, the objective of this work was to evaluate the potential of ATR-FTIR for the discrimination between defective and non-defective coffee beans. For this purpose, two different methodologies were developed and evaluated, one based on the analysis of the coffee powders and other on the analysis of the brewed coffees.

MATERIALS AND METHODS

Arabica green coffee samples were acquired from a Coffee Roasting Company located in Minas Gerais (MG) State, Brazil (Café Fino Grão, Contagem, MG). Beans were manually sorted (by a professional trained and certified for green coffee classification) into five lots: non-defective, immature, black and sour (separated into light and dark coloured). Coffee samples (25 g) were taken from each lot and submitted to roasting in a convection oven (Model 4201D Nova Ética, São Paulo, Brazil), at 220, 235 and 250^{o}C. After roasting, samples were ground and submitted to color evaluation using a tristimulus colorimeter (HunterLab Colorflex 45/0 Spectrophotometer, Hunter Laboratories, VA, USA) with standard illumination D_{65} and colorimetric normal observer angle of 10^{o}. Roasting conditions were established for each specific lot, given that defective coffee beans have been reported to roast to a lesser degree than non-defective coffee beans when submitted to the same processing conditions. Roasting degrees were then defined according to luminosity (L*) measurements similar to commercially available coffee samples, corresponding to light ($23.5 < L^* < 25.0$), medium ($21.0 < L^* < 23.5$) and dark ($19.0 < L^* < 21.0$) roasts. The corresponding roasting

times ranged from 7 to 10 min (250 ^{o}C), 9 to 16 min (235 ^{o}C) and 12 to 33 min (220 ^{o}C), with the smaller and larger times for a given temperature corresponding to the light and dark roasts, respectively. This way, 9 roasting conditions were obtained for each of the 5 sample classes. Thereafter, samples were sieved (0.15 < particle diameter < 0.25 mm). The brew coffee samples were obtained putting an erlenmeyer with 5 g of coffee powder and 25 mL of distilled water into water bath at 90 °C for 20 min, filtering the extract with coffee filter paper, and cooling down to room temperature.

A Shimadzu IRAffinity-1 FTIR Spectrophotometer (Shimadzu, Japan) with a DLATGS (Deuterated Triglycine Sulphate Doped with L-Alanine) detector was used in the measurements that were all performed in a dry atmosphere at room temperature (20 ± 0.5 °C). A horizontal ATR sampling accessory (ATR-8200HA) equipped with ZnSe cell was employed. Measurements were performed using approximately 2 g of coffee powder and 2 mL of brewed coffee. The empty recipient was used to obtain the background spectrum. Samples were analyzed in triplicate, obtaining a total of 270 spectra, 135 for each of the methodologies evaluated. Regardless of the sample preparation procedure, all spectra were recorded within a range of 3100–800 cm-¹ with a 4 cm-¹ resolution and 20 scans and submitted to background subtraction.

Prior to statistical analysis, the pre-treatments mean centering and Savitzky-Golay derivative transformations were applied to raw data. The software Matlab (The MathWorks, Co., Natick, MA) using the statistical package PLS Toolbox (Eigenvector Research, Inc.) was employed for the chemometric calculations.

RESULTS AND DISCUSSION

Average spectra obtained for non-defective coffee are shown in Figure 1. Several differences between coffee powder and brewed coffee spectra can be observed. The peaks at 2930-2830 cm^{-1} are assigned to asymmetric stretching of C–H bonds. Although they have already been reported for both Arabica and Robusta roasted coffee samples, no identification was attempted. Due to the substantial decrease in the absorbance intensity after aqueous extraction, we believe that lipids contribute the most to these peaks. The decrease in the absorbance intensity of the peak at 1745 cm^{-1}, related to C=O bond stretching from lipids, reinforces this assumption. Brewed coffee spectra also exhibited some noise in the region of 900-800 cm^{-1}. Considering this observations, the spectral ranges selected to PCA analysis were: (a) the whole spectra (3100-800 cm^{-1}) for coffee powder analysis, and (b) the region of 1900-900 cm^{-1} for brewed coffee analysis.

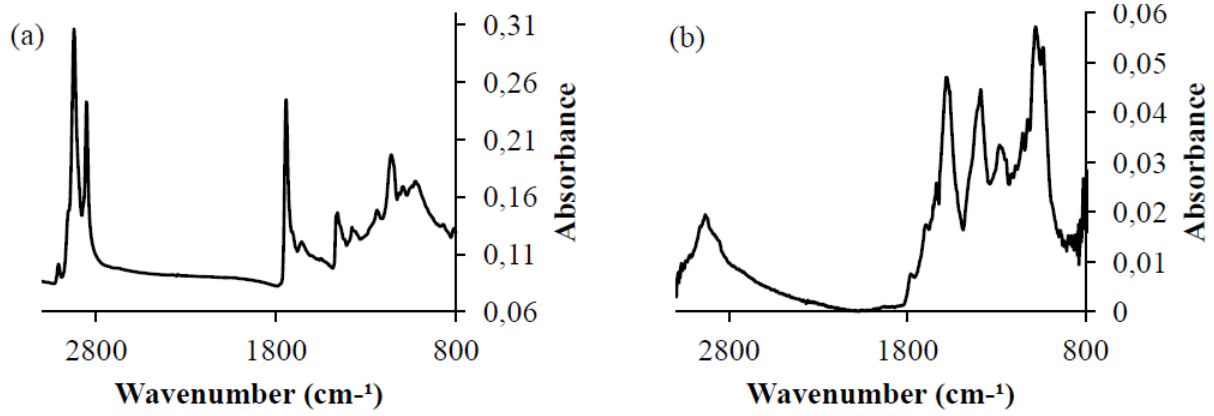

Figure 1. Typical untreated spectra of (a) coffee powder and (b) brewed coffee obtained by ATR-FTIR.

1st and 2nd derivatives were applied to data using Savitzky-Golay algorithm and PCA analysis was performed (Figure 2). In both powder and brewed coffee, PCA provided a visual discrimination of samples in two groups: (a) non-defective and light sour and (b) black, immature and dark sour. This discrimination seems to be clearer for coffee powder analysis. Evaluation of the loadings plot of PC1 and PC2 (not shown) indicated that, in the coffee powder analysis, only two spectral ranges contribute to the discrimination of the samples: 2930-2830 cm^{-1} and 1750-1700 cm^{-1}. As described before, these regions are mainly related to the presence of lipids. In the case of brewed coffee analysis, although all the region between 1700-1000 cm^{-1} exhibited high loadings values, the major contribution on the sample discrimination came from the region of 1450-1250 cm^{-1}, where chlorogenic acids, quinic acid, caffeine and trigonelline can absorb.

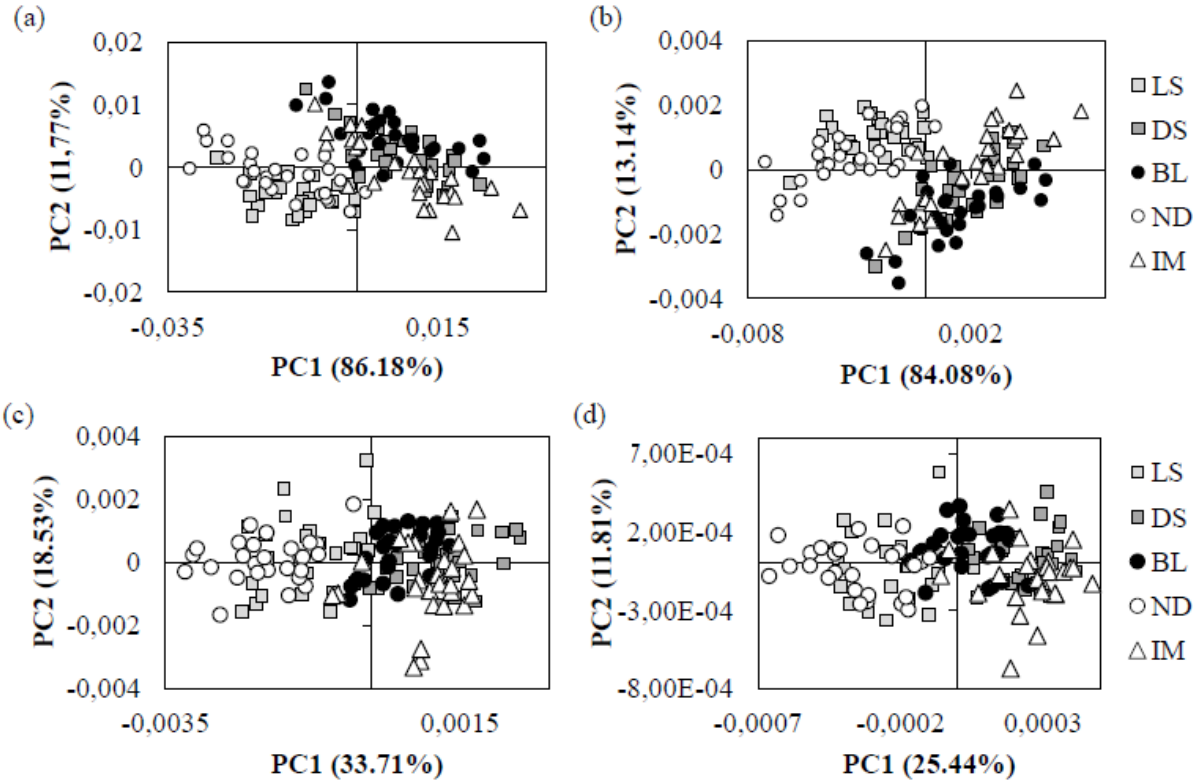

Figure 2. PCA scores scatter plot (PC1 vs. PC2) based on ATR-FTIR spectra of (a,b) coffee powder and (c,d) brewed coffee after the following pretreatment steps: mean centering and Savitzky-Golay (a, c) 1st and (b, d) 2nd derivatives. LS=light sour, DS=dark sour, BL=black, ND=non-defective and IM=immature.

The results of this work indicate that, except for light sour beans, it is possible to dicriminate between defective and non-defective coffees by ATR-FTIR, using either solid or liquid samples. It is important to consider that coffee powder analysis is faster and simpler, being more suitable for routine analysis. Other studies from our group demonstrated that light sour could not be discriminated from non-defective ones, before and after roasting, which indicates the chemistry similarity of them. Also, the separation of sour beans in light and dark is a procedure that has been introduced by our research group. Therefore, there is a need to further evaluate the sensory impact of light sour beans in the coffee beverage.

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The authors acknowledge financial support from the following Brazilian Government Agencies: CAPES, CNPq and FAPEMIG.

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Oxidation of Lipids and Proteins in Green Arabica Coffee During the Storage Period

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SUMMARY

Changes in the coffee beverage quality during the storage of the green coffee had been attributed to the reactive compounds from the lipids oxidation. These reactive compounds on its turn could generate changes in other compounds of coffee beans. In this work we followed the modifications in the natural and pulped natural coffee bean composition which could be related to these processes. The concentration of the free fatty acids, thiobarbituric acid reactive substances (TBARS), protein carbonyl groups and 5-caffeoylquinic acid (5-CQA) during 15 month of storage were measured. Results showed an increase in the free fatty acid concentration and a decrease in the content of 5-CQA during all the storage period. The TBARS content increased until the third month, whereas the content of the protein carbonyl groups increased until the sixth month in the pulped natural coffee, and until the ninth month in the natural coffees. Thus the results suggested the development of oxidation process during storage of green coffee involving lipids, protein and 5-CQA.

INTRODUCTION

During the long storage time of coffee beans, even if under optimal conditions, the flavor of this beverage changes and becomes woody. Studies on the coffee beans behavior during the storage suggested that the oxidation of lipids is responsible for such changes, considering that active lipases, hydroperoxides and unsaturated free fatty acids were found in the coffee beans.

The association of lipids and proteins in oleosins may enable the oxidative process of coffee proteins, as occurred with soy protein as a result of the linoleic acid oxidation. Therefore the oxidative process is not restricted to lipids and may be extended to other compounds of the bean. The objective of the present study was to monitor in coffee beans the changes in the levels of free fatty acids, TBARS, carbonyl groups and 5-CQA during 15 months of storage.

MATERIALS AND METHODS

Ripe fruits of Arabica coffee cultivar IPR 98 (Timor hybrid x Villa Sarchi) were collected in 2009 in Apucarana (Paraná State 23°31’42”S and 51°28’27”W, 734m). One half of the fruits were processed as natural coffee (CN) and the other half as pulped natural coffee (CD). The coffee samples were dried under shade up to around 12% moisture. For the resting period (three first months of storage), non hulled coffee was stored at room temperature (22-26 °C) and relative humidity (RH) 60-80%. After this period, the coffee samples were hulled, placed in small jute bags and stored for 12 months under controlled conditions (22 °C and 64 % RH).

At the beginning of the storage (after the end of the drying period) and at every 3 months until to complete 15 months of storage we quantified the concentration of:: free fatty acids, using C_{11} and C_{13} as standards and expressing the results in mg g^{-1} lipids, tiobarbituric reactive substances (TBARS), expressed in nmol of MDA g^{-1} dry weight (dw), the carbonyl groups in the proteins, extracted in shaker and expressed in nmol of carbonyls mg^{-1} protein and 5-CQA, expressed in g 100 g^{-1} (dw).

RESULTS AND DISCUSSION

Considering the importance of free fatty acids (FFA) in the cellular oxidative process they were quantified during storage. Table 1 shows that the FFA content ranged from 1.89 to 3.67 mg g^{-1} of lipid during the storage of the CN coffee and from 1.37 to 3.84 mg g^{-1} of lipid during the storage of the CD coffee. Other authors also reported increase of FFA during the storage of green coffees and attributed the behavior to the presence of lipase or reactive oxygen species (ROS).

The oxidation of lipids in coffee beans may result from the enzymatic action and from ROS, thereby generating secondary products that can react with the thiobarbituric acid. Theproximity of proteins and lipids inside the cells, may enable the oxidation of proteins as a consequence of the lipid oxidation. The presence of carbonyl groups in the proteins is an indicative that this oxidation could be generated by ROS or by oxidation of lipids.

Table 1. Content of free fatty acids, thiobarbituric acid reactive substances (TBARS), carbonyl groups and 5-caffeoylquinic acid (5-CQA) in natural coffee (CN) and pulped natural coffee (CD) during the storage of non hulled coffee (first 3 months at 22-26 °C and 60-80% RH) and hulled coffee (12 months after at 22 °C and 64% RH).

Compound	Sample	Start	3 months	6 months	9 months	12 months	15 months
Free fatty acids	CN	1.89^{e}	2.42^{d}	2.81^{c}	2.90^{c}	3.38^{b}	3.67^{a}
(mg g^{-1} lipids)	CD	1.37^{e}	1.80^{d}	2.53^{c}	2.76^{c}	3.17^{b}	3.84^{a}
TBARS	CN	8.86^{c}	9.26^{ba}	9.10^{bc}	9,41^{a}	9.46^{a}	9.48^{a}
(nmol MDA g^{-1},dw)	CD	9.24^{d}	9.98^{ba}	9.86^{b}	10.03^{ba}	10.18^{a}	10.22^{a}
Carbonyl groups	CN	2.64^{c}	2.83^{bc}	2.91^{b}	3.15^{a}	3.34^{a}	3.36^{a}
(nmol mg^{-1} protein)	CD	2.80^{c}	3.13^{b}	3.35^{a}	3.42^{a}	3.44^{a}	3.46^{a}
5-CQA	CN	5.17^{a}	5.15^{a}	4.88^{b}	4.79b	4.68^{c}	4.61^{c}
(g 100 g^{-1},dw)	CD	4.94^{a}	4.83b^{a}	4.73^{cb}	4.65^{dc}	4.57^{ed}	4.52^{e}

Mean values followed by the same letter in the lines do not differ significantly by Tukey test at 5% probability.

The concentration of TBARS and carbonyl groups in the CN and CD coffees at the beginning of the storage period (Table 1) were in the interval reported in the literature. The presence of TBARS and carbonyl groups at the beginning of the storage suggested the occurrence of oxidative process during the drying. This event could be consequence of a stress by the loss of water.

The concentration of TBARS and carbonyl groups in CN and CD coffees increased during the first months and stabilized at the end of the storage. This behavior can be explained by the occurrence of respiration of the beans, which can lead to the formation of ROS and consequently to oxidation of lipids and proteins (Table 1). The respiration of the beans stops as the storage elapses and consequently the formation of ROS and the oxidation of lipids and proteins would also stop. The formation of Schiff compounds involving carbonyl groups and

secondary products from the oxidation of lipids could be another explanation for the stabilization observed in the last months of storage.

The major chlorogenic acid in coffee beans is the isomer 5-CQA, and chlorogenic acids are compounds known for their antioxidant capacity. The concentration of 5-CQA measured in CN and CD coffees at the beginning of storage (Table 1) are close to that presented in the literature. Results in Table 1 showed the reduction in concentration of 5-CQA during the storage. Probably it was a consequence of the non-enzymatic and enzymatic oxidation. The results of the present study suggested the development of oxidation process during storage of green coffee involving lipids, protein and 5-CQA.

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High-Resolution ^{1}H-NMR Characterization of Stimulated Whole Human Saliva Before and After *espresso* Coffee Drinking

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SUMMARY

Saliva is a complex biological fluid involved in food oral processing and in food sensing. It is present as a thin film covering the surface area of our mouth. Saliva is responsible for the high resistance to infections of the oral cavity. Its action is manifested both through a physical mechanism because it ensures the self-cleaning of the oral cavity and the wetting of oral mucosa, and through the antibacterial activity ensured by the presence of specific components. Coffee beverage, particularly *espresso* coffee, is known to strongly interact with human oral cavity. This interaction leads to several peculiar characteristics of the beverage including long lasting aftertaste, tongue and teeth staining ability and anti-cariogenic potential thanks to its anti-adhesive action against *Streptococcus mutans*, which is considered as the major causative agent of human dental caries. In order to put in evidence possible molecular interactions between oral cavity and coffee compounds, the combined mechanically stimulated secretions of the major (parotid, submandibular and sublingual) and minor salivary glands known as "stimulated whole saliva" of 5 healthy subjects have been characterized before and after *espresso* coffee consumption. *Espresso* coffee sample was the same for all the subjects. By adopting a strict saliva sampling protocol, the characterization has been performed by high-resolution ^{1}H-NMR. Before *espresso* coffee drinking, the CPMG-NMR spectrum of stimulated whole saliva shows many components present in all samples. Main peaks present in the spectra have been assigned by ^{1}H,^{1}H COSY, ^{1}H,^{1}H –ROESY and ^{1}H,^{13}C-HSQC experiments while the CPMG-NMR experiments allows a better comparison of spectra as it filters out protein components altering the baseline. After coffee drinking, the concentration of some species widely varies from subject to subject. Caffeine persists in saliva more than other coffee compounds, after consumption.

INTRODUCTION

Coffee beverage is known to strongly interact with human oral cavity. This interaction leads to several peculiar characteristics of the beverage. From the sensory point of view, it is well known that the taste and aroma of coffee can be savored for a long time, up to 20 minutes in case of *espresso* coffee, after it has been drunk. On the basis of dynamic tensiometric characterization, it has been suggested that the prolonged taste perception after *espresso* coffee consumption could be related to beverage good wetting properties for the oral cavity. Human saliva was found to contain polyphenols after consumption of coffee, and these compounds have been found to persist a long time after ingestion. Secondly, coffee is well known as staining agent for restorative and veneering materials as well as for denture base resins in Dentistry. In comparison with tea and coke, coffee resulted to be the most chromogenic beverage for a range of denture teeth materials. Finally, several investigations put in evidence the anti-cariogenic potential of coffee thanks to its anti-adhesive action against *Streptococcus mutans*, which is considered as the major causative agent of human

dental caries. It has been shown that trigonelline, caffeine and chlorogenic acid were the coffee components which exhibited the highest anti-adhesive activity. By resorting to different coffee preparation methods it has been shown that in all cases coffee solutions were able to reduce significantly *S. mutans* adherence to dental enamel and dentine.

The combined secretions of the major (parotid, submandibular and sublingual) and minor salivary glands are known as "whole saliva". Stimulated whole saliva is produced primarily by parotid glands and is released upon smell, taste, mechanical or pharmacological stimulus. This type of combined secretions is primarily associated with the alimentary functions of saliva. In the present work, mechanically "stimulated whole saliva" of 5 healthy subjects have been characterized before and after *espresso* coffee consumption by high-resolution ^{1}H-NMR spectroscopy. This technique offers many advantages over alternative time-consuming, labor-intensive analytical methods since it permits the rapid, non-invasive, and simultaneous study of a multitude of components present even in the lack of knowledge on the chemical composition prior analysis. Moreover, NMR spectroscopy has been already exploited to characterize human saliva even from a diagnostic point of view. In order to highlight the changes in the saliva composition, the mathematical difference of CPMG-NMR spectra recorded after and before *espresso* coffee consumption has been studied and compared with the *espresso* coffee spectrum. As far as we know, it is the first time the molecular interactions between oral cavity and coffee beverage have been investigated resorting to ^{1}H-NMR saliva metabolomics approach.

MATERIALS AND METHODS

5 healthy individuals (2 male age: 50 and 38, and 3 female age:28, 32, 33) were asked to rinse with deionized water, chew on a 2 x 1 cm piece of Parafilm "M" (American Can Co., USA), and expectorate all available saliva into a Falcon type tube for a time sufficient to collect a sample volume of 5 mL. All samples were collected between 10 am and 11 am to avoid possible diurnal variation in saliva production and composition.

Immediately after *espresso* coffee drinking individuals were asked to chew on a 2 x 1 cm piece of Parafilm "M" for 2 min swallowing saliva according to own natural frequency and then expectorate all available saliva into a Falcon type tube as previously described between 11 am and 12 am. Home *iperespresso*® coffee machine (mod. Y1, FrancisFrancis, Italy), pure *C. arabica* blend medium roasted coffee capsules and tap water (60 mg/L Ca++, 12 mg/L Mg++ and 190 mg/L bicarbonate) were used to prepare 5 regular beverages (22 mL). The 5 *espresso* coffees were mixed in a beaker and were offered to the individuals for drinking at the same time.

All NMR measurements were performed at 300 K on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer provided with a two-channel Broadband Inverse probe. All samples were diluted in 7.4 pH buffer (TSP 3.2 mM; Na2HPO4 37.5 mM, NaN3 3mM).

Assignment of both saliva and coffee components were accomplished by a series of 2D spectra, namely 2D-correlation spectroscopy (COSY), 2D-rotating frame Overhauser spectroscopy (ROESY) and 1H,13C-heteronuclear single quantum coherence (HSQC).

RESULTS AND DISCUSSION

The CPMG-NMR spectrum of stimulated whole saliva shows many metabolites, mainly representing small organic acids and amino acids, present in all samples. ^{1}H-CPMG experiment was used to simplify the spectrum, filtering out-signals from high molecular

weight molecules which severely alter the baseline in the regular ^{1}H-NMR spectrum. The concentration of some species widely varies from subject to subject and this may reflect several factors like lifestyle, habits and oral microbiota. Figure 1 shows the sum of NMR spectra of saliva samples from 5 individuals (2 males and 3 females).

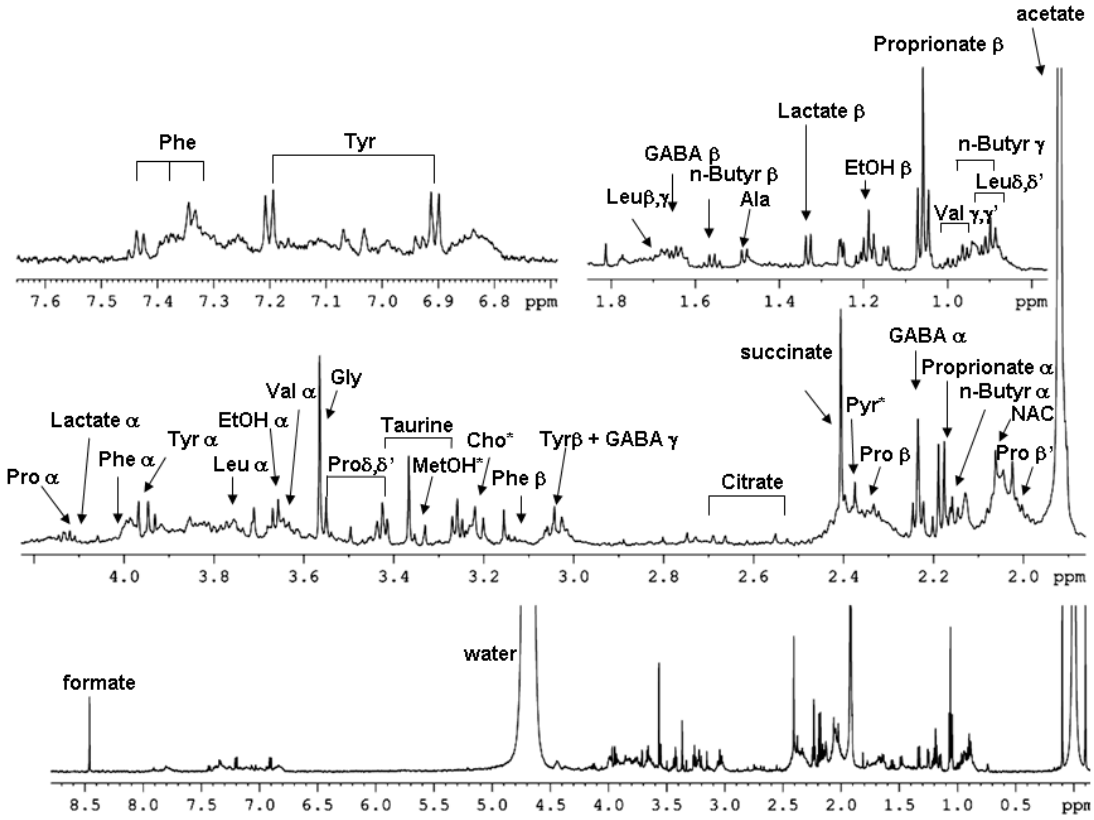

Figure 1. Main species in the NMR spectrum of saliva taken from 5 individuals. Abbreviations. GABA=γ-aminobutyric acid; n-Butyr=n-Butyric acid; Cho=Choline $N^{+}(CH_3)_3$; NAC=acetamido methyl groups of N-acetyl sugars.

The figure shows the assignment of the main peaks. The assignment was based on ^{1}H,^{1}H COSY, ^{1}H,^{1}H –ROESY and ^{1}H,^{13}C-HSQC spectra and facilitated by previous studies. CPMG experiments were repeated on samples collected after 2 minutes from coffee degustation. In order to highlight the changes in NMR spectra, we analysed the mathematical difference of CPMG-NMR spectra recorded after and before coffee consumption (Figure 2). In the figure, the spectrum of coffee is also reported for comparison.

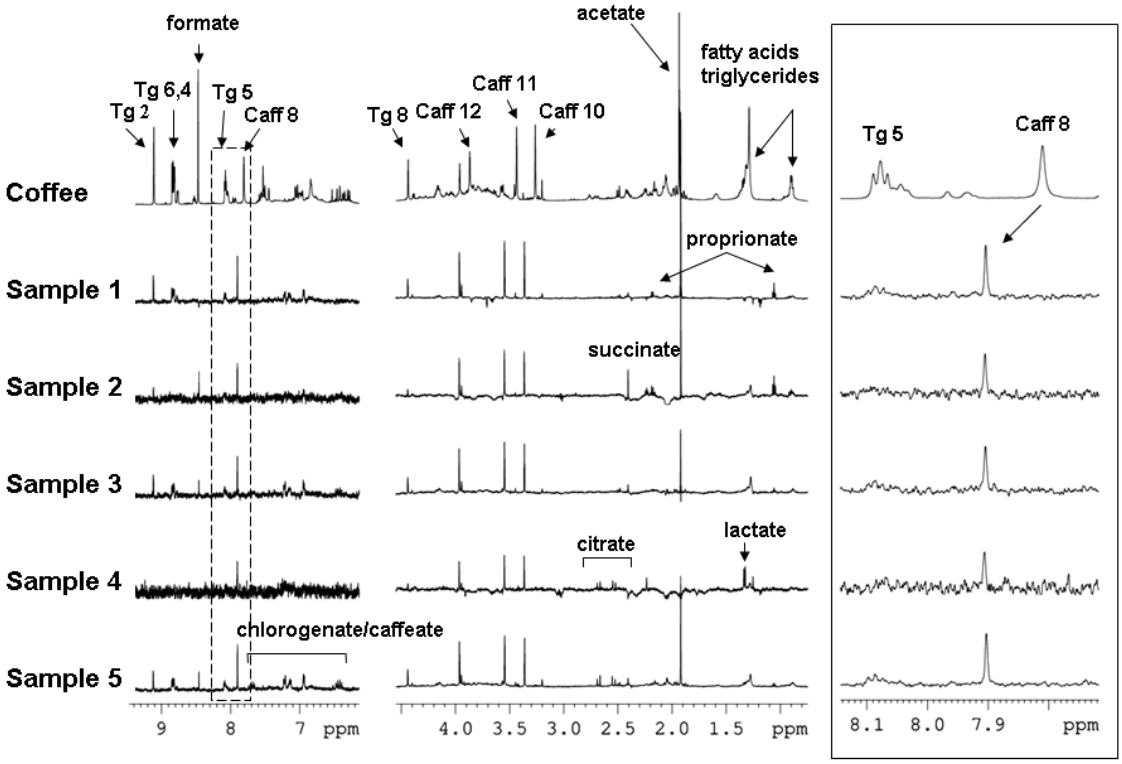

Figure 2. Mathematical difference of CPMG-NMR spectra of saliva recorded after and before coffee consumption. In the figure, the spectrum of coffee is also reported for comparison with the assignment of the main species.

The analysis of the difference spectra clearly shows how caffeine is still present in the difference spectrum, indicating its tendency to remain in the saliva. This is not a simple artifact of dilution as the difference spectra are not just the spectrum of coffee scaled in intensity; furthermore the effect is observed for caffeine more than the other coffee components. This is evident in the framed enlargements of figure 2. While in spectrum of coffee the signal of trigonelline and caffeine has similar intensities, in the difference spectra practically only caffeine is retained. The enlargement also show a shift of the caffeine signal. The NMR chemical shift of caffeine in coffee brews is largely dependent on its molar fraction complexed with chlorogenic acid. However the observed shift is not only due to the disappearance of chlorogenic acid, because the same effect would be obtained by simply diluting the complex (it is estimated that the dilution of caffeine is around 3%). Chlorogenic acids and trigonelline are also retained but to a minor extent. Interestingly the content of some saliva metabolites after espresso coffee consumption shows a remarkable inter-individual variability. Whether this variability is related to the variability observed before coffee drinking or it is induced by coffee drinking has to be ascertain with further studies.

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The Potential of Some *Coffea canephora* Coffees for Industrial Uses

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SUMMARY

Traditionally, coffee grains have been mainly used for coffee beverages and as a caffeine source for medicine and food industries. Nowadays, they have also been considered as raw material for other nutraceuticals and cosmeceuticals ingredients. In this work the potential of industrial use of some *Coffea canephora* grains under selection at the Agronomic Institute of Campinas was evaluated. The content of caffeine, 5-caffeoylquinic acid (5-CQA) and soluble solids in the endosperm, as well as the beverage quality were considered for that. Concentration values ranged from 2.26 up to 4.28% (db) for caffeine, 29.68 up to 35.69% (db) for soluble solids and 4.52 up to 7.53% (db) for 5-CQA. The overall quality of the evaluated beverages was between 75.2 and 83.5 in a 100 points full scale. High acidity and flavors like fruit, flowers, vanilla, nuts, caramel, honey, chocolate and strawberry were perceived in the beverages.

INTRODUCTION

Around 10% of the total robusta coffee (*Coffea canephora*) produced in Brazil nowadays are exported. The 90% remaining in the country are consumed exclusively as roasted and ground coffee and as soluble coffee.

The improvement of the beverage quality and innovative technologies for other uses of the robusta coffee will add values to the product and stimulate its production.

Although the beverage quality of the robusta coffee is less appreciated than that of the arabica coffee beverage, it is necessary to consider the genetic diversity among the coffee plants of the *C. canephora* species and also that it is possible to improve its technological characteristics and sensory quality through the clone selection and plant breeding.

From the point of view of the sensory quality of the beverage, the genetic improvement and selection of the robusta coffee may be guided by the chemical composition of the raw grain, since it is a direct consequence of the content and constitution of the proteins, lipids, carbohydrates and chlorogenic acids, and also from the trigonelline and caffeine contents.

Coffee breeding based on the chemical composition of coffee grains enable also the indication of coffee plants to satisfy interests of industrial exploitation beyond those for consumption as roasted and ground or as soluble coffee. In this scope, are the extraction of caffeine and chlorogenic acids for the production of energetic and antioxidant beverages and the use of oils and components of the lipid fraction of the grain for use in pharmaceutical and cosmeceutical industries.

The aim of this work was to evaluate the genetic variability of the *Coffea canephora* plants in process of improvement for industrial uses, either as material suitable for the production of grain of high quality beverage, either by the potential for industrial exploitation as a source of raw material for pharmaceuticals and food industries.

MATERIALS AND METHODS

Plant Material

80 *C. canephora* coffee plants: 6 Kouillou, 69 Robusta, 2 Laurentii and 3 hybrids were used in the study. Full ripe fruits from 60 coffee plants were prepared as natural coffee and full ripe fruits from the other 20 coffee plants were prepared as pulped coffee. The coffee beans were dried under the Sun until nearly 11% moisture, according measure done after their maintenance by 16 hr at 105 ^{o}C. All coffee samples were ground for chemical analyses.

Chemical analyses

Caffeine and 5-cafeoilquinic acid (5-CQA) in the coffee grains were extracted in 70% methanol solution and simultaneously quantified by high pressure liquid chromatography. 30% methanol solution was used as eluent at a flow rate of 1mL min-1. The compound concentrations were determined based on the area of the same pure compounds. The soluble solids content were determined by the methodology AOAC 973.21 (2002).

Sensory analysis

The pulped coffee beverages were assessed by specialists in the evaluation of quality of robusta coffee. The evaluations were in the DQA system in a 0 to 100 points scale. Only the results of overall quality were considered in this work. For this attribute, notes between 90 and 100, indicate beverage of exceptional quality; between 80 and 90 indicate fine coffee; between 70 and 80 indicate very good coffee; between 60 and 70 indicate good coffee and the coffee which beverage is evaluated with note 60 is considered a marketable coffee.

Statistical analysis

The results of the chemical analyses were submitted to analysis of variance and averages were compared through the test of Scott & Knott. Correlation analysis was done between the overall quality of the beverages and the results of chemical analysis.

RESULTS AND DISCUSSION

Although being *C. canephora* allogamus species the beans from of the analyzed plants presented only moderate variability in relation to soluble solids. Seven coffee groups with statistically significant difference ($p > 0.05$) were established by the Scott & Knott test. A sample (Kouillou) stood out by giving 35.70% (db) of soluble solids. Three samples contained between 35.02 and 34.29% (db); 13 samples between 33.75 and 32.9% (db); 21 samples between 32.80 and 32.05% (db); 24 samples between 32.00 and 31.05% (db); 10 samples from 30.86 to30.52% (db) and 8 samples between 30.18 and 29.7% (db).

According to Scott & Knott test, the 80 results of caffeine content in raw grain were classified into 17 groups that statistically differed ($p > 0.05$) from each other: 1 sample (Robusta) presented concentration of caffeine equal to 4.28% (db); 2 samples with concentrations between 4.18 and 4.16% (db); 1 sample with a concentration equal to 3.79% (db); 5 samples

with concentrations between 3.68 and 3.58% (db); 2 samples with concentrations between 3.51 and 3.52% (db); 4 samples with concentrations between 3.46 and 3.40% (db); 8 samples with concentrations between 3.37 and 3.29% (db); 5 samples with concentrations between 3.24 and 3.20% (db); 5 samples with concentrations between 3.17 and 3.15% (db); 13 samples with concentrations between 3.12 and 3.01% (db); 11 samples with concentrations between 2.97 and 2.91% (db); 4 samples with concentrations between 2.89 and 2.81% (db); 7 samples with concentrations between 2.79 and 2.73% (db); 2 samples with caffeine concentration 2.60% (db); 4 samples with concentrations between 2.53 and 2.45% (db); 4 samples with concentrations between 2.42 and 2.37% (db) and 1 sample with caffeine concentration 2.26% (db).

The concentration of 5-cafeoylquinic acid ranged from 7.53 (Kouillou) and 4.52% (db) (Robusta). The coffee samples were classified in 9 groups with 5-CQA with concentrations of 5-CQA statistically different, according to the Scott & Knott test: 3 samples with concentrations between 7.53 and 7.47% (db); 6 samples with concentrations between 7.25 and 7.16% (db); 2 samples with concentrations between 7.10 and 7.06% (db); 5 samples with concentrations between 6.92 and 6.84% (db); 3 samples with concentrations between 6.79 and 6.72% (db); 9 samples with concentrations between 6.63 and 6.47% (db); 11 samples with concentrations between 6.41 and 6.27% (db); 8 samples with concentrations between 6.24 and 6.13% (db); 12 samples with concentrations between 6.10 and 5.94% (db); 5 samples with concentrations between 5.83 and 5.57% (db); 4 samples with concentrations between 5.57 and 5.43% (db); 4 samples with concentrations between 5.36 and 5.28% (db); 4 samples with concentrations between 5.03 and 4.94% (db) and 1 sample with 4.52% (db).

Beverages of 20 coffee samples were graded between 83.50 (Robusta) and 75.25 (Laurentii). Therefore, the coffee beverage quality was between fine and very good coffee. Nine samples were classified as fine coffees with overall quality varying between 83.00 and 80.00. High acidity and flavor of fruits, vanilla, nuts, caramel, honey, chocolate and strawberry were perceived in the beverages. There was not significant correlation between the overall quality of the beverage and the concentrations of the quantified chemical compounds. The variable that most influenced the overall quality was the 5-CQA concentration. In conclusion, all analyzed coffee plants have potential for industrial uses as a source of coffee grains for high quality robusta beverage. Half of them producing grains for fine robusta beverage. Four of the studied material showed high potential use in soluble coffee industry. The coffee fruits with highest potential as a source of 5-CQA and caffeine are plants of Kouillou and Robusta varieties, respectively.

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Venezuelan Coffee from Biscucuy: the First Sensorial Evaluation of its Aroma Potential

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SUMMARY

In the western part of Venezuela, producing arabica coffee around the town of Biscucuy is traditional. The production area is located in the Andean piedmont, on the municipality of Sucre, in the state of Portuguesa.

A development plan was set up by the municipality of Sucre to improve the agronomic practices and the quality of coffees with the aim of a better valorisation. The purpose of this study is to get a first insight in the aroma potential of these coffees, quite unknown in the coffee world till now. This study shows that coffees from Biscucuy area may have a good sensorial potential. The variability of the cultivars seems to involve differences in the sensorial profiles much more important than the variability due to the altitude or location.

INTRODUCTION

In Venezuela, producing coffee around the town of Biscucuy is traditional (Figure 1). The production area is located in the Andean piedmont, on the municipality of Sucre, in the state of Portuguesa (Figure 2).

Figure 1. Harvest of coffee ("Casa da cultura" in Biscucuy).

Figure 2. Map of the western part of venezuela.

This area is the second coffee producing zone in Venezuela with a production of 9 000 tons per year (about 10% of the national production). Some 15 000 families are dependent on this production. In the frame a the development plan and with the aim of increasing their income, the producers decided to create a cooperative with the target of producing a specific quality coffee so to value their know-how. This coffee growing area is highly contrasted in terms of cultivars (Bourbon, Caturra, Catuaï, Typica…) and altitude (from 700 m to 1450 m).

The aroma potential is determined by sensorial profiles carried out by 10 panellists, trained in the sensorial laboratory of CIRAD, France. The descriptors are those usually used to describe the basic tastes and the specific flavours of coffees. Twenty four coffee samples are taken in the Biscucuy area. They illustrate the diversity of the 24 locations according with various factors such as cultivars, altitude, fertilization and shade.

MATERIALS AND METHODS

The coffee samples are taken from farmers, members of the “Grano de Oro” cooperative. The farmers usually produce 2 types of coffee: the best quality for the cooperative, well sorted and sun dried (in the red frame) and the “conventional” (in the blue frame) (Figures 3 and 4).

Figure 3. Two types of coffee.

Figure 4. Sun dried coffee.

The members producing coffee for the cooperative "Grano de Oro" have to respect a technical procedure. A special care is taken to the harvest. Only ripe cherries at the beginning of the harvest period are picked up. Then coffee pulp is removed, the beans are washed and sun dried. Once dried, parchmin coffee is stored at the cooperative to be roasted and sold.

For the study, 24 samples from 7 locations in a radius of 20 km around Biscucuy are taken in the batches intented for the cooperative. All the samples are hulled, sorted, roasted and cupped.

The samples (100 g of each) are roasted (medium roasting) on Probat® laboratory roaster (PRZ02). Coffee is roasted one day before cupping session. The parameters of the roasting are 9 minutes and 180° Celsius.

Coffees are prepared with Bodum® system (brewing method) and served according to the ISO NF standard 6668-2008.

Evaluation is carried out by 10 panellists. Quotation is done on a zero to ten scale for the usual sensory attributes: aroma intensity, aroma quality, body, acidity, bitterness, astringency, sourness, fruity taste, harsh, greenish, earthy, chemical, aroma persistance, global quality.

RESULTS AND DISCUSSION

Global evaluation

The figure 5 shows the medium profile of sensory evaluation of Biscucuy coffees (mean, minimum and maximum scores).

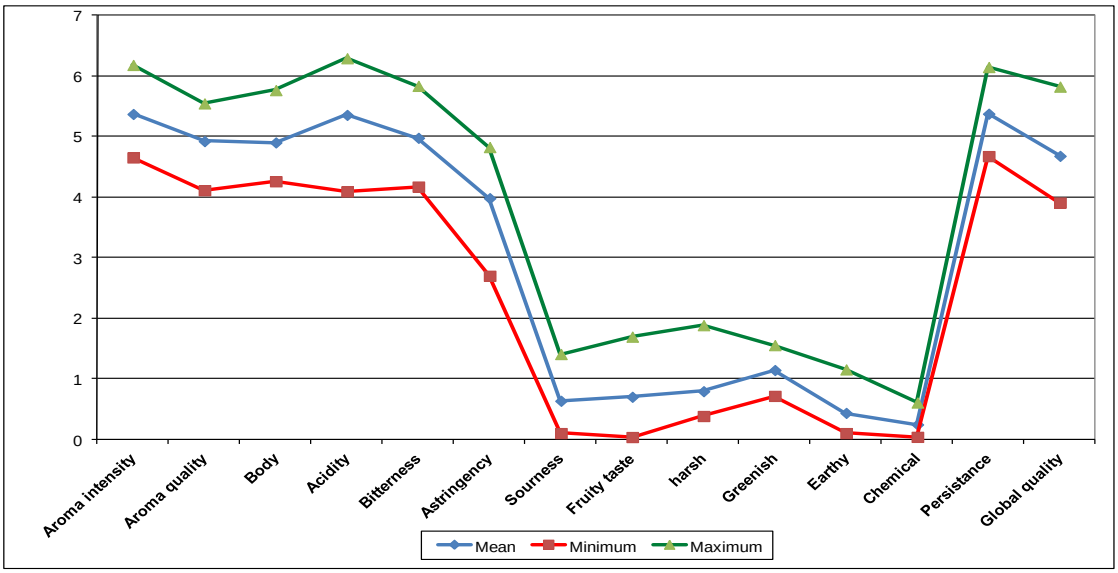

Figure 5. Mean, minimum & maximum scores of samples for each sensory attribute.

The level of aroma intensity is quite high but the aroma is of medium quality. The coffees have a medium body and are slightly more acid than bitter. They are quite astringent and and greenish. The fruity taste is present but light. Most of the coffees are well balanced between acidity and bitterness. The defects (chemical, earthy, greenish) get very low scores.

The Biscucuy coffees can be considered as medium quality coffees.

Groups of coffees

To built groups of coffees, the dynamical clustering analysis is used (XLSTAT® software).

The best division gives 4 groups of coffees (Table 1).

Table 1. Significant differences between groups for the main descriptors.

descripteur	Group 1	Group 2	Group 3	Group 4
Number of samples	10	3	3	8
Aroma intensity	+	+ -	-	-
Body	-	+	-	-
Acidity	+	-	+	+
Bitterness	-	+	-	-
Fruity taste	-	-	+	-
Global quality	-	-	+	-

variance analysis followed by mean tests.

The groups 1 and 4 contain most of the coffees. These coffees differ with aroma intensity (more or less) and a good level of acidity.

In the group 2 (Bourbon and Typica varieties), three coffees have a strong body and a high level in bitterness. On the contrary, the three coffes of group 3 (Caturra and catuaï) are more acid than bitter, with a light body and a clearly perceptible fruity taste.

The coffees grown and processed in the Biscucuy area are not similar, some of them are intersting in term of sensorial quality, either for their body and bitterness or for their fruity taste. The figure 6 shows the sensorial profiles of the coffee groups.

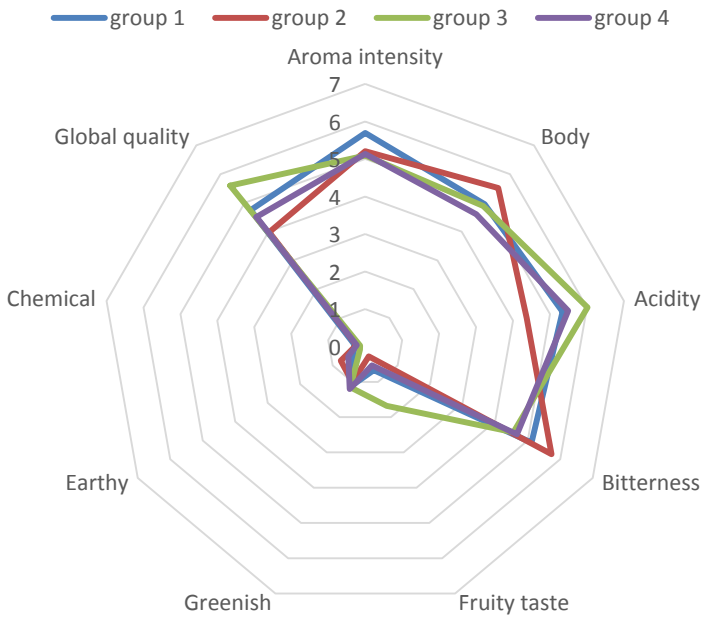

Figure 6. Sensorial spider graphs of the 4 groups.

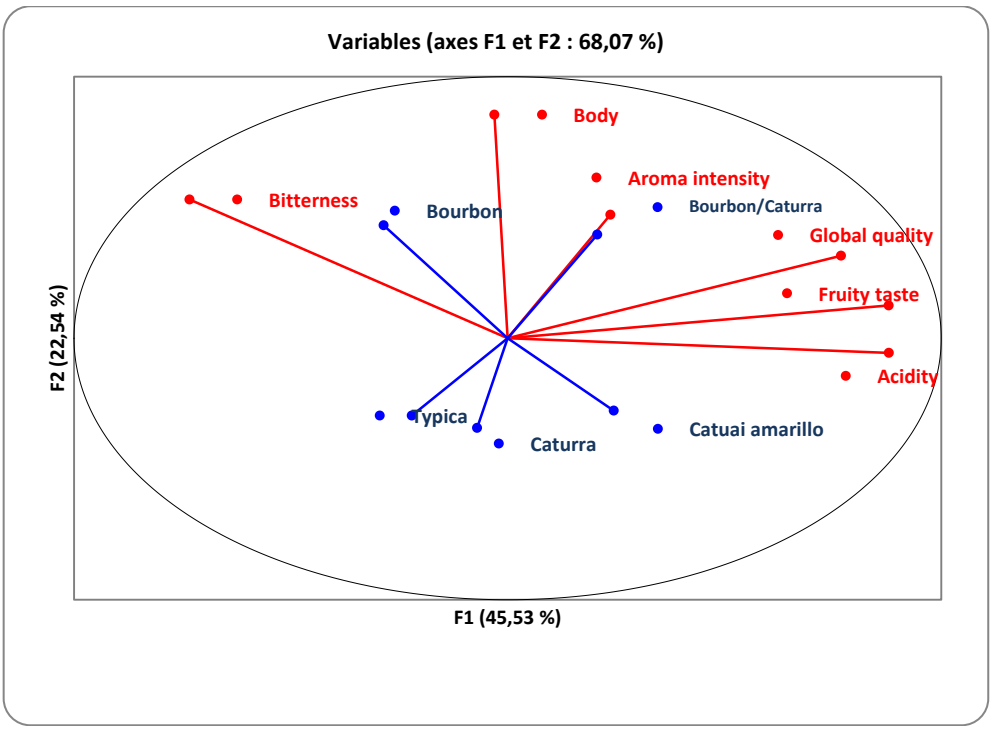

Figure 7. PCA (circle of correlations with descriptors and varieties).

The Principal Components Analysis (PCA) (Figure 7) confirms the results of variance analysis. The Bourbon variety seems to give a coffee with high aroma intensity and a strong body. For the Typica variety, the bitterness is higher than the acidity. Caturra and Catuaï have a higher acidity than Bourbon and Typica.

The results have to be taken cautiously because of the low number of samples. For example, it is impossible to highlight in a significant way the part of altitude on the sensorial quality. The same goes for the locations of the farms where the samples have been taken.

CONCLUSIONS

Coffees from Biscucuy area are globally evaluated as medium. They appear to be balanced with acidity and bitterness. Some of them have a good sensorial potential (either for the body or for the fruity taste). The variability of the cultivars seems to involve differences in the sensorial profiles much more important than altitude or location. Based on these preliminary results, a project has been set up to identify and quantify all the factors impacting the sensorial quality.

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Optimization of the Phenolic Compounds Extraction from Ground and Brew Coffee

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SUMMARY

Several ways to extract phenolic compounds from coffee samples have been reported in the literature, but many differences are observed in the described conditions. Due to that, the aim of this study was the optimization of the extraction of phenolic compounds from roasted coffee ground and brew. A full factorial experiment was designed to evaluate the number of sequential batches of extraction; the extraction solvent used and the standing time before the centrifugation step. The amount of phenolic compounds was estimated by the classical Folin-Ciocalteu spectrophotometric method, and the treatment that presented the highest value of absorbance was considered the best condition of extraction. No difference was observed for the coffee brew considering the extraction factors that were evaluated. It was concluded that the brewing process was able to extract the most of the phenolic compounds and that was not necessary an additional extraction step. However, for the ground coffee, the best condition was: 3 sequential extraction batches with water at 90 ºC, followed by centrifugation without any standing time between the extraction batches. The guarantee that the largest possible amount of the phenolic compounds was extracted is relevant for the food and nutraceutycal industry, which should take advantage of the antioxidant property of phenols.

INTRODUCTION

Several ways to extract phenolic compounds from coffee samples have been reported in the literature (Goldstein e Swain, 1963; Araújo 2007; Vignoli, 2009; Lima et al., 2010; Abrahão et al., 2010). However, many differences are observed in the described conditions, such as the solvents, time, temperature and number of extractions batches. Due to that, the evaluation of the different parameters of the extraction is necessary to obtain an optimized method and to ensure the best condition to achieve a large yield of phenolic compounds.

The aim of this study was the optimization of the extraction of phenolic compounds from roasted coffee ground and brew.

METODOLOGY

A full factorial experiment was designed to evaluate the following factors: 1 or 2 sequential batches of extraction; water, 50% methanol or 80% methanol as extraction solvents at room temperature; and 20, 40 or 60 minutes of standing time before the centrifugation step (Table 1). The identification of the different treatments are demonstrated in Table 2.

Considering the results obtained in the first experiment for the ground coffee, additional experiments were designed to evaluate other factors such as: 2, 3 or 4 sequential batches of

extraction; 0 or 20 minutes of standing time before the centrifugation step; and room or 90°C temperature of water (Table 3).

Table 1. Studied factors for the extraction of phenolic compounds (brew and ground coffee).

Factor	Levels
Extraction batches	1 or 2
Solvent at room temperature	Water, 50% Metanol, 80% Metanol
Standing time (min)	20, 40, 60

Table 2. Treatments identification for brew and ground coffee full factorial experiment.

Treatment	Factors
1	1 Ext, Water, T20
2	2 Ext, Water, T20
3	1 Ext, Water, T40
4	2 Ext, Water, T40
5	1 Ext, Water, T60
6	2 Ext, Water, T60
7	1 Ext, Met 40, T20
8	2 Ext, Met 40, T20
9	1 Ext, Met 40, T40
10	2 Ext, Met 40, T40
11	1 Ext, Met 40, T60
12	2 Ext, Met 40, T60
13	1 Ext, Met 80, T20
14	2 Ext, Met 80, T20
15	1 Ext, Met 80, T40
16	2 Ext, Met 80, T40
17	1 Ext, Met 80, T60
18	2 Ext, Met 80, T60

Table 3. Studied factors in the additional experiments for ground coffee, considering water as the extraction solvent.

Factor	Levels
Extraction batches	2, 3 or 4
Temperature	Room temperature, 90°C
Standing time (min)	0, 20

The amount of phenolic compounds was estimated by the classical Folin-Ciocalteu spectrophotometric method, and the treatment that presented the highest value of absorbance was considered the best condition of extraction.

Statistical analysis were performed by ANOVA, followed by the Tukey test and the Student t test (α=0,05).

RESULTS AND DISCUSSION

No difference was observed for the coffee brew considering the extraction factors that were evaluated in the 18 treatments (p>0,05). It was concluded that the brewing process was able to extract the most of the phenolic compounds and that was not necessary an additional extraction step. However, for the ground coffee, the best condition was: 3 sequential extraction batches with water at 90 ºC, followed by centrifugation without any standing time between the extraction batches, as described in Figures 1, 2 and Table 4.

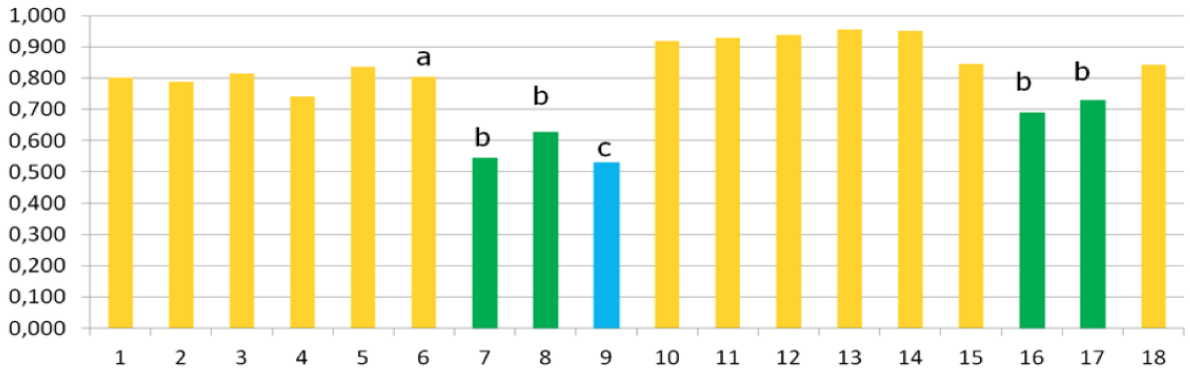

Figure 1. Ground coffee full factorial data considering the identification numbers from table 2. *Difference from another treatments. **Lowest value obtained.

Table 4. Additional experiment from ground coffee: Standing time test at room temperature.

	3 extractions Standing Time (minutes)		4 extractions Standing Time (minutes)	
	0	20	0	20
Absorbance (nm) Mean ± SD	0,423 ± 0,017[a]	0,461 ± 0,033[a]	0,447 ± 0,028[a]	0,461 ± 0,026[a]

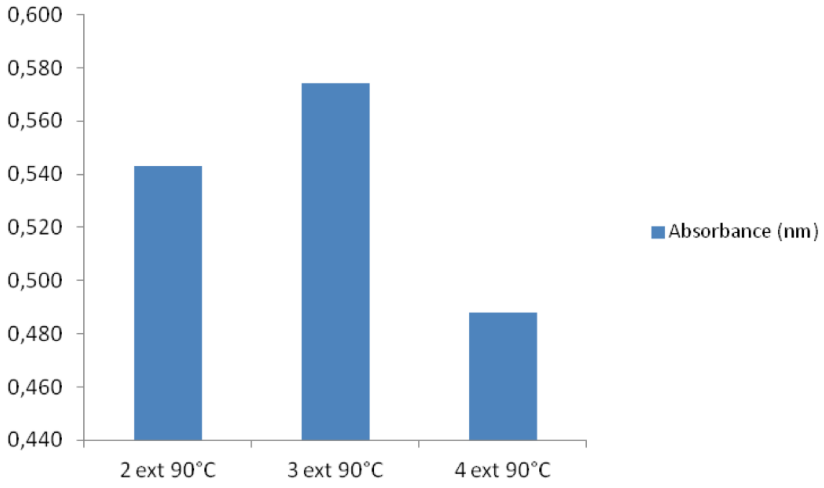

Figure 2. Additional experiment from ground coffee: number of extractions test at 90ºC.

CONCLUSIONS

The guarantee that the largest possible amount of the phenolic compounds was extracted is relevant for the food and nutraceutycal industry, which should take advantage of the antioxidant property of phenols. Although the phenolic extract is recognized as a mixture of phenols soluble in the solvent system, the standardization of the extraction process enables a reliable comparison of the phenolic compounds levels from different coffee samples.

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Changes in Flavor Characteristics of a Chilled-Cup Coffee Beverage During Storage

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SUMMARY

Changes in flavor characteristics during storage of a chilled ready-to-drink (RTD) black coffee beverage were quantitatively investigated using analytical sensory evaluation, gas chromatography/olfactometry (GC/O), and GC/mass spectrometry (GC/MS). Analysis of variance (ANOVA) of sensory scores showed that 12 sensory attributes were significantly ($p < 0.05$) changed during storage. Volatile compounds of retronasal aroma (RA) were examined using a RA simulator (RAS). GC/O analysis of RAS volatiles detected 20 odorants and the changes in 16 identified compounds were analyzed using GC/MS. ANOVA of the peak areas showed that 12 compounds decreased significantly ($p < 0.05$) during storage. In addition, Principal component analysis (PCA) of sensory and GC/MS data demonstrated greater changes in overall flavor and volatile profile between 0- and 1-month-stored samples than between those stored for 1 and 2 months.

INTRODUCTION

RTD chilled coffee beverages dispensed in plastic cups with a straw (chilled-cup coffee) are popular in the Japanese coffee beverage market. Change in flavor during storage of RTD coffee beverages is an important issue, as these products are often stored for a long period of time before consumption. Pérez-Martínez *et al*. investigated the changes occurring in the volatile fraction of coffee brews stored at 4 °C and 25 °C for 30 days. However, because chilled-cup coffee is usually consumed using a straw, it is important to focus on its retronasal aroma, which is caused by flavor compounds traveling from the mouth to the nasal cavity via the nasopharynx and the lungs.

This study was conducted to demonstrate changes in the flavor characteristics of chilled-cup coffee beverages during storage, by means of sensory evaluation and analyses of the odor compounds related to retronasal perception. The results provide essential information for preserving the freshness of chilled-cup black coffee.

MATERIALS AND METHODS

Sample preparation

Espresso coffee was brewed from *arabica* coffee beans using an industrial-scale extractor at a plant (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) and diluted to a solute concentration of 1.2 °Brix. After ultra-high temperature processing sterilization, the diluted espresso coffee was aseptically filled in plastic cups with an attached straw. The cups had both gas-barrier and

shading property, and the headspace in the cups was replaced with N_2 gas after filling with coffee. The samples were then cooled and stored at 10°C.

Sensory evaluation

Sensory descriptive analysis was performed by 14 expert panelists (eight males and six females between 24 and 40 years old) who had worked in the quality control department of a coffee-roasting company. Hedonic rating, flavor intensity, and 17 sensory attributes were rated on a 7-point scale. Each panelist tasted the samples using a straw, comparing the standard sample (shortly after preparation, 0M) with those stored for 1 and 2 months (1M and 2M). All descriptors of the standard sample were given four points as reference.

RAS volatiles sampling

Aroma compounds of chilled-cup black coffee stored for 0, 0.5 (0.5M), 1, and 2 months were collected using a RAS coupled to solid phasemicroextraction (SPME) fiber (Figure 1), composed of50/30 μm DVB/Carboxen/PDMS fiber (Sigma-Aldrich Co.,St. Louis, MO, USA) 2 cm in length. After sampling for 2 min, the SPME fiber was injected into the GC inlet port and the analyte was desorbed at 225 °C or 250 °C.

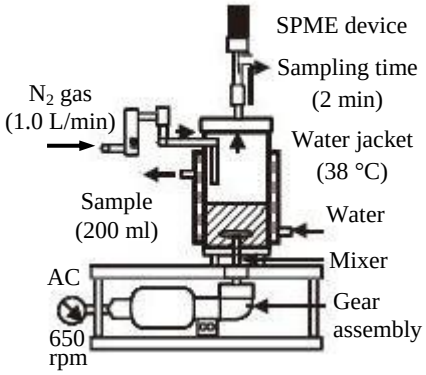

Figure 1. Diagram of RAS.

GC/O analysis

GC/O analysis was conducted in triplicate using CharmAnalysisTM on a 6890 GC (Agilent Technologies, Santa Clara, CA, USA) modified by Datu, Inc. (Geneva, NY, USA). A 15 m × 0.32 mm id DB-WAX column with 0.25 μm film thickness was used. Seven odor descriptors were used to describe potent odorants. The odor activities were represented as charm values.

GC/MS analysis

A 7890A GC (Agilent Technologies) coupled with a 5975C MS detector (Agilent Technologies) was used to quantitate the RAS volatiles. A 60 m × 0.25 mm id DB-WAX column with 0.25 μm film thickness was used. The analysis was carried out in triplicate with selected ion monitoring.

Statistical analysis

ANOVA, Tukey-Kramer honestly significant difference (HSD) test, and PCA were conducted with JMP8 software package (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSON

Sensory evaluation

Twelve sensory attributes were significantly changed during storage ($p < 0.05$; ANOVA) (Table 1). PCA was applied to examine alterations in overall flavor characteristics. Three principal components (PC) with eigenvalues > 1 were obtained. The fresh samples were on the right of the PC1 axis, but they moved to the left with storage time (Figure 2). In addition, the change in the overall flavor between 0- and 1-month-stored samples was greater than between those stored for 1 and 2 months, indicating that the flavor characteristics of chilled-cup black coffee underwent non-linear changes.

Table 1. PC loadings of sensory attributes using PCA of sensory data.

Sensory attribute	PC1	PC2	PC3
Flavor intensity			0,59
Soy-sauce-like	-0,77		
Bitter	0,81		
Roast	0,77		
Fruity	-0,73		
Floral	-0,78		
Green	-0,72		
Full-bodied	0,55		
Light	-0,67		
Mild		0,84	
Balanced		0,71	
Watery	-0,54		
Eigenvalue	5,1	2,1	1,4
Proportion (%)	42,1	17,3	11,4

GC/O

Twenty potent odorants were detected by the GC/O analysis and classified into seven odor descriptions (Table 2). Charm values in each description decreased during storage and total charm value was reduced by about half.

Table 2. Potent odorants found by GC/O analysis in RAS volatiles of chilled cup black coffee and the change in charm values during storage.

Description	Compound	Retention index	Storage period			
			0M	0.5M	1M	2M
Buttery-oily	2-Methylbutanal	975	18	5	6	5
	3-Methylbutanal	994	131	98	82	86
Sweet-fruity	Methyl 2-methylbutyrate	1033	-	11	15	9
	Ethyl butyrate	1060	-	-	-	13
	Beta-damascenone	1789	105	79	99	63
Nutty-roast	Unknown	1234	9	-	-	-
	Unknown	1243	14	5	-	-
	Unknown	1330	7	-	-	-
	2-Ethyl-3,5-dimethylpyrazine	1443	117	91	87	66
	2,3-Diethyl-5-methylpyrazine	1475	119	62	35	19
	2-Acetylpyridine	1569	17	6	5	5
Smoke-roast	Furfurylthiol	1411	17	5	3	3
	Unknown	1533	8	6	15	-
	Methyl 2-methyl-3-furyl disulfide	1639	36	23	29	21
Green-earthy	2-Methoxy-3-isopropylpyrazine	1417	14	11	-	6
	2-Isobutyl-3-methoxypyrazine	1506	156	130	71	59
Phenolic	Guaiacol	1827	456	506	329	266
	4-Ethylguaiacol	1996	416	424	349	318
	4-Vinylguaiacol	2161	47	26	12	6
Other	Dimethyl trisulfide	1349	37	28	22	16
	Total charm value		**1724**	**1516**	**1158**	**961**

GC/MS

Sixteen identified odorants detected by the GC/O analysis were measured using GC/MS analysis with selected ion monitoring, resulting in the quantitation of 13 odorants (Table 3). ANOVA and the Tukey-Kramer HSD test showed that 12 odorants, except for ethyl butyrate, decreased significantly ($p < 0.05$) during 2-month storage. By means of PCA, two PCs with eigenvalues > 1, explaining 95.2 % of the total variance of the data, were obtained. The scatter plot (Figure 3) was similar to that of the sensory data (Figure 2). In addition, the 0.5-month-stored samples were midway between the 0-month and 1-month samples.

Table 3. PC loadings of volatile compounds using PCA of GC/MS data.

Volatile compound	PC1	PC2
2-Methylbutanal	0,94	
3-Methylbutanal	0,96	
Methyl 2-methylbutyrate	0,96	
Ethyl butyrate		0,92
Beta-damascenone	0,97	
2-Ethyl-3,5-dimethylpyrazine	0,98	
2,3-Diethyl-5-methylpyrazine	0,98	
2-Acetylpyridine	0,95	
2-Isobutyl-3-methoxypyrazine	0,99	
Guaiacol	0,99	
4-Ethylguaiacol	0,99	
4-Vinylguaiacol	0,92	
Dimethyl trisulfide	0,98	
Eigenvalue	11,4	1,0
Proportion (%)	87,5	7,7

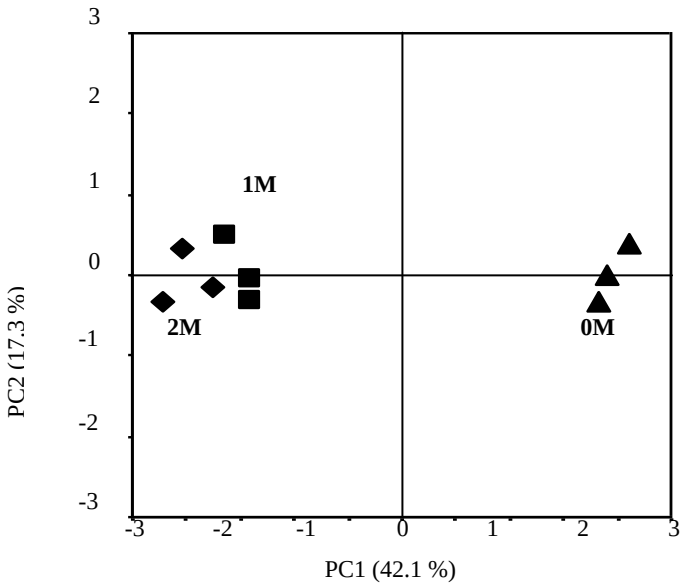

Figure 2. Scatter plot of PC1 and PC2 scores obtained by PCA of sensory data.

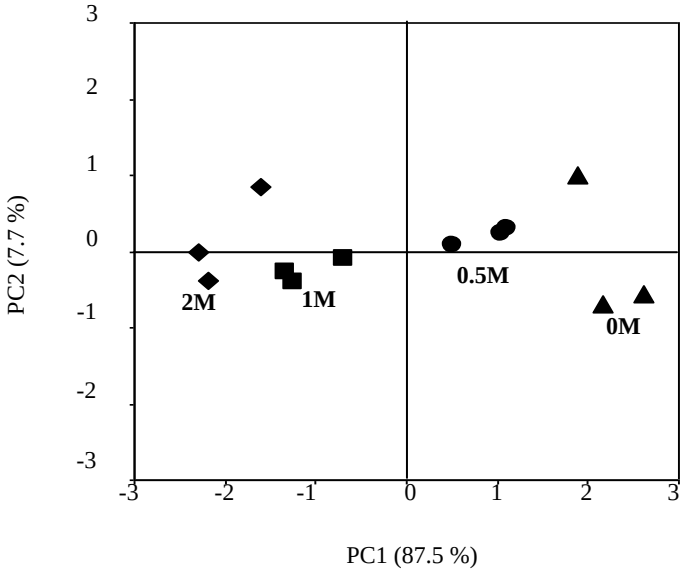

Figure 3. Scatter plot of PC1 and PC2 scores obtained by PCA of GC/MS data.

CONCLUSIONS

During 2-month storage, 12 RAS volatile compounds of a chilled-cup black coffee beverage decreased significantly ($p < 0.05$). PCA of sensory and GC/MS data showed greater changes in the overall flavor and volatile profile between 0- and 1-month-stored samples than between those stored for 1 and 2 months. Change in RAS volatiles during the first month of storage is particularly important to sensory quality of chilled-cup black coffee.

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Quality Evaluation of Coffee Extracts Concentrated Using Various Methods

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SUMMARY

Several methods were investigated for the manufacture of high-quality coffee extract concentrate as an ingredient for coffee beverage products. Coffee extract (CE) brewed using a column extractor was concentrated by progressive freeze-concentration (PF) and reverse osmosis concentration methods. In addition, another coffee concentrate (extract) was brewed using a 1.5-times longer column extractor. The concentrates were evaluated using sensory ranking tests for similarities between the standard sample (CE) and each diluted concentrate sample. Instrumental analyses were also performed using gas chromatography/mass spectrometry (GC/MS), a taste sensing system, TS5000Z, and an electronic nose (E-nose) system, αFOX4000. Furthermore, coffee beverages containing milk and sugar and having the same quantity of coffee solids from each coffee concentrate were subjected to sensory descriptive analysis. Sensory tests, volatile profile obtained from GC/MS data, and principal component analyses of responses from both the taste sensing and the E-nose systems showed that the coffee concentrate created via the PF method was the most similar to the CE. Moreover, the change in quality using this method, before and after concentration, was the smallest among the three concentration methods.

INTRODUCTION

Although multiple-column extraction and evaporation are widely used for the concentration of coffee extract; these methods can cause a cooked flavour and lead to quality degradation. Freeze concentration is known to be superior to the other methods in terms of product quality. However, the practical application of freeze concentration is very limited due to the high start-up costs. In practice, reverse osmosis is most frequently used, although it is intermediate both in quality and cost between evaporation and freeze concentration.

Two methods of freeze concentration are presently used. The conventional method involves suspension crystallization, and has been applied to concentrate products such as coffee extract and fruit juice. In contrast, progressive freeze-concentration (PF) is based on a completely different concept in the crystallization process. In this method, a large single ice crystal, as compared to the many small ice crystals in the suspension crystallization method, is formed and grown on the cooling surface in a crystallization vessel, resulting in ease of separation between the ice crystal and the mother solution. As this system is very simple, processing costs are expected to decrease substantially. To increase productivity, a tubular ice system which can increase the cooling surface area was developed and applied to the concentration of liquid food.

In this study, to produce high-quality coffee concentrate as an ingredient for ready-to-drink (RTD) coffee beverages, we comparatively evaluated the quality of concentrates produced by PF, reverse osmosis concentration (RO), and 1.5-times longer column extraction-concentration (LC) methods using sensory evaluation, GC/MS analysis, as well as taste sensing and E-nose analyses.

MATERIALS AND METHODS

Coffee extraction and concentration

Using a column extractor (Figure 1, Morinaga Milk Industry Co., Ltd.), coffee extract of 5.3 ˚Brix (CE) was brewed from arabica roasted coffee beans (Brazil no. 2-3, L value = 17.8) with hot water (100˚C). The extract was cooled to less than 10˚C, and then concentrated to about 1.6-times concentration (8.3, 8.2 ˚Brix) by PF and RO methods, respectively. Also, coffee extract (concentrate) of 7.8 ˚Brix was brewed from the same coffee beans using a 1.5-times longer column extractor.

A schematic of the experimental apparatus, consisting of the tubular ice system with interior flow circulation, is shown in Figure 1. The apparatus consists of two straight pipes, bent pipes at the top and the bottom, and a pump for circulation. The straight pipes were cooled from the outside by a coolant (ethylene glycol; cooled down at 1˚C/10 min after holding at –3˚C for 10 min). The ice crystal grows on the inside surface of the cooled pipe.

RO was performed using an experimental apparatus, RUW-5CH (Nitto Denko Matex Corp., Tokyo), equipped with RO membrane NTR-759HG S2F (Nitto Denko Matex Corp.; effective membrane area, 1.8 m^2; material, aromatic polyamide). Test conditions were as follows: initial temperature, 13˚C; flow rate, 7 L/min; and pressure, 4 MPa.

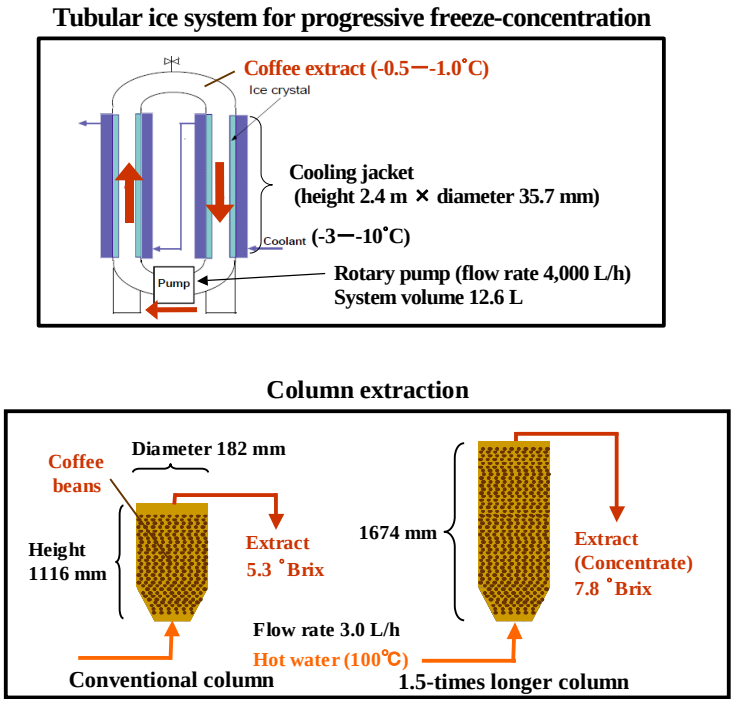

Figure 1. Schematic of the experimental concentration apparatus used in this study.

Sensory evaluation

Sensory ranking tests for similarities between the standard sample (CE, 10˚C) and various concentrate samples (10˚C) diluted to 1.5 ˚Brix, dispensed in white plain plastic cups, were carried out by 11 panelists (10 males and 1 female; age range, 29-45), who had previous experience with beverage development for Morinaga Milk Industry.

Coffee beverages with milk and sugar (coffee green bean, 10.7%; milk fat, 1.6%; non-fat solids, 2.6%), which contained the same quantity of coffee solids from each coffee concentrate, were subjected to a 7-point sensory descriptive analysis for hedonic rating and 13 sensory descriptors by the 11 panelists. Each beverage sample was compared with the standard beverage sample containing CE, which was given four points. Significant differences in sensory characteristics among the beverages were revealed by analysis of variance (ANOVA) of the sensory scores.

GC/MS analysis

Aliquots (10 mL) of the diluted samples were placed in 20-mL vials and held at 35˚C, and the headspace volatile compounds were then captured using a solid-phase microextraction (SPME) fiber (50/30 μm DVB/Carboxen/PDMS; Sigma-Aldrich Co., St. Louis, MO, USA) for 30 min. GC/MS analyses were performed on a 6890 gas chromatograph equipped with a HP5973A mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). The capillary column was HP-INNOWax (30 m × 0.25 mm, 0.50 μm film thickness, Agilent Technologies Inc.), and the flow rate of helium carrier gas was maintained at 1.2 mL/min. The oven temperature was programmed at an initial 40˚C for 2 min, then increased at 4˚C/min to 120˚C, 6˚C/min to 240˚C and next held at 240˚C for 10 min. The injection port was maintained at 250˚C. The inlet was operated in the pulsed splitless mode (30 psi, 3 min). GC/MS analyses were carried out using the scan mode. The compounds were identified by comparison of the mass spectra with data in the Wiley Online Library. The GC peak areas were classified into each functional group and summed to create the volatile profile.

Taste sensing analysis

Diluted concentrate samples were analyzed using the taste sensing system TS-5000Z equipped with five lipid/polymer membranes sensors (AAE, CTO, CAO, COO, and AE1 (Intelligent Sensor Technology Inc., Kanagawa, Japan), at room temperature and in quadruplicate for each sample. Details of the system, the lipid components of the sensors, and the method used to measure the sensitivity and the sensitivity of adsorption (CPA) of the samples are described in a previous paper. Principal component analysis (PCA) was applied to the sensor responses in order to generate a taste profile of the samples.

E-nose analysis

Headspace air of the diluted concentrate samples was injected into the E-nose system αFOX 4000 equipped with 18 metal oxide semiconductor (MOS) sensors (Alpha M.O.S.; Toulouse, France), at room temperature and in triplicate for each sample. Details of the analysis method are described in a previous paper. PCA was applied for the maximum sensor responses to generate an E-nose profile of the samples.

Statistical analysis

Data analyses were conducted with the JMP8 software package (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Sensory evaluation

Similarity ranking obtained from sensory tests showed that the PF coffee concentrate was the most similar to CE (Table 1). In addition, similar results were obtained from sensory descriptive analysis of coffee beverages with milk and sugar (Figure 2).

Table 1. Sensory ranking for similarity between column extract (standard) and various coffee concentrates obtained from sensory tests.

Concentration method	Total score[a]	Persons who evaluated as most similar to column extract
PF	17	7
RO	22	3
LC	27	1

[a]*Score: most similar to the flavour of standard sample (column extract), 1 point; secondary similar, 2 points; not similar, 3 points.*

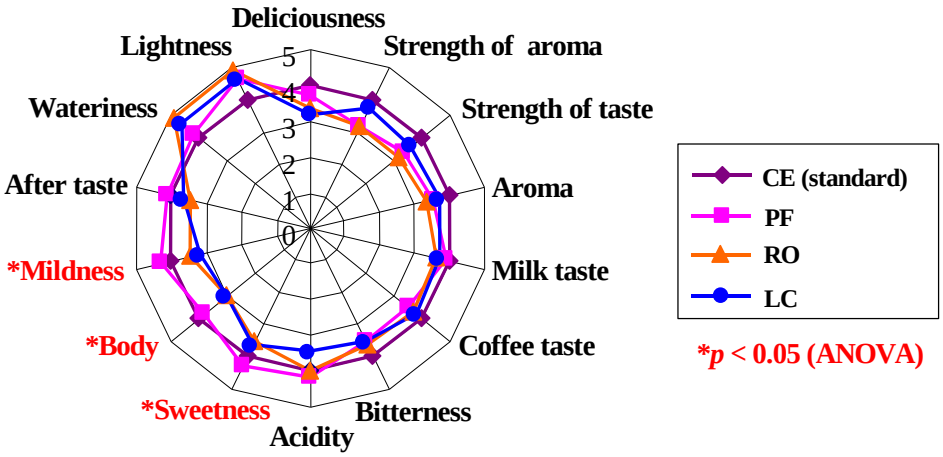

Figure 2. Analytical sensory profile of coffee beverages with milk and sugar using various coffee concentrates. Score: weak, 1 point; strong, 7 points.

GC/MS analysis

Figure 3 shows the total peak area of volatile compounds classified into seven functional groups. The volatile compound profile of the PF coffee concentrate was the most similar to CE. Meanwhile, the total peak area of the 58 volatile compounds of the coffee concentrate sample derived from the LC method was almost the same as CE. Peak area was in the rank order: CE > LC > PF > RO.

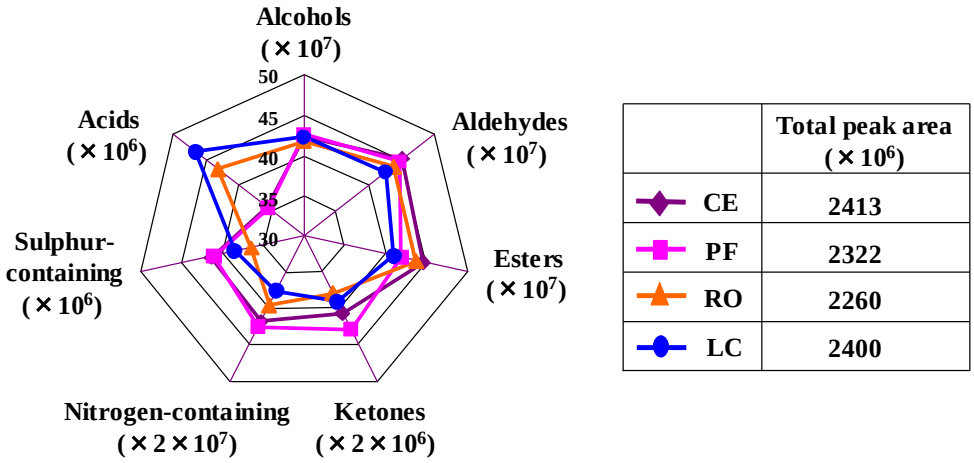

Figure 3. GC/MS profile of various coffee concentrates by peak areas of functional groups.

Taste sensing and E-nose analyses

PCAs of responses from the taste sensing system and the E-nose system showed that the PF coffee concentrate was the most similar to CE (Figures 4 and 5). Tables 2 and 3 list the PC loadings of the taste sensing and E-nose sensors, respectively.

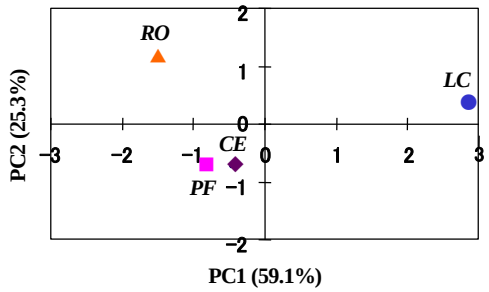

Figure 4. Scatter plot of PC1 and PC2 scores obtained using PCA of taste sensor responses for various coffee concentrates. Average PC score (*n* = 4) for each sample is shown.

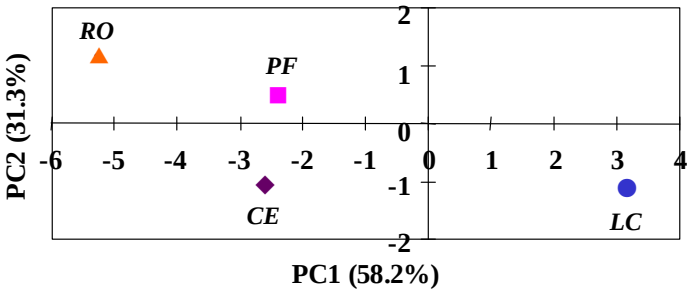

Figure 5. Scatter plot of PC1 and PC2 scores obtained using PCA of E-nose sensor responses for various coffee concentrates. Average PC score (*n* = 3) for each sample is shown.

Table 2. PC loadings of taste sensors using PCA of sensor responses. These sensor responses showed significant differences ($p < 0.05$) among the concentrates using ANOVA. CPA = change of membrane potential caused by adsorption, corresponding to aftertaste.

Sensor (corresponding taste)	PC1	PC2
AAE (Umami)	0.55	
CTO (Saltiness)		-0.74
COO (Bitterness)	0.93	
AE1 (Astringency)	0.89	
CPA (COO)(Bitter aftertaste)		0.63
CPA (AE1) (Astringent aftertaste)	0.89	
Variance	3.5	1.5
Contribution (%)	59.1	25.3
Cumulative contribution (%)	59.1	84.4

Table 3. PC loadings of E-nose sensors using PCA of sensor responses.

Sensor	PC1	PC2
LY2/LG	0.88	
LY2/G	-0.89	
LY2/AA	-0.89	
LY2/GH	-0.89	
LY2/gCTl	-0.86	
T30/1	0.99	
P10/1		0.96
P10/2		0.95
P40/1		0.84
T70/2	0.99	
PA/2	0.96	
P30/1	0.98	
P40/2	0.97	
P30/2	0.98	
T40/2		0.68
T40/1		0.94
TA/2		0.90
Variance	10.5	5.6
Contribution (%)	58.2	31.3
Cumulative contribution (%)	58.2	89.6

CONCLUSIONS

Sensory evaluation, GC/MS analysis, as well as taste sensing and E-nose analyses showed that the change in quality, before and after concentration, using the PF method was smaller than with the RO and LC methods. PF was evaluated to be the best method among the three concentration methods in terms of coffee extract quality.

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Coffee Extraction Process: Yield, pH and Antioxidant Capacity Studies.

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SUMMARY

Once the roasted coffee is obtained, the extraction process is the main operation in the production of soluble coffee because is there where the final sensorial and chemical characteristics of the instant product are defined. This research studied three variables in the extraction process: extract drawoff, feed water temperature and extract flow and their effects in the antioxidant activity, pH and the yield of the process. A replicated full factorial experiment design was performed in an extraction pilot plant. 16 runs in all the experiment were done with Arabica Colombian coffee with 25-26 color (L) in Hunterlab scale. The results indicate that when high yield is the target, high water temperature and high extract drawoff are required. High values of antioxidant activity (Total Polyphenols, FRAP and ABTS) and pH are found when the process is set in low water temperature. The water flow did not affect any of the studied parameters. The feed water temperature is the main variable on the coffee extraction process and modifies all the characteristics of the final product.

INTRODUCTION

In recent year studies have been conducted regarding the benefits of coffee consumption in human health: cardiovascular diseases, Parkinson´s, liver disease, diabetes, among others. The high content of polyphones in a coffee beverage contributes to reduce the quantity of free radicals, avoiding cell damages. These studies indicate that both green and roasted coffee beans are knowledge as an antioxidant compound source. Green coffee beans contain antioxidants such as chlorogenic acids, polyphenols and alkaloids. The amount of these antioxidant compounds suffer a reduction in the roasting process, however the Maillard reaction can generate products such as the melanoidins which exhibit antioxidant activity . Phenolic compounds are also found in instant coffee. Due to the importance of the instant coffee in the Colcafe´s portfolio, this research is carrying on.

The extraction process defines the physicochemical and the sensorial parameters in the final powder product. This research focus the analysis on the extraction process considering three variables feed water temperature, extract drawoff and extract flow and their effect in the yield, pH and antioxidant activity. FRAP, ABTS and total polyphenols methods are use to measure the antioxidant activity.

MATERIALS AND METHODS

Raw material

Arabica Green Coffee; Colombian origin.

Roasting Process

- Industrial scale Roaster (PROBAT).
- Roasting batch: 140 kg.
- Roasting degree: L*= 25 – 27.
- Final moisture levels: 5,5 – 6,5%.

Extraction

The whole research was done in pilot plant, with a soluble solid capacity of 200–600 kg/day. Each run consisted of 20 continues extractions. After plant stabilization several drawn extracts were mixed forming a representative sample for further analysis.

Table 1. Parameters level in extraction process

Parameter	Low values	High values
Feed water temperature (°C)	125-155	170-200
Extract flow (L/h)	150-250	300-400
Ratio Extract drawoff	130-230	300-400

Measurement of Antioxidant activity, Yield and pH

Antioxidant activity (FRAP, ABTS and Total Polyphenlos) was measured spectophotometrally by standards.

Coffee soluble solids concentration was measured by refractometry (Mettler Toledo RE40) and pH was in a pHmeter.

Statistical analysis

The effect of the extractions variables feed water temperature, extract drawoff and extract flow on the response variables extraction yield, pH, and antioxidant activity of the obtained coffee extract was studied with a 2^3 experimental design with repetition.

Obtained data was analyzed by means of the statistical software Minitab 14.

RESULTS AND DISCUSSION

Statistical Analysis Yield

Table 2. Anova results; second order models.

Variable	P-Value			R^2	$R^{2\,(adj)}$
	Water Temp.	Extract drawoff	Extract flow		
Yield	0,000	0,000	0,194	98,85	98,08
pH	0,000	0,004	0,218	93,40	88,99
Total polyphenols	0,000	0,099	0,255	90,36	83,93
ABTS	0,000	0,807	0,426	87,72	72,53
FRAP	0,000	0,087	0,454	78,16	72,70

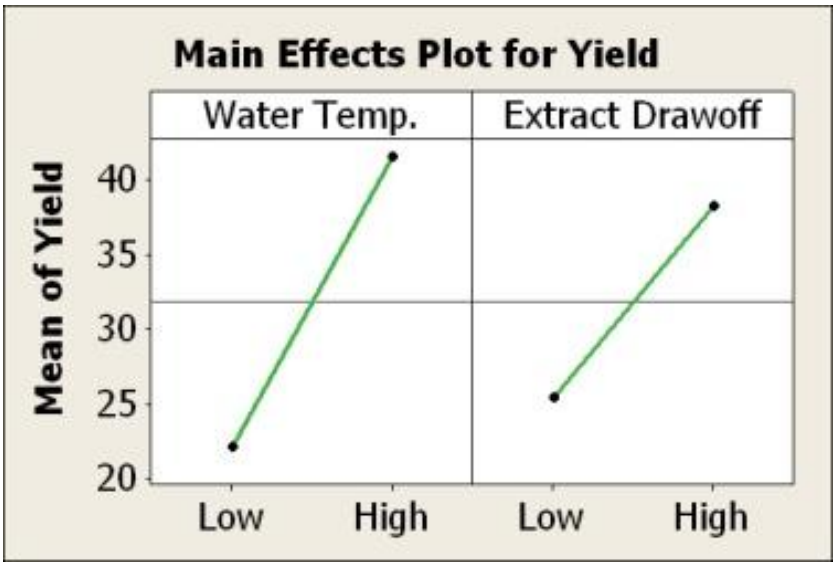
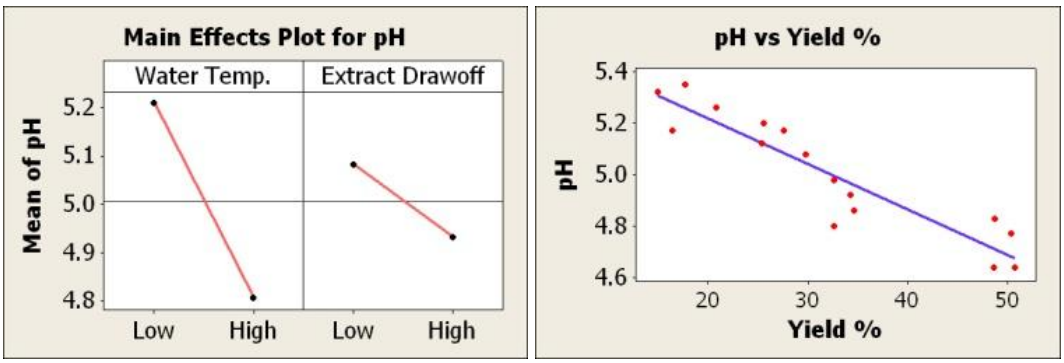


Figure 1. Main effect for yields: Water temperature and extract drawoff.

High temperatures produce higher solubility of the soluble solids , as a result higher values of yield are obtained (Figure 1). At high temperatures there is a reduction of the extract viscosity which improves the diffusivity . With higher values of yield non desired compounds can be obtained, but the cost will be lower. The extract flow does not show a significant effect in the process yield.

pH

According to Sivetz, temperature is the main factor in the extraction hydrolysis. The pH values in instant coffee powder are result of the formed acids in the hydrolysis step . When high yield are fixed (30 to 50%) the acid compounds will be different and the pH will be lower (Figures 2, 3) compared with roasted coffee beverage.

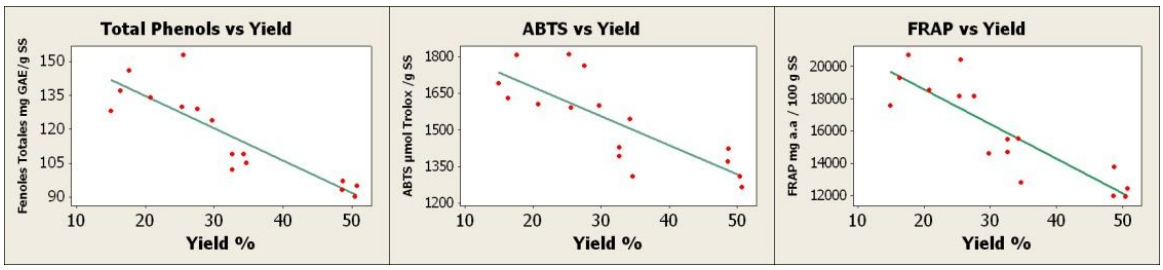


Figures 2, 3. pH main effects: Water temperature and extract drawoff; correlation yield vs. pH.

Antioxidant Activity

Many studies correlated the antioxidant capacity with coffee species, roasted bean grade and preparation methods.

A significant effect for extract antioxidant activity (Total Polyphenols, FRAP y ABTS) was found with feed water temperature. Low temperatures let high antioxidant values in the extract. When yield is correlated with temperature, antioxidant activity can be shown in figures 4, 5 and 6.



Figures 4, 5, 6. Antioxidant activity correlations vs process yield: Total polyphenols, ABTS and FRAP.

Vignoli , evaluated the effects in antioxidant activity in freeze dried coffee considering raw material, extraction process and roasted grade; this research show a similar antioxidant activity behavior when the extraction process condition were modified. Total Polyphenols values obtained in this paper are consistent with Sánchez-González and Brezová researches . Low Total Polyphenols values can be related with soluble solid solubility but this will be study in next researches.

CONCLUSIONS

Eventhough antioxidant capacity of coffee is modified during the roasting process, it is clear that extraction conditions also affects the final antioxidant activity of the obtained soluble coffee and thus of the final beverage.

Extracts obtained at low temperatures and low extract drawoff, lead to coffee extracts with high antioxidant activity values expressed as Total polyphenols, ABTS and FRAP measures.

Yield is the most important parameter in extraction process. Low yields values mean high production costs, higher pH, higher Antioxidant activity and good extract quality flavours.

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Sensory Properties of Under-Roasted Coffee Beverages

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SUMMARY

In the early stages of roasting process (under-roasting conditions) non-enzymatic browning is only partially developed, aroma is almost completely contributed by unaffected precursors and pyrolysis is just at its beginning being dehydration the prevailing phenomenon. The effect of these conditions on sensory properties of coffee beverages has not yet been investigated. The goal of the present study is to fulfil the gap between green and roasted coffee through the definition of a set of sensory attributes describing the sensory profile of beverages prepared by using under-roasted coffee.

Coffea arabica from two different geographical origins (Brazil and Guatemala) was thermally treated (temperature ranging from 140°C to 165°C for a total of 6 different samples) for 20 minutes.

The sensory profile of samples was described by a panel of 14 assessors, with no previous experience in coffee sensory evaluation, by using the descriptive analysis.

The dataset obtained from this work leads us to conclude that when non-enzymatic browning is not fully developed, the sensory attributes used to describe beverages obtained by conventional roasted coffee are not appropriate. Thus, a new set of sensory descriptors with appropriate reference standards should be used to properly describe the specific sensory properties of beverages obtained from coffee treated by under-roasting conditions.

The results show that there is a minimum process temperature below which these products, are not described as coffee. In this case aroma and flavour are dominated by peanuts, vegetables and cereal notes.

INTRODUCTION

It is well known that roasting is a process during which coffee beans are brought for a given time (typically 10-15 min) to a temperature in the range 170-240°C. It has been reported that during the process, the drying step occurring up to 130°C is followed by coffee bean softening due to glass-to-rubber transition of coffee polymers and then by the actual roasting which starts, at 160-170°C. When roasting reactions take place, non-enzymatic browning and pyrolysis with formation of aroma volatile compounds and large quantities of gas, mainly CO_2, change completely both physical and sensory properties of the coffee beans.

The heat treatment induces the evolution of the sensory profile from green coffee to the stage which characterizes and makes recognizable the product in its common meaning.

Roasting conditions therefore, in addition to specie/variety, geographical origin and post-harvest processing, remarkably affect the characteristic aroma and flavour of coffee.

Both green and roasted coffee have been extensively investigated highlighting the deep modifications of chemical and sensory properties induced by conventional roasting process. The influence of roasting degree and roasting process on sensory properties of coffee beverages has been widely studied. Sensory profile of beverages prepared by using green coffee has also been investigated, albeit not extensively.

The adoption of conditions milder than those of the conventional roasting (under-roasting , 140-160°C) allows to limit both thermal degradation of coffee compounds such as chlorogenic acids trigonelline and many others and consequent generation of a plethora of compounds including melanoidins, chlorogenic acid lactones and aroma volatile compounds. For the latter, it has been recommended "gently roasting conditions" (140°C for 20 min) to reduce the formation of the heat-induced toxicant furan (C_4H_4O). However effects of these conditions on sensory properties of coffee beverages, have not been investigated yet.

In this work we studied the relationships between the roasting temperature and the sensory profile of beverages prepared by using coffee roasted in under-roasting conditions.

MATERIALS AND METHODS

Coffea arabica from two different geographical origins (Brazil and Guatemala) was thermally treated (temperature ranging from 140°C to 165°C for a total of 6 different samples for each) for 20 minutes in a 10 kg roasting plant.

Each coffee sample, characterized by a set of physical measurements (moisture, total weight loss and colour), was used to prepare beverages by means of a plunger coffee maker.

A panel consisting of 14 subjects (5 males and 9 females, age range 22-44 years) regular coffee consumers and with no prior experience in coffee sensory evaluation was employed to describe the beverages by mean of descriptive analysis. Subjects were told that the aim of the evaluation was the description of vegetable-based infusions. Thus, any direct reference to the coffee was avoided.

Six training sessions were run. Subjects were trained to recognize and rate the intensity of sweetness, sourness, bitterness, and astringency in standard solutions. A 9-point category scale (1:extremely weak; 9:extremely strong) was used.

After completing the general training step assessors developed a vocabulary describing differences among samples according to the Generic Descriptive Analysis. During the term generation phase, assessors were presented samples in pairs. Three pairs were presented in each session.

The panel reached a consensus on a list of 28 attributes describing aroma, mouthfeel, and flavour sensations of the samples.

Finally assessors participated to seven evaluation sessions: six for under-roasted coffee samples and one for a reference sample represented by a conventionally roasted *C. arabica* blend (medium roast). In each session six samples were evaluated.

Assessors had 2 min break after each sample and a further 15 min break every two samples. Each sample was evaluated 3 times. The samples were presented one by one in 100cc closed bottles coded with 3-digit random numbers. Sample presentations was balanced across subjects. For each samples assessors were asked to rate the intensity of aroma descriptor first. Then they were asked to take a sip of the sample and rate the intensity of flavor attributes. Finally they had to take a second sip and rate mouthfeel attributes.

All ratings were expressed on a 9-point category scale. In the adopted experimental conditions aroma evaluation was performed at 64-66°C and the in-mouth evaluation at 54-56 °C.

The evaluations were run in individual booths under red light, and the data were collected with the software Fizz (ver.2.40.G, Biosystemes).

RESULTS AND DISCUSSION

The relationships between thermal treatment performed at fixed time (20 min) on coffee and sensory properties of resulting beverages were investigated. As far as beverage preparation is concerned, the infusion method has been chosen thanks to its reproducibility and to its extensive use in coffee cup testing.

Product differences for each attribute were assessed by a three-way ANOVA mixed model using assessor and replicate as random factors, while sample was the fixed factor. A significant sample effect ($p \leq 0.05$) was found for 20 out of 28 attributes. Differences among samples from descriptive analysis were studied by means of Principal Component Analysis (PCA). PCA models were computed on panel averages of each significant attribute arising from the ANOVA models. Samples were included as dummy variables (down-weighted in the data matrix) to improve the visual interpretation. The full cross validation was computed to validate the interpretation of the first two components. Results are summarized in the correlation plot of Figure 1.

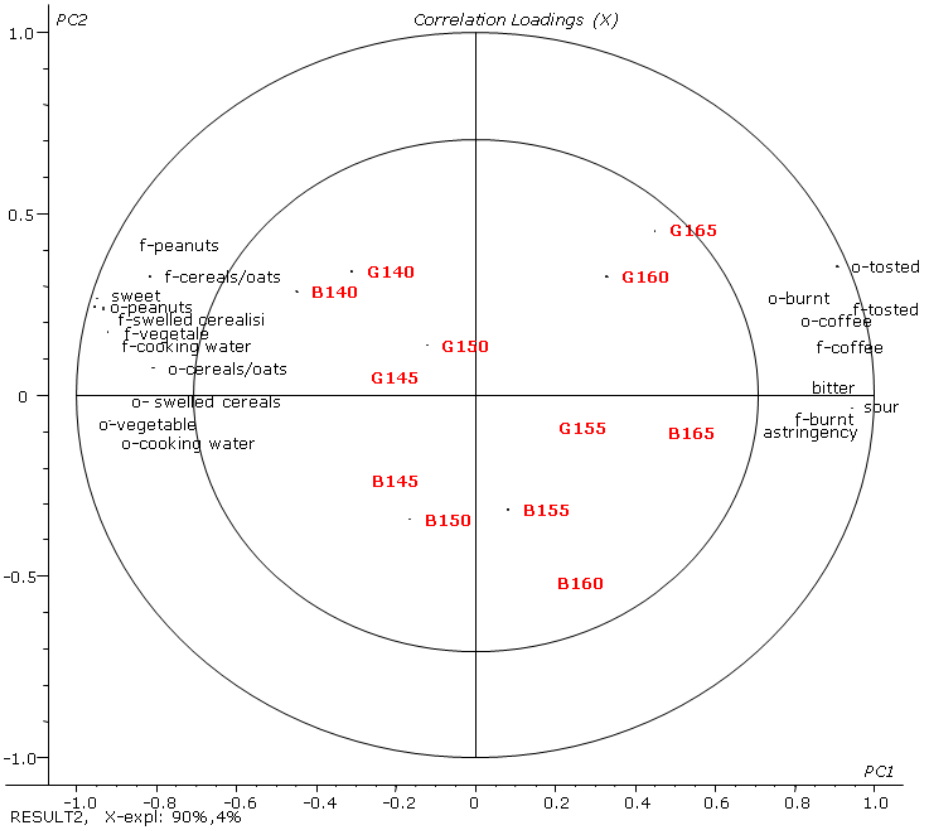

Figure 1. Bi-plot from Principal Component Analysis of mean intensity values of significant attributes for under-roasted coffee samples from Brazil (B) and Guatemala (G). Process temperatures are indicated.

Along the first component samples are discriminated according to their process temperature.

Samples thermally processed at a temperature equal or below 150 °C are positioned on the left side of the map while those thermally processed at a temperature equal or higher than 155°C are located on the right. The first component is negatively associated to a group of 11 attributes (aroma and flavor of peanut, vegetable, cereals/oats, puffed cereals, cereal broth and sweet taste) and positively correlated to a group of 9 attributes (aroma and flavor of coffee, roasted and burnt, bitter, sour and astringent).

High correlation values were found both between attributes negatively associated to process temperature (Cronbach $\alpha = 0.97$) and between those positively associated to process temperature (Cronbach $\alpha = 0.98$). Thus two mega-attributes (sum of the intensities of the attributes within each group) were defined and called “non coffee” and “coffee” respectively. Intensity data of mega-attributes were independently submitted to a three-way ANOVA mixed model. Mean intensity values of mega-attributes "non coffee" and "coffee" of reference and unroasted samples from Brazil and Guatemala are reported in Figure 2 (a and b). Samples processed at a temperature equal or lower than 150°C are characterized by mean values of the mega-attribute “non-coffee” systematically and significantly higher than the reference sample. On the contrary, samples treated at temperatures equal or higher than 155°C do not differ from the reference sample for this attribute. Moreover, Guatemala sample treated at 165 °C and Brazil samples processed at both 160 and 165°C do not significantly differ from the reference samples in relation to the “coffee” mega-attribute.

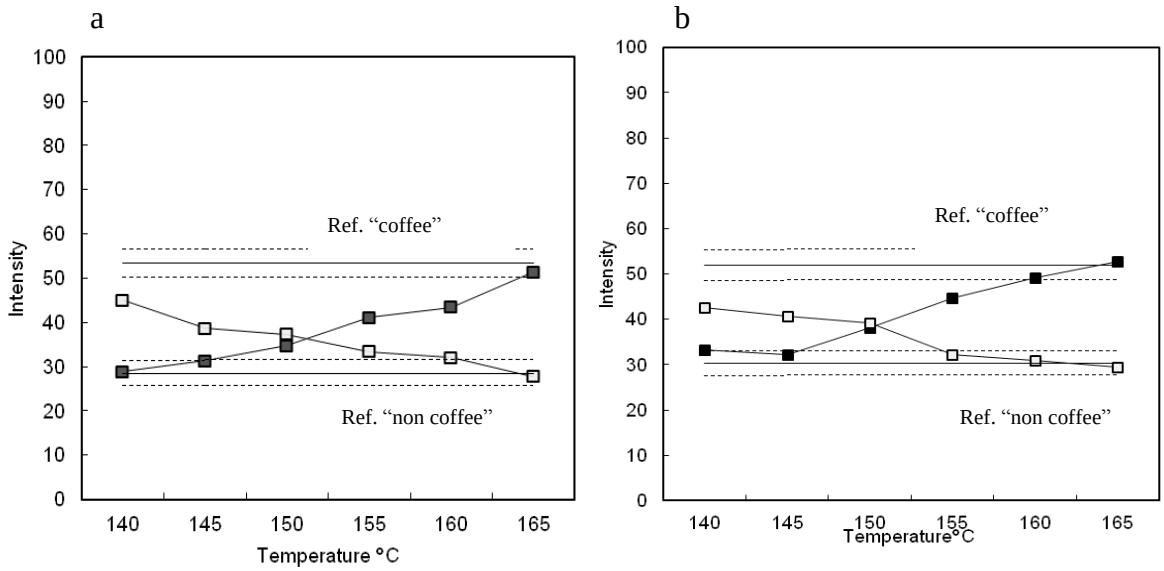

Figure 2. Mean intensity values of mega-attributes "non coffee" (□) and "coffee" (■) in samples from Brazil (left) Guatemala (right) processed at different temperatures. Lines indicate mean intensity values of mega-attributes in the reference sample processed in standard roasting conditions. Dotted lines represent standard error in reference sample.

The conditions of coffee thermal treatment needed to obtain beverages with the sensory characteristics of standard coffee were clearly identified. Attributes commonly used to evaluate the sensory properties of conventional roasted coffee do not describe the main sensory characteristics of products obtained in "under-roasting" conditions. Thus, a new set of sensory descriptors with appropriate reference standards, should be used to describe the specific sensory properties of beverages obtained from coffee treated by under-roasting conditions.

Furthermore, for the first time, we found out that there is a minimum process temperature below which these products, are not described as coffee. In particular, the results indicate that beverages from coffee processed for 20 min at temperatures below 150°C (e.g. under conditions suggested to minimize furan formation), cannot be recognized as coffee flavoured beverages. In this case aroma and flavour are dominated by peanuts, vegetables and cereal notes.

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Potential of Biogenic Amines Evaluation of Immature Coffee Quality

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SUMMARY

Immature coffee processing contributes to a high amount of defective beans which determines a significant amount of low quality coffee sold in the Brazilian internal market. Unripe beans processing was tested, taking the levels of bioactive amines as criteria for evaluating the extent of fermentation and establishing the differences between different processing methods. The beans were processed by the dry method and after being mechanically de-pulped, immediately after harvest or after a 12h resting period in a dry pile or immersed in water. Seven bioactive amines were quantified: putrescine, spermine, spermidine, serotonin, cadaverine, histamine, and tyramine, with global amounts ranging from 71.8 mg/kg to 80.3 mg/kg. The levels of spermine and spermidine were lower in the unripe de-pulped coffee than in the natural coffee. The specific conditions processing also influenced cadaverine levels and histamine was reduced in unripe depulped coffee. A resting period of 12 h does not induce significant alteration on the beans and can be improved if performed in water. These results confirm that peeling immature coffee can decrease fermentation processes while providing uniform drying, reducing defects and potentially beverage quality.

INTRODUCTION

Coffee is a natural beverage, with several compounds that promote human health and performance. However, some compounds present in the grains may interfere with its quality and safety. Given the current trade barriers and restrictions on food marketing, concerns are increasing about coffee quality and safety. Meanwhile, consumers themselves are also becoming more selective. One of the biggest problems in the Brazilian coffee production chain is the high amount of defective beans, which contributes to a significant amount of low quality coffee sold in the internal market. The presence of defects affects coffee quality, and these defects can arise from problems that occur during harvesting, processing, or drying. “Green defects” are associated with the presence of immature fruits, with leased to increased stringency and have been described as an important factor for coffee classification.

The key difference between the classical dry and wet processing methods is pulp removal: in the wet method, the fruit pulp is separated from the seeds before drying by a combination of mechanical de-pulping and fermentation, whereas in the dry method occurs only after a prolonged period of drying the entire cherries. Processing of unripe coffee beans by the wet method is seen as a potential way to minimise the negative impacts of defective “green” beans. The drying step after pulp removal is more uniform and results in fewer black, green, and sour defects, producing improved coffee with higher commercial value. The quality of coffee beverages is related to the chemical composition of the roasted beans, which, in turn, is affected by the chemical composition of the green beans and by post-harvesting processing

conditions. The criteria commonly used to evaluate the quality of coffee include bean size, color, shape, processing method, crop year, flavor or cup quality and presence of defects (5). Among those, cup quality and presence of defects (type classification) are the most important criteria employed worldwide in coffee trading. The variations in processing, and also of the fact that the content of amines is known to be related to quality of food products in general, an investigation of the profiles of amines in coffee samples of different qualities, as well as the effects of processing on those compounds are relevant. Because defective beans are known to have altered levels of bioactive amines and the concentrations of such amines can depend upon the processing method, the present study aims to evaluate the effect of different methods on the levels of bioactive amines in unripe coffee beans processing.

MATERIALS AND METHODS

Coffee samples

Coffee fruits (*Coffea arabica L.)* were grown in the UFLA site. After cleaning and hydraulic separation, the proportion formed by cherry and green fruits was pulped without a counter-weight. This method regulates the pressure of the drum pulper, allowing no more than 10% of cherry fruits to exit with the green fruits. The pulped cherry beans were dried and the rejected mixture with 10% cherries was used in this study. A part of this portion was naturally processed and used as control (A). Another part of the mixture was pulped using a counter-weight to regulate the pressure, resulting in a pulped parcel (B) and a natural parcel (C) that were also dried. A third part of the mixture with 10% cherry fruits was put in two boxes and allowed to rest for 12 hours. One of the boxes was filled with water. After the resting period, both portions were pulped with a counter-weight, giving rise to pulped and natural immature coffee samples after resting in water (D and E) and pulped and natural immature coffee samples after resting in the open air (F and G). The experimental parcels were dried on patios until water content of the coffee beans was about 10-12% (wb).

Sample preparation

After drying, samples were ground to a fine grain for a period of 2 minutes in a refrigerated mill. A second milling step was performed with a grinding ball mill using liquid nitrogen for 1 minute, and a 0.75 mm sieve was used to isolate the smallest grains. Samples were preserved by freezing and then freeze-dried for extended preservation.

Chromatographic analysis

Analyses were performed on an HPLC system with dual pumps, an auto-sampler and a diode array detector. The column used was a reversed-phase Kromasil 100 C_{18} (5 mm x 250 mm x 4.6 mm), and it was operated at room temperature. The elution was performed with a linear gradient of 0.05 M phosphoric acid and methanol / acetonitrile (1:1) at 1 mL/min with gradient elution. Compounds were identified by chromatographic comparison with standards and by co-elution, taking as reference published data on green coffee. Detection was performed using the DAD at 254 nm. All procedures were performed at least in duplicate, and the results were expressed as mg/kg of dried unripe coffee.

Statistical analysis. All data were subject to analysis of variance (ANOVA). The GLM procedure of the SAS was used to evaluate the statistical significance of the differences among mean values. The F-test was also applied, and Tukey test was used to compare means.

RESULTS AND DISCUSSION

In this study, immature cherries (with up to 10% ripe cherries) were processed by the classical dry method (A, B, C and D) and by the wet method, in which the cherries are mechanically de-pulped before being dried (E, F, G). Shell removal in immature beans is a step that induces intense mechanical stress and wear in the pulping equipment. In order to reduce the peel adhesion and facilitate pulp removal the immature cherries were left to rest for 12 h either placed in dry piles or immersed in water. A resting period like is often necessary due to processing constraints, as mature beans are typically given higher priority and are de-pulped sooner. These organic compounds are usually formed by free amino acids decarboxilation as a consequence of physical-chemical and biochemical processes that take place during coffee bean processing. These fermentations can be intentionally carried out by the producer, as in the wet processing method, in order to remove the mucilage remains. Some fermentation can also occur during the drying steps, used on both wet and dry processing methods, although the nature of the process depends on the coffee beans: because of the lack of external layers, the wet processed beans dry faster and more uniformly, resulting in higher quality beans. In the dry method, the beans are dried still within the cherry which results in reduced drying efficiency and consequently undesired fermentation.

Variations can be seen as a consequence of differences in resting period, drying method, drying period, and the maturity of the cherries. In this study, immature cherries were separated before being processed, allowing specific adjustments to be made in the drying step for each batch (i.e., each batch was either pulped or dry-processed). When the different processes are compared (dry vs. wet), all naturally processed batches (had increased amounts of total bioactive amines compared to those that were previously de-pulped, the result was not found to be statistically significant. Quantities of total bioactive amines ranged from 71.8 mg/kg in the control (A) to 80.3 mg/kg in treatment C. Putrescine levels varied from 53.8 mg/kg in the controls (A) to 61.5 mg/kg in the immature cherries with a 12 h dry rest before drying (C). These values are within the range of the only reference values available in the literature for immature beans (average value of 53.9 mg/kg). The polyamines spermidine and spermine are formed sequentially from putrescine, and these also occur naturally in fruits.

Changes in their concentrations can be observed in response to a variety of stresses, including mechanical damage, carbonic gas treatment and temperature fluctuations. In this study, spermine and spermidine levels in immature coffee beans averaged 6.8 mg/kg and 7.3 mg/kg, respectively. This result was only significant ($p<0.05$) for the samples processed immediately (B and E); the increased concentrations in the naturally processed coffees may be due to the longer drying periods required for this type of coffee. The three bioactive amines (histamine, cadaverine and tyramine) were detected in reduced amounts, below 1 ppm. The most toxic of these compounds is histamine; temperature is recognized as a critical factor in histamine formation. Thus, when immature fruits were piled for 12 h, the temperature increase may have favoured histamine formation. Additionally, the drying time of natural coffee is longer compared to depulped coffee, mainly due to presence of the exocarp, allowing more time for the formation of histamine in the bean. The higher amine levels in the procedure without water may be due to other factors that influence this amine, such as pH variation, temperature, oxygen levels, and the concentrations of free amino acids. The average level of tyramine found in immature seeds was around 0.38 mg/kg. Cadaverine showed average levels of 0.49 mg/kg in immature coffee beans. Vasconcelos et al. did not detect the presence of this amine in coffee associated with “green” defects, and the tyramine levels they found were slightly higher than the 0.20 mg/kg found in normal Arabica coffee beans. In the present work, lower cadaverine levels were found in the procedures that lacked a resting step. However, when immature coffee beans remained at rest, increased concentrations were observed when de-

pulped after 12 h of resting, both with and without water. This may be related to various physiological processes in fruit that result from stress due to the accumulation of grains in a box. It was observed in this study that the specific conditions of unripe coffee beans processing influenced the resulting concentrations of biogenic amines, particularly spermine, spermidine, histamine and cadaverine. The depulping of unripe beans reduces fermentation and favours uniform drying, contributing to a reduced formation of biogenic amines. However, if cherries could not be de-pulped immediately and had to be reserved, then the 12 h resting period was not responsible for a substantial increase in the concentrations of bioactive amines amounts. Nevertheless, a benefit could still be observed if the cherries are immersed in water. When the two processing methods are compared – natural vs. wet – the total amount of bioactive amines is smaller in the latter, and the amount of defective beans after processing is also reduced. Treating immature beans separately and using adjusted depulping could provide additional improvements to the quality of immature beans and reduced the levels of biogenic amines, similar to those observed in ripe coffee. The depulping step allows for a shorter and more uniform drying period, reducing the extent of fermentation and reducing the number of defective beans found after processing, and increasing the beverage quality, regarding the amines composition in the unripe coffee beans. These results indicate that the use of more appropriate techniques for processing and drying lead to a reduction in polyamine levels. Whether this reduction is associated with increased cup quality or not is an issue that deserves further attention.

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Acrylamide in Immature Coffee: Formation and Potential Strategies of Control

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SUMMARY

Natural coffee produced in Brazil shows a pattern of highly variable quality. The presence of the green defect is associated with factors arising from the harvesting, processing and drying techniques. Green coffee resulting from this process has a lower commercial value because it contains defective grains. During post-harvest coffee processing, techniques such as piling and immersion in water may be used to store the coffee prior to pulping. These processes result in coffee with a different physical-chemical composition and higher sensory quality than the green grains that result from natural processing. The amino acid asparagine is the main precursor of acrylamide, a potentially harmful substance to human health. Asparagine levels become relevant in coffee composition because asparagine concentration is higher in immature fruits. Peeling of unripe coffee enables a reduction in asparagine levels. Different post-harvest processing and degrees of roasting were compared with the aim of minimizing acrylamide formation in roasted unripe beans. It was concluded that levels are even smaller for dark-roasted beans and pulped unripe fruits present lower levels of acrylamide, showing promising results in the control and formation of this substance.

INTRODUCTION

The presence of a high amount of immature fruit in Brazilian coffee is one of the reasons for the low quality of a final product. There is no doubt that factors such as grade and homogeneity of the material affect the attributes of this commodity. Although the majority of fruits are ripe at harvest, there will always be a significant proportion of immature and overripe fruit because most of the coffee in Brazil is harvested by strip-picking, in which all kinds of fruits are processed together. In the dry or natural process, whole ripe and unripe cherries are dried on patios after harvest. The so-called washed or wet process requires a raw material composed of only ripe cherries. After passing through the washer-separators and before pulp removal, the separation of the green immature fruits from the ripe fruits can be performed using differences in pressure in the separator. In this method, the cherries are pulped and the mucilage is removed from the parchment mechanically or by the use of fermentation. The pulpers used in the preparation of pulped coffees are equipped with pressure separators to divide immature grains from mature ones. Immature fruits must be dried separately; coffee parchment with unremoved mucilage must be immediately dried. However, after this operation, the batch consisting predominantly of green fruits has a low potential of producing high-quality coffee. Pulping of the green fruit has emerged as a way to improve coffee quality and add value to immature coffee beans. Grains resulting from this process have a higher quality than those obtained by drying the entire green fruits. In addition

to shortening the fermentation process and encouraging a more uniform and faster drying, reducing the defects of the immature coffee.

It is well known that the mode of coffee processing (i.e., the wet or dry method) determines the quality of the corresponding green coffees and establishes characteristic flavor differences. However, differences in the constitution of coffee beans may be related to the presence or absence of skin tissues, as reported by. Furthermore, specific and low molecular flavor precursors, i.e., carbohydrates and free amino acids, are different in differently processed green coffees. Among the amino acids present in raw coffee grain, asparagine is the major precursor of acrylamide, a potentially carcinogenic substance. The presence of acrylamide in foods, including coffee, may present risks to human health. The toxicological potential of acrylamide in foods is related to the concentrations of its precursors in the raw material, which may vary significantly between species, cultivation practices and processing techniques.

Qualitative analysis has shown that asparagine is the main amino acid present in immature coffee grains. More recent work has shown that the pulping of immature fruits allows a reduction in asparagine levels. Both the low yield of the asparagine/acrylamide reaction and significant reduction of acrylamide during roasting may be the reason that the correlation between asparagine and acrylamide in coffee is less prominent than in other foods. Although coffee beans are roasted at high temperatures, the amount of acrylamide found in roasted beans and ground coffee is reported to be low. Reports on commercial market samples of roasted coffee confirm lower levels of acrylamide for dark-roasted in comparison to lighter-roasted coffees. However, some post-harvest practices were reported to interfere with the free amino acid content of green coffee beans, which might offer the opportunity for further investigations. The aim of this study is to assess the effect of post-harvest coffee processing on acrylamide formation in immature coffee, relating it to asparagine concentrations in the raw material. The post-harvest processing conditions of immature coffee beans are also related to the degree of roasting and acrylamide formation in order to improve the quality and value of the final product.

MATERIALS AND METHODS

Coffee samples

Coffee fruits (*Coffea arabica L.)* were grown in the UFLA site. After cleaning and hydraulic separation, the proportion formed by cherry and green fruits was pulped without a counter-weight. This method regulates the pressure of the drum pulper, allowing no more than 10% of cherry fruits to exit with the green fruits. The pulped cherry beans were dried and the rejected mixture with 10% cherries was used in this study. A part of this portion was naturally processed and used as control (A). Another part of the mixture was pulped using a counter-weight to regulate the pressure, resulting in a pulped parcel (B) and a natural parcel (C) that were also dried. A third part of the mixture with 10% cherry fruits was put in two boxes and allowed to rest for 12 hours. One of the boxes was filled with water. After the resting period, both portions were pulped with a counter-weight, giving rise to pulped and natural immature coffee samples after resting in water (D and E) and pulped and natural immature coffee samples after resting in the open air (F and G). The experimental parcels were dried on patios until water content of the coffee beans was about 10-12% (wb).

Roasting process

Coffee samples were roasted in a batch roaster using medium and dark roast. The roasting process started at 150ºC, and the final point was determined by visual and instrumental examination with a colorimeter. The average temperature reached was 220ºC after 9 minutes. The roasted beans were vacuum packed in aluminum packages, sealed and stored until analysis. The roasted beans were ground in an electric grinder at 20 mesh to perform the analysis.

Statistical analysis

All data were subject to analysis of variance (ANOVA). The GLM procedure of the SAS was used to evaluate the statistical significance of the differences among mean values. The F-test was also applied, and Tukey test was used to compare means.

RESULTS AND DISCUSSION

The acrylamide concentrations are presented for the two roasting levels tested, as well as the asparagine levels obtained from each procedure applied to the unripe coffee. Higher amounts of asparagine correspond to increased levels of acrylamide in roasted coffee. The coffee beans presenting higher levels of acrylamide are those submitted to medium roast and obtained from natural or dry process for all procedures: control (A), natural (C), 12 hours immersed in water (E) and 12 hours without water (G). This fact may be related to the higher amounts of asparagine present in natural coffee compared with pulped coffee, as reported by Dias et al.. Unripe natural coffee grains submitted to medium roasting showed higher levels of acrylamide compared with unripe, pulped beans. These levels are directly related to asparagine quantities. The results are in accordance with other studies, in which asparagine was reported as limiting factor in acrylamide formation during the roasting process.

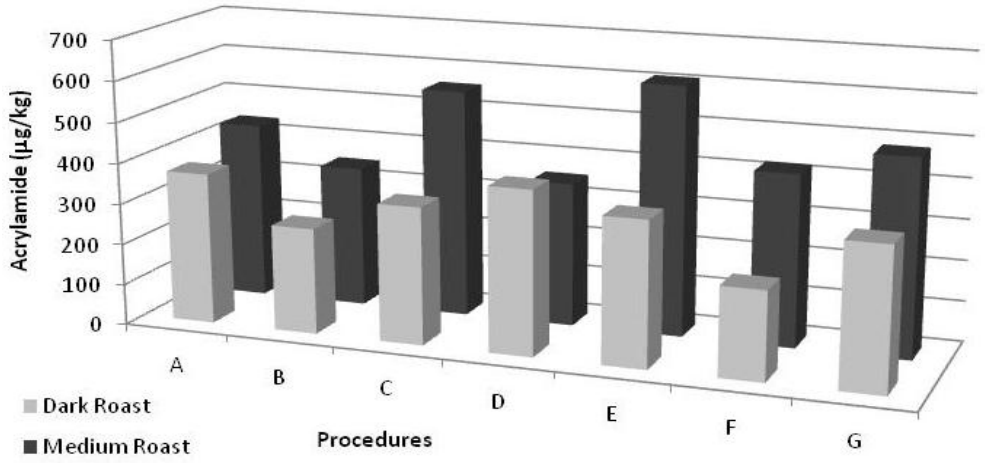

Figure 2. Formation of acrylamide in unripe coffee differently processed and roasted at medium and dark degrees. Storage procedures: A - Control; B - Unripe pulped; C - Unripe natural; D - Unripe pulped 12 hours in water; E - Unripe natural 12 hours in water; F - Unripe pulped 12 hours piled; G - Unripe natural 12 hours piled.

No significant differences in acrylamide levels were detected in the dark roasts of natural and pulped immature coffee. In this experiment, the highest concentrations of acrylamide were obtained at low temperatures (220°C) and a short roasting time (9 minutes). During more intense roasting, the acrylamide degrades until it can no longer be detected. Coffee is typically

roasted at temperatures in the range of 200–230°C. The average amount of acrylamide found in immature coffee beans was 458.2 µg kg^{-1} for medium roasting and 326.8 µg kg^{-1} for dark roasting. Roasted coffee beans can produce levels of acrylamide between 40 and 400 µg kg^{-1} with a mean value of 200 µg kg^{-1}. At the end of the roasting process, according to data from the European Commission, the beans present an average level of acrylamide between 265 and 290 µg kg^{-1}. In dark-roasted immature coffee beans, mean levels found are close to those made public by the European Commission. It can conclusively be said that the food industry, in a joint collaborative effort, could show moderate success in the relative reduction of acrylamide by establishing several collective measures for the treatment of certain foods, e.g., raw material selection, adapting processing parameters and guidance on final food preparation. Alterations in post-harvest coffee will define the potential formation of acrylamide from changes in concentration of its precursor. Acrylamide content is altered by the levels of asparagine and the type of processing, showing a significant difference after coffee roasting. Currently, there are no options available for acrylamide reduction in the process of roasting coffee beans. Controlling the levels of acrylamide's precursors in the raw material seems to be the most effective way to reduce acrylamide during roasting. The pulping of immature coffee contributes to decreased asparagine levels, and consequently acrylamide levels, for both levels of roasting. Whatever the processing type, acrylamide content is lower after dark roasting when immature coffee beans are processed in the same day or when the fruits are stored piled in a box for 12 hours. The procedures discussed in this work can form the scientific basis for the control and generation of new technologies in post-harvest coffee treatments, minimizing the potential formation of harmful chemicals such as acrylamide and improving the quality, safety and economic viability of immature coffee.

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Characterization of Fatty Acids Profile and Oxidative Process in Green Coffee Beans during Storage

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SUMMARY

The objective of this research was to characterize the profile of fatty acids in green coffee beans to evaluate the influence of these profile changes on the beverage quality during the storage period. Coffee samples (natural coffee) and wet (pulped coffee) were submitted to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C until the grains reached moisture content of 11% (wb). When the natural and parchment coffee dried reached has hygroscopic equilibrium were packaged in jute bags and stored at environment without control of temperature and humidity relative. To evaluate the effects of the post-harvest processing and drying methods on coffee bean quality the analyses were done at zero, six and twelve months after the storage. After processing the grains were classified by size in screens with circular and oblong perforations and coffee samples free of damaged or defective beans were submitted to lipids extraction by cold press being the free fatty acids identified and quantified by gas chromatography, and another part of beans were submitted to sensory analysis. It were identified and quantified twelve fatty acids and into the lipid composition the main acid saturated was the palmitic acid and among the unsaturated acids prevails the linoleic acid followed by oleic acid. The variations in the fatty acids profile contributed for significant sensory changes, particularly in the natural coffee processing procedure, which presented unpalatable flavor and undesirable aroma, independent of the drying method.

INTRODUCTION

The storage of coffee beans is considered a critical phase for the beverage quality maintenance. It is very known that during storage the coffee beans suffer a fast deterioration due several biochemical and sensory alterations culminating in loss of quality and undervalued the coffee price. The presence of compounds that determine the flavor and aroma of the coffee beverage of the differentiated quality is necessary to expand the consumer market, both Brazilian and the international sector, ie, its quality depends on genetic, environmental and technological. The procedures of processing, drying, and storage can harm or affect the quality of coffee. Thus, besides the conduct and management of the farming, harvesting, different processing techniques and reducing the water content, by a drying method influence the quality, should be considered for safe storage of coffee, because, among other things, reduces risks breathing, oxidation and fermentation in grains stored.

The properties of the fatty acids present in oil from of coffee grains are important for coffee to drink, and for pharmaceutical and cosmetic formulations, but the fatty acid composition of arabica coffee grains suffers variation significant effect due to defects grains, its biosynthesis

depends temperature, especially the biosynthesis of polyunsaturated fatty acids. Some chemical alterations detected in the green coffee grain are usually associated to oxidative processes, in special the oxidation of lipids that contributed to form unpleasant flavours and undesirable aromas in roasted coffee beans, depreciating the cup quality. However, it is not yet well understood how this process occur and how extent is its effects on sensory quality. Therefore, the objective of this research was to characterize the profile of fatty acids in green coffee beans and to evaluate the influence of these profile changes on the beverage quality during the storage period.

MATERIALS AND METHODS

Arabica coffee samples obtained by dry (natural coffee) and wet (pulped coffee) processing procedures were submitted to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C until the grains reached moisture content of 11% (wb). Natural (whole fruits) and pulped (parchment) coffee samples with moisture content of 11% (wb) were packaged in jute bags and stored at environment without control of temperature and humidity relative. To evaluate the effects of the post-harvest processing and drying methods on coffee bean quality the analyses fatty acids were done at zero and twelve months and sensory were done at zero, six and twelve months after the storage. After processing the grains were classified by size in screens with circular and oblong perforations and coffee samples free of damaged or defective beans were submitted to sensory analysis and the fatty acids characterization by gas chromatography. The lipids extraction was by cold press being the free fatty acids identified and quantified by gas chromatography (Official Methods and Recommended Practices of the American Oil Chemists Society - AOCS method 1-62). The sensory analysis was performed according the Specialty Coffee Association of America (SCAA) procedures.

RESULTS AND DISCUSSION

The storage time in each processing method led to the fatty acid composition to small variations from zero to twelve months, independent of the drying method, being more pronounced the variation in the twelve natural coffees (Table 1). Among the identified fatty acids, the linoleic stands out from the unsaturated and saturated, palmitic acid, followed by moderately oleic and stearic acid and small amounts of other acids (Table 1). The predominance of the four main acids in the oil of coffee beans corroborates other authors.

Unsaturated fatty acids are highly susceptible to degradation reactions, among them, the linoleic acid. During storage their percentage in the oil was reduced, consequently increasing the content of palmitic acid. Although small, to twelve months, the variations (Table 1) indicate that there was degradation for all components of the oil, these changes occurred during the aging of the coffee beans during drying and not these. Important to emphasize, the results suggest that the release of fatty acids is not uniform and that the degradation occurs differently from a fatty acid to another, which agrees with findings.

The degradation of the quality of acids caused changes in the quality of the coffee beverage as noted in the sensory analysis (Table 2) indicating that there was oxidative deterioration of some chemical constituents of stored grain, especially polyunsaturated acids evidenced by lower content of the oil at twelve months (Table 1). The oxidations form undesirable compounds to the taste of the drink, among them taste of stale coffee and rancid odor, indicating loss of product quality. In this study, changes in taste and smell caused significant reductions in notes during the sensory evaluation of coffee (Table 2). The variation in fatty acids indicates latent damage arising from processing and drying processes, and that

associated with variations of the water content of grains stored degraded to a greater degree unsaturated fatty acids, especially the natural coffee (Table 1) shown in this study for greater reduction in sensory quality coffees in these storage function (Table 2).

Table 1. Fatty acids of oil from coffee beans (µg 100 µg-1), the natural and pulped coffee submitted to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C, to zero and twelve months of storage.

PROCESSING	Natural coffee				Pulped coffee			
DRYING	Concrete yard		air at 40°C		Concrete yard		air at 40°C	
Fatty acids	Storage time (months)				Storage time (months)			
	zero	twelve	zero	twelve	zero	twelve	zero	twelve
Miristic (14:0)	0,086	0,095	0,087	0,099	0,086	0,094	0,082	0,092
Palmitic (16:0)	35,433*	35,562*	35,339*	35,474*	35,462	35,521	35,467	35,517
Estearic (C18:0)	7,098	7,163	7,049	7,102	7,358	7,166	7,204	7,138
Oleic (C18:1)	8,109	8,114	7,977	8,167	8,175	8,103	8,375	8,302
Vacenic (18:1 c11)	0,477	0,469	0,528	0,42	0,52	0,431	0,475	0,425
Linoleic (C18:2)	43,863*	43,251*	43,858*	43,264*	43,862	43,802	43,813	43,796
α - linolenic (C18:3 n-3)	1,371	1,332	1,389	1,375	1,345	1,344	1,349	1,348
Araquidic (C20:0)	2,551	2,485	2,479	2,402	2,543	2,412	2,494	2,208
Gadoleic (C20:1);	0,265	0,292	0,265	0,293	0,284	0,273	0,294	0,283
Eicosadienoic (C20:2)	0,041	0,036	0,047	0,046	0,048	0,041	0,042	0,042
Araquidonic (C20:4)	0,697	0,768	0,751	0,693	0,788	0,76	0,762	0,756
Docosadienoic (C22:2)	0,178	0,213	0,208	0,197	0,242	0,233	0,224	0,221

**By Tukey test, p <0.05 on the lines significant differences.*

Table 2. Mean values of sensory analysis (notes), the natural and pulped coffee to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C to zero and twelve months of storage submitted.

TREATMENT	Storage time (months)		
	zero	six	twelve
Natural coffee concrete yard	82,22 a A	80,58 a B	77,57 b C
Natural coffee air at 40°C	82,42 a A	80,83 a B	77,50 b C
Pulped coffee concrete yard	82,38 a A	80,75 a B	79,99 a C
Pulped coffee air at 40°C	82,54 a A	80,84 a B	80,13 a C

Means followed by same letters, the letters in the column between lines within each processing method of drying between columns and capitals for storage times do not differ at 5% probability by Tukey test. p < 0,05. Among the drying methods did not significantly.

Importantly, since the unsaturated fatty acids are related to the formation of aldehydes, the higher linoleic acid content the better the quality of the beverage. It is believed that the oxidation of the compound affected the quality of coffee to twelve months, and these changes in the grain decreased quality of the drink of coffee (Table 2). The variation of the composition of unsaturated fatty acids observed in this study is justified by a factor of storage time since these acids are more likely to react to oxygen and thus undergo oxidation, which agrees with found that reducing unsaturated acids as a function of storage time.

In the results obtained in this study were identified and quantified twelve fatty acids and into the lipid composition the main acid saturated was the palmitic acid and among the unsaturated acids prevails the linoleic acid followed by oleic acid. The variations in the fatty acids profile contributed for significant sensory changes, particularly in the natural coffee processing

procedure, which presented unpalatable flavor and undesirable aroma, independent of the drying method.

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Green Bean Physical Characteristics and Beverage Quality of Promising Low Caffeine Arabica Coffee Genotypes in Brazil

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SUMMARY

Considering the scarcity of information concerning the coffee genotype effects on sensory profile and green bean physical characteristics in Brazil this study aimed to evaluate the size and shape of green beans and beverage quality of several Coffea arabica genotypes under selection at Agronomic Institute, concerning to identify progenies with good physical characteristics, distinctive cup quality and naturally low level of caffeine content in the beans. The experiment was carried out at Agronomic Institute (IAC) in Campinas-SP during the 2010/2011 crop year. It were analyzed fifty five coffee bean samples from different genotypes in F2 and F1BC1 generations between wild coffee genotypes naturally caffeine-free and elite Brazilian commercial cultivars, being each treatment constituted by the respective progeny. Healthy and ripe coffee fruits were hand harvested and prepared by semi washed process (pulped natural coffee) and the wet parchment coffees were sun dried over elevated screens until the grain reached moisture content of 11% (wb). After hulling the clean coffee beans were classified by size to determine the percentage of retention in screens. Coffee samples from up to 15 inch sieve size without defective beans were submitted to sensory analyses according to Specialty Coffee Association of America (SCAA) procedures. The results indicated that there are differences for both green bean size and cup quality among the progenies. This confirms the existence of genetic variability for physical and sensory traits into the studied genotypes, indicating the feasibility of selection of new cultivars in the future. Some progenies showed qualitative characteristics better than the commercial cultivars used as check, revealing the real intrinsic genetic potential of these genotypes to produce naturally low caffeine coffee beans with sensory profile differentiated by flavor and aroma.

INTRODUCTION

Sensory profile, green bean size and green bean shape & make are physical characteristics so important to determine the coffee price at market, where generally large beans receive better prices than small beans. It is reported that intrinsic sensory coffee quality depends on a combination of factors including the green bean chemical composition and green bean physical characteristics. The differentiated quality of coffee, especially the arabica species has accelerated Brazil's insertion in the market for specialty coffees. The coffee grains size and physical characteristics are very important to coffee quality and coffee prices determination in the market, and the presence of compounds that determine the flavor and aroma of the coffee beverage, ie, its quality depends on genetic, environmental and technological. It is also important to emphasize that the procedures for processing and drying, and storage affect the quality of coffee. Thus, besides the conduct and management of the farming, harvesting, different processing techniques and the drying methods, should be considered for maintenance the quality and the safe storage of coffee, because, among other things, reduces risks breathing, oxidation and fermentation in grains stored.

However the relations between these green bean physical characteristics and the sensory profile of coffee as well as its effects on beverage quality have not yet been fully elucidated. In this context arabica coffee cultivar that presents genetic potential to produce coffees beans with differentiated cup quality constitute an important alternative for coffee breeding programs aiming to improve cultivars for specialty coffee production. Considering the scarcity of information concerning the coffee genotype effects on sensory profile and green bean physical characteristics in Brazil this study aimed to evaluate the size and shape of green beans and beverage quality of several Coffea arabica genotypes under selection at Agronomic Institute (IAC), concerning to identify progenies with good physical characteristics, distinctive cup quality and naturally low level of caffeine content in the grains.

MATERIALS AND METHODS

The experiment was carried out at Agronomic Institute (IAC) in Campinas-SP during the 2010/2011 crop year. To characterize the quality of the drink of the genotypes selected by the Genetic Improvement Program of the IAC, are being evaluated numerous progeny and genotypes in the form of treatments, each consisting of a population of plants with different genetic compositions. The genetic heritage contributes to an uneven ripening of the fruits, and the harvest extends over a long period during the year, once to evaluate the quality of the coffee were used only fully ripe fruit (cherries). It were analyzed fifty five coffee bean samples from different genotypes in F2 and F1BC1 generations between wild coffee genotypes naturally caffeine-free and elite Brazilian commercial cultivars, being each treatment constituted by the respective progeny. Healthy and ripe coffee fruits were hand harvested and prepared by semi washed process (pulped natural coffee) and the wet parchment coffees were sun dried over elevated screens until the grain reached moisture content of 11% (wb). After hulling the clean coffee beans were classified by size in several screens with circular perforations of 19, 18, 17, 16, 15, 14 and 13/64 inches and three screens with oblong perforations of 11, 10 and 9 x ¾ inches to determine the percentage of retention. Coffee samples classified in sieve size up to 13 inch without, defective beans were submitted to sensory analyses descriptive, according procedures to Specialty Coffee Association of America (SCAA). The sensory analysis was performed by Judges Certificates by SCAA, based on the protocol for sensory analysis of Specialty Coffee Association of America (SCAA), according to the methodology proposed by. It was evaluated five cups per sample and the correspondent whole bean of each cup was ground after running a cleansing quantity of the sample through a laboratory grinder and then grinding each cup's batch individually into the cupping glasses, ensuring that the whole and consistent quantity of sample gets deposited into each cup, according to the procedure described by. The roasting process of the samples was done with 100 grams of grain, by monitoring the temperature and time roasting that was between eight and twelve minutes . All samples were roasted with a minimum of twelve hours before the tasting. Were evaluated and graded with grading on a scale of zero to ten points the fragrance / aroma, acidity, body, flavor, aftertaste, sweetness, consistency, clean cup, and global impression balance in each genotype. The cumulative score and the final result obtained of the sensory evaluation were described according SCAA.

RESULTS AND DISCUSSION

The beverage quality assessment done by trained coffee tasters and the prescribed sensory evaluation procedures method provides specific and reliable data about each coffee genotype and can be used successfully for screening in breeding selections to coffee quality improvement. The results indicated that there are differences for both green bean size and cup quality among the progenies (Table 1), corroborating with other studies. This result confirms

the existence of genetic variability for physical and sensory traits into the studied genotypes, indicating the feasibility of selection of new cultivars in the future.

Table 1. Average percentage of grains retained in sieves with circular screeners (Flat beans) and sieve with oblong screeners (Peaberry) and the SCAA scores for sensory analysis of *Coffea arabica* genotypes, the crop year 2010/2011 (Agronomic Institute (IAC), Campinas-SP).

Tratamento	Percentage of grains retained in screens				Overall SCAA Sensory
	Flat beans		Peaberry		
	P ≥16	P<16	P≥10 x 3/4	P9 x 3/4	Score
1	43,80	48,63	5,02	2,56	84,06
2	43,29	46,92	6,93	2,86	83,88
3	80,64	10,11	7,68	1,56	83,50
4	70,86	18,41	8,97	1,76	80,63
5	50,46	41,36	5,06	3,12	81,81
6	62,09	32,53	3,24	2,13	82,94
7	64,45	25,34	8,81	1,40	84,50
8	59,84	30,47	7,25	2,44	82,88
9	73,08	12,58	13,74	0,61	84,00
10	72,09	18,91	7,23	1,78	83,79
11	59,87	32,60	6,15	1,38	79,13
12	80,64	10,11	7,68	1,56	81,88
13	74,16	12,86	12,22	0,77	80,88
14	83,89	9,14	6,58	0,39	80,75
15	33,18	58,04	5,49	3,29	79,00
16	34,47	56,76	7,07	1,70	79,25
17	63,68	24,70	9,33	2,28	82,44
18	70,26	22,75	5,41	1,59	81,63
19	74,09	14,01	8,88	1,13	80,75
20	48,49	38,40	11,97	1,14	84,63
21	37,67	53,41	5,90	3,02	84,69
22	87,21	3,46	8,79	0,20	83,42
23	86,16	9,42	4,18	0,23	78,38
24	68,41	23,09	6,60	1,90	79,44
25	81,47	9,69	8,51	0,32	79,63
26	81,66	10,07	7,79	0,48	80,88
27	83,48	6,81	9,15	0,57	80,83
28	84,44	4,88	10,34	0,34	81,06
29	85,14	6,23	8,26	0,36	81,46
30	83,34	8,83	7,63	0,21	81,29
31	82,90	4,02	12,80	0,28	83,88
32	66,15	24,35	8,19	1,31	83,25
33	76,84	15,02	8,03	0,10	80,13
34	85,47	9,34	4,26	0,94	81,75
35	77,91	12,35	9,45	0,28	78,38
36	82,78	8,64	8,05	0,53	79,58
37	63,10	29,51	5,69	1,70	81,06
38	85,35	9,21	4,79	0,66	83,88
39	71,67	18,71	8,46	1,16	82,25
40	58,12	34,95	5,99	0,95	80,25
41	79,89	15,61	4,16	0,34	79,88
42	50,99	41,64	5,41	1,96	80,88
43	76,62	15,49	6,78	1,11	83,56
44	84,43	10,36	4,85	0,36	80,06
45	82,11	6,71	10,80	0,39	82,13
46	83,43	3,30	12,95	0,32	84,58
47	84,19	10,98	4,47	0,35	82,25
48	85,11	8,45	6,02	0,42	82,44

Beverage quality is an important attribute of coffee and contributed for price determination. Therefore, it is very know that cup quality is influenced by large number of factors including genotype, environmental, processing and their interactions. To get reliable information about beverage quality by genotype comparisons, this first phase all genotypes have been cultivated in the same site and have received the same post harvest processing were been studied progenies in field trials to identify genotypes for the production of coffee naturally with low caffeine and to avoid possible interferences in the cup quality and from the results in second phase expand the tests in different Brazilian conditions.

Some progenies showed qualitative characteristics better than the commercial cultivars used as check, revealing the real intrinsic genetic potential of these genotypes to produce naturally low caffeine coffee beans with sensory profile differentiated by flavor and aroma, offering new perspectives for the production of differentiated quality coffees in Brazil.

ACKNOWLEDGEMENTS

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Sensory Quality Alterations and Activity of Leaf Proteins during the Coffee Beans Storage

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SUMMARY

The oxidative process occurs when there is imbalance between the antioxidant defense system and the production of reactive species of oxygen and nitrogen. If these reactive substances are predominant, they react with other proteins, carbohydrates and lipids, causing changes in the mechanisms of cell homeostasis that leading to loss of functionality. This study aimed to characterize the LEA proteins profile in *Coffea arabica* L. beans and to verify its relationship with the loss of beverage quality during the storage period. Natural (whole fruits) and pulped (parchment) coffee samples with moisture content of 11% (wb) were packaged in jute bags and stored at environment without control of temperature and humidity relative. To evaluate the effects of the post-harvest processing and drying methods on coffee bean quality the analyses were done at zero, three, six, nine and twelve months after the storage. After processing the grains were classified by size in screens with circular and oblong perforations and coffee samples free of damaged or defective beans were submitted to sensory analysis and the LEA proteins characterization. The pulped coffee beans showed better cup quality than the natural coffee beans independent of the drying method. The LEA proteins profile was influenced by post-harvest processing procedures and its activity had strong association with the sensory coffee quality.

INTRODUCTION

The intrinsic quality of coffee is an important factor for the price determination at market. The procedures for processing and drying, and storage affect the quality of coffee. The post-harvest procedures can cause damage to membranes promoting metabolic dysfunctions and structural in the grains resulting in chemical changes, affecting the quality of the coffee, particularly, the effects on the integrity of membrane systems because of the drying temperature and due to the storage time by changing the biochemical component in the grains responsible by aroma and characteristic flavor of the drink coffees. The main quality changes of coffee beans during the storage period are associated to fluctuations in temperature and relative humidity of the storage environment that affect the intensity and speed of the grain deterioration. The oxidative process occurs when there is imbalance between the antioxidant defense system and the production of reactive species of oxygen and nitrogen. If these reactive substances are predominant, they react with other proteins, carbohydrates and lipids, causing changes in the mechanisms of cell homeostasis that leading to loss of functionality. The Maintaining of the quality of the coffee depends upon of the drying storage conditions. The *LEA* proteins synthesized and accumulated in the later stages of seed development are

cited as defense mechanisms before or during drying. In coffee seeds, the modifications on *LEA* proteins as a function of drying, are reported with low seed quality physiological. The defense mechanisms of grain are extremely important to keep the quality, especially when the objective is the storage for a long time. And it is assumed that the *LEA* protein also acts as a protective mechanism against stress on seed storage. However, the potential of the protein as an indicator of spoilage during storage needs studies. This study aimed to characterize the LEA proteins profile in *Coffea arabica* L. beans and to verify its relationship with the loss of sensory quality during the storage period.

MATERIALS AND METHODS

Arabica coffee samples obtained by dry (natural coffee) and wet (pulped coffee) processing procedures were submitted to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C until the grains reached moisture content of 11% (wb). When the dried fruit coffees and the parchment coffees reached hygroscopic equilibrium they were packaged and stored at lab conditions without environment control. The beans were hulled and graded by size in several screens with circular and oblong perforations and samples of flat beans retained over screens up to 16/64 inch sieve size and free of damaged or defective beans were submitted to lab analyses. The the water content in the grains, the sensory analysis was performed according the Specialty Coffee Association of America (SCAA) procedures, and the *LEA* proteins extracting, the electrophoresis and the visualization of bands were done according the methods described by, respectively. To evaluate the effects of the post-harvest processing on coffee bean quality the analyses were done during one year at zero, three, six, nine and twelve months after the storage.

RESULTS AND DISCUSSION

Changes were observed in the activity of *LEA* proteins due to the storage time. Regarding the influence of storage time, it was verified the intensification of bands, except at twelve months (Figure 1). The progressive variations in intensity of bands of coffee beans during the storage period are associated to fluctuations in temperature and relative humidity of the storage environment.

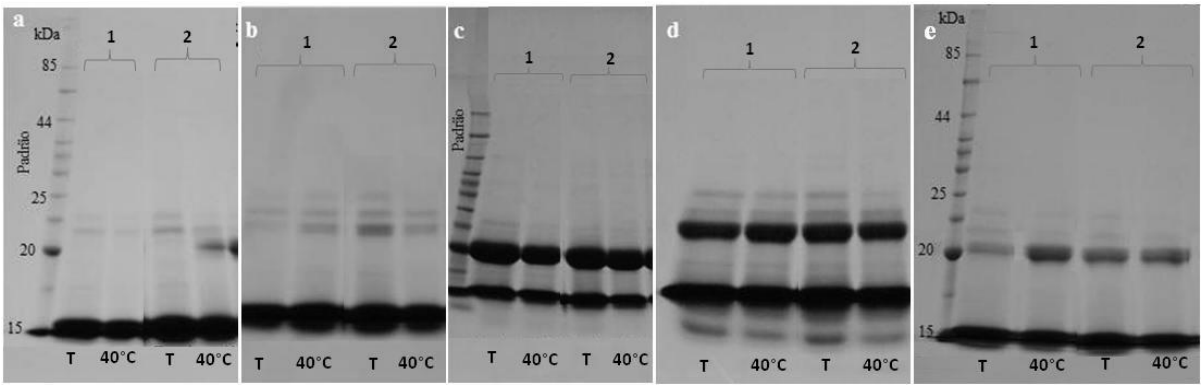

Figure 1. Electrophoresis pattern of LEA proteins in coffee grains throughout storage, of pulped (1) and natural coffee (2), dried in terrace and under heated air at 40°C.

Regarding the influence of storage time, it was verified the intensification of bands, except at twelve months (Figure 1). At six months there was variation on the intensity of bands, however, without significant differences among coffees in the same drying conditions. At nine months the higher activity was noted in the visible bands. On the other hand, the activity

of visible bands decreases at twelve months, besides have been differentiated between natural pulped coffees. The activity of *LEA* proteins was similar to the observed in cucumber seeds by the *Revista. Brasileira de sementes.*

As the storage time advanced, the degradation of grains occurred in function of oxidative processes and production o free radicals, represented by the reduction of *LEA* activity in the end of storage. Increasing the activity and number of bands up to nine months in storage, can be associated to defense mechanisms activated during the drying process. However, the decreased activity of *LEA* proteins to twelve months may have occurred because of stress storage since some enzymes linked to the membrane system reduced the efficiency of protection in phospholipids. The modifications on *LEA* proteins evidence the progressive deterioration of stored grains, altering the sensory quality the café (Table 1). It was concluded that: the pulped coffee beans showed better cup quality than the natural coffee independent of the drying method; the *LEA* proteins profile was influenced by post-harvest processing procedures; the intensity and variation of the LEA proteins had strong association with the sensory coffee quality; the cup quality of pulped coffee was less affected by the interaction effects of processing, drying and storage period than the natural coffee.

Table 1. Mean values of sensory analysis (notes), the natural and pulped coffee submitted to drying in concrete yard and in a mechanical dryer operating with hot air drying at 40°C to zero, six and twelve months of storage.

	Storage time (months)		
TREATMENT	**zero**	**six**	**twelve**
Natural coffee concrete yard	82,22 a A	80,58 a B	77,57 b C
Natural coffee air at 40°C	82,42 a A	80,83 a B	77,50 b C
Pulped coffee concrete yard	82,38 a A	80,75 a B	79,99 a C
Pulped coffee air at 40°C	82,54 a A	80,84 a B	80,13 a C

Means followed by same letters, the letters in the column between lines within each processing method of drying between columns and capitals for storage times do not differ at 5% probability by Tukey test. p < 0,05. Among the drying methods did not significantly.

From the hypothesis that *LEA* proteins has acted against heat stress in drying, the metabolic processes due to the variation of water content in the grains as a function of temperature and relative humidity, can justify the actions of the *LEA* during storage. Corroborating with who resulted that in different seeds, reported the alteration in the seed o with low quality physiological.

ACKNOWLEDGEMENTS

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Principal Component Analysis of Physical and Chemical Characteristics of Coffee Submitted to Different Post-Harvest Processes

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SUMMARY

The present work aimed to develop a methodology in order to aid the cup test by means of principal component analysis (PCA) of physical and chemical characteristics of coffee submitted to different postharvest procedures. Lots of coffee with parchment and cherry coffee were dried under temperatures of 40, 50 and 60 °C. To the samples dried at 40 °C were added different levels of green coffee (0, 10, 20 and 30 %). After twelve months of storage, samples were subjected to sensorial analysis and analyses of color, electrical conductivity, bulk density, one thousand mass and gas chromatography. By means of comparison between PCA of physical and chemical properties of coffee grain and PCA of sensorial analysis, it was observed a similarity among analyses. It was concluded that PCA of physical and chemical characteristics was compatible to sensorial analysis, allowing this procedure to aid the cup test, permitting less subjectivity of this test.

INTRODUCTION

Coffee quality is a funcion of grains chemical composition, genetics, cultural management, harvest procedure, processing, moisture content and temperature during storage. This quality is related to taste and fragrance, which depends on the chemical components complexity present in the coffee beverage.

Procedures that are used nowadays in order to evaluate coffee quality are based on empirical and subjective variables. Classification of coffee using the aspects or type and drink classification by means of cup test could be complemented with physical and chemical methods which would aid this evaluation, aiming to decrease subjective evaluation.

Being that stated, the goal of the present work was to use the principal component analysis of physical properties (one thousand grain mass, density, moisture content, cell membrane integrity and color) and chemical properties (identification and quantification of volatile compounds) of coffee grain produced at Minas Gerais, Brazil.

MATERIALS AND METHODS

Green and cherry coffee (cv. Catucaí) was used. These were harvested at Braúna Farm, located at Araponga city, MG, Brazil. After harvest, part of cherry coffee was immediately dried at 40, 50 and 60 °C (samples C40, C50 and C60). Remaining portion of cherry coffee

were dehulled and dried at the same temperatures stated before (samples P40, P50 and P60). Samples denominated C10, C20 and C30 represents grains dried in its integral form at the temperature of 40 °C with addition of 10, 20 and 30 % of green coffee. All samples were dried in ovens of air-forced circulation until final moisture content of 11 % w.b. Afterwards, samples were stored under controlled temperature during twelve months. After storage and processing, samples were sent to physical and chemical characterization. Moisture content, bulk, one thousand mass grain was quantified, electrical conductivity, color and volatile compounds were obtained. Due to the amount of variables analyzed, the technique of principal component analysis (PCA) was employed in order to group the samples with similiar physical and chemical properties. A matrix of 27 x 12 (9 samples with 3 repetitions and 12 variables) was used to physical and chemical properties analysis. Sensorial evaluation used a matrix of 9 x 13 (9 samples and 13 variables).

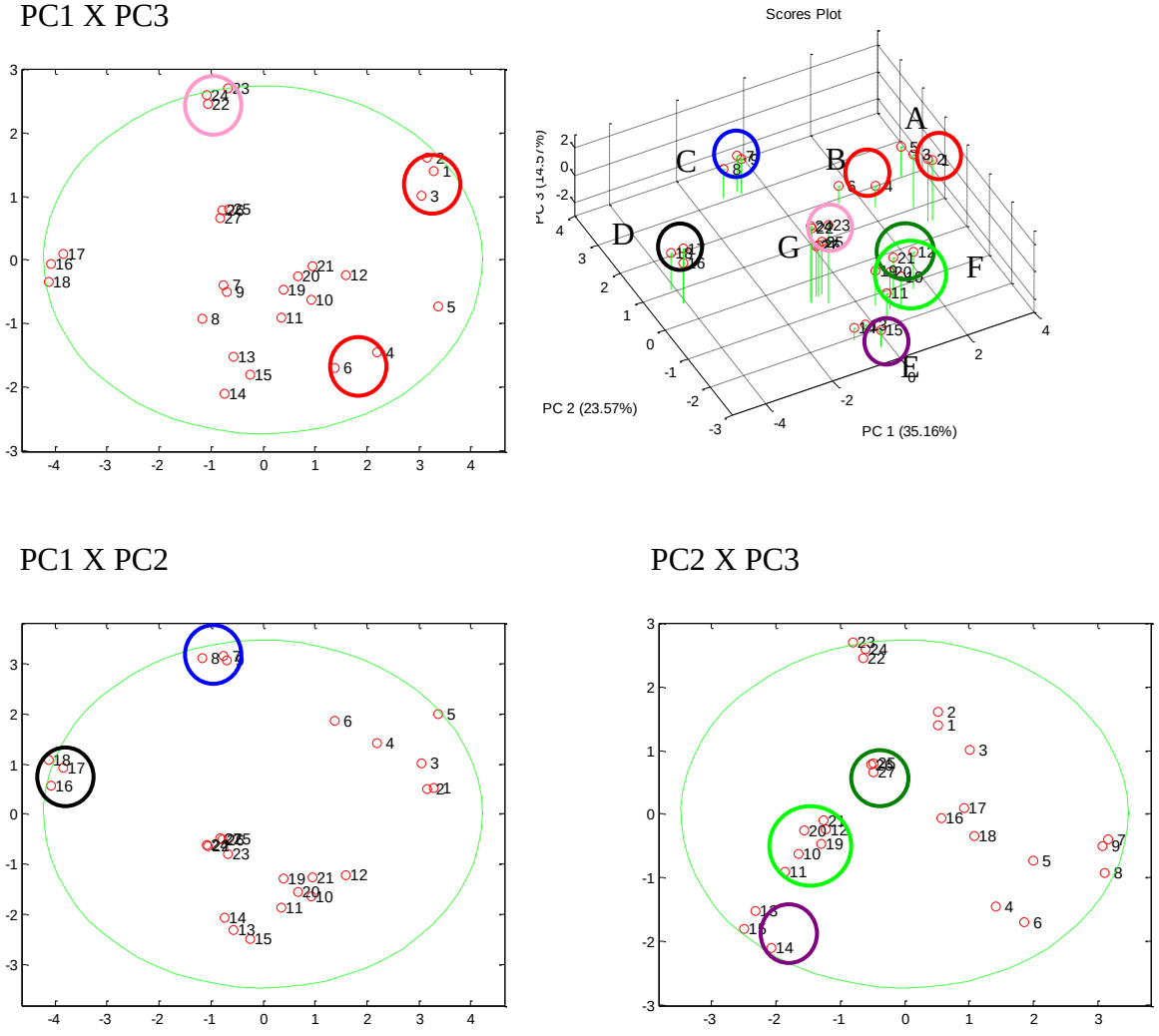


Figure 1. Principal component analysis of physical and chemical properties of coffee after twelve months of storage.

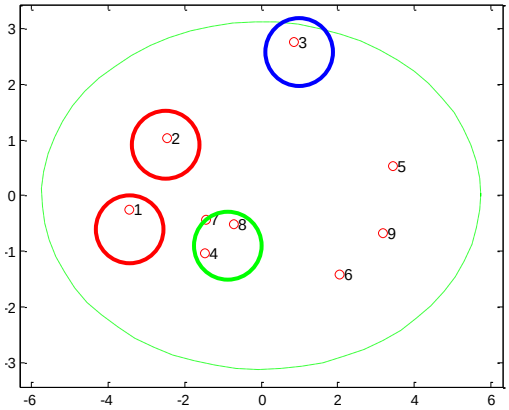
RESULTS AND DISCUSSION

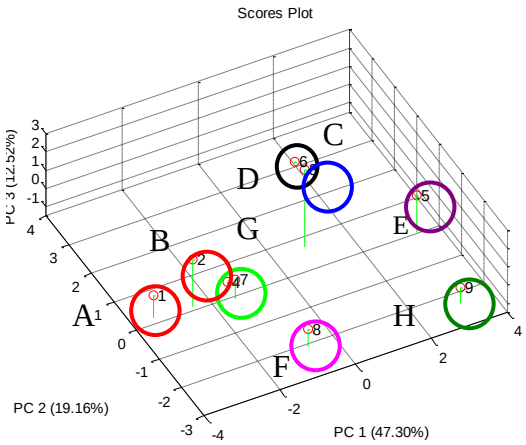
Principal components observed at Figure 1 explains 73.30 % of total data variance. Principal component 1 (PC1) explans 35.16, PC2 explains 25.57 % and PC3 14.57 % of data variance. These differentiate coffee into eight groups (A,B, C, D, E, F, G and H).

It can be noticed from Figure 1 that interaction between PC1 and PC2 was responsible for separation of samples C and D. It was verified that these groups represents samples dried at 60 °C with parchment and as cherry, respectively. This interaction denotes the resemblance among dried grain at the same temperature, regardless of the processinng procedure. Also, interaction between PC1 and PC3 explains the separation of A, B, G and H groups. A and B groups are grain with parchment dried at 40 and 50 °C, demonstrating that after twelve months of storage these grains presents physical and chemical characteristics different from the remanining samples. Differentiation between samples that were added green coffee can be seen at groups G and H. Because of this trend, the presence of immature grain affects physical and chemical properties of coffee.

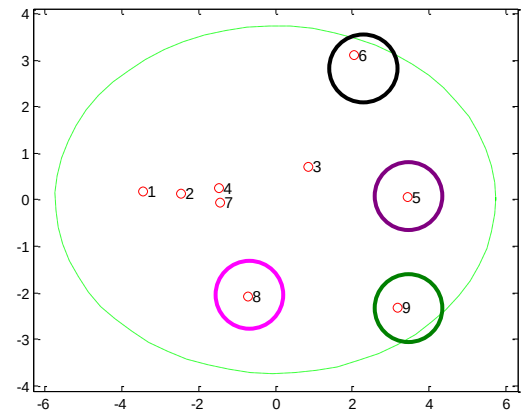
Figure 2 presents the principal component analysis of sensorial analysis of coffee after twelve months of storage.

PC1 X PC3





PC1 X PC2



PC2 X PC3

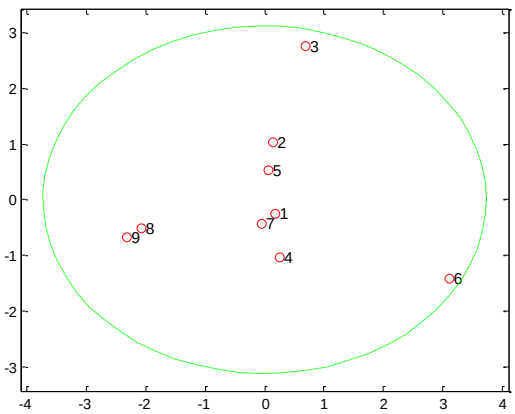


Figure 2. Principal component analysis of sensorial analysis of coffee after twelve months of storage.

Interaction between PC2 and PC3 was responsible to segregate E and F groups (cherry coffee dried at 40 °C and sample with 10 % of green coffee). This trend denotes the resemblance among physical and chemical properties of these samples, suggesting that the presence of a

small amount of green coffee does not affect directly physical and chemical properties of coffee.

Principal components explain 78.98 % of total data variance. PC1 explains 47.30 %, PC2 was 19.16 % and PC3 explained 12.52 % of data variance. These differentiated samples into eight groups (A to H). From Figure 2, it can be observed that interaction between PC1 and PC3 was responsible to separate samples A, B, C and D from the remaining ones. Interaction between PC1 and PC2 separated C, D, E, G and H groups. It was verified that, after twelve months of storage, all existing groups from PCA of physical and chemical analysis (Figure 1) can be found at PCA of sensorial analysis (Figure 2), denoting a compatibility among these two analysis. Thus, characterization of coffee using Principal Components Analysis is a potential technique to be used along with the cup test.

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Evaluation of Green Bean Physical Characteristics and Beverage Quality of Arabica Coffee Varieties in Brazil

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SUMMARY

In the specialty coffee market are preferred coffee beans that present good physical characteristics and high cup quality with distinctive sensory attributes, emphasizing the fragrance, aroma, flavor, sweetness, acidity and overall score. The objective of this research was to evaluate physical characteristics of the green beans and to describe the sensory profile of some Brazilian arabica coffee varieties released by Instituto Agronômico de Campinas (IAC) and to identify the promising ones for specialty coffee production. It was analyzed twenty four *Coffea arabica* genotypes during three crop years in the Northwest Sao Paulo State, Brazil. Healthy and fully ripe fruits were selectively handpicked and prepared by semi washed processing procedures and the wet parchment coffees were sun dried over elevated screens until the grain reached moisture content of 11.5%. After grading the green coffee beans from up to 16/64 inch sieve size without defective beans were submitted to sensory evaluation according to Specialty Coffee Association of America (SCAA) procedures. Considering that all coffee genotypes were obtained in the same environmental conditions and the same post harvest processing procedures, it is supposed that the prominent differences in beverage quality could be attributed to the genetic effects, corroborating other results cited in literature.

INTRODUCTION

The specialty coffee demand is growing both in traditional and emerging market as disposable income increasing but the supply of high quality coffee is reaching its upper limit. Without an increase in the total world volume of specialty-grade green coffees, the specialty coffee industry will face to a problem in the not too distant future, that could constitute a possible coming specialty coffee supply crisis.

It is very known that beverage quality is influenced by large number of factors including environmental, processing and their interactions, but scientific researches have demonstrated that genetic constitution of arabica coffee genotype lead the predominant effects in the cup quality, determining flavor and other sensory attributes of coffee. For some specialty coffee market the cup quality is considered more important than green bean physical attributes regarding the coffee price determination.

Since the coffee genotype is considered an important factor for cup quality expression several efforts have been done in Brazil to improve the coffee quality and to get reliable information about beverage quality by genotype comparisons it is so important that all varieties have been cultivated in the same site and the beverage quality assessment should be done by trained

coffee tasters. The prescribed sensory evaluation procedures method provides specific data about each coffee genotype and can be used successfully for screening in breeding selections to coffee quality improvement.

MATERIAL AND METHODS

Study site and test varieties

The experiment was carried out in 2009, 2010 and 2011 crop years at Recreio Farm Estate Coffee in the Northwest São Paulo State, Brazil. A total of twenty four *Coffea arabica* genotypes were used in this study. Many of them are elite arabica coffee varieties including 'Mundo Novo' and 'Catuaí Vermelho', which are the most representative Brazilian cultivars used as reference for quality evaluation. Each one of these genotypes was represented by ten plants per plot disposed in the field in a Randomized Complete Block Design with three replications.

Harvest and processing

Fruits samples were collected during the peak harvesting period of June-July. Ripe health cherries were harvested by hand from each of the experimental plot and processed using semi washed processing procedures (natural pulped coffee). The full ripe fruits were pulped and the parchment coffees were sun dried over elevated screens until the green beans reached moisture content of 11.5% (wb). After hulling the beans were graded by size using round perforations screens with circular perforations of 18, 17 and 16/64 inches.

Sensory evaluation

The sensory analyses were done according to the Specialty Coffee Association of America (SCAA) prescribed procedures by trained coffee tasters certified by SCAA. The beverage quality assessment was done by descriptive analysis of cup quality for each coffee sample from respective experimental plots. Roasting of green coffee beans was done to attain a light to light-medium roast within 24 hours of evaluation and allowed to rest for at least eight hours. The samples were weighed out to the predetermined ratio of 8.25g of ground coffee per 150 ml of water (5.5% mass:volume) and grounded immediately prior to cupping. Ten sensory attributes including uniformity, sweetness and cleanliness were assessed and rated on a 10-point scale. For the attributes fragrance, aroma, flavor, aftertaste and balance, 1=very poor and 10=outstanding, while for acidity 1=very flat and 10=outstanding and for body 1=very thin and 10=very bright. Other differentiated and specific attributes were also described. The data were organized and each coffee genotype was analyzed by their cumulative sensory score, according to SCAA descriptions for specialty coffees.

RESULTS AND DISCUSSION

The results indicating that there were significant genotype effects both on coffee bean size and overall sensory quality (table 1). Considering the average values for percentage of beans retained over 18, 17 and 16 screen size it was observed that genotype 5 (Icatu Precoce IAC 3282) showed 83% of beans from up to 16, but with no significant difference from the genotypes 1 (Yellow Bourbon Epamig), 6 (Yellow Bourbon Procafé), 8 (Red Bourbon São João Batisa), 9 (Yellow Bourbon Betânia), 10 (Yellow Bourbon Daterra), 11 (Yellow Bourbon IAC J9) and 21 (Mundo Novo Amarelo Monte Deste) that presented values between 79% e 82%. There were not significant environmental and genotype × environmental interaction effects indicating that in this study the green bean size should be conditioned

priority by genetic constitution of the coffee variety. Thus, the green bean size could be consider an important criterion for coffee plant selection aiming to improve the green bean quality since the evaluations and comparisons have occurred in the same environmental conditions and in the same post harvest processing procedures.

Table 1. Effects of *Coffea arabica* L. genotypes on the green bean characteristics and sensory quality. São Sebastião da Grama, São Paulo, Brazil.

Genotype[3]	Flat beans - screens 18+17+16 (%)				Overall SCAA Score			
	2009	2010	2011	Average	2009	2010	2011	Average
01. Yellow Bourbon Epamig	75.47	80.58	80.58	78.88 ab	79.04 a	82.25 abc	82.00 ab	81.10
02. Mundo Novo IAC 502/9	72.10	73.86	79.21	75.06 abc	81.54 a	83.17 abc	81.30 ab	82.00
03. Catuaí Vermelho IAC 144	76.31	73.38	71.53	73.74 bc	79.67 a	81.50 abc	81.42 ab	80.86
04. Red Bourbon Procafé	77.47	75.37	79.47	77.44 abc	79.92 a	81.38 abc	82.96 ab	81.42
05. Icatu Precoce IAC 3282	83.04	82.93	83.14	83.04 a	79.00 a	81.92 abc	82.75 ab	81.22
06. Yellow Bourbon Procafé	79.92	77.95	81.89	79.92 ab	82.13 a	83.50 ab	85.25 a	83.63
07. Yellow Bourbon Bom Jardim	77.66	78.25	77.07	77.66 abc	78.63 a	84.25 a	84.44 ab	82.44
08. Red Bourbon São João Batisa	82.99	75.78	78.22	78.99 ab	79.54 a	81.83 abc	81.58 ab	80.98
09. Yellow Bourbon Betânia	82.08	78.71	80.83	80.54 ab	80.79 a	82.83 abc	82.29 ab	81.97
10. Yellow Bourbon Daterra	82.63	76.51	82.70	80.61 ab	82.59 a	82.92 abc	81.29 ab	82.27
11. Yellow Bourbon IAC J9	76.92	79.51	80.90	79.11 ab	81.21 a	81.92 abc	82.96 ab	82.03
12. Yellow Bourbon Toriba	77.06	74.76	79.35	77.06 abc	82.46 a	80.75 abc	83.94 ab	82.38
13. Yellow Bourbon IAC J10	74.29	73.13	75.46	74.29 abc	80.50 a	80.50 abc	82.46 ab	81.15
14. Yellow Bourbon Castro	80.01	78.98	75.95	78.31 abc	81.08 a	81.00 abc	81.80 ab	81.29
15. Yellow Bourbon Nogueira	64.84	55.14	66.12	62.03 d	83.13 a	81.58 abc	81.29 ab	82.00
16. Yellow Bourbon Paixão	62.33	57.86	64.14	61.44 d	80.75 a	77.13 c	82.09 ab	79.99
17. Italiano Monte Alegre	74.93	78.96	76.58	76.83 abc	81.83 a	80.83 abc	83.94 ab	82.20
18. Trigo Monte Alegre	70.38	80.30	69.78	73.48 bc	82.88 a	77.67 bc	84.31 ab	81.62
19. Limoeiro Monte Alegre	73.99	76.09	70.51	73.53 bc	80.00 a	82.08 abc	83.75 ab	81.94
20. Yellow Bourbon Samambaia	79.22	76.68	76.30	77.40 abc	81.83 a	81.42 abc	82.71 ab	81.99
21. Mundo Novo Monte Deste	81.55	83.29	80.02	81.62 ab	81.29 a	79.50 abc	81.96 ab	80.92
22. Mundo Novo IAC 4266	72.74	73.68	71.80	72.74 bc	81.33 a	81.33 abc	81.33 ab	81.33
23. Yellow Caturra IAC 476	76.10	77.14	69.14	74.12 abc	81.08 a	79.33 abc	78.80 b	79.74
24. Red Caturra IAC 477	68.76	71.82	68.57	69.72 cd	83.58 a	78.25 abc	79.50 ab	80.44
Means	75.95	75.44	75.80	75.73	81.08	81.20	82.34	81.54
$F_{genotype}$	-	-	-	10.56^{2}	1.75^{ns}	2.49^{2}	2.03^{1}	0.84^{ns}
$F_{environment}$	-	-	-	0.18^{ns}	-	-	-	4.57^{2}
$F_{g\ x\ e}$	-	-	-	0.85^{ns}	-	-	-	2.42^{2}
CV_e (%)				3.77	2.19	2.40	2.22	2.40

[1] and [2] = significant differences by the F test at level of 5% e 1% of probability, respectively; [ns] = not significant differences; Values followed by the same small letter in the columns did not present significant difference by the Tukey test at level of 5% of probability.

[3]Genotypes number 1, 4, 6, 7, 8, 9, 10, 12, 15, 16, 17, 18, 19, 20 and 21, followed by the origin name, are under selection at Agronomic Institute (IAC). Genotypes number 2, 3, 5, 11, 13, 22, 23 and 24 are Brazilian cultivar released by IAC.

For sensory quality evaluation it was observed significant effects both for environmental and genotype × environmental interaction, indicating that any variety selection should be done based on genotype performance in each crop year. In 2009 crop year it was not observed any significant genotype effects on coffee beverage quality. In 2010 the higher overall SCAA score it was observed in genotype 7 (Yellow Bourbon Nogueira) differing from the genotype 16 (Yellow Bourbon Paixão) whose overall score was the worst, but with no significant

difference from the other genotypes that showed intermediate overall scores varying between 83.5 and 78.25 points, except for the genotype 18 (Trigo Monte Alegre) which showed the second worst overall score. In 2011 it was observed the higher overall SCAA score in genotype 6 (Yellow Bourbon Procafé), differing significantly from the genotype 23 (Yellow Catura IAC476). The remaining genotypes showed intermediate overall scores, but without difference from the better or from the worst genotypes. Since there were no significant differences for cup quality between the control cultivars (Mundo Novo and Catuaí Vermelho) and Yellow Bourbon genotypes, it was not yet possible to conclude if Bourbon genotype has superior beverage quality than the other genotypes. However, considering that only Yellow Bourbon genotypes showed overall SCAA score up to 84 points in at least two consecutive crop years, this constitutes an important indicative of the best quality of this genotype, revealing its high intrinsic genetic potential for specialty coffee production and confirming the literature.

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Descriptive and Quantitative Sensory Profiles of the Coffee from Sucre Municipality, Portuguesa State Venezuela

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SUMMARY

Sensory quality of coffee depends on a number of factors ranging from planning to planting of the coffee to the method of preparation of coffee. In this paper we studied the quantitative descriptive sensory profiles of coffee from Sucre municipality of Portuguesa state in Venezuela. The samples studied were mixtures of Arabica coffee beans harvested in the golden crop cooperative 2009-2010. The study of sensory profiling was performed by an analytical sensory panel in the laboratory of the Research Center for Development (CIRAD), Montpellier, France. Data were analyzed by Friedman test to determine the significance of the data and graphs. The quantitative profile of the attributes studied showed that the intensity of odor and the body of the coffee cups were the descriptors that were more important and a balance of acidity and sour descriptors. The coffee samples presented different descriptive sensory profiles of the attributes body, acidity, green, chemical and overall quality, while the attributes odor intensity, bitter, sour, fruity, woody, earth, chemical, fermentation, harsh and astringent were the same for all samples.

INTRODUCTION

Coffee is a very desired drink by its organoleptic characteristics, making it one of the most consumed in the world, it contains a vast array of chemical compounds responsible of sensory quality and its physiological effects. Coffee is very important in Venezuela because it is grown mainly by small farmers with a long tradition in this area and depends almost entirely for their livehood. The coffee grown on the Sucre municipality, Biscucuy, Portuguesa State, takes precedence in Venezuela for its quality, but despite this, there are some producers that do not apply best practice in crop management, postharvest and storage, which tends to cause poor quality coffees. The present work aims to provide a description of the quantitative descriptive sensory profiles of coffees from Sucre municipality, Biscucuy, Portuguesa state.

MATERIALS AND METHODS

Parchment coffee samples donated by coffee grower from the golden grain cooperative of Sucre municipality, Biscucuy, Portuguesa state-Venezuela were used, whose coffee plantations are located at an altitude between 900 and 1500 m above sea level. Ten (10) samples of Arabica coffee 180 g each, fully randomized, harvested fully ripe, fermented pulped, made the same day of harvest and dry in the sun, belonging to the 2009-2010 coffee harvest were taken for analysis. The samples were transported to the laboratories of the Center for International Cooperation in Agricultural Research for Development (CIRAD), Montpellier France. Approximately 150 g of coffee per sample where dehulled to get green coffee. An average roasted process was applied to the green coffee samples, subsequently

ground and infusion was prepared to 100°C and served to the analytical sensory panel of CIRAD at a temperature of 80°C. The panelists evaluated by duplicate the descriptors body, acidity, green, chemical, global quality, odor intensity, bitter, sour fruity, woody, earth, chemical, fermentation, rough and astringent. Data were analyzed through non parametric statistical test of Friedman and Descriptive Profile.

RESULTS AND DISCUSSION

The study of the sensorial attributes of coffee samples indicated that the coffees presented significant differences regarding body characteristics, acidity, green, burnt and overall quality (Figure 1). This phenomenon could be because coffee organoleptic properties are affected by factors such as variety, region/growing conditions, postharvest handling, grinding and brewing. Although, the aroma profiles of a coffee cup are more influenced by roasting and brewing than by varieties.

Figure 1. Descriptive sensory profile of the ten studied samples of Arabica coffee (from A to J).

In the present study a group of samples were found with statistically the same sensory attributes of odor intensity, bitter, fruity, sour, woody, earthy, chemical, fermentation, rough and astringent (Figure 2). This indicates that coffee growers have been implementing similar postharvest practices, which enable them to obtain quantitative descriptive sensory profiles of coffee samples, demonstrating the advance of producers toward uniformity of their attributes and improving regular quality coffee in the Sucre municipality, Biscucuy, Portuguesa state, Venezuela.

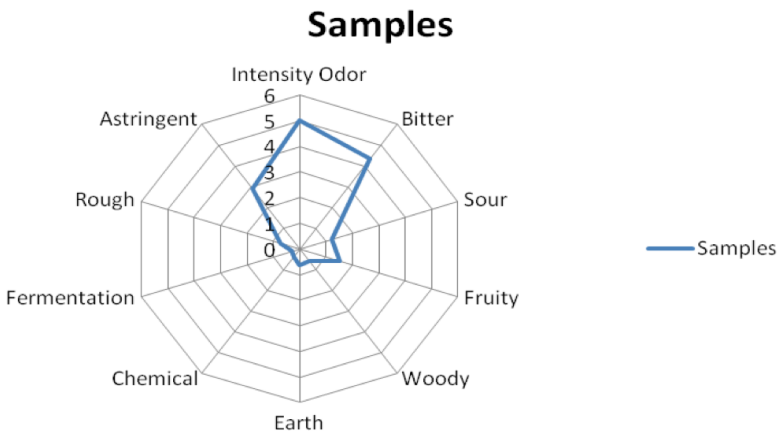

Figure 2. Descriptive profiles of similar samples.

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Effect of Roasting Conditions on Coffee Beverage: Exploring Consumer Perception

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SUMMARY

This study aimed at investigating the effect of roasting coffee conditions on sensory characteristics of the brews by consumers. Green Brazilian Arabica coffee beans were roasted in a plant scale semi-fluidized bed roaster at different temperature gradients to obtain three speed conditions (slow, medium and fast), and two roasting degrees (moderately light and dark), yielding six different types of beans. The beans were ground to medium grid and 57 coffee drinkers participated in the study. They evaluated the six beverages in terms of liking, and also using check-all-that-apply (CATA) questions, which comprised 20 hedonic and sensory attributes. They were related to *aroma, flavor, mouthfeel, aftertaste*, and *texture*. Data were analyzed using frequency of mention of each word of the CATA question by counting the number of consumers who used that term to describe each coffee beverage followed by Multiple Factor Analysis (MFA) on the frequency table. Consumer overall liking scores were considered as supplementary variable. Brews from light roasting (slow and fast speeds) were described by the following attributes: characteristic coffee aroma and flavor, aromatic, fragrant, sweet aroma, weak aroma, low body, which were positively correlated to overall liking. Brews from dark coffee roasted at slow and medium speeds were perceived by participants as having burnt aroma and flavor, rubbery, astringent, and unpleasant aftertaste. On the other hand, brew from the dark roast (fast speed) was perceived as having body, intense aroma, acid taste, and stale flavour. Medium light brew stood between light (slow and fast speeds) and dark (slow, medium, and fast speeds). The results indicated that consumers perceived samples differently depending on roasting process conditions. Colour played an important role on the brews' description, suggesting that moderately light roast has pleased consumers. However, further studies with a larger number of participants, and taking into account the granulometry of the samples have to be carried out.

INTRODUCTION

The sensory characteristics of coffee beverages play an important role on coffee drinkers' experience. Previous studies have reported that several factors affect coffee sensory properties, such as: plant varieties, growing region/conditions, processing, roasting degree, grinding size, and brewing methods. Extensive studies have been carried out to explore the relationship between those variables and the coffee aroma and flavor, as well as overall consumer acceptance in ground roasted coffee. However, only few studies have focused on the role of roasting conditions on consumer perception of brewed coffee. In addition, the majority of them either has used trained people to profiling samples, or investigated only consumer liking. Although sensory profiling is a useful technique for the food industry in several steps of the process optimization, it is time consuming. Moreover, trained panels often

describe the product differently or take into account attributes that may not be relevant to consumers.

The way consumers perceive the sensory characteristics of products is particularly important to the food industry in order to create products that meet consumer expectations. Therefore, alternative methods have been developed to achieve this goal, and the check-all-that-apply (CATA) question is one of them. This study aimed at investigating the effect of roasting coffee conditions on sensory characteristics of the brews by consumers.

MATERIALS AND METHODS

Brazilian good quality green Arabica coffee beans (São Paulo, Brazil) were roasted in a plant scale semi-fluidized bed roaster by varying the roasting speed condition (slow, medium, and fast) and colour (moderately light and dark), yielding six different types of roasted coffee beans. Samples were ground in a discos grinder (Gourmet M-50, LEOGAP Ind. e Com. de Máquinas, Curitiba, PR, Brasil, grid # 6) for brews' preparation. Coffee beverages were prepared at 10% (weight/volume), with mineral water in electrical coffee makers, and kept up to 20 min at 68˚ ± 2˚C; after this time they were discharged. Although it has been previously reported [5] that some brewed coffee sensory attributes changed after 45 min, we decided to avoid any possible alteration, and use a fresher beverage.

Fifty-seven coffee consumers were invited to participate in the study. They worked at Embrapa Food Technology, Rio de Janeiro Brazil, consumed at least one cup of black coffee a day, and aged from 18 to 65 years old. They evaluated samples regarding pre-established sensory attributes and hedonic terms following the CATA question standard procedures. These attributes and terms were elicited from previous studies carried out in Embrapa Food Technology, and also from the literature. They were related to *aroma* (characteristic, weak, burnt, chocolate, aromatic, sweet, fragrant, intense); *flavor, mouthfeel, and aftertaste* (characteristic, burnt, rubbery, stale, acid, very bitter, bad flavor, very astringent, intense residual flavor, unpleasant aftertaste); *texture* (low body, full-bodied). Participants were instructed to mark all terms they perceived in each coffee beverage. Samples were monadically presented in 50mL porcelain cups coded with three digit numbers, presented at 68°±1°C. They were evaluated in sensory booths under white light. Sample presentation order was balanced to prevent carry over effects, and water and biscuits were provided to participants as a cleanser between samples.

Data were analyzed using frequency of mention of each word of the CATA question by counting the number of consumers who used that term to describe each coffee beverage, followed by Multiple Factor Analysis (MFA) on the frequency table. Consumer overall liking scores were considered as supplementary variable.

RESULTS AND DISCUSSION

Results from the MFA are presented in Figure 1 (a and b).

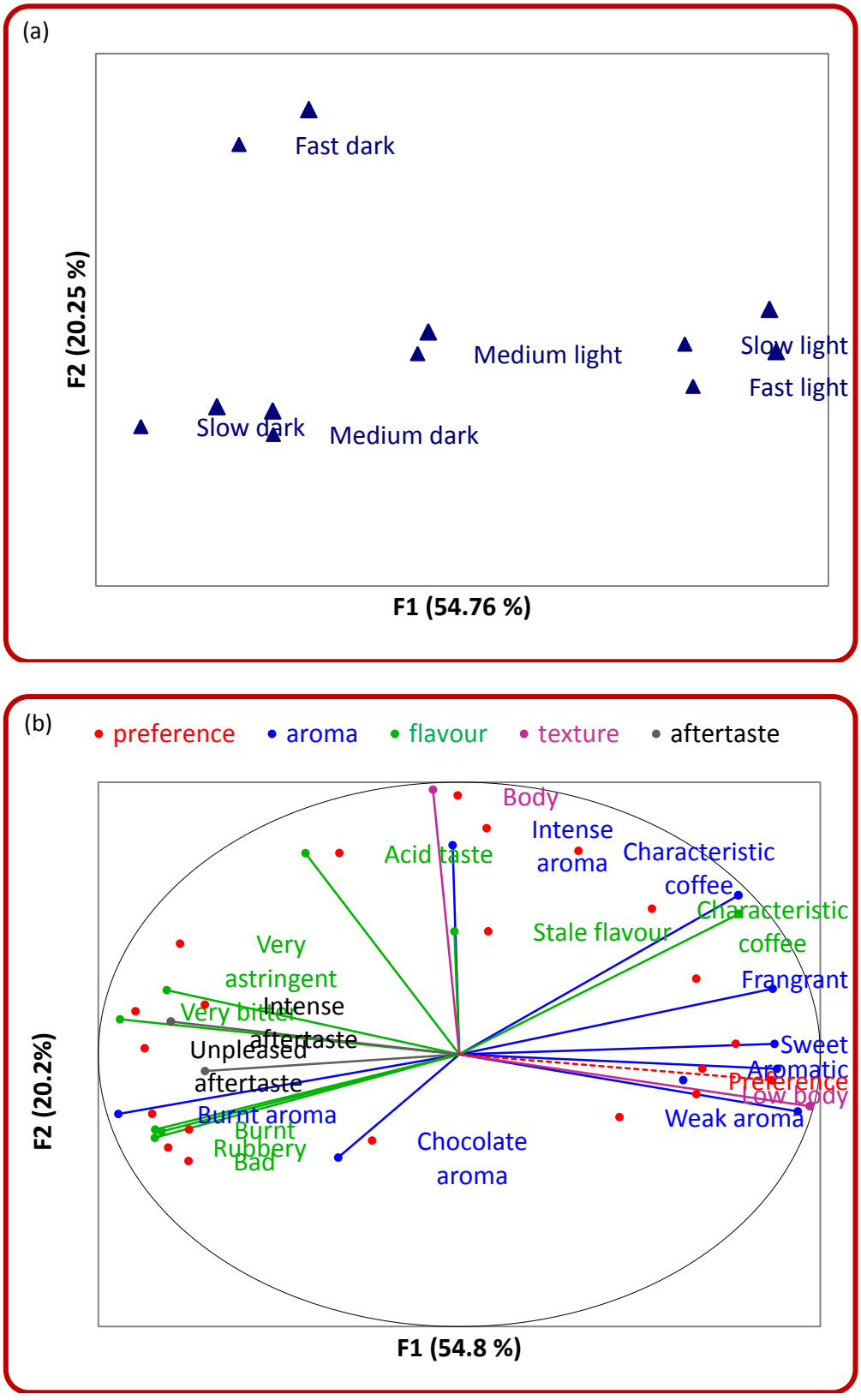

Figure 1. (a) Representation of the coffee beverages processed at different roasting conditions and colour degrees in the first two dimensions of the Multiple Factor Analysis. (b) Terms and sensory attributes of the coffee beverages.

The first two dimensions of MFA accounted for by 75% of the variance. The first dimension separated moderately light roasting (slow and fast speeds) from medium speed and dark roasting (slow, medium, and fast speeds) (Figure 1a). Brews from moderately light coffees roasted at slow and fast speeds were described by the following attributes: characteristic coffee aroma and flavor, aromatic, fragrant, sweet aroma, weak aroma, low body, which were positively correlated to overall liking (Figure 1b). Medium light coffee presented the same attributes but less intensely.

Brews from dark coffee roasted beans at slow and medium speeds were perceived by participants as having burnt aroma and flavor, rubbery, astringent, and unpleasant aftertaste (Figure 1b). Beverages made from dark beans (fast and medium speeds) were perceived as intense aroma, stale flavour, body, and acid taste.

The results indicated that consumers perceived samples differently depending on the roasting process conditions. The sensory characteristics and terms closer to the Preference vector, which it is shown in red in Figure 1b, have driven consumer liking for the coffee beverages. Colour played an important role on the brews' description, suggesting that moderately light roasting has pleased participants. However, further studies with a larger number of coffee consumers, and taking into account the granulometry of the samples and amount used for beverage preparation should be carried out.

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Thermal Stability Study of Ochratoxin A During Roasting Coffee (*Coffea Arabica*)

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SUMMARY

Two samples of contaminated coffee (5.3 and 57.2 ppb of OTA) were roasted at 230°C using two methods: rotating cylinder (RC) and fluidized bed (FB). Samples were taken every 3 and 0.9 min from RC and FB respectively. Each sample was analyzed for OTA content. The results showed that the process by RC was more effective on OTA reduction than FB for a same degree of roasting with 67% OTA reduction against 36% for the medium roast. The thermal degradation rate of pure OTA and the OTA mixed with the components of coffee (5 sugars, 3 amino acids, caffeine and chlorogenic acids) were determined, showing that interactions took place dependent themselves on the conditions of pH and pKa values of the components tested, in this case by influencing by the reactivity and the rate of degradation of OTA. A transformation product (TP) was observed in the chromatograms obtained from the interaction of OTA with the components of coffee. A test of alkalinization and warming of pure OTA confirmed that the TP comes from the structural modification of the OTA molecule and is not a product of interaction with the natural components of coffee.

The TP was purified to carry out its chemical elucidation. The chemical nature of compound transformation and spectroscopic data such as UV-Vis (λmax: 237nm), the affinity with the mobile phase of the OTA, the analysis of alkalinization (OTA regeneration phenomenon and TP), the analysis of stable isotopes (SIDA's) and the mass spectrum (molecular ion M +: 420 m/z), suggest that the TP of OTA during the roasting process corresponds structurally to an analogue of OTA which retains its acidic carboxyl group and in accordance to fragmentation corresponds to the Hydroxi-Ochratoxin A (OH-OTA), as well as minor amounts of OTA and its isomers.

INTRODUCTION

Ochratoxin A (OTA) is a metabolite produced by species of *Aspergillus* and *Penicillium* and has been associated with nephrotoxic, immunotoxic, teratogenic and carcinogenic effects. Roasting coffee is thermal processes that have an effect on the OTA content. Before 1988 it was thought that the OTA was destroyed during roasting, nevertheless concentrations superior to the 20 ppb have been reported in commercial roasted coffee. Several reports concerning the roasting impact on OTA content in coffee beans have shown contradictory results. Such variability could be related to the different analytical conditions or roasting process or heterogeneity in toxin distribution. Some explanations for OTA reduction during roasting were suggested: isomerization into other diastereomers; the existence of reactions that "masked" OTA or rearrangements of the OTA molecule at the roasting temperatures. Because there are no conclusive data regarding the effect of roasting on OTA in grain and the need for scientific bases for establishing regulations for export of green coffee, the objective of this

work was to study the impact of different types of roasting on the thermal stability of OTA in coffee and chemical elucidation of the transformation products.

MATERIALS AND METHODS

Two levels of contamination were obtained by the contamination of coffee with a strain of *A. westerdijkiae* (5.3 and 57.2 ppb of OTA). These lots were roasted at 230°C using two methods: drum rotation (RC) and fluidized bed (FB). Samples were taken every 3 min from RC and every 0.9 min for FB to quantify the OTA residual.

Fo the study of thermal transformation of the OTA pure during heating in model systems, 10 uL of a solution of OTA (400 ng/L) were deposited on a filter paper disk, each disk was added equal amount of the following solutions: arabinose, galactose, arginine, glucose, glutamic acid, maltose, phenylalanine, sucrose, chlorogenic acid and caffeine. The system was heated at 250 ° C for: 0, 6, 12, 18, 24 and 30 min. Every time a disc was removed and the residual OTA was quantified by HPLC.

For the identification of (the) product (s) of transformation of OTA during roasting, we used the following analysis tools: UV-visible spectrum (λmax: 237nm), chromatographic parameters (HPLC-fluorescence detector) or the affinity with the mobile phase of the OTA, the analysis of alkalinization (OTA regeneration phenomenon and PT), analysis by stable isotope dilution (SIDA's) when an isotope labelled OTA (d5-OTA) in the functional group of the phenylalanine was dissolved in methanol at a concentration of 100 ug / mL, a portion of this sample was mixed with the extract obtained OTA and PT (1:1). Subsequently, this mixture was analyzed by HPLC/MS-MS, which allowed differentiation between isotopologues. A pattern of internal standard was used to discard error signals by intensity difference is compared whether the processing compound is structurally similar to isotope labelling OTA and finally an analysis HPLC/MS-MS was developed (molecular ion M +: 420 m/z).

RESULTS AND DISCUSSION

The effect of two types of roasted on the thermal degradation of the OTA, as well as the impact on the degree of roasting on residual OTA content was studied. The roasting process by FB was less efficient to reduce to safe levels the OTA and the kinetics sigmoid exhibit the same behavior of the exposure time dependent characteristic of organic compounds with a final reduction ratio of 75% toxin for both levels of contamination. In the case of RC, we observed greater efficiency in the final degradation of OTA and 90% for the two marked kinetic degradation rates were present: a slow followed by rapid degradation (Figure 1).

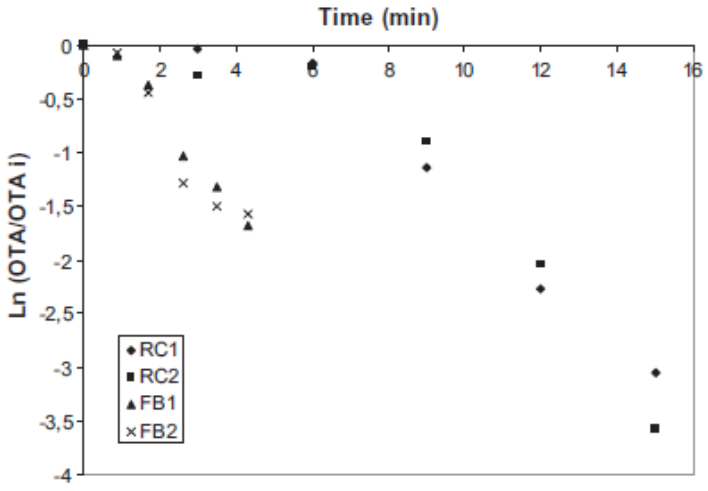

Figure 1. Kinetics of OTA reduction at 230°C in roasted coffee beans contaminated at two contamination levels (1: 5.3 ppb; 2: 57.2 ppb of OTA) in rotating cylinder (RC) or fluidized bed (FB).

By comparing the graphs of the degradation kinetics of OTA for both processes, we observed a similar behavior of the exposure time dependent. That is, both processes have the same performance; however, its effectiveness is favored by RC due to longer exposure to heat. This evidence can provide information on the most effective method of degradation of OTA in coffee and based on these results, it can be predicted by modeling the potential risk of exposure to the toxin, considering the operating conditions.

In model systems (conjugate-OTA), all showed a protective effect on the degradation of the toxin and the formation of a new product proved to the appearance in the chromatograms (Figure 2a). The complexes or matrices showed interactions are performed dependent on the conditions reaction as pH, reaction time and pKa values of each of the reactants or reactivity of the functional groups of the molecule OTA as the halogen atom, which is critical in other groups covalently nucleotides. However, it is possible that the formation of the product of transformation of the OTA is due to interactions with other components of the coffee, since the conjugate-OTA complexity within this matrix involves other interactions with other compounds that are produced during the treatment thermal (Figure 2b).

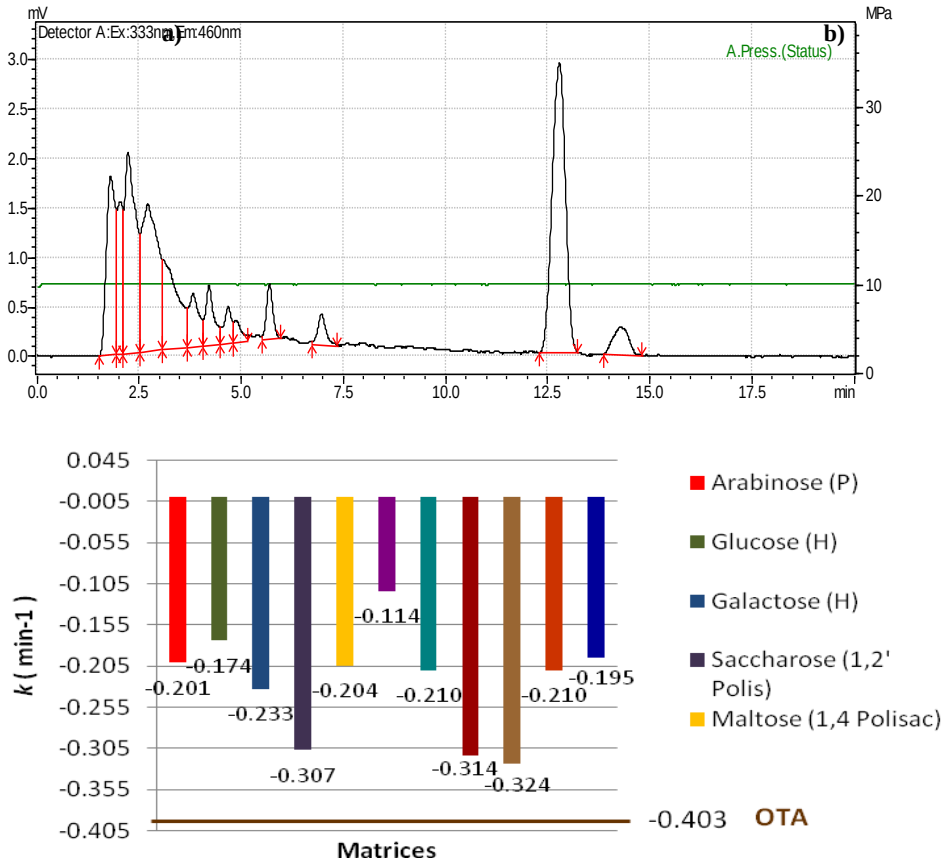

Figure 2.The thermal degradation rate of OTA pure and OTA in models systems: a) Chromatograms that provid the apparence of PT; b) OTA degradation rate in model systems.

The results obtained corroborate that PT previous findings comes from the OTA according to spectroscopic data specific rules UV absorption by polyene systems (double bonds), derived from aromatic carbonyl groups and confirmed by the characteristic absorption bands of the aromatic compounds in both coumarin (237 nm and 272 nm). The results chromatographic of separation showed that the OTA eluted was quickly and so show a more polar character. In addition, the PT was less polar than the OTA and so him was eluted then of the toxin. The SIDA's allowed confirming that the PT corresponded to one of the peaks of the sample marked OTA. Finally, the tool holder was higher fluid analysis coupled to mass therein OTA structurally elucidated in coffee roasting and processing product (PT) in roasted coffee. The fragmentation spectrum of HPLC/MS-MS revealed that the OTA in roasted form was transformed into its lactone ring open (OP-OTA), this molecule has the ability to regenerate back to OTA. For the PT, the fragmentation pattern revealed a complex fragmentation where the hydroxy-ochratoxin A (OH-OTA) could correspond to the PT also other fragmentation patterns were observed as the OP-OTA, 14-DC-OTA and 14R-OTA. These last two as a result of isomerization reactions transformation during the roasting process OTA pure (Figure 3).

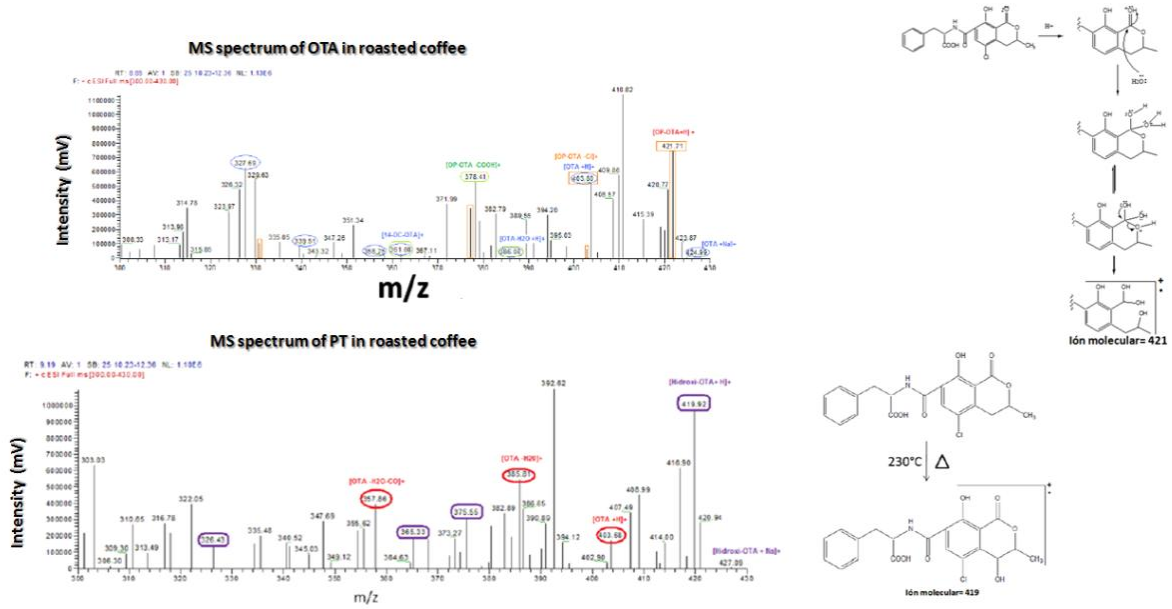

Figure 3.The fragmentation spectrum HPLC/MS-MS of OTA and PT formation ion moleculare during the process of roasting coffee.

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Morphological and Molecular Characterization of Ochratoxin A Producing Strains of *Aspergillus* Isolated of Coffee (*Coffea Arabica*) from Veracruz in México

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INTRODUCTION

The section *Circumdati* is a group of organisms especially well known for its production of ochratoxin A (OTA), *A. ochraceus* is a member has attracted much interest by its role in the ochratoxin contamination of coffee. Interestingly several isolated have now been reidentified as *A. westerdijkiae* [3], so many of the species reported as *A. ochraceus* may indeed be *A. westerdijkiae*, so identification of the most common and often important species remains problematic due to the variability in the phenotypic characters. New trends are based on a polyphasic approach using phenotypical characters together with physiology, multigene DNA sequences to identification successful[2]. Here we describe the results of our survey on the occurrence about type OTA producing strains of *Aspergillus* isolated of coffee using polyphasic approach. In this way we have an idea about kind of microorganism infects the coffee for this location.

MATERIALS AND METHODS

OTA producing *Aspergillus* strains were isolated from coffee (*C. arabica*) from Veracruz, México. They were named: AsCBa, AsCBb, AsCS, AsCV, AsCP51 and AsCP55. *A. ochraceus* MULC 44640 identified by morphological techniques was studied too[7]. All cultures were tested for OTA production on CMEA (Coffee Meal Agar) by the agar plug technique[1]. For the morphological characterization, conidia were inoculated on malt extract agar (MEA), Czapek yeast extract agar (CYA) and CYA with 20% sucrose (CY20) and incubated for 7 days at 25 °C and 37°C. The morphological characterization was made based on identification Pitt keys[5]. The primers used to amplify Internal Transcribed Spacer[8] were: ITS1 5′-TCCGTAGGTGAACCTGCGG 3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′; partial sequence of the beta tubulin gen[4]: Bt2Aw-F 5′-TGATACCTTGGCGCTTGTGACG-3′ and Bt2Aw-R 5′CGGAAGCCTAAAAAA TGAAGAG-3′ and the Translation Elongation Factor[6] (EF-1α): TE1R 5′-GCCATCCTTGGAGATACCAGC-3′ and EF1728F 5'-CATCGAGAAGTTCGAGA AGG-3'. The products were lyophilized and sequenced by the IBT (UNAM, Cuernavaca, Mexico). The alignments of the sequences were carried out with the ClustalW2 software (http: www.ebi.ac.ukl).

RESULTS AND DISCUSION

Morphological characterization

AsCS, AsCP51, AsCBb and *A. ochraceus* MULC 44640 strains were growth similar in CYA and MEA medium (7 days, 25 °C). Colony diam.: CYA 38-52 mm, MEA 31-42 mm. CYA: good sporulation yellow, mycelium white, inconspicuous; sclerotia sparsely produced on edges, reverse creme brown, soluble orange pigment present; not growth at 37 °C(Figure 1 A). MEA: moderate to good sporulation, mycelium cottony, yellow ochre, no sclerotia produced, reverse ochre yellow, no soluble pigment present(Figure 1 B). Figure 1 C and D show microscopic structure: conidial heads radiate, splitting into columns; stipes up to 1600 µm in length, uncoloured to yellow pigmented; vesicles globose to spathulate, 16 x 25-32 µm; biseriate; metulae covering the entire vesicle, measuring 4-6 x 8-12 µm; phialides 2.1-3.1 x 7-11 µm; conidia predominantly globose, 2.9-3.0 x 3.1-3.3 µm. sclerotya sparcely produced, white to cream. Septate hyphae. These strains were identified as *A. westerdijkiae* by identification keys Pitt[5].

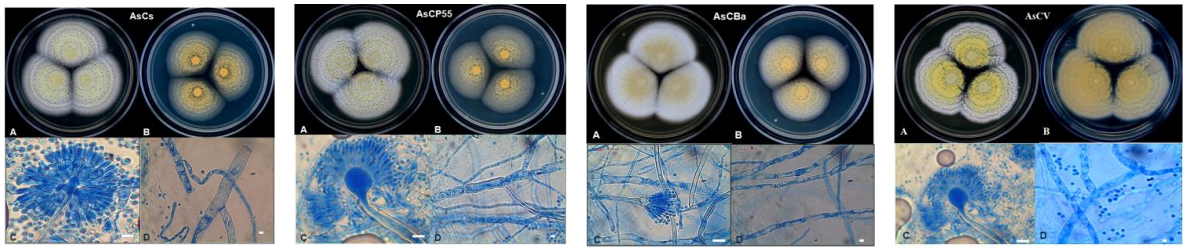

Figure 1. Seven day-old cultures on (A) CYA and (B) MEA, (C) Conidiophores, (D) Septate hyphae. Photos were taken with 1000X objecyive optical microscope. Scale bars: C=10µm and D=3µm.

Molecular characterization

All sequences were searched by BLAST, ITS rDNA sequence was searched showed an homology of 95 % with *A. ochraceus* and *A. westerdijkiae* species, meanwhile TB sequence showed a high reliably relation, 99-100% only with *A. westerdijkiae* specie, so these OTA producer strains were located in *Circumdati* section. *A. ochraceus* NRRL 398, UEL 14A and *A. westerdijkiae* ITAL 234, NRRL 3174, CBS 112791 ITS rDNA sequences were taken as reference from Genbank and were alignment join our OTA producing strains trough Clustal W2 (Figure 2). A homology of 100% into 5.8S gene was researched. 5.8S gene homology was of 100% over all strains, variation was located into intron 1 and 2. Still in strains reported as *A. ochraceus* a variation was found, different bases were located up 170 for *A. ochraceus* NRR 398 and UEL 14A, in similar way is *A. westerdijkiae*, so our results shown ITS rDNA analysis it is not enough for closely species identification [4]. Figure 3 shows OTA producing strains, *A. ochraceus* and *A. westerdijkiae* from Genbank alignment with β-tubulin partial sequence gene. Region target was intron 4 because Moreira *et al*., suggest it is enough for find *A. westerdijkiae* into *A. ochraceus*. Exons 4 and 5 was gotten too with Bt2aw-F y Bt2aw-R primers, however were excluded of analysis because their homology was full. GGAAA sequence was seen as a conserved sequence only by strain reported *A. ochraceus*, so the sequences without GGAAA belong to *A. westerdijkiae*. On this way our isolated were identified like *A. westerdijkiae*. β–tubulina analysis shows high reliably, so with it the variation is for each, instance in *A. ochraceus* C base is in place of T of *A. westerdijkiae*.

Morphological differences were found only on CYA medium. *A. ochraceus* MULC 44640, AsCs, AsCP55, AsCBb and AsCP51 were identified as *A. westerdijkiae*. The AsCBa isolated was producing micelium more than conidias although molecular analysis was on *A. westerdijkiae*, so we considerate is a variation into *A. westerdijkiae* specie. AsCV strain was a high production on conidias but the most importan was its growth at 37 °C, according to Frisvad[3] it is a skill exclusive to *A. ochraceus* specie, however in TB-ITS analysis it was identified as *A. westerdijkiae*. ITS rDNA sequence was searched for BLAST and showed a homology of 95 % with *A. ochraceus* and *A. westerdijkiae* species, meanwhile TB sequence showed a high reliably relation of 99-100% only with the *A. westerdijkiae* specie. FE 1-α sequence for species of section *Circumdati* is not reported yet, so relation was of 94-97 % with *A. terreus* and *A. fumigatus*, however FE 1-α analysis showed differences between strains that belong to the same species.

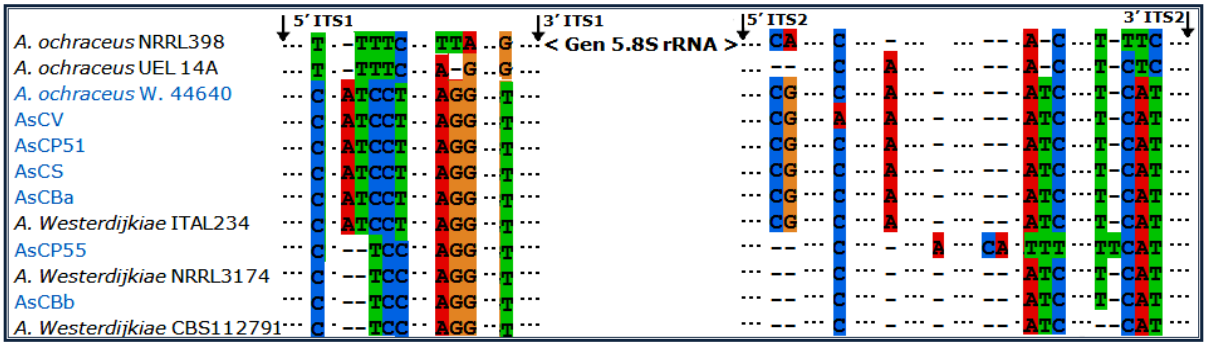

Figure 2. Alignment of complete OTA producing stains ITS1-5,8-ITS2 regions illustrating the sequence divergence among *A. ochraceus* and *A. westerdijkiae* species. The highly conserved region of the 5,8 gene intervening between the ITS1 and ITS2 regions has been omitted.

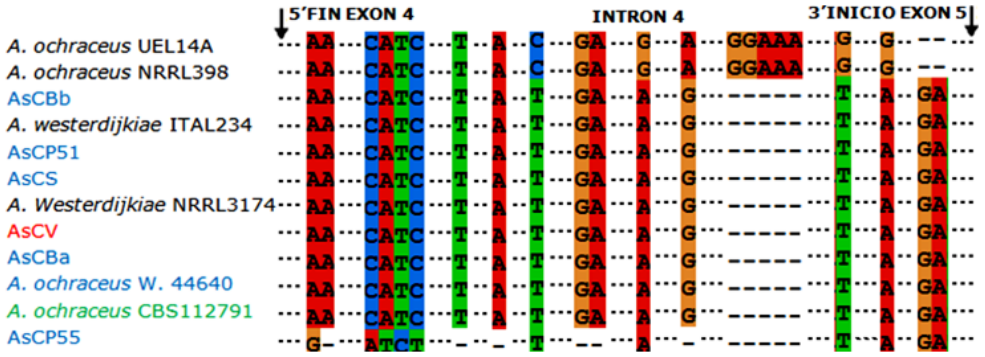

Figure 3. Alignment of OTA producing strains β-rubulin gene partial illustrating the sequence divergence among *A. ochraceus* and *A. westerdijkiae* species. Analysis is just on itrón 4.

CONCLUSIONS

This survey showed that *A. westerdijkiae* is predominant than *A. ochraceus* in coffee. This is not desirable because *A. westerdijkiae* is the highest OTA producing specie and it is very morphological similar to *A. ochraceus* and they are confused.

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Antifungal Properties of Lipophilized Green Coffee Chlorogenic Acid

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SUMMARY

Chlorogenic acid (CGA) extracted from Mexican green coffee beans (*Coffea arabica*) was esterified to alcohols of different chain length (methanol, butanol, octanol, and dodecanol). CGA and lipophilized CGA (L-CGA) antifungal activities were tested on five toxigenic moulds from the *Aspergillus* genus (*A. flavus*, *A. nomius*, *A. ochraceus*, *A. parasiticus*, and *A. westerdijkiae*) using the microdilution method and the minimum inhibitory concentrations (MIC_{50} and MIC_{90}) were determined. All molecules presented antifungal activity, however, only dodecyl chlorogenate and octyl chlorogenate showed a MIC_{50} for all fungi. Octyl chlorogenate had a MIC_{90} for *A. flavus*, *A. parasiticus*, and *A. nomius*. Lipophilization improves hydrophobicity of CGA resulting in amphiphilic molecules with antifungal properties.

INTRODUCTION

Chlorogenic acids (CGAs) are potent natural antioxidants that occur ubiquitously in plants. They are a family of esters formed between certain *trans*-cinnamic acids (i.e. caffeic, ferulic, sinapic, or *p*-coumaric acid), being the 5-*O*-caffeoylquinic acid (5-CQA) (IUPAC numbering; IUPAC, 1976) the commonest individual CGA. Because of their polar nature, they can not be easily used in emulsified systems. To increase their lipophilicity, they can be esterified to a fatty alcohol, for exemple, chemically or enzymatically. This reaction is called lipophilization, and the resulted esters conserve their functional properties, including antimicrobial activity. Ma et al. (2007) synthesized chlorogenic acid analogues possessing a lipophilic chain, including an amino acid group, which presented antifungal activities. Alkyl gallates is another exemple of lipophilized phenolic acids which present antimicrobial activity. The antimicrobial activity increases with the extension of the alkyl chains in the hydrophobic parts of the molecules until it reaches the maximum and then it falls. To the best of our knowledge, 5-CQA and L-5-CQAs antifungal activities on mycotoxin producer moulds have not been published. The aim of this work was to determine the antifungal activity of 5-CQA before and after lipophilization with different alcohols (methanol, butanol, octanol, and dodecanol) on five mycotoxin producer *Aspergillus* moulds.

MATERIALS AND METHODS

Two-steps method was followed to synthesize chlorogenates. A chemical step to obtain the methyl chlorogenates using Amberlite IR120 H was followed by an enzymatic transesterification with each alcohol, using *Candida antarctica* lipase B. The obtained products were characterized by TLC and NMR (Figure 1) The acylation reactions of methyl

chlorogenate were carried out in 50 mL of fatty alcohol (butanol, octanol or dodecanol) and and 20 mg/mL of molecular sieves. Samples of the reaction mixture were dissolved in methanol and filtered. The filtrate was analyzed by TLC and HPLC. Butyl, octyl, and dodecyl chlorogenates were purified in a two steps procedure (liquid-liquid extraction, silica gel 60 column. Collected fractions were followed by a TLC, HPLC and NMR analysis. Antifungal activity was tested on 5-CQA and L-5-CQAs (methyl, butyl, octyl, and dodecyl chlorogenates). Five toxigenic moulds (*A. flavus*, *A. nomius*, *A. ochraceus*, *A. parasiticus*, *A. westerdijkiae*) were tested, and the MIC_{50} and MIC_{90} for each compound were determined.

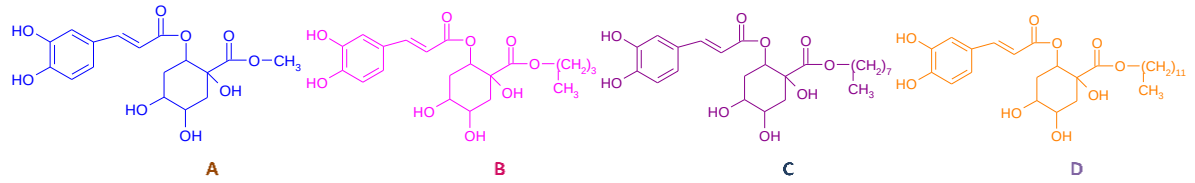

Figure 1. Methyl (A), Butyl (B), Octyl (C) and Dodecyl chlorogenates.

RESULTS AND DISCUSSION

All compounds (5-CQA and L-5-CQAs) presented antifungal activity for the five fungi at the tested concentrations. MIC_{50} values for octyl and dodecyl chlorogenates were inferior to 1.5 mg/mL for all strains. The octyl ester exhibited the highest antifungal activity. These results agree those from Kim et al. (2010) who observed that esterification with octanol greatly enhanced the antifungal activity of gallic acid against *A. fumigatus*, *A. flavus*, and *A. terreus*. The lowest MIC values were observed when applying octyl chlorogenate on *A. parasiticus* for MIC_{50}, and on *A. parasiticus* and *A. westerdijkiae* for MIC_{90}, respectively. The highest MIC_{50} value for octyl and dodecyl chlorogenates were observed with *A. nomius* for the first, and with *A. westerdijkiae* and *A. nomius* for the last, reflecting a relatively higher resistance level of these strains to these compounds. It was demonstrated that lipophilization improved the antifungal activity of 5-CQA on five mycotoxin producer *Aspergillus* moulds. *A. ochraceus* was the most sensible to 5-CQA and *A. parasiticus* was the most sensible to L-5-CQAs. The antifungal activity of the homologous series of 5-CQA alkyl esters was quasi-parabolically dependent on alkyl chain length with a maximum at 8 carbons.

Table 1. Minimum inhibitory concentration (MIC_{50} MIC_{90}) of octyl (CO) and dodecyl (CD) chlorogenates.

Mould	MIC_{50} mg/mL	MIC_{90} mg/mL
Aspergillus westerdijkiae	0,5 CO	X
	1,25 CD	
Aspergillus ochraceus	0,75 CO	X
	0,75 CD	
Aspergillus flavus	0,5 CO	1,5 CO
	0,75 CD	
Aspergillus parasiticus	0,5 CO	1,5 CO
	0,75 CD	
Aspergillus nomius	0,75 C0	1,5 CO
	0,75 CD	

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Development of Primer Sets for Detection of Corn and Barley in Roasted Coffee by Real Time PCR

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SUMMARY

The present study focused on the development and validation of real-time Polymerase Chain Reaction (PCR) systems for the detection of barley and corn in roasted coffee. For this purpose, the extraction of DNA from barley, corn and coffee was performed using a modified version of the DNeasy mini plant Kit protocol. GenBank was accessed to define the target genes for each adulterant and the primers were designed using GeneFisher2 and named CEVADA3 and ZEINA2 for barley and corn, respectively. Primers specificity was demonstrated by using real time PCR with SYBR Green dye. The limits of detection (LOD) of barley and corn DNA were 5,0pg and 0,1pg, respectively, while the limits of quantification (LOQ) were 8.1pg and 0.3pg, respectively. Reaction efficiencies were 104% and 95%, for barley and corn. Despite the low quality of the DNA isolate caused by roasting at high temperatures, it was possible to detect specific DNA sequences from corn and barley in roasted coffee.

INTRODUCTION

Coffee is one of the most popular beverages in Brazil and worldwide. The Brazilian *per capta* coffee consumption in 2012 reached 80 liters, which corresponds to 19.72 million bags. In order to increase profit, during processing, coffee can be adulterated with cheaper materials, especially cereals. The most common adulterants found in roasted coffee worldwide are barley and corn. Although mixing cereals with coffee does not imply in reduction of coffee's nutritional value, adulteration leads to unfair competition and is characterized as a crime under the normative instructions around the world, including in Brazil. In order to guarantee the purity of coffee, highly sensitive and selective methodologies for detection of adulterants must be developed, being DNA analysis a promising tool for this purpose. For this purpose, DNA markers (target) need to be defined and primers designed to delimit endogenous and specific DNA sequences for each species. The primers design take into consideration some important aspects such as the amount of guanine and cytosine (GC), repeated base sequences, as well as other aspects that will affect the temperature of hybridization and possible formation of undesirable primer dimmers.

Considering the needs for sensitive and specific methods to detect commercial coffee adulteration in different markets around the world, the present study focused on the

development and validation of primer sets for the detection of barley and corn, using real time PCR.

MATERIALS AND METHODS

Fresh samples of barley (*Hordeum vulgare*), corn (*Zea mays*) were used as specific target and rice (*Oryza sativa*), wheat (*Triticum aestivum*) and soy (*Glycine max*), were used as non-specific targets. All grains were purchased at a local market and were not genetically modified. Ten green coffee samples (four *C. arabica* and six *C. canephora*) were obtained directly from producers in São Paulo, Espírito Santo and Minas Gerais, Brazil. Coffee was roasted in a fluidized bed roaster (I-roast, USA) at 230°C to give dark colour degree (# 35 Agtrom-SCAA); barley and corn were roasted in a microwave oven to reach the same colour as coffee. Samples were ground in a mill (IKA A11basic to pass a 500µm sieve) Roasted samples were submitted to DNA extraction with DNeasy kit/ buffer CTAB. DNA concentrations were determined in all *in natura* and roasted samples by spectrophotometer (Shimadzu UV-1800 Japan) at 260nm and the absorbance ratio 260:230 was calculated to evaluate the quality of isolated DNA.

Tree DNA sequences corresponding to the endogenous genes for barley, corn and coffee were surveyed from Genbank (accession number NC008590.1, M60837.1, EF044213.1, respectively). Sequences were submitted to the program Basic Local Alignment Search Tool (BLAST) to analyze the similarity among other species. The primer pairs were designed using Genefisher software (Giegerich et al., 1996), setting up the size amplicon of 100 pb. Primers were synthesized by Eurofins MWG Operon and their amplification was confirmed using *in silico* PCR runs at BIOINFX (http://www.bioinfx.net/).

Dilution series of template DNA (0.0002%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100%) were assayed in 10 replicates for each primer pair to obtain standard curves of barley, corn and coffee.

PCR runs were achieved using the SDS-ABIPRISM 7000 (Applied Biosystems). The reaction mixture contained 1 x Power SYBR Green Master Mix (Applied Biosystems) 240nM primers and 50ng DNA in 25µl final volume. Thermal conditions were as follows: 10 min at 95º C, 45 cycles of 15 s at 95º C and 1 min at annealing temperature (Tm) of each primer pair.

RESULTS AND DISCUSSION

Despite the high temperatures applied for coffee roasting, it was possible to extract a satisfactory amount of genomic DNA. According to Martellossi et al., phenolic compounds are PCR inhibitors and because coffee is rich in polyphenols, The ratio between the absorbances at 260 nm and 230 nm in roasted coffee was low (Table 1), indicating that the isolated DNA was not pure. Despite this fact, it was possible to perform the PCR runs.

The primers designed (Table 2) when subjected to analysis *in silico*, with their respective targets, showed only one PCR product, as expected. This result confirms the specificity of the selected primers.

Table1. Coffee Genomic DNA quantification and purity.

Samples	Abs_{260}	Abs_{230}	Ratio Abs_{230}/Abs_{260}	[DNA] ng/µL
Green coffee	0,097±0,025	0,051±0,060	1,94	127,5 ± 35,5
Roasted coffee	0,043±0,016	0,068±0,030	0,63	33,7 ± 17,3

Results are mean ± SD; n = 10.

Table 2. Primer parameters for barley, corn and coffee markers.

Food	Primers	Sequence	Tm (°C)	Efficiency(%)	R^2	LOD (pg)	LOQ (pg)
Barley	Cevada 3	F: CCGGACCAGAACTTCTTG R: CCTGAAGCACGATTTCTG	60	104	0,99	5,0	8,1
Corn	Zeina 2	F:CAGGCTCCAACAAGCAATG R:GCAACTGTTGTGCCCTGATG	62	95	0,99	0,1	0,3
Coffee	Café 1	F:TTCCGAAGTCCTGGAGAG R:CGGAGGATATCTCAATCG	60	88	0,99	0,7	2,4

F/R= Forward, Reverse primers. Tm = annealing temperature. LOD and LOQ= Limit of detection and limit of quantification. R^2- Correlation coefficient.

The obtained data for efficiency, correlation coefficient of the standard curves, LOD and LOQ were very acceptable when compared to data reported in established protocols for validation of analysis for detection and quantification of DNA sequences, being evident that the use of this molecular tool has potential applications for quality control in the coffee industry. The melting curves (Figure 2) indicate if the amplification is specific or nonspecific using a temperature ramp where the expected product presented a sharp peak with specific temperature. The melting curve for Cevada3, Zeina2 and Coffe1 demonstrated that these primer pairs were specific for barley and corn detection and nonspecific amplification peaks were attenuated by increasing Tm.

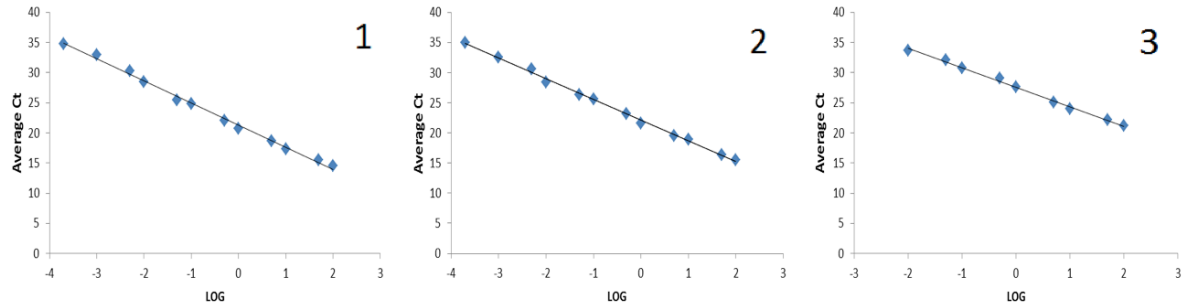


Figure 1. Standard Curves for coffee (1), barley (2) and corn (3) was used for determination of LOD and LOQ. Dilution series of DNA template: corn 0,01% to 100% of 50 ng (m/v), barley and coffee 0,0002% to 100% of 50 ng(m/v). Ct = average of 10 replicates.

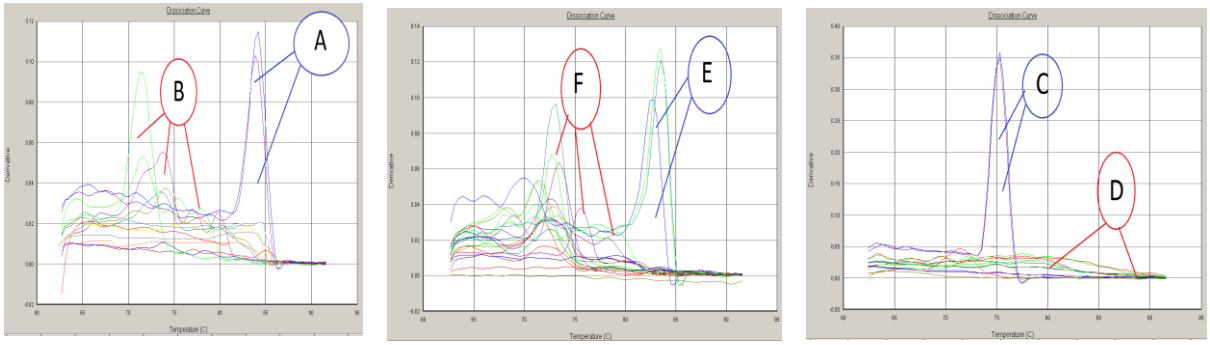
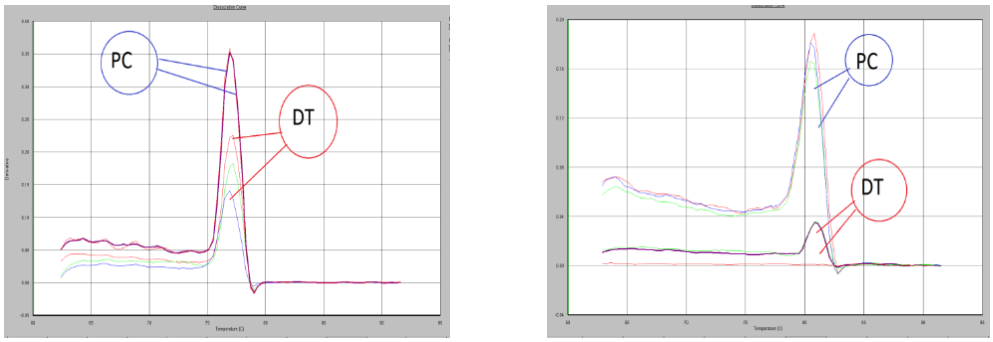


Figure 2. Primer Cevada3 (AB), Café1 (CD) and Zeina2 (EF). Letters A, C and E show specific amplifications and letters B, D and F show nonspecific amplifications like primer-dimer. Each primer pair was tested with genomic DNA of target and non-target foods (rice, wheat, soy, corn, barley, coffee *arabica* / *robusta*).

Even in a highly degraded DNA, the markers (primers) were able to detect the presence of adulterants in coffee intentionally adulterated with barley and corn as shown in Fgure3.



1 2

Figure3. PC-Positive control, DT- Detection of adulterant. (1) primer for roasted barley (Cevada3); (2) primer for roasted corn (Zeina2).

In the present work, it was possible to analyze the genomic DNA from processed coffee, barley and corn. The designed primers proved to be sensitive and specific for each target, being able to detect adulterated coffee samples. Therefore, real time PCR showed to be a promising tool in the identification of barley and corn as coffee adulterants in the market.

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Characterization of Fiber Modified Spent Coffee Grounds for Treatment with Alkaline Hydrogen Peroxide

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SUMMARY

The objective of this study was to modify the hydration properties of spent coffee grounds and characterize it. The coffee ground treated was analyzed for chemical composition, dietary fiber, fiber fractions and water retention capacity, all conducted at the Vegetable Products Laboratory, Department of Food Science, Federal University of Lavras. The modification of the coffee grounds fiber caused a 30% increase in water retention capacity compared to untreated coffee grounds. This treatment allowed obtaining a product with high protein content (13.16%), fat (17.89%) and total dietary fiber (64.19%), which is composed of soluble dietary fiber (0.58%) and insoluble dietary fiber (63.61%). The fiber fraction was composed of cellulose (45.46%), hemicellulose (2.61%) and lignin (21.48%). Therefore, by its chemical composition characteristics, the modified coffee grounds may be used as a source of fibers for the manufacture of products with functional appeal.

INTRODUCTION

Currently, researchers and the food industry have shown interest in the study and development of food that features, besides nutritional and sensory value, substances that facilitate biological functions to decrease the risk of disease and promote the health of consumers. Foods containing such components are termed functional foods, the fibers of which stem from the ingredients used in these foods.

The technological application, when trying to formulate a product with high fiber content, it presents some problems that often limit its use. Thus, there is often a need to modify the functional characteristics of these materials prior to incorporation in food. In lignocellulosic materials, the negative effects can be minimized by the application of treatments, physical or chemical, to simultaneously solubilize the lignin and decrease the crystallinity of cellulose. This increases the hydration capacity of these materials, improving therefore the sensory characteristics of the final product will be used where the fiber, especially in terms of tactile sensation in the mouth.

Currently, DF-rich ingredients mainly come from cereals because of their low cost and flexibility of use. However, other raw materials can be considered, such as coffee, whose byproducts and waste are low-cost and available worldwide.

Coffee grounds are the residue obtained during the processing of coffee powder with hot water when preparing soluble coffee. Almost 50% of world coffee production is processed for the preparation of soluble coffee. Therefore, coffee grounds are generated in large quantities, with an annual output of 6 million tons worldwide. Some attempts to reuse SCG have been made, however, none of these strategies has yet been routinely implemented, and most of these residues remain unutilized, being discharged to the environment where they cause

severe contamination and environmental pollution problems. The scientific literature does not mention the specific use of coffee grounds as a source of dietary fiber, however, studies such as those of Diaz-Rubio and Saura-Calixto and Borrelli et al. indicate the potential use of coffee and its byproducts as a source of dietary fiber.

The objective of this study was to modify the hydration properties of spent coffee grounds and evaluate the possibility of use as a source of dietary fiber for production of functional foods, studying its chemical characteristics.

MATERIALS AND METHODS

The spent coffee grounds used in the study was donated by the company Soluble Coffee Brasilia, located in Varginha / MG, Brazil. The coffee residue was treated with a solution of alkaline hydrogen peroxide for modification of the physical and functional properties. The spent coffee grounds was treated with 1% alkaline hydrogen peroxide for 5 hours at 30°C. Samples of coffee grounds treated were dried in an oven (65°C) for 24 h before the analyses.

Dietary fiber and chemical composition of the samples were determined according to the methods described by AOAC. The fiber fraction was determined by a modified procedure of Van Soest. The Water Holding Capacity (WHC) was determined according to Robertson et al.

RESULTS AND DISCUSSION

The composition of spent coffee treated is shown in Table 1.

Table 1. Chemical Composition of Coffee Grounds Treated (CGT).

parameters	Protein	fat	ashe	carbohydrates[b]
CGT	13.16±0.74	17.89±0.30	2.13±0.07	37.48±1.20

[a] *Values are given per 100 g of dry matter.* [b] *Calculated by difference.*

The coffee grounds treated (CGT) had a significant amount of protein (13.16%). The value obtained in this study was lower than that reported by Ravindranath et al. about 14%, but was higher than the value reported by Lago et al. which varied between 6.7% and 9.9%. Differences in the chemical composition of CGT may occur according to the variety of beans utilized and the roasting and extraction process to which they were submitted. Coffee bean oil is rich in fat, and to produce soluble coffee, during processing, only a part of this fat is used for instant coffee (0.1%), the remaining fat is found in SCG (20%).24 In the CGT analyzed, the oil content was found to be 17,89%, Lago et al. observed an CGT oil content that ranged from 19.90 to 27.83%. According to Turatti the oil present in CGT can be used in food products as a filling for candy or even as a flavor enhancer in soluble coffee, as well as use in formulation of coffee liqueurs. Kondamudi et al. reported that the oil of CGT may be converted into a similar amount of biodiesel using transesterification methods. Coffee grounds treated also contain ash. According Arya and Rao after extraction with hot water to prepare instant coffee, most of the mineral elements contained in the CGT are easily extracted, but a small amount still remains in the CGT, where potassium is predominant. It was observed that CGT exhibit a high carbohydrate content (37.48%) (Table 1). According Mussato et al. CGT are rich in polymerized structures of cellulose and hemicellulose, the hemicellulose is composed of three sugars, mannose (46.8%), galactose (30.4%) and arabinose (30.8%), and cellulose is composed of glucose (19.0%).

Spent coffee grounds show a high fraction of Insoluble Dietary Fiber (63.61%), which corresponds to 99% of Total Dietary Fiber (Table 2). After comparing the data from the CGT with those of the other ingredients of cereals, it is clear that Soluble Dietary Fiber has an equivalent level of Total Dietary Fiber (64.19%). The amount of Soluble Dietary Fiber (0.58%) in CGT is low compared with that in the other ingredients. Insoluble Dietary Fiber is important in regulating bowel functions. Several studies have shown that Insoluble Dietary Fiber (lignin, cellulose, etc.) plays a role in the prevention of intestinal cancer, possibly because of the improvement in intestinal motility, meanwhile, soluble dietary fiber reduces cholesterol and glucose absorption. Although coffee grounds tend to be low in soluble fiber, they can be used as functional ingredients to develop foods that are rich in insoluble fiber and thus prevent constipation.

Table 2. Dietary fiber content of coffee grounds treated and different types of cereals[a].

	CGT	wheat bran [b]	Oat bran [b]
Total dietary fiber	64.19±1.01	44.00	23.80
Soluble Dietary Fiber	0.58±0.01	2.90	3.60
Insoluble Dietary Fiber	63.61±1.02	41.10	20.2

[a] *Values are given per 100 g of dry matter.* [b] *Data from ref Food Res. Int., 1998.*

Fractionation of dietary fiber in coffee grounds treated is presented in Table 3.

Table3. Fiber composition of the coffee grounds treated (CGT)[a].

parameters	Cellulose	Hemicellulose	Lignin
CGT	45.34±0.25	2.61±0.27	24.38±0.14

[a] *Values are given per 100 g of dry matter.*

The main advantage of fractionation is additional information about the fiber components, facilitating the understanding of the variability in the physiological and physicochemical properties of different food-induced fiber sources, since the amounts of dietary fiber alone cannot predicting the actual functional property of the fiber.

Azevedo determined the fraction of fiber in coffee grounds treated and found cellulose 43.40%, soluble hemicellulose 2.25% and lignin 23.30%, results close to those reported in this study.

Comparison of the results of water retention capacity (WHC) of the coffee grounds, before and after treatment is shown in Table 4.

Table 4. Effect of treatment with alkaline hydrogen peroxide in water retention capacity of the spent coffee grounds.

CGT	WHC
without treatment	2.49
with treatment	3.23

Treatment improved by 30% the water retention capacity of the coffee grounds treated compared to untreated coffee grounds. The increase in the CRA is consistent with the idea that these treated materials have more open structure with porosity increased, which facilitates retention of water.

The results presented in this study show that treatment allows the obtention of a product with a water retention capacity greater than the untreated material, coffee grounds presented himself as a byproduct rich in its composition, can be used by various sectors of industry. By removing the oil from coffee grounds for the production of biodiesel or for use as a flavor enhancer in food products, we obtain cake-rich insoluble fibers and significant amounts of protein. Owing to its unique chemical composition, coffee grounds could be used as a source of fibers for the manufacture of products with functional appeal.

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Optimisation of Chromatographic Techniques for the Isolation and Analysis of Volatile Aromatic Compounds in Commercial Coffee Samples

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SUMMARY

The extraction method chosen for coffee volatiles in this project was the simultaneous distillation extraction (SDE). The concentration of the extract was compared using two methods: nitrogen gas (N_2) evaporation and Kuderna-Danish concentration. It was found that N_2 evaporation could concentrate the extract more efficiently and to a smaller volume. Tetradecane was the internal standard used for quantitation of the aroma compounds. The extracted volatiles were separated and identified using an optimised Gas Chromatography Mass Spectrometry (GC-MS) method. The extraction method was used to characterise the aroma profiles from commercial samples of coffee from various origins: Zentveld's *Ernesto Roast* (Australia, standard); *Highlands Coffee* (Vietnam); *Kapal Api* (Indonesia); *Suan Ya Luang* (Thailand); *Cafe Canecão* (Brazil); *Cafe Aguila Roja* (Colombia); *Kopi Luwak* (Indonesia); *Loa Sinuak* (Lao); and *Goroka Coffee* (Papua New Guinea). Important character-impact aromatic compounds (furans, pyrazines, pyridines, pyrroles, phenols and esters) were found in all samples. In contrast, sulphur containing compounds varied more, and affect more the perceived aroma of the coffee. The sulphur compounds, present in each of the coffees although at much lower concentration, are more powerful and have a potential to influence coffee aroma. It is likely that the presence of sulphur strengthens the character impression of the overall aroma and strikes a familiar scent. The ability of sulphur compounds to have an 'odour addition', or synergism to other aromatic compounds, could indicate that the particular concentration and mix of the sulphur compounds in a coffee, will distinctly define its aroma.

INTRODUCTION

Coffee is a widely consumed beverage, and contains one of the world's most widely consumed psychoactive stimulant compounds, caffeine. When consumed by humans, caffeine stimulates the central nervous system, reduces drowsiness temporarily, and increases alertness and heart rate. As the effects of caffeine in coffee appear to be sought out, the unique essence of flavour and distinct pungent aroma of coffee are other attributes that are a pleasure to taste and smell to consumers, and non-consumers. Long-time consumers take preference of certain brands, blends and roasting grades. Next to quality and quantity of colour pigments, the composition of various aroma substances in a food product can greatly affect consumer preference and inherently the commercial value of it. The food industry is quickly catching onto the impact flavour and aroma can affect the consumer. The ability to be able to replicate nature-identical flavours and aromas is of high value in the food flavour industry. Advances in chemical analysis and organic chemistry has paved the way for this technology. In a sense, to know the composition of an aroma, is to have the means to produce a close replicate, and an insight to those precious few compounds that could make all the difference to consumer preference. The flavour and aroma of coffee are complex. A vast number of compounds

contribute to coffee aroma and flavour and even its texture. Compounds that exist in coffee are acids; aromatic compounds (volatiles of high and low volatility), and bitter substances (acids, proteins, furans, pyrazines, caffeine). Coffee aroma and flavour have been of interest to researchers for some time. There are many methods which were developed to analyse the chemical composition of coffee aroma and new improved methods continue to be developed. The aroma analysis includes the extraction and the identifications steps.

The aim of the project was to optimise existing flavour extraction and identification methods and to analyse and compare *Arabica* coffee beans from different regions. An important stage of this analysis was the concentration of the extracted volatiles. This step has always been a challenge with volatiles, as they need to be treated carefully to minimise loss into the surrounding environment. This study compares two existing methods, one very common (nitrogen gas) and another, supposedly more efficient, Kuderna-Danish concentrator. Gas chromatography program was optimised. Comparative testing was carried out to display the difference in chromatograms of a coffee sample treated in the same way, but over a range of different oven programs. The optimised method was used to analyse regional coffee samples.

MATERIALS AND METHODS

The coffee samples were roasted *Arabica* coffee beans sourced from various locations:

- Standard sample: Australia, Zentveld's Coffee Ernesto Roast.
- Vietnamese – *Highlands Coffee*, Loai.
- Indonesia – *Kapal Api*, Kopi Medan.
- Thailand – *Suan Ya Luang.*
- Brazil – *Cafe Canecão*, Tradicional.
- Colombia – *Cafe Aguila Roja.*
- Indonesia – La Jaranica *Kopi Luwak.*
- Laos – *Lao Sinouk*, Scandinavian Roast.
- Papua New Guinea – *Goroka Coffee.*

The equipment used in the experiments consisted of:

- Sunbeam™ (Botany, Australia), domestic coffee grinder for sample preparation.
- SDE (Likens & Nickerson) apparatus; 2 × 500 mL round bottom flasks; 2 pear shaped flasks; conical flask; extractor; condenser and 2 electric jackets for aroma extraction.
- Kuderna-Danish concentrator with a 500 mL flask (Sigma-Aldrich), 1 × 1000 mL beaker; 3 boiling chips; 1 × 15 mL condenser tube graduated with hooks and connecting springs; 3 ball macro-economy Snyder column; condensate trap and condenser.
- GC-MS Agilent Technologies Inc. model 6890 N (Wilmington, DE, USA) with 7683B Series Injector, 6890N Network GC system and 5975 Inert Mass Selective Detector.
- GC column SGE (Ringwood Vic, Australia) Solgel-wax™ Model SGE054796, stationary phase: polyethylene glycol; 30.0 m (L) × 250 μm (ID) × 0.25 μm (FT).

The chemicals included solvent, dichloromethane (CH_2Cl_2), internal standard: tetradecane (C_{14}), anhydrous Na_2SO_4, acetone (Sigma-Aldrich), and dry CO_2 (BOC).

The extraction procedure in the SDE apparatus included placing approximately 24 g of ground coffee dissolved in 200 mL of distilled water in the lower positioned flask and 80 mL of dichloromethane in the higher positioned flask. The top extremity of the extractor was connected to a cold trap condenser containing dry ice and acetone. The lower part of the

extractor was connected to a water-inlet pipe whilst the higher part was connected to a water-outlet pipe. The extraction process commenced with heating the solvent flask with dichloromethane using medium to high temperature. When the U bend in the apparatus began to fill up with the dichloromethane, the coffee sample was brought to boiling. The extraction time was set to 2 h. If any water seeped into the extracted sample, anhydrous Na_2SO_4 was used to absorb the water At the end of the extraction process internal standard was added to the solvent flask. The extract was concentrated by solvent evaporation. The methods compared were: Kuderna-Danish vs N_2 gas. Only standard sample was used for the comparison. GC/MS was used for the identification of the aroma compounds. Four methods were applied to the standard sample (see Table 1).

Table 1. GC-MS method parameters.

Method/ Injection/ flow rate	Solvent delay (min)	Start (°C)	Hold (min)	1st Rate (°C/min)	2nd (°C)	Hold (min)	2nd rate (°C/min)	3rd (°C)	Hold (min)	3rd rate (°C/min)	4th (°C)	Hold (min)
A: 4 µL, split, 1.2 mL/min	4.10	30	0.50	50	60	0.00	3	250	10.00	~	~	0.00
B: 5 µL, splitless, 1.0 mL/min	3.80	30	0.50	2	70	10.00	4	250	0.00	~	~	0.00
C: 5 µL, splitless, 1.2 mL/min	3.80	30	0.50	2	70	0.00	3	130	0.00	4	250	10.00
D: 5 µL, splitless, 1.2 mL/min	3.80	30	0.50	2	120	0.00	4	250	5.00	~	~	0.00

The identification of significant compounds in each sample was conducted with the use of the mass spectral library WILEY275.

The quantitation of the compounds was related to the concentration of internal standard. Each compound of interest was manually integrated using the Agilent software. The concentration of that compound was then calculated by multiplying the known concentration of the internal standard, by its ratio to the internal standard integral.

RESULTS AND DISCUSSION

The Likens and Nickerson extraction method that was used to isolate and extract the volatiles present in the sample proved to be well suited to the aim as the aromatic compounds were not affected by the excessive heating. The method was simple and effective, and upon repetition, the apparatus gave consistent volumes of extract and mass spectral results.

The compound identification was primarily done using WILEY275 spectral library and most compounds had identifications of between 80 to 98% quality. It is not uncommon for the MS software to 'misread' the ion spectra, and name the compound incorrectly. The identifications were double-checked by examination of the spectra.

The chromatograms produced after injection of the concentrated extracts obtained by Kuderna-Danish and nitrogen gas are shown in Figure 1, A and B respectively. The Kuderna-Danish concentrator did not yield as great abundance of the aromatic compounds as the

nitrogen gas concentration method. Similar result was obtained at lower temperature and thus the latter could be eliminated as a possible cause.

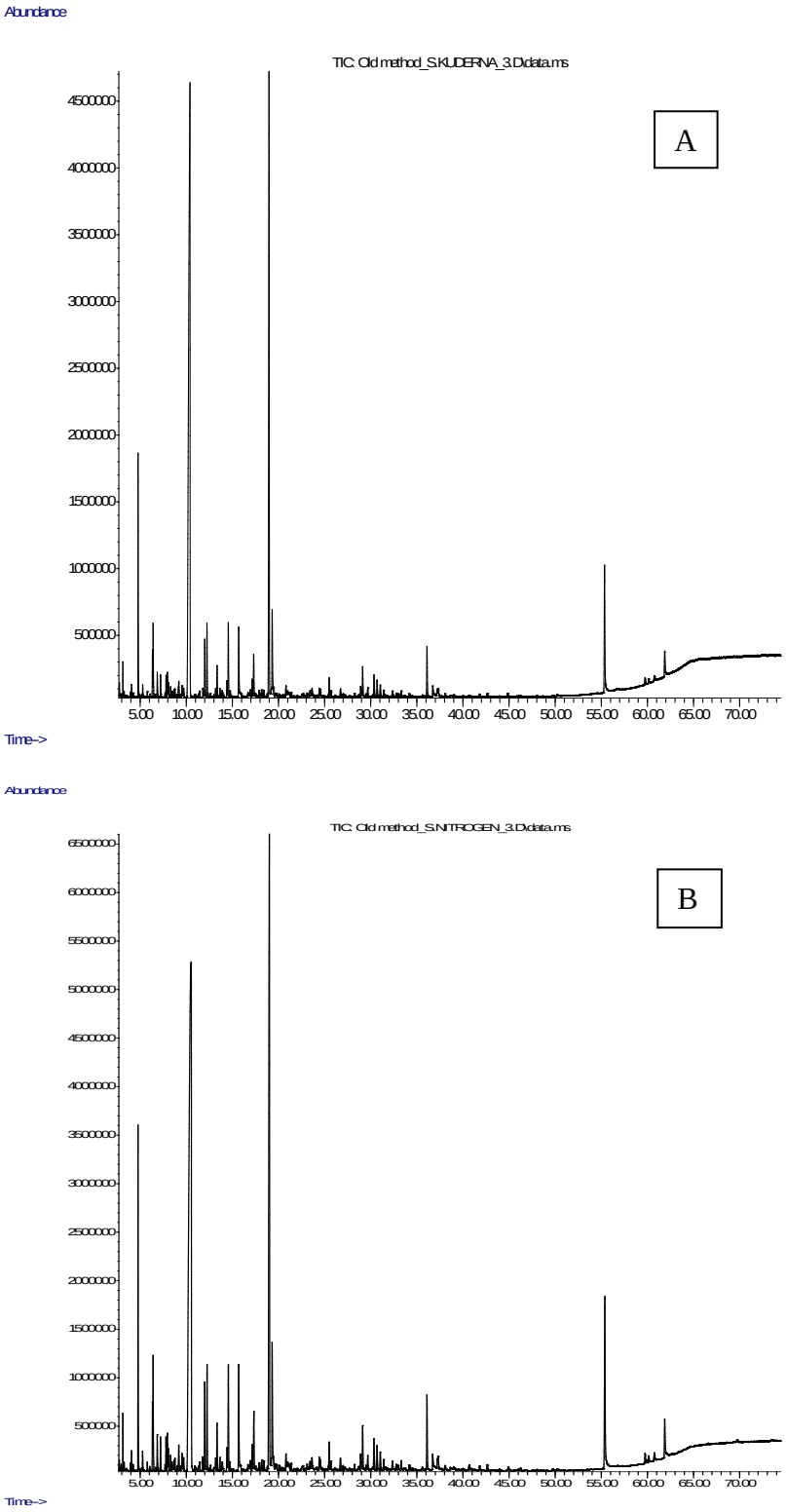

Figure 1. Comparison of abundance of aroma compounds in standard coffee (Ernesto Roast, Zentveld's, Australia) after concentration with time in minutes. A: Kuderna-Danish; B: nitrogen gas.

To optimise the GC-MS program, four methods (Table 1) were applied to the standard sample. Method A, described in the *Proceedings* of the *International Workshop on*

Agricultural and Bio-systems Engineering, intended to increase peak resolution and reduce overlapping. This method was the first tested, and gave a very neat chromatogram, with clear resolution, and no overlapping peaks. The method did not give many peaks, however. For this reason, method A was used as a focus for the optimisation of solvent evaporation. Methods B, C, and D were developed from method A, by alteration of certain oven, inlet and flow rate parameters. The spreading of the chromatogram peaks was not achieved very well with method B but the amount of compounds that the MS detected was significantly higher. This might have been due to the steady temperature increase rate and lack of temperature rate change between 70 and 250 °C. Method C employed an extra temperature rate to spread out and sharpen peaks in the middle of the chromatogram. This was achieved well. To reduce the baseline and further increase resolution along the chromatogram, the initial slow temperature increase rate interval was extended in method D (Figure 2), producing an exceptional chromatogram, with sharps peaks and almost no baseline. As a result, method D was used in the other tests.

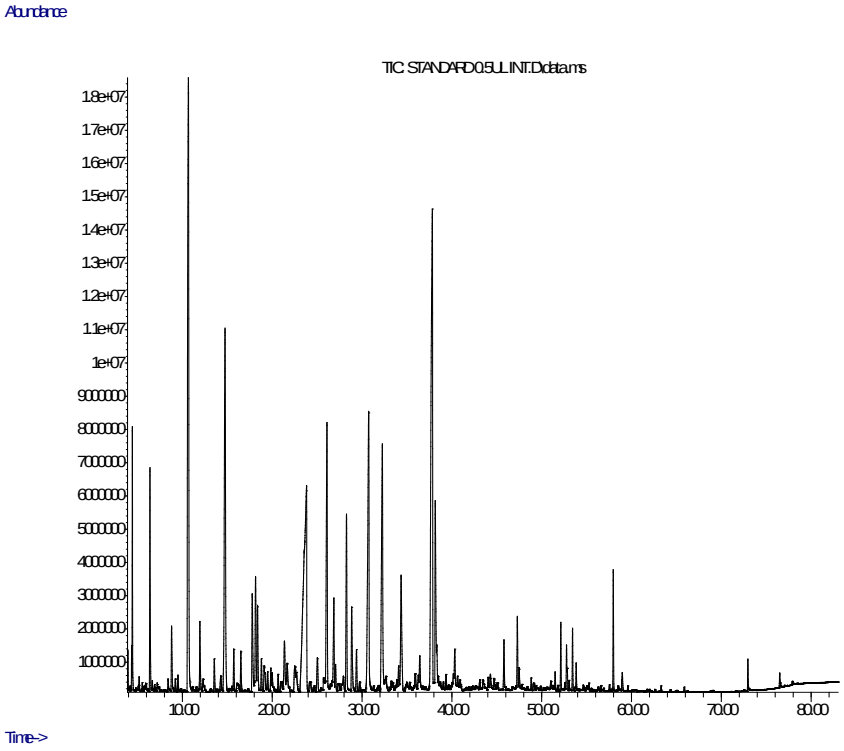

Figure 2. Chromatogram of standard sample, GC-MS method D, Abundance vs. Time (min).

As far the determination of character impact compounds in the regional samples is concerned, the aromatic compounds identified were, in the majority, expected and most compounds appeared consistently across most of the samples (Figure 3). These appear to be generic or standard aromatic compounds present in *arabica* coffee. Some samples, however, presented smaller profiles and uncommon compounds. The Indonesian *Kopi Luwak* is one of these samples. The coffee itself has a unique harvest source and claims to have a very specific "smooth, caramel and chocolate" flavour. The amount of caffeine present in the coffee is certainly low enough to be at an almost negligible level, and its absence might also contribute to supposed higher quality taste. Another coffee of small aromatic profile was the *Goroka* Coffee, an organic sample form Papua New Guinea.

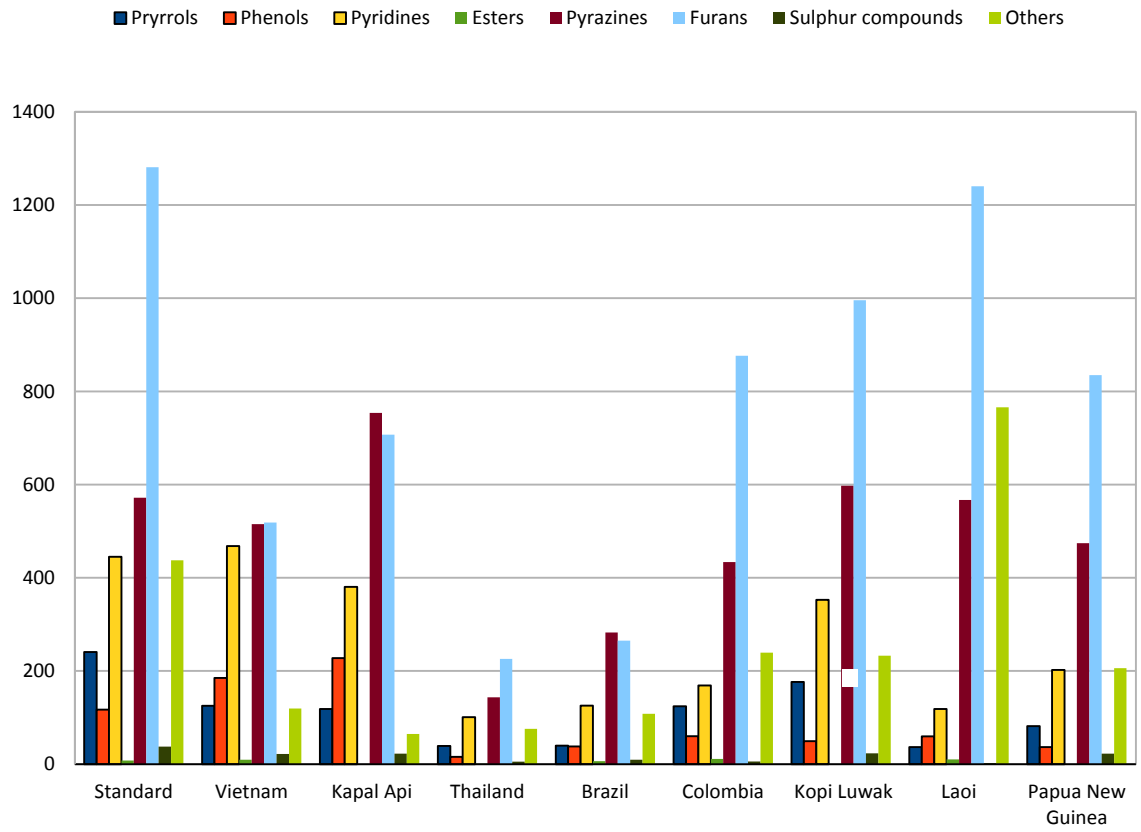

Figure 3. Aromatic compounds in regional coffee (µg/mL). Kapal Api/Kopi Luwak are from Indonesia.

Figure 3 shows the compounds considered to be the most representative of the coffee aroma profile. Not every coffee sample will possess the same volatile compounds just at different levels. This project reveals that the origin of the coffee might well very much affects the compounds it possesses and their concentrations. The concentration of aromatic compounds has a strong influence on the entire aroma of the produce. The perceived scent of a compound can change from extremely pungent and unpleasant at 100% concentration to light and pleasing aromas when at concentrations as low as < 10%. The 'others' category in Figure 3 refers to the remainder of the compounds identified, but not considered as character impact aroma compounds. Furans, pyrazines and pyridines are the volatile aromatic compounds that dominate each coffee sample.

Pyrolytic degradation of sugars is responsible for the formation of furans. Their presence in coffee is due to roasting, and is considered to be important for quality of coffee flavour and aroma. Furfuryl, a product of caramelisation is a common flavour constituent, characteristic if a toasty odour. Furanones are other significant furans that have a sweeter and fruitier scent, also giving burnt caramel-like nutty impressions, but similar still to furfuryls. 1-(2-furyl)-2-propanone gives a rum-like aroma.

Pyrazines are a class of compounds believed to contribute to the desireable flavour of foods. Alone, pyrazine is more bitter sweet in scent. Alkyl pyrazines have a higher concentration at lower roasts and give the nutty, roasted and burnt note. The odour attribute given to the coffee by methyl pyrazine is a mixture of chocolate, nutty, earthy and green.

Pyridine, a pyrolysis product, is made with the thermal reaction between sugars and amino acids, or alkanals with amino acids comparing with other classes of food flavour/aroma compounds, relatively few pyridines occur naturally, but are still considered to have a significant organoleptic contribution. Pyridines will appear as a 'basic' food aromatic compound, but will be mostly dominated by pyrazine. Pyridine has been described as a pungent, penetrating and somewhat nauseating odour, but in dilution it becomes much less offensive, and has a warm, burnt and smoky character. With respect to the coffee samples analysed, pyridine itself appears in every coffee, as the majority in its class, and again, the other most abundant pyridines identified were alkyl-substituted pyridines. Pyridine will give a green and bitter astringency whereas the alkyl substitutions can give buttery and caramel odour qualities, although keeping the astringency.

Pyrroles, found more in coffee than any other food, are produced thermally, like furans, pyrazines and pyridines, from a reaction between amino acids and sugars. The concentration of pyrroles is proportional to the roasting degree, increasing with it, and aroma will vary depending on the degree of roast also. The aromas that pyrroles can emit are mushroom and oily scents, green and hay-like, sweet, corn-like or slightly burnt (alkylpyrroles). Structurally, they are closely related to furans.

Phenol concentration increases with roasting degree, and is mostly described as having a 'smoky' aroma.

Esters in certain combinations play a strong role in fruit, cocoa and tea flavours and aromas. It appears that most esters give sweet floral scents suggestive of fruit and herbs. Some, however, can have pungent and bitter astringencies. Esters are of lesser representation in coffee and are not considered potent contributors to coffee aroma.

The aroma of a coffee can be defined by a spectrum of certain compounds at certain concentrations and 'odour addition' mixtures, or synergisms made possible by sulphur compounds. Figure 4 shows the major sulphur compounds found in the regional coffee samples.

Contributing to the pleasant and unpleasant flavours and odours, sulphur compounds are essential for character impact of roasted coffee. Sulphur groups on any compound will strongly bind to the olfactory receptors, and therefore have a very high aroma impact. Most sulphur compounds thermally produced contain heterocyclic sulphur. Over 250 thermally produced sulphur compounds have been identified, and most present in coffee, and meat. At high concentrations, stench aromas are produced from many sulphurous compounds – so much so that they are added as odorants to natural gases so that leaks can easily be detected by the stench.

Meaty characteristics are given by furans with thiol, methylthio and disulfide substituents. Sulphur compounds with a thiol function are well represented in wine aroma and flavour. Berger mentions research conducted by a French enologist, Emile Peynaud and quotes him *"fermentation brings out the primary aroma hidden in the fruit* [grape]*"*. This refers to specific enzymic activity exhibited by yeasts during fermentation which are responsible for the release of thiol odorants.

The odour threshold value of simple alkylthiazoles has a range of 1 to 1000 µg/kg, typically with a green, vegetable-like, cocoa, nutty, and some meaty characteristics. Furans with thiol, sulphide or disulphide substitution are noted as important meat and coffee volatiles.

Formation of these involves interaction with hydrogen sulphide, dicabonyls, furanones and furfuryls.

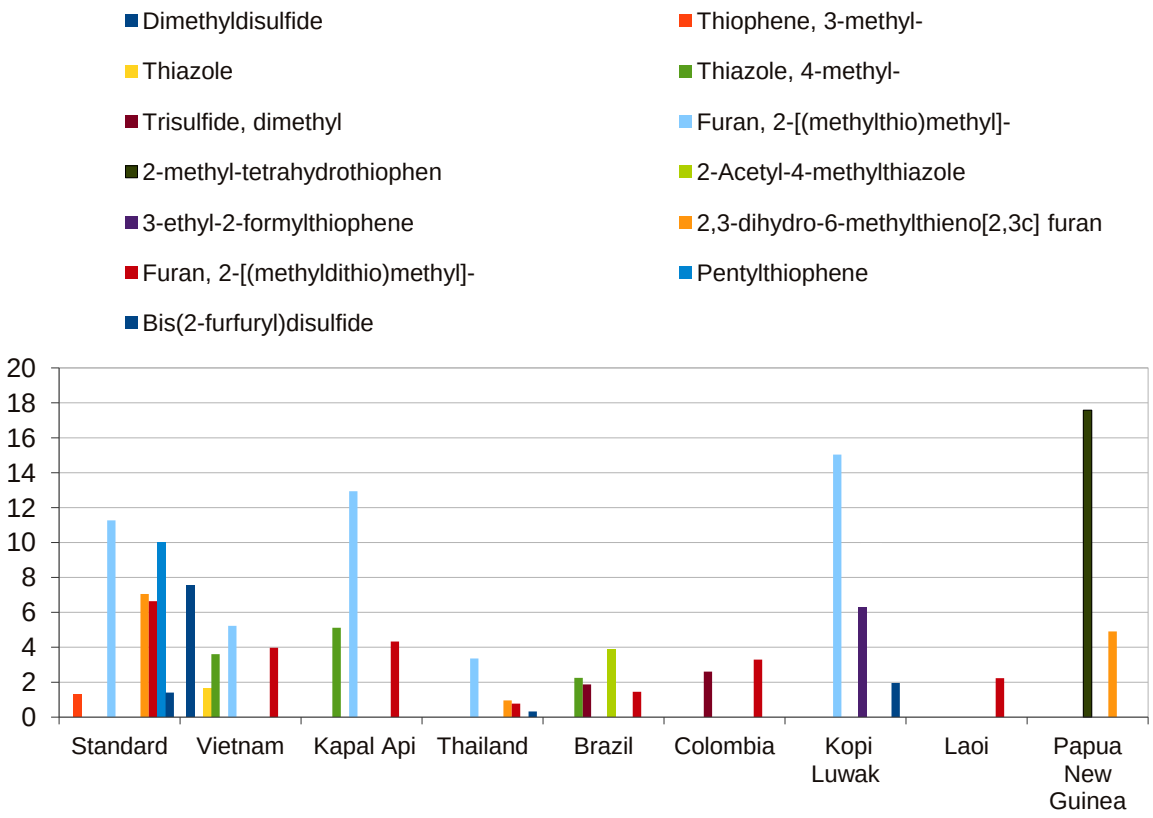

Figure 4. Concentration (µg/mL) of all sulphur compounds identified in each coffee sample.

CONCLUSIONS

The comparison of concentration methods (Kuderna-Danish vs. nitrogen gas) showed the advantage of nitrogen gas over macro Kuderna-Danish concentrator by extracting volatile compounds with a higher abundance. However, a micro Kuderna-Danish concentrator may be able to concentrate to smaller volumes more easily than the macro and should be compared with the N_2 concentration method.

The GC-MS methods were optimised as much as possible, giving a reasonable run time and exceptional peaks. Slowing temperature rates any further, will risk an overloaded internal standard peak.

As for character-impact odorants of each coffee sample shows that the most abundant classes were furans, pyrazines and pyridines respectively, with pyrazines showing in a higher proportion in the Brazil and *Kapal Api* samples, but only marginally (Figure 3). These classes of compounds are all present in the roasted coffee beans as products of the thermal reactions that take place during roasting. Due to the similarity in trend of these classes in each coffee, it is assumed that the aroma they contribute to each individual coffee are similar, however varying in intensity.

The sulphur compounds present in each of the coffees, although at much lower concentration, are more powerful and in effect have a potential to have a greater influence on aromatic character. The standard Australian Zentveld's Ernesto Roast coffee shows the highest amount

of sulphur containing volatiles (Figure 5) inherently giving it the most diffusive aroma. The Vietnamese Highlands; Indonesia's *Kapal Api* and *Kopi Luwak*, and Papua New Guinea's Goroka Coffee coffees contain almost the same concentration of aromatic sulphur compounds. It is possible that the presence of sulphur in the volatiles strengthens the character impression of the overall aroma and strikes a familiar scent. The ability of sulphur compounds to have an 'odour addition', or synergism to other aromatic compounds, could indicate that the particular concentration and mix of the sulphur compounds in a coffee will define its aroma distinctly.

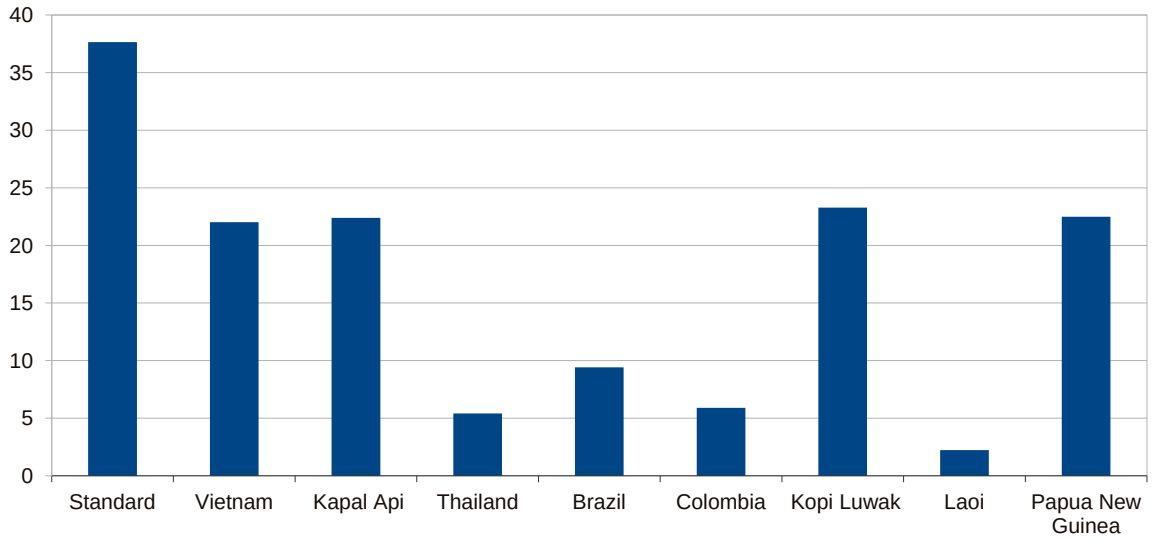

Figure 5. Total concentration of Sulphur compounds in each coffee (µg/mL).

ACKNOWLEDGEMENTS

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Gasification of By-Products from the Coffee Processing for Power Generation

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SUMMARY

This project consists of a pilot plant for the use of coffee parchment husk and dry coffee pulp, as biomass to fuel a thermal unit fixed bed gasification, low cost that can generate electricity supplying a generator with a capacity of 60 kW, with the aim of moving part of the production of a coffee Processing plant in the Los Santos, Costa Rica. The type of gasifier developed fixed bed is the descendant flow (Downdraft) in this system are passed biomass by high temperature zone of the gasifier, known as combustion or gasification of the system, making this biomass into carbon monoxide and hydrogen. The gas is collected from the bottom of the gasifier and the combustion residues are collected in the base of the gasifier.

INTRODUCTION

The efficient use of energy resources requires conducting research using coffee by-products in the most efficient possible economic and technical. This leads to conversion of biomass to cleaner energy such as electricity generation that drives the process of coffee processing in the country, to start and be energy self-sufficient approach.

Gasification of biomass is a process pyrolytic optimized, by means of which a liquid or solid substance with a high content of carbon, is transformed by its combustion in a combustible gas mixture to obtain a gas similar to other conventional gases.

Unlike other biomass utilization processes such as incineration or combustion direct using normally by Milling companies where complete combustion takes place, the gasification is controlled in the presence of oxygen to promote the reduction of organic matter. Additionally, gasification optimizes the use of biomass between 30 and 40 percent compared to combustion with excess oxygen used, also optimizing the results of the transformation process.

In essence the gasification process is the conversion of solid biomass into a fuel gas containing carbon monoxide, hydrogen and methane primarily through a thermochemical process. This process is carried out in a closed and sealed chamber that operates slightly below atmospheric pressure and takes place in several stages as shown in Figure 1.

Environmentally does not destroy the fuel gasification, organic compounds dissociate and become gas, which is channeled to a combustion engine or a gas turbine, which results in a much more energy efficient process.

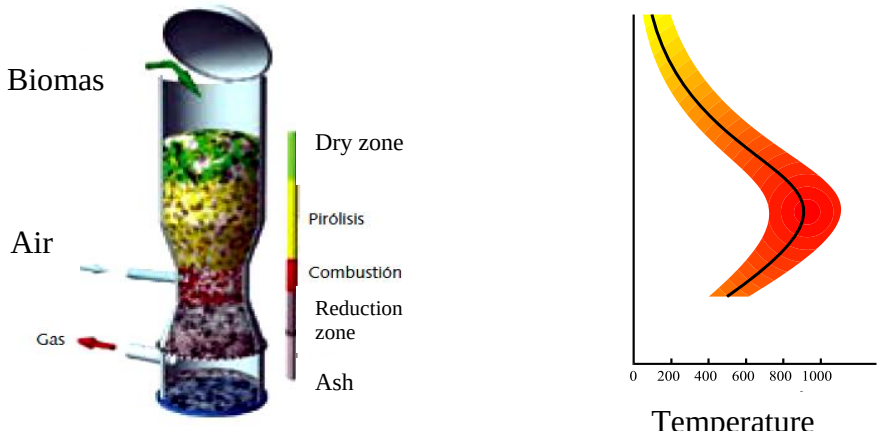

Figure 1. Process description downflow fixed bed gasification and its temperature profile.

The present work is the development of a pilot plant for biomass gasification process by-products of coffee processing (dry pulp and husk), to power an electric generator 60 kW Generac brand.

MATERIALS AND METHODS

This project involves the design and technical assistance in the manufacture of a pilot gasifier Milling Coope Dota RL located in Santa Maria de Dota, San Jose Costa Rica. To this end we implemented a construction process that included the use of parts and materials in the processing plant seeking to minimize the cost and ease of manufacture. The raw material consists of dry brush and coffee husks, and as a gasifying agent for thermochemical reactions inside the gasifier is atmospheric air.

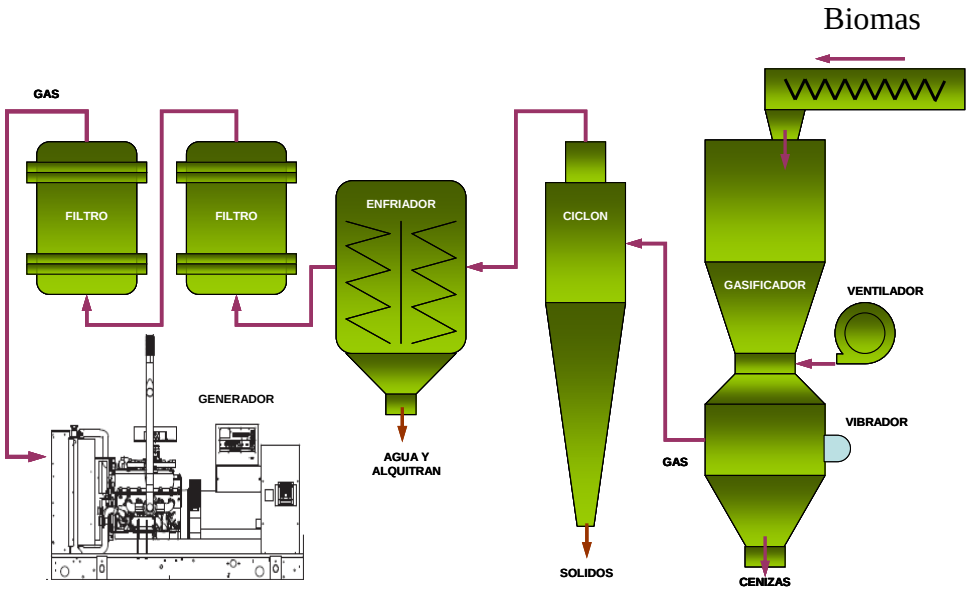

Figure 2.

The type of gasification technology is the type selected Downdraft. This has several advantages with respect to other technologies gasifiers, such as: obtaining a gas with low content of tar, simple manufacturing configuration, possibility of using biomass with low apparent density of 200 to 250 kg/m3.

The pilot plant consists of a downflow gasifier "downdraft" biomass fed into the top by helical elevator, forced draft centrifugal fan for combustion air insufflation, electromechanical vibrator to give downward movement to the inner material. The selected conditioning system for the gas produced in the gasifier consists of a cyclone and gas cooler condensate separation, and two water holding filters and tars "packed" with charcoal. Figure 2 shows the functional diagram of this system.

Was generated design and construction plans, to perform manufacturing facilities RL Coopedota Benefit personnel thereof, Figure 3. Through visits to company monitoring and assistance given to the construction of equipment and incorporated details required, using parts recycling unused industrial equipment available in the Mill. Once fabricated and assembled the team proceeds to dry biomass assessment by setting preliminary tests and commissioning, using dry brush and coffee husks. The moisture content of the biomass was performed in the Chemical Laboratory CICAFE and gas flow measurement, calorific and chemical analyzes were performed by external laboratory services (Laboratory Lambda Costa Rica).

Figure 3.

RESULTS AND DISCUSSION

The characteristics of the biomass used is presented in Table 1, the moisture content of the coffee husks can vary between 9% and 11%, this was used as obtained from the stripping of coffee, without any conditioning and heating power of 4100 Kcal / kg. The dry pulp was proceeded to sun drying through the traditional method of drying coffee yard, to bring it to a moisture level of 10.5% and with calorific BH of 3886 Kcal / kg. In the elemental analysis shows the levels of reduced sulfur thus eliminating problems would be caused by SO2

Table 1. Chemical analysis of biomass to fuel gasifier.

Elemental Analysis		
Element	***Dry Pulp***	***Hulls***
Carbon	44,26	45,32
Hydrogen’	5,64’	5,90
Nitrogen’	2,15’	0,97
Sulphur’	0,1	0,06

Based on the assessments made, was obtained by the consumption of biomass gasifier 71.43 kg / hr, with a total production of 183.6 m3/hr gas with a calorific value of 1402 kcal/m3 using husk as biomass, achieving a performance of 86.8% gasification. With gas outlet temperatures of 350 º C to 400 º C, with a generation of condensed water and tar from 0.15 to 0.2 l / kg biomass. Table 2.

Table 2. Gasifier operating parameters fed coffee husk.

Gasification Operation Data	
Biomass Consumption	71.43 kg/hr
Conversion Ratio	2.57 m^3/Kg biomass
Caloric Power Gas	1402.00 Kcal/m^3
Performance	88.84%

The chemical composition of the obtained gas mainly composed of carbon monoxide, methane, hydrogen, carbon dioxide and other gases, is within the expected concentrations for this technology as shown in Table 3.

Table 3. Chemical composition of the gas produced by gasification of coffee husks.

Gas Produced	**Concentration (vol/vol)**
Carbon monoxide (CO)	18,10 %
Methane (CH_4)	2,85 %
Hydrogen (H_2)	9,10 %
Carbon dioxide	11,60 %

The electricity generated by this system will be used to feed a small processing plant in the wet stage of coffee processing capacity of 7500 kg / hr coffee fruit that includes: 1 pump for fruit, 2 pulpers coffee fruit, 1 screening for pulping coffee, 1 desmucilage vertical upflow, 1 pump for parchment coffee first, 1 pump for lower grades, 1 and 1 elevator auger screw. For a total installed power of 39 kW.

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The Effect of Effective Microorganism (EM) for Cleaning Effluent from Coffee Washing Mills in Ethiopia

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SUMMARY

Effective and Beneficial Microorganisms (EM) are a mixed culture of, fermentative, soil-based, beneficial microorganisms which can be applied to many environments to break down organic matter. The objective of the experiment was to evaluate the efficiency of Effective Microorganisms (EM) in managing coffee processing effluent generated from wet mill stations The experiment was conducted in constructed coffee effluent holding concrete pits and plastic tank in three replications under controlled green house condition at Jimma Research Center. The treatment of EM decreased COD, BOD, TDS. DO and pH but did not affect the temperature of waste water. The effect of EM on the pH showed that there was a significant difference between the different concrete ponds in application of EM. However, it was observed that tank treated with EM had neutral pH (7.37). The result also showed that with no application of EM the pH tended to rise to 8.37 towards the end of experiment. The COD, BOD and TDS increased 11 days after EM treatment. Fifteen days after EM treatment the BOD and VOD value of coffee waste effluent was reduced by 80 percent and 87 percent respectively. In addition, the ammonia, Orthophosphate, Nitrate, Nitrite and Total phosphate content, was also decreased by 98, 71, 49, 46 and 67 percent respectively, due to the microbial breakdown of organic matter. Physico-chemical characteristics of coffee effluent of the two treatments were significantly different before and after application of EM. The levels of ammonia, BOD, COD, orthophosphate, Nitrate and Nitrite were low and the pH values were within acceptable range. Without application of EM in final effluent allows little or no larva re-growth compared with EM treated coffee effluent pond. This result suggests that EM can control the coffee effluent and had significantly reduced coffee effluent waste odour. These reductions could be attributed to the microbial utilization of these nutrients, thereby making the water suitable for recycling for agricultural purposes. EM has proved to be an effective biological agent in waste treatment system.

INTRODUCTION

During wet processing of coffee (pulping, fermentation, and washing) huge amount of waste is produced from pulping stations in coffee growing areas. This operation requires a lot of water. Wet processing produces large quantities of liquid wastes from the pulping, fermentation and washing stages. Effluent disposal pits are consequently sited very close to watercourses mostly on sloppy ground, as conveyance of the effluent into them is dependent on gravity. This poses threat to the ecosystem since the effluent discharged from the coffee factories can easily reach the rivers when there is overloading of seepage pits during the peak season or through run off during heavy rains.

Poor coffee waste management in the area has led to the rise of critical anaerobic conditions in rivers flowing from coffee producing areas where this water is used for irrigation, livestock, and human consumption. The coffee industry was using more volume of water

required. Surface water bodies were being contaminated with high BOD (during the pulping season were BOD measured as high as 1600 mg/l) soluble solids due to the decomposition of coffee pulp waste (JARC, 2003). Due to wet coffee processing, rivers and streams in the region have come under immense pressure and serious contamination with organic wastes (coffee effluent and pulp). Solutions of Effective microorganisms, developed in Okinawa in the 1970's have been used for environmental management which contain naturally occurring microbes (Higa, 1996). EM has also been described as a multi-culture of coexisting anaerobic, aerobic beneficial microorganisms and has the ability of reducing the biological toxicity of waste water (EM Trading, 2000). Hence, EM WOLJEEJII a private enterprise introduced (Bokashi and EM) biological eco friendly water treatment to be evaluated for its effectiveness.

Although, EM is successfully used in many countries, no attempt has been done in this regard in Ethiopia. Hence proposing and conduction a research project aimed at evaluating efficiency of EM on overcoming pollution of coffee effluent has become important realizing the present escalating problem of environmental pollution. Therefore, this proposal was initiated to evaluate the efficiency of Effective Microorganisms (EM) in managing coffee processing effluent generated from wet mill station by injection of EM solution and Bokashi balls directly in the coffee effluent holding pits for exploring the possibility of recycling the waste water.

MATERIALS AND METHODS

The experiment was conducted in concrete pond and green house at Jimma Research Center. For the concrete pond experiment EM was applied on coffee processing effluent obtained from wet mill station. Concrete pits were constructed at Jimma research Center. In another experiment in the laboratory water samples from coffee effluent of 100 lt plastic tank was collected and arranged in three replications. EM was treated in the plastic tanks in order to know the application of EM under controlled laboratory conditions.

In-situ and Ex-situ measurements from each tank were sampled weekly until 40 days for both studies of the concrete and plastic tanks in the laboratory and kept in 1 litre polypropene containers to minimize contamination. The portion of the samples was analyzed directly at the site for pH, dissolved oxygen and Biological oxygen demand using pH meter and OD/BOD meter respectively, equipped with electrode and probe respectively. The other portion of the samples was kept in a cold storage to minimize microbial degradation. These samples were analyzed later for Total Dissolved Solids (TDS), chemical oxygen demand (COD) and biological oxygen demand (BOD) and for soluble Nitrates, Phosphates, and EC in the laboratory. The presence of larva was checked and counted in the laboratory weekly using mesh wire. The analysis of the water samples were made following the standard methods for the examination of water and wastewater (APHA, 1998) at Jimma Research Center and Jimma University laboratories.

Materials for EMAS are EM.1 (EM stock solution), molasses, water, air tight plastic container, plastic tube of small diameter (for letting air out of plastic container) and a small bottle filled with water into which the other end of an outlet tube is placed. The proportion of the mix was 5% EM, 1.5 % molasses and 90% water. The mixture was kept under shade for 2 weeks. EMAS is expected to be ready for use when it had a sweet fermented smell and a pH value of less than 4. EM mud balls were made manually or mechanically using steel mold and a hammer. EM mud balls were made by mixing 10 kg of soil, 1 kg of rice bran and 2 L of EMAS. Application of EM mud balls was recommended after white fungi have covered the balls.

For one cubic meter area one ball EM bokasha was added to a concrete pond once a week. Water sample at different stages initial and weekly bases after treatment was taken from all treatments. The treatment was applied according to the standard procedure (EM WOLJEEJII, 2009). EM solution and Bokasha ball for polluted water was prepared and also be provided by EM WOLJEEJII.

RESULTS AND DISCUSSION

From the present investigation it was found that under green house, the fresh effluent waste from plastic tank showed high concentration of COD (1165 mg/l), BOD (524 mg/l), orthophosphate (0.158 mg/l), nitrate (0.996 mg/l), nitrite (0.019 mg/l) and Total phosphate (0.52 mg/l (Table 1). These concentrations were much higher than the permissible limits prescribed by WHO. Physico-chemical characteristics of coffee effluent with plastic tanks were significantly different before and after application of EM. The levels of ammonia, BOD, COD, orthophosphate, Nitrate and Nitrite were low and the pH value was within acceptable ranges (Table 1). More importantly, the BOD and COD values showed the benefits of treating waste water with EM. The treatment of EM tended to decrease COD and BOD but did not affect the temperature of waste water. Application of EM to the tanks reduced the BOD and COD values of water by 80 percent and 87 percent, respectively. In addition, the ammonia, Orthophosphate, Nitrate, Nitrite and Total phosphate content, was also reduced by 98, 71, 49, 46 and 67 percent respectively, due to the microbial breakdown of the organic matter (Table 1).

Table 1. Influence of Water Treatment with EM on Physico-chemical characteristics of coffee effluent.

Water Quality Parameter	Initial (Fresh effluent)	After		Efficiency EM (%) [(b/a)*100]
		Without EM (a)	*With EM (b)*	
Ammonia	0.105	0.41	0.810	98
Orthophosphate	0.158	0.066	0.019	71
Nitrate	0.996	0.357	0.181	49
Nitrite	0.019	0.013	0.007	46
COD	1165	1150	172	85
Total phosphate	0.52	0.321	0.107	67
BOD	524	532	107	80

All units except pH are in mg/l (ppm)

The presence of both nitrogen form (nitrate and Nitrite) is a major problem that reduce the recycle of waste water. However, application of EM into the second pond reduced the nitrogen and phosphorous contents significantly (Table 1). The rate of reduction of nitrogen was 49 and 46 percent in the treated water for nitrite and nitrate respectively, in comparison to that of untreated water. A similar result was also observed for phosphorus with 71 percent and 67 percent for orthophosphate and total phosphorus in the treated ponds respectively. These reductions could be attributed to the microbial utilization of these nutrients, thereby making the water suitable for recycling for agricultural purposes.

Figure 1 shows the comparison between the pH levels of the two ponds. The effect of EM on the pH suggests that there was a significant difference between ponds in application of EM. However, it can be seen that pond treated with EM had neutral pH (7.37). After the

experiment was completed, observations showd that under no application of EM the pH tended to rise up to 8.53 suggesting that EM treated led to alkaline condition.

The TDS content (Figure 2) of fresh effluent waste was 214 ppm, which showed that the content of suspended organic material in fresh effluent waste was relatively low. The DO content (Figure 3) of fresh effluent waste was found to be 0.4 mg/l. Analysis of the electrical conductivity showd that after EM application, decreased EC 856 to 303 ds m3 during applications of EM. A general trend of decreasing EC after completion of dosages is also illustrated (Figure 4).Results obtained from the study supports claims of reduced coffee effluent pollution, after the final application of EM. Hence, results suggest that the EM does have some affect upon the liquid waste content of the ponds by EM. Based on the results obtained from this experiment, following final application of EM, that a decreasing trend in pH, EC, DO and TDS.

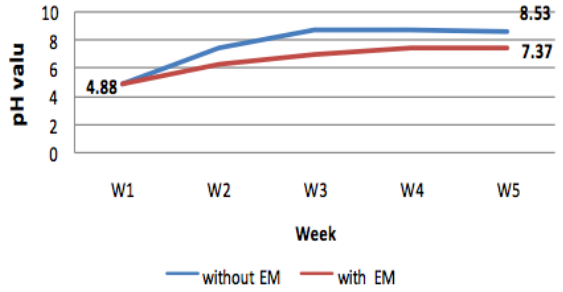

Figure 1. Effect of EM on pH.

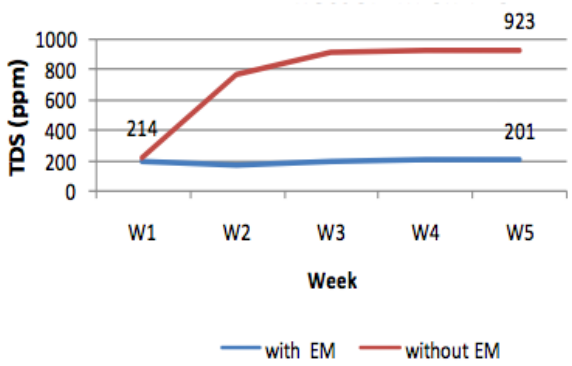

Figure 2. Effect of EM on TDS.

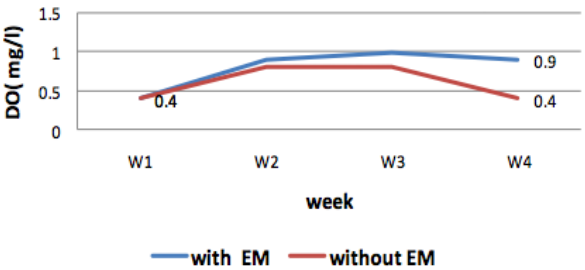

Figure 3. Effective of EM on Dissolved Oxygen (DO).

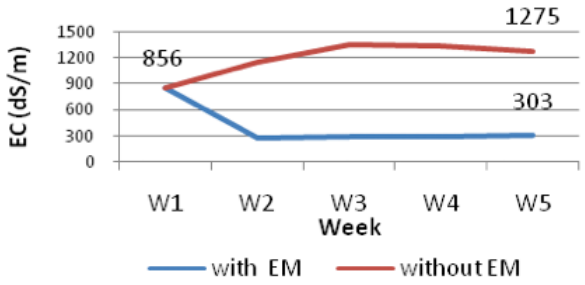

Figure 4. Effect of EM on EC.

For two months close observation was made on the effect of EM on larva is shown in Figure 5. The larva population count was 54 and 37 ten days after first treatment for with no EM and with EM, respectively. After 30 days from the first treatment, pond treated with EM showed higher population of larvae suggesting that population of larva increased with time.

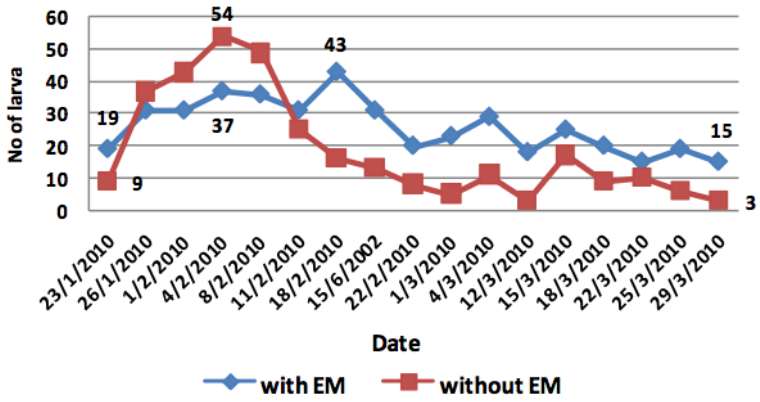

Figure 5. Effect of EM on larva population.

The effect of EM on increased larva population could be associated with population of high DO. In conclusion it can be said that effluent treated With EM showed high larva population compared with out EM. This condition therefore, allows the re-use of effluent at different time without the fear of the development of bad odour. In addition, after EM application, different unidentified floating materials were observed on the surface of the pond compared with pond treated with EM.

CONCLUSIONS

The present investigation has revealed that EM is effective biological agent for waste treatment system. Pond treated with EM has showd higher number of larva population and also the population of larva tended to increase with prolonged time. It can be concluded that EM can control the coffee effluent pollution around wet mill station by breaking down coffee by products and suppressing pathogens thereby eliminating the foul smelling odour caused by mainly high levels of ammonia, hydrogen sulphide and methane. As a result, the water can be recycled and used for agriculture. The activity of EM can significantly improve the water quality of the rivers and the overall contribution towards creating safe environment is

tremendous. In conclusion, EM is effective simple for handling and application, safe and also cost effective to be adopted in coffee growing areas of the country.

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Study of Green Coffee Processing and Brewing Variability Across Species, Origins, Grades and Time

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SUMMARY

Like most agricultural products, green coffee varies due to natural conditions. This natural variability leads to variations in processing and brewing performance. The present work aims to quantify these natural variations across a broad range of bean origins and grades over a period of four months. Processing and brewing performance was assessed with regards to organic roast loss upon roasting, total solids extractable at atmospheric pressure and foaming properties of the extracts. It was observed that differences across green coffee origins were of the order of 30%, and that the variations within green coffee origins ranged from 3% to 20%. These results indicate a significant impact of raw material differences and seasonal variability on industrial performance, and should therefore be taken into account when aiming to achieve consistent product quality.

INTRODUCTION

The quality of green coffee is subject to natural variations like most agricultural raw material. Maintaining a consistent finished product quality has always been a key concern of the coffee roasting industry which has over the years developed a lot of expertise in purchasing and blending coffee seeds. With the recent rise in demand for coffee products driven by developing markets and increased speculation on green coffee seeds due to uncertainties in the global trade of financial products, the volatility of coffee prices has markedly increased. Coffee roasters are facing availability issues and are under pressure to opportunistically manage the cost of the raw materials they use, making it even more important to control the processing performance of these raw materials in order to ensure consistent finished product quality.

Roast coffee in-cup flavour is considered the most important attribute to define its quality, so major emphasis is rightly placed on selecting raw materials with the right flavour characteristics. However a recent and ever increasing shift of consumption habits towards convenience and personalisation is driving an increased use of high-end instant coffee products and on-demand brewing products (i.e. individual cup extraction systems at home). Ensuring consistency in the manufacturing and use of these products requires the control of key products processing characteristics that go beyond flavour attributes. There is a gap in the systematic reporting of the processing performance of various origins of green and roast coffee, as well as their variation through time.

This study was designed to quantify the differences in processing performance between different types and origins of coffee, as well as the variation through time for each of those coffees. The performance indicators studied are the level of organic material losses during roasting, the content of total water-extractable solids in the roast product, and the quality of the foam formed from the water extract. A key preliminary to this work is the development of

processing methods with sufficient levels of repeatability and reproducibility in time, so as to ensure the observed variation is driven by changes in the raw material rather than the processing itself.

MATERIALS AND METHODS

Coffee processing and extraction

Roasting

Batches of 1000±0.15g of green beans were roasted in a Rotating Fluidised-Bed roaster (model RFB10, Neuhaus Neotec GmbH, Ganderkesee, Germany). The roast time was set to 140s with constant air temperature adjusted in order to obtain a roast colour of 12.0±0.5La (La Unit, Dr Langer LA100 colour measurement unit) which is commercially described as a medium roast. A water quench was applied immediately after roasting immediately followed by fluidised bed air cooling with ambient air for 120s so as to achieve 4.0±0.5% moisture in the final roast beans after cooling.

Grinding

Beans were ground using a DK5LS toothed disk grinder (Mahlkönig GmbH & Co., Hamburg, Germany) to a median particle size of x_{50}=450±25µm.

Extraction

15±0.01g of the ground coffee was placed in a 500ml conical flasks with 300±0.5ml of water at 85°C and agitated for 15min in a thermostatic water bath (85°C, 110rpm agitation). The resulting extracts were cooled in icy water for 5 minutes (to roughly ambient temperature) and immediately filtered on folded paper filters (model 315, VWR International, Leuven, Belgium).

Measurements performed on coffee samples

Measurements performed on whole beans

The weight loss of organic material during roasting, known as Organic Roast Losses (ORL) was calculated based on accurate measurements of beans batch weight and moisture contents before and after roasting using **Errore. L'origine riferimento non è stata trovata.**. Moisture content of green and roast whole beans was determined by the gravimetric method (oven drying at 105°C for 24h).

$$\mathbf{ORL} = \frac{\mathbf{Batch\ weight}_{\mathbf{initial,db}} - \mathbf{Batch\ weight}_{\mathbf{final,db}}}{\mathbf{Batch\ weight}_{\mathbf{initial,db}}} \quad (1)$$

Where *Batch weight* $_{initial,\ \boldsymbol{db}}$ and *Batch weight* $_{final,\ \boldsymbol{db}}$ are respectively the weights (in g) of the batches before and after roasting, on a dry basis (*e.g.* corrected for their respective relative humidities).

Measurements performed on ground coffee

Median particle size of the roast and ground coffee was assessed by laser diffraction using a Helos KF equipped with a Rodos M dry disperser (Sympatec GmbH, Clausthal-Zellerfeld, Germany). Duplicate measurements were taken on about 10g of sample each.

Roast colour: The ground same sample was then placed in a Dr. Langer LA 100 monochromatic colorimetric unit which allowed roast colour instant reading with a 2-point calibration. The value read is expressed in a specific La (Langer) unit which expresses the sample's degree of light reflectance (the smaller the number, the darker the sample).

Measurements performed on coffee water extracts

Total solids content was assessed by pouring 10ml of liquid extract in a vial and analysing it in a Density / Specific Gravity Meter (model DA-520, Kyoto Electronics Co. Ltd, Kyoto, Japan). It measured the density difference between de-ionised water and the sample, expressing its total solids content in g per 100g of samples.

Foam quality is a generic term which refers to the quality of the foam obtained from a coffee brew. In this study, foam was produced in a controlled way using an eductor designed to incorporate air into a solution flow thanks to Venturi effect. Hot coffee extract (74±1°C) was pumped through a peristaltic pump at approximately 140ml/min during 20s, going through the eductor and falling in a 100ml beaker or a 100ml graduated cylinder (see
Figure 1).

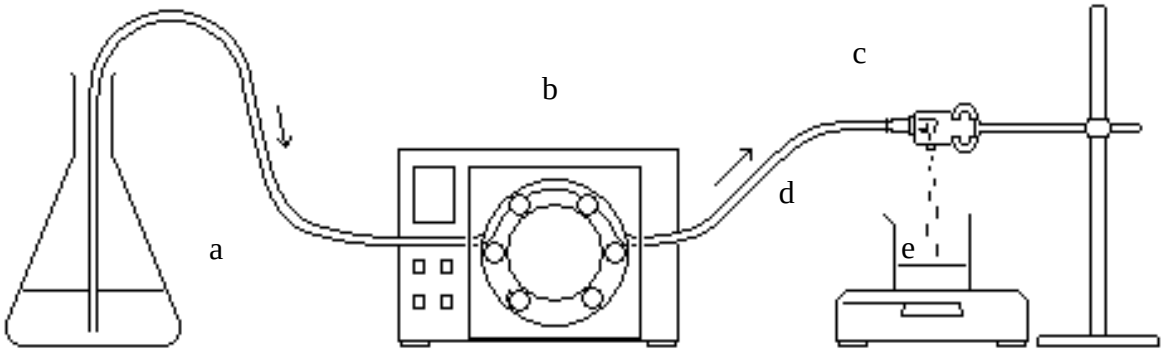

Figure 1. Foam generation setup. a) Hot coffee extract. b) Peristaltic pump. c) Venturi eductor. d) Beaker / Graduated cylinder. e) Weighting scales.

An arbitrary Foam Quality index (FQ) gets calculated as per **Errore. L'origine riferimento non è stata trovata.**.

$$\boldsymbol{FQ = \frac{\varphi_{air} \cdot \tau}{d_{50}}} \quad (2)$$

The total volume of foam (V_{total}) recovered in the graduated cylinder was read immediately after forming the foam (at t=0). The volume of extract used to form this foam was calculated based on the measured weight of extract (m_e) and the extract density (ρ_e) measured by the density meter used for total extracted solids measurements (see above). The relative volume of air incorporated in the extract (φ_{air}) was calculated as per

(3)

.

$$\varphi_{air} = \frac{V_{total} - (\frac{m_e}{\rho_e})}{m_e} \quad (3)$$

As the foam destabilises a visible interface between the floating foam and the drained liquid phase underneath formed, allowing the observer to record the volume of drained extract over the course of 300 seconds from t=0. After that time the interface reached a relatively stable level. The evolution of the relative extract volume drained (V_d) over the first 300 seconds can be fitted to an exponential equation as per

$$V_d(t) = V_{min} + (V_{max} - V_{min}).(1 - e^{-\frac{t}{\tau}}) \quad (4)$$

, whereby V_{max} is V_d at t=300s and V_{min} is V_d at t=0.

$$V_d(t) = V_{min} + (V_{max} - V_{min}).(1 - e^{-\frac{t}{\tau}}) \quad (4)$$

The liquid drainage kinetic constant τ increases when drainage slows down (i.e. increased stability of the foam).

The bubble size distribution of the foam was calculated by image analysis based on digital pictures taken in standardised conditions. The median bubble size d_{50} was used as the characteristic dimension.

RESULTS AND DISCUSSIONS

Repeatability and reproducibility of the methods

Repeatability and Reproducibility of a variable were respectively calculated as percentages of the mean for repeated independent measurements on the same day (

$$\text{Repeatability} = \frac{\sum_{\text{duplicates}} \frac{|\text{Difference between duplicates}|}{\text{Average of duplicates}}}{\text{Number of duplicated measurements}} \quad (5)$$

) and independent batches produced on different weeks (

$$\text{Reproducibility} = \frac{\sum_{\text{batches}} \frac{|\text{Difference between batches}|}{\text{Average of batches}}}{\text{Number of independent batches}} \quad (6)$$

).

$$\text{Repeatability} = \frac{\sum_{\text{duplicates}} \frac{|\text{Difference between duplicates}|}{\text{Average of duplicates}}}{\text{Number of duplicated measurements}} \quad (5)$$

$$\text{Reproducibility} = \frac{\sum_{\text{batches}} \frac{|\text{Difference between batches}|}{\text{Average of batches}}}{\text{Number of independent batches}} \quad (6)$$

Table 1. Repeatability and reproducibility of the performance measurements

	Roasting ***ORL***	***Extraction*** ***Total solids***	***Foaming*** ***Quality index***
Repeatability			
Arabica (Brazil)	3.8%	3.0%	10.2%

Robusta (Indonesia)	7.1%	2.5%	6.2%
Reproducibility			
Arabica (Brazil)	12.1%	4.0%	13.0%
Robusta (Indonesia)	22.1%	2.4%	14.2%

Processing standardisation and control allows for relatively low levels of internal variability, although Organic Roast losses and Foam Quality index are inherently difficult to accurately reproduce through time.

Those levels of internal variation are to be contrasted with the variability and differences observed between different origins and over the time span of this study (see subsequent section 0).

Coffee performance variability through time: comparison of different coffee origins

Variability was assessed for various species and countries of origins as indicated in Table 2. For each of those origins samples were obtained over a period of 4 months at their arrival in Bremen harbour in Germany.

Table 2. Codes used for each type of coffee defined in terms of category (specie and process) and country of origin (see codes used in figures throughout).

Coffee category	Code	Country of origin
Robusta (dry process)	Ind	Indonesia
	Vnm	Vietnam
	Cam	Cameroon
	Brz	Brazil
Natural Arabica (dry process)	Brz	Brazil
	Eth	Ethiopia
	Png	Papua New Guinea
Washed Arabica (wet process)	Hon	Honduras
	Per	Peru
	Chi	China
	Ken	Kenya

Variability in Organic Roast Losses

Organic Roast Losses (ORL) is the decrease in dry matter weight observed during roasting expressed as a percentage of the original dry matter prior to roasting. It corresponds to the chaff losses (which should not exceed 0.5% of total dry matter) and CO2 evaporation following thermally induced chemical reactions that occur when the beans are roasted.

ORL tends to be on average higher across the range of robusta coffee (averaging 8%) than in arabica coffee (averaging 6.3% and 7% for wet and dry processed arabica respectively) as can be seen in
Figure 2.

The range of time driven variability in ORL (i.e. max value – min value observed) varies also widely across the range of origins and tend to be greater for robusta (between 6% and 60% of the mean) than in arabica coffee (between 6% and 34% of the mean). Both dry and wet processed arabica observed showed similar average ORL levels, apart from dry processed Ethiopian which had levels of ORL comparable to those observed in robusta.

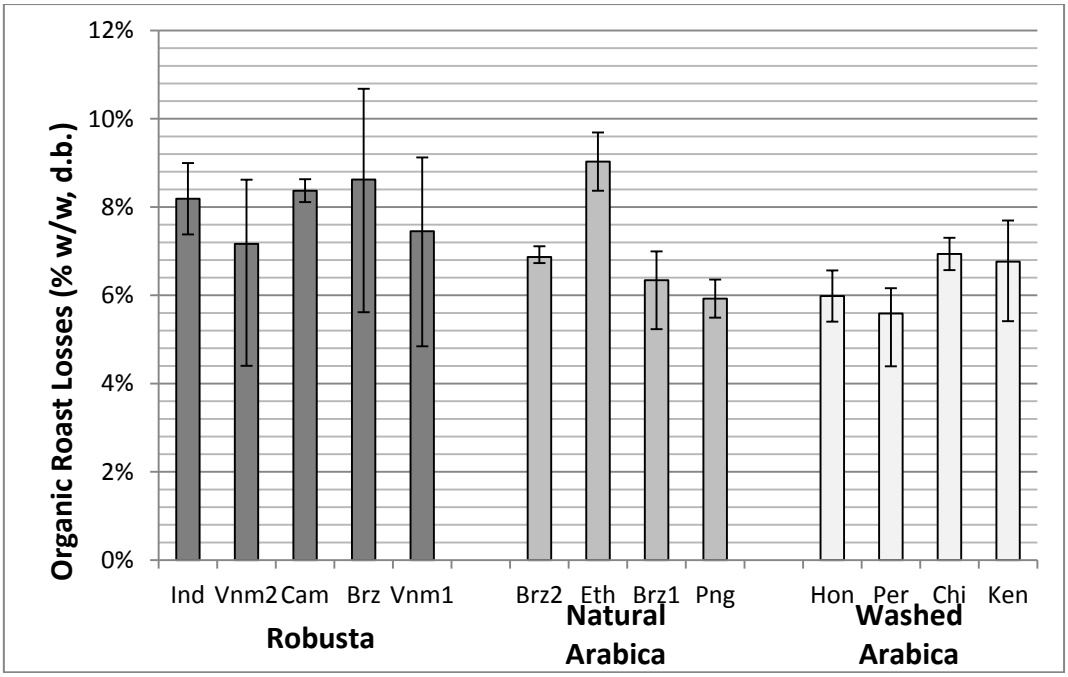

Figure 2. Average Organic Roast Losses of various coffees collected over a period of four months. Error bars indicate highest and lowest values measured over that period.

Variability in Total Extractable Solids

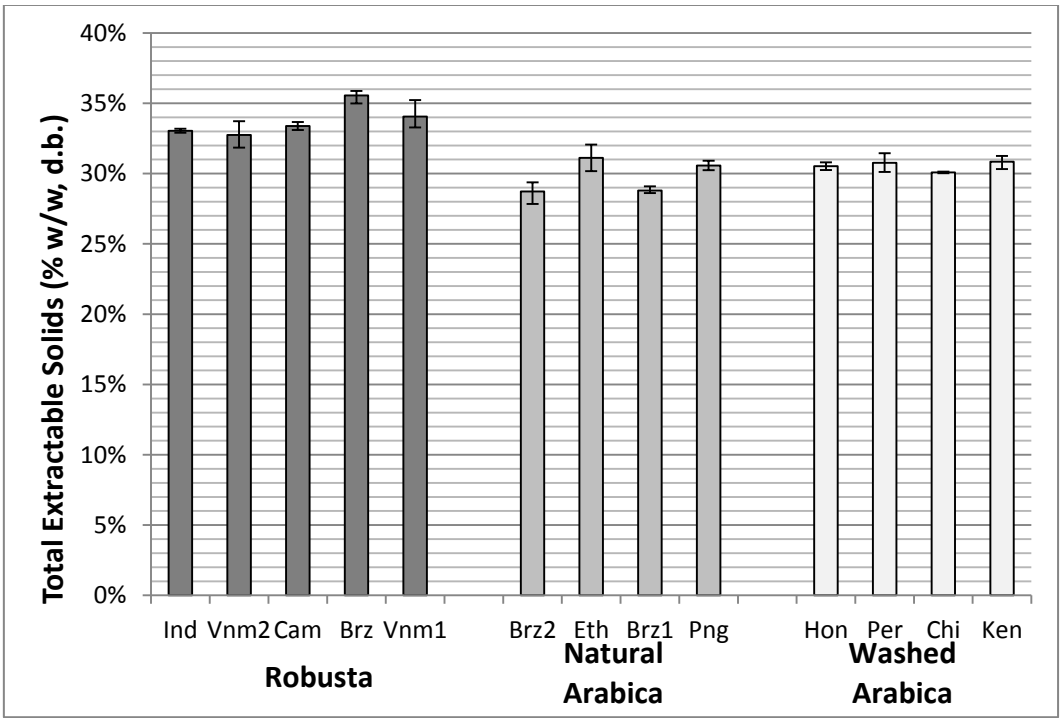

Figure 3. Average Total Extractable Solids of various coffees collected over a period of four months. Error bars indicate highest and lowest values measured over that period.

The Total Extractable Solids (TES) content of a roast coffee is the weight fraction of total solids (in %) that can be extracted with water in the standard extraction conditions used (see section 0). It is of primary importance for products brewed in individual portions as it determines the amount of roast coffee required in the brewing capsule in order to reach the desired level of coffee solids in the consumed beverage.

Robusta coffee contain on average 34% extractable solids which is more than arabica coffees which averages only 30% (see
Figure 3). The observed differences in average TES between origins within each category of coffee are small (maximum 8% of the mean). The range of time driven variability in TES across the whole range of coffee types analysed (i.e. max value – min value observed) is also rather small and consistent within the range (between 1% and 6% of the mean value, averaging 3%). These results suggest that the sometimes larger variability observed in brews obtained from commercial systems may be mostly driven by variability in the brewing conditions rather than the raw material.

Variability in Foam Quality

The quality of the foam that forms at the surface of a cup of coffee upon brewing is an important driver of consumer-liking. Although some progress has been made in understanding the chemistry involved in coffee foam formation and stabilisation (see Nunes et al, 1997 and Schokker et al., 2002), systematic coffee foam characterisation remains a challenging area because of its unstable and delicate nature. Numerous methods are at use with varying degrees of repeatability/reproducibility, making comparison between methods and sources of results a difficult subject matter.

The Foam Quality index presented here is an attempt at expressing with a single figure the overall foam ability and stability of the foam obtained from the various coffees, in order to simplify the comparison. It assumes that the quality of coffee foam increases when:

- - the average size of the bubbles it contains decreases.
- - the drainage velocity decreases (i.e. increased stability).
- - the volume fraction of air incorporated increases.

Very strong correlations observed between those three foam characteristics support the use of a single number to represent “overall” foam quality.

On average it is observed (see
Figure 4) that robusta coffee tends to yield a higher foam quality index (average 41) than Arabica coffee (average 31). Large differences in FQ index can be observed between the different origins of the robusta range as well as a large range of time driven variability representing between 5% and 77% of the corresponding mean FQ value. Although robusta from Indonesia and Cameroon proved more stable over the course of the four months considered, these results suggest that the geographical origin of a robusta coffee is not a reliable indicator of its foaming ability. Wide variations in foaming performance can reasonably be expected from most robusta origins.

The observed FQ within the Arabica range is more consistent with a range of average values representing around 23% of the overall average across the various Arabica.

Despite the relatively high level of internal method variability (see chapter 0) these results suggest that differences attributed to the type and origin of the coffee (as opposed to those due to preparation conditions) lead to significant differences in foam quality.

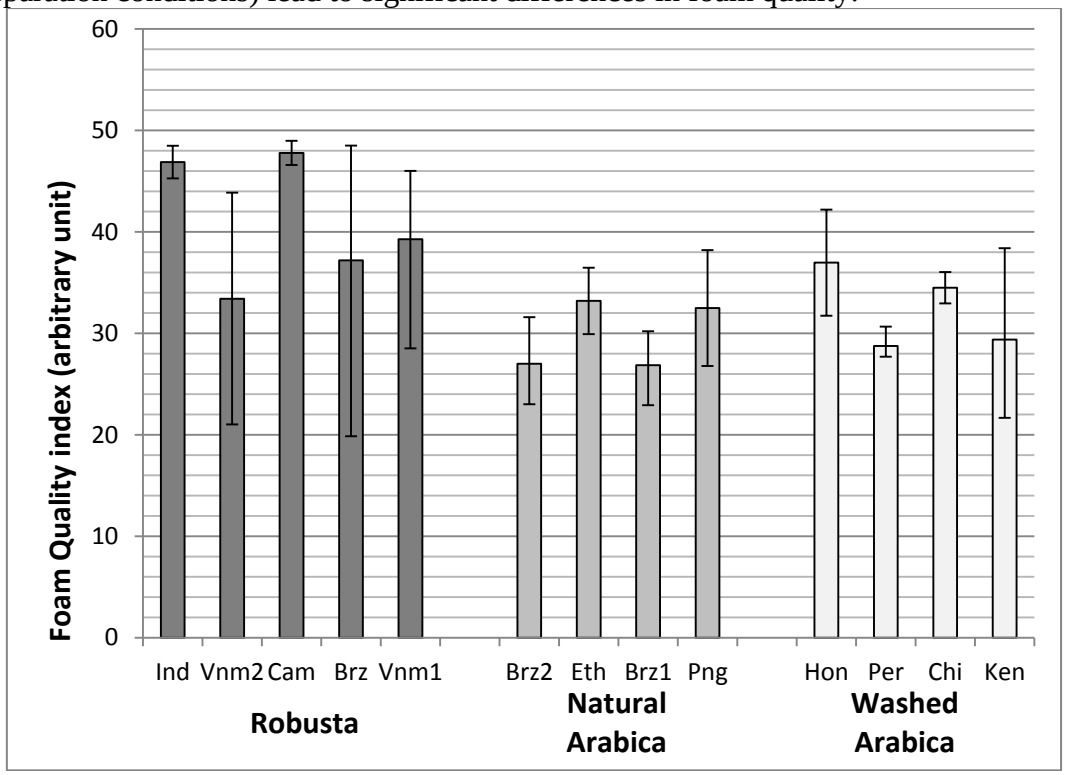

Figure 4. Average Foam Quality index of various coffees collected over a period of four months. Error bars indicate highest and lowest values measured over that period.

CONCLUSIONS

This study shows marked differences in processing performance between different coffee types and origins, the magnitude of which suggests that they would significantly impact the product manufacturing cost (based on ORL differences) as well as the sensorial quality of the resulting product (based on FQ differences). Although differences in TES appeared relatively small and fairly stable in time, differences in ORL and FQ were highly variable in time over the 4 months period covered by the present work, suggesting a significant seasonal variation with regards to the reproducibility of the methods used to evaluate the performance. It is however difficult to definitely conclude on the significance of differences between origins as the seasonal variability considered in this study (i.e. over 4 months) represents only a third of the total annual crop cycle of the plant. It can be expected that over the course of a full year, observed internal variability would be higher than those observed in the present work. More extensive data collections are required to build confidence in our understanding of differences in processing performance between different origins of coffee.

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Enhancing Arabica Coffee Cup Taste Profile by Involving Biological Agents during Fermentation Process

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SUMMARY

One of a rare, distinct and unique coffee in the world is 'Kopi Luwak' which is produced by involving a native animal behavior of "*Luwak*" (*Paradoxorus hermaproditus*), a civet cat. "Luwak" is native animal in South East Asia region particularly in Sumatra and Java islands. An experiment on fermentation Arabica coffee involving biological agent such as luwak animal had been done at Andungsari Experiment Station of ICCRI during harvesting period of 2010. Material used in this fermentation trial was fresh depulped Arabica coffee beans. The depulped beans were fermented by several different treatments involving fermentation methods, biological agents and duration of fermentation. The fermentation methods consisted of underwater fermentation (A1) and dry fermentation (A2). The biological agent treatments used in this trial were no involvement of biological agent (B0), fresh "Kopi Luwak" (B1), yeast of a traditional fermented cassava cake (*Sacharomyces sp*.) (B2), fungus of "*Tempe*" (*Rizopus sp*.) (B3) and bacteria of yogurt (*Lactobacillus sp*.) (B4). Duration of fermentation treatments were 12 – 14 hours (C1) and 36 – 38 hours (C2). The fermented coffee parchments were washed and followed by fully sundried until 12 % of moisture content. After deparchment the green coffee samples were roasted at medium level (Agtron Scale at 65#) for cupping test involving 5 specialists at ICCRI laboratory by using SCAA's protocol. The results showed as follows (1) dry fermentation performed better flavors than that of underwater fermentation, (2) treatments with biological agent during fermentation indicated better Flavour than that of no biological agent involvement, (3) bacteria of yogurt (*Lactobacillus Sp*.) provided the best effect on Flavour enhancement that that of other microbe inoculums, (4) fermentation duration of 12 hours indicated better Flavour than that of 36 hours, (5) "'Kopi Luwak" performed the best Flavour than that of all fermentation treatments. It was assumed that higher fermentation temperature inside "Luwak" ingestion (35 - 37 ^{o}C) than that of common fermentation process (21 – 23 ^{o}C) as well as a complexity relationship between other unknown factors provided a significant effect in generating an excellent Flavour on Arabica coffee.

INTRODUCTION

One of a rare, distinct and unique coffees in the world is 'Kopi Luwak' which is produced by involving a native animal behavior of "*Luwak*" (*Paradoxorus hermaproditus*), a civet cat. "Luwak" is native animal in South East Asia region particularly in Sumatra and Java islands. During ingestion in the civet cat stomach, coffee parchment has a specific fermentation process. Normaly, coffee fermentation is the bio-chemical decomposition of the mucilage covering the pulped beans, which constitutes an obstacle to drying. Coffee fermentation is produced by natural occurring micro biota for varying lengths of time depending on climatic conditions and fruit ripeness. The micro flora during the fermentation of coffee is constituted by pectolytic bacteria *Erwinia herbicola* and *Klebsiella pneumonia*, with optimal activities at pH 8.5, whereas fermentation conditions are acidic (pH 5.3-3.5). The most frequent lactic acid

bacteria isolated were *Leuconostoc mesenteroides, Lactobacillus plantarum* and *Lactobacillus brevis.* The yeast *Kloeckera, Candida* and *Cryptococcus* genuses have been isolated from coffee fermentation bacs. These microorganisms could be responsible for the alcoholic taste of the coffee beverage after over fermentation. The mucilage decomposition seems to be correlated to acidification and not to enzymatic pectolysis.

The microbial control of coffee fermentation microflora would be possible in order to limit the off-flavour development, to reduce the growth and production of toxin by toxigenic fungi and to standardize the final coffee quality. The use of an inoculum of lactic acid bacteria would be preferable in order to stay as close as possible to the natural fermentation, where the acidification is important. The LAB have shown to restrict the growth of the most important toxigenic fungi thereby reducing the formation of harmful toxins during the malting and brewing. Mixed inocula of LAB for use as inoculants of coffee fermentations would be to significantly reduce growth and OTA production by *A. Ochraceus*.

An experiment of fermentation Arabica coffee involving biological agent such as luwak animal had been done at Andungsari Experimental Station of ICCRI during harvesting period of 2010. Material used in this fermentation trial was fresh depulped Arabica coffee beans. The depulped beans were fermented by using several different treatments involving fermentation methods, biological agents and duration of fermentation.

MATERIALS AND METHODS

Mature Arabica cherries collected from Andungsari Experiment Station of the Indonesian Coffee and Cocoa Research Institute (ICCRI) were used in this experiment. The fresh coffee cherries were pulped and fermented by various methods. The fermentation methods consisted of underwater fermentation (A1) and dry fermentation (A2). The biological agent treatments used in this trial were B0 (no involvement of biological agent), B1 (100 gram of fresh "Kopi Luwak"/10 kg fresh parchment), B2 (100 gram of *ragi* of a traditional fermented cassava (*Sacharomyces sp*., and other molds/yeast)/ 10 kg fresh parchment), B3 (100 gram of "*Tempe*" (*Rizopus sp*.)/ 10 kg fresh parchment), and B4 (65 ml of yogurt (*Lactobacillus sp*.)/10 kg fresh parchment). Duration of fermentation treatments were 12 – 14 hours (C1) and 36 – 38 hours (C2). The fermented coffee parchments were washed and followed by fully sundried until 12 % of moisture content. After deparchment the green coffee samples were roasted at medium level (Agtron Scale at 65#). From each sample, 5 (five) cups of coffee were prepared and tested. The coffee cup taste was evaluated by idetifying its fragrance, aroma, flavor, aftertaste, acidity, body, uniformity, balance, clean cup, sweetness and overall/preference. This procedure was developed by Specialty Coffee Association of America. The cupping test involving 5 specialists at Sensory Laboratory of ICCRI.

RESULTS AND DISCUSSION

Fermentation Methods

The effect of fermentation method on cup profile of Arabica coffee is presented at Table 1.

Table 1. The effect of fermentation.methods on cup taste profile of Arabica coffee (Compared to "Domesticated Luwak Coffee").

Flavour Atributes	Underwater Fermentation (in Plastic Bucket)	Dry Fermentation (in Plastic Sack)	Domesticated Luwak Coffee
Fragrance/Aroma	7.5	7.46	7.58
Flavor	7.4	7.5	7.81
Aftertaste	7.39	7.49	7.81
Acidity	7.06	7.25	773
Body	7.41	7.44	769
Uniformity	10	9.9	983
Balance	7.31	7.42	775
Clean Cup	10	10	10
Sweetness	9.9	9.92	10
Overall	7.36	7.47	7.85
Total Score	**81.33**	**81.85**	**84.07**

Note: Based on the Spesialty Coffee Association of America method. Quality scale: 6.00 – 6.75 = Good; 7.00 – 7.75 = Very Good; 8.00 – 8.75 = Excellent; 9.00 – 9.75 = Outstanding. Total Score = 80.00 or more belonged to specialty grade.

The results showed as follows (1) dry fermentation performed better flavors than that of underwater fermentation. In washed coffee production, final quality among others is greatly dependent upon the fermentation process. Improvement of quality has also been observed by under-water soaking for 24 hours. Under-water soaking (fermentation) of washed wet parchment for specified period was reported to improve raw and liquor quality of coffee by way of leaching some of the chemical compounds (diterpenes, poly phenols, tannins etc.) responsible for bitterness and browning of coffee beans. It was reported that parchment soaked under basic conditions give dull parchment whereas it is very clean when soaked under acidic conditions. The process of dry fermentation of parchment followed by underwater soaking is popularly called the "Two stage fermentation procedure" and is widely followed in Kenya. It has been confirmed that under-water soaking following 'dry' fermentation, i.e., two-stage fermentation enhances the appearance of both raw and roast coffees compared to 'dry' fermentation only. But, it had been reported that the method of removing the mucilage (dry fermentation, under water fermentation, peptic enzyme accelerated fermentation or chemical cleaning) has no effect on the liquor quality and there is no evidence that any one method can produce significantly better liquors than another. The authors also indicated that high levels of coffee skins in fermenting coffee produces inferior raw, roast and liquor qualities compared to skin-free controls, with the liquors adversely affected by the development of off-flavors variously described as coarse, bitter, fruity, or unclean.

The effect of acid soaking (fermentation) on coffee processed by 3 different demucilization methods were in the order of Citric > under-water soaking > Ascorbic > Phosphoric > Malic > Lactic > No soaking. In general coffee processed by natural fermentation + manual wash +

acid soaking performenced good body, fair acidity and slight bitter and/or medicinal taste. Coffee processed by enzyme + machine wash+ acid soaking (fermentation) recorded fair to good body, fair to fair plus acidity and sweetish flavour. Coffee processed by machine wash alone recorded, fair to good body, fair to good acidity and slight bitter and/or harsh taste.

Treatments Of Biological Agent During Fermentation

The effect of biological agents during fermentation on cup taste profile is presented in Table 2.

Table 2. The effect of biological agents during fermentation process on cup taste profile of Arabica coffee (compared to "Domesticated Luwak Coffee").

Flavour Atributes	B0	B1	B2	B3	B4	Domesticated Luwak Coffee
Fragrance/Aroma	7.41	7.42	7.52	7.5	7.56	7.58
Flavor	7.38	7.4	7.5	7.46	7.51	7.81
Aftertaste	7.35	7.39	7.51	7.45	7.5	7.81
Acidity	7.13	7.1	7.16	7.15	7.24	7.73
Body	7.27	7.46	7.4	7.47	7.54	7.69
Uniformity	10	9.96	9.96	9.83	10	9.83
Balance	7.28	7.28	7.45	740	7.41	7.75
Clean Cup	10	10	10	10	10	10
Sweetness	9.79	10	9.75	10	10	10
Overall	7.34	7.41	7.43	7.4	7.51	7.85
Total Score	80.95	81.43	81.67	81.65	82.27	84.07

Remarks: B0 - No involvement of biological agent; B1 - Fresh "Kopi Luwak"; B2 - Yeast of a traditional fermented cassava cake (Sacharomyces sp.); B3 - Fungus of Tempe (Rizopus sp.); B4 - Bacteria of yogurt (Lactobacillus sp.).Cup taste scoring based on the Spesialty Coffee Association of America Method: 6.00 – 6.75 = Good; 7.00 – 7.75 = Very Good; 8.00 – 8.75 = Excellent; 9.00 – 9.75 = Outstanding. Total score = 80.00 or more belonged to specialty grade.

The results showed treatments with biological agent during fermentation indicated better Flavour than that of no biological agent involvement, bacteria of yogurt (*Lactobacillus Sp*.) provided the best effect on Flavour enhancement that that of other microbe inoculums.

In natural condition, the coffee fermentation can be characterized as a mixed yeast/bacterial fermentation. *Kloekera apiculata* (*Hansenispore apiculata* or *Saccharomyces apiculatus*) and *Hansenispore uvarum* are reported to dominate the yeast population with other yeasts such as *Pichia kluyveri* (*P. fermentans*) and *Kluyveromyces marxianus* (*Candida kefir* or *C. bulgericus*). The yeast species are fermentative and the dominant species share the characteristic of only assimilating and fermenting glucose amongst the usual sugars tested to identify yeasts. The bacterial side of the fermentation is conducted by lactic acid bacteria, some Enterobacteriaceae and *Bacillus*. The most common bacteria to produce pectolytic enzymes are *Pseudomonas* (*P. fluorescens*, for example) and *Erwinia (E. carotovora*, for example). Of these only *Erwinia* is fermentative and, in fact, the presence of *Pseudomonas* is difficult to demonstrate in fermentation liquors. In general, the lactic acid bacteria have been reported to be more numerous than the Enterobacteriaceae. Analysis of several fermentations under the project "Enhancement of Coffee Quality Through the Prevention of Mould Formation" has shown that the balance between yeasts and bacteria can vary widely, such that

some are primarily bacterial and others dominated by yeast. It is not clear how the outcomes of these two types differ, or why it should differ.

It has been shown that the native flora from fermented coffee reduces the *A. ochraceus* growth, a dangerous fungi species. Three predominant lactic acid bacteria strains were isolated and identified as *Leuconostoc mesenteroides* dextranicum, *Lactobacillus brevis* and *Lactobacillus plantarum*. The isolated bacteria were able to inhibit growth of *A. ochraceus* and OTA production. Also, the three lactic acid bacteria and the cell-free supernatant were able to prevent spore germination of A. *ochraceus* in MRS broth. Coffee fermentations performed using a starter culture of three lactic acid bacteria and an inoculum of *A. ochraceus* confirmed a significant reduction of OTA production by *A. Ochraceus*. This study offers evidence to use a starter culture of lactic acid bacteria (*Yakult*) in coffee fermentation is able to improve the coffee flavor. The Yakult contains the unique Lactic Acid Bacteria, *Lactobacillus bulgaricus*) and *Lactobacillus casei* Shirota strain.

"Kopi Luwak" performed the best Flavour than that of all fermentation treatments. It was assumed that higher fermentation temperature inside "Luwak" ingestion (35 - 37 ^{o}C) than that of common fermentation process (21 – 23 ^{o}C) as well as a complexity relationship between other unknown factors provided a significant effect in generating an excellent Flavour on Arabica coffee. A scientific literature on Kopi Luwak had been published by Marcone. The title is 'Composition and properties of Indonesian palm civet coffee (Kopi Luwak) and Ethiopian civet coffee", which reported on various physicochemical properties of Palm Civet coffee (Kopi Luwak) and its comparison to African civet coffee. There are some major physical differences between them that include Colour differences, where Kopi Luwak was found to be higher in red Colour hue and was overall darker than control beans. Scanning electron microscopy revealed that all Palm Civet beans possessed surface micro-pitting caused by the action of gastric juices and digestive enzymes during digestion. Large deformation mechanical rheology testing revealed that Luwak coffee beans were harder and more brittle than their controls indicating that digestive juices were entering into the beans and modifying the micro-structural properties. SDS-PAGE electrophoresis also showed a difference by revealing that proteolytic enzymes were penetrating into Luwak coffee beans and causing substantial breakdown of storage proteins. Doing a complete proximate analysis, Luwak coffee beans were found to be lower in total proteins, which means that proteins were partially broken down and leached out during the digestion process inside the animals' gastrointestinal tract. Since proteins are responsible for much of the flavor, particularly bitterness, it is clear that the lower protein content of Kopi Luwak is one reason for a less bitter coffee. Also after roasting, it was noted that there were significant differences in the Flavour profile of the Kopi Luwak vs. the controls when analyzed by an electronic nose for volatile aroma compounds. Accordingly, understanding of the chemical and physical principles behind the transformation of the beans through the Luwak digestion can provide a process for treating coffee beans to produce Kopi Luwak, or similar quality coffees, without the Luwak or Asian Palm Civet. Such a method would provide a more controlled, consistent, and economical way to obtain high quality coffee with a unique flavor, aroma, texture, body, smoothness and richness.

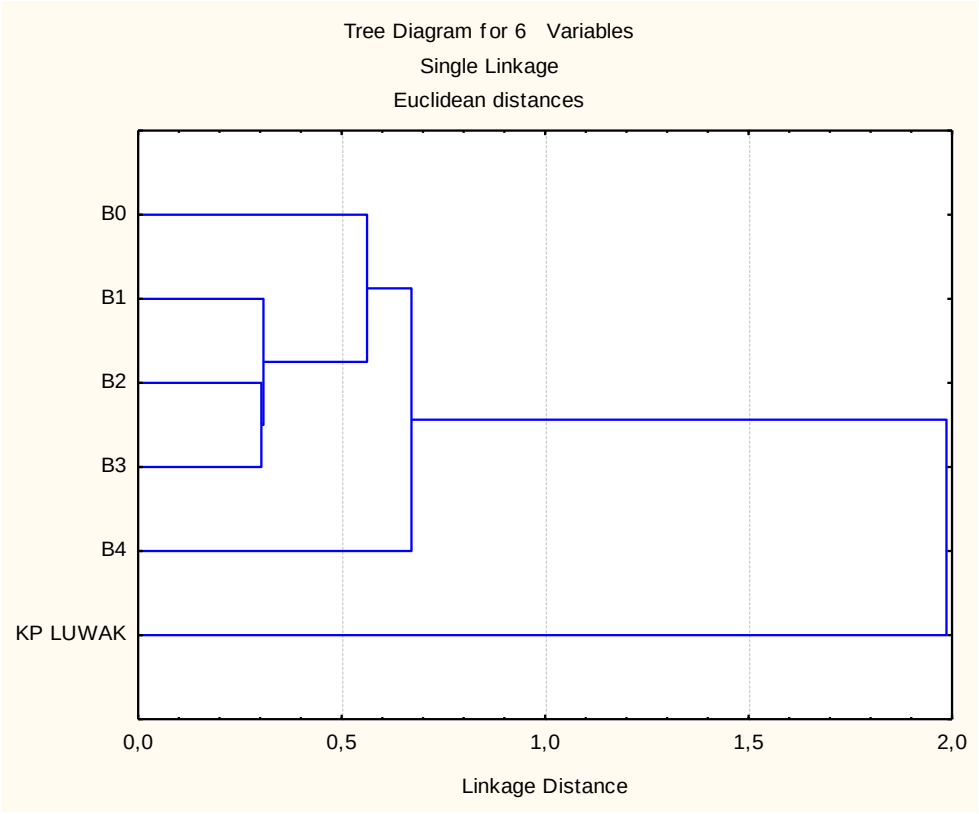

Figure 1. Clustering the effect of biological agent treatments on the cup taste profile. Remarks: B0 - No involvement of biological agent; B1 - Fresh "Kopi Luwak"; B2 - Yeast of a traditional fermented cassava cake (Sacharomyces sp.); B3 - Fungus of Tempe (Rizopus sp.); B4 - Bacteria of yogurt (Lactobacillus sp.).

Fermentation periode

The results showed treatments fermentation duration of 12 hours indicated better Flavour than that of 36 hours. In Indonesia, coffee small holders mostly use 12 hours of fermentation period, but the large coffee plantation use 36 hours of fermentation period. In Africa, farmers were generally aware about the fermentation process but the periods taken in this process were diverse. One quarter of the farmers visited undertook the fermentation process for the recommended 72 hours. The rest took various periods ranging from 12 hours to 60 hours. The average deviation from the recommendations was 17 hours. It had been reported that's post fermentation soaking for 24 hours produced better raw and roast appearance than either 8 or 16 hours soaking, but extending the soak to 48 hours did not cause any further improvement to the green coffee and actually reduced the roast quality.

Table 3. The Flavour atributes of Arabica coffee were differentiated by Fermentation period (Compared with "Domesticated Luwak Coffee") *).

Flavour Atributes	Fermentation period		Domesticated Luwak Coffee
	12 hours	*36 hours*	
Fragrance/Aroma	7,48	7,48	7,58
Flavor	7,47	7,43	7,81
Aftertaste	7,48	7,40	7,81
Acidity	7,23	7,08	7,73
Body	7,44	7,41	7,69
Uniformity	10,00	9,90	9,83
Balance	7,42	7,31	7,75
Clean Cup	10,00	10,00	10,00
Sweetness	9,90	9,92	10,00
Overall	7,44	7,39	7,85
Total Score	81,87	81,32	84,07

Note: Basic on the Spesialty Coffee Association of America Method. Quality scale (1) 6.00 – 6.75 = Good (2) 7.00 – 7.75 = Very Good (3) 8.00 – 8.75 = Excellent (4) 9.00 – 9.75 = Outstanding. Total Score >= 80.00 Specialty grade.

CONCLUSIONS

The results showed as follows (1) dry fermentation performed better flavors than that of underwater fermentation, (2) treatments with biological agent during fermentation indicated better Flavour than that of no biological agent involvement, (3) bacteria of yogurt (*Lactobacillus Sp*.) provided the best effect on Flavour enhancement than that of other microbe inoculums, (4) fermentation duration of 12 hours indicated better Flavour than that of 36 hours.

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Drying Rate and Quality of Natural Coffee

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SUMMARY

This study was carried out for the purpose of assessing the effects of temperature and drying rate on the quality of natural coffee. Ripe fruits of coffee (*Coffea arabica* L. cv. Mundo Novo) were sun dried on a suspended bed until reaching a moisture content of approximately 30 - 35% (wb) and then dried with heated air. Three dry bulb temperatures (35, 40 and 45 °C) and three drying rates for each dry bulb temperature were studied. The effects of treatments on coffee quality were studied through physiological, chemical and sensory analysis. The observed data showed that the temperature of 35 and 40°C are recommended for the production of natural specialty coffees; the temperature of 45 °C is not recommended for the production of natural specialty coffees; the drying rate affects the quality of natural coffee; raising the drying rate at temperatures of 35 °C and 40 °C has a negative effect on the quality of natural coffee; the quality of natural coffee is affected mainly by thermal damage in dry air heated to 45 °C.

INTRODUCTION

The market value of coffees is based on their quality. “Specialty Coffees” are usually distinctive beverages offering the consumer a unique sensory perception and experience, and they attain higher prices than commodity coffee. The production of coffees with a high standard of quality depends on various factors, such as crop management and harvesting, processing, drying and storage procedures.

Coffee processing through the dry method (natural method) proves to be more sustainable as compared to the wet method because of its low water consumption and wastewater production. On the other hand, due to a lack of care and inadequate management of natural coffee during post-harvest, natural coffee is usually associated with poorer quality when compared to coffees processed by the wet method. However, correct management of harvesting, processing and drying of the fruits allows for the production of natural specialty coffees.

The effect of drying temperature on coffee quality has been well studied. Recent research shows that natural coffee loses quality when dried at temperatures above 40 °C. On the other hand, the drying rate of grains and seeds is able to be increased and the quality of the grains maintained by reducing the relative humidity of the air, particularly when using low drying temperatures. However, few studies describe the effects of interactions between temperature and relative humidity of the air used in drying, and the effects of the drying rate on the quality of natural coffees.

Drying is, without doubt, the key point in the production of natural specialty coffees because the presence of the husk and the mucilage with high sugar content reduces the drying rate and increases the risk of occurrence of undesirable fermentations, affecting their quality. Evaluation of coffee quality is traditionally performed by sensory analysis. For specialty coffee, the beverage is evaluated from the description and scoring attributes of fragrance / aroma, uniformity and absence of defects, sweetness, flavor, acidity, body, aftertaste, balance and overall impression. In addition to sensory analysis, other analyses may be used to complement the understanding of changes in the coffee in response to different factors, such as thermal damage to cell membranes or even changes in their chemical composition.

This study was carried out for the purpose of assessing the effects of temperature and drying rate on the quality of natural coffee.

MATERIALS AND METHODS

Coffee fruits of the Mundo Novo cultivar (*Coffea arabica* L.) were harvested in the 2010 crop year in the southern part of the state of Minas Gerais, Brazil (Latitude: 21° 27' 45" S, Longitude: 45° 19' 17.8" W, altitude: 1100 m). The fruits were selectively harvested and then subjected to hydraulic separation for the removal of fruits with lower mass densities (dry pods, floaters, malformed beans, etc.) and subjected to manual selection for the removal of immature and overripe fruits. After that, the selected ripe berries were sun dried in a suspended bed until they reached a moisture content of approximately 30 - 35% (wb) and then dried in mechanical dryers with heated air.

The coffee was dried for 14-hour periods, alternating with 10-hour intervals for rest. The speed of drying air was kept constant at 0.33 $m.s^{-1}$, corresponding to the flow of $20 m^3. min^{-1}.m^{-2}$ of perforated sheeting. Knowing the mass and initial moisture content of the coffee, the drying rates were obtained by the gravimetric method (Equations 1, 2 and 3). The trays containing the samples were removed from the dryer and weighed every two hours on an analytical balance with 0.01 g precision until reaching the moisture content of 11±0.5% (wb).

$$MC_{db} = \frac{WM}{dm} \quad (1)$$

$$MCt = \frac{WMi - (CMi - CMt)}{dm} \quad (2)$$

$$DR = \frac{MCpr - MCt}{\Delta t} \quad (3)$$

where:

- MC_{db}: Moisture Content in dry basis ($kg.kg^{-1}$)
- WM: water mass (kg)
- dm: dry mass (kg)
- MCt: Moisture Content at time “t” ($kg.kg^{-1}$)
- WMi: initial water mass (kg)
- CMi: initial coffee mass (kg)

- CMt: Coffee mass at time "t" (kg)
- DR: Drying rate (g. kg $^{-1}$.h^{-1})
- MCpr: Previous Moisture Content (kg.kg^{-1})
- MCt: Moisture Content at time "t" (kg.kg^{-1})
- Δt = time interval between weighings (hours)

The effects of the three dry bulb temperatures (Tdb) and three drying rates (DR) for each dry bulb temperature, obtained by the combination between the dry bulb temperatures and dew point temperatures (Tdp), were studied (Table 1).

Table 1. Dry bulb temperature, dew point temperature and relative humidity of the drying air.

Tdb (°C)	Tdp (°C)	Relative	Average DR	Maximum DR
35	16.2	32.7	4.68	11.34
35	10.8	23.0	5.61	13.35
35	2.6	13.1	6.41	14.59
40	16.2	25.0	6.57	15.89
40	10.8	17.5	7.38	21.22
40	2.6	10.0	8.51	23.14
45	16.2	19.2	10.60	23.19
45	10.8	13.5	12.13	29.82
45	2.6	7.7	13.27	53.12

The effects of treatments on coffee quality were studied through analysis of total, reducing and non-reducing sugars, electrical conductivity, leaching of potassium, free fatty acid profile and sensory analysis. Analyses were made at two distinct time periods: soon after drying, and after seven months of storage in a chamber with a controlled environment at a temperature of 10 °C and relative humidity of 50%, packed in brown single-layer kraft paper bags and transparent polyethylene plastics sacks. Analyses were only made on coffee bean samples from 16, 17 and 18 size screens to ensure roasting uniformity. Moreover, defective beans, such as poorly formed, badly filled, and unripe beans, husks, and broken and insect-bored beans were discarded to avoid external effects that might interfere with the treatments used.

RESULTS AND DISCUSSION

The contents of total, reducing and non-reducing sugars and free fatty acids did not show significant differences due to temperature and drying rate, regardless of the period of analysis, before or after storage.

The results of electrical conductivity, potassium leaching and sensory analysis of natural coffee according to temperatures and drying rates are shown in Tables 2 and 3, respectively.

Table 2. Electrical conductivity, potassium leaching and sensory analysis of natural coffee according to drying temperature.

Storage (months)	Tdb (°C)	Electrical conductivity ($\mu S.cm^{-1}.g^{-1}$)	Potassium leaching (ppm)	Sensory analysis
0 (zero)	35	56.22 a	17.62 a	82.40 a
	40	61.18 b	18.77 a	82.00 a
	45	72.93 c	21.50 b	80.44 b
7 (seven)	35	67.23 a	20.36 a	80.41 a
	40	77.54 b	23.51 b	79.87 a
	45	81.99 b	25.12 b	79.19 a

Mean values followed by the same small letters in the column do not differe among themsleves (P>0.05) by the Tukey test.

It may be observed in Table 2 that, regardless of the time period in which analyses were made, before or after storage, the values of electrical conductivity and potassium leaching of the coffees showed a clear tendency of increasing with the increase in temperature, so that the coffees dried at a temperature of 35 °C had lower values of electrical conductivity in comparison with coffees dried at a temperature of 45 °C and coffees dried at a temperature of 40°C had intermediate values. It may also be observed that when sensory analysis was made soon after drying, the coffees submitted to drying temperatures of 35 °C and 40 °C showed significantly greater scores when compared to coffees submitted to a drying temperature of 45 °C. After seven months of storage, a tendency of reduction in the scores was observed with the increase of temperature. Nevertheless, these differences were not statistically significant. These results prove the negative effects of high temperature for production of natural specialty coffees.

Table 3. Electrical conductivity, potassium leaching and sensory analysis of natural coffee according to drying rate.

Storage (months)	Tdb (°C)	Drying rate (g water.kg^{-1}.h^{-1})	Electrical conductivity (µS.cm^{-1}.g^{-1})	Potassium leaching (ppm)	Sensory analysis
0 (zero)	35	11.34	53.36 a[1]	16.88 a	83.94 a[1]
		13.35	53.53 a[1]	16.88 a	82.87 ab
		14.59	61.77 b	19.11 a	80.37 b
	40	15.89	56.74 a	17.96 a	82.68 a
		21.22	66.21 b	20.25 a	81.00 a
		23.14	64.62 b	18.11 a	82.31 a
	45	23.19	78.61 b[1]	20.71 a	79.25 a
		29.82	63.79 a	20.63 a	83.00 b
		53.12	76.41 b[1]	23.16 a	79.06 a
	Sun dried		65.10	20.92	80.25
7 (months)	35	11.34	62.12 a	18.63 a	81.79 a[1]
		13.35	61.82 a	17.94 a	80.67 ab
		14.59	77.75 b	24.53 b	78.79 b
	40	15.89	62.92 a	19.07 a	81.74 a
		21.22	88.33 b[1]	26.77 b[1]	79.04 ab
		23.14	81.36 b[1]	24.68 b[1]	78.83 b
	45	23.19	85.23 a[1]	26.27 a[1]	78.66 ab
		29.82	75.04 a	23.34 a	81.41 a
		53.12	85.23 a[1]	25.74 a[1]	77.50 b
	Sun dried		67.06	20.88	78.5

Mean values followed by the same small letters in the column do not differ among themselves (P>0.05) by the Tukey test; [1]mean value differs statistically (p>0.05) by the Dunnett test in relation to coffee dried completely in the sun.

In Table 3, it may be observed, regardless of the time period in which the coffee was analyzed, that the increase in the drying rate negatively affects coffee quality and its effect depends on the temperature. For the temperatures of 35 °C and 40 °C, in general, the negative effects of increasing the drying rate on coffee quality are more pronounced, causing the increase of electrical conductivity and potassium leaching and reduction in sensory analysis scores. For the temperature of 45 °C, both the high drying rate and the greater exposure time to this temperature, when the coffee is subjected to a lower drying rate, cause physiological damage to coffee. That way, the effects of the drying rate are overlaid by the effects of thermal damage, making them not very evident.

It may also be observed that the coffees dried at a temperature of 35 °C and the drying rate of 11.34 g.kg^{-1}.h^{-1} show significantly greater sensory scores when compared to the coffee completely dried in the sun, which does not show any differentiation from the other treatments.

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Identification of Biochemical and Physiological Markers Related to Green Coffee under Storage

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SUMMARY

Cup quality of coffee is affected during storage due to various factors such as air temperature and humidity. This is an important constraint for the coffee beverage industry due to the loss of quality during storage, particularly affecting premium coffee. The study aims to identify the influence of storage conditions (temperature, humidity, oxygen) and their effects on physiological parameters, biochemical and sensory profiles.

One Arabica green coffee batch is divided into 11 samples stored under different conditions, trying to accelerate or slow down the aging of coffee. Our results indicate two different mechanisms of green coffee modification under storage. In one hand, there is a decrease over time in chlorogenic acids and total sugars contents but the decrease is related neither to a specific storage conditions nor to the viability of the beans. In contrast, the acid value and acetic acid increases, seed viability loss, and the increase of some volatile compounds in green coffee are related to the storage conditions. Furthermore, these biochemical and physiologiocal changes are associated to the global sensory deterioration of green coffee. The above-mentioned parameters are potential markers for green coffee quality control.

INTRODUCTION

Coffee flavour is associated with the chemical composition and physical characteristics of green coffee. In the two last decades, scientific interest has grown in the physiology and biochemistry of green coffee beans and their role in the quality of final roasted coffee.

Different factors like air temperature and humidity can modify the coffee cup quality over time and also its biochemical composition. Selmar and co-workers investigated the impact on the coffee quality of different post-harvest processing (wet, dry, semi-dry processing) under standard storage conditions for 2 years. Storing coffee beans within the parchment keep the viability of the beans for 1 year. In comparison, hulled coffee beans lost viability within the first 6 months of storage. A decrease in glucose, fructose and glutamine is observed but the decrease is not correlated with the viability of the coffee beans.

It is already demonstrated that the bean life and the cup quality can be extended by storing the beans in appropriate conditions. According to Wajda and co-workers, the acid value is a function of the age of coffee beans, the acid value increased over time for the coffee beans stored under tropical conditions for up to 4 years due to high temperature and humidity, which correlated negatively with sensory quality. In comparison, samples stored under European conditions showed lesser increases in acid value over 5 years and lesser sensory changes.

The roasted coffee storage has also an impact on the volatile compounds profile, Pérez-Martínez M and co-workers, investigated changes in volatile profiles of Arabica coffee brews

during storage at 4 and 25°C. Seven compounds are proposed to monitor the age and the sensory quality of stored coffee brews.

The aim of the current study is to identify chemical and physiological modifications in green coffee during storage under different conditions. These modifications are compared over time to the sensory quality in order to identify potential markers for the coffee aging.

MATERIALS AND METHODS

Green coffee samples

The experiment is organized with one single batch of *Coffea arabica* Var. Typica produced in Ecuador with a fully washed process at 1,300 m asl. Prior to the experimental phase, the coffee batch is maintained in parchment at 9°c and 65 % RH. The batch is hulled and then divided into 11 samples stored at different conditions which are a combination of 3 ambient parameters: temperature, humidity, and oxygen (Table 1). To obtain the different conditions, samples are maintained in a temperature-controlled heated oven at 25 or 40°C and in a temperature-controlled cold room or refrigerator at 9 and -25°C. To obtain the relative humidity, two salts are used: $MgCl_2$ for 33% RH and $Mg(NO_3)_2$ for 53% RH. Nitrogen cylinder containing 2% oxygen is used to maintain oxygen at 2%. To simulate warehouse storage, one sample is kept at ambient temperature. Portions of all samples are withdrawn at 3, 6 and 9 months for chemical, physiological and sensory analyses.

Table 1. Storage conditions for Arabica green coffee sample.

Sample name	T°C	Humidity (%)	Oxygen (%)	
A	25	33	21	
B	25	33	2	
C	25	53	21	
D	25	53	2	
E	40	33	21	
F	40	33	2	
G	40	53	21	
H	40	53	2	
I	9	65	21	
J	-25	65	21	Control sample
K	20-25	20-50	21	Room Ambient conditions

Chemical analyses

Different extraction and analyses methods are used such as HPLC for chlorogenic acids (CA) analysis; ion exchange Dionex ICS5000 for carbohydrate analysis; oil extraction is done using Buchi extractor B-811and then titration with an ethanolic potassium hydroxide in presence of phenolphthalein for acid value determination, and headspace solid-phase microextraction coupled to gas chromatography – mass spectrometry (SPME-GC-MS) for the volatile compounds in green coffee samples. Results are shown as the means of extraction and analysis of two replicates, expressed as % of dry matter (DM).

The viability of green coffee beans are checked using TTC reagent (2,3,5-triphenyltetrazolium chloride) following the protocol reported by Dias and Da Silva. Qualitative information is obtained based on the staining results using a scale from 0 (non-viable bean) to 2 (viable

bean). TTC (2,3,5-triphenyltetrazolium chloride) is a redox indicator used to differentiate between metabolically active and inactive tissues. The bean red colour after staining indicates the viable beans (1 'light red colour to 2 'intense red colour') and no colour change after staining indicates non-viable beans (0).

Table 2. TTC results for viable and dead beans.

Positive test (viable bean)	Negative test (dead bean)
	

Sensory analysis

Sensory properties of samples were evaluated using a standardized procedure. An internal panel of 4 selected and trained panelists evaluated the samples. Tasting sessions were organized with two successive tables of four samples and average calculated on the score of the 4 panelists.

Sensory attributes such as coffee aroma, flavor, body, acidity, aromatic intensity, bitterness and defectives notes were evaluated on a scale from 0 to 5. Positive and negative attributes were used to build a sensory index using the following formulas:

- Positive attributes = Coffee aroma + flavor + Body + acidity + aromatic.
- Negative attributes = Bitterness + defective notes.
- Sensory Index = Positive attributes – Negative attributes.

RESULTS AND DISCUSSION

Effects of the factors under study

The effect of the storage duration (periods of sampling), the oxygen content in the gaseous phase, the air humidity and the ambient temperature are statistically analysed by ANOVA on the main parameters observed during the experimental phase (Table 3). There are highly significant differences induced by the storage duration and the temperature for almost all parameters under consideration, except the total chlorogenic acids content (Total CA) of green coffee in the case of temperature. Except on viability, there are no significant differences induced by the percentage of oxygen in the atmosphere. It is suspected that the lower level at 2 % is still too high to induce significant differences.

Statistical differences are also observed on the acid value and hexanal concentration in the headspace when different ambient humidities are applied during storage.

Table 3. Effect of the different factors investigated during coffee storage (p value – ANOVA).

Factors	Number of replications	Cup tasting	Biochemical composition				Physio.	Volatiles	
		Sensory index	Total sugars	Acetic acid	Total CA	Acid value	Viability	Hexanal	Pentyl-Furane
Time (month) (0, 3, 6, 9)	11	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
Oxygen (2 & 21%)	16 or 8	0.195	0.801	0.558	0.777	0.549	**0.013**	0.085	0.305
Humidity (33 & 53%)	16 or 8	0.536	0.640	0.187	0.782	**0.000**	0.748	**0.004**	0.136
Temperature (-25, 9, 22, 25 & 40°c)	4 or 2	0.066	**0.010**	**0.004**	0.677	**0.004**	**0.003**	**0.019**	**0.000**
Temperature (25 & 40°c)	16 or 8	**0.001**	0.064	0.545	0.107	**0.000**	0.335	0.809	**0.005**

Significant p values are in bold character

Effect of temperatures over the time

The sensory value of all treatments is decreasing over time with strong drop after 6 months (Figure 1). The sensory decrease is stronger at higher temperature but even detectable at –25°c.

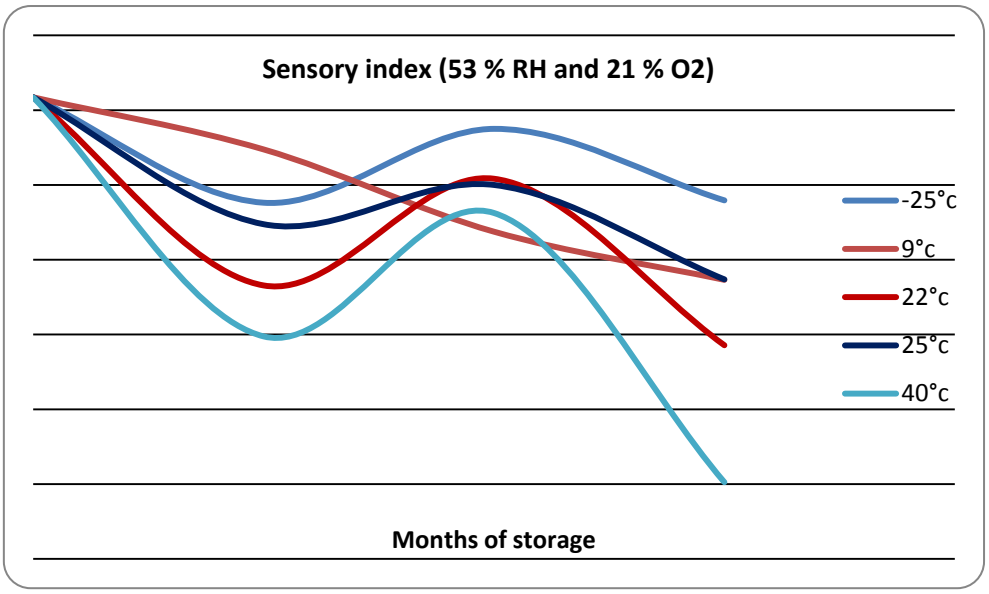
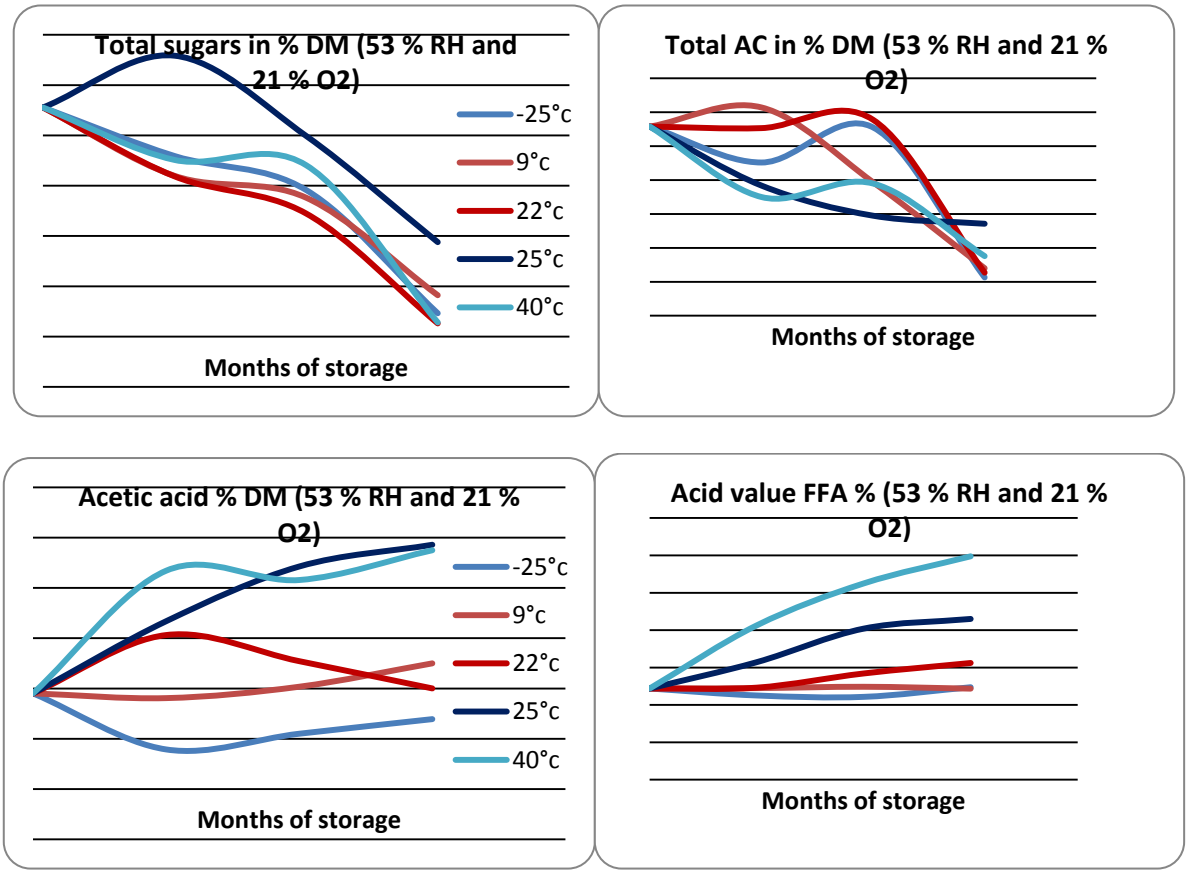


Figure 1. Sensory index fluctuations over time at different storage temperatures.

There are also significant changes in the composition of the green coffee as illustrated in figures 2 to 5. Total sugars and chlorogenic acids contents are decreasing over time in all coffees under storage with a strong drop for the chlorogenic acids after 6 months of storage. The treatment at 25°C keeps significantly more sugars than other treatments when all temperatures are compared, but this difference is no more significant when the comparison is

done between 25°C and 40°C with more replications (Table 2). The acid value which is an indicator of the lipids degradation is significantly increasing over time for the treatments at 22°c and over. When lower temperature are applied (9°C and – 25°C), the acid value is stable over the periods under consideration. There is a significant increase in the acetic acid content of the green coffee stored at high temperatures (25°C and 40°c), but a slight increase was also detected for the treatment 22°C after 3 months. For the acetic acid content at lower temperatures it is nevertheless difficult to conclude as these coffees were not maintained under confinement as they were for the highest temperatures (25°C and 40°C). It can be suspected that the acetic acid accumulation observed at these temperatures is not detected for the other treatments which were not confined (22°C, 9°c and – 25°C).



Figures 2 to 5. Biochemical modifications of green coffee over time at different storage temperatures.

Viability test using TTC shows that the green coffee beans are no more viable after 3 months at 25°C and 40°C (Figure 6). While the green coffee beans stored at low temperatures (-20°C and 9°C) are still viable after 9 months. The viability is also decreased for green beans stored at room temperature.

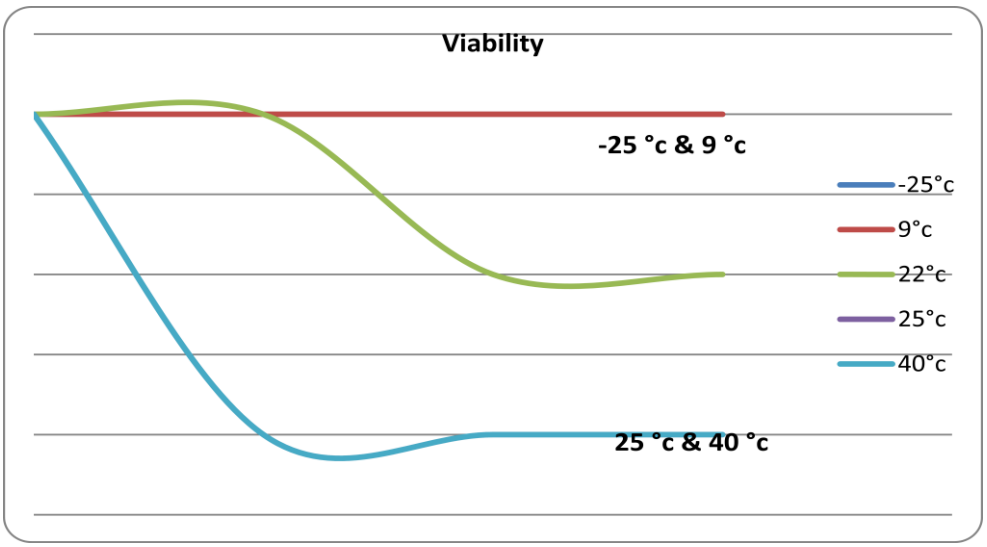
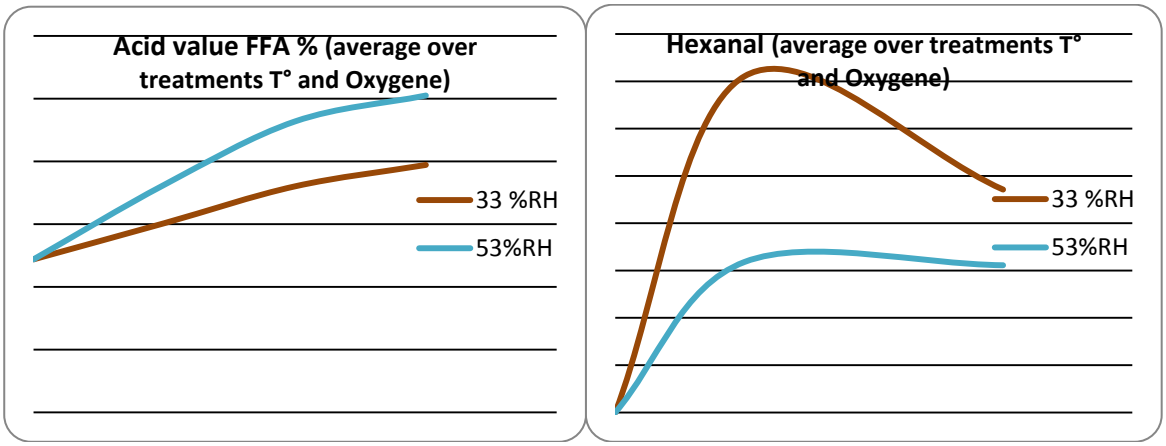


Figure 6. Viability test of coffee beans over time at different storage temperatures.

Effect of the humidity over time

There are significant differences between the two treatments under consideration (33 & 53 % RH) for the acid value of the green coffee lipids fraction and the volatile compound hexanal (Figures 7 & 8).



Figures 7 & 8. Changes over time in acid value and hexanal at different relative humidities.

Once again the acid value is increasing over time and this phenomena is more important at high humidity (53% RH). Changes in the concentration of several volatile compounds are observed. Some volatile compounds are increasing in intensity over time during storage such as aldehydes, and furans. Two main compounds are detected: hexanal and 2-pentylfuran.

CONCLUSIONS

The cup quality of green coffee is decreasing under all storage conditions, but this trend is significantly amplified at higher temperatures. This quality decrease is likely related to numerous physiological and chemical changes happening in the coffee beans during the storage period. Our results indicate two different mechanisms of green coffee modification under storage.

In one hand, there is a decrease over time in chlorogenic acids and total sugars contents but the decrease is related neither to a specific storage conditions nor to the viability of the beans. In contrast, the acid value and acetic acid increases, seed viability loss, and the increase of some volatile compounds in green coffee are related to the storage conditions, mainly temperature and humidity. The increase of the acid value and the hexanal is resulting of lipids degradation over time most probably leading to off-flavor notes affecting the green coffee cup quality. Hexanal is known to give a rancid off-note to coffee and it is formed after the oxidation of polyunsaturated fatty acids such as linoleic acid. The 2-pentylfuran content is increasing only at 40°C. The furans are formed during the degradation of carbohydrates, lipids and ascorbic acid at high temperature.

The current work is only describing the changes, more investigations are required to understand the mechanisms behind these changes. Nevertheless, some enzymes such as polyphenol oxydase, lipase and invertase are known to degrade phenolic compounds, lipids and carbohydrates respectively.

In the light of our results, acid value and the viability test are potential markers for green coffee aging control. For the acid value, it can be tentatively considered to use the limit of 2.0 FFA % as the maximum acceptable value for green coffee quality. This marker highlights the age and the storage conditions of coffee.

Work is in progress to confirm the chemical and physiological changes identified in the current study using various green coffee beans with different biochemical compositions. Further investigation will be carried out to better understand physiological, biochemical and sensory changes:

- Reactive oxygen species formation (ROS), they play an important role in the regulation of seed aging and ultimately leading to the seed death.
- Lipid peroxidation: e.g. malondialdehyde analysis.
- Protein oxidation: reversible and irreversible oxidation of some amino acids.
- Thermodynamic property of coffee beans using DSC technique (differential scanning calorimetry).

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Physico-Chemical Characterization of the Waste Produced By Mechanical Pulping of Robusta Coffee

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SUMMARY

This work that aims to characterize the residue of Robusta coffee for use as an ingredient in the industry, two samples of coffee bean were received. These grains were selected, followed by extraction of the pulp and as a result, we obtained three different samples: coffee pulp, coffee aqueous residue and Finisher's residue. These samples were subjected to laboratory tests: soluble solids content, total polyphenol, total solids, total anthocyanins, lipid content, fructose, glucose, tannin, chlorogenic acids, caffeine and trigonelline, which allowed the study of the viability of using the Rosbusta coffee residue for industrial new beverage production.

INTRODUCTION

In coffee producing countries, waste and by-products of this chain are a major source of contamination and a serious environmental problem. Therefore, since the middle of last century have been striving to develop methods to use them as raw material for the production of feed, beverages, vinegar, biogas, caffeine, pectin, pectin enzymes, protein and organic fertilizer. In addition, by-products used as ingredients for other sectors have a source of income for the coffee production chain with high added value. Specifically in the case of coffee pulp or mucilage, there are studies that show results of the chemical composition of pulp that indicate its use as an ingredient for the food and drinks. In the case of Robusta coffee, where there is mechanical pulping, there is a substantial amount of waste produced. O use of products such as the pulp of coffee cherries brings both environmental and economic advantages since it is an alternative to add value to the productive chain of coffee. However, for the application of slurry in the food and drinks you need a careful study of the intrinsic characteristics of the product. In this context studied was the byproduct of the pulping waste of Robusta coffee. At this stage of the research studies have been conducted on their physico-chemical composition, as well as preliminary studies of income and technology industrialization of waste.

MATERIALS AND METHODS

Were evaluated two different Robusta coffee clones from the collection of Campinas Agronomic Institute (IAC), identified the samples as 67-9 and 69-1. For the extraction of the pulp/mucilage from the insoluble material (Skin), was added water in the ratio 1:1 (weight/weight). After that, the material was placed in a container for the extraction of soluble solids under mild agitation, without heat. Then, the samples were passed on Finisher equipment (FMC, model 35/cm-876) and using sieve size 40'. This way, three samples were

initially evaluated for this stage of research: residue of coffee extractor into peeler (pulping) with traditional mechanical addition of water, it was called pulp; the aqueous residue extracted into coffee peeler (pulping), it was called the aqueous extract; and the residue retained on the sieve of the pulper unending screw called Finisher's residue. The three treatments described above were evaluated for Robusta coffee clones 67-9 and 69-1. The physicochemical evaluation for this six samples were performed according the literature: Soluble Solids Content was determinated by Carls Zeiss refractometet, model 32-G110d (Jena); pH was assessed by a Digimed brand, model DM20 pH-meter; Humidity/Moisture and total solids; Anthocyanins; Polyphenol content; Sugars was meadured by liquid chromatography; Dietary fiber and total tannins; Total lipids was made by combined methods; Trigoneline, caffeine and chlorogenic acids.

RESULTS AND DISCUSSION

Were collected 14,700g of Robusta coffee variety of 67-9, and 925g were discarded in the manual selection; 12,555g were used for extraction of the pulp, giving 5,885g of skin, with a yield of 46.8%. In the selection of this coffee beans, we could observe that it's larger and with fewer defects in comparison with the other clone.

As for 69-1 variety, it were harvested 12,900 grams of coffee, and in manual selection, 1,405g were discarded; then, 8,705g were used for pulp's extraction, giving 3,700g of skin, with a yield of 42.5%. In this selection we could notice that this coffee the grains were smaller and more defects than the other sample.

The physicochemical measurements made in each sample are presented in Table 1.

The analysis of anthocyanins for the six samples tested showed no representative quantity that the used method could determine in the following treatments: pulp 67-9, *Finishe*r's residue 67-9 and aqueous extract 69-1. For the other three samples was observed a small amount of anthocyanin. This first analysis has indicated that the product studied had no significant amounts of total anthocyanins.

The results show that the pH of coffee bean 67-9 is less acid than the 69-1, and in both clones there was a slight decrease in pH after completion of the procedures, particularly by addition of water. 67-9 pulp has less soluble solids than the 69-1, and in both of them we could realize a higher concentration of soluble solids in the residual skin than in theirs aqueous extract. The results of polyphenols are shown in Table 1, and they were analyzed in BioEstat 5.0 software, which was made using ANOVA and Tukey Test ($p<0.001$).

The results presented in Table 1 demonstrate that, in all treatments, the clone 67-9 showed higher values compared to total polyphenols in the clone 69-1. Among the treatments, the *Finisher*'s residue 67-9 had the highest value, differing from the rest. Was found to each clone of coffee, that the *Finisher's* residue had more polyphenols than the other treatments, indicating that the residue could be better used in terms of extraction and processing technology.

Table 1. Determinations of physicochemical Robusta coffee samples.

Sample	pH	Soluble Solids (°Brix)	Moisture bs (%)	Polyphenols (mg/100g d.b.)[1A]
Pulp 67-9	5.13	8.40	82.94	1140.98a
Pulp 69-1	5.33	10.0	80.66	688.16b
Aqueous Extract 67-9	5.28	2.50	97.60	1640,22c
Aqueous Extract 69-1	5.07	3.34	96.72	698.16b
Finisher's Residue 67-9	5.25	7.80	83.75	1866.00d
Finisher's Residue 69-1	5.07	6.70	77.18	1117.83e

[1]*Different letters indicate statistical difference (p<0.001)* [A]*db=dry base.*

The results obtained for sucrose (g/100g) and chlorogenic acids (mg/100g) were not detected in the sample of Robusta Coffee.

The results for lipids, fructose, glucose, tannin, caffeine, trigonelline and dietary fiber are show in Table 2.

Table 2. Determinations of physicochemical in Robusta coffee samples.

Treatments	Total lipids (g/100g)	Fructose (g/100g)	Glucose (g/100g)	Tannins (mg/100g)	Caffeine (mg/100g)	Trigonelline (mg/100g)	Dietary Fiber (mg/100g)
Pulp 67-9	0.21c	1.76b	1.38b	38.71b	37.49d	128.13b	8.77d
Pulp 69-1	0,25b	2.31a	1.86a	64.46a	70.52b	228.64ab	9.85
Aqueous extract 67-9	ND < 0.05	0.69e	0.56e	42.54b	4.19e	32.52e	ND
Aqueous extract 69-1	ND < 0.05	0.99d	0.82d	22.98c	5.55e	57.99d	ND
Finisher's Residue 67-9	0.30a	1.23c	0.97c	34.31b	41.06c	119.16c	9.39c
Finisher's Residue 69-1	0.31a	1.35c	1.03c	62.70[a]	109.64a	231.47a	13.59a

[2]*Different letters indicate statistical difference (p<0.001). ND = Not Detected.*

By observing the results obtained for total lipids, we see an insignificant quantity in samples of the aqueous extract of the two clones of coffee, which was expected, because the extraction was performed only with water. We note that the pulp coffee clone 69-1 contains more lipids than noted in coffee 67-9, however, for the *Finisher's*residue insignificant differences were noted between the two clones. As expected, the aqueous extract of both clones has shown no significant result of dietary fiber, since most of the fiber is in the concentrated *Finisher'*s residue. The presence of fiber is important, since they contribute to cake formation and retention of noxious compounds.

The results presented in Table 2 demonstrate, in all treatments, that the clone 69-1 has showed higher values of fructose and glucose when compared to the total in 67-9. It was also noted that much of fructose and glucose is retained in the Finisher during the processing, since the aqueous shows lower values than those presented for the *Finisher's*residue. This fact indicates that the extraction of soluble solids sugar carried out with water can be improved, since the *Finisher's*residue presented a high number of sugars. Stand out in Table 2 show the levels of

tannin, caffeine and trigonelline presented for pulp and *Finisher's*residue clone 69-1, which differed significantly from other treatments, suggesting that this clone is superior to the use of these compounds and its extraction.

Statistical analysis of results of instrumental color, shown in Table 3, was performed for each treatment of both clones, since the treatments have different physical characteristics.

Table 3. Instrumental color in Robusta coffee samples.

Treatments	**L***	**a***	**b***
Pulp 67-9[3]	16.84 b	9.47 a	8.27 b
Pulp 69-1[3]	20.72 a	9.34 a	8.82 a
Aqueous extract 67-9[3]	25.58 a	4.31 b	6.26 b
Aqueous Extract 69-1[3]	26.70 a	5.42 a	7.34 a
*Finisher's*Residue67-9[3]	21.21 a	12.3 a	7.45 b
*Finisher's*Residue69-1[3]	22.82 a	8.43 b	11.00 a

Comparing the samples, we found that for parameter L* (Black to White), three samples of clone 69-1 showed brighter color than the samples from 67-9. For the parameter a* (green to red), we see that the pulp and *Finisher's*residue 67-9 were more red color than the 69-1, but for the aqueous extract the results were reversed. As for parameter b* (blue to yellow), all the treatments of clone 67-9 were less yellow than the coffee samples 69-1.

Different letters indicates statistical difference ($p<0.001$)

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Comparison of Seepage Rate of Raw and Treated Coffee Processing Effluent

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SUMMARY

The coffee processing effluents can pollute the environment and in particular the natural surface water ways. That has been mainly attributed to the clogging of seepage pits by suspended solids in the effluent thereby impairing their performance. As a result, raw effluent can easily overflow from a pit into the surface water way until its performance is restored during the offseason maintenance. For that reason, development of a strategy to remove suspended solids from the effluent before disposal into the seepage pits is necessary. This study compared the percolation of raw versus treated effluent using scaled down seepage pit models. The results indicate that seepage of treated effluent was from the pits 2 to 7 times faster than that of raw effluent.

INTRODUCTION

The by-products of the wet coffee processing include coffee pulp and the effluent. The processing effluent is normally discharged into seepage pits from to percolate into the soil. However, the disposal of the effluent has occasionally been of great concern mainly with respect to pollution of the, water ways which also cater domestic and coffee processing needs. Pollution occurs if the raw effluent overflows from the pits into the natural surface water ways due to mainly pit clogging caused by suspended solids (Adams and Dougah, 1981 and Wood *et al.*, 2000). Meanwhile, the sludge is only removed from the pits during the offseason maintenance to restore their performance but recurs in the following season. In view of that, a large area is required to accommodate more seepage pits than ideally specified for the available limited space. Consequently, the additional seepage pits in some cases tend to extended beyond the stipulated critical distance between them and the river. Therefore, an upgrade in the pit performance would effectively contain the effluent within a smaller area besides keeping the required safe distance from the surface water way.

Currently, minimization of processing water use followed by removal of suspended solids from the effluent prior to their discharge into the seepage pits have been considered as the most viable solution to that problem (Wood *et. al.*, 2000). Such a strategy expected to translate into fewer and efficient seepage pits to prevent the effluent form access the surface waterways from the pits. This study compared the seepage rate of raw and treated effluent from the pits.

MATERIALS AND METHODS

The experimental trials for this study were sited in areas where the type of soil was in conformity with specifications for coffee cultivation (Michori and Kimeu, 1980). The experimental pit model was derived from the improved pit design (Wood *et. al.*, 2000) into a

scaled down model with a diameter of 0.5 m and a depth of 0.75 m comprising the actual depth of filling the effluent, 0.5 m and a free board, 0.25 m. At each site, six (6) model pits were dug 10 m apart along a contour and used to study the seepage of raw and treated effluent.

The raw effluent was sampled as it was discharged from the factory during the coffee processing operations. Treated effluent was collected from the effluent treatment trials which were in progress at the same site. The different effluents were then poured gently from buckets into three (3) separate and randomly selected pits to fill the effective depth. The drop in the effluent level in each pit was immediately after timed and measured from a datum above the pit at preset time intervals. The seepage was allowed to continue until there was no significant difference in two consecutive records. The pit was immediately thereafter refilled again and the cycle repeated several times. Considering the seepage of raw effluent for instance, the average of every set of readings recorded from the 3 pits at the same time from the beginning were calculated. A similar procedure was applied to the data relating to the treated effluent.

RESULTS AND DISCUSSION

The average seepage values were plotted against time to generate the general seepage rate trend curves (Figure 1). The results show that 47 cm of the treated effluent had percolated out of the pit after 3 hours compared to 7.5 cm of the raw effluent. The implication of these results is that the percolation rate of treated effluent from a pit was six (6) times faster than that of raw effluent. Such a large difference between the seepage of the raw and treated effluent confirmed the importance of removing the suspended solids from the effluent prior to its discharge into the disposal pits.

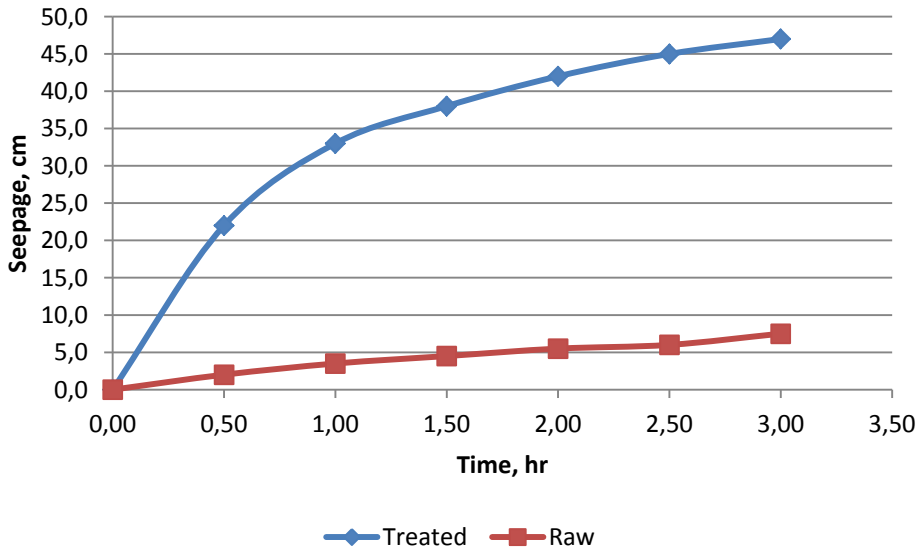

Figure 1. Seepage of raw and treated effluent.

The data responsible for the trend of each cycle of seepage of the effluent against time (Figure 1) was subjected to logarithm transformations according to Little and Hills (1972) in order to derive a general expression describing the process. The transformed data was then plotted and fitted approximately with straight lines whose r=0.987 for the treated and r=0.996 for the raw effluent (figure 2). The corresponding regression equations implied that the raw effluent

surface level from the top of the pit (z, cm) could be expressed as $z= k_r t^{0.706}$ and that of the treated effluent as $z=K_c t^{0.418}$ Where t is the time taken for the effluent surface to change by z cm from the datum while K_r=antilog(0.524) and Kt=antilog(1.491) were the infiltrations constants for the raw and treated effluent respectively.

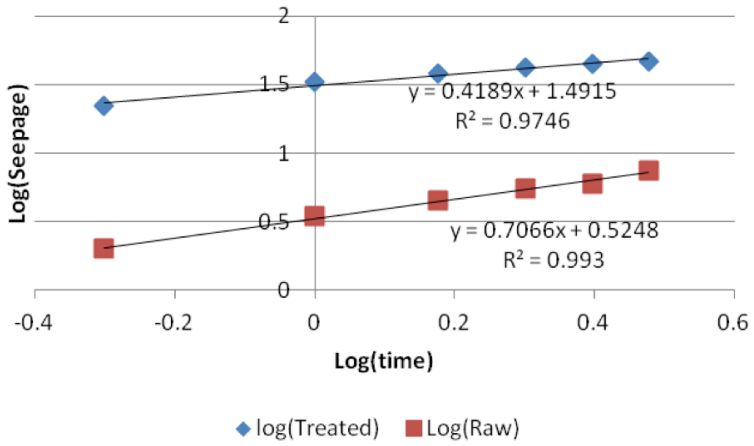

Figure 2. Relationship between log (seepage) versus log(time).

Having analyzed one seepage cycle in detail, it was important to confirm the sustainability of the pit's performance level. In view of that, it was noted that recharging the seepage pits with treated effluent every time it neared empty and timing the process resulted in cycles as shown in figure 3. The five (5) representative recharge cycles in that case demonstrated consistency in the trend after every cycle. Based on that, it could be inferred that the improved seepage of the effluent from the pits would be sustainable for quite long to last possibly a full season without clogging the pits. The pits would also be conveniently sited beyond the stipulated critical distance (150m) between the factory and the processing water source. An upgrade in the performance of the seepage pits would also release substantial space to other coffee processing operations in some factories.

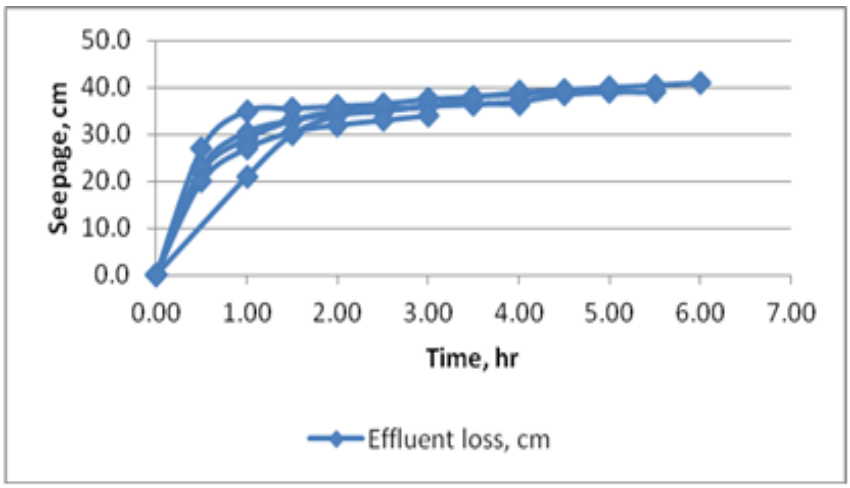

Figure 3. Multi-recharge seepage cycles.

The improved percolation of the effluent into the ground after removal of suspended solids might not only be beneficial to the vegetation close to the pit but could also stimulate other options for its utilization like irrigation. The improved performance of the seepage pits could also be important to the preceding treatment process depending on the material used to remove the solids from the effluent. For instance, the solids remover and the settled sludge could become invaluable by-products for further utilization.

CONCLUSIONS

It has been demonstrated that removal of the suspended solids from the coffee processing effluent prior to its discharge to the seepage pits can improve the performance of the latter to the extent of replacing six (6) pits with one (1). Consequently, the introduction of such an intervention in the primary coffee processing factories can ease the disposal of the effluents within the limited factory space without compromising the environment.

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Comparison of TPC and FRAP Assays for estimating Antioxidant Activities of different Roasts of Arabica Coffee Beans

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SUMMARY

Samples of Arabica coffee were harvested at Zentveld's Plantation in Newrybar (northern NSW) depulped, demucilaged and dried under controlled temperature and relative humidity conditions. The temperature was set to 60 °C and the relative humidity was maintained at 15%. These coffee beans were subjected to various degrees of roasting including light roasting, medium roasting and dark roasting. The unroasted samples (green coffee) were used as a control treatment. The standard definition of roasting degree is characterised by temperature, roasting time, colour and percentage of weight loss. The total phenolic composition (TPC) and ferric reducing antioxidant power (FRAP) were determined. Two-way ANOVA revealed that there were significant effects of roasting process for TPC and FRAP.

INTRODUCTION

Coffee is among the most popular beverages in the world. Recently, coffee has become the second most important commodity traded worldwide behind petroleum and its present value is $ 10 billion annually. There are coffee crops in more than 70 countries. The leaders among coffee producing countries are Brazil, Columbia, Ethiopia, and India.

In modern societies, consumers pay much attention to health and nutritional aspects of coffee. Focus on drawbacks of coffee drinking and caffeine intake that was brought up by epidemiological studies in the past was caused by combination of overconsumption of coffee and unhealthy behaviour, namely smoking and lack of exercise.

However, various studies demonstrated advantages of coffee consumption. Coffee activates the central nervous system. This leads to alertness and lower incidence of Parkinson disease. Moreover, coffee consumption also prevents diabetes type 2 and colorectal cancer.

There is currently a growing interest in antioxidants in food and beverages. Antioxidants are accepted as playing a significant role in protecting normal cells from reactive oxygen species (ROS), which are hazardous by-products from the respiration of regular cells.

With regard to postharvest operations, coffee processing can follow one of the following two methods which are wet processing and dry processing. Dry processing is practiced in the countries which have lower labour cost. The dry processing method can also be applied to unripe and overripe fruits. This involves just a simple drying and hulling of the coffee cherries. In contrast, the wet procedure requires a strict grading of the fruits prior to processing, because wet process involves mechanical depulping that can only be applied to fully ripe coffee cherries. The coffee processed by the wet method has a better quality which specially applies to a smooth Arabica coffee. Roasting step plays an important role in producing quality coffee.

The complicate part of roasting coffee beans consists in applying heat quickly and uniformly. This generates the aroma and flavour compounds. During the roasting process, coffee beans are exposed to dry air at 200-250 ºC for 10-20 min. This results in the loss of water and organic matter. Moreover, there is also a change of colour, volume, flavour and various compounds.

This study aims at determining antioxidant activity in fermented and non fermented coffee and to compare them within the different roasting degrees.

MATERIALS AND METHODS

Two batches of demucilaged coffee cherries weighing 500 g each were thawed at room temperature overnight. The first batch was non-soaked. The mucilaginous parchment beans were washed manually and the water was changed three times. The second batch was soaked. The coffee beans were fermented at ambient temperature for 10 h with the water to coffee ratio of 1:3.

In cases of drying process, the parchment coffee beans were dried in a cabinet dryer at 60 ºC and 15% relative humidity until a water content of 12% (wet basis) was achieved.

The roasting processing and grinding treatments: The beans were stored at -20 ºC prior to roasting. The coffee beans were manually dehulled and roasted. The treatments of roasting was divided into unroasted, light roasted, medium roasted and dark roasted. Samples were roasted using Precision Coffee Roaster® (USA). As for grinding, green coffee was frozen with liquid nitrogen and ground. Roasted coffee was also ground. Coffee was ground by Krupp, Burr Grinder GVX, China. The granule size of all samples was 1 mm.

In terms of colour measurement, the measurements were carried out on ground coffee using Minolta Data Processor Dp-301 (Japan).

The extraction stage: Coffee was brewed with deionised water using a reflux distillation apparatus. The ratio of ground coffee to water was 1:10 (w/v). The residues were re-extracted three times. The extracted samples were instantly cooled to ambient temperature in an ice bath and stored at 4 °C until required for pH determination and antioxidant activity evaluation.

In TPC determination, the Folin-Ciocalteu (FC) colorimetry was used. It is based on a chemical reduction of the mixed reagent. The products of the metal oxide reduction have a blue colour that presents light absorption at 765 nm. This intensity of light absorption is used for the estimation of the concentration of phenols.

In FRAP determination, the total antioxidant potential of the coffee samples was determined using a modification of the ferric reducing ability of plasma (FRAP) assay by Benzie and Strain (1996). FRAP reagent was prepared from 300 mmol/L acetate buffer, pH 3.6, 20 mmol/L ferric chloride and 10 mmol/L 2,4,6-tripyridyl-s-triazine made up in 40 mmol/l hydrochloric acid. All three solutions were mixed together in the ratio 100:10:10 (v:v:v). The FRAP assay was performed after preheating to 37 °C. A 2900 µL volume of reagent was added to 100 µL of sample and then incubated at 37 °C for 2 h. Absorbance at 593 nm was measured relative to a blank also incubated at 37 °C. Coffee samples were diluted 1:50 prior to assay. Antioxidant potential of samples was determined against a standard curve of Trolox. The results were expressed in µM TE/mg samples.

The data were compared using analysis of variance (ANOVA) and Duncan, multiple range test at 5% level of significance using SPSS.

RESULTS AND DISCUSSION

Antioxidant potentials of soaked and non-soaked coffee at different roasting degree were compared. The TPC and FRAP assays from a single extract were done in triplicate in order to check the reproducibility of the assays. Non-soaked green coffee showed a significantly higher antioxidant activity than soaked green coffee. Non-soaked light roasted coffee had considerably higher antioxidant activity than fermented light roasted coffee. The antioxidant activity of non-soaked medium roasted coffee was similar to that of fermented green coffee. Overall, the non-soaked coffee presented a higher level of antioxidant activity than the soaked coffee.

Table 1. Level of antioxidant activity (mean ± standard deviation) of brews obtained from beans subjected to different degree of roasting.

Roasting	TPC (mg GA/ mg)		FRAP (µmole TE/mg)	
	Soaked	Non-soaked	Soaked	Non-soaked
Green Coffee	47.02±3.18	77.15±1.28	79.37±2.48	143.13±3.17
Light Roasting	67.38±3.09	91.64±1.86	150.19±2.99	185.44±2.51
Medium Roasting	38.77±0.85	72.11±1.79	32.46±1.91	77.20±4.36
Dark Roasting	34.61±1.62	66.97±0.52	21.25±1.78	25.21±2.09

This study shows that the antioxidant activity in non-soaked coffee beans was significantly different from that in soaked coffee beans. The results could lead to conclusion that temperature of roasting affected the antioxidant activity. Antioxidant activity in coffee is an appealing feature to consumers. Any postharvest treatments that maintains the level of compounds known for their antioxidant activity is likely to be of interest to the consumers and to the food industry.

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Quality of Conilon coffee dried on a concrete terrace in a greenhouse with early hulling

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SUMMARY

Reducing the time required to dry coffee while maintaining its quality is a challenge for coffee producers. This study was performed to quantify the decrease in drying time as a result of early hulling of conilon coffee (*Coffea canephora*) dried on a concrete terrace in a greenhouse, as well as to determine the quality of the dried coffee.

The coffee was harvested at more than 80% ripe fruits and immediately placed on concrete terraces in greenhouses for drying. The parcels were hulled when 22%, 19%, 16% and 13% (wb) moisture levels were reached. Then, the parcels hulled at 22%, 19% and 16% moisture were returned to the greenhouse until they reached the desired moisture level of 13% (wb). The time spent on drying was quantified, and coffee quality was assessed through sensory, chemical and physiological analyses. The study was conducted in a randomized block design with four replications and the results submitted to the F test (ANOVA) and mean test (Tukey 5% probability).

The analyses showed the same trends for all treatments applied. In addition, the Potassium Leaching (KL) result was lower for the coffee hulled at a moisture content of 22% (wb). The coffee hulled at 22% (wb) moisture content presented a considerably shorter drying time (6 days shorter) than the coffee hulled at 13% (wb) moisture content. The early hulling of conilon coffee did not affect the final quality. Thus, early hulling is a practical and significantly favorable technique for coffee farmers because it decreases drying time and improves the use of terrace space without diminishing coffee quality.

INTRODUCTION

Conilon coffee (*Coffea canephora*) is one of the major species of the genus *Coffea* and is of great economic and social importance for Brazil and the world. Approximately 35% of the coffee produced in Brazil and more than 34% of the coffee marketed in the world is conilon coffee, while the remainder is arabica (*C. arabica*). For many years, conilon coffee has been added to arabica coffee to enhance its flavor for the production of soluble coffee, justifying its industrial production in Brazil and worldwide. Recently, this practice has been intensified, and there are several good of conilon/arabica coffee blends available.

It is known that coffee flavor and aroma are strictly related to the chemical composition of the fruit, and they depend on the complexity of more than 800 volatile compounds present in the grains. These characteristics are influenced by climate and soil conditions, coffee species and variety, crop management, harvest time, type of postharvest hulling, and drying and storage conditions. Within this context, drying is one of the key steps of postharvest coffee processing. Appropriate drying contributes to the preservation of the physiological characteristics of the coffee and guarantees a longer storage time.

Drying is one of the key stages that can overtax the costs of coffee production. The time spent on this step and the coffee quality are the fundamental factors for the choice of coffee drying procedure. Significantly reducing the drying time while maintaining coffee quality is fundamental for the best use of the drying structure and labor force and consequently for the profitability of the coffee producer. Thus, the aim of this study was to quantify and relate the reduction of drying time of early hulling of conilon coffee in terraced greenhouses with the final quality of the grains.

MATERIALS AND METHODS

The experiment was conducted in Vila Valério, in northern Espírito Santo, Brazil. The conilon coffee was harvested at more than 80% ripe fruits and placed in concrete terraces with a transparent plastic cover (terrace in a greenhouse, hangar type). The moisture of coffee was systematically monitored posteriorly using a moisture measurer, and when coffee reached 22%, 19%, 16% and 13% moisture, it was sent to a coffee peeler to be hulled. The coffee hulled at 22%, 19% and 16% of moisture was returned to the terrace until it reached 13% moisture. This procedure was performed in four distinct periods during the harvest, and four repetitions were performed in blocks.

The time required for the full drying of the coffee was quantified (before and after hulling). The sensory quality of the beverage (cup test/taste) was evaluated by a company that specializes in conilon cup tests using criteria established by the Coffee Quality Institute.

Samples of coffee were tested for electrical conductivity and potassium leaching as described by (2005), total titratable acidity and total reducing sugars using the procedures described by (1990) and soluble solids in a benchtop refractometer. The F (ANOVA) and Tukey (at 5% of probability) tests were performed to compare the evaluated characteristics among groups. The authors thank Conilon Brasil for performing the sensory analyses and Universidade Federal do Espírito Santo, Universidade Federal de Lavras and Metalurgica Fardin.

RESULTS AND DISCUSSION

Early hulling of conilon coffee reduced the drying time in a greenhouse terrace by more than six days when hulled at 22% moisture (Table 1). The average gain in drying time was approximately two days among treatments. Faster drying occurred due to the absence of pericarp, a natural barrier to the exchange of heat and water. Therefore, the area of the terrace may be reduced, as can the labor force required for the drying of the same amount of coffee per crop, allowing a significant economy of resources and work force.

Table 1. The mean values of total time spent for drying of coffee hulled at 22%, 19%, 16% and 13% of moisture and the associated quality (cup test). Vila Valério-ES, Brazil.

Hulling	Drying time (days)	Quality scale Grade[1]	Quality scale Type[1]
22%	7.20a	73.31a	Very good
19%	9.21b	75.00a	Very good
16%	11.00c	75.06a	Very good
13%	13.25d	75.50a	Very good
CV (%)	7.92	2.48	-

Means followed by the same letter were not significantly different (5% probability, Tukey's test). [1] *Quality scale used by Coffee Quality Institute.*

Despite of the early removal of the pericarp, the quality of beverage was not reduced significantly (Table 1), and all treatments received a mean grade superior to 73, considered very good by the Coffee Quality Institute.

In addition to the advantages mentioned, the use of a greenhouse terrace did not require night work and did not pollute the environment. This arrangement may also be used to securely and efficiently dry other products, such as black pepper, corn, beans, or cocoa, among others.

The content of soluble solids, electrical conductivity and titratable acidity were equal in all treatments (Table 2). Potassium leaching was lowest in coffee hulled at 22% moisture, tending to increase as moisture decreases. These results indicate that early hulling did not negatively affect the coffee quality, suggesting that it may be a more efficient way to achieve the desired characteristics.

Table 2. Mean values of electrical conductivity, potassium leaching, total titratable acidity and soluble solids after total drying in coffee hulled at 22%, 19%, 16% and 13% moisture. Vila Valério - ES, Brazil.

Hulling	Soluble solids (%)	Electrical conductivity (μS cm^{-1} g^{-1})	Potassium leaching (ppm)	Total titratable acidity (NaOH 0,1 N/100 g)
22%	1.31a	56.68a	15.48a	238.12a
19%	1.30a	79.19a	17.48ab	212.50a
16%	1.21a	69.61a	18.83ab	228.75a
13%	1.29a	86.28a	23.56b	213.12a
CV (%)	6.41	20.87	18.50	14.44

Means followed by the same letter were not significantly different (5% probability, Tukey's test).

Early hulling did not alter the content of reducing, non-reducing and total sugars (Table 3). Reducing sugars react with amino acids through the Maillard reaction, producing undesirable compounds. Generally, higher contents of total and non-reducing sugars are found in higher-quality drinks, which are responsible for the production of a caramel flavor, providing a sweetness to the drink. The reducing sugar content generally decreases with the temperature used for drying, but this was not affected by the early hulling.

Table 3. Mean values of reducing, non-reducing and total sugars after total drying in coffee hulled at 22%, 19%, 16% and 13% moisture. Vila Valério - ES, Brazil.

Hulling	Reducing sugars (%)	Non-reducing sugars (%)	Total sugars (%)
22%	**0.52a**	4.30a	4.82a
19%	0.45a	4.48a	4.93a
16%	0.49a	4.31a	4.80a
13%	0.54a	3.88a	4.42a
CV (%)	31.1	13.78	11.52

Means followed by the same letter were not significantly different (5% probability, Tukey's test).

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Drying Kinetics of Natural Coffee for Different Temperature and Relative Humidity

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SUMMARY

The purpose of this study was to evaluate the thin-layer drying kinetics of coffee berries (*Coffea arabica* L.) and to adjust different mathematical models to experimental values as a function of dry bulb and dew point temperatures of the drying air. The ripe fruits were dried in a thin layer in mechanical dryers until they reach a moisture content of 0.14 $kg.kg^{-1}$ (db). The effects of three dry bulb temperatures and three dew point temperatures of the drying air were evaluated. The modified Midili and Successive Residues (with two terms) models were the most adequate for describing the drying process. A rise in temperature and reduction in dew point temperature increased the effective diffusivity coefficient and drying rate and reduced drying time of the coffee.

INTRODUCTION

Drying is, without doubt, the key point in the production of natural specialty coffees, because the presence of the shell and the mucilage with high sugar content, reduces the drying rate and increases the risk of undesirable fermentations occur, affecting their quality. The drying rate is influenced mainly by the temperature of the drying air. However, if coffee bean temperature exceeds 40°C during drying, coffee quality is harmed. On the other hand, the drying time of grains and seeds may be significantly reduced and maintained its quality, by reducing the relative humidity of the drying air, particularly when using relatively low drying temperatures. In light of that, a mathematical model that represents the drying kinetics of coffee fruits becomes necessary for determining the ideal operating conditions of the drying system so as to ensure the least time and, consequently, lowest power consumption. Numerous models have been proposed to describe the drying rate in thin-layer drying of biological materials. These studies are fundamental for the development of mathematical models for simulating the drying of agricultural products.

Considering the importance of the theoretical study of the drying processes of agricultural products, the purpose of this study was to evaluate the thin-layer drying kinetics of coffee fruits of the Mundo Novo cultivar (*Coffea arabica* L.) and to adjust different mathematical models to experimental values as a function of the dry bulb temperature and dew point temperature of the drying air.

MATERIALS AND METHODS

The ripe coffee cherries of the Mundo Novo cultivar (*Coffea arabica* L.) were harvested by hand picking, in the 2010 crop year, in the southern part of the state of Minas Gerais, Brazil (Latitude: 21° 27' 45" S, Longitude: 45° 19' 17.8" W, altitude: 1100 m). Then the cherries

were processed by dry method. The cherries were sun dried in a suspended bed until they reached moisture content of 0.5 (db.) and then dried with heated air in a thin layer in mechanical dryers until they reach moisture content of 0.14 (db). The effects of the three dry bulb temperatures and three dew point temperatures of the drying air on the drying kinetics of natural coffee were studied. Different values for the relative humidity of the air were obtained as a result of the combinations of dry bulb temperatures and dew point temperatures (Table 1). Air velocity was kept constant at 0.33 m s^{-1} throughout the entire drying period, which corresponds to the airflow $20 m^3.min^{-1}.m^{-2}$.

Table 1. Dry bulb temperature, dew point temperature and relative humidity of the drying air.

Dry BulbTemperature (°C)	**Dew Point Temperature** (°C)	**Relative Humidity** (%)
35	2.6	13.1
35	10.8	23.0
35	16.2	32.7
40	2.6	10.0
40	10.8	17.5
40	16.2	25.0
45	2.6	7.7
45	10.8	13.5
45	16.2	19.2

The gravimetric method was used to calculate the moisture content during drying. The trays with coffee samples were removed from the drier and weighed each two hours until reach the final moisture content of 0.14 (db).

The moisture ratio (MR), which was calculated by equation 1, is essential for describing the different models of thin-layer drying.

$$MR = \frac{(M - Me)}{(Mi - Me)} \quad (1)$$

where MR is moisture ratio (non-dimensional); M is product's moisture content at time t (db); Me is product's equilibrium moisture content (db) and Mi is product's initial moisture content (db).

For each drying time, the moisture content of coffee was correlated with the initial moisture content and equilibrium moisture content for the specific drying conditions. Thus, under all conditions evaluated, the values for moisture ratio in relation to drying time were adjusted to the different models proposed in the literature used for describing the drying kinetics of coffee fruits (Table 2).

Table 2. Mathematical models used to predict the drying phenomenon.

Model Designation	**Model**
Lewis	$MR = exp(-kt)$
Page	$MR = exp(-kt^n)$
Thompson	$MR = exp\{[-a-(-a^2 + 4bt)^{0,5}] (2b)^{-1}\}$
Modified Midilli	$MR = exp(-kt^c) + bt$
Verma	$MR = -a\ exp\ (-kt) + (1-a)\ exp(-k_1 t)$
Successive Residues	$MR = a\ exp(-(b\ exp(-c\ T^{-1}))\ t) + d\ exp\ (-(e\ exp\ (-f\ T^{-1}))t)$
Modified Henderson & Pabis	$MR = a\ exp\ (-kt) + b\ exp\ (-k_0\ t) + c\ exp\ (-k_1\ t)$

where t is drying time (h); T is dry temperature (° C); k, k0 e k1 are constant drying and; a, b, c, d, e, f, n are model coefficients.

For adjustment of the mathematical models, nonlinear regression analyses were performed by the Gauss-Newton method, using STATISTICA 5.0 ® software (Statsoft, Tulsa, OK, USA). The choice for the best model was based on joint analysis of the following statistical parameters: standard deviation of the estimate (SE), mean relative deviation modulus (P) and coefficient of determination (R^2). The standard deviation of the estimate and mean relative deviation modulus were calculated using equations (2) and (3), respectively.

$$SE = \sqrt{\frac{\sum_{i=1}^{n}\left(Y - \hat{Y}\right)^2}{DF}} \tag{2}$$

$$P = \frac{100}{n}\sum_{i=1}^{n}\left(\frac{\left|Y - \hat{Y}\right|}{Y}\right) \tag{3}$$

where SE is standard deviation of the estimate (decimal);Y is experimentally observed value; Y^ is value calculated by the model; DF is model's degrees of freedom (number of parameters of the model minus one); P is mean relative deviation modulus (%) and n is number of observed data.

The effective diffusivity coefficient for the drying conditions used in this study was calculated by adjusting the model based on the theory of liquid diffusion (equation 12) to the observed data by means of nonlinear regression, using STATISTICA 5.0® software (Statsoft, Tulsa, OK, USA). This equation is the analytical solution for Fick's second law, considering the geometric shape of the product to be spherical, disregarding the volumetric shrinkage of the fruits and considering the boundary condition of the known moisture content on the surface of the product.

$$MR = \frac{M - Me}{Mi - Me} = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left[\frac{-n^2\pi^2 D_{eff}}{R_e^2}t\right]$$

where Deff is effective diffusivity coefficient ($m^2.s^{-1}$); Re is equivalent radius of the coffee fruits (m); n is number of terms and t is time (s).

RESULTS AND DISCUSSION

The statistical parameters, coefficients of determination (R^2), standard deviations of the estimate (SE) and mean relative deviation modulus (P), the modified Midilli model constants and coefficients adjusted to the observed and the effective diffusivity coefficients obtained for the drying of coffee fruits for the different combinations of dry bulb and dew point temperatures used in this experiment, are shown in Table 3.

Table 3. Statistical parameters, constant drying, model coefficients fitted for the modified Midilli model to the experimental data and effective diffusivity coefficient (Deff) of coffee berries.

Model	Tdb (°C)	Tdp (°C)	RH (%)	R^2(%)	P(%)	SE	k	c	b	D_{eff} x 10^{11} (m^2/s)
Modified Midili model	35	2.6	13.1	99.95%	1.16	0.0234	0.0227	1.1131	0.0008	1.460
	35	10.8	23.0	99.97%	0.98	0.0185	0.0281	1.0660	0.0005	1.586
	35	16.2	32.7	99.96%	1.00	0.0236	0.0311	1.0800	0.0005	1.810
	40	2.6	10.0	99.92%	1.51	0.0227	0.0420	1.0286	0.0012	1.927
	40	10.8	17.5	99.93%	1.41	0.0218	0.0569	0.9626	0.0008	2.214
	40	16.2	25.0	99.97%	0.90	0.0167	0.0576	0.9693	0.0003	2.350
	45	2.6	7.7	99.89%	2.21	0.0213	0.0600	1.0153	0.0010	2.517
	45	10.8	13.5	99.95%	1.23	0.0161	0.0738	1.0407	0.0026	3.338
	45	16.2	19.2	99.98%	0.87	0.0103	0.1698	0.6873	-0.0021	3.993

Coefficient of determination (R^2) greater than 90% and mean relative deviation modulus (P) values below 10% are considered acceptable values for describing the drying phenomenon, and the capacity of a model to faithfully describe a particular physical process is inversely proportional to the standard deviation of the estimate (SE). Among the models used in this experiment to describe the drying of coffee fruits, the modified Midilli model presented the best fits. On average, these models achieved the highest values for the coefficient of determination ($R^2 > 99.89\%$) and the lowest values for mean relative deviation modulus and standard deviation of the estimate ($P < 2.21\%$ and $SE < 0.024$).

ANOVA showed that the dry bulb temperature and the dew point temperature, and consequently the relative humidity of the air, had a significant effect on the effective diffusivity coefficient ($P < 0.0001$). The effect of dry bulb temperature was expected, given that an increase in temperature reduces water viscosity, which directly affects fluid resistance to draining; therefore, its decrease in viscosity facilitates the diffusion of water molecules in the capillaries of the product. Another factor that can be attributed to the increase of the effective diffusivity coefficient is that with increasing temperature, there is an increase in the level of vibration of water molecules, which also contributes to faster diffusion. The decreasing of dew point temperature for a given dry bulb temperature resulted in an increased effective diffusivity coefficient. Regarding the effect of relative humidity, results obtained in this study are consistent with those obtained by Ondier et al., who found significant effects of the reduction in relative humidity on the drying time of rice.

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Aerodynamics Properties of Coffee Cherries from *Coffea Canephora*

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SUMMARY

Designing the equipment for processing, sorting, sizing and other post-harvesting equipment of agricultural products requires information about their physical properties. Among the properties, terminal velocity is very important. Terminal velocity was measured with a designed vertical wind column using the suspension velocity method. In this study, several physical properties of *Coffea Canephora* clone 83 cv. EMCAPA 8141-Robustão Capixaba in two ripeness stages; green ripe and hard ripe cherries were evaluated as a function of moisture content in the range of 11.90–62.10% (w.b.) and 12.65–66.41% (w.b.) respectively. In green ripe cherries, the average experimental bulk density, true density and porosity were found to be in the range of 500.06 to 642.75 kg m^{-3}, from 799.98 to 1114.85 kg m^{-3} and from 37.49 to 43.58% respectively. The terminal velocity increased from 8.47 to 11.27 m s^{-1} as the moisture content increased. In hard ripe cherries, the average experimental bulk density, true density and porosity were found to be in the range of 488.42 to 636.66 kg m^{-3}, from 770.51 to 1101.36 kg m^{-3} and from 36.56 to 43.68% respectively. The terminal velocity increased from 9.68 to 12.85 m s^{-1} as the moisture content increased. The corresponding value of drag coefficient varied from 0.62 to 0.75 and 0.54 to 0.68 respectively.

INTRODUCTION

Information on physical and aerodynamic properties of agricultural products is needed in design and adjustment of machines used during harvesting, separating, cleaning, handling and storing of agricultural materials. The properties which are useful during design must be known and these properties must be determined at laboratory conditions.

Bulk density, true density and porosity are major considerations in designing the drying, aeration and storage systems, as these properties affect the resistance to air flow through the grain mass.

In handling and processing of agricultural products, often air is used as a carrier for transport or for separating the desirable products from unwanted materials, therefore the aerodynamic properties such as terminal velocity and drag coefficient are needed for air conveying and pneumatic separation of materials.

The objective of this study was to determine some physical and aerodynamic properties of coffee cherries to develop appropriate technologies in design and adjustment of machines used during harvesting, separating, cleaning, handling and storing of agricultural materials.

MATERIALS AND METHODS

This work was developed in the Physical Properties and Quality Evaluation Laboratory of the *Centro Nacional de Treinamento em Armazenagem* – CENTREINAR, of *Universidade Federal de Viçosa*, Minas Gerais, Brazil. There had been used coffee fruits from the *Coffea Canephora* species, clone 83 of clonal variety EMCAPA 8141, collected in an experimental farm of *EPAMIG*, in the municipality of Leopoldina. Green ripe and hard ripe cherries have been used in the experiment.

The drying process was carried out in forced convection oven at the temperature of 60 ± 3 °C. The water content of the product has been determined by the oven method, with temperature of 105 ± 3°C, with three replicates.

The true density (ρ), which is defined as the ratio of the mass of a sample to its solid volume was determined by using the liquid displacement method. For this purpose, pycnometer and vegetal oil were used. The bulk density (ρ_b) of coffee fruits based on the volume occupied by the bulk sample was measured by filling of a 500 mL cylinder from a height of 15 cm, and weighing the content.

The porosity (ε) of the bulk is the ratio of the volume of internal pores in the particle to its bulk volume and was determined as,

$$\varepsilon = 1 - \left(\frac{\rho_b}{\rho}\right) \quad (1)$$

The experimental apparatus used to determine the terminal velocity (V_t) consists of a fan, electronic revolution regulator, electric motor, plenum chamber, airflow straightener, vertical transparent tube which diameter was 100 mm. A sample was placed on the plenum chamber and the fan speed was gradually increased by an electronic revolution regulator until the fruit was floated in a level, at the level the air velocity was measured by a hot wire anemometer.

The following equation was used to calculate the drag coefficient of coffee fruits.

$$C_D = \frac{2\, m\, g\, (\rho - \rho_a)}{\rho\, \rho_a\, {V_t}^2\, A_p} \quad (2)$$

Where,

- g: Gravitational acceleration, m s^{-2}.
- m: Mass of the coffee fruit, kg.
- ρ_a: Air density, kg m^{-3}.
- C_D: Drag coefficient.
- A_p: Projected area of the coffee fruit, m^2.

RESULTS AND DISCUSSION

Physical properties for two ripeness stages of *Coffea Canephora* clone 83 cv. EMCAPA 8141-Robustão Capixaba as true density, bulk density, porosity and terminal velocity were determined as shown in figure 1 and 2.

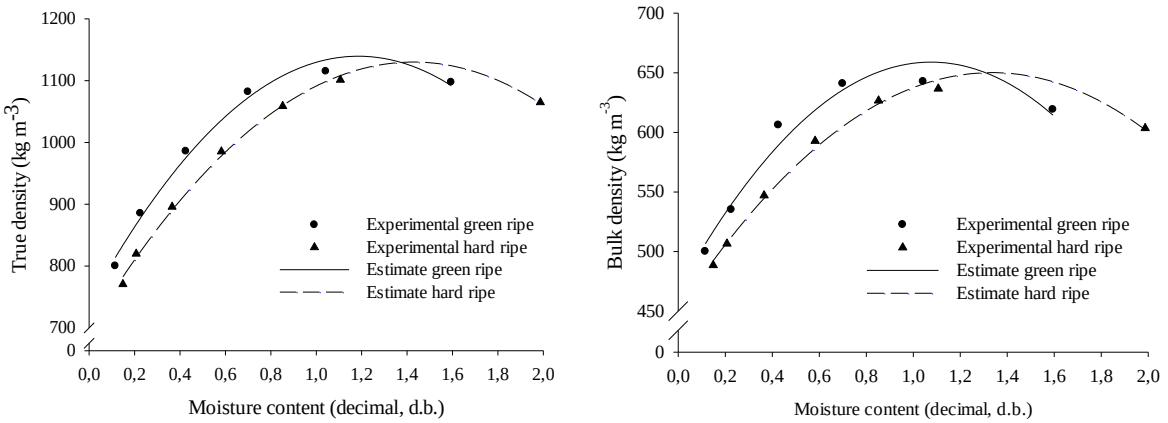

Figure 1. True and bulk density of *Coffea Canephora* clone 83 cv. EMCAPA 8141-Robustão Capixaba in two ripeness stages.

Figure 1 shows that the true density for green ripe and hard ripe cherries were increased from 799.98 to 1114.85 kg m-3 and 770.51 to 1101.36 kg m-3 respectively; and the bulk density had identical behavior increasing from 500.06 to 642.75 kg m-3 and 488.42 to 636.66 kg m-3 respectively.

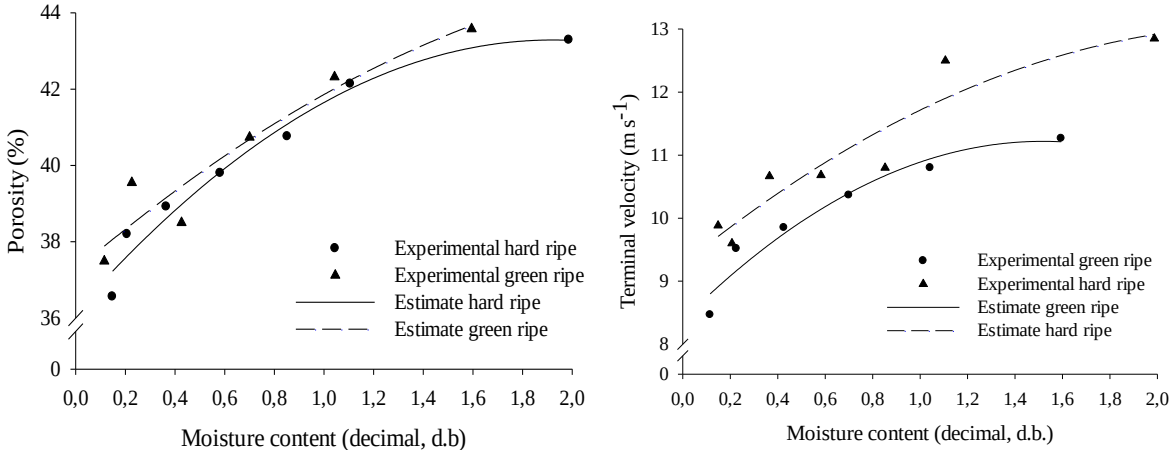

Figure 2. Porosity and terminal velocity of *Coffea Canephora* clone 83 cv. EMCAPA 8141-Robustão Capixaba in two ripeness stages.

The porosity calculated from the relevant data was increased from 37.49 to 43.58% for green ripe cherries and from 36.56 to 43.68% for hard ripe cherries, as the cherry moisture content increased from 11.90 to 62.10% (w.b.) and from 12.65 to 66.41% (w.b.) respectively.

The terminal velocities of two ripeness stages were obtained as a function of moisture content. Terminal velocity of green ripe and hard ripe cherries varied nonlinearly from 8.47 to 11.27 m s^{-1} and 9.68 to 12.85 m s^{-1}, respectively.

Regression modeling for two ripeness stages of *Coffea Canephora* clone 83 cv. EMCAPA 8141-Robustão Capixaba had shown correlation between the physical properties and moisture content as follows in the table 1.

Table 1. Statistical correlations between the physical properties and moisture content (Mc).

Phys. property	Green ripe cherry	R2	Hard ripe cherry	R2
True density (kg m-3)	739.7+674.5Mc-284.4Mc2	0.977	696.9+607.3Mc-212.8Mc2	0.956
Bulk density (kg m-3)	467.6+355.6Mc-165.3Mc2	0.957	450.1+299.9Mc-112.4Mc2	0.992
Porosity (%)	37.25+5.59Mc-0.98Mc2	0.912	36.16+7.41Mc-1.93Mc2	0.969
Ter. Velocity (m s-1)	8.38+3.74Mc-1.23Mc2	0.946	9.27+3.07Mc-0.62Mc2	0.888
Drag coefficient	0.752-0.179Mc-0.062Mc2	0.91	0.71-0.074Mc-0.005Mc2	0.932

It is shown that with increase of moisture content for green ripe and hard ripe cherries the physical properties studied increases. Research papers have reported an increase in the physical properties studied with the increase in the moisture content.

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Mathematical Modeling of Drying Natural Coffee for Different Temperature and Low Relative Humidity

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SUMMARY

Due to the great importance of the drying process and its repercussion in the final quality of the product, the present study aimed at evaluating the kinetics of coffee drying in different air temperatures for low temperatures of dew point for cherries of Arabic coffee (*Coffea arabica* L.) processed in the dry method. The cherries were harvested manually, and only the ripe cherries were selected and subjected to hydraulic separation. The freshly-picked cherries presented water proportion of 1,9 ± 0,1 $kg.kg^{-1}$ (db) and were subjected to drying in mechanical dryers under three temperatures of dry bulb (35, 40 e 45 °C), for the same temperature of dew point (2,6 °C) of the drying air, obtaining, respectively, three relative levels of humidity (13,1; 10,0 and 7,7%). Mathematical models were adjusted to the experimental data to characterize the drying process of the coffee cherries. The choice of the best model was based on the statistical parameters: estimated standard deviation (SE), relative average error (P), coefficient of determination (R^2) and tendency of distribution of residues. It was concluded that, the modified models of Henderson and Pabis and of Successive Residues with two terms were the most adequate to describe the drying process. The modified version was chosen because it is the most simple. The elevation of the temperature of the dry bulb in low temperatures of dew point causes an increase in the coefficient of effective diffusivity, of the drying index and it also reduces the drying time. For the conditions which were studied, the effective coefficient of diffusivity of water for coffee cherries varied between 1,908 and 3,721 x 10^{-11} $m^2.s^{-1}$ for the equation of Arrhenius was of 52,89 $kJ.mol^{-1}$.

INTRODUCTION

The freshly-picked coffee, due to the fact that it was a product that brings some peculiarities, such as the high proportion of water, approximately 60% (wb), and lack of uniformity in relation to the maturation, differs from the other cherries cultivated in large scale. Therefore, the drying process is a fundamental phase to avoid the attack of microorganisms and the fermentations which can hazard the quality of the coffee.

According to the conditions of the drying process, different models can be adjusted to properly describe the kinetics of hygroscopic porous capillary products. The models of Lewis, Page, Thompson, Modified Midilli, Verma, Successive Residues and modified Henderson and Pabis have frequently been adjusted to predict the drying process of seeds and fruits.

It was not possible to find in the scientific literature, information on models which describe the natural drying of coffee in different temperatures and low relative air humidity. This way the present study aimed at evaluating the drying kinetics in different temperatures of the drying air for a low temperature of the dew point of cherries of Arabic coffee processed by the dry method.

MATERIALS AND METHODS

In order to conduct the experiment ripe coffee cherries (*Coffea arabica* L. Cv, Mundo Novo) were used. They were manually harvested with a proportion of water of 1,9 ± 0,1 dry base(db). After the harvest, the hydraulic separation was made followed by the manual selection to eliminate the immature and overmature fruits. Later the drying was held in mechanic dryers until the fruits reached water proportion of 0,50 ± 0,05 (db). The next 14 hours of drying alternated with intervals of 10 hours rest, until the fruit reached water proportion of approximately 0,14 (db), which corresponded to water proportion of branded coffee, of 11±0,5% (wb).

The water proportion of the branded coffee at the beginning and at the end of the mechanic drying was determined by the standard method ISO 6673 (INTERNATIONAL ORGANIZATION FOR STANDARDIZATION – ISO, 2003). The follow-up of the reduction of the proportion of water in the fruits during the drying process was made by using the gravimetric method (loss of mass), using an analytical scale with precision of 0,01 g.

In order to evaluate and reach the expected water proportion in the coffee cherries, the water proportion of the drying of the product, according to the following expression.

$$Tx.Sec. = \left(U_{ant} - U_{at}\right)/Dt \quad (1)$$

where Tx. Sec.: drying index (g. kg $^{-1}$.h^{-1}); U_{ant}: water proportion in the previous period (g. kg $^{-1}$ (db)); U_{at}: current water proportion (g. kg $^{-1}$ (db)); and Δt: time interval between weighing (hours).

Before the beginning of the mechanical drying the equivalent ray of the coffee cherries, defined as the ray in a sphere with equivalent volume to the cherry volume. For the calculation of the volume, a sample of 100 fruits was taken. Their measures were taken by using a digital paquimeter with the precision of 0,01 mm, being the coffee cherries (V) calculated by Equation 2.

$$V = \left(4/3\right)\rho\, abc \quad (2)$$

in which V: volume of the cherries (m³); a: length (m); b: width (m); and c: density (m).

The characteristics of the drying air were controlled by an Air Conditioning System of the Lab Air (SCAL), according to *Engenharia Agrícola*. The equipment allows the precise control of the flow, temperature (T) and the relative humidity (UR) of the drying air.

For all the treatments the drying air speed was monitored with the use of an anemometer of paddles being kept in a constant of 0,33 m.s^{-1}, equivalent to the flow of 20 m³.min^{-1}.m^{-2}. Three temperatures of the dry bulb were used (35; 40 and 45 °C) and one temperature of dew point (2.6 °C), corresponding to three relative humidity levels of the drying air (13,1; 10,0 e 7,7%) respectively. For each treatment four repetitions were held.

In the analysis of the drying data the ratio of humidity (RU) is essential to describe different patterns of drying in thin layers. The ratio of humidity during the drying process, due to the variables which were evaluated was determined by Equation 3. For the tested conditions, the

values of the ratio of humidity in relation to the drying time were adjusted to the models used to describe the kinetics of the drying of the coffee cherries presented in Table 2.

$$RU = \left(U - U_e\right) \Big/ \left(U_i - U_e\right) \quad (3)$$

in which RU: ratio of humidity (adimentional); U: proportion of water of the product in time t (decimal, db); U_e: proportion of balanced water of the product (decimal, db); U_i: initial proportion of water in the product (decimal db).

Table 2. Mathematical models used to predict the kinetics of drying.

Designation of the model	Model	Equation
Lewis	$RU = \exp(-kt)$	(4)
Page	$RU = \exp(-kt^n)$	(5)
Thompson	$RU = \exp\{[-a-(-a^2 + 4bt)^{0,5}](2b)^{-1}\}$	(6)
Midilli Modificado	$RU = \exp(-kt^c) + bt$	(7)
Verma	$RU = -a\exp(-kt) + (1-a)\exp(-k_1 t)$	(8)
Resíduos Sucessivos	$RU = a\exp\{-[b\exp(-c\,T^{-1})]\,t\}+d\exp\{-[e\exp(-f\,T^{-1})]t\}$	(9)
Henderson & Pabis Modificado (KARATHANOS,1999)	$RU = a\exp(-kt) + b\exp(-k_0 t) + c\exp(-k_1 t)$	(10)

in which RU: Ratio of humidity, t: drying time (h); T: temperature of drying (° C); k, k_0 and k_1 are constant in the drying; and a, b, c, d, e, f, n are coefficient of the models.

To adjust the mathematical models, non-linear analysis of regression were held by the Gauss-Newton method, using the STATISTICA 5.0® *software* (Statsoft, Tulsa, USA). The choice of the best method was based on the statistic parameters: deviation tendency of residue distribution. The patterned deviation of the estimative and the relative average error were calculated respectively by the equations 11 and 12.

$$SE = \sqrt{\sum \left(Y - \hat{Y}\right)^2 / GLR} \quad (11)$$

$$P = \left[(100/n) \sum \left(\left|Y - \hat{Y}\right| / Y\right)\right] \quad (12)$$

in which SE: patterned deviation of the estimative (decimal); Y: value observed experimentally; $\hat{Y}$: value calculated by the model; GLR: degrees of freedom of the model; P: relative average error (%); and n: number of observed data.

The coefficient of effective diffusion for the conditions of drying used in the present study was calculated by adjusting the model, based on the theory of liquid diffusion (Equation 13) to the observed data, by means of non-linear regression using the STATISTICA 5.0® *softwar*e. This equation is the analytical solution for the second law of Fick.

$$RU = \frac{U - Ue}{Ui - Ue} = \frac{6}{\rho^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left[-\frac{n^2 \rho^2 D_{eff}}{R^2} t\right] \quad (13)$$

in which D_{eff}: of effective diffusion ($m^2.s^{-1}$); R: equivalent ray of the coffee cherries (m); n: number of terms; and t: time (s).

For the calculation of the energy of activation of the kinetics of drying of the coffee cherries the Arrhenius equation (Equation 14) was used. That equation shows the relation between the energy of activation and the speed in which the reaction happens.

$$D_{eff} = D_0 \exp\left(-Ea/RTa\right) \quad (14)$$

in which D_0: pre-exponential factor;; E_a: energy of activation (KJ mol^{-1}); R: universal constant.

RESULTS AND DISCUSSION

According to what was defined in the methodology, from the volume calculated by Equation (2), the equivalent ray of the coffee cherries calculated was of 6,51 x 10^{-3} m.

The elevation of the air temperature raises the drying index, the coefficient of effective diffusivity and reduces the drying time for the coffee cherries. The values of the coefficient of effective diffusivity obtained from the coffee cherries ranged from 1,91 e 3,72 x 10^{-11} $m^2.s^{-1}$.

From Equation 14 the activation energy (Ea) calculated for the liquid diffusion of the cherries is of 52,89 $kJ.mol^{-1}$, being inside the band of energy values of activation for agricultural products.

Among the models used in the present study, to describe the drying process of the coffee cherries, the models of Successive Residues with two terms, and modified Henderson and Pabis were the ones which presented the best adjustments, higher values of coefficients of determination (R^2 > 99,98%), and lower values of the relative average error and patterned deviation of estimative (P < 4,16% e SE < 0,012).

Although all models used in the study have shown values of coefficient of determination, patterned deviation of estimative and relative average error which are considered satisfactory, the modified Henderson and Pabis models and the Successive Residues with two terms presented higher randomization in the residue distribution, indicating that those models are the appropriate one for the description of the phenomenon of drying coffee cherries in the conditions considered in the present experiment, according to Acta Scientiarum Agronomy.

Due to the smaller number of parameters and the higher simplicity the modified Henderson and Pabis's model was chosen to describe the kinetics of drying of coffee cherries in this experiment.

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Opposed Drum Roaster

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SUMMARY

Small drum roasters have a much greater surface-area-to-volume ratio and a lower tangential velocity than large drum roasters – this results in less uniform mixing and more variation in heat exposure for the coffee beans during a roast. A small opposed drum roaster with improved mixing ability was designed and evaluated. This roaster uses two counter-rotating horizontal drums facing each other. The time required to achieve a uniform mixture increased with degree of fill in this roaster design and roasting times were slightly shorter than those for conventional drum roasters.

INTRODUCTION

Micro-roasters use small, rotating drum, batch roasting machines to provide freshly roasted coffee to local customers. These roasting machines typically have a roasting capacity of 0.5 to 15 Kg. of green coffee beans. Large coffee roasters use much larger roasting machines with a typical capacity of 30-120 Kg.

Micro-roasters are at a disadvantage because small drum roasting machines provide less bean mixing and less uniform heat exposure to the coffee beans than large drum roasting machines, when comparing two drum roasters of the same design.

The smaller drum roaster would have a much larger Surface Area to Volume Ratio (SA:V) than the larger drum roaster, as shown in Figure 1. As a result of increased SA:V in the smaller roaster, there would be much more contact between the bean pile and the drum walls. Where a single wall metal drum is used, this could lead to bean scorching.

Small drum roasters have a lower tangential velocity than large drum roasters, even when operated at a higher rpm in order to achieve the same rotational Froude number, as shown in Figure 2. Since the kinetic energy of beans at the drum wall varies with the square of tangential velocity, this could result in less uniform mixing in small drum roasters. There would be much less energy available to do the work of mixing. The Froude number, when used with granular media in rotating drums, is a ratio of inertial forces to gravitational forces. The Froude number has been used to characterise flow regimes in rotating drum coffee roasters and has been used to scale granular flow in rotating drums. At Froude numbers above 1.0, most beans are centrifuged against the inside wall and there is little tumbling or cascading of beans.

Mixing of the bean pile is required during coffee roasting so that all beans receive equal heat exposure. Studies conducted on granular media have shown that the effective thermal conductivity of granular media increases with increased mixing and increased shear rate.

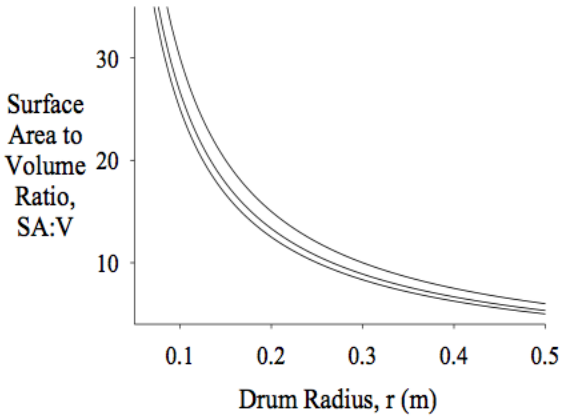

Figure 1. Surface Area to Volume Ratio versus Drum Radius for Cylindrical Drums (for drum length/diameter = 1,15 and 2).

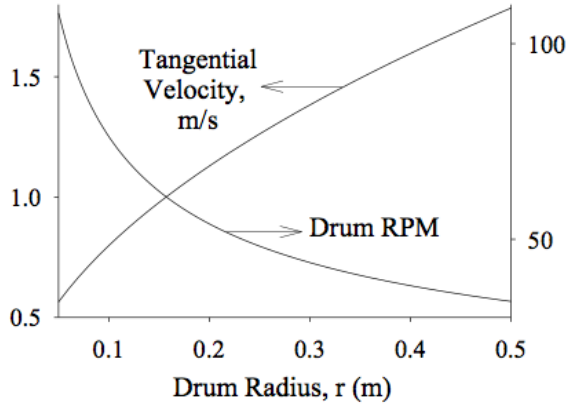

Figure 2. Tangential Velocity and Drum RPM versus Drum Radius for Froude Number = 0,65 Tangential Velocity = (radius, r) (angular velocity, ω) Froude Number, Fr = $\omega^2 r/g$

The objective of this study was to design and evaluate a small drum roaster with improved mixing ability in order to provide more uniform heat exposure to coffee beans during roasting.

MATERIALS AND METHODS

The roaster is an opposed drum roaster, with two counter-rotating horizontal drums facing each other. Figure 3, below, illustrates the two counter-rotating drums as well as the direction of bean flow during operation. Coffee beans are propelled from one drum to the other by tapered vanes attached to the inside of the drums. Intensive mixing takes place where opposing streams of beans meet in the middle of the roaster. Emptying the roaster is done by reversing the rotation of the front drum.

Before fabricating the roaster in steel, acrylic models of counter-rotating horizontal drums with various vane designs were constructed. In order to compare different designs as well as to determine how mixing ability was affected by degree of fill, one drum was filled with dried navy beans (white in color) and the other drum with dried black beans. With a bulk density of 842 kg/m^3, these were slightly heavier than green coffee beans. These beans were similar in size to Colombian Excelso green coffee beans, with 67-70% by weight through a 17 screen and retained on a 15 screen. A video was shot of the bean mixing that took place when power was supplied to the drum motors. The video was reviewed to determine the mixing time – that

is, when the mixture of white and black beans appeared to be uniform. Before and after images of the acrylic drum model are shown in Figure 4, below. The drums had an inside radius of 0.14 m and the combined length of the two drums was 0.25 m. The drums were rotated at 45 RPM. This rotation rate was the maximum rate before significant numbers of beans were propelled aloft by the drum vanes.

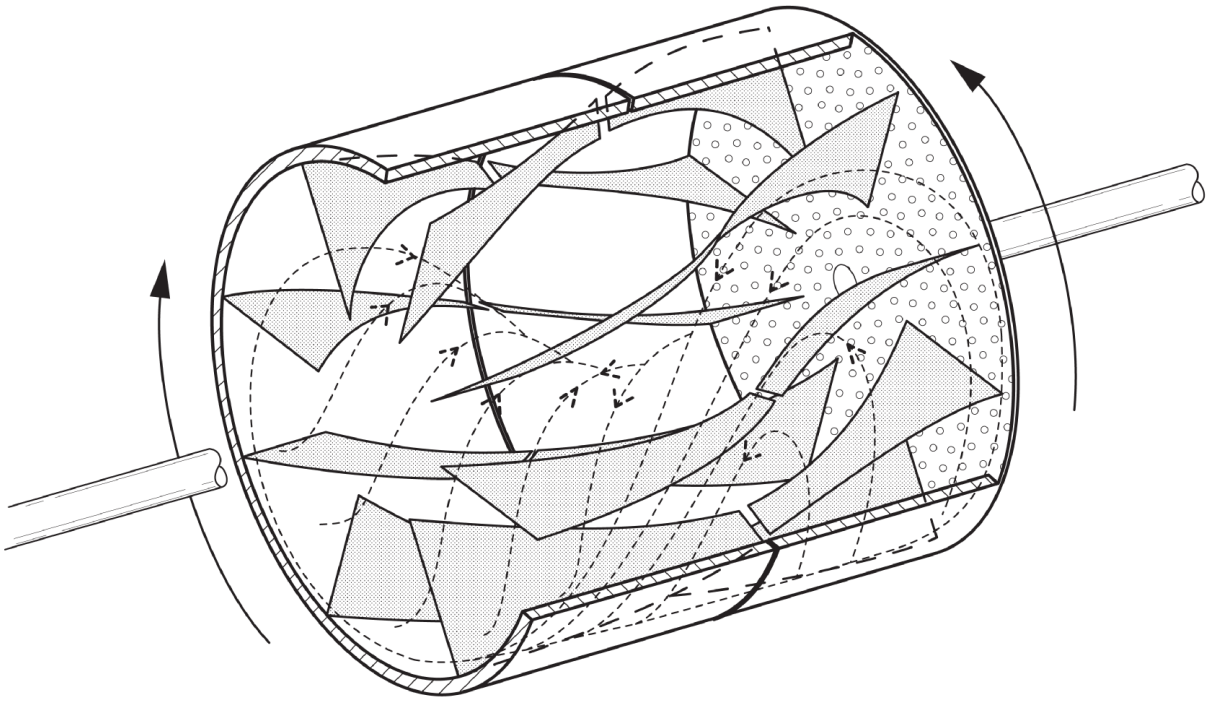

Figure 3. Coffe bean movement inside an opposed drum roaster. The bean flow paths are shown by the dotted lines with short line segment.

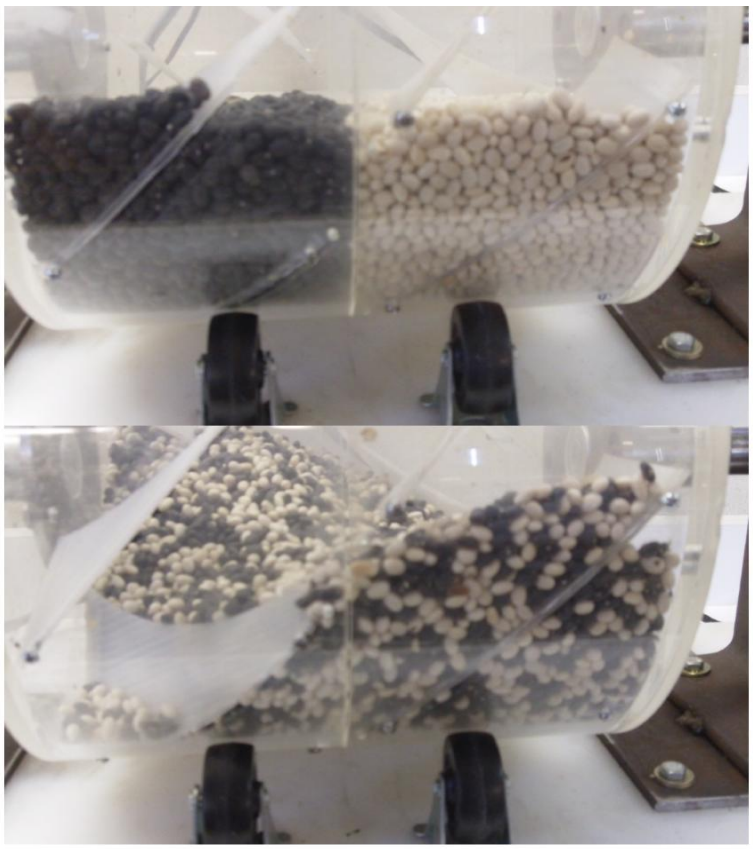

Figure 4. Acrylic model of the opposed drums before and after mixing of dried navy and black beans.

After the vane/drum design had been finalized, an opposed drum roasting machine was fabricated in steel. This roaster had a capacity of 2.2 kg. of green beans at 20% degree of fill. The drums had the same dimensions as the acrylic drums and were single wall, 6.4 mm wall thickness, and fabricated from C1020 carbon steel tubing. Eight vanes tapering from 7.6 cm height to 2.5 cm. height were used in each drum. Each drum was attached using struts to a central cantilever shaft, which was belt driven. Each drum had its own drive motor and speed controls. The rest of the roaster was of standard design, with bean inlet and bean outlet through the front plate and a propane burner beneath the rotating drums. The temperature of the burner exhaust gases at the rear of the roaster was 315°C or less and the maximum flow rate of gases through the drums was 5.8 L/s during roasting.

RESULTS AND DISCUSSION

Table 1. Mixing Time vs. Degree of Fill for Opposed Drum Acrylic Model.

Degree of Fill (%)*	Mixing Time (s)**
19.6	5.9
20.0	6.7
25.0	9.0
30.0	11.2
37.2	12.7

*% of drum volume occupied by beans

**time required to achieve uniform mixture (average of 3 observations)

The results of the mixing trials undertaken with the final vane/drum design in the acrylic model at 45 RPM are shown in Table 1, at right. Mixing time increased with degree of fill. This is in line with anecdotal accounts of increased variation in roasting degree within a coffee roast as the green bean charge weight is increased when using drum roasters. More variation in heat exposure and therefore more variation in roasting degree within a roasting batch would be expected when mixing time increases.

The roasting times for the shortest and longest roasts performed with the actual roaster over a four month period are shown in Table 2, below. The roasting times were measured from when the roaster was charged, not from when the bean pile temperature bottomed out. 2.2 kg. of green coffee beans were used in each roast. These roasting times are slightly shorter than roasting times anecdotally reported for conventional drum roasters.

Table 2. Length of Roast for the Opposed Drum Roaster – shortest and longest roasts.

		Roasting time (minutes)		
Coffee type	***Type of roast***	***Average***	***Std. Dev.***	***n***
Sumatra Mandheling SWP Decaf	Medium: 60s>1st crack	8,8	0,2	17
Sumatra Gayo Linge	Dark: 30s into 2nd crack	11,9	0,9	147

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Evaluation of GAB Model to Describe the Desorption Isotherms of Coffee in Different Processing Levels

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SUMMARY

Desorption isotherms of coffee in three different forms (green, pulped and coffee fruit) were obtained. Desorption was accomplished by static method using saturated salt solutions, providing relative humidity range of 11 to 95 %. Five different temperatures were utilized (10, 20, 30, 40 and 50 °C). Equilibrium moisture content data were correlated by the Guggenheim–Anderson–de Boer model, which presented good fit to the data, according to statistical procedures. Equilibrium moisture content ranged from 4.34 to 10.75 g/100g dry solids.

INTRODUCTION

Brazil is one of the main green coffee producers eithin the world, being also a high consumer (per capita consumption 5.64 kg). Thus, it is required the study of the different factors that affect cup quality of coffee.

One factor is the different processing procedures, in which coffee can be processed in two different ways: ‘dry method’ or natural form and ‘wet method’ or hulling. In the dry process, the entire cherry after harvest, unwashed in natural form, is first cleaned and then placed in the sun to dry on tables or in thin layers on patios. In the Wet Process, the skin and some pulp covering the seeds/beans is removed before they are dried. This is done either by the classic ferment-and-wash method or a newer procedure called machineassisted wet processing, aquapulping or mechanical demucilaging.

The processing procedure also affects post-harvest techniques. In order to perform drying and storage procedures, sorption isotherms, which describe the equilibrium relationship between moisture content and relative humidity of the environment, are indispensable and unique for each product.

Being that stated, this work aimed to determine and model the sorption isotherms of green coffee, pulped coffee and coffee fruit for various temperature and relative humidity conditions using GAB model.

MATERIALS AND METHODS

Coffee fruits (*Coffea arabica* L.), variety “Catuai Vermelho” were used. They were manually harvested during cherry stage. During harvest, unripened, deteriorated and injured coffee was

eliminated. Afterwards, fruits were washed and selected for better characterization of the raw material. In order to obtain different means of processing coffee, part of coffee fruits was hulled (pulped coffee) while the rest was processed (green coffee). Due to the high initial moisture content of coffee and in order to reduce the risk of microorganisms development, coffee fruits and pulped coffee were submitted to partial drying on yards.

Coffee fruits and pulped coffee were dried until moisture content reached 45 % dry basis, and green coffee was dried until 11 % dry basis. This process was obtained by low temperature drying using an air conditioner unity manufactured by Aminco, model Aminco-Aire 150/300. At the end of drying process, the material had its parchment and silver pellicle which involves coffee pulped removed by hand.

Equilibrium moisture content (X_{eq}) of green, pulped and coffee fruit were determined using the static method, which is based on the use of saturated salt solutions to achieve constant water activity of samples. Salt solutions used to obtain constant relative humidity were NH_4Cl, KBr, KNO_2, KNO_3, K_2SO_4,$CaCl_2$, $Ca(NO_3)_2$, $Na_2Cr_2O_7$, $MgCl_2.6H_2O$, LiCl, $LiCl.H_2O$ and NaCl. This group of salts allowed a wide range of relative humidity, from 11 % to 95 % RH. Sorption isotherms of green, pulped and coffee fruit were acquired at 10, 20, 30, 40 and 50 °C.

Experimental data of equilibrium moisture content were fitted to the Guggenheim–Anderson–de Boer (GAB) model. GAB model has the advantage that its parameters has physical meanings, and its adjustment in describing experimental data is up to 0.90 of water activity:

$$X_{eq} = \frac{X_m C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)}$$

where X_{eq} is the equilibrium moisture content, g/100g dry solids; X_m is the monolayer moisture content, g/100g dry solids; C, K are model constants that are related to monolayer and multilayer properties, respectively, dimensionless and, a_w is the water activity, decimal.

The GAB parameters were estimated using a nonlinear regression by Gauss-Newton approximation method, which minimizes the sum of square errors in a series of interactive stages. The adequacy of the model was analyzed based on the values of mean relative percent deviation (P), the standard error (SE), determination coefficient (R^2) and residual plots:

$$P = \frac{100}{n} \sum_{i=1}^{n} \left(\frac{\left| Y - \hat{Y} \right|}{Y} \right)$$

$$SE = \sqrt{\frac{\sum_{i=1}^{n} \left(Y - \hat{Y} \right)^2}{DF}}$$

where P is the mean relative per cent deviation, %; n is the number of observed data; SE is the standard error, g/100g dry solids; Y is the observed value; $\hat{Y}$ is the estimated value through the model; and DF is the degrees of freedom of the model.

RESULTS AND DISCUSSION

Statistical parameters in order to analyze model adequacy are presented in Table 1.

Table 1. Parameter estimated values, mean relative error (P), standard error of estimate (SE), determination coefficient (R^2) and residual plots for GAB model to coffee sorption in different processing levels.

Temperature (°C)	Parameters			R^2 (%)	SE (g/100g dry solids)	P (%)	Residual plots
	X_m	C	K				
Coffee fruits							
10	10.75	22.30	0.76	99.75	0.40	2.05	Random
20	10.14	32.08	0.75	99.72	0.33	1.30	Random
30	9.63	14.24	0.76	99.95	0.15	0.98	Random
40	9.31	12.67	0.75	99.92	0.18	1.24	Random
50	7.64	13.23	0.82	99.66	0.34	2.78	Random
Pulped Coffee							
10	9.99	6.16	0.67	99.69	0.35	2.86	Random
20	8.36	6.55	0.73	99.76	0.27	1.04	Random
30	7.39	5.71	0.79	99.89	0.19	1.74	Random
40	7.13	5.76	0.74	99.65	0.28	3.41	Random
50	4.34	12.29	0.92	99.07	0.43	5.85	Random
Green Coffee							
10	7.92	12.59	0.74	99.89	0.19	1.44	Random
20	7.60	9.99	0.75	99.54	0.35	1.30	Random
30	7.54	8.29	0.75	99.95	0.12	1.05	Random
40	6.37	11.70	0.77	99.77	0.21	2.31	Random
50	5.67	9.69	0.81	98.82	0.47	5.96	Random

GAB model presented random residual plots of Xeq experimental data of green, pulped and coffee fruit at all temperatures investigated at the present work. Also, low values of P and SE were encountered along with high values of R2. These results indicate that GAB model is adequate to estimate equilibrium moisture content of coffee in its different forms of processing.

The number of water molecules that are strongly adsorbed in specific sites at the food surface are indicated by the monolayer moisture content. This parameter tended to decrease as temperature increased (Table 1), varying between 10.75 and 4.34 g/100g dry solids. This trend can be explained by the number of active sites that are reduced with increased temperature, resulting into modifications of the physical and chemical characteristics of the product.

The monolayer moisture content is the safest moisture content for storage purposes, providing increased time period with minimum quality loss at a certain temperature during preservation. Values below monolayer moisture content slow down deteriorative reactions, pest attacks and respiration rate of the product. Water activity values corresponding to the monolayer moisture content of coffee were obtained from the data presented in Table 1 and is tabulated in Table 2.

Table 2. Corresponding water activity (a_w) values for safe storage of green, pulped and coffee fruits at different temperatures.

Product	Temperature (°C)				
	10	***20***	***30***	***40***	***50***
Coffee fruits	0.229	0.201	0.276	0.291	0.264
Pulped Coffee	0.429	0.383	0.375	0.399	0.242
Green Coffee	0.297	0.319	0.344	0.294	0.301

It can be noticed that coffee fruits had lower values of a_w than pulped coffee and green coffee, meaning that its storage is more difficult to accomplish at these temperatures, requiring more energy and care. This fact is expected since that the presence of hulls on coffee fruits ends up on increased energy to dry this product, increased time to this product reach equilibrium with the surrounding environment. In addition, coffee fruits also have mucilage, a component of high sugar content, leading to a high probability of microorganism and insect attack. These facts also may explain the non-patterned values of water activity for increased temperatures.

Water activity of green coffee may be assumed to be constant at all temperatures investigated (Table 2), due to absence of hull and mucilage. Pulped coffee presented a decrease on water activity values with temperature increase, probably due to the presence of mucilage.

The C constant is associated with the chemical potential differences between the monolayer and superior layers. No defined behaviour could be concluded for this parameter. Probably this fact is due to the heterogeneous characteristic of coffee product, which components variance among layers may lead to the trend noticed, observed both for the same product but between temperatures and between different products.

The K constant is correlated to the chemical potential difference between the monolayer and free water state. This parameter presented a slightly increase at temperature of 50 ºC to the same product, being considered constant for the remaining temperatures. Higher levels of temperature (50 ºC) lead to a rapid water molecules transition between multilayer and free water.

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Thermodynamic Properties Analysis from Drying Process of Coffee in Different Processing Levels

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SUMMARY

Thermodynamic properties of coffee in three different forms (green, pulped and coffee fruit) were obtained during the desorption process. Desorption was accomplished by static method using saturated salt solutions, providing relative humidity range of 11 to 95%. Five different temperatures were utilized (10, 20, 30, 40 and 50°C). Enthalpy values for each model coefficient were estimated, ranging from -8.09 to 82.64 kJ kg^{-1}. Differential enthalpy and differential entropy increased with decreased equilibrium moisture content, which was also found for Gibbs free energy. Coffee fruits presented higher values of these thermodynamic parameters.

INTRODUCTION

Brazil is one of the main green coffee producers eithin the world, being also a high consumer (per capita consumption 5.64 kg). Thus, it is required the study of the different factors that affect cup quality of coffee.

One factor is the different processing procedures, in which coffee can be processed in two different ways: ‘dry method’ or natural form and ‘wet method’ or hulling. In the dry process, the entire cherry after harvest, unwashed in natural form, is first cleaned and then placed in the sun to dry on tables or in thin layers on patios. In the Wet Process, the skin and some pulp covering the seeds/beans is removed before they are dried. This is done either by the classic ferment-and-wash method or a newer procedure called machineassisted wet processing, aquapulping or mechanical demucilaging.

The processing procedure also affects post-harvest techniques. In order to perform drying and storage procedures, sorption isotherms, which describe the equilibrium relationship between moisture content and relative humidity of the environment, are indispensable and unique for each product. Sorption isotherms are also efficient tools to determine thermodynamic interactions between water and food components. Thermodynamic properties provide useful information for the development and improvement of dryers and for studies on water properties on food surface.

Being that stated, this work aimed to calculate the thermodynamic properties during the drying process of green coffee, pulped coffee and coffee fruit for various temperature and relative humidity conditions.

MATERIALS AND METHODS

Coffee fruits (*Coffea arabica* L.), variety "Catuai Vermelho" were used. They were manually harvested during cherry stage. During harvest, unripened, deteriorated and injured coffee was eliminated. Afterwards, fruits were washed and selected for better characterization of the raw material. In order to obtain different means of processing coffee, part of coffee fruits was hulled (pulped coffee) while the rest was processed (green coffee). Due to the high initial moisture content of coffee and in order to reduce the risk of microorganisms development, coffee fruits and pulped coffee were submitted to partial drying on yards.

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Equilibrium moisture content (X_{eq}) of green, pulped and coffee fruit were determined using the static method, which is based on the use of saturated salt solutions to achieve constant water activity of samples. Salt solutions used to obtain constant relative humidity were NH_4Cl, KBr, KNO_2, KNO_3, K_2SO_4,$CaCl_2$, $Ca(NO_3)_2$, $Na_2Cr_2O_7$, $MgCl_2.6H_2O$, LiCl, $LiCl.H_2O$ and NaCl. This group of salts allowed a wide range of relative humidity, from 11% to 95% RH. Sorption isotherms of green, pulped and coffee fruit were acquired at 10, 20, 30, 40 and 50°C.

Experimental data of equilibrium moisture content were fitted to the Guggenheim–Anderson–de Boer (GAB) model:

$$X_{eq} = \frac{X_m C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)}$$

where X_{eq} is the equilibrium moisture content, g/100g dry solids; X_m is the monolayer moisture content, g/100g dry solids; C, K are model constants that are related to monolayer and multilayer properties, respectively, dimensionless and, a_w is the water activity, decimal.

Thermodynamic parameters, such as differential entropy of desorption (ΔS), differential enthalpy (ΔH) and Gibbs free energy (ΔG) were obtained by means of a known methodology, which an approximate (1-α)100% confidence interval for isokinetic temperature is used.

$$\ln a_w = \pm\left(\frac{\Delta H_{st}}{RT} - \frac{\Delta S}{R}\right)$$

$$\Delta H = \Delta H_{st} - \Delta H_{vap}$$

$$\Delta G = \pm RT \ln a_w$$

where ΔH is the isosteric heat of sorption, kJ kg^{-1}; ΔH_{vap} is the latent heat of vaporization of pure water, kJ kg^{-1}; ΔH_{st} is the net isosteric heat of sorption, kJ kg^{-1}; ΔS is the differential entropy of sorption, kJ kg^{-1} K^{-1}; and ΔG is the Gibbs free energy, kJ kg^{-1} mol^{-1}.

RESULTS AND DISCUSSION

Figure 1 presents the values of differential enthalpy according to the equilibrium moisture content.

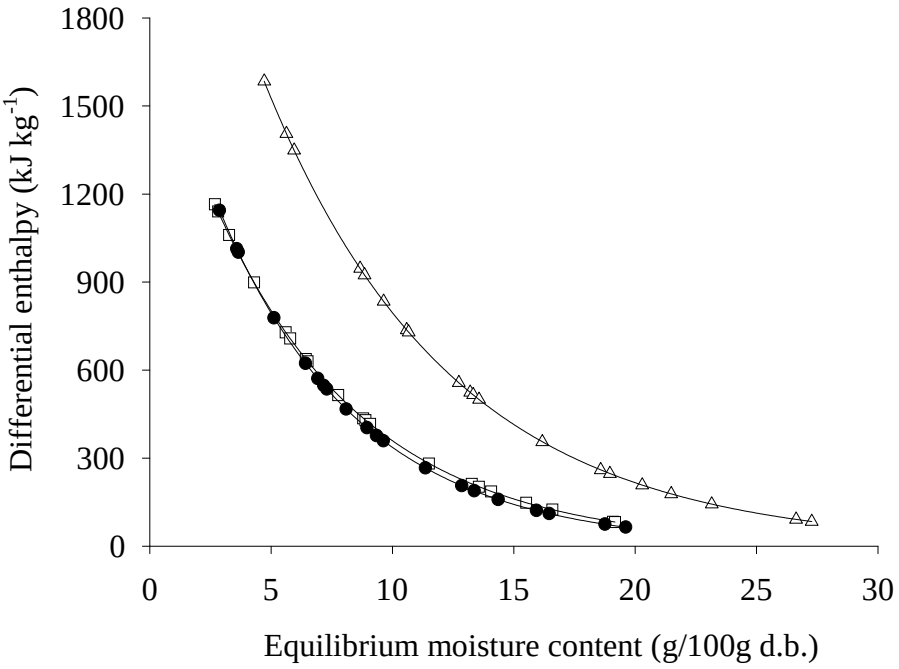

Figure 1. Observed and estimated values (—) of differential enthalpy in function of equilibrium moisture content of green (●), pulped (□) and coffee fruits (Δ).

According to Figure 1, it is observed a reduction of coffee X_{eq} in its different types of form. The required energy to remove water from the product is higher in lower levels of X_{eq}. Elevated values of differential enthalpy at low values of X_{eq} can be explained by the differences of bonding forces between moisture and adsorbent surface of the product. At initial stages of sorption (low values of moisture content), there are polar sorption sites highly active, of elevated interaction energy, which are covered by water molecules, forming the monomolecular layer. Throughout time, which water molecules are being chemically connected to the sorption sites highly active, sorption starts to occur at sites less active (higher moisture content), with lower interaction energy and, consequently, lower differential enthalpy.

Values of differential enthalpy of coffee fruits are higher than those found for green and pulped coffee, at all X_{eq} range. These results are similar to those found in previous work with hazelnuts, peanuts and almond. According to these authors, it was because of enhancement of lipid–lipid interaction after dehulling of these products, which would increase the hydrophobicity of cellular components. Furthermore, because of the low hygroscopicity of the layer that involves pulped coffee, values of differential enthalpy of green and pulped coffees were similar.

Figure 2 presents differential entropy of sorption values (kJ kg^{-1} K^{-1}), in function of equilibrium moisture content (g/100g dry solids) for green, pulped and coffee fruit.

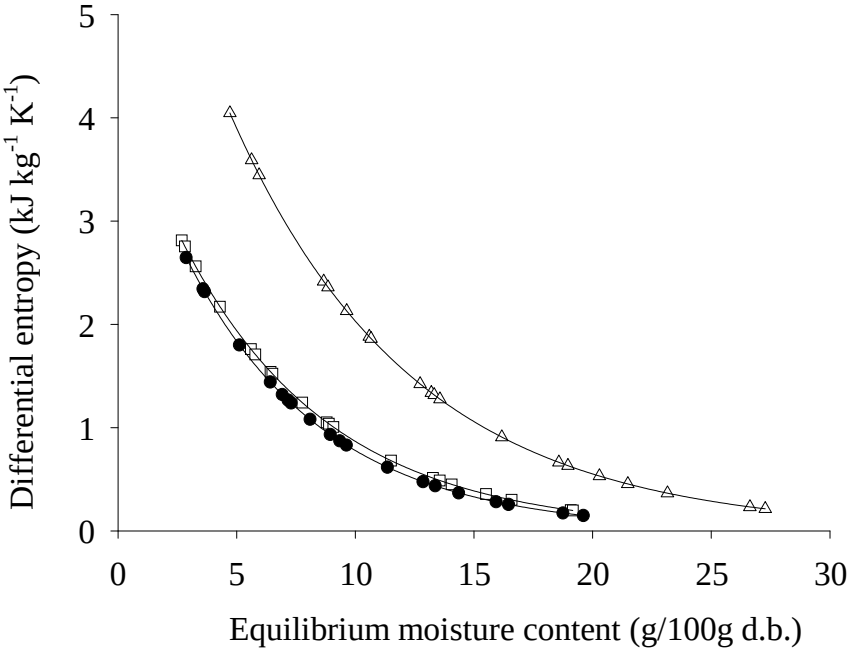

Figure 2. Observed and estimated values (—) of differential entropy of sorption of green (●), pulped (□) and coffee fruits (Δ).

The relationship between differential entropy and equilibrium moisture content presented similar behavior observed in differential enthalpy values. Coffee fruits also presented higher values of differential entropy than pulped and green coffee. The number of available desorption sites corresponding to a specific energy level is proportional to the differential entropy, which describes the degree of disorder and motion randomness of water molecules. Therefore, ΔS values of coffee fruits are expected since this product possesses the hull, an increase of area where water molecules may move, in which leads to higher number of desorption sites at a certain X_{eq} value, regarding green and pulped coffee.

Based on the second law of thermodynamics, a process is reversible when the sum of all entropy changes for all subsystems in a process is constant. Thus, coffee sorption in its different types of processing is irreversible (hysteresis effect), because entropy is produced during the process. However, at high values of X_{eq} in which differential entropy tends to become constant, sorption process may be reversible.

Gibbs free energy increased with decreased equilibrium moisture content. These values are expected, because in higher values of moisture content, there is lower necessity of work to make the sites available to sorption, because they are already available. This trend is observed by lower values of ΔG.

It is observed that temperature influence over Gibbs free energy is higher at lower values of X_{eq}, in which lower temperatures provides higher values of ΔG. This trend can be correlated to the vibration of water molecules within the product, where at high temperatures this vibration (movement) is higher; this leads to lower work required to the product layers to become available to sorption. At high levels of X_{eq}, temperature influence becomes negligible due to the sorption sites are already available. Positive values of ΔG are also expected, as it characterizes endothermic reaction, or else, reactions that require energy from the environment to occur.

Green and pulped coffee presented similar values of Gibbs free energy, while coffee fruit presented higher values of this parameter. This trend may be explained by the presence of the hull in coffee fruits, as stated before at the differential entropy discussion. ΔG values become similar between different types of coffee in high levels of X_{eq} (above 20 %).

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The Behavior of the Antioxidant Enzymes and H_20_2 Content During the Drying of Arabica Coffee Beans

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SUMMARY

Studies related to antioxidant enzymes during the drying of coffee were not found in the literature. Considering their importance, the enzymatic activities of superoxide dismutase (SOD), guaiacol-peroxidase (GPOX) and glutathione reductase (GR) as well as the hydrogen peroxide content were evaluated during the drying of coffee beans processed as natural coffee and pulped natural coffee. The results revealed low SOD, GPOX and GR enzymatic activities in natural coffee compared to pulped natural coffee during the drying period. Moreover, the hydrogen peroxide content in natural coffee was more prominent than in pulped natural coffee. These results suggested the development of oxidative stress during the coffee drying process.

INTRODUCTION

When the coffee fruit is freshly picked the beans moisture is around 49-58% whereas after drying the moisture level drops to approximately 12%.

Some studies performed during the drying of coffee beans have shown the development of germination process and drought stress. These events imply respiration in the coffee beans that could facilitate the generation of reactive oxygen species (ROS) and also oxidative stress. Fortunately the plants have developed enzymatic (through antioxidant enzymes), and non-enzymatic defense systems for the detoxification of ROS.

Several studies have been performed during the drying of coffee beans but studies related to the evaluation of antioxidant enzymes were not found. The aim of the present study was to follow the activities of the enzyme superoxide dismutase (SOD), guaiacol peroxidase (GPOX), glutathione reductase (GR) and hydrogen peroxide content during the drying of Arabica coffee beans.

MATERIALS AND METHODS

Ripe fruits of *Coffea arabica* cv. IAC Mundo Novo were picked up and selected in Campinas, São Paulo. Half of the fruits were processed as natural coffee (CN) and half as pulped natural coffee (CD). The processed coffees were sun-dried up to approximately 12% moisture.

Determination of the enzymatic activities and hydrogen peroxide production were performed in the beans of freshly harvested fruits (at the beginning of drying) and at four different moisture levels during the drying period. The performed analyses were: moisture; extraction

and quantification of proteins; activity of superoxide dismutase (SOD) by electrophoretic analysis; Guaiacol peroxidase (GPOX) activity, which was expressed in unit (U) and to which one enzyme activity unit corresponded to an increase of 0.001 in absorbance per minute; Glutathione reductase (GR) activity, which was measured by the rate of reduction of glutathione oxidized (GSSG) at 412 nm in 1 min and quantification of hydrogen peroxide. All measurements were performed in triplicate and analyzed independently for each coffee (CN and CD) by ANOVA and Tukey test (p<0.05) using Statistica software package (v10, Statsoft).

RESULTS AND DISCUSSION

SOD is considered the first line of defence against ROS, and electrophoresis of the enzyme exhibited 4 isoenzymes as was reported in the literature. However, the number of SOD isoenzymes was significantly smaller than that of the coffee cell suspension cultures, in which up to 9 distinct isoenzymes were observed. This electrophoresis also showed an oscillatory activity behavior during the drying, thereby the enzyme appeared to be more active in the first days of the drying in pulped natural coffee than in natural coffee (Figure1).

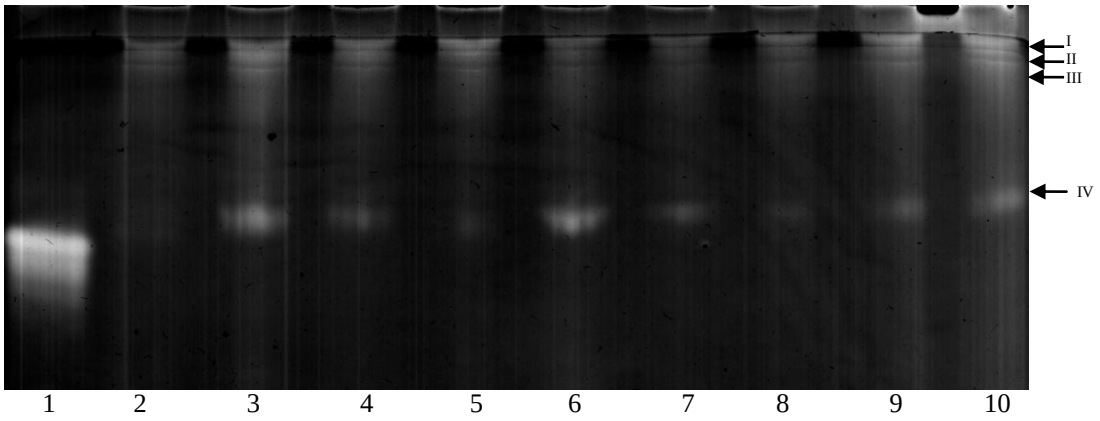

Figure 1. Electrophoresis of superoxide dismutase (SOD) in coffee beans processed as pulped natural coffee (CD) and natural coffee (CN) during the drying. 1- standard bovine SOD; 2- start of the drying (fruit); 3- pulped natural coffee after 2 days of drying (CD40); 4- pulped natural coffee after 5 days of drying (CD30); 5- pulped natural coffee after 6 days of drying (CD20); 6- pulped natural coffee after 8 days of drying (CD10); 7- natural coffee after 5 days of drying (CN40); 8- natural coffee after 6 days of drying (CN30); 9- natural coffee after 8 days of drying (CN20); 10- natural coffee after 13 days of drying (CN10).

Natural coffee (CN) exhibited GPOX and GR activities during the whole drying period but there was no significant increase (p>0.05) compared to the sample with 50.6% moisture (Figure 2). It was also detected the presence of H_2O_2 inside the beans, whose concentration increased from the start (50.6% moisture) to CN40 (41% moisture) and decreased from there until the end of the process. The decreasing of H_2O_2 content at the end of drying period in natural coffee could be explained by the possible presence of the catalase. Another alternative for this behavior could be the formation of hydroxyl radical from hydrogen peroxide.

Figure 3 revealed significant increasing in GPOX and GR during the drying of the pulped natural coffee. Thereby the lowest concentrations of H_2O_2 measured in CD at the end of the drying period might be due to the changes observed in GR and GPOX activities.

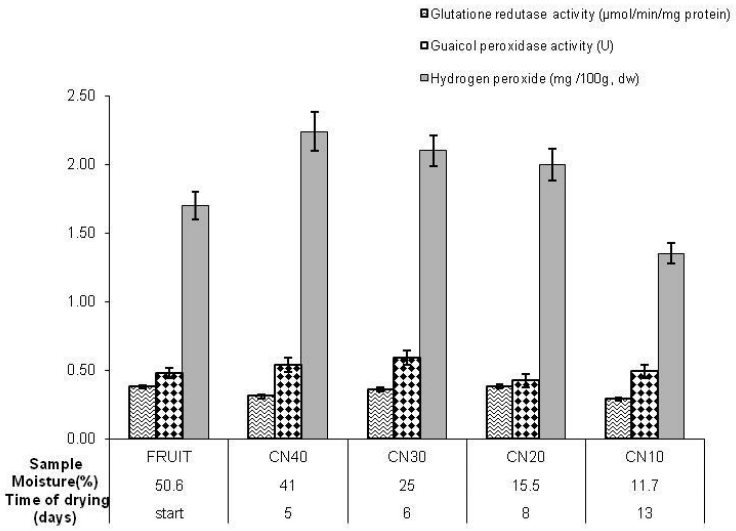

Figure 2. Content of hydrogen peroxide and activity of glutathione reductase and guaiacol peroxidase in the beans of natural coffee (CN) during the drying period.

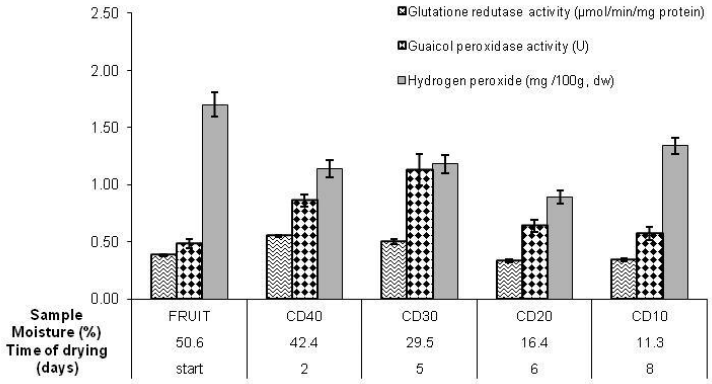

Figure 3. Content of hydrogen peroxide and enzymatic activity of glutathione reductase and guaiacol peroxidase in the beans of pulped natural coffee (CD) during drying period.

The differences in the activity of GR and GPOX for CN and CD coffees may be a consequence of the absence of pulp in CD coffee. Endosperm of the pulped coffees would be more susceptible to the effect of the sun during the drying, as observed in pea and wheat seedlings. Moreover, the composition of the pulp present in natural coffee would cause different stimulus for coffee bean during the drying.

In summary, the results for the antioxidant enzyme activities in CD coffee and the H_2O_2 concentrations in CN coffee suggested the development of oxidative stress during the coffee drying.

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Quality Coffee (*Coffea Arabica* L.) Subjected to Two Processing Types

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SUMMARY

This study aimed to differentiate the quality of coffee (*Coffea arabica* L.) among genotypes subjected to dry (natural coffee) and semi washed (pulped natural coffee) processing in southern Minas Gerais State, Lavras, Brazil. Six coffee genotypes were characterized and sensory and chemical analyses were performed.

An effect of genotype was evident and the Bourbons exhibited a greater potential to generate better quality beverages compared to the other cultivars (Mundo Novo, Red Catuai and Icatu Precoce). The chemical components 5-CQA and trigonelline contributed to separating the superior quality coffees from the other coffees. In the present study, the natural coffees also had more body compared to the pulped natural coffees. The form of processing is not allowed to discriminate against commercial genotypes (Mundo Novo IAC 502/9, Red Catuai IAC 144, and Icatu Precoce IAC 3282), which were worst sensory quality.

INTRODUCTION

A high beverage quality is essential for consumption and appreciation of coffee. Beverage quality is associated with several biochemical constituents present in green beans that are transformed into a thousand of compounds during the roasting. Some compounds could have effect on, as trigonelline, sugars, soluble solids, chlorogenic acids and caffeine.

Various factors affect the coffee quality expression, as for genetic (species and varieties), environmental (soil, climate, altitude), genotype-by-environmental interactions and post-harvest procedures, in especial the processing method, drying, and storage. These factors have been considered to be the most important determinants for the sensory profile of coffee beverage.

Coffee fruits need to be processed in the post harvest phase to turn the beans ready for consumption. There are two different methods for coffee processing: the wet processing (washed coffee) and the dry processing (natural coffee). Post harvest processing methods are recognized to modify the biochemical composition of green coffee beans and affect in some extent the sensory attributes.

The Bourbon cultivar is highly valued in the specialty coffee markets due its intrinsic quality and represent a genetic group that hold great potential for achieving a high beverage quality . In Brazil is so rare information related to beverage quality comparison among different arabica coffee cultivars as well as the correlation between beverage quality and effects of different post harvest processing techniques. Thus, the objectives of this current research were to evaluate the biochemical composition of green coffee submitted to different post-harvest

processing methods and to examine the relationships between these quality attributes and organoleptic characteristics in some arabica coffee cultivars in Lavras, Southern Minas Gerais State, Brazil.

MATERIALS AND METHODS

A total of six arabica coffee genotypes of five-year old including three Yellow Bourbon collected from major Brazilian coffee growing areas and three commercial cultivars named Mundo Novo IAC 502/9, Catuai Vermelho IAC 144 and Icatu Precoce IAC 3282 which served as reference in quality comparison. All cultivars were submitted to two different post harvest processing methods, dry (natural coffee) and semi washed (pulped natural coffee) described in the Table 1.

Sensory evaluation was conducted by a panel of trained cupping judges SCAA. Seven sensory descriptors were assessed by a trained panel of judges and rated on a 10-point scale according to the SCAA. The sensory descriptors included fragrance/aroma intensity and quality, flavour, aftertaste, acidity, body, balance, overall and final score. The green coffee beans chemical composition (total titratable acidity, total soluble solids, total sugars, reducing sugar, non-reducing sugars) were determined according to AOAC. The chlorogenic acid (5-CQA) and trigonelline) were determined by HPLC, according to Food Chem. All laboratory determinations were performed in triplicate.

To examine the correlations between the sensory attributes and the chemical characteristics of the coffees, principal component analysis (PCA) was conducted, using the software SAS statistic (ver. 8. Cary, USA, 1999).

Table 1. Arabica coffee genotypes evaluated in the experiment carried out in Lavras, southern Minas Gerais.

Treatment	Genotype	Origin	Processing
1	Mundo Novo IAC 502/9	Epamig – Machado/MG	Pulped
2	Catuai Vermelho IAC 144	Epamig – Machado/MG	Pulped
3	Icatu Precoce IAC 3282	Procafé – Varginha/MG	Pulped
4	Yellow Bourbon[1]	Procafé – Varginha/MG	Pulped
5	Yellow Bourbon IAC J9	IAC – Campinas/SP	Pulped
6	Yellow Bourbon[2]	Carmo de Minas/MG	Pulped
7	Mundo Novo IAC 502/9	Epamig – Machado/MG	Natural
8	Catuai Vermelho IAC 144	Epamig – Machado/MG	Natural
9	Icatu Precoce IAC 3282	Procafé – Varginha/MG	Natural
10	Yellow Bourbon[1]	Procafé – Varginha/MG	Natural
11	Yellow Bourbon IAC J9	IAC – Campinas/SP	Natural
12	Yellow Bourbon[2]	Carmo de Minas/MG	Natural

IAC – Instituto Agronômico, Epamig – Empresa de Pesquisa Agropecuária de Minas Gerais, Procafé – Fundação Procafé, Origin – refers to the institution, city and state (Brazil) where the genotypes were collected to be used in the experiment carried out in Lavras. [1] and [2] – not identified lineage of Yellow Bourbon from Varginha and Carmo de Minas, respectively.

RESULTS AND DISCUSSION

The first principal component (Figure 1A) separated the treatments into two groups: G1 (Bourbon genotypes – treatments 4, 5, 6, 10, 11 and 12) and G2 (Mundo Novo, Catuai

Vermelho, and Icatu Precoce cultivars – treatments 1, 2, 3, 7, 8 and 9). It appears that all of the sensory attributes were scored for the coffees included in G1 group, discriminating the Bourbon genotypes from the other cultivars.

Due to the beverage sweetness, taste and peculiar aroma, many coffee farmers are switching to planting Bourbon cultivars that produce the highest quality coffee and are considered one of the best coffee types in the world for producing specialty coffees. This study demonstrated that the Bourbon genotypes differed from the other arabica coffee cultivars, confirm that present a greater potential to produce specialty coffees (Figure 1A).

The relationship between the 5-CQA content and the coffee quality is still unclear and can even be considered controversial. In this study, this chemical component contributed to separating the superior quality coffees from the other coffees.

The second principal component also separates the coffees into two groups: G3 (pulped coffees – treatments 1 to 6) and G4 (natural coffees – treatments 7 to 12) (Figure 1B). The total sugar content was the principal discriminator for the pulped coffees (G3). The body and the total titratable acidity, higher in the G4 group, allowed the discrimination of the natural coffees. The commercial cultivars (treatments 7, 8, 9) processed by the dry method showed higher levels of titratable acidity and lower scores in sensory evaluation when compared with the genotypes of Bourbon.

Several metabolic processes that significantly alter the green coffee bean chemical composition occur inside the beans during the post harvest processing. Thus, the coffee beans obtained from different processing procedures have distinct sensory attributes in their beverage quality.

Dry processed coffees are considered to have high body than those obtained through wet processing. In the present study, the natural coffees also had high body values compared to the pulped coffees.

Figure 1C shows the formation of three groups (G1, G2 and G3) according to the similarities in the chemical composition and sensory profiles of the coffees. Treatments 6, 10 and 12 (Yellow Bourbon genotypes), which belong to G1 group, are clearly distinguished from the coffees in G3 group (Treatments 1 and 7 – Mundo Novo; Treatments 2 and 8 – Catuai Vermelho; and Treatments 3 and 9 – Icatu Precoce). An intermediate group (G2) is also formed by the Bourbon genotypes (Treatments 4, 5 and 11).

The coffees in G1 group showed higher values for aftertaste, flavor, fragrance, and final score, and were characterized as higher quality coffees. The Yellow Bourbon from Carmo de Minas-MG belongs to this group regarding the processing method (treatment 6 -pulped coffee and treatment 12 – natural coffee). However, the Yellow Bourbon from Varginha-MG had a better sensory quality when submitted to dry processing method (treatment 10 – natural coffee). The Bourbon coffees belonging to G2 group had promising quality sensory attributes, but less intense compared to the Bourbon coffees in G1 group. The G3 group coffees, constitutes by Mundo Novo, Catuai Vermelho and Icatu Precoce cultivars, had a lower quality compared with the other coffees, regarding the post harvest processing method.

It was observed evident genotype effect on coffee quality and the Bourbon cultivar exhibited a greater potential to produce better beverage quality compared to the other arabica coffee cultivars (Mundo Novo, Catuai Vermelho and Icatu Precoce). The Yellow Bourbon from Carmo de Minas/MG showed better coffee quality regarding the post harvest processing

method. The Bourbon genotypes processed by dry method showed better beverage quality than the washed method. The total sugar, body and total titratable acidity content allowed for the discrimination between the pulped and natural coffees.

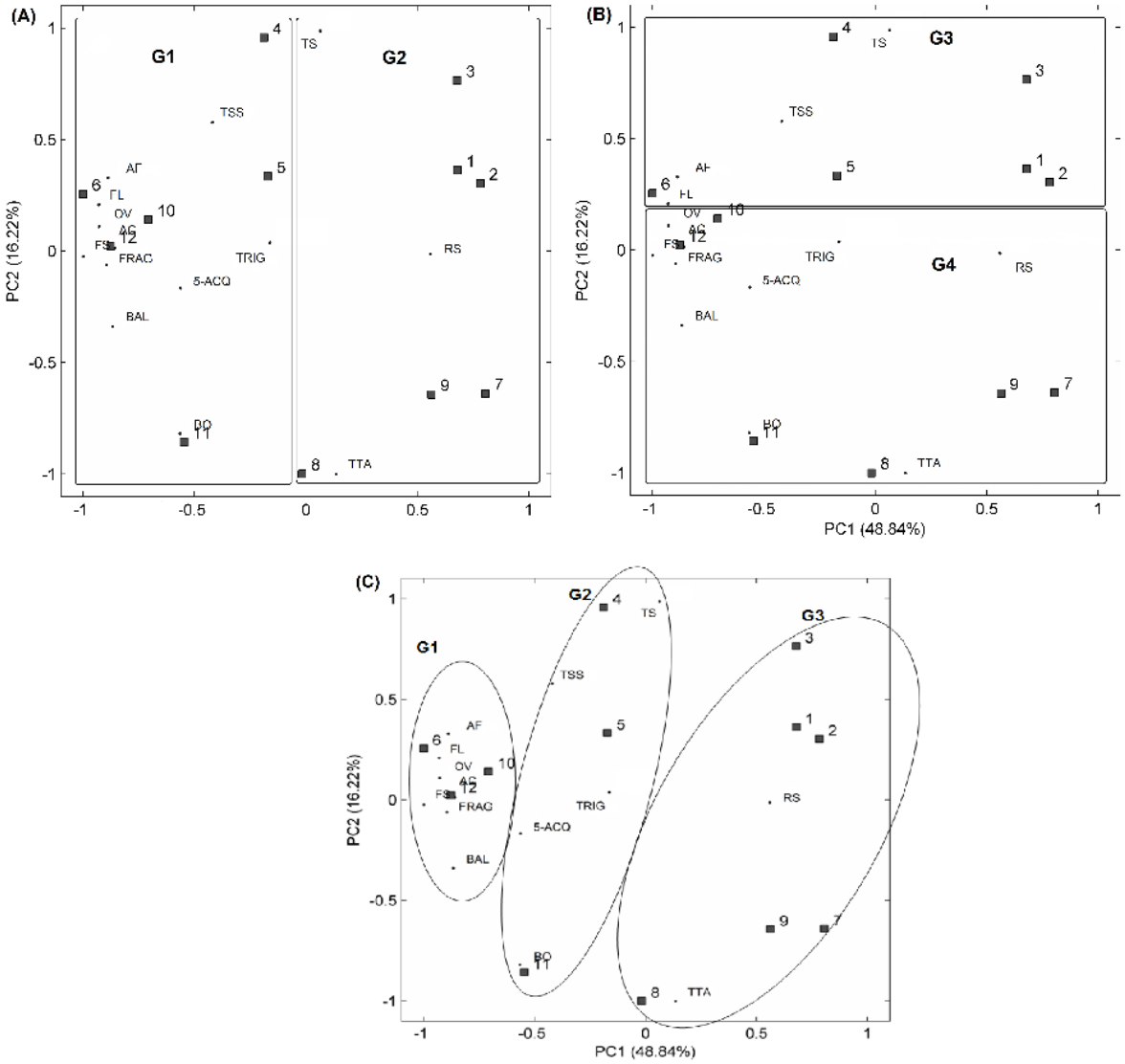

Figure 1. (A). First principal component scores separated into two groups based on PC1: G1 and G2. (B) First principal components separated into two groups based on PC2: G3 and G4. (C). Separation of coffees into three groups (G1, G2, and G3) by PCA according to their chemical and sensory profiles. (Fragrance = Frag; Flavor = Fl; Acidity = Ac; Body = Bo; Aftertaste = Af; Balance = Bal; Overall = Ov; Final score = FS; Trigonelline = Trig; 5-CQA; Total sugars = TS; Reducing sugars = RS; Total soluble solids = TSS and Total titratable acidity = TTA).

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Development of a Hybrid Roasting Process for the creation of new roasting - and flavor-profiles with special attention to the option of Short - Middle - and Long - Term Roasting

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SUMMARY

In many cases the demand is placed to carry out also long-term roasts (12 – 20 minutes) on short-term roasters (roasting times of 1.5 – 4 minutes). PROBAT has successfully developed a hybrid roasting system which offers this flexibility for a high individuality of roasting combination.

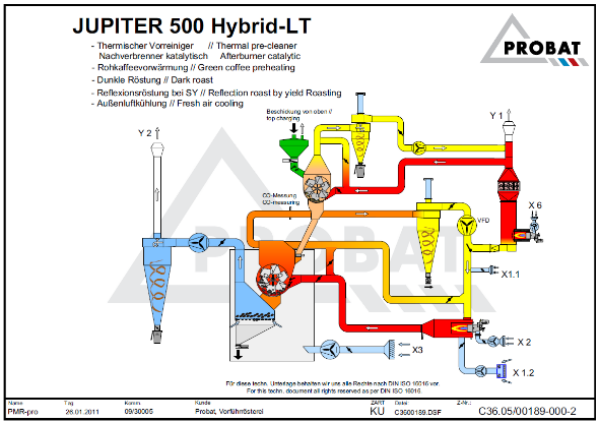
INTRODUCTION

The possibility of a flexible setting of roast supply air temperature, roasting air volume and bean distribution in the roasting chamber was analyzed in the course of more than 1,200 trial roasts. Sensory and chemical analysis techniques were applied for the appraisal. Hot air feeding system, mixing elements and water quenching were redeveloped within the scope of transfer to an industrial roasting machine. The entire procedural process management as well was adapted to the target requirements. The cooling process in combination with water quenching was optimized through the application of new thermal analytical options.

RESULTS AND DISCUSSION

First analyses of trial roasts by means of HPLC, GC-MS and cupping groups show that it is possible to achieve the classic short- and long-term roasting profiles. At the same time it is possible to achieve comparable flavor notes as on a drum roaster. Owing to the flexible combination of setting parameters also other roasting profiles with new taste profiles can be achieved. This flexibility is of particular advantage since specific chemical components regarded as requested resp. unrequested when discussing about health effects can be specifically influenced.

The already available results are to be confirmed and deepened by further trials and analyses. Already now it becomes clear that new options for the creation of roasting profiles as well as of the flavor development are resulting from this new “hybrid” roasting process.



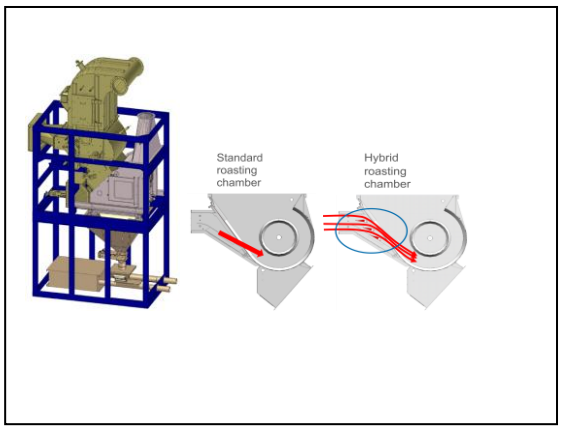
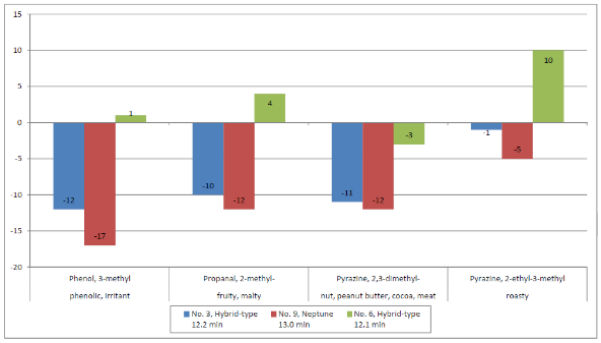


Figure 1. The PROBAT Hybrid Roaster.

Figure 2. Jupiter Standard and Hybrid.



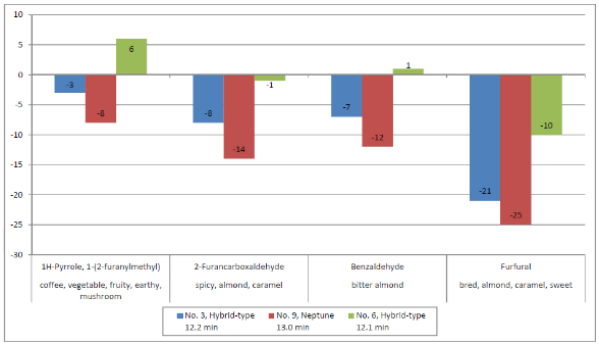
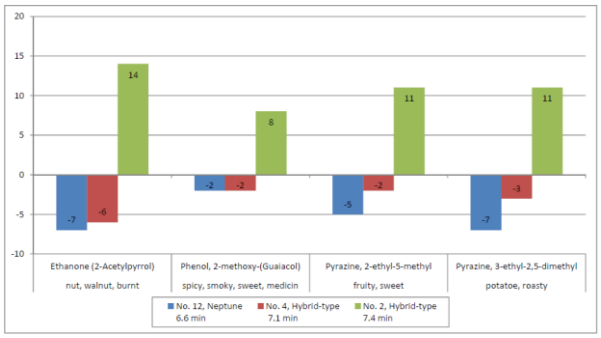


Figure 3 and 4. Drum-roast in comparison to Hybrid-roast: 13 min (GC-Analysis Data from Alexia N. Glöss: ZHAW/ICBC).



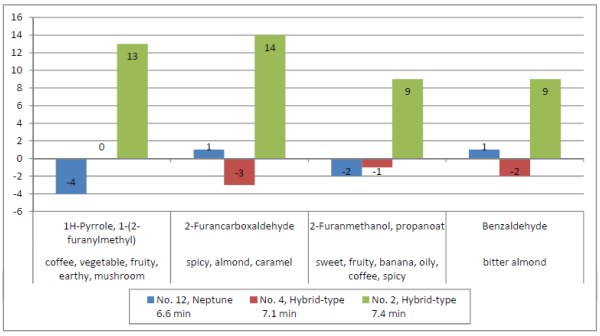


Figure 5 and 6. Drum-roast in comparison to Hybrid-roast: 7 min (GC-Analysis Data from Alexia N. Glöss: ZHAW/ICBC).

CONCLUSIONS

Roasting Profile => Coffee taste

The Hybrid Roaster offers the possibility to create a very wide range of taste profiles - with one roasting system

With this roaster it is possible

- to run the standard Jupiter - process and
- at the same roasting time, to achieve comparable flavor notes to drum roaster products.

The Hybrid roaster offers the highest flexibility for flavor development

With the Hybrid roaster our customers are able to meet all market requirements, e.g.

- roasting of difficult product (e.g. low quality beans, broken beans,…).
- blends with different bean sizes and densities.
- up to high quality- and Gourmet-Coffee.

Optimising Coffee Brewing Using A Multiscale Approach

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SUMMARY AND INTRODUCTION

We report measurements and a model study of espresso style brewing as a coupling of processes occurring at two different scales: diffusion from grains (~μm) and convection within a packed bed (~cm).

Measurement of grain porosity and permeability

We measured the porous microstructure of coffee grains. Porosity and skeletal density were assessed using a combination of Hg porosimetry and He pycnometry. We found that the porosity, ϵ_g, of the open pore space (those reachable from the outside) in a series of grains of different grind size with values ranging from 0.5 to 0.65; decreasing with increasing grind size. The bed packing determines the resistance to flow, hence the pressure head required to achieve a given drink volume in a given time (typical 40 ml in 10 sec for an espresso brew). The permeability (inverse of hydraulic resistance) of beds was measured using a custom pressure rig). The flow rate Q and pressure head ΔP are related by:

$$Q = \frac{KA}{\mu L}\,\Delta P \qquad (1)$$

where A and L are the bed area and height, and μ is the viscosity of the pumped fluid. One may back out the, permeability, *K,* from the experiment, typical values of K lie in the range 10^{-12}-10^{-14} m^2. The experimental measurement of porosity can be used the well-known Kozeny-Carman, Carman (1937), permeability relation to compare with experiment. The permeability, *K*, is highly sensitive to the bed porosity which in turn depends on the grain porosity and the bulk density. Equation (1) can be used to predict the drink volume achievable under a given pressure head for typical coffee bed. In practice pressure heads are not all applied across the bed, in some systems significant pressure drops occur at the exit for example. Using pore length scales, we estimate that the flow in espresso style coffee bed pores is in the low Reynolds (viscous, low inertial, non -turbulent) regime.

Mean Field Model of extraction

We built a simple model predicting the concentration in the pore space of a bed, C(t), combining a flux out of the particles and extraction uniformly from the bed:

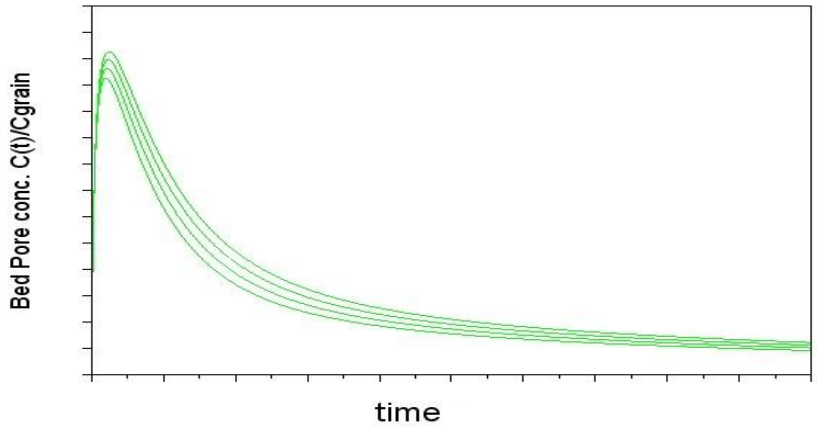
$$\frac{\partial C}{\partial t} + \frac{3(1-\varepsilon)}{\varepsilon} J(t, C, v, \varphi) - G = 0 \; and \; G = \frac{Q(t)C(t)}{\epsilon V_{bed}} \quad (2)$$

Where the second term is the flux from the grains per unit volume of the bed pore space of porosity, ε, it depends on the grain particle size distribution φ and v the mean fluid velocity in the bed pores. The third term, *G*, models extraction from the bed pore space, V_{bed} is the volume of the bed. Grains are modelled as spheres of porosity, ϵ_g , and we solve for the diffusive flux equation , J(t), out of spheres explicitly solving the radial concentration profile C_g *(r,t)* of a 'representative' diffusing species. The model was coded and solved using the SCILAB (2012) package. We approximate a coffee grind particle size distribution by just two particles a coarse particle of radius, r_{coarse} and a fine particle of radius r_{fine} = 20 μm with a fraction of grind volume in the fines of ϑ_{fine}. Thus the flux term is approximated by

$$J(t) = \frac{3(1-\epsilon_{bed})}{\epsilon_{bed}} \left[\frac{j_{fine}(t)\vartheta_{fine}}{r_{fine}} + \frac{j_{coarse}(t)(1-\vartheta_{fine})}{r_{coarse}} \right] \quad (3)$$

RESULTS

Figure 1 shows the model estimate of the concentration against time within the pore space of a highly packed bed normalised to an initial grain concentration. The concentration rapidly rises to a peak with the initially high flux from the grains; the curve turns over as the extraction pulls the bed concentration down by the end of the extraction. This is in accord with what we visually experience when we brew: the initial fluid emerging is very dark, but very quickly becomes much lighter.
Figure 1.



DISCUSSION AND CONCLUSIONS

We have developed a simple model of extraction and are developing a set of experiments with which to parameterise it. The whole research program is giving us many new insights into coffee extraction. In particular in agreement with Spiro (1993) we find it unnecessary to invoke a so-called short time wash phases: as is evident in figure 1 the diffusion model accounts well for the initially high flux out of the grains. Our current understanding is that the role of pressure in extraction is limited to that of being necessary to achieve the required flow thru a packed bed and that flow is laminar not turbulent. In the current model, the prime effect of flow rate on extraction (and hence that of pressure) is via the pore bed concentration; it also has a secondary but smaller effect through its determination of boundary layer thickness.

Although the geometric and single species limitations of the model can be improved directly, there are many complex physical and chemical complications in real systems: grain swelling, bed permeability and boundary layer fluctuations coupled to fines levels, the release of gas by the grains when initially wet, and many more. The next challenge for modelling is to include some of these effects.

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Storage of Green Coffee in Hermetic Packaging

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SUMMARY

The aim of the present study was to evaluate changes in the color and beverage quality green coffee beans *(Coffea arabica* L.) after 12 months of storage in an artificial atmosphere. The evaluation consisted of a 2 x 2 factorial design with three replicates, two storage conditions (hermetic big-bags with and without CO_2 injection, i.e., a modified atmosphere and a controlled atmosphere, respectively) and two sampling positions (upper and lower). Two additional treatments were studied as controls: jute sacks and GrainPro sacks. The beans were qualitatively evaluated for their color and for their beverage quality attributes including their sweetness, acidity, flavor, body and aftertaste. The evaluation was performed by a group of 13 judges certified by the Specialty Coffee Association of America (SCAA). The beans packaged in impermeable containers were more frequently given classifications of green color and current crop. The frequencies for the attributes of sweetness and acidity were consistently lower when the beans were packaged in jute sacks.

INTRODUCTION

Coffee is an agricultural product with a quality-based price. The value of coffee increases significantly with improvements in quality, which are necessary to obtain new markets. During roasting, the taste and aroma of coffee develop from ingredients originally present in raw beans. Taste and aroma are the principal factors affecting beverage quality. The presence and development of the taste and aroma precursors in raw coffee beans depend on genetic, environmental, and technological factors. Traditionally, green coffee beans have been stored in jute sacks. Jute is most frequently used because it is readily adaptable to smallscale commerce and because it is easily sampled for lot inspections. Recent studies have demonstrated the feasibility of using hermetic big-bags with an application of CO2 to preserve the physical, chemical and sensory quality of coffee. However, dissemination and application of new technologies for coffee storage require a validation of laboratory results on a broader scale with reference to the international market. Thus, new scientifically based studies using a sensory panel that consists of a larger number of trained tasters are extremely important. Therefore, this study was conducted with the objective of commercially validate the effects of an artificial atmosphere on the color, flavor and aroma of coffee stored for 12 months. The coffees were evaluated by a sensory panel composed of 13 tasters who were judges certified by the Specialty Coffee Association of America and who operate commercially in various coffee-producing regions of Brazil.

MATERIALS AND METHODS

The experiment was conducted in a commercial warehouse in southern Minas Gerais State, Brazil. The coffee used in the experiment was obtained from a lot taken from the 2008 (*Coffea arabica* L.) crop and passed through 17 and 18 screens. The beverage made from this

lot had a score of 80 points, classifying it as good-quality coffee according to the Specialty Coffee Association of America (SCAA) scale.

For this study, the coffee was bagged in 60-kg quantities in conventional jute sacks, in hermetic plastic sacks (GrainPro) or in 840-kg quantities in big-bags. The big-bags, consisting of two layers of high-density polyethylene and a layer of PVC, were constructed according to the specifications of patent PI 0903676-8 filed with the INPI (National Institute of Industrial Protection). A bag with this structure is impermeable to water and gases, including carbon dioxide (CO_2), oxygen (O_2) and nitrogen (N_2). Bags measuring 1.0 m x 1.10 m x 1.30 m were fitted with an interior system of valves for the injection, distribution, monitoring and repositioning of CO_2. An exterior nylon wrap placed over the hermetic big-bags made them resistant to mechanical stress. Thus, the bags were protected both from atmospheric influences and from mechanical damage.

The CO_2 was injected into the bottoms of the big-bags. The injected gas progressively displaced the intergranular air until the CO_2 reached the desired 60% level. The level of CO_2 was measured using an Anagas-CD98 instrument. The concentration of CO_2 in the packaging was monitored biweekly. We reintroduced gas when a reduction below the desired level was detected. This procedure served to maintain a constant concentration of CO_2 inside the bags.
The coffee samples were randomly collected using a grain sampler in the jute sacks and GrainPro. However, in the hermetic big-bags, the sampling was performed at two depths: 0.30 m (high) and 0.60 m (medium). For all packages, 500 g of coffee beans was collected after 12 months of storage.

The quality analysis was performed by a group of 13 SCAA-certified judges who operate commercially in various coffee-producing regions in Brazil. The samples were qualitatively evaluated for the sensory characteristics of the beverage and for bean coloration. The evaluators indicated the predominant color of the beans: blue-green, green, yellowish, mottled or bleached. The sensory ratings of the beverage were performed by evaluating its attributes: sweetness, acidity, flavor, body and finish. The sensory analysis was performed by cupping judges certified by the Specialty Coffee Association of America (SCAA), according to methodology proposed by Lingle. In this evaluation, grades on a scale of zero to ten were awarded for the attributes. The grains were roasted in a Probat roaster, model "Probatino," which has a capacity of 150 g.

The experiment used a completely randomized design with three replications and a 2 x 2 factorial design. The factors were the two packaging methods (hermetic big-bags with and without the CO_2 injection) and the two sampling positions. In addition to the treatments included in the factorial design, two additional treatments were used: storage in GrainPro sacks and conventional storage in jute sacks.The results of the qualitative analysis are represented on graphs showing the frequency.

RESULTS AND DISCUSSION

A higher frequency of green coloration was attributed to the coffee packaged in hermetic big-bags with an injection of 60% CO_2 at the upper position, and the lowest frequency was found in the coffee packaged in the jute sacks (Figure 1a). A greater frequency of coloration denoted as yellowish, mottled and bleached was found in the jute sack packaging. The beans packaged in the hermetic big-bags with a CO_2 injection at the middle position, hermetic big-bags without a CO_2 injection and GrainPro bags had a frequency of bluish-green coloration ranging from 14 to 16%.

Packaging coffee in these packaging maintained the color of the beans, with frequency values higher than 60% for green coloration and the absence or low frequencies of yellowish, mottled and bleached colorations.

Maintaining the green, greenish or bluish-green coloration of the coffee beans during storage is extremely important, given that this visual characteristic often determines the acceptance or rejection of the product during commercialization. The loss of coloration by green coffee beans during storage is a phenomenon known as whitening, which can result in the product becoming worthless. In studying the effects of different packaging methods on the coloration of coffee beans, concluded that the use of hermetic big-bags intensified the green color of the beans during storage. Notably, as shown in Figure 1(b), the coffee packaged in jute sacks had the highest frequency of flavor classified as poor. Conversely, the flavors classified as good, very good or excellent were predominant in the other types of packaging.

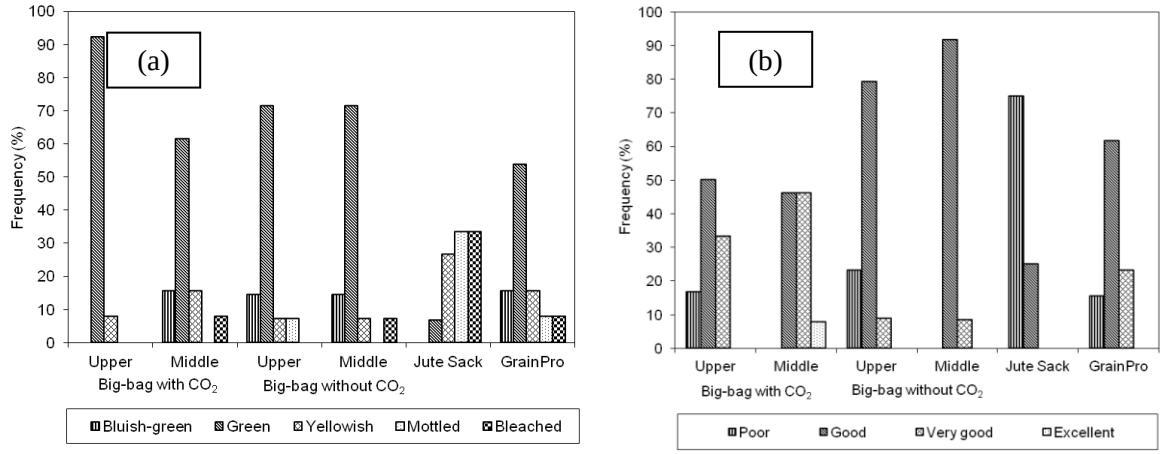

Figure 1. Frequency of the coloration (a) and flavor (b) after 12 months of storage in the different packaging.

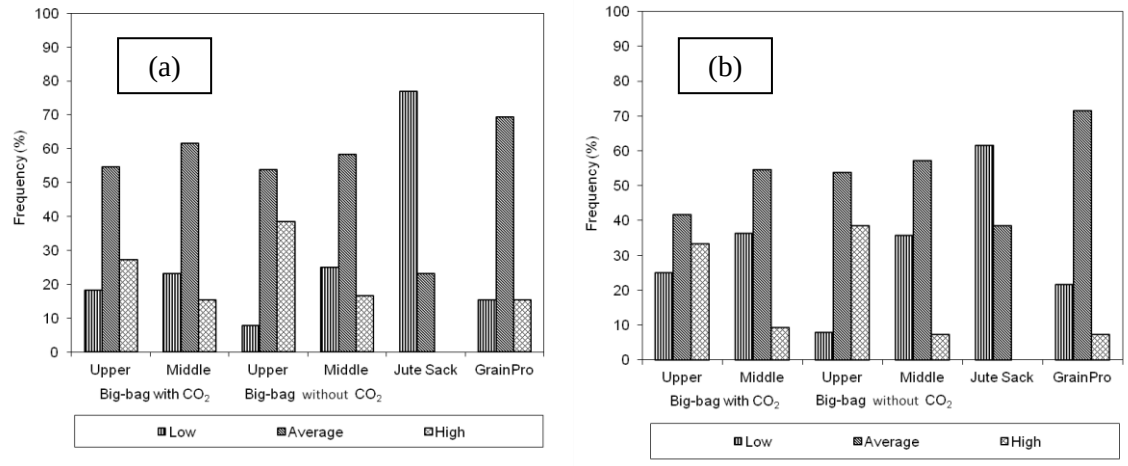

Figure 2. Frequency of the sweetness (a) and acidity (b) attributes after 12 months of storage in the different packaging.

As observed in Figure 2(a), the frequency of the sweetness attribute was predominately average, with minor variations in the impermeable packaging. However, low sweetness was observed more frequently in the coffee packaged in jute sacks.

The acidity attribute was predominantly average in the impermeable packaging. Notably, a higher frequency of low acidity was found in the coffees packaged in jute sacks (Figure 2b). Acidity can be pleasant or unpleasant. A pleasant acidity contributes to the vivacity of the coffee, increases the perception of sweetness and gives a fresh-fruit characteristic. The body attribute consists of the tactile perception of the liquid in the mouth. Most of the samples with an intense body can also have a high score in terms of quality due to the presence of more solids dissolved in the beverage. Aftertaste is defined as the persistence of flavor. If the aftertaste leaves a very brief or unpleasant sensation, a low score can be given.

The graph in Figure 3(a) shows that the body attribute is predominantly average, with some fluctuations among the different types of packaging studied. The high body attribute was not observed for the coffee packaged in jute sacks. In addition, the low body attribute was not observed in the coffee packaged in big-bags with CO_2 in the upper position. In Figure 3(b), the finish attribute was predominately classified as good, with some fluctuations for the impermeable packaging. A high frequency of an excellent aftertaste was found in the coffee packaged in big-bags without CO_2 in the upper position, and high frequencies of a poor aftertaste were found in the beans packaged in jute sacks.

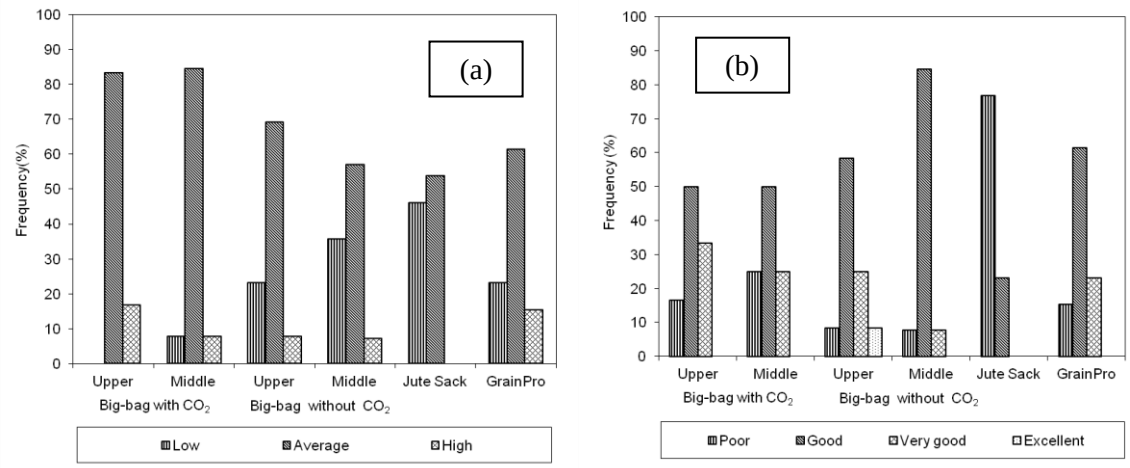

Figure 3. Frequency of the body (a) and finish (b) attributes after 12 months of storage in the different packaging.

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Cup Quality Assessment of 5 Primary Processing Methods on Indonesian Arabica Coffee

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SUMMARY

Research conducted in Flores (Marsh, Yusianto, Mawardi ASIC 2010) on the effect of primary processing on cup taste demonstrated that under Flores conditions, *Pulp Natural (PN)* processing created a highly preferred coffee cup profile compared to *Full-Washed* (*FW*), *Wet-Hulled (WH)* processing. *PN* processing was ranked the most preferred processing method by cuppers across all 3 Arabica varieties tested. This new research investigated if these findings could be verified in a different location in Indonesia. Five coffee processing methods were evaluated including the same 3 processing methods used in the Flores research with the addition of the processing methods of *Pulp Natural/Wet Hulled (PN/WH)* and by use of *Demucilager* (*DM)* processing.

Fresh coffee cherry of a single Arabica variety was harvested and processed to dry coffee by 5 different processing methods and then assessed by 13 cuppers. Coffee samples were assessed when fresh by 7 cuppers and then by 6 cuppers when the coffee was older.

Cupper's preferences and cupping notes revealed that processing method together with coffee freshness clearly influenced coffee cup taste.

- *PN* was shown to improve in Ranking Preference as it aged. PN received no Most Preferred rankings by the 7 cuppers when assessed fresh (within 3 months of processing). The 6 cuppers who assessed the matured samples (6 to 9 months after processing) all ranked *PN* the highest preference. Sweetness, floral notes and complexity were the significant descriptors used in the cuppers notes to describe the *PN* coffee.

- In the fresh cuppings *FW* received the second highest number of "Most Preferred" ranking by 3 cuppers with *DM* receiving 2 "Most Preferred" rankings, and *WH*, *PN/WH* and *DM* all receiving 1 "Most Preferred" ranking indicating all coffees had positive attributes that appealed to at least some of the 7 individual cuppers.

The results indicate that processing plays a clear effect in the cup quality and flavour profile of coffee. For the variously called "Pulp Natural" / "Descascado" / "Honey Coffee" aging or maturing would appear to be a very important component of the flavour development process. While this maturing process is an essential step in developing this unique cup quality, the exact process is not understood and more research is required to be able to more fully manage this process.

MATERIALS AND METHODS

During the 2010 coffee season 3 x 500 kg harvests of Arabica coffee were collected from a 2 ha plot of well managed Arabica coffee of the catimor variety "*Andung Sari II" at* an altitude of 1200m in the Jember Distinct of East Java, Indonesia.

Harvested coffee was divided into 5 equal lots and the 5 methods of primary processing were carried out on the plantation and wet, freshly pulped coffee was then transported to the main ICCRI research station at Kaliwining (Jember) altitude 65m for further processing, hulling, drying, sorting and storage. Dry coffee went into storage in late September 2010.

Coffees samples were processed by the 5 methods, using 100kg fresh cherry from each of 3 replications.

- Full-Washed: (*FW)* Coffee is pulped, fermented over night, washed, dried to 12% mc. This process was used as a "Control" as it gives a clean acidic coffee familiar to most cuppers.
- Wet-Hulled: (*WH)* Coffee is Pulped, fermented 1 night, dried for 3 days to 30% mc then wet hulled, to produce green bean and then dried to 12% mc.
- Pulp-Natural / Descascado: (*PN)* Coffee is pulped and dried with mucilage intact, without washing to 12% mc. (Replicating local traditional processing in Flores).
- Pulp Natural and then Wet Hulled. (*PN/WH)* Coffee is Pulped and dried with mucilage intact to 30% mc and then wet hulled and dried to 12% mc.
- Demucilaged: (*DM)* Coffee is pulped and passed directly through a demucilager twice, removing an estimated 98% of the mucilage, no fermentation, no washing, then dried to 12% mc.

When dried the 3 replications of 5 sets of samples were cupped by the ICCRI in-house cupping team to ensure each process gave a consistent cup across the 3 replications. 5 composite samples were prepared representing each of the 5 processing methods.

The storage conditions of the samples were not recorded. It is known that the coffees were stored as per normal procedure at the ICCRI research centre cupping laboratory. *FW*, *DM* and *PN* Coffees were stored as parchment till required for cupping. *WH* and *WH/PN* coffee where stored as green bean.

Assessment was conducted by 4 separate blind cuppings using 13 cuppings in Jember, Jakarta and Sydney.

- Cupping 1: 12/10/2010. ICCRI, Jember, East Java with 3 cuppers.
- Cupping 2: 8/12/2010. SCAI, Jakarta with 4 cuppers.
- Cupping 3: 15/3/2011. Mountain Top Coffee, Sydney with 3 cuppers.
- Cupping 4: 18/6/2011. ICCRI, Jember, East Java with 3 cuppers.

Coffee samples were cupped in 4 cuppings due to availability of cuppers. This resulted in the first two cuppings to be grouped as fresh coffee (within 3 months of processing) while cuppings 3 and 4 grouped as matured coffee (6 to 9 months after processing).

Cupping was blind and cuppers were asked to rank coffees from "Most Preferred" to "Least Preferred" and to give reasons for rankings.

RESULTS

Comments from cuppers indicated that all coffees were clean and of general high commercial or specialty quality without defects. Many comments from cuppers indicated these coffees were typical of Indonesian specialty coffee with relatively good body and the Indonesian characteristics.

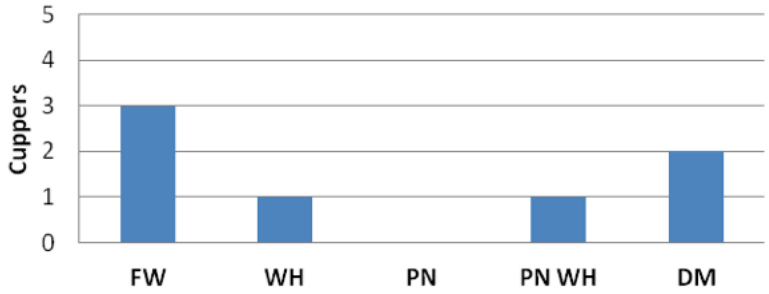

Figure 1. Most Preferred Ranking for Fresh Coffeee Samples.

Cupping notes indicated PN was either harsh or astringent and had high, unbalanced acidity and consequently received no Most Preferred rankings by the 7 cuppers in the 1st and 2nd cuppings when the coffee was fresh.

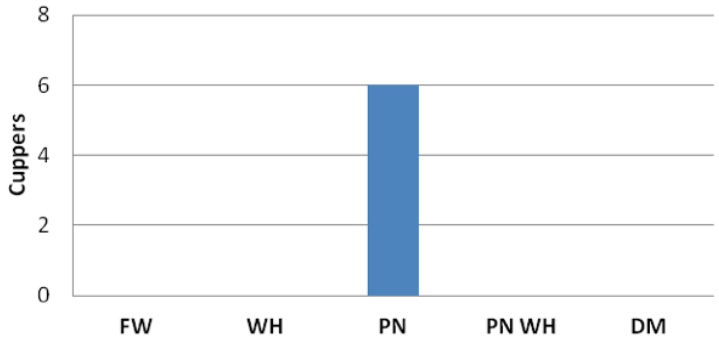

Figure 2. Most Preferred Ranking for Mature Coffee Samples.

PN received 100% of the Most Preferred rankings in the 3rd and 4th cuppings (Mature coffee) Cupping notes indicated high balanced acidity, floral notes, sweetness and complex body.

Table 1. Selected Comments by Cuppers.

Email comments from Australian Specialty Coffee Importer, Mountain Top Coffee who conducted the 3rd cupping. *"We cupped the 5 samples today in our offices, and we were also joined by one of our clients. All cupping was done blind. It was an interesting flight, and although there was some disagreement on the order of preferences, we all agreed upon Treatment C (Descascado) being the top preferred lot. There was consensus between the three of us that this lot had the most balanced profile in comparison with the other samples. On the whole we did also agree that for coffees from Java, although all samples did somewhat display varying degrees of what we referred to as 'typical Indonesian earth/funk', these samples were quite clean and balanced.*
Cupping notes from the 4th cupping conducted in the ICCRI cupping lab identified *PN* had sweet and floral characteristics. The following comment was made by an experienced international cupper. "*Treatment C (PN) was interesting, acid, very floral and aromatic aftertaste, like a Rwandan coffee"*

DISCUSSION

The *FW* processing demonstrated proven ability to produce a balanced coffee with good acidity as a fresh coffee. *WH* processing also demonstrated it can produce good coffee as is proven through its overwhelming use throughout the Indonesian Specialty coffee industry. The experimental processing method of *PN/WH* also appeared to be a viable processing method and deserves more research as it is an extremely fast process, requiring little water and no fermentation. Demucilaging technology is used extensively around the world and in this cupping assessment was clearly preferred by some cuppers.

The most notable result from this research was that *PN* processing combined with longer storage times can have an extremely positive impact on cup quality. As a fresh coffee, *PN* created cup profiles that were too fruity, too acid, unbalanced and harsh or astringent. However, after a period of longer storage the flavor profile of PN evolved to a balanced, complex sweet and floral cup. Clearly storage was an integral and active component of this method of processing.

Storage conditions during the trial were not studied but comparison of the PN and PN/WH preferences indicate that the flavor profile improvement occurred during the dry storage of the parchment with mucilage intact. Both coffees began the process as a Pulp Natural, but *PN/WH* had dried mucilage and parchment removed after 3 days of drying to produce naked green bean. It would seem, intuitively, that the mucilage adhering to the *PN* parchment during storage imparts the sweetness and floral notes of the cup and complexity in the body. While there may also be an amount of mucilage still adhering to the naked bean on the *PN/WH* due to the process of hulling, this did not produce the same high preference ranking as the PN.

CONCLUSIONS

Good storage of specialty coffee is understood to be an integral part of the process to maintain quality. This research has demonstrated that storage has a strong influence on not only maintaining but also developing high cup quality of the PN process. PN process is growing in popularity due to its complex, sweet and floral cup profiles created as demonstrated in this research. This processing system shows great promise but the storage phase of this PN process system requires greater understanding to maximize quality and to provide consistent cup profiles.

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Differential Expression of WRKY Transcriptional Factors in Endosperm Tissues during Stress and Ontogeny of Fruits of *Coffea Canephora* with Respect to Caffeine Biosynthesis

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SUMMARY

Caffeine biosynthesis in coffee involves the sequential methylation of xanthosine precursor, is catalyzed by enzymes of the SAM-dependant N methyltransferase (NMT) multigene family. Regulation of caffeine biosynthesis however remains to be illustrated, although preliminary data suggest that the age of plant organs as well as light may play an important role. The promoter of theobromine synthase-like gene of *Coffea canephora* carry several motifs for light and WRKY family of transcription factor and this forms the basis of the present analysis. Many of these transcription factors are known to work downstream of salicylic acid signaling pathway. Treatment of leaves of *Coffea canephora* with salicylic acid and methyl jasmonate augmented the biosynthesis of methyxanthines (theobromine and caffeine) correlating with the increased expression of the NMT genes as well as over expression of certain WRKY. We also studied the expression pattern of the WRKY factors during the ontogeny of endosperms. Theobromine synthase is repressed in endosperms at the early dry weight accumulation phase until fruit maturity concomitant with the repression of *CcWRKY-19*. This factor is over-expressed also during SA and MeJ treatments coinciding with over-expression of NMT genes. The involvement of this factor in transcriptional regulation of theobromine synthesis needs to be ascertained.

INTRODUCTION

The core caffeine biosynthetic pathway begins with the sequential methylation of the committed precursor xanthosine. Theobromine is the major intermediate of caffeine biosynthetic pathway (Ogita et al., 2004) and is produced as a result of the catalytic activity of the second NMT (NMT-2) ie., theobromine synthase. Four exclusive theobromine synthase genes have been identified from *C. arabica*, first two (*CaMXMT1*, *CTS1*) encoding a 378 amino acid enzyme and other two (*CaMXMT2*, *CTS2*) encoding a 384 amino acid enzyme. To-date the theobromine synthase-like genes isolated from *C. canephora* all encode for 378 amino acid (Satyanarayana et al., 2005). Theobromine synthase genes of *C. arabica* exhibit a spatio-temporal expression pattern-usually expressing at high levels in younger tissues like young leaves and immature berries (Ogawa et al., 2000; Uefuji et al., 2003). Apart from this, light irradiation enhances synthesis of purine alkaloids in coffee suspension cultures (Kurata et al., 1997). However, the mechanism of transcriptional regulation of theobromine synthase has not been addressed. The promoters of *C. canephora* theobromine synthase-like genes carry numerous motifs for light responsive transcription factors, WRKY responsive W-boxes (four motifs) and jasmonate responsive elements (Satyanarayana et al., 2005). WRKY family of transcription factors is a large group comprising 72 member genes in *Arabidopsis thaliana* (Eulgem et al., 2000) and 103 in *Oryza sativa* (Wu et al., 2005). Salicylic acid and pathogen/wounding stimuli are known to act through WRKY family of transcription factors that are implicated in stress and developmental response. Ramiro et al., (2010) identified in *C.*

arabica, 22 WRKY unigenes encompassing the 60 amino acid WRKY domain that binds to W-Box on the WRKY responsive promoters and studied their expression profile during pathogen resistance response. However, there are no studies on WRKY genes in *C. canephora* and especially on their expression pattern in correlation to theobromine synthase expression. Thus, such a study would benefit our understanding of co-expression regulatory networks associated with caffeine biogenesis. We thus, undertook this study to see if the members of this family share co-expression pattern with theobromine synthase gene during the ontogeny of endosperms and during responses to salicylic acid (SA) and methyl jasmonate (MeJ) treatment.

MATERIALS AND METHODS

WRKY genes were mined from the Solanaceae Genome Network (SGN) *Coffea canephora* database, Cornell University, by multiple tBLASTn searches using *Arabidopsis thaliana* WRKYs as query. The 60 amino acid WRKY domain of the *Arabidopsis* genes and the coffee unigenes was used for construction of phylogenetic tree using PhyML program. Gene specific primers were designed for the 19 coffee unigenes as well as for the NMT genes using the available sequences. *Coffea canephora* var. *robusta* S-274 endosperms were collected during two fruiting seasons and grouped into ten different growth phases. For stress treatment, one-year-old seedlings were used. The green house acclimatized seedlings were placed in 200ml of 1X nutrient Hoagland's liquid medium in plastic pots. Salicylic acid was administered directly to the medium at 50 and 500µM concentration (SA-50 and SA-500), whereas for methyl jasmonate treatment the plants were enclosed in plastic chambers and exposed to fumes from 1µL or 10µL (MeJ-1 or MeJ-10) undiluted methyl jasmonate spotted on tissue paper. Untreated plants, used as controls (C), were maintained at appropriate conditions. Second leaf pairs from apices were sampled at 6hrs and 12hrs post-treatment. After 12hrs the treated plants were subjected to control conditions devoid of the stress inducing factors for 48hrs (R). RNA was isolated from endosperms and the leaves of the seedlings using modified CTAB method and subsequently treated with DNAaseI. Reverse transcription was primed with oligo-dT primer on 1µg of total RNA using Verso cDNA synthesis kit (Thermo scientific co.) and the cDNA was diluted 1:29 in deionized water. Semi-quantitative RT-PCR was carried out to study the expression patterns of eighteen WRKY genes and the three NMTs during ontogeny and in stress conditions in a total reaction volume of 10µL using parameters optimized separately for each primer set. The reactions were normalized with *GAPDH* and/or *Ubiquitin* genes.

Leaf samples collected at the same time as for RNA extraction, were dried at 37^{o}C overnight. 500mg leaf samples were ground to fine powder using mortar and pestle and the methylxanthines were extracted by boiling in 4mL 50% methanol. The samples were centrifuged at 10000rpm for 10 minutes. The pelleted was re-extracted with 1mL of 50% methanol twice and the extract was pooled. Samples were dried using Rotavapour. The residues were finally dissolved in 5ml of 50% methanol, filtered through 0.4µM syringe filters and the methylxanthines were estimated by RP-HPLC on C-18 column. Parameters were controlled by a Shimadzu LC 10 as liquid chromatograph equipped with a dual pump and a UV spectrophotometer detector (model SPD 10 A) (270nM) and the recorder C-R7a Chromatopac. Separation was carried out in 0-40% gradient of methanol in 50mM sodium acetate pH 5.0 at a flow rate of 1mL/Min.

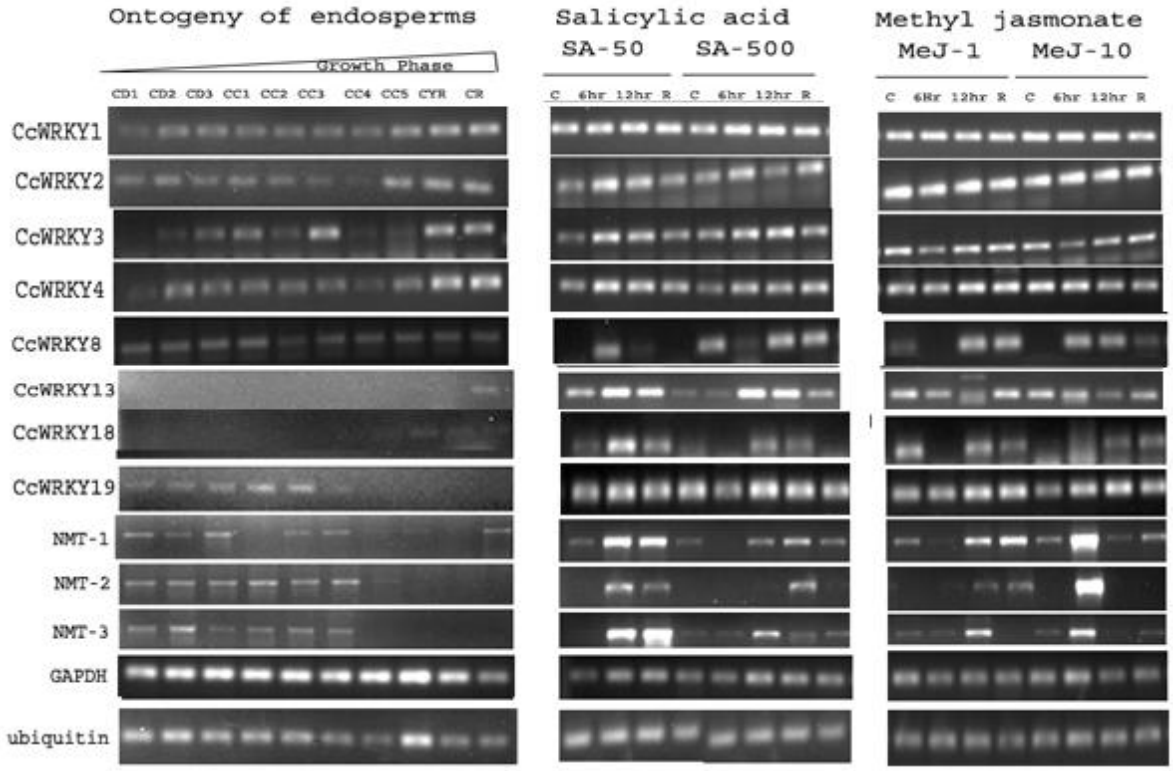
RESULTS AND DISCUSSION

We identified 31 WRKY unigenes from the *C. canephora* database at the SGN initiative and the NCBI database. Twenty-one of the WRKY unigenes had the sequence information for the

60 amino acid WRKY domain and these were named as *CcWRKY1-21*. Eighteen identified WRKYs (*CcWRKY*1-*19* except *CcWRKY-15*) were used in the differential expression study. The phylogenetic tree indicated that the *C. canephora* WRKY (*Cc*WRKY) represented all major classes as defined for the *A. thaliana* counterparts (*At*WRKY). Biochemical analysis of stress experiments clearly indicate the augmentation of caffeine in leaves upon SA and MeJ exposure. As a further control for the experimental setup, we used rescued plants (R), where the treated plants were acclimatized to control conditions for 48hrs prior to sampling. It is clear that the expression of all the three NMTs (NMT-1, NMT-2 and NMT-3) increased several folds upon both SA and MeJ exposure and fell down to normal levels in rescued plants at most cases (Figure 1). Of the eighteen WRKY, twelve (*CcWRKY-1*, *2*, *3*, *4*, *5*, *6*, *8*, *9*, *10*, *13*, *18* and *19*) showed over expression during at least one of the concentrations of SA. *CcWRKY-1*, *2*, *3*, *4*, *13*, *18* and *19* over-expressed only upon induction and dropped back to basal levels in the rescued plants. In contrast, only five WRKY genes (*CcWRKY-4*, *5*, *8*, *10* and *19*) responded for MeJ stimuli, the most promising candidate being *CcWRKY-8* and *19*.

We further extended this work to study the expression pattern of NMTs and WRKYs during the ontogeny of endopserms. The endopserms were grouped into ten growth phases (CD1, CD2, CD3, CC1, CC2, CC3, CC4 CC5, CYR, CR) beginning from 3 months post anthesis until maturity. CD1-CD3 is discoid endopserm of size range 0.2-0.5cm diameter, 0.5-0.8cm diameter and 0.8-1cm diameter. The endosperm covers <50% total fruit space in CC1, ~50% in CC2, ~70% in CC3 and almost entire space in CC4. CC5 marks the beginning of dehydration phase. CYR and CR correspond to endosperm from yellow-red and red berries, respectively. Biochemical profiling for methylxanthines during ontogeny of fruits has been previously documented (Koshiro et al., 2006). Maximum percentage of caffeine accumulation was recorded in seeds from stage E fruits, which, correlates with the start of dry weight accumulation in endosperm. This stage corresponds to CC5 stage where we noticed complete shutdown of transcription of the N-methyltransferse genes especially the second and third NMT of the pathway. Unlike the expression pattern of the NMTs, expression level of most of the WRKY factors increased as the fruit reached maturity, which may be accounted for stress encountered by dehydration. We argue that transcription factors dedicated for the transcriptional regulation of theobromine synthase would also share the same expression pattern as of theobromine synthase even if partly for few of the experiments, considering different mechanisms of SA, MeJ and developmental responses. Out of the eight promising transcription factors (*CcWRKY-1*, *2*, *3*, *4*, *8*, *13*, *18* and *19*), *CcWRKY-19* correlated with expression of theobromine synthase gene in all three experiments. Preliminary *in silico* co-expression analysis clusters the closest *Arabidopsis* homolog of *CcWRKY*19 among defense related genes as well as the gene of flavanol synthesis. We select these factors as promising candidates for further validation using yeast-one hybrid assays.

Figure 1. Differential expression of selected WRKY factors and NMT genes during ontogeny of endosperm and in leaves after SA and MeJ stimuli. SA-50- salicylic acid 50µM, SA-500- salicylic acid 500µM, MeJ-1-1µL neat methyl jasmonate, MeJ-10- 10µL neat methyl jasmonate, C-control (untreated plants), R- rescued (treated plants recovered in control conditions for 48hrs).



The work concludes with the expression profiling of WRKY factors during stress response and ontogeny of endosperm. It also further strengthens our hypothesis of involvement of SA and MeJ in augmentation of caffeine, thus validating the *in silico* analysis of promoter. Since the candidate WRKY genes over express in response to SA and MeJ, we can anticipate similar effects on SA and MeJ treatments on whole berries. It would be interesting to study if SA and MeJ treatment on berries is able to remove the block responsible for theobromine synthase expression shutdown beyond the CC5 stage and if *CcWRKY*19 is involved in the crosstalk.

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Ancestral Synteny Is Shared Between Distantly Related Plant Species from the Asterid and Rosid Clades

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SUMMARY

A set of 867 COSII markers allowed to established macrosynteny between coffee, tomato and grapevine. Coffee and tomato genomes shared 318 orthologous markers and 27 conserved syntenic segments (CSSs), coffee and grapevine shared a similar number of syntenic markers (299) and CSSs (29). Despite important genome macrostructure reorganization, several large chromosome segments showed exceptional macrosynteny, shedding new insights into chromosome evolution between flowering plants. In addition, we analyzed a BAC sequence of 174 kb containing the *Ovate* gene, conserved in a syntenic block between the three plants, that showed a high microsynteny conservation. Surprisingly, a higher level of conservation was observed between coffee and grapevine, both ligneous and perennial plants, than between coffee and tomato, an annual plant. Out of 16 genes identified in coffee on this segment, 7 and 14 showed complete synteny between coffee and tomato or grapevine, respectively. A significant conservation was found at the macro- and micro-structure levels between distantly related species from the Asterid (*Coffea canephora* and *Solanum* sp.) and Rosid (*Vitis vinifera*) clades. Conservation did not decline at the *Ovate* locus in relation to phylogenetic distance, suggesting that the time factor alone does not explain all the divergences. These results are considerably useful for syntenic studies between supposedly remote species for the isolation of important genes for agronomy.

INTRODUCTION

Coffea genus belongs to the Rubiaceae family, the fourth largest family of angiosperms. To date, this genus encompasses 124 perennial species, all native of Africa, Madagascar, the Mascarene and Comoros Islands, Asia and Australia. It includes two economically important species: *C. arabica* and *C. canephora*, which represent a major agricultural commodity in world trade. The Rubiaceae is related to the Solanaceae family that contains economically important crop species such as tomato, tobacco, and potato, all being annual. Both families belong to the Asterid clade, and diverged from their common ancestor approximately 83-89 million years ago. Rubiaceae and Solanaceae are frequently considered as “sister” plant families based on genetic similarities observed between coffee and tomato (genome size and chromosome number).

The Vitaceae is an economically important family of angiosperms. It includes the *Vitis vinifera* species, a perennial plant, used as fruit or for beverage production. Recent phylogenetic analyses have placed the Vitaceae family as the earliest diverging lineage of the Rosid clade. Grapevine and coffee diverged from their last common ancestor approximately

114-125 MYA, a long period of evolution that should allow numerous chromosomal rearrangements to accumulate.

So far, very few comparative genome mappings have been performed between distantly related species belonging to two different clades. Extensive pairwise comparative studies have been performed within the Solanaceae and between tomato and coffee. However, no multiple comparisons have been conducted using Solanaceae, coffee and grapevine to study the pattern of chromosomal evolution between species that have not experienced recent polyploidization. Recently, good conservation of the microsynteny was described at one locus between coffee and grapevine. Such microsynteny raised the question of the extent of genome microstructure conservation and the possible presence of macrostructure conservation between distantly-related genomes.

To better understand the structural relationships between the coffee, tomato and grapevine genomes we combined macro- and microsynteny studies to evaluate the genome structure conservation between these distantly-related species. 867 genetically-mapped COSII markers in coffee and tomato identified blocks of synteny with the grapevine pseudo-chromosomes, giving a significant but fractioned conservation of genome macrostructure between the three species.

MATERIALS AND METHODS

COSII markers were mapped into the coffee mapping population (BP409 X Q121) developed by Nestlé R&D (France) in collaboration with the Indonesian Coffee and Cocoa Research Institute (ICCRI). The tomato genetic map, used in this study, including all COSII markers, is available at http://solgenomics.net/cview/map.pl?map_version_id=52. The grapevine reference genome sequences and annotated coding sequences representing 26,346 gene models were downloaded from http://www.cns.fr/externe/GenomeBrowser/Vitis/. (Accessions: FN597015FN597047).

Coffee and tomato unigene sequences for each COSII loci were retrieved from the SOL Website http://solgenomics.net/. Orthologous COSII gene sequences were identified based on sequence similarities between coffee or tomato sequences and the grapevine genome using BlastN and BlastX analyses. A conserved syntenic segment (CSS) was defined by a minimum of three coffee or tomato COSII sequences that map to the same grapevine region at a maximal distance of 3,2 Mbp between pairs of markers.

The *C. canephora* BAC Clone 111O18, containing the *Ovate* gene, was sequenced using the Sanger method (Accession#: HM635075). The final BAC sequence was analyzed and annotated similarly as in *BMC Plant Biology* and *The Plant Journal*. Local conservation of gene order and orientation was investigated between the coffee tree and Solanaceae by direct comparison of orthologous BAC sequences. Sequence comparisons were computed by Dotter and BLAST. The orthologous grapevine *Ovate* region was identified by BLAST searches using predicted coffee coding regions as queries.

RESULTS AND DISCUSSION

Using the updated coffee and tomato genetic maps, the coffee-tomato comparison was reassessed and shared 318 common markers leading to a total of 27 syntenic blocks. The highest number of syntenic relationships was established between coffee Linkage Group (LG) G and tomato LG 2 (Figure 1) with 16 COSII orthologous relationships (over 50 cM for

coffee and 63.5 cM for tomato). In four cases, all the syntenic blocks defined on a single coffee LG were related to a single tomato LGs: D and 3, H and 8, J and 4, K and 5.

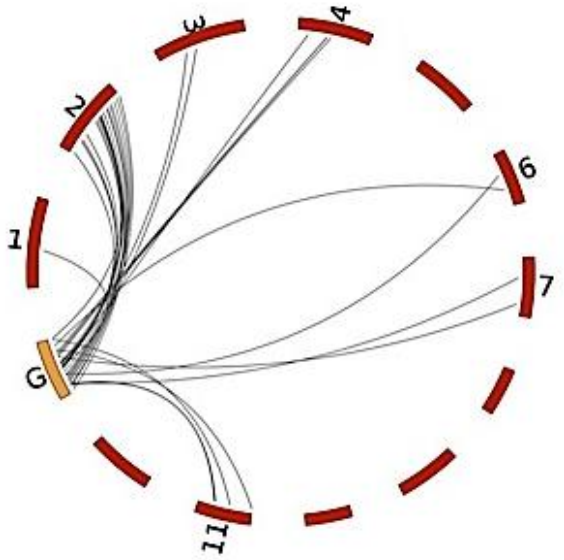

Figure 1. Syntenic relationship between coffee linkage group G and all tomato linkage groups, 16 COSII are common between coffee LG-G And tomato LG 2.

430 COSII loci mapped in coffee, 356 (83%) were found conserved on grapevine genomic sequences, out of them 299 (84%) mapped to a single locus on the grapevine genome, These findings are consistent with an absence of recent polyploidy in the grapevine genome. Out of the 299 single-locus grapevine COSII sequences, 282 were found on assembled pseudochromosomes. Four putative orthologs fell into segments assigned to known chromosomes but with unknown positions and 13 orthologs were found in contigs unallocated to specific grapevine chromosomes. The distribution of COSII sequences conserved along coffee and grapevine chromosomes showed that the putative orthologs are distributed all along the coffee linkage groups. Despite differences between chromosome numbers and genome size between coffee and grapevine (respectively ~704 Mbp for 11 chromosomes and ~475 Mbp for 19 chromosomes), the synteny relationships are substantial but fractionated, through the conservation of 29 syntenic blocks spread between the 11 coffee LGs and the 19 grapevine pseudo-chromosomes.

In most of the cases, coffee LGs contain syntenic blocks that map to several grapevine chromosomes indicating numerous rearrangements such as translocations and inversions. In the most extreme case the syntenic blocks of coffee LG B, the longest linkage group, were found conserved in 9 different grapevine chromosomes. In contrast, evidence is also given for significant conservation of coffee LGs with grapevine chromosomes: LG C and grapevine chromosome 5, LG J and chromosome 18 and LG K and chromosome 1 (Figure 2) suggesting that the chromosomal organization of these LGs might be ancestral to the Asterids and Rosids lineages.

567 (75%) COSSII loci mapped in the tomato were found conserved on the available grapevine genome. Out of them 470 (82.9%) mapped to a single locus. A total of 45 syntenic blocks were identified between the two species. Unlike coffee, no conservation between specific tomato LG and grapevine chromosome was observed as tomato CSSs from a unique LG always mapped to several grapevine chromosomes.

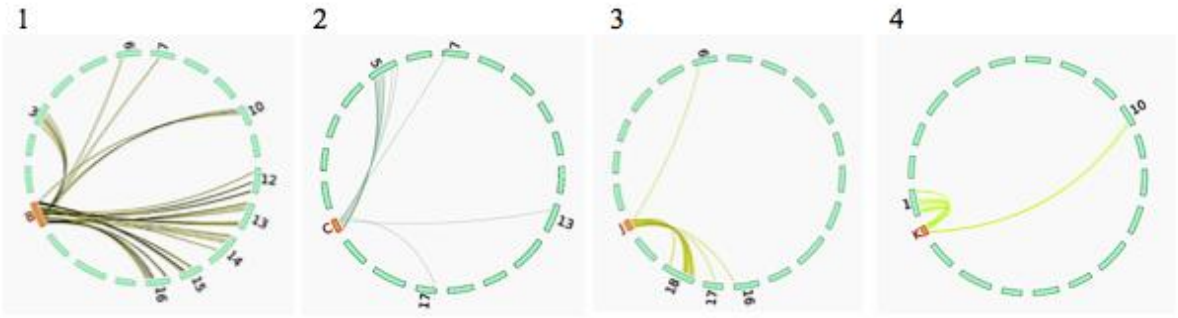

Figure 2. Macrosyntenic relationships between coffee linkage groups and grape pseudo-chromosomes. (1: dispersed, 2-3-4: conserved).

To determine if agronomical important genes are syntenic between the coffee tree, the tomato and the grapevine, we identified, mapped and compared the *Ovate* region in coffee, grapevine and several Solanaceae. The Ovate locus, identified in tomato, co-localizes with a major QTL affecting fruit shape and we showed that it is conserved within a syntenic block between the coffee tree, the tomato and the grapevine. We compared genomic sequences between the coffee tree, several Solanaceae (tomato, petunia, eggplant, pepper and a wild potato) and the grapevine at the ovate region. Pairwise comparisons revealed that nucleotide conservation was strictly limited to the exons. The overall gene content and order was found conserved. The *Ovate* gene was found at an orthologous location among the analyzed BAC sequences. However, local missing or extra genes and a local gene inversion created small differences between coffee and five Solanaceae species (figure 3). Despite small differences, the comparison between coffee and Solanaceae at the ovate locus reveals strong overall conservation. Surprisingly, the level of conservation appears fairly high between coffee and grapevine, since a total of 20 coffee genes encounter putative orthologs along the segment on grapevine chromosome 4 (Figure 3) and gene order and orientation appeared strictly conserved. In conclusion, the overall conservation analysis of the gene content, order and structure among five Solanaceae species, the coffee tree and the grapevine suggests that microstructure may be conserved in syntenic blocks among coffee, tomato and grapevine genomes. Surprisingly there is a better conservation of the microsynteny between the coffee tree and the grapevine than between the coffee tree and the Solanaceae at the Ovate locus.

In conclusion, we used a large set of genetically mapped COSII loci on coffee tree and tomato and the public release of the grapevine genome to evaluate the synteny relationships between these distantly related genomes from species of agronomic interest. A significant synteny can be detected through numerous small blocks, allowing a first analysis about the divergent chromosomal histories between tomato, coffee trees and grapevine. In addition, based on the observation of the *Ovate* locus, syntenic blocks appear to be associated with an extensive conservation of the microstructure. However, Solanaceae microstructures appear less conserved than the coffee tree and grapevine ones, suggesting a divergent and specific evolution of the locus in the Solanaceae prior to the separation with the Rubiaceae and that time factor alone does not explain the divergences. From an applied perspective, the detailed analysis of blocks of synteny over these distantly related plant species is certainly of great interest in comparative genomic approaches to identified genes of interest.

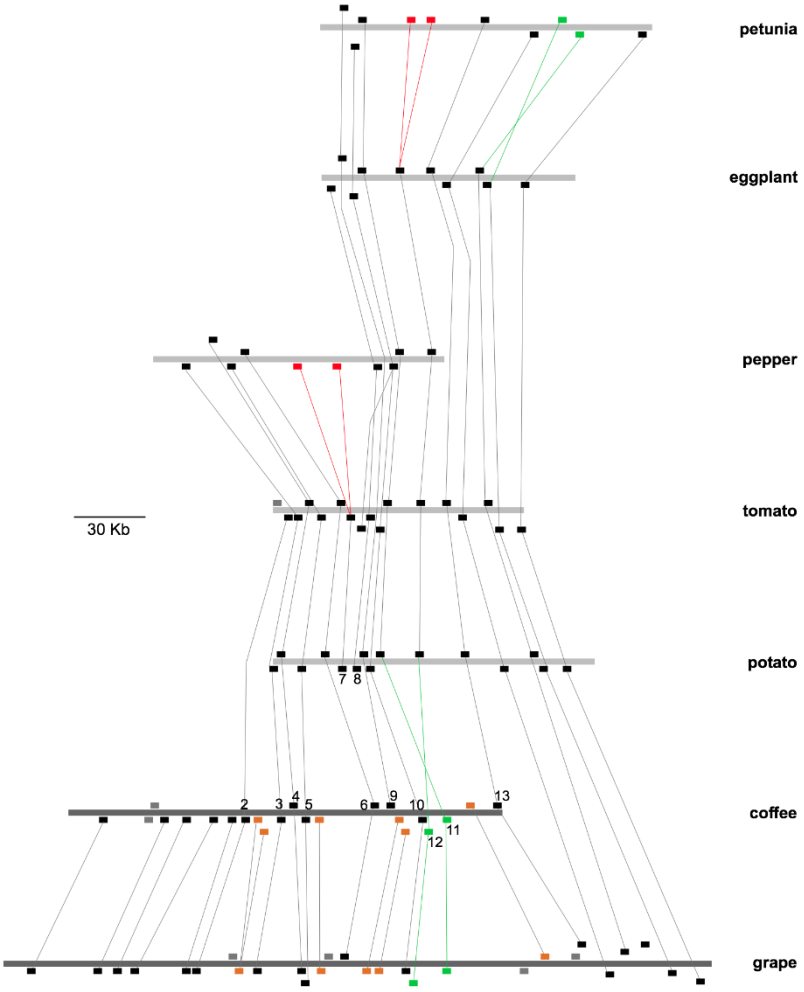

Figure 3. Microsynteny between five Solanaceae species, coffee and grape.

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Interaction between Coffee Plants and the Insect Coffee Berry Borer, *Hypothenemus Hampei*

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SUMMARY

No sources of natural resistance have been identified in *Coffea* species against Coffee Berry Borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: *Curculionidae*), the most important coffee pest in Colombia. However, *Coffea liberica* has shown some levels of antibiosis against the insect. In order to understand the interactions between the coffee plant and CBB, to characterize the response of coffee plants under CBB attack and the bases of the *C. liberica* antibiosis, a functional genomics study was carried out using ESTs libraries, cDNA microarrays, and an oligoarray containing 43.800 coffee sequences. The results allowed the comparison of the *C. liberica* vs. *C. arabica* fruit responses to insect infestation after 48h. Out of a set of 2,500 plant sequences that exhibited differential expression under insect attack, 895 were induced in *C. liberica*, at least 2 times more than in *C. arabica.* In order to validate some of the induced genes, quantifications through real-time PCR were done. At least four genes: an isoprene synthase gene, a patatin-like protein gene, a hevein-like protein sequence, and a trypsin inhibitor known also as miraculin-like gene, *CoMir*, were highly upregulated in *C. liberica* at 24 and/or 48 h after insect infestation compared to *C. arabica*. Functional annotations indicate that they participate in separate defense plant processes such as volatiles synthesis, lipid or chitin degradation, and proteinase inhibition, suggesting the activation of different metabolic pathways and plant defense mechanisms in the coffee plants in response to the insect attack. One of those processes is the pathway that leads to the production of isoprene. The effect that isoprene has on the CBB was measured by monitoring the development of the insect from egg to adult, on coffee-artificial diets amended with increasing concentrations of isoprene. Concentrations of isoprene above 25 ppm caused mortality and developmental delay in all insect stages from larva to adult, as well as the inhibition of larvae molting. In conclusion, functional and comparative genomics of coffee accessions allowed the identification of at least one possible mechanism of insect induced response, providing new tools to screen and utilize the genetic resources available, as well as revealing unknown mechanisms of production of volatile substances in the coffee plant with negative effects on CBB that may be applied for pest control purposes and possible interaction with other pests.

INTRODUCTION

Colombia is one of the most important producers of *Coffea arabica*, with 914,000 ha planted in 2010 (FNC, 2010). The coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolitidae), is the most common pest affecting coffee cultivation in Colombia and it is found in all of the coffee-growing regions of the world. The female

insects encounter coffee fruit and bore into the pericarp until they reach the endosperm, where they lay their eggs. The larvae emerge and feed on the endosperm, resulting in decreased coffee yields due to the abscission of berries, the loss of fruit weight, and a decrease in the quality and therefore the price of coffee. The coffee berry starts a vulnerability period 120 to 150 days after flowering and as it reaches ripeness; it becomes more attractive to the insect, due to the release of volatiles.

A variety with reduced susceptibility or moderate resistance to the CBB could be part of IPM practices. Varieties with high incomplete resistance or complete resistance to CBB would mean a great competitive advantage. These varieties would alleviate the balance of expenses of the coffee growers and the negative effects that the chemical control practices have in the environment and the families who inhabit the farms.

Thus far, no natural resistance to CBB has been identified in either cultivated or uncultivated coffee plants. However, studies have shown differences in the susceptibility of various species of coffee to this insect. Romero & Cortina reported that female insects feeding on the fruit of *C. liberica* produced 30% fewer eggs in the F2 generation than did females feeding on the fruit of *C. arabica*, resulting in a lower reproductive rate and a decrease in the intrinsic growth rate of these insects. These differences can be related to the presence of antimetabolic substances, the absence of appropriate nutrients or differences in the structure of the cell wall that directly affect the insect's digestion.

Based on these findings, differential expression studies were done and cDNA expression libraries obtained from the fruits of both *C. arabica* var. *Caturra* and *C. liberica* harboring the CBB were used to identify some of the genes responsible for the differences between these two species, in insect behavior and coffee plant susceptibility. Differences in the transcript abundance of several genes were observed.

To confirm and to identify new transcriptional changes in the coffee berries that were induced by the attack of the insects and to compare the responses between *C. arabica* and *C. liberica* to *H. hampei*, the expression of more genes was studied using oligoarray analysis and qRT-PCR with the goal of identifying the genes or specific metabolic pathways of the plant's response to insects to be used in coffee breeding programs against the CBB or as new insect control strategy.

MATERIALS AND METHODS

Plant material

The experiment plants were obtained from the Colombian Coffee Germoplasm Collection the at Cenicafe experimental station in Chinchiná, Caldas, Colombia. For each genotype, *C. arabica* var. Caturra and *C. liberica*, three trees were selected with fruits in an active transcription state, at 120-150 days after flowering for *C. arabica* and 150-180 days after flowering for *C. liberica*. All fruits were counted in four selected branches from each tree. The selected branches were covered with entomological sleeves, and in three of the branches female borers were added at a 3:1 insect to fruit ratio. The other branch was left without insects as a control treatment. The infested fruits were collected at 48 hour after infestation. The CBB were obtained from a colony previously established at Cenicafé. The eggs used for the dietary bioassays were obtained from the same colony.

The collected coffee fruits were cleaned with RNase AWAY (Molecular BioProducts, USA) and stored in liquid nitrogen. The fruit samples were macerated in liquid nitrogen. One gram

of macerated tissue obtained from three fruits for each replicate was used for RNA extraction using the RNeasy® Plant Mini Kit (Qiagen, Valencia, California, USA). The integrity and purity of the RNA were assessed using agarose gel electrophoresis and a TBS-380 fluorometer (Turner Biosystems, Sunnyvale, California, USA).

Sequence information

A 4X44K oligoarray was designed, with 45,220 possible oligos sequences, 1.417 corresponding to control samples and 43.803 sequences corresponding to coffee sequences from which: 22.464 belonged to the *C. arabica* species, 8.896 to *C. canephora*, 22 to Timor Hybrid, 1.276 to *C. kapakata*, and 11.145 to *C. liberica*. All the sequences were obtained from the GenBank and sequences from libraries obtained at Cenicafe. All the sequences used had more that 200 bp.

The oligo design and slide hybridizations were done at the BIO5 Institute at the University of Arizona. The oligo slides with synthetized sequences were manufactured by Agilent Technologies USA. The slides were hybridized with RNA obtained from the infested coffee fruits from *C. arabica* or *C. liberica* with CBB after 48h infestation time. As control the RNA obtained from non infested fruits was used.

For each genotype, six RNA extractions were done coming from three infested vs. non infested fruits. For each extraction corresponding to a biological replica, 5 µg of RNA was obtained. The total study included 24 independent biological samples, 12 from each one of the genotypes *C. arabica* and *C. liberica*, which had or had not been infected by the coffee borer (n=6). The samples were hybridized within genotype.

Oligoarray Analysis

Analysis was implemented in R2.11.1 software. The $\log_2$ transformed data were normalized within slides using a global loess with the normexp background correction (due to high correlations between background and spot; in limma. Data were scaled between slides. For differential expression analysis, a mixed model ANOVA using shrinkage estimators with infection status, genotype, infection status by genotype interaction, and dye as fixed effects and slide (four arrays with separate samples hybridized to them were printed on each physical slide) and array as random effects was fit (R/maanova). Permutation-based p-values (from 1000 samples) for fixed effects yielded results in this data set, so the tabular values were preferred. P-values were adjusted for the large number of probes tested (43,803 probes; false discovery rate (fdr)). We examined pairwise contrasts comparing infected and healthy plants within each genotype as a planned contrast with subsequent fdr. Effect sizes were estimated by mean and standard error values after normalization.

Quantitative Real-Time PCR assays

In order to validate the expression of some of the genes identified in the oligoarrays, new coffee artificial infestations with CBBs were carried out in the same plant material used for the oligoarray hibridizations. Three adult trees from each genotype were selected. In each tree, two branches were selected and infestations were done. In the other branch no insects were added (control treatment). In the first tree, the berries from the infested and non infested branches were collected after 24h. In the other trees the berries were collected after 48 and 72h pos-infestation. After collection, berries were stored in liquid nitrogen. The frozen berries were ground in a mortar. The powder obtained from 2 berries (1 g) was used. Total RNA extraction was done as indicated above. First-strand cDNA synthesis was performed with

oligo (dT) and a Sensiscript kit (Qiagen, Valencia, CA) using 50 ng of total RNA as a template in a 25 µl reaction mixture. The cDNA samples were then diluted (1:25), and 2 µl of the dilution was used in a SYBR Green PCR , QuantiTect SYBR Green PCR Kit (Qiagen). Gene-specific primers were designed (Primer3plus; Untergasser et al. 2007) using a stringent set of criteria: predicted melting temperature of 61-62°C, primer length of 19-23 nucleotides, guanine-cytosine contents of 40-60%, and PCR amplicon lengths of 70-150 bp.

Thirty genes were chosen for quantitative Real Time PCR amplification among them are Hevein-like protein, Isoprene synthase, Trypsin inhibitor Kunitz, S-Adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, Patatin-like protein, already reported. PCR was carried out in an optical 96-well plate with chromo 4 (MJ Research, Waltham, MA) sequence detection and the data analysis was done following the methodology reported before.

Bioassays with artificial diets

To determine the effects of isoprene on CBB, 10 ml of a meridic coffee-based diet containing ground parchment coffee. was added to a Petri dish (35x10 mm) and incubated until 47% humidity was reached. A piece of the diet (1 cm in diameter) was removed from the middle the dish, creating a hole in the diet. Fifty CBB eggs were placed on the diet in each dish. Isoprene (99%) (Aldrich Chemical Co., Milwaukee, USA) was diluted in 5% ethanol to obtain concentrations of 500, 200, 100, 25, 7, 5, and 0.5 ppm. To each Petri dish, 50 µl of the different concentrations of isoprene was added daily to the hole in the middle of the diet. As a control, 50 µl of 5% ethanol or distilled water was added to the diet. Each treatment was repeated at least three times. The dishes were incubated at 26 ± 1°C in dark conditions and kept closed with vinilpel paper.

The development of the insects from egg to adult was monitored, including counting the numbers of alive and dead insects every two days, for 22 days. The response variable was defined as the number of dead insects and the developmental state of each of the insects in each treatment. The average number of dead insects was estimated for each treatment in the three replicates. To determine significant differences among the treatments, an analysis of variance (ANOVA) was performed for a completely randomized design (CRD) with a significance level set at 0.05. A Duncan test was used to compare the treatments.

RESULTS AND DISCUSSION

The oligoarray results showed an interaction between genotype and infection in 2.424 (5.5%) probes tested . Of these, 571 (23.6%) probes responded to infection in both *C. arabica* and *C. liberica*, but the magnitude of the response varied by genotype. Another 957 (39.5%) probes responded significantly in *C. arabica* but not *C. liberica*, while 895 (36.9%) responded significantly in *C. liberica* but not *C. arabica*. Among the probes that did not have an interaction between genotype and infection, 8205 (19.8%) had a significant overall infection response, with 4.089 (49.8%) of affected probes being down-regulated by infection and 4.116 (50.2%) of affected probes up-regulated by infection in both genotypes. We also observed a genotype effect in 27,335 (66.0%) of the probes with no genotype by infection effect, where 16,777 (61.4%) of probes that varied by genotype were expressed in higher amounts in *C. liberica* and 10,558 (38.6%) of affected probes expressed more highly in *C. arabica*.

Among the genes that showed the highest level of induction were found: -Patatin like protein (lipid acyl hidrolase) sequence. Patatin had been identified before for being lethal to spotted cucumber larvae, stunting the growth of survivors by preventing maturation, resulting in no

reproduction. Also in the findings were present a sequence of -Caffeic acid O-methyltransferase II related to the chlorogenic acid pathway, - trypsin inhibitor Kunitz gene know as miraculin-like gene *CoMir* - a wound induced protein WIN1-hevein, and at least 2 new sequences: -mannose/glucose-specific lectin and germin-like protein 4.

For the Real Time PCR validation, nineteen genes out of thirty evaluated showed over expression in *C. liberica* compared to *C. arabica* and were annotated with insect defense functions. The results from two of the genes are showed in Figure 1 and 2. In Figure 1, *C. liberica* showed a high over-expression of the isoprene synthase gene at 24h after infestation with almost 20 times over-production compared to the control. This induction decreases at 48h, and at 72h the levels are similar to the controls. In *C. arabica* no over-expression of the gene is observe at any time.

The expression pattern of sequence annotated as a Patatin – like protein is showed in Figure 2, a fivefold over-expression in *C. liberica* at 24h with compared to the controls. After 48h the overproduction increased 30 times more and the gene was then repressed at 72h. It is the gen that over-expresses the most of all evaluated. On the contrary *C. arabica* showed very low levels of expression at 24 and 48h compared to the controls, and at 72h the expression is even lower.

In addition, the following genes were also over expressed: -WIN1-hevein, -Trypsin inhibitor Kunitz *CoMir*, -S-adenosyl-L-methionine: Salicylic acid carboxyl , -No apical meristem (NAM) protein: induce jasmonic acid, -Membrane protein family Band7, -auxin/indoleacetic acid (Aux/IAA) sequence, -Aspartate aminotransferase, -Glicosido hidrolasa, -SAM metiltransferase 1, -Germin like storage protein, – A CPRD12 coupi gen, - A SC24 seed lectin like protein, -Putative epidermal glycoprotein EP1, that can acts as a barrier, -Glyoxalase/bleomycin resistance protein/dioxygenase, -Mannose/glucose-specific lectin, -Berberine like protein involve in the synthesis of alkaloids with repellant functions and, - 1-aminocyclopropane-1-carboxylate ACC oxidase related to the ethylene production. The genes over-expressed in the *C. liberica* genotype in response to the CBB attack can be potential new tools to widen the genetic basis of the cultivable varieties in the effort to improve their resistance to pests and diseases.

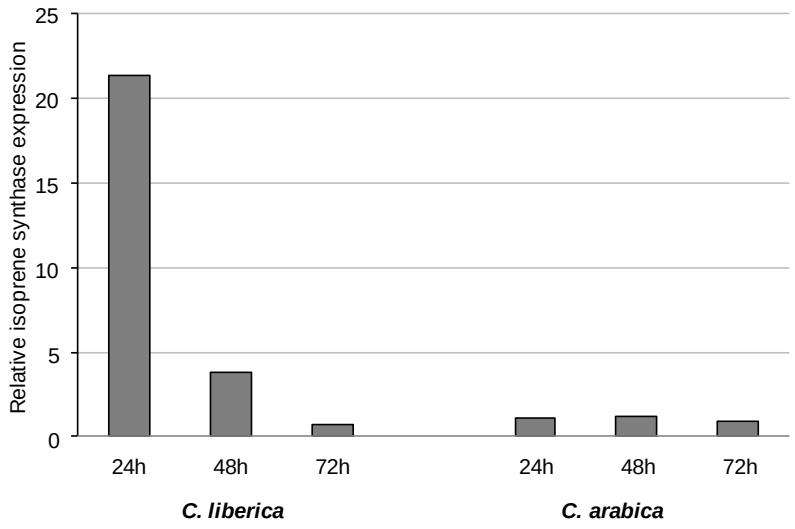

Figure 1. Relative expression of isoprene synthase gene by quantitative real-time PCR. Quantitative real-time PCR was performed with cDNA from green coffee berries at 24h, 48h and 72h after infestation of CBB in *C. liberica* and *C. arabica*.

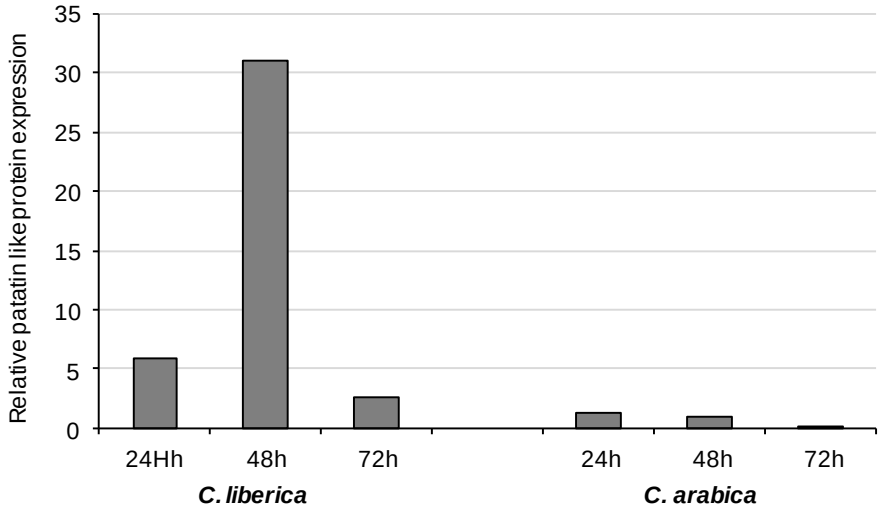

Figure 2. Relative expression of patatin like protein by quantitative real – time PCR. Quantitative real-time PCR was performed with cDNA from green coffee berries at 24h, 48h and 72h after infestation of coffee berry borer in *C. liberica* and *C. arabica*.

In order to validate the activity of the candidate genes on the CBB, and considering that one of those genes, the isoprene synthase genes is an enzyme that catalyzes the conversion of dimethylallyl diphosphate (DMADP) to isoprene and pyrophosphate and that the isoprene volatile is involved in plant-insect herbivory interactions and it acts as a deterrent to insects, the effect of isoprene was evaluated directly on the CBB.

The dietary bioassays (Figure 3) showed that the percentage of larval mortality under the presence of different isoprene concentrations was significantly different among the treatments ($F = 15.46$, $P=0.0001$). The Duncan analysis divided the experiment into three treatment groups. Group A, with the lowest mortality rates, corresponded to the two control treatments (water and 5% ethanol). In this group, the mortality rates at the end of the experiment were lower than 20%, indicating that 5% ethanol had no effect on the insects; and therefore it can be used to dilute isoprene. Group B consisted of all the samples treated with different dilutions of isoprene, and the mortality rates ranged between 40 and 80%. Finally, Group C consisted of the samples treated with 99% isoprene, in which no insects survived; 100% mortality was reached after 11 days. In the last group, the larvae exposed to the isoprene treatment began to die after day 2, and all of the deaths occurred at the first or second larval instar stage.

The evaluation of the effects of isoprene on CBB larvae using artificial diets demonstrated that different concentrations of isoprene could cause insect mortality ranging between 40% and 80% under experimental conditions, whereas pure isoprene was highly lethal within days. Furthermore, this substance affected insect metamorphosis and inhibited insect molting; the larvae that were in contact with high concentrations of isoprene were unable to reach the adult stage and eventually died after 40 days. The presence of isoprene negatively affected insects development and survival either by reducing food intake, inhibiting normal development, or by preventing larval development when the volatile interacts with the adult stages of the insect. Isoprene has also been reported to have insect repellant properties and this

characteristic is of great interest in the management of insect pests, particularly CBB, because controlling infestation by this insect is difficult: the insect is considered to be an internal parasite because part of its life cycle occurs inside the coffee fruit. Only a small number of insecticides and biocontrolers such as the entomopathogenic fungus *B. bassiana*, combined with cultural practices and crop agronomical management, have been capable of controlling successfully this insect.

It has been demonstrated that CBB responds to olfactory stimuli in order to locate and colonize fruits; therefore, it is likely that the plant exploits this fact to defend against and repel CBB. Mathieu *et al*. reported that the physiological state of the coffee berry borer affects its response to volatile compounds emitted by the fruit and observed that colonizing female insects are the primary group attracted to the fruits. Thus, isoprene represents a new compound with toxic and repellent effects that could be used as part of a novel strategy to control CBB infestation.

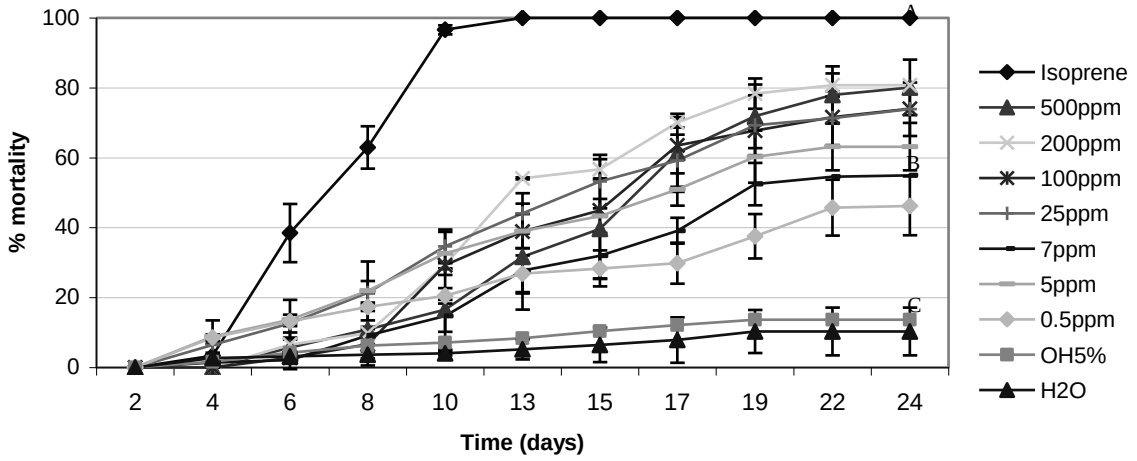

Figure 3. Effect of isoprene on coffee berry borer mortality, evaluated in artificial diets. Letters A, B and C indicate statistical differences between the treatments according to Duncan test at 5%.

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Integration of Genetic Linkage Maps of *Coffea arabica*

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SUMMARY

A total of 110 molecular markers were used for screening 247 plants of the H511-1 population. The resulting partial map consisted of eight linkage groups (LGs), comprising 99 markers and covering 1,340.23 centimorgan (cM), with an average distance of 14.73 cM between markers. For a second partial map, 217 plants of the H419-1 population were genotyped using 125 molecular markers, of which 101 were distributed in 13 LGs, covering 1,426.87 cM, with an average distance of 16.21 cM between markers. Integration of both data sets was enabled by the existence of 12 loci common to both sets (i.e. anchor markers). It was possible to combine five LGs from the map of H511-1 with six LGs from that of H419-1. The analysis resulted in a final linkage map comprising 14 LGs, four of which corresponding to the integrated LGs. The integrated map contained 188 markers, and covered 1,973.35 cM of the *C. arabica* genome. The average interlocus distance was 11.34 cM, and only 18.97% of these intervals exceeded 20cM. This map was used to identify QTLs involved in resistance to coffee leaf rust (*Hemileia vastatrix*). For this analysis, plants from both populations were inoculated with race II of *H. vastatrix*. Five QTLs were identified: two QTLs with major effect were detected in LG3, and explained 50.0% and 54.8% of the phenotypic variation; and two minor QTLs were detected in LG2 and one in LG4, explaining 7.0%, 9.4%, and 9.5% of the phenotypic variation, respectively. Map integration enhanced the genetic resolution, and proved to be a promising strategy for obtaining a *C. arabica* map that can be useful for current and future genomic research, such as QTL analysis and positional cloning, in addition to marker-assisted selection in coffee breeding programs.

INTRODUCTION

The demand for improved coffee cultivars has required refined tools of genetic analysis, able to provide detailed information about the genome. These tools have promoted enhanced knowledge about this species, in addition to generating basic information for gene manipulation. Molecular markers and genetic linkage mapping represent some of the most important available tools. Advances in the area of molecular mapping have increased the understanding of genomic structure, and genetics and genes that control traits of agronomic importance. This way, locating and mapping genes and QTLs, as well as cloning genes based on genetic maps, have become important complements for breeding programs. Nevertheless, the construction of genetic maps in *Coffea arabica* has been challenging, owing to the polyploid nature and the low polymorphism rate in the germoplasm with breeding interest for this species.

An alternative to minimize the difficulties found in the construction of saturated maps that widely cover the genome of *C. arabica* is the construction of partial maps, using different populations and classes of DNA markers, with subsequent integration of these maps. An integrated map increases the saturation, reduces the number of gaps, and increases the density of markers as well as total covered length of the map. In order to enhance genome coverage, the integrated map increases the availability of markers for application in breeding and genetic analysis of the traits. Moreover, the integration of a high number of markers in a single map increases the chances of finding informative markers in any part of the genome, as well as in different genetic backgrounds.

This way, the present work aimed at constructing an integrated genetic map for *C. arabica* making use of two F_2 populations, as well as identifying QTLs associated to resistance to coffee leaf rust.

MATERIALS AND METHODS

Two mapping populations were used to construct the integrated map. Map 1 (population 419-1) was obtained based on 217 F_2 individuals, resulting from the crossing of cv Catuaí Amarelo UFV 2143-235 with Híbrido de Timor UFV 445-46. Map 2 (population 511-1) was constructed based on 247 F_2 individuals from the crossing of cv Catuaí Amarelo UFV 2148-57 with Híbrido de Timor UFV 443-03. Both populations are being used in the Coffee Breeding Program of the Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG)/Universidade Federal de Viçosa (UFV), Brazil.

The maps were constructed based on genotyping data of the F_2 populations. For map 1, 125 molecular markers (99 RAPD, 25 SSR, and one RGA) were genotyped, whereas for map 2, 110 molecular markers (31 SSR, 74 AFLP, one RGA, and four RAPD) were used. The linkage groups (LGs) were formed and ordered using a minimum LOD score of 3.0 and maximum recombination of 40%. The estimated recombination frequencies were converted into genetic distances, in centimorgans (cM).

The maps were integrated using the GQMOL software, according to methodology proposed by Salgado et al. The GQMOL program is available for free download at http://www.ufv.br/dbg/gqmol/gqmol.htm.

RESULTS AND DISCUSSION

For construction of the integrated linkage map, two partial maps were initially obtained, based on data from two F_2 populations of *C. arabica*. Map 1 (population H419-1) was constructed from 101 molecular markers allocated in 13 LGs, covering 1,426.87 cM, with an average distance of 16.21 cM between markers. In map 2, 99 markers were distributed in eight LGs, covering 1,340.23 cM of the genome, with an average distance of 14.73 cM between markers.

Based on the partial maps, 12 common markers were identified between both maps. To efficiently integrated partial maps, it is necessary that a group of markers located in the different LGs be present in both maps, serving as anchors for the integration. Distinct maps can only be represented together when a minimum number of anchor markers is available. In the present work, the 12 anchor markers enabled the integration of six LGs from map 1 with

five from map 2: LGs 2 and 5 of the first map were integrated with LG1 of the second map; LG3 and LG6 of map 1 joined LG2 from map 2; LG1 from map 1 was incorporated with LG3 of map 2; and LG11 of map 1 was integrated with LG4 and LG6 of the second map (Figure 1). The further LGs were not integrated due to the absence of markers that would allow the anchoring process.

After identification of the anchor markers, the partial maps were aligned. In this process, one marker was established as reference point and, based on information from the LGs, all further markers were positioned in relation to the reference. This way, a map was obtained which considered neither the distances nor the most appropriate positioning. All markers were present in the aligned map, including repetitive anchor markers. Next, the aligned map was ordered, in that the mean distance was established between each pair of markers. The ordered map thus comprised all markers present in both partial maps, with anchor markers now appearing only once. The following step consisted of obtaining the effective integrated map, with the correct order and measurement of distances were calculated by multilocus analysis. It is important to highlight that, in this analysis, mapping functions have a great influence on the final result. The adoption of one among the different mapping functions thus depends on the presuppositions concerning the distribution of crossing-overs, the degree of interference, and the length of the considered chromosomic segment.

Using this strategy, a genetic map was obtained consisting of 14 LGs, of which four corresponded to integration of the partial maps. Saturation in the final map was of 188 markers covering 1,973.35 cM of the *C. arabica* genome. The mean interlocus distance was 11.34 cM, and only 18.97% of the intervals between markers exceeded 20 cM.

This map was employed to identify QTLs involved in resistance to coffee leaf rust (*H. vastatrix*). For this analysis, plants from both populations were inoculated with the race II of *H. vastatrix*. Five QTLs were identified: two with major effect were detected in LG3, explaining 50.0% and 54.8% of the phenotypical variation, as well as three with minor effect, being two in LG2 and one in LG4, explaining 7.0%, 9.4%, and 9.5% of the phenotypical variation, respectively (Figure 1).

Increasing the genetic resolution constituted a promising strategy for obtaining the map of *C. arabica*, which can be applied in future genomic researches, such as identification of QTLs, positional cloning, and assisted selection by molecular markers in breeding programs.

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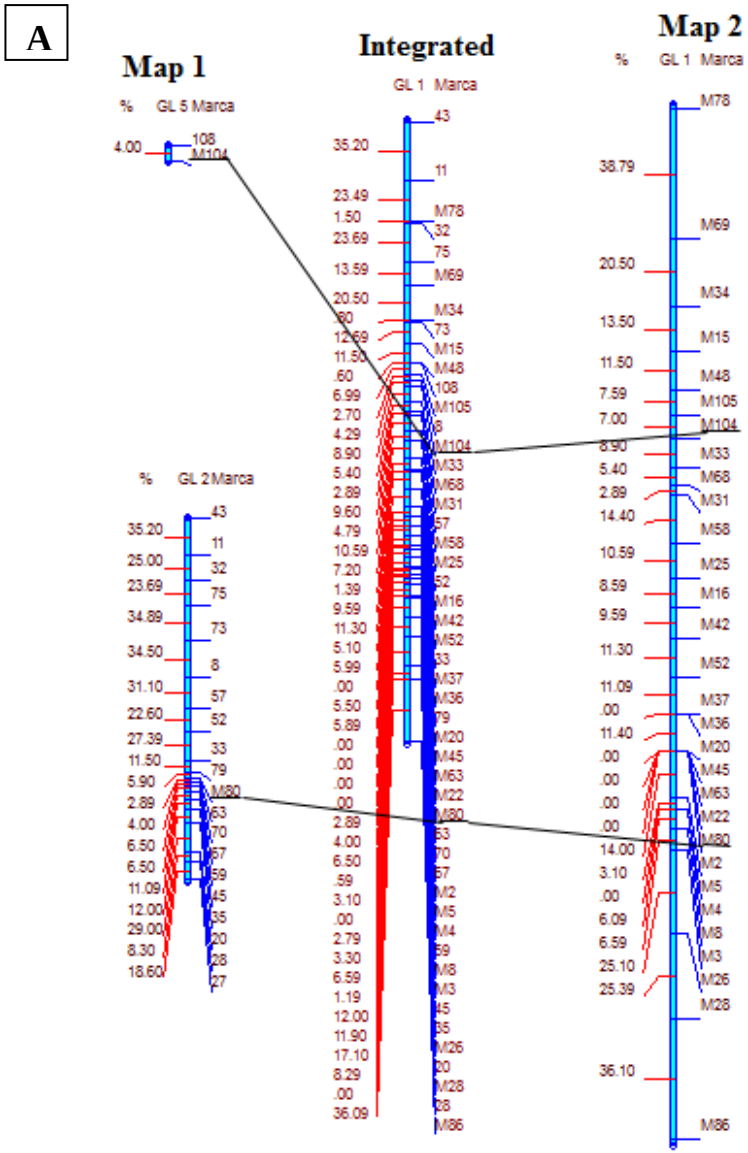

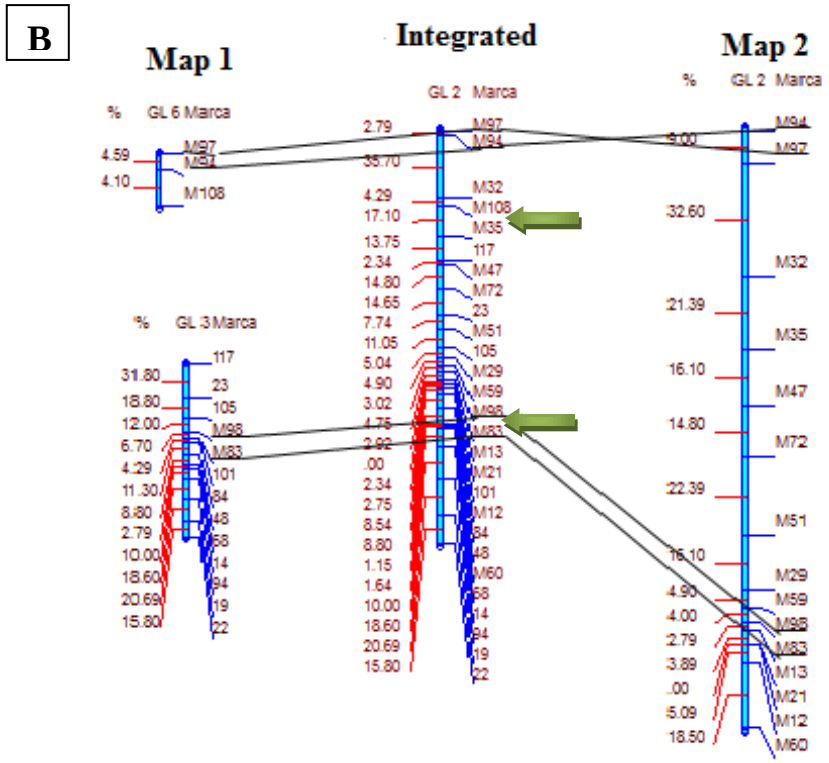

Figure 1. A – Integrated linkage group (LG) 1, resulting from the integration of groups 2 and 5 of the map1 and group 1 from the map2. B – Integrated LG2, originated from the groups 3 and 6 of map1 and group 2 of map2. C – Integrated LG3, came from the group 1 of map1 and 3 of map2. D – Integrated LG4, originated from the group 11 of map1 and groups 4 and 6 of map 2. Black lines indicate the position of anchor markers in the partial and integrated maps. Green arrows indicate the QTL position.

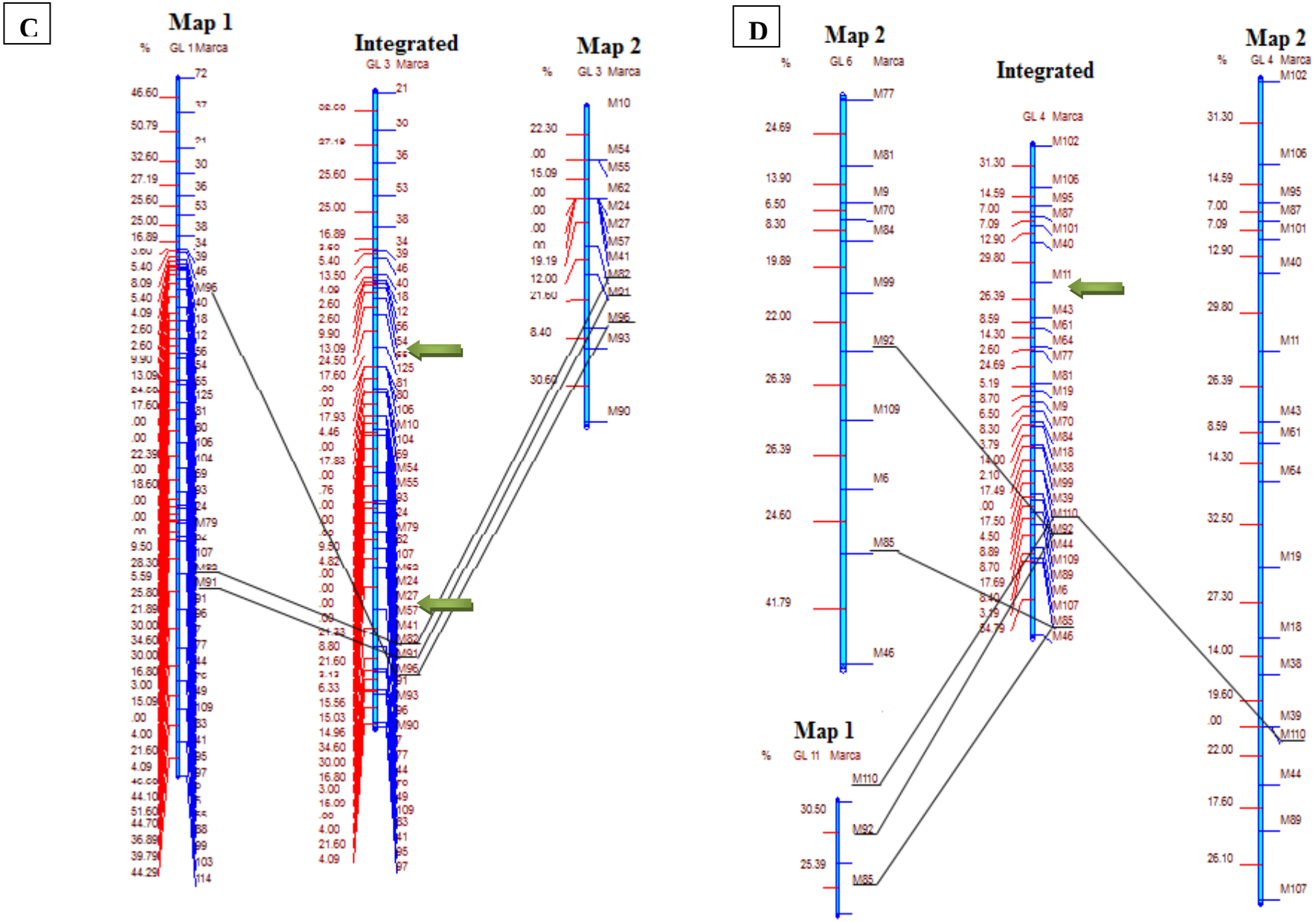

Figure 1. Continue.

High Density Genetic Map of Robusta Coffee

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SUMMARY

Management of genetic and genomic data for coffee is becoming a crucial step in order to improve the efficiency of breeding for this important agricultural and economic crop for tropical countries.

Genetic map is one of the core genetic resources for synteny study and dissection of complex agricultural or technological traits of importance. Usually, the genetic mapping workflow is starting with a discovery phase where genetic markers, such as RFLPs, SSRs or SNPs, are mined from genomic or EST libraries from different institutes (IRD, CIRAD, Trieste University, Cornell University, Cenicafe and Nestlé). Using this classical strategy a first genetic map was obtained on a F1 progeny, performed by ICCRI, using two elite Robusta, highly heterozygous, clones BP409 and Q121, respectively, determined as a Congolese type and a hybrid between Conilon and Guinean genotypes. The segregating progeny include 93 individuals which were used to create a genetic map including a first set of 1550 loci covering 1400cM.

A newly developed sequence-based marker technology, Restriction Associated DNA sequencing (RADseq), enables synchronous SNP marker discovery and genotyping using massively parallel sequencing. Using RAD sequence data from the segregating population previously selected a total of 1700 RADseq markers were added. The final high density Robusta map comprises 3250 loci with an average density close to one marker every 220 kb.

Our F1 high density genetic map was dedicated to facilitate comparative genomic studies based on synteny and provides the opportunity for anchoring and ordering the numerous scaffolds arising from the Robusta genome sequencing. This data shows that the DNA sequence anchored so far is covering approximately 75% of the genetic map.

Finally, this Coffee genetic map could serve as a bridge to allow identification of loci associated with quantitative traits (QTLs) , thus facilitating the gene discovery or speed up the breeding process. This high density Robusta genetic map is also being used as source of new markers for the development of the Arabica coffee genetic map.

INTRODUCTION

Genetic linkage maps can facilitate a large range of genetic analyses in plants. Indeed, such resources play a major role in the genetic dissection of complex traits (QTLs) and also in comparative syntenic analyses of genome structure, as well as in molecular breeding efforts. Until recently, these genetic maps were typically based on relatively low throughput marker technologies, which tended to limit marker density across the genome. In the last decade, a

number of molecular marker technologies have been developed, including RFLP, AFLP, RAPD, SSR or SNP. RAPD and AFLP have proven not to be reliable across different mapping populations. Moreover, these two types of markers mainly detect a single allele at the locus analysed, this "dominant" character is difficult to manage in an allogamous plant species such as Robusta. However, with the advent of next generation sequencing technologies, as well as the development of high throughput genotyping platforms, it is now possible to simultaneously interrogate thousands of single nucleotide polymorphisms (SNPs) from the genome of interest. These methodologies make possible the efficient development of increasingly dense genetic maps. With the improved sequencing technology, the last two years have been seen the development of NGS combining restriction-site associated DNA (RAD) for SNP testing.

One of the goals of the research reported here, was to generate a sufficient number of molecular markers for saturing the Robusta genome and to produce a high density genetic map that could be used for coffee breeding purposes and also for further analysis of the tetraploid Arabica coffee genome. Another interest of this high genetic mapping in Robusta is to use this genetic framework to anchor the genome sequence data generated recently from this coffee species.

MATERIALS AND METHODS

A consensus international reference map of *C. canephora* has been developed in Nestlé R&D Centre in collaboration with the Indonesian ICCRI institute. This map derived from a F1 cross between two highly heterozygous genotypes, a Congolese group genotype (BP409) and a Congolese x Guinean hybrid parent (Q121). The segregating population is composed of 93 F1 individuals. Several types of markers were used (RFLPs, SSRs, SNPs and RADs) into this coffee mapping population.

RFLP mapping was performed using eight restriction enzymes (*Dra*I, *Eco*RV, *Hin*dIII, *Hae*III, *Rsa*I, *Sca*I, *Hin*cII and *Pvu*II). Several sources of DNA were used from EST and genomic libraries.

SSR primer pairs were designed to obtain a range of PCR amplicon length from 100 to 300bp in genomic enriched libraries and EST databases. The alleles detection was obtained using an ABIPrism 3500 analyzer.

SNPs identified in sequenced amplicons from the two parents were mapped using the MGB TaqMan or HRM technologies. Progenies genotyping was performed using allelic discrimination assays. Analysis of results has been done with LightCycler 480.

The RAD libraries are made from digestion of DNA using two restriction enzymes, *Nsi*I (6 base cutter) and *Mse*I (4 base cutter). The fragments (150 - 500 bp) were selected to ligate to two adaptors, and one of them with tag for each progeny. Equal amount of amplicons from each individual was pooled to make Illumina RNAseq library with individual tags for each library. Co-segregating markers within 50 kb region (< 1 cM) based on the aligned template scaffold are sorted as bin. One marker from each bin was selected for mapping.

The linkage analysis and map construction were performed using JoinMap software version 4.1 using LOD threshold of 5 and Kosambi's function to calculate genetic distance between two loci. The Robusta consensus genetic map was built using the F2 segregating loci as anchor markers in order to merge the two homologous parental linkage groups.

RESULTS AND DISCUSSION

A first set of molecular markers including RFLP, SSR and SNP were used to provide the framework of the Robusta high density genetic map. Some of these markers which are segregating in both parental linkage maps were used as anchors to merge the two homologous genetic groups. In a second step, we integrated the RAD markers according to their polymorphic parental origin (BP409 or Q121). Because a large number of the SNPs detected in the RAD sequences are specific to each parent the strategy conduct to map these markers following a backcross segregation.

A total of 3230 loci were mapped on the Robusta consensus map from the F1 segregating progeny (BP409 x Q121). These loci were distributed on 11 linkage groups coded from A to K (Table 1). The genetic map is covering 1471 cM with an average interval between two adjacent mapped markers of 0.46. The linkage group length varying from 221 cM (group B) to 93 cM (group I). Only one important genetic gap (12 cM) is observed on the linkage group J (Figure 1). The total physical size of the Robusta genome was estimated to be closed to 720 Mb, meaning that each cM is equal to 490 kb.

Table 1. International reference genetic Robusta map characteristics. The eleven linkage groups (A to K) were described according to their size (cM), number of markers, density and origin of the markers (RADs, RFLPs, SSRs and SNPs). The estimation of the sequencing scaffolds coverage is also given.

Linkage group	Size (cM)	Nb. Markers	Density	RADs	RFLPs,SSRs, SNPs	Scaffold coverage (cM)	Scaffold coverage (%)
A	136	320	0.43	157	163	86	63
B	221	520	0.43	304	216	152	69
C	127	235	0.54	136	97	90	71
D	111	274	0.41	149	125	81	73
E	117	266	0.44	140	126	84	72
F	169	349	0.49	168	181	123	73
G	124	305	0.41	156	149	98	79
H	137	271	0.51	168	103	76	55
I	93	180	0.52	98	82	68	73
J	124	286	0.44	156	130	88	71
K	112	224	0.50	115	109	77	69
Total	**1471**	**3230**	**0.46**	**1747**	**1481**	**1023**	**70**

In order to investigate in detail the relationships between the physical and genetic maps we determine the physical location of the mapped molecular markers with a significant match on the sequenced genome. Up to now, a total of 349 scaffolds comprising 364.3 Mb were anchored and represent approximately 64% of the genome assembly Approximately 80 % of the mapped loci were located without ambiguity on the eleven linkage groups of the Robusta genome allowing to anchor and orient 139 scaffolds covering 289.5 Mb which is about 51% of the genome assembly. These scaffolds were covering 1023 cM (70%) on the high density genetic Robusta map (Figure 1).

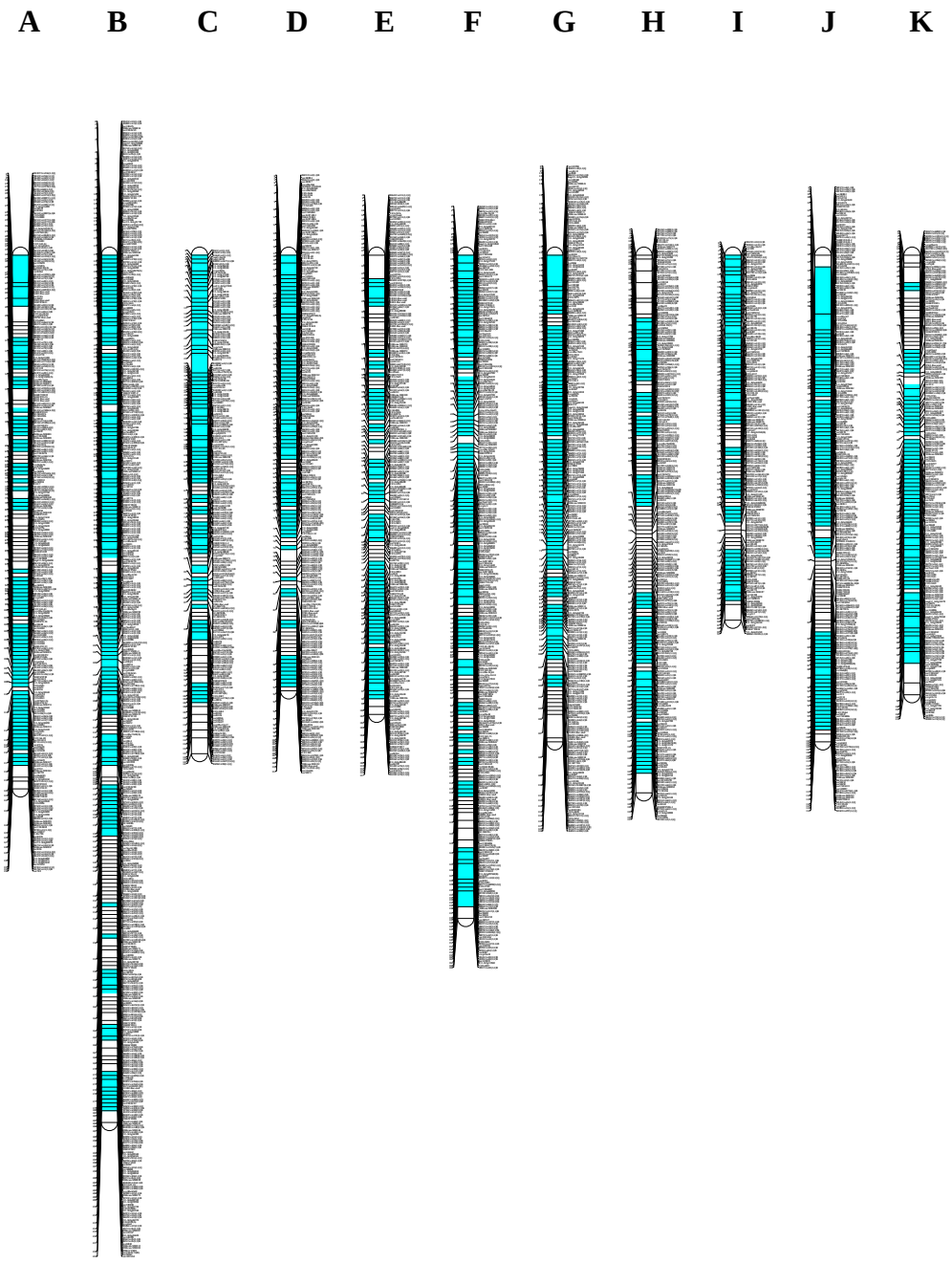


Figure 1. High density genetic map of the Robusta genome using RFLP, SSR, SNP and RAD molecular markers.

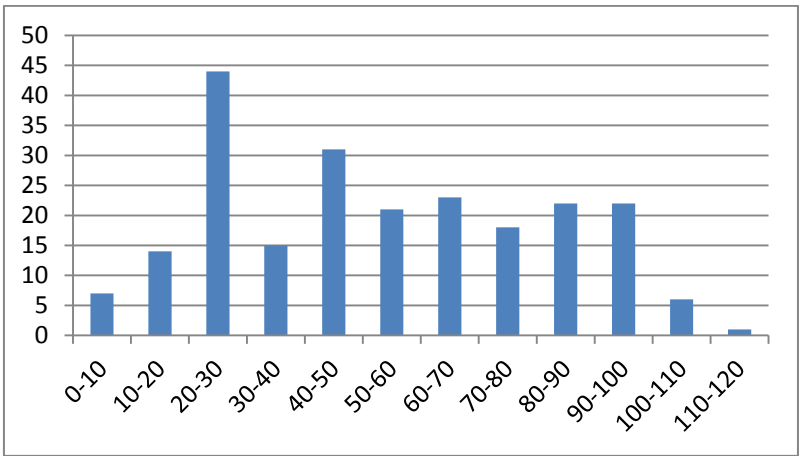
The distribution of loci along the eleven linkage groups was clearly uneven (Figure 2A). Some areas of higher density inferring presence of centromeric regions, were delimited most of the time as a single region on each of the eleven groups.

Using both physical and genetic maps, for the sequenced Robusta genome parts, it is possible to estimate the cM value on each point of the linkage group analysed (Figure 2B). For the genetic linkage group K these cM values are fluctuating from 170 kb to 1100 kb in the centromeric area where there is the higher marker density. These results indicate that the relationship between genetic distances in cM and physical distances in base pairs varied

greatly across each genetic linkage group. Moreover, the estimated average value of 490 kb per cM previously calculated seems to be realistic.

In Arabidopsis this ratio varied from 30 to 550 kb per cM for the chromosome 4 but in wheat the variation was even more extreme with 1 cM corresponding to the range of 118 to 22000 kb.

A



B

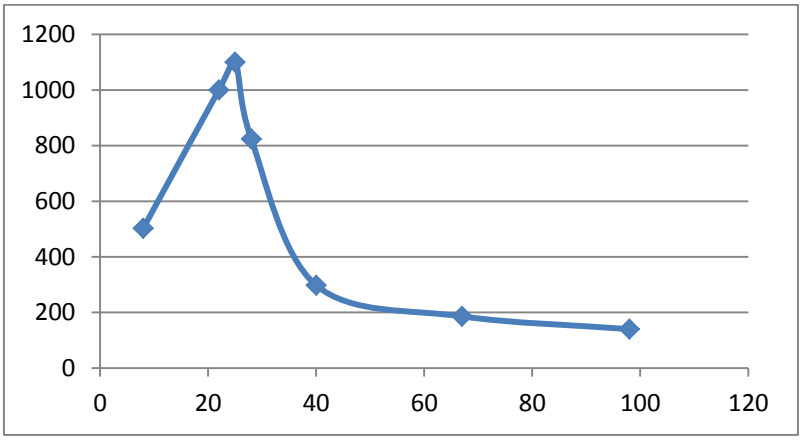


Figure 2 A & B. A, Distribution of loci number on Robusta linkage group K according to map intervals (cM). B, Estimation of the value of the physical distance (Kb) for one centimorgan along the linkage group K.

In conclusion, the construction of this international Robusta high density consensus map is a highly valuable resource for different applications including the transposition to other Robusta mapping populations to optimize the choice of panels of well distributed markers for the construction and assembly of these new genetic maps. It also provides a genetic framework that can be used for various QTL studies, genome structure comparisons and can be used as a draft for Arabica mapping.

According to this analysis it is also evident that the RAD sequencing is a powerful strategy for genotyping and provides a large access to SNP detection.

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Coffee Cysteine Proteinases and Related Inhibitors with High Expression during Grain Maturation and Germination

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SUMMARY

We have characterized cDNA encoding two cysteine proteinases (CP) and 4 cysteine proteinase inhibitors (CPI) from coffee. Detailed expression analysis of the genes CcCP1 and CcCP4 in Robusta using quantitative RT-PCR showed that the respective transcripts accumulate primarily during grain maturation and germination/post germination. The corresponding proteins were expressed in *E. coli* and purified, but only one, CcCP4, which has a KDDL/KDEL C-terminal sequence, was found to be active after a short acid treatment. The temporal expression of the CcCP1 gene suggests it is involved in modifying proteins during late grain maturation and germination. The expression pattern of CcCP4, and its close identity with KDEL containing CP proteins, implies it may play a role in protein and/or cell remodelling during late grain germination, and may play a strong role in the PCD associated with the post-germination phase of coffee seedling development. Quantitative RT-PCR expression analysis of the cysteine proteinase inhibitor genes in different Robusta tissues showed that CcCPI-1 is primarily expressed in developing and germinating grain and CcCPI-4 is very highly expressed during the late post germination period, as well as in mature, but not immature leaves. Transcripts corresponding to CcCPI-2 and CcCPI-3 were detected at relatively similar, but generally low levels in many tissues. The expression patterns suggest that CcCPI-1 could be involved in modulating a grain CP activity; while CcCPI-4 may play a role modulating CP activity in young coffee seedlings, as well as protecting the seedlings from insects and pathogens. CcCPI-2 and CcCPI-3, having lower and more widespread expression, could be to be more general “house-keeping” CPI genes. Most recently, we have examined the expression of the CPI genes in roots and have begun to explore the functionality of the coffee CPI proteins *in-vitro* by over-expressing both CPI-2 and CPI-3 as a fusion protein in *E.coli* and showing that this fusion protein can inhibit recombinant CcCP-4 proteinase activity *in-vitro*.

INTRODUCTION

Cysteine proteinases (CP) represent a large group of proteins in plants, with over 140 genes found in the model plant Arabidopsis, and have been shown to participate in processes ranging from tissue development to mobilizing storage proteins during seed germination . As well as being interesting proteins to study from a biological perspective, it is likely that cysteine proteinases with high expression in coffee beans (grain) can be expected to contribute to the quantity and spectrum of peptides and amino acids found in developing and mature coffee beans. Because such molecules are potent maillard flavor precursors, it is possible that significant differences in protease activity during the development of coffee beans could contribute to differences in the flavour potential of these beans. We have recently isolated and characterized cDNA encoding two cysteine proteinases which are expressed in the coffee bean during development and studied the quantitative expression of these genes in various coffee tissues. We also produced both proteins *in-vitro* and were able to demonstrate

that one of these proteins (CcCP-4) had proteinase activity after an "acid activation" step that caused the release of the N-terminal CP inhibitor peptide (containing a cathepsin propeptide I29 inhibitor domain) from the recombinant CcCP-4 pro-peptide. This release is thought to be produced by an acid induced autocatalytic activity intrinsic to the precursor CcCP-4 polypeptide.

Plant cysteine proteinase inhibitors, including those of coffee, are of agronomic interest because they may possess biological control activity against fungi and other pathogens. For example, a strawberry cysteine proteinase inhibitor has been found to limit the growth of two strawberry fungal pathogens in-vitro. In addition, a tomato cysteine proteinase inhibitor has been found to be active against digestive cysteine proteinases of insect herbivores, and that mutations within the "inhibitory" sequence could increase this inhibitory activity further. More recently, expression of two potato proteinase inhibitors (serine proteinase inhibitors) in transgenic cotton plants improved the resistance of these plants to insect damage in open fields. A short, concise overview of the potential of using cysteine proteinase inhibitors for pest control in the future has also been presented. To begin studying the possible roles of such proteins in coffee, we characterized cDNA for 4 cysteine proteinase inhibitor (CPI) genes from Robusta, and we examined their quantitative expression in a number of coffee tissues. We found that CcCPI-1 is primarily expressed in developing and germinating grain and CcCPI-4 is very highly expressed during the late post germination period, as well as in mature, but not immature leaves. CcCPI-2 and CcCPI-3 transcripts were detected at relatively similar, but generally low levels in the tissues examined. We proposed that CcCPI-1 could be involved in modulating a grain CP activity; while CcCPI-4 may play roles modulating grain CP activity and possibly play some role in the protection of young coffee seedlings from insects and/or other pathogens. CcCPI-2 and CcCPI-3, having lower and more widespread expression, could be to be more general "house-keeping" CPI genes.

In order to explore the possibility that one or more CPI genes could be involved in protecting coffee from pathogens in the soil, we have examined the expression of these genes in more detail in different Robusta root samples. Furthermore, to investigate the possible functional activity(s) associated with the coffee CPI proteins in detail, we set out to express and purify these polypeptides from *E.coli* and then test some of their proposed activities. Here we present the production and purification of a recombinant fusion protein containing CPI-2 and CPI-3 and show that this fusion protein is capable of inhibiting the activity of recombinant coffee cysteine proteinase CcCP-4.

MATERIALS AND METHODS

Plant material

Coffea canephora (FRT07) tissues (roots system) were collected in 2012 from young plantlets grown in the greenhouse. The tissues were then put into liquid nitrogen, then kept at -80°C until use. Coffee cherries of C*offea canephora* (FRT05) used to obtain the germinating beans were harvested at maturity from field grown trees in Equator in 2008, and sent to Tours (France) at room temperature. On arrival, they were manually depulped, washed and the light grain removed by floating. The remaining beans were dried and the teguments were manually removed. Subsequently, the grain were sterilized by a 1 h treatment in calcium hypochlorite (50 g/l), followed by three washes using sterile water. The beans were then incubated *in vitro* on Heller medium without added sugar or hormone (Agar 7 g/l), at room temperature (25°C). Later, bean radicles with their developing roots were harvested from five germinated beans 1 month after the first sign of radical emergence, and from five other germinated beans 2

months after the first sign of radical emergence. Note that the first sign of radical emergence appeared approximately 12 days after bean imbibition.

RNA preparation

The various tissue samples were reduced to a powder under liquid nitrogen using a mortar and a pestle. The powders obtained were then stored at -80°C until the total RNA was extracted. Total RNA was extracted and purified from the stored powders as previously described using the RNeasy Plant minit Kit (Qiagen) and an on-column DNase treatment. The quality and the quantity of the final RNA samples obtained were examined by agarose gel electrophoresis, and by absorbance using spectrophotometer respectively.

cDNA synthesis and quantitative RT-PCR experiments

The method used to make the cDNA was identical to that in reference "Coffee cysteine proteinases and related inhibitors with high expression during grain maturation and germination". The cDNA samples generated were then diluted twenty fold in sterilized water and aliquots were stored at -20°C for later use. Quantitative PCR measurements were done using the same TaqMan probes, primers and the reactions as described earlier. Three repetitions were done for each sample. Transcript levels for the different test genes were normalized to the levels of the constitutively expressed ribosomal rpl39 gene.

DNA sequence analysis

Plasmid DNA was purified using Qiagen kits according to the instructions given by the manufacturer. Prepared plasmid DNA was then sequenced using the dideoxy termination method [Sanger et al. 1977] by GATC Biotech AG (KONSTANZ, Germany). Computer analyses were performed using the Laser Gene software package (DNASTAR, version 8.1.4).

Construction of the HIS-TEV-CPI2-Xa-CPI3 Expression Vector

The expression vector used to produce the HIS-TEV-CPI2-Xa-CPI3 recombinant fusion protein (pSL5) was generated using Invitrogen Gateway® Technology by first cloning the sequence encoding the protein into the entry vector pENTR/TEV/D-TOPO, then moving the insert by recombination into the expression vector pDEST17. Briefly, two independent PCR were first conducted to amplify CPI-2 and CPI-3. The complete coding sequence of CPI-2 (minus its ATG codon and its stop codon) was amplified from the plasmid pcccs30w24l11 with the primers "CACC-CPI2-PF" (5' CACCGCAAAAGTTGGTGGGATCA 3'), and "CPI2-PR" (5'AGCCAT*ACGTCCATCTAT*GGCACTAGTATCACCAGCA 3'). The complete coding sequence of CPI-3 was amplified from the plasmid pcccs30w24e12 using the primers "CPI3-PF" (5' AGTGCC*ATAGATGGACGT*ATGGCTTCTGCCTTTCCC 3') and "CPI3-PR" (5' TCAGGGAAGTTTCCTGAAG 3'). (Note: A 12bp sequence shown in italic/bold (5' *ATAGATGGACGT* 3') encoding the Xa cleavage sequnce was included in the primers CPI2-PR (reverse primer) and CPI3-PF (forward primer) in order to be able to cut the recombinant CPI-2-Xa-CPI-3 fusion protein into the individual CPI-2 and CPI3 proteins later). Both PCR reactions were performed in a 50µL final volume and contained: 1µL of plasmid (diluted 100X), 5µL of 10X PFU Buffer, 1µL of dNTP mix (10mM, Promega), 2.5µL of each primer (1µM) and 1.5µL PFU DNA polymerase (Promega M7741, 3U/µL). The PCR program used was: 3min 94°C, the 35 cycles of 1min 94°C, 30sec 55°C, 1min 72°C, and a final cycle of 7min 72°C. All PCR products had the expected sizes when run on agarose gels. A second PCR was the performed to fuse the CPI-2 and CPI-3 polypeptides on both sides of the Xa factor cleavage sequence as follows in a 50µL final PCR reaction mix: 1µL of

both 100 fold diluted PCR reactions, 5µL PFU buffer, 1µL dNTP mix (10mM), 2.5µL of primers CACC-CPI2-PF and CPI3-PR and 0.5µL of PFU DNA polymerase and water. The PCR program used was: 3min 94°C, then 35 cycles of 1min 94°C, 10sec 55°C, 2min 72°C, and a final cycle of 7min 72°C. Agarose gel analysis of the new PCR product showed that it had the expected size. This PCR fragment was then gel-purified, put into the "entry" plasmid pENTR/TEV/D-TOPO (Invitrogen #K2525-20) and transformed into One Shot® TOP10 Chemically Competent cells (Invitrogen #C4040-10) as recommended by the manufacturer. This generated the plasmid pSL2. The insert was then recombined into the pDEST17 expression vector to place the 6xHis tag of the pDEST17 vector N_terminal to the TEV-CPI2-Xa-CPI3 fusion sequence in pSL2. The product of the recombination reaction was then transformed into One Shot® TOP10 Chemically Competent cells. The resulting final expression plasmid which carried ampicillin resistance, was called pSL5. This plasmid was then isolated and its insert completely sequenced for verification. Finally, pSL5 was transformed into *E. coli* BL21-AI™ One Shot® Chemically Competent cells (Invitrogen, #C6070-03) for use in the protein production step.

Production, purification and refolding of HIS-TEV-CPI2-Xa-CPI3

Protein production was launched using the BL21-AI *E. coli* containing the pSL5 expression plasmid (ampicillin resistant) with arabinose induction as described in the Gateway protocol. Protein extraction, His-Tag column purification and dialysis/refolding were performed as previously described for the production of recombinant CcCP-4.

Cysteine protease assay and testing inhibition by recombinant HIS-TEV-CPI2-Xa-CPI3 polypeptide

The recombinant coffee CcCP-4 protein was produced as described previously. To test the effect of the recombinant HIS-TEV-CPI2-Xa-CPI3 polypeptide on the activity of recombinant CcCP-4, following experiment was carried out: either 10µL water (in the case of the control experiment) or 10µL His-tag purified and dialyzed recombinant HIS-SUMO-CP4 protease (1.3µg) were mixed with 20µL 50mM sodium formate buffer (pH3), then incubated 30 sec at 37°C for CP4 activation. This was immediately followed by the addition of either 10µL water (control) or 10µL HIS-TEV-CPI2-Xa-CPI3 protease inhibitor (3µg), followed by the addition of 6.7µL BSA reaction buffer (1% BSA, 1xPBS and 6 mM L-cysteine, pH7.5) to all the reactions. The enzyme reactions were subsequently incubated at 37°C and 3 µL aliquots were taken at different times and added to sample loading buffer, heated for 7 min at 95°C, then run on SDS-PAGE gels and stained with coomassie blue.

RESULTS AND DISCUSSION

The recent study of coffee CPI gene expression indicated that transcripts from all 4 genes could be detected in whole root samples taken from the Robusta variety BP409, with CPI-1 having the lowest expression. Expression analysis of a second Robusta sample showed relatively similar results, except that few CPI-4 transcripts were found in this second sample. The fact that the other 3 CPI genes showed low but clear expression in Robusta roots suggests that the corresponding proteins could help protect roots from attack by pathogens in the soil. To gain more information on the expression of the CPI genes in roots, we decided to analyse the expression of these genes in the roots of less developed plants growing in the greenhouse and in the radicles of germinating coffee beans (grain). The expression of the four CPI in the roots of a young FRT07 plant are seen in Figure 1 (Panel A) and show that transcripts representing all four of the CPI genes are detected in this whole root sample (similar results were seen in an independent sample from the same variety), although the expression levels

were several fold lower than seen earlier in more mature roots. In this sample, expression of CPI-2 was slightly higher than the other CPI genes. The analysis of CPI expression in the radicles of germinating beans is seen in Figure 1 (Panel B). This data again indicates that all 4 genes are expressed, and show a similar expression pattern to that seen for the young FRT 07 roots above, but with slightly higher levels of expression. The data presented in Figure 1 indicates that all 4 cysteine proteinases may play a regulatory role in the roots vis-a-vis the CP proteins expressed there, although it appears that they are not involved with controlling either CP-1 or CP-4 proteinases because no transcripts for either gene were detected in these samples (data not shown). The presence of transcripts for all 4 CPI genes also confirms the idea that the corresponding proteins could help protect the roots from pathogen attack. Given this later possibility, a future goal is to explore whether there could also be an induction of one or more the CPI's in roots when they are subjected to attack by, for example, fungi or nematodes. If such an induction is found, it will then be interesting to determine whether this induction could be variety specific, thus possibly contributing to variety specific differences in pathogen sensitivity.

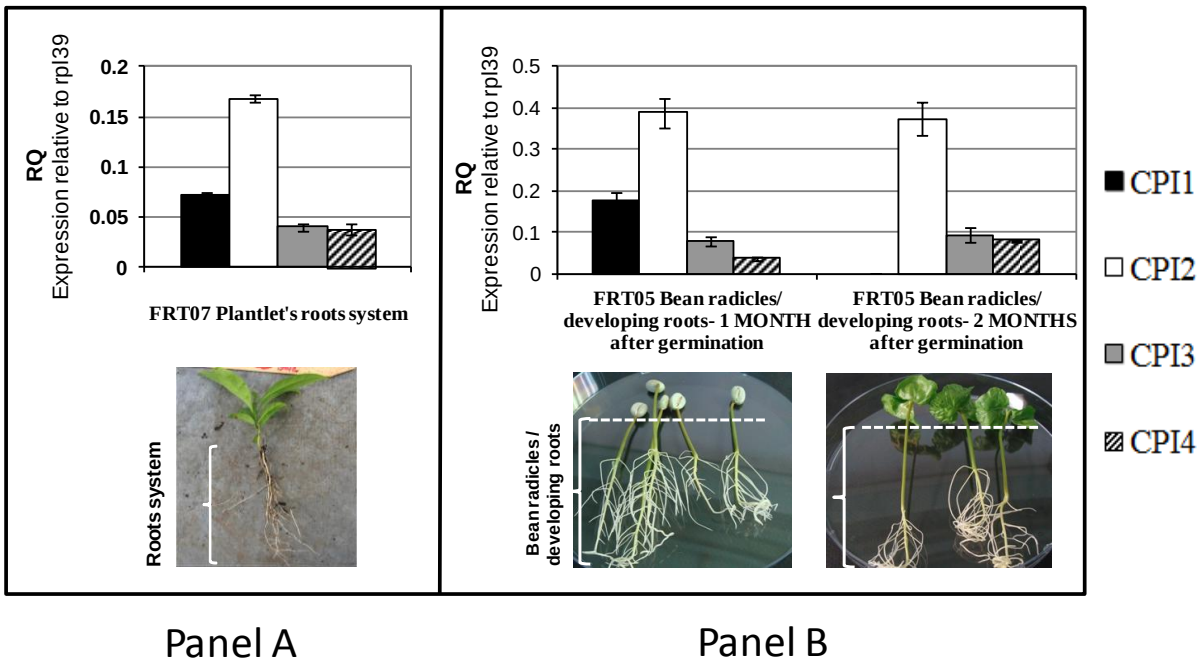

Figure 1. Quantitative gene expression analysis of the coffee cysteine proteinase inhibitors CPI-1, CPI-2, CPI-3 and CPI-4 in *Coffea canephora* roots and in radicles showing early root development.

RQ is the expression level of each gene relative to the constitutively expressed gene RPL39 in that sample. Panel A: The relative expression of each CPI gene was measured in the root system of a *Coffea canephora* FRT07 plantlet harvested in the greenhouses from Nestlé Tours R&D. Panel B: The relative expression of each CPI gene was measured in radicles showing early root development taken from germinating beans (grain). The samples were from *in-vitro* germinating beans of *Coffea canephora* FRT05 taken either one month, or two months, after the first observation of radicle emergence.

To learn more about the functions of the coffee CPI, we are working towards producing recombinant forms of these proteins in *E.coli* and then testing them for their effect on cysteine proteinases *in-vitro*, and eventually on different plant fungal pathogens. Our first work in this area is the construction of a plasmid (pSL5) to express a fusion polypeptide called HIS-TEV-CPI2-Xa-CPI3 that contains both CPI-2 and CPI-3 (separated by a short linker peptide

containing a sequence specifically cleaved by the selective Xa protease). Figure 2 (Panel A) shows that the HIS-TEV-CPI2-Xa-CPI3 fusion protein is relatively well expressed in "induced" cells of the E.coli strain BL21-A1. However, this data also indicates all the protein was insoluble. However, we were able to solubilize and purify the recombinant HIS-TEV-CPI2-Xa-CPI3 using its associated HIS tag, and then renature the protein as described in the materials and methods section, leading to the purified fraction seen in Figure 2 (Panel B).

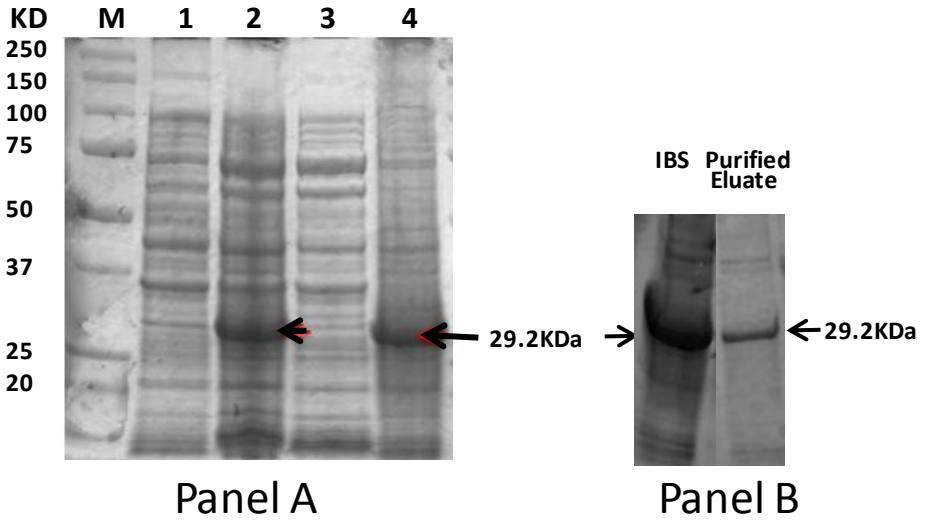

Figure 2. Coomassie SDS-Page gel analyses of the recombinant HIS-TEV-CPI2-Xa-CPI3 fusion protein from BL21-AI cells.

Protein samples were run on SDS-PAGE gels and stained with coomassie blue. The black arrows indicate the expected length of the recombinant protein (29.2KDa). The marker used was Precision Plus Protein^{TM}.

Panel A

- Lane M. Molecular mass marker.
- Lane 1. Non Induced BL21-AI + pSL5 whole cell lysate.
- Lane 2. Induced BL21-AI + pSL5 whole cell lysate.
- Lane 3. Induced BL21-AI + pSL5 soluble fraction.
- Lane 4. Induced BL21-AI + pSL5 insoluble fraction. Soluble and insoluble fractions were obtained by centrifuging the induced whole cell extract.

Panel B

IBS; washed inclusion bodies obtained from the Induced BL21-AI + pSL5 insoluble fraction; Purified Eluate**;** eluate of the purified recombinant HIS-TEV-CPI2-Xa-CPI3 fusion protein of the His tag column.

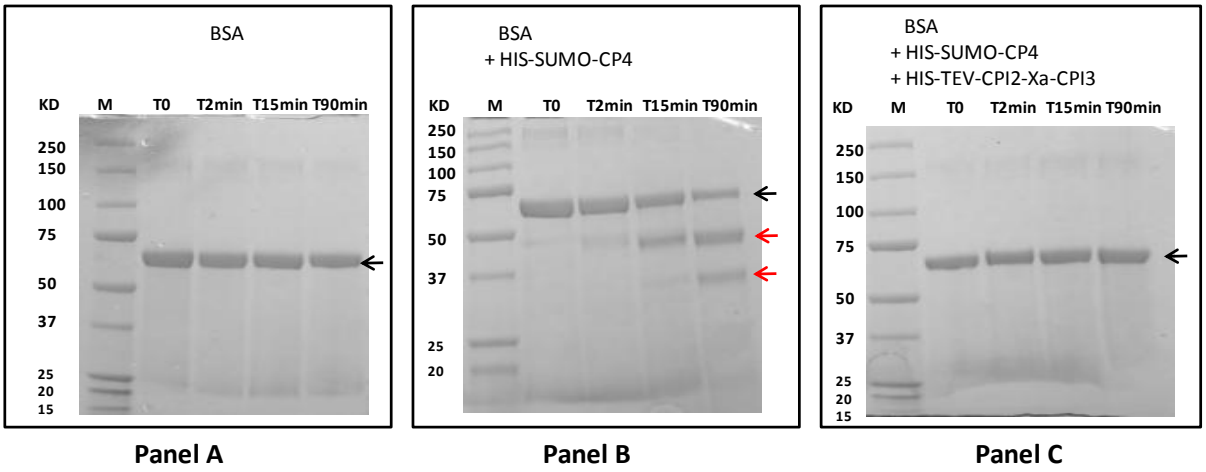

Figure 3. HIS-SUMO-CP4 protease activity assay and the inhibition of CP4 activity by the HIS-TEV-CPI2-Xa-CPI3 recombinant protein.

The assays were performed as described in the Material and Methods. For each assay, 3µL reaction samples were taken at the start (T=0), and at T=2 min, T=15 min, T=90 min, and immediately added to 5µL 5X SDS gel loading buffer. The samples were then run on 8-16% SDS-PAGE gels followed by coomassie staining. The markers (lanes M) are as in Figure 2. T=reaction times with BSA (bovine serum albumin; Sigma-Aldrich #A7906-100G). The black arrow indicate intact BSA (mol wt ~66 kDa), whereas red arrows indicate detection of bands deriving from BSA degradation.

The purified HIS-TEV-CPI2-Xa-CPI3 polypeptide was then tested for its ability to inhibit the recombinant coffee CcCP-4 activity (produced in E. coli as previously described). Figure 3 (Panel B) shows that activated recombinant CcCP-4 efficiently cleaves BSA and Figure 3 (Panel C) clearly demonstrates that addition of the purified HIS-TEV-CPI2-Xa-CPI3 polypeptide completely blocks the activity of the CcCP-4 proteinase. This result confirms that the fusion polypeptide HIS-TEV-CPI2-Xa-CPI3 can act as a cysteine proteinase inhibitor. Future experiments will be directed at cleaving the fusion polypeptide with Xa protease, purifying the individual inhibitors and retesting them against CcCP-4 activity and other cysteine proteinases. We also hope to express the other two inhibitors in *E. coli* and to compare their inhibitory activity with recombinant CPI-2 and CPI-3 polypeptides. Eventually we wish to test these recombinant inhibitors against different plant pathogenic fungi to determine if they have any antifungal activity, and if so, what is the specificity of the anti-fungal activity found.

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High-throughput Sequencing of cDNA Shows That cv. Rubi and IAPAR59 of *Coffea arabica* Have Different Molecular Response to Water Privation

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SUMMARY

It is well known that drought periods affect coffee plant development, leading to plant death and abortion of developing fruits in case of severe drought. In relation to coffee genetic diversity, several works reported the identification of plants of *C. canephora* conilon susceptible or tolerant to drought which were analyzed at the physiological level and also used to identify candidate genes underlying stress responses. Even narrow, a genetic diversity for drought tolerance also exist in the species *C. arabica*. In addition to the identification of undiscovered transcripts, the recent development of low-cost, high throughput next-generation (NGS) sequencing technologies now opens the way to perform expression profiling and to identify gene presenting differential expression patterns by comparing the frequency of reads obtained after sequencing. In order to initiate such kind of approach in coffee, RNA of meristematic tissues from Iapar59 (I59, drought tolerant) and Rubi (R, drought sensible) cultivars of *C. arabica* grown under field-grown with (I) or without (NI) irrigation were extracted and used to generate cDNA that were further sequenced. The sequencing data revealed 282.213, 135.304, 345.751 and 230.064 reads obtained from I59-I, I59-NI, R-I and R-NI samples, respectively, totalizing more than 255Mb. These data was compared with all available transcriptome *Coffea* data, including the EST sequences from both *Coffea arabica* and *C. canephora*. The results of these comparisons will be presented as well as the preliminary data of an electronic northern performed in order to identify differentially expressed genes between the two cultivars in both conditions (NI or I). General numbers show that Rubi has a more intense molecular response to drought, with many genes over-expressed. Also functional analysis of biological processes shows an enrichment of stress response genes in Rubi non-irrigated while in I59 there is a decrease in expression in genes related to carbohydrate metabolism and cell wall organization. It is worth noting the number of genes up-regulated in Rubi non irrigated (NI) and involved with drought stress response, like *RD22*, *PDIR10* (dirigent-like protein), *MYB* and caffeine synthase for example, while these genes were not detected under this stress in I59. In other hand, many drought genes related to drought-stress tolerance were detected in the I59 cultivar involved in biosynthetic process such as sugar transporters, some proteins related with abiotic stress tolerance (i.e. osmotins), water channel protein, LEA proteins (implicated in detoxification and alleviation of cellular damage during dehydration) and heat shock proteins (HSPs). In the I59 cultivar, genes coding for enzymes involved in the ABA synthesis (isopentenyl diphosphate isomerase, geranylgeranyl reductase), which causes stomatal closure, precursor and enzymes, were also

highly expressed. Q-PCR experiments were also performed and allowed to confirm the data obtained *in silico* for several genes. These results will be presented and discussed.

INTRODUCTION

In coffee, drought is a limiting factor affecting flowering, bean development and consequently coffee production. As a consequence of global climate change, periods of drought may become more pronounced and coffee growing geographical regions could suffer important delocalization, leading to environmental, economical and social problems. In this context, adaptation of coffee plantations to water constraint becomes a major concern for coffee producing countries. Within the coffee genus it is well known that genetic variability exists regarding drought tolerance for example in the species *Coffea arabica*. If drought tolerance mechanisms have been widely studied at the molecular level in model plants, molecular basis of drought tolerance in *Coffee sp.* are very scared.

In a first attempt to investigate the effects of water stress on gene expression, we recently identified a set thirty genes showing different expression in leaves of drought-tolerant and susceptible clones of *C. canephora* grown under controlled (greenhouse) and different water conditions. In *Coffea sp.*, EST resources have been developed for various species, tissues, such as roots, leaves and fruits, but no genomic resources are available for shoot apices which are important organs in the perception of environmental stimuli. The aim of this study was to compare the transcript profiles in the plagiotropic apices from IAPAR59 (drought-tolerant) and Rubi (drought-susceptible) cultivars of *C. arabica* grown in field-grown conditions and submitted or not to drought were collected at the end of the dry season. These data, which are the first one of coffee meristems from plants subjected to water limitation.

MATERIALS AND METHODS

Plant material

The cultivars of *C. arabica*, Rubi MG1192 (R hereafter) and IAPAR59 (I59 hereafter) differ in their growth rates and water use efficiency (WUE, estimated by isotopic carbon composition) at maturity, I59 being superior to R. Plants (four years old) of both cultivars were established in the field of Embrapa Cerrado Center (Planaltina-DF) and grown with (I) or without (NI) irrigation.

Sampling procedures

For qPCR experiments, plagiotropic apices were collected during the dry season (august 2011) from I and NI plants of both R and I59 cultivars. Water stress was evaluated by measuring predawn leaf water potentials (Ψ_{pd}) with a Scholander-type pressure chamber. For both cultivars, Ψ_{pd} values were closed to -0.20 and <-3.50 Mpa for I and NI condition, respectively. After freezing in liquid nitrogen, apices were further conserved at-80°C.

RNA isolation and Real-time RT-PCR assays

Samples were ground in liquid nitrogen, total RNA was extracted and qPCR were carried as previously described. Gene expression was normalized (SDS 2.1 software) with the expression of ubiquitin (*UBI:* SGN-U347154) reference gene.

RESULTS AND DISCUSSION

From the NGS 454 data, 597.669 cleaned reads of 300 bp average length were generated for the four (I59I, I59NI, RI and RNI) cDNA libraries. A total of 55.578 clusters (54.628 contigs + 950 singlets) was obtained (http://bioinfo03.ibi.unicamp.br/cirad/). These libraries were further compared leading to the identification of 213 contigs that presented *in silico* differential expression between the cultivars or/and the irrigation condition. These contigs were then used (i) to screen the Sol Genomics Network (SGN, http://solgenomics.net/content/coffee.pl) in order to verify the accuracy of the NGS sequences and (ii) also to identify their putative function by searching sequence homologies (BLAST) in the GenBank database. The list of 213 contigs was further reduced to 83 unique contigs which were used to design 85 primer pairs for 75 of them. After testing the primer pairs for their specificity and efficiency with a mixture of cDNA, useful results of qPCR experiments were finally obtained for 38 contigs and classified as follows:

- down-regulation of gene expression under drought, independently of the cultivar (n=6).
- up-regulation of gene expression under drought, independently of the cultivar (n=7).
- down-regulation of gene expression with drought with higher expression of the contig in plagiotropic apices I59 than in those of Rubi (n=5).
- up-regulation of gene expression under drought with higher expression of the contig in plagiotropic apices I59 than in those of Rubi (n=19).
- up-regulation of gene expression under drought with higher expression of the contig in plagiotropic buds Rubi than in those of I59 (n=1).

For some of these contigs, qPCR expression profiles are presented and discussed below.

Up-regulation of gene expression under drought was observed for several contigs coding for putative “Heat Shock Proteins” (also called HSP) such as 17kDa (referred as small HPSs), 70kDa (DnaK family) and 83kDa (Hsp 90 family) (Figure 1). Heat shock proteins (also known as molecular chaperones) have been previously reported to be involved in protein folding as well as in the removal and disposal of non-functional proteins under drought stress in higher plants. The expression profiles presented here were similar to those previously reported to occur for HSP proteins-encoding genes in leaves of *C. canephora* submitted to drought. In that sense, they confirmed that NI plants were really under drought stress (positive control of the experiment) conditions. However, no significant differences of gene expression profiles were observed between R and I59 cultivars.

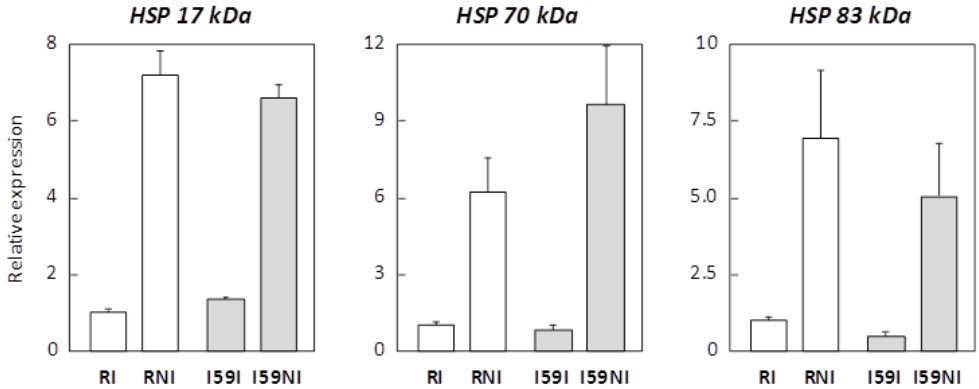

Figure 1. Expression profiles of HSP-encoding genes in plagiotropic apices of cultivars Rubi (R) and IAPAR59 (I59) cultivars of *C. arabica* subjected (NI) or not (I) to drought.

The expression of the ubiquitin-encoding gene was used as a reference to measure the relative quantification that corresponds to the mean of three technical repetitions ± SD. Results are expressed using RI as an internal calibrator (relative expression = 1).

The contig coding for a putative jasmonate *O*-methyl transferase (JMT) was down-regulated under drought but showed higher expression in plagiotropic apices of I59 than in those of Rubi under both I and NI conditions (Figure 2). This enzyme use SAM and jasmonate to synthesize methyl jasmonate (MeJA) which is important cellular regulators involved in diverse plant developmental processes but also in plant defense responses to insect wounding, attack by various pathogens, and water deficit. It was recently shown that transgenic rice over-expressing *Arabidopsis thaliana* jasmonic acid carboxyl methyltransferase gene increased levels of methyl jasmonate (MeJA) but also of abscisic acid (ABA) in young panicles grown in non-drought conditions suggesting that MeJA, rather than drought stress, induced ABA biosynthesis under drought conditions. It should be of a particular interest to evaluate MeJA/JA and ABA levels in R and I59 cultivars to know if they are or not correlated with the differences of JMT gene expression and in order to see if they could explain the physiological differences observed between these cultivars.

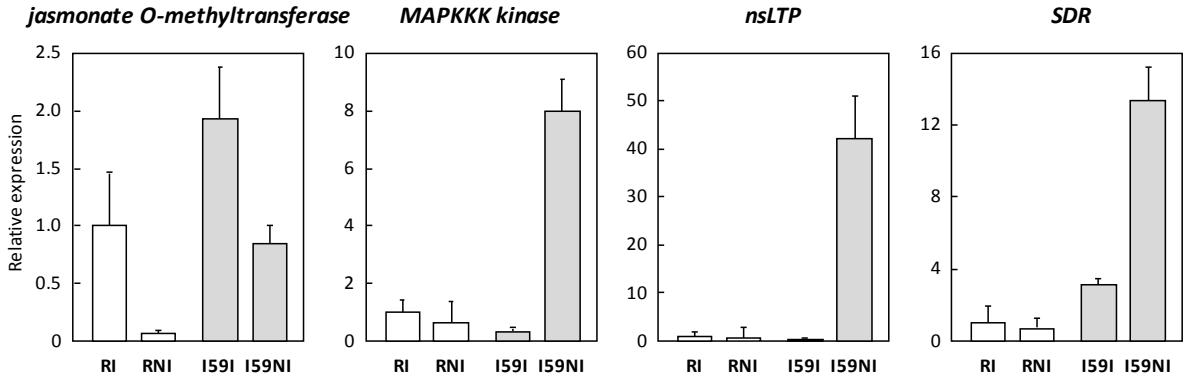

Figure 2. Expression profiles of jasmonate *O*-methyl transferase (JMT), MAPKKK kinase nsLTP and SDR genes in plagiotropic apices of cultivars Rubi (R) and IAPAR59 (I59) of *C. arabica* subjected (NI) or not (I) to drought. (same legend as in Figure 1).

Another interesting profile concerns the expression of the contig coding for a putative serine/threonine MAPKKK (Figure 2) which was greatly up-regulated under drought in plagiotropic apices of I59 but not in those of the Rubi cultivar. Mitogen activated protein kinases (MAPK) are important mediators in signal transmission, connecting the perception of external stimuli to cellular responses. Plant MAPKs were shown to be activated or transcriptionally induced in response to various environmental factors, such as mechanical stress, pathogen attack and also abiotic stresses, like drought. Could the high expression of MAPKKK gene in I59NI be related to the drought-tolerance of this cultivar? The question remains open.

The contig coding for non-specific lipid transfer proteins (nsLTP) was also highly up-regulated under drought in plagiotropic apices of I59 but not in those of the Rubi. In addition to their role in mediating phospholipid transfer, nsLTP are also be involved in plant defense activity against fungal and bacterial pathogens, participating in the assembly of hydrophobic protective layers of surface polymers like cuticle or waxes. In tree tobacco (*Nicotiana glauca*), like in other plants, LTP was predominantly expressed in the epidermis and induced under drought stress concomitantly to wax deposition. The same seems to occur also in coffee

since the wax thickness on adaxial leaf surface was greater for I59 than for Rubi [J.L Verdeil, unpublished results].

The expression profile of the contig coding for the putative secoisolariciresinol dehydrogenase (SDR) also followed those observed for the two previous genes. This enzyme produces matairesinol, a central precursor of numerous lignans essential compounds of plant cell walls. In addition to their important roles in determining physicochemical and mechanical properties of cell walls, lignans can act as antioxidants, biocides (fungicides, bactericides and antiviral agents). They are synthesized by the phenylpropanoid pathway which was reported to be up-regulated under drought in higher plants, like in *Citrullus lanatus* sp. where growth reduction and tolerance to desiccation were associated with more lignin in the roots. As a constituent of the cell wall, lignans are expected to influence wood density which is an important indicator of the hydraulic properties in trees and has been shown to affect conductivity and drought survival by prevention of vessel cavitation. Regarding the differential expression profile presented here for SDR-encoding gene between the clones and irrigation condition, I59 should have higher amount of lignans than the drought-sensitive cultivar Rubi. Either acting as antioxidant or improving cell wall rigidity, this could (in part) explain the higher tolerance to drought of the I59 compared to the Rubi cultivar.

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Coffea L. Genome Size and Genetic Diversity using Nuclear Microsatellite Markers across Africa and the Indian Ocean Islands

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SUMMARY

Due to its immense economic importance, *Coffea* has been the focus of numerous cytogenetic and genetic diversity studies, but despite this effort it remains insubstantially studied. In this study we investigate the genetic diversity and genome size of *Coffea* across Africa and the Indian Ocean Islands namely Mascarocoffea.

Genomes size of Mascarocoffea varied from 0.96 to 1.41 pg/2C. The overall DNA values corresponded to two partially overlapped normal distributions: one harboring species from East Africa and the Indian Ocean Islands, the other harboring species from West/Central Africa. Genome sizes increased following a North to Southeast gradient in Madagascar and an East to Central-West gradient in Africa.

From genetic diversity analysis across Africa and the Indian Ocean Islands using nuclear microsatellite variation, *Coffea* comprises a closely related group of species with an overall pattern of genotypes running from west to east. Genetic structure was identified in accordance with pre-determined geographical regions. The genetic diversity among African and Indian Ocean *Coffea* species is high in terms of number of alleles detected, and Madagascar appears to represent a place of significant diversification in terms of allelic and species richness.

INTRODUCTION

The coffee genus (*Coffea L.; Rubiaceae*) comprises 124 species, and occurs naturally in Africa, Madagascar, the Comoros Islands, Mascarene Islands, Indian subcontinent, south tropical Asia, south-eastern Asia and Australasia (Davis, 2011). There are two main coffee crop species, *Coffea arabica L.* and *C. canephora Pierre ex A.Froehner*, which provide a global commodity second only to oil, accounting for exports worth an estimated US$ 15.4 billion in 2009/10 (International Coffee Organization (ICO) 2012). Given the immense economic importance, *Coffea* has been the focus of numerous cytogenetic and genetic diversity studies.

All *Coffea* species are diploids with 2n=2x=22 (Bouharmont 1963; Louarn 1972), except for *C. arabica*, an amphidiploid with 2n=4x=44 (Bouharmont 1959; Lashermes et al. 1996, 1999). Previous studies (restricted to the genus *Coffea* s.s.) performed on fresh leaves revealed 2C DNA content variations (1.03 to 1.76 pg) among a set of 23 African *Coffea* species (Noirot et al. 2003) and two Mascarocoffea species (Cros et al. 1995).

Even though extensive multiple sampling of natural populations in Africa and in Madagascar was carried out between 1960s and 1980s, detailed analyses of genetic diversity with molecular markers have only included a relatively small sampling of species. Studies were largely focused on *C. arabica* and *C. canephora* and their close relatives (reviewed by de Kochko et al., 2010).

Our objectives were to:

- develop a methodology that permits reliable estimations of genome size and investigations on large panels.
- assess the genome size of a broad sample of Mascarocoffea and to investigate possible association with species geographic distribution.
- investigate the inter- and intra-specific genetic diversity across the African and the Indian Ocean Islands.
- study species relationships generated by nuclear microsatellite (SSR) data.

MATERIALS AND METHODS

The study uses a total of 728 individuals of wild origin obtained via germplasm collections: 421 individuals, from a total of 51 populations, representing 39 species from Comoros, Mauritius and Madagascar and 307 individuals from a total of 36 populations representing 21 African species (60 species in total). Leaves from Madagascan and Comorian species were obtained from the Kianjavato Coffee Research Station (KCRS) in Madagascar. The African and the Mascarene (Mauritian) species were sampled from the *Coffea* collection maintained by IRD at the Armeflhor de Bassin Martin Station, Saint-Pierre, Reunion (originating from representatives of the international *Coffea* collection maintained at the Divo's research station, Ivory Coast (Hamon et al., 1998). These *Coffea* germplasm collections, assembled between 1960s and 1980s, represent exceptional taxonomic and geographical coverage for Africa, Madagascar and the Mascarenes. They were created without taxonomic, commercial, or regional bias, and in most cases include multiple population representatives. Their accessions have been verified and vouchered by *Coffea* taxonomists. The identifications of a large proportion of the KRCS and IRD collections have been corroborated using sequence data (Maurin et al., 2007). Mature leaves were sampled from one to 14 trees per species or population (depending on the availability), lyophilized and stored until use.

DNA content was measured using flow cytometry at the Imagif Cell Biology platform (Gif-sur-Yvette, France) according to Marie and Brown (1993).

Genetic data are produced using 13 polymorphic nuclear microsatellite markers (SSRs) (Plechakova *et al.* 2009) including seven EST-SSRs and, the data analyzed using model and non-model based methods.

RESULTS AND DISCUSSION

Genetic diversity

The genetic structuring and differentiation for West/West Central Africa (W/WCA) is better than all the other three regions (East Africa EA; Madagascar MAD, Mascarene MAS), as shown by the higher number of private alleles (PA), and lower percentage of shared alleles (SA). W/WCA also possesses the highest level of expected heterozygosity (He). If we consider that the mutation rate for a given SSR would be similar for both the source and tested species (Harr et al., 1998) and that the number of mutations should increase with the time of divergence, we could infer that the speciation in EA, MAD and the MAS was posterior to that in W/WCA, or that the selective pressure in W/WCA was stronger than in the other regions, although both of these suggestions are highly speculative. For the four regions, W/WCA, EA, MAD and MAS, He is higher than observed heterozygozity (Ho), which means that there is a strong differentiation among the regions. We know that this is congruent with the fact that each region includes more than one true species (i.e. there are species radiations within these areas). Inter-regional differentiation and regional unity is also supported by the numbers of PA and SA obtained for each of the four regions (Figure 1).

For each sub-region and all loci, the Hardy-Weinberg equilibrium was rejected and a heterozygote deficit was observed. This deficit was partly the result of the presence of null alleles, intra- regional/sub-regional differentiation and/or inbreeding, although the role of each of these factors cannot be determined.

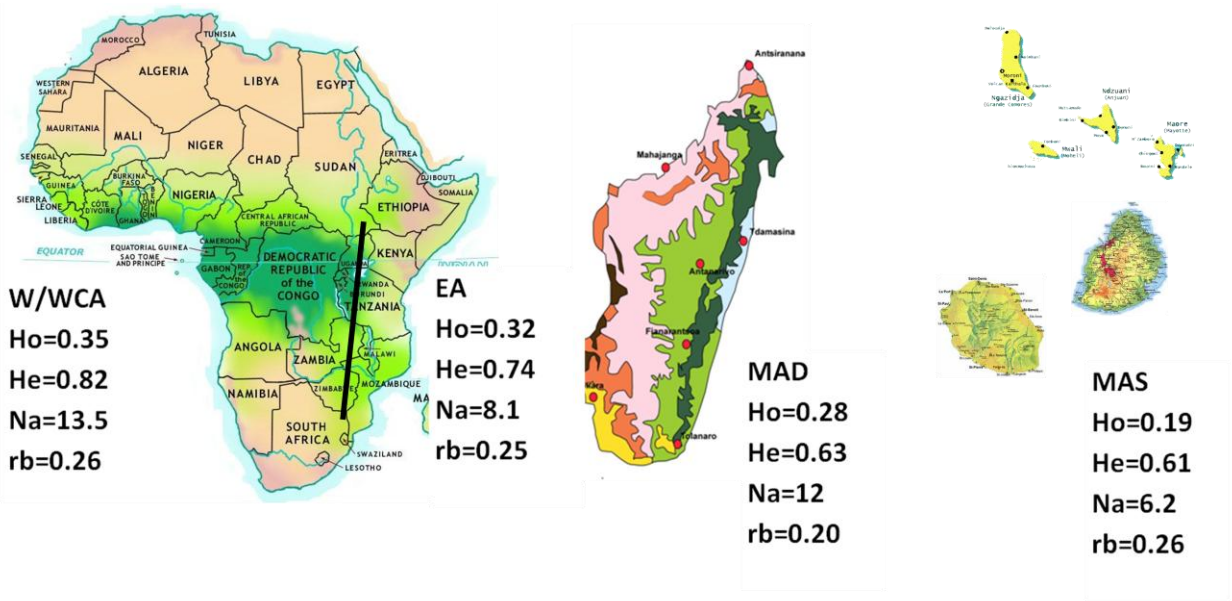

Figure 1. Genetic parameters for the different predefined geographical areas/regions/sub-regions. Each parameter was averaged on the 13 loci analyzed. Na: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity and rb: null alleles rate.

Genetic relationship

The tree of genetic relationships (Fig. 2) based on shared allele distance (*D*SA) is congruent with phylogenetic studies based on plastid and ITS sequencing (Maurin *et al.*, 2007; Davis *et al.*, 2011), although bootstrap support for most groupings above the level of species is zero to

negligible. There is a clear separation between Africa and the IOIs, as retrieved on the basis of ITS and plastid sequencing (Davis *et al.*, 2011) and Ty1-copia LTR-retrotransposon data (Hamon *et al.*, 2011), although this does not agree with the paraphyly of African and IOIs species identified using low copy nuclear marker sequencing (Nowak *et al.*, 2012).

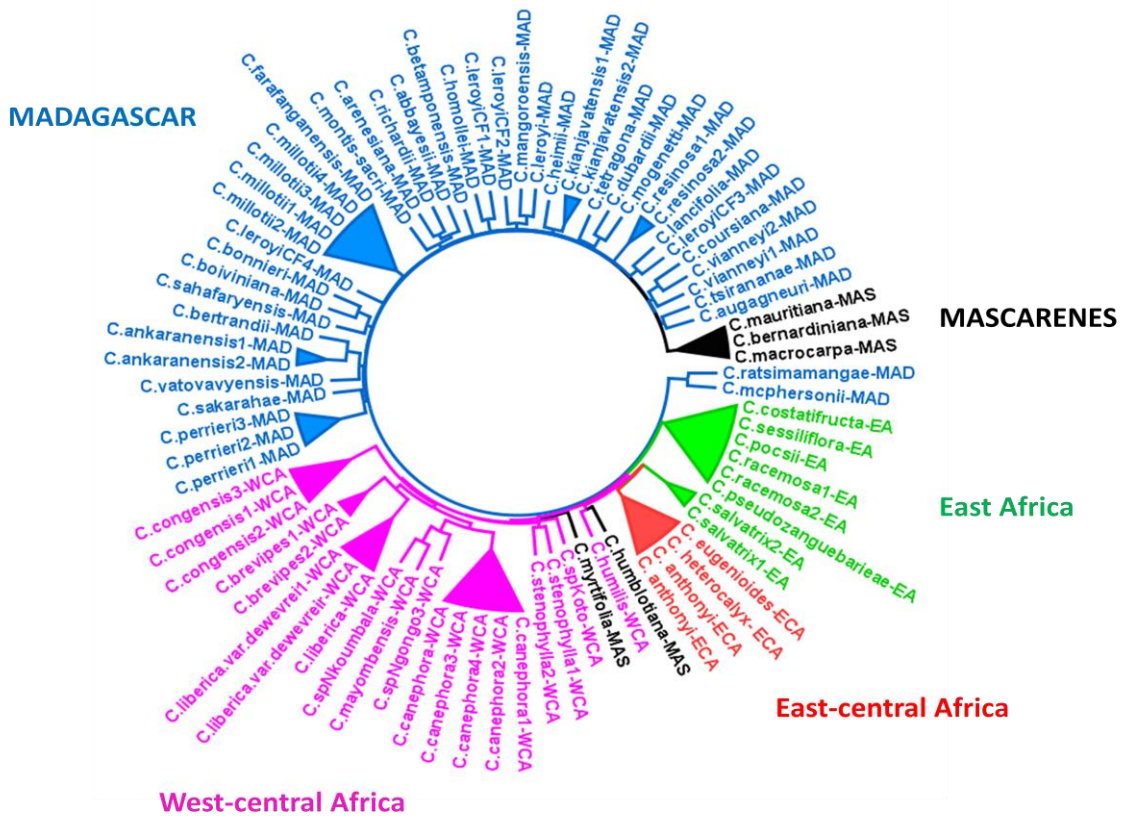

Figure 2. Unrooted neighbour-joining trees based on 13 microsatellite markers, using shared allele distance (*D* $_{SA}$) (Chakraborty and Jin, 1993), to show the genetic relationships among 81 African and Indian Ocean *Coffea* populations. Trees obtained from 500 bootstrap iterations. West and West-Central Africa [pink];East central Africa [red], East Africa [green]; Madagascar [dark blue]; and Mascarenes including the Comoros [black] (Razafinarivo et al 2012b).

Mascarocoffea genome size variation

Mascarocoffea lyophilized leaves (LyL) turned out to be suitable materials for flow cytometric analyses. Most measurements revealed CV (coefficient of variation) values of around 3%, comparable with those generally obtained from fresh material. Overall, a very high linear correlation was obtained between measurements of Fresh leaves (FL) *vs.* LyL of a set of eight Malagasy and six African species used as references (R^2=0.96). Hence, for coffee trees, the relationship between 2C values obtained from the two data sets (LyL *vs* FL) was as follows: 2C value (in pg) LyL = 1.052 * 2C value FL - 0.029. This was used to predict the 2C DNA values that would be obtained using LyL from data obtained previously with FL of African species (Noirot et al. 2003).

DNA content variation between populations within a species was not significant. This allowed determination of 2C DNA values per species by a simple arithmetic mean. Although 2C DNA values ranged from 0.96 (*C. mauritiana*) to 1.41 pg (*C. millotii* ex *C. dolichophylla*),

discrimination between all species, based only on genome size, was impossible. The global 2C DNA values distribution (this study and values calculated from Noirot et al. 2003) was bimodal and was obtained by overlapping two curves, one corresponding to Mascarocoffea and the other to African species.

Genome size and biogeography

Regarding their biogeography, the results highlighted global trends (Figure 3): i) the smallest genomes were native to Mauritius and Comoros; ii) in Madagascar, 2C DNA values tended to increase from north to southeast; iii) considering the species habitat reported by Leroy (1972), Charrier (1978), Davis et al. (2006), (F Rakotonasolo, Royal Botanic Gardens, Antananarivo, Madagascar, 'pers. Comm.'), genomes of the species that occur in dry forests (yellow boxes, Figure 3) covered the lower-middle part of the total size variation range, while genomes of species from humid forests (green boxes, Figure 3) mainly represented the middle-upper part of the genome size spectrum.

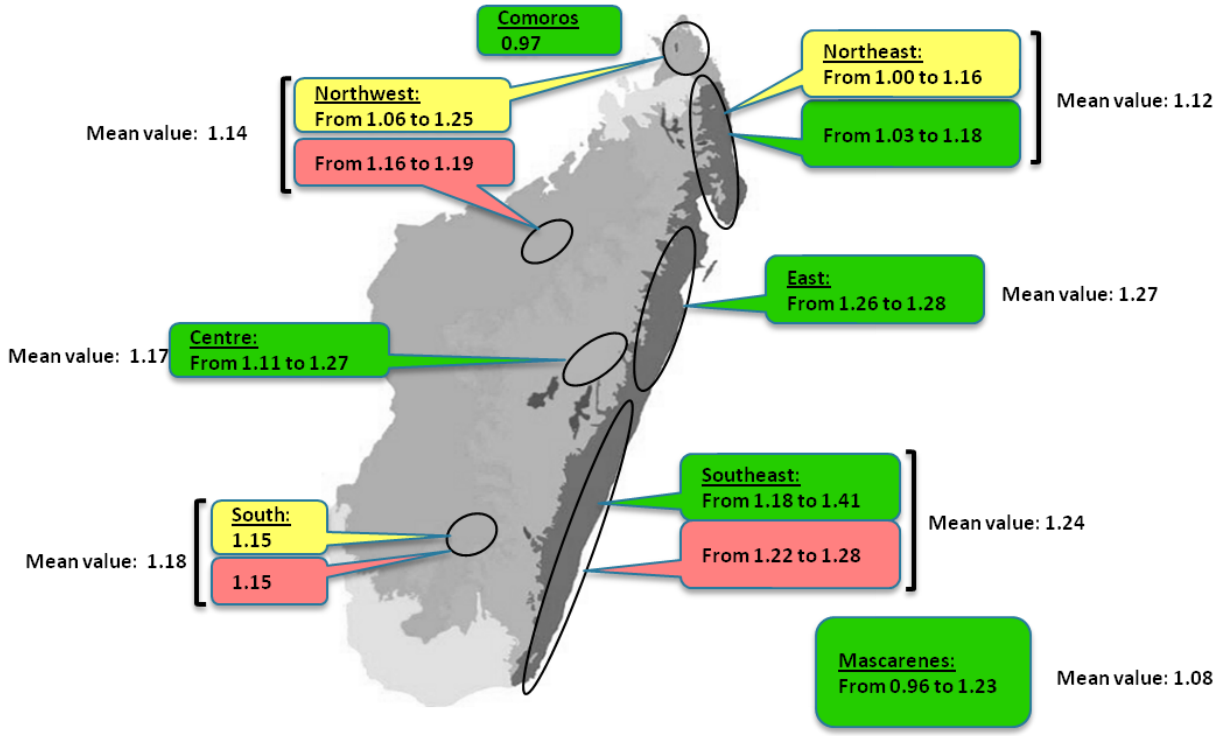

Figure 3. 2C DNA content distribution. Box color indicates the habitat: in green, humid forest; yellow, dry forest; orange; red, gallery forest. For details on species/populations values see Razafinarivo et al. (2012a).

Genome size and species relationships

There is no association between *Coffea* genome size and species relationships established from their genetic distances. Relationships among species can also be estimated through the cross-hybridization success rate or through the F1 hybrid pollen fertility rate. For instance in Madagascar, two pairs of species, i.e. *C. resinosa* and *C. millotii* with a 31.4% cross hybridization success rate and *C. vatovavyensis* and *C. bertrandii* with a 73% F1 hybrid pollen fertility rate (Charrier 1978), should be considered as relatively close while they differed significantly by their 2C DNA content (1.27 vs. 1.40 pg for the former and 1.03 vs. 1.22 pg for the latter).

CONCLUSIONS

For Mascarocoffea, the global trend is that species with small genomes grow in dry areas of northern Madagascar, while species from humid forests of eastern and southeastern Madagascar have bigger genomes.

The genetic diversity is associated to a high morphologic diversity in Madagascar. However, a high number of species have disappeared or are endangered due to the strong deforestation, political problems, poverty in Madagascar and lack of national and international funds (thanks to UCC for their help until end 2012).

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An Inhibitor from *Lupinus Bogotensis* Seeds Effective against Aspartic Proteases from *Hypothenemus Hampei*

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SUMMARY

The coffee berry borer, *Hypothenemus hampei* is one of the most devastating coffee pests. Digestion in the midgut is facilitated by aspartic proteases. The greatest inhibitory activity of aspartic proteases was found in *L. bogotensis* seeds. The aspartic protease inhibitor from *Lupinus bogotensis* (LbAPI) who had the highest inhibitory activity was purified and characterized. LbAPI exhibits a molecular mass of 12,84 kDa and consists of a single polypeptide chain. LbAPI was highly effective against aspartic proteases with a IC_{50} of 2.9 μg. Its amino-terminal sequence has 76% identity with vicilin. The gene encoding LbAPI was expressed in *E. coli*, and the effect of recombinant LbAPI (rLbAPI) showed that an artificial diet containing 1.5% and 1% rLbAPI produced 98% and 50%, respectively, mortality of the larvae. These results suggest that *LbAPI* may be promising for obtaining a coffee plant resistant to *H. hampei.*

INTRODUCTION

The coffee berry borer, *Hypothenemus hampei* (Ferrari), is the insect pest that causes the highest economic losses in the world's coffee crop. Many coffee farmers currently rely on the application of synthetic insecticides, such as clorpirifos, methyl pirimifos, and fenitrothion, which are toxic to insects. The growing concern of environmentalists and the increased resistance of this pest to insecticides have prompted research to be conducted on environmentally friendly control strategies. Among these, several plant proteins have the potential to control the insect, including protease inhibitors (PIs). The ability of PIs to interfere with insect growth and development has been attributed to their ability to bind the active sites of digestive proteases and block, alter, or impede access to the substrate, thus reducing the availability of essential amino acids derived from ingested protein. The aspartic proteases of *H. hampei* digest food protein in the insect's midgut. Therefore, the inhibition of these proteases provides a promising defense mechanism for the control of this insect. The present study describes the purification and characterization of an inhibitor, which represents the first aspartic protease inhibitor isolated from *Lupinus bogotensis* (LbAPI) with inhibitory activity against the aspartic proteases of *H. hampei*. In addition, to evaluate the effect of LbAPI in artificial diets, the expression and purification of the recombinant LbAPI (rLbAPI) in *Escherichia coli* was achieved, and the *in vivo* effects on the development, growth, and mortality of larvae insects were also investigated.

MATERIALS AND METHODS

Purification of LbAPI. The inhibitors of *H. hampei* aspartic proteases were obtained from the defatted flour of *Lupinus bogotensis* seeds according to the methods in Pestic. Biochem. Physiol. Inhibitory activity assay. Inhibitor activities against proteases of *H. hampei* and pepsin were determined by monitoring the hydrolysis of hemoglobin at 280 nm in 0.2 M citric acid, 0.1 M NaCl, pH 2.5.

cDNA cloning and determination of nucleotide sequence of *LbAPI* gene. The isolation of total RNA from *L. bogotensis* seeds was performed using the RNeasy Maxi Kit (Qiagen). An RT-PCR reaction was performed to synthesize the cDNA using 1 μg of total RNA. To obtain the complete sequence of the *LbAPI* gene, genomic DNA was isolated from *L. bogotensis* seeds using the DNeasy Plant Mini Kit (Qiagen). The PCR product was amplified using specific primers.

Cloning of *LbAPI* gene into the pET15b expression vector. The 260 pb *LbAPI* fragment encoding the mature LbAPI protein was subcloned into Nde I/BamH I-linearized pET15b. The resulting construct, pET-*LbAPI*, was introduced into *E. coli* strain BL21 Star^{TM}(DE3)pLysS (Invitrogen). Expression was initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM and culture was incubated for 4 h. The bacterial cells were lysed by sonication. The clear lysate, obtained after centrifugation at 10,000 *g* for 15 min at 4°C, was mixed with 5 ml equilibrated nickel- nitrilotriacetic acid affinity resin (Ni-NTA agarose (Qiagen) for 1 h at 4°C. The recombinant LbAPI protein was eluted in 30 ml of lysis buffer containing 250 mM imidazole.

Feeding test of rLbAPI against the coffee berry borer. The rLbAPI was incorporated into the diet at concentrations of 0, 1.0 and 1.5% (w/w). During the 30 days of the life cycle of the berry borer, the dead larvae were counted, and the surviving larvae were weighed. In the control treatment, distilled water or BSA at the same inhibitor concentrations was added to the artificial diet. Each treatment was replicated three times, using 96 larvae per replicate. The bioassay was maintained under controlled conditions at 28 °C and 55% stable humidity. A completely random design was used and the means of the larval mortality treatments were compared using contrast.

RESULTS AND DISCUSSION

Seeds extracts of *Lupinus bogotensis*, *Brachiaria humidicola*, *Amaranthus hypochondriacus*, *Phaseolus acutifolius*, *Phaseolus coccineus*, *Hyptis suaveolens*, *Centrosema pubescens*, and *Trifolium repens* were evaluated to identify aspartic protease inhibitors. The greatest inhibitory activity of aspartic proteases was found in *L. bogotensis* extract with a specific activity of 74.1 IU/mg, compared with extracts of *H. suaveolens*, *B. humidicola*, and *A. hypochondriacus* that showed a lower inhibition of aspartic proteases. The proteolytic activity of *H. hampei* was inhibited by 90% with 100 μg of crude extract of *L. bogotensis*, whereas it took 1 mg of crude extract of *H. suaveolens* and *B. humidicola* to inhibit the activity at 70 and 60% respectively.

Aspartic protease inhibitors were purified using a combination of chromatography methods, which were monitored by measuring the inhibitory activity of the fractions against aspartic proteases from *H. hampei*. The protease inhibitors were eluted from a Q sepharose anionic exchange column using a linear NaCl gradient at 141, 192, 230, 258, and 282 mM NaCl, and named as follows: *Lupinus bogotensis* aspartic protease inhibitor (LbAPI) 1, LbAPI2,

LbAPI3, LbAPI4, and LbAPI5, respectively. The amino-terminal amino acid sequences for the five polypeptides were identical or nearly identical to each other. LbAPI4, which had the highest inhibitory activity, was named the *L. bogotensis* aspartic protease inhibitor (LbAPI), this inhibitor exhibited a molecular mass of 12.84 kDa, as determined by MALDI-TOF, and consists of a single polypeptide chain with an isoelectric point of 4.5. In thermal activity experiments, the stability was retained at pH 2.5 after heating the protein at 70°C for 30 min, but it was unstable at 100°C. The protein was stable at a broad range of pH, from 2 to 11, at 30°C. In *in vitro* assays, LbAPI was highly effective against aspartic proteases from *H. hampei* guts with a half maximal inhibitory concentration (IC_{50}) of 2.9 µg. (Figure 1). Its amino-terminal sequence had 76% identity with the seed storage proteins vicilin and β-conglutin. The similarity of LbAPI to vicilins from *Lupinus albus* L. suggests that they may also serve as storage proteins in the seed. The gene *LbAPI* corresponds to a sole open reading frame of 354 nucleotides that code for a polypeptide of 117 amino acids and has no introns. The gene *LbAPI* was expressed in *E. coli* and purified (Figure 2), and the effect of recombinant LbAPI (rLbAPI) was tested using bioassays. rLbAPI had an apparent molecular mass of 15 kDa and consisted of a single polypeptide chain with an isoelectric point of 4.5. Our *in vitro* experiments showed that rLbAPI was highly efective for inhibit aspartic protease activities in the crude gut extracts of the coffee berry borer. Dietary utilization experiments showed that an artificial diet containing 1.5% and 1% rLbAPI produced 98% and 51%, respectively, mortality of the larvae, whereas only 6.5% mortality for the control without rLbAPI (Figure 3). The LbAPI action against the larvae of *H. hampei* may involve the inhibition of aspartic proteases and binding to the chitin components of the peritrophic membrane in the midgut of the beetle. These results suggest that *LbAPI* may be promising for obtaining a coffee plant resistant to *H. hampei*.

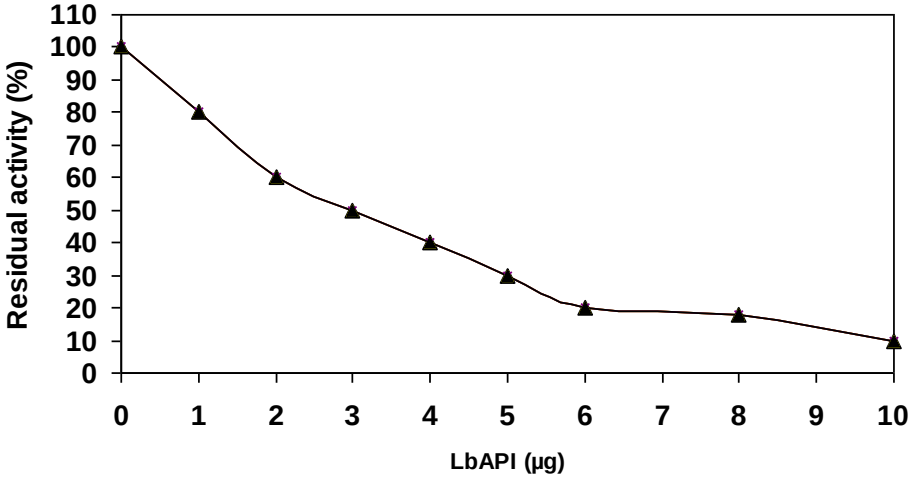

Figure 1. LbAPI inhibition of the midgut aspartic-like proteolytic activity (hemoglobin assay) from adult *H. hampei* (▲).

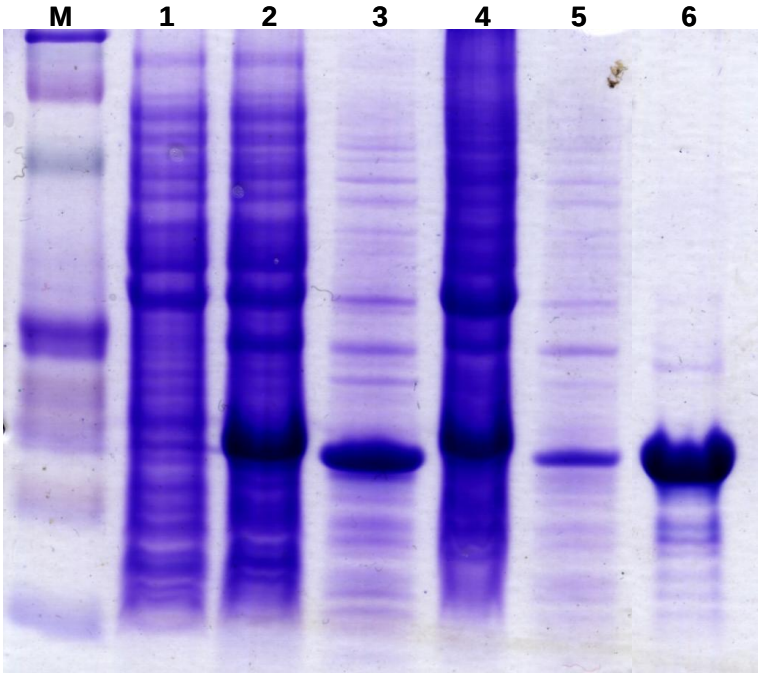

Figure 2. Overproduction and purification of recombinant LbAPI *in E*. coli cells BL21 Star™(DE3)pLysS transformed with the pET15b/ LbAPI expression plasmid. M, molecular weight markers; 1, total cells extracts of *E. coli* cells without induction; 2, total cell extracts after induction by IPTG; 3, soluble fraction of *E. coli* cells after sonication; 4, insoluble fraction after sonication; 5, elution with 20 mM imidazole; 6, purified rLbAPI.

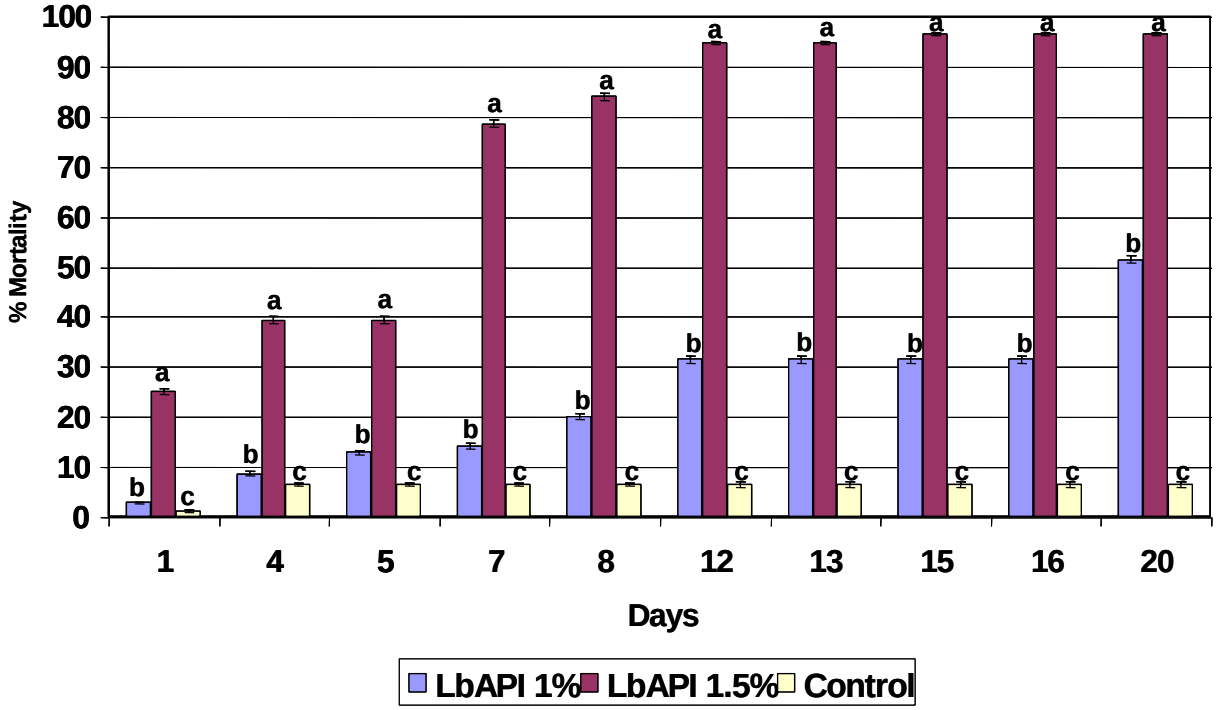

Figure 3. The inhibitory effects of rLbAPI on the mortality of *H. hampei* larvae. rLbAPI was included in an artificial diet at a final concentration of 1.5%. Each measurement was performed in three replicates. The error bars represent the standard deviation.

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Analysis of the Expression Pattern of miRNAs, by Stem-Loop RT-PCR, in Coffee Species with Different Ploidy Levels

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SUMMARY

MicroRNAs (miRNAs) are small non-coding RNAs that play a key role in negative control of gene expression. Using the information stored in the coffee ESTs database, it was possible to identify eight MIR families involved in the transcriptome modeling of this crop. In this study, the sequence and secondary structure of miR172 are presented. Furthermore, the expression pattern of this miRNA was evaluated in two coffee species with different ploidy level. The assessment of the role of microRNAs combined with characterization of the main regulated transcription factors can provide new perspectives and opportunities for the next efforts involving the manipulation and development of coffee cropping.

INTRODUCTION

The phenomenon of interference RNA mediated by microRNAs is an important pathway for the stability and regulation of messenger RNA translation. Through the pairing of specific bases, these short sequence (20 - 22 nucleotides) transcripts inhibit the translation process and/or promote cleavage of their target mRNAs. In plants, miRNAs play an important role in multiple biological and metabolic processes. Identified miRNA functions include control of tissue (leaf, root, stem, and flower) differentiation and development, phase switch from vegetative growth to reproductive growth, phytohormone signaling, and response to different biotic and abiotic stress.

Many experiments have also demonstrated that small RNAs, the microRNAs (miRNA), small interfering RNAs (siRNA), trans-acting siRNAs (ta-siRNA) and natural antisense siRNAs (nat-siRNA), are associated with the main cellular processes involved with the functionality and stability of the genomes, acting on the control of epigenetic alterations that occur in response to the hybridization processes. Such responses include alterations in the structure of the chromatin, rearrangements or cytosine methylation, gene silencing, transposon activation and of DNA deletion.

In hybrid genomes, the loci of inherited miRNAs are subject to gain new expression patterns in specific organs/tissues or development stages. Like protein coding genes, promoters of miRNA loci contain canonical cis-promoter elements, such as TATA box and transcription initiator, and various transcription factor responsive elements. In the union process of the parental genomes in a single nucleus, cis and trans elements of the promoter sequences of those genes can undergo alterations. Also some genes can be lost in the speciation process.

It is important to note that changes in the expression pattern of regulatory genes, as are MIR genes and transcription factors, can influence many other genes involved in different metabolic pathways. A single miRNA can regulate hundreds of genes involved in the transition from one developmental stage to another. This shows the great importance of the biogenesis control of these RNAs, the differential expression of target regulated genes can bring adaptive advantages for the polyploid, as well as disadvantages, bringing for instance, genome instabilities, reduction of fitness or the hybrid energy. This information points to the great importance that small RNAs have for cellular dynamics.

In this study, previously known plant miRNAs were used to search the *Coffea arabica* miRNA homologs in the publicly available EST sequence (www.lge.ibi.unicamp.br/cafe). We presented, for the first time, the sequence, structure and expression pattern of miR172 in in tetraploid species *Coffea arabica* and diploid species *Coffea canephora*.

This first study about gene silencing by microRNAs performed on coffee provides new perspectives for the next efforts involving the development of coffee cropping. This knowledge may help in understanding of the various mechanisms that the cells provide to control metabolic, reproductive, stress response and polyploidization processes in two important species of coffee, both with respect to the economy as well as genomic research studies.

MATERIALS AND METHODS

In silico analysis

The methodology adopted to characterize the coffee miRNAs, was described by Zhang et al. and has been used thoroughly in the characterization of small RNAs homologs in plant species. Figure 1 represents an outline of the methodology. A total of 1,549 mature miRNAs, belonging to 20 plant species, among them Arabidopsis thaliana and Oryza sativa, were used in the characterization of the potential miRNAs contained in the coffee EST library. The sequences of these RNAs were taken from the miRBase (Release 13.0, March, 2009). To avoid overlapping among the data, redundant sequences were eliminated and 809 sequences remained and were defined as the reference database (query sequences).

Through the Gene Project on-line interface (www.lge.ibi.unicamp.br/cafe) Coffea arabica reads containing singlets or contigs were searched to characterize probable MIR candidate genes. Only coffee sequences presenting less than 4 mismatches, including nucleotide inserts and/or deletions, regarding the miRNAs query, appeared as candidates. The 769 refined sequences were confronted with mRNAs sequences stored in the NCBI data platform, to eliminate the coding sequences proteins.

Finally, the precursor sequences of approximately 400 nt were extracted (200 nt upstream and 200 nt downstream from the BLAST hits) and used for hairpin structure predictions using MFOLD3.2 algorithm [35]. All of the parameters of the software were used in (its)standard format. Free energy aspects (ΔGkcal / mol), the nucleotide makeup (A, G, C and U) and the location of the region corresponding to mature miRNA were analyzed.

However, only the sequences of RNA that were framed in the following criteria were considered candidates: (1) the structure of the mature miRNA to be predicted should not contain more than four mismatches in comparison with previously known mature miRNA structure; (2) the sequence of RNA should fold in an appropriate secondary structure (adequate hairpin structure); (3) contain the mature miRNA sequence in one arm of the

hairpin and an miRNA* sequence in the other; (4) it should not have more than six mismatches among mature miRNA sequences and their opposite sequence in the secondary structure; (5) loops or ruptures in the miRNA sequences were also not allowed; (6) and finally, the predicted structures should present an MFEI over 0.85.

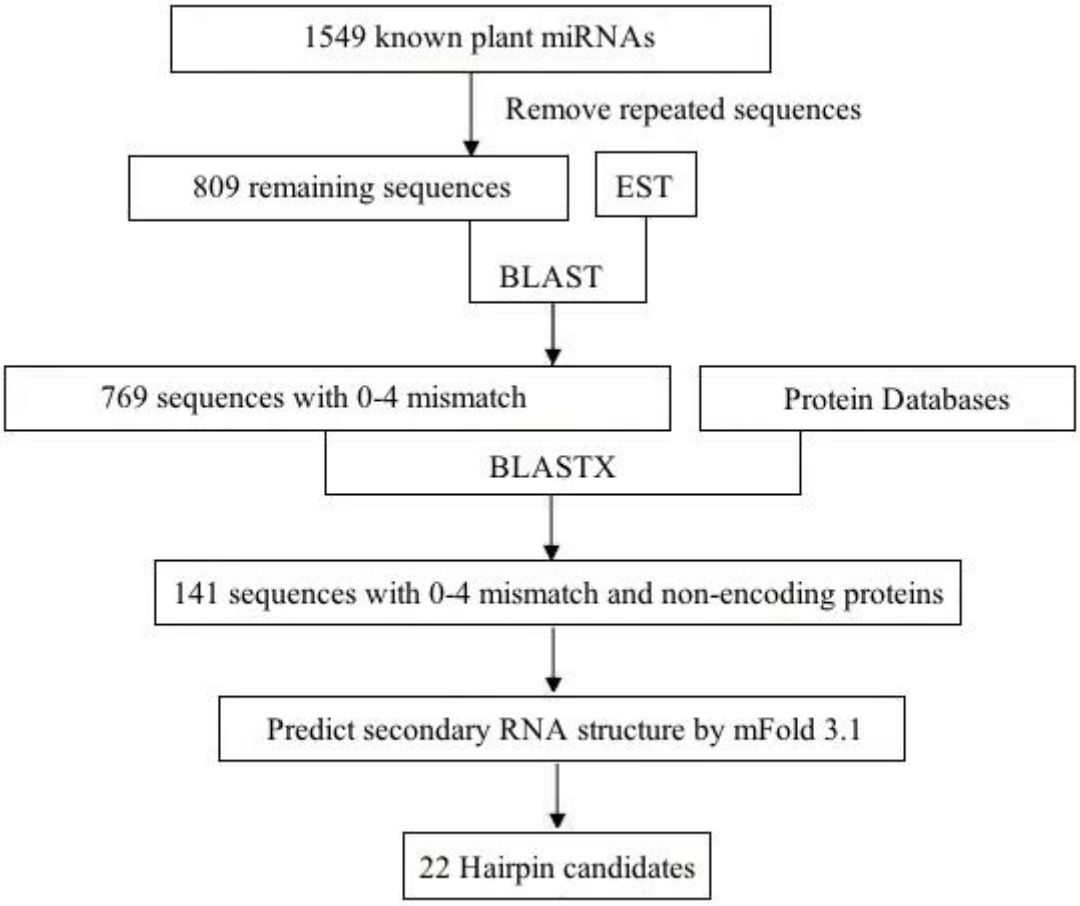

Figure 1. Flowchart showing the methodology used in the discrimination of possible coffee miRNAs.

In vitro analysis

Stem–loop RT and qPCR primers for coffee miR172 (table 1) were designed according to Varkonyi-Gasic, et al.. Real-time PCR was performed using a standard SYBR Green PCR kit protocol on an Applied Biosystems ABI PRISM 7500 Real-Time PCR. The reactions were conducted in a final volume of 10 µL, using 1.0 µL cDNA L in the dilution 1:25 (v:v), 0.3 µl of each initiator at a concentration of 10mM, 5.0 µL of SYBR® Green PCR Master Mix (Applied Biosystems) and 3.4 µL of milli-Q water, for each reaction. The reaction mixture was incubated at 94ºC for 5 min, followed by 40 cycles at 95ºC for 15s, 60ºC for 10 s and 72ºC for 15 s. Then the samples were heated from 55 to 99ºC with an increase of 0.1ºC/ s to acquire the denaturing curve of the amplified products.

All reactions were run in triplicate. For the calculation of the relative expression, we used the comparative CT method. Each sample is normalized with the endogenous controls, using the equation ΔCT = CT (target gene) - CT (endogenous control). The normalization factor was the arithmetic mean of the $-actin gene expression and Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH), the relative quantification was obtained by the formula 2^(-delta delta Ct).

Table 1. Primers designed for to detect mature miR172.

miR172	miR sequence	UGAAUCUUGAUGAUGCUACAC
	Forward primer	CCTGAGTGAATCTTGATGATG
	Reverse Universal	GTGCAGGGTCCGAGGT
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACATGTAG

RESULTS AND DISCUSSION

From 1,549 initial sequences, only 141 sequences met the alignment conditions , showing being distinct from codants RNAs . After analyzing secondary structure criteria, the total number of candidate miRNAs was drastically reduced:to a total of 22 possible miRNAs that were characterized for the coffee plant. These RNAs were classified in 8 different families, and then distributed in a non-uniform way among them. For the miR172 family, 3 members were described. Their predicted structure is represented in figure 2.

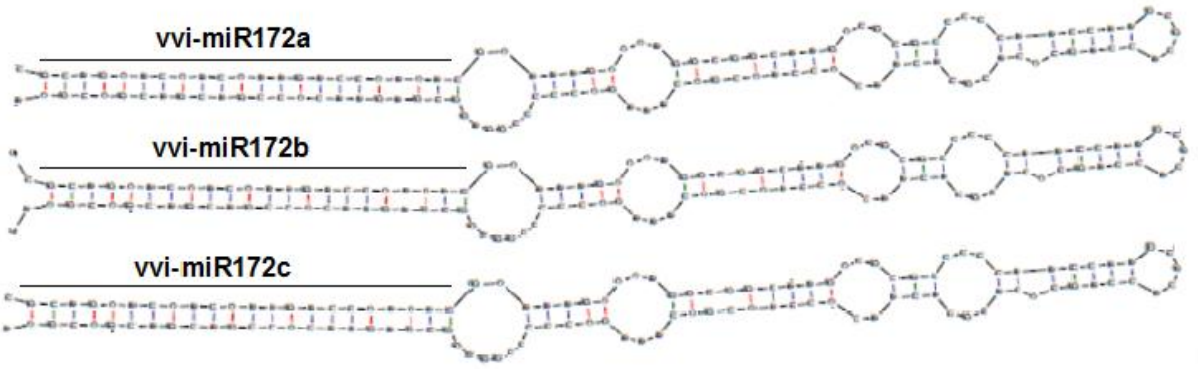

Figure 2. Secondary structure proposed for miR172 by MFOLD software (Zuker 2003).

Concerning the expression experiment, the stem-loop RT- qPCR assay showed that with coffee, the expression of these miRNAs is proportional to the ploidy level of the genome. In agreement with the additive model of expression in *Coffea canephora*, which presents number of chromosomes 2n = 2x = 22, the amount of accumulated miRNAs miR172 corresponds to values very close to half of the expression verified in the *Coffea arabica* species 2n = 4x = 44 (Figure 3).

Different expression patterns have been demonstrated in hybrid plants. For example, in an analysis of the siRNA and miRNA expression in *Arabidopsis thaliana, Arabidopsis arenosa* and in allotetraploid lineages (from F1 to F7) derived from those species, the authors found that the miRNA expression patterns were highly variable between the allotetraploid and their progenitors. Most of the tested miRNAs were expressed non-additively and the regulation of the target genes clashed with what is found for *Arabidopsis thaliana* as well as *Arabidopsis arenosa*. On the other hand, corroborating our results in a study using diploid Aegilops *tauschii* (genome DD), tetraploid *Triticum turgidum* ssp. (AABB), their triploid hybrid (ABD), and derived hexaploid (AABBDD), most miRNAs are found to be expressed in the mid-parent value. The authors relate that, while the siRNAs percentage decreased, the number of small RNAs corresponding to miRNAs, with a few exceptions (up regulation) in the allopolyploids, increased in according to the ploidy level.

Veitia et al. demonstrated that in the case of allopolyploids, the additive expression can supply the necessary molecular base for balance and compensation of the extra dose of redundant genes assembled in a single nucleus. That expression pattern can confer stability to the development of the hybrid species.

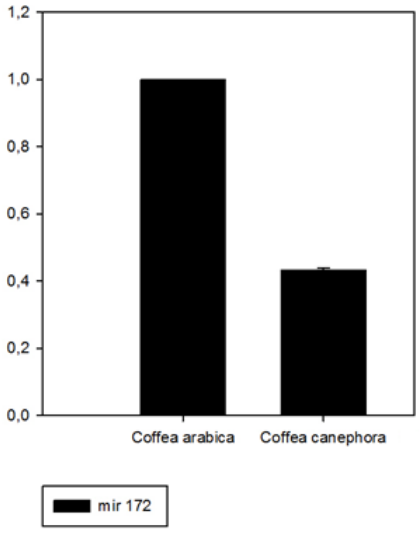

Figure 3. Profile of relative expression of miR172 in leaves of *Coffea arabica* and *Coffea canephora*. The transcript level is represented as a ratio (relative expression) of the absolute value of the expression of the MIR gene for the absolute value of the expression of the β-actin and GAPDH normalizing genes. In the graph, *Coffea canephora* expression values were plotted having the *Coffea arabica* expression value as reference. The vertical bars represent the standard deviation of two biological samples.

CONCLUSIONS

Using publicly available ESTs databases, coffee miRNA precursors were identified and classified into 8 families. The qPRC results indicated that the expression level of the MIR genes increased proportionally with ploidy level of the genome. In *Coffea arabica* the accumulation value of having transcripts relative to the miRNAs was approximately double of that found for the diploid species. These data may suggest a potential correlation between miRNA expression levels and different phenotypes in hybrids and allopolyploids, but the molecular link between miRNAs and a specific trait, such as fertility, flowering time, hybrid vigor or improved yield, has yet to be demonstrated.

Our dataset provides a framework for future research into the functional roles of miRNAs and their targets in coffee. In these future studies more inclusive analyses, such as metabolomic, proteomic and RNAseq evaluations, should be adopted to investigate the global effects of the miRNA responses to the different stimuli or environmental conditions. It is important that we have, in a clear manner, the quali-quantitative measure of the general response of the coffee plants to changes in the microtranscriptome.

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Restructured Sampling Plan Enables the Characterization of More Virulence Genes of *Hemileia Vastatrix* in Kenya

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SUMMARY

Arabica coffee was introduced in Kenya at about 1900 near the coast and the crop has since spread across the country. The first report of Coffee Leaf Rust (CLR, caused by *Hemileia vastatrix*) on cultivated coffee in the country was made in 1913 and breeding for resistance against the disease has been a priority. In addition to conventional *Coffea arabica* varieties, derivatives of Timor Hybrid (HDTs) that are natural hybrids between *C. arabica* L. and *C. canephora* Pierre have been being introduced into the country and used in breeding programmes since 1960s. The HDTs received major genes responsible for rust resistance (S_H6, S_H7, S_H8, S_H9, $S_H?$) that present in the *C. canephora* germplasm introgressed into the *C. arabica*. No new virulence genes and/or races of the pathogen have been reported amongst rust samples collected from Kenya since 1977 when four virulence genes (*v2, v3, v4* and *v5*) plus the uncharacterized one(s) in race XX were reported. This was despite countrywide sampling and analysis of CLR and it prompted redesigning of the survey strategy in order to increase the chances of detecting new virulence genes and races of the pathogen. Sampling was therefore conducted targeting coffee germplasm conservation plots, breeding fields and commercial farms planted with mixed coffee varieties. The samples were characterised at the Coffee Rusts Research Centre (CIFC) and the results revealed three new virulence genes (*v1, v7* and *v8*) with the possibility of a fourth one (*v9*). The virulence genes *v7, v8* and *v9* are able to overcome the resistance genes derived from HDTs and this presents a threat to the resistant varieties that have been developed. The above results also demonstrates the need for changing survey strategies especially in Africa where the actual number of CLR races appear to be understated compared to the fact that currently there over 45 known races of the pathogen. This paper discusses the outlook possible implications of such a status.

INTRODUCTION

Arabica coffee (*Coffea arabica* L.) is native to the highlands of Ethiopia, Southern Sudan and Northern Kenya (Mount Marsabit) (Anthony et al., 2002). In Kenya, cultivation of coffee was started at about 1900 at the coast where it was introduced by French Missionaries and later spread westwards into the country. However the disease is reported to have been observed in uncultivated coffee in 1861 in Western Kenya near Lake Victoria (Ferreira SA and Boley, 1991).). This was most likely on a *Coffea* species other than *C. arabica*. Nevertheless, coffee and Coffee Leaf rust (CLR), caused by *Hemileia vastatrix* Berk & Br., have co-existed for at least a Century in Kenya. Currently, more than forty five (45) have been characterised in different coffee growing countries (Varzea and Marques, 2005, Bhat *et al*. 2010). The development of new races is affected by the number of resistance genes that the pathogen is exposed to. In Kenya, the main commercial varieties are susceptible (SL28, SL34), tolerant (K7) or resistant (Ruiru 11 and Batian) to the disease under field conditions. In germplasm conservation fields, there are resistant accessions that are used for breeding purposes. One

notable source of disease resistance is Timor Hybrid (HDT) that is natural hybrid between *C. arabica* L. and *C. canephora* Pierre ex Froehner. The first introductions of this lineage were in 1960s from CIFC as pure derivatives and in 1970s as Catimors from Colombia which are crosses between HDT and Caturra. Subsequent breeding work lead to the release of the cultivars Ruiru 11 in 1986 (Nyo) and Batian in 2010 (Gichimu et al., 2010) both of which are resistant to the disease. HDT received major genes responsible for rust resistance (*S_H6*, *S_H7*, *S_H8*, *S_H9* and others not yet identified) from *C. canephora* (Várzea & Marques, 2005, Diniz *et al*. 2012) and the exposure of the pathogen to these genes would be expected to stimulate the development of new races. Previous work reported the existence of six (6) races [I (v2,5), II (v5), VII (v3,5), XV (v4,5), XX (v?), and XXIV (v2,4,5)] in rust samples from Kenya (Thitai and Okioga, 1977). Subsequent work based on surveys in commercial farms has not reported new races. This raised a concern because elsewhere, especially in India and Brazil, new races were being reported with the ability to infect even HDT derivatives (Gichuru, 2005; Varzea and Marques, 2005, Silva et al., 2006 Bhat *et al*. 2010, Diniz *et al*., 2012). The objective of this work was therefore designed to enhance the possibility of encountering new races/resistance genes that may exist in Kenya.

MATERIALS AND METHODS

Sampling was focused on two types of coffee fields: (i) coffee germplasm conservation plots at Coffee Research Station, Ruiru (latitude 1^o 06'S, longitude 36^o 45'E and altitude of 1620 meters above sea level) with attention to accessions that have been known to be resistant to CLR (ii) coffee breeding fields involving donors of resistance to CLR in the same location. In the breeding fields, the proportion of infected plants was assessed to determine if the infected plants were likely to be missing the resistance gene(s) by normal segregation. If the number was higher than would be expected, samples were collected from the infected plants of that population.

Unlike in previous work where the collection of spores aimed at single leaves, in this work leaves from the same variety or breeding population in one plot were bulked together. The infected leaves were dried for one week at room temperature while pressed between newspapers. The dried leaves were then sent to Coffee Rusts Research Center (CIFC) for rust race characterization, according the method described by D'Oliveira & Rodrigues Jr. (1960). Twenty three (23) samples were used for this study (Table 1).

Table 1. Coffee accessions from which the rust samples were obtained and races characterized. THE CLR phenotype refers to the observe red field reactions in Kenya.

Accession	Type	CLR Phenotype	Virulence genes	Race	Race Status
1. Pretoria	Museum Accession	Tolerant	v2,4,5,8	XXXVI	New
2. Bourbon	Museum Accession	Tolerant	v2,5,8	XLI	New
3. Pretoria x SL 28	Breeding line	Tolerant x Susceptible	v2,4,5,8	XXXVI	New
4. Rume Sudan x SL 28	Breeding line	Resistant x Susceptible	v2,4,5,8	XXXVI	New
5. HDT x SL 28	Breeding Plot	Resistant x Susceptible	v2,5,8	XLI	New
6. CIFC 110/2	Museum Accession		v2,5,7,8 or v2,5,7,8,9	XLII	New
7. CR-27	Breeding line	Resistant x Susceptible	v2,4,5,8	XXXVI	New
8. Ennareta	Museum Accession	Tolerant	v2,5,8	XLI	New
9. K7 x SL 28	Breeding line	Tolerant x Susceptible	v2,5,8	XLI	New
10. Rume Sudan	Museum Accession	Resistant	v2,5,8	XLI	New
11. Geisha 9	Museum Accession	Tolerant	v1,5	III	New
12. Purpurascens	Museum Accession	Tolerant	v2,5,8	XLI	New
13. Geisha 10	Museum Accession	Tolerant	v2,4,5,8	XXXVI	New
14. Marsabit	Museum Accession	Tolerant	v2,4,5,8	XXXVI	New
15. Mokka 1	Museum Accession	Tolerant	v2,5,8	XLI	New
16. Mundo Novo	Museum Accession	Tolerant	v1,2,5	XVII	New
17. S.333	Museum Accession	Tolerant	v2,5,8	XLI	New
18. B.A. 36	Museum Accession	Tolerant	v1,2,4,5	XXIII	New
19. Blue Mountain	Museum Accession	Tolerant	v2,5	I	Old
20. Laurina	Museum Accession	Tolerant	v2,5,8	XLI	New
21. Rume Sudan x SL 28	Breeding Line	Resistant x Susceptible	v2,5,8	XLI	New
22. HDT x SL 28	Breeding Line	Resistant x Susceptible	v2,5	I	Old
23. Catimor x SL 28	Breeding Line	Resistant x Susceptible	v2,5,8	XLI	New

RESULTS

From the samples analyzed, 21 (91.3%) revealed the existence of new races while the remaining 2 samples were characterized as race I, which is the most common one in the country (Table 1). The races detected were III (*v*1,5), XVII (*v*1,2,5), XXIII (*v*1,2,4,5), XXXVI [*v*2,4,5, (8)], XLI [*v*2,5, (8)] and XLII (v2,5,7,(8) or v2,5,7,(8,9) (assortment of the virulence genes previously detected in the country) and three new races carrying the genes *v1*, *v7*, *v8* and possibly *v9*. The virulence gene *v6* was not detected in the characterized samples.

DISCUSSION

There are more than 45 races of *H. vastatrix* characterized in the world which have different combinations of the 9 virulence genes (*v*1 to v9) and unidentified ones (*v*?) (Varzea and Marques, 2005). Twenty of these races are virulent to HDT derivatives. In India, there used to

be 14 races of the rust up to 1980s and afterwards increased to 37 races (Prakash, 2005). It needs to be observed that HDT derivatives were introduced into India at the same time as in Kenya. By harmonizing the reports by Zambolim *et al.* (2005) and Cabral *et al*. (2009), there are 18 races of CLR in Brazil. It is therefore not surprising that there have been concerns about the status of races of this pathogen in Kenya where despite several surveys, no new races had been detected since 1977 (Gichuru, 2005). The strategy applied in this study whereby the sampling was restructured to intensify efforts on the germplasm diversity rather than geographical representation was very successful with 91.3% of the samples revealing new rust races and three new virulence genes (*v1, v7, v8*) and possibly *v9*). These findings are similar with the results obtained in other countries. These races are able to break some of the resistance in HDT and this presents a threat to sustained production of cultivars derived from this genotype.

This study allowed characterizing of six new races of *H. vastatrix* in Kenya. Information on the current number of races of this pathogen in many coffee growing countries, especially in Africa, is lacking. This is a major gap considering that the co-existence of the pathogen and various *Coffea* species and varieties is longest in this continent. One limitation to conducting a wide coffee leaf rust survey in African coffee growing countries is the inadequacy of resources to develop collaborative work with CIFC including training of African scientist in this subject as well as the creation of appropriate infrastructures in these countries.

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New Cytological and Molecular Data on Coffee - *Colletotrichum Kahawae* Interactions

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SUMMARY

Colletotrichum kahawae is the causal agent of Coffee Berry Disease (CBD), the most devastating Arabica coffee disease in Africa at high altitude, and represents an imminent threat for cultivation in America and Asia. In Kenya, the hybrid commercial variety, Ruiru 11, was bred for resistance to CBD and coffee leaf rust. Its female parent consists on lines of the cultivar Catimor (from Colombia) selected for resistance to both diseases in Kenya. One such line is Catimor 88, which is still being used in breeding programmes. Molecular studies using a F2 mapping population of the susceptible cultivar SL28 and the resistant line Catimor 88, identified and mapped a major gene (Ck-1) responsible for resistance to CBD.

In this study we aimed to start the cytological and molecular characterization of C. kahawae interaction with Catimor 88 vs a susceptible coffee variety (Caturra).When challenged with C. kahawae isolate Que2 (from Kenya), the majority of Catimor 88 hypocotyls presented scab lesions, whereas hypocotyls of the variety Caturra exhibited dark sunken lesions with sporulation, five to six days after inoculation. Light microscopic studies showed, in the susceptible genotype, the fungus growing without apparent inhibition and feeding on living host cells (biotrophy) before switching to necrotrophy. Reversely, in the resistant genotype fungal growth was frequently restricted to a single epidermal cell and plant response occurred early with accumulation of phenolic compounds and deployment of hypersensitive-like response (HR).

Gene expression studies using quantitative real time PCR (qRT-PCR) were developed to characterize these interactions. This is a sensitive technique for quantifying gene expression, and its success depends on the stability of the reference genes used for data normalization. To our knowledge this is the first study on validation of coffee reference genes during C. kahawae infection time-course. Eight candidate reference genes (namely ubiquitin and gapdh) were selected and their expression stability was evaluated by qRT-PCR in healthy and infected tissues of Caturra and Catimor 88, at 12h, 48h and 72h after inoculation. NormFinder and geNorm algorithms were used to select the appropriate reference genes. Validation of such qRT-PCR reference genes were discussed as the basis for the following gene expression analyses regarding this pathosystem.

INTRODUCTION

Coffee Berry Disease (CBD) caused by the hemibiotrophic fungus *Colletotrichum kahawae* J. M. Waller & P. D. Bridge, has so far remained restricted to Arabica coffee in Africa, where

50-80% of crop losses may be registered, if chemical control measures are not applied. The severe socio-economic repercussions of CBD in Africa and its potential dispersal to other coffee growing countries led to the need of focusing research efforts on exploiting and characterising available genetic resources and using this information in breeding programmes. In this work, the coffee genotype Catimor 88, which exhibits field resistance in Kenya, was selected for resistance characterization to *C. kahawae*, comparatively with a susceptible variety, through an integrative cytological and molecular approach.

MATERIALS AND METHODS

Hypocotyl inoculation

Resistant hypocotyls of Catimor 88 (from Kenya) as well as susceptible hypocotyls of cultivar Caturra (CIFC 19/1) were inoculated with the *C. kahawae* isolate Que2, from Kenya. Hypocotyls at the soldier stage were inoculated according to the technique previously described with slight modifications.

Light microscopy

Conidia germination and appressoria formation were observed on hypocotyl pieces, 3h, 6h, 12h and 24h after inoculation, as previously described. For time course studies of fungal growth and plant cell responses, cross sections of infected hypocotyl fragments, made with a freezing microtome, were submitted to cotton blue lactophenol staining and epifluorescence test. Observations were made with a microscope Leica DM-2500 equipped with a mercury bulb HB 100W, u.v. light and blue light.

RNA extraction

RNA was extracted from inoculated resistant and susceptible hypocotyls with Spectrum™ Plant Total RNA Kit (Sigma) according to manufacturer's instructions. cDNA was synthesized as previously described.

Candidate reference gene selection and validation

Eight candidate genes were selected based on their previous description as good qPCR reference genes (RG) for *Coffea* spp. and for biotic stress studies: 40S ribosomal protein S24 (*S24*), 14-3-3 protein (*14-3-3*), 60S ribosomal protein L7 (*RPL7*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Ubiquitin (*UBQ*), V-type proton ATPase (*VATP16*), *SAND* family protein (*SAND*) and Ubiquinol-cytochrome c reductase complex chaperone (*UQCC*). Expression stability was evaluated by qRT-PCR (Applied Biosystems) at 12h, 48h and 72h after inoculation. NormFinder (NF) and geNorm (GN) algorithms were used to select the appropriate reference genes. Pathogenesis-related protein 10 (*PR10*) and receptor-like kinase (*RLK*) gene expression was used to validate the selected RGs.

RESULTS AND DISCUSSION

Microscopic analysis

The development of pre-penetration fungal growth stages was similar in resistant and susceptible genotypes. Conidia germination and appressoria differentiation were initiated at 3h and 6h after inoculation, respectively. At 12h after inoculation, 40-50% of the appressoria were melanised, extending to 90-100% of appressoria by 24h (Figure 1A). At this time, the

percentages of germinated conidia and appressoria formed ranged from 70-80%. In both coffee genotypes, the melanised appressoria began to penetrate inside the epidermal cells with formation of a globose infection vesicle, between 24h and 48h. Hyphae developed from the vesicle grew either intra- and intercellularly colonizing epidermal (Figure 1B,C) and cortex cells. As previously reported, in the susceptible genotype the fungus pursued its growth feeding on living host cells (biotrophy) before switching to necrotrophy, at 72h after inoculation. On the contrary, resistance was characterized by restricted fungal growth (hyphae were more frequently confined to the epidermal cells) (Figure 1C). As shown in Figure 2 the hyphal length inside host tissues was significantly higher in the susceptible than in the resistant hypocotyls at 72h after inoculation. In the susceptible hypocotyls the necrotic cells associated with the entanglement of the fungal hyphae originated by different infection sites did not allow the quantification of fungal growth inside hypocotyls tissues beyond 72h.

As shown in Table 1, in the resistant genotype, the first cytological changes were displayed at 24h after inoculation in 3% of infection sites and corresponded to autofluorescence (AF) of epidermal cell walls. At 48h the AF of both cell walls and cytoplasmic contents was detected in the epidermal cells and at 72h, this response spread to adjacent cells of the epidermis and of the first layer of cortex.

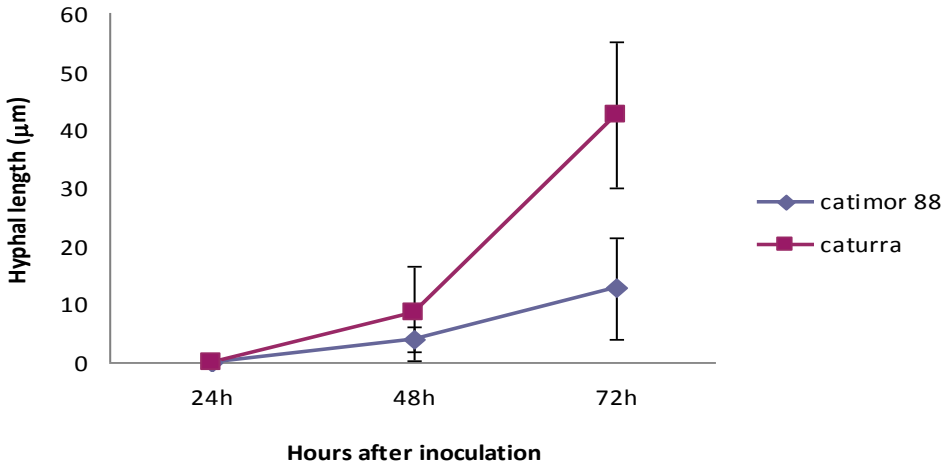

Figure 2. Hyphal length in resistant (Catimor 88) and susceptible (Caturra) hypocotyls, at different time points. Each value is the mean±standard deviation of 3 different experiences (60-80 infection sites were observed per experiment per time). The mean values of hyphal length did not differ significantly at 48h after inoculation (t= 1.49) but were significantly higher in the susceptible that in the resistant hypocotyls (t= 4.25; P≤0.001).

Table 1. percentage of infection sites with cytological responses induced by *C. Kahawae* in coffee hypocotyls, at different times after inoculation.

Time after inoculation (hours)	Autofluorescence and/or browning of epidermal and cortex cells	Coffee genotype		t test*
		Catimor (Resistant)	Caturra (Susceptible)	
24	W	3±2	1±1	1.43 ns
	w+ CC	0	0	-
48	W	19±12	1±1	3.01**
	w + CC	11±8	3±2	3.08**
72	W	14±7	1±1	5.63***
	w + CC	42±15	9±7	5.85***

W=cell wall; cc=cytoplasmic contents.
x ± SD=Mean ± Standard Deviation.
All data were pooled from three esperiments; 60-80 infection sites were observed per experiment per time.
** Student test [(ns-non significative; ** (P≤0.01); ***(P≤0,001)].*

Autofluorescence (under blue light epifluorescence) is thought to indicate the presence of phenolic-like compounds and cytoplasmic autofluorescence and/or browning is frequently associated with plant cell death (hypersensitive response - HR). These responses (AF of cell wall only and AF of cell wall and cytoplasmic contents) were also detected in the susceptible genotype, but in a significantly lower percentage of infection sites, at 48h and 72h after inoculation (4% and 10%), comparatively with the resistant genotype (30% and 54%). Cell wall modifications, including the accumulation of phenolic compounds, and the hypersensitive-like cell death have been also associated with host resistance in different plant-pathogen interactions.

Reference gene selection and validation

Based on the microscopic analysis, 12, 48 and 72 hours after inoculation were chosen for gene expression studies. From the eight RG tested, *VATP16*, *SAND* and *UQCC* were discarded due to non-specific amplification and primer dimmer formation on the amplicon region. Interestingly, despite belonging to important classes involved in cellular functioning and being previously described as good RGs for different coffee tissues, *GAPDH* and *UBQ9* were the least stable genes for coffee hypocotyls inoculated with *C. kahawae*. These results are in accordance with those obtained for common bean hypocotyls inoculated with *C. lindemuthianum*, which also pointed out ubiquitin as an unstable gene. Considering the entire dataset and the analysis by geNorm and NormFinder, *RPL7* appears as one of the most stable genes, together with *14-3-3*. *PR10* and *RLK* gene expression was assessed to validate the selected RG since they were previously described to be induced during coffee infection with *H. vastatrix*. Both genes appear induced after *C. kahawae* infection, being the expression pattern over-estimated when using the two least stable genes (*GADPH* and *UBQ9*) (Figure 3). These results confirm that a careful validation of reference genes is essential for each experimental condition. With the proper reference genes chosen, gene expression analyses will be performed to unravel the molecular mechanisms involved in coffee resistance to *C. kahawae.*

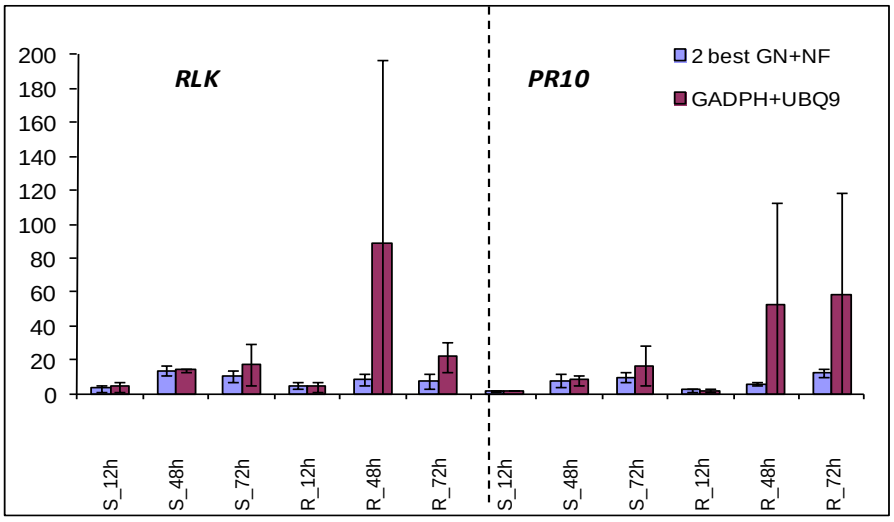

Figure 3. RLK and PR10 expression (fold change) in Catimor 88 (R) and Caturra (S) inoculated with C. Kahawae at 12h, 48h and 72h after inoculation,compared to non-inoculated samples. Two normalization strategie are presented. Median and SD values of two biological replicates are presented.

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Relative Influence of Plot and Landscape Scale Factors on Coffee Berry Borer Abundance: A Variation Partitioning Hierarchical Approach

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SUMMARY

We assessed the relative influence of plot and landscape scale factors on coffee berry borer abundance using a variation partitioning approach. We used data from 50 coffee plots, located in Turrialba, Costa Rica, which were characterized for their management, environmental, plant status and landscape attributes. Our results indicated that the number of remaining infested coffee fruits per tree after harvest was the most important variable with 22 % of the coffee berry borer abundance variation explained, followed by the number of fruiting nodes (16 %), altitude (associated to temperature, 13 %), and in a lesser degree, shade type (11 %), spatial dependence between plots (9 %) and proportion of pasture in a 400 m radius (7 %). Our results indicate that picking the remaining infested coffee fruits after harvest is the most important cropping practice for the control of coffee berry borer. However, control can be maximized by integrating plot and landscape scale management activities.

INTRODUCTION

Romero, Romero *et al*. and Avelino *et al*. demonstrated that the abundance of coffee berry borer *(Hypothenemus hampei*; Coleoptera, Curculionidae) in coffee plots was positively correlated to the proportion of coffee area in the landscape, possibly because coffee berry borer (CBB) survival was improved during the postharvest period in landscapes with more resources. This result raised the question of the importance of landscape effects on the pest in comparison with effects of plot scale factors, particularly those related to pest control practices. We studied that question through a variance partition approach.

MATERIALS AND METHODS

We used data from Romero, where 50 coffee plots (120 coffee trees each) from the Turrialba region (Costa Rica) were characterized for their management, environmental, plant status and landscape attributes, as well as for the CBB abundance. Turrialba is located within the Volcánica Central-Talamanca biological corridor. This region is propitious to CBB development. Mean annual rainfall is 2700 mm with no marked dry season and annual average temperature is 22 °C. Flowerings are multiple, spreading normally from December to May, and of low intensity, resulting in multiple fruit cohorts and harvest rounds from July to December.

Analyses were performed in two steps:

- We first employed a variation partitioning technic based on a redundancy analysis. Our purpose was to assess the relative contribution of groups of variables, characterizing management and environment, to CBB abundance variance.

- We then applied a hierarchical variation partitioning technic based on multiple generalized linear models. Our purpose was to assess the individual contribution of each variable to CBB abundance variance.

RESULTS

Relative influence of environment and management on CBB abundance (variation partitioning technic

Variables belonging to the environment group, together, explained a larger variance proportion of CBB abundance than those belonging to the management group (Figure 1c). Within environment (Figure 1a), elevation contributed most to total variance (F=18.7, P=0.005), followed by annual rainfall (F=5.9, P=0.010), landscape context (F=5.8, P=0.017), geographic distance between plots (space, F=3.7, P=0.025) and soil chemical and physical characteristics (F=3.8, P=0.015). Within management (Figure 1b), the initial CBB abundance showed the larger contribution to the total variance of the CBB abundance (F=33.6, P=0.005), followed by the plant status group (F=23.5, P=0.005), the coffee plot structure (F=6.0, P=0.005) and cropping practices that did not show a significant contribution.

Relative influence of individual variables on CBB abundance (hierarchical variation partitioning technic)

The initial abundance of CBB (i.e. the number of bored berries remaining on the plant after harvest) was the variable with the larger contribution to the total variance, followed by the number of fruiting nodes, shade type, elevation and percentage of the area with pasture in a radius of 400 m (Figure 2).

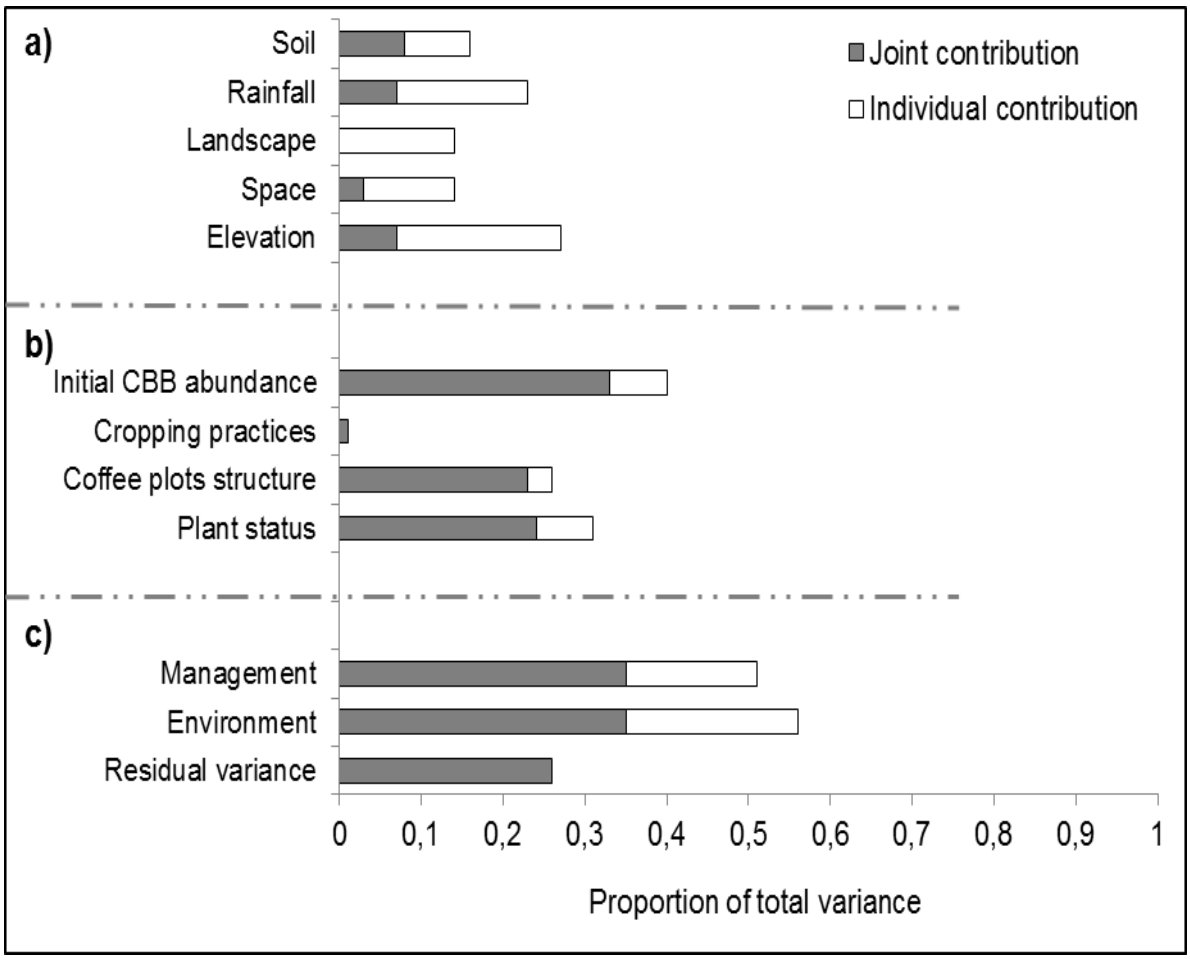

Figure 1. Total (full bar), individual and joint contributions of a) environmental factors, b) management factors, and c) the whole set of environment and management factors, to CBB abundance.

DISCUSSION AND CONCLUSIONS

Our results indicate that picking the remaining infested coffee fruits after harvest is the most important cropping practice for the control of CBB. Landscape context attributes also explained the abundance of CBB, but their low contribution to the total variation of the pest abundance suggests that control practices at landscape scale should only have slight effects on CBB abundance and therefore on its control. However, the amount of variance of the CBB abundance that is jointly explained by management and environment variables (including metrics of landscape context) suggests that control of CBB can be maximized taking into account both scales (landscape and plot).

Splitting the variance of the CBB abundance according to the unique and joint contribution of environment and management variables can improve the understanding of the patterns and processes that contribute to the CBB abundance and re-focus the control strategies.

Partition of variance is a useful tool to assess the relative contribution of factors at multiple scales.

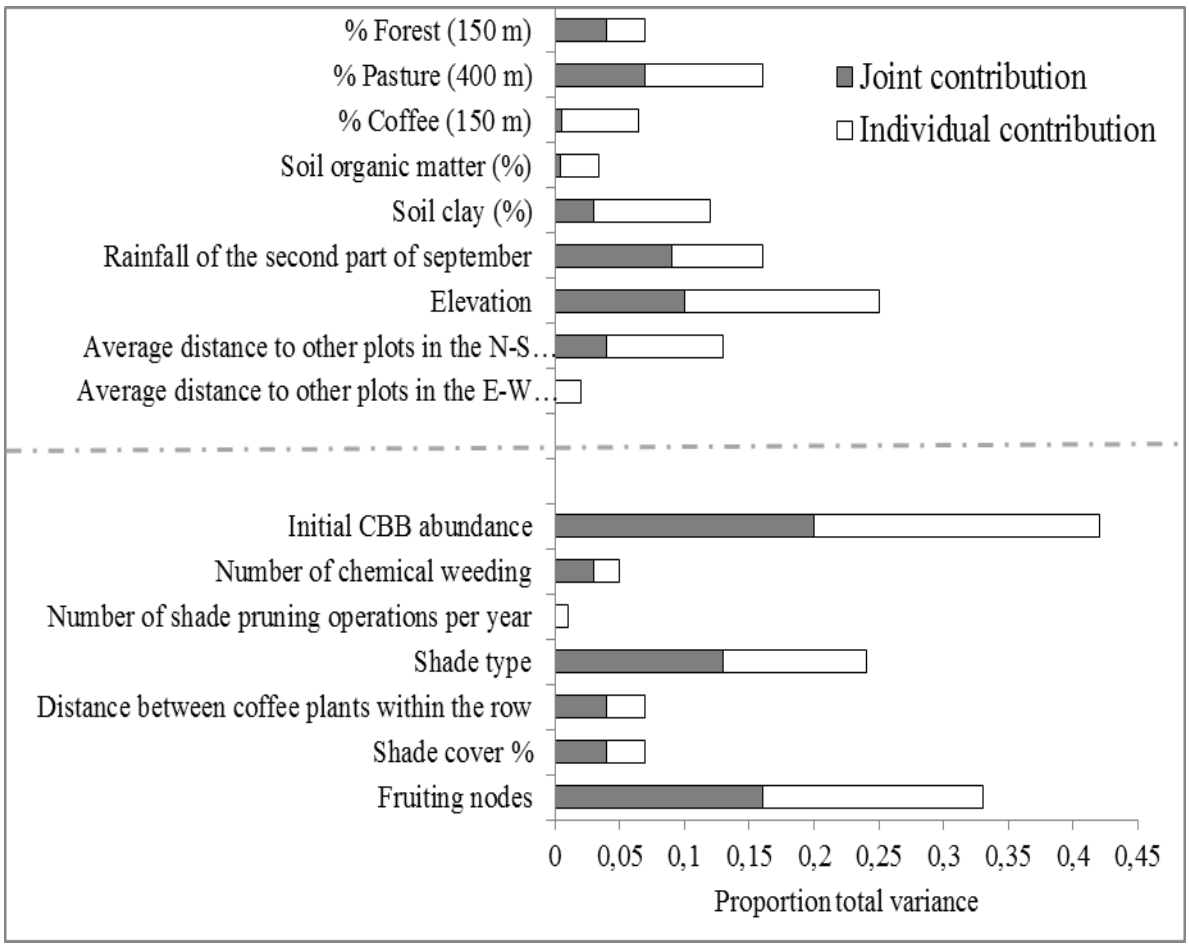

Figure 2. Total (full bar), individual and joint contributions of specific variables to CBB abundance.

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The Changing Climate for Sustainable Coffee

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SUMMARY

There is growing evidence that climate change is affecting coffee production; the lower limit for growing coffee has risen visibly in some countries, the proportion of Robusta to Arabica is increasing in others and reports of extreme weather encountered by coffee farmers are becoming common.

Climate change poses a severe challenge to our current concepts of sustainable coffee production. Over the past two decades various sustainable schemes have arisen to provide the consumer with ethical and environmentally friendly products. These schemes however were designed and instigated before the threat of climate change became apparent and this presentation argues that a major rethink is now required if they are to cope with rapidly changing conditions in the field. There are two main reasons – scale and time:

- Scale: the farm is the unit of certification, but various environmental challenges can only be partially combatted at the farm level. Certifications do not deal with wider problems such as depletion of ground water (e.g. Vietnam), erosion and landslides (e.g. Uganda), proliferation of diseases (much of Africa) and deforestation (e.g. Latin America and SE Asia).

- Time: over the past ten years certified coffee has grown from 1% of global sales to 10%. But during that period, volume of coffee produced has increased by more than this, with much of the extra supply coming from newly planted land, some of it recently deforested.

Thus the current commercial trends in sustainability are too small and too slow to cope with the level of change now becoming apparent in many coffee countries. A shift away from farm-scale towards landscape-scale issues that identify and remedy major limiting factors to production now needs to be considered. Standards cannot provide comprehensive solutions that accommodate the wide range of local and varying threats to production and livelihoods that coffee farmers are increasingly facing. This means defining limits for what standards can reasonably achieve and establishing roles and modalities for other stakeholders to ensure effective and long-term support for hard-pressed farmers.

INTRODUCTION

Are sustainability standards working? That is, are they delivering long-term sustainability for global coffee – in the sense that stakeholders (farmers through to consumers) can have some confidence that the various threats to coffee farmers' livelihoods are being fully addressed?

This is not an easy question to answer. There is no denying that sustainable certifications have had a major impact on the coffee industry. Over the last 15 years they have grown from a tiny industry niche – less than 1% of global production – to a position where they now account for

about 8% or more of sales But what impact has this growth had on the range of complex issues that encompass the concept of sustainability?

There are two categories of questions that need to be asked about standards' effectiveness:

- Do they deliver improvements in sustainability according to their own criteria?
- Are they coping with the major global threats to sustainability that are getting steadily worse due to (*inter alia*) population pressure and climate change?

This presentation deals with these two questions, especially in regard to the increasing reality of climate change that now is starting to severely test the concepts of sustainability. So we start here by looking at the first question:

DO SUSTAINABLE CERTIFICATIONS DELIVER SUSTAINABILITY ACCORDING TO THEIR RESPECTIVE CODES?

Sustainability is usually divided into three broad categories – environmental, social and economic. A recent major review of standards states the following:

"It is difficult to firmly attribute observed sustainability impacts to certain practices, and thus to determine whether standards systems are achieving their intended objectives.' 'The effects of standards on yield and quality are variable and difficult to attribute to the standard per se, since most study designs are confounded by possible differences in preexisting conditions."

In the case of coffee, we have evidence that standards do have positive effects. Thus in Costa Rica, organic certification improves coffee growers' environmental performance by significantly reducing chemical input use and increasing adoption of some environmentally friendly management practices. Advantages also accrue to Peruvian Fair Trade cooperatives, though differences can be quite subtle. Likewise for Mexican Fair Trade coffee advantages are measurable but often quite small.

Others however conclude that '*benefits for producers remain unclear'*, or even, in the case of Nicaraguan Fair Trade producers, farmers failed to increase per hectare profits or per capita incomes and that over a period of ten years, organic certified producers became relatively poorer than their conventional neighbours.

There are rather few comprehensive studies of the environmental effects of certified coffee. One of the most detailed found no significant differences between farms participating in organic and/or Fair trade certification programs and uncertified farms in terms of shade management or species richness of ants and birds.

As one recent review of sustainable standards concluded:

"*The overall conclusion […] it is hard to draw firm conclusions, and that there is little evidence to support strong inference that certification schemes do impact on sustainability in a positive way*".

Yet another review found a number of weaknesses in the existing literature on the sustainability of coffee production which focuses exclusively on narrow measures of sustainability such as the presence of shade and the use of small, non-random samples that do not control for confounding factors. As that review indicates, the central difficulty of all studies to date is the 'counterfactual' problem – i.e. they are all essentially *post hoc* studies

that deal with a self-selected group (farmers who have decided to joint certification schemes) that are then retrospectively compared to farmers who have not joined the schemes.

Hence from a strict scientific viewpoint, the evidence for sustainable standards delivering significant and reliable improvements, even according to their own criteria, is far from compelling. It seems very likely that some aspects of sustainability are improved by certification in some cases, but this does not constitute measurable and significant progress in environmental, social or economic improvement at scale.

A major unanswered question is whether the entirety of sustainable farms constitutes more than the sum of their parts. Ecological theory, in the shape of the species-area law for instance, would militate against many small parcels of coffee land having significant overall impact on species survival. Another question is whether the total cost of sustainable certifications (a very large but unknown sum borne mostly by farmers and consumers) is money well spent.

It is to these broader issues of sustainability and coffee's global significance and value that this paper now turns as it examines other aspects of sustainability that have not been well addressed by sustainable certifications.

COFFEE AND GLOBAL SUSTAINABILITY

There are a number of serious global challenges that face humanity, caused primarily by increasing human populations and the pollution they create. These include climate change itself as well as growing water shortages through intensive groundwater extraction, water pollution, energy shortages, biodiversity declines, soil erosion, and pests and diseases, both indigenous and invasive. Many of these have become noticeably more problematic over the past decade or so and coffee production could variously be both a cause and a victim of these changes. The substance of this presentation is that the coffee industry can only claim it is serious about sustainability if it is addressing these fundamental issues: hence through its current focus on farm-by-farm certification (a micromanagement or 'micro-sustainability' approach) it is not addressing global concerns (a 'macro-sustainability' approach) that may well be more important.

So how acute are the problems? The simple answer is that we do not know because we cannot quantify them; the necessary mechanisms are not in place to provide accurate data and few coffee scientists or sustainability professionals are concerned about them. Because of limited space, the following only briefly covers some of the issues that need addressing.

Land use and land use change

Basic data about the amount of land dedicated to coffee production is uncertain. The area can be estimated from FAOSTAT, which shows a decline since 1990, even though during this period global production has risen by over 50% (Figure 1). We know that production has risen sharply in a few countries and fallen in others. Through inspection of national production figures it is certain that a substantial proportion of increases must have come from extending the growing area of coffee. There have also been productivity increases in some countries such as Brazil but the extent of this contribution cannot account for more than a fraction of global increases. It seems likely that:

- National reporting of total coffee land area is in some/many cases inaccurate.
- Total land-flux of coffee (i.e. amount entering and leaving coffee production) is rising due to abandonment of coffee production at low altitudes due to global warming and occupation of new land to supply increasing demand.

Accurate data on these issues is urgently needed if we are first to describe coffee's changing global footprint and then seek the most cost-effective ways of maintaining maximum sustainability.

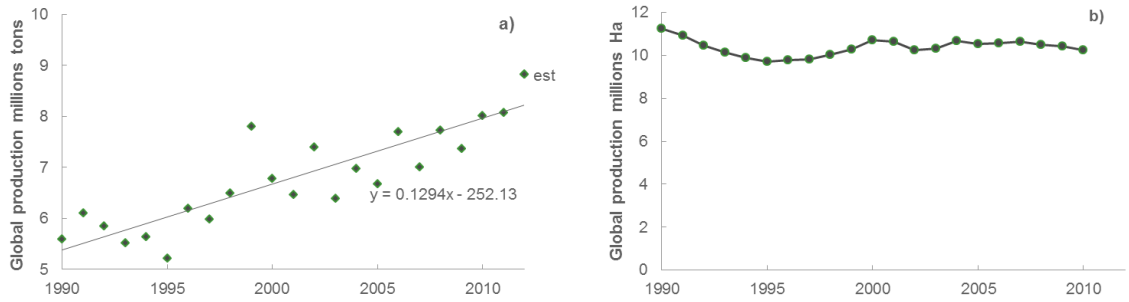

Figure 1. Global coffee production since 1990. a) tonnage (www.ICO.org data); b) area (FAOSTAT)

Deforestation and carbon

Scientific data is scant but there are enough press and unpublished reports to suggest that there are substantial plantings of coffee on recently deforested land. The yearly accrual of this new land is likely to be of the same order of magnitude as any certification of newly shaded coffee lands. Because the biodiversity and carbon lost from deforestation is greater than can be replaced by shade coffee, it seems almost certain that coffee is becoming less sustainable at the global level in regard to biodiversity and carbon footprints.

Research is needed to assess coffee's global carbon footprint and determine where the greatest fluxes of carbon (release and sequestration) are occurring. Only when that data is available could an effective plan be made to reduce carbon footprint. I.e., it may well be more cost-effective to concentrate on reducing carbon losses (through restricting deforestation and increasing per hectare productivity) than by encouraging many small local carbon sequestration projects that will have little overall impact on climate change. Lifecycle analysis is currently used by the coffee industry to assess carbon footprint, but such analysis is limited to the visible coffee chain and does not take into account land use change. Such limitations need to be addressed in the future to more accurately reflect full carbon fluxes.

Research is also needed to assess the long-term effects of certification on enhancement or protection of biodiversity, as compared with biodiversity loss through deforestation. I.e., it might be more cost-effective to dedicate premiums from sales of sustainable coffee to protect or buy forested land than to spend it on shade projects.

Water use

Substantial water pollution occurs through wet processing and there is no evidence that sustainable certifications have had a significant effect on cleaning up streams and rivers; for

example, there is no indication that certifiers or any *post-hoc* evaluators collect water samples and measure pollution levels during and immediately after the harvesting period.

Irrigation is common in several major coffee countries, including India, Brazil and Vietnam. In Vietnam there is evidence that farmers are using unsustainably high amounts of groundwater, yet there seem to be no provisions to assess this in any current sustainable certification scheme. Indeed the ability of audit regimes to improve sustainable water management has been seriously questioned with suggestions that over-extraction can only be addressed through catchment-wide planning initiatives that go far beyond the current capabilities of sustainable schemes.

Because of increasing global shortages of water in many areas, there is therefore an urgent need to reassess coffee's troubled relationship to water and consider large scale means to reduce pollution and often excessive water wastage. Technology exists that can fix these issues, but currently sustainable certifications take insufficient steps to evaluate problems and introduce solutions that have real catchment-scale benefits.

Pests and diseases

These cause substantial but largely un-quantified losses to coffee production that inevitably increase coffee's global environmental footprint. Recent upsurges in Coffee Leaf Rust (CLR) have affected more than 30% of Colombia's national production. In Africa, Coffee Wilt Disease (CWD) may have caused more than one billion dollars' worth of losses to farmers over the past 20 years. However, there are no attempts by standards setters to limit the spread of these diseases or their effects on farmers. Hence standards do not attempt to investigate or improve the effectiveness of quarantine services. Nor have they actively promoted CLR-resistant varieties that could have ameliorated serious outbreaks in Central America and Colombia in recent years.

Major erosion and landslides

Landslides have caused the deaths of hundreds of coffee farmers and their families over the past five years. Because of increasingly intensive rainfall, erosion problems will get worse. Many of the major events give prior signals of failure, but cannot be tackled at the farm level, i.e. through farm-level certification. This is a classic landscape-level issue that requires a different approach to sustainability.

Biodiversity

There is no credible evidence that sustainable certifications are having material effects on protecting biodiversity and endangered species. Indeed, one of the original reasons to promote shade coffee – to protect neotropical bird migrants whose over-wintering habitats were threatened by intensified coffee – was overstated. More recent work found that the abundance of birds recorded in the N. American Breeding Bird Survey decreased by up to 18% between 1966 and 2005. But on disaggregating this data the abundance of US and Canadian resident species was found to decrease by 30%, and that of migrants within the US and Canada decreased by 19%. By contrast, neotropical migrants increased by up to 20%. It is evident from this and studies referred to previously, that coffee's relationship to biodiversity is much more complex than originally assumed. Moreover, even though un-shaded coffee supports less biodiversity than shaded coffee, climatic strictures (e.g. increasing humidity leading to increasing fungal diseases) mean that re-shading is not a serious agronomic option and the alternative – turning coffee lands into pasture – is a much worse option.

Global impact of sustainable certifications

Without better quality data it is difficult to assess the global impact of coffee production and because of this, any ameliorating impact of sustainable certifications is likewise difficult to evaluate. The available data however would suggest that the overall impact of certifications on global coffee is still small. We know that over the past 15 years, certifications of all types have risen to about 18% of world production of which about a half is actually sold as such. But during this time, coffee production has expanded by more than 20% - so there is actually more uncertified coffee than there was when certifications started. And because we do not know the total flux of coffee lands, we are unable to calculate the total land use change caused by coffee during that time.

Scale and time

To play a significant role in global sustainability, standards will need to be adopted widely within a relatively short time-frame – i.e. substantially faster than the rate of new coffee land expansion. In order to achieve this they would have to roll-out a simple set of universal codes. However as a recent study points out, "*attempts to reduce the management issues of complex landscape-level processes to a supply chain compliance checklist will do little to address critical sustainability issues in producing regions".* The argument is that supply chain audit regimes are fundamentally ill-suited to the requirements of natural resource management, which are socially and ecologically embedded within local landscapes in diverse and intricate ways. This view is echoed from a new and masterly treatise on standards which questions the validity of globally mandated standards. Indeed to build standards that are fair, equitable and effective, a principal recommendation is to allocate the responsibilities for policy formation and implementation to the lowest level of governance possible. This means that local knowledge, customs and norms take precedence over that from some larger geographic area unless there is a compelling reason why this should not be the case. In the case of coffee standards therefore, if they are to be effective, they need to be tailored to local needs and conditions, i.e. a problem-oriented focus which militates against a universal standard regime.

CONCLUSIONS

Coffee is a complex business. It is a component of many systems –climate, land use, water, carbon and nutrient cycles, biodiversity and ecosystem services, as well as trade and geopolitical systems. Many of these systems are undergoing fundamental change, with population pressure as the ultimate driver. It is therefore vital to acknowledge and understand them and how they interact to affect the coffee industry. Standards aim to codify behaviour of the coffee system – this is a noble undertaking but to date they have been highly partial and limited in what they have managed to achieve. The main contention here is that standards have failed to take a fully scientific approach to deal with the inherent complexity of interacting systems. And there is a danger that the industry will come to rely upon them for issues of scale for which they were not designed. As such they cannot be expected to do the 'heavy lifting' to secure the long-term sustainability of the coffee industry. A more scientific and fully quantifiable approach to both standards and sustainability is required, drawing on a range of disciplines and making full use of a systems approach.

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Integrated Climate Change Impact Modeling of the Coffee Sector

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SUMMARY

Here we present a spatially explicit integrated climate change impact assessment of the coffee sector using the global recursive dynamic partial equilibrium model of crop, livestock and forestry production GLOBIOM. We define two production systems for green coffee, labeled Arabica and Robusta, employing the agro-ecological niche model Maxent. Using this machine learning based algorithm we spatially disaggregate FAO production data and estimate current and future yield potential for the coherent integration in the GLOBIOM baseline data. The results indicate a likely regional shift of coffee production towards South East Asian regions, a higher Robusta share in the production and a concentration of production in the remaining areas with highly favorable climatic conditions. As such areas are projected to migrate to higher altitudes in the future this raises concerns over increased deforestation pressure.

INTRODUCTION

Pro-active adaptation to climate change anticipates future risks and attempts to benefit from opportunities by taking actions before major damage is experienced. This is in contrast to re-active adaptation that takes place after impacts are experienced (Füssel 2007). Various biophysical impact models project a highly negative impact of climate change on the coffee crop with the likely result of reduced global yields (e.g. Gay Garcia et al. 2006, Zullo et al. 2011, Davis et al. 2012). As plantations have a lifespan of several decades and adaptation strategies such as breeding efforts are difficult to implement there is a compelling argument that adaptive action in coffee production must be pro-active and needs to be initiated now. Thus, there is a need for research in the global coffee system that effectively guides adaptation policy. Such climate change impact assessments should include global market feedbacks into the analysis as this enables the prioritization of adaptation pathways based on economic efficiency considerations (e.g. Josling et al 2010).

This work represents the first integrated climate change impact assessment for the coffee sector using a combination of agro-ecological niche modeling and the global recursive dynamic partial equilibrium model GLOBIOM. The GLOBIOM model has been designed to provide policy analysis concerning land use competition between major land-based production sectors such as agriculture, bioenergy and forestry. It combines spatially explicit information on land uses, agronomic and forestry potentials as well as production costs with regional demand and bilateral trade flows. A general description can be found in Havlik et al. (2010). A description of the effect of demand side changes can be found in Schneider et al. (2011) where the model is used to research the effects of population growth on land use change. However, like other models GLOBIOM does not include a model of the coffee sector despite the importance of this crop in regions that are likely to be affected by population growth, climate change and increasing livestock product demand and are thus deforestation hot spots (van Vliet et al. 2012). The explicit inclusion of coffee production into the model is

therefore likely to give additional insights into the dynamics of land use change and the effects of climate change on food security.

METHODS

The crop model in GLOBIOM requires as inputs spatially explicit production statistics and current yield potentials to model the spatial equilibrium of price and quantity at regional levels. In our scenario future yield potentials as a result of climate change and technical progress and future demand are imposed and the new equilibrium is computed. GLOBIOM uses the MapSpam database of crop distribution (You et al. 2012), and the EPIC model (Williams in Singh 1995) to estimate yield potentials. Future productivity changes are estimated based on ordinary least squares regression on FAO time series for the years 1980 to 2010. Demand is modeled as in Alexandratos et al. (2006). To include a coffee model into GLOBIOM it is therefore necessary to model current distribution of coffee production, current yield potentials, future yield potentials, and future productivity and demand changes. We use a modified Maxent approach as described by Ovalle et al (2012) to model distribution and climatic yield potential.

The Maxent ecological niche model

We define two Maxent models for each coffee species (*Coffea arabica* and *Coffea canephora*), one to disaggregate harvested area statistics, the other to model yield potential. The Maxent ecological niche approach assumes that a species can be found in places where environmental conditions are suitable. It is differentiated between the potential and the realized environmental space. The potential distribution is the abiotic environmental space where the species could theoretically exist; the realized distribution is further restricted by biotic and migrational constraints. Assuming that coffee is produced in regions where climatic yield potential is high, we argue that it is possible to estimate coffee yield potential from the climatic suitability index as modeled by Maxent when the model only uses climatic variables. This model is similar to the one presented in Ovalle et al. (2012). As further conditions for actual presence of coffee production are arguably the presence of agricultural land and market incentives we attempt to approximate the realized distribution by supplementing the climatic variables with spatially explicit variables for land use intensity and market access as a modeling basis for Maxent. We use this agro-ecological suitability model as a prior probability distribution for the disaggregation step.

As climatic input rasters we include the 19 bioclimatic variables that can be downloaded from the Worldclim database (Hijmans et al. 2005). Some authors argue that spatial multicollinearity should be eliminated from the input variables by means of principal components analysis to avoid overestimation of the influence of a single variable. Others disagree as such an approach would result in variables that are less meaningful for results interpretation and argue for the use of only variables that have a known influence on the modeled species. We argue that this neglects the possible influence of biotic constraints which could implicitly be modeled by the inclusion of the entire bioclimatic variable dataset. Maxent tests and weighs each variable in a jack-knife test to determine the final suitability function. We rely on this approach to limit coefficient overestimation and include all 19 bioclimatic variables.

Globiom has previously been applied for a climate change impact study. Yield potential changes where calculated using the EPIC model for the CNRM CM3, MRI CGCM 2.3.2 and UKMO HadGem1 global climate models for the A2 scenario (ofter referred to as “business as

usual scenario"). We therefore run Maxent projections on downscaled climate data for these models as described in Ovalle et al. (2012).

Yield potential and technical progress

The Maxent model that only employs climatic variables is our basis to estimate yield potential. In the literature stated values for maximum yields under optimal management for Arabica are estimated to be around 2000kg/ha and about 2300kg/ha for Robusta systems (Wintgens 2009). We assume that such yields can be achieved in locations with optimal climatic suitability. The Maxent output based on bioclimatic variables only is an estimate of the likelihood that the climate at a specific location is suitable for a species. We thus use equation [1] to calculate spatially explicit yield potentials for the Arabica and Robusta systems both for current and future climatic conditions.

$$Y_i = Y_{max} \times (1 - (1 - Suit_i)^2) \quad (1)$$

Y_i is the yield potential in cell i, Y max the maximum achievable yield and $Suit_i$ the suitability index from the Maxent model in cell i. The resulting function is shown in Figure 1.

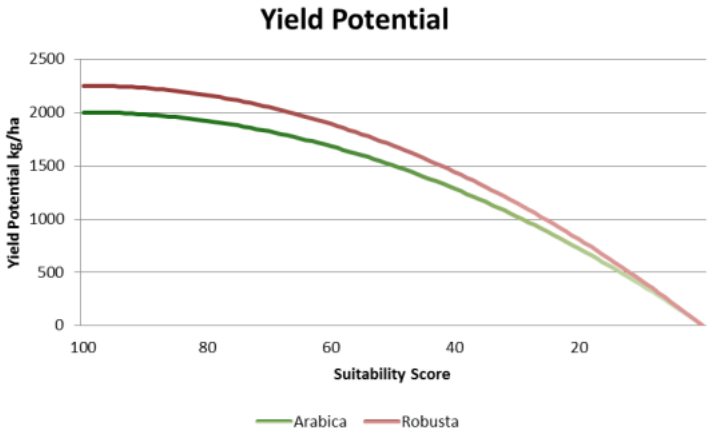

Figure 1. Yield Potential and Suitability

As the yield potential distribution for Arabica production systems is identical to the one shown in Ovalle et al. (2012) here only the Robusta data is shown. The current distribution is shown in
Figure 3.

For each Globiom simulation unit the relative change of yield potential is calculated and included in the model scenario. The mean change of yield potential of units with a potential above 200kg/ha by 2050 for Arabica is -57% and -21% for Robusta. However, we assume a global increase of one percent per year for all simulation units and production systems.

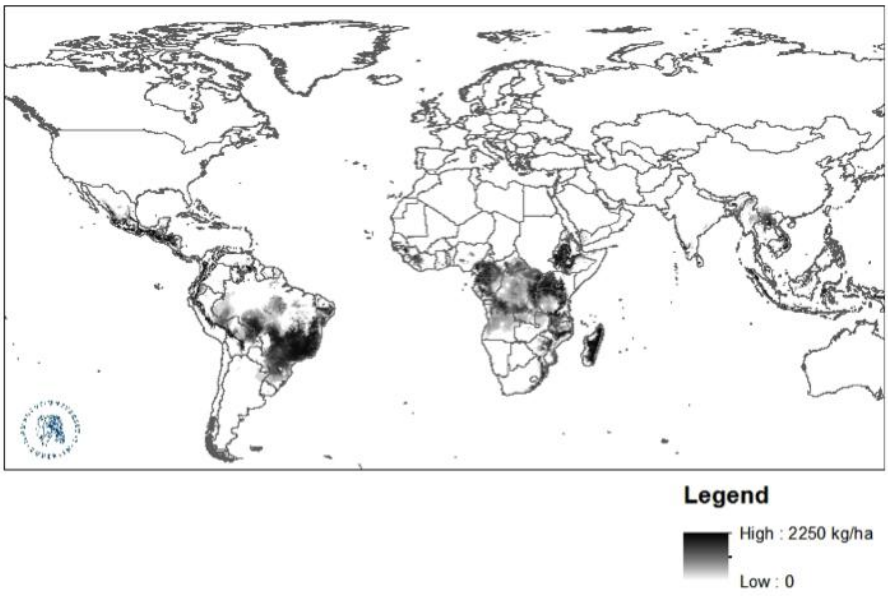

Figure 2. Current distribution of Robusta yield potential.

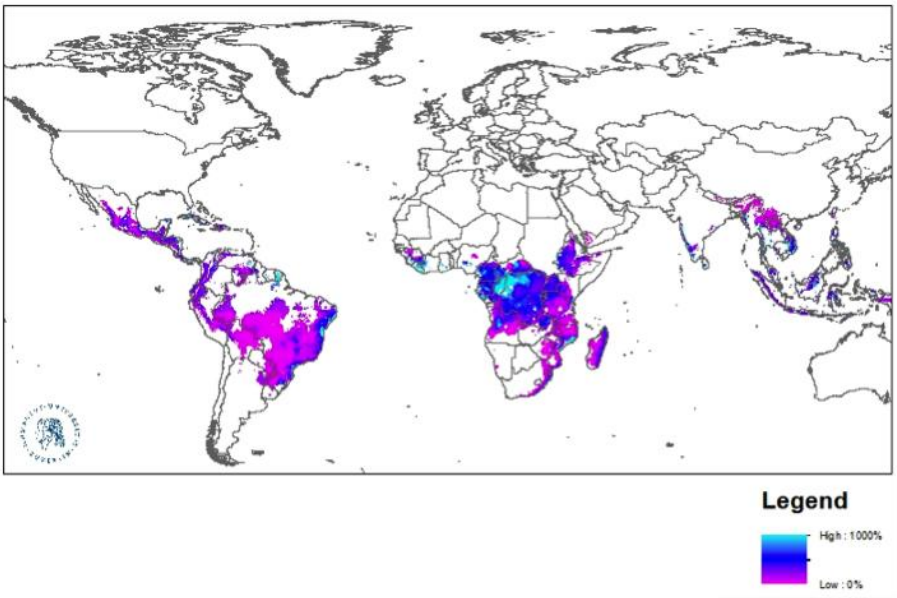

Figure 3. Relative yield potential for Robusta UKMO HadGem1 A2/2050.

Downscaling of FAO data

GLOBIOM makes use of the MapSpam database of spatially disaggregated national and subnational crop production statistics. This database allocates acreage and yield values to global grid cells. This is done based on a variety of input data such as land use statistics, population statistics, and agro-ecological suitability. Each crop can be produced in four prototypical production systems that are described as subsistence, low- and high input rainfed and irrigated. A detailed documentation can be found on MapSpam.info (You et al. 2012). Even though MapSpam already features coffee data this is limited to "Green Coffee" production in the aforementioned prototypical production systems.

We argue that for a climate change impact assessment of the coffee sector a differentiation between Arabica and Robusta production systems is more meaningful than the MapSpam "green coffee" aggregation. The two coffee species differ in the range of environmental conditions in which they prosper while no such clear distinction can be found between low- and high input production systems. In order to integrate coffee coherent with this database into GLOBIOM we therefore implemented a process similar to the MapSpam model for these systems. We recognize the widely employed systems description for Arabica production by Moguel and Toledo (1999) and intend to improve our work in this direction in future work as shade cover and irrigation extend the environmental range for both species.

As GLOBIOM largely uses FAO data we download national level production statistics (Total production and Area harvested) for "green coffee" from FAO (2012) for the years '98-'02 and average over this period. We divide this accumulated dataset into Robusta and Arabica production systems by calculation production shares by system based on the USDA statistics (USDA 2012). This second dataset distinguishes between Arabica and Robusta production but cannot be directly used as no acreages are reported. We assume a 25% higher yield for the Robusta system per hectare harvested, calculate harvested area shares and apply these to the FAO data.

To model the prior probability distribution we supplement the climate variables from WorldClim with the GLC2000 Global Land Cover dataset (European Commission 2003) and spatially explicit market data taken from (Verburg et al. 2011). This is intended to model the dependency of commercial coffee production on agricultural land and market access. The resulting probabilities of presence are used as a prior probability distribution. We minimize the sum of the squared differences between the share of area A in a cell i of the total area in a country and the share of the suitability score of cell i and the sum of suitability scores of a country.

$$\min_{Country} \sum_i \left(\frac{A_i}{\sum_{Country} A} - \frac{Suit_i}{\sum_{Country} Suit} \right)^2 \quad (2)$$
$$\text{s.t.} \sum_i A_i = A_{Country}$$
$$\sum_i A_i - A_{Available} \leq 1$$

This is subject to the conditions that the entire harvested area is spatially disaggregated and that only available cropland is assigned. Available cropland is calculated from the Globiom database to ensure that no conflicts are created.

Future demand

Demand is modeled as a function of GDP per capita with elasticities defined for low- middle- and high income countries. The classification of countries follows the World Bank definition as in Soubottina (2004). We assume a GDP per capita elasticity of 0.3 for low income countries, 0.4 for middle income countries and 0.6 for high-income countries.

MODEL RESULTS

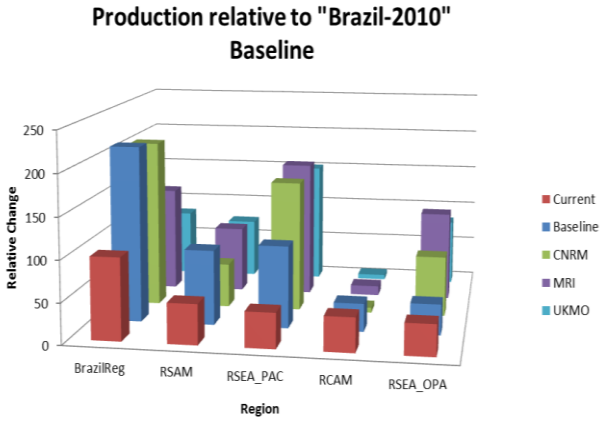

Figure 4. Regional shift of coffee production by climate model.

The model projects a demand increase until 2050 by about 130% while prices could decrease by 10 to 25% depending on the climate change model output. Figure 4 shows the regional shift of production of coffee until 2050 relative to the Brazilian 2010 model year production. While in the baseline no-CC scenario Brazilian production would double, under climate change production shifts increasingly towards the South East Asian region (RSEA_PAC).

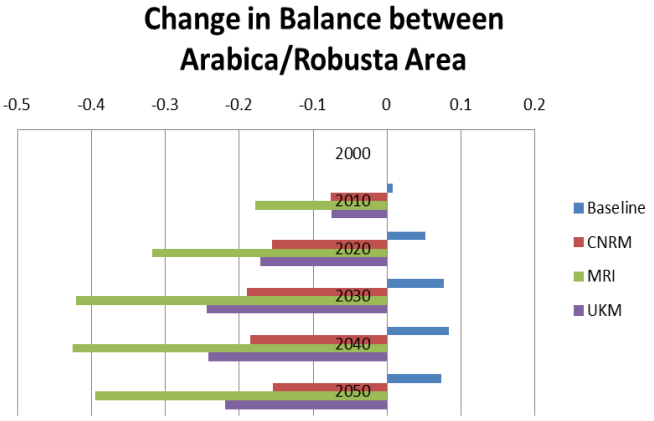

Figure 5. Change in Balance of Arabica/Robusta area by scenario.

As the Arabica production system is much worse affected by climate change than the Robusta system there is a shift towards the latter. In the baseline no-CC scenario Arabica production would even further increase. In the MRI CGCM 2.3.2 model scenario the shift is most pronounced and by 2050 75% of production would stem from Robusta systems (Figure 5).

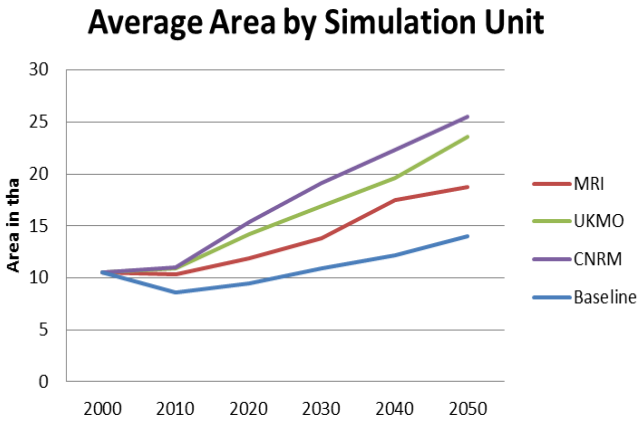

Figure 6. Development of average area for coffee production in simulation units.

Another key trend that can be observed in the climate change scenarios is the increasing concentration of coffee production in fewer simulation units. Figure 6 shows the average area devoted to coffee production by simulation unit. Under the baseline no-CC scenario the average area would remain almost constant. Depending on the scenario the concentration in fewer simulation units may almost double by 2050 compared to the baseline.

DISCUSSION

This study presents the methodology and the initial results of a spatially explicit integrated climate change impact assessment of the coffee sector using the global recursive dynamic partial equilibrium model of crop, livestock and forestry production GLOBIOM. We integrate two production systems for green coffee, Arabica and Robusta employing the agro-ecological niche model Maxent. Using this machine learning based algorithm we spatially disaggregate FAO production data and estimate current and future yield potential for the coherent integration in the GLOBIOM baseline data. This model allows us to gain new insights into climate change impacts in coffee production. The production follows the shift of climatic suitability for coffee production form South American locations to South East Asia. Furthermore, an increasing concentration of coffee production in areas with suitable conditions may be observed. As such regions can increasingly be found in higher altitudes (Ovalle et al. 2012) this finding indicates that deforestation in favorable sites could be driven by coffee demand.

Our model in its current states lacks the integration of water demand restrictions which has been mentioned as a concern for South East Asian locations. Additionally we intend to integrate data on carbon stocks and carbon emissions of the systems in order to enable the modeling of trade-offs between adaptation and mitigation strategies. Also a further specification of production systems into high and low input systems and or irrigated systems could further improve the accuracy of results. Currently such work could be limited by data availability. On the demand side we intend to differentiate demand into Arabica and Robusta varieties to allow for industry specific future scenarios.

Our work points out the importance of a systemic view of sustainability. Even locally sustainable production techniques may result in unintended natural resource exploitation in other regions by market feedback effects if demand cannot be met otherwise. The assumed increase in global yield by 1% per year will only be possible if there are increasing investments in coffee research. Unless this goal can be met the indicated impact on

deforestation and sustainability and the general transformation of the sectors structure may well be more drastic than discussed here.

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Greenhouse Gases Emissions of Green Coffee Production in the State of Minas Gerais, Brazil: Measurement and Mitigation

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SUMMARY

In recent years, the debate about environmental impacts and the sustainability of agricultural products has increased. Consumers are more and more concerned about the quality of food products and now looking for those with a low environmental impact, with a particular attention to carbon emissions. There are few studies regarding the greenhouse gases emissions associated with the Brazilian green coffee production. Located in the southeast region, Minas Gerais is the largest coffee-growing State in Brazil. It accounts for nearly 70% of the national coffee production. The objective of this study was to determine the main greenhouse gases (GHG) sources and to measure the carbon footprint of coffee production in the State of Minas Gerais. Three coffee farms located in the three typical production regions of Minas Gerais were selected: Cerrado Mineiro (CM), Matas de Minas (MM) and Sul de Minas (SM). Data from crop years 2009/2010 and 2010/2011 were considered. The carbon emission sources considered within the coffee farm were mobile and stationary combustion, nitrogen and organic fertilizers, lime and electricity. The upstream emissions related to the production and transport of agricultural inputs have not been accounted. Total emissions were different in the three different regions. However, besides the total emissions during a crop year, it is important to consider the emissions per unit of green coffee and per area cultivated with coffee in order to give a more accurate representation of the impacts in the production system. Total emissions have been therefore weighted by quantity of green coffee produced and by production area. Results show that the major source of carbon emissions was nitrogen soil fertilization. Fuel consumption for farm operations and lime application are also shown to be important. Electricity was not significant as most of the energy supply in Brazil comes from low GHG emission sources (e.g. hydroelectric and biomass). Additionally, some mitigation strategies related to nitrogen fertilization were proposed, such as the substitution of urea for lower GHG emission sources (e.g. ammonium nitrate) and the correction of fertilization rates and methods. This study is to be considered as a pioneering initiative in Brazil and it intends to provide baseline carbon footprint data, upon which emissions reduction plans and further research can be built. With these results it will be possible to design management strategies to promote sustainability along the coffee production chain and to add value to the final product.

INTRODUCTION

In recent years, the debate about environmental impacts and the sustainability of agricultural products has increased. Consumers are more and more concerned about the quality of food products and now looking for those with a low environmental impact, with a particular attention to carbon emissions.

Coffee has marked out the route for sustainability of tropical crops already back in the eighties. This tradition has grown inside the coffee market giving life to many sustainability voluntary and corporate standards and in general putting the issue of sustainable coffee production on the table of the entire sector. Statistics show that today 9% of the total coffee consumption is certified as sustainable and 16% of the global coffee production is actually certified as sustainable (The Coffee Barometer, 2012). At recent growth rates and in light of big players' claims, in 2015 market growth is expected to reach 20-25% of the global coffee trade. As a consequence, there will be a saturation of the actual certified coffee production which therefore needs to be expanded to meet the future demand. These sustainability standards have moved from specific issues like social fairness, environmental protection or traceability to embrace the complexity of sustainable agriculture. They are all more recently dealing with the growing importance of climate change. It is indeed of international concern the impact of climate change on agriculture and on how to mitigate and adapt to it. This issue is debated by the civil society which is extremely concerned about the future food supply. The main indicator for assessing the impact of agriculture on climate change is carbon footprint. Carbon footprint is the measure of the total emissions from a defined system of the greenhouse gases, listed by the IPCC, in CO_2-equivalents according to each 100 years global warming potential (GWP100). Coffee urgently needs studies on this topic to anticipate the adoption of future standards and to grow in harmony with the modern concept of a profitable agriculture respecting and preserving the environment as well as the people.

There are few studies regarding the greenhouse gases emissions associated with the Brazilian green coffee production. Located in the southeast region, Minas Gerais is the largest coffee-growing State in Brazil. It accounts for nearly 70% of the national coffee production. The objective of this study has been to determine the main greenhouse gases (GHG) sources and to measure the carbon footprint of coffee production in the State of Minas Gerais.

MATERIALS AND METHODS

Primary data for the crop years of 2009/10 and 2010/11 were collected in three different coffee farms in the following regions of the Minas Gerais State in Brazil: Cerrado Mineiro (CM), Sul de Minas (SM) and Matas de Minas (MM). In Table 1, we present data on location, production area and yield in each of the farms. Data are presented for the crop years of 2009/10 and 2010/2011 and for two consecutive years representing the 2010-2011 biennium. The crop yield is the average of the entire farm, from coffee plants in the growing phase, pruned and in full production.

Table 1. Location of the studied farms, production areas and crop yields in each of the farms evaluated in two crop seasons (2009/10 and 2010/11) and in the 2010-2011 biennium.

Region	City	Production Area (ha)		Yield (60 kg bags/ha)		
		Crop year 2009/10	Crop year 2010/11	Crop year 2009/10	Crop year 2010/11	Biennium 2010-2011[1]
Cerrado Mineiro (CM)	Presidente Olegário	372	375	27,3	31,8	59,1
Matas de Minas (MM)	São João Manhuaçú	578	575	22,5	40,0	62,5
Sul de Minas (SM)	Cabo Verde	134	127	41,0	27,6	68,6

[1] *Sum of yields from the crop years of 2009/10 and 2010/11.*

The most important greenhouse gases for the agricultural sector - carbon dioxide (CO2), methane (CH4) and nitrous oxide (N2O) - were assessed in each of the studied farms. The main sources of emissions are listed below (Table 2).

Table 2. Emission sources and greenhouse gases considered in this inventory.

	Emission Source	GHG
	Direct	
Mobile combustion	Diesel, biodiesel, ethanol e gasoline	CO2, CH4, N2O
Stationary combustion	Cooking gas, Coffee parchment, Eucalyptus wood and Coffee wood (from pruning)	CO2, CH4, N2O
Fertilizers	Synthetic fertilizer	CO2 e N2O
Fertilizers	Organic fertilizer	N2O
Soil additive	Lime	CO2
	Indirect	
Electricity	Purchased electricity	CO2

In the case of fossil fuels, we considered the emissions of CO2, CH4 and N2O. For biomass fuels (firewood, ethanol and biodiesel), we considered only the CH4 and N2O emissions. The CO2 was disregarded, assuming the biogenic CO2 is absorbed by the next crop through the process of photosynthesis.

N2O from the application of nitrogen fertilizers include direct emissions that occur after application through reactions of nitrification and denitrification, and indirect emissions, where part of the nitrogen is lost through volatilization, leaching or runoff, and is subsequently emitted as N2O. We also considered the CO2 emissions arising from the application of agricultural lime and urea. CO2 emissions derived from generation and distribution of electric energy consumed by these farms were incorporated in the inventories.

The upstream emissions related to the production and transport of agricultural inputs have not been accounted.

The methodology used for estimating GHG emissions was the activity data multiplied by emission factors. The results for N2O and CH4 were converted into CO2 equivalent, considering the concentration and Global Warming Potential (GWP) of each gas in the backdrop of 100 years, following the Fourth Assessment Report (AR4) of the IPCC.

RESULTS AND DISCUSSION

Agricultural crops tend to exhibit great variability of production due to weather conditions, management of soil fertility, pest management and economic conditions. The coffee crop has other peculiarities, especially with regards to the production biannuality.

Usually in Brazil, the coffee crop has a high yield in a crop year and a low yield in the following one. Given this characteristic, GHG emissions were assessed for the production processes carried out in the crop years of 2009/10 and 2010/11.

Total GHG emissions and emissions by sources for each of the three farms evaluated are shown in Table 3.

Table 3. GHG emissions by emission source in three coffee producing regions in the state of Minas Gerais in the 2010-2011 biennium.

Source	CM	MM	SM
	tCO2e		
Fertilizers	1,905	2,112	263
Mobile Combustion	514	339	101
Stationary Combustion	78	10	10
Electricity	38	16	8
Lime	252	352	62
Total	2,787	2,830	444

The farm located in the south of Minas Gerais (SM), had the lowest GHG emissions in the biennium (444 tCO2e) compared to other producing regions of the state. The farms located in regions of the Cerrado Mineiro and Matas de Minas did not differ much in relation to total GHG emissions in the biennium (2,787 and 2,830 tCO2e, respectively).

These results, however, should be analyzed with caution since they are directly related to the size and profile of the farms and the average productivity of the crop in each situation. In this case, more important than the total GHG emissions are the relative share (%) of the emission sources in each coffee production region.The comparative GHG emissions for each region in the 2010-2011 biennium and the participation of each emission source in the final amount of emissions are shown in Figure 1.

The majority of GHG emissions was derived from the application of synthetic fertilizer. The high impact on the application of nitrogen fertilizer is due to the N2O emissions after the fertilizer reacts in the soil. The global warming potential of this gas is about 300 times higher than CO2, which enhances the degree of importance of N2O emissions in agricultural systems.
The second largest source of GHG emissions was fossil fuel burning in agricultural operations and transport, followed by the application of lime to correct soil acidity. Electricity consumption in the farms evaluated resulted in relatively low emissions. This is due to the

fact that the energy matrix in Brazil is primarily composed of energy sources with low greenhouse gas emissions, such as hydropower and biomass.

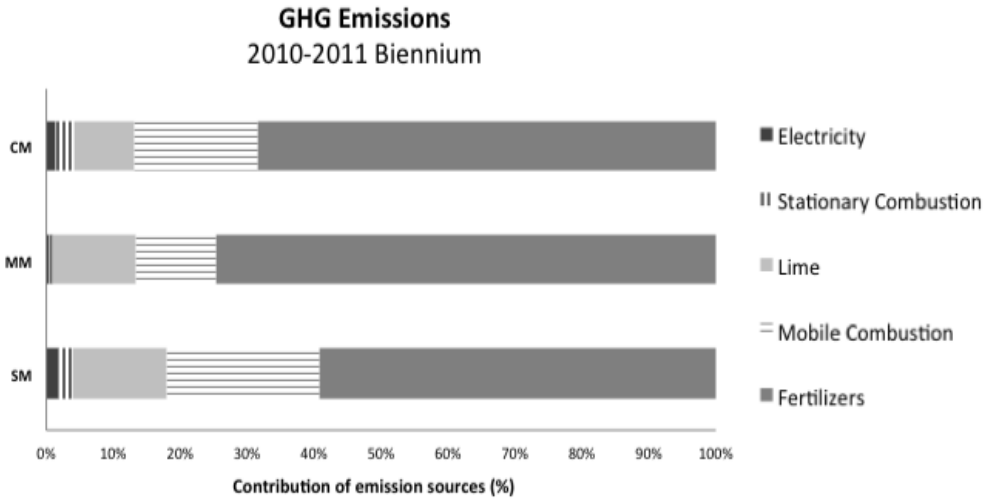

Figure 1. Contribution of emission sources (%) in three coffee producing regions in the state of Minas Gerais in the 2010-2011 biennium.

Besides the total amount emitted by the farms in a given period, it is important to assess the amount of GHG emitted per unit of final product and production area to avoid comparisons of farms with different sizes. The weighting of emissions per unit of product or production area also allows for monitoring the environmental efficiency of the farm, enabling the establishment of targets to reduce emissions and the carbon footprint.

Taking into account the coffee production of each farm during the period of assessment, we calculated the GHG emissions per area (tCO_2e/ha), per bags of coffee (kg CO_2e/60 kg bag) and per tonne of grain (tCO_2e/ ton) for the 2010-2011 period (Table 4).

Table 4. GHG emissions per area and yield (60kg bag and tons) in three producing regions in the state of Minas Gerais in the 2010-2011 biennium.

Region	GHG Emissions (2010-2011)		
	tCO2e/ ha	kg CO2e/ 60 kg bag	tCO2e/ ton
CM	3,7	139	2,32
MM	2,5	79	1,31
SM	1,7	49	0,82

CM had the highest intensity of GHG emissions per area, per bag of coffee and per tonne of grain. The emissions per bag and per tonne of coffee in this farm are almost 80% higher than those observed in MM, even though the total GHG emissions of these farms were numerically very close (2,787 and 2,830 tCO_2e, respectively).

It is important to note that the information presented does not necessarily represent the emissions standard of the regions surveyed, but mainly a diagnosis of major emission sources in each locality. Associating the results with the production profile of each region may result in errors, since no information were obtained from a representative number of farms in each region.

Nevertheless, the results indicate that nitrogen fertilizers are the main source of GHG emissions from the production of coffee in the state of Minas Gerais, which allows recommending some alternatives for reducing emissions in the production process.

Mitigation opportunities

As seen, the main source of GHG emissions in three farms evaluated in the 2010-2011 biennium was the application of nitrogen fertilizers.
It is known that the coffee crop requires large amounts of nitrogen in order to achieve optimum levels of productivity, resulting in emissions of large quantities of N_2O into the atmosphere.
Previous studies have reported that 84% of the annual emissions of N_2O from soil occur after application of the nitrogen fertilizer. According Hergoualc'h et al. (2008, 2012) the influence of nitrogen fertilization on N_2O fluxes is more pronounced in the first weeks after fertilizer application.

The coffee agricultural sector can embrace some measures to reduce GHG emissions, considering the supply of coffee's nutritional needs and maintenance of the productivity. Some suggestions are presented below:

Source

The first measure that can be adopted is the substitution of the nitrogen source applied to the soil.

Urea, the main fertilizer used in the coffee farms, contributes to the emission of large amounts of GHG into the atmosphere. Because it contains carbon in its composition, after the reaction occurs in the soil this carbon is emitted as CO_2. Therefore, replacing or reducing the use of urea by other nitrogen sources such as ammonium sulfate and organic waste, can contribute to reducing GHG emissions.

Since organic residues are not capable of supplying the quantity of nutrients necessary to maintain high levels of productivity in coffee, we recommend using organic waste from the processing of coffee beans in addition to the synthetic fertilizer applied to the soil.

Application

The surface application of nitrogen in the soil should be avoided because it results in higher N_2O emissions to the atmosphere. The application in furrows, injected or drip can significantly reduce emissions, compared to surface application in the soil.

Nitrogen application rates

Regarding the quantity of nitrogen fertilizer being applied, there is evidence that there is a direct relationship between the quantity of fertilizer added to the soil and N_2O emissions to the atmosphere.

Therefore, application rates that match the nutritional needs of the plantation, without excesses, can contribute to reducing emissions.

Agroforestry systems

Another recommended measure for the reduction of GHG emissions to the atmosphere is the adoption of agroforestry coffee system.

Preliminary studies in Costa Rica (Hergoualc'h et al., 2012) reported that the system conversion from monoculture to agroforestry contributed to the reduction in the balance of GHG emissions to the atmosphere.

In Brazil, coffee has been cultivated mainly in monoculture systems (full sun). In many other producer countries, however, the coffee has been traditionally cultivated under a canopy with different tree species.

These trees provide shade (Moguel and Toledo, 1999) and create microclimate conditions compatible with the ecophysiology of coffee (Da Matta, 2004). Furthermore, the root system of trees protects the soil against erosion and provides a continuous input of organic matter to the soil.

The soil quality in tropical agroecosystems depends to a large extent, on the biomass produced, the entry of plant waste (Tian et al., 2007) and the residence time of the litter (Hairiah et al., 2006) that provides protection to the soil and food for soil organisms, which contribute to the improvement of soil structure, moisture retention and soil nutrient supply (Kibblewhite et al., 2008).

Based on successful examples of coffee agroforestry systems, the study by Souza et al. (2012) showed that there is potential to combine coffee agroforestry systems in Brazil with the maintenance of soil quality, biodiversity conservation from the point of view of climate change and contribute to supporting ecosystem services.

CONCLUSIONS

The analysis of the GHG emissions in the 2010-2011 biennium allowed for a more consistent assessment of farms with different agricultural managements and with different crop yields between years.

However, the results are specific to the farms inventoried and do not necessarily reflect the reality of GHG emissions in the regions evaluated. Most of all, this study is a pioneering initiative and represent an initial estimate of GHG emissions of coffee production in Brazil.
Nevertheless, the results indicate that nitrogen fertilizers are the main source of GHG emissions, which allows recommending some alternatives for reducing emissions and the carbon footprint in the production process. Reducing emissions could give these farms greater added value to the coffee produced and even advantage over competitors in the market.

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Climatic Factors Directly Impact the Biochemical Composition and the Volatile Organic Compound Fingerprint in Green Arabica Coffee Bean as well as Coffee Beverage Quality

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SUMMARY

This study was aimed at determining whether climatic conditions during bean development affected the chemical composition of the seed, the sensory perception of the coffee beverage and combinations of volatile compounds in green coffee. Using 16 experimental plots in Reunion Island displaying broad climatic variations, we showed that chlorogenic acids and fatty acids in the seed were controlled by the mean air temperature during seed development. By contrast, total lipid, total soluble sugar, total polysaccharide and total chlorogenic acid contents were not influenced by climate. Volatiles were extracted by solid phase micro-extraction and the volatile compounds were analysed by GC-MS. The results revealed that, among the climatic factors, the mean air temperature during seed development greatly influenced the sensory profile. Positive quality attributes such as acidity, the fruity character and flavour quality were correlated and typical of coffees produced at cool climates. Two alcohols (butan-1,3 diol and butan-2,3 diol) were closely correlated with a reduction in aromatic quality, acidity and an increase in earthy and green flavours. We assumed that high temperatures induce accumulation of these compounds in green coffee, and would be detected as off-flavours, even after roasting. Climate change, which generally involves a substantial increase in average temperatures in mountainous tropical regions, could be expected to have a negative impact on coffee quality.

INTRODUCTION

The coffee seed contains all the necessary precursors to generate coffee aroma during roasting. The major storage compounds of mature Coffea arabica seeds are cell-wall polysaccharides (CWP, 48–60% DM), mainly galactomannans and arabinogalactan-proteins, lipids (13–17% DM), proteins (11–15% DM), sucrose (7–11% DM) and chlorogenic acids (CGA, 5–8% DM). Each of these major storage compounds plays several crucial roles in the complex roasting chemistry. Volatile aroma compounds are secondary metabolites mostly generated from isoprenoids, phenylpropanoids, amino acids, and fatty acids. Although a single fruit or vegetable synthesizes several hundred volatiles, only a small subset generates the flavour fingerprint. The volatile composition of roasted coffee is likely one of the most important factors determining coffee character and quality. Some compounds that are present in clean green coffee are also subsequently found in roasted coffee. According to and, out of the 300 volatile compounds detected in clean green coffee, around 200 are still present after roasting.

Elevation is the environmental factor most frequently mentioned with respect to origin effects. However, it was not possible to determine whether the positive effects of elevation were due to a temperature gradient, to other climatic variables or even to specific soil conditions. The purpose of our work was to assess, for the first time, whether climatic conditions during bean development affect volatile compounds in green coffee, and whether such modifications are reflected in the sensory perception of the coffee beverage. In the following study, we analysed volatile organic compounds from green coffee samples derived from 16 Arabica coffee plots located throughout Reunion Island and encompassing a wide range of tropical climatic conditions.

MATERIALS AND METHODS

Biological material

Experiments were carried out in Reunion Island on seeds of *Coffea arabica* cv. Laurina (a natural dwarf mutant of cv. Bourbon) displaying two specific traits that have been identified so far: a rather low caffeine content and a typical oblong shape. The experimental plots (described in and were planted in 2003 without shade and were in their second year (2006) of production (about 2 m high). Plant spacing was 2 m between rows and 1 m along rows. Fertilisers were applied and pests and diseases were controlled according to locally recommended practices. Out of 107 experimental plots available throughout the island, 16 that maximize variations in elevation (150–1032 m a.s.l.) and climatic conditions were selected (Table 1). The survey unit was a compact plot with a stand of about 240 coffee trees.

Meteorological observations and harvest dates

The island of Réunion hosts a dense meteorological network of more than 50 automated stations dedicated to sugarcane production. The experimental design included 16 coffee plots that were carefully selected to present a very broad range of climatic variations (Table 1). Temperatures were also recorded locally (under the coffee canopy) using portable temperature recorders. The average temperature (Taver) gradient ranged from 13.4°C to 25.3°C, thus covering what is considered to be the optimum growing range for this species (15-24°C). Tropical climates are characterized by a reduced seasonal temperature variation, with elevation being the main factor modulating this physical variable. As expected, Taver, Tmin and Tmax were highly correlated with elevation ($R>0.92$, $P<0.0001$) and highly intercorrelated ($R>0.95$, $P<0.000$). Only the average temperature (Taver) was thus taken into account in the rest of this study. In most Arabica coffee growing areas, annual rainfall is in the 1500-2500 mm range. In our network plots, rainfall mostly ranged from 807 to 1918 mm/year, with two outlier spots located on the eastern rainy coast where annual rainfall was around 5000 mm. Solar radiation, which ranged from 973 $J.cm^{-2}.d^{-1}$ to 1783 $J.cm^{-2}.d^{-1}$ was negatively correlated with elevation ($r=-0.72$) due to the frequent cloudy weather in the highlands, and positively correlated with temperature ($R= 0.83$, $P< 0.01$). It should be noted that the network was set up without shade, whereas C. arabica evolved in a shady environment in the Ethiopian highland forest region. Potential evapotranspiration ranged from 1.9 to 4.6 $mm.d^{-1}$ and did not show any correlation with other climatic parameters. Finally, the water temperature observed during postharvest processing (Tferm) ranged from 19°C to 25.3°C (Table 1) and was disconnected from climatic variables measured in fields. Harvest dates differed depending on the location.

Table 1. Geographical and climatic data for the last 5 months preceding harvest.

Plots	Harvest Day	Alt	Tmin	Taver	Tmax	R	SR	PET
1	04-2006	150	21.8	25.3	28.6	3.8	1728	4.6
2	04-2006	270	22.5	23.9	26.4	9.0	1783	2.1
3	07-2006	315	16.3	19.3	24.3	4.7	1284	2.5
4	08-2006	463	18.4	21.2	25.2	1.5	1488	2.5
5	05-2006	500	18.0	22.0	26.6	2.7	1150	2.9
6	08-2006	549	15.8	19.5	24.1	3.8	1374	3.4
7	07-2006	585	14.6	18.9	23.8	3.0	1043	2.2
8	09-2006	661	12.6	16.2	20.9	5.1	1199	2.4
9	09-2006	757	13.7	17.0	21.1	4.2	1148	2.6
10	10-2006	790	10.8	14.4	19.2	2.7	1164	2.7
11	08-2006	805	14.0	17.5	22.0	3.0	1031	2.2
12	09-2006	880	12.3	16.4	21.6	1.4	1270	1.9
13	09-2006	980	9.6	13.4	17.3	2.6	1023	1.9
14	10-2006	985	10.3	14.3	19.1	2.9	973	2.2
15	09-2006	1014	10.7	14.9	20.0	4.2	1133	2.6
16	12-2006	1032	9.9	14.0	17.7	2.0	1349	3.3
Cv (%)			28.2	20.2	15.0	51.1	19.0	26.0

Plots: date of the harvest; Alt: elevation above sea level (m); mean of minimum, average, and maximum daily temperatures (°C) (Tmin, Taver and Tmax); R: rainfall (mm.d-1); SR: solar radiation (J.cm-2.d-1); PET: potential evapotranspiration (mm.d-1).

Berry harvest and processing

Coffee berries were collected at the harvest peak for each plot (April to December 2006), when the coffee quality was maximal. Only just-ripe cherries (when the pericarp turns purple) were harvested. Fruits were harvested in the morning (2000 g of coffee cherries), transported to the CIRAD processing factory (Ligne Paradis – Saint Pierre), and directly processed in the afternoon by the wet method (depulping, fermentation and drying). Temperature probes inserted inside the batch (Tferm) were used to record temperatures during the fermentation process (Table 1), which was divided into three successive steps: dry fermentation with only mucilage (24 h), maceration by adding water to cover the seeds (15 h) and finally washing, while replacing the water three times during this step (11 h). The green coffee was screened through a size 15 sieve and defective beans were discarded, so as to obtain a batch of approximately 500 g of green coffee beans (11% moisture) that was used for both chemical and sensorial analyses. The samples were frozen at -80°C in plastic flasks pending their use.

Lipid, sugar, caffeine and chlorogenic acid determination

Lipids, sugars, caffeine and chlorogenic acids were determined as described in Joët et al. 2009). Roughly, fatty acid methyl esters (FAMEs) were prepared according to the ISO-5509 standard and measured by GC. Sugars were determined by High-Performance Anion exchange Chromatography coupled with Pulsed Amperometric Detection (Dionex Chromatography Co., Sunnyvale, USA). The seed CWP content was estimated by measuring the defated alcohol insoluble residue (DAIR). Chlorogenic acids and caffeine were analysed by HPLC at 325 and 276 nm, respectively. All metabolites were analysed in triplicate (from 3 different extractions) using a completely random experimental design.

Extraction of volatile compounds from ground coffee by headspace-SPME

Green coffee beans samples were ground and brought to room temperature for 90 min prior to sampling for headspace analysis. A Carboxen/poly(dimethylsiloxane) (CAR/PDMS) type 75 µm SPME fibre (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the coffee headspace as its affinities for all classes of aroma compounds found in coffee have been previously documented, and notably for trace compounds or low molecular weight compounds. One gram of ground coffee was placed in a 2 ml hermetically sealed glass flask, which corresponded to a headspace of 1/3 of the sampling flask. The flasks were placed for 30 min in a thermostatically regulated oven until the sample headspace equilibrium was reached. The equilibration time and temperature, were fixed in accordance with prior studies undertaken by this laboratory. Then volatile compounds were extracted by placing the SPME fibre in contact with the headspace for 15 min at equilibrium temperature. For compound desorption, the fibre was placed in the GC injector and heated to 250°C for 4 min. All samples were extracted in duplicate.

Combined gas chromatography-mass spectroscopy

The coffee SPME extracts were analysed on a GC–MS apparatus (HP-6890A GC connected 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. Injection was performed in splitless mode for 4 min at 250°C with a Supelco specific SPME insert of 0.75 mm i.d. The carrier gas (helium) flow rate was 1.5 ml/min. The column temperature was programmed from 44 to 170°C at 3°C/min, followed by a rise from 170 to 250°C at 8°C/min. The electronic impact ionisation method was used with an ionisation energy of 70 eV. The mass range scanned was 40 to 350 amu at a scanning rate of 2.89 scans/s. The transfer line temperature was 260°C.

Identification of volatile compounds

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 the linear alkanes (Retention Index Standard, Sigma).

Analysis of sensory characteristics

The coffee beverage was prepared by brewing 50 g of roasted coffee in 1 l of water for 5 min. The cup quality of the coffee samples was assessed twice by 12 expert tasters using 8 sensory criteria: aroma (intensity and quality), body, acidity, bitterness, astringency, sourness, flavour attributes (green, earthy, fruity, medicinal). A hedonic assessment score, i.e. an overall appreciation, was also assigned to the coffee beverage. Scoring was on a scale of 0 to 10, where a score of 0 corresponded to the total absence of the criterion in the coffee.

Statistical analysis

Following ANOVA, the sensory and volatile compound values were compared by the Duncan test ($P \leq 0.05$). Pearson's correlation coefficients (r) were calculated between variables. Principal components analyses (PCA) were applied to the means of the sensory, climatic and volatile compound data.

RESULTS AND DISCUSSION

No environmental factor significantly influenced the accumulation of the four main classes of storage compounds studied (total CWP, total lipids, total free sugars and total CGA). However, within a given chemical family (CGA, lipids or free sugars), some components were significantly influenced by the environment. Out of the 28 metabolites analyzed, half showed significant correlations with TAVER during the last five months of seed development i.e. the period when storage compounds accumulate in the seed. In contrast, no significant correlation was found between rainfall or potential evapotranspiration. Within a given chemical family, when significant ($P < 0.01$), the slope of regression equations could differ among the compounds of the family concerned. For instance, 3-caffeoyl quinate (3-CQA) and 4-CQA content were positively correlated with temperature while the reverse trend was observed for 5-CQA. Interestingly, the same phenomenon was found for di-CQA: i.e. di3.4-CQA and di-4.5-CQA were positively influenced by temperature while a negative correlation was observed for di3.5-CQA. These results suggest that temperature may act directly on routing towards the different isomers within the CGA metabolic pathway without affecting the final CGA content. A similar routing regulation was observed within the FA biosynthetic pathway. Indeed, the relative contents of the two major FA, namely linoleic and palmitic acids (35–45% each), were negatively correlated with environmental temperature.

Sensory attributes of coffee beverage quality linked with climatic factors

We showed (Table 2) that, temperature variations played a paramount role in quality generation since it was correlated with six (of the eight) sensory attributes. Solar radiation also seemed to play an important role as three sensory attributes were correlated with this factor. The other climatic variations seemed to play a minor role.

Table 2. Correlation matrix (Pearson's r linear-correlation coefficients) between significant sensory attributes and mean environmental factors as calculated during the last 5 months of fruit development.

Sensory attributes	Taver	Tferm	R	SR	PET
Aroma quality	-0.57	-0.47	-0.10	-0.51	-0.55
Body	-0.00	0.36	-0.45	0.02	-0.20
Acidity	-0.51	-0.48	-0.40	-0.45	-0.18
Bitterness	0.08	0.48	**-0.59**	0.10	-0.18
Fruity	**-0.67**	-0.30	0.22	-0.51	-0.22
Green	0.61	0.46	0.19	**0.66**	**0.78**
Earthy	**0.83**	0.45	-0.13	**0.73**	0.47
Overall quality	-0.59	-0.51	-0.30	-0.54	-0.31

Underlined r values indicate P values <0.05 and r values in bold indicate P values < 0.01. Taver, average daily temperatures; Tferm, mean temperature during wet processing; R, rainfall; SR, solar radiation; PET, potential evapotranspiration.

Volatile compound composition of green coffee beans as a function of climatic conditions

Of the 44 volatile compounds tested, 18 were not correlated with the climatic variables. Among the climatic variables, temperature appeared again to be the predominant factor since it was correlated with 21 volatile compounds. Solar radiation, as a temperature co-variable, was found to be less accurate in explaining the variance in volatile compounds since significant correlations were only noted for seven volatile compounds. Rainfall was linked to

four volatile compounds (2-ethyl-1-hexanol, 3-methyl-2-butenoate, methane and gamma valerolactone, respectively), since PET was linked only with butan-2,3-diol and butan-1,3-diol. Finally, temperature during wet processing was positively correlated with pyrrole and pentan-2-ol contents.

Strikingly, in the alcohol class, only positive correlations were detected, indicating that these compounds, notably 2-butoxyethanol, and diols such as butan-2,3 diol and butan-1,3 diol, were positively affected by warm climatic conditions. Among the aldehydes, benzaldehyde was positively correlated with temperature while, by contrast, ethanal accumulated in cool climates. Other compounds were found to be negatively correlated with temperature, i.e. 2-methylfuran, 2-butanone and dimethylsulfide. Finally, it should be noted that the highest correlations (r^2>0.7) were found for one hydrocarbon (2-phenylethanol), one aliphatic acid (methyl acetate) and one lactone (gammabutyrolactone) that accumulated in warm climates.

Volatile compounds as possible indicators of sensory attributes

The volatile compounds were correlated with the sensory data. The majority of compounds that were positively affected by warm temperatures (namely, 2-butoxyethanol, 2-ethyl-1-hexanol, butan-2.3-diol, butan-1.3-diol, benzaldehyde, 2-hexanol, methyl acetate, 3-methyl-butanoate, benzyl alcohol, 2-phenylethanol 2-butanone, butan-2.3-dione, and gamma-butyrolactone) were also negatively correlated with aroma quality, acidity, fruity character or overall quality. Moreover, we found that these compounds (except for 3-methyl butanoate) had high positive correlations with the negative earthy attribute, indicating that these compounds, if not directly involved in off-flavours were at least indicators. From this standpoint, butan-1.3-diol and butan-2.3-diol showed the highest correlation coefficients with the earthy attribute, with r2 values of around 0.86. Conversely, among the five compounds that were associated with cool climatic conditions (ethanal, 2-methylfuran, 2-butanone, acetone and dimethylsulfide), 2-butanone was negatively correlated with the earthy attribute while ethanal was associated with the fruity attribute. Moreover, some volatile compounds that were not clearly correlated with the climatic parameters recorded, such as ethanol, 2-furanmethanol, hexanal, 2-methyl-1-propanal, 3-methylfuran and toluene, presented close correlations with body, acidity and bitterness attributes.

To draw up a summary representation of our results, the sensory analysis variability structure was analyzed by principal component analysis. In this analysis, the volatile compounds and climatic variables were considered as supplementary variables. The graphic representation (Fig. 1) is based on the first two principal components, which accounted for 84.5% of the overall variance. As expected, the aromatic quality, fruity character, overall quality attributes contrasted with the negative attributes (earthy and green), while acidity contrasted with bitterness and body. The earthy and green attributes appeared to be linked to high temperatures, solar radiation and PET. Volatile compounds such as butan-2,3-diol, butan-1,3-diol and 2-phenylethanol, appeared to be indicators of the earthy attributes, whereas toluene, 2 methyl-1-propanal and hexanal appeared to be indicators of body and bitterness. High fermentation temperatures seemed to be linked with an increase in pyrrole, benzaldehyde and 2-furanmethanol and contrasted with the fruity character and overall quality. Finally, acidity, overall quality, fruity character and aroma quality were located opposite high temperatures and solar radiation and close to acetone and ethanal. These latter volatile compounds could be considered as highland coffee indicators.

CONCLUSIONS

Our results indicated that environmental temperature during seed development negatively influenced the sensory profile. Recent studies have shown that even minimal increases in mean temperatures due to climate change will have disastrous consequences on coffee production. In some regions, climate change will considerably reduce the area presently suitable for coffee production. Our study suggests that climate change, which in mountainous tropical areas leads to a marked increase in average temperatures, will also very likely have a substantial negative impact on the quality of highland coffees.

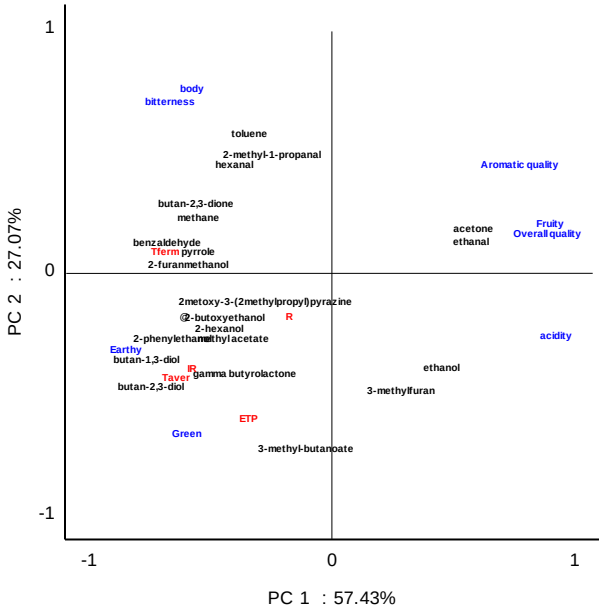

Figure 1. Scatterplot of a principal components analysis based on sensorial analysis findings. The PCA resulted from the sensorial analysis of beverages produced from green coffee samples harvested on 16 plots representative of the usual full range of variations in elevation (150 to 1032 m a.s.l.) and climatic conditions on coffee plots. The first two components which accounted for 84.5% of total variability are represented. Sensory criteria (active variables) are represented in blue. Volatile compounds and climatic variables are supplementary variables (respectively in black and red). Taver, average daily temperatures; Tferm, mean temperature during wet processing; R, rainfall; SR, solar radiation; PET, potential evapotranspiration. The @ symbol represents 2-ethyl-1-hexanol and benzylalcohol with similar patterns.

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Development and Release of Coffee Berry Disease Resistant Varieties to Specialty Coffee Producing Regions in Ethiopia

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SUMMARY

Ethiopia is acknowledged for the production and supply of world renowned specialty coffees among which Harar, Sidamo, and Yirgacheffe have been trademarked and licensed by a number of local and international companies like Illy, Starbucks, Caribou and others. The demand for these finest coffees has been tremendously increasing in the specialty market although the crop yield is dwindled due mainly to major diseases like coffee berry disease (CBD) and vascular wilt. With the objectives of developing disease resistant specialty coffee varieties and boost the yield with their intrinsic cup quality, a multidisciplinary research program (disease resistance, yield and quality) have been designed and implemented between 1997 and 2010. During these periods, a large number of mother coffee trees were selected from Sidamo and Yirgacheffe/Gedeo (101), Wollega (361) and Hararghe (1013) and their seedlings were planted out in a single tree progeny plot (6 – 10 tree) in the respective regions to evaluate their reaction to diseases (CBD, wilt and leaf rust), yield and quality traits. Based on three to four years data, thirteen to fifteen most promising selections were promoted to verification trials and planted (20 - 150 trees/plot) in a randomized complete block design with three replications at least in two farmers' fields representing their respective original environments. With regard to pathological parameters, attached berry test for CBD and seedling inoculation tests for both CBD and wilt were employed to verify the resistance of all the promising selections under field and growth room or greenhouse conditions. Based on the results a total of the best eleven CBD resistant coffee varieties were officially approved for production in the major specialty coffee growing areas of Ethiopia in 2010. These varieties are designated as '*Fayate*', '*Odicha*' and '*Koti*' for Sidamo/ Yirgacheffe, four varieties, namely, '*Haru-1'*, '*Challa*', '*Sende*' and '*Menesibu*' for Wollega/Ghimbi; and four varieties '*Harusa*', '*Mocha*', '*Mechara-1'* and '*Bultum*' for Hararghe areas. The overall mean percentage of CBD infection on Wollega varieties varied from 11 to 15 in ABT in the fields; while Harar varieties have shown less than 10% infection at Hararghe with contrasting values between 13 and 45% at Jimma. The Sidamo/ Yirgacheffe varieties had the best performance possessing moderate to high levels of double resistances to both CBD and CWD infections (< 11%). All of them demonstrated good yield and typical quality profiles of their origin like 'fruity', 'floral'/'spicy', and 'mocha' for Sidamo/Yirgacheffe, Wollega and Harar specialty coffees, respectively. Above all, more than 15 million seeds and seedlings of these resistant specialty coffee varieties have been distributed to coffee farmers in the regions.

INTRODUCTION

The endowment of Ethiopia with diverse coffee types and agro-ecology allowed production of high quality coffee (Coffea arabica L.) demanded by consumers. Production of specialty coffee varieties adaptable to specific agro-ecological niches allows farmers to supply high quality coffee at premium prices. The dynamic shift from traditional/commercial to specialty market also reduces farmers vulnerability to slump in coffee prices and the encouraging prices enhance quality coffee production efficiency (Wollni and Brümmer, 2012). The existence of similar but distinctive inherent coffee quality profile across regions in Colombia has differentiated geographic origins of specialty coffee (Oberthür et al., 2011). Nevertheless, so far Ethiopian coffee quality profiles such as Harar, Sidamo, Yirgacheffe and Gimbi specialty coffees known in the world market, trademarked and licensed by a number of local and international companies have been produced using unimproved or low yielding land races that are prone to various diseases. Coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR) caused by Colletotrichum kahawae, Gibberella (Fusarium) xylarioides and Hemileia vastatrix, respectively, are the major ones. Development of coffee for multiple disease resistance while maintaining the inherent physical and cup quality characteristics is quite challenging unless at least supported by coffee quality profile mapping of each locations. We are privileged in location specificity of Arabica coffee under defined agro-ecology, growing demand for specialty coffee in the international market, and existence of genetic diversity in major coffee growing areas to gear our coffee research system towards specialty coffee variety development for each agro-ecological niche of the country.

The development of disease resistant specialty coffee varieties addressing quality issues was started simultaneously in the four major regions, namely; Wollega/Ghimbi, Sidamo, Yirgacheffe and Hararghe some 15 years back in Ethiopia. The coffee varieties were originally selected and/or collected from farmers' coffee fields in semiforest or garden production systems and intensively evaluated and tested for their reaction to economically important diseases in the country and assessed for yield potential and quality profiles in the respective geographic areas. In this manuscript, the development and release of coffee berry disease (CBD) resistant coffee varieties in specialty coffee producing regions of Ethiopia, pertaining to pathological investigations are briefly reported.

MATERIALS AND METHODS

Descriptions of the study sites

The laboratory and greenhouse/growth room studies were undertaken at Jimma Agricultural Research Center (JARC) and the field studies were conducted in the Research Centers in the respective geographic regions in west, south and eastern parts of Ethiopia (Table 1). JARC is located 358 km away to southwest of the capital, laying at about 1750 m altitude receiving almost year round rainfall amounting 1550 mm annual average, with 12.8 and 26.7 ^{0}C minima and maxima temperatures, respectively. The field research activities were conducted at different localities, namely, Haru and Mugi research stations for coffee growing areas of Gimbi and Wollega; Awada and Wonago stations meant for developing Sidama/Yirgacheffe specialty coffee profiles in the Southern Region. Mechara Research Center is destined for the famous Harar coffee with '*Moka*' flavour; segregate in the eastern escarpment adapted to more marginal coffee growing region of the country. As opposed to the south, southwest and western regions, coffee trees grow in areas characterized by erratic rainfall, high temperature, on eroded soil but in highly managed garden system. Detailed descriptions of the study sites are presented in Table 1.

Table 1. Descriptions of the study sites for developing specialty coffee varieties in Ethiopia.

Region	Zone	Research center	Verification site	Altitude (average)	Rain fall[2] (mm)	Temperature (^{0}C) [2]	
						Min	*Max*
Oromiya	Jimma	JARC/ Melko	-	1750	1550	12.8	26.7
	West Wollega	Haru	Erecha	1750	1727	16	27
	West Wollega	-	Duchi	1800			
	Kelem Wollega	Mugi	Mugi	1550	1655		
SNNPR[1]	Sidama	Awada	Korkie	1740	1342	12.6	26.2
	Gedeo	Wonago	Konga	1850	-	-	-
Oromiya	East Hararghe	Mechara	Mechara	1750	1100	14	29
		-	Micheta	1815			

[1] Southern Nation Nationalities People's Region; [2]Data from nearest meteorological station, = no data

Wollega Specialty Coffee Variety Development

A total of 361 coffee accessions collected from West Wollega in 1998 were established at Haru Research Subcenter in July 1999 in simple lattice design with seven trees per plot at a spacing of 2m x 2m. Of these, 81 promising coffee accessions were intensively tested under field and growth room/ greenhouse conditions for CBD and CWD resistance. Among these, based on three to five years data, thirteen promising coffee accessions were promoted and planted out in three varying localities, Duchi, Erecha (1700 – 1800 m asl) and Mugi (1550 m asl) in randomized complete block design (RCBD) with three replications consisting of 40 to 60 trees per block to verify their resistance to CBD and other diseases and yield and quality traits. Two CBD resistant varieties 74110 and 75227 were included as standard checks. All the routine coffee management practices were uniformly applied accordingly.

Sidama/Gedeo Specialty Coffee Variety Development

A total of 101 coffee accessions (57) collected from Sidama in 1997 and (44) from Gedeo areas in 1985 were planted at Awada (1740 m asl) and Wonago (1850 m asl) in variety trials with 6 – 10 trees per plots with three to three to four replication in RCBD. After rigorous testing under field and growth room conditions, 12 promising coffee selections and three standard checks (1377, 744, 75227) were advanced to variety verification trials in two localities at Korkie (Sidama zone) and Konga (Yirgacheffe, Gedeo zone) consisting of 66 to 75 trees per plot in RCBD with three replications in July 2004.

Harar Specialty Coffee Variety Development

A large number of coffee accessions (1013) were massively collected from major Hararghe coffee growing areas in 1998 to save the endangered Harar coffee types owing to booming of chat (*Catha edulis*) production and then the majority of most of them were well- established at JARC/Melko in single plot with 10 trees per plot/accession. Among these, 60 accessions were selected and then continuously evaluated for their resistance to major coffee diseases both under field and glasshouse conditions since 2003. Fourteen accessions with promising performances were selected and planted in two fields at Mechara and Micheta (West Hararghe) in order to verify their performance in their original agroecology. The trials were consisted of 150 trees per accession/plot in RCBD with three replications; and the coffee trees were properly managed accordingly.

Testing for Resistance to Coffee Berry Disease and Coffee Wilt Disease

The modified seedling hypocotyls inoculation technique was adopted for CBD resistance test (Girma and Chala, 2009; Adugna et al., 2009). Four to six weeks old hypocotyls (soldier stage) seedlings of each accession was inoculated by brushing the stem of each seedlings using camel brush at spore concentration adjusted to 2 x 106 conidia/ml. The inoculated seedlings were arranged on the bench in a growth room and covered with plastic sheet for 48 hours to maintain about 100% relative humidity and temperature adjusted to 21 – 22°C to favor infection. After three weeks incubation, the number of infected seedlings per box were scrutinized and recorded using 0 – 4 scales, and a disease index expressed as percentage of CBD infection was computed for each cultivar per box (Van der Graaff, 1981; Tefesetewold, 1995; Adugna et al., 2009). The stem nicking procedure was employed in testing all the promising accessions for resistance to coffee wilt disease at seedling stage in the greenhouse. Each seedlings were inoculated with a conidial suspension of 2 x 106 per ml and the cumulative percentages of dead seedlings and incubation periods (day) were used for analyses (Adugna et al., 2009).

Besides, attached berry test (ABT) technique was used to verify the CBD resistance levels by artificially inoculating expanding coffee berries on three primary branches selected from the middle canopy layer of a sample coffee tree (3 – 5 trees/plot) in the fields. These branches were inoculated after 5:00 PM by spraying 2 x 106 conidia/ml using manually pressurized hand sprayer. Each inoculated branch was immediately covered with plastic sleeves misted with water over night to favor infection and removed the next day before 8:00 AM. Three weeks later, the number of healthy and CBD infected berries per branch were recorded to calculate percentage berry infection. The percentages of rusted leaves and CBD infection per tree were estimated visually.

RESULTS AND DISCUSSION

Wollega Specialty Coffee Varieties

The results indicated that there were significant differences ($p < 0.01$) among the progenies of Wollega coffee selections in their resistance to CBD in ABT in the field at Haru and the overall mean CBD infection ranged between 1.8 (W265/98) to 88.9% (F-59, not shown) as compared to the standard check 74110 (10.7%) (Table 2). Among the 13 promising selections promoted to verification plots, all except W4/98, W105/98, W170/98 and W175/98 manifested consistently lower CBD severity (< 11%) at Duchi and Erecha, which was comparable to the standard check 74110. Coffee selections W13/98, W33/98 and W66/98 showed lower CBD infection although higher disease was recorded on most of the selections in seedling inoculation test in the growth room. Testing of these coffee selections for CWD resistance showed significant differences in percent dead seedlings and incubation period that varied from 0% (W196/98) to 92% (W92/98) and 31 to 139 days, respectively (Table 2). Based on their overall consistent performances comparable to the standard CBD resistant check 74110 both in the field and growth room results, four selections W66/98, W76/98, W92/98 and 78/84 were approved and released in similar coffee growing areas of West Wollega zone.

Sidama/ Yirgacheffe -Gedeo Specialty coffee varieties

The mean CBD infections among 1997 coffee selections varied from 10% (resistant check 75227) to 47.7% (9744) at Awada. Similarly those selections at Wonago showed CBD infection ranging from 15.7 (75227) to 39.7 (85294) as compared to the released checks 744

(22.6%) and 1377 (27.3%) (Table 3). Significantly lower level of CBD infection (0 - 10%) were recorded on selections promoted to verification plots at Korkie in Sidama and Konga in Yirgacheffe/Gedeo zones in visual assessment. The CBD seedling inoculation test also showed significant differences ranging from 12.8 (741 resistant check) to 96% (370 susceptible check). Selections 971 (28 – 33%) and 85257 (51 – 56%) showed consistently lower CBD severity in repeated hypocotyls inoculation tests while selection 974 demonstrated slight variation across years. Similarly, significant differences were observed in mean percent wilted seedlings varying from 2.9% (971) to 93.7% (979). Selections 971 and 974 showed consistently low (<16%) seedling death in repeated inoculation tests for wilt resistance (Table 3). In addition, these selections showed comparatively lower level of CBD infection in ABT (<35%) under field conditions and in seedling inoculation test (<47%) showing multiple disease resistance nature. Adugna et al. (2008) also reported the existence of such kind of resistance in Arabica coffee gremplasm collections. Visual score for CLR under field condition at Awada and Wonago indicated acceptable level of rust infection, but warn its future economic importance on selection 971 (upon its large scale cultivation) and 9722 (data not shown).

Harar Specialty Coffee varieties

Among 60 coffee accessions that were intensively tested for CBD resistance in ABT, 14 accessions showed mean CBD infection between 1% and 50% in the progeny plot at JARC (Melko). All these selections except H823/98 showed less than 16% CBD infection in ABT in the verification plots at Mechara and Micheta. Selection H739/98 and H857/98 consistently demonstrated lower (0 – 14%) CBD severity levels in repeated field inoculation tests in progeny and verification plots. Selection H674/98 expressed lower level of CBD in verification plot in Hararghe than in the progeny plot at Jimma (14 – 26%) (Table 4). However, the coffee selections had exceptionally higher infection and inconsistent performance in CBD under growth room condition than in the fields. There were also differences among the selections varying from moderate to high susceptibility CWD but H823/98 showed about 25% wilted seedlings which is better than the tolerant check Catimore J21 (35%) (data not shown). Coffee leaf rust infection ranged from 7 to 54% in 2004 at Jimma while few rusted leaves were observed in verification plots at Mechara and Micheta that might be due to variation in rust pathogen populations across regions as reported by Girma and Chala (2009); Adugna *et al*. (2009) and Jefuka *et al*. (2009).

In conclusion, based on the detailed analyses of the results of field evaluations and greenhouse tests, eleven CBD resistant coffee varieties, some of which showed multiple resistance, were developed and officially approved for production in the respective specialty coffee producing regions of Ethiopia in 2010 (MoAR, 2010). These included four coffee varieties designated as W66/98 '*Haru-1'*, W76/98 '*Challa*', W92/98 '*Sende*' and 78/84 '*Menesibu*' for Wollega/Ghimbi areas; three varieties namely; 971 '*Fayate*', 974 '*Odicha*' and 85257 '*Koti*' for Sidamo/ Gedeo zones; and four varieties H674/98 '*Harusa*', H739/98 '*Mocha*', H823/98 '*Mechara-1'* and H857/98 '*Bultum*' for Hararghe agroecologies. The overall mean percentage of CBD infection on Wollega varieties varied from 11 to 15 in ABT in the field (progeny and verification plots); while Harar varieties have shown less than 10% infection in verification trials at Hararghe with contrastingly values ranging from 13% to 45% in progeny plots at Jimma. The Sidamo/ Yirgacheffe coffee varieties (971 and 974) had the best performance with moderate to high levels of multiple resistances to both CBD and CWD infections (< 15%). These released resistant coffee varieties found to possess better yield and typical quality profiles of their origin like 'fruity', 'floral'/'spicy', and 'Mocha' for Sidamo/Yirgacheffe, Wollega and Harar specialty coffee, respectively. Above all, more than

15 million seeds and seedlings of these materials were distributed to coffee farmers in the regions.

Table 2. The resistance of Wollega coffee selections to CBD in the progeny plot at Haru and in verification plots at Duchi and Erecha and to CBD and CWD seedling inoculation tests at JARC.

Coffee Selection	Haru (2 years mean, %)	Duchi	Erecha	Seedling test for CBD (%)	Seedling test for CWD (%)	Incubation Period (days)
W4/98	29.4 (32.3)	32.7 (34.62)	23.68 (29.29)	74.2 (59.6)	50.4 (45.3)	74.7
W6/98	14.1 (16.2)	15.98 (22.22)	7.56 (14.6)	87.4 (69.2)	Nt	Nt
W10/98	4.1 (10.6)	4.79 (9.85)	9.56 (19.82)	73.7 (59.2)	49.7 (45)	120.0
W13/98	18.7 (22.9)	9.04 (16.35)	4.4 (12.13)	60.9 (51.3)	84.5 (67)	66.7
W14/98	10.9 (14.9)	5.2 (12.2)	5.08 (14.27)	85.2 (67.5)	23.9 (29.2)	102.0
W33/98	4.0 (8.1)	7.6 (14.0)	13.17 (24.02)	25.6 (30.4)	59.2 (51.3)	83.7
W66/98	2.0 (5.6)	8.96 (17.2)	15.3 (23.45)	47.5 (43.6)	45.4 (42.3)	88.7
W76/98	8.0 (16)	5.26 (13.2)	6.26 (14.47)	49.8 (44.7)	88.3 (70.7)	83.3
W92/98	9.7 (14.5)	18.1 (25.2)	18.55 (27.49)	95.5 (78.2)	91.7 (80)	62.0
W105/98	22.3 (24.9)	7.3 (14.7)	8.75 (18.13)	83.6 (66.2)	30.2 (32.9)	65.0
W170/98	23.4 (27.6)	6.5 (14.3)	30.98 (37.13)	84 (66.6)	90.8 (72.6)	55.3
W175/98	32.5 (33.4)	2.96 (9.7)	7.77 (17.3)	62.8 (52.5)	54 (47.5)	108.0
W265/98	1.8 (6.2)	2.18 (8.4)	5.09 (12.3)	47.4 (43.5)	80.8 (64.4)	69.0
78/84	Nt	2.31 (8.7)	13.27 (23.2)	86.2 (68.5)	33.6 (34.8)	112.3
74110[1]	10.7 (18.2)	5.35 (13.1)	28.4 (36.9)	Nt	76 (61.8)	111.3
741[1]	Nt	Nt	Nt	8.4 (16.5)	8.9 (14.3)	31.3
754[1]	Nt	Nt	Nt	28.5 (32.2)	Nt	Nt
370[2]	Nt	Nt	Nt	100 (90)	48.9 (44.6)	139.0
N	15	15	15	84	81	81
Mean	(41.2)	(15.58)	(21.6)	(58.7)	(55.5)	83.4
LSD	(14.5)	(10.06)	(8.94)	(7.9)	(17.0)	44.5
CV (%)	(30.6)	(38.61)	(24.71)	(8.4)	(19.0)	33.1

[1]741, 7454, 74110, and [2]370 CBD resistant and susceptible checks, respectively. Means in the bracts were arcsine transformed values before analysis. Nt = Not tested.

Table 3. The performance of Sidama/Gedeo specialty coffee selections in reaction to major coffee diseases at Awada and Wonago in Southern Ethiopia.

Coffee	Attached berry test (2 years mean %)		Seedling inoculation tests (%) at Jimma		
Selection	*Wonago*	*Awada*	*CBD*	*CWD*	*IP (days)*
85237	29.1	Nt	53.0	59.0	85.0
85257	26.3	Nt	51.3	40.3	90.0
85238	32.1	Nt	40.7	14.6	56.7
85259	21.8	Nt	23.3	57.1	76.0
85294	39.7	Nt	70.0	60.6	82.3
1377 [b]	27.3	Nt	62.0	Nt	Nt
744 [a]	22.6	15.2	35.8	Nt	Nt
75227 [a]	15.7	10.0	39.4	Nt	Nt
741 [a]	Nt	Nt	20.9	Nt	Nt
754 [a]	Nt	Nt	24.1	Nt	Nt
971	Nt	30.0	32.2	4.35	57.0
974	Nt	32.9	46.8	15.41	90.0
979	Nt	41.4	46.6	93.94	85.0
9718	Nt	42.2	46.2	14.76	83.0
9722	Nt	42.6	37.3	49.1	85.0
9744	Nt	47.7	47.0	17.77	85.0
Catimor J21[d]	Nt	Nt	Nt	40.32	81.0
SN 5[e]	Nt	Nt	Nt	85.94	81.0
Mean	32.6	40.93	49.5	48.9	80.5
LSD (5%)	11.3	15.0	10.7	24.6	NS
CV (%)	30	32.4	11.9	29.9	25.8

Means in the brackets are actual values, [a] CBD resistant check; [b] CBD resistant released variety; [d,e] CWD susceptible and resistant checks, respectively; Nt: not tested; NS: not significantly different.

Table 4. The performance of Harar specialty coffee varieties in reaction to CBD in the progeny plot at Jimma, verification plots at Mechara and Micheta, and to CBD under laboratory condition at JARC.

Coffee	Progeny plot (ABT, %)	Verification plots (ABT, %)		Seedling test (%)
Selection	*Jimma (2 years means)*	*Mechara (2 years means)*	*Micheta*	*CBD (3years mean)*
H618/98	3.6 (9.0) - 15.3 (22.5)	4.8 (10.3) - 15.2 (20.1)	2.2 (6.9)	42.7 - 91.8
H622/98	4.8 (10.4) - 50.4 (45.3)	3.6 (9.8) - 9.4 (14.3)	Nt	66.6 - 93.5
H674/98	14.0 (20.6) - 26.5 (30.4)	4.1 (11.5) - 5.9 (13.7)	5.1 (12.7)	90.6 - 95.2
H739/98	0.0 (0.0) - 8.5 (15.2)	3.0 (9.6) - 13.4 (21.4)	1.8 (6.3)	71.6 - 83.3
H822/98	2.1 (4.9) - 10.1 (18.5)	4.1 (10.4) - 5.4 (12.6)	3.7 (10.9)	67.0 - 92.0
H823/98	9.6 (14.8) - 14 (21.8)	1.7 (7.3) - 31.6 (33.6)	2.3 (8.6)	72.7 - 85.3
H856/98	1.0 (3.3) - 9.4 (14.4)	0.6 (3.5) - 5.2 (12.2)	5.6 (9.8)	60.2 - 96.7
H857/98	3.8 (9.2) - 5.0 (10.5)	5.1 (12.5) - 14.9 (22.5)	1.8 (6.0)	82.8 - 92.0
H858/98	7.3 (14.7) - 39.8 (42.1)	11.5 (19.5) - 5.9 (36.7)	2.0 (4.7)	46.3 - 93.8
H915/98	0.0 (0.0) - 1.1 (3.5)	8.1 (13.2) - 10.5 (18.8)	3.9 (6.8	63.7 - 96.3
H929/98	1.7 (6.1) - 19.9 (21.8)	8.3 (14.4) - 15.5 (22.1)	1.7 (5.6)	70.9 - 74.4
H968/98	2.1 (4.9) - 15.7 (14.5)	0.0 (0.0) - 5.6 (12.8)	0.3 (1.9)	40.8 - 94.7
H980/98	0.9 (3.2) - 14.5 (21.3)	3.3 (10.1) - 9.7 (6.9)	3.2 (6.5)	73.8 - 82.4
H981/98	2.1 (4.85) - 4.0 (9.3)	4.0 (10.2) - 12.1 (20.3)	3.7 (11.1)	45.0 - 97.5
74110 [a]	Nt	0.0 (0)	0.0 (0.0)	15.3
Mean	21.2 - 21.4	10.4 - 18.9	7.37	84.74 - 79.6
LSD (5%)	13.8 - 21.6	11.6 - 13.8	NS	12.29 - 15.8
CV (%)	39.9 - 62.3	43.8 – 66	72.7	8.96 - 14.0

Means in the brackets were arcsine transformed of actual values; [a] CBD resistant varieties, Nt: not tested; NS: not significant.

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Coffee Insects in a Changing World[1]

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SUMMARY

Global environmental changes (GEC) such as climate change (CC) and climate variability have serious impacts in the tropics, particularly in Africa. These are compounded by changes in land use/land cover, which in turn are driven mainly by economic and population growth, and urbanization. These factors create a feedback loop, which affects ecosystems and particularly ecosystem services, for example plant-insect interactions, and by consequence agricultural productivity. We studied effects of GEC at a local level, using a traditional coffee production area in greater Nairobi, Kenya. Using the coffee berry borer, the most serious biotic threat to global coffee production, we show how environmental changes and different production systems (shaded and sun-grown coffee) can affect the crop. We combined detailed entomological assessments with historic climate records (from 1929-2011), and spatial and demographic data, to assess GEC's impact on coffee at a local scale. Additionally, we tested the utility of an adaptation strategy that is simple and easy to implement. Our results show that interactions between CC and migration/urbanization, with its resultant landscape modifications, adversely affect agroecosystems such as coffee. Bio-diverse shaded coffee proved far more resilient and productive than coffee grown in monoculture, and was significantly less harmed by its insect pest. Thus, a relatively simple strategy such as shading coffee can tremendously improve resilience of agro-ecosystems, providing small-scale farmers in Kenya with an easily implemented tool to safeguard their livelihoods in a changing climate.

INTRODUCTION

The Intergovernmental Panel on Climate Change (IPCC) predicts increases in the mean global temperature of up to 5.8 ºC by 2050, as well as more frequent ENSO (El Niño / La Niña) events, with climatic conditions expected to become generally more variable. As a consequence of these global environmental changes (GEC) and increasing temperatures the life history traits of indigenous and invasive species may be impacted.

In addition to global warming caused by greenhouse gases, the effects of changes in land use/ land cover on climate are an important part of GEC which, unfortunately, are frequently overlooked For example, land use changes have been linked to alteration in surface energy and water balance, changes in land surface temperatures and habitat degradation and loss of biodiversity. As a result, modifications in local conditions may have an important impact on

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ecosystems and ecosystem services, for example plant-insect interactions, and ultimately on agricultural productivity.

Thus, there is a need to better understand the interactions between agricultural intensification and GEC to meet the challenge of developing resilient production systems for important agricultural commodities like coffee.

Using the coffee berry borer (*Hypothenemus hampei*), the most important coffee pest worldwide, we document how environmental changes - particularly changes in temperature and rainfall – and coffee system type (shaded or sun-grown coffee) affect this major coffee pest. Starting in 1929 British entomologists investigated the eco-climates of coffee plantations in the Kiambu area of then colonial Kenya. Almost 100 years later we revisited the same coffee plots where those studies were conducted, to find out if and how changes in temperatures and land use pattern in the area have affected the cultivation of coffee in Kiambu.

MATERIALS AND METHODS

The study was conducted in a commercial coffee plantation in Kiambu district (Central province), Kenya.

Data on *H. hampei* infestation level were collected every two weeks from June 2009 to June 2011. In both the shaded and the sun-grown coffee plots.

To assess the temperature in the plots, data loggers (HOBO U12 J, K, S, T Thermocouple data logger, Onset Computer Corporation MA, USA) were installed in each plantation type (shaded or sun-grown) in June 2009. Temperature was recorded every half hour for the whole study period. In addition, historic climatic data for the farm and for the Kiambu area (Tmax, Tmin, Tmean, and precipitation) were gathered from the studies on eco-climate of coffee plantation by Kirpatrick and from McDonald, and provided by the Kenya Meteorological department.

The satellite data used in this analysis came from the Landsat-TM on December 17, 1984 and on August 19, 2010, and the Landsat-ETM on February 21, 2000. Additionally, topographic maps, Africover land cover data, and SRTM 90m digital elevation data for study area were used. To create a multi-temporary remote sensed data set for change detection, all images were geometrically corrected to the Universal Transverse Mercator coordinate system (zone 37) and then radiometrically normalized. ETM+ image were rectified to the same geo-referencing system as TM image using a geometric polynomial transformation model (first degree) and nearest neighbor resampling method.

RESULTS AND DISCUSSION

An important consequence of global climate change is human migration. Climate change consequently leads to internal displacement of people, and is hence a new key determinant of urbanization. At the same time, urbanization is an example of how land use change modifies regional climate. This interaction between global climate change, human migration/urbanization, economic development and the inherent modification of the landscape can severely affect ecosystems and people.

In sub-Saharan Africa, Kenya and its capital Nairobi are an example of such situation. The population in the country has noticeably grown during the last century from 2.5 million

inhabitants in 1897 to 40 million in 2010. Since the latter part of the 20th century the population of Kenya, and in particular that of Nairobi, has gone up sharply; that of Kenya from 15.3 million in 1979 to 40 million in 2010, and that of Nairobi from 827,775 in 1979 to 3.1 million in 2009. The Kiambu area in the outskirts of Nairobi has been traditionally and for many decades, one of the most important coffee production areas of the country. A consequence of this accelerated population growth and urbanization process of the last decades, has been the transformed landscape of Kiambu. The sharply increasing human population densities of Kiambu - 194 people/ km^2 in 1969 to 638 people/ km^2 in 2011 – are fueling the pressure on the land. Recently, coffee production has started to be replaced by upstream market real state developments and the few coffee farms that remain have responded by increasing management intensification in order to maintain their productivity.

Our analyses of land use change in Kiambu confirm the dramatic transformation since 1984, with the most noticeable changes in vegetation having occurred in areas around the larger coffee plantations. These plantations were originally pre-colonial "white settlements" and coffee estates. Kiambu's large coffee estates were and still are characterized by an intensified cultivation scheme under very low or no shade (Fig. 2). On the other hand, small-scale diverse production systems that include not only coffee but also maize, beans, timber and fruit trees (upper middle section of Figs. 1-2) show little vegetation change over time, which can be attributed to normal fluctuations in vegetation index or abandonment of coffee farms, indicating high resilience in the system. The small-scale farms around the original pre-colonial "white settlements" usually belong to numerous African families that with time have divided their land into very small units (of max. 1-2 acres each) where every member of the family (siblings) cultivates coffee and food crops in a diversified manner.

The striking changes in land use as a consequence of urbanization have had drastic effects on the prevalent temperature conditions of the Kiambu area (Fig 3). Our analysis of 82 years (1929-2011) of location specific climate data indicates an increase in temperature at a rate of 0.005°C per year, matching the IPCC estimates for Africa. It is noteworthy to mention that temperature recordings for the study area were gathered from weather stations within the areas dominated by large-scale coffee estates or directly at the farm where the field study was conducted.

Ecosystems are influenced by the dynamic interactions between climatic factors, plants, pests, their natural enemies and the surrounding ecosystem including humans. GEC together with changes in land use influence population dynamics at all trophic level. In agricultural systems, particularly that of coffee, herbivorous insects can have significant impacts on plant productivity and can become a constant problem for farmers. Pioneering work on the effects of eco-climatic conditions on insect pests in shaded and sun-grown coffee, was carried out by Kirkpatrick in the 1930s, in the same coffee plantation in Kiambu where our field study was conducted.

We were interested in studying how coffee is affected by changing environmental conditions via the indirect effects of a herbivore. We used the coffee berry borer because of its economic importance.

In order to simulate contrasting microclimatic conditions and management intensification levels and their effects on the coffee plant and the pest, two different coffee systems – shaded and sun-grown plantations – were compared, with the objective of investigating whether shaded coffee is indeed more resilient to climatic variability than sun-grown.

During the course of the study, mean temperatures in the sun-grown plantation were roughly 2°C higher than in the shaded one. Minimum temperatures were higher in the shaded system, on the other hand, indicating that the shaded system was less prone to drastic temperature fluctuations.

According to the reported thermal tolerance of coffee berry borer, the temperatures we recorded imply that *H. hampei* could develop in both plantation types, but that borer development would be much faster in the sun-grown system. Jaramillo et al. calculated that for every 1°C rise in the thermal optimum, the maximum intrinsic rate of increase of the pest would increase by an average of 8.5%. Consequently pest populations in the sun-grown plantation would rise 17% more than in the shaded one. We recorded a 12.4-fold increase in berry infestation and a 18.2-fold increase in the cumulative number of female beetles in the sun-grown versus the shaded plantation, confirming results of Jaramillo et al. model as well as corroborating observations made in coffee plantations in Mexico. Finally, pest infestation levels in the sun-grown plantation exceeded the 5% economic threshold on nearly all sampling dates, whereas in the shaded plantation this threshold was never reached. We also noticed a marked influence of rainfall pattern on the *H. hampei* infestation level but interestingly only under sun-grown conditions (Fig. 4). Rainfall triggers colonization flights of *H. hampei* females; an effect that is enhanced by high temperatures in the plantation.

Not only infestation of but also damage to the berries was more significant in sun-grown coffee. Here, colonizing females were more frequently found inside the berries constructing galleries and ovipositing, while females in the shaded plantation were more often found in the exocarp. Additionally, considerably more berries with a hole in the exocarp but without the insect were found in the shaded plantation, implying that the colonizing females probed the berries but did not find suitable conditions for gallery construction and egg laying. Delayed development and maturation of berries under shade and consequent changes in their final biochemical composition may explain this finding, as well as changes in the emission of host location olfactory clues used by the colonizing females.

In conclusion, our study illustrates the remarkable changes in human population density, vegetation cover and land-use, local climate and the interconnections of all these factors in the peri-urban environment of an East African capital over nearly 100 years. The study not only demonstrates the urgent need to study climate-change at regional spatial scales, but also the importance of local factors. Moreover, we were able to illustrate how these effects can affect agricultural productivity, mainly through their impacts on higher trophic levels like insect herbivores.

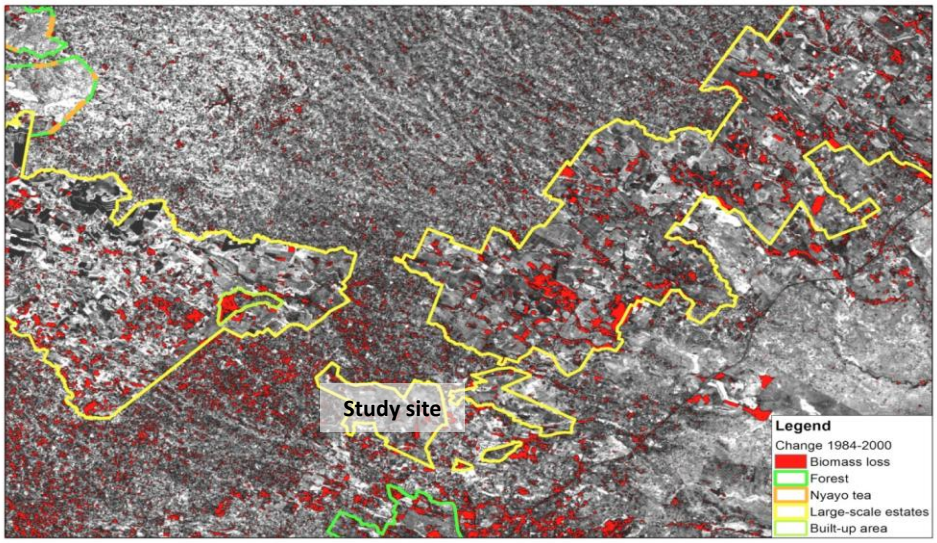

Figure 1. Land use change image 1984-2000 for the Kiambu area (Kenya) (background scene: Brightness image 1984).

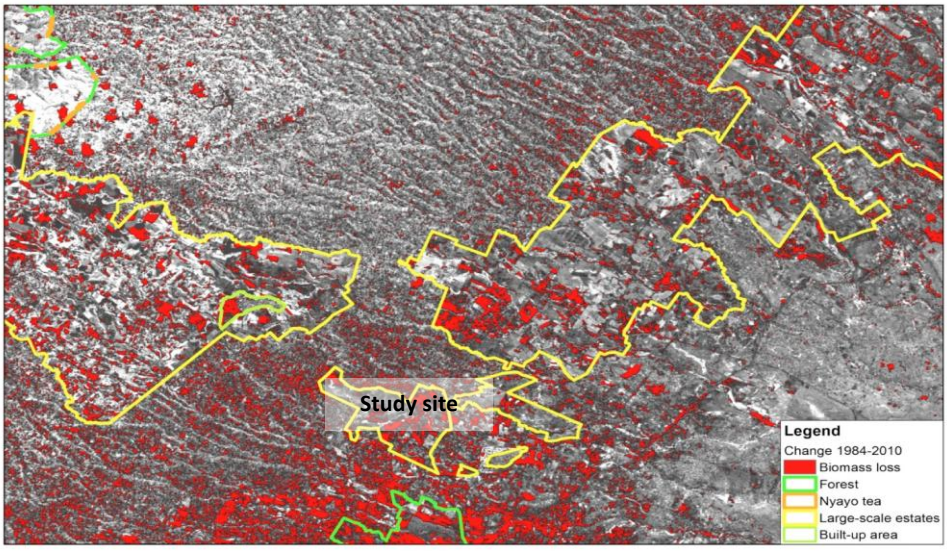

Figure 2. Land use change image 1984-2010 for the Kiambu area (Kenya) (background scene: Brightness image 1984).

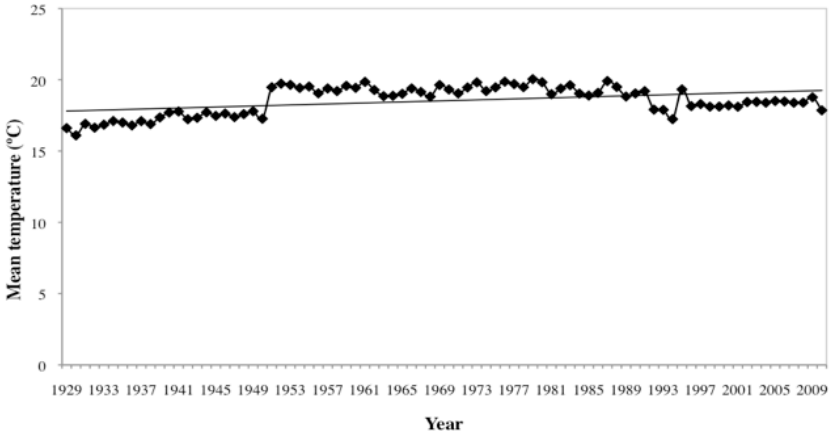

Figure 3. Mean temperature in Kiambu area (Kenya) during the period 1929 – 2011.

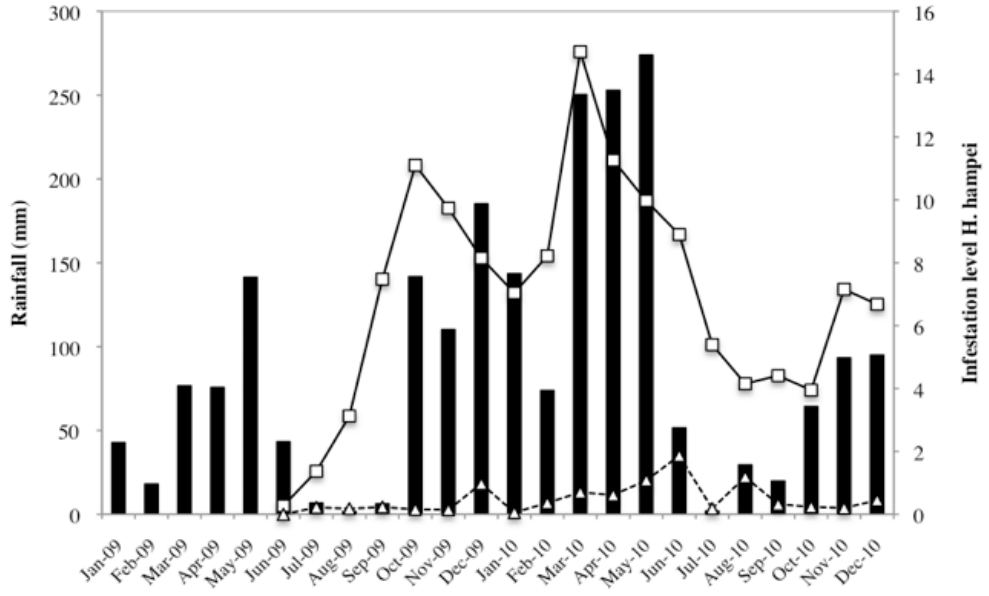

Figure 4. Effect of rainfall (mm) on *Hypothenemus hampei* infestation level under shaded and sun-grown coffee plantations in Kiambu (Kenya) between June 2009 and June 2011.

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Pyramiding of Rust Resistance Genes as an Approach for Durable Resistance in Arabica – Indian experience

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SUMMARY

Coffee leaf rust (CLR) caused by the obligate parasitic fungus *Hemileia vastatrix* Berk & Br. is the major disease of concern for arabica coffee (*Coffea arabica* L) cultivation, leading to severe crop losses and escalation of production costs. Resistance to CLR is reported to be conditioned by at least nine genes, S_H1 to S_H9, either singly or in combination; the corresponding virulence factors in pathogen are designated as v1 to v9. Of the nine S_H genes reported so far, S_H1, S_H2, S_H4 and S_H5 were identified in the tetraploid *C. arabica* where as S_H6, S_H7, S_H8 and S_H9, were introgressed to *C. arabica* from *C. canephora*, a diploid species while S_H3 was introgressed from another diploid species, *C. liberica*. Considering the durable nature of S_H genes of diploid origin, systematic breeding programme had been undertaken in India for the last 12 years to transfer the rust resistance genes of diploid origin into arabica using the *C. canephora* and *C. liberica* introgessed lines. The present work was aimed at assessing the field performance of the hybrids between Catimor and *C. liberica* introgressed lines with respect to yield potential and resistance manifestation. This communication highlights the leads achieved in pyramiding various rust resistance genes in *C.arabica* cultivars and successful application of marker assisted selection for tracking S_H3 gene in new breeding lines.

INTRODUCTION

Coffee is one of the important plantation crops grown in India, covering an area of about 0.4 mln ha of which Arabica and Robusta coffee types share 49% and 51% of the area, respectively. The annual production is around 0.31 mln MT, approximately 4% of world's total production. Coffee cultivation in India is unique as it is grown under two tier shade canopy in simulated micro-climate. This eco-friendly manner of coffee cultivation is instrumental for maintaining the forest cover and also in preserving rich bio diversity of flora and fauna. On the other side, coffee is cultivated in relatively harsh environmental conditions in India compared to other major coffee growing countries. In addition, there are some specific problems of diseases and pests associated with the climatic conditions prevailing in Indian coffee tracts that necessitate ideal shade management and appropriate growing practices.

Among various diseases affecting arabica coffee, coffee leaf rust (CLR) caused by the obligate parasitic fungus *Hemileia vastatrix* is the most serious disease of concern, leading to severe crop losses to the tune of 70% in susceptible cultivars, if timely control measures are not adopted. Considering the economics of disease management and also to discourage the chemical control measures, development of disease tolerant cultivars is the most preferred and viable option for sustainable coffee cultivation. Breeding for rust resistance undertaken in

different countries though resulted in development of several resistant varieties, breakdown in resistance has been commonly observed from time to time due to the evolution of new virulent races of CLR pathogen. Nevertheless, this phenomenon has been more commonly observed in India because of the favourable climatic conditions for rust disease build up and selection pressure on the pathogen due to the spread of several rust tolerant cultivars in field. Therefore, breeding for durable rust resistance is a continuous process and a crucial priority in the Indian context.

Resistance to coffee leaf rust is reported to be governed by atleast nine resistance genes, S_H1 to S_H9, either singly or in combination while the corresponding virulence factors in the pathogen are referred as v1 to v9. Of these nine resistance factors, S_H1, S_H2, S_H4 and S_H5 were identified in the tetraploid *C. arabica* while S_H6, S_H7, S_H8 and S_H9, were introgressed to *C. arabica* from the diploid species *C. canephora.* The S_H3 gene was introgressed from another diploid species *C. liberica.* The adaptive capacity of the *H. vastatrix* with an ability to overcome host resistances has resulted in the gradual loss of resistance in commercially grown cultivars and all the nine major S_H genes (S_H1 to S_H9) have been overcome by gradual appearance of new physiological races of rust pathogen. So far, 45 races of rust are known to be distributed in different coffee growing countries. However, the virulence of each race varies depending on the genotype and particular environment. In general it was observed that the resistance genes identified in *C.arabica*, used either singly or in combination, have not provided durable resistance to most of the races of rust fungus. On the other hand, the genes introgressed from the diploid species such as the S_H3 gene from *C. liberica* as well as certain genes from *C.canephora* are found to be more durable and have provided long - lasting protection to arabica under field conditions, as compared to the genes identified in tetraploid *C. arabica* itself. Hence, pyramiding resistance genes or the combined use of resistance genes of diploid species in a selected arabica genotype or using them in composite varieties are some of the promising approaches to achieve durable rust resistance.

For the last 12 years, systematic breeding efforts have been undertaken in India to transfer the rust resistance genes of diploid origin into arabica through *C. canephora* and *C. liberica* introgessed lines and several hybrid progenies have been developed. In the present paper we report the field performance of the hybrids between Catimor and *C.liberica* introgressed lines. Furthermore, the leads achieved in India in marker assisted selection for tracking S_H3 gene in donor parents as well as hybrid lines and its implications in breeding for achieving broad spectrum of rust resistance, are detailed and discussed.

MATERIALS AND METHODS

Plant material

With an objective of integrating S_H3 gene of *C. liberica* origin with the genes of robusta origin (S_H6 – S_H9) for achieving broad spectrum of resistance in semi-dwarf genotypes, crosses were effected between Catimor line derived from crosses between Caturra x HDT (CIFC 1343) and selected Indian cultivars, S.1934 and S.2931. Both S.1934 and S.2931 are the *C. liberica* introgressed lines and are used as donors for S_H3 gene while, the Catimor line possess the genes S_H6, S_H7, S_H8 and S_H9 introgressed from Timor hybrid (CIFC 1343). Four F_1 hybrid progenies (S.4814 to S.4817) generated during 1999 and field planted in 2000 at Central Coffee Research Institute formed the material for the present study.

All the genotypes were planted in compact plots at a spacing of 1.8 x 1.8 m, under a mixed canopy of shade. The plants were trained on topped single stem system and standard agronomic practices were adopted. The progenies have been evaluated for field tolerance to

rust, yield and bean characteristics. Observations on incidence of leaf rust disease were carried out during Oct-Nov, the peak months of disease build up. Individual plants with even few pustules were treated as susceptible for scoring the percentage of resistant/susceptible types. Further, vigour of the plants and retention of infected leaves was also taken into consideration for assessing the resistance manifestation and average of three years data was taken into account for selection. Washed clean coffee samples of each genotype were prepared from 6 kg of uniformly ripened fruits by following the standard method and the same was used for analysing the bean parameters like percentage of "A" grade beans (retained on 17 no sieve – 6.65 mm) and weight of 100 "A" grade beans.

Marker analysis

DNA was isolated from the fresh leaves using CTAB method following the protocol of Krizman *et al.* with modifications. The selected plants in both the progenies with superior agronomic characteristics were subjected to molecular assays with two sequence-characterized DNA markers (BA-124-12K-f, Sat244) closely linked to S_H3 gene. In the first step, all the selected plants were subjected to analysis with SCAR marker BA-124-12K-f in order to confirm the presence or absence of S_H3 gene. In the second step, plants that possess S_H3 gene were subjected to marker assays with marker Sat244 to establish the homozygous or heterozygous status of the gene. The PCR assays using specific primer pairs and electrophoresis conditions were followed as described by Combes *et al.* and Mahe *et al.*

Towards durability of resistance in "Chandragiri" variety

With an objective of pyramiding S_H3 gene with rust resistance genes S_H6, S_H7, S_H8, S_H9 in "Chandragiri" variety, crosses were effected between "Chandragiri" and S.3827, a hybrid of (Caturra x Ciocee) x (Caturra x S.795) used as donor parent for S_H3. The F_1 progeny established in field during 2010-11 season, also formed the material for molecular analysis.

RESULTS AND DISCUSSION

Evaluation of field performance

All the four F_1 hybrid progenies (S.4814 to S.4817) evaluated in the present study exhibited vigorous vegetative growth and plant ideotype was more towards semi-dwarf with compact stature as the crosses were made between semi-dwarf and tall phenotypes. Among the four progenies, S.4814 recorded the maximum projected yield of 1546 kg/ha clean coffee while the progeny, S.4817 recorded the next best yield of 1200 kg/ha. The genotypes differed significantly in their field reaction to leaf rust as studied for three years. The data indicated two progenies S. 4816 and S.4815 to manifest high rust incidence of 43.24% and 33.33% of population, respectively. However, the progenies S.4814 and S. 4817 recorded low incidence as mean per cent (3 years) of rust susceptible population varied between 2.32 to 5.15%.

As regards to bean grades, S.4817 recorded maximum per cent of "A" grade beans (67.6) while S.4814 recorded 64.75%. The weight of 100 "A" grade beans ranged between 18.23 to 18.65 gm indicating good bean density in both the lines. The evaluation of liquor profile revealed FAQ (Fair Average Quality) to FAQ+ with a overall rating of 85.5 and 87%. The liquor of S.4814 was reported to be quiet, balanced, with good sweetness and in S.4817, the liquor was clean with balanced profile and some distinctive honey taste.

Marker assays

In the present study, a total of two sequence characterized genetic markers Sat244, BA-124-12K-f, reported to be closely linked to SH3 leaf rust resistance gene were used for marker assays. Both the markers gave clear amplification profiles that could distinguish the plants for presence or absence of SH3 gene. In the preliminary assays, the analysis of the F1 hybrid progenies with marker BA-124-12K-f resulted in direct polymorphism with presence and absence of the 320 bp size band (figure 1). In secondary analysis of the selected plants that proved positive for SH3 (as revealed by the first marker) with marker Sat 244, the amplification product of 300 to 305 bp size is seen in plants possessing SH3 resistance gene, while in plants lacking SH3 resistance gene, a band of slightly lesser size (~ 300 bp) is amplified at the same locus (figure 2). Further, in plants homozygous to SH3, one intense band is seen, while in plants heterozygous to SH3, two less intense bands are seen (figure 2). Thus, the marker Sat 244 is more informative as it is possible to distinguish the plants homozygous and heterozygous for SH3 gene, based on the amplification profile.

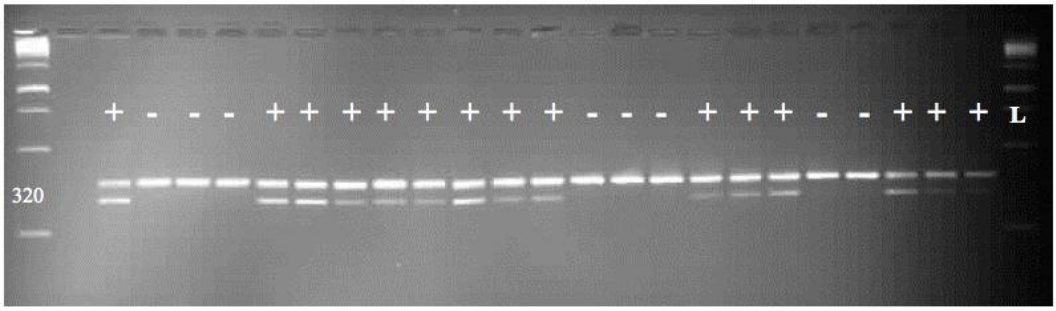

Figure 1. Amplification profile of F_1 hybrid progeny with SCAR - BA 124 12K showing presence (+) and absence of S_H3.

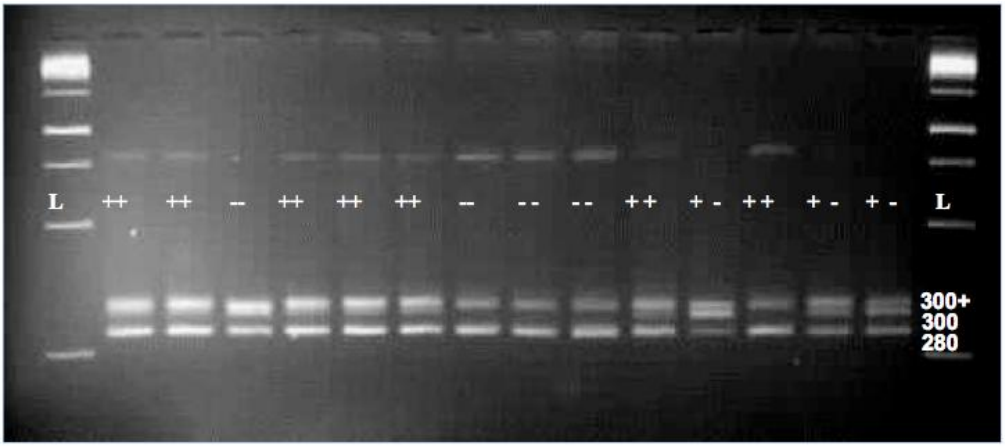

Figure 2. Amplification profile of F_1 hybrid progeny with SCAR Sat.244 showing homozygous and heterozygous status of S_H3.

Based on individual plant yield, field tolerance to rust and bean quality traits coupled with SCAR marker analysis, elite plants homozygous to S_H3 were marked and selfed among different progenies. The selfed progenies raised from individual F_1 plants were established in four different locations for simultaneous evaluation and selection for further exploitation.

Integration of S_H3 in Sarchimor derivatives

"Chandragiri", a Sarchimor derivative was released for commercial cultivation in India during 2007. The field performance of this variety is very encouraging with high yield potential and field tolerance to rust. The manifestation of high tolerance levels to rust pathogen is primarily due to the rust resistance genes of robusta (S_H6, S_H7, S_H8 and S_H9) introgressed from Timor hybrid (CIFC 832/2). Hence, there is likely risk that the races of "Catimor" may try to adapt on "Chandragiri variety" when expanded in larger areas. As a pre-emptive breeding strategy, efforts were made to transfer the S_H3 gene to "Chandragiri" variety to provide broad spectrum of resistance and S. 3827, a cross bred derivative of (Caturra x Ciocee) x (Caturra x S.795) was selected as S_H3 donor parent. Elite plants homozygous to S_H3 were selected in S.3827, based on agronomic characters and through marker assisted selection. Reciprocal crosses were made between "Chandragiri" and S.3827 during 2010 and six progenies were established in field. SCAR assays of the randomly selected plants of the F_1 progeny revealed the presence of S_H3 gene and detailed analysis of the field performance has been undertaken.

The results obtained in the present study established that the approach of integrating S_H3 with S_H genes of robusta origin is very promising for achieving broad spectrum of resistance in Catimor and Sarchimor derivatives. Rodrigues *et al.* reported that S_H genes are dominant and condition total susceptibility to compatible races and specific resistance to incompatible races. Coffee plants with the S_H3 resistance gene are expected to manifest susceptibility to the corresponding virulence factor (v3). Further, on studying the segregation pattern of the S_H3 in F_2 hybrids, Kukhang *et al.* reported a variable rust reaction to a virulent rust isolate and suggested that S_H3 resistance was more complex that could be explained by a single dominant gene and attributed the same to the relative durability of this resistance under field conditions in India. The segregation data of AFLP markers and rust screening of F_2 population derived from a cross between two *Coffea arabica* inbred lines, Matari and S.288, Prakash *et al*. also confirmed the hypothesis of a single dominant gene for the S_H3 resistance factor. Furthermore, even though virulent rust races with gene combinations comprising of v3 virulent gene are distributed across the Indian coffee tracts, to the genotypes with S_H3 gene combinations tend to exhibit the ability to with stand the onslaughts of rust so that the rust management in these genotypes is possible with timely prophylactic sprays. Thus, because of the complex nature of resistance manifested by S_H3 gene under field conditions and present only in early Indian arabicas like S.795, there exists a great scope for its exploitation in breeding for enhancing the spectrum of resistance in several semi-dwarf genotypes.

It may be pertinent to mention that the rust pathogen could be able to overcome the resistance of Catimor genotypes derived from Timor Hybrid (CIFC 832/1) in almost all the coffee growing countries. Consequently, the Sarchimor derivatives evolved from Timor Hybrid (CIFC 832/2) gained popularity and being cultivated across several coffee growing countries. As the resistance in Sarchimor genotypes is mainly imparted by the genes of robusta origin introgressed from Timor hybrid (CIFC 832/2), there is a likely possibility that the races prevalent on Catimor derivatives may try to adapt on Sarchimor genotypes. Hence, the selected strategy of integrating SH3 with that of other robusta genes in Sarchimor is a promising approach for durable rust resistance. In this direction, the leads obtaining in the present study in successful transfer of SH3 gene into Catimor and Sarchimor derivatives, has great promise in developing new lines with broad spectrum of resistance to leaf rut.

The availability of SCAR markers linked to SH3 gene, facilitated the marker assisted selection for pyramiding of S_H3 gene with other rust resistance genes. However, efforts to develop markers linked to other rust resistance genes, especially that of *C. canephora* origin are not very successful. The recent report of Dhiora *et al.* on AFLP markers linked to

resistance genes of *C. canephora* origin (S_H7, S_H8, S_H9) is the second report and the utility of these markers for Marker assisted selection needs to be validated. In conclusion, the study clearly demonstrating the scope and potential of the approach and marker assisted selection for pyramiding of leaf rust resistance genes in arabica coffee.

CONCLUSIONS AND PERSPECITVES

The results obtained in the present study are very interesting. The important conclusions from the study and future perspectives are as follows.

- Integration of S_H3 in Catimor and Sarchimor derivatives has been successfully achieved
- The SCAR markers for S_H3 proved very efficient for MAS.
- The elite plants identified in F_1 progenies of Catimor, can be exploited for commercial cultivation by mass propagation through tissue culture.
- Potential scope also exists in deriving a composite variety.
- The S_H3 introgressed Sarchimor lines have potential implications in providing enhanced and long lasting resistance to leaf rust pathogen.

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New Record and Outbreaks of Bacterial Blight of Coffee (Pseudomonas Syringae) in Southern Ethiopia: Impact of Climate Change Scenarios

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SUMMARY

So far, apart from the three calamitous diseases, namely coffee berry disease, coffee wilt and leaf rust, others are unimportant on Arabica coffee (*Coffea arabica* L.) in Ethiopia. There have been, however, sporadically severe outbreaks impacting the crop production and the case in point is bacterial blight of coffee (BBC). Bacterial blight of coffee also known as Elgon or Solai dieback, caused by *Pseudomonas syringae* pv *garcae* van Hall, is reported as a serious disease of Arabica coffee in Kenya and Brazil. This bacterial pathogen was earlier noted to be associated with bean rot or discoloration of coffee in Ethiopia. In May 2008, outbreaks of uncommon disease of coffee with blight syndrome were observed for the first time in two districts in southern Ethiopia where the famous Sidamo and Yirgacheffe specialty coffees are produced by small-scale farmers. Since then, detailed investigations including diagnostic surveys for assessing the disease occurrence, severity and incidence accompanied by collecting different parts of infected coffee trees in the fields were conducted along with isolation, identification and characterization of the causal pathogen in the Plant Pathology Laboratory at Jimma Research Center. The results of three years (2008 – 2011) study showed that the disease syndrome on young, mature and older coffee plants were similar with bacterial blight of coffee documented elsewhere. The disease invariably attacks coffee leaves, branches and shoots with characteristic blight symptoms. The infected branches and shoots start die-back from the point of infection towards the tip while coffee berries on infected braches are also completely destroyed leading to total crop failure. The isolation and identification of the causal pathogen from samples of leaves, berries, branches and shoots consistently produced a bacterial species which belongs to *Pseudomonas syringae* that further proved by characterization and pathogenicity tests. The disease incidence and severity during the first outbreak (2008) was about 90 and 68 percent, respectively. In subsequent years, in addition to the disease prevalence in already affected areas, fresh BBC outbreaks were discovered on coffee trees in 11 sub-counties of the neighboring districts with mean severity that ranged from 6.4 to 38.4 and 2.6 to 45.7 percent in *Aletawondo and Aletachuko* respectively, resulting in considerable damages. Among others, climatic factors mainly unusual rainfall with hailstorm might favoured the disease outbreaks implicating the present climate change *scenarios* are upsetting Arabica coffee production in east Africa.

INTRODUCTION

Bacterial blight of coffee (BBC) caused by the phytopathogenic bacterium *Pseudomonas syringae* pv *garcae* van Hall is an important disease of Arabica coffee in Kenya and Brazil. It is known with different names Elgon or Solai dieback in the former country while it is called halo or aureola leaf spot in Brazil. Copper fungicides are intensively applied to reduce heavy crop loss or eliminate damage on coffee trees and harvest good yield (Kairu *et al.,* 1985; Kairu and Muthamia, 1989; Kairu, 1991). Itao *et al.* (2008) also reported that some coffee cultivars IAPAK 59, IPR103, IPR104, and IPR108 showed resistance to halo blight after evaluating under field conditions.

The pathogen *Pseudomonas syringae* has a very wide hosts ranging from annual herbaceous monocots to perennial woody fruit trees with many kinds of symptoms, which is extremely difficult to control and result in significant economic losses (Kennelly *et al*., 2007). Bacterial blight of coffee is little known and not reported so far from Ethiopia, although it is presumed to induce berry rot and/or bean darkening perhaps in association with infestation of antestia bug (*Antestiopsis spp.*) mainly in lower altitude coffee belts of the country (Eshetu *et al*. 2000). In 2008, unknown disease with blight syndrome was widely observed on coffee trees in some districts of the major coffee producing regions of southern Ethiopia. Subsequently, detailed survey works and investigations were undertaken to detect the disease and determine causes of the problem, and thus findings of the research work on the nature of the disease and its causal agent, occurrence and distribution in relation to some influencing (weather) factors are elucidated in this document.

MATERIALS AND METHODS

Disease survey and assessments

Since the first in its kind of occurrence and the disease outbreak was so terrifying that diagnostic surveys were systematically designed and executed to detect and assess the magnitude of damage, accompanied by critical observations that involved diagnosis of infected coffee trees, symptom descriptions following the disease development. Alongside, a number of queries on important factors such as agronomic practices undertaken by the farmers and any deviations in weather conditions that supposed to influence the disease occurrence were noted in questionnaires.

Disease diagnosis and detection- symptoms description

How the syndrome starts on and progressive symptom development was studied on different parts of infected coffee trees (leaves, twigs, branches and berries, and vertical heads) after labeling each part at very early stage of infections. Those typical symptoms were progressively captured with digital camera and then analyzed in comparison with published works (CABI, 2006).

Disease assessment

Five to ten samples of affected coffee fields in three affected villages were systematically selected in the three outbreak districts (*Dara, Aletawondo and Aletachuko*) for disease assessment. Number of infected leaves, branches, twigs or damaged heads on each sample tree were counted and recorded. Besides the sample based assessment, detailed baseline data and information (questionnaire-based survey) were collated by extension agents and experts in almost all affected farmers' coffee field after having trained them about the disease.

Disease severity (number of infected leaves per branch, number of infected branches and/or twigs per tree), incidence (number of trees with infected leaves, branches/twigs per total sampled trees in a field) and prevalence (number of infested coffee fields per village '*kebele*') were computed and disease distribution was analyzed and finally mapped.

Isolation and identification of the causal agent

About 12 samples of leaves, branches/ twigs and berries were randomly collected from symptomatic blighted/ infected coffee trees in almost all the assessed fields. They were kept in perforated plastic bags, labeled and transported to Plant Pathology Laboratory at Jimma Research Center. The specimens were maintained in refrigerator at 4 ^{o}C until isolation. Three culture media (Pseudomonas agar base, nutrient agar, and King's B medium were prepared and used to isolate and grow for identification and characterization of the pathogen isolates accordingly (Lelliott and Stead, 1987; Kairu 1997).

Two isolation procedures, namely, direct plating of the specimens and dilution method were employed. Small symptomatic sections/ pieces were taken from leaves, branches/ twigs and berries were disinfected with 5% sodium hypochlorite solution (laundry bleach), dried under running laminar hood and then four pieces were plated on each culture media. In the dilution technique, about three branches or twigs were kept in sterile test tubes with distil sterilized water for 2 hrs and then from each suspension three plates were streaked with sterile wire loop on each of the above mentioned media.

The plates were incubated at 28 ^{o}C for 24 to 48 hrs while each plate was examined for any microorganisms emerging from the plated specimens or streaks. Those putative bacterial colonies were purified on the same media from a single cell colony and incubated for the same period, and the isolates were identified based on colony pigments (presence/absence of fluorescent) and morphology, and cellular structures under microscope. Representative strains of all the survey sites were further characterized on Pseudomonas base agar and King's medium (Lelliott and Stead, 1987; Kairu 1997).

Pathogenicity test

The pathogenicity of two representative bacterial strains were tested on young coffee seedlings raised from seeds of two known coffee varieties *viz.* '971' and '85257' (released as specialty coffee varieties in Sidama/Gedeo areas in 2010, Jefuka *et al*. this proceeding). The seedlings were grown in polyethylene box (5.8 L volume) containing heat sterilized sandy soil in the lath house at Jimma Research Center in 2009/2010.

The coffee seedlings were inoculated with two groups of bacterial isolates at three to four pairs of true leaves stage. The two isolate groups were those bacterial colony showing typical yellowish green (fluorescent) pigment and the grayish-white (nonfluorescent) on Pseudomonas agar and King's B medium. Bacterial suspension was prepared from cultures of each group grown on nutrient agar for 36 hr at 28 ^{o}C. The suspension was diluted in distilled sterile water to 10^{9} cfu/ml, and a pair of fully expanded young leaves of four seedlings (per coffee variety) were pricked and inoculated with one milliliter of the suspension using hypodermic syringe (Kairu, 1997). A coffee seedling with the same number of leaves was pricked with sterile water as a negative control. All the inoculated seedlings were immediately misted with sterile water, covered with transparent plastic sheet and maintained in air-conditioned growth room at 22 ± 1 ^{o}C. The water spray was applied every morning and evening in order to maintain high humidity during the study period. Disease infection and

symptom appearance were observed throughout the study period and finally re-isolation and characterization was conducted in the laboratory.

RESULTS AND DISCUSSION

Symptoms of the disease syndrome on infected coffee trees in the field

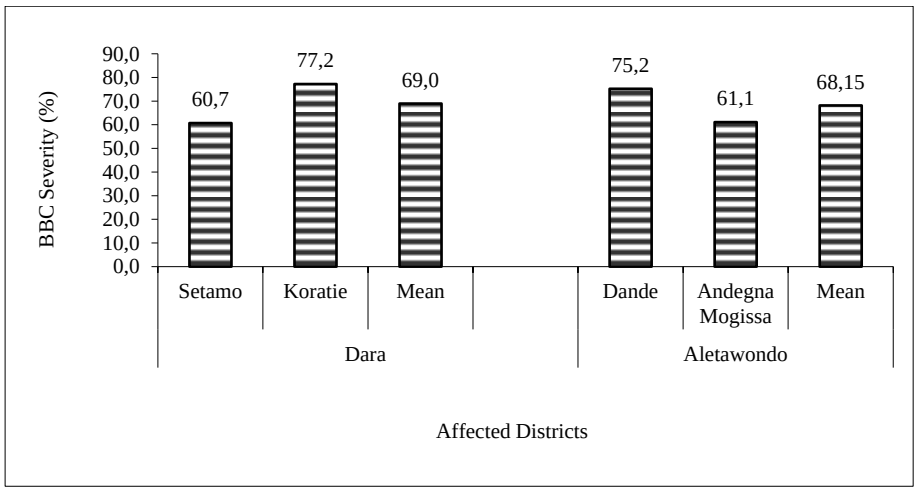
The first symptoms of the disease were blackening of nodes that progressed to internodes and darkening of petioles and basal parts of the leaves lamina attached on the same node of infected branches or twigs. During the rainy and wet season, most of the infected parts show clear water-soaked lesions. As the disease develops, these symptoms eventually cover the whole leaves turning dark brown rolling inward and often remain attached to the dying branches or twigs. In some cases, as the infected branches and twigs dieback, the axial leaves or tips turn yellow and finally become brownish black. The immature berries at pinhead and expanding stages are also very susceptible and become shriveled.

The disease usually begins on the upper or middle canopies of coffee trees with apparently succulent and actively growing vegetative parts of the plant that spread down the vertical heads with primary or secondary branches leading to complete death. At first glance, all the attacked trees in the field appear as if burnt by fire. When the rain stops or became erratic for some time (a week or more), the disease progress also cease and fresh symptoms may not be seen, in stead new flash of suckers grow just below the cessation point that may be re-infected when the favorable conditions occurred again. In such a way, multiple infections are possible per season that finally deteriorated or debilitated the coffee trees although it did not entirely kill the plant unlike vascular wilt or root rot diseases.

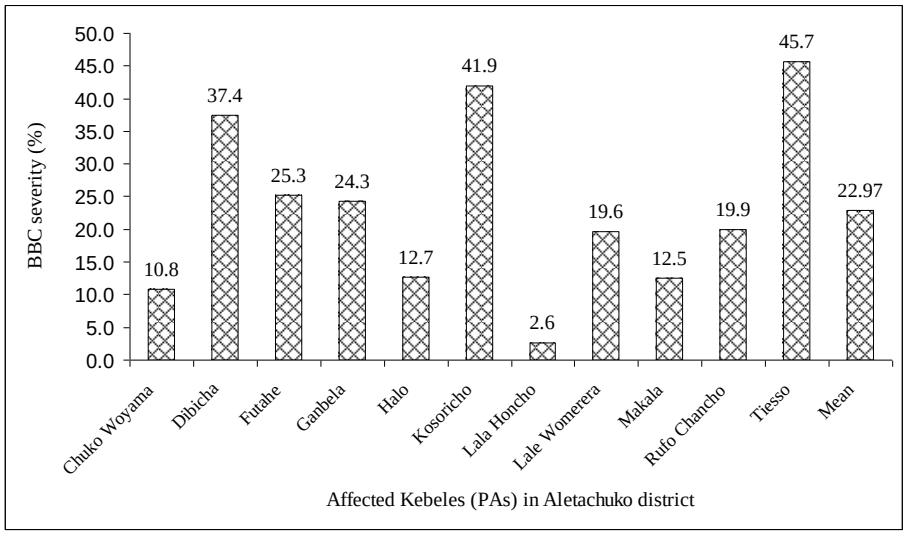
Such symptoms are similar to that of the disease known as bacterial blight of coffee (BBC) caused by *Pseudomonas syringae* documented elsewhere (Ramos and Shavida, 1976; Kairu, 1997) but not officially reported on Arabica coffee in Ethiopia. However, the bacterial pathogen *P. syringae* was recorded to be associated with coffee bean darkening or berry rot. The observed symptoms and dieback is different from branch dieback incited by thread blight (*Corticium koleroga*) and that of leaf blight (*Ascochyta terda*) commonly encountered during wet seasons and in highland coffee growing areas of Ethiopia (Eshetu *et al.* 2000).

Occurrence and distribution of bacterial blight of coffee

The disease syndrome was observed for the first time on few coffee trees in some farmers' fields in two adjacent coffee producing *weredas* (= districts) known as *Dara* and *Aletawondo* in Sidama zone (*ex* Sidamo) of southern region of Ethiopia in 2008. However, some coffee farmers reported that they have noticed similar symptoms one or two years earlier. During the first outbreak in 2008, the disease incidence ranged from 80 to 100 percent per sample plot with average severity was about 68 percent (Fig. 1a). In the next year, in addition to its prevalence and increased intensity in already affected areas, the disease outbreak was further noted in another nearby coffee growing district called *Aletachuko* with mean incidence and severity of 95 and 60%, respectively. Besides the sample based survey, comprehensive data analysis indicated significant severity that ranged from 2.6 to 45.7 percent in *Aletachuko* resulting in considerable damage (Fig. 1b).



(a)



(b)

Figure 1. Severity of bacterial blight of coffee in three districts of Sidama zone; Dara and Aletawondo in 2008 (a) and Aletachuko in 2009 (b) in southern Ethiopia.

The disease attacked the coffee trees at all stages although there were variations among the trees that perhaps attributed to genetic differences among the coffee types under field conditions. It was rather more severe and caused extensive damage on susceptible trees resulting in complete crop failure destroying coffee berries. The destruction was so serious in certain areas that forced some farmers' neglect their coffee fields while others cut down and uprooted those damaged trees and grown beans or other vegetable crops.

Predisposing factors for the occurrence and outbreaks of BBC in Ethiopia

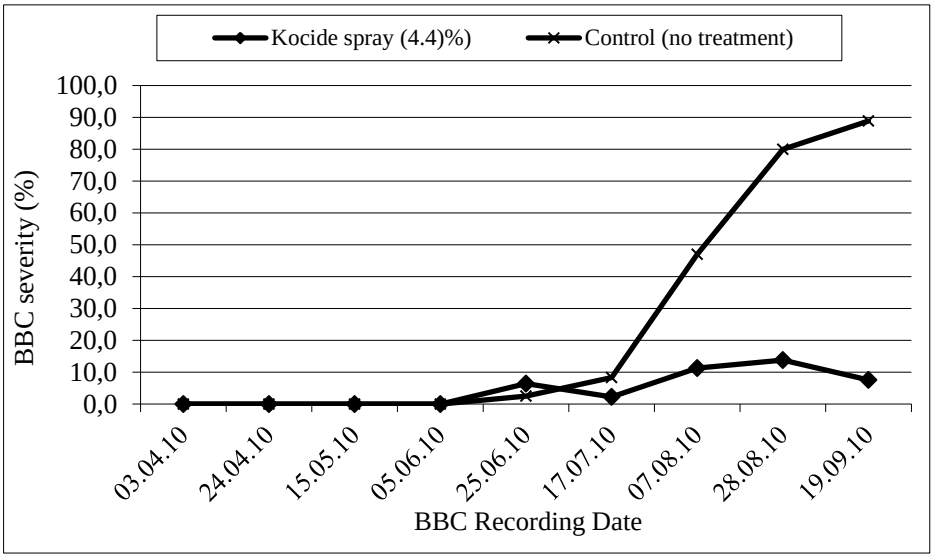
During this study, interviews with most farmers elucidated that they did not experience such type of disease that 'burn' their coffee and they claimed severe and prolonged drought preceding the disease occurrence. Some farmers also agreed or attributed that the occurrence and outbreak of the disease followed the unusual torrential rainfall with heavy hailstorm destroying most of their crops including coffee. There have been hailstones injury observed on most of the infected branches/succulent twigs during the disease assessments. These observations suggested that such weather calamities might have predisposed the coffee plants to bacterial infections. Kairu (1994) reported similar observations that frost damage provided the pathogen with infection entry avenues and in one case in Kenya, frost injury was followed by severe BBC leaf and shoot infection. Ramos (1979) demonstrated the epiphytic survival of

Pseudomonas syringae pv *garcae* on coffee in field inoculation and the result showed that dispersal of inoculum occurs by rain splash.

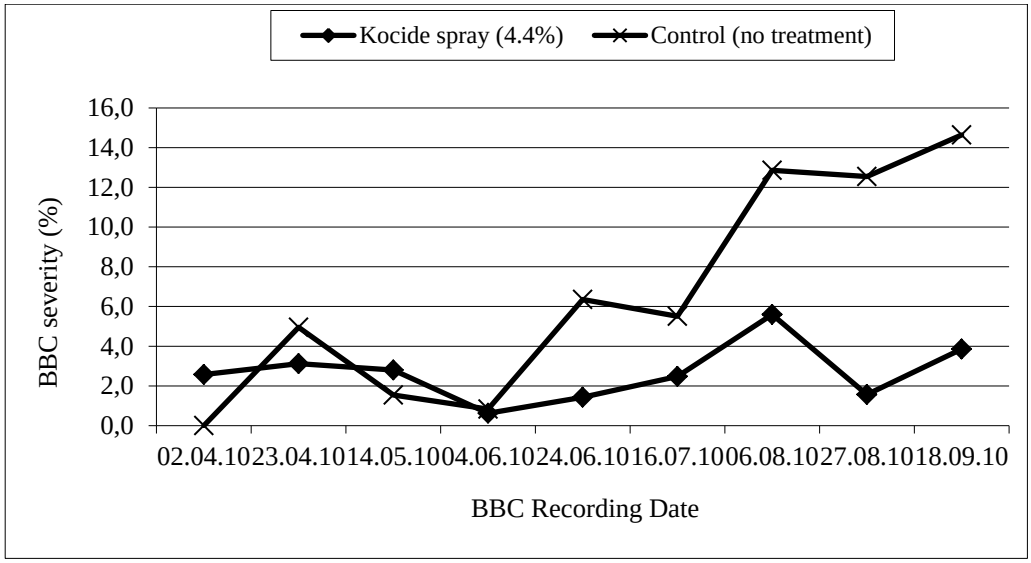
The disease progress was studied on randomly selected coffee trees treated with fungicide (Kocide) (Kairu and Muthamia, 1989) and untreated control plots under field conditions at two locations. There was no or few bacterial blight symptom recorded both on treated and untreated coffee trees in April and May 2010 during which little or no rain was recorded in the field at Tiesso in Aletachuko. While the disease started in early June and sharply increased, especially on untreated coffee trees (no kocide spray and no pruning), in July and August 2010 (Fig. 2a). In the second field, at Dande (Aletawondo), the disease infection was present throughout the study period although its severity increased as of early June 2010 (Fig. 2b). These variations in bacterial blight epidemics can be ascribed to the prevailed weather conditions mainly rainfall pattern for the bacterial pathogen infection and disease development. Ramos and Kamidi (1981) stated that BBC fluctuation, epiphytotic inoculum abundance, and subsequent disease increase, which occurred during maximum extension growth and flowering of trees, were determined by the intensity and duration of rainfall.

Identification and characterization of bacterial isolates from specimens of coffee

Almost all the specimens of leaves, branches, twigs and berries collected from infected coffee trees in all sample fields of affected localities namely, Dara, Aletawondo and Aletachuko, consistently produced bacterial colony typically similar in color and morphology to the *Pseudomonas spp*. on both nutrient agar and Pseudomonas base media after 24 hr incubation at 28 ^{o}C. Pure colonies of 39 bacterial isolates were broadly categorized into two groups fluorescent type (yellowish green colony) and grayish white (nonfluorescent) colony based on pigments they exhibited on King's B medium and Pseudomonas base agar. The bacterial isolates showed Gram negative reaction and were characterized with conventional procedures and pathogenicity test on the host. Barta and Willis (2005) also observed variation among strains of *Pseudomonas syringae* pv. *garcae* as fluorescence (1399, 2710 and 2708) and non fluorescence group (512, 588 and 589).



(a)



(b)

Figure 2. Seasonal patterns of bacterial blight of coffee on mature trees at (a) Tiesso, Aletachuko (b) and Dande, Aletawondo in southern Ethiopia (2010).

Pathogenic reactions of the bacterial strains on coffee seedlings

In the pathogenicity test, the two bacterial groups yellow green fluorescent colony and gray white nonfluorescent strain inoculated with leaves of young coffee seedlings showed typical leaf blight symptoms of BBC infection on both coffee varieties (971 and 85257) (Figure 3). The symptoms were characteristically related to those observed on infected mature coffee trees in the field and similar to the disease symptoms described on Arabica coffee in Kenya (Ramos and Shavdia, 1976; Kairu, 1997). Both bacterial strains at first produced small dark necrotic water-soaked lesions just at the point of inoculation that eventually covered all over the leaf as the disease developed. Surprisingly new infections were also initiated around apex of inoculated leaves (away from pint of inoculation) indicating that the bacterial cells washed down with droplets of water from the preceded spreading necrosis and incited infection (Plate 1). This showed that the inoculum from the initial infection at the top or middle canopies can be sources of infections for plant parts at lower canopy under field conditions.

Figure 3. Typical symptoms of bacterial blight of coffee on artificially inoculated coffee seedlings 'cv. 971' with *Pseudomonas syringae* isolates in the growth room at Jimma (2010).

In general, the symptoms reproduced by both strains of *P. syringae* in our pathogenicity test are similar to the type of symptoms observed on infected coffee trees in the field during the study periods which is in agreement with the bacterial blight of coffee present for many years in Kenya (Firman, 1963; Ramos and Shavdia, 1976; Kairu 1997). It was, however, different from halo blight or bacterial spots on Arabica coffee reported in Brazil (Itao *et al.*, 2008; Destéfano *et al*. 2010). Besides, in our study, the Ethiopian *Pseudomonas syringae* isolates are proved to be pathogenic and more closely resembles that of Kenyan strains reported by Kairu (1997). Kairu (1997) indicated that *Pseudomonas syringae* pv. *garcae* from Kenya and Brazil differed in pathogenic and biochemical characters. Destéfano *et al*. (2010) recently reported a new pathovar *P. syringae* pv. *tabaci* isolated from Arabica coffee seedlings with leaf spot in the nursery and found the same symptoms on artificially inoculated leaves of coffee seedlings, although he mentioned that these symptoms were similar to those caused by *Pseudomonas syringae* pv. *garcae* or *Burkholderia andropogonis*.

In conclusion, our findings evidenced the first occurrence and wide spread of bacterial blight of coffee caused by the bacterial pathogen *Pseudomonas syringae* in southern Ethiopia. The disease epidemics is found to be favoured by torrential rainfall associated with hailstorm and prevalence of wet and humid conditions, that perhaps reflects one of the climate change *scenarios*. The disease has been causing severe damage to Arabica coffee since 2008 or earlier impacting production of Sidamo and Yirgacheffe speciality highly demanded in the world market. Thus, further in-depth research on the disease epidemiology and control practices are required along with exploring and developing resistant varieties against the emerging bacterial disease of coffee. While strong efforts to contain its large-scale spread and damage to unaffected coffee growing areas especially in the west and southwest Ethiopia where the wet and humid climatic conditions are more prevalent. Detailed characterization of the bacterial strains collected from Arabica coffee in Africa (Ethiopia and Kenya) and Latin America like Brazil may resolve the present controversies in the pathogen population structure in relation to the disease symptoms.

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Application of Coffee Rust Genomics to the Understanding of its Evolution

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SUMMARY

We have applied genomics to study population evolution of coffee rust pathogen due to a recent outbreak of the disease in Colombia. A deep DNA and RNA sequencing was carried out in 8 and 3 *H. vastatrix* isolates respectively. According to the results using the software CEGMA, a first estimation of the *H. vastatrix* genome size resulted in over 250Mb, with our sequencing data covering around 92% that exhibited a GC content of 32%. Most *H. vastatrix* sequences had a homologous sequence with other fungal sequence or were uncharacterized. *H. vastatrix* isolate/race diversity at the transcriptome level is significant. We have also identified that over 60% of the *H. vastatrix* genome contains putative transposon sequences. Even though the identification of differences in secretomes of several *H. vastatrix* isolates is in progress, we already have identified a set of *H. vastatrix* sequences, which are presumably involved in pathogenesis of the fungus. Molecular markers and aggressiveness tests performed in coffee rust isolates suggested that the recent epidemic of the disease in Colombia was caused by race II or a close genetic variant of this race. Genome sequencing is a powerful tool for the study of *H. vastatrix* mechanisms to infect coffee and to identify genetic differences between races and isolates of the pathogen.

INTRODUCTION

Coffee rust, caused by the fungus *Hemileia vastatrix*, leads to widespread damage to crops worldwide. The pathogen has been reported to cause losses of up to 30% in susceptible varieties of the species *Coffea arabica*, if it is not controlled. The disease is polycyclic, which means that it undergoes several cycles during the cultivation period, where these can overlap. The fungus is present in almost every coffee-producing country in the world. In Colombia, it was reported for the first time in 1983 in the central coffee-producing zone of the country, and since then, it has quickly spread to all other coffee-producing areas of the country. A recent outbreak of the disease has affected major areas of coffee production in Colombia. It seems that this new epidemic was caused by increased patterns in rainfall in the country and it is necessary to continue monitoring for the emergence of new races of the pathogen.

It is well established that there are multiple races that exist in coffee-producing countries throughout the world, and studies from the CIFC (Coffee Rust Research Center) in Portugal have demonstrated the presence of more than 30 races of the pathogen identified from a series of more than 40 types of coffee. Historically, race II has been predominant in most countries, and it attacks all cultivated varieties of the species *C. arabica* that have not been genetically enhanced for pathogen resistance. In Colombia, 6 pathogenic races have been identified with confidence, and some other unknown races have also been detected. However, it is estimated that there are more than 10 different races attacking the different coffee varieties grown in this

country. The changes in virulence of the pathogen have been reflected in the appearance of new isolates that have been able to infect plants that were originally resistant.

Rusts are some of the most devastating diseases of crops and the recent emergence of a new race of the stem rust fungus that threatens to destroy wheat worldwide and new epidemics of coffee rusts are signs that show us that we have to study these destructive pathogens in detail. The study of the genome of these pathogens is giving insights of their evolution mechanisms and the repertoire of genes that are involved in pathogenesis. Among the rust fungi (Pucciniales) with genomic studies, the poplar rust genome *Melampsora larici-populina*, sequenced by the DOE Joint Genome Institute (JGI), has been completed by a shotgun method. This genome assembled into 462 main genome scaffolds totaling 101.1 Mb.

Genomic analysis is, similarly, likely to open up new avenues for the discovery of virulence determinants in other pathogens. The Broad Institute released the *Ustilago maydis* genome sequence. *U. maydis* is an important model on the study of plant-fungi interactions with an estimated genome size of 20.5Mb, contains 6,902 predicted protein-encoding genes and lacks pathogenicity signatures found in the genomes of aggressive pathogenic fungi, for example a battery of cell-wall-degrading enzymes. Deletion of individual clusters altered the virulence of *U. maydis* in five cases, ranging from a complete lack of symptoms to hypervirulence [10]. Despite years of research into the mechanism of pathogenicity in *U. maydis*, no 'true' virulence factors had been previously identified. Thus, the discovery of the secreted protein gene clusters and the functional demonstration of their decisive role in the infection process illuminate previously unknown mechanisms of pathogenicity operating in biotrophic fungi. Other basidiomycetes fungi have been recently sequenced including *Coprinus cinerea* with a 37-megabase genome assembled into 13 chromosomes.

The first *H. vastatrix* genome data identified 6763 genomic sequences that were assigned to the *H. vastatrix* transcriptome. That study predicted secreted proteins possibly involved in *H. vastatrix* infection processes of coffee tissues. We have obtained genome and transcriptome sequences of the coffee rust *H. vastatrix* and these data will allow the identification of potential molecular markers for the study of the rust races. Also, the knowledge of its secretome is a start for the understanding of the mechanisms used by the fungus in the colonization of coffee tissues and its similarity to other plant rusts pathogenicity processes.

MATERIALS AND METHODS

H. vastatrix urediniospores samples were taken from coffee infected leaves taking care of sampling vey young pustules with no evidence of the presence of the parasite fungus Lecanicillium lecanni. The samples of H. vastatrix genome were sequenced by Illumina™ and ROCHE™ 454 technologies. Reads were subjected to quality control with FastQC (Babraham Bioinformatics, Babraham Institute). Then, they were trimmed (CLCbio script), masked or filtered by low complexity end regions and reads shorter than 70 nucleotides were discarded. Mdust and SeqClean were used for the cleaning process (The Gene Index Project, Harvard University). Genome sequences were assembled with the CLC Assembler (CLC bio, Aarhus, Denmark). The quality of the assembly was assessed with CLC tools and in-house R scripts. For prediction of gene models we followed the ‘align then assemble’ approach.

We aligned RNAseq data to the genome with Cufflinks and we predicted proteins in the mapped contigs using Augustus. Gene families from predicted proteins larger than 70 amino acids were annotated with OrthoMCL. H. vastatrix predicted proteins were used for classification into secreted and non-secreted proteins. For this task the programs SignalP 4.0

and PProwler were used to predict secreted proteins. A 0.9 probability cut-off was used for PProwler predictions. A set of secreted proteins predicted previously for H. vastatrix was compared with our predictions. Shortly, a Blastp was performed between our set of H. vastatrix proteins and Fernández et al. (2012) predictions. Finally, a set of proteins that showed similarity with the proteins predicted as secreted proteins was obtained. Multiple comparisons of the three sets of secreted proteins were performed (SignalP, PProwler and Fernández-Blastp).

For genome annotation we used standard perl scripts and basic bioinformatics software such as BLAST; BLAST homologies were visualized using a BLAST Output Visualization Tool. The databases we used for comparisons corresponded to 67.118 Pucciniales proteins comprising 16,694 amino acid sequences from Melampsora larici-populina, 22,815 Puccinia striiformis f. sp. tritici sequences, 15,979 P. graminis f. sp. tritici sequences, and 11,630 P. triticina bbbd race 1 isolate 1-1 sequences.

We also aligned H. vastatrix genome contigs against the genomes of *P. graminis*, M. laricis-populina and U. maydis using Mauve. The Low Collinear Blocks (LCB) values were set by visual inspection searching the best block size for each pair of alignments (largest coverage of both genomes). Finally, values used for LCB were: *P. graminis* 12154, M. laricis-populina 10409 and U. maydis 1203. We surveyed the frequency and classes of TE-like elements present in the coffee Rust assembled contigs using RepeatMasker. We also manually annotated retrotransposon families identified after prediction of gene families with OrthoMCL.

RESULTS AND DISCUSSION

We constructed single libraries for 454 sequencing and paired libraries for Illumina sequencing. We obtained 412 million short-reads from Illumina and 5.8 million reads from 454. The quality analysis of the 454 reads showed low quality before base at position 200. The mean of the quality value was below 20 around base position 600 and a bias of nucleotide composition before this position was identified. The mean read quality was considered acceptable.

For Illumina reads, the mean quality value was above 20 for all isolates, although some reads had low quality after base at position 95. Reads had little bias in nucleotide composition at the beginning of the read. The mean read quality was very high for all isolates. The level of read duplication was low for 454 reads. Illumina reads showed some level of duplication, approximately 20% of reads had 10 or more reads duplicated, this probably was an artifact of sequencing and was taken into account for the read cleaning process.

We performed a hybrid 454-Illumina assembly of all genomic *H. vastatrix* and of HvCat sequences and Illumina individual assemblies of the seven remaining isolates sequenced with the CLC Assembler (CLC bio, Aarhus, Denmark) (Table 1). We assessed the completeness of the genome by running the software CEGMA. We estimated a size of the *H. vastatrix* genome of 362,7Mb, which would make it one of the largest available fungi genomes. Preliminary flow-cytometry analysis of rust urediniospores estimated its genome size between 440-610Mb (Anikster Y., The Institute for Cereal Crops Improvement, Tel Aviv University, Ramat Aviv, Israel). Rust genomes are very variable in size ranging from 67Mb for *P. graminis* to 414Mb for *Uromyces appendiculata*.

Table 1. Statistics of genome assemblies of 8 *H. vastatrix* isolates and a hybrid assembly including all isolates genomes.

Sample IDs	Contigs	Average Size	%GC	Assembly	Completeness
HvHybrid	**396264**	**841,5**	**32,77**	**333,258,024**	**91,94%**
HvCat 454					
HvCat Illumina	254645	622	32,50	122,820,521	57,26%
Hv387	211495	713	32,61	150,707,107	44,35%
Hv494	211700	653	32,53	138,293,025	35,08%
HvDQ952	197927	612	32,78	121,119,448	31,85%
HvH_179	203770	646	32,36	131,574,289	31,05%
HvH_569	202168	660	32,31	133,358,100	37,50%
HvH_701	215628	734	32,49	158,292,515	56,86%
HvMar_1	203360	619	32,58	125,814,765	22,18%

A total of 23.2% of the paired reads mapped in the same contig. Most unpaired reads (66.8%) matched two different contigs (useful for scaffolding, data not shown). Most of contigs showed a high coverage. Some contigs had coverage greater than 100X. We decided to use the hybrid assembly to perform the next bioinformatics analysis given that it was the only assembly displaying a satisfactory level of completeness.

We used Cufflinks to map three RNA-seq data sets against the HvHybrid genome assembly and we identified a total of 21,345 clones that matched RNA-seqs. This set of clones was selected for gene prediction with Augustus. For annotation of *H. vastatrix* proteins, we used the program already trained on *Sacharomyces sequences*. A total of 18,234 sequences were predicted that encode 70 o more amino acids with an average length of the gene models predicted of 1,047bp. We filtered this set of sequences to remove transposon sequences identified in RepBase Release 17.01 and this final set contained 14,445 predicted protein sequences. This set of predicted proteins was used to perform BLASTp (e-value=1e-3) homology searches against Pucciniales sequences (67,118 seqs) and we identified 13,796 hits (73,86%). We carried out homology annotation with BLASTp against rust protein datasets described above and *U. maydis* annotated proteins (Table 2).

Table 2. BLASTp homology sequence analysis of *H. vastatrix* RNA-seq sequences and other rust predicted protein datasets. E-value = 1e-3.

		***H. vastatrix* RNA-seq samples**			
Rust protein datasets	**Predicted proteins**	**HvCat Nor**	**Hv701**	**Hv955**	**HvFernandez**
***P. triticina* 1-1 bbbd race 1_1**	11,630	42.2% (18703)	40% (22240)	36.6% (23705)	29.4% (1987)
P. graminis* f.sp. *tritici	15,979	43.8% (19411)	41,5% (23129)	37.8% (24480)	31.9% (2160)
P. striiformis* f.sp. *tritici	22,815	43.5% (19257)	40.4% (22544)	36.9% (23874)	29.9% (2025)
M. laricis populina	16,694	39.2% (17385)	40.8% (22765)	37.8% (21302)	29.3% (1987)
U. maydis	6,787	34.6% (15352)	38% (21224)	34.7% (22481)	24.5% (1659)

Using RepatMasker we identified interspersed repeats in 74,4% of the *H. vastatrix* genome sequences with a large proportion of repeats (38,7%) classified as LTR elements. A smaller proportion (7,2%) of repeats were classified as DNA elements and only 2,1% as LINEs. A total of 26,3% of repeats was unclassified.

Using the set of 14,445 predicted proteins we run the algorithm OrthoMCL to cluster *H. vastatrix* genes and we identified 3921 gene families (> 2 genes) and 2,103 orphan genes. A large proportion of families contained fewer than 5 genes (95%) and the family with the most genes had a homologous only in *P. graminis* f.sp. *tritici*.

We predicted 659 secreted proteins using PProwler and 775 secreted proteins with the SignalP algorithm. A total of 180 proteins in our *H. vastatrix* set presented similarity with secreted proteins predicted by Fernández et al. (2012). A Venn diagram (Figure 1) showed shared and unique coincidences between the three sets of data, including the proteins extracted by comparison with Fernández et al (2012). The SignalP and PProwler methods shared 483 proteins and with Fernández et al. (2012) dataset a total of 44 proteins.

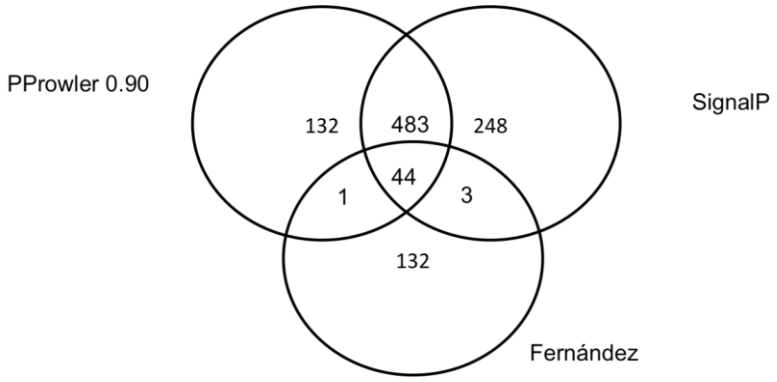

Figure 1. Venn diagram of consensus secreted predicted proteins. SignalP and PProwler (0.9 probability cut-off) programs were used to predict which proteins are secreted. A set of 14445 putative proteins was used for classification into secreted or non-secreted proteins.

In Melampsora more than half of the predicted secretome members encoded lineage-specific proteins, and similarities with experimentally characterized fungal effectors were also identified. A SAGE analysis indicated a strong stage-specific regulation of transcripts encoding secreted proteins. The average sequence identity of putative secreted proteins to their closest orthologs in the wheat stem rust *P. graminis* f. sp. *tritici* was dramatically reduced compared with non-secreted ones. A comparative genomics approach based on homologous gene groups unravelled positive selection in putative members of the secretome.

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Arabica Coffee Production in the Yunnan Province of China

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SUMMARY

Arabica coffee was introduced into Yunnan Province of China in 1892 by French missionaries. In 1950s, the coffee growing area at Yunnan was about 4,000 ha of Typica and Bourbon varieties introduced from Burma. Mainly due to coffee leaf rust (CLR) the coffee growing area decreased to 270 ha in 1970s. In the 1980s and 1990s the Dehong Tropical Agriculture Research Institute of Yunnan (DTARI) released two rust resistant cultivars, respectively S288 introduced from India and Catimor (Caturra 19/1 x HDT 832/1) from the Coffee Rust Research Center (CIFC)/ Tropical Research Institute (IICT), in Portugal. Due to low yield, poor cup quality and susceptibility to coffee leaf rust (CLR) and stem borer, the cultivar S288 has been gradually replaced by Catimor.

In 2011, the coffee area at Yunnan Province had expanded to 43,000 ha, with 90% of Catimor cultivar planted, namely line CIFC 7963. In 2010 the production of green coffee reached 49,400 tons. DTARI recorded in their experimental fields with Catimor yields of 3 to 5.25 tons/ha/year on average, with an occasional top year of 7.5 tons/ha. These yields are obtained at a plant density of 4995–6240/ha, with inputs of fertilizers 2 times/year, and of pesticides 3 times/year, to control stem borers. According to the production plans of Yunnan Province, the coffee-growing area will increase to 66,700 ha by the end of 2015, with an expected annual output of some 96,000 tons of green coffee. Nowadays, there are more than 200,000 coffee growing households in Yunnan and nearly 700,000 persons benefit directly or indirectly from the coffee industry.

The major coffee regions are located at altitudes between 700 and 1700m and latitudes 21°N (Menghai County, Xishuangbanna District) and about 26°N (Huaping County, Lijiang District). In these regions average temperatures are 18.9-22.4ºC, annual rainfall 700-1700mm and 1800-2300 sunshine-hours/year.

The cup quality of Catimor coffees produced in Yunnan reached the international standard of B++ to A++ depending on cultivation altitude. Three coffee seed samples from a Catimor coffee plantation at Lujiangba have also been sent to the SCAA (Specialty Coffee Association of America) for cup testing and received quality scores of 77, 79 and 81.

Unfortunately, over the past few years the cultivar Catimor has become susceptible to CLR. Recently, rust races not yet earlier found in China were characterized at CIFC: race VIII

(v2,3,5) from cultivar S288 and races XXXIII (v5,7 or v5,7,9), XXXIV (v2,5,7 or v2,5,7,9) and XLII (v2,5,7,8 or v2,5,7,8,9) from Catimor. A new rust race with the virulence genotype (v1,5,7 or v1,5,7,9) was also detected in Catimor samples. Meanwhile, DTARI and CIFC have increased a cooperative programme to introduce new coffee germplasm with broad spectra of resistance to leaf rust and guidelines have been given to prevent or delay the emergence of new rust races.

INTRODUCTION

Coffee was first successfully cultivated in China's Taiwan Province in 1884 and it was introduced in Yunnan Province in 1892 by French missionaries, who brought Arabica coffee (*Coffea arabica* L.) seeds and planted them at Zhukula village in the mountain valleys near Binchuan. Robusta coffee (*Coffea canephora* Pierre ex Froehner) was brought to Hainan Province, the only tropical area in China, from Malaya by Chinese people in 1908.

Yunnan's coffee production began in 1914 with the introduction by Jinpo border officials of Arabica coffee varieties as Typica and Bourbon from Myanmar (Burma) that were cultivated in Nongxianshan village of Ruili County. Arabica coffee was then distributed to other regions with sporadic planting in some provinces with suitable climatic conditions as Lujianba, Baoshan District.

This work attempts to give a panoramic view of the coffee production in China with particular emphasis on the production plans of Yunnan Province. The collaborative activities between Dehong Tropical Agriculture Research Institute of Yunnan (DTARI) and Coffee Rust Research Center (CIFC)/ Tropical Research Institute (IICT) in Portugal are also described.

Coffee growing in China

Yunnan Province is a mountainous area with the Tropic of Cancer running trough southern part. It is located in the southwest part of the People's Republic of China, has an area of 394,000 km^2 approximately, and borders Myanmar (Burma) in the west, Laos in the south and Vietnam in the southeast. The capital of the province is Kunming.

The major Arabica coffee growing area in Yunnan is located from 21°08'41.60" to 26°37'47.59" North longitudes, 101°43'16.74" to 101°15'56.95" East latitudes. Coffee is grown at altitudes between 700 and 1700m with average annual temperatures between 18.9-22.4ºC, annual rainfall of 700-1700mm and 1800-2300 sunshine-hours/year.

Yunnan's main coffee growing districts are Puer, Baoshan, Dehong, Xishuangbanna, Lincang, Wenshan, Honghe and Nujiang comprising 43,065 ha (Table 1).

During 1950s, Arabica coffee plantation in Yunnan occupied about 4,000 ha but this area decreased to around 270 ha in the 1970s mainly due to serious attack by coffee leaf rust (CLR) and also by marketing problems and social political policy.

In the 1980s and 1990s the DTARI released two rust resistant varieties, respectively S.288 introduced from India in 1963, and Catimor (Caturra 19/1 x HDT 832/1) from CIFC in Oeiras, Portugal. Catimor was first introduced in Hainan, Yunnan's Lujianba, Ruili and other places for adaptability growth experiment.

In 1988 the efforts of the Yunnan Provincial Government with the Nestlé Company, resulting in an UNDP project, developed the planted coffee area in Yunnan reaching 7,800 ha in 1997

and expanded to 43,000 ha in 2012 with 90% of Catimor, namely line CIFC 7963. Coffee production in Yunnan Province reached 53,240 tons in 2011 compared to 49,334 tons in 2010.

In 2010 the production of green coffee reached 49,334 tons with an increased annual area of 8068 ha and harvested area of 24,933 ha (Table 1).

Table 1. Yunnan's planted area, increased annual area, harvested area and coffee production in 2010.

Location	Total area (ha)	Increased annual area (ha)	Harvested area (ha)	Production (t)
Puer	18,413	3,561	11,127	21,200
Baoshan	9,180	667	6,553	14,200
Dehong	8,966	2,660	3,927	9,300
Xishuangbanna	3,347	680	2,253	3,600
Lincang	1,413	373	500	565
Wenshan	753	67	213	200
Honghe	673	27	100	45
Nujiang	320	33	260	224
Total	43,065	8,068	24,933	49,334

In 1993 a demonstration farm was implemented at Jia Zhong (Hogood Coffee Co. Ltd.) with the main objective to know the yield behavior of Catimor and to supply know how to the farmers and motivate them to grow this cultivar and to replace the traditional S.288 by Catimor.

In this experiment (6 years harvested) the average yield was 12.465 kg/ha (coffee parchment), with a pick of 15840 kg/ha in the 4th year yield (Table 2) and with a plant density of 2 x 0.8m. The following scheme of fertilizing was used: after harvest (organic, 10kg/plant); before flowering and in harvest season. Bordeaux mixture was used 2 times/year.

Table 2. The Catimor CIFC 7963 production in Hogood Coffee Co. Ltd.

Cultivar	Area (ha)	Production (parchment coffee) at different harvested year (kg/ha)					
		1st	2nd	3rd	4th	5th	6th
Catimor	0.93	13365	12375	14850	15840	5940	12420
S.288	6.4	830	1280	2320	3090	1695	1980

Nowadays the majority of farms reached on average 3 to 5.25 tons/ha/year (clean coffee). More than 99% per cent of the coffee enterprises belongs to private sector.

According to the production plans of Yunnan Province, the coffee-growing area will increase to 66.700 ha by the end of 2015, with an expected annual output of 96.000 tons of green coffee. Nowadays, there are more than 200,000 coffee growing households in Yunnan and nearly 700,000 persons benefit directly or indirectly from the coffee industry.

Robusta coffee is mainly grown in Hainan Province. According to historical records in 1908 Chinese people brought Robusta coffee from Malaya to Zhanzhou County, Hainan Province. In 1988, coffee (Arabica and Robusta) cultivation area in this Province reached 15,850 ha.

However, that area decreased to 999 ha in 1998 and in 2010 only had 220 ha with harvested area of 99 ha, production of 156 tons, with the output value of 2.34 million Yuan. Selection and breeding of Robusta coffee have been done by Spice and Beverage Research Institute, Chinese Academy of Tropical Agricultural Sciences and eight high yield clones 24, 24-1, 24-2, 24-10, 24-11, 6, 26 and 27 were obtained with 3000kg clean coffee bean per hectare.

Characteristics of coffee from Yunnan Province

Coffee processed by the wet method, associated with favorable climatic conditions provided the excellent cup quality classified by the international standard as B^{++} to A^{++} degree depending on the altitude of coffee growth (Table 3). Catimor 7963 was tested for cup quality and the samples were classified as A for 1100m from Lujiang got grade A^{++} for 1100m altitude (Table 3). For further confirmation, three Catimor seed samples from coffee plantations at Lujiangba (Baoshan District) were analyzed by SCAA (Specialty Coffee Association of America) for cup quality, and obtained 77, 79 and 81 score points.

Table 3. Coffee cup quality of Catimor 7963 from different regions in Yunnan.

Location	Exterior colour	Taste	Moisture content (%)	Flavour description	Quality	Altitude (m)
DTARI	Light blue	Normal, Beany flavour	11.5	General aroma (burnt flavour), strong sharp acid, bitter medium (Chinese herb bitter), flavour as roasted sweet potato, body medium	B++	800
Experimental farm, DTARI	Blue	Normal	10.3	Aroma intensity (flower potpourri style), strong fruit acid, bitter more than medium, fast and incredible lingering aftertaste, rich mouth feeling	A	1100
Nanjingli, Ruili	Blue	Normal, Beany flavour	10.6	Aroma medium, strong acidity, bitter more than medium	B++	1260
Laoshuijing, Lujiang farm	Light blue	Normal	10.9	Strong aroma but slight weak than the same origin of Typica, bitter strong, high fruit flavour acid, incredible lingering aftertaste, rich mouth feeling	A++	1100
Sanli, Liuku	Blue, clean, large and uniform grain size	Normal	11.2	Aroma strong, bitter medium, acid strong, mild acerbity, incredible lingering aftertaste, rich mouth feeling	A++	1200
Babianjiang, Ninger	Blue	Normal	10.9	Very strong aroma, strong bitter, fast, good lingering aftertaste, flavour in roasted sweet potato, acid medium, rich mouth feeling	A++	890

Coffee disease and pests

The major coffee diseases in Yunnan's coffee plantations are coffee leaf rust (*Hemileia vastatrix* Berk & Br.), leaf spot (*Cercospora coffeicola* Berk. & Cooke), anthracnose (*Colletotrichum gloeosporioides* Penz) and coffee physiological dieback diseases.

The main local pest is the coffee stem brown borer (*Acalolepta cervina* (Hope) Synonym: (*Dihammus cervinus*) although stem white borer (*Xylotrechus quadripes* Chev.), red coffee borer (*Zeuzera coffeae* (Nietner)), mealy bug (*Planococcus lilacinus* Cockrell) and green scale (*Coccus viridis* (Green)) are also present.

Collaborative activities between DTARI and CIFC/IICT

The most serious disease in Yunnan's coffee plantations is CLR caused by the fungus *H. vastatrix*. Recently, Catimor became susceptible to CLR, with high levels of incidence at coffee plantation in Hogood Coffee Co. Ltd (Table 4).

Table 4. Comparison of coffee leaf rust incidence on Catimor CIFC 7963 in different geographical locations in 2011.

Location	Altitude (m)	Incidence (%)	Incidence index
Dehong DTARI	810	58.9	26.4
Dehong Hogood	820	88.8	62.7
Yingjiang County	900	84.5	43.4
Puer´s Nadaohe	1040	91.3	53.3
Puer´s Laobentian	788	100	62.5

This phenomenon is related with the increasing of virulence of *H. vastatrix* (development of new rust races) in these coffee regions, confirmed by coffee leaf rust survey. Several CLR samples from China coffee fields were characterized at CIFC (Portugal), in the set of coffee differentials, as previously described. The first rust samples, from Caturra, Typica and Bourbon were characterized in 1994 as races: I (v2,5), II (v5), XV (v4,5), XXIII (v1,2,4,5) and XXIV (v2,4,5). Recently, six CLR samples from S.288 and Catimor were characterized respectively as race VIII (v2,3,5) and races XXXIII (v5,7 or v5,7,9), XXXIV (v2,5,7 or v2,5,7,9). A new rust race, from Catimor, never characterized before showed the following genotype of virulence: (v1,5,7 or v1,5,7,9).

In several coffee regions of Yunnan, Catimor did not show yet CLR infection. Similar situation has been described for the same Catimor as well as in other HDT derivatives in other coffee growing countries, like Brazil, Colombia, India and Philippines among others.

In the last years DTARI and CIFC have increased the cooperation to develop new resistant coffee cultivars as well as to implement some strategies to delay or to prevent the appearance of new rust races in Yunnan coffee regions. This collaborative research work includes also training on different subjects related with control of CLR by resistant varieties, a rust race survey in different coffee regions, introduction of new coffee genotypes and selection of coffee genotypes with large spectra of resistance to CLR.

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Susceptibility Assessment of Coffee (*Coffea* Spp) Genotypes to the *Mycena Citricolor* Fungus and Determination of Candidate Defense Genes

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SUMMARY

In Costa Rica, the presence of the fungus *Mycena citricolor* in coffee (*Coffea arabica*) plantations, had reduced the production and elevated the costs due it´s control. Therefore, the genetic resistance is the best option for the disease management. It was evaluated in the laboratory the susceptibility of 33 accessions including nine species of the genera *Coffea*, against one highly pathogenic isolate of *Mycena citricolor* previously cultivated under *in vitro* conditions. In the study of pathogen interaction, samples were evaluated at 4, 7 and 10 Days After Inoculation (DAI), the percentage of lesions per leaf, spored lesions and lesion diameter at 10 DAI. It was obtained 100% of lesions in the *Coffea arabica* genotypes at 4 DAI, however less than 55% in *C. canephora*. Spore production in new lesions was manifested after 7 DAI, especially in *C. arabica* genotypes. The lesion´s diameter at 10 DAI was bigger for *C. eugenioides* and lesser for *C. canephora*, *C. congensis* and *C. arabica*. None of the genotypes exhibit resistance to the pathogen, however different response levels against the fungi were observed. After those pathogenic analyses, 22 genotypes with variable level of susceptibility were used for molecular analysis, using 12 conserved primer pairs for NBS and LRR motif candidate resistance genes, oxalate oxidase and enzymes with known appearance in defense mechanisms. A variable level of polymorphism (between 0 to 67%) was obtained for 9 primer pairs. Products with similar bands reported in literature for other crops were not detected; however 16 of the bands could be associated to tolerance or susceptibility to the pathogen, becoming possible resistance molecular markers. Further analysis of the obtained sequences and comparison in gene databases would determinate it´s function in the host-pathogen interaction.

INTRODUCTION

The *Mycena citricolor* is a very versatile saprophytic and pathogen fungi, with a broad range of genera and species that affect, besides of coffee (*Coffea* sp) (Barquero, 2009). Since very decades, American Leaf Spot, an american endemic disease, with special distribution in the central american area, had affected the coffee culture, especially in highlands, with shadow and humidity.

It´s agronomical control is based for instance in cultural practices, like management of shadow, reduce of plants density, pruning, selection of few axes, drainage and organic decomposition reduction; however, when infestation is high, chemical control is needed, with agronomical viable effectiveness, as long as it´s application is in time, doses and compound, adequate (Barquero, 2009). Despite of that, the presence of the fungi is persistent, so investigation of control alternatives´ is required.

To date, no commercial cultivated coffee is resistant to the pathogen, nevertheless, horizontal or polygenic resistance to the pathogen can´t be discarded in the *Coffea* genera, under the premise that several other resistance genes to wide range of diseases and plagues can intermediate in the tolerance.

The research on other defense mechanisms to phytopathogenic fungus in plantae, showed the existence of host-pathogen-environment interaction, with the intervention of resistant proteins or "R", with correlated relationship function in response to different pathogens, including virus, bacteria, fungus, nematodes and insects.

The evolution of those types of proteins and their respective genetic sequences, had demonstrated to be phylogenetically ancient, even previously than the divergence of angio- and gymnosperms so, have been used as molecular markers in multiplicity of species of plants to relate phenotypes with expressed proteins previously studied in genomes and documented in gene banks, due it´s high conserved domains (Hu *et. al*. 2003, He *et. al*. 2004, Ramalingam *et. al*. 2003).

The majority of proteins synthesized from resistance genes in plants, encodes for Nucleotide-Binding Sites, NBS and Leucine-Rich Repeats, LRR, which function is related to recognition of the pathogen and activation of the transduction signal that undergoes the resistance (Scherer *et al*., 2005). Their importance refers to the comprehension of the resistance mechanisms and their use as defense candidate genes approach in breeding and phytopathology research.

The existence of coffee genotypes with different defense expression to the attack of *Mycena citricolor*, gave the opportunity to explore different ways to limit the damage in this dicotyledonous plant, with possibilities that thru a breeding program, can obtain less susceptible genotypes.

OBJECTIVES

- Evaluate the response of several available accessions of coffee.
- Determinate with PCR based technology, the existence of possible candidate defense genes.
- Determinate the phenotypic response with possible candidate defense genes.

MATERIALS AND METHODS

Susceptibility Assessment

Leaves from 33 coffee genotypes, belonging to 9 species of the *Coffea* genera, where evaluated against a highly pathogenic isolate of *M. citricolor*. The reaction was made under humidity and temperature controlled conditions inside the laboratory. Thirteen accessions of *Coffea* sp including (*C. canephora, C. liberica, C. racemosa, C. stenophylla, C. eugenioides, C. salvatrix, C. congensis* and *C. dewevrei*) and 10 *C. arabica* accessions where used from the germplasm bank in CATIE (Turrialba) and 9 more of *C. arabica* and one of *C. canephora* from CICAFE (Barva).

For each genotype, one representative plant was selected, in a vigorous and healthy physiological stage and taken 5 leaves in two separated months. In the laboratory, each leave was washed with water and inoculate 5 gems. At 4, 7 and 10^{th} day after inoculation (DAI), was evaluated the number of lesions (incidence), gems formed (spore production), and

diameter of lesion at 10th day. Data was analyzed with an average percentage corresponding to each parameter and estimated the "Disease Index", calculated according to the formula:

$$IE = PI_{10DDI} + PLE_{10DDI} + PA_{4DDI} \times DL_{10DDI}$$

where PI is infection percentage at 10 DAI, PLE is spore production percentage at 10 DAI, PA disease advance percentage at 4 DAI and DL diameter of lesions at 10 DAI.

Molecular Assessment

Twenty two representative accessions with different levels of susceptibility against *M. citricolor*, were analyzed in PCR using 12 set of primers reported in literature, to detect possible resistance genes analogues (Wu *et al.*, 2004; Watanabe *et al.*, 2008; Ramalingam *et al.*, 2003; Shen *et al.*, 1998).

DNA isolation was carried out according to Fast DNA SPIN Kit protocol (FastDNA® Kit, MP Biomedical, EEUU). Products were electrophoretically separated in agarose gel at 2,0 % with TBE 1X buffer, and dyed with 1 µl de GelRed ® (SYBR, Molecular Probes, Inc. EEUU). Photos revealed were digitally analyzed with the UVIDoc program from UVITec ® (Cambridge, England).

Further results of the bands obtained for each primer set were manually analyzed and marked its presence or absence condition, with 1 or 0, respectively, used to design a dendrogram with the Statistica 6.0 program (StatSoft, Inc, EEUU).

RESULTS AND DISCUSSION

Susceptibility Assessment

According to the results, the response of the different genotypes of coffee against the fungus *Mycena citricolor*, had infestation levels that varied according to every of the parameters evaluated. The infection at first 4 DAI was superior for 80% of all genotypes besides Robusta and *C. dewevrei* accessions, with fewer than 55%. At 10 DAI the genotypes showed lesser appearance of lesions spored, with the lowest levels in Robusta, *C. racemosa*, *C. liberica* L3477, *C. canephora* and *C. liberica* L3478 samples, with a range between 0 and 12%, been for the rest superior than 25%, with emphasis in Eugenioides, that gave a 100%. Diameter of lesions determinated at 10 DAI varied between 0,40 and 2,44 cm, with less than 0,83 cm samples of Robusta and Arabica's T8667, Caturra, Java, RS 4 and E531.

According to the "Disease Index" (Figure 1), only two samples of Robusta where at least tolerant, been the other species less tolerant or very susceptible. The Eugenioides sample, Stenophylla and E416 (*C. arabica*), where the most susceptibles.

Even though diversity of commercial cultivars is reduced in arabica´s, the present study had determinate that the response of coffee against the American Leaf Spot, is differentially expressed both intra- as interspecifically. Those results implicate that should exist multiple sets of genes that could interact and activate differentially according to signal-response mechanisms, involving each specie´s configuration, and express different levels of defense to the fungus.

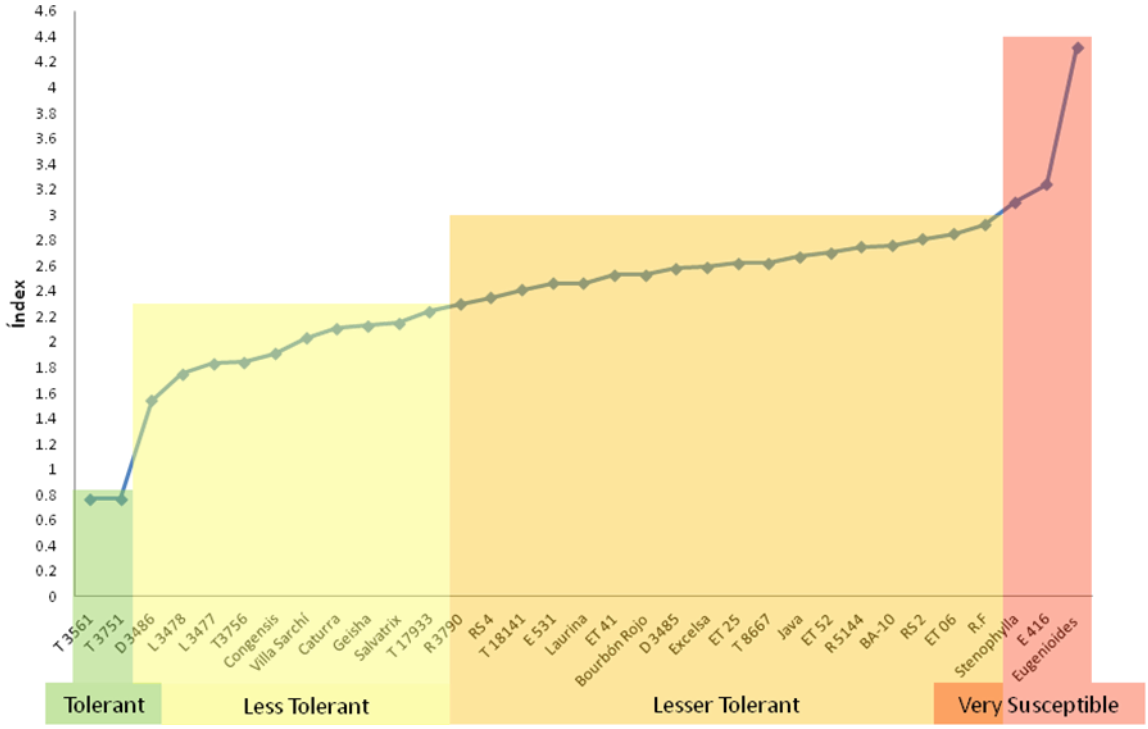

Figure 1. Accession´s Disease Index.

Molecular Assessment

The information obtained from the selected primers, was significant for 9 of the 12 pairs, with a variable number of fragments obtained, ranging from 11-99, and an estimated weight of 34-3000 bp.

The Figure 2 shows an example of the OXO1 primer, that generate a high level of polymorphism (44%), with fragment lengths around 764 and 104 pb. A fragment of 246 pb was very intense and generalized in 20 samples of coffee and control potato, which indicates possible a constituent fragment, referred to the gen that synthesize oxalate oxidase.

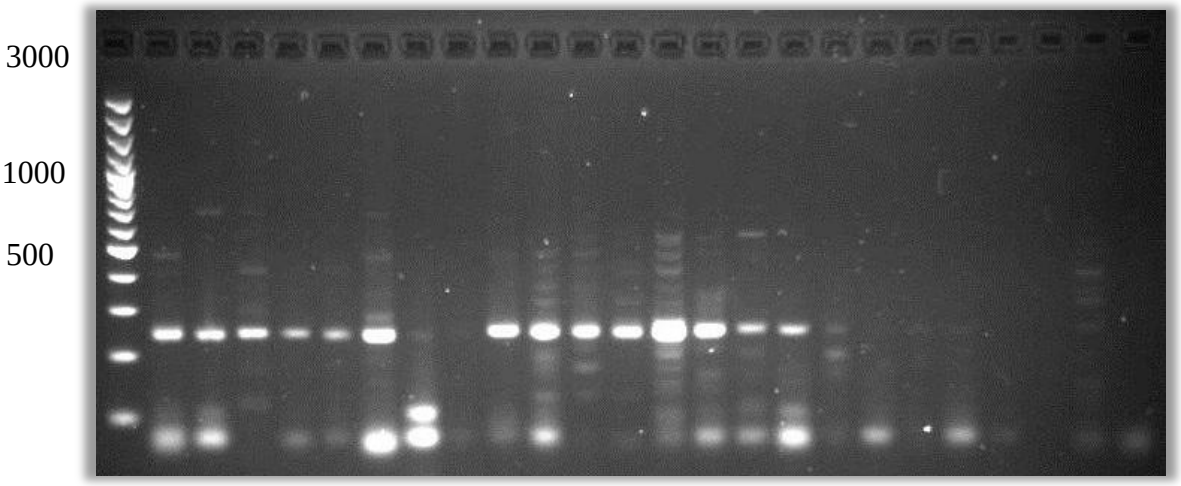

Figure 2. Pattern of fragments obtained for the OXO1 primer.

A global phylogenetic analysis and primer specific dendrograms, evidenced that exists variation in the genetic samples and grouped related genotypes, however, no relationship by this method, was obtained to the phenotipic response to the fungi (Figure 3); instead, fragment and manual selection was accomplished and reveals 16 phenotipic bands possibly related to the defense response, and 2 more as constitutive bands (Table 1).

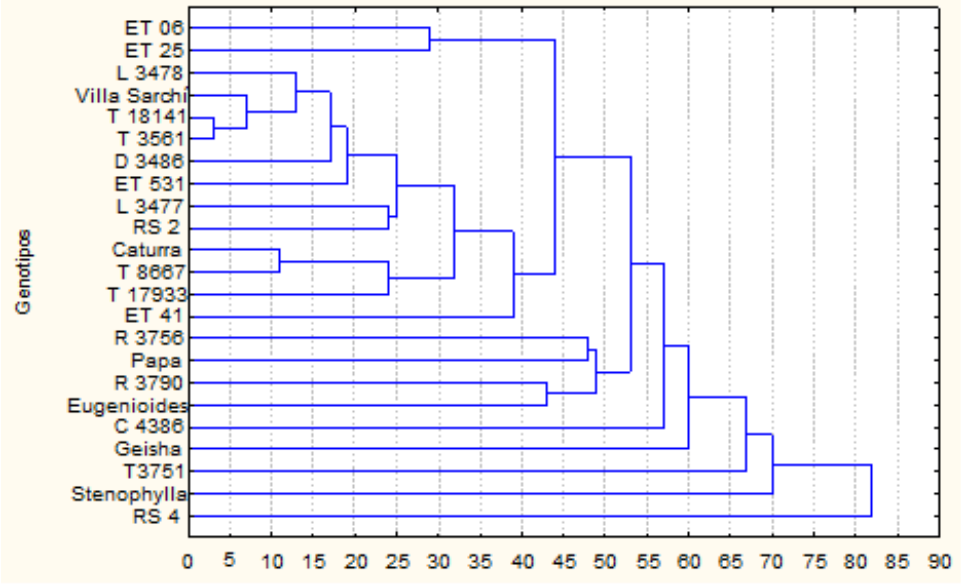

Figure 3. Global dendrogram obtained for all 9 sets of primers for phylogenetical analysis.

Dendrogram of Figure 3 shows that T3561 and T18141 (Sarchimor; Timor Hybrid CIFC 832/2 x Villa Sarchí CIFC 971/10) are up to 96% similar according to the used primers, and so, Villa Sarchí is proximately 91% similar to both mentioned genotypes. The Caturra sample showed 87% of similarity with T 8667 (Catimor; Timor Hybrid CIFC 832/1 x Caturra CIFC 19/1), and both a 71% compared to T 17933 (Catimor; Timor Hybrid CIFC 1343 x Caturra CCC135). Even that information is useful for phylogenetic analysis, the association with phenotype behavior against *M. citricolor* was not precise, so searching in the fragments obtained by each primer, was the approach for determination of possible association with specific DNA bands. In Table 1 is resumed the different fragment lenghs (bp) of the possible related sequences or candidate genes involved in the host-pathogen interaction.

Table 1 Size of primer products and possible association according to phenotype.

Primer	Fragment Lengh (bp)	Possible Relationship
RGA14	2498	Tolerance
	937	Tolerance
	571	Tolerance
	740-720	Susceptibility
OXO1	246	Oxalate oxidase
	426	Tolerance
	300	Tolerance
	176	Tolerance
	120	Tolerance
GOXO1	630	Tolerance
RPS2N	564	GENERAL
	243	Susceptibility
	152	Tolerance
NLRR1	1403	Tolerance
	1242	Tolerance
	721	Tolerance
	258	Susceptibility
LRR	620	Susceptibility

Information obtained from this study, revealed certain fragments obtained for specific primers, and it´s the possible association of tolerance or susceptibility to some coffee genotypes against the fungus *Mycena citricolor*.

CONCLUSIONS

- Coffea samples evidenced differential biological behavior in response to Mycena citricolor, with more than 94% of the population evaluated characterized as susceptible.
- Products obtained from the selected primers, varied between 0 to 67% of informative bands.
- The study detected 9 bands with possible relationship to tolerant phenotypes and 7 for susceptible ones.
- A direct relationship between a specific marker and a phenotype, still not could be determinated.
- Utilized primers were also effective for phylogenetic analysis.

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Strengthening Producer Organizations to Speed up the Multiplication of Improved Hybrid Coffee Varieties in Tanzania

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SUMMARY

Recent progress in coffee research by the Tanzania Coffee Research Institute (TaCRI) has led to the release of 15 Arabica hybrid coffee varieties that are resistant to coffee berry disease (CBD) and coffee leaf rust (CLR) and 4 Robusta hybrid coffee varieties that are resistant to coffee wilt disease (CWD) that threaten both Arabica and Robusta coffee growers in the country. The improved coffee varieties are the foundation for a coffee green revolution in Tanzania as they are high yielding, resistant to the major diseases, have good cup quality and are adapted to a wide range of environments. However, since they are hybrids their multiplication is through vegetative propagation. TaCRI has perfected two methods, clonal propagation and grafting for their multiplication, which go together with the provision of training courses to producer organizations to accelerate the multiplication of improved hybrid seedlings. Linkages have been established in 40 coffee growing district councils and established over 600 nurseries of the improved hybrid varieties for both Arabica and Robusta coffee varieties across the country which has the capacity of multiplying over 18 million seedlings per year. The decentralization of seedlings multiplication programme as well as participation and strengthening of producer organizations in the management of their own nurseries has resulted into increased number of seedlings multiplication for replanting programmes. This paper outlines the progress achieved in hybrid seedlings multiplication through strengthening producer organizations.

INTRODUCTION

Tanzania produces two types of coffee, namely Arabica coffee (*Coffea arabica)* and Robusta coffee (*Coffea canephora*). The total area under coffee production is estimated to be 265,000 ha (TCB, 2012), of which 70% is under Arabica and the remaining 30% is under Robusta. Coffee contributes about 115 million USD to the export earnings and provides direct employment to over 450,000 farm families. About 90% of coffee in Tanzania is produced on a small scale in plots size of not more than 2 hectares and the remaining 10% is grown on large scale (estates).

The current coffee production in Tanzania stands at 50,000 tons on average fluctuating from 33,000 to 68,000 tons with yield levels being less than 400 g/plant on average (TCB, 2012).The coffee industry is faced by many challenges that include low productivity, lack of enough improved cultivars, insect-pests attacks, diseases attack, low use of inputs and unreliable market prices. Coffee research progress by the Tanzania coffee research institute (TaCRI) has led to the release improved hybrid coffee varieties that are resistant to coffee berry disease (CBD) and coffee leaf rust (CLR) for Arabica coffee varieties and coffee wilt disease (CWD) that threaten Robusta coffee growers in the country.

The new varieties have the following exceptional characteristics, which are high yielding, excellent beverage quality and they are well adapted to a wide range of environments. However, since they are hybrids, their multiplication is not through seed which is a challenge for their multiplication and availability to coffee growers. Packaging and dissemination of appropriate technologies especially the multiplication and distribution of the new planting materials is the most priority needed by coffee growers to speed up the rejuvenation of the coffee industry in the country TaCRI is currently addressing this by strengthening a number of producer organizations which include coffee co-operatives, coffee unions, district councils and organized farmer groups.

Recent research progress has shown that in many cases, individual farmers can not afford the technologies being promoted than if they work together in organized groups (Abaru *et al.,* 2006). The more farmers work collectively the more they pool resources and gradually help each other to adopt such technologies thus become vibrant and hence accessing a number of benefits such as research technologies and access to credit organizations.

Similar findings have been reported by Rondot and Collion, (2012) that helping and strengthening the capacity of producer organizations is one of the strategies to fight against rural poverty. Farmers through their organizations should be the natural partners of researchers and their institutions for mutual exchange of modern and traditional knowledge including translating formal research results into real life production. TaCRI is also putting much emphasis on strengthening producer organizations (coffee co-operatives, coffee unions, farmer groups, district councils) by developing their capacity to access good agricultural practices including knowledge on multiplication of improved planting materials with noticeable achievements.

COMPONENTS OF STRENGTHENING PRODUCER ORGANIZATIONS

Producer organizations

Reported by Gianatti and Llewellyn, (2012); Magesa *et al.*, (2010), the most successful groups are those that are fully involved in the planning, implementation and monitoring of their own developmental activities. TaCRI is working with over 700 producer organizations ranging from coffee co-operatives, coffee unions, district councils, coffee estates and farmer groups of 20-30 members who produce 90% of coffee in Tanzania. Some of the groups are well structured in terms of administration with their constitutions in place to guarantee responsibilities. Norms and regulations for the organizations are part of the groups to ensure successful performance of the groups. The need for a certain knowledge building is initiated by the groups, with TaCRI providing technical backstopping to monitor their progress especially on hybrid seedlings multiplication including the construction of propagation units and establishment of clonal nurseries.

Figure 1. Harvesting of shoots in a clonal nursery (left); planting of cuttings in the propagation boxes (right).

Village based training of producer organizations

It has shown that training of farmers raises the level of technical know-how, improves their capacities to analyze and synthesize, stimulates their critical spirit and facilitates dialogue (Wolf, 1995). Experience shows that the farmer's potential for progress is linked to his general level of education, and top down approach should no longer be encouraged for training of farmers. Research works have revealed that making farmers to be part in the technology dissemination increases the success of the technologies being promoted (TaCRI, 2011).

TaCRI is empowering coffee farmers with technologies on the multiplication of improved seedlings of coffee varieties through participatory approach which encourages horizontal information exchange between researchers-extension agents and farmers. Through field observation, different agronomic practices are selected as the main topic for discussions; ideas are collected, analyzed and come with simple solutions for complex problems. Farmers are given freedom to present the field problems and they are free to propose the type of technology they want thus creating ownership of that particular technology.

Figure 2. Training of producer organizations on hybrid seedlings multiplication.

Backstopping to producer organizations

Frequent meetings in the field between researchers, extension agents and farmers encourage communication and interaction, and leads to complicity between the groups, which contributes to progress. Regular backstopping remains the core function of the extension officers. TaCRI through a programme of technology transfer and training works closely with producer organizations (coffee co-operatives, coffee estates, district councils and farmer groups) to provide them with up-to-date information from the research institution through regular backstopping. A number of activities are conducted during backstopping including provision of technical advices to farmers on specific technologies such as establishment of nurseries for the multiplication of hybrid seedlings. It also includes discussion with farmers, collecting the existing problems observed in the field, documenting and providing feedback solutions.

Training of extension officers

Supriadi and Chamala (2012) reported a number of roles of the extension officers which includes but not limited to empowering, community organizing, problem solving and education. More recently, extension has been asked to play a "technology development role" by linking research with community group needs and helping to facilitate appropriate technology development. Training of extension officers at various levels while encouraging the use of participatory approach has been one of the strategies to strengthen producer organizations. Extension officers are responsible for monitoring the nurseries (district and farmer groups' nurseries) including provision of technical skills to producer organizations in their area of work.

Building of technical knowledge needed to advise farmers on topics related to solving their immediate farming problems so that they can manage their natural resources effectively and increase production (FARA, 2006). This does not require deep specialized knowledge on certain commodity crops, but rather broad knowledge on a particular technology that is being disseminated. The joint participation of various players in research-extension–production limits the deadlocks and facilitates the dissemination of technologies to the target group.

Exchange and study visits

These form part of training course whereby representative of the producer organizations are selected for a field visit in the areas where there is already impact seedlings multiplication and the way the groups are working together. Conclusions drawn from these field visits are used to concentrate efforts on priority problems.

Training of farmer promoters (farmer to farmer extension)

Provision of training and education has a direct impact on the technology being disseminated (FARA, 2006). Magesa *et al* (2010) reported increase in the adoption of technology when farmer to farmer approach was used in the dissemination of coffee technologies to coffee growers in Tanzania. Similar approach is being used by TaCRI to speed up the dissemination of coffee technologies by working with farmer promoters. Farmer promoters are farmers with knowledge on a particular technology that is being disseminated to farmers, and have undergone training courses by extension officers. Each farmer promoter is assigned a particular area to promote seedlings multiplication and brings back to the extension officers the feedback for discussion.

Linkages and partnerships

Improved communication including strengthening linkages between partners/producer organizations is important for effective research dissemination of technology for sustainable development. Continuous interaction between partners is the foundation for the development of agricultural rural development. Strengthening linkages with producer organizations is important in order to provide them with up to date information on new technologies being developed by the research institutions (Smith *et al.,* 2004). Several periodic structured visits to various producer organizations have been conducted with the aim of creating awareness about new coffee technologies, and there is increased demand for technologies in areas where researchers have interacted with stakeholders.

Provision of inputs (fertilizers, mother plants, polythene bags, etc)

The government of Tanzania has subsidized the coffee seedlings. The seedlings are currently multiplied by clonal propagation and grafting techniques developed by Tanzania coffee research institute. Seedlings produced for planting in the nurseries to support seedlings multiplication are free of charge. In addition to this, building materials for construction of propagation boxes and fertilizers needed in the nurseries are provided free of charge.

RESULTS

Producer organizations' nurseries

TaCRI is working with 40 with district councils and the numbers of farmer groups have increased to 647 groups in the six coffee growing zones Figure 3.

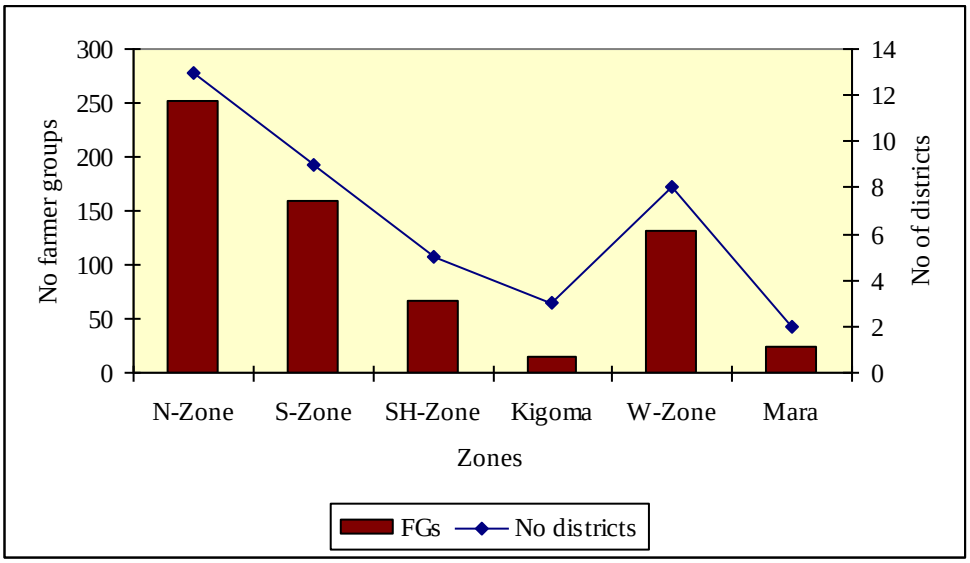

Figure 3. The number of farmer groups working with TaCRI. N= Northern; S= Southern; SH= Southern highlands; W= Western; FGs= Farmer groups.

Hybrid seedlings multiplication

TaCRI has strengthened the capacities to its stakeholders to support the multiplication of 18 million seedlings of improved coffee varieties annually. This is based on the number of nurseries, number of mother plants and propagation boxes available to producer

organizations. A higher proportion of seedlings multiplied come from small scale producers who contribute about 90% of the total country annual coffee production. This group has been the central focus of TaCRI as helping this group means increasing coffee production, poverty reduction of the coffee growers and increased national income.

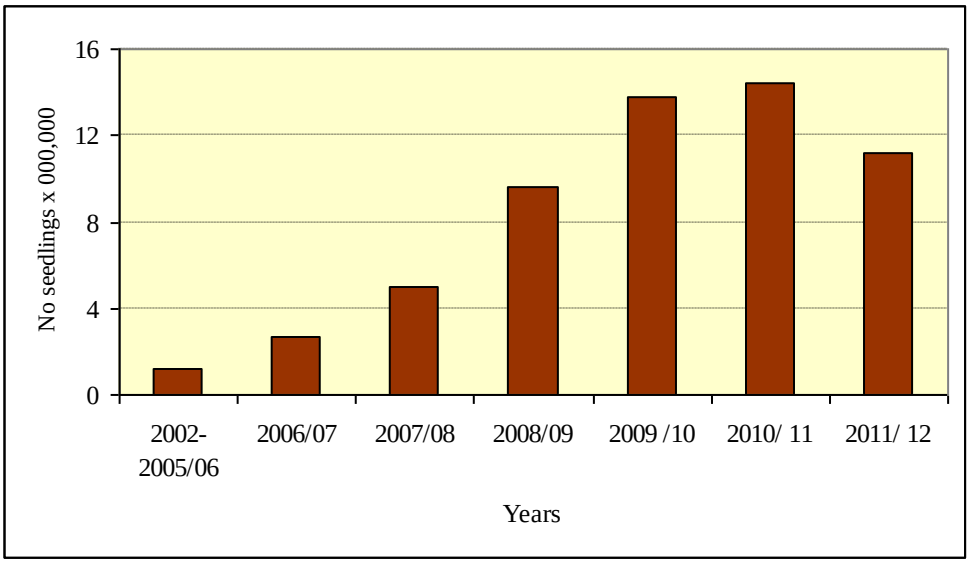

Figure 4. The number of farmers trained in seedlings multiplication.

Village based training of producer organizations

Since 2002 we have trained, 313,051 farmers (80.29% male and 19.72% female) on seedlings multiplication (Figure 5).The current trend shows an increase in the number of trainees with women farmers coming in as leaders in coffee production.

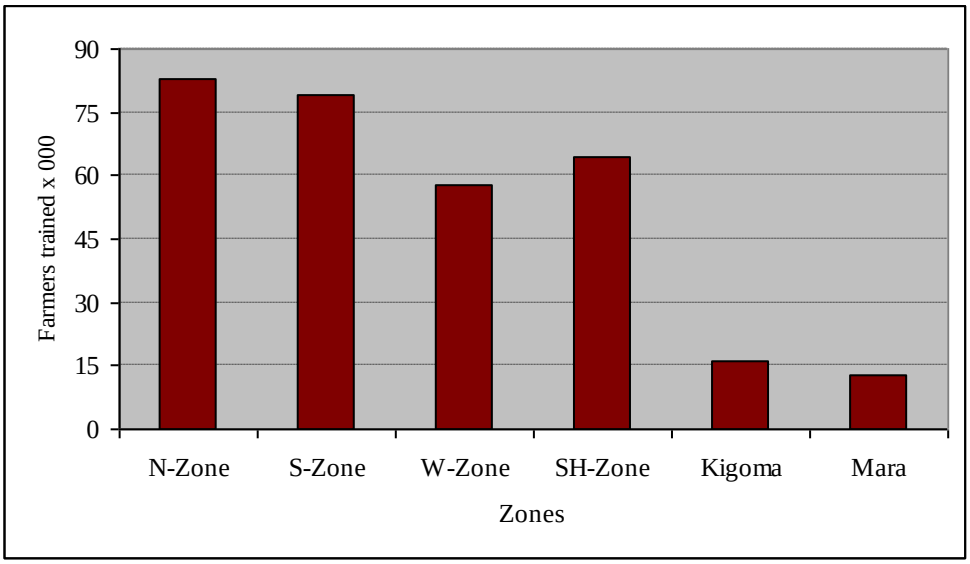

Figure 5. The number of farmers trained in hybrid seedlings multiplication. N= Northern; S= Southern; SH= Southern highlands; W= Western.

Capacity to multiply hybrid seedlings

The available capacity to support hybrid seedlings multiplication has increased up to 18 million seedlings from exiting over 350,000 mother plants. The highest number of seedlings is produced by small scale farmers (Figure 6) who have about 152, mother plants.

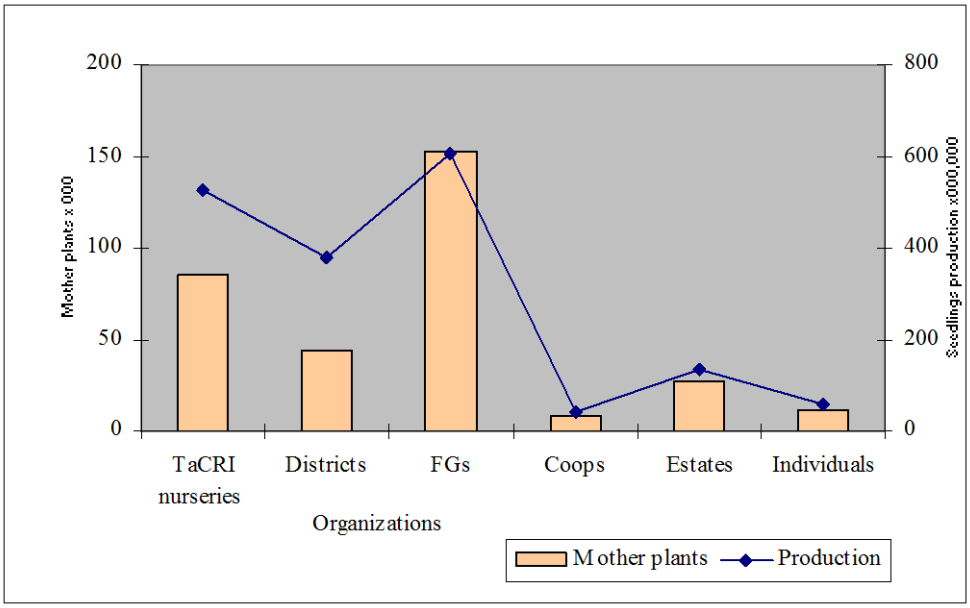

Figure 6. The available capacity of hybrid seedlings multiplication. Key: FGs= Farmer groups/small scale coffee growers.

Training of extension officers and farmer promoters

TaCRI has strengthened a total of 5,430 farmer promoters and 3,493 extension officers who are participating in seedlings multiplication activities (Figure 7)

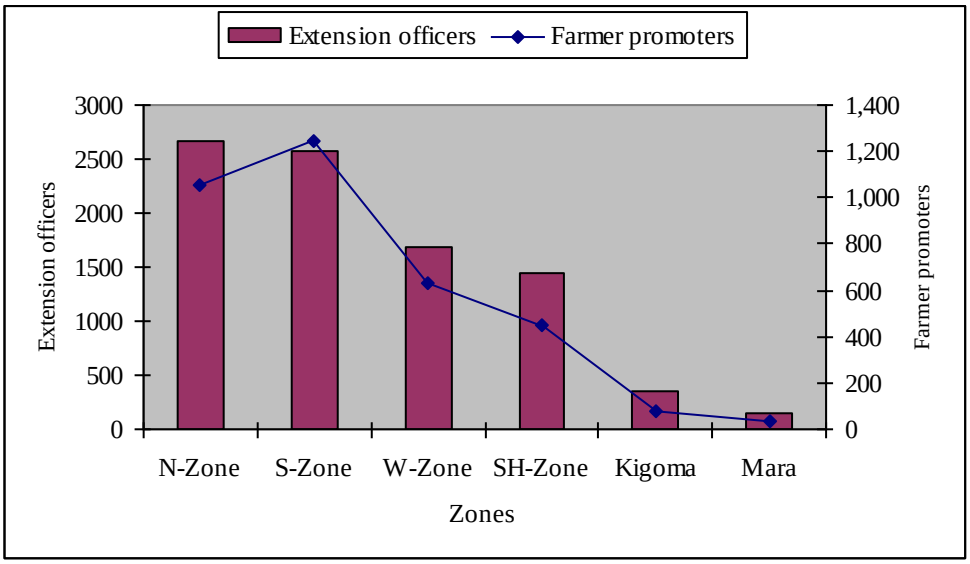

Figure 7. The number of farmer promoters and extension officers trained. Key: N= Northern; S= Southern; W= Western; SH= Southern highlands.

CONCLUSIONS

From these findings, it shows that producer organizations are effective partners in dissemination of technologies and are good channel for communication between farmers and researchers. Therefore, collective approach through organizing and strengthening producer organizations is important for successful adoption of technologies developed and it is a cost effective approach in terms of resource use.

ACKNOWLEDGEMENTS

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Participatory Breeding on Arabica Coffee to Obtain Superior Local Variety in order to Support Origin Specialty Coffee Products Development in Indonesia

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SUMMARY

A participatory breeding on Arabica coffee involving farmers and plant breeders to select superior local variety(s) has been conducted Lintong highland (North Sumatra), Gayo highland (Central Aceh, Northern Sumatra) and Kendenan Plateau (Enrekang, South Sulawesi). The aim of applying this method was to find out superior variety(s) of Arabica coffee having appropriate specific traits and adaptable to such location. It can also be expected to accelerate the process of variety adoption by local farmers then. Collaboration between local farmers and coffee breeder in participatory breeding is also an important step to eliminate farmer's worry in adopting new national variety(s) released by the government whether suitable to their local conditions or not. Selection process was usually initiated by the farmer(s) based on high yielding performance by using their native experiences. The next step was conducted by plant breeder in cooperation with coffee farmers and other relevant scientists to evaluate more detail of cup quality, resistance to leaf rust and/or parasitic nematode as well as yielding capacity and agronomic traits. Cup profiles were evaluated by ICCRI's specialists and overseas professionals. Yielding ability was observed on its parent-offspring populations for 3 – 4 years. Selected candidate varieties were compared to commercial varieties (S 795 and Typica) at different farmer's sites for several years observations. The results showed as follows: (1) Three selected varieties namely Sigarar Utang, Gayo 1 and Gayo 2 already been released by Minister of Agriculture for commercial planting at North Sumatera and Gayo highland. The three varieties performs excellent cup profile with high yielding ability (2 tones/ha) and tolerant to leaf rust disease. (2) Observation over selected local variety at Kendenan Plateau namely Salongge variety performed lower yielding ability (below 1 ton/ha) and less stable yield over years as well as more heterogenous population in their offspring than that of existing released varieties planted at the same location. (3) Participatory breeding approach in form of collaboration between farmers and coffee breeders is able to accelerate in developing local specific varieties.

INTRODUCTION

Indonesia is well known as Robusta coffee producer, however in the future the government wish to increase Arabica production due to higher value than that of Robusta. Area of Arabica coffee growing in Indonesia is increasing so far, that the proportion of Arabica growing areas increases from about 10 % in 1980's to be about 20 % of the national coffee growing areas with the total production about 150,000 tons at the moment. Most of Arabica coffees from Indonesia are marketed to specialty segment by using local origin names such as Toraja coffee, Gayo coffee, Java coffee, Bali Kintamani coffee, Lintong coffee, Kalosi coffee, etc.

Superior planting material having good adaptability to certain local coffee growing areas will be a key role on the enlargement of Arabica coffee growing in Indonesia. Conventional breeding program for Arabica coffee to find out superior planting materials has been conducted by ICCRI since the early of 1900's, but developing certain planting materials adaptable to such local agro-ecology by using participatory breeding approach can be an effective approach in the country. The reasons are (1) availability of morphological variants on Arabica coffee farm derived from seedling population, and (2) in the country Arabica coffee grown at strictly different climate and soil types. Coffee farmers and extension service specialists will play their important roles. Coffee farmers normally conduct early selection of potential genetic variants based on visual evaluation, such as plant vigor, cherries size, resistance to pest and disease as well as yield, assisted by local extension specialists.

Chiffoleau and Desclaux (2006) mentioned that conventional breeding at a research center usually does not sufficient to support planting materials for certain specific locations, so that participatory breeding by involving farmers can be applied in order to find out planting materials suitable for specific purposes.

Participatory on plant breeding is defined as a form of collaboration between farmers and plant breeders in order to obtain superiors varieties appropriate to certain agro-ecological (Stirling and Witcombe, 2004). In the collaboration farmer and plant breeder are able to exchange knowledge and experience each other. Farmer has a lot of experiences on characteristics of planting materials, whereas plant breeding has a lot of experience on genetics resource and plant breeding method.

Participatory breeding on Arabica coffee has been applied in Indonesia namely in Lintong Nihuta (North Sumatra Province), Gayo (Aceh Province – Northern Sumatra), and Kendenan-Enrekang highland (South Sulawesi Province). The results of the activities are briefly presented in this paper.

MATERIALS AND METHODS

Participatory breeding on Arabica coffee was conducted at 3 growing areas namely (1) at Lintong Nihuta (1400 m – 1600 m altitude, andisol, wet climate), Bukit Barisan highland, North Sumatera Province; (2) at Gayo highland (1200 m – 1500 m altitude, ultisol, wet climate), Aceh Province – Northern Sumatra; dan (3) at Kendenan-Enrekang (1100 m – 1350 m altitude, latosol, dry climate), Latimojong highland, South Sulawesi Province).

Coffee farmers firstly selected certain mother trees based on vigor, yield, cherry size as well as pest and disease attacks at their farms. Availability of morphological variants on Arabica commercial planting was very often observed due to seedling originated population and it’s chance for out crossing up to 10 % (Van der Vossen et al., 2000). Local extension specialists then facilitated to make communication with ICCRI to follow up the farmer’s finding.

Several steps took by ICCRI to make precise evaluation on desirable characteristics namely (1) cup taste evaluation, (2) detail evaluation on morphological characteristics, yield and its stability over years, resistance to main pests and diseases as well as establishing seed garden. The promising mother tree(s) should be compared to recommended variety dominantly grown by neighboring farmers. The evaluation normally consumed time about 3 – 4 years. For cup taste profile evaluation ICCRI also involved international specialists.

The promising variety will be recommended for local commercial planting if it’s key characteristics perform better than that of the commercial variety(s) grown by local farmers.

This new local variety will also be involve on national multi location trials in order to evaluate broad range adaptability. ICCRI will provide technical assistance to establish a seed garden before releasing new variety developed by participatory breeding for commercial planting.

ICCRI will collect selected tree(s) does not appropriate for commercial plating if it bears certain interesting characteristic(s). The approach of participatory breeding on coffee conducted by ICCRI as mentioned in Figure 1.

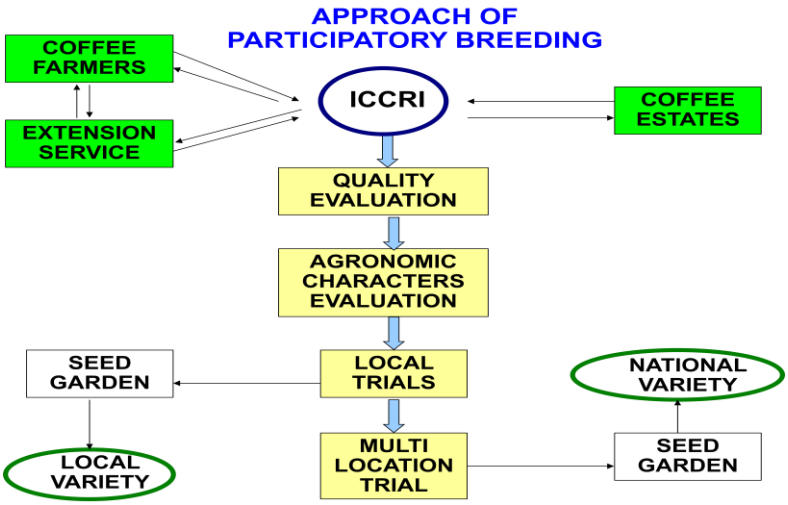

Figure 1. Approach of participatory breeding on coffee at ICCRI.

RESULTS AND DISCUSSION

Participatory breeding on Arabica coffee at Lintong Nihuta, Bukit Barisan highland, North Sumatra.

North Sumatra Province is one of the main Arabica producing areas in Indonesia with the total growing about 58,000 ha in 2010. At the end of 1970's coffee farmers at Lintong Nihuta received grant of Arabica coffee seedlings from a Government project, which the seeds originated from East Timor. There were significant morphological variants on the population that one of coffee farmers selected a unique semi dwarf tree having higher yield compare to their old tall type commercial varieties. The new semi dwarf tree also performenced earlier cherries bearing than that of the old one. The farmer then collected a single tree origin seeds for his new planting. In his new planting area he got not only higher yield but also earlier harvest than that of traditional planting, so that he could pay his financial debt immediately. He mentioned that the new dwarf type coffee was similar to 'debt payment' or in local bataknese language called 'Sigarar Utang'.

Detail observation on mother tree as well as it first progeny population was aimed to conform desirable characteristics of the promising tree such as good cup taste, high yielding potential as well as resistant to leaf rust disease. Cup taste quality was observed by ICCRI's panelists. Yield observation was also conducted at 50 different farms which the seeds were originated from identified 'Sigarar Utang' population. Table 1 shows summary of five year observations result on promising 'Sigarar Utang' variety compared to commercial 'Sumatra Typica' variety.

Table 1. Evaluation of cup test, bean and agronomic traits as well as yield on promising 'Sigarar Utang' mother tree and it progenies nominated by farmers at Lintong Nihuta, Bukit Barisan highland, North Sumatra (1,400 m altitude, wet climate, andisol).

Selection criteria	Mother tree and progenies of 'Sigarar Utang' variety (promising)	Sumatra Typica (commercial variety)
Bean performance	Rectangle, medium – large size	Roundish, medium – large size
Cup taste	Excellent	Very good
Yield	1.2 ton/ha	0.4 ton/ha
Yield stability	Stable	Stable
Growth	Semi dwarf	Tall
Internodes	Small	Medium
Cherry cluster	Medium – big	Small – medium
Resistance to leaf rust	Moderately resistant	Susceptible
Resistance to nematode	Highly susceptible	Susceptible
Resistance to coffee berry borer (CBB)	Susceptible	Susceptible

After passing several steps, particularly the establishment of seed garden, the promising 'Sigarar Utang' has been recommended as a local commercial variety by the Ministry of Agriculture since 2004 by using same name. Variety of Sigarar Utang is dominating Arabica coffee growing in North Sumatra at the moment.

Participatory breeding on Arabica coffee at Gayo highland of Aceh Province, Northern Sumatra.

In 2010 Arabica coffee growing area in Gayo highland was reported about 60,000 ha. A number of varieties were grown by coffee farmers at this area. A number of local farmers in the area also developed 'new local varieties' of Arabica coffee after founding an interesting variant(s) in their farms, particularly in yielding capacity. A lot of varieties grown by the farmers may be serious effects on quality issue, particularly its consistency.

In 2006 – 2007 harvesting seasons, a project on economic development funded by UNDP in cooperation with ICCRI did evaluation on Arabica coffee cup quality over 17 different varieties grown by the farmers in Gayo highland consisted of national recommended varieties, 'new local varieties' and promising varieties resulted from conventional breeding program. The result showed that there were significant different on cup taste profile between varieties, and three varieties performed highest ranks. The three varieties sequentially were two of 'new local varieties' namely Borbor and Timtim, as well as a Catimor type promising variety introduced from Thailand namely P 88.

Table 2. Evaluation of cup quality, agronomic traits and yield on mother trees and its progeny of Borbor and Timtim 'promising local varieties' at Gayo highland (1,400 m altitude; wet climate; andisol).

Traits observed	Timtim (local promising)	Borbor (local promising)	S 795 (National recommended)
Bean performance	Roundish, large size	Rectangle, large size	Roundish, medium size
Cup taste	Very good	Excellent	Very good
Yield (1,600 trees/ha)	1.2 ton/ha	1.1 ton/ha	0.7 ton/ha
Yield stability	Stable	Stable	Stable
Growth type	Tall	Tall	Tall
Internodes	Long	Medium	Medium
Cherry cluster	Medium – Big	Medium – Big	Small – Medium
Resistance to leaf rust	Resistant	Moderatly resistant	Resistant
Resistance to nematode	Moderate	Moderately resistant	Susceptible

ICCRI continued the work by doing intensive evaluation on agronomic characteristic, resistance to main pests and diseases as well as capacity and stability of yield for Borbor and Timtim with recommended variety of S 795 grown by farmers as a control. The choice on the two varieties was mainly based on the farmer's preference. The evaluation was conducted for three years at several coffee farms. Summary of the observation done over Borbor and Timtim varieties as mentioned in Table 2.

Based on Table 2, local promising varieties of Borbor and Timtim performed better traits than that of national recommended variety of S 795 under Gayo highland condition. The two varieties already grown by several farmers and demand on seed is increasing so far. Seed gardens for the two varieties have been established each in order to avoid incorrect seed distribution.

In 2010 Ministry of Agriculture has officially released 'local promising varieties' of Timtim and Borbor for local commercial planting with new name of Gayo 1 for Timtim and Gayo 2 for Borbor, respectively.

Participatory breeding on Arabica coffee at Latimojong highland of Enrekang district, South Sulawesi Province.

In 2010 Arabica coffee growing area in Latimojong highland of Enrekang district was reported about 11,911 ha. This area produces a well-known specialty Arabica coffee called Kalosi coffee. Coffee farmers in Kendenan village of the area developed a 'new local variety', the seeds originated from an interesting variant tree called Salongge variety, particularly due to visually high yielding capacity.

Table 3. Evaluation of cup quality, agronomic traits and yield on mother trees and its progeny of 'Salongge promising local varieties' at Latimojong highland of Enrekang (1.000 m altitude; latosol; dry climate).

	Salongge (Local promising)	**S 795 (National recommended)**
Bean performance	Roundish, medium size	Roundish, medium size
Cup taste	Very good	Very good
Yield	0.7 ton/ha	1,0 ton/ha
Yield stability	Not stable	Stable
Growth	Tall	Tall
Internodes	Long	Medium
Cherry cluster	Medium	Medium
Resistance to leaf rust	Susceptible	Resistant
Resistance to nematode	Susceptible	Susceptible

Observation on 'Salongge local promising variety' was conducted intensively on mother tree and 9 farms for 3 year harvests continuously. Parameters used on the observation were cup taste quality, agronomical characteristics, resistance to main pests and diseases as well as yielding potential and its stability. S 795 was used to be control variety, because it dominantly grown by the farmers. Result of the observation is summarized at Table 3.

Progenies of 'Salongge local promising variety' performed very heterogeneous agronomical traits. Some population showed better cup taste quality than that of S 795, but in general the taste of Salongge population was similar to S 795. Yield potential of Salongge populations were lower and less stable than that of S 795.

Based on the observation results it was concluded that 'Salongge local promising variety' not feasible to be promoted for commercial releasing by the government. Several selected mother trees already propagated clonally for germplasm collection and further observation.

CONCLUSIONS

- Participatory breeding is a useful approach in order to accelerate obtaining of superior local variety(s) of Arabica coffee.

- Application of participatory breeding on Arabica coffee in Indonesia has resulted local recommended varieties such as Sigarar Utang variety for Northern Sumatra highland as well as Gayo 1 and Gayo 2 for Gayo highland.

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Impact of Long-Term Increased Ambient CO_2 on the Photosynthetic Functioning of *Coffea arabica* and *C. canephora* leaves

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SUMMARY

Plants of *Coffea arabica* (Icatu, IPR108) and *C. canephora* cv Conilon Clone 153 were grown under 380 or 700 μLCO_2 L^{-1} air, in order to evaluate the long term impacts of elevated CO_2, particularly at the photosynthetic apparatus level. Under elevated CO_2, changes occurred in the photosynthetic apparatus functioning, with the net photosynthetic rate (P_n) showing increased values (between 80 and 155%) at 700 relatively to 380 μL L^{-1}. Since the stomatal conductance to water vapor (g_s) was maintained (Icatu) or showed increases lower than that of Pn (Cl 153; IPR 108), water use efficiency increased. Stomata density and size were also affected decreasing and increasing, respectively, in the leaves of high CO_2 grown plants of Cl 153 and Icatu. The plants grown at 700 μL L^{-1} showed as well increased electron transport rates at photosystem I (between 5-20%) and II (between 10-30%) level with Cl 153 displaying the highest increases. Enhancements were also observed on the total activities of ribulose 1,5-biphosphate carboxylase oxygenase (between 12-27%) and in Ru5PK (between 24-35%). Only minor changes were observed on the contents of photosynthetic pigments between [CO_2] treatments. It is concluded that P_n values were related to changes in some components of the photosynthetic apparatus (reflecting an acclimation ability), concomitantly to a higher functioning efficiency of the photosynthetic structures (*e.g.*, possibly through the inhibition of photorespiration at high external [CO_2]). There were no obvious differences in the way how the studied *C. arabica* (Icatu, IPR 108) and *C. canephora* (Cl 153) genotypes responded to increased atmospheric [CO_2]. Further studies are running, considering a wide physiological, biochemical and molecular analysis, allowing an integrated view of the plant responses to high CO_2 availability.

INTRODUCTION

Coffee business involves around 100.000 million USD and employs (directly or indirectly) *ca*. 500 million people worldwide, based in the trading of *C. arabica* and *C. canephora* beans. In the field plants are often exposed to various environmental stresses that limit plant growth and photosynthesis, and impose changes at cell and whole plant metabolism, namely, of carbon and nitrogen. That is likely to become exacerbated in many regions as a consequence of climate changes. To a certain extent, plants are able to cope with environmental limitations and to maintain an efficient functioning due to a wide range of complementary mechanisms that protect the cell as a whole and the photosynthetic apparatus in particular, with the latter being used as a tolerance probe to environmental stresses. Therefore, in the context of climate changes, this work aims at providing practical knowledge concerning the impact on the coffee plant of the predicted increase of atmospheric CO_2. That will provide insights concerning plants ability to cope with these environmental changes, something that was only barely dealt with and limited to the use of climate models.

MATERIALS AND METHODS

Potted plants (in 12 L pots) with 1.5 years from *C. arabica* (cv. Icatu and cv. Catucaí IPR 108) and *C. canephora* cv. Conilon Clone 153 (Cl 153) were transferred from a greenhouse into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown under environmental controlled conditions of temperature (25/20 ºC, day/night), RH (75%), irradiance (*ca*. 800 μmol m^{-2} s^{-1}) and photoperiod (11.5 h), exposed to two defined CO_2 concentrations CO_2 (380 or 700 μL L^{-1}). After 8 months, several parameters (from morphological features up to gene expression) were evaluated in the leaves developed under these conditions.

Leaf determinations included stomatal size (SS) and density (SD) observations, gas exchanges, thylakoid electron transport rates, total activities of ribulose 1,5-biphosphate carboxylase-oxygenase (rubisco) and ribulose 5-phosphate kinase (RuB5PK), as well as photosynthetic pigments (spectrophotometrically), performed as previously described.
Here we present only a small part of the ongoing experiments.

RESULTS AND DISCUSSION

The first results (2012) pointed to plant growth (height and leaf area) increases under high CO_2 conditions, whereas membrane permeability did not suffer modifications with elevated CO_2 (data not shown), but changes occurred in the photosynthetic apparatus functioning.

Under enhanced CO_2 availability the net photosynthetic rate (P_n) increased significantly (80-155%) when compared to normal CO_2 (Figure 1). That was consistently observed both in the morning and afternoon periods (data not shown). Also in the 700 μL L^{-1} grown plants, the leaf stomatal conductance to water vapor (g_s) differed minimally in Icatu or increased in Cl 153 and IPR 108 when compared to the 380 μL L^{-1} plants. Nevertheless, in all cases g_s varied to a lower extent than P_n, what provoked an increase in the water use efficiency. Anyhow, g_s could also be influenced by stomatal density (SD) and size (SS) that followed somewhat divergent patterns. In Cl 153 and Icatu SD tended to decrease 10% and 21%, respectively, whereas the opposite trend was followed by SS, which displayed increases of 6% and 7%. In IPR 108 both SD and SS were almost unresponsive to the CO_2 treatments (Figure 2).

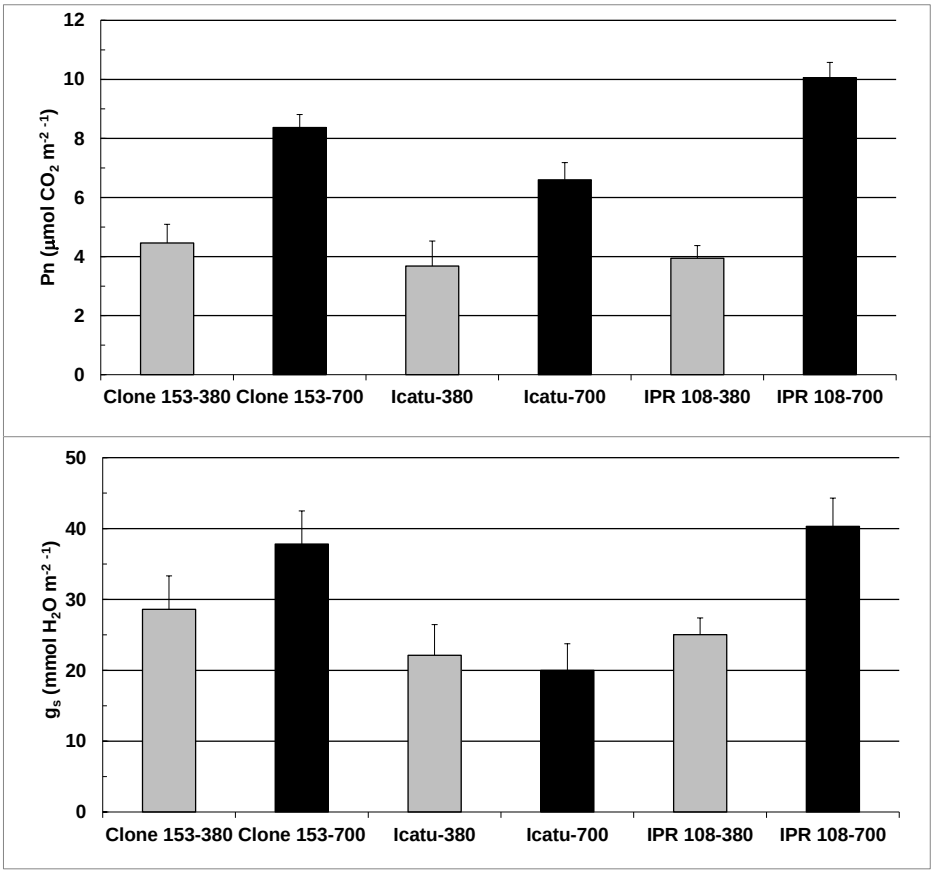

Figure 1. Net photosynthesis (P_n) and stomatal conductance to water (g_s) in leaves of plants grown under two [CO_2] (380 and 700 µL L^{-1}), in the three studied *Coffea* cultivars.

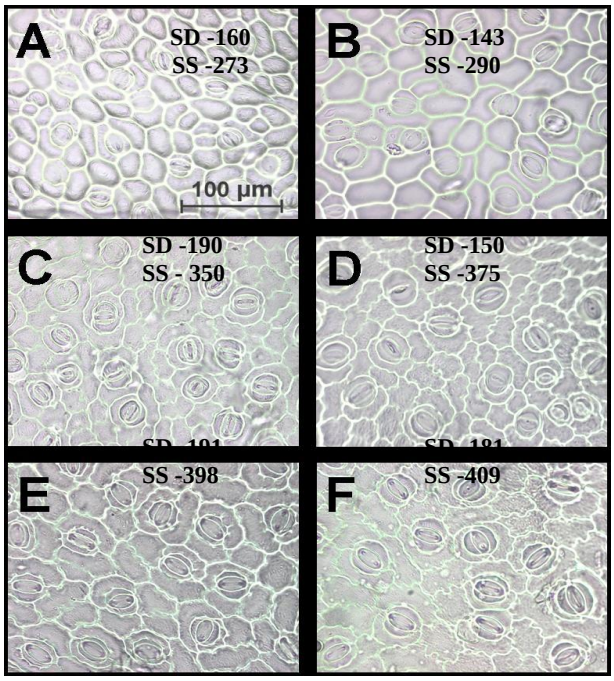

Figure 2. Stomata density (SD – number mm^{-2}) and size (SS – μm^2), as well as stomatal impressions of leaf surface representing an example of fully developed leaves under 380 (left) and 700 µLCO_2 L^{-1} (right) in Cl 153 (A,B), Icatu (C,D) and IPR 108 (E,F) cultivars.

Stomatal size and density determine maximum leaf diffusive (stomatal) conductance to CO_2. (g_{cmax}). Also, smaller stomata provide higher stomatal conductance for the same total pore area because of the shorter diffusion path length. In evolutionary terms it is known that high densities of small stomata are the only way to attain the highest g_{cmax} values required to counter CO_2 decrease on atmosphere along evolution. This explains cycles of stomata increasing density and decreasing size evident in the fossil history under the CO_2 impoverished atmospheres of the Permo-Carboniferous and Cenozoic glaciations. However, the pattern was reversed under rising atmospheric CO_2 regimes. In fact, an enrichment in the mole fraction of CO_2 in controlled environments, under range 350-700 $\mu LCO_2\ L^{-1}$, was found to cause an average decline of 9% in SD, although not in all species. Such decrease was in the range of that observed in both Cl 153 and Icatu.

Under elevated CO_2, the P_n rise was probably related to a new balance considering a higher carboxylation rate and a lower photorespiration rate. However, that P_n increased could also be promoted by the changes observed in some photosynthetic components, namely in the potential electron transport involving both photosystem (PS) I and II and in ribulose 1,5-biphosphate carboxylase-oxygenase (rubisco) and ribulose 5-phosphate kinase (Ru5PK) activities (Figure 3). That would support the observed increase up to 25% in the photosynthetic capacity (A_{max}) (data not shown). In fact, the thylakoid electron transport rates involving the PSII (with and without the oxygen evolving complex) increased between 10% in *C. arabica* cultivars and 25-30% in Cl 153, whereas electron transport rate involving PSI increased between 5 and 20%, in the same cultivar order. Also, the activities of enzymes related to photosynthesis increased between 12% (IPR 108) and 27% (Icatu) in rubisco, and between 24% (Icatu) and 35% (Cl 153) in Ru5PK. Such higher investment in photosynthetic components would therefore contribute to support the higher P_n values, allowing also the higher photosynthetic capacity and a saturating irradiance values observed for the 700 $\mu L\ L^{-1}$ plants (data not shown).

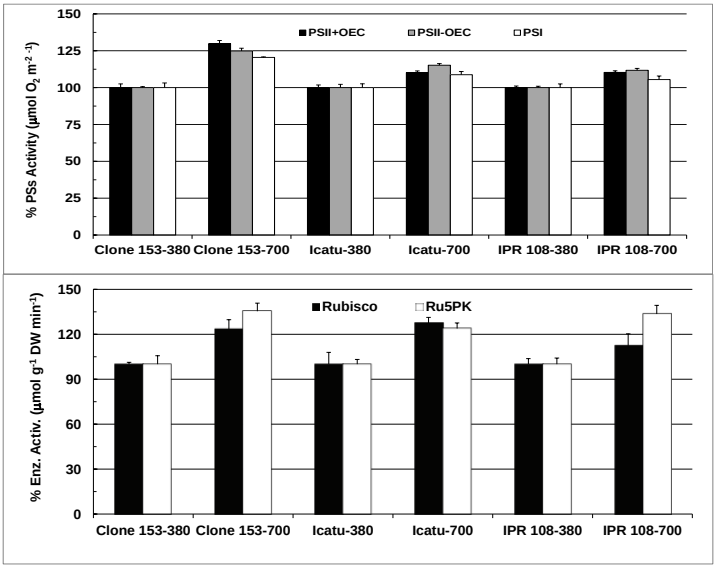

Figure 3. Variation in percentage (in relation to 380 $\mu L\ L^{-1}$ treatment) of photosystems I and II activities (from $\mu molO_2\ m^{-2}\ s^{-1}$), as well as for total rubisco activity and Ru5PK (both from $\mu molCO_2\ g^{-1}$ dw), in leaves of plants grown under two [CO_2] (380 and 700 $\mu L\ L^{-1}$), in the three studied *Coffea* cultivars.

Several works reported that, under optimal conditions elevated CO_2 may increase the photosynthetic rate more than 50% as compared to plants grown under normal CO_2

concentrations, although the impact on the relative growth rate was near 10% rise. That was probably due to a limitation to fully use the enhanced carbohydrate contents or due to nutrient or intrinsic internal growth limitations. Such P_n rise, promoted by long-term air CO_2 enrichment, was previously reported in several trees and potato, but no effects were observed on growth stimulation, carboxylation efficiency (V_{cmax}) or maximal electron transport (J_{max}) driving ribulose-1,5-bisphosphate (RuBP) regeneration. In some cases Rubisco content was even reported to decrease and the photosynthetic performance was not increased under high CO_2.

Small changes were observed in response to a higher CO_2 availability in the photosynthetic pigments, both in their total contents and the ratio between them (total chlorophyll/total carotenoids) (Figure 4), although *C. arabica* cultivars presented slight reductions. These results agree with previous observations that chlorophyll (a + b) levels were unaffected by CO_2 treatment in other woody species.

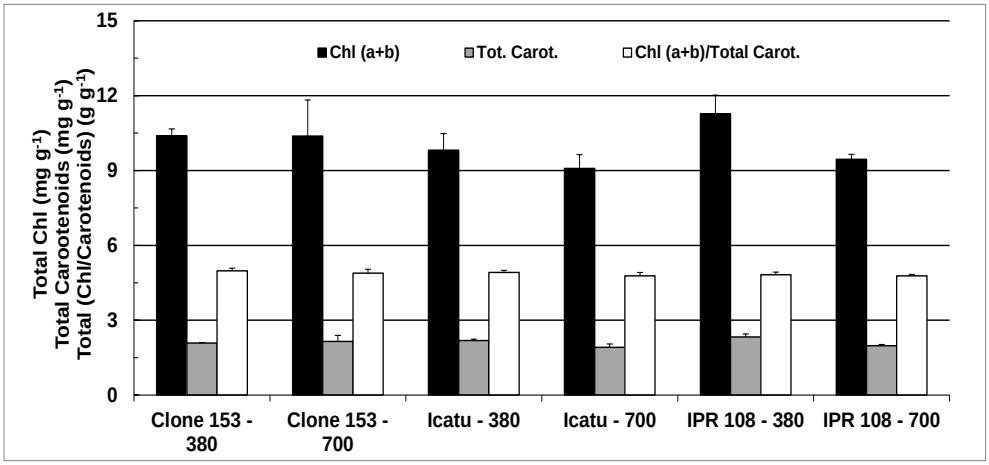

Figure 4. Total chlorophylls, Total Carotenoids and their ratio, in leaves of plants grown under two [CO_2] (380 and 700 μL L^{-1}), in the three studied *Coffea* cultivars.

In conclusion, under adequate temperature (25/20 ºC), a higher photosynthetic functioning apparatus was observed (with positive implications on water use efficiency) in the plants grown under high CO_2 availability (700 μL L^{-1}). The increased P_n and A_{max} values would be associated also with the enhancement of the thylakoid electron transport and enzyme (rubisco and Ru5PK) activities (long-term acclimation ability), as well as with an expected inhibition of photorespiration, directly promoted by the higher CO_2 availability. However, those enhancements in the photosynthetic rates were not related to the ability to capture photons, since photosynthetic pigments did not suffer significant changes, at least under the used irradiance conditions (similar to moderate shade). Phenotypic changes (stomata size and density) could also be involved in leaf gas exchanges modifications.

Based on the presented parameters, under temperatures close to optimum there were no clear differences between the studied *C. arabica* (Icatu, IPR 108) and *C. canephora* (Cl 153) genotypes concerning their responses to increased atmospheric [CO_2]. Taking into account that temperature is expected to rise concomitantly to the [CO_2] increase, other studies are currently in progress under adequate and stressful environments. That will help to provide an integrated view at physiological, biochemical and molecular levels, characterizing the impacts and responses of the functioning of the photosynthetic apparatus driven by high environmental CO_2 availability.

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