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Table of Contents

VOLUME 1

COMMUNICATIONS AND POSTERS

Coffee consumption and human physiology

- Evaluation of the *in Vitro* Inhibitory Effects of an Extract of Decaffeinated Green Coffee Beans on Lipid Degradation
  Kitaura, K. Iwai, K., Fukunaga, T., Kimura, R., Nakagiri, O. (Japan)

- Urinary Excretion of Chlorogenic Acids and Metabolites in Humans after Green and Roasted Coffees Consumption
  Monteiro, M. , Marques,V., Farah, A. (Brazil)

- The Bioavailability of Chlorogenic Acids from Coffee Is Not Affected By the Simultaneous Consumption of Soybean Extract
  Monteiro, M. , Duarte, G., Felberg, I., Farah, A. (Brazil)

- Coffee is the Main Contributor to the Dietary Antioxidant Capacity in Brazil
  Torres. T., Farah, A. (Brazil)

- Activation of the Nrf2/ARE Pathway and ARE-Dependent Gene Expression by Different Coffee Constituents
  Boettler, U., Volz, N., Stiebitz, H., Bytof, G., Lantz, I., Somoza, V., Lang, R., Hofmann, T., Marko, D. (Germany)

Chemistry

Communications

- High-Molecular Weight Components in Coffee Beverages and Their Degradation by Human Microbiota
  Gneichwitz, D., Reichardt, N., Blaut, M., Bunzel, M. (USA)

- Biological Activities of Arabinogalactan-proteins Isolated from *Coffea arabica* Freeze Dried Instant Coffee
  Navarini, L., Suggi-Liverani, F., Paulovicova, E., Prisenznakova, L., Nosal'ova, G., Matulova, M., Capek, P., Coradini, D., Casarsa, C. (Italy)

- Lipids in Coffee Brews - Impact of Grind Size, Water Temperature, and Coffee/Water Ratio on Cafestol and the Carboxylic Acid-5-Hydroxytryptamides
  Buchmann, S., Zham, A., Kölling-Speer, I., Speer, K. (Germany)

- Analysis of Diterpens in Green and Roasted Coffee of *Coffea arabica* Cultivars Growing in the Same Edapho-Climatic Conditions
  Kitzberger, C.S.G., Scholz, M.B.S., Pereira, L.F.P., Vieira, L.G.E., Sera, T., Silva, J.B.G.D., Benassi, M.T. (Brazil)

- Recent Developments in Coffee Flavour Formation Using Biomimetic In-Bean Experiments
  Poisson, L., Kerler, J., Davidek, T., Blank, I. (Switzerland)

- Advanced Analytical-Sensory Correlation – Towards a Better Understanding of Coffee Flavor Perception
  Baggenstoss, J., Poisson, L., Glabasnia, A., Moser, M., Rytz, A., Thomas, E., Blank, I., Kerler, J. (Switzerland)

- Characterization of Non-Defective and Defective Coffee Beans by Fourier Transform Infrared Spectroscopy (FTIR)
  Oliveira, L., Craig, A.P., Franca, A.S. (Brazil)

- Improved Chemometric Model for Prediction of Arabica Coffee Overall Quality Using Diffuse Reflectance near Infrared Spectroscopy
  Ribeiro, J.S., Salva, T.J.G. (Brazil)

- Chemical Stability of Roasted Coffee Aqueous Extracts
  Silanes-Kenny, J., Navarini, L., Suggi-Liverani, F., Melrose, J. (UK)

- Pesticides Contamination of Coffee in East Africa
  Ishiwaki, T., Yamaishi, E., Tanabe, Y. (Japan)

Posters

- Gas Chromatography – Mass Spectrometry in Tandem with Sensory Analysis for Identification of Luwak Arabica Coffee
  Ismayadi, C., Ariza Budi, T.S., Misnawi (Indonesia)

- Potential of Fourier Transform Infrared Spectroscopy (FTIR) for Detection of Adulteration in Roasted Coffees
  Franca, A.S., Oliveira, L.S., Brito, M.S. (Brazil)

- Defective Coffee Beans as Raw Materials For Adsorbent Production By Thermo-Chemical Activation
  Franca, A.S., Clark, H.L.M., Oliveira, L.S. (Brazil)

- Production of Adsorbents from Spent Coffee Grounds: Effect of Thermal/Chemical Treatments on Phenol Adsorption
  Oliveira, L.S., Silva, L.C.O., Franca, A.S. (Brazil)

- Taste Compounds Affecting Sensory Characteristics of Ready-to-Drink Chilled Espresso
  Miyai, T., Akiyamam, M., Michishita, T., Katakura, T., Ikeda, M., Araki, T., Sagara, Y. (Japan)

- Ethyl Formate As a Marker for the Fermented Off-Note in Coffee
  Lambot, C., Husson, J., Bedon, L., Michaux, S., Goulois, E., Lindinger, C., Broun, P. (France)

- Effect of Roasting Variables in Antioxidant Properties of Coffee Beverages
  Naranjo, M., Tilano, A., Gaviria, C., Velez, T., Rojano, B. (Colombia)

- Differentiation of Brazilian Arabica Crude Coffees Using Diffuse Reflectance near Infrared Spectroscopy and Chemometrics
  Ribeiro, J.S., Salva, T.J.G. (Brazil)

- Chemical Characterization of *Espresso* Coffee Foam: Influence of the Extraction Method
  Navarini, L., Lonzarich, V., Colomban, S., Rivetti, D., De Angelis, E., D'Amelio, N., Suggi-Liverani, F. (Italy)

- Oil Bodies Ultrastructure in Green Coffee Seeds of Different Geographical Origin
  Crisafulli, P., Silizio, F., Pallavicini, A., Navarini, L. (Italy)

- Comparison among Different Discrimination Tests: The Case of *Espresso* Coffee
  Barnaba, M., Umech, N., Navarini, L., Suggi-Liverani, F. (Italy)

- Evaluation of *Coffea arabica* and *Coffea canephora* Beverages Using Electronic Tongue (ET)
  Santos, E.S.M., Paterno, L.G., Fonseca, F.J., Mattoso, L.H.C., Freitas, D.D.G.C., Deliza, R. (Brazil)

- Fast Method for the Quantification of 16-O-Methylcafestol in Roasted Coffee
  Buchmann, S., Küchenmeister, G., Kölling-Speer, I., Speer, K. (Germany)

- Coffee Aroma – Influence of the Brewing Method and the Used Coffee
  Zahm, A., Speer, K. (Germany)

- Identification of C-5-HT Degradation Products Using Pyrolysis-GC/MS
  Zahm, A., Speer, K., Peters, J. (Germany)

- Serotonin and 5-Hydroxyindole in Green and Roasted Coffee
  Zahm, A., Ronczka, R., Speer, K. (Germany)

- Chemical Stability and Consumer Acceptance during Storage of a Cold Instant Soy Coffee Beverage
  Felberg, I., Deliza, R., Farah, A., Calado V., Silva, J.P.L., Toci, A.T., Freitas, S.C., Donangelo, C.M. (Brazil)

- Soluble Coffee Classification through Rapid Scanning Methodologies
  Fisk. I.D., Virdie, A., Kenny, J.S., Ullrich, F. (UK)

- Contents of Chlorogenic Acids, Caffeine, Trigoneline and Sucrose in Brazilian Defective Seeds
  Toci, A.T., Farah, A. (Brazil)

Processing

Communications

- Climate Change, Harvesting Systems and Sustainability
  Brando, C.H.J. (Brazil)

- Coffee Processing Water Minimization Options
  Mburu, J.K. (Kenya)

- Assessing the Performance of Ecological Wet Processing Plants under Tanzanian Conditions
  Maro, G.P., Teri, J.M., Mushi, I.K., Kimaro, W. (Tanzania)

- Flavor Profiles of Robusta Coffee Processed by Wet Hulling and Pulped Natural Method
  Yusianto, Mawardi, S. (Indonesia)

- Single Coffee Bean Roast Gas Measurements with Microprobe Single Photon Ionization Time-of-Flight Mass Spectrometry
  Herz, R., Zimmermann, R. (Germany)

- Case Study of Biogas Production from Plant-Based Materials and Animal Manure Resources Available in the Coffee Farm
  Mulato, S., Suharyanto, E. (Indonesia)

- Identification of Variety and its Suitable Cherries Processing Method for Improving Specialty Arabica Coffees from Dry Climate Area at Flores Island of Indonesia
  Mawardi, S., Yusianto, Mars, A. (Indonesia)

- Cup Profile Similarity and Uniqueness of Specialty Arabica Coffees from Different Origins in Indonesia
  Yusianto, Mawardi, S., Sulistyowati, Ismayadi, C. (Indonesia)

- A Novel Approach to Triphenyltetrazolium Staining As a Quality Control Tool for Green Coffee: Correlation with Aging, Geographical Origin and Sensory Analysis
  Del Terra, L., Barnaba, M., Silizio, F., Navarini, L. (Italy)

Posters

- Effects of Soaking Parchment on Sensory and Some Biochemical Components of *Coffea arabica* L. Produced in Kenya
  Kathurima, C.W. (Kenya)

- Development of a Grinder for the Special Requirements of Ground Coffee Used in Capsules or As Coffee Powder Admixture to Other Foodstuff
  Koziorowski, T., Schneider, T., Baumeister, H., Beckmann, C., Möller- Willenberg, U. (Germany)

- Optimizing of Single Column Reactor for Robusta Coffee Beans Decaffeination
  Widyotomo, S., Mulato, S., Purwadaria, H.K., Syarief, A.M. (Indonesia)

- Development a Portable-Direct Digital Display of Coffee Beans Moisture Tester
  Sri-Mulato, Suharyanto, E. (Indonesia)

- *Coffea arabica* Beans Microstructural Changes Induced by Roasting: an X-Ray Microtomographic Investigation
  Frisullo, P., Del Nobile, M.A., Barnaba, M., Navarini, L., Suggi-Liverani, F. (Italy)

- Characteristics of Coffee Extraction Process in a Single Column Reactor
  Hananto, Y., Mulato, S., Purwadaria, H.K. (Indonesia)

- Characteristics of Crystallization Process for Low Caffeine Instant Ginseng Coffee
  Tirtaguna, R., Mulato, S., Purwadaria, H.K. (Indonesia)

- Chorogenic Acids and Lactones in Coffees Decaffeinated by Water and Supercritical CO_2 and Roasted in a Pilot Plant Scale Fluidized Bed Roaster
  Farah, A., Perrone, D., Fernandes, J., Silanes, J. (Brazil)

- Study of the Impact of Extrusion Cooking Conditions on the Atmospheric Extractability of Roast Coffee Solids
  Guilmineau, F., Saldanha do Carmo, C. (UK)

- KENCO Coffee, 100% Committed to Sustainability: How Innovation Enables the Commercialisation of Sustainable Coffee in the Mainstream Retail UK Market
  Guilmineau, F., Hett, M., Clark, J.A. (UK)

Genomics & Genetics

Communications

- Expression Analysis and Nucleic Polymorphism of Candidate Genes for Drought Tolerance in Coffee
  Freire, L., Vieira, N., Vinecky, F., Alves, G., Leroy, T., Pot, D., Elbelt, S., Marques, T., Rodrigues, G., Marraccini, P., Andrade, A. (Brazil)

- MOCCADB - An Integrative Database for Functional, Comparative and Diversity Studies in the Rubiaceae Family
  Poncet, V., Plechakova, O., Benedet, F., Cuyot, R., Couderc, M., De Block, P., Hamon, P., Campa, C., de Kochko, A., Hamon, S., Tranchant-Dubreuil, C. (France)

- A Coffee High Density Genetic Map for Quantitative Trait Loci Analysis on Agronomical, Technological and Biochemical Characteristics in Robusta and Arabica
  Crouzillat, D., Rigoreau, M., Lefebvre-Pautigny, F., Priyono, Broun, P., Lambot, C. (France)

- Functional Markers in Assisted Selection – Study of an Expressed SSR Locus Associated with Leaf-Miner Resistance
  Molino, J., Silvestrini, M., Martinati, J., Guerreiro-Filho, O., Maluf, M. P. (Brazil)

- Sequencing the Coffee Genome: Overall Strategy and Progress Made in the Frame of ICGN
  Lashermes, P., Argout., Y., Crouzillat, D., Priyono, Yepes, M., Wincker, P. (France)

- Overview of the Functional Virulent Genome of the Coffee Leaf Rust Pathogen *Hemileia vastatrix*
  Talhinhas, P., Azinheira, H. G., Loureiro, A., Batista, D., Vieira, B., Pina- Martins, F., Tisserant, E., Petitot, A.S., Paulo, O.S., Duplessis, S., Silva, M. d. C., Fernandez, D. (Portugal)

Short communications

- Molecular Genetic Diversity of Arabica Coffee (*Coffea arabica* L.) Using ssr Markers
  Alemayehu, T., Pétiard. V., Broun, P., Crouzillat, D. (Ethiopia)

- The Effect of Low Temperature on Metabolism of Membrane Lipids in *Coffea* spp. and Associated Gene Expression
  Batista-Santos, P., Goulao, L.F., Fortunato, A.S., Pais, I.P., Campos, P.S., Partelli, F., Ribeiro, A.I., Ramalho, J.C. (Portugal)

- Coffee Control Quality on Geographic and Genetic Origins Determined By DNA Traceability
  Morel, E., Rigoreau, M., Rodriguez, A., Lambot, C., Broun, P., Crouzillat, D. (France)

- Coffee Seeds Gene Expression Analysis in Different Post-Harvest Processes
  Modonut, M., Del Terra, L., Barnaba, M., Graziosi, G., Pallavicini, A. (Italy)

- Screening of SSR Markers for Diversity Studies in *Coffea arabica*
  Dos Santos, M., Ferreira, R., Sera, T., Pot, D., Pereira, L.F., Charmetant, P., (Brazil)

Labels – Quality - Breeding

Communications

- Quality Upgrading in Specialty Coffee Chains and Smallholder Livelihoods in Eastern Indonesia: Opportunities and Challenges
  Neilson, J., Arifin, B., Fujita, Y., Hartatri, D.F.S. (Australia)

- Evaluation of Organic Coffee Farming Potential from Perspectives of Production and Natural Resources Management in Yayu District, Southwestern Ethiopia
  Tesfaye, A., Bobe, B., Ketema, B. (Ethiopia)

- Sustainable Coffee Farming Framework: a Chain View
  Celi, G., Liverman, D., (Italy)

- Performance of *Coffea arabica* F1 Hybrids in Comparison with American Pure Line Varieties
  Bertrand, B., Alpizar E., Llara L., Santacreo R., Hidalgo M., Quijano J.M., Charmetant P., Montagnon C., Georget F., Etienne, H. (France)

- An Example of Successful Technology Transfer: *Coffea arabica* Propagation by Somatic Embryogenesis
  Georget, F., Bertrand B., Malo, E., Montagnon, C., Alpizar, E., Dechamp, E., Jourdan, I., Etienne, H. (France)

- The Impact of Participatory Village Based Training for Increased Coffee Productvity, Production and Income in Tanzania
  Magesa,J.M., Swai, F.B., Mushi, I.K., Ng'Homa, N.M., Mdemu, S., Teri, J.M. (Tanzania)

Coffee Agronomy. Pathology - Breeding

Communications

- Integrated Control of Coffee Berry Borer (*Hypothenemus hampei*) on Arabica Coffee in South Sulawesi, Indonesia
  Wiryadiputra, S., Jahuddin, R., Mainsese, I.L., Oduor, G.I., Sastroutomo, S.S. (Indonesia)

- Nematodes: an Emerging Constraint to Coffee Production in Kenya
  Gichuru, E., Maundu, J.N., Mugo, H.M., Mburu, J.N., Gitonga, K.T., Kimenju, J.W., Mutua, G.K. (Kenya)

- Population Structure of *Colletotrichum kahawae*, the Causal Agent of Coffe Berry Disease (CBD): Insights from a Multi-Locus Approach
  Silva, D., Talhinhas, P., Várzea, V., Loureiro, A., Silva, M.d.C., Paulo, O.S., Batista, D. (Portugal)

- The Regional Coffee Wilt Programme: Where Do We Go from Here?
  Phiri, N., Baker, P., Rutherford, M., Flood, J., Musoli, P., Mbuyid, K., Kilambo, D., Adugna, G., Hakiza, G., Pinard, F., Oduor, G. (Kenya)

- Progress in Breeding for Resistance to Coffee Wilt Disease (Tracheomycosis) in Tanzania
  Kilambo, D., Ng'homa, N.M., Mtenga, D.J., Teri, J.M., Phiri, N. (Tanzania)

- Nematode Diversity after Forest Conversion into Coffee-Based Agroecosystems
  Gede Swibawa, I., Hairiah, K., Suprayogo, D. (Indonesia)

Short communications

- The Raise of Eco-Label Coffee: a Comparative Study of Their Effects on Costa Rican and Kenyan Cooperative Coffee Sector
  Le Coq, J.F., Pinard, F., Kirimba, E.G., Soto, G. (France)

- Comparative Analysis of Coffee Eco-Certification Initiatives
  Pinard, F., Kirumba, E. (Kenya)

- The CAFNET/Coffee-Flux Project: Evaluating Water, Carbon and Sediment Ecosystem Services in a Coffee Agroforestry Watershed of Costa Rica
  Roupsard, O., Gomez-Delgado, F., Charbonnier, F., Benegas, L., Taugourdeau, S., Kinoshita, R., Moussa, R., Dreyer, E., Lacointe, A., Rapidel, B., Perez, A., Robelo, A., Barquero, A., Rivera Wilson, C., Navarro, M.N.V., Jourdan, C., Le Maire, G., Thaler, P., Bonnefond, J-M., Harmand, J-M., Vaast, P. (France)

Coffee Agronomy. Sustainability – Agro-ecology

Communications

- Climate Change - Impact on Coffee Pests
  Vinod Kumar, P.K. (India)

- The Socio-Economic Impact of Climate Change on Mesoamerican Coffee Production
  Läderach, P., Ovalle, O., Baca, M., Eitzinger, A., Oberthür, T. (Nicaragua)

- Climate Change: Potential Impact on Eastern Africa Coffees
  Walyaro, D. (Kenya)

- An Assessment of Land Degradation in Kilimanjaro Region, Tanzania, and Its Implications to Coffee Sustainability
  Maro, G., Kitalyi, A., Nyabenge, M., Teri, J.M. (Tanzania)

- Reducing N-NO_3^- Leached on Coffee Based Agroforestry System by Impede Nitrification
  Kurniawan, S., Hairiah, K., Widianto, Suprayogo, D., Wandri, R. (Indonesia)

- Carbon Stock Assessment for a Forest-to-Coffee Conversion Landscape in Kalikonto Watershed (East Java, Indonesia): Scale up from Plot to Landscape Level
  Hairiah, K., Kurniawan, S., Aini, F.K., Lestari, N.D., Lestariningsih, I.D., Widianto, Zulkarnaen, T., Van Noordwijk, M. (Indonesia)

Polyphenol Exposure from Coffee and Cardiovascular Health

K.D. CROFT

School of Medicine and Pharmacology, University of Western Australia

WHAT IS ATHEROSCLEROSIS AND WHAT ARE THE RISK FACTORS FOR HEART DISEASE?

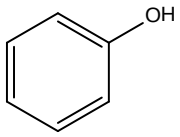
Atherosclerosis is the build-up of cholesterol in the arteries. It is a complex disease that occurs over a long time period and involves inflammation, oxidative stress and cell activation (Libby, 2002). The build-up of fatty lesions in the blood vessel can constrict normal blood flow and if the lesion ruptures and prothrombotic material is exposed, a thrombosis may result which blocks blood flow to the vessel. This could result in myocardial infarction or heart attack, where part of the heart tissue is damaged due to lack of normal blood flow. Heart disease is more common in men and increases with increasing age. A family history of heart disease is considered a strong risk factor for this disease. There are also a number of modifiable risk factors for heart disease related to life style and nutrition. These include smoking, high blood cholesterol, physical inactivity (overweight), high blood pressure and diabetes.

Population studies suggest that diets rich in fruit and vegetables are associated with reduced risk of heart disease (Huxley and Neil, 2003). Since the oxidation of lipoproteins (particles that transport cholesterol in the blood) is thought to play a role in atherosclerosis (Stocker and Keaney, 2004) and fruits and vegetables contain compounds that appear to be strong antioxidants, it has become commonly assumed that dietary 'antioxidant' must be the reason why these foods protect against heart disease. However to date there is no strong evidence to suggest that antioxidant supplements protect against heart disease (Halliwell et al., 2005). While there is ample evidence to show that many polyphenols compounds found in fruits and vegetables are strong antioxidants in the test tube, their role as antioxidants in the human body is much less established. Due to the often low bioavailability and metabolic transformation of these compounds, it is likely that they do not act as simple antioxidants but rather have specific activity against key enzymes that may influence cardiovascular health (Crozier et al., 2009).

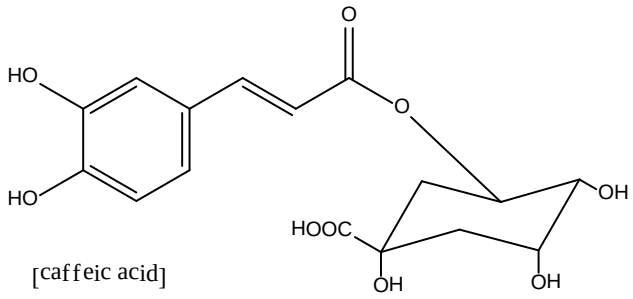
POLYPHENOLIC COMPOUNDS

The term polyphenol refers to a large group of naturally occurring compounds that possess one or more phenolic structures (see Figure 1). The flavonoids represent a very large class of compounds that are commonly found in fruits, tea, cocoa etc. This group of compounds has attracted considerable attention due to the potential bioactivity of some flavonoids and isoflavonoids. A review of bioactive flavonoids is outside the scope of this article and the reader is referred to several comprehensive reviews in this area (Crozier et al., 2009). Polyphenolic compounds can fall into a number of chemical classes. Some examples of these are listed in Table 1 which indicates some examples of each class and dietary sources in which they are found. Coffee is a major source of chlorogenic acid which is a phenolic acid. In some populations coffee is likely to be the major source of dietary polyphenols such that

several cups of coffee is likely to provide approx. 1 g of chlorogenic acid (Crozier et al., 2009).



phenolic group



[caffeic acid] [quinic acid]

Chlorogenic acid, 5-O-caffeoylquinic acid

Figure 1.

Table 1. Some examples of dietary polyphenols with biological activity.

Chemical class	Examples	Source
Flavonoids	Catechins, quercetin, isoflavones	Widespread in the plant kingdom
Phenolic acids	Chlorogenic acid	Fruits, coffee
Lignans	Sesamin, isolariciresinol	Sesame, flax seed, bran
Stilbenes	Resveratrol	Red wine, peanuts
Phenylpropanoids	Curcumin, zingerone	Tumeric, ginger
Terpenoids	Oleuropein	Olives

DIETARY POLYPHENOLS AS ANTIOXIDANTS

Polyphenolic compounds are redox active, they can act as antioxidants or prooxidants depending on the experimental conditions (Halliwell et al., 2005). There is no question that they are strong antioxidants in vitro. However, they may not be good antioxidants in vivo. The main reasons for this are the low bioavailability of many polyphenolic compounds. This means that the concentrations that are often tested in vitro can never be achieved in vivo. In addition most polyphenols undergo metabolic transformation so that the metabolites in the circulation may have different structure and properties form the parent compounds (Kroon et al., 2004). It is reasonable therefore to study the bioactivity of the metabolites rather than the parent compound. It is also more likely that phenolic compounds or their metabolites have

more specific activity against key enzymes or act as signalling molecules rather than as simple antioxidants (Lotito and and Frei, 2006).

POLYPHENOLS IN ANIMAL MODELS OF ATHEROSCLEROSIS

Since atherosclerosis is a disease that takes many decades to develop in humans, animals models are often used to study the pathogenesis of this disease. One of the most common models is the apo E knock out mouse. This mouse lacks the gene for apolipoprotein E which is critical for the binding and uptake of lipoproteins particles in the liver. As a result these animals have very high circulating concentrations of cholesterol and develop lipid lesions in the aorta within a few months or even earlier if they are placed on a high fat diet. Using this model we and others found that polyphenols in dealcoholized wine could significantly reduce lesion development over a period of months and this effect was independent of any antioxidant activity (Waddington et al., 2004). Polyphenols from a number of dietary sources have been shown to reduce lesion formation in this and other animal models of atherosclerosis (Tijburg et al., 1997). Most of these studies use crude extracts which contain many different compounds and it is uncertain if some polyphenols have more activity than others. Some studies have shown protective effects of individual flavonoids such as catechin or quercetin (Hayek et al., 1997) but generally few studies have used pure compounds. In order to compare different classes of polyphenols we fed apo E KO mice diets incorporating small amounts of either quercetin, epicatechin, theaflavin, sesamin or chlorogenic acid over a period of 20 weeks. Lesion formation in the aortic sinus and the thoracic was assessed and compared to apo E KO mice fed the control diet without any added polyphenols. In both areas of the aorta quercetin and theaflavin (a black tea polyphenol) significantly reduced lesion formation (Loke et al., 2010). Chlorogenic acid reduced lesion formation by 40% in the thoracic aorta but less so in the aortic sinus. Interestingly the effects on lesions did not seem to be related to antioxidant activity per say but more so by alleviating inflammation and endothelial dysfunction (Loke et al., 2010).

DIETARY POLYPHENOLS AND CARDIOVASCULAR RISK IN HUMANS

There are a number of population studies that link increased dietary intake of flavonoids or polyphenols with reduced risk of cardiovascular mortality (Huxley and Neil, 2003).Such associations do not prove a direct link and therefor it is important to conduct intervention studies in an attempt to provide direct evidence for an effect of dietary polyphenols on cardiovascular disease risk factors. Early dysfunction of the blood vessels has been linked with later development of atherosclerosis and heart disease (Celermajer, 1997). So called endothelial dysfunction can be assessed no invasively in humans by flow mediated dilation (FMD) of the brachial artery (Celermajer, 1997). Using this technique intervention studies have demonstrated that flavonoids found in cocoa and tea can improve endothelial function both acutely and long term (Heiss et al., 2003; Hodgson, 2006). There have been few studies examining the effects of coffee or coffee polyphenols on FMD but one recent study suggests that effects may be confounded by the presence of caffeine (Buscemi et al., 2010). Studies on blood pressure are also complicated by caffeine with most studies showing a blood pressure raising effect of caffeine (Higdon and Frei, 2006). Some dietary polyphenols have shown a modest reduction in blood pressure in short-term human intervention trials as shown in a recent meta-analysis (Hooper et al., 2008).

COFFEE POLYPHENOLS

Coffee is an infusion prepared from ground roasted coffee beans. This extract is likely to be a complex chemical mixture containing not only phenolic compounds but also lipids, carbohydrates, alkaloids (caffeine) etc (Higdon and Frei, 2006). By far the major phenolic compounds in coffee are the esters of quinic acid with caffeic acid (chlorogenic acids, Figure 1). The major isomer in coffee is the 5-O-Caffeoylquinic acid. The absorption and metabolism of chlorogenic acids is complex and has been studied in detail (Stalmach et al., 2009). Chlorogenic acids are well absorbed but extensively metabolised, with some metabolites reaching maximal concentration in plasma at 1 hr after consumption and others taking up to 4 hours to reach maximal concentrations. Some of the urinary metabolites may act as good biomarkers of coffee consumption (Stalmach et al., 2009). While chlorogenic acid is a good antioxidant in vitro there is less evidence for antioxidant effects in vivo. In the apo E KO mouse, chlorogenic acid incorporated into the diet for 20 weeks had no significant effect on aortic tissue f2-isoprostanes (a biomarker of oxidative stress) (Loke et al., 2010). In a recent human clinical trial drinking up to 8 cups of coffee per day for 1 month significantly reduced plasma F2-isoprostanes compared to baseline (Kempf et al., 2010). However, interpretation of this finding is limited because this was not a placebo controlled study. It would therefore seem reasonable not to focus just on antioxidant activity of coffee polyphenols. For example, a recent study in mice suggests that coffee metabolites in plasma may enhance high density lipoprotein (HDL)-mediated cholesterol efflux (Uto-Kondo et al., 2010). This suggests that coffee may have antiatherogenic properties. These findings need to be supported by human intervention studies.

Studies on the effects of coffee consumption and cardiovascular health have been complicated by the presence of diterpenes extracted during some methods of coffee extraction. Boiled coffee contains kahweol and cafestol which can elevate serum cholesterol levels, and this may explain why some earlier studies liked increased coffee consumption with high serum cholesterol (Jee et al., 2001). Paper filters remove these compounds from coffee and espresso style coffee also contains lower amounts. Interestingly large prospective cohort studies form the Health Professionals follow-up and Nurses Health study show a modest benefit of coffee consumption on cardiovascular and all-cause mortality (Lopez-Garcia et al., 2008). The CARDIA study (coronary artery risk development in young adults) followed up your adults after 20 years and found no significant association between coffee and caffeine intake and coronary or carotid atherosclerosis (Reis et al., 2010).

Probably the most striking association between coffee intake and health is the observation that individuals with the highest level of coffee intake have approx. one-third the risk of developing type 2 diabetes compared with those at the lowest level of consumption (van Dam and Hu, 2005). These observations have recently been supported by a meta-analysis of 18 studies with information on over 450,000 participants which found that for every additional cup of coffee consumed in a day was associated with a 7% reduction in relative risk of developing type 2 diabetes (Huxley et al., 2009). Similar associations were seen for decaffeinated coffee which suggests that caffeine is not likely to be the active component of coffee giving this effect. These putative protective effects of coffee need to be confirmed in randomised intervention trials.

The mechanism of the potential effect of coffee on type 2 diabetes remains to be established. Chlorogenic acid (1 g) reduced early glucose and insulin responses during a 2 hour oral glucose tolerance test but had no significant effect on area under the curve during the test compared to placebo (van Dijk et al., 2009). There is some suggestion that chlorogenic acids

can inhibit hepatic glucose-6-phosphatase activity (Henry-Vitrac et al., 2010) however a trial which involved consuming 4 and the 8 cups of coffee for consecutive 4 week periods showed no significant effect on glucose metabolism or response to an oral glucose tolerance test (Kempf et al., 2010). There is some evidence that coffee constituents can modulate Nrf2 nuclear translocation and antioxidant response element (ARE) – dependent gene expression which may alter cellular antioxidant defence (Boettler et al., 2010). However, it remains to be establishedin carefully conducted human intervention trials if coffee polyphenols can provide protection against oxidative or inflammatory stress in subjects at risk of developing type 2 diabetes.

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Studies on Coffee/Caffeine in Alzheimer's Disease and Other Neurodegenerative Disorders

A.D. MENDONÇA[1] AND R.A. CUNHA[2]

[1]Institute of Molecular Medicine and Faculty of Medicine, University of Lisbon, Portugal. E-mail: mendonca@fm.ul.pt
[2]Center for Neuroscience and Cell Biology of Coimbra and Faculty of Medicine, University of Coimbra, Portugal. E.mail: cunharod@gmail.com

SUMMARY

Caffeine is the most widely consumed psychoactive drug worldwide, however its potential beneficial effect for maintenance of proper brain functioning has only recently begun to be adequately appreciated. This has mainly resulted from the convergence of conclusions from epidemiological studies and from fundamental research in animal models. Epidemiological studies first revealed an inverse association between the chronic consumption of caffeine and the incidence of Parkinson's disease. This was paralleled by animal studies of Parkinson's disease showing that caffeine prevented motor deficits as well as neurodegeneration. On the other hand, epidemiological studies showed that the consumption of moderate amounts of caffeine was inversely associated with the cognitive decline associated with aging as well as the incidence of Alzheimer's disease. Again, this was paralleled by animal studies showing that chronic caffeine administration prevented memory deterioration and neurodegeneration in animal models of aging and Alzheimer's disease. Many of the aspects were reviewed in a recent issue of *Journal of Alzheimer's Disease* (http://tiny.cc/oqw3l).

Given the limitation of epidemiological studies to demonstrate causal relationships, the next step should be moving from observational studies to randomized, placebo-controlled clinical trials. For the planning of such clinical trials, caffeine as an experimental drug represents a unique opportunity and also a special challenge.

In selecting participants, the expected biological *window of opportunity* must be considered, that is to say, it should be determined *when* the administration of caffeine would be most effective in counteracting the neurodegenerative process. In this respect, caffeine could potentially interfere with the initial stages of the neurodegenerative processes involving the neurovascular unit, blood-brain barrier dysfunction, insulin regulation, and synaptotoxicity.

The proposed dosage is worth while mentioning. Evidence from some but not all epidemiological studies may suggest a beneficial effect for moderate doses of caffeine but a possible deleterious effect for larger doses. The main target of caffeine action must be determined to define the optimal dosage for therapeutic use.

Another key question to consider is the population that should be targetted. People above a certain age, at which cognitive decline is more probable, would be a preferential population. It should be taken into account that the effects of caffeine might be different in men and women, as well as in individuals carrying particular polymorphisms of the adenosine A_{2A} receptor.

The issue of the primary outcomes must be considered. Either conversion to dementia/AD or cognitive decline, for instance in a specific memory test, could be used. Although conversion to dementia/AD is a very significant outcome from a clinical point of view, the use of cognitive decline as the primary outcome is a sensitive and objective method, reducing the number of participants and the time of follow-up.

Of particular concern is the fact that caffeine is widely available, so that the placebo group could not be free from the experimental drug, and the treated group would have variable amounts of caffeine added to the experimental dose. Fortunately, since caffeine has been widely consumed for many years, concerns about toxicity are minimal.

Despite these methodological difficulties, if the putative neuroprotective effects of caffeine could be tested in appropriate clinical trials, important consequences for health recommendations and prevention of neurodegenerative disorders might result.

The Antibacterial Effect of Coffee against S*treptococcus mutans* Is Enhanced By the Addition of Caffeine and Other Coffee Natural Compounds

A.P. ALMEIDA[1], C.C. NAGHETINI[1], V.R. SANTOS[2], A. FARAH[3], M.B.A. GLÓRIA[1]

[1]LBqA, Faculdade de Farmácia, Universidade Federal de Minas Gerais, MG, Brazil.
E-mail: mbeatriz@uai.com.br
[2]Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, MG, Brazil
[3]Instituto de Química, Universidade Federal do Rio de Janeiro, RJ, Brazil

SUMMARY

In the present study, the inhibition of *Streptococcus mutans* by coffee chemical compounds and by these compounds added separately to *Coffea arabica* extract was investigated by the disc diffusion method. Additionally, the turbidimetric test was used to verify the influence of caffeine concentration on the inhibition of the growth of *S. mutans* for up to 24 h. The inhibitory activities of different commercial Arabica blends, extraction solvents (water or ethanol) and brewing methods (filtered or espresso) were also compared. All tested coffee extracts showed antibacterial activity, which was affected by the type of extraction solvent, but not by the brewing method and variations in blends. Plain caffeine, trigonelline, caffeic, protocatechuic and chlorogenic acids at 2.0 mg/mL showed similar antimicrobial activity (~ 7.50 ± 0.50 mm). The turbidimetric assays indicated that caffeine at concentrations found in the beverage (0.5 mg/mL to 1.0 mg/mL) inhibited *S. mutans* temporarily (4 h). Higher concentrations were needed to obtain a stronger and longer lasting inhibition. When caffeine, caffeic acid, protocatechuic acid and trigonelline were added separately to a *Coffea arabica* extract, a similar improvement of its antibacterial effect was observed suggesting a synergistic effect.

INTRODUCTION

Streptococcus mutans is the main microorganism that causes dental caries, dental plaque, and cariogenicity (Yim et al., 2010). Some natural products can affect survival and virulence factors of *S. mutans*. Medicinal plants and foods have been recently investigated as adjunctive therapeutical tools to control caries disease (Duarte et al., 2006; Smullen et al., 2007; Antonio et al., 2010). The inhibitory effects of cranberry, propolis, coffee, wine, cocoa, oolong tea, and reconstituted dried milk on *S. mutans*, in *vitro* and in experimental animal studies, have been reported by different authors (Antonio et al., 2010; Yamanaka-Okada et al., 2008; Almeida et al., 2004; Furiga et al., 2008). Except for milk, in all cases it was suggested that polyphenols were the main antibacterial agents (Antonio et al., 2010; Almeida et al., 2006; Smullen et al., 2007). Other natural substances such as trigonelline, caffeine and α-dicarbonil compounds have also shown antibacterial activity against *S. mutans* (Antonio et al., 2010; Almeida et al., 2004; Daglia et al., 2007). However, the results involving caffeine have been controversial. Cogo et al. (2008) and Antonio et al. (2010) did not find antibacterial effect using 400 μg/mL and up to 1 mg/mL of plain caffeine, respectively, in susceptibility tests. Daglia et al. (2007) found inhibitory activity from 5 mg/mL up to 12.5 mg/mL, the latest showing bactericidal effect. Antonio et al. (2010) observed that decaffeinated extracts showed lower antibacterial activity

against *S. mutans* compared to the respective non-decaffeinated extracts. Therefore, studies clarifying the role of caffeine and other natural coffee compounds on the antibacterial activity of coffee are still needed.

The purposes of this study were to investigate the *in vitro* antibacterial activity of bioactive coffee chemical compounds against *S. mutans*, including caffeine at different concentrations; to investigate the inhibitory effect of different coffee extracts made from commercial blends against *S. mutans*; to evaluate the effect of the addition of plain natural coffee chemical compounds to coffee extracts on their antibacterial activity; to compare the use of ethanol and water as extraction solvents; and to compare the brewing methods used.

MATERIAL AND METHODS

Caffeic acid, protocatechuic acid, trigonelline (Sigma, St Louis, MO, USA), 5-caffeoylquinic acid (Aldrich, Milwaukee, WI, USA), and caffeine (Reagen, RJ, Brazil) were used. 3-caffeoylquinic and 4-caffeoylquinic acids were obtained from 5-caffeoylquinic (Farah et al., 2006). Four commercial roasted *Coffea arabica* blends were used. Aqueous extracts (filtered coffee), ethanolic extracts and Espresso coffee were prepared using 8.0 g of ground coffee and 40 mL of water. Aqueous filtered coffee extract from one coffee blend was supplemented separately with 2.0 mg/mL of trigonelline; chlorogenic acid (5-CQA); caffeine; caffeic acid; and protocatechuic acid. The *S. mutans* strain was ATCC 25175. Bacteria were grown under microaerofilia at 36.5 ± 1.0 °C/48 h on BHI agar (Dialab MG, Brazil) and kept at 4 °C.

The extracts were analyzed for chlorogenic acids, caffeic acid, caffeine and trigonelline by reverse-phase HPLC methods (Farah et al., 2006; 2005). The pH was measured by pH meter (DM20 Digimed, SP, Brazil).

The *in vitro* antibacterial activity of plain coffee chemical compounds and coffee extracts against *S. mutans* was investigated by the agar diffusion method (NCCLS, 1993). The turbidimetric method (USP, 2006) was used to determine the influence of caffeine concentration on the inhibition of *S. mutans* growth for up to 24 h contact. Every experiment was performed in triplicate.

The results were submitted to Anova and the means compared by Tukey or Bonferroni tests (4.03 GraphPad Prism) at 5% significance level.

RESULTS AND DISCUSSION

All extracts showed *in vitro* antibacterial activity against *S. mutans* (Table 1). No significant difference was found on the activity among coffee blends and brewing processes used. However, larger diameters of inhibition zones were observed for the ethanolic extracts.

The percent inhibition of *S. mutans* in the presence of different concentrations of caffeine and at different contact times are indicated in Table 2. Higher inhibitions were observed at 4.0 and 6.0 h of contact (exponential bacterial growth) when using 8.0 mg/mL of caffeine (> 80% inhibition). However, at 24 h contact, considering the same concentration of caffeine, there was a significant decrease in the inhibition, reaching percentages near the IC_{50} (inhibition of 50% of the growth). The significant decrease in the percent inhibition with contact time suggests that caffeine had a bacteriostatic effect on *S. mutans*.

Table 1. Antibacterial activity of *Streptococcus mutans* exposed to ethanolic and aqueous (filtered and espresso) extracts of different blends of Arabica coffee.

EXTRACTS	Inhibition zones (mm) / Arabica coffee blends[a]				
	A	B	C	D	Average
Ethanolic	8.33 ± 1.61	9.00 ± 1.0	9.33 ± 0.58 [a]	8.33 ± 0.58	8.75 ± 0.50 [a]
Filtered	7.33 ± 1.26	6.92 ± 0.88	7.00 ± 1.00 [b]	7.00 ± 1.00	7.06 ± 0.18 [b]
Espresso	7.33 ± 1.53	7.17 ± 0.76	7.00 ± 1.00 [b]	7.17 ± 0.76	7.17 ± 0.13 [b]

[a]*Means (± standard deviations) with distinct letters in the same column are significantly different (Tukey test, $p \leq 0.05$). No significant difference was observed among blends using the same extract. Diameters of inhibition zones: ethanol and water = 6.00 mm; amoxilin clavulanic acid = 23.0 mm.*

Table 2. Percent inhibition of *Streptococcus mutans* exposed to different concentrations of caffeine up to 24 h contact.

Contact	Percent inhibition of *S. mutans* growth / Caffeine concentration (mg/mL)[a]				
time (h)	8.0	4.0	2.0	1.0	0.5
4.0	81.7 ± 2.6 a,x	75.0 ± 1.0 b,x	48.7 ± 3.2 c,x	48.5 ± 2.2 c,x	13.8 ± 1.9 d,x
6.0	89.4 ± 0.5 a,x	68.5 ± 2.5 b,y	42.0 ± 3.6 c,x	41.8 ± 2.8 c,x	9.0 ± 1.8 d,y
8.0	77.3 ± 1.5 a,y	42.0 ± 2.8 b,z	18.1 ± 1.1 c,y	17.9 ± 1.0 c,y	4.3 ± 0.3 d,z
24.0	47.8 ± 2.1 a,z	44.8 ± 4.5 a,z	6.6 ± 0.6 b,z	6.6 ± 0.6 b,z	3.9 ± 0.1 b,z

[a]*Means of triplicates (± standard deviations) with distinct letters in the same line (a,b,c,d) and in the same column (x, y, z) are significantly different (Bonferroni test, $p \leq 0.05$).*

Individually tested coffee chemical compounds (2.0 mg/mL) produced similar diameters of inhibition zone against *S. mutans* (Table 3). However, when these compounds were added to the coffee extract, there was a significant increase in the antimicrobial activity, with exception of chlorogenic acid that showed a non-significant increase. When the concentration of trigonelline was increased from 0.82 to 2.82 mg/mL, there was a significant increase (14%) in the diameter of the inhibition zone. Also, the increase in the caffeine content from 2.1 to 4.1 mg/mL, produced an increase of 11.1% in the diameter of the inhibition zone.

The addition of natural coffee chemical compounds enhanced the antibacterial activity of coffee extracts. Moreover, the results confirm the antibacterial effect of Arabica roasted coffee extracts against *S. mutans*, irrespective of the blend and brewing method. This characteristic, in addition to the anti-adhesive properties on *S. mutans* reported in the literature (Landucci et al., 2003), make coffee extracts potential inhibitors of dental caries. It is anticipated that in the future, by determining the concentration of effective compounds, the antimicrobial activity of a certain type of coffee will be possibly predicted.

Table 3. Antibacterial activity of *Streptococcus mutans* exposed to 2.0 mg/mL of selected coffee chemical compounds and to *Coffea arabica* aqueous extract (blend B) supplemented with 2.0 mg/mL of the referred compounds.

Chemical compounds	Inhibition zones (mm)[a]	
	Supplemented coffee extract	Aqueous solutions
Chlorogenic acid (5-CQA)	8.25 ± 0.66 a	7.58 ± 0.38 a
Caffeic acid	8.67 ± 0.29 a	7.33 ± 0.29 b
Protocatechuic acid	8.50 ± 0.50 a	7.50 ± 0.50 b
Caffeine	8.33 ± 0.38 a	7.50 ± 0.50 b
Trigonelline	8.33 ± 0.58 a	7.33 ± 0.29 b

[a]*Means (± standard deviations) followed by distinct letters in the same line are significantly different (Tukey test, $p \leq 0.05$). No significant difference was observed among chemical compounds (same column). Diameter of inhibition zones: amoxilin and clavulanic acid = 23.0 mm.*

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Extract of Decaffeinated Green Coffee Beans Improves Postprandial Hyperglycemia in Rats and Humans

K. IWAI, T. FUKUNAGA, O. NAKAGIRI, M. HIRANO, M. IKEGUCHI

R&D center, UCC Ueshima Coffee Co.Ltd., 3-1-4, Zushi, Takatsuki-shi, Osaka, 569-0036, Japan, Research and development division, Toyo Shinyaku Co. Ltd., 7-28, Yayoigaoka, Tosu, Saga, 841-0005, Japan

SUMMARY

The objectives of this investigation were to assess the influence of extract of decaffeinated green coffee beans (EDGCB) on postprandial glucose and insulin responses in rats and humans. In SD rats, EDGCB and carbohydrate (EDGCB 500 mg/kg and sucrose, maltose or soluble starch, 2 g/kg body weight in 10 ml/kg water) were administered orally by direct stomach intubation. EDGCB significantly decreased the postprandial blood glucose at 30 min compared with the control group. It is suggested that inhibitory effect of alpha-glucosidase by chlorogenic acids is a possible mechanism of the antihyperglycemic effect of EDGCB, after confirmation by in vivo evaluation following fractionation of EDGCB. In a human study, test meals of 200 g plus one of two doses of EDGCB (100 or 300 mg/20 0ml water) or a control were administered in a double-blind trial. Plasma glucose after 30 min was significantly lower after EDGCB-containing beverages (300 mg) than the control (n = 41). No significant differences were observed in plasma insulin profiles and plasma glucose AUC over the course of the experiment. In the group of high glycemic response subjects (n = 18), plasma glucose level at 30 min later was significantly reduced after EDGCB doses (100mg and 300mg, respectively) in comparison with the placebo group. Also, plasma glucose AUC from 0 to 120 min was significantly reduced after EDGCB doses (100 mg). No significant differences were observed in plasma insulin profiles over the course of the experiment. These results suggested that EDGCB works by inhibiting amylolytic enzymes and attenuating intestinal glucose absorption.

INTRODUCTION

Recently, the number of diabetic patients has been increasing, especially in Asia, due to the prevalence of a western-type lifestyle (Zimmet et al., 2001). In Japan, the number of diabetic patients is estimated to be over 6.8 million and more than 12 million people are hyperglycemic (Wild et al., 2004; Kawamori, 2002); it is expected to increase at the fastest rate worldwide. The rising rates of diabetes in Japan may also be attributed to genetic factors. Generally, one out of three Japanese have a prevalence of at least three genes that code for proteins thought to play key roles in lipid and glucose metabolism: the beta 3- adrenergic receptor, PPAR gamma, and calpain-10 (Kawamori, 2002).

A feature of Japanese hyperglycemic people is that they are relatively lean with normal fasting glucose levels but increased postprandial hyperglycemia. It is therefore speculated that impaired insulin secretion is the primary metabolic defect underlying glucose intolerance in the Japanese population (Kawamori, 2002; Kanauchi et al., 2002). People without diabetes but with impaired insulin secretion may have serious diabetes complications in the future because they have no subjective symptoms.

Recently, there has been increasing interest in polyphenols, since some studies suggest an association between the prevention of lifestyle diseases, such as type 2 diabetes, and the administration of polyphenols (Thompson et al., 1984; Bryans et al., 2007); for example, hydroxycinnamic acids, one of the main group of polyphenols, are the most studied polyphenol group, are rich in foods of plant origin, and are frequently found in green coffee beans. Green coffee beans contain considerable amounts of hydroxycinnamic acid derivatives (chlorogenic acids), including 4.3-7.2% caffeoylquinic acids, 0.3-1.2% feruloylquinic acids, and 0.8-2.5% dicaffeoylquinic acids (Clifford, 1985). A previous study demonstrated that chlorogenic acids have potent inhibitory activities against pancreatic alpha-glycosidase activity *in vitro* (Rohn et al., 2002; Ishikawa et al., 2007). Moreover, Welsch et al. reported that 1mM chlorogenic acid reduced glucose uptake by around 80% in an in vitro brush border membrane system by dissipation of the Na+ gradient (Welsch et al., 1989). Only one previous study has shown that green coffee bean extract is possibly effective against weight gain and fat accumulation in mice (Shimoda et al., 2006). However, it is not clear whether chlorogenic acids suppress postprandial hyperglycemia after carbohydrate loading, such as sucrose, maltose, starch, and other even more carbohydrate-rich food. The aim of this study were to assess the influences on postprandial blood glucose responces in rats and humans.

MATERIALS AND METHODS

Sample Preparation

Coffee beans (*Coffea canephora* var. *robusta*), obtained from Vietnam in 2006, were used. First, caffeine and fat were extracted from ground coffee beans by a supercritical CO_2 extraction system, after which, decaffeinated green coffee beans (EDGCB) were extracted by 56% (v/v) aqueous ethanol. The extracts were filtered, evaporated and spray-dried. Chlorogenic acid content in EDGCB was determined by HPLC analysis (Matsui et al., 2007). The nutrient composition (carbohydrate, protein, fat, fiber, mineral and energy), caffeine and chlorogenic acids of the EDGCB are shown in Table 1.

Table 1. The nutrient compositions of EDGCB (3.0% moisture).

Nutrient compositions	g/100 g
Chlorogenic acids	40.0
Carbohydrate (sucrose, glucose)	22.3
Protein	10.4
Potassium	6.7
Trigonelline	3.2
Fiber	2.2
Fat	0.2
Others	1.2
Ener	14.3 kJ/g

In vivo studies in SD Rats

Specific pathogen-free 6-week-old male Sprague-Dawley rats were purchased from Kyudo Co. Ltd. (Kumamoto, Japan), and were acclimatized for 1 week in polycarbonate flat-bottomed cages. Each rat was fed a laboratory diet (MF; Oriental Yeast, Tokyo, Japan) and given water *ad libitum* until experimental use. After an acclimatization period of 1 week, they were randomly divided into five groups; each group consisted of six rats. Subsequently, carbohydrate tolerance tests were carried out after 16-h food deprivation. After ingestion, blood samples were collected from the tail vein and immediately subjected to blood glucose level measurement by a disposable glucose sensor (Glutest Pro; Sanwa Chemical Research, Co., Tokyo, Japan) before administration (t = 0) and at t = 30 min, 60 min, and 120 min, where t = 0min was the start of EDGCB and carbohydrate ingestion.

Effects of EDGCB on the glycemic response after carbohydrate (sucrose / maltose / soluble starch / glucose) administration

EDGCB and carbohydrate (EDGCB 500 mg/kg body weight and sucrose, maltose soluble starch and glucose, 2 g/kg body weight in 10 ml/kg water) were administrated. The control group was administered the same volume of carbohydrate solution without EDGCB and a blank group was administrated EDGCB (500 mg/kg in 10 ml/kg water) only.

***In vivo* Studies in Humans**

The study, which was conducted in accordance with the concept of the Helsinki Declarations, was approved by the Institutional Review Board (chairperson, Tetsuo Hosoya, MD) of Suda Clinic (Tokyo, Japan). Subject recruitment, blood sampling, measurements of blood pressure, pulse rate, body weight and height, and a medical examination were conducted at Samoncho Clinic (Tokyo, Japan: Main Investigator, Yoshiaki Kikuchi). Blood analyses were conducted by Mitsubishi Chemical Medience Co., Ltd., (Tokyo, Japan). The following subjects were excluded: those who did not satisfy the selection criterion and those who met the exclusion standard. Based on the results of the recruiting subjects, 45 subjects were selected for the study after clinical analysis.

Study Schedule and Method of Ingestion

This study was a randomized, double-blind, placebo-controlled, single-ingestion crossover clinical trial. The design and purpose of the study were explained to the subjects both orally and in writing. The controller, who did not participate directly in the examination, divided the subjects into three random groups. The tests were conducted 1 week apart, in the same time zone, and with the same diet. The subjects were instructed to consume their normal quantities of food and drink, and to take normal exercise, and to abstain from excess eating or drinking or exercise during the study period. All subjects were instructed to finish dinner by 21:00 on the day before the test, and were instructed not to eat, except to drink water, from 21:00 until the test. On the test day, the subjects did not smoke from 1 hour before the test. The subjects ingested two pieces (total weight: 200 g) of Onigiri (a snack of rice formed into a triangle and wrapped in edible seaweed) along with the test beverage (100mg or 300mg EDGCB/200ml water or 200ml water as a control) in 10 minutes. The Onigiri as loading diets were filled with salted salmon and salted edible kelp. The nutrient totals for the loading diets were as follows: (salmon) energy, 774 kJ; carbohydrate, 38.9g; protein, 5.2 g; fat, 1.0 g; (edible kelp) energy, 757 kJ; carbohydrate, 40.6 g; protein, 3.8 g; fat, 0.4 g. The test beverages and the placebo as

the control were packaged by the controller so that the contents could not be identified by the staff and subjects.

Medical Examination

After ingestion, antecubital venous blood was collected to measure plasma glucose and serum insulin concentrations before the meal and at t = 30, 60, 90 and 120 min, where t = 0 was the start of test meal ingestion. All subjects were interviewed by the doctor about their health and subjective symptoms just before the test and 120 min after the test had ended. Samples collected for glucose and insulin analysis were centrifuged immediately and the plasma was separated, aliquoted and frozen at -20 °C until analysis. Plasma glucose was measured using the glucokinase method. Insulin was determined from serum samples using chemiluminescent immunoassay. The areas under the blood concentration-time curve (AUC) for blood glucose and insulin were calculated according to the trapezoidal rule. The technicians who analyzed the plasma samples were blinded to the identity and treatment regimen of subjects, and the outcome was determined before statistical analysis.

Statistical Analyses

Changes in blood glucose and glucose AUC re presented as the mean ± SD. All statistical analyses were performed with SPSS 16.0J for Windows (SPSS Inc., Chicago, IL). Differences in mean values were statistically evaluated by Paired t-test. Differences among groups at various times were analyzed by a repeated ANOVA followed by the Dunnett's multiple comparison. P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Effects of EDGCB on the glycemic response after carbohydrate (sucrose / maltose / soluble starch/ glucose) administration

Figure 1 shows the changes of blood glucose levels in rats after oral administration of carbohydrate (sucrose or maltose or soluble starch or glucose) at 2g/kg with or without EDGCB. When 500 mg/kg EDGCB was orally administrated simultaneously with sucrose, maltose, soluble starch or glucose, the blood glucose level at 30 min (5.93 ± 0.67 mmol/L, 7.17 ± 0.87 mmol/L, 6.92 ± 0.80 mmol/L and 8.04 ± 0.98 mmol/L respectively) compared with the control group at 30 min (8.11±1.01mmol/L, 8.42±0.85mmol/L, 8.24 ± 0.75 mmol/L and 9.60 ± 1.07 mmol/L respectively) after administration were significantly suppressed ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). EDGCB showed no effect on the glucose tolerance curve when only EDGCB was administrated (Figure. 1D). No significant differences were observed in plasma glucose AUC from 0 to 120 min over the course of these experiments.

***In vivo* studies in Humans.**

Forty-five subjects were selected and randomly assigned. The doctor confirmed that no subjects were in ill health during the test and none of the randomly assigned subjects dropped out of the trial; however, data from one subject were not included in the efficacy evaluable population, because of difficulty with sampling during the test. Moreover, in statistical analysis, subjects in whom blood glucose increases were under 20 mg/dL (1.11 mmol/L; two men and one woman) were excluded. Of the efficacy evaluable population (41 subjects), 22 subjects were men and 19 were women. The subjects had a mean age of 34.8 ± 8.0 years old,

60.4 ± 8.9 kg weight, with a BMI 22.0 ± 3.1 kg/m^2, 24.5 ± 8.3% body fat, fasting glucose 4.88 ± 0.36 mmol/L, 110.5 ± 11.6mmHg systolic blood pressure, and 58.8 ± 9.0 mmHg diastolic blood pressure.

Changes in postprandial glucose and glucose AUC after ingestion in this group are shown in Table 2. In the efficacy evaluable subjects (41 subjects), plasma glucose after 30 min was significantly (P <0.05) lower after EDGCB-containing beverages (7.24 ± 0.86 mmol/L, 300 mg) compared with the control (7.56 ± 1.13 mmol/L). No significant differences were observed in plasma insulin profiles and plasma glucose AUC from 0 to 120 min over the course of the experiment.

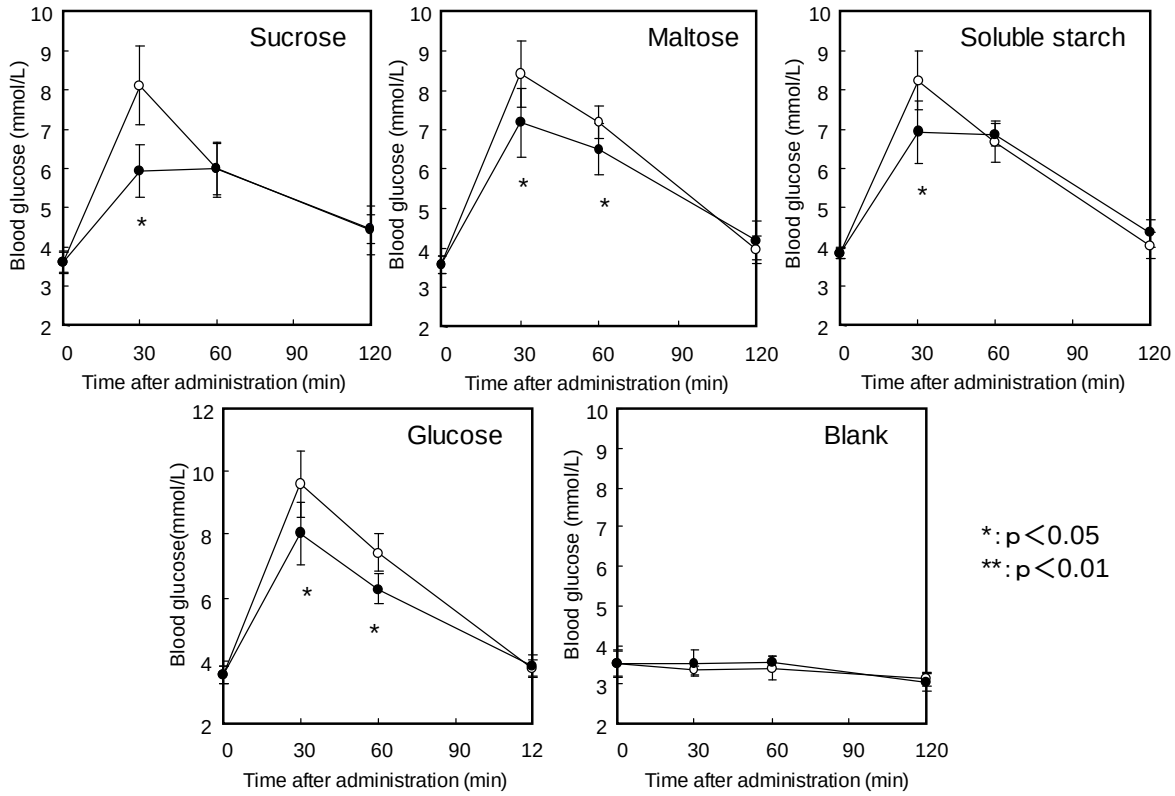

Figure 1. Effects of EDGCB on the glycemic response after carbohydrate (sucrose / maltose / soluble starch/ glucose) administration in SD rats (n=6). *P < 0.05, **P < 0.01 versus controls. (paired t-test). SD rats were food deprived for 16 h and then administered sucrose or soluble starch or maltose (2g/kg) without (open circles) or with 500mg/kg (closed circles) of EDGCB. A blank group was administrated without (open circles) or with EDGCB (500mg/kg in 10ml/kg of water) (closed circles) only. At each time point to 120 min, blood samples were collected from the tail vein, being immediately subjected to a blood glucose levels measurement by disposable glucose sensor.

Table 2. Changes in postprandial glucose, insulin responses and glucose AUC after ingestion in all subjects (n=41).

Treatment	Parameter	Time					Glucose AUC 0-120 min (mmol*min/L)
		0	30	60	90	120	
Control	Glucose (mmol/l)	4.89±0.36	7.56±1.13	6.94±1.68	6.07±1.33	5.93±1.05	779.20±126.08
	Insulin (mU/ml)	3.7±1.6	37.3±19.4	30.5±16.5	22.6±12.6	19.3±10.2	
100 mg EdGCB	Glucose (mmol/l)	4.91±0.38	7.33±1.07	6.65±1.34	5.97±1.13	5.85±0.85	760.16±105.69
	Insulin (mU/ml)	3.9±1.6	36.7±18.6	31.8±19.4	23.0±12.1	22.0±11.1	
100 mg EdGCB	Glucose (mmol/l)	4.88±0.32	7.24±0.86*	6.67±1.47	5.96±1.32	5.92±0.83	758.07±108.01
	Insulin (mU/ml)	3.9±2.0	37.6±22.8	28.7±15.5	22.1±10.4	19.5±10.3	

*Data are means±SEM. Significant difference a repeated ANOVA followed by the Dunnett's multiple comparison. *p<0.05, **p<0.01 vs. control.*

Table 3. Changes in postprandial glucose, insulin responses and glucose AUC after ingestion in high glycemic response group (n=18).

Treatment	Parameter	Time					Glucose AUC 0-120 min (mmol*min/L)
		0	30	60	90	120	
Control	Glucose (mmol/l)	5.13±0.27	8.60±0.85	7.98±1.69	6.94±1.24	6.50±1.11	879.68±105.49
	Insulin (mU/ml)	3.9±1.6	40.5±22.2	33.7±13.5	27.7±13.8	22.6±10.4	
100 mg EdGCB	Glucose (mmol/l)	5.14±0.30	7.98±1.07**	7.27±1.27	6.45±1.07	6.20±0.82	821.26±93.60*
	Insulin (mU/ml)	4.0±1.6	39.3±23.5	33.3±14.6	26.3±14.0	24.4±11.0	
100 mg EdGCB	Glucose (mmol/l)	5.01±0.32	7.91±0.77**	7.64±1.52	6.72±1.39	6.27±0.91	837.22±97.74
	Insulin (mU/ml)	3.6±1.5	40.9±27.9	33.4±15.2	26.6±11.0	21.7±11.7	

*Data are means±SEM. Significant difference a repeated ANOVA followed by the Dunnett's multiple comparison. *p<0.05, **p<0.01 vs. control.*

It is generally believed that the peak postprandial glucose level varies according to the individual. We then carried out statistical analysis using the group in which the postprandial glucose level with the loading diet (placebo food) was higher after 30 minutes than the mean value in all subjects. This high glycemic response group (n=18, 10 men and 8 women) averaged 35.1 ± 8.5 years old, 58.9 ± 9.2 kg, with BMI 21.4 ± 3.0 kg/m2, 23.6 ± 8.1% body fat, fasting glucose 4.89 ± 0.36 mmol/L, 111.7 ± 11.5 mmHg systolic blood pressure, and 57.6 ± 8.4 mmHg diastolic blood pressure.

In the high glycemic response group as shown in Table 3., plasma glucose after 30min was significantly ($P < 0.01$) lower after both EDGCB-containing beverages (7.98 ± 1.07 mmol/L, 100mg and 7.91 ± 0.77 mmol/L, 300 mg) compared with the control (8.60 ± 0.85 mmol/L). EDGCB reduced plasma glucose AUC from 0 to 120 min (100mg and 300mg). In the high glycemic response group, the glucose AUC from 0 to 120 min was significantly ($P < 0.05$) reduced after EDGCB doses (100 mg) in comparison with the control group. No significant differences were observed in plasma insulin profiles over the course of the experiment.

It is well-known that dietary carbohydrate is not directly absorbed from the intestine unless it has been subjected to the action of saliva and pancreatic alpha-glycosidase. The inhibition of alpha-glycosidase activity would therefore be an effective approach to control hyperglycemia (Matsui et al., 2001). Acarbose, Voglibose and Migritol, specific pancreatic alpha-glycosidase inhibitors, are clinically used to prevent hyperglycemia (Van de Laar et al., 2005).

Recently, there has been increasing interest in natural and neutraceutical products for diabetes control, Yeh et al. (2003) reviewed the published literature from 1960 to March 2002 on the efficacy and safety of 36 herbal therapies and 9 vitamin/mineral supplements for glucose control in patients with diabetes. The efficacy of natural and neutraceutical products for diabetes control has also been evaluated (Fujita et al., 2001; Hosoda et al., 2003; Yamada et al., 2005; Maki et al., 2007).

As shown in Table 1, EDGCB contains a high concentration of chlorogenic acids, over 40%. Rohn et al. (2002) reported that the reaction of porcine pancreatic alpha-amylase with chlorogenic acid and soluble starch from potatoes affected enzyme activity, which was significantly reduced *in vitro.* In our study, EDGCB suppressed the rise in blood glucose levels after the administration of sucrose, maltose, soluble starch and glucose.

Our findings suggest that the mechanism by which EDGCB suppressed the postprandial blood glucose level involves both suppression of intestinal blood glucose transport and the inhibition of alpha-amylase.

We then examined if the same postprandial hyperglycemia-suppressive effect of EDGCB could be observed in humans. Rats and humans do not always respond similarly; however, the results of the present human trial demonstrated that the ingestion of either 100 mg (40 mg as chlorogenic acids) or 300 mg (120 mg as chlorogenic acids) EDGCB and a carbohydrate-rich meal significantly blunted postprandial glucose. This is a new finding that EDGCB is effective in lowering plasma glucose levels in a group in which the peak postprandial glucose level is higher than the mean value in all subjects.

In addition, hydroxycinnamic acid derivatives in EDGCB interact with the Na+-dependent glucose transporter as antagonist-like molecules, possibly playing a role in controlling dietary glucose uptake in the intestinal tract (Ishikawa et al., 2007; Welsch et al., 1989). The

difference in plasma insulin profiles did not result in any difference in the present study. We therefore suggest that the main action mechanism of the glucose-lowering effect of EDGCB was not promoted by insulin secretion.

No evidence of a dose response was identified in the current study, but this finding speculates that the acute effects may be maximal at the 100 mg dose, indicating that low doses of EDGCB may be effective for preventing type 2 diabetes. Rodriguez de Sotillo and Hadley (2002) reported that an infusion of chlorogenic acid improved glucose tolerance, and decreased plasma and liver lipids in Zucker (fa/fa) rats. Further investigations are needed to evaluate the longer-term effects of EDGCB on glucose homeostasis following oral administrations in humans.

In conclusion, the results show that EDGCB possibly works by inhibiting amylolytic enzymes and attenuating intestinal glucose absorption. These results provide us with significant hints for the development of health-conscious food, soft drinks and supplements.

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The Human Urinary Excretion of Chlorogenic Acids and Metabolites after Coffee Consumption May Be Affected By the Simultaneous Consumption of Milk

G.S. DUARTE, A. FARAH[1,2]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil
[2]Instituto de Nutrição, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil. E-mail: afarah@iq.ufrj.br

SUMMARY

Coffee consumption has been associated with health benefits attributed to the antioxidant activity as well as other biological activities of chlorogenic acids (CGA), the major bioactive compounds present in coffee brew. It is known that these compounds are bioavailable in humans, but *in vitro* studies have shown that milk proteins may bind CGA and decrease their absorption. Considering that adding instant or brewed coffee to milk is a common practice in Brazil, the second major consumer country in the world, the aim of the present study was to evaluate the effect of the simultaneous consumption of coffee and milk on the urinary excretion of CGA from coffee and metabolites in humans. This was a cross-over study in which each volunteer (n=5) participated in three different treatments, consisting of consumption of water, instant coffee dissolved in water, and instant coffee dissolved in whole milk, in different occasions. CGA compounds were analyzed by HPLC-DAD-MS. On average, the total urinary CGA and metabolites recovery after coffee and coffee-milk consumption was higher than after water consumption. However, the total urinary excretion after coffee-milk consumption was consistently lower in all volunteers compared to coffee, with an average decrease of 33 ± 15% ($p=0.009$). After coffee consumption, 68 ± 12% of total CGA ingested was recovered in urine, in cinnamic and quinic acids moieties derivatives, while after coffee-milk consumption only 40 ± 8% was recovered. In conclusion, the practice of simultaneous consumption of instant coffee and whole milk may produce a negative impact on the bioavailability of coffee CGA in humans.

INTRODUCTION

Chlorogenic acids (CGA) have been studied for more than a century as the main components of the phenolic fraction of green coffee seeds (Clifford, 1979). They include different classes of compounds formed by the esterification of one molecule of (-)-quinic acid and one to three molecules of *trans*-hydroxy-cinnamic acids (Clifford, 2000). CGA are known to be important determinants of coffee flavor, contributing to the acidity, astringency and bitterness of the beverage (Clifford, 2000; Trugo and Macrae, 1984a; Clifford and Wight, 1976; Farah et al., 2006b). Additionally, a series of health benefits have been associated with CGA consumption in the last few years, related to their antioxidant and anti-inflammatory activities, among others (Farah, 2009) Coffee is an excellent dietary source of CGA (35-400 mg/100 mL cup) (Farah, 2009). The bioavailability of CGA has been reported in humans after green and roasted coffee consumption (Monteiro et al., 2007; Farah et al., 2008; Renoulf et al., 2010). However, it has been previously reported in previous *in vitro* digestion studies that milk proteins may bind CGA and decrease their bioavailability. This has also been observed for

other phenolic compounds from different food products like tea and cocoa (Farah et al., 2006; Mullen et al., 2009). In many countries the habit of adding coffee to milk is frequent. In Brazil, the second coffee consumer country in the world, very often brewed or instant coffee is added to milk for breakfast and other meals. Therefore the objective of this study was to investigate the effect of the simultaneous ingestion of coffee and milk to CGA bioavailability.

MATERIALS AND METHODS

The present study´s protocol was approved by the Ethical Committee of the UFRJ Clementino Fraga Filho Hospital.

On separate days, after a 48h free phenolic diet and at 10h fasting, subjects (n=5) ingested the same volume (200 mL) of either water, instant coffee diluted in water or instant coffee diluted in whole milk. The same diet was repeated prior to each test day and during urine collection.

Urine samples were collected at baseline (-4-0 h) and at intervals 0-4 h, 4-8 h, 8-12 h and 12-24 h after each beverage consumption. CGA compounds (caffeoylquinic, feruloylquinic and dicaffeoylquinic acids) and metabolites (caffeic, vanillic, ferulic, isoferulic, p-coumaric, gallic, 4-hydroxybenzoic, dihydrocaffeic, syringic, sinapic, 2,4-dihidroxybenzoic, hippuric, 3,4-dihidroxyphenilacetic, 3-(4-hydroxyphenil) propionic, trans- 3-hydroxycinnamic and benzoic acids) were determined in coffee brew and urine by HPLC and LC-MS as previously reported (Farah et al., 2008; Farah, 2009).

Urinary recovery calculations were made considering the total number of equivalent moieties of cinnamic and quinic acids in the test coffee beverage and the total number of phenolic and quinic acid moieties recovered in the 24h urine collected after each treatment, as a percentage (Monteiro et al., 2007).

The software GraphPadPrism® (version 5.1) was used for statistical analyses. The comparison between treatments was performed using the ANOVA factorial and one-way ANOVA with post-test of Fisher. Differences were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Coffee Brew

Nine major CGA compounds were quantified in the 4g portion of instant coffee offered to subjects in both coffee treatments (Table 1). Caffeoylquinic acids represented most of CGA composition (~84%), followed by feruloyquinic acids (~12%) and dicaffeoylquinic acids (~4%), which is in accordance with contents reported in the literature for coffee brews (Farah, 2009). The mean total amount of CGA in the instant coffee portion was 561µmol, which corresponds to 1.14 mmol in equivalent moieties of cinnamic and quinic acids.

Urinary excretion of CGA compounds and metabolites after water, coffee and coffee-milk consumption

Six CGA (3-caffeoylquinic acid, 4- caffeoylquinic acid, 5- caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid e 4,5-dicaffeoylquinic acid) and 16 phenolic compounds (caffeic, vanillic, ferulic, isoferulic, p-coumaric, gallic, 4-hydroxybenzoic, dihydrocaffeic, syringic, sinapic, 2,4-dihidroxybenzoic, hippuric, 3,4-dihidroxyphenilacetic,

3-[4-hydroxyphenil] propionic, trans- 3-hydroxycinnamic and benzoic acids) were identified in the baseline urine and in all intervals up to 24h after the consumption of all treatments.

Table 1. Contents of the main CGA compounds in the instant coffee portion (4g) offered to the subjects (n=5) in both coffee treatments[1].

Compound	Contents (µmol/ portion)
3-caffeoylquinic acid	151.2 ± 7.9
4-caffeoylquinic acid	149.2 ± 6.9
5-caffeoylquinic acid	172.5 ± 7.9
Total CQA	**472.9 ± 13.8**
3,4-dicaffeoylquinic acid	9.3 ± 0.4
3,5-dicaffeoylquinic acid	6.3 ± 0.3
4,5-dicaffeoylquinic acid	5.7 ± 0.2
Total diCQA	**21.2 ± 0.1**
3-feruloylquinic acid	28.0 ± 1.6
4- feruloylquinic acid	21.2 ± 0.9
5- feruloylquinic acid	17.9 ± 1.2
Total FQA	**67.1 ± 1.6**
Total CGA	**561.0 ± 12.3**

[1]*Results are mean ± SD, n=3.*

Regarding baseline and water treatment results, Nurmi et al. (2006) have previously identified 10 phenolic compounds in the baseline urine samples of 12 subjects. In our study, although subjects were advised not to consume food sources of phenolic compounds during the 48h prior to each experiment and throughout the 24 h of the experiment days, the presence of phenolic compounds in baseline and in all urine intervals of water treatment is not surprising, since CGA has been identified in human digestive fluids after 12 h fasting, which reinforces the hypotheses of long half-life through enterohepatic recycling (Monteiro et al., 2007) and/or storage and slow release of CGA in the human body, earlier proposed by Booth et al. (1957).

A large inter and intra-individual variability was observed on the content of CGA and metabolites excreted in all treatments, probably due to differences on the subjects capacity to absorb and or metabolize these compounds. However, despite this variability, the qualitative pattern of excretion was similar for all subjects in each coffee treatment.

As shown in Figure 1, Hippuric acid (N-benzoyl-glycine) was the major compound excreted in the urine samples of subjects after ingestion of water, coffee and coffee-milk, representing respectively 87, 8 and 79.2% of total phenolic compounds identified after 24 hours of ingestion of coffee and coffee-milk beverages, respectively. The predominance of hippuric acid excretion after the intake of dietary polyphenols in humans had previously been reported in the literature (Dupas et al., 2006b; Olthoff et al., 2003; Clifford, 2000). 3,4-

dihydroxyphenylacetic acid was the second major compound excreted in the urine of subjects 24 hours after consumption of coffee (0.5 ± 0.3 mmol) and coffee-milk (0.2 ± 0.1 mmol) beverages. As hippuric acid, it has been suggested that 3,4-dihydroxyphenylacetic acid may derive from the action of intestinal bacteria on the molecule of caffeic acid (Renoulf et al., 2010), for the first time, this compound was identified and quantified in the urine of humans after ingestion of brewed coffees. High levels of dihydrocaffeic and vanillic acids were also present in the urine of subjects after consumption of both coffee beverages. The majority excretion of the dihydrocaffeic and vanillic acids after coffee brew consumption has also been reported in the literature (Rechner et al., 2004; Rechner et al., 2001; Monteiro et al., 2007).

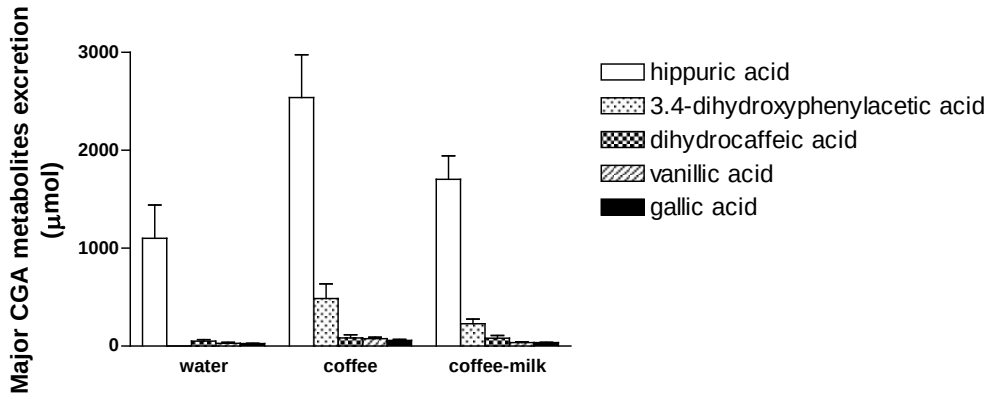

Figure 1. Urinary excretion of major CGA metabolites after water, coffee and coffee-milk consumption.

The total urinary excretion of phenolic compounds in the water treatment was 1.3 ± 0.8 mmol. Higher contents of CGA and metabolites were excreted in all subjects after coffee (3.3 ± 1.4 mmol) and coffee-milk (2.2 ± 0.6 mmol) consumption. The total content of phenolic compounds excreted after coffee-milk consumption was, on average, approximately 1.5 times (33%) lower ($p = 0.009$) than the total excreted after plain coffee consumption. This lower excretion was consistent in all subjects. As the subjects ingested the same levels of CGA in both treatments and repeated their diets in all three treatments, this result indicates that the simultaneous ingestion of whole milk and coffee may decrease the absorption and/or metabolism of these compounds in the human body.

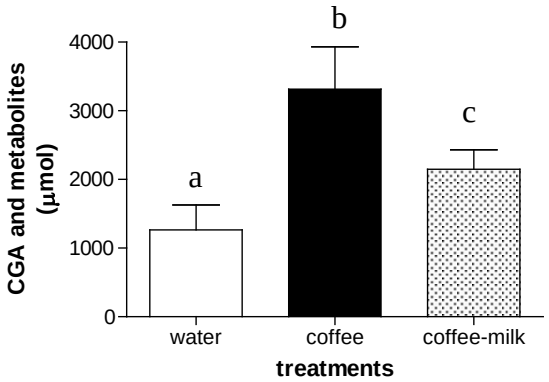

Figure 2. Total urinary excretion of CGA and metabolites after water, coffee and coffee-milk consumption in humans (n=5).

Concerning the urinary recovery of the consumed phenolic and quinic acid moieties though the coffee beverages, subjects excreted from 420 to 1310 μmol of these compounds during the 24h after the consumption of plain coffee, with average of 776 mmol, which represents 68% of consumed CGA. On the other hand, after coffee-milk consumption only 325 to 640 μmol of phenolic compounds were excreted, with average of 446 mmol, which represents 40% of ingested CGA, a reduction of 28% comparing to plain coffee.

Considering that this is the first study to quantify CGA and metabolites in human urine both in baseline and during 24h after the consumption of water, coffee and milk added of instant coffee, it is difficult to make comparisons, but our results are in accordance with findings reported by Mullen et al (2009), who investigating the impact of milk addition on the absorption and metabolism of flavonoids from a chocolate drink in humans, reported that milk addition to cocoa significantly decreased the excretion of urinary metabolites of flavonoids comparing to cocoa and water. According to these authors, this result seems to be a direct consequence of interactions between milk constituents with flavonoids present in chocolate, which probably would have caused changes in the mechanism of transport through the intestinal wall of these compounds into the bloodstream. This finding also corroborates the results shown by Dupas et al. (2006), in which about 17% of 5-CQA initially introduced in an in vitro system remained complexed to milk proteins or peptides until the end of digestion, modifying, hypothetically, its absorption.

CONCLUSION

The present study confirms that CGA are absorbed and extensively metabolized in the human body, that they circulate in the body for at least 72h after their consumption and, finally, the present study demonstrates that mixing milk and coffee in the same beverage may decrease the bioavailability of CGA. This effect, however, most probably will depend on the proportion milk to coffee used. Additionally, it is noteworthy to comment that the amount of CGA provided by coffee is considerably high and that even considering the decrease in bioavailability caused by milk components, the absorbed amount should be still high comparing to other food sources of these compounds.

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In Vitro Antibacterial and Antidemineralizant Activity of *Coffea canephora* Extract

A.G. ANTONIO[1], N.L.P. IORIO [2], A. FARAH[3], K.R.N. DOS SANTOS[2], L.C. MAIA[1]

[1]Dept. Ortodontia e Odontopediatria, Faculdade de Odontologia, Universidade Federal do Rio de Janeiro (UFRJ), RJ, Brazil
[2]Dept. Microbiologia Médica, Instituto de Microbiologia Prof. Paulo de Góes, UFRJ, RJ, Brazil
[3]Instituto de Química and Instituto de Nutrição Josué de Castro, UFRJ, RJ, Brazil

SUMMARY

The antibacterial activity of a *Coffea canephora* extract was evaluated *in vitro* against *Streptococcus mutans* and *Streptococcus sobrinus*, the main causative agents of caries disease. The viability of planktonic cells was analyzed by susceptibility tests (MIC and MBC) and time-kill assay that were monitored for 3h, against different concentrations of the extract. The demineralization inhibition effect of the extract was also investigated after it had been applied on the biofilm of deciduous teeth. MICs and MBC values, 0.7 ± 0.2 (mg/mL) and 16.0 ± 0 (mg/mL), respectively, showed that the *C. canephora* extract at 20% was active against *S. mutans*, although it had no significant effect on *S. sobrinus*. The extract produced a 4-log reduction in the number of colonies of *S. mutans* after 3 h treatment ($p < 0.05$). The difference among both controls and coffee plaque pH (~ 4.8) was not statistically significant after the 7th day of treatment. The demineralization in the fragments subjected to the coffee extract was lower than in the negative and blank controls ($p < 0.05$). Our data suggest that *C. canephora* extract at 20% is not only beneficial as an anticariogenic agent but also as an antidemineralization agent.

INTRODUCTION

Coffee is not only the most popular and widely consumed beverage throughout the world, but it is rich in polyphenols as well (Farah et al., 2006). Studies have shown the possibility of dental caries prevention by coffee's antibacterial activity against *Streptococcus mutans* (Daglia et al., 2007; Antonio et al., 2010), which is a member of the endogenous oral microflora and also a contributor to biofilm formation in the oral cavity (Bradshaw et al., 2002).

According to the literature, there is no evidence that *C. canephora* extracts exert the same activity against cariogenic bacteria other than *Streptococcus mutans*. Another important aspect that has not been investigated is the effect of coffee on the deremineralization process of caries disease. Therefore, in the current study we first evaluated, *in vitro*, the antibacterial effect of a *C. canephora* extract against *S. mutans* and *S. sobrinus*. Following, we investigated the demineralization effect of the extract on deciduous teeth after *ex vivo* treatment of mixed biofilm.

MATERIALS AND METHODS

Coffea canephora extract

Regular *Coffea canephora* cv. Conillon beans were roasted in a fluidized bed roaster to produce a light roasting degree (Antonio et al., 2010). An 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.

Characterization of phenolic compounds and caffeine from *Coffea canephora* extract

The contents of caffeic and chlorogenic acids were determined by gradient LC-DAD-ESI-MS according to Farah et al. (2006). The contents of caffeine were determined by LC-ESI-MS according to Perrone et al. (2008).

Bacterial strains and culture to evaluate the Minimum Inhibitory Concentration (MIC)

An inoculum with 4-5 x 10^6 CFU/mL of both bacteria: *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33478 were prepared according to Antonio et al. (2010).

MIC and Minimum Bactericide Concentration (MBC) determination

MIC was evaluated by the dilution method in Mueller-Hinton broth medium according to Antonio et al. (2010), with concentrations ranging from 5 to 160 mg/mL of the 20% aqueous coffee extracts. Samples from tubes where the MIC results showed no bacterial growth were inoculated onto a blood agar plate, and incubated for 48 h aiming at identifying the MBC.

Killing kinetics assays

This experiment was described by Alviano et al. (2008). Coffee extracts at 1%, 16% and 20% were tested against *S. mutans* ATCC 25175. Chlorexidine at 0.05% (positive control) and Milli-Q water (negative control) were also tested .After the inoculum addition to the tubes containing the substances, 100 µL aliquots of each system were collected at 30 min intervals up to 3 hours, to enumerate viable cells by serial dilution (10^{-3} to 10^{-8}) and spreading of 100 µL of each dilution in blood agar plates. The blood agar plates were incubated in microaerofilia for 48h to determine the CFU/ml.

Tooth selection and sample preparation for biofilm plate assay

Twelve exfoliated primary first molar teeth having no structural alterations were sectioned mesiodistally, resulting in 24 fragments. Each fragment was coated with an acid-resistant varnish leaving a window (21 mm^2) of exposed tooth. All fragments were submitted to ethylene oxide sterilization prior to the experiment.

Inoculum to form biofilm on tooth fragments

The inoculum comprised unstimulated whole mixed saliva, collected from three volunteers aged 25–36 years. The saliva produced in the first 30 seconds was discarded and then, it was collected for exactly 5 minutes. The saliva (1 mL) from each volunteer was placed into a tube, which was mixed using a vortex, resulting in an inoculum with 2 x 10^8 CFU/mL (dilution of 1:200).

Biofilm plate assay

The biofilm model was conducted in polystyrene 24-well tissue-culture plates. The tooth fragments were fixed inside the wells, which were completed with BHI media (1485 µL/well) already containing the inoculum (15 µL/well). The system was incubated in microaerofilia for 10 days so as to produce biofilm. Three different types of treatment on the biofilm were performed (6 teeth for each treatment): (1) 50 µL of *C. canephora* extract at 20%; (2) 50 µL of Milli-Q purified water (negative control); and (3) 50 µL of chlorhexidine at 0.12% (positive control). Six biofilm-covered teeth, which did not receive any treatment, were considered the blank control. The treatment procedure was performed once a day, at the same hour, during a week.

Plaque pH measurements

The plaque pH measurements were performed with a touch microelectrode. A group of three teeth from the six treated with each substance and also from the six blank controls had their biofilm evaluated. The plaque pH was measured on the 7^{th} day of treatment at time intervals of 0 and 5 minutes after substances' application.

Cross-sectional microhardness test of teeth fragments

At the end of the experimental phase, the tooth fragments were prepared to the cross-sectional microhardness (CSMH) test, which was performed according to Hara et al. (2003). The hardness of the untreated enamel that was covered with the nail varnish was used as control.

Mineral contents of the *C. canephora* extract and the BHI media

An aliquot of 3 mL from *C. canephora* extract at 20% and from BHI growth media was sampled and centrifuged. The supernatant was treated with 250µL of 65% of HNO_3. The contents of Zn, Sr, Si, S, P, Na, Mn, Mg, K, Fe, Cu, C, B, Bo and Al in the coffee and in the BHI media were analyzed by atomic absorption spectroscopy. Fluoride was analyzed using an ion specific electrode.

Statistical analysis

The Kruskal-Wallis test was used for statistical comparison of Killing kinetics assays results and also for the effect of *C. canephora* extract on acidogenicity of biofilm. For analyses of CSMH values, ANOVA and Tukey test were used to detect differences among treatments. A 5% significance level was considered.

RESULTS AND DISCUSSION

The polyphenols, caffeine and minerals concentrations in the *C. canephora* aqueous extract at 20% are presented in Tables 1 and 2, respectively.

MIC and MBC values, 7 ± 2 (mg/mL) and 160 ± 0 (mg/mL), respectively, showed that the coffee extract is active against *S. mutans.* No antibacterial (MIC) and bactericidal (MBC) activities were found against *S. sobrinus* at tested concentrations.

In the current study, *C. canephora* extract did not show bacteriostatic activity against *S. sobrinus* at tested concentrations. Although it might be possible at higher concentrations, we

opted for testing concentrations that still could be regularly consumed (up to 20%). Additionally, this concentration has already shown antibacterial properties against enterobacteria (Almeida et al., 2006)

.

The MIC of coffee extract for *S. mutans* growth was 7 ± 2 mg/mL. A similar result (MIC = 5 mg/mL) has been previously observed by Antonio et al. (2010). Regarding the MBC (160 mg/mL) of this extract against *S. mutans*, it was higher than MIC in about 4 to 5 dilutions. Comparable results were obtained in a similar study investigating another natural product (Castro et al., 2009).

The different concentrations of coffee extract (1%, 16% and 20%) acted differently on *S. mutans* when compared to the controls and one to another. *C. canephora* aqueous extract at 16% and 20% ($p > 0.05$) demonstrated a 4-log reduction in the growth of *S. mutans* after 3h treatment, compared to the untreated control ($p < 0.05$).

Table 1. Contents of polyphenols and caffeine in *C. canephora* extract at 20%.

Chemical compounds	**(µg/mL)**
Total polyphenols (cinnamic acid derivatives)	3650.8 ± 74.0
3-caffeoylquini acid	808.6 ± 16.8
4-caffeoylquinic acid	863.4 ± 22.3
5-caffeoylquinic acid	1342.2 ± 52.7
3-feruloylquinic acid	136.6 ± 7.0
4- feruloylquinic acid	181.08 ± 7.4
5- feruloylquinic acid	78.7 ± 6.5
3,4-dicaffeoylquinic acid	125.30 ± 4.4
3,5-dicaffeoylquinic acid	42.75 ± 0.4
4,5-dicaffeoylquini acid	72.14 ± 1.1
Caffeic acid	87.33 ± 35.0
Caffeine	2110 ± 0.4

Results are shown as mean of triplicate extraction, espressed as mean ± SD.[a,]

Chlorexidine at 0.05% could reduce the CFU count of *S. mutans* below the detection limit (50 CFU/ml) from 30 min on ($p < 0.05$). *C. canephora* extract at 1% was not able to reduce the initial bacterial population after 3h treatment in the same proportion of the extracts with the highest concentrations ($p < 0.05$). Our data suggests that the consumption of coffee can prevent the colonization of *S. mutans*, since coffee has been widely and safely consumed as a beverage for a long period of time. Signoretto et al. (2006) observed a positive correlation between the consumption of coffee and oral health in terms of reduction of plaque deposition and lower counts of *S. mutans*.

Considering the results of the cross-sectional microhardness test, it showed that the *C. canephora* extract at 20% inhibited the demineralization of enamel at depths up to 30µm from the enamel surface. The process of enamel demineralization involves the dissolution of enamel apatite crystals, and the diffusion of ions into and out of the enamel microstructures. It

has been suggested that the ion diffusion pathway in enamel is controlled by the organic matrix network, which occupies the enamel tissue (Zhang et al., 2009). In the present study, the chemical analyses of the coffee extract showed a large amount of calcium and phosphorus in the referred extract. So, the authors supposed that the *C. canephora* extract could interact with the enamel organic matrix through its mineral contents, inhibiting the decomposition of the organic matrix during the acid attack by the microrganisms.

Considering the acidogenecity of biofilm, although we observed a small raise (from 4.81 ± 0.10 to 4.95 ± 0.05) of biofilm pH 5 min. after the last treatment with the *C. canephora* aqueous extract at 20%, it was not significant when compared to the treatment with negative control (from 4.89 ± 0.11 to 4.84 ± 0.13) and to blank control (4.83 ± 0.14 to 4.85 ± 0.16). The *C. canephora* extract studied was made from a light roasted ground coffee with weak acidic properties (pH 5.28 ± 0.8). Although it prevented the rising of dental biofilm pH, it has a large amount of phenolic compounds and caffeine that favors its antimicrobial action (Antonio et al., 2010).

In view of the presented data, a light roasted *C. canephora* aqueous extract can be considered as a potential anticariogenic substance due to its capacity of preventing the growth of *S. mutans* and of inhibiting dental demineralization.

Table 2. Mineral contents in *Coffea canephora* extract at 20% and in BHI medium.[a]

Minerals	Content in Coffea canephora extract (20%) (µg/mL)	Content in BHI medium (µg/mL)
Zinc	0.52 ± 0.03	1.57 ± 0.02
Strontium	1.11 ± 0.05	0.04 ± 0.01
Silicon	6.66 ± 0	4.05 ± 0.04
Sulfur	314.66 ± 9.6	243.73 ± 3.2
Phosphorus	491.67 ± 22.77	294.82 ± 10.54
Sodium	26.66 ± 1.67	2714.8 ± 97
Manganese	1.94 ± 0.03	0.04 ± 0.01
Magnesium	589.69 ± 18.32	14.04 ± 2.12
Potassium	10173.58 ± 182	759.35 ± 19.43
Iron	3.22 ± 0.15	0.8 ± 0.01
Copper	0.46 ± 0.02	0.2 ± 0
Calcium	216.71 ± 11.93	10.8 ± 3.2
Barium	0.14 ± 0	0.04 ± 0.01
Boron	4.23 ± 0.16	0.10 ± 0
Aluminum	0.083 ± 0.08	0.3 ± 0
Fluoride	0.018 ± 0	0.044 ± 0.003

[a] *Results are shown as mean of duplicate analyses, expressed as mean ± SD.*

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FINANCIAL SUPPORT

Faperj (Brazil).

Energy Value of Soluble Coffee for Nutrition Labeling

V. LELOUP, F. LE DERFF, I. BLANK

Nestlé Product Technology Center Orbe, 1350 Orbe, Switzerland

SUMMARY

Nutritional declaration has become mandatory in many countries for all food, including coffee. In this context, the energy value of soluble coffee was reviewed taking into account the specificity of the chemical composition of coffee products. A wide range of commercial soluble coffees were analysed to establish a statistical distribution of coffee nutrients. The energy value of soluble coffee was calculated taking into account the recent regulatory recommendations. The statistical evaluation of the nutrient distribution showed a limited variation indicating a generic composition for energy declaration. Globally, at a dosage of 2 g/100 mL, a cup of soluble coffee provides 0.0 g fat, 0.2 g proteins, 0.1 g carbohydrates, and 0.4-0.7 g fibers. Depending on the local regulatory directives, the energy values vary in the range of 1-4 kCal/cup. Thus, soluble coffee has only a marginal contribution to the energy balance of a diet. On the other hand, the coffee beverage is a major source of dietary antioxidants.

INTRODUCTION

The methodology to define the nutrition labeling is described in EU directives and relates to the energy value of food constituents, in particular protein, fat, carbohydrates, dietary fibers, and a few other minor components (Directive E 90/496/CEE, 1990). Although this methodology may be appropriate for many foodstuffs, it leads to a systematic over-evaluation of the energy value when dealing with thermally treated products such as roasted coffee and soluble coffee. In this context, the energy value of soluble coffee was reviewed taking into account the chemical composition of coffee products (Leloup, 2006)[2]. In particular, the direct quantification of amino acids, carbohydrates, and dietary fibers by chromatographic methods was proposed to avoid over/under-evaluation of coffee nutrients. It was shown that the contribution of soluble coffee to the energy balance of a diet is minute, i.e. ~1 kCal/cup brought by 0.1 g proteins and <0.2 g carbohydrates. In recent years, the situation regarding fibre definition (i.e. DP>3 or DP>10) and energy value (i.e. 0, 2 or 4 kCal/g) has also evolved depending on local legislation.

A wide range of commercial soluble coffees were analysed with the methodology specifically developed for coffee to establish a statistical distribution of coffee nutrients. An *in-vitro* digestibility test was performed on a soluble coffee to assess the enzymatic release of low molecular weight compounds that would be available for the small intestine absorption. Finally, the energy value of soluble coffee was calculated taking into account the recent regulatory recommendation.

MATERIAL & METHODS

About 100 commercial soluble coffees representative of product diversity were obtained from markets worldwide.

The composition in nutrients was determined using the methodology specifically developed for soluble coffee (Leloup, 2006). As concerns dietary fibres, DP>10 (i.e. CODEX definition) were determined using the AOAC 985.29 method, while DP>3 (i.e. European definition) were calculated from the total carbohydrate composition. The energy values were calculated as recommended by EU directives.

RESULTS & DISCUSSION

The amounts of the different energy-contributing compounds follow normal distribution for the overall range of soluble coffees (Figure 1). For each nutrient, the average and standard deviation were calculated. The standard deviations show a limited variation (Table 1).

For each nutrient, the Min/Max values corresponding to a confidence level of 99.72% (± 3 sigma) were calculated from these distributions. Min/Max values are within the tolerance range calculated from CIAA recommendations. Based on these statistical values, a unique composition for the nutritional declaration of soluble coffee is well justified.

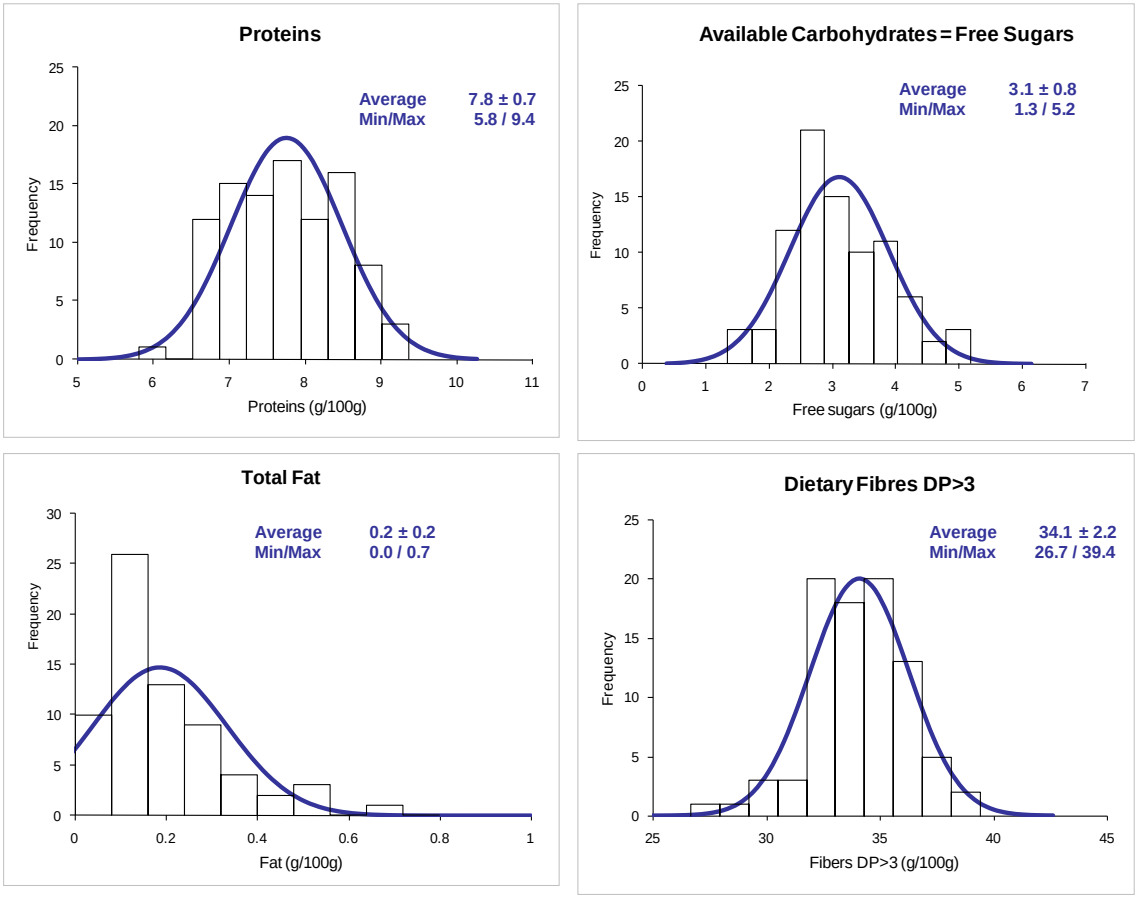

Figure 1. Distribution of some energy-contributing compounds evaluated for the whole range of soluble coffees (average as well as Min/Max values are given in g/100 g soluble coffee).

Table 1. Average and standard deviation of energy-contributing compounds in soluble coffees.

Compounds	Unit	Content per 100 g Average ±3StD[1]				Tolerance[2]		
	g	Average	StD	Min	Max		Min	Max
Protein	g	7.8	0.7	5.7	9.9	±20%	6.2	9.4
Total carbohydrates	g	3.1	0.8	0.7	5.5	±30%	2.2	4.0
of which sugars	g	3.1	0.8	0.7	5.5	±30%	2.2	4.0
Fat	g	0.2	0.2	-	-	discretion	-	-
of which saturated	g	0.1	0.1	-	-	discretion	-	-
Dietary fibers DP>10	g	34.1	2.2	27.5	40.7	±20%	27.3	40.9
Dietary fibers DP>3	g	17.5	3.5	7.0	28.0	±20%	14.0	21.0
Sodium	mg	100	-	-	-	±50%	50	150

[1]*Average ±3StD corresponds to the confidence interval of 99%.*
[2]*Tolerance as currently recommended by CIAA.*

Table 2. Consolidated nutritive values of soluble coffees depending on fiber definition and energy value (Big 8) expressed for 100 g powder and per cup (dosage 2 g/100 mL).

Compounds	Unit	Fibers DP>10		Fibers DP>3	
	g	g/100 g pwd	g/100 mL*	g/100 g pwd	g/100 mL*
(1) Energy (if fiber 0 kCal)	kCal kJ	50 210	1 4	50 211	1 4
(2) Energy (if fiber 2 kCal)	kCal kJ	85 351	2 7	118 484	2 9
(3) Energy (if fiber 4 kCal)	kCal kJ	120 508	2 10	186 791	4 15
Protein	g	7.8	0.2	7.8	0.2
Total carboydrates	g	3.1	0.1	3.1	0.1
of which sugars	g	3.1	0.1	3.1	0.1
Fat	g	0.2	0.0	0.2	0.0
of which saturated	g	0.1	0.0	0.1	0.0
Dietary fibers	g	17.5	0.4	34.1	0.7
Sodium	mg	100	2.0	100	2.0

CONCLUSION

The content in energy-contributing compounds was determined for a representative range of pure soluble coffees. The statistical evaluation of the nutrient distribution showed a limited variation supporting the proposition of a generic composition for energy declaration. Comprehensive labeling propositions are provided to take into account dietary fiber definition (i.e. DP>3 or DP>10) and energy value for dietary fiber (i.e. 0, 2 or 4 kCal/g) depending on

local regulatory requirements (Table 2). Globally, at a dosage of 2 g/100 mL, a cup of soluble coffee provides 0.0 g fat, 0.2 g proteins, 0.1 g carbohydrates, and 0.4-0.7 g fibers. Depending on legislation for labeling in the local markets, the energy values vary from 1 to 4 kCal/cup. Soluble coffee has only a marginal contribution to the energy balance of a diet. However, the coffee beverage is a major source of dietary antioxidants (Fukushima et al., 2009) and also contributes to hydration.

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Quantification of Compounds in Coffee Involved in Gastric Acid Regulation

E.DOSSIN, V. LELOUP, I. BLANK

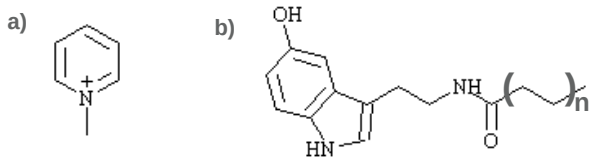
Nestlé Product Technology Center Orbe, 1350 Orbe, Switzerland

SUMMARY

Some compounds present in coffee (e.g. $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides, caffeine, pyrogallol, and catechols) have been suggested to induce stomach irritation after coffee consumption. On the other hand, *N*-methylpyridinium has recently been shown exhibiting protective effect towards gastric acid secretion. In model brews and soluble-type coffees, *N*-methylpyridinium was found in the range of 13-115 mg/mL, its concentration being the highest in dark roasted brews. The $^{\beta}$*N*-alkanoyl-5-hydroxytryptamide concentrations range between 15-218 mg/L and are particularly enriched in green coffee brews. Based on this work, one could expect lower gastric acid secretion for coffee beverages prepared from dark roast.

INTRODUCTION

Coffee is one of the most popular beverages highly appreciated for its stimulating effect. Some consumers, however, experience symptoms of gastric irritation after coffee consumption, which might be caused by an increased acid secretion in the human stomach (Boekema et al., 1999; Nilsson et al., 2004). Complex mechanisms of gastric acid regulation have been described involving many cell receptors such as histamine H2, acetylcholine M3, cholcystokinin B or somatostatin receptors. The receptor diversity could explain the difficulty in identifying putative stomach irritating compounds in coffee beverage. In particular, $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (C-5HT), caffeine, pyrogallol, and catechols were incriminated for the gastric irritation[3]. In other studies, *N*-methylpyridinium (NMP), a trigonelline derivative, was shown to induce chemopreventive activities in-vivo and in-vitro, possibly also decreasing gastric acid secretion (Rubach et al., 2010; Somoza et al., 2003). The objective of this work was to investigate the levels of NMP and C-5HT derivatives (Figure 1) with the aim of mapping these stomach irritating / protective compounds in model coffees.



C18-5HT	N-steraoyl 5-hydroxytryptamide
C20-5HT	N-arachinoyl 5-hydroxytryptamide
C21-5HT	N-heneicosanoyl 5-hydroxytriytamide
C22-5HT	N-behenoyl 5-hydroxytryptamide
C23-5HT	N-tricosanoyl 5-hydroxytryptamide
C24-5HT	N-lignoceroyl 5-hydroxytryptamide

Figure 1. Chemical structures of compounds assessed in the coffee extracts: a) N-methylpyridinium, b) $^{\beta}$N-alkanoyl-5-hydroxy-tryptamides

MATERIAL AND METHODS

Sample preparation

A Vietnamese Robusta and a Colombian Arabica were roasted at three different levels (i.e. light, medium, dark) in a Probat RT3 roaster and finely ground. The roast and ground (R&G) coffees were extracted using a Dionex extractor in a single-step (i.e. 100 °C/10 min) or two-step (i.e. 100°C/10min + 180 °C/10 min) procedure to prepare brew-type and soluble-type coffees, respectively. The extracts were freeze-dried prior to analysis.

Quantification *N*-Methylpyridinium (NMP)

The chromatographic separation was achieved on a Kinetex 2.6 µm HILIC (100x2.1 mm) (Phenomenex) column coupled to a QToF 6520 (Agilent). A binary gradient of A/B (A: 2.5mM ammonium acetate with 0.1% acetic acid 90% ACN/10% water, B: 2.5 mM ammonium acetate with 0.1% acetic acid 10% ACN/90% water) was applied as follows: 0-2 min 100/0; 2-5.5 min 55/45; 5.5-6 min 0/100; 6-7 min 0/100. The flow rate was 700 µL/min. The column temperature was kept at 30 °C. The sample concentration was approximately 10 mg/mL in water. The injection volume was 2µL.

NMP was identified by combining retention time and exact mass (<3 ppm). Quantification was performed by external calibration. The spiked reference material showed excellent recovery (102%). The mass spectrometer QTOF 6520 (Agilent) was used in positive ESI-mode and was operated in MS mode to scan the 25-1600 *m/z* mass range. Nitrogen was used as drying gas (value of 10L/min) and the nebulizer was set at 35 psig. The source was operated at 300 C with Vcap at 5000 V, the fragmentor at 120V, the skimmer at 70V and the octopole at 300 V.

Quantification $^{\beta}$*N*-alkanoyl-5-hydroxytrypamides (C-5HT)

The chromatographic separation was performed using an Ultimate 3000 (Dionex) on a Luna 3µm C8 (150x3 mm) (Phenomenex) column. The isocratic condition 01% formic acif 93% MeOH/7% water was applied for 9 min. The flow rate was 450 µL/min. The column temperature was kept at 40°C. The sample concentration was approximately 6mg/mL in 33% THF/33% MeOH/33% water. The injection volume was 5 µL.

The mass spectrometer 4000QTrap (Applied Biosystems) was used in positive ESI-mode and operated in MRM mode to follow the $^{\beta}$*N*-alkanoyl-5-hydroxy-tryptamides derivatives ($[M+H]^+ \rightarrow$*m/z* 160 and $[M+H]^+ \rightarrow$ *m/z* 177). Nitrogen was used as the curtain (value of 22) and collision gas was set to high. The source was operated at 420 °C with ionspray at 5500 V; the nebulizer gas was set at 45 psi, the declustering potential at 100 V and the collision energy at 40e V. The C-5HT derivatives were identified and quantified by combining retention time and two distinct mass transitions. Quantification was performed by external calibration.

NMP and C-5HT levels in coffee beverages

The concentration in coffee beverage was further calculated for an average cup dosage, i.e. brew-type prepared at 50 g R&G/L, and soluble-type coffee at 15 g/L.

RESULTS AND DISCUSSION

N-Methylpyridinium (NMP) and $^{\beta}$*N*-alkanoyl-5-hydroxytrypamides (C-5HT) were quantified in coffee powders by advanced LC-MS techniques. The expected concentrations in the brew-type (11.2-13.7 g/L) and soluble-type coffee (15 g/L) were calculated as shown in Table 1. The roasting degree was by far the main factor affecting NMP and C-5HT concentrations in these coffee preparations. The effect of extraction type and coffee species was moderate.

NMP results from trigonelline degradation during roasting. NMP concentrations in coffee preparations significantly increase with roasting level from 13-25mg/L in light roast to 68-115mg/L in dark roast. NMP concentrations are lower in soluble-type preparations. Only small amounts of NMP (i.e. <15%) are additionally obtained at high temperature (i.e. 180 °C), the major part of NMP being already extracted at moderate temperature (e.g. 100 °C). At comparable roast level and extraction conditions, preparations from Arabica coffee are slightly richer in NMP.

C-5HT stands for a series of fatty acid amides of serotonin. C-5HT concentrations in coffee preparations drastically decrease with the roasting level from 149-218 µg/L in green coffee to 15-50 µg/L in dark roast. Major C-5HT components in green coffee brew-type are C_{20}-5HT (35%), C_{22}-5HT (55%) and C_{24}-5HT (5%). Increasing roasting level and/or extraction temperature (i.e. soluble-type) leads to a change in the C-5HT profile with increased level of shorter fatty acids, i.e. C_{18}-5HT (>10%), C_{20}-5HT (35-50%), C_{22}-5HT (20-50%), and C_{24}-5HT (<5%). At comparable roast level and extraction conditions, preparations from Robusta tend to be richer in C-5HT.

CONCLUSION

NMP concentrations in model brews and soluble-type coffees were in the range of 13-115 mg/mL. NMP concentration is highest in dark roasted brews. At this level, NMP in coffee matrix was shown to reduce the gastric secretion[3].

C-5HT concentrations in model brews and soluble-type coffees range between 15-218 mg/L. C-5HT concentrations are highest in green coffee brews. Such C-5HT concentrations were reported to already induce gastric acid secretion (Lang et al., 2010). Upon roasting and/or at high temperature extraction, the proportion of C-5HT derivatives with shorter fatty acid chains increase. This could reduce the impact of coffee beverage on the gastric acid secretion, as shown by Lang et al. (2010) when comparing activity on gastric secretion of C_{20}-5HT vs. C_{18}-5HT[5].

Based on this work, one could expect lower gastric acid secretion for coffee beverages prepared from dark roast. However, the mechanisms of the physiological response and the coffee molecules involved in receptor activation are far from being well understood.

Table 1. Concentration of N-methylpyridinium and βN-alkanoyl-5-hydroxytrypamides in Arabica and Robusta coffees roasted at different levels (i.e. light to dark) and extracted under different preparation conditions (i.e. brew-type and soluble-type).

Coffee	Roasting	Yield (g/100g)	Type	Cup Dosage (g/L)	N-methyl pyridinium (mg/L)	C18-5HT (ug/L)	C20-5HT (ug/L)	C21-5HT (ug/L)	C22-5HT (ug/L)	C23-5HT (ug/L)	C24-5HT (ug/L)	Total C-5HT (ug/L)
Arabica	Green	23.0	Brew	11.5	0.0	3.0	74.9	1.3	124.0	1.7	12.7	217.6
Arabica	Light	22.4	Brew	11.2	16.9	1.6	34.8	0.8	68.4	1.0	7.8	114.4
Arabica	Medium	23.1	Brew	11.6	39.4	0.8	15.7	0.4	19.1	0.3	2.5	38.8
Arabica	Dark	24.1	Brew	12.0	114.5	0.4	6.6	0.3	7.0	0.1	0.8	15.2
Robusta	Green	20.2	Brew	10.1	0.0	14.3	85.1	0.6	45.5	0.2	3.3	149.0
Robusta	Light	24.8	Brew	12.4	25.3	14.8	12.9	0.6	9.0	0.1	0.8	38.3
Robusta	Medium	26.6	Brew	13.3	51.8	17.0	12.9	0.0	6.9	0.1	0.5	37.5
Robusta	Dark	27.4	Brew	13.7	114.3	3.6	13.5	0.4	18.8	0.3	3.2	39.7
Arabica	Green	42.6	Soluble	15.0	0	3.5	71.0	1.2	105.2	1.4	10.6	192.9
Arabica	Light	47.6	Soluble	15.0	12.5	1.7	29.4	0.7	51.8	0.7	5.7	90.2
Arabica	Medium	49.0	Soluble	15.0	28.1	1.2	18.6	0.5	15.9	0.2	2.0	38.3
Arabica	Dark	49.6	Soluble	15.0	77.9	0.7	10.2	0.4	7.9	0.1	0.8	20.0
Robusta	Green	38.8	Soluble	15.0	0.0	21.7	94.4	0.9	45.5	0.3	3.2	165.8
Robusta	Light	53.8	Soluble	15.0	17.4	20.0	26.6	0.8	12.3	0.1	0.9	60.8
Robusta	Medium	55.7	Soluble	15.0	35.1	19.1	19.7	0.6	7.6	0.1	0.5	47.6
Robusta	Dark	57.3	Soluble	15.0	67.6	4.3	19.4	0.4	22.0	0.4	3.5	50.0

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Evaluation of the *in Vitro* Inhibitory Effects of an Extract of Decaffeinated Green Coffee Beans on Lipid Degradation

K. KITAURA, K. IWAI, T. FUKUNAGA, R. KIMURA, O. NAKAGIRI

R&D center, UCC Ueshima Coffee Co., Ltd., 3-1-4, Zushi, Takatsuki-shi, Osaka, Japan

SUMMARY

Green coffee beans contain many types of polyphenolic chlorogenic acids (CGAs). Polyphenols are known to inhibit lipid degradation, but the effects of coffee and CGAs are yet to be fully elucidated, and the detailed mechanisms by which this occurs are not studying enough, too. In this study, we examined the biochemical activities of extracts of decaffeinated green coffee beans (EDGCB) and studied their ability to disrupt lipid metabolism. We found that compared to a catechin positive control, EDGCB and CGAs had little effect on micelle particle size distribution. In contrast to CGA-depleted fractions (CGA (-)), EDGCB and CGA-enriched fractions (CGA (+)) prepared from EDGCB markedly inhibited pancreatic lipase. These results suggested that the inhibitory effects of EDGCB on pancreatic lipase are due to CGAs. When individual CGAs were tested, the IC_{50} values for dicaffeoylquinic acids (diCQAs) were lower than that of caffeoylquinic acids (CQAs) and feruloylquinic acids (FQAs) (i.e., diCQA < CQA < FQA). These results suggest that EDGCB inhibits lipid degradation not by altering micelle particle size distribution, but by inhibiting pancreatic lipase, specifically by its CGA component. In addition, the inhibitory effects on pancreatic lipase markedly varied among CGAs isomers.

INTRODUCTION

In recent years, much attention has been directed to the effects of polyphenols on reducing neutral fat by inhibiting lipid degradation The effects of various types of polyphenols on neutral fat reduction have been reported (Shishikura et al., 2006; Nakai et al., 2005; Sugiyama et al., 2007; Raghavendra et al., 2007). These studies indicate that the inhibitory effects of polyphenols on lipid degradation may involve two possible mechanisms. One mechanism involves changing the size of lipid micelle substrates (Shishikura et al., 2006), and the other involves the inhibition of pancreatic lipase activity (Nakai et al., 2005; Sugiyama et al., 2007). Green coffee beans are enriched with many chlorogenic acids (CGAs) and contain a high percentage of CGAs compared to other. Furthermore, it has been reported that 5-caffeoylquinic acid (5-CQA), considered to be a prototypical CGA, inhibits lipase activity (Raghavendra et al., 2007).So we produced an extract of decaffeinated green coffee beans (EDGCB) as a functional food material, and focused on lipid degradation by lipase, clarified the effects of EDGCB and CGAs as its active components on the micelle particle size distribution and their inhibitory effects on pancreatic lipase, and evaluated their *in vitro* inhibitory effects on lipid degradation.

MATERIALS AND METHODS

Preparation of extract of decaffeinated green coffee beans (EDGCB)

EDGCB was prepared by defatting and decaffeinating green coffee beans harvested in Vietnam (*Coffea canephora*, known as robusta) with supercritical CO_2, followed by ethanol extraction, extract concentration, and spray drying. EDGCB is involved about 40% chlorogenic acids (CGAs) and low caffeine (<1%).

Evaluation of effects of EDGCB and CGAs on micelle particle size

The effects of EDGCB and CGAs on micelle particle size distribution were evaluated by the method of Shishikura et al. (2006) using an artificial small intestinal model. First, a standard olive oil emulsion was prepared: 20% (v/v) olive oil, 4% (w/v) phosphatidylcholine (PC) in 2 mM Tris/HCl buffer (pH 7.5). PC was dispersed in buffer before adding olive oil. The mixture was sonicated after adding olive oil. To prepare the artificial small intestinal model, the standard olive oil emulsion prepared as described above was diluted with a stock solution of sodium salt of taurocholic acid (NaT) containing sodium chloride and calcium chloride to give the final composition as 8 mM NaT, 1% (v/v) olive oil, 2.3 mM PC, 10 mM $CaCl_2$, 150 mM NaCl, and 2 mM Tris/HCl buffer (pH 7.5). Samples were dissolved in 3 mL water and 20 mL of the artificial small intestine model was added to the sample. We used distilled water as a control. After adding the sample, the pH of the sample mixture was readjusted to 7.5 with $NaHCO_3$. The samples were incubated at 37 °C with constant shaking (100 strokes/min) for 3 h and micelle particle size distribution, mean particle size, and specific surface area were measured using a laser scattering particle size distribution analyzer (HORIBA LA-920 (HORIBA, Ltd., Japan)). EGCg ((-)-epigallocatechin gallete) and catechins (Catechin Mixture From Green Tea) were used as positive controls. CQAs- and feruloylquinic acids (FQAs)-rich fraction (CQAs & FQAs) and dicaffeoylquinic acids (diCQAs)-rich fraction were prepared from EDGCB by Toyopearl HW-40F chromatography (Tosoh Co., Ltd Japan).

Measurement of pancreatic lipase activity

Inhibition of pancreatic lipase was evaluated by applying the method of Han et al. (2005) using an artificial small intestine model prepared as follows: 80 mg triolein, 10 mg lecithin, and 5mg sodium salt of taurocholic acid were added to 9 mL 0.1 M HEPES buffer (pH 7.0) and sonicated for 10 min. This mixture (20 μL) was added to 20 μL of test sample and incubated at 37 °C for 30 min, followed by addition of 10 μL of pancreatic lipase solution (15,000 unit/mg) and incubated at 37 °C for another 30 min. After incubation, the reacted solutions were boiled for 3 min to stop the reaction. The amount of free fatty acid released by lipase was measured using NEFA C (Wako Pure Chemical Industries Ltd., Japan). The test materials included EDGCB along with the CGA (+) and CGA (-) fractions derived from EDGCB by Toyopearl HW-40F chromatography. Each of the CGA fractions was further subjected to Toyopearl HW-40F chromatography and preparative HPLC.

RESULTS AND DISCUSSION

EDGCB had minimal effect on micelle particle size distribution, mean particle size, and specific surface area of an artificial small intestinal model. This effect was much lower than that of catechins (Figure 1, Table 1). CGAs also had little effect on micelle particle size distribution. These results suggest that EDGCB and CGAs do not directly affect micelle particle size distribution in this artificial system.

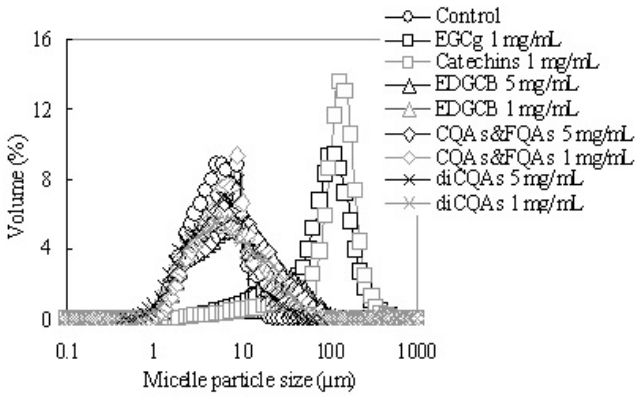

Figure 1. Changes in micelle particle size distribution in the presence of EDGCB and CGAs. Values are the means, n=3.

Table 1. Micelle particle size and specific surface area of artificial small intestinal model with EDGCB and CGAs.

Samples	Micelle particle size (μm)	Specific surface area (m^2/cm^2)
Control	5.18±0.09	1.58±0.02
EGCg 1 mg/mL	97.23±4.05	0.15±0.01
Catechins 1mg/mL	122.93±6.99	0.11±0.01
EDGCB 5 mg/mL	13.01±7.43	1.20±0.36
EDGCB 1 mg/mL	12.07±6.59	1.22±0.34
CQAs&FQAs 5 mg/mL	11.95±3.68	1.12±0.23
CQAs&FQAs 1 mg/mL	8.14±4.15	1.29±0.25
diCQAs 5 mg/mL	6.50±2.11	1.70±0.22
diCQAs 1 mg/mL	9.32±4.33	1.40±0.39

Values are mean ± SD, n=3.

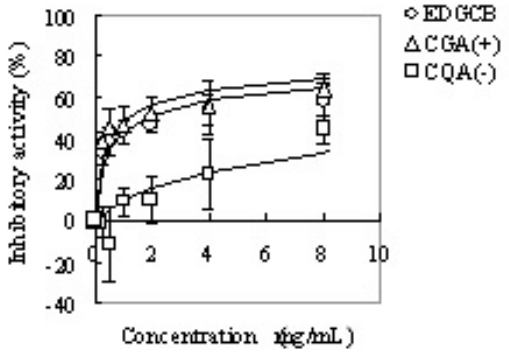

Figure 2. Inhibitory effects of EDGCB and CGA (+), (-) fractions on pancreatic lipase. Values are the means ± SD, n=5.

Table 2. The IC_{50} values of EDGCB, CGA (+) and CGA (-).

Samples	IC_{50} (mg/mL)
EDCGB	1.74
CGA (+)	1.11
CGA (-)	>8

On the other hand, EDGCB inhibited the activity of pancreatic lipase (Figure 2, Table 2). Both CGA (+) and EDGCB showed similar high levels of inhibition compared to that of CGA (-). The inhibitory effect of CGA (+) on pancreatic lipase was a little higher than that of EDGCB, presumably because it is relatively enriched in CGAs compared to EDGCB, the starting material used for fractionations. We conclude, therefore, that the CGA component of EDGCB is responsible for inhibiting pancreatic lipase.

The measurement of pancreatic lipase activity of each of the CGAs contained in EDGCB, they had inhibitory effects on pancreatic lipase activity, and their effects markedly varied among CGAs (Figure 3). Among the CGAs, the IC_{50} values of diCQAs were lower than that of CQAs and FQAs (Table 3).

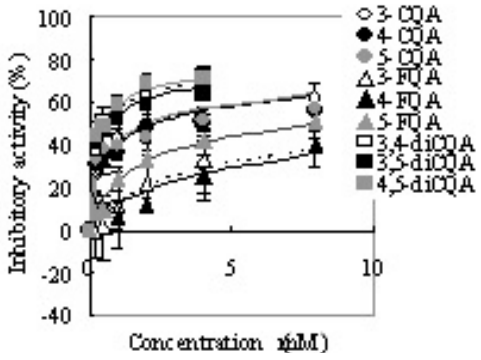

Figure 3. Inhibitory effects of CGAs on pancreatic lipase. Values are mean ± SD, n = 5.

Table 3. The IC_{50} values of CGAs.

Samples	IC_{50} (mM)
3-CQA	2.23
4-CQA	2.39
5-CQA	2.12
3-FQA	>8
4-FQA	>8
5-FQA	>8
3,4-diCQA	0.54
3,5-diCQA	0.71
4,5-diCQA	0.43

These results suggested that the CGA component of EDGCB interferes with lipid degradation by inhibiting pancreatic lipase rather than by a mechanism involving disruption of micelle integrity. Among CGAs, diCQAs had the highest inhibition on pancreatic lipase.

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Urinary Excretion of Chlorogenic Acids and Metabolites in Humans after Green and Roasted Coffees Consumption

M. MONTEIRO[1], V. MARQUES[1], A. FARAH[1,2]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro (UFRJ), Brazil
[2]Instituto de Nutrição, Universidade Federal do Rio de Janeiro (UFRJ), Brazil.
E-mail: afarah@iq.ufrj.br

SUMMARY

The aim of this study was to compare the bioavailability of CGA from green and roasted coffee extracts in humans by measuring the urinary excretion of chlorogenic acids (CGA) and metabolites after the extracts consumption. Capsules of placebo, green coffee and roasted coffee were consumed in a randomized order by seven volunteers, after 10h fasting and 48h phenolic free diet. Three caffeoylquinic acids, three dicaffeoylquinic acids and thirteen metabolites were identified and quantified in all urine samples. The average total urinary excretion of phenolic compounds after consumption of green coffee (2.4 ± 0.9 mmol) and roasted coffee (2.3 ± 0.6 mmol) was significantly higher than after placebo consumption (1.3 ± 0.6 mmol), with no significant difference between both coffee treatments. Our results indicate that CGA from green and roasted coffees are similarly bioavailable in humans. However isomerization of some compounds may occur in the digestive tract.

INTRODUCTION

Chlorogenic acids (CGA) are a family of phenolic compounds derived from the esterification of hydroxycinnamic acids such as caffeic, ferulic and *p*-coumaric acids with quinic acid. The major CGA groups in coffee are caffeoylquinic acids (CQA), feruloylquinic acids (FQA) and dicaffeoylquinic acids (diCQA), with at least three isomers per group. Coffee is one of the main food sources of these compounds (Clifford, 2000). A series of health benefits have been associated with CGA consumption, both in isolated forms or as key ingredients of plant extracts (Farah, 2009). Their bioavailability in humans has been reported in different studies after roasted and green coffee consumption (Monteiro et al., 2007; Farah et al., 2008). However, despite the drastic changes produced in coffee matrix during the roasting process, the effect of such changes on CGA bioavailability has not yet been investigated in a single study. In the present study, we compared the bioavailability of CGA and metabolites in humans after green and roasted coffees consumption, measured by urinary excretion of CGA and metabolites.

MATERIALS AND METHODS

Each volunteer (n=7) consumed, in different days and in a randomized order, capsules of freeze-dried green and roasted arabica coffee extracts containing 9.8 µmol of CGA/kg weight, and placebo. Urine samples were collected at baseline (10h fasting) and at intervals of 0-4 h; 4-8 h; 8-12 h and 12-24h after each treatment. The analyses of CGA in coffee and CGA and metabolites in urine were performed by HPLC-DAD according Farah et al. (2005) and Monteiro et al. (2007). Peaks identification was confirmed by LC-MS according to Farah et

al. (2006) and Perrone et al. (2008). Chromatographic data were treated by ANOVA and results were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Nine CGA compounds were identified in the green and roasted coffee extracts offered to the volunteers. Although the total amount of CGA ingested was the same for both green and roasted coffee extracts, a difference was observed between the distributions of individual isomers in both extracts (Table 1).

Table 1. Contents of caffeoylquinic, dicaffeoylquinic, feruloylquinic and total chlorogenic acids in green and roasted extracts offered to volunteers (n=7).

Compound	Green coffee	Roasted coffee
	µmol/g (db)	
3-caffeoylquinic acid	58.3[a]	78.8[b]
4-caffeoylquinic acid	79.5[a]	90.2[b]
5-caffeoylquinic acid	266.4[a]	148.4[b]
3,4-dicaffeoylquinic acid	9.0[a]	5.0[b]
3,5-dicaffeoylquinic acid	7.6[a]	3.4[b]
4,5-dicaffeoylquinic acid	7.0[a]	3.8[b]
3-feruloylquinic acid	6.9[a]	4.8[a]
4 +5-feruloylquinic acids	6.1[a]	7.2[b]
Total chlorogenic acids	467.3[a]	370.4[a]

Different letters on the same row indicate significant differences (ANOVA), $p \leq 0.05$.

Six CGA compounds, that included 3-, 4- and 5-caffeoylquinic acids and 3,4-, 3,5- and 4,5-dicaffeoylquinic acids and thirteen phenolic acids (caffeic, ferulic, isoferulic, vanillic, gallic, *p*-hydroxybenzoic, dihydrocaffeic, syringic, sinapic, hippuric, benzoic, 3,4-dihidroxyphenilacetic and *trans*-3-hydroxycinnamic acids) were identified and quantified in all urine samples.

The presence of CGA and phenolic acids in baseline urine, even after the volunteers were for 48h on a phenolic-free diet before each treatment, as well as the presence of these compounds in the urine from placebo treatment corroborate the hypotheses proposed by Booth et al. (1957), Baer-Dubowska and Szaefer (1998) and Farah et al. (2006) that these compounds have a long half life in the human body due to entero-hepatic circulation and possibly some storage in body tissues.

There was a large inter and intra individual variation on the urinary excretion of CGA and metabolites. The average total urinary excretion of phenolic compounds after green (2.4 mmol) and roasted (2.3 mmol) coffees consumption was significantly higher (p<0,000) than after the consumption of placebo (1.3 mmol), with no significant difference between both coffee treatments (Figure 1).

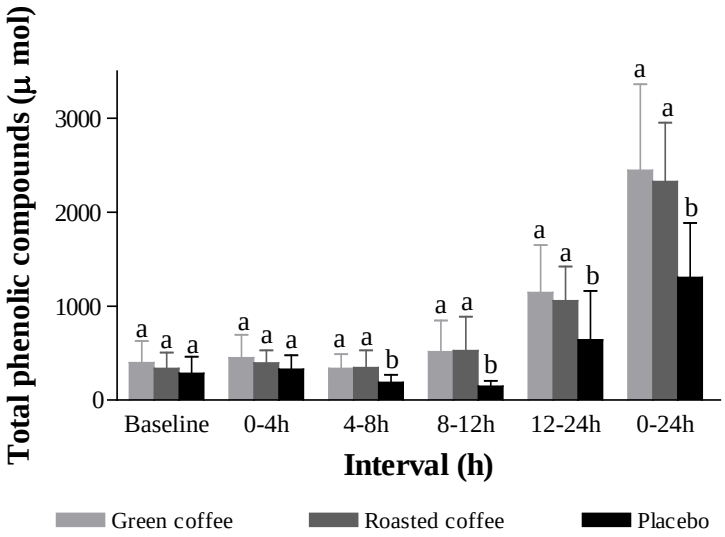

Figure 1. Average urinary excretion of total phenolic compounds at baseline and at intervals after green coffee, roasted coffee and placebo consumption in humans. Values are mean ± SD, n = 7. Different superscript letters in the same interval show significant differences.

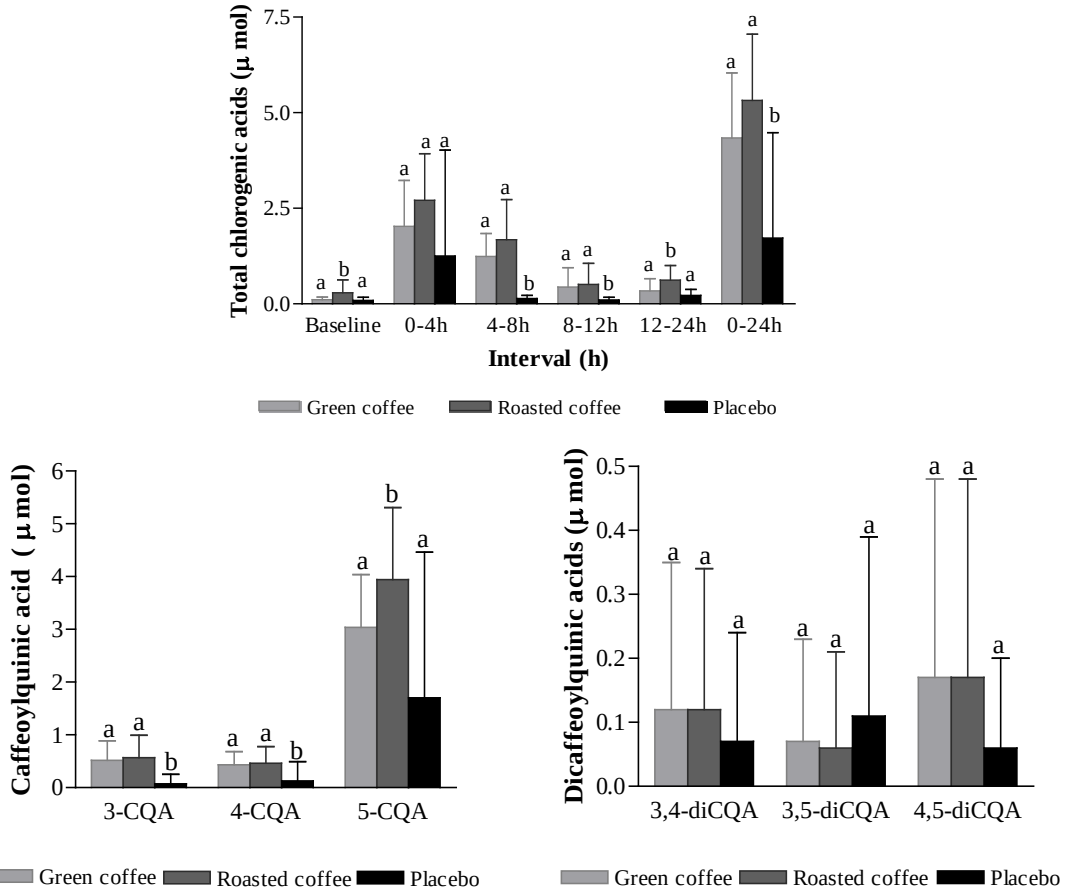

Figure 2. Average urinary excretion of chlorogenic acid compounds for 24h after consumption of green coffee, roasted coffee and placebo in humans. Values are mean ± SD, *n* = 7. Different superscript letters in the same interval show significant differences (ANOVA, $p \leq 0.05$). CQA = caffeoylquinic acids, diCQA = dicaffeoylquinic acids.

Although no significant differences were observed between the excretion of total CGA and metabolites in urine during the 24h after administration of the dry extracts of booth green and roasted coffees, the same was not observed for the individual isomers. Although the intake of 5-caffeoylquinic acid through green coffee was significantly higher compared to the intake through roasted coffee (Table 1), the 24h urinary excretion of 5-caffeoylquinic acid after green coffee consumption was lower when compared to roasted coffee treatment (Figure 2), which could indicate the bioavailability of 5-caffeoylquinic acid incorporated into melanoidins during the roasting process. Additionally, despite the higher intake of the isomers 3-caffeoylquinic acid and 4-caffeoylquinic acid through roasted coffee compared to the intake through green coffee, no significant differences in the urinary excretion of these isomers were found in the 24h urine after the consumption of both extracts, which could indicate isomerization of 5-caffeoylquinic acid present in green coffee during the digestion process. Indeed, Farah et al. (2006) observed the isomerization of 5-CQA when incubated with human and artificial intestinal digestive fluids.

The individual recovery of total hydroxycinnamic and quinic acid moieties' derivatives varied from 20 to 47% after green coffee consumption and from 25 to 47% after roasted coffee consumption, with average recoveries of 33% and 37%. Our results indicate that the drastic changes produced in coffee matrix by roasting process do not affect the total bioavailability of CGA in humans.

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The Bioavailability of Chlorogenic Acids from Coffee Is Not Affected By the Simultaneous Consumption of Soybean Extract

M. MONTEIRO[1], G. DUARTE[1], I. FELBERG[1,2], A. FARAH[1,3]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro (UFRJ), Brazil
[2]EMBRAPA Agroindustria de Alimentos – CTAA, Brazil
[3]Instituto de Nutrição Josué de Castro, UFRJ, Brazil

SUMMARY

The aim of the present study was to compare the urinary excretion of chlorogenic acids (CGA) and phenolic metabolites in humans after the consumption of plain coffee, soybean extract with coffee, plain soybean extract and water consumption. Three caffeoylquinic acids, three dicaffeoylquinic acids and fifteen metabolites were identified and quantified in all urine samples. The average total urinary excretion of phenolic compounds after consumption of plain coffee (3.2 ± 1.1 mmol) and soybean extract with coffee (3.1 ± 0.6 mmol) were significantly higher than after the consumption of plain soybean extract (1.29 ± 0.5 mmol) and water (1.33 ± 0.5 mmol), with no significant difference between both coffee treatments. Our results indicate that the simultaneous consumption of soybean extract and coffee (in coffee beverages) does not affect the bioavailability of CGA in humans.

INTRODUCTION

Studies have shown that soy and coffee are associated with reduced risk of several chronic diseases (Kang et al., 2010; Farah, 2009). Soybean extracts contain proteins, carbohydrates, lipids and polyphenols, mainly isoflavones. It is known that these constituents may influence the absorption and metabolism of phenolic compounds in humans (Manach et al., 2004). Chlorogenic acids (CGA) are a family of phenolic compounds derived from the esterification of hydroxycinnamic acids such as caffeic, ferulic and p-coumaric acids with quinic acid. The major CGA classes in coffee are the caffeoylquinic acids, feruloylquinic acids and dicaffeoylquinic acids, with at least three isomers per group. Coffee is one of the major sources of CGA in nature and it is commonly used in soybean drinks to improve their taste and increase consumer acceptance. Since both food products have been considered for many as functional foods because of their phenolic composition, the aim of this study was to evaluate the effect of the simultaneous consumption of soybean extract and coffee on the urinary excretion of CGA and metabolites in humans.

MATERIALS AND METHODS

Each volunteer (n=5) consumed in four different randomized occasions 200 mL of (a) 2% of instant coffee dissolved in mineral water; (b) a soy-coffee drink containing 2% of instant coffee and 11.5% of instant soybean extract dissolved in mineral water , (c) a soy drink containing 11.5% of instant soybean extract dissolved in mineral water and (d) plain mineral water. Urine samples were collected at baseline (10h fasting) and at intervals of 0-4 h; 4-8 h; 8-12 h and 12-24 h after the consumption of each beverage. CGA and metabolites were measured in coffee and urine by HPLC and LC-MS, according to Farah et al. (2005) and Monteiro et al. (2007). Results were treated by ANOVA to evaluate the influence of treatment

on the urinary excretion of CGA and metabolites. Values were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Nine CGA (3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,4-diCQA and 4,5-diCQA) were identified in the roasted coffee offered to the volunteers and in the soy-coffee drink. The total CGA content in the coffee drink was 561.2 μmol. Caffeoylquinic, feruloylquinic and dicaffeoylquinic acids represented respectively 84.2%, 12.0% and 3.8% of the CGA quantified in roasted coffee. No CGA compound was identified in the soybean extract.

Six CGA, including 3-, 4- and 5-caffeoylquinic acids and 3,4-; 3,5- and 4,5-dicaffeoylquinic acids (Figure 1), and fifteen phenolic acids (caffeic, ferulic, isoferulic, gallic, *p*-hydroxybenzoic, dihydrocaffeic, vanillic, syringic, sinapic, 3,4-dihidroxyphenilacetic, hippuric, 2,4-dihidroxybenzoic, 3-(4-hydroxyphenil)propionic, *trans*-3-hydroxycinnamic and benzoic acids) were identified in all urine samples before and after each treatment. The concentrations of isoflavones and their metabolites were not evaluated in this study.

The presence of CGA and phenolic compounds in baseline urine, even the volunteers are on a diet free of phenolic compounds in 48 hours before the each treatment, and after treatment with water corroborates the hypothesis proposed by Baer-DuBowski and Szaefer, 1998 and Farah et al. (2006) that these compounds have a long half-life through entherohepatic circulation and that they may be stored in the liver and/or other body tissues

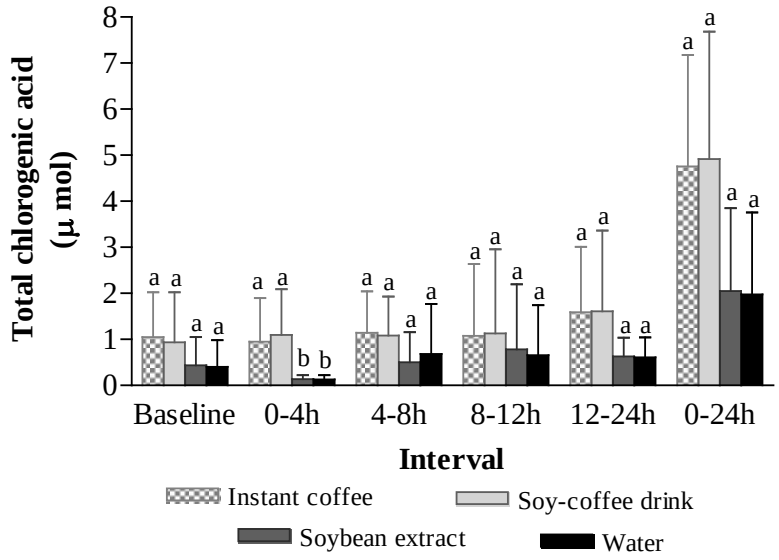

Figure 1. Average urinary excretion of total chlorogenic acids at baseline (-4-0h) and at each interval after instant coffee, soy-coffee drink, soybean extract and water administration in humans. Values are mean ± SD, *n* = 5. Different superscript letters in the same interval show significant differences.

The average total urinary excretions of the evaluated phenolic acids after the consumption of coffee (3.2 mmol) and soy-coffee drink (3.1 mmol) were significantly higher ($p < 0,000$) than those after the consumption of instant soybean extract (1.33 mmol) and water (1.29 mmol), with no significant difference between the first two treatments (Figure 2). It was observed that

the urinary excretion of total phenolic acids was higher at interval 12-24 h after treatments when compared to interval 0-12 h (Figure 3).

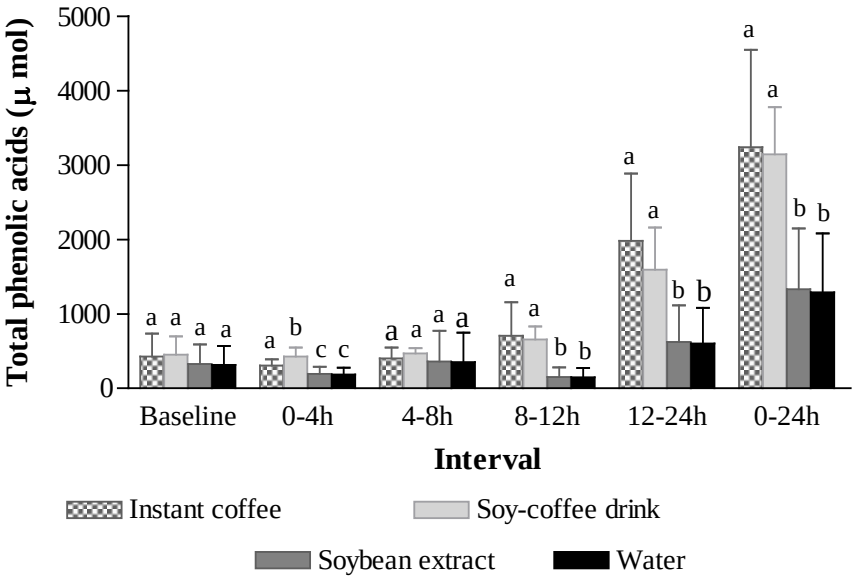

Figure 2. Average urinary excretion of total chlorogenic and phenolic acids at baseline (-4-0h) and at intervals after instant coffee, soy-coffee drink, soybean extract and water administration in humans. Values are mean ± SD, n = 5. Different superscript letters in the same interval show significant differences.

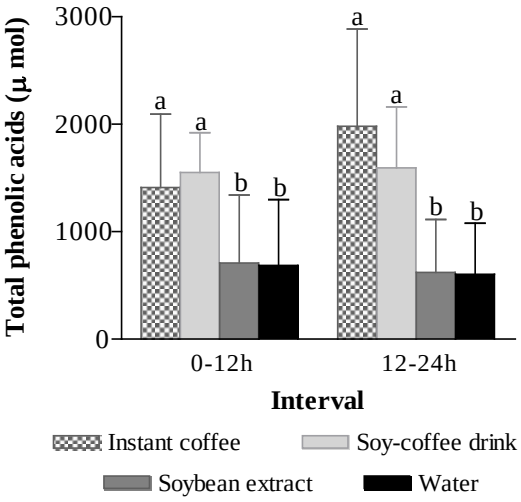

Figure 3. Average urinary excretion of total chlorogenic and phenolic acids at intervals 0-12 h and 12-24 h after instant coffee, soy-coffee drink, soybean extract and water administration in humans. Values are mean ± SD, *n* = 5. Different superscript letters in the same interval show significant differences.

On average, after the consumption of instant coffee and coffee added to soybean extract, respectively, 62% and 57% of total hydroxycinnamic and quinic acid moieties' derivatives were recovered in urine. Our results indicate that the consumption of soybean extract with coffee does not affect the metabolism of CGA in humans compared with plain coffee.

ACKNOWLEDGEMENTS

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Coffee is the main Contributor to the Dietary Antioxidant Capacity in Brazil

T. TORRES[1] AND A. FARAH[1,2]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, Ilha do Fundão, RJ, Brazil
[2]Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Ilha do Fundão, RJ, Brazil. E-mail: afarah@iq.ufrj.br

SUMMARY

Considering that Brazil is currently the second coffee consumer in the world and that coffee is known to present a high antioxidant capacity, mainly due to its phenolic composition, the aim of the present study was to evaluate the relative contribution of coffee to the Brazilians' dietary antioxidant capacity. For this, the 41 plant food products most consumed in Brazil among other consumed products known as good antioxidant sources were evaluated considering the annual *per capita* consumption database from the National Survey Institute "IBGE". These food products, accounted theoretically for 95% of the contribution to dietary antioxidant capacity in Brazil. All food products were prepared as usually consumed and the antioxidant capacity was measured by two methods (TEAC e FRAP). Coffee presented the highest antioxidant capacity of all evaluated food products, followed by mate tea, red wine and açaí – an Amazonian fruit, while cola beverages, refined wheat flour and cachaça – a distilled alcoholic beverage, presented the lowest. Taking into account the annual *per capta* consumption, coffee was the most important contributor to the dietary antioxidant capacity in Brazil, contributing with 67% and 70% of dietary antioxidant capacity when evaluated by TEAC and FRAP, respectively. The other beverages (wine, fruit juices, mate tea, among others) contributed in total, with 15% and 12% to dietary antioxidant capacity, respectively. Beans contributed with 10% and 9%, while cereals and derivatives with 3% and 4% respectively. Fruits contributed only with 3% of the dietary antioxidant capacity, when evaluated by both TEAC and FRAP and vegetables with only 2%.

INTRODUCTION

Diets based mainly on the food intake of antioxidant compounds have been associated with potential benefits to human health, including protection against chronic diseases (Pellegrini et al., 2003). A possible mechanism mediating the protective effect is the reduction of oxidative stress by bioactive compounds from plant foods (Svilaas et al., 2004). Plants are the major carriers of antioxidant compounds and in general, different antioxidants compounds with different chemical characteristics act synergistically integrated to protect the human body against oxidative damage (Parras et al., 2007). Therefore, assessment of dietary antioxidant capacity through plant foods consumption is considered an important tool to monitor this protective effect (Saura-Calixto and Goni, 2006). Brazil is the world's largest coffee producer and currently the second largest consumer market (ABIC, 2010). Given this high consumption of the beverage as well as its high antioxidant capacity reported in the literature, the purpose of this study was to evaluate the relative contribution of coffee to the dietary antioxidant capacity in Brazil.

MATERIALS AND METHODS

The selection of plant foods of major relevance in Brazilian's diet was based on the national survey data base from the Brazilian Institute of Geography and Statistics – IBGE (2010). The selection considered the highest *per capita* consumption of plant foods in Brazil and the different feeding patterns of the five major regions of the country. The list resulted in the 24 most consumed plant food products in Brazil, with 3 samples for each food product. Additional 17 products also consumed by Brazilians and known as good antioxidant sources were included in the study. Together, these food products accounted for 76% of all plant food products consumed in Brazil and 58% of all food intake. Also, considering the antioxidant capacity data available in the literature as well as the National survey data base, the evaluated food products theoretically accounted for about 95% of the dietary antioxidant capacity in Brazil. The evaluated food products were prepared as commonly consumed by Brazilians (BRAZIL, 2005) and the antioxidant capacity was evaluated by two different *in vitro* assays, TEAC (Re et al., 1999) and FRAP (Benzie and Strain, 1996). The antioxidant capacity results before and after being associated with the corresponding estimated intake were presented on wet basis, as mean ± SD and by food groups (beverages, cereals products, beans, fruits and vegetables), representing Brazil as a whole, or separated by large regions (South, Southeast, Midwest, North, Northeast) or family income. The association between methods of *in vitro* analysis (TEAC and FRAP) was assessed by Pearson correlation test ($p < 0.05$).

RESULTS AND DISCUSSION

The analyzed food products were classified in an antioxidant capacity list as presenting either a very high, high, medium high, medium low or low antioxidant capacity, according to the average percentage values of TEAC and FRAP ($r = 0.97$, $p < 0.0001$) in relation to the total antioxidant capacity of all analyzed food products (Figure 1). Coffee presented the highest antioxidant capacity among all food products analyzed, followed by mate tea – commonly consumed in the South region of Brazil, red wine, and açaí – an Amazonian fruit commonly consumed in the North region of Brazil. It is noteworthy the fact that the antioxidant capacity of coffee was equivalent to 2 ½ times those of mate tea and red wine, which are also known as potential sources of antioxidants. The lowest antioxidant capacity values were observed for soft drinks, refined wheat flour and cachaça - a sugar cane distilled alcoholic beverage.

Very High	High	Medium High	Medium Low	Low
Coffee, Mate, Red wine Açaí (Amazonian fruit)	Beetroot Pinto beans, black beans, Pineapple, Apple, Orange, Tangerine, Papaya, Grape green, Tomato (extract), Black-eyed beans, Cookies, Corn	Pumpkin, Potato, Cassava, Onion, Carrot, Tomato, Cabbage, Corn meal, Cassava meal, Beer, Bottled juice (Orange)	Coconut, Watermelon, Banana, Chayote, Lettuce, Bread, Biscuits, Wheat flour	Pasta, Rice polished, Cola beverage, Guarana beverage, Cachaça (sugar cane brandy)

Figure 1. Classification of the analyzed food products according to their antioxidant capacity evaluated by FRAP and TEAC (data on wet, ready to consume basis).

The high antioxidant capacity of coffee can be attributed mainly to its phenolic compounds, among other compounds with antioxidant activity. Green coffee contains high amounts of chlorogenic acids (commonly from 5-9 g/100 g) and despite their thermolability, a light roasted coffee may commonly contain up to 4 g/100 g (Farah, 2009). Although the coffee

brands evaluated in our study were roasted to moderately dark to dark degrees, they still contained ~1-2 mg of chlorogenic acids per100g of coffee, which is a considerable amount comparing to other food sources of phenolic compounds in nature.

In addition to presenting the highest antioxidant capacity among all analyzed food products, considering the high annual *per capita* consumption, coffee was the most important contributor to Brazilians dietary antioxidant capacity, regardless of family income and diversified food patterns of major regions of the country, except in the South region where mate tea and coffee showed a similar percentage contribution to the dietary antioxidant capacity. Nationally speaking, although other beverages (wine, fruit juices, mate tea, among others), beans, cereals and derivatives, fruits and vegetables also contributed to the dietary antioxidant capacity, their percentage contributions were way bellow that of coffee (Figure 2).

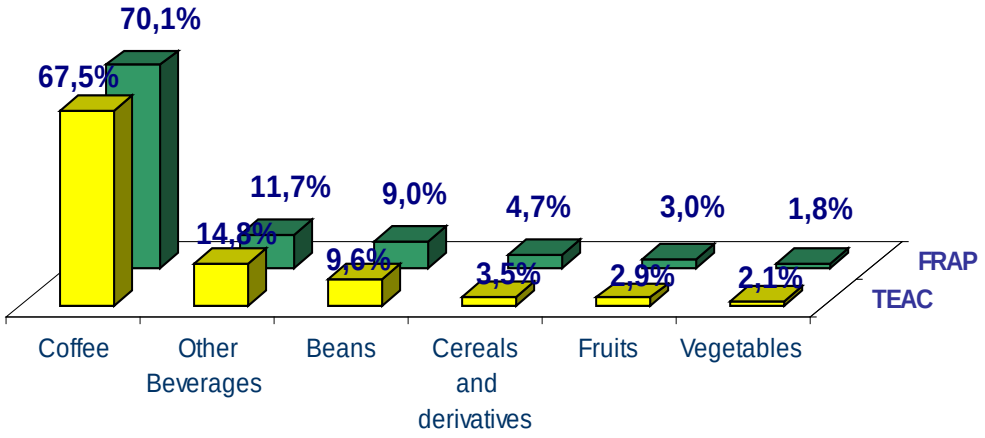

Figure 2. Percent contribution of coffee and other food products to the dietary antioxidant capacity in Brazil.

The importance of coffee to the dietary antioxidant capacity in Brazil was expected, given its high consumption in the country as well as the fact that similar studies performed in countries where coffee is commonly consumed like Norway (Svilaas et al., 2004), Italy (Pellegrini et al., 2003) and USA (Wu et al., 2004) have observed the same nature of results, even though not always using the same analytical methods.

In conclusion, our results show that coffee is the main contributor to the dietary antioxidant capacity in Brazil. However, it is important to remember that the consumption of food products rich in antioxidant compounds should vary in order to optimize the health benefits of nutrients and other bioactive compounds contained in these foods.

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Activation of the Nrf2/ARE Pathway and ARE-Dependent Gene Expression by Different Coffee Constituents

U. BOETTLER[1], K. SOMMERFELD[2], N. VOLZ[1], N. TELLER[1], V. SOMOZA[3], H. STIEBITZ[4], G. BYTOF[4], I. LANTZ[4], R. LANG[5], T. HOFMANN[5], D. MARKO[1]

[1]University of Vienna, Department of Food Chemistry and Toxicology, Vienna, Austria
[2]Institute of Applied Biosciences, Universität Karlsruhe, Karlsruhe, Germany
[3]Research Platform Molecular Food Science, University of Vienna,Vienna, Austria
[4]Tchibo GmbH, Hamburg, Germany
[5]Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Freising, Germany

SUMMARY

We investigated whether coffee extracts (CEs) of different proveniences or selected coffee constituents influence the Nrf2/ARE pathway, a major antioxidative and chemopreventive detoxifying mechanism in cells.

An activation of the pathway by different CEs was observed by Western blot analysis in human colon carcinoma cells (HT29), apparent as an increase in nuclear levels of Nrf2 protein, the key element of this pathway. In addition to the known Nrf2 activator 5-*O*-caffeoylquinic acid (CGA) *N*-methylpyridinium ion (NMP) was identified as potent activator of Nrf2 nuclear translocation and ARE-dependent gene transcription of selected antioxidative phase II enzymes. In contrast, trigonelline significantly suppressed Nrf2 activation and furthermore decreased the NMP-mediated induction of Nrf2/ARE-dependent gene expression in HT29 cells.

In conclusion, several coffee constituents, partly already present in the raw bean but also some that are only generated during the roasting process contribute to the Nrf2-translocating properties of consumer-relevant coffee. Thus, a fine tuning in the degradation/formation of activating and deactivating constituents during roasting appears to be critical for the chemopreventive properties of the final coffee product.

INTRODUCTION

Diet and life style are key factors for the development of many life-threatening diseases. Thus, the interest in food and foodstuffs, supporting the body's defences against such diseases has increased during the last years. One pivotal mechanism to guard against cancer is the protection of cells against ROS-induced damage. In this regard cells have developed defence mechanisms, such as the increased expression of phase II detoxifying enzymes. The expression of many phase II genes is regulated by an antioxidant response element (ARE), which is activated via binding of the transcription factor Nrf2 (Nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2). Activation by ROS or upstream protein kinases induce the nuclear translocation of Nrf2, its binding to the ARE and the onset of the transcription of phase II enzymes such as NAD(P)H-quinone oxidoreductase 1 (NQO1) or heme oxygenase 1 (HO1, Figure 1) (Wilkinson and Clapper, 1997; Lam et al., 1987). The increase of phase II

enzyme activity in mice liver after feeding of diets containing coffee has already been described, proposing the Nrf2/ARE pathway as potential mechanism (Boettler et al., 2010).
We investigated the induction of Nrf2 translocation into the nucleus by different coffee extracts (CE) in HT29 cells. Furthermore, the impact of various constituents of both, the phenolic fraction of coffee with CGA and the cationic fraction of coffee including trigonelline and its degradation products was determined.

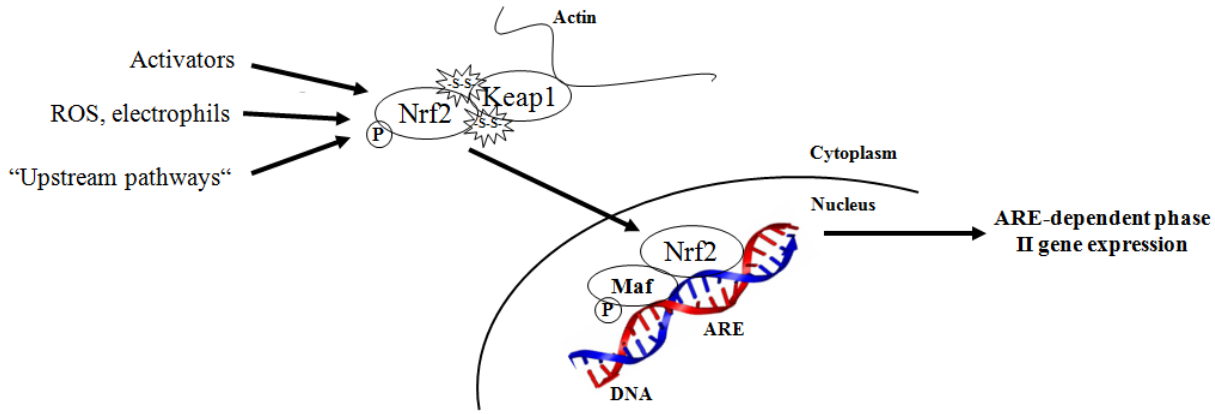

Figure 1. Activation of the Nrf2/ARE pathway. ARE: Antioxidant response element; Nrf2: Nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2; P: Phosphate; ROS: Reactive oxygen species.

To underline potential health benefit we investigated whether the modulation of Nrf2 nuclear translocation is reflected subsequently by enhanced transcription of selected ARE-dependent genes.

MATERIALS AND METHODS

Coffee beverages were prepared using a common coffee drip filter machine (TCM, Germany) with 48 g of roast coffee powder and 900 mL of bd water. An aliquot (500 mL) of the freshly prepared coffee beverage was frozen with liquid nitrogen and lyophilized (0.77 mbar, 48 h) to yield the coffee extracts (CEs) (Lang et al., 2008). All constituents and extracts were dissolved in DMSO (1 % final conc.) and adopted in serum free media.

Cell culture

HT29 cells (ATCC 299) were obtained from DSMZ (Germany). Cell culture media and supplements were purchased from Invitrogen Life Technologies, Germany and cultivated according to Lam et al. (1987).

Western blot analysis

4.5 x 10^6 HT29 cells were grown for 48 h and subsequently incubated for 3 h with the different compounds and 100 U/mL catalase, preventing hydrogen peroxide accumulation. Thereafter cells were rinsed with PBS, abraded on ice to further separate the nuclear fraction from the cytosolic fraction, separated by SDS-PAGE (10% polyacrylamide gel) and processed to Western blot according to Lam et al. (1987). Chemoluminescent signals (Lumi-GLO, Cell Signaling Technology) were analysed for quantification and light units were plotted as test over control [%].

RNA Extraction and realtime PCR

1.6 x 10^6 HT29 cells were grown for 48 h. After incubation total RNA was extracted with the RNeasy® Mini Kit. Reverse transcription as well as realtime PCR were performed as described in Lam et al. (1987). Fold changes in expression of the target gene relative to the internal control gene (ß-actin) were analyzed using Bio-Opticon Software. Data of all assays were analyzed by the $2^{-\Delta\Delta CT}$ method.

RESULTS AND DISCUSSION

Three different coffee extracts (Table 1) were investigated on their impact on the Nrf2/ARE pathway in HT29 cells. Since no changes in the cell growth were observed, a modulation due to cytotoxic effects might not be of relevance (data not shown). The *coffea robusta* extract R. India (RI) was most effective in increasing Nrf2 nuclear protein levels, displaying an U-shaped curve progression with two peak maxima at 10^{-1} and 100 µg/mL, respectively (Figure 2). It has already been reported that *robusta* samples contained higher amounts of reducing constituents and were more effective regarding *ex vivo* protective activity than *arabica* samples (Daglia et al., 2002). The two *arabica* extracts A. Columbia (AC) and A. Brazil (AB) differed regarding their potential. AC induced Nrf2 nuclear translocation only in low concentration ranges (data not shown), whereas AB displayed one maxima in low (100 pg/mL) and on in high AB concentrations (250 µg/mL, Figure 2). Since both coffees were roasted to the same degree, the different modulations of AC and AB might be a result of a different constituent pattern.

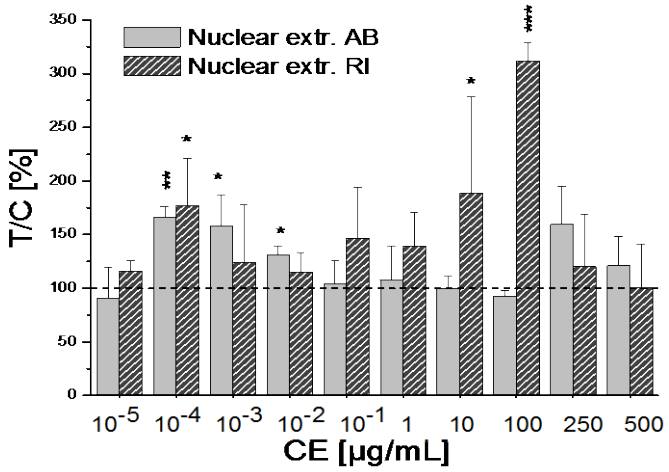

Figure 2. Impact of AB and RI on nuclear Nrf2 protein levels in HT29 (3 h). Data are presented as T/C [%], 1 % DMSO as control. Mean ± SD of three independent experiments. Significances are calculated using Student's *t* test (* = p < 0.05; * = p < 0.01; *** = p < 0.001).

Table 1. Content of NMP and CGA in the CEs (Lang et al., 2008).

CE	CGA [mg/L]	CGA [mM]	NMP [mg/L]	NMP [uM]
AC	743	2.1	22	234
AB	693	1.9	27	287
RI	640	1.8	30	320

The three coffee extracts AB, AC and RI differ in the concentration of CGA and NMP (Table 1) (Boettler et al., 2010). Therefore, we investigated the influence of these constituents on Nrf2 translocation in HT29 cells. CGA was identified as a potent inducer of Nrf2 translocation significantly enhancing Nrf2 nuclear levels at concentrations ≥ 10 nM (Figure 3). These findings are also supported by Daglia et al. (2002). The coffee extract AC, richest in CGA induced Nrf2 nuclear translocation in HT29 cells exclusively in pg/mL conc. corresponding to pM conc. of CGA, questioning the role of CGA in the effects entailed by the CEs. These results are supported by data of Feng et al., (2005), indicating that *in vitro* antioxidant activity was lower in green coffee, rich in CGA, in comparison to roasted coffee. Trigonelline did not increase Nrf2 nuclear translocation in HT29 cells up to 500 µM. Of note, a significant drop in Nrf2 total protein was observed at concentrations ranging from 10 pM-100 nM trigonelline (Figure 3). In contrast, the degradation product NMP significantly increased Nrf2 nuclear translocation in a U-shaped curve marked by a potent induction at 100 nM NMP and 100-500 µM NMP (Figure 3).

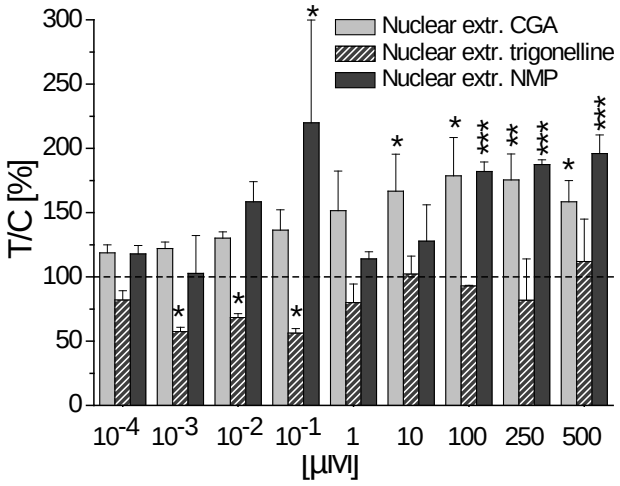

Figure 3. Impact of CGA, trigonelline and NMP on nuclear Nrf2 protein levels in HT29 cells (3 h). Data are presented as T/C [%], 1% DMSO as control. Mean ± SD of at least three independent experiments. Significances are calculated by Student's *t* test (* = p<0.05; ** = p<0.01; *** = p<0.001).

To determine whether the detected effects are passed downstream on gene transcription level rt PCR was performed. NMP and CGA did not influence NQO1 gene transcription in the tested concentration ranges but significantly increased HO1 gene transcription (NMP: 10^{-2}-10 µM and CGA: 10 µM). In contrast, trigonelline significantly decreased both, NQO1 and HO1 transcript levels, however co-incubation with NMP abrogated the suppressing properties of trigonelline to the no effect level (Figure 4). Yet, it has to be underlined that nM concentrations of trigonelline were sufficient to compensate the effect of 10 µM NMP,

indicating that the content of these two coffee constituents with controversial effects on Nrf2 signalling might be critical for the biological activity of the whole CE. Both, CGA and NMP (10 uM) significantly increased Nrf2 gene transcription, thereby influencing Nrf2 *de novo* synthesis, which seems to be essential for the activation of the Nrf2/ARE pathway (Lam et al., 1987). Trigonelline did not modulate Nrf2 gene transcription, however a slight reduction was apparent after co-incubation with NMP in low concentrations (10^{-2} uM, Figure 4).

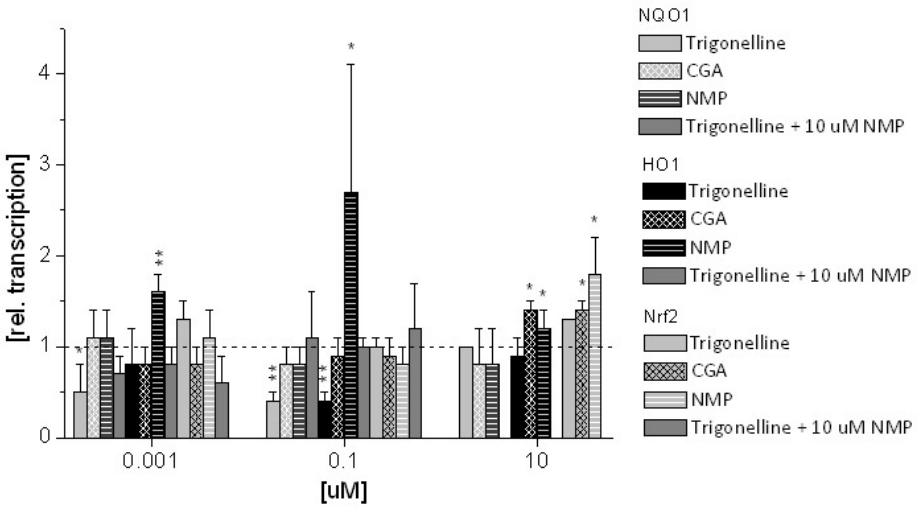

Figure 4. Gene transcription of HO1, NQO1 and Nrf2 in HT29 cells after incubation with CGA, NMP and trigonelline (3 h). Mean ± SD, normalized on ß-actin and presented as rel. transcription of the control (1 % DMSO) = 1. Significances are calculated by Student's *t* test (* = p<0.05; ** = p<0.01).

In summary, our data show a potent induction of Nrf2 translocation by different CEs in HT29 cells. CGA and NMP were identified as potent inducers of the pathway. The NMP-precursor trigonelline potently suppressed Nrf2 signalling, compensating the effects of NMP. Thus, a fine tuning in the degradation/formation of activating and deactivating constituents of the Nrf2/ARE pathway appears to be critical for the potential chemopreventive properties of the final coffee product.

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High-Molecular Weight Components in Coffee Beverages and Their Degradation by Human Microbiota

D. GNIECHWITZ[1], N. REICHARDT[2], M. BLAUT[2], M. BUNZEL[3]

[1]Institute of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany
[2]German Institute of Human Nutrition Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany
[3]Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108, USA

SUMMARY

Coffee beverages are a source of well-fermentable dietary fiber. Soluble dietary fiber contents can be influenced by factors such as coffee variety, degree of roast, and brewing procedure. For example, soluble dietary fiber contents of coffee beverages from a medium roasted, medium ground arabica coffee ranged, depending on the brewing procedure, between 0.26 and 0.38 g/100 mL. Soluble coffee dietary fiber is readily fermented by human fecal bacteria, resulting in the production of acetate, propionate, and butyrate as short-chain fatty acids. Fermentation of soluble coffee fiber goes along with an increase of bacterial species belonging to the *Bacteroides-Prevotella* group. In addition to soluble dietary fiber, coffee brews contain fiber-like materials which escape the analytical determination of soluble dietary fiber. This high molecular weight material, which is ethanol soluble but resistant against enzymatic degradation using protease, α-amylase, and amyloglucosidase, contains relatively low amounts of polysaccharides, mostly arabinogalactans, but is predominantly made up of unknown components, likely Maillard reaction products. The carbohydrate portion of this material is well fermentable, too, but NMR experiments also hint that the unknown components are at least partially degraded by human fecal bacteria.

INTRODUCTION

In the past, coffee consumption was often associated with negative health effects. However, more recent studies did not confirm several of these assumptions. Nowadays, it is widely accepted that a moderate coffee consumption of about three to four cups of coffee per day does not trigger negative health effects (not considering risk groups such as elderly, children, pregnant women, and people already suffering from high blood pressure). Moreover, animal experiments and epidemiologic studies even suggest several health benefits from coffee consumption. Potential effects include the reduction of the risk of type 2 diabetes (Higdon and Frei, 2006), certain cancers including colorectal cancer (Giovannucci, 1998), as well as Alzheimer (Arendash and Cao, 2010) and Parkinson's disease. Naturally, potential health beneficial effects of the coffee beverage are based on one or several constituents of the coffee brews. About 40% of the coffee beverage dry weight is made up of known, low-molecular weight compounds such as chlorogenic acids, caffeine, trigonelline, etc. Another 30% of the coffee brew dry weight contains polysaccharides and proteins, leaving about 30% of the dry matter unknown. Low-molecular weight Maillard reaction products as well as melanoidins, brown colored, high-molecular weight Maillard reaction products, are found in the group of "unknowns". In the coffee beverage it is also hard or sometimes even impossible to

differentiate between pure polysaccharides, proteins, and melanoidins as these coffee brew polymers extensively interact with each other. For example, coffee melanoidins are discussed to be composed of several structural components, such as poly- and/or oligosaccharides, proteins and/or peptides, phenolic compounds, and chromophores. Most likely different melanoidins populations with different ratios of the mentioned structural components exist (Nunes and Coimbra, 2010), making these compounds a hard to characterize group of coffee brew constituents. Polysaccharides found in coffee brews are galactomannans and arabinogalactans/arabinogalactan-proteins (Nunes and Coimbra, 2002). While galactomannans from coffee brews are generally only little substituted, arabinogalactans are highly branched polymers in both green and roasted coffee (Redgwell et al., 2002) (Figure 1), although roasting has major effects on arabinogalactan structures.

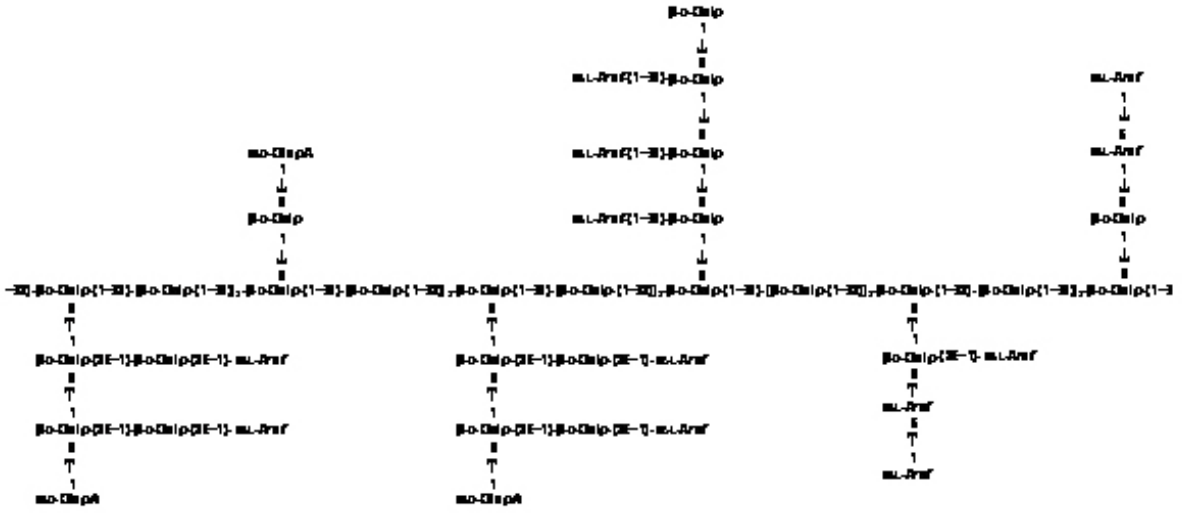

Figure 1. Potential structural unit of green coffee arabinogalactans (adapted from Redgwell et al., 2002).

Although not recognized for a long time, both galactomannans and arabinogalactans from coffee brews should be regarded as dietary fiber. According to the dietary fiber definition of the American Association of Cereal Chemists International "dietary fiber is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (DeVries, 2001). Next to arabinogalactans and galactomannans, which cannot be degraded by the intestinal enzymes and thus reach the large intestine, melanoidins might be another dietary fiber source from coffee. We isolated and characterized high molecular weight components of coffee beverages, which potentially qualify as dietary fiber, and described their structural characteristics. In addition, we analyzed the fermentability of different high-molecular weight fractions of coffee brews and studied the influence on the intestinal microorganisms. The results of these studies are detailed in a series of papers (Gniechwitz et al., 2006; 2007; 2007; 2008; 2008; Reichardt et al., 2009) and certain aspects will be summarized in the following.

MATERIALS AND METHODS

Detailed descriptions of the methodologies are given in previously published papers (Gniechwitz et al., 2007; 2007; 2008).

RESULTS AND DISCUSSION

The concentration of soluble dietary fiber in a coffee brew prepared from a medium-roasted and medium ground arabica coffee (50 g/L) was determined to 0.35 g/100 mL (AOAC method 985.29) (Figure 2).

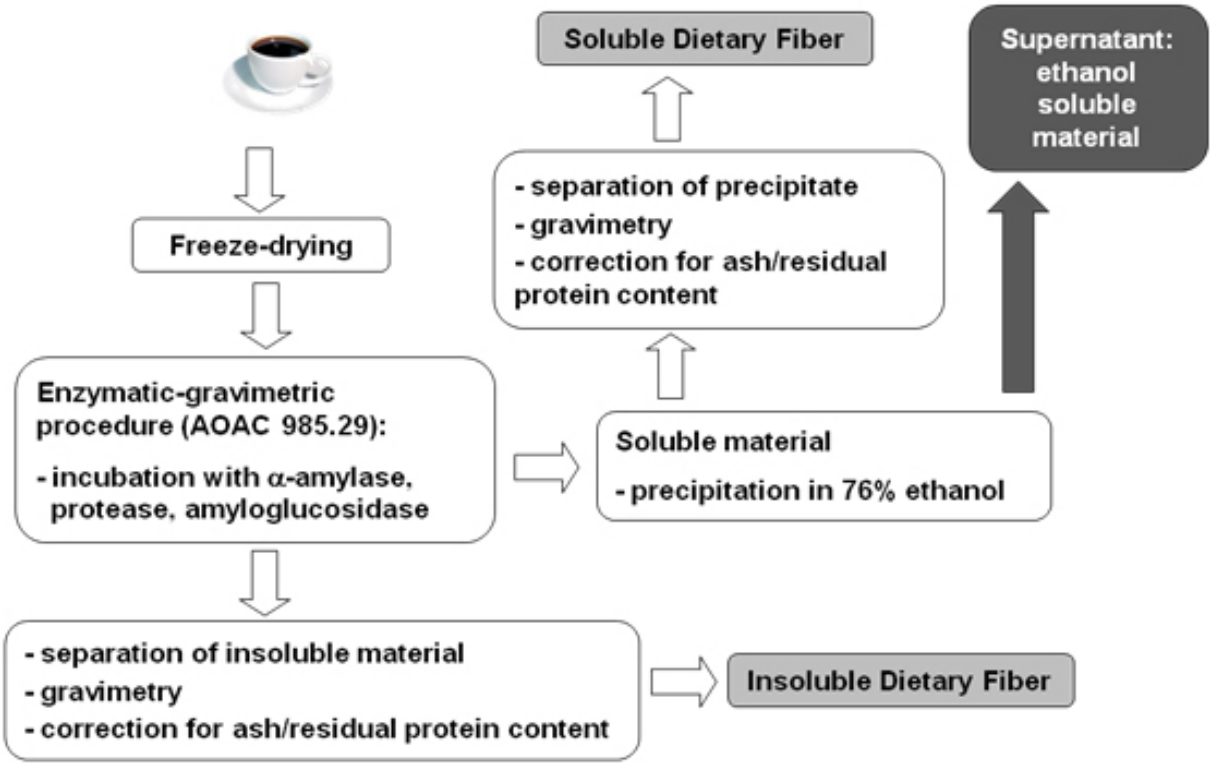

Figure 2. Determination of soluble (and insoluble dietary fiber) from coffee brews. The ethanol-soluble material is further analyzed for "fiber-like materials" as detailed below.

Chemical identification of the soluble dietary fiber demonstrated that 62% of the fiber was made up of polysaccharides with the remainder potentially composed of Maillard reaction products and other non identified substances. Analysis of the neutral monosaccharides as monomers of the coffee fiber showed a composition of 50.6% mannose, 38.9% galactose, 8.8% arabinose, 1.0% rhamnose, and 0.7% glucose. Methylation analysis revealed that galactomannans and type II arabinogalactans were present in almost equal proportions (galactomannan/arabinogalactan ratio 1.3). The galactomannans showed a low substitution degree of 4%. The arabinogalactans of the soluble coffee fiber were made up of 27% arabinose, with 75% of those being terminal units and 25% being (1→5)-linked units of the arabinan side-chains. About 25% of the galactose units were branching points in the structure of the arabinogalactans as revealed by being (1→3,6)-linked.

Coffee fiber was isolated on a preparative scale and used for fermentation with gut bacteria. Fermentation experiments were carried out under anoxic conditions at 37 ºC using cell pellets from stool samples of four healthy volunteers as a source of gut bacteria. Aliquots of the fermentation suspension were taken after 0 h, 3 h, 6 h, 12 h, and 24 h of incubation and were chemically and microbiologically analyzed. As a reference, long-chain inulin was fermented using similar conditions as applied for coffee fiber.

The optical density of the cell suspension reflecting the bacterial cell density increased over time for both coffee fiber and inulin reaching a maximum after 12 h of fermentation. Between 12 and 24 h the optical density slightly decreased for both substrates, potentially as a result of substrate depletion or bacterial toxin formation. The maximum optical density was 24% higher for inulin as compared to coffee fiber, possibly reflecting that inulin is a pure polysaccharide while coffee fiber was only made up of about 62% carbohydrates. After 24 h of fermentation 8.7% (determined as alditolacetates) or 14.7% (determined by using the phenol-sulfuric acid methodology), respectively, of the coffee fiber polysaccharides were not degraded by the fecal bacteria. Within 24 h of fermentation 93% of the polysaccharide mannosyl and galactosyl residues, but only 84% of the arabinosyl residues were utilized by the fecal bacteria. This indicates that galactomannans are more susceptible to bacterial degradation than arabinogalactans. More detailed information about the carbohydrate degradation was obtained from the methylation analysis, revealing the linkage positions of the monosaccharides in the residual polysaccharides after fermentation. Between 0 and 6 h a slight decrease of the ratio total Man*p*/4,6-Man*p*, representing the substitution of the galactomannans with galactose, was determined. This decrease indicates that within the first 6 h of fermentation unbranched regions of the galactomannans are faster degraded than the regions carrying side-chains. An increasing Man*p*/4,6-Man ratio after 12 h of fermentation, however, shows that possible hindrances due to the side chains were overcome. The overall branching degree of the galactan chains can be determined by using the (3-Gal*p* + 6-Gal*p*)/3,6-Gal*p* ratio. An increase of this ratio over the course of the fermentation reveals that side chains were easily removed from the galactans. A decreasing T-Ara*f*/5-Ara*f* with increasing fermentation time shows that single arabinosyl units linked to the galactan backbone are more quickly degraded than arabinan side chains composed of 5-linked arabinosyl units. Potential termination of these arabinans by a rhamnosyl unit might explain the unexpected stability of the arabinans towards microbial degradation.

Polysaccharide fermentation went along with the formation of the short chain fatty acids acetic, propionic and butyric acid. The short chain fatty acid concentrations after 24 h of fermentation were about 9.2 mM for coffee fiber and 10.7 mM for inulin. Acetic acid was the dominant short chain fatty acid for both fiber sources; significantly higher amounts of butyric acid were formed from inulin.

Fluorescence *in-situ* hybridization was performed to monitor changes in the relative proportions of the dominant population groups over the course of coffee fiber or inulin fermentation, respectively. Fermentation of both fiber sources led to an increase in bacteria belonging to the *Bacteroides-Prevotella* group by about 60% when compared to the control, thus becoming the dominant bacterial group. The increase of bacteria of the *Bacteroides-Prevotella* group is logical as members of the genus *Bacteroides* are known to degrade a large variety of complex carbohydrates. While the proportions of the *Clostridium coccoides-Eubacterium rectale* group decreased during the fermentation of coffee fiber, no changes were monitored for lactobacilli. Also, no bifidogenic effect was found for either coffee fiber or long-chain inulin.

As it was known that polysaccharide extractability from coffee beans depends on several factors, the influence of factors such as coffee variety, roasting and grinding degree, and brewing procedure on dietary fiber contents and composition of the coffee brews was investigated. It was demonstrated that drip brew coffee from an arabica coffee (Colombia, medium roast, medium ground, 50 g powder/L) contained significantly more soluble dietary fiber (350 mg/100 mL) than a comparable robusta coffee (India) (302 mg/100 mL). Studying the mentioned arabica coffee, using, however, different degrees of roast, it was shown that the

dietary fiber content increased from a light roasted to a medium roasted coffee (289 vs. 350 mg/100 mL). Further roasting (dark roast) did not further increase the soluble dietary fiber content of the coffee beverage. The influence of the brewing procedure on the dietary fiber content was also studied by using the arabica coffee mentioned before (medium roast). Distinct differences were found for drip brewed coffees of different preparation volumes. By increasing the preparation scale (1 L, 10 L, or 20 L of the beverage were prepared by using always the same coffee water ratio of 50 g/L) the dietary fiber contents were significantly decreased (350 vs. 299 vs. 259 mg/100 mL). Preparation of 10 L and 20 L of the beverage was performed in a coffee urn, a large capacity coffee maker often found in gastronomy. The reduced contact times of coffee powder and water due to exceptionally short brewing times in the coffee urn (10 min for 10 L, 14 min for 20 L) are likely to be responsible for the reduced extraction of coffee fiber, especially fiber polysaccharides. Also, by using a French press system lower contents of soluble dietary fiber were extracted. The extraction time (brewed for either 3 or 6 min) did not significantly affect the fiber content (263 and 268 mg/100 mL, respectively). Highest amounts of soluble fiber were obtained in the brew by using a stovetop espresso maker (Italian moka) (375 mg/100 mL). Different from our hypothesis, the degree of grinding had not a statistically significant effect on the soluble dietary fiber contents, neither in a drip brew coffee beverage nor in an Italian moka. Although the higher surface area of a finer ground coffee should increase the extraction of polysaccharides and potential other fiber compounds from the coffee powder, we did not see this effect in our studies.

Variations in the soluble dietary fiber contents of the coffee brews also went along with different galactomannan/arabinogalactan ratios in the fiber as well as with structural changes in the polysaccharides. As a general trend for the different extraction procedures it was observed that higher contents of soluble dietary fiber were accompanied by higher proportions of galactomannans in the fiber. Also, an increase in the galactomannan contents went along with a decrease in the mannan substitution degree, demonstrating that less substituted galactomannans are harder to extract than galactomannans carrying more galactose side-chains.

The isolation procedure of soluble dietary fiber involves the precipitation of water-soluble material (“soluble dietary fiber”) by using 76% (v/v) ethanol (Figure 2). However, it is well known that oligosaccharides but also certain polysaccharides may escape precipitation in ethanol. Thus, the ethanol soluble material was further analyzed for compounds that potentially add to the dietary fiber content of coffee brews. The ethanol soluble material went through the process of starch and protein digestion as mandatory steps for the soluble dietary fiber preparation. Generally, compounds with molecular weights >1 kDa are assumed to be excluded from passive absorption in the small intestine and could potentially add to the fiber content of coffee brews. Dialysis with a molecular weight cut-off (MWCO) of 2 kDa was used to separate potential fiber compounds from low-molecular weight coffee brew constituents. The ethanol-soluble high molecular weight (>2 kDa) material (HMWM) potentially adds between 0.11 and 0.19 g/100 mL to the fiber contents of coffee brews (prepared from medium ground arabica coffee (origin Colombia)), depending on the degree of roast of the coffee used. While an increase of the ethanol-soluble HMWM was noticed when light and medium roasted coffees were compared, no further increase was detected comparing medium and dark roasted coffees. Thus, a similar trend was observed as for the soluble dietary fiber contents. The ethanol-soluble HMWM of the medium roasted coffee, however, only contains about 13% percent carbohydrates. The monosaccharide composition demonstrated that arabinogalactans dominate this fraction (ca. 45% galactose, 35% arabinose, and 8% rhamnose, but only 5% mannose).

For a more detailed structural characterization of the ethanol-soluble HMWM and to perform microbiological studies, the material was isolated from the medium roast coffee on a larger scale by using ultrafiltration (MWCO 3 kDa) instead of dialysis. The monomer composition of the carbohydrates in this fraction was slightly different than in the dialysis fraction; however, the average yield of this fraction was comparable to the dialysis fraction. Also, arabinose and galactose contributed about 80% to the carbohydrates of the ultrafiltration ethanol-soluble HMWM just as for the dialysis fraction. Methylation analysis was performed by using the ethanol-soluble HMWM from ultrafiltration and the data were compared to those from the polysaccharides of the soluble dietary fiber. It was demonstrated that the arabinogalactans of the ethanol-soluble HMWM were characterized by lower galactose/arabinose ratios and that the arabinogalactans were less branched as indicated by a higher (3-Gal*p* + 6-Gal*p*)/3,6-Gal*p* ratio (2.7 vs. 1.9). Also, the arabinogalactans from the ethanol-soluble HMWM show a lower T-Ara*f*/5-Ara*f* ratio (1.5 vs. 3.6), indicating longer arabinan stretches in these arabinogalactans. The ethanol solubility of these arabinogalactans might be related to these structural differences when compared to the soluble dietary fiber arabinogalactans; however, association with melanoidins might also be responsible for preventing them from being precipitated out by using 76% (v/v) ethanol. The molecular weights of the ethanol-soluble HMWM are extended over a wide range with the majority of compounds having molecular weights either higher than 100 kDa or in between 3 and 10 kDa. Other characteristics of this fraction were a protein content of 20%, determined as the sum of amino acids, a non-amino acid nitrogen content of 2%, and high absorptions of the solutions at both 405 and 280 nm (three to four times higher than the absorptions of comparably concentrated solutions of the soluble dietary fibers).

As the ethanol-soluble HMWM contains about 20% protein the potential contribution of this fraction to the dietary fiber content of coffee brews should be accordingly reduced. Proteins are per definition not part of the dietary fiber complex as, for example, fermentation of proteins in the large intestine can lead to the formation of potentially cytotoxic compounds such as ammonia.

The ethanol-soluble HMWM was subjected to an *in-vitro* fermentation experiment with human fecal bacteria. As also described above for the soluble coffee dietary fiber the optical density over the course of the fermentation increased, indicating the utilization of the ethanol-soluble HMWM by fecal bacteria. However, the increase was modest if compared to the increase of the optical density for the fermentation of the soluble coffee dietary fiber. As the ethanol-soluble HMWM contains less carbohydrates than the soluble coffee fiber, this points out that non-carbohydrate components are less readily utilized. The fermentation of the ethanol-soluble HMWM was accompanied by the formation of the short-chain fatty acids acetic acid, propionic acid, and butyric acid in a ratio of about 68:21:11. Formation of these short-chain fatty acids is most likely due to the fermentation of the carbohydrates in this fraction, which were degraded to 45% after 24h and 66% after 48 h. Although the carbohydrates of this fraction were thus extensively degraded, they were less efficiently utilized than the carbohydrates in the soluble coffee fiber (see above). The fermentation process of the carbohydrates in the ethanol-soluble HMWM was followed by using the two-dimensional HSQC NMR-experiment, demonstrating the utilization of the carbohydrates in this fraction and also showing different degradation rates for the various and differently linked monomers of the polysaccharides. Also by using the HSQC experiment, we demonstrated that structural components of the ethanol-soluble HMWM, which neither represent carbohydrates nor proteins, were degraded by the bacteria. It is assumed that these unknown components represent Maillard reaction products formed during roasting.

Again, fluorescence *in-situ* hybridization was performed to monitor changes in the relative proportions of the dominant population groups over the course of the fermentation (0, 24, and 48 h) of the ethanol-soluble HMWM. The proportion of members of the *Eubacterium rectale-Clostridium coccoides* group and of bifidobacteria decreased, whereas the proportion of the members of the *Bacteroides-Prevotella* group was comparably stable over 48 h of incubation. These results hint that members of the *Bacteroides-Prevotella* group are able to utilize the ethanol-soluble HMWM as a substrate.

In conclusion, we demonstrated that coffee beverages are sources for soluble dietary fiber and may additionally add fiber-like materials which escape the analytical determination of soluble dietary fiber. The carbohydrates of both the soluble coffee fiber but also of the ethanol-soluble HMWM are well fermentable by human fecal bacteria, leading to the formation of short-chain fatty acids. Maillard reaction products also contribute to coffee fiber and are likely, at least partially, fermentable, too. The contents of soluble dietary fiber from coffee brews can be influenced by factors such as coffee variety, degree of roast, and brewing procedure. These data clearly indicate that fiber intake from coffee beverages should not be neglected when average daily intakes of dietary fiber are calculated.

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Biological Activities of Arabinogalactan-proteins Isolated from *Coffea arabica* Freeze Dried Instant Coffee

L. NAVARINI[1], F. SUGGI-LIVERANI[1], L. PRISENŽŇÁKOVÁ[2], E. PAULOVIČOVÁ[3], G. NOSAL'OVÁ[2], M. MATULOVÁ[3], P. CAPEK[3], D. CORADINI[4], C. CASARSA[5]

[1]illycaffè S.p.A., Via Flavia 110 – Trieste, Italy
[2]Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Sklabinska 26, 037 53 Martin, Slovakia
[3]Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dubravska 9, 845 38 Bratislava, Slovakia
[4]Department of Work Medicine "Clinica del Lavoro L. Devoto", Section of Medical Statistics and Biometry "G.A. Maccacaro", University of Milano, Milano, Italy
[5]Department of Life Sciences, University of Trieste, Trieste, Italy

SUMMARY

The *in vitro* proliferative activity of two arabinogalactan-proteins (AGP1 and AGP2), isolated from pure Arabica freeze dried instant coffee and differing in molecular mass (5.400 and 19.600), were studied on human tumor HepG2 (hepatocarcinoma) and CaCo2 (colon carcinoma) cell lines, by WST-1 assay. After a 7-day treatment HepG2 cells growth was statistically ($P<0.01$) reduced by 1 mg/mL AGP2 while AGP1 was not active. On the contrary, CaCo2 cells were significantly inhibited only by the highest concentration of AGP1 (1 mg/ml), with a cell growth decrease of 18.5% compared to control.

Ex vivo experiments using Balb/c mouse splenocytes model showed an active immunomodulation of splenocytes of AGP1, the compound with the lower molecular mass, at the concentration range of 1 to 100 μg/mL. A concentration-dependent TNFα, IFNγ and IL-2 cytokine secretion was observed using ELISPOT and/or ELISA methods. Preliminary results on AGP1 revealed the effective acceleration of pro T_H1 cytokine production by stimulated mouse splenocytes and indicated an immunomodulating activity.

When tested on the citric acid-induced cough reflex AGP1 showed a significant cough-suppressing effect, which started 30 min after its application and lasted during the entire experiment course (5 h). Preliminary comparative tests with codeine, a drug commonly used in clinical practice, revealed that antitussive activity of coffee AGP1 was higher than that of opioid receptors agonist codeine without any effect on airways smooth muscle.

INTRODUCTION

Arabinogalactan-proteins (AGPs) are complex glycoproteins of high molecular mass belonging to structural components of the plant extracellular matrix. It is assumed that they play important roles in many life functions of plant cells, including cell growth and development, programmed cell death. In addition, they are presumably involved in cell interactions as signalling molecules even though their precise biological functions have not yet been determined (Brecker et al., 2005).

It is well known that instant coffee powder, prepared from coffee beans, contains carbohydrate polymers among which AGP moieties represent a relevant fraction. Recently characterized from pure Arabica soluble coffee) (Wolfrom and Anderson, 1967; Capek et al., 2009; 2010), AGPs have been found to have a relatively lower molecular mass in comparison with native ones due to drastic industrial processes used for preparation of instant coffee powder and to be composed of galactose (85.0-85.2%) and arabinose (8.2-8.3%) with small amounts of accompanying sugars and low protein content (1.6-1.7%). They contain a β-1,3-linked galactose backbone branched at C-6 by side short galactose and galactose-arabinose chains. Coffee AGPs represent a divers group of natural polymers which are regularly consumed by human being.

As it has been reported that many polysaccharides showed a great variety of biological activities including antitumor, immunostimulating, anti-inflammatory, anticoagulant and antitussive effect (Capek et al., 2003; Prisenžňáková et al., 2009; Nosáľová et al., 2006), aim of present study was to verify the possible proliferation activity of AGPs on two human tumor cell lines, namely HepG2 (derived from a hepatocarcinoma) and CaCo2 (derived from a colon carcinoma), as well as their immunomodulating and antitussive activities on splenic cells in term of tumor necrosis factor α (TNF-α), interferon γ (IFN-γ) and interleukin 2 (IL-2) production, and on guinea pigs reflex and airway smooth muscle activity.

TNF-α regulates the cytokine and chemokine gene expression in several cell types (T- and B-lymphocytes, macrophages, and NK cells) involved in host response to infection by triggering a cascade of events among which fever induction, acute-phase protein release and endothelial cell activation.

Activated monocytes or macrophages represent the primary source for TNF-α especially after priming by IFN-γ which is the key regulator of other pro-inflammatory cytokines including IL-1α, IL-6 and IL-8. (Locksley et al., 2001; Old, 1985) IFN-γ, in fact, has potent immunoregulatory effects on a wide range of immune competent cells, it activates macrophages and NK cells, blocks T_H2 cell formation, enhances the production of complement, the binding of IgG_{2a}, and cytotoxic activity of NK cells (Belardi, 1995; Trinchieri and Perussia, 1985). IFN-γ has also demonstrated antiviral and microbicidal function (Sechler et al., 1988).

Once expressed, TNF-α promotes inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, it acts as a chemotactic agent for monocytes and it activates phagocyte killing mechanisms (via increased $NO^{2-}/O^{2-}/H_2O_2$) (Vassalli, 1992).

IL-2 is a cytokine synthetized and secreted primarily by T-helper lymphocytes, that have been activated by stimulation with certain mitogens or by interaction with antigen/MHC complexes on the surface of antigen–presenting cells (Hatakeyama and Taniguchi, 1990).

MATERIALS AND METHODS

Raw material

Dark brown freeze-dried instant coffee powder prepared from 100% *Coffea arabica* blend was used for isolation of two arabinogalactan-proteins (AGP1 and AGP2).

Cell lines and animals

Human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma cell lines (CaCo2) were purchesed from the American Type Culture Collection (LGC Standards s.r.l., Milan, Italy). Female Balb/c mice aged 8-12 weeks were obtained from the breeding facility VELAZ, Prague (Czech Republic). The adult male Trik strain guinea pigs, weighing 150-350 g and used in the experiments, were obtained from Department of Experimental Pharmacology Toxicology, Slovak Academy of Sciences, Dobra Voda, Slovakia. Animal experiments were conducted in compliance with GLP and OECD guidelines, according to the ethical guidelines issued by the Research Base of Slovak Medical University, Institute of Preventive and Clinical Medicine (Bratislava, Slovakia). The animals were housed in approved animal holding facility and had water and food ad libitum. The experiments were carried out after prescribed adapting period to laboratory conditions.

Arabinogalactan-proteins (AGP1 and AGP2) have been isolated from dark brown freeze-dried instant coffee powder of *Coffea arabica* blend according to already described procedure (Wolfrom and Anderson, 1967). AGP1 is the same sample named as AG1 in Capek et al. (2009) and named AGP in Capek et al. (2010). AGP2 chemical composition is very close to that of AGP1 but it contains more protein (3.9%) and 2.6% of rhamnose not present in AGP1.

In vitro experiments

HepG2 and CaCo2 cell lines were maintained respectively in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Milan, Italy) supplemented with 4.5 g/L glucose, 10% heat inactivated fetal bovine serum (Sigma, Milan, Italy), and in Eagle's Minimum Essential Medium (Gibco) supplemented with 20% heat inactivated fetal bovine serum (Sigma, Milan, Italy) and 2 mM l-glutamine. The cells were grown in 75 cm^2 T-flasks (Cellstar, Sigma) in an incubator whose environment was maintained at 37 °C along with 5% CO_2. Medium was changed twice weekly. HepG2 cells were passaged twice a week, while CaCo2 cells once a week. Confluent monolayers were harvested by washing the cells with Dulbecco's Phosphate saline (DPBS, Sigma) followed by trypsin-EDTA solution (Sigma).

Harvested cells were seeded into 96-well plate (5×10^4 cell/well) and allowed to attach for 24 h. The seeding medium was then removed and replaced by a medium supplemented with AGP1 and AGP2 (range 1 μg/mL - 1 mg/mL), 1mg/mL (+)-arabinogalactan from Larch wood (ARBG, Fluka) or growth medium as control (CTRL) for 1 week.

The effect of compounds on cells growth was measured by WST-1 reagent (Roche, Milan, Italy) according to manufacturer's instructions using a microplate reader at 450 nm (605 nm reference filter). WST–1 (4-(3-4-iodophenyl)-2-(4-nitrophenyl)- 2H-5-tetrazolio)-1,3-benzenedisulfonate) is a tetrazolium salt that form soluble formazan products by active mitochondrial dehydrogenase of living cells. The optical density (O.D.) value of study groups was divided by the O.D. value of control and calculated as percentage of control. Results are presented as O.D. ratio.

Preparation, treatment, and analysis of splenic cells

Immunomodulating activity was investigated using ELISPOT or ELISA method. Briefly, spleens were removed under sterile condition and splenocytes were carefully washed out from spleens with complete RPMI-1640 (Sigma, Stockholm, Sweden) containing 10% fetal calf serum (FCS) (Gibco, Berlin, Germany). Washing was performed by centrifugation

(1500 g/5min, 4 °C), cell pellet was adjusted by growth medium approx to $4x10^6$ cell/mL and 100 µL aliquots were specifically stimulated with AGP1 (concentrations 1-100 µg/mL) in 12-well tissue culture plates (Sarstedt, USA). As positive control, polyclonal stimulation with Concanavalin A was performed. Plates were incubated in humidified 37 °C CO_2 incubator for 24h. After then, $5x10^4$ cells were plated for the enumeration of TNF-α and IL-2 producing cells onto anti-mouse TNF-α or IL-2 precoated PVDF-backed microplate. Following steps and procedures were according to manufacturer's recommendation (ELISPOT Mouse TNF-α, R&D Systems, UK and Mouse IL-2 ELISPOT, BenderMedSystems, Austria).

Polyclonal stimulation

Quantitative evaluation of spots and enumeration of TNF-α and IL-2 producing cells was performed via KS ELISPOT 4.10 running under AxioVision software using Imager A.1 Microscope (Zeiss, Germany).

Quantitative detection of mouse TNF-α and mouse IFN-γ after specific stimulation of isolated splenocytes in culture media was performed by enzyme-linked immunosorbent assay Mouse TNF-α Instant ELISA and Mouse IFN-γ Instant ELISA (BenderMedSystems, Austria). All the data are shown as means±SEM and were analyzed using ANOVA and multiple comparison tests. Changes were considered statistically significant at P-value < 0.05.

Antitussive activity tests as well as the airway smooth muscle reactivity were effected according to Šutovská et al. (2009).

RESULTS AND DISCUSSION

The *in vitro* activity of the isolated AGP1 and AGP2 arabinogalactan-proteins (with a molecular mass of 5.400 and 19.600, respectively) were studied on two human tumor cell lines HepG2 (hepatocarcinoma) and CaCo2 (colon carcinoma), by WST-1 assay. In this study, various concentrations of arabinogalactan-proteins, ranging from 1 µg/mL to 1mg/mL, were used. As shown in Figure 1A, after a 7-day treatment HepG2 cells growth was significantly reduced ($P<0.01$) by 1 mg/mL AGP2. However, this decrease appears to be very modest (-14%) compared to control. Interestingly, CaCo2 cells growth (Figure 1B) was significantly inhibited only by the maximum concentration of AGP1 (1 mg/mL), with a decrease of 18.5% compared to both control and ARBG, and 21% with respect to the same dose of AGP2 ($P<0.001$). No specific antiproliferative/cytotoxic effect was observed by WST1 assay using the concentrations range 0.25 - 1 mg/mL on both cell lines (data not shown).

The comparison between the behaviour of larch wood arabinogalactan (ARBG) and that of coffee AGPs is very interesting. In facts, it is well known that larch wood arabinogalactan structure is characterized by a β-D-(1→3)-galactopyranan main chain, comprising about one-third of the molecule, with nearly every main chain residue (1→6)-linked to a side chain consisting of single sugar residues, dimeric structures or more complex oligosaccharides (Prescott et al., 1995; Ponder and Richards, 1997). This polymer does not contain protein moiety and it has a maximum molecular weight of about 37.000. As far as we know, larch wood arabinogalactan does not show antitumor activity. In comparison to ARBG, both AGP1 and AGP2 are characterized by a β-D-(1→3)-galactopyranan main chain much less substituted (about 10-15 times more →3)-Gal*p*-(1→ linkage). Triple helical conformation adopted in aqueous solutions by some polysaccharides is regarded as an important structural feature for their antitumor activity. This is the case of (1→3)-β-glucans (Zhang et al., 2007).

It has been shown that (1→3)-β-galactan can assume the same triple-helical structure of (1→3)-β-glucans and that the galactan triple helix can also accommodate a disaccharide Gal-(1→6)-Gal substituted at the 6th position in every Gal unit in the main chain (Chandrasekaram and Janaswamy, 2002). The very modest activity detected for both AGP1 and AGP2 could be related to these structural features, whereas the different response for the two cell lines may reflect chemical and physico-chemical differences of the two coffee polymers.

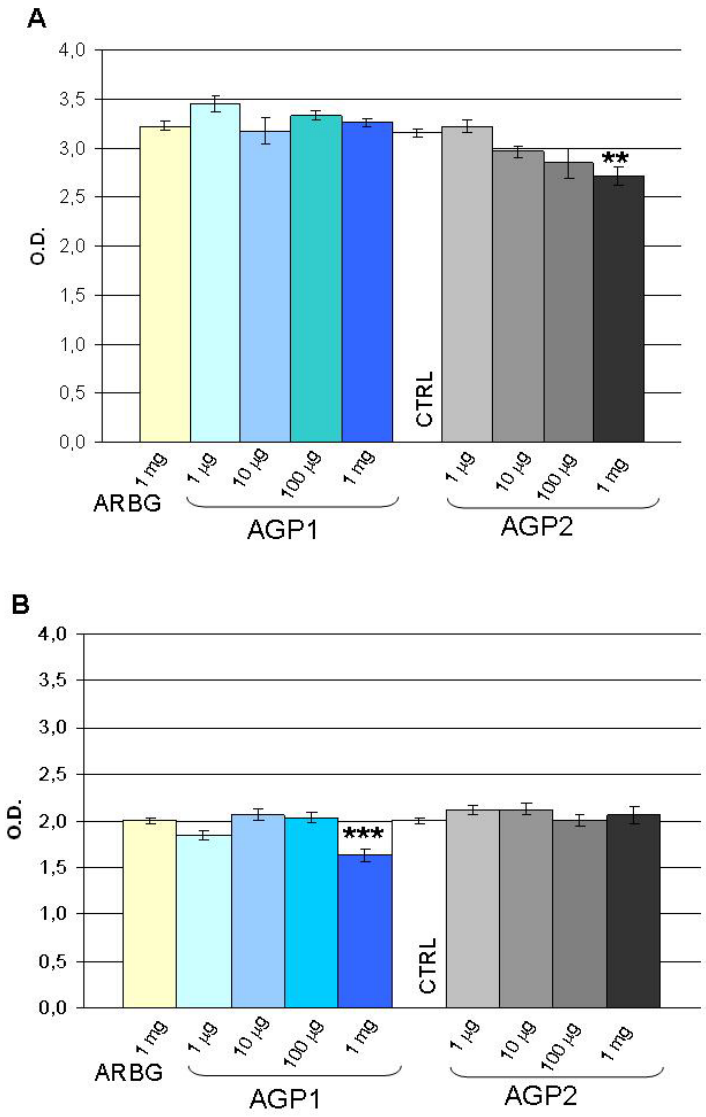

Figure 1. AGP1 and AGP2 activity on the growth of HepG2 (A) and CaCo2 (B) cell lines. Values are means±SEM (n = 8). ** P < 0.01 and *** P < 0.001 compared to control (CTRL).

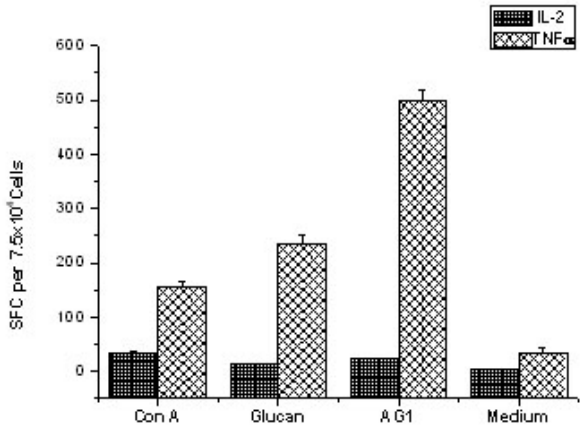

Figure 2. Enumeration of IL-2 and TNF-α secreting cells among splenocytes from mice treated with 100 µg/mL of AGP1, Concanavalin A (Con A) or yeast β-D-glucan. The results are expressed as mean ± SEM.

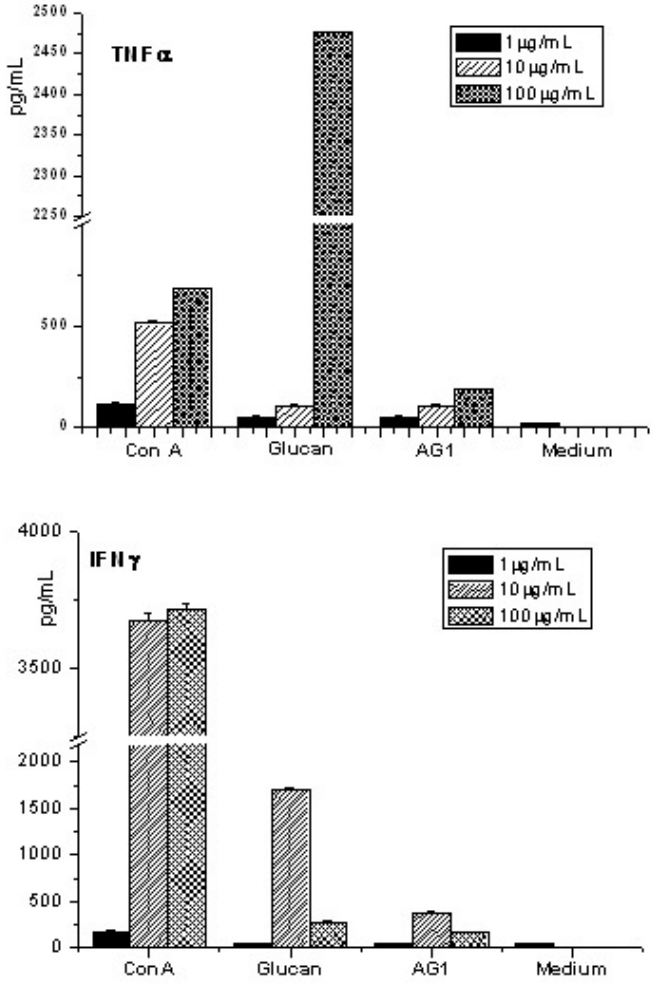

Figure 3. Concentration-dependent pattern of cytokine release by murine splenic cells induced by AGP1, Concanavalin A or yeast β-D-glucan. The results are expressed as mean ± SEM.

When the influence of AGP1 (molecule mass 5.400) on some mediators of immune system was investigated on mouse splenocytes it was observed an effect on *ex vivo* TNF-α and IL-2 production similar to that of β-D-glucan (*C. albicans* serotype A) and mitogen Concanavalin A. Maximal efficacy was revealed with 100 μg/mL AGP1 (Figure 2). The maximal secretion of cytokines TNF-α and IFN-γ were observed with AGP1 1 mg/mL. The TNF-α levels secreted in the medium after specific stimulation represented 7.6% and 24.7% of those observed after the stimulation with either β-D-glucan (immunomodulator and biological response modifier) or mitogen Concanavalin A, respectively. Conversely, the secretion of IFN-γ, induced by AGP1 represented ~ 60% that of β-D-glucan and over 4% that of Concanavalin A (Figure 3).

When coffee powder AGP1 was tested on the citric acid-induced cough reflex in guinea pigs and reactivity of airways smooth muscle *in vivo* conditions, a significant cough-suppressing effect of AGP1, which started 30 min after its application and it lasted during the entire experiment course (5 hours) it was observed. Preliminary comparative tests with codeine, a drug commonly used in clinical practice, revealed that antitussive activity of coffee AGP1 was higher than that of opioid receptor agonist codeine (Figure 4). Moreover, it was found that the reactivity of airway smooth muscle was not affected by coffee AGP1 (Figure 5) similarly as codeine.

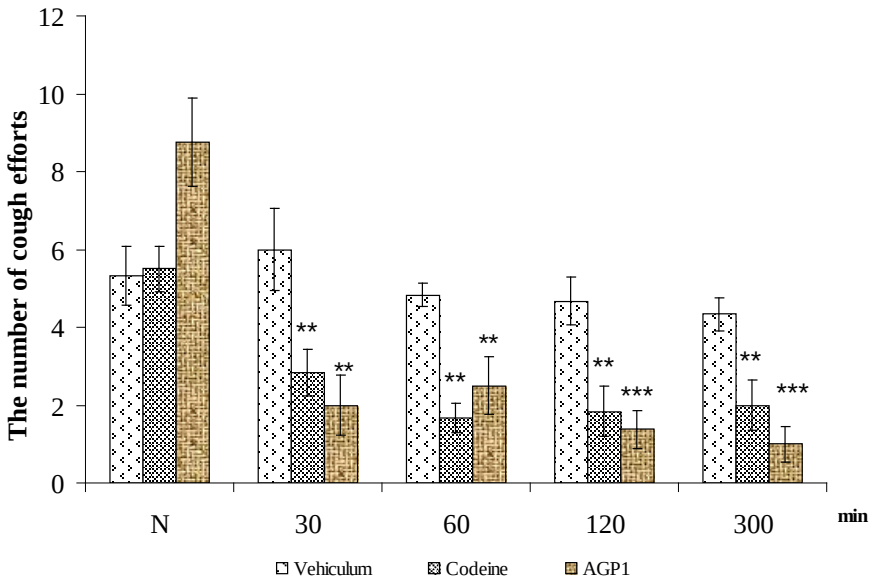

Figure 4. The cough suppressive effect of AGP1 polysaccharide in comparison to codeine and vehiculum. * p< 0.05; ** p< 0.01 and *** p < 0.001 vs N values (t-test).

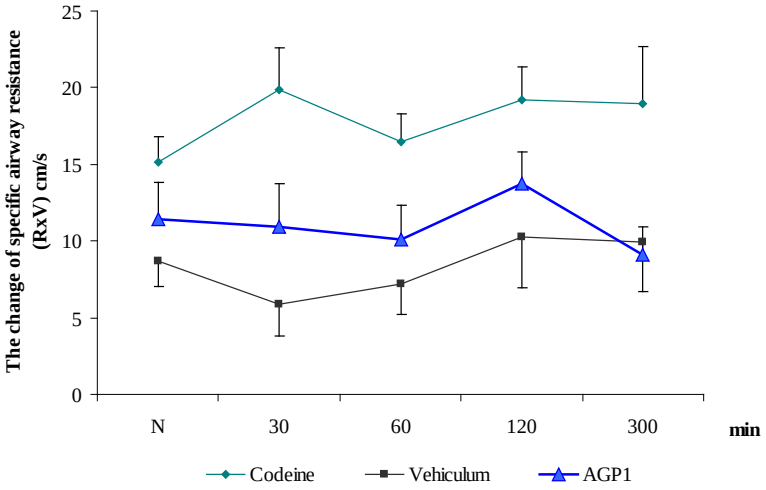

Figure 5. The influence of AGP1 (blue) on the value of specific airway resistance (RV) in comparison with codeine (grey) and vehiculum (black).

CONCLUSION

Biological tests on two coffee arabinogalactan-proteins (AGP1 and AGP2) indicate that: 1. at the highest concentration (1 mg/mL) the compounds were able to reduce tumor cells proliferation; 2. TNF-α, IL-2 and IFN-γ were closely related with the reactivity of AGP1 and its immunomodulating properties. In particular, AGP1 seems to be a good inductor of both pro-inflammatory cytokine TNF-α and IFN-γ and less potent in TNF-α induction in comparison to β-D-glucan, a strong immunomodulator and biological response modifier. According to evident induction of TNF-α, IL-2 and IFN-γ, pro-T_H1 polarization and bioimmunological efficacy of AGP1 could be proposed, with emphasis on cellular immunity; 3. AGP1 showed a relatively high efficacy on the citric acid induced cough reflex in guinea pigs test system. Noteworthy, AGP1 antitussive activity was higher then that of codeine, centrally acting antitussive agent widely used in clinical practice. Besides, reactivity of airway smooth muscle was not affected by coffee arabinogalactan-protein indicating that arabinogalactan-protein did not provoke bronchoconstriction, a very unpleasant side effect showed by many cough suppressive agents.

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Lipids in Coffee Brews - Impact of Grind Size, Water Temperature, and Coffee/Water Ratio on Cafestol and the Carboxylic Acid-5-Hydroxytryptamides

S. BUCHMANN, A. ZAHM, I. KÖLLING-SPEER, K. SPEER

Technische Universität Dresden, Food Chemistry, Germany

SUMMARY

The diterpene cafestol, the carboxylic acid-5-hydroxytryptamides (C-5-HT) and other lipid constituents may pass into the beverage during the preparation of the coffee brew. Cafestol is responsible for an increase in the serum cholesterol level, and the C-5-HT are known to cause stomach irritation as well as gastric lesions, therefore, conditions for reducing these components in the brew are of interest. In this study, the influence of the particle size, the water temperature, and the water/coffee ratio on the transfer of the lipids into the brew were studied on Fresh Brew coffee and Espresso, due to its increasing popularity.

Exemplarily, a MAAS SL 1000 vending machine was used to prepare the different Espresso and Fresh Brew coffees. The impact of the preparation parameters depends on the brewing method.

It was shown that in addition to the particle size, the coffee/water ratio was the most important factor influencing the cafestol and the C-5-HT contents in Espresso. The water temperature impacts only the extraction yields of cafestol and C-5-HT in Fresh Brew coffees. By varying the preparation parameters, it is possible to reduce the cafestol and C-5-HT content in Espresso from 2.7 to 0.9 mg/cup and from 290 to 40 µg/cup while in Fresh Brew coffees the contents are reduced from 2.6 to 0.7 mg/cup and from 135 to 25 µg/cup, respectively.

INTRODUCTION

In Germany, coffee consumption increased to 150 L per capita in 2009. One reason was the rising popularity of Espresso and Espresso preparations such as Latte Macchiato and Cappuccino. Lately, the Consumentenbond of the Netherlands reported that coffee prepared in vending machines might increase the serum cholesterol level. The discussion was resumed over the physiological effects of coffee (Consumentenbond Automatenkoffie, 2007). The results of several working groups indicated that the diterpenes cafestol and, to a lesser extent, kahweol were responsible for raising the serum cholesterol level (Urgert and Katan, 1996; Van der Wouw et al., 1994). The intake of five cups of coffee with a content of 1.5 mg cafestol raised the cholesterol level by 0.1 mmol/L. The most influencing factor concerning the content of lipids, and especially diterpenes as a part of the coffee oil in the beverage, is the brewing method (Sehat et al., 1993).

Concerning the physiological effects of coffee consumption, the so called carboxylic acid-5-hydroxytryptamides (C-5-HT), as part of the lipid fraction, must also be considered (Wurziger and Harms, 1968). The group of the C-5-HT is located in the waxy layer of the outermost part of the bean and is known to cause stomach irritations as well as gastric lesions (Rösner et al.,

1971; Fehlau and Netter 1990). Until now, only little is known on the C-5-HT content in coffee brews (Zahm and Speer, 2008).

Due to the negative physiological effects, conditions for reducing these components in the coffee brew are of interest. As a result of the increasing market share of vending machines, the aim of our research was to study the cafestol content as well as the C-5-HT levels in differently prepared Espresso and Fresh Brew coffees.

MATERIALS AND METHODS

Commercially roasted Arabica coffee was ground using an industrial centrifugal mill (VTA6S, Stahwert Mühlenbau, Germany). The particle size distribution was determined by employing laser diffraction particle size analyzer (LS 13320**,** Beckman Coulter).

The coffee brews were prepared using a MAAS SL 1000 vending machine (Espresso and Fresh Brew unit). In addition to the grind size, the variable preparation parameters were the water temperature (80, 95 and 110 °C), the amount of coffee, and the amount of water. Table 1 gives a summary of all the preparation parameters,

Table 1. Preparation parameters.

Parameter	Fresh Brew	Espresso
Temperature	80/95/110 °C	
Coffee	8/9/10 g	6.5/7.5/8.5 g
Water	70/125/180 ml	40/65/90 ml

In this particular vending machine, the Espresso is brewed in counter-current extraction. At first, the coffee powder is filled into the espresso unit and compressed. Subsequently, the hot pressurized water extracts the coffee powder (max. 12 bar) within about 30 seconds.

In contrast to the Espresso, the Fresh Brew is made without pressure. The coffee powder is loosely filled into the unit, and afterwards the defined volume of hot water is added. After about 10 seconds of static extraction, the brewed coffee is backed out with an air current.

The coffee brews were lyophilised immediately. The free and esterified cafestol contents were analysed according to DIN 10779 (1999). Briefly, the dry residues were saponified and then extracted with liquid-liquid extraction. For quantification, an HPLC system with UV-detection was applied. The results are given as mg cafestol per cup.

The determination of the C-5-HT content was based on the published literature (Zahm and Speer, 2008; Hinkel, 2009). Briefly, the lyophilised coffee brew was extracted by means of accelerated solvent extraction (ASE 200, Dionex). Afterwards, the extract was cleaned up by means of automated SPE. The quantification was accomplished by RP-HPLC equipped with a fluorescence detector. The total C-5-HT content per cup was calculated as the sum of the single C-5-HT. As internal standard, behenic acid-5-methoxytryptamid was used.

Statistic screening designs were employed to optimise the extraction parameters and to evaluate the main effects, interaction effects, and quadratic effects. The optimal and recommended operation conditions were attained by using a Box–Behnken design.

The Box–Behnken design was specifically selected since it requires fewer runs in the case of three or four variables than an on-factor-at-a-time-experiment. This cubic design is characterised by a set of points lying at the midpoint of each edge of a multidimensional cube and the centre point replicates (n = 3). Altogether, 6 models for each grinding grade were developed with the amount of coffee, water temperature, and water amount as variables. The coefficient for the determination (R^2) of all the models was above 0.95, indicating that the models adequately represented the actual relationship between the parameters chosen. Also, the results of the error analysis indicated that the lack of fit was insignificant ($p > 0.05$). 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

To ensure comparable conditions, one charge of a roasted Arabica coffee was used throughout this study. The cafestol level was determined at 4.6 g/kg, and the total C-5-HT level was 750 mg/kg. The roasted Arabica coffee beans were ground with a centrifugal mill at three settings. The obtained charges were analysed in regard to the particle size distribution. Obviously, there were only slight differences between finely-ground and medium-ground coffee. The particle size distribution maxima were at 375 and 650 µm, respectively. In contrast, coarsely-ground coffee had a wider range in its particle size distribution with a maximum at 1150 µm (Figure 1).

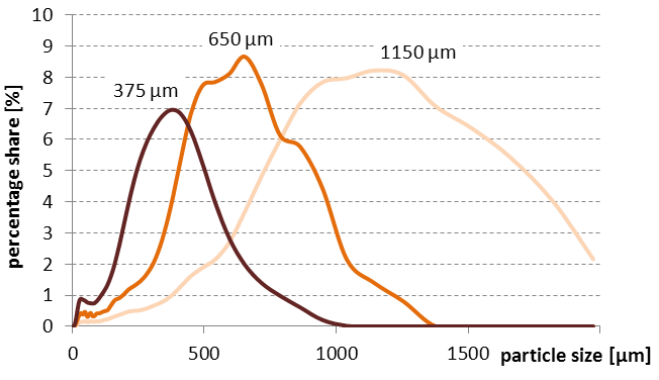

Figure 1. Particle size distribution of the ground coffee.

A typical HPLC chromatogram of the major diterpenes kahweol and cafestol obtained from an Espresso is presented in Figure 2. Also, a fluorescence chromatogram of all the C-5-HT is shown. In roasted as well as in green coffee the major constituents were arachidic- (1), behenic- (2) and lignoceric acid-5-HT (3).

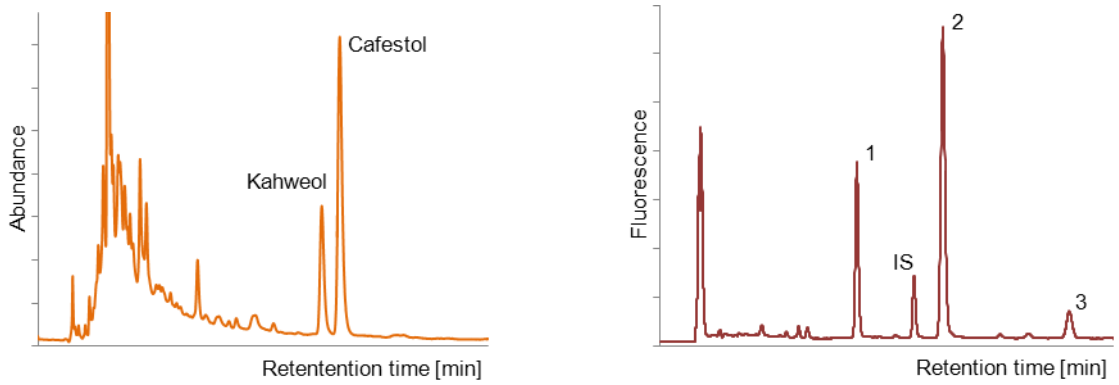

Figure 2. HPLC Chromatogram of cafestol (left) and C-5-HT (right) in coffee brew.

Espresso

The impact of the grind size was studied on an Espresso prepared with 7.5 g coffee and 65 ml water at 95 °C (Figure 3). The finer the particle size, the higher was the extraction yield. Regarding the cafestol content, especially the difference between the medium and the coarse grinding grade was remarkable. A decrease from 2 mg/cup to 0.6 mg/cup was observed. The total C-5-HT content showed the most differences between finely-ground and medium-ground coffee. This fact may be due to the allocation of the compounds. Cafestol is distributed through the whole coffee bean whereas the group of the C-5-HT is located in the waxy layer on the surface of the coffee bean. However, for Espresso, the coarsely-ground coffee was inapplicable. Therefore, the following studies were carried out with the grinding grades medium and fine.

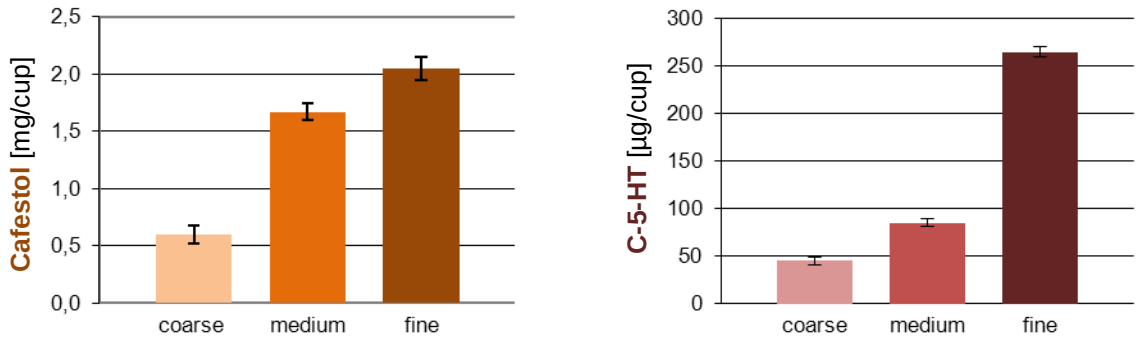

Figure 3. Impact of grinding grade on the cafestol and C-5-HT content in Espresso.

The correlation between the water temperature, the cafestol, and the C-5-HT content was studied on an Espresso prepared with 65 ml water and 7.5 g coffee (Figure 4). The temperature hardly affected the cafestol and the total C-5-HT yields of the coffee brews.

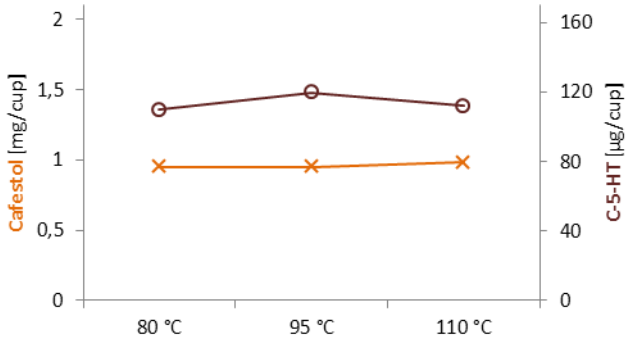

Figure 4. Impact of water temperature on cafestol (x) and total C-5-HT (o) level in Espresso.

Exemplarily, in Figure 5, the 3D response surface plot for the medium grind size is presented. With the increase in the amount of coffee, the cafestol and C-5-HT yield rose almost linearly. The extraction yields reached a maximum, especially, with a simultaneous increase in the water.

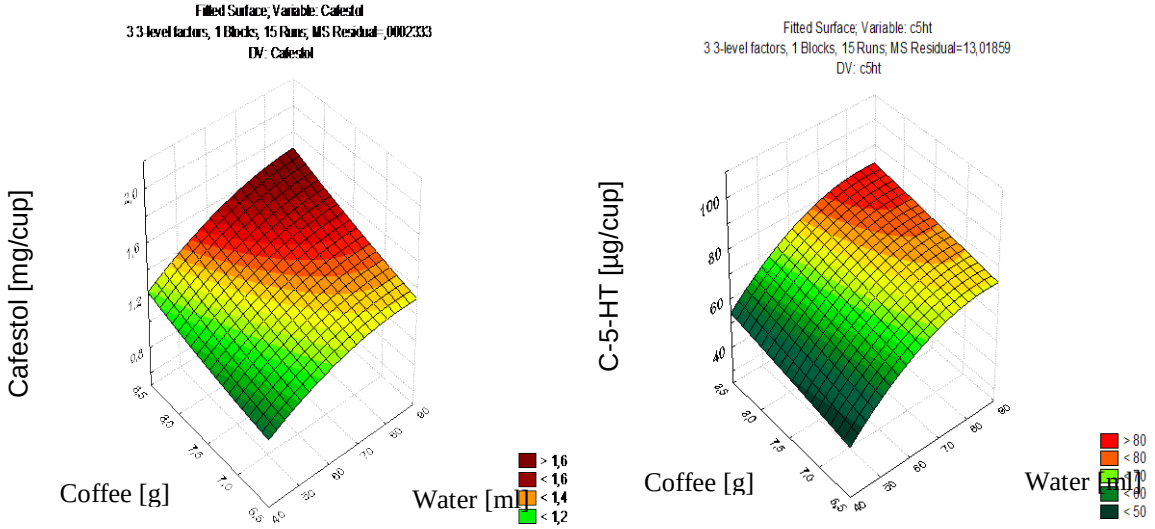

Figure 5. 3D response surface plot for Espresso with grinding grade medium at 95 °C

In general, an increase in the water quantity led to higher cafestol and C-5-HT contents in the coffee brew, but the correlation between the extraction yield and the amount of water was not linear. Caused by the filter effects of the coffee powder, especially, the run of the C-5-HT content flattened out at about 70 ml of water.

As expected, the finely ground coffee led to an overall higher C-5-HT and higher cafestol contents. Due to the enlarged surface of fine coffee powder, the extraction yield increased.

Regarding the impact of the coffee amount at a constant water quantity, the cafestol level was affected more than the total C-5-HT content. The highest extractions yields of 2.7 mg cafestol and a total C-5-HT content of 290 µg per cup were reached with 8.5 g of coffee and a 90 ml cup size. In contrast to the medium grinding grade with an increasing water quantity and a constant amount of coffee, only a slight flattening in the run of C-5-HT and the cafestol level was observed.

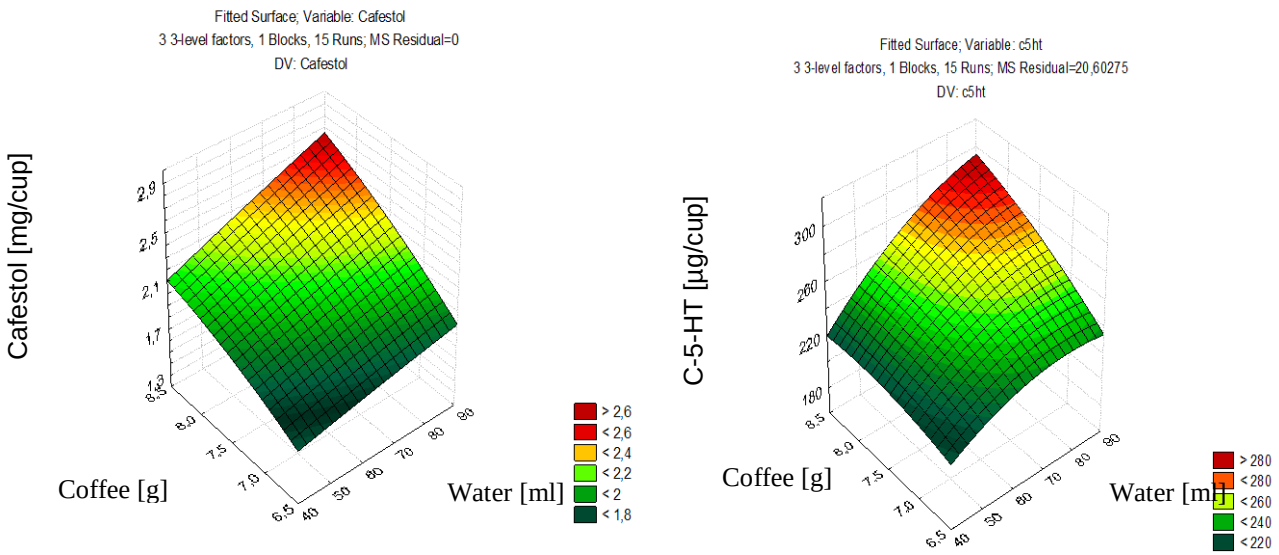

Figure 6. 3D response surface plot for grinding fine grade coffee at 95 °C.

Fresh Brew

The impact of the grind size was studied on Fresh Brew coffee prepared with 9 g coffee and 125 ml water at 95 °C. In contrast to the Espresso, the extraction yields for Fresh Brew showed a smaller range, indicating a minor impact of the particle size. It was remarkable that the highest amounts of cafestol and C-5-HT per cup were detected for the medium grind size.

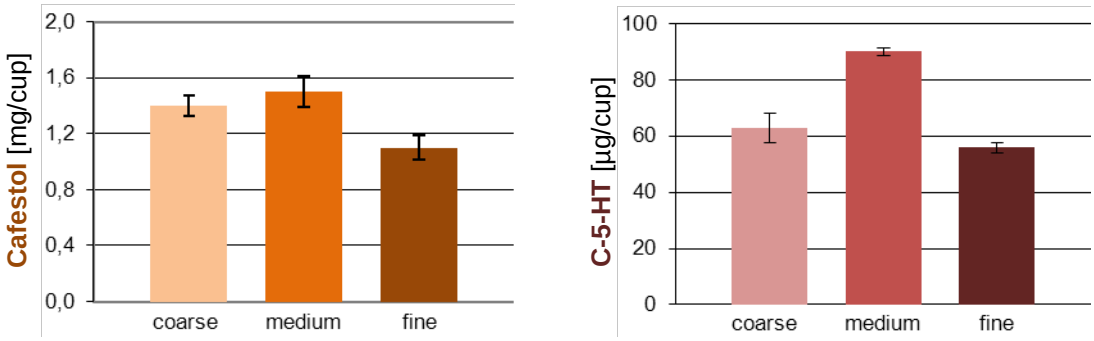

Figure 7. Impact of grinding grade on the cafestol and C-5-HT content in Fresh Brew coffee.

In Figure 7, the 3D response surface plot for Fresh Brew coffee at medium grind size is presented. For both, the Fresh Brew and the Espresso, an increase in the amount of water caused an increase in the cafestol and the C-5-HT content per cup However, the Fresh Brew was more impacted by the volume of water than the Espresso. There was barely any effect of the coffee amount on the extraction yields. Furthermore, the range between the maximum and the minimum value was about 30 % wider than for the Espresso.

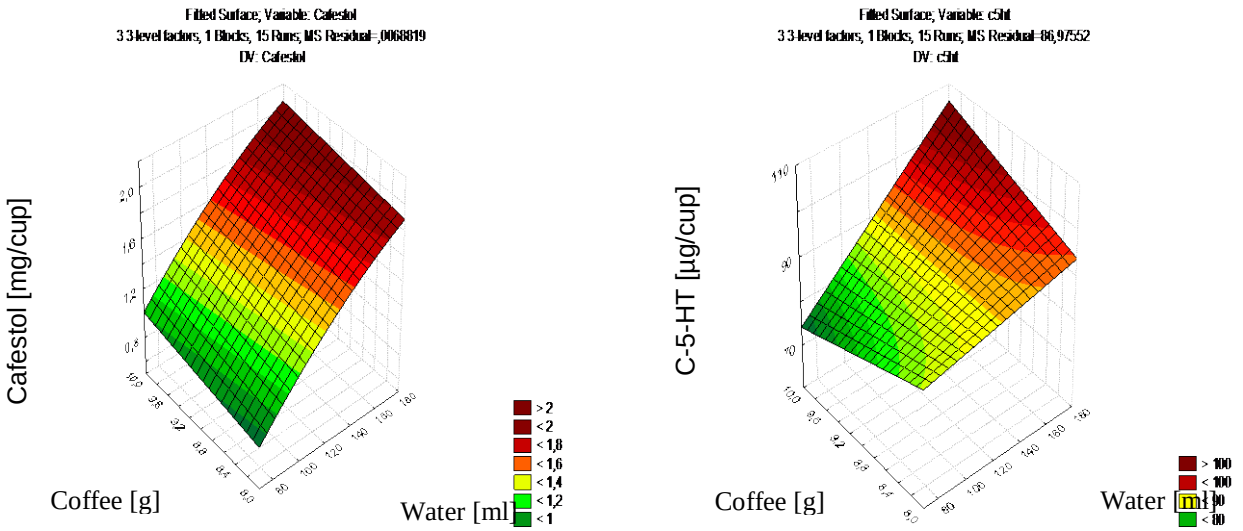

Figure 8. 3D response surface plot for Fresh Brew with grinding grade medium at 95 °C.

The influence of the water temperature on cafestol and C-5-HT in Fresh Brew was remarkable. Increasing the water temperature from 80 °C to 110 °C caused nearly three times higher cafestol content whereas the C-5-HT amount doubled. This effect was caused by the better solubility of the lipids in hot water and the thermodynamic properties of the extraction process.

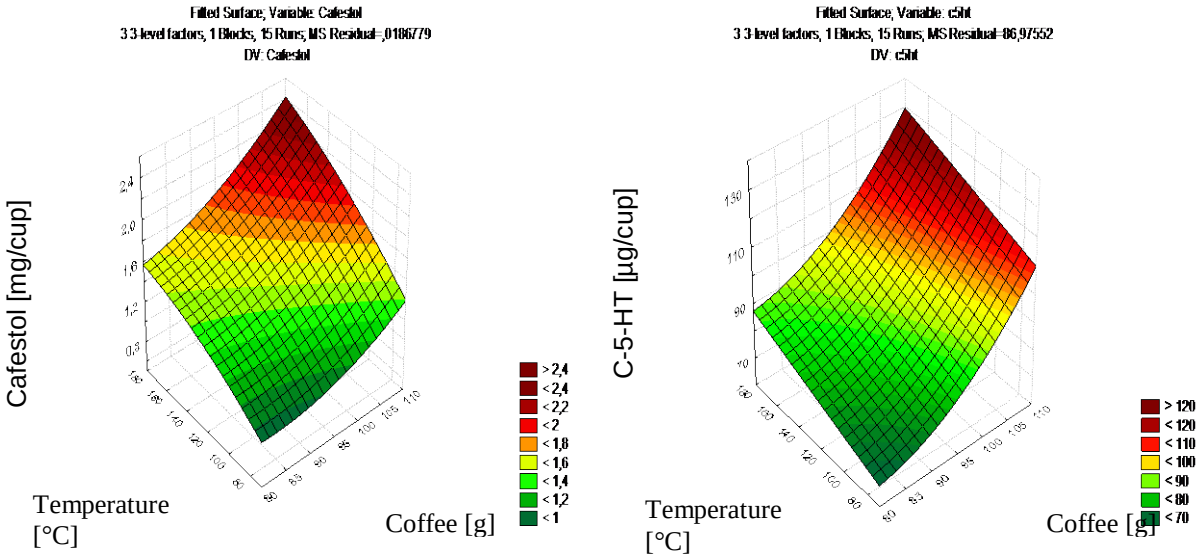

Figure 9. 3D response surface plot for Fresh Brew with grinding grade medium at 95 °C.

In summary, the impact of four preparation parameters for Espresso and Fresh Brew on the cafestol and C-5-HT levels per cup were studied. The Box-Behnken design proved to be a capable method for evaluating the main and the interaction effects of the preparation parameters.

Espresso showed an overall higher cafestol and C-5-HT content per cup. Especially, Espresso brewed with finely ground coffee led to outstanding, high extraction yields. In contrast to the Espresso, the Fresh Brew was hardly affected by the particle size of the coffee powder.

The coffee/water-ratio showed itself to be the most important parameter influencing the cafestol as well as the C-5-HT content in both brewing methods. For the Fresh Brew, an increase from 80 °C to 110 °C water temperature caused an enormous increase in the extraction yields of cafestol and C-5-HT.

The minimum and the maximum values of the extraction yields are presented in Table 2. It is possible to reduce the cafestol and C-5-HT content per cup at an average of 75 % by changing the preparation parameters.

Table 2. Minimum and maximum values of cafestol and C-5-HT amount per cup.

Espresso*	Cafestol [mg/cup]	C-5-HT [µg/cup]
minimum (medium/ 80 °C/ 6.5 g/ 40 mL)	0.9	40
maximum (fine/ 110 °C/ 8.5 g/ 90 mL)	2.7	290
Fresh Brew		
minimum (coarse/ 80 °C/ 8 g/ 70 mL)	0.7	25
maximum (medium/ 110 °C/ 10 g/ 180 mL)	2.6	135

***** *For Espresso, the coarsely-ground coffee was inapplicable.*

Furthermore, the cafestol content in the brew is influenced by the cafestol level in the roasted coffee, which ranged from 2.2 to 9.1 g/kg.

To which extent the aroma is influenced was studied by HS/SPME-GC/MS. In Figure 10, two chromatograms for Espresso with a minimum (A) and a maximum (B) cafestol content are presented. Along with the minimum cafestol content, a significantly lower amount of volatiles was observed. This is in accordance with the sensory evaluation by a non-trained panel of test persons, who estimated the flavour as poor. Summarizing, a compromise will need to be found between a low cafestol content and an acceptable flavour.

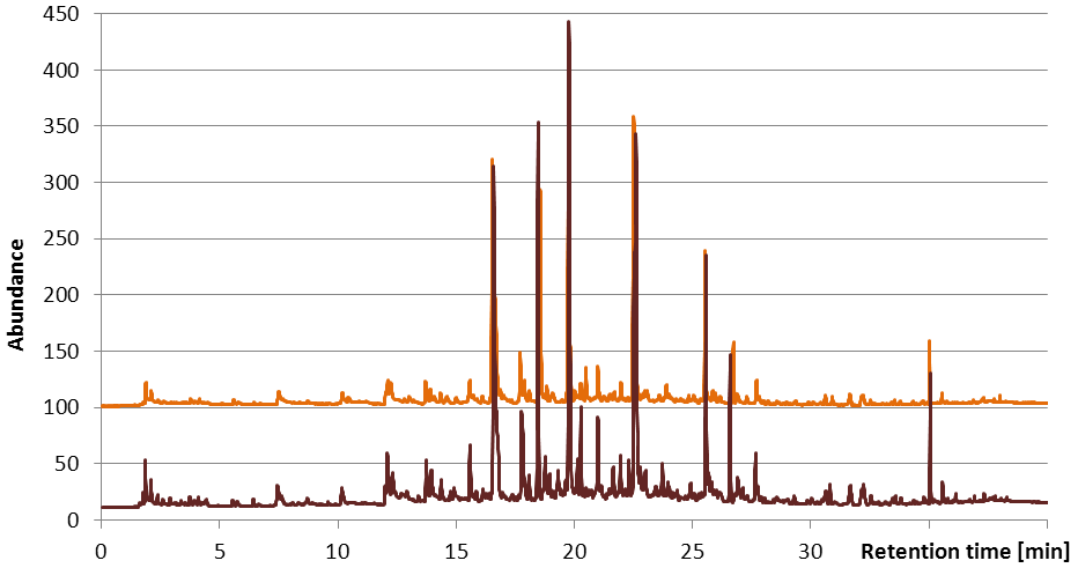

Figure 10. HS/SPME-GC/MS Chromatograms of volatiles in the Espresso with the minimum (A) and maximum (B) cafestol amount in Espresso.

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Analysis of Diterpens in Green and Roasted Coffee of *Coffea arabica* Cultivars Growing in the Same Edapho-Climatic Conditions

C.S.G. KITZBERGER[1], M.B.S. SCHOLZ[1], L.F.P. PEREIRA[2], L.G.E. VIEIRA[1], T. SERA[1], J.B.G.D. SILVA[3], M.T. BENASSI[4]

[1]Instituto Agronômico do Paraná, Londrina, PR, Brazil
[2]EMBRAPA Café, Londrina, PR, Brazil
[3]Centro Tecnológico Cocari, Mandaguari, PR, Brazil
[4]Universidade Estadual de Londrina, Londrina,PR, Brazil

SUMMARY

Lipids are important components of coffee beverage flavor and aroma. Coffee oil is rich in diterpens of the kaurane family, mainly cafestol ($C_{20}H_{28}O_3$) and kahweol ($C_{20}H_{26}O_3$), which have increasingly received attention in recent years due to their physiological effects in human health. However, few studies have been conducted on the effects of the genetic variability for those lipids in *Coffea arabica*. In this work we initiate the characterization of cafestol and kahweol in different cultivars of *Coffea arabica,* growing in the same edaphoclimatic conditions. Mature coffee fruits from cultivars Catuaí, Icatu and three Catucaí derived the cultivars IPR 100, IPR 102 and IPR 106. They were harvested at the Agricultural Field Station of the 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 five samples were subjected to medium roasting for 8 to 11 minutes at 200-210 °C, until the degree of roasting light/media (L* around 28). The extraction of diterpens was carried out in green or roasted coffee by direct saponification with KOH, extraction with terc-butyl methyl ether, and clean up with water. A reverse-phase HPLC column with isocratic elution with acetonitrile/water (55/45 v/v) was used for detection and quantification of kahweol at 290 nm and cafestol at 220 nm. In green beans, the level of kahweol was higher than cafestol, for all three IPR cultivars. Meanwhile, the inverse was observed for green beans cultivars Catuaí and Icatu, where cafestol levels were higher than kahweol. The higher levels of kahweol in relation to cafestol were again observed in roasted coffee of the three IPR cultivars. In cultivars Icatu the values for kahweol and cafestol were similar (635 and 683 mg/100 g, respectively). The highest levels of kahweol were observed in cultivar IPR 106 (1096 mg/100 g). The cultivar IPR 102 showed the highest level of cafestol (394 mg/100g). Association of this data with gene expression profile can be useful to find genes involved in cafestol and kahweol metabolism as well as to develop molecular markers for diterpens in coffee.

INTRODUCTION

Coffee is the most consumed beverage in the world, and its quality and functional properties are being widely exploited and associated with compounds of interest like caffeine, minerals, amino acids, lipids and sugars (Higdon and Frei, 2006; Rufián-Henares and Morales, 2007; Marinova et al., 2009).

The lipid composition of coffee has been described as a major influence on human health. Compounds of the unsaponifiable fraction such as cafestol and kahweol had desirable effects against cancer (Roos et al., 1997; Cavin et al., 2002), induced degradation of toxic substances and protection against aflatoxin B1 (Cavin et al., 2002), and presented antioxidant and antiinflammatory action (Kim et al., 2006) and hepatoprotective effect (Lee et al., 2007). However, undesirable effects of cafestol on human health has also been reported as cholesterol raising factor (Urgert and Katan, 1997).

Coffee oil is rich in diterpens from kauren family, specially cafestol ($C_{20}H_{28}O_3$) and kahweol ($C_{20}H_{26}O_3$). Diterpens are pentacyclic alcohols based on fusion of isoprene units (C5) to form the skeleton of 20 kauren carbons. Kahweol differs from cafestol by a double bond between carbons 1 and 2 leading to a spectrum with maximum peak absorption at a different wavelength (Figure 1). Analysis of cafestol in coffee demonstrated a concentration higher in *Coffea arabica* than in *Coffea canephora* (Speer and Kölling-Speer, 2006). Kahweol was reported to be specific to arabica coffee beans (Campanha et al., 2010, Dias et al., 2010) or detected only in traces in *C. canephora* (Speer and Kölling-Speer, 2006,

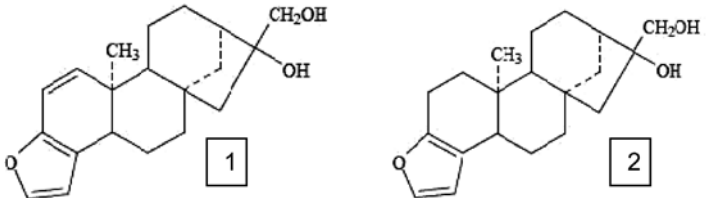

Figure 1. Structural formulas of kahweol (1) and cafestol (2).

From the factors affecting the composition of coffee, genetic variability has been highlighted for contributing directly to the diversity in terms of acidity, sugars, fat and caffeine (Sholz et al., 2000) and sensory quality (Medina Filho, 2007). It is also known that parameters such as altitude and temperature affect the composition in a different way (cell wall carbohydrates, chlorogenic acids, lipids and caffeine) depending on the variety (Jöet et al., 2010).

As for diterpens, there is little information about the influence of genetic variability in *C. arabica* cultivars, the aim of the study was to characterize the cafestol and kahweol contents in different coffee cultivars grown under the same edapho-climatic conditions.

MATERIALS AND METHODS

The samples of arabica coffee species were collected in Mandaguari – Paraná – Brazil at the Agriculture Technologic Park of Coop COCARI. Five cultivars were used: Catuaí vermelho, Icatu amarelo, IPR 100, IPR 102 and IPR 106 (Eira et al., 2007; Sera et al., 2005; Alteia et al., 2001; Ito et al., 2007). The samples were harvested from May to July 2009 at latitude 23°32'52" (South), altitude of 650 m and average annual temperatures of 22 to 23 °C. Cherry fruits were manually selected, washed and sun-dried in patio. The samples were processed, standardized in Grade 16 sieve size (6.5 mm) and characterized for their number of deffective beans (Brasil, 2003).

The samples of green coffee beans were frozen (-18 °C) and grounded (0.5 mm particles) in the disk mill (Perten 3600, Sweden) immediately prior to testing and using liquid nitrogen to prevent oxidation of compounds in the matrix (Dias et al., 2010). For roasted coffee, a roaster (Rod-Bel, São Paulo, Brazil) was used for 8-11 minutes at temperatures of 200 to 210 °C,

reaching light to medium roasting degree (L* about 28) as described by Scholz (2008). The samples were grounded in a manual disk grinder (FAMA, Indaiatuba, PR), stored in plastic bags and kept in a freezer (-18 °C).

For diterpens extraction, the samples were subjected to direct saponification with KOH and then extracting the unsaponifiable matter with t-BME, in duplicate. They were cleaned up with water after the extraction (Figure 2). The green coffee samples were weighed directly into the centrifuge container with 2 mL of KOH to prevent diterpens oxidation (Dias et al., 2010).

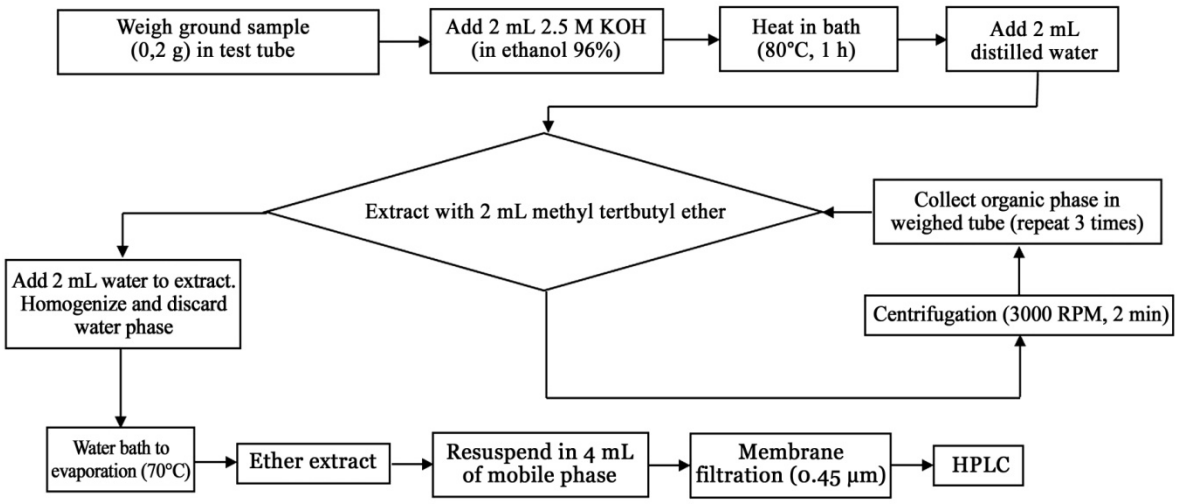

Figure 2. Flowchart of unsaponifiable coffee matter extraction (DIAS et al., 2010).

The analysis were performed in a High Pressure Liquid Chromatography Surveyor Plus (San Jose, USA) consisting of an autosampler with Peltier temperature control for rack of samples and oven integrated, quaternary gradient pump, diode-array detector, chromatography data system for ChromQuest 5.0 integrated via the network.

The analysis was conducted as described by Dias et al. (2010) using reversed-phase Spherisorb ODS 1 column (250 mm x 4.6 mm id 5 mm). It was used as mobile phase isocratic elution of acetonitrile/water (55/45 v/v) in 0.9mL/min flow rate. Detection was performed at 220 and 290 nm for cafestol and kahweol, respectively. It was applied oven temperature of 25 °C, and 20 minutes of running time. The identification was based on retention time comparison and coelution with the authentic standards. All samples were expressed on a dry basis (drying in the oven at 105 °C for 3 hours) and submitted to duplicate extraction and injection.

The quantification was carried out by external standardization, generating calibration curves for the compounds to be studied, with six different concentrations of standards (in triplicate) in the most appropriate concentration ranges. Calibration curves were constructed on concentrations of cafestol and kahweol (50-1000 mg/100 g of coffee). Data were analyzed by one-way ANOVA, considering the cultivar as the source of variation, and Tukey test ($p \leq 0.05$), using the Statistica 6.1 software.

RESULTS AND DISCUSSION

A great variability in the composition of diterpens was observed for both green and roasted coffee of different cultivars (Table 1). Roos et al. (1997) had already reported large differences in levels of cafestol and kahweol between coffees from different species grown in the same place. In this study, since the growing conditions, harvesting and processing were the same for all the plants and harvested coffee, the differences can be mainly attributed to the particular characteristics of each cultivar.

Table 1. Content of kahweol and cafestol (mg/100g) in green and roasted coffees of different cultivars on dry base*.

Cultivars	Green			Roasted		
	Cafestol	Kahweol	Total Diterpens	Cafestol	Kahweol	Total Diterpens
Catuaí	604±8a	371±6c	975±13b	668±52a	439±43c	1107±96b
Icatu	501±37a	433±18bc	934±56b	683±50a	635±51b	1318±99ab
IPR 100	328±32b	892±59a	1221±91ab	339±17b	939±25a	1278±42ab
IPR 102	356±34b	605±52b	960±86b	394±7b	691±4b	1086±3b
IPR 106	325±14b	986±46a	1312±60a	357±5b	1096±83a	1453±88a

**Mean of four values. Different letters in the column indicate significant differences ($p \leq 0.05$).*

For green coffee, there was a difference in the level of cafestol and kahweol between the new IPR cultivars and both Catuai and Icatu (Table 1). IPR 100 and IPR 106 also showed a high level of kahweol when compared with the other cultivars. The concentration of cafestol ranged from 325 to 604 mg/100 g of green coffee (1.8 times) with the highest value observed for the variety Catuai and the lower values for IPR 100 and 106. Kahweol showed even higher variation 371-986 mg/100 g of green coffee (2.6 times greater), with the highest content found in IPR 106. However, no difference was observed in total diterpens between the cultivars, with the exception of IPR 106. Kurzock and Speer (2001) reported values around 270 to 670 and 110 to 350 mg/100 g for cafestol and kahweol, respectively. Interestingly, Catuaí and Icatu have values near this range, but the IPRs have a different profile. Among IPRs, the IPR 102, derived from a cross of Icatu and Catuaí, showed behavior closer to traditional cultivars than to cultivars IPR 106 (only Icatu genetic background) and 100 (Catuaí genetic background). Total concentrations are found in the range of 934 to1312 mg/100 g and are among the levels reported in the literature (700 to 1300 mg/100 g) by Speer; Kölling-Speer (2006).

For roasted coffee, the cafestol content ranged from 339 to 683 mg/100g and the kahweol from 439 to 1096 mg/100 g (Table 1). Campanha et al. (2010), observed in Brazilian arabica coffees levels of 360 to 478 and 661 to 866 mg/100g for cafestol and kahweol respectively. Nicolau-Souza et al. (2010) analyzed five brands of gourmet coffee (100% Arabica), and observed cafestol concentration from 460 to 470 mg/100 g and kahweol from 570 to 800 mg/100 g. Our results were slightly above the range of those reports. However, Campanha (2008) reports lower values of cafestol (275 to 282 mg/100g) and higher values of kahweol (787 to 933 mg/100g) for *C. arabica* cv IAPAR 59, with different degrees of roasting, in a diterpens profile, similar to that observed for IPRs.

We note that the content of cafestol and kahweol increased when compared to green coffee in all varieties (increase of 3 to 36% for cafestol and 5 to 47% for kahweol). The relative increase in concentration can be attributed to degradation of thermolabile constituents from the roasting (Table 1). There is disagreement in literature over the stability of diterpens with the processing. Some authors report that diterpens could form dehidro derivatives (dehydro cafestol and kahweol) and other degradation products from roasting, reducing their levels (Kurzock and Speer, 2001, Speer and Kölling-Speer, 2006). Urgert et al. (1995) evaluated the behavior of kahweol and cafestol in *C. arabica* of intense roasts (26.5% weight loss), and concluded that roasting did not reduce the concentration of these compounds. Campanha et al. (2010), working with three degrees of roasting for different blends of coffee, reported that increasing in the degree of roasting did not lead to a reduction in diterpenes content. DIAS (2009) evaluated the degradation of cafestol and kahweol (time of 2-10 min, maximum temperature of 230 °C) and observed that the levels of cafestol and kahweol remained stable, even though dehidro derivatives occurred with more intense processes of roasting, due to increased concentration of lipids during the roasting process.

The ratio of kahweol/cafestol for the different cultivars was calculated (Figure 3). For the cultivars IPR the kahweol/cafestol ratio ranged approximately from 1.70 to 3.07 for both green and roasted coffee, since the amount of cafestol was always higher than kahweol on those cultivars. An opposite behaviour was observed for the cultivars Catuaí and Icatu which showed a kahweol/cafestol relation of approximately 0.61 to 0.93 for both green and roasted coffee.

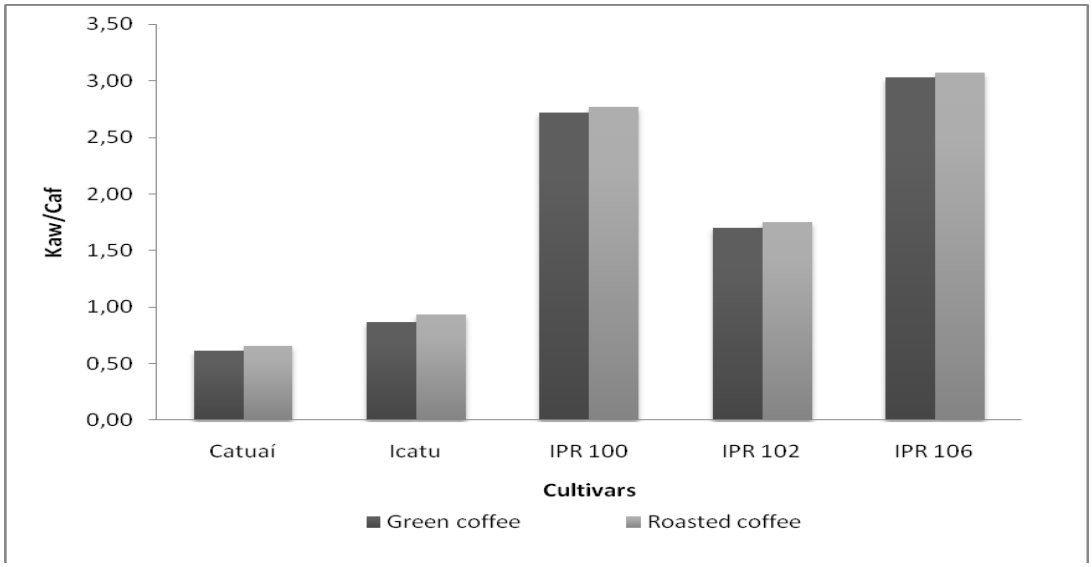

Figure 3. Relation kahweol/cafestol for green and roasted coffees.

This relation kahweol/cafestol was not affected by the roasting process, indicating that it could be used to characterize the cultivars regardless the degree of roasting (Figure 3).

It can be observed that the cultivars IPR 100 (Catuaí genetic background) and IPR 106 (Icatu genetic background) showed kahweol/cafestol relation around 3. Meanwhile, Catuaí and Icatu showed the lowest ratio. The cultivar IPR 102, which has both cultivars, Icatu and Catuaí in the genetic background showed a intermediated ratio.

Considering health issues, the crosses that generated the cultivars IPR were interesting as they showed higher levels of kahweol and, conversely, the levels of cafestol were reduced compared to traditional cultivars. Kahweol has been associated to beneficial health effects, but it has been proven that cafestol has a roll in raising cholesterol (Higdon and Frei, 2006), so the balance between cafestol and kahweol in cultivars IPRs, without altering the total amount of diterpens, can brings positive impact for consumers. Further work in the characterization of gene expression on those cultivars, associated to of diterpens profiles may be useful for finding genes involved in metabolism of cafestol and kahweol and to develop molecular markers for diterpens in coffees.

ACKNOWLEDGMENTS

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Recent Developments in Coffee Flavour Formation Using Biomimetic In-Bean Experiments

L. POISSON, J. KERLER, T. DAVIDEK, I. BLANK

Nestlé Product Technology Center Orbe, 1350 Orbe, Switzerland

SUMMARY

The formation of key odorants, such as α-diketones and 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone, was studied upon coffee roasting. The approach involved the incorporation of potential precursors in green coffee beans by means of biomimetic in-bean and spiking experiments. Both labelled and unlabelled precursor molecules were used, and the target analytes in the roasted coffee samples were characterized in terms of their isotope labelling pattern and abundance. The biomimetic in-bean experiments evidenced that the pathways proposed for the formation of 2,3-butanedione, 2,3-pentanedione and 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone are also valid in the complex system as presented in green coffee. The role of amino acids, for example, alanine, and free sugars was largely substantiated. The results underscore the potential of this methodology to provide better understanding of the formation pathways occurring in complex food systems, which may be different from those obtained in model experiments.

INTRODUCTION

The desirable aroma of coffee is mainly generated during the roasting step at high temperature beyond 200 °C from precursors that are present in the green beans. Sugars, proteins, free amino acids, chlorogenic acids, and trigonelline are the principal flavor precursors in green coffee. Maillard-type reactions play a central role comprising a wide variety of chemical transformations taking place during roasting of coffee. Therefore, special emphasis has been devoted to studying the generation of Maillard-derived aroma compounds such as thiols, diketones, cyclic enolones, and pyrazines (Yaylayan and Keyhani, 1999; Tressl et al., 1993; Amrani-Hemaimi et al., 1995).

Despite numerous studies performed in model systems under dry heating conditions (Yaylayan and Keyhani, 1999; Tressl et al., 1993; Amrani-Hemaimi et al., 1995), flavour formation pathways in coffee are still not well understood, mainly because results of model systems can hardly be extrapolated to complex food products. The complexity of the green coffee composition as well as the chemical and physical transformations that the coffee beans undergo during roasting cannot be sufficiently well reproduced in model systems. As an example, the importance of the bean structure integrity has been evidenced by demonstrating that roasting of ground green coffee resulted in different analytical data as compared to whole green coffee (Schenker, 2000). Hence, the so-called biomimetic in-bean experiments have been developed to study the importance of precursors for the formation of key aroma compounds during coffee roasting under realistic conditions. This approach is based on the use of green coffee beans as “mini-reactors” for model reactions, which allows a more realistic evaluation of potential precursors and provides a more precise insight into formation pathways (Milo et al., 2002; Mueller et al., 2006). It consists of (i) extraction of the soluble part of the green coffee beans with hot water and (ii) drying of the depleted beans. Various

subsequent studies can be envisaged with this “raw material”. To test the overall concept of in-bean experiments, a complete compounded equivalent of the natural extract can be incorporated into the exhausted beans. This biomimetic recombinate can be selectively omitted or fortified in certain precursors. Similarly, labelled precursors may be incorporated for mechanistic studies. In addition, spiking of untreated green coffee beans with precursors or precursor groups (sugars, amino acids) represents another straightforward approach to study modulation of coffee flavour.

The aim of the study was to assess the role of main precursors in coffee, such as sugars and amino acids, in the formation of Maillard derived aroma compounds such as α-diketones and 4-hydroxy-2,5-dimethyl-3[*2H*]-furanone, as well as the elucidation of their intrinsic formation pathways. A combination of biomimetic in-bean experiments and spiking of green coffee with unlabelled and stable isotope labelled precursors was performed.

MATERIAL AND METHODS

Biomimetic in-bean experiments

Biomimetic in-bean experiments (hot water extraction of green coffee beans, incorporation of biomimetic recombined extract, omission experiments, spiking of precursor compounds, and mechanistic studies) were performed according to the literature (Poisson et al., 2009).

Instrumental analysis

Identification and quantification of odorants was performed by Solid Phase Micro Extraction (SPME) combined with gas chromatography Mass Spectrometry (GC/MS). Gas chromatographic and mass spectrometric conditions were applied according to literature (Poisson et al., 2009). Quantified concentrations of the assessed compounds are expressed as relative amounts compared to the reference set at 100% (roasted biomimetic recombined beans or roasted green coffee).

RESULTS AND DISCUSSION

α-Diketones

To study the role of the overall soluble precursor part of the green coffee on the generation of α-diketones, the aroma impact odorants 2,3-butanedione and 2,3-pentanedione were quantified in the roasted water-extracted beans and compared to the reference roasted green beans (Figure 1). The diketones decreased considerably in the exhausted beans as a result of the water extraction of soluble precursors, similarly to the majority of aroma compounds analyzed (7). In particular the 2,3-pentanedione content was significantly reduced (- 83%), but also 2,3-butanedione with a decrease of 40%. Previously, Milo et al. (2002) showed similar results in in-bean experiments where diketones were reduced by more than 50% in the water-extracted beans. To better understand the role of precursors involved in the formation of these α-diketones, either free amino acids or free sugars were omitted in the biomimetic recombined beans. Quantitative analysis of the roasted beans revealed that the free amino acids did not influence the content of the α-diketones, as their concentrations were not affected by the omission of amino acids (Figure 1). Furthermore, spiking of green coffee with an excess of L-alanine (Figure 2) resulted in only small increase of said compounds (7% and 17%, respectively). These results demonstrate that bound amino compounds like peptides and

proteins can be considered as the main source of *N*-containing species involved in the formation of α-diketones.

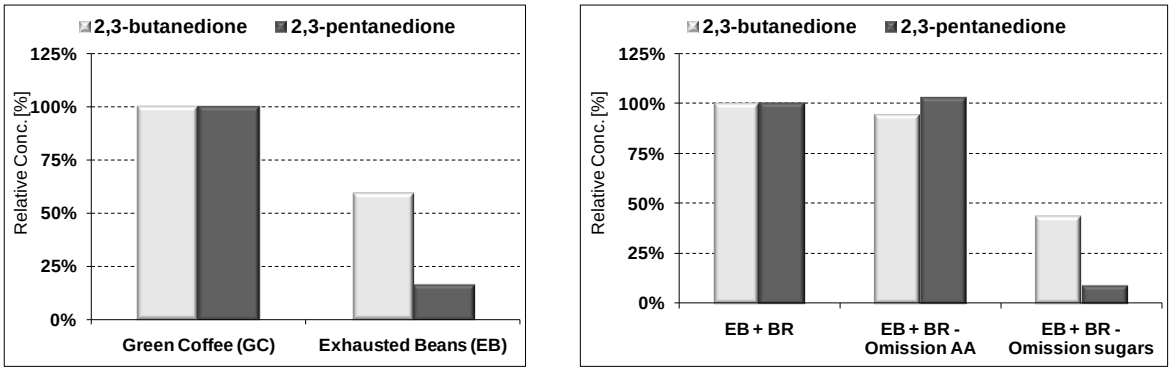

Figure 1. Concentrations of α-diketones in roasted green coffee beans (GC) compared to roasted water-exhausted beans (EB; left), and roasted water-exhausted beans reconstituted with biomimetic recombinate (BR) omitted in amino acids or sugars (right).

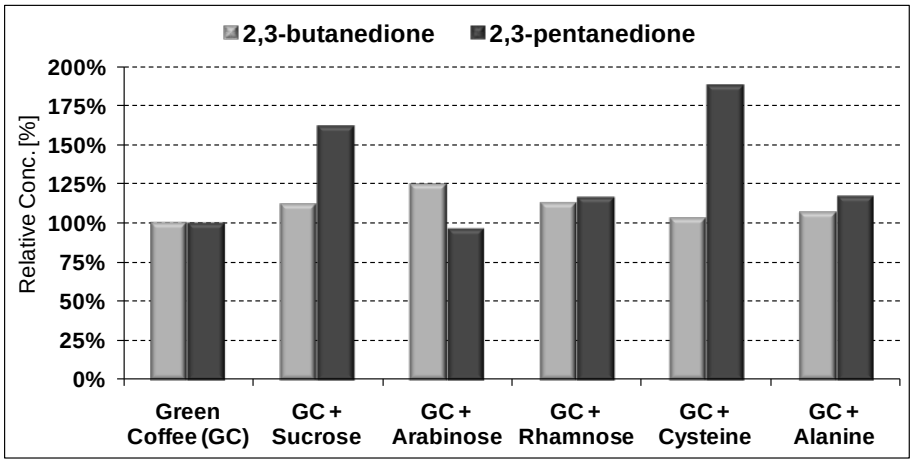

Figure 2. Concentrations of aroma compounds obtained by roasting of reference green coffee beans spiked with precursors (sugars, amino acids).

However, spiking of green coffee with an excess amount of cysteine led to an unexpected result showing a strong increase of 2,3-pentanedione by 88%, but not of 2,3-butanedione (Figure 2). These data indicate different formation pathways leading to 2,3-butanedione and 2,3-pentanedione, which mainly depends on sugars as a direct source of C-fragments. The *N*-pool seems to have an indirect role by affecting the overall turnover of sugar degradation. Cysteine seems to reduce the complexity of chemical reactions, thus leading to fewer competing mechanisms, thus allowing more 2,3-pentanedione to be formed. However, free cysteine is present only in trace amounts in green coffee and thus probably does not influence the generation of diketones to the same extent as shown in this model experiment.

In contrast to the free amino acids, free sugars were found as a limiting factor in the formation of both α-diketones. Their omission resulted in considerable reduction of α-diketones, i.e. 2,3-butanedione decreased by about 60% and 2,3-pentanedione by 91% (Figure 1). On the other hand, significantly higher amounts of 2,3-pentanedione were found in coffee spiked with sucrose (+62%), whereas 2,3-butanedione increased by only 12% (Figure 2). Other sugars

like arabinose and rhamnose had only a minor effect, leading to slight increase of single compounds by up to 25%.

The incorporation of labelled precursors underlined the role of free sugars in diketone formation. Spiking of green coffee with an excess of L-[3-^{13}C]-alanine (0.48 g/150 g beans) resulted in only 5% $^{13}C_1$-labeled 2,3-pentanedione (Figure 3). On the contrary, incorporation of [$^{13}C_6$-Fru-$^{12}C_6$-Glu]-sucrose (17% of natural content) yielded considerable amounts of fully labelled 2,3-pentanedione (10%, m/z 105) and to a lesser extent partially labelled isotopomers ($[M+2]^+$: 2% and $[M+3]^+$: 4%) (Figure 4). Unlike 2,3-pentanedione, almost no labelled 2,3-butanedione isotopomers were determined (<1%). Yaylayan et al. (1999) suggested on the basis of model studies with labelled precursors that the major part of 2,3-pentanedione (90%) is formed by recombination of a C2-C3 moiety of L-alanine and a C_3 carbon unit from D-glucose. Besides the direct formation from the carbohydrate skeleton, the recombination of transient intermediates has been discussed (Schenker, 2000) as well. Our results indicate that a recombination of C_2 moiety of L-alanine and C_3 moiety from labeled sucrose (fructose-part) does occur. However, 2,3-pentanedione is formed to a larger extent from the C_6 skeleton of fructose.

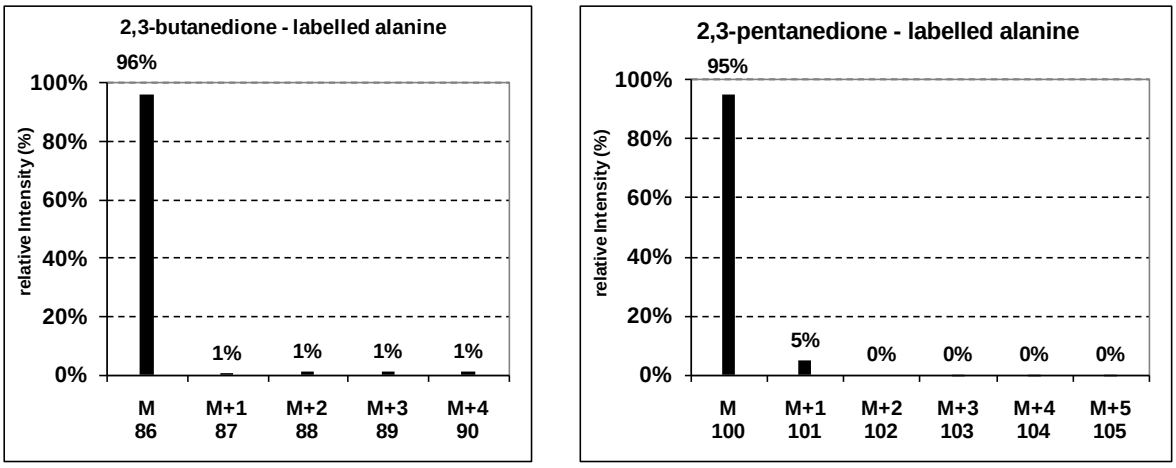

Figure 3. Percentage labelling distribution of 2,3-pentanedione, generated from isotope-labelled L-[3-^{13}C]-alanine (0.48 g/150 g beans) upon coffee roasting.

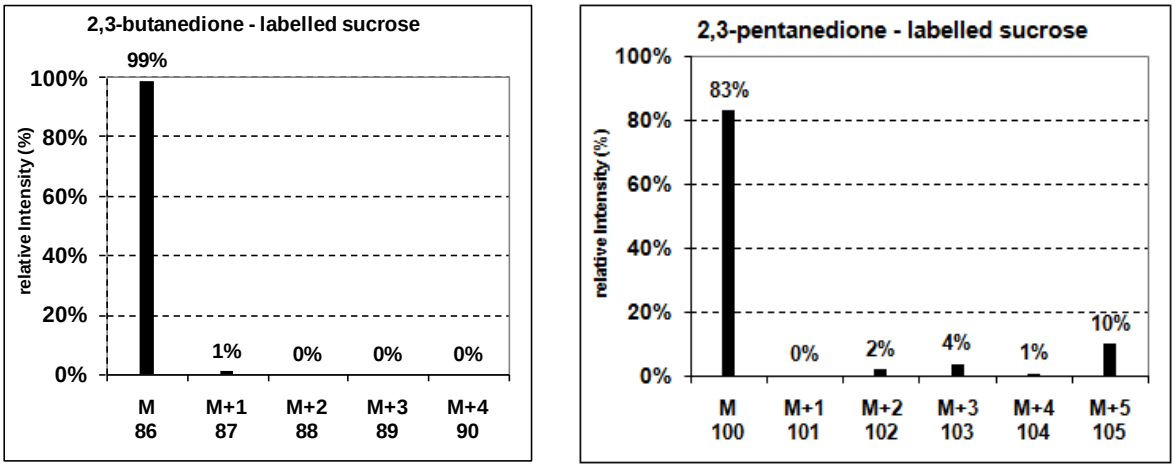

Figure 4. Percentage labelling distribution of 2,3-butanedione, generated from isotope-labelled [$^{13}C_6$-Fru-$^{12}C_6$-Glu]-sucrose (17% of natural content) upon coffee roasting.

4-Hydroxy-2,5-dimethyl-3[2H]-furanone

Figure 5 shows various potential precursors of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (HDMF). With respect of the composition in green coffee, the hexoses and fragments of sugars are supposed to be as most important precursors for HDMF. Figure 6 shows the effect of water extraction of all soluble precursors from the green bean on the generation of HDMF. Similar to the diketones, HDMF significantly decreased by 35% as compared to the reference roasted green bean. Particularly the free sugars play a major role in the formation of HDMF. The omission of all free sugars in the biomimetic recombinate resulted in significantly decreased concentrations of HDMF (-55%). However, the omission of the amino acids promoted the generation of HDMF, as the concentration increased significantly by 62%. This is probably due to (i) fewer degradation reactions of HDMF that are favoured by *N*-compounds and/or (ii) less trapping of reactive C_3 fragments by free amino acids.

The incorporation of [$^{13}C_6$-Fru-$^{12}C_6$-Glu]-sucrose into the sugar-free biomimetic recombinate led to the exclusive generation of fully labelled molecules (m/z 134, 16% yield) and fully unlabeled molecules. Schieberle (8) showed by carbon module labeling (CAMOLA) studies that under roasting conditions HDMF is solely formed via the intact C_6-glucose skeleton. As shown by the in-bean experiment with labelled sucrose in the present study (Figure 7), this is probably also valid under coffee roasting conditions. As shown before, free sugars (mainly sucrose) contribute to about half of the amount of HDMF in roasted coffee. In addition, the data also indicate that fructose is a much more efficient precursor as compared to glucose because only one-sixth of the total sucrose amount was added as labelled isotopomer, hence yielding 16% labelled HDMF. Spiking with rhamnose resulted in very high levels of HDMF (Figure 8), which is not surprising as this monosaccharide is known as a direct precursor of HDMF (9, 10).

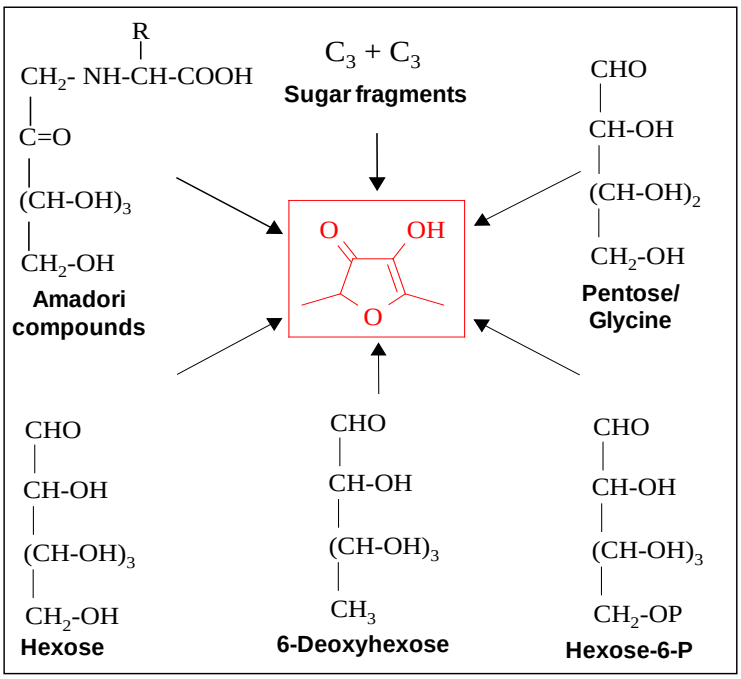

Figure 5. Potential precursors in the formation of the caramel-like smelling 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone. R: Rest of the amino acid; P: Phosphate moiety.

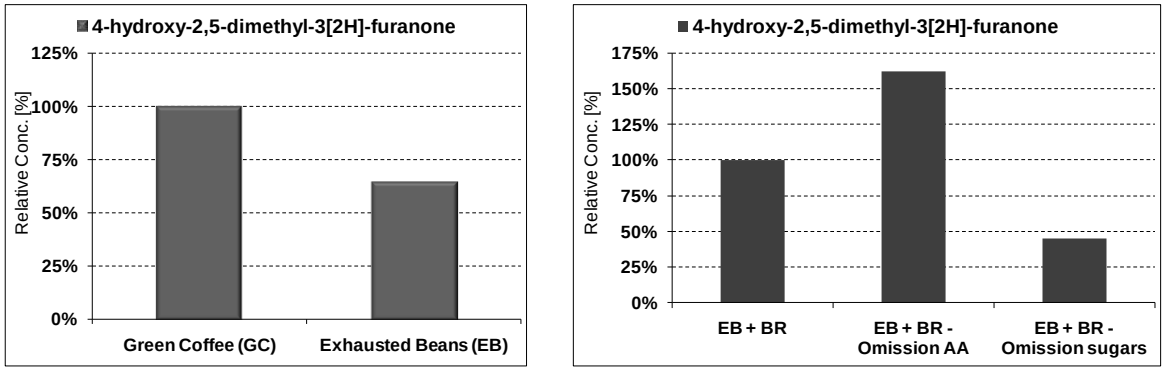

Figure 6. Concentrations of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone in roasted green coffee beans (GC) compared to roasted water-exhausted beans (EB; left), and roasted water-exhausted beans reconstituted with biomimetic recombinate (BR) omitted in amino acids or sugars (right).

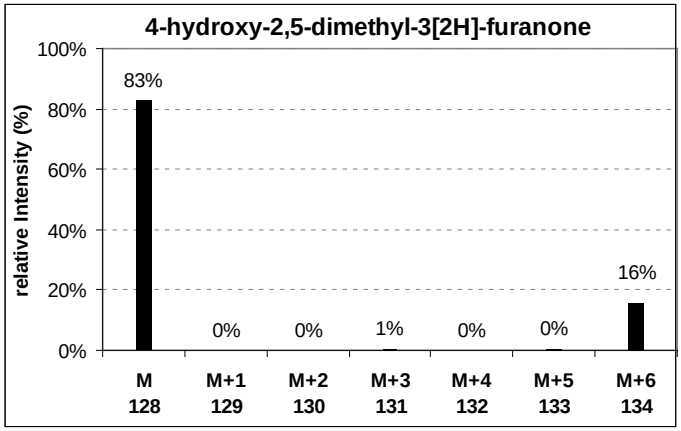

Figure 7. Percentage labelling distribution of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone, generated from isotope-labelled [$^{13}C_6$-Fru-$^{12}C_6$-Glu]-sucrose (17% of natural content) upon coffee roasting.

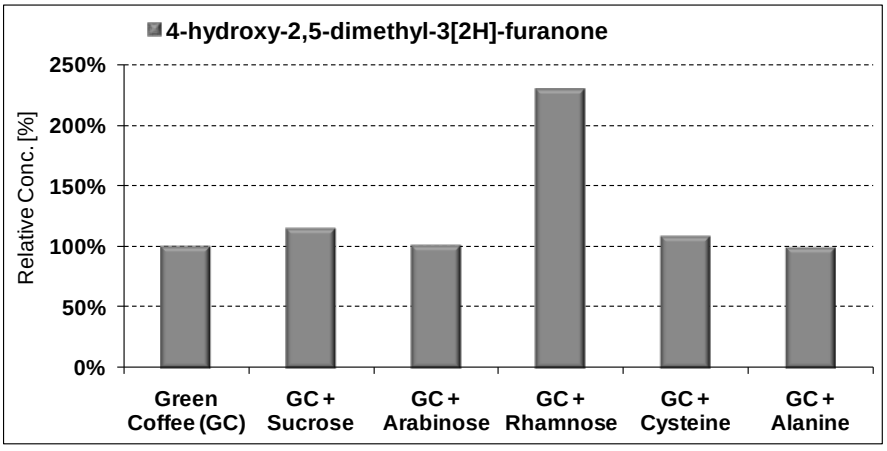

Figure 8. Concentrations of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone obtained by roasting of reference green coffee beans spiked with different precursors (sugars, amino acids).

CONCLUSION

This study revealed that the biomimetic in-bean approach is a powerful tool for the elucidation and verification of formation pathways of aroma compounds upon coffee roasting. It was demonstrated that combinations of omission, spiking, and mechanistic experiments under real food matrix conditions are very useful in providing further and more precise insights into Maillard-type reactions and formation mechanisms. The formation pathways of several key aroma compounds, such as α-diketones and 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone proposed by model systems could be largely confirmed by in-bean coffee roasting experiments.

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Advanced Analytical-Sensory Correlation – Towards a Better Understanding of Coffee Flavor Perception

J. BAGGENSTOSS[1], L. POISSON[1], A. GLABASNIA[1], M. MOSER[2], A. RYTZ[2], E. THOMAS[3], I. BLANK[1], J. KERLER[1]

[1]Nestlé PTC Orbe, Switzerland
[2]Nestlé Research Center, Vers-chez-les-Blanc, Switzerland
[3]Nestlé Nespresso S.A., Lausanne, Switzerland

SUMMARY

To better understand the link between sensory perception of coffee and quantitative analytical data, 12 coffee blends were assessed by instrumental analysis and sensory profiling, and the two resulting datasets were statistically correlated. Several of the 42 aroma and 12 taste compounds analyzed in this study exhibited a good correlation with specific sensory descriptors and may therefore be used as chemical markers for the characterization of flavor profiles and for new product developments.

INTRODUCTION

Besides the stimulating effect of a cup of coffee, the main drivers for its consumption are the complex aroma and the powerful taste of the beverage. During the last decades scientific knowledge has considerably advanced. In the headspace of coffee, hundreds of substances have been identified, and the ones mainly responsible for the aroma, the so-called key impact compounds, have been elucidated by gas chromatography – olfactometry and omission experiments (Blank et al., 1992; Czerny et al., 1999; Grosch, 1998; Mayer et al., 2000; Semmelroch and Grosch, 1996)). In addition, significant progress was made in the identification of key taste compounds. Caffeoyl quinic acid lactones (Frank et al., 2008; 2006), 4-vinylcatechol oligomers (Frank et al., 2007), diketopiperazines (Ginz and Engelhardt, 2001), and recently (furan-2-yl)methylated benzene diols and triols (Kreppenhofer et al., 2010) were identified as compounds with a major impact on coffee bitterness.

Although the individual flavor qualities of the identified single compounds are known, their individual contribution to a complex mixture such as coffee flavor remains unclear and might depend on various effects. Until today, sensory profiling remains the most accurate method for describing coffee flavor. However, for coffee research and development, more objective methods would be desirable, which statistically link sensory descriptors to the concentration of flavor compounds. Recently, a statistical model for the prediction of coffee aroma was developed based on sensory profiling and analytical headspace measurements with proton transfer reaction – mass spectrometry (PTR-MS) (Lindinger et al., 2008). PTR-MS measurements are fast, and the model described in the study was very reliable. However, quantitative PTR-MS measurements are difficult to carry out and identification of compounds can sometimes be ambiguous, hence a causal correlation of flavor compounds in coffee and the sensory perception is still missing.

The aim of this study was to identify the flavor compounds that are best correlated to sensory perception. For this purpose, 12 espresso coffees of different cup sizes were analyzed – 3 Ristrettos (25 mL), 6 Espressos (40 mL) and 3 Lungos (110 mL).

MATERIALS AND METHODS

Preparation of Espresso Coffees

Coffees were extracted with Acqua Panna water (Sanpellegrino S.p.a., Milano, Italy) using a commercial coffee machine (Type C190 plus). The extraction volume was 25 mL for Ristrettos, 40 mL for Espressos, and 110 mL for Lungos. The volume was controlled indirectly *via* the mass of the beverage. The extraction time was controlled manually, and samples with too short or too long extraction time were rejected.

Flavor analysis

42 key aroma compounds and 12 taste compounds were analyzed quantitatively with various analytical techniques (Table 1).

Quantification of volatile compounds with solid phase microextraction (SPME) GC-MS

The extracted coffees were diluted (if necessary), and, after cooling, spiked with defined amounts of stable isotope labelled standards (Table 1). After 10 min of stirring, the solution was transferred to headspace vials. The coffee volatiles were then extracted by solid phase microextraction (SPME) for 10 min at 40 °C with a Supelco 50/30 µm StableFlex DVB/CAR/PDMS fiber (Supelco, Buchs, Switzerland). The fiber was desorbed in the injection port of the gas chromatograph at 240 °C with a splitless time of 3 min. Helium was used as a carrier gas. The volatile compounds were separated and quantified with

a) a 60 m x 0.25 mm x 0.25 µm ZB-Wax column (Phenomenex, Aschaffenburg, Germany) on a Thermo Trace gas chromatograph coupled to a DSQ mass spectrometer (Thermo Electron, Reinach, Switzerland) (analytes **1**-**17**),
b) a 60 m x 0.25 mm x 1.4 µm ZB-624 column (Phenomenex, Aschaffenburg, Germany) on a Thermo Trace gas chromatograph coupled to a MD800 mass spectrometer (Thermo Electron, Reinach, Switzerland) (analytes **18**-**34**)
c) a 60 m x 0.25 mm x 0.25 µm DB-FFAP column (Agilent, Palo Alto, CA, USA) for the first dimension separation, and a 2 m x 0.1 mm x 0.1 µm DB-1701 column (Agilent, Palo Alto, CA, USA) for the second dimension in a two dimensional LECO Pegasus 4D GCxGC-TOF MS system (Leco Corp., St. Joseph, MI, USA) (analytes **35**, **36**, **38**)
d) a 60 m x 0.25 mm x 0.25 µm Equity 1701 column (Supelco, Buchs, Switzerland) for the first dimension separation, and a 2 m x 0.1 mm x 0.1 µm DB-WAX column (Agilent, Palo Alto, CA, USA) for the second dimension in a two dimensional LECO Pegasus 4D GCxGC-TOF MS system (Leco Corp., St. Joseph, MI, USA) (analyte **37**).

Table 1. Analytes and standards used for quantification of coffee flavor compounds.

	Analyte	**Quantification**		**Analyte**	**Quantification**
volatile compounds			28	furfural	a
1	methanethiol	a	29	furfuryl acetate	f
2	dimethyl sulfide	a	30	phenylacetaldehyde	f
3	2-furfurylthiol	a	31	2-methoxyphenol	a
4	3-mercapto-3-methylbutyl formate	a	32	4-ethyl-2-methoxyphenol	a
5	hexanal	a	33	4-vinyl-2-methoxyphenol	a
6	ethyl 2-methylbutanoate	a	34	p-cresol	g
7	ethyl 3-methylbutanoate	a	35	3-methyl-2-butene-1-thiol	a
8	2-ethyl-3,5-dimethylpyrazine	a	36	2-methyl-3-furanthiol	a
9	2-ethyl-3,6-dimethylpyrazine	b	37	3-(methylthio)propanal	a
10	2-ethenyl-3,5-dimethylpyrazine	b	38	*(E)*-β-damascenone	a
11	2,3-diethyl-5-methylpyrazine	a	39	2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol^{TM})	a
12	trimethylpyrazine	a	40	4,5-dimethyl-3-hydroxy-2(5H)-furanone (sotolon)	a
13	2-acetylpyrazine	a	41	3-hydroxy-2-methyl-4H-pyran-4-one (maltol)	a
14	2-acetylpyridine	c	42	4-hydroxy-3-methoxybenzaldehyde (vanillin)	a
15	2-acetylthiazole	c	*non-volatile compounds*		
16	2-isopropyl-3-methoxypyrazine	a	43	3-caffeolyquinic acid (3-CQA)	h
17	2-isobutyl-3-methoxypyrazine	a	44	5-caffeolyquinic acid (5-CQA)	h
18	acetaldehyde	a	45	4-caffeolyquinic acid (4-CQA)	h
19	propanal	d	46	3-caffeoylquinic acid lactone (3-CQL)	h
20	methylpropanal	a	47	4-caffeoylquinic acid lactone (4-CQL)	h
21	2-methylbutanal	e	48	5-feruloylquinic acid (5-FQA)	h
22	3-methylbutanal	a	49	4-feruloylquinic acid (4-FQA)	h
23	2,3-butanedione	a	50	caffeine	i
24	2,3-pentanedione	a	51	cyclo-Val-Pro	j
25	dimethyl trisulfide	a	52	cyclo-Ala-Pro	j
26	*N*-methylpyrrole	a	53	cyclo-Pro-Leu	j
27	pyridine	a	54	cyclo-Phe-Pro	j
a	with isotope labelled analogue		**f**	with 28-[$^{2}H_4$]	
b	with 8-[$^{2}H_6$]		**g**	with 31-[$^{2}H_3$]	
c	with 13-[$^{2}H_5$]		**h**	with external standard (3-CQA)	
d	with 20-[$^{2}H_7$]		**i**	with external standard (caffeine)	
e	with 22-[$^{2}H_2$]		**j**	with external standard (analogue)	

Quantification of volatile compounds with solid phase extraction (SPE) GC-MS

For compounds **39**-**42,** the coffee brews were spiked with the corresponding stable isotope labelled standards, then polysaccharides were precipitated with methanol, and extraction was carried out with LiChrolut EN Standard PP-Cartouches (Merck, Darmstadt, Germany). The resulting solvent extract was analyzed with a Thermo Trace DSQ GC-MS (Thermo Electron, Reinach, Switzerland) equipped with a 60 m x 0.25 mm x 0.25 µm ZB-1701 column (Phenomenex, Aschaffenburg, Germany).

Quantification of non-volatile compounds with HPLC-DAD and LC-MS

For the analysis of non-volatile compounds, coffees were rapidly cooled to room temperature, and then diluted by a factor of 10 (for the analysis of caffeoylquinic acids **43**-**45**, caffeoylquinic acid lactones **46**-**47** and feruloylquinic acids **48**-**49**), 50 (caffeine), or 1 (diketopiperazines **51**-**54**). Then the solutions were passed through a 0.45 µm Spartan syringe filter (Whatman, Kent, UK). Caffeoylquinic acids, feruloylquinic acids and caffeoylquinic acid lactones were analyzed with reversed phase HPLC-DAD on a Zorbax XDB-C18 column, 150 x 3 mm, 5 µm particle size using a methanol / aqueous formic acid gradient with a flow rate of 250 µL/ min. Detection was carried out at a wavelength of 325 nm and the analytes were quantified *via* an external 5-CQA standard. Caffeine was separated and detected with the same setup, but the detector wavelength was 270 nm and quantification was carried out with an external caffeine standard.

The diketopiperazines **51**-**54** were separated with a Zorbax XDB-C18 column, 150 x 3 mm, 5 µm particle size using a methanol / aqueous formic acid gradient with a flow rate of 250 µL/ min. Quantification was carried out with the corresponding diketopiperazines as external standards using the following mass transitions in MRM mode: m/z 245 → m/z 217 (cyclo-Pro-Phe), m/z 197 → m/z 169 (cyclo-Pro-Val), m/z 169 → m/z 70 (cyclo-Ala-Pro), and m/z 211 → m/z 184 (cyclo-Leu-Pro).

Sensory profiling of Espresso coffees

Sensory profiling was carried out by 12 trained panellists. Each coffee was assessed twice. The following sensory descriptors were evaluated: ‘roasty’, ‘bitter’, ‘acid’ (basic coffee attributes, rated on a scale from 1 to 10), and the aroma descriptors ‘fruity-floral’ (notes of red fruits, citrus fruits, floral notes like bergamot and jasmine), ‘green-vegetal’ (fresh herbs, grass, vegetables), ‘dry-vegetal’ (dry wood, malt, cereal), ‘vegetal-humus’ (earthy, humus, mushroom), ‘cocoa’ (roasty, cocoa), ‘sweet’ (vanilla, caramel, honey). The aroma descriptors were rated on a scale from 0 to 5.

Statistical methods

The statistical model used to correlate the sensory and analytical datasets was adapted from an earlier study (Lindinger et al., 2008). Briefly, the model was built as follows:

Preprocessing of analytical and sensory data

a. Transformation of sensory data: each attribute was normalized (subtraction of the mean and division by the standard deviation)
b. Transformation of instrumental data:

i. The logarithm (base 10) was taken for each compound, and then a normalization step was performed as described before.
ii. On the transformed dataset, the intensity was computed for each coffee by taking the average of the normalized values of all compounds.
iii. For each coffee blend, the intensity was then subtracted from each individual flavor compound.

Principal components regression

a. A new sensory set was built after subtraction of the linear effect of intensity (this step was carried out in order to base the model on differences in flavor quality rather than on differences in flavor intensity)
b. A principal component analysis (PCA) is performed on this new sensory set and the transformed analytical data was projected on it by principal components regression (PCR) using the 2 first principal components.

RESULTS AND DISCUSSION

In order to correlate a sensory with an instrumental dataset, all information other than quality should be filtered out from both datasets (Lindinger et al., 2008). If intensity was not subtracted, a PCA would result with clustering of coffees mainly as a function of their cup size, i.e. their concentration. A similar effect would be observed for the instrumental data, if the subtraction of intensity was not carried out. The different flavor compounds would be inter-correlated and more or less strongly correlated to intensity. Because of the strong correlations of intensity to the concentration of flavor compounds, most of the information on the more subtle relationships between individual flavor compounds and sensory descriptors would be obscured.

If the linear effect of intensity is subtracted from the sensory data as described in the preceding paragraph, a PCA plot results, in which the coffees of different cup sizes are separated by their flavor composition rather than by their concentrations (Figure 1). Two main opposing axes can be observed: the descriptors related to flavor notes that are developed in a rather late stage of the roasting process (bitter, cocoa, dry-vegetal, roasty, and sweet), and, in the other direction, the green-vegetal, acid, and fruity-flowery notes, which are related to a light degree of roast. In fact, if the bean color expressed on the CTn scale is projected on the PCA plot, a perfect correlation with acidity results. One of the sensory descriptors, vegetal-humus, seems to be far less dependent on the degree of roast than the others, and it might therefore be concluded that vegetal-humus is a descriptor that is better related to blend specificities than the others.

On the PCA plot, which is based on the sensory data, the different flavor compounds can be projected using principal component regression (PCR, Figure 1). The flavor compounds which are best correlated to the sensory descriptors can be used as chemical markers for the different sensory descriptors assessed by the sensory panel. These markers are summarized in a flavor wheel representation (Figure 2). Some of the correlations obtained in this study are in line with what would be expected from the odor quality of the single compounds. Hexanal and methional are correlated to the green-vegetal notes; p-cresol is correlated to vegetal-humus; dimethyl trisulfide and the guaiacols are between the roasty / bitter and vegetal-humus descriptors; phenylacetaldehyde, pyridine and 2-acetylpyridine are correlated to sweet notes, and 3-methyl-2-butene-1-thiol and *N*-methylpyrrole are related to the roasty / dry vegetal / cocoa / bitter descriptors. The existence of such intuitive statistical correlations, however,

does not prove their causality. In order to evidence a causal relationship between a flavor compound and sensory perception, olfactometric experiments would be helpful because they allow determining the impact of a given single aroma compound on the total aroma of coffee as a function of concentration.

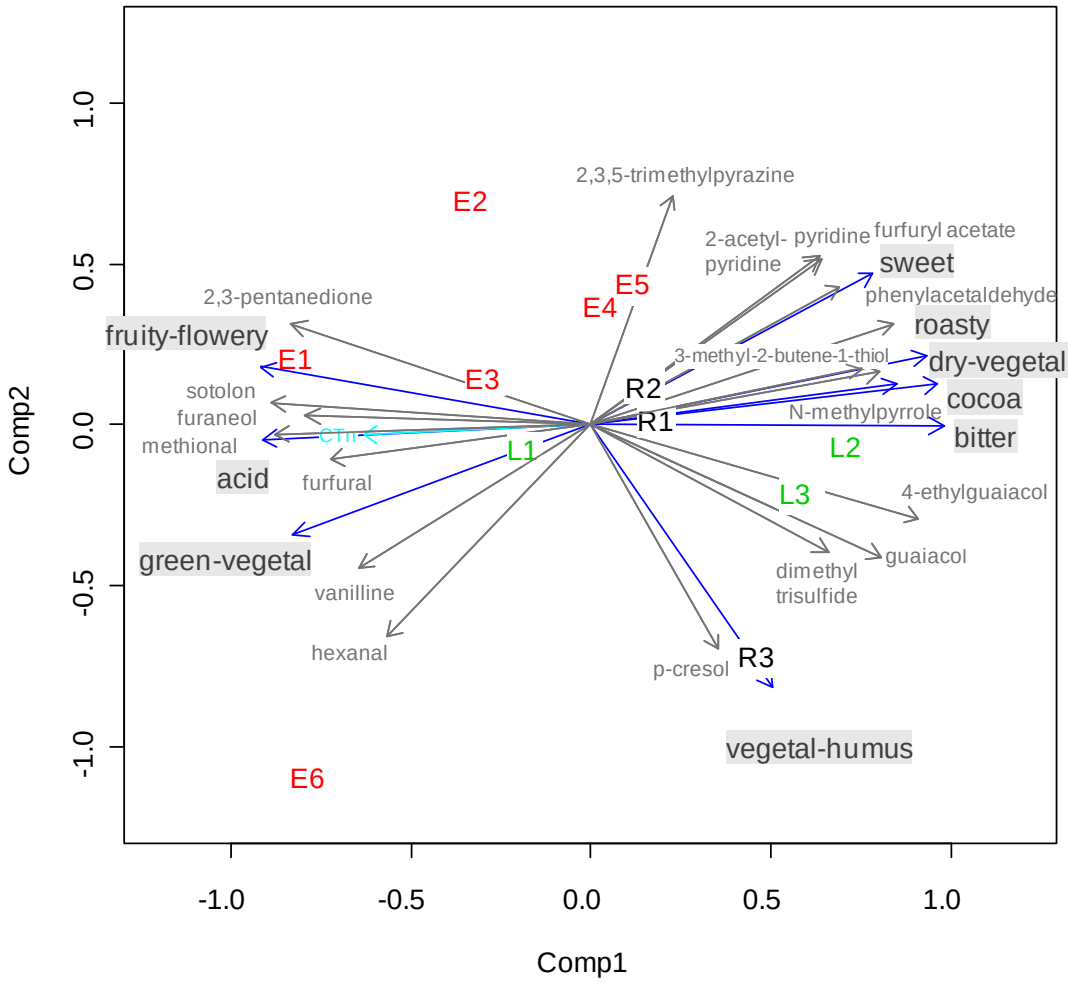

Figure 1. PCA plot based on sensory data after subtraction of intensity, and projections of flavor compounds. The longer an arrow is, the better it is represented on the PCA plot.

On the other hand, several of the correlations found in this study seem to be counter-intuitive. In particular, from their individual aroma qualities, sotolon and furaneol would rather be related to spicy and sweet descriptors; 2,3-pentanedione and vanillin to sweet, furfural to sweet / roasty, and trimethylpyrazine to earthy. There are two possible reasons for these seemingly counterintuitive correlations: first, the correlation may be purely statistical, meaning that there is no causal effect behind the correlation. And second, in a complex aroma mixture, the impact of a single compound may go well beyond the contribution of its intrinsic flavor note; it may enhance or suppress other flavor notes, play as an antagonist or exhibit synergistic effects with other compounds.

None of the bitter compounds analyzed in this study exhibited a strong correlation to the ‘bitter’ sensory descriptor. The current knowledge about bitterness in coffee is still fragmentary, and until now, no clear correlations between bitter compounds and coffee bitterness were found, implying that yet unknown compounds might be key contributors to

coffee bitterness (Hofmann, 2009). Although more than 30 bitter taste receptors are known, and although these receptors recognize a wide range of chemical classes (Temussi, 2009), it is believed that our sense of bitter taste is not very specific and its capability of discriminating bitter compounds is rather poor (Chandrashekar et al., 2006). To find a clear correlation for bitterness, it might therefore be necessary to identify a majority of the bitter compounds and to correlate the sum of their concentrations over threshold to the sensory perception.

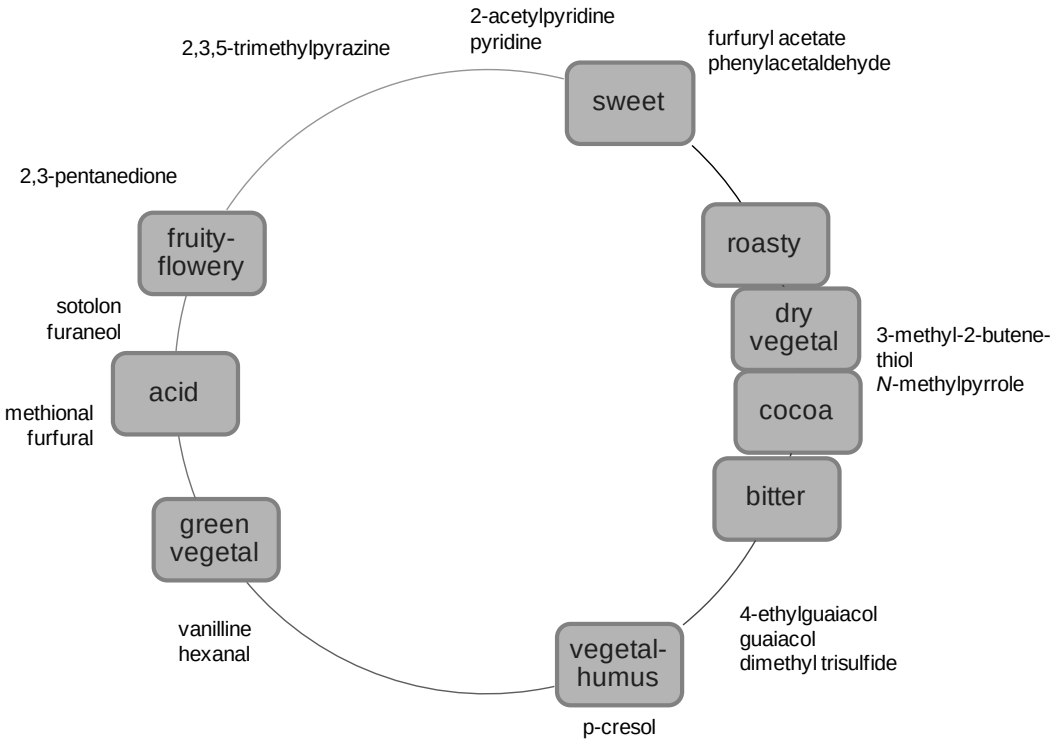

Figure 2. A 'flavor-wheel' representation of the best correlations between flavor compounds and sensory descriptors.

In a future step, the correlations found in this study might be tested for causality by further sensory and olfactometric experiments. In addition, a predictive model based on the sensory and analytical dataset could be built, and the predictions of the model be tested against the results of a trained sensory panel.

Such a predictive sensory-analytical model will have its applications in a more molecular-sensory guided development of coffee blends. Together with knowledge on green coffee specificity, effect of precursor compounds, and coffee roasting dynamics, the model will indicate the routes to be taken to generate specific flavor profiles.

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Characterization of Non-Defective and Defective Coffee Beans by Fourier Transform Infrared Spectroscopy (FTIR)

L.S. OLIVEIRA[1,2], A.P. CRAIG[2], A.S. FRANCA[1,2]

[1]Dep. Engenharia Mecânica
[2]Programa de Pós-Graduação em Ciência de Alimentos
Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901,
Belo Horizonte, MG, Brazil. E-mail: adriana@demec.ufmg.br

SUMMARY

The objective of this work was to evaluate the potential of Fourier Transform Infrared spectroscopy (FTIR) in the characterization and separation of defective and non-defective coffee beans. Defective (black, immature and sour) and non-defective Arabica coffee beans were manually separated. Ground coffee samples were then submitted to FTIR analysis employing (a) an attenuated total reflectance (ATR) accessory and (b) KBr disks. Multivariate statistical analysis (PCA) was performed in order to verify the possibility of discrimination between defective and non-defective coffee samples. A clear separation between defective and non-defective coffee beans was observed. Such results indicate that FTIR analysis presents potential for the development of a fast and reliable analytical methodology for detection of defective beans in roasted and ground coffee.

INTRODUCTION

The presence of defective coffee beans depreciates the quality of the coffee beverage consumed worldwide (Franca and Oliveira, 2008). The intrinsic defects (sour, black and immature beans) are the ones that, when roasted, contribute the most to the depreciation of the coffee beverage quality. These beans represent about 20% of the total coffee produced in Brazil and, although they are separated (color sorting) from the non-defective beans prior to commercialization in external markets, the majority of these beans are dumped on the Brazilian internal market. Thus, the roasting industry in Brazil has been using these defective beans in blends with healthy ones, and, overall, a low-grade roasted coffee is consumed in the country. Furthermore, the color-based separation procedure is not efficient, especially in the case of immature beans (Mendonça et al., 2009). Recent studies have shown that several physical and chemical parameters could be employed for separation between defective and non-defective green coffee beans of a given variety (Arabica or Robusta). Examples include color and size, that are effective if immature beans are not present (Mendonça et al., 2009), volatile components (Mancha Agresti et al., 2008), levels of histamine (Vasconcelos et al., 2007) and ESI-MS profiles (Mendonça et al., 2008). However, most of the employed instrumental techniques and analytical procedure are time demanding, expensive and require a considerable amount of qualified manual labor.

Recent studies have also shown that FTIR-based methods, in combination with chemometric techniques, can be successfully applied in the food industry, in association with detection of substances that affect the quality of food products or are employed for adulteration (Franca and Oliveira, 2010). FTIR-based methods are fast, reliable, simple to perform and do not require sample pre-treatment. Such technique provides simple and reproducible means of

handling food products with nondestructive analyses, with the sampling/analysis procedure usually taking less than 5 min.

There are a few studies that have focused on FTIR applied to coffee analysis (Kemsley et al., 1995; Briandet et al., 1996; Wang and Jun, 2009). The specific applications were separation between Arabica and Robusta (Kemsley et al., 1995), adulteration of freeze-dried instant coffees by glucose, starch or chicory [8], and geographical discrimination of Arabica coffees grown in Hawaii (Wang and Jun, 2009). Thus, the objective of this work was to evaluate the potential of Fourier Transform Infrared spectroscopy (FTIR) in the characterization and discrimination between defective and non-defective coffee beans.

METHODOLOGY

Arabica green coffee samples were acquired from Café Fino Grão (Belo Horizonte, MG). These correspond to coffee beans that were rejected by a color sorting machine. Black, sour, immature and non-defective beans were manually separated to constitute four sampling lots and ground to a particle diameter of 0.42 mm. 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}. Ground coffee samples were then submitted to FTIR analysis (IRAffinity-1 FTIR Spectrofotometer, Shimadzu, Japan). Preliminary tests were performed in order to define the methodology for sample preparation and spectra collection: (i) transmission reading from KBr disks: each disk was prepared by careful abrasion of KBr (150 mg) with the ground coffee sample (3 mg) and then pressed at 78 atm; (ii) direct reflectance readings employing attenuated total reflectance (ATR) accessory. ATR-based spectra presented a lot of noise and variability within a given sample, whereas KBr-based spectra presented no noise when a clear (no coffee) KBr disk was employed as a background (see Figure 1). Thus, KBr disks were chosen for the remaining tests. Further processing of the spectra included baseline correction and first derivative. For principal component analysis (PCA), data matrices were assembled so that each row corresponded to a sample and each column represented the spectra data at a given wavelength.

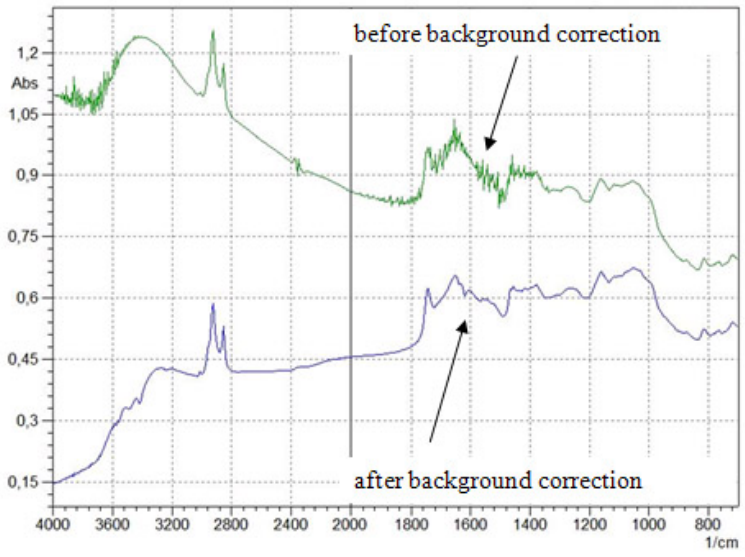

Figure 1. Typical KBr-based FTIR spectra of green coffee before and after background correcti--

RESULTS AND DISCUSSION

Average values of measured color parameters for non-defective and defective coffee samples are shown in Table 1. Measurements were based on the CIE $L^*a^*b^*$ three dimensional 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. However, chromaticity can be better represented and discussed in terms of polar coordinates, so a^* and b^* values were converted to chroma (c^*) and hue angle (h), representing color saturation and tone, respectively:

$$c^* = \left[a^{*2} + b^{*2}\right]^{1/2} \quad [1]$$

$$h = \tan^{-1}\left[b^*/a^*\right] \quad [2]$$

Results presented in Table 1 show that black beans presented lower luminosity values than non-defective, immature and sour ones, indicating that this parameter can be successfully employed only to separate black defects prior to roasting. Such results are in agreement with previous studies on physical attributes of defective coffee beans (Mendonça et al., 2009). Values for color parameters are similar to those reported on previous studies (Mendonça et al., 2009; Franca et al., 2005), with higher hue values for immature and black beans in association with a greenish tone.

Table 1. Average color attributes of non-defective and defective coffee samples.

Sample	Luminosity (L*)	Hue angle (h)	Chroma (c*)
Non-defective	59	79	20
Black	38	87	14
Sour	51	80	19
Immature	64	89	22

Typical FTIR spectra obtained for defective and non-defective coffee samples are shown in Figure 2. A full assignment of the spectral bands is quite a challenging problem and is not the objective of this work. Furthermore, FTIR literature data on coffee is only available for roasted samples, so a direct comparison cannot be done. Nonetheless, a few qualitative aspects of the spectra can be discussed. The two sharp bands in the 2800-3000 cm^{-1} range have also been reported for both Arabica and Robusta roasted coffee samples, but no identification was attempted (Kemsley et al., 1995). Nonetheless, studies of FTIR analysis of caffeine on soft drinks have also reported two sharp peaks at 2829 and 2882 cm^{-1}, with the later one being correlated with the asymmetric stretching of C–H bonds of methyl ($–CH_3$) group in the caffeine molecule (Paradkar and Irudayaraj, 2002). Lipids in general exhibit a characteristic band arising from the carbonyl (C=O) vibration at 1750 cm^{-1}, that can be clearly identified in the spectra shown in Figure 2 and has also been reported for both Arabica and Robusta roasted coffees (Kemsley et al., 1995). The bands in the in the 900-1500 cm^{-1} region are probably associated to carbohydrates.

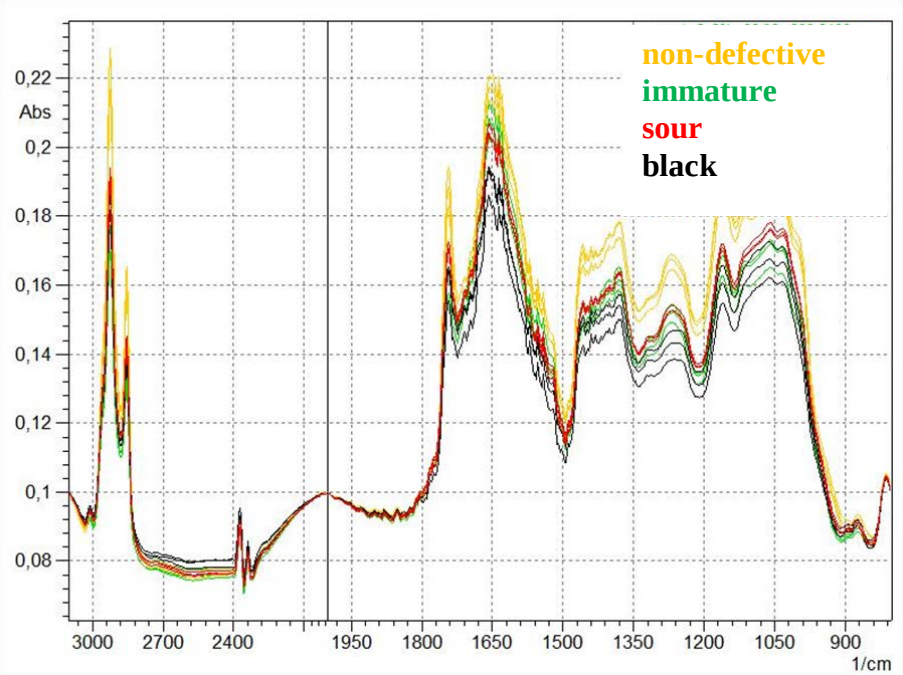

Figure 2. Typical FTIR spectra of non-defective and defective coffee beans.

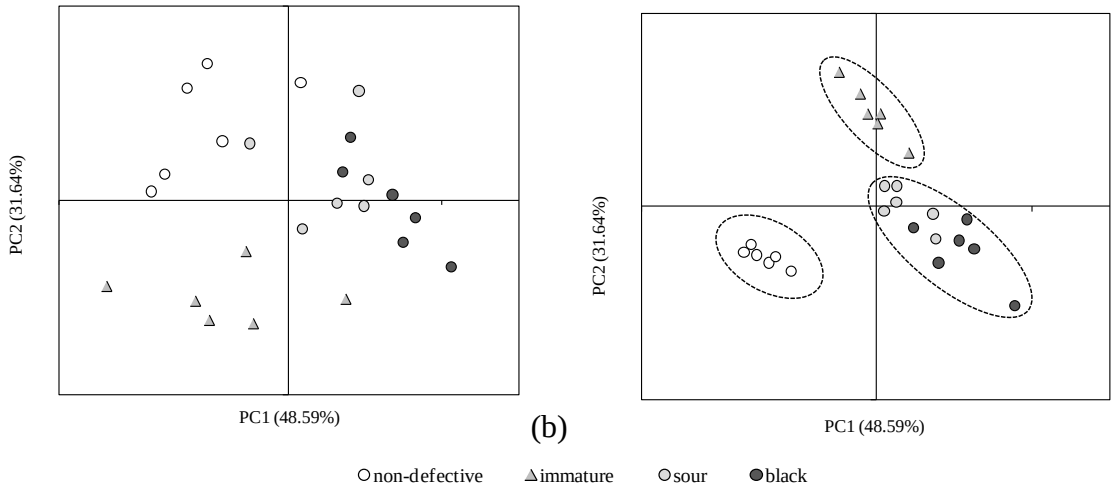

Figure 3. PCA scores scatter plots of (a) FTIR spectra and (b) first derivatives of FTIR spectra obtained for non-defective coffee (•) and coffee husk (o) samples.

PCA analyses of all samples, based on raw spectra and also on the first derivatives, are displayed in Figure 3. Analyses were based on a 24 x 1192 data matrix. In the case of PCA based on the baseline-corrected spectra (Figure 3a), the two first components accounted for 80.23 % of the total sample variance. A certain amount of sample separation can be observed, mainly for non-defective (negative PC1, positive PC2) and immature (negative PC1, negative PC2) beans, but sample scattering is significant. In the case of PCA based on the first-derivative of the spectra (Figure 3b), the first and second principal components accounted for 31.18 and 21.55 % of the total sample variance, respectively. In this case, sample scattering diminished considerably and there is a clear separation between non-defective and defective coffee beans. Three separate groups can be identified: (i) non-defective, (ii) immature and (iii) black/sour.

CONCLUSIONS

The feasibility of employing FTIR as a methodology for the separation between defective and non-defective coffees was evaluated. PCA results based on first-derivatives of the spectra showed that non-defective and defective coffee samples were clearly separated into distinct groups. A certain degree of sample separation was observed among the defective coffee samples, with two separate clusters respectively corresponding to immature and to fermented (black/sour) coffees. The preliminary results obtained in the present study confirm that FTIR analysis presents potential for the development of an analytical methodology for discrimination between defective and non-defective coffee beans. Further studies will be conducted employing a larger set of samples in order to develop predictive calibration models. The methodology will be also tested for roasted coffee samples.

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Improved Chemometric Model for Prediction of Arabica Coffee Overall Quality Using Diffuse Reflectance near Infrared Spectroscopy

J.S. RIBEIRO, T.J.G. SALVA

Agronomic Institute of Campinas - Coffee Center, P.O. Box 28, 13012-970, Campinas, SP, Brazil

SUMMARY

Beyond the use of the near infrared spectroscopy for coffee species identification, for the degree of roasting determination and for the quantification of some chemical compounds of the coffee beans, the near infrared analysis has been also tried as an objective method for beverage quality evaluation. In this work, a chemometric model based on coffee beverage sensory data and diffuse reflectance near infrared spectroscopy is proposed to predict the score of overall quality of coffee beverage. Partial least squares regression (PLSR) was the multivariate method used to construct the model. The ordered predictor selection (OPS) algorithm was applied to select the wavelengths for the regression model in order to take into account only significant wavelengths. The final model was constructed with 7 latent variables and 363 variables. The root mean square error of cross validation (RMSECV), correlation coefficient (r_{cv}) and root mean square error of prediction (RMSEP) values computed by the model established were 0.61 ± 0.02, 0.96 ± 0.01 and 0.52, respectively. When using a scale ranging from 1 to 10 points for the beverage overall quality evaluation, the mean error of the previewed values was 0.52, while in the cupping evaluation the mean error was 0.5.

INTRODUCTION

The consumption of coffee beverage represents about 90 billion dollars annually, from the choice of the beans to be planted until the final commercialized product. Around the world, specialists and scientists search for ways to recognize, to promote and to valorise standards of quality for the best coffee beverages. Normally, the beverage coffee quality is evaluated by experts through the cupping test.

Despite the reliability of the cupping method for commercial purposes, scientists are searching for more objective, simpler and faster analytical methods that could be easily used in routine coffee beverage analyses.

Near infrared spectroscopic (NIRS) has been tentatively tried for qualitative and quantitative chemical analyses and also in sensory evaluation of foods and beverages (] Li et al., 2006; Wang et al., 1999; Siebielec et al., 2004; Reich, 2005; Roggo et al., 2007; Chen et al., 2006; Cozzolino et al., 2006; Karoui et al., 2006; Cen and He, 2007; Pizarro et al., 2007; Pedro and Ferreira, 2009).

This work aimed to improve previous chemometric model (Ribeiro and Salva, 2009) for the prediction of beverages overall quality score using the correlation between NIR spectra of green coffee samples and the scores from sensory analyses.

MATERIALS AND METHODS

One hundred and fifty one Brazilian Arabica green coffee samples previously submitted to sensory analysis for overall quality attribute were analysed by diffuse reflectance near infrared spectroscopy (DRIFTS). All samples were ground in Perten grinder model Mill 3600 Instruments, Sweden) until granulometry between 0.5-1 mm.

Diffuse reflectance spectra of ground coffee were obtained using a near-infrared spectrophotometer (Foss NIRSystems 6500, Raamsdonksveer, Netherlands) equipped with a reflectance detector and sample transport module. Each spectrum was profiled from 256 scans from 1100 up to 2500 nm at 2 nm resolution. Three different aliquots of the 151 samples were analysed resulting 453 spectra.

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.) (Wise et al., 2004).

In the present study three pre-treatments were applied to the original data matrix: Normalization by norm one (unit area), Savitzky-Golay smoothing with a window size of 5 points and first derivative (Savitzky and Golay, 1964).

The algorithm ordered predictor selection (OPS) was used for variable selection (Teófilo et al., 2009), according to the following sequence:

- Step 1 – Selection of an informative vector that contains information about the location of the best independent variables for prediction;
- Step 2 - Differentiation of the original variables according to the corresponding values of the informative vector selected in step 1;
- Step 3 - Sorting variables in decreasing order of magnitude;
- Step 4- Building and evaluating the multivariate regression models through a cross validation strategy.

The partial least squares method (PLS) was the regression method used for modelling (Ferreira et al., 1999; Ribeiro et al., 2009). The confidence limit used for the value of leverage was of 95% and two standard deviations were used for the Student residue.

All samples were submitted to a sensory evaluation by experts using 10 g of roasted and ground coffee in 100 mL of hot water. The cup quality was assessed through the overall quality attribute, which means the joint perception of the flavour, aromas and other attributes like bitter, acidity and body evaluated during the sensory analysis (www.abic.com.br, accessed in April 2008). The overall quality attribute was evaluated through an arbitrary scale varying from 1 to 10 points, where 1 point was conferred to a very bad overall quality and 10 points to an excellent overall quality.

RESULTS AND DISCUSSION

The original spectra of the ground green coffee samples were organized into a format X matrix (453 x 700) and then the data pre-treatment was performed (X_p). Figure 1 shows the original (A) and pre-treated (B) spectra.

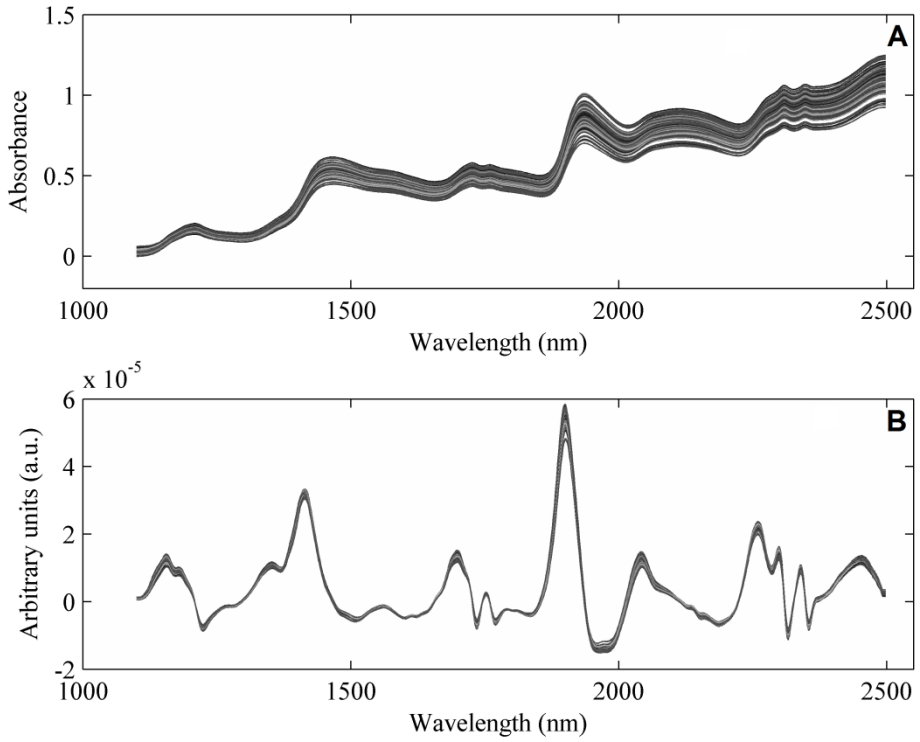

Figure 1. Original (A) and pre-treated (B) diffuse reflectance spectra of the ground green coffee samples.

The OPS algorithm selected 363 variables (29 wavelength ranges) from a total of the 700 available as indicated by the vertical lines in Figure 2. The variable selection consisted in the choice of spectra regions composed by wavelengths highly correlated with the response studied. In this case, the adequate selection of spectral regions allows to minimize the calibration errors (Teófilo et al., 2009), improving significantly the efficiency of the generated model. The spectra regions selection allows the construction of robust and easily interpreted models with high-precision predictions.

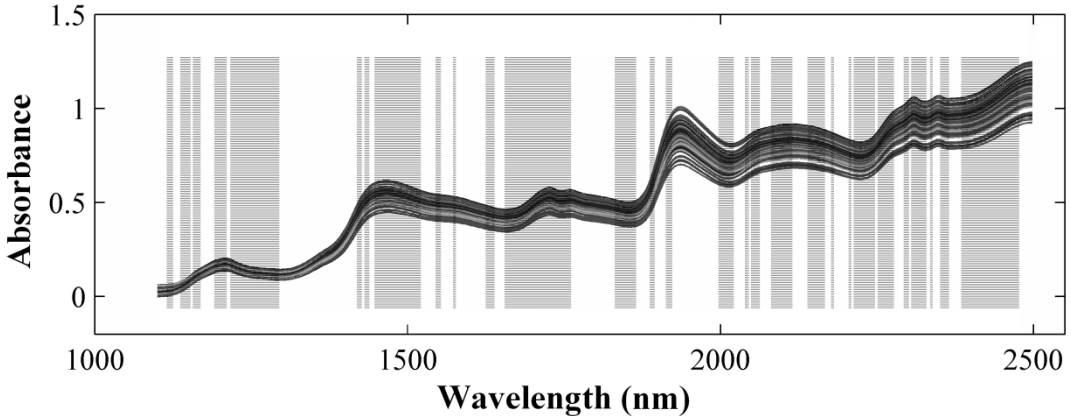

Figure 2. Variables selected and used to construct the PLS model.

For the regression model development, the average scores for the beverage overall quality conferred by the experts were used as the dependent variables (**y**) and the pre-treated infrared spectra (matrix $\mathbf{X}_p$) of the ground green coffee samples was used as the independent variables.

The data set was split as follows: 136 samples (408 spectra) were randomly selected to be the calibration set, and the remaining 15 samples, corresponding to 45 spectra, were used for external validation. Leave five out cross-validation (three replicates of five samples were left out at a time) was the method used to select the number of components in the models.

The leverage vs. Student residual plot provides important information about the presence of outliers for the PLS models construction. In this sense, samples that simultaneously present elevated values of Student residual and leverage must always be excluded and the calibration model reconstructed. In this study, there were some replicates showing high values of leverage, whereas others showed high values of Student residual (Figure 3). However, there were no replicate that presented simultaneously high values for leverage and for Student residual. Therefore, there were not atypical samples.

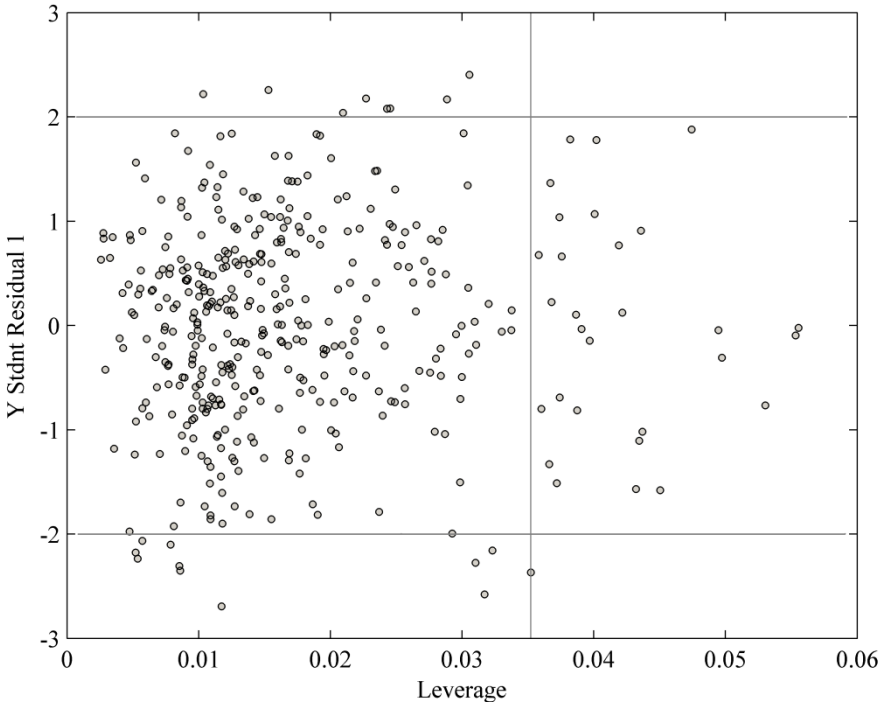

Figure 3. Leverage vs. Student residual plot for the samples used in the construction of the PLS regression model.

Using 7 latent variables it was possible to describe 93.11% and 96.76% of the variance used in blocks **Y** and **X**, respectively. The root mean square error of cross validation (RMSECV), correlation coefficient (r_{cv}) and root mean square error of prediction (RMSEP) values computed by the model established were 0.61 ± 0.02, 0.96 ± 0.01 and 0.52, respectively. The description of the variances for each latent variable used is indicated in Table 1.

Table 1. Variance percentage obtained for each latent variable of the PLS model.

Nº LV	Block **X**		Block **Y**	
	% variance	**% Cumulative**	**% variance**	**% Cumulative**
1	50.75	50.75	36.33	36.33
2	11.67	62.42	17.34	53.67
3	12.32	74.74	12.49	66.16
4	13.43	88.17	11.11	77.27
5	4.30	92.47	8.94	86.21
6	2.48	94.95	3.78	89.99
7	1.81	96.76	3.12	93.11

Figure 4 shows the experimental values for the sensory attribute vs. the respective values estimated in cross validation step. The model was also validated by the external data set (15 samples). The predicted values for the external validation samples were also included in this figure to show that they were well predicted.

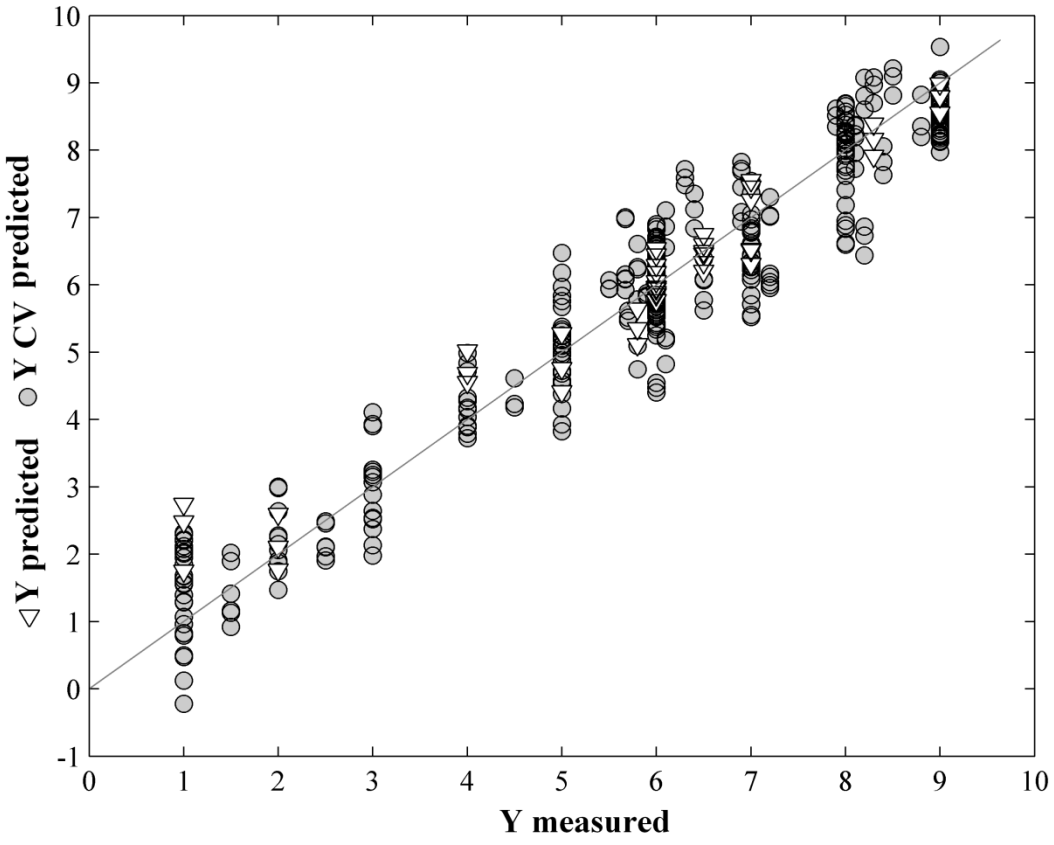

Figure 4. Plots of measured vs. predicted values for the calibration (○) and prediction (Δ) sets.

Table 2. Scores of the beverage overall quality as provided by the sensory analises and the scores predicted by regression model.

Prediction samples	Mean notes from experts	Mean notes from model
1	7.0 ± 0.5	6.45 ± 0.12
2	7.0 ± 0.5	7.42 ± 0.15
3	6.0 ± 0.5	6.15 ± 0.26
4	1.0 ± 0.5	2.32 ± 0.51
5	5.0 ± 0.5	4.81 ± 0.54
6	4.0 ± 0.5	4.75 ± 0.24
7	8.3± 0.5	8.15 ± 0.24
8	6.0 ± 0.5	5.86 ± 0.10
9	6.0 ± 0.5	6.30 ± 0.13
10	6.5 ± 0.5	6.43 ± 0.20
11	6.5 ± 0.5	6.51 ± 0.21
12	6.0 ± 0.5	6.12 ± 0.36
13	5.8 ± 0.5	5.37 ± 0.26
14	9.0 ± 0.5	8.78 ± 0.22
15	2.0 ± 0.5	2.15 ± 0.42

Table 2 presents the values for beverage overall quality of the 15 samples used in the external validation step provided by the experts as well as the values predicted by the PLS model.

According to the results showed herein, there is high linear correlation between the beverage overall quality scores provided by the sensory analyses and specific wavelengths of the near infrared spectra of the ground green coffee. The PLS model constructed from the 363 variables as selected by the OPS algorithm predicted the overall quality scores with mean error of 0.52 in a scale from 1 to 10, while the mean error of the scores attributed by the experts was of 0.5 in the same scale.

The PLS model published previously (Ribeiro and Salva, 2009), was constructed with 119 samples and 24 spectral regions. That model was built with 5 latent variables, RMSECV = 0.70 ± 0.04 r_{vc} = 0.93 ± 0.01 and RMSEP = 0.4. Now, this improved model was 151 samples of different years and the results are very similar. Almost all the spectral regions selected before were selected again in this model.

CONCLUSIONS

The PLS regression model using NIR spectroscopy profiles of crude ground Arabica coffees was able to predict the beverage overall quality scores with a mean error of 0.52 when the mean error provided by the sensory analyses for this attribute was 0.50 for the same set of samples. Variable selection using the OPS algorithm was a primordial step to find out the spectral regions that would best describe the sensory attribute studied.

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Chemical Stability of Roasted Coffee Aqueous Extracts

J. SILANES-KENNY[1], L. NAVARINI[2], F. SUGGI-LIVERANI[2], J. MELROSE[1]

[1]Kraft Foods, Global Coffee Technology Centre, Banbury, United Kingdom
[2]illycaffè S.p.A., Via Flavia 110 – Trieste, Italy

SUMMARY

It is well known that storage of roasted coffee aqueous extracts causes a set of chemical and physical changes leading to a remarkable deterioration of their organoleptic characteristics. These changes include acidity and turbidity development as well as time-dependent colour and flavor modifications reaching an overall sensory unacceptability in a period of time which depend on storage conditions. Early studies put in evidence that most of the chemistry involved in the phenomenon was confined into hydrolytic reactions producing acids from quinic acid lactones and chlorogenic acid lactones formed during roasting, from low molecular weight esters and from thermal degradation products of chlorogenic acids. Unfortunately, this chemistry cannot explain in detail the origin of the instability, which it is still largely unknown in spite of the efforts of several research groups over the last two decades. Moreover, it is not yet clear whether acidity and turbidity development and time-dependent colour and flavor modifications are concomitant or interconnected phenomena.

The present work offers an up-dated overview of literature data on this important aspect of coffee technology. Emphasis has been paid to report on the role played by raw materials, storage conditions and technological processes in affecting the chemical stability. Possible precursors and mechanisms proposed to interpret the phenomenon are reviewed and critically discussed.

INTRODUCTION

Roasted coffee aqueous extracts show a very low time-dependent chemical stability characterized by development of perceived sourness and turbidity as well as by changes in colour and in the aroma profile. The most evident effects of such instability, in addition to the sensory deterioration, are the pH decrease, corresponding to an increase in titratable acidity, and the sediment formation. The complex set of chemical reactions at the basis of the instability of liquid coffee as well as of coffee concentrates, starts immediately after brewing and proceeds relatively rapidly during storage even at sub-zero temperatures. In spite of remarkable effort by several research groups in understanding the origin and the related mechanisms of such phenomena, up to now, the chemistry governing the instability is far from clear. Several precursors have been suggested as possible sources of chemical instability: chlorogenic acids, quinides, carbohydrates, melanoidins and other non-enzymatic browning products, but unfortunately the problem is still open and very challenging. Very intriguing and still undisclosed is the interplay between changes of volatiles and non-volatiles during the storage. Only recently, the interactions between key aroma compounds and non-volatiles as well as the chemical stability of selected volatiles in aqueous model systems have been studied to ascertain possible molecular mechanisms at the basis of coffee brews staling.

In the past, a pH value of 4.8 has been considered as the limit of acceptability for stored coffee brews but, nowadays, in addition to empirical assessment, an emerging body of findings demonstrate that pH cannot be considered as the unique criterion for coffee brew acceptance (Perez-Martinez et al., 2008). In particular, the concomitant evolution of pH, aroma profile and colloidal state and their interactions with the human oral cavity system have to be investigate to establish more solid acceptance criteria.

In addition to the effort in understanding the chemistry behind the chemical instability of roasted coffee aqueous extracts, no technological strategy has been fully successful in preventing the problem without affecting the product quality.

ACIDITY DEVELOPMENT DURING STORAGE OF AQUEOUS EXTRACTS

It is well known that organic acids are naturally occurring compounds in green coffee beans and that the roasting process induces the increase/decrease of some of them or the formation of new ones through thermal decomposition of precursors. So far, 38 acids have been identified in coffee of which 17 seemed to be generated from carbohydrate precursors present in the green bean (Bahre and Maier, 1999). In addition to citric and acetic acids, suggested by Engelhardt and Maier (1985) as key contributors to acidic taste in coffee beverages, Zehentbauer et al. (2004) found quinic, lactic, formic, fumaric, malic and phosphoric as key acidic compounds.

Early studies on the acidification of coffee brews induced by aging have been focussed on the relationships between pH decrease and increase of organic acids.

Sivetz and Desrosier (1979) showed that the decline in pH (as a result of the formation of caffeic and quinic acids as breakdown products of chlorogenic acid following aldehydic oxidation) accelerated with increasing storage temperature.

Maier et al. (1984) reported on the variation of selected relevant organic acids on aging (see Table 1). The average increase after 72 h at 80 °C was found to be 29% with a remarkable increase of phosphoric acid (+54%). It has been observed by the same authors that the increase of the organic acids is more pronounced by increasing the coffee roasting degree and that chlorogenic acids and quinic acid showed the tendency to increase, as well.

Table 1. Organic acids variation (100 = initial value) of coffee infusions aged at 4 different temperatures (Maier et al., 1984).

Temperature, °C	20	40	60	80
Time, h	72	72	72	72
Malic acid	99	104	108	115
Formic acid	101	105	117	130
Citric acid	97	103	104	118
Acetic acid	98	98	109	127
Glycolic acid	101	107	110	128
Phosphoric acid	99	102	107	154

Van der Stegen and van Duijn (1987), with a similar approach but under different experimental conditions, reported comparable data, as shown in Table 2., even if the increase of quinic and glycolic acids was higher than that previously reported (Maier et al., 1984). Moreover, chlorogenic acids, showed a slight increase (+ 2%) after 24 h at 95 °C.

Table 2. Increase of individual acids after 24 hours aging (van der Stegen and van Duijn, 1987).

Acids	**Van der Stegen and Van Duijn 1987 95 °C (meq/L)**	**Maier et al. 1984 Boiling (meq/L)**
Quinic	2.8	1.6
Acetic	1.0	1.1
Succinic	0.9	-
Formic	0.5	0.6
Glycolic	0.4	-0.1
Lactic	0.3	0.6
Citric	0.2	0.6
Malic	0.1	0.2
Phosphoric	-	1.1

In view of the parallelism between increase in titratable acidity and increase of organic acids content, van der Stegen and van Duijn (1987) suggested that the acidification of coffee beverage on standing is due to hydrolytic reactions involving ester compounds and quinic acid lactones formed during roasting process.

Verardo et al. (2002), on coffee extracts aged 72 h at 65 °C, found that even in green coffee aqueous extracts there was a significant increase in some organic acids, namely formic, acetic and quinic acids and that, in the case of roasted coffee extracts, in confirming the tendency to a general increase of individual organic acids, formic and acetic acids showed a remarkable increase followed by quinic, phosphoric, glycolic and lactic. The same group, in order to confirm the possible role played by ester compounds, compared the results obtained after aging (65 °C, 6 h) of the same coffee extract with and without initial pH correction to 2.5 with 0.3 M HCl. In spite of the different hydrolytic starting conditions, there was no evidence of large difference between the two set of data. On the contrary, the addition of a reducing sugar (galactose) into the coffee extract before aging (65 °C, 72 h), produced a significant increase of formic, acetic, glycolic and lactic acids in respect to the same coffee extract aged without the initial addition. These results are evidence of the complexity of the phenomena and suggeste the possible involvement of both sugar degradation and Maillard reaction in explaining the acidification.

Severini et al. (1993), investigating the storage of a coffee infusion at 40 °C up to 120 h, observed no variations in the content of chlorogenic, malic, lactic and citric acids. In another study (Migliosi et al., 1998), the storage at low temperature (4 °C) up to 2 months of a coffee aqueous extract, produced no variations in the content of 3-, 4- and 5-caffeoylquinic acids in spite of the observed pH decrease, suggesting that chlorogenic acids were not involved in the

acidification. On the other hand, a coffee brew kept 4 h at 80 °C showed a significant decrease of 5-caffeoyl-quinic acid content and a concomitant increase of 3- and 4-caffeoyl-quinic acids concentration and decrease (up to 60% of initial amount) of corresponding lactones (Schrader et al., 1996). These changes were attributed to acid-catalysed isomerisation of 5-caffeoyl-quinic acid and to the hydrolysis of the intramolecular ester bond of the lactones. However, in view of the total decrease of the sum chlorogenic acids and corresponding lactones, the authors suggested that other reactions such as polymerization or degradation, took place during the aging. Isomerization of 5-caffeoyl-quinic acid has been suggested as a mechanism for conversion into 3- and 4-caffeoyl-quinic acids after retort sterilization (124 °C for 18 min) of a canned coffee beverage (Yamada et al., 1997). The same canned coffee beverage stored up to 20 days at 60 °C showed a decrease of chlorogenic acids and an increase of their degradation products: caffeic and quinic acids, 4-vinyl-cathecol and 4-vinyl-guaiacol. Moreover, it was confirmed an increase of the phosphoric acid as a decomposition product of inositol-hexaphosphoric (phytic) acid, even if, it has been suggested that the phosphoric acid represent only a minor source of hydrogen ions in coffee (Griffin and Blauch, 1999).

Very recently, Perez-Martinez et al. (2008), followed the evolution of several compounds during storage of a coffee brew at 4 and 25 °C (with and without oxygen) for 60 and 30 days, respectively. In all cases the pH dropped (more rapidly at 25 °C with oxygen) but both caffeine and trigonelline were unaffected by aging. 5-caffeoyl-quinic acid significantly increased during the first 3 days of storage (except for the brew stored a 4 °C without oxygen). This increase has been attributed to hydrolysis of chlorogenic acid lactones or to release of chlorogenic acids from melanoidins. After this increase, 5-caffeoyl-quinic acid was maintained up to 20 and 30 days when a decrease was observed in the coffee brew stored without oxygen at 25 and 4 °C, respectively. Storage time hardly affected the amount of both caffeic and ferulic acids. Moreover, 4-vinyl-guaiacol showed a significant decrease during storage, and this, in view of a larger extent in the brew with oxygen, has been ascribed to possible oxidation to vanillin and vanillic acid.

It seems clear that the effect of aging on chlorogenic acids is different if storage is performed in the temperature range 4-40 °C or at higher temperatures. Cammerer and Kroh (2006) reported a different behaviour of coffee beverages held for several hours at 70 °C or at 40 °C, as far as free chlorogenic acids is concerned. In particular, whereas at 70 °C a significant increase of free chlorogenic acids content was observed, at 40 °C only negligible changes were detected.

Ginz et al. (2000) in a detailed study on the formation of aliphatic acids by carbohydrate thermal degradation (up to 280 °C), found that formic, glycolic acetic and lactic acids were invariably produced independently on sugar type (sucrose, glucose, fructose arabinose, arabinogalactan), possible mixing with amino acids (sucrose/glycine mixtures), previous thermal dehydration (1,6-anhydroglucose) and the presence/absence of oxygen. Similar studies gave evidence for the relevance of sugar fragmentation in the Maillard reaction as far as short-chain carboxylic acids formation is concerned, and the coexistence of several reaction mechanisms both hydrolytic and oxidative leading to carboxylic acids (Davidek et al., 2006a; 2006b).

Some results reported in coffee literature (Verardo et al., 2002; Anese and Nicoli, 2003) suggest, somewhat surprisingly, that non-enzymatic browning reactions (caramelization and Maillard) seem to be involved in the acidification of coffee brews on storage: caramelization can represent a source of acids and acidic precursors (in the case of glucose: 1-

deoxyglucosone and 3-deoxyglucosone) which can contribute to develop acidity during storage; in addition melanoidins (even those derived from sugar breakdown products (Cammerer et al., 2002)) with their not negligible content of covalently associated phenolic compounds (Nunes and Coimbra, 2010) and incorporation of chlorogenic acids (Bekedam et al., 2008; Leloup et al., 1995), may release acidic compounds (quinic acid or hydroxycinnamic acids) during aging. Moreover, sugar esters in green coffee beans (Weckerle et al., 2002) and acetylated polysaccharides (Oosterveld et al., 2004; Nunes et al., 2005), present in both green and roasted coffee beans, may represent additional sources of acidic compounds released in the course of aging.

In all cases, the acidification process, is not stopped by changing one or more of the most usual technological parameters which can be controlled during industrial production, parameter such as: raw materials (including water), roasting degree and roasting process, brewing and extraction conditions, coffee soluble solids concentration, usual ingredients (including buffering agents), headspace atmosphere, process and storage temperature (For the latter, the replacement of pasteurization (25 min at 65 °C) with no-thermal sterilization processes (Severini et al., 1995) as well as freezing and storage at -20 °C do not stop the acidity increase of aqueous coffee extracts (Nicoli et al., 1991)). However these parameters may however effect the rate of acidifications and the overall quality effects although zeroth order kinetics remain.

COLOUR AND TURBIDITY CHANGES

Pangborn (1982) observed visually a strong increase in intensity of color and turbidity in the first hour of keeping a coffee beverage at 95 °C. At 60 and 80 °C the observed changes were rather small. A sugared and pasteurized (70 °C) coffee beverage, stored in the range 4-40 °C for up to 12 days showed a colour change with time (from light to dark) with a profile very similar to that of pH change (Dalla Rosa et al., 1990). In investigating the role played by oxygen in colour and turbidity changes of coffee beverage during storage (20 and 80 °C up to 5 hours), Steinhart et al. (1995) clearly showed that darkening and turbidity development during hot storage were remarkably increased by passing oxygen through the system. In the absence of oxygen (very limited headspace) both darkening and turbidity showed small changes. Interestingly, the system stored with oxygen at low temperature showed the darkening observed in hot storage but a decrease of the intensity of turbidity. According to the authors, first of all melanoidins are thermally unfolded, secondly phenolic groups contained into melanoidins are subjected to oxidation reactions which promote aggregation. Both oxidative aggregation and electrostatic interactions lead melanoidin micelles to a growing tendency towards insolubility.

Colour and turbidity changes have been reported in the case of coffee beverages (pasteurized 15 min at 85 °C) stored 64 days at two different temperatures: 4 and 20 °C (Pizzirani et al., 1996). In particular, in the course of the storage, the colour, initially black, progressively shifted to brown, and the turbidity (accompanied by sediment formation) regularly increased, with a shape close to that of the pH vs time profile. Low temperature storage resulted in a less remarkable effects albeit very well detectable.

Similar behaviour has been reported by Migliosi et al. (1998) during storage up to two month at 4°C of coffee beverages prepared by using different type of water and different botanical species. Interestingly, the increase of turbidity in the course of the storage was higher in the case of *Coffea canephora* var robusta in respect to that of *C. arabica.*

A progressive increase of browning during storage in ordinary atmosphere (up to 17 days at 30 °C) of coffee brews prepared by using coffee at three different roasting degree (light, medium and dark) has been reported (Anese and Nicoli, 2003). However, during storage of the coffee brews in nitrogen, no colour changes have been observed. In the same study, no differences have been observed in the pH drop of coffee brews maintained under ordinary or nitrogen atmosphere, being the kinetics expressed as rate constants not statistically different. According to the Authors, the presence of oxygen trigger the generation of radical species involved in further developing of the Maillard reaction. Maillard browning and the degradation of Amadori compounds are known to be enhanced in the presence of molecular oxygen and metal ions (Rizzi, 2003). It is well known that free radicals are present in both roasted and instant coffee and the related electron spin resonance signal persist in solution for several weeks with no evidence of hyperfine splitting (Troup et al., 1988). Moreover, it is also well known that free radical reactions occur in the beverage and that hydrogen peroxide is generated in coffee brews, mainly due the metal ions-catalyzed autooxidation of polyphenols, with a concentration increasing as a function of time (Pascual et al., 2002; Akagawa et al., 2003; Nagao et al., 1986). It has been demonstrated that radicals in coffee are derived from sugars or other carbohydrates and not from phenols (Gonis et al., 1995). This finding suggests that the effect of reactive oxygen species in colour changes could be related to the carbohydrate moieties of melanoidins and/or to sugar degradation products rather than to direct action on incorporated chlorogenic acids. Polyphenols may play an indirect role on colour changes through their capability to generate hydrogen peroxide. As matter of fact, the production of hydrogen peroxide has been shown to be completely inhibited under nitrogen atmosphere (Akagawa et al., 2003).

SEDIMENT FORMATION

As already mentioned in the previous paragraph, turbidity development and sediment formation have been usually observed during storage of roasted coffee aqueous extracts. Sediment is sometimes observed in coffee concentrates, and this represent a quality defect of the product since limits their applications. In spite of this important aspect, coffee literature has limited reports of such types of instability. Storage of solubilised instant coffee at elevated temperatures (up to 80 °C for 72 h) led to an increase in sediment yield (Bradbury and Atkins, 1997). Holding the same system at 60 °C up to more than 150 h led to a steady increase of sediment yield. In all cases the yield of sediment was paralleled by yield of precipitated mannan polysaccharide, which was shown to be a major component (ca. 60%) of insoluble sediment formed from soluble coffee (Bradbury and Atkins, 1997). According to the Authors, galactomannan naturally present in the aqueous extracts, gradually converts to an insoluble crystalline form, characterized by X-ray diffraction pattern identical to that of Ivory Nut β1→4 mannan, resulting in precipitation. Recently, Delgado et al. (2008) in a detailed investigation on the chemical composition of fractions from coffee extracts and sediments, substantially confirmed early observations. In particular galactomannan was found to be the main polysaccharide component of the insoluble fractions and probably responsible for sediment formation. However proteins and considerable amounts of lignin and lipids in some fractions, have also been found. The galactomannan present in the insoluble fractions had a lower galactose:mannose ratio than that found in the whole extract and this has been related to the lower polysaccharide solubility leading to sediment formation. To enforce this view, the Authors were successful in achieving a remarkable reduction of the sediment by treatment with a commercial galactomannanase enzyme. Unfortunately, no studies have been devoted to investigate sediment formation together with the evolution of the other chemical and physical changes during storage. However, it could be very hard to believe that sediment formation and the other types of instability are completely independent phenomena.

AROMA DEGRADATION

Aroma is one of the key drivers of consumer liking of roast coffee beverages, and this is no exception in beverages prepared from aqueous extracts. Therefore, in order to produce a ready to drink liquid coffee beverage or a concentrate from which to prepare said beverage that is acceptable to consumers, one must solve the problem of aroma degradation which readily occurs in a few hours in an unmodified liquid coffee beverage.

Coffee aroma is composed of approximately 800 compounds (Czerny et al., 1999) although only representing around 0.2% of the coffee's dry matter, they play a critical role in the acceptability of the beverage by consumers world wide. Coffee aroma degradation has been shown to proceed in very short time frames, of hours or even minutes (Hofmann and Schieberle, 2002), and have been shown to be heavily dependant on temperature (Manzocco and Nicoli, 2007).

Studies by Charles Bernard et al. (2005) showed the degradation of sulphides, pyrroles and diketones in the coffee brew, and further attempted to understand the role of different coffee fractions and compound classes on aroma degradation. The authors concluded that chlorogenic acids may indirectly contribute to thiol degradation by formation of quinones ion in the presence of oxygen and radical formation during Fenton reaction.

It has also been shown that weaker, hydrophobic, binding can occur with lipids or "hydrophobic pockets of proteins" in coffee brews (Denker et al., 2006). However, such binding is likely reversible and should not be considered in the context of aroma degradation in coffee brews.

Of more interest is the role of the complex Maillard reaction compounds defined generally as melanoidins. Melanoidins are brown coloured compounds that have generally been difficult to characterise due to their heterogeneity and complexity. Kroh et al. (2008) described melanoidins as products of α-dicarbonyl compounds polymerization in a carbohydrate-based aldol condensation, whereas Tressl et al. (1998) postulated melanoidins to be pyrroles- and/or furans-based polymer-like material formed during the advanced stages of the Maillard reaction. The work of Bekedam and Schols (2008; 2008) in the University of Wageningen showed the complexity of coffee melanoidins, and also identified chlorogenic acids esterified in the melanoidin complex, and Nunes and Coimbra (2007) showed phenolic groups covalently incorporated into it.

In summary, melanoidins are coloured compounds of a heterogenous nature that are primarily derived from protein and carbohydrate during roasting, but will also incorporate other structures such as chlorogenic acids and phenols. Hofmann's group (2002) further identified the presence of Maillard derived pyrazinium compounds derived from CROSSPY (1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium) cation radicals. This particular moiety is of interest because it was shown in the author's paper that this structure is capable of covalently bonding with furfurylthiol and thus reducing the sulfury, fresh and roasted odours typical of the fresh brew.

Further work by Hofmann's group (2005) identified new sites for binding of furfurylthiol in the coffee bean by means of "in bean" model experiments in which extracted green coffee beans were re-infused with certain precursors to understand which would yield thiol binding moieties. This work enabled the authors to conclude that chlorogenic acids, and even more caffeic acid, roasted in the presence of metal ions generated thiol binding structures with an

activity very close to that measured in the authentic coffee brew, possibly pointing that these acids are critical to the generation of active sites that must be located in coffee melanoidins. A later study by the same group (Muller et al., 2006) identified several derivatives from chlorogenic acids and caffeic acid (catechol, pyrocatechol, 4-ethylcatechol, hydroxyhydrobenzene, 4-methylcatechol and 3-methylcatechol) in the coffee bean and was able to identify their conjugates in the aged brew. However, Hofmann et al. did not report on the relative importance of CROSSPY derived moieties and chlorogenic and caffeic acid derived moieties in binding thiols in the coffee brews.

Charles Bernard et al. (2005) identified two differentiated mechanisms for thiol degradation, either nucleophilic addition or radical mechanism. This means that not only non volatile compounds, but also other aroma compounds as playing a role in reacting with these fresh thiols. According to the authors, aldehydes present in the coffee aroma, mainly derived from Maillard reaction or sugar degradation, are nucleophillically attacked by the free electron pair on the sulphur atom in the thiol, yielding a non odour active compound, and therefore effecting the loss of fresh aroma typical of newly brewed coffee brews. The findings of this study were apparent in US patent (Zheng et al., 2006) "stabiliser free stabilised aroma", and others which disclose the removal of the aldehydes by means of contacting the coffee aroma with a nucleophile bound to a column in order to stabilise the odour active thiols.

Another industrial approach to dealing with this issue has been disclosed by Kraft (Silanes-Kenny, 2010). This consists in enzymatically reacting to molecules of thiol to yield a disulfide. These disulfides have the advantage of not having a reactive free sulphur, but still possessing a fresh coffee aroma which is able to sustain the freshly brewed character of the liquid coffee during its shelf life, and deals with the issue of reaction with volatile compounds and non volatile compounds simultaneously.

However, both these attempts (Charles-Bernard et al., 2005; Silanes-Kenny, 2010) to stabilise coffee aroma have been limited to dealing only with the issue of thiol degradation, which in reality seems to be but one of the processes occurring during coffee brew storage.

A possible interplay between pH and aroma degradation has been described by Kumazawa and Masuda (2003). In particular, it has been shown that the stability of 2-furfurylthiol during heat processing has a close correlation with the pH of the aqueous solution. In the pH range of 5.0-7.0, normally used after adjustment before the sterilization of canned coffee drinks, model solutions of 2-furfurylthiol heated at 121 °C for 10 min contained very low residual amount of this potent odorant and several of its degradation products (difurfuryl disulfide, furfural and furfuryl alcohol). The same model solutions kept in the pH range 4.0-5.0, after heating showed a limited degradation only. In the same study, the comparison before and after heat processing (121 °C for 10 min) of canned coffee drink samples revealed that methanethiol (putrid), acetic acid (sour), 3-methylbutanoic acid (sour), 2-furfuryl methyl disulfide (meaty) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (caramel like) increased after heating whereas 2-furfurylthiol (roasty), methional (potato like) and 3-mercapto-3-methylbutyl-formate (roasty) decreased.

Similar studies carried out on coffee brews to follow changes of volatile compounds during storage (4 and 25 °C) showed that no new volatile compounds were detected at the end of the storage time (30 days) in spite of the fact that the stored coffee brews (especially at higher temperature) lose part of their aroma intensity and freshness acquiring some nondesirable notes such as rancid aroma (Perez-Martinez et al., 2008). By resorting to aroma indices to

follow the coffee brews staling, the authors found very highly significant ($p < 0.001$) and excellent ($r > 0.75$) correlations between seven aroma indices and rancid aroma: acetaldehyde/2,3-butanedione; acetaldehyde/2,3-pentanedione; acetaldehyde/dimethyl disulfide; acetaldehyde/ formic acid, methyl ester; acetaldehyde/2-methylfuran; acetaldehyde/3-methylfuran and acetaldehyde/2-vinylfuran. The same seven aroma indices exhibited significant ($p < 0.05$) or very significant ($p < 0.01$) and good ($0.5 < r < 0.75$) negative correlations with aroma intensity and very significant positive correlations with sour taste (Perez-Martinez et al., 2008).

As discussed in detail above, it has been shown extensively (Verardo et al., 2002) that a number of volatile acids, such as formic and acetic acid, are also generated during storage, most likely due to acid hydrolysis of complex carbohydrates, shown by Redgwell et al. (2002) to contain acetic and formic acid moieties, but also possibly by Maillard reaction. As well as the acidic nature of these compounds, which will be discussed in detail, acetic acid can also play a significant role in coffee aroma, producing a vinegary and slightly acrid aroma that can also be detected in aged brews.

Further studies have also shown the generation of other potent odorants during coffee brew storage, such as 4-vinylguaiacol (Yamada et al., 1997) - 4-Vinylguaiacol is a clove like odorant naturally present in coffee (Czerny et al., 1999) and it can be generated as a degradation product of cholorogenic acids, but also vanillin or vanillic acid.

Moreover, Bradbury et al. (1998) also identified that when the coffee brew's acidity is stabilised via the accelerated degradation of all acidity precursors and the brought back to the original pH, the aroma of the coffee is kept fresh even under very high temperature conditions. This effect is very surprising, as in effect all of the thiols generated in the brew are still degraded during storage, and therefore loss of freshness should ensue.

However, as a matter of speculation, rather than the degradation of thiols and the generation of further aroma compounds, it is possible that the flat and not fresh aroma perceived when tasting a stored coffee brew may have more to do with interactions between acidic compounds and aroma compounds in mouth and tongue, as well as to different partitioning of aroma compounds in the acidic environment, Of course, *supra* and *infra* additive sensory interactions have been reported previously extensively, even in coffee (Miyazawa et al., 2009).

In summary, the chemical change in aroma compounds in a coffee brew during storage is a complex issue that makes the development of coffee brews with stable aroma without the use of ingredients or technologies that would otherwise impact the quality of the final beverage very difficult indeed. Studying the physical, chemical and cognitive interactions between compounds responsible for acidity and freshness of aroma in the coffee brew may be the key to understanding the development of liquid coffees with stable and desirable aromas without an ingredient line.

CONCLUSION

In spite of the effort of several Academic and Industrial research groups, the chemistry behind the instability of roasted coffee aqueous extracts is still undisclosed. From one hand, the strategy adopted up to now, seems not fully appropriate to unravel the complexity of the system. It could be very interesting to adopt other strategies, like for instance molecular network mapping approach (cf as used in metabolomics) , to identify evidence of possible

interconnections among the observed phenomena. The increasing role played by melanoidins in interpreting the phenomena at the basis of the chemical instability of coffee brews and extracts, makes it essential to further elucidate the chemical structure of this class of polymeric compounds. Up to now, the molecular weight has been considered as the main discriminative criterion of melanoidins, but it seems clear that this is not sufficient and that the chemistry has to be taken into the account. Several types of melanoidins (protein-like, polysaccharide-like, polymers built up of different types of repeating units, etc.) could be involved in the whole set of reactions occurring during storage. Each type may show different behaviour or reactivity towards changes of temperature, pH, oxygen, radicals, molecular and electrostatic interactions.

This challenging topic, being a fundamental aspect related not exclusively to technological implications, should be faced by the entire coffee science community regardless to possible proprietary interest.

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Pesticides Contamination of Coffee in East Africa

T. ISHIWAKI*, E. YAMAISHI, Y. TANABE

Research and Development Office, S. ISHIMITSU & CO., LTD.,
4-40 Iwayaminami-machi,Nada-ku Kobe, 657-0856 Japan.
*E-mail: t-ishiwaki@ishimitsu.co.jp

SUMMARY

The residual pesticides in green coffee beans have been one of the biggest problems for importers and roasters in Japan. At the last ASIC conference in Brazil, the risks of contamination of pesticides were reported by the same authors. After the conference, a detailed research of the risk in African coffee was conducted by the authors and JETRO (Japan External Trade Organization).

Samples of jute bags, dusts in dry mills, soils, and waters in rivers, green beans, parchment coffees in cherries on trees were collected in Kenya, Tanzania and Ethiopia. Residual pesticides were checked by QuEChERS method. Many kinds of pesticides were detected from almost all of the samples.

To reduce the risks, it is important to know more about each risk. The purpose of this study is to inform the risk factors in coffee industry in East Africa.

INTRODUCTION

On May 29, 2006 the Ministry of Health, Labour and Welfare (MHLW) in Japan introduced the positive list system for pesticides, feed additives and veterinary drugs remaining in foods (http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228). Strict standards, less than 0.01 ppm, were applied to more than 600 pesticides for green coffee beans. If the residual levels get over the standards, the product is enjoined from domestic distribution without any risk evaluation for human health. An accidental contamination could cause violation of the law. And any trivial contamination could lead to a serious problem. At the last ASIC conference in Brazil, the risks of contamination of pesticides were reported by the same authors (Ishiwaki et. al., 2008).

Ethiopian coffee is one of the most popular brands in Japan. Unfortunately, in 2008 the Japanese government found, γ-BHC, Heptachlor, and Chlordane over the maximum residue levels (MRLs) in almost all of the Ethiopian coffee that was imported. The volume of Ethiopian coffee imported into Japan was about 30000 metric tons before 2007, but it decreased to about 1000 metric tons in 2009 (http://coffee.ajca.or.jp/English/related/e02.html). This problem became a nontariff barrier. A detailed research of the risk in African coffee was conducted by the authors supported by JETRO (Japan External Trade Organization) in 2009 and 2010.

The purpose of this study is to inform the risk factors in coffee industry in East Africa.

MATERIALS AND METHODS

Green coffee beans, fresh coffee cherries, and parchment coffee were collected in Kenya, Tanzania, and Ethiopia. Both fresh coffee cherries and parchment coffees were hulled by hands and separated into coffee beans and pulps or parchments. All parts were analysed separately.

Dust samples and bags samples for export and domestic distribution were collected at dry mills, cooperatives, or warehouses. And bags samples for the other crops, like maize were purchased at markets.

Soils, waters, and wastes samples were taken at farms, warehouses, and near there. The list of the samples are shown in Table 1.

The procedure of pre-treatment by STQ method, which is an arranged QuEChERS（Quick Easy Cheep Effective Rugged and Safe）method (http://www.quechers.com/default.htm; Anastassiades et al., 2003) with semi-automatic processer (STQ-L200, AiSTI SCIENCE CO.,Ltd) is shown in Figure 1. All of reagents for the process were purchased from Wako Pure Chemical Industires, Ltd. All of cartridges for the solid phase extraction were products of AiSTI SCIENCE CO.,Ltd. After the pretreatment, the residual pesticides were analysed by GC/MS (GCMS-QP2010, SHIMADZU CORPORATION). The informations about the target pesticides are summarized in Table 2.

Table 1. Types and numbers of samples for analysis.

	Ethiopia	Tanzania	Kenya	Total
Green coffee beans	70	78	22	170
Parchments	6	2	0	8
Fresh coffee cherries	5	1	0	6
Bags for export	5	8	7	20
Bags for domestic distribution	38	18	7	63
Bags for the other crops	3	7	0	10
Dusts in factories	3	13	8	24
Soils	1	3	0	4
Waters	2	1	0	3
Wastes of animals	3	0	0	3

Table 2. Target pesticides and their quantification limits (QL).

Compound	QL (ppm)	Compound	QL (ppm)
Aldrin	0.01	Endrin	0.01
Allethrin	0.01	EPN	0.01
Atrazine	0.01	Etrimfos	0.01
α-BHC	0.01	Fenitrothion	0.01
β-BHC	0.01	Fenvalerate	0.01
γ-BHC	0.01	Fipronil	0.01
δ-BHC	0.01	Flucythrinate	0.01
Chlordane	0.01	Flutriafol	0.01
Chlorpyrifos	0.01	Heptachlor	0.01
Chlorpyrifos-methyl	0.01	Iprodione	0.01
Cyfluthrin	0.01	Isoprocarb	0.01
Cyhalothrin	0.01	Malathion	0.01
Cypermethrin	0.01	Parathion	0.01
o,p'-DDD	0.01	Parathion-methyl	0.01
p,p'-DDD	0.01	Permethrin	0.01
o,p'-DDE	0.01	Phenthoate	0.01
p,p'-DDE	0.01	Piperonyl butoxide	0.01
DDT	0.01	Pirimiphos-methyl	0.01
Deltamethrin	0.01	Pyraclostrobin	0.01
Dichlorvos	0.01	Pyrethrin	0.01
Dieldrin	0.01	Thiamethoxam	0.01
Endosulfan	0.01	Triadimenol	0.01

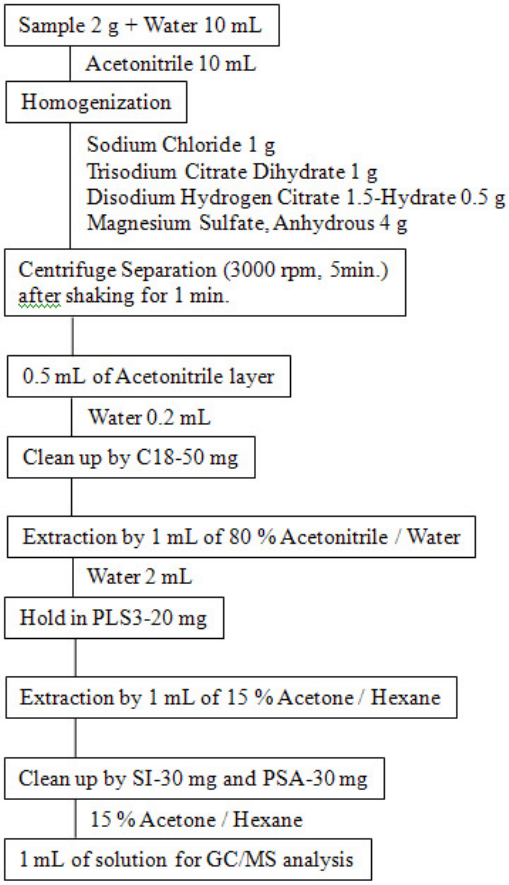

Figure 1. Procedure of pre-treatment for GC/MS analysis.

RESULTS AND DISCUSSION

The results are summarized in Table 3. Many kinds of pesticides including POPs (Persistent Organic Polutants) were detected from Ethiopian coffee. Pirimiphos-methyl and Chlorpyrifos were frequently detected from Tanzanian coffee. In some cases, DDT and Pirimiphos-methyl were detected over the MRLs in Japan.

The pesticides detected in the fresh coffee cherries were considered to be resulted from absorption via the roots. Trace amounts of Chlorpyrifos and DDT were detected from the samples in Ethiopia, in spite of the fact that the farmers did not use such pesticides. It was assumed that the pesticides were from environmental source, because those were also detected from soils, waters, and wastes of animals.

Table 3. Detection rates, and concentrations of pesticides detected.

a) Ethiopia	Sample	Pesticide	Detection rate (%)	Average concentration (ppm)	Maximum concentration (ppm)
	Green coffee beans	Pirimiphos-methyl	20	Trace	0.02
		Chlorpyrifos	17	Trace	Trace
		γ-BHC	10	Trace	Trace
		Chlordane	16	Trace	Trace
		DDT	26	Trace	0.05
	Parchments	Pirimiphos-methyl	50	Trace	Trace
		Chlorpyrifos	33	Trace	Trace
		γ-BHC	50	Trace	Trace
		Chlordane	50	Trace	Trace
		DDT	50	Trace	Trace
	Fresh coffee cherries	Chlorpyrifos	20	Trace	Trace
		DDT	40	Trace	Trace
	Bags for export	Pirimiphos-methyl	40	Trace	Trace
		Chlorpyrifos	40	Trace	Trace
		γ-BHC	20	Trace	Trace
		Chlordane	20	Trace	Trace
		DDT	40	Trace	0.01
	Bags for domestic distribution	Pirimiphos-methyl	53	0.11	1.86
		Chlorpyrifos	79	0.08	1.85
		γ-BHC	68	Trace	0.02
		Chlordane	82	0.01	0.05
		DDT	100	0.02	0.12
		Heptachlor	38	Trace	1.68
	Bags for the other crops	Pirimiphos-methyl	67	Trace	0.01
		Chlorpyrifos	67	Trace	Trace
		DDT	67	0.06	0.11
	Dusts in factories	Pirimiphos-methyl	100	0.01	0.01
		Chlorpyrifos	100	0.01	0.01
		Chlordane	100	0.03	0.04
		DDT	100	0.02	0.02
		Dieldrin	15	Trace	0.09
	Soils	Chlordane	100	Trace	Trace
		DDT	100	0.02	0.02
	Waters	γ-BHC	50	Trace	Trace
		Chlordane	50	Trace	Trace
	Wastes of animals	Pirimiphos-methyl	67	Trace	0.02
		Chlorpyrifos	33	Trace	Trace
		Chlordane	33	Trace	Trace
		DDT	33	Trace	Trace

b) Tanzania	Sample	Pesticide	Detection rate (%)	Average concentration (ppm)	Maximum concentration (ppm)
	Green coffee beans	Pirimiphos-methyl	47	Trace	0.01
		Chlorpyrifos	53	Trace	0.02
	Parchments	Pirimiphos-methyl	100	Trace	Trace
		Chlorpyrifos	100	Trace	Trace
	Bags for export	Pirimiphos-methyl	50	Trace	Trace
		Chlorpyrifos	50	0.03	0.12
		DDT	13	Trace	0.06
	Bags for domestic distribution	Pirimiphos-methyl	100	0.01	0.01
		Chlorpyrifos	100	0.02	0.04
		γ-BHC	61	Trace	0.01
		Chlordane	67	Trace	Trace
		DDT	78	0.02	0.06
	Bags for the other crops	Pirimiphos-methyl	100	0.15	0.29
		Chlorpyrifos	100	0.02	0.04
	Dusts in factories	Pirimiphos-methyl	100	0.03	0.07
		Chlorpyrifos	100	0.23	0.08
		γ-BHC	85	0.01	0.13
		Chlordane	100	0.01	1.42
		DDT	100	0.03	0.08
	Soils	Pirimiphos-methyl	100	Trace	0.01
		Chlorpyrifos	100	Trace	0.01
		γ-BHC	100	0.02	0.04
		DDT	100	0.01	0.05

c) Kenya	Sample	Pesticide	Detection rate (%)	Average concentration (ppm)	Maximum concentration (ppm)
	Green coffee beans	Pirimiphos-methyl	23	Trace	0.01
		Chlorpyrifos	32	Trace	0.02
	Bags for export	Pirimiphos-methyl	30	Trace	Trace
		Chlorpyrifos	70	Trace	Trace
		γ-BHC	14	Trace	0.04
		DDT	14	Trace	Trace
	Bags for domestic distribution	Chlorpyrifos	14	Trace	Trace
		DDT	30	Trace	Trace
	Dusts in factories	Pirimiphos-methyl	38	Trace	Trace
		Chlorpyrifos	100	Trace	Trace
		γ-BHC	25	Trace	Trace
		DDT	13	Trace	Trace

In many cases, the residual pesticides were considered to be resulted from contamination during the course of distribution, because the concentrations in bags or parchments were higher than the concentrations in green coffee beans. The Ethiopian government incinerated all of the old bags in 2008, some of which contained higher levels of pesticides more than 10 ppm. The result showed that such highly contaminated bags disappeared from the market, but contamination of the bags still continued. The bags could be the serious source of contamination, because up to 10% concentrations of pesticides in bags were detected in green beans inside. Pirimiphos-methyl in Tanzanian coffee was assumed to be resulted from bags too. The farmers don't use it for coffee growing. The concentration in bags for domestic distribution was not so high, but 0.29 ppm of Pirimiphos-methyl was detected from bags for maize at maximum. According to the result of hearing investigation, the farmers use this insecticide for the storage of maize, and they sometimes stock their parchment coffees in maize bags.

The pesticides contained in dust at factories have two aspects. Dust was assumed to be one of the sources of contamination of bags and parchment coffees or dried cherries if it remained in huller. An example of the effect of cleaning is shown in Table 4. The manager of A keeps the factory clean with air compressors and vacuum cleaners, in spite that the manager of B does not care for cleaning.

Table 4. Difference in pesticides concentrations in stocked bags between factories.

Detected pesticide	Factory A (ppm)	Factory B (ppm)
Heptachlor	trace	1.68
Chlorpyrifos	trace	1.85
Pirimiphos-methyl	0.01	1.86

Dust was also important for the healthy aspects of the workers. The ADI (Acceptable Daily Intake) for the pesticides are shown in the left part of Table 5. The ratios to ADIs in the right part of the table were calculated on the assumption that a 50 kg weight worker intakes 1 g of dust in a day. It is considered that dusts are undesirable not only for coffee but also for human health.

Table 5. Effects of dusts on human health evaluated by ADIs.

Pesticide / ADI (mg/kg/day)	Maximum concentration (ppm) / Ratio to ADI (%)
Chlorpyrifos / 0.01	0.08 / 0.016
Pirimiphos-methyl / 0.025	0.07 / 0.006
DDT / 0.005	0.08 / 0.032
Chlordane / 0.0005	1.42 / 5.68
Dieldrin / 0.0001	0.09 / 1.8

All of the pesticides detected in this study exist in Africa (Haylamicheal et al., 2009). The FAO promotes a project to take the POPs away from Africa (http://www.fao.org/docrep/w8419e/w8419e00.HTM). But, it is not enough for reducing the risk, because very small amounts of pesticides in the bags or the environments lead to violation. It is considered to be

the most important counterplan for this issue is education about the pesticide management for the farmers and cleaning at the factories.

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Gas Chromatography – Mass Spectrometry in Tandem with Sensory Analysis for Identification of Luwak Arabica Coffee

CAHYA ISMAYADI, ARIZA BUDI TUNJUNG SARI, MISNAWI

Indonesian Coffee and Cocoa Research Institute, Jl. PB Sudirman No. 90,
Jember 68110, Indonesia

SUMMARY

Luwak coffee has gained indispensable attention in the cluster of specialty coffee. The price of Luwak coffee gains a special premium at highest level compared to others. Luwak coffee is produced by collecting coffee beans originated from droppings of natural digestion of coffee berries through digestive track of civet cat (*Paradoxorus hermaphroditus)*. Selection of optimum ripeness of coffee berries and involvement in Luwak metabolism along digestive track has been suggested to build the uniqueness of the coffee. Current identification of Luwak coffee is base on trust to the producer or trader, no scientific analysis and verification to recognize the genuine of Luwak coffee. This research aimed to develop a methodology to identify the uniqueness of Luwak coffee that can be used for detection of its originality. Result of the study showed that Luwak coffee considerably possessed a distinct low acidity and full body with a slight earthy flavor. However, these characters can be vague due to certain coffee such as Mandheling coffee those exhibiting the similar character. Further identification by using gas chromatography – mass spectrometry volatile compound analysis extracted with Solid Phase Microextraction showed 27 volatile compounds those presences in Luwak coffee but not in non Luwak coffee, despite high similarity chromatogram between the two coffees. Two of these compounds were n-hexadecanoic acid and 4-hidroxy-3-methylacetophenone which were presence significantly. Other compounds were of alcohol, ketone, andehyde, pyrazine, furane, pyridine, pyrimidine, thiophene, and carboxylic acid. These finding clearly indicated that tool for the detection of Luwak coffee instantly can be developed by elaboration of combination of sensory analysis and gas chromatography identification.

INTRODUCTION

Luwak coffee is well-known as the rarest and most expensive coffee in the world. The retail price for roasted Luwak coffee reaches $200 per kilogram or even more (Amarta, 2005) It is not its exotic location of origins but rather its unusual and quite unexpected method of production which generate a rare and unique coffee as it is processed in the digestive system of the indigenous civet (Marcone, 2004).

Luwak coffee is produced when the civet eats ripe Arabica coffee cherries, which are one of its favorite foods.

The common civet, *Paradoxurus hermaphroditus* (Pallas; Family: Viverridae, Order: Carnivora) or toddy cat as is commonly called, is one of the least studied mammals due to its strictly nocturnal and highly secretive nature. Three species of *Paradoxurus* have been recognized, including *P. jerdoni* of South India, *P. zeylonensis* of Sri Lanka, and *P.*

hermaphroditus, the distribution of which ranges from Sri Lanka and India through China, South East Asia, and Philippines (Krishnakumar, 2002).

Authentication of Luwak coffee is approached by several method. Sensory evaluation as the most common way to identify coffee characteristics found that in the cup of Luwak coffee there is a heavy body and low acidity, similar to other Indonesian coffees. The flavor is sweet and mild, yet distinctive. Coffee experts believe that the digestive process may leach potassium out the beans, resulting in a unique flavor profile and sweet after-taste.
Study on compositions and properties analysis of Luwak coffee which involve colour analysis, micro surface analysis, micro-structural analysis and substantial analysis has been conducted on green Luwak robusta coffee bean (Marcone, 2004) as an approach to trace authenticity of Luwak coffee.

MATERIAL AND METHOD

Sample preparation

Luwak Arabica coffee beans were obtained from Andungsari Coffee Plantation area owned by Indonesian Coffee and Cocoa Research Institute, located in district of Bondowoso, East Java Province. Droppings of domesticated Luwak were collected, washed and undergone wet-hulling process which comprises process of drying and dehulling. Green beans then were roasted at 120°C for 8-9 minutes until reached medium dark color, and were ground to fine grind in which approximately 70-75% of all the particles passed sieve no.20. The ground roasted coffee were packed in sealed plastic bag and stored in refrigerator prior to use.

Volatile compounds extraction

Volatile compounds of Luwak coffee beans were extracted using Solid Phase Micro-extraction in headspace technique. Samples were placed in capped glass vial, and needle of SPME apparatus (Supelco) then penetrated through the cap. When the plunger was pushed, a cylinder of Polydimethylsiloxane-divinylbenzene polymer was ejected and being exposed on to headspace area. Exposal of PDMS-DVB is a process of obtaining volatile compounds which was allowed for 30 minutes before being analyzed.

Gas Chromatography-Mass Spectometry Analysis

Essential of analysis was employing Gas Chromatography (GC Agilent 7890A) coupled with Mass Spectrometry detector (Agilent 5975C) equipped with 100% dimethyl polysiloxane column (dim 0.25 mm, length 30 m, film thickness 0.25 µm). The initial temperature was 60 °C and was raised 5 °C /min until reached 220 °C and being hold for 20 minute. The SPME needle was injected to inlet port of GC and desorpting the analyte in splitless 260 °C injector condition.

Sensory evaluation

Sensory evaluation was conducted using standard protocol of coffee cuptesting. About 10 gram of ground roasted coffee was brewed by 95 °C hot water and was allowed 5 minute prior to tasting. Evaluation was done by 3 trained panelists with at least 15 year experience.

RESULT AND DISCUSSION

Use of GC-MS for identification of coffee volatiles

Lopez-Galilea (2006) suggested 47 volatile compounds constitute in volatile compounds of coffee. Those compounds include aldehydes, ketones, furans, pyrroles, pyrazines, pyridine, phenolic compounds, and indole. Akiyama et al. (2003) reported 66 compounds were detected in roasted arabica coffee including those classified as myrcene and limonene. Detection of GC-MS in this research found compounds suggested either by Lopez-Galilea (2006) and Akiyama (2003), covered the classes of alkanes, aldehydes, ketones, alcohols, amides, carboxylic acids, esters, furans, pyrazines, pyridines, phenols and pyrroles. Other compounds followed included carbondioxide, siloxane, furfural, succinonitrile, diazine, indazole, imidazole. Indole compounds were found in the type of 2,3-dihydro-1H-inden-5-ol and 1H-indol-5-ol.

Pyrazines possessed intense peak area and frequency, compared to other compounds. Either in Luwak coffee or in non-Luwak coffee, there were 10 pyrazines detected, in which 2,6-dimethyl-pyrazine, 2-ethyl-6-methylpyrazine, trimethylpyrazine, 2,6 diethylpyrazine and 2-acetyl-3-methylpyrazine were found in both type of coffee. While 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 5H-5-methyl-6-7-dihydrocyclopentapyrazine, and N,N-dimethyl-2-pyrazinamine were found only in Luwak coffee, as well, 3-ethyl-2,5-dimethyl pyrazine, 2-acetyl-3-methylpyrazine, 2-methyl-5-(1-propenyl)-(E)-pyrazine that were found only in non-Luwak coffee.

Distinguishing Luwak coffee and non-Luwak coffee

Attempt is required by Luwak coffee user as well as by its supplier and trader to find trustworthy indicator of Luwak coffee. Marcone (2004) suggested that Luwak coffee has higher level of red green hue and being overall darker from ordinary coffee. Scanning electron microscopy revealed that all Luwak coffee beans possessed surface micro-pitting (as viewed at 10,000 magnification) caused by the action of gastric juices and digestive enzymes during digestion. Large deformation mechanical rheology testing revealed that civet coffee beans were in fact harder and more brittle in nature than their control counterparts indicating that gestive juices were entering into the beans and modifying the micro-structural properties of these beans. SDS–PAGE also supported this observation by revealing that proteolytic enzymes were penetrating into all the civet beans and causing substantial breakdown of storage proteins.

This research complements the research of Marcone (2004) by identifying volatile compounds of Luwak arabica coffee concurrently with non-Luwak arabica Coffee. To increase sensitivity, the detection level was brought lo larger amplitude, thus allowed acquisition of minor compounds. Luwak coffee had more amide, carboxylic acid, esters and phenol constituents, and less of aldehyde, alcohol, alcane, furan and pyrrole constituents, compared to non-Luwak coffee.

After digestion of Luwak, groups of compounds in arabica coffee subsequently disappeared or being decomposed and resulted in absence or formation of new compounds. Compounds such as 2-methoxy-4-vinylphenol, alpha-methyl-2-pyrimidine-methanamine,1-(4-ter-butyl-phenyl) -3-(1-methyl-2-phenylethyl) urea and the other 21 compounds were detected solely in arabica coffee and absent in Luwak coffee. Nevertheless, 25 compounds presented in Luwak coffee that was not detected in non-Luwak coffee. Those 25 compounds included n-hexynoic acid, 2-

methyl isonicotinamide, 2,6-dihydroxinaphtalene, di(3-methylbutyl)amine, n-hexadecanoic acid and 4-hidroxy-3-methylacetophenone.

Among those distinguished compounds, n-hexadecanoic acid and 4-hidroxy-3-methylacetophenone are compounds those significantly detected. Metabolism of Luwak is suspected to influence the existence of n-hexadecanoic acid (palmitic acid) in coffee volatile, considering it is a constituent of lipid fraction of coffee (Carisano and Gariboldi, 1964) and hardly found in studies of roasted coffee volatile employing headspace extraction. The distinctive of palmitic acid, has made it being recommended by Martin *et al.* (2001) as chemical descriptor to differentiate coffee varieties, along with the other fatty acid.

Neither Lopez-Galilea (2006) nor Akiyama (2003, 2005) found 4-hidroxy-3-methylacetophenone in their studies. Unsual process undergone by Arabica coffee beans after consumed by Luwak was supposed to have contribution. This compound was found by Lee and Shibamoto (2002) in analysis of volatile components isolated from Hawaiian green coffee beans.

Sensory Characteristics

Sensory evaluation noted a perfect high body and somewhat earthiness in Luwak Coffee. Acidity was reported low but favorable. The taste of Luwak coffee is close to Sumatera/Mandheling coffee where fermentation and pulping of semi-dried coffee often results in intense body and gentle acidity.

CONCLUSION

Gas chromatography is prospective to be an appropriate method for Luwak coffee identification. Acquisition of distinctive compounds indicates possibility to use aroma as indicator of Luwak coffee in conjunction with physical identification. Sensory evaluation might be the first screening layer of Luwak coffee identification, which subsequently followed by physical and aroma analysis.

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Potential of Fourier Transform Infrared Spectroscopy (FTIR) for Detection of Adulteration in Roasted Coffees

A.S. FRANCA, L.S. OLIVEIRA, M.S. BRITO

Departamento de Engenharia Mecânica Universidade Federal de Minas Gerais,
Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil.
E-mail: adriana@demec.ufmg.br

SUMMARY

The objective of this work was to evaluate the potential of FTIR for the identification and quantification of coffee husks as adulterants in roasted and ground coffees. Roasted and ground samples were analyzed by Fourier Transform Infrared spectroscopy (FTIR) integrated with an attenuated total reflectance (ATR) accessory. Multivariate statistical analysis (PCA) was performed in order to verify the possibility of discrimination between coffee beans and coffee husks. The analysis was based on original spectral data and also on the first derivatives of the spectra. A clear separation between coffee and coffee husks was observed. Such results indicate that FTIR analysis presents potential for the development of an analytical methodology for detection of adulteration in roasted and ground coffee.

INTRODUCTION

As a product of consumption, ground roasted coffee is quite vulnerable to adulteration since it presents physical characteristics (particle size, texture and color) that are easily reproduced by roasting and grinding a variety of biological materials (cereals, seeds, roots, parchments, etc). Thus, this food product has been the target of fraudulent admixtures with a diversity of cheaper materials, such as twigs, coffee berry skin and parchment, spent coffee, roasted barley, maize, cocoa, soybean and others (Oliveira et al., 2009). There are several recent studies regarding the application of Fourier Transform Infrared Spectroscopy (FTIR) in the field of food adulteration, with many applications in wine analysis and olive oil adulteration (Franca and Oliveira, 2010). There are a few studies on the application of FTIR to roasted and instant coffee analysis, mainly targeting varietal and geographic differentiation (Briandet et al., 1996; Kemsley et al., 1995; Wang et al., 2009). Thus, the objective of the present study was to evaluate the potential of FTIR in the identification and quantification of coffee husks as adulterants of roasted and ground coffees.

METHODOLOGY

Arabica green and roasted coffee samples were obtained from local markets and coffee husks were provided from Sindicafé-MG (Minas Gerais State Coffee Roasters Union). Coffee husks were submitted to oven roasting at 200 °C at roasting times ranging from 30 to 50 min. Green coffee beans were submitted to oven roasting at 250 °C with roasting times ranging from 9 to 10 min. 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 °C. Roasted and ground coffee and coffee husk samples were analyzed by Fourier Transform Infrared spectroscopy (FTIR) integrated with an attenuated total reflectance (ATR) accessory (IRAffinity-1 FTIR

Spectrofotometer, Shimadzu, Japan). For principal component analysis (PCA), data matrices were assembled so that each row corresponded to a sample and each column represented the spectra data at a given wavelength.

RESULTS AND DISCUSSION

Average values of color parameters for coffee and coffee husks samples are shown in Table 1. Coffee husks presented slightly higher average luminosity values in comparison to coffee samples, given the inclusion of samples submitted to lighter roasts. Differences in the other parameters were considered less significant in comparison to luminosity, given that this is the most important color parameter in the evaluation of roasted coffee (Mendonça et al., 2009).

Table 1. Average color attributes of roasted coffee and coffee husk samples.

Sample	L*	a*	b*
Coffee - commercial	21.09±1.32	9.27±0.49	10.49±0.92
Coffee – lab roasted	21.35±1.90	8.89±1.39	10.90±0.92
Coffee husks	22.48±1.12	6.31±0.29	8.22±1.02

Typical FTIR spectra obtained for coffee and coffee husk samples are shown in Figure 1. A qualitative comparison of both spectra indicates several differences.

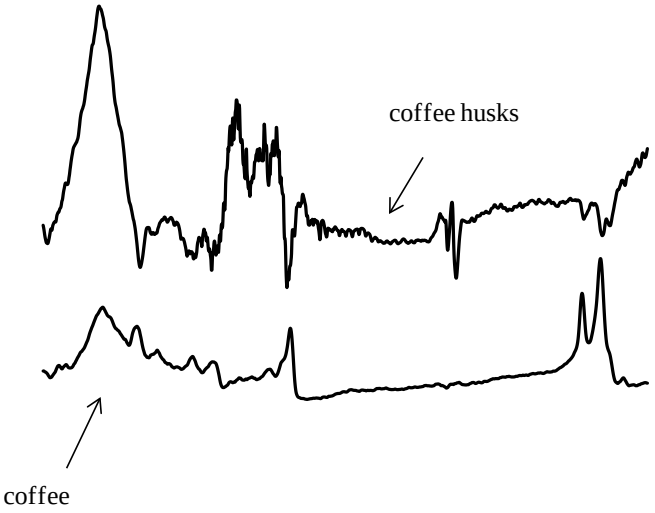

Figure 1. Typical FTIR spectra of roasted coffee and coffee husks samples.

PCA analysis of all samples, based on raw spectra and also on the first derivatives, is displayed in Figure 2. Analysis was based on a 42 x 1192 data matrix corresponding to 18 and 24 samples of roasted coffee husks and coffee, respectively. In the case of PCA based on the raw spectra (Figure 2a), the first principal component accounted for 99.47% of the total sample variance. Roasted coffee husks are clearly separated from roasted coffee samples, mainly based on the second component. However, scattering of the data points is significant. In the case of PCA based on the normalized spectra (Figure 2b), the first and second principal components accounted for 75.41% of the total sample variance. In this case, sample scattering diminished and a better separation, based on PC1, can be viewed. In the case of PCA based on

the first-derivative of the spectra (Figure 2c), the first and second principal components accounted for 53.74% of the total sample variance. Sample scattering diminished further and a clear separation between coffee and coffee husks is obtained based on PC2.

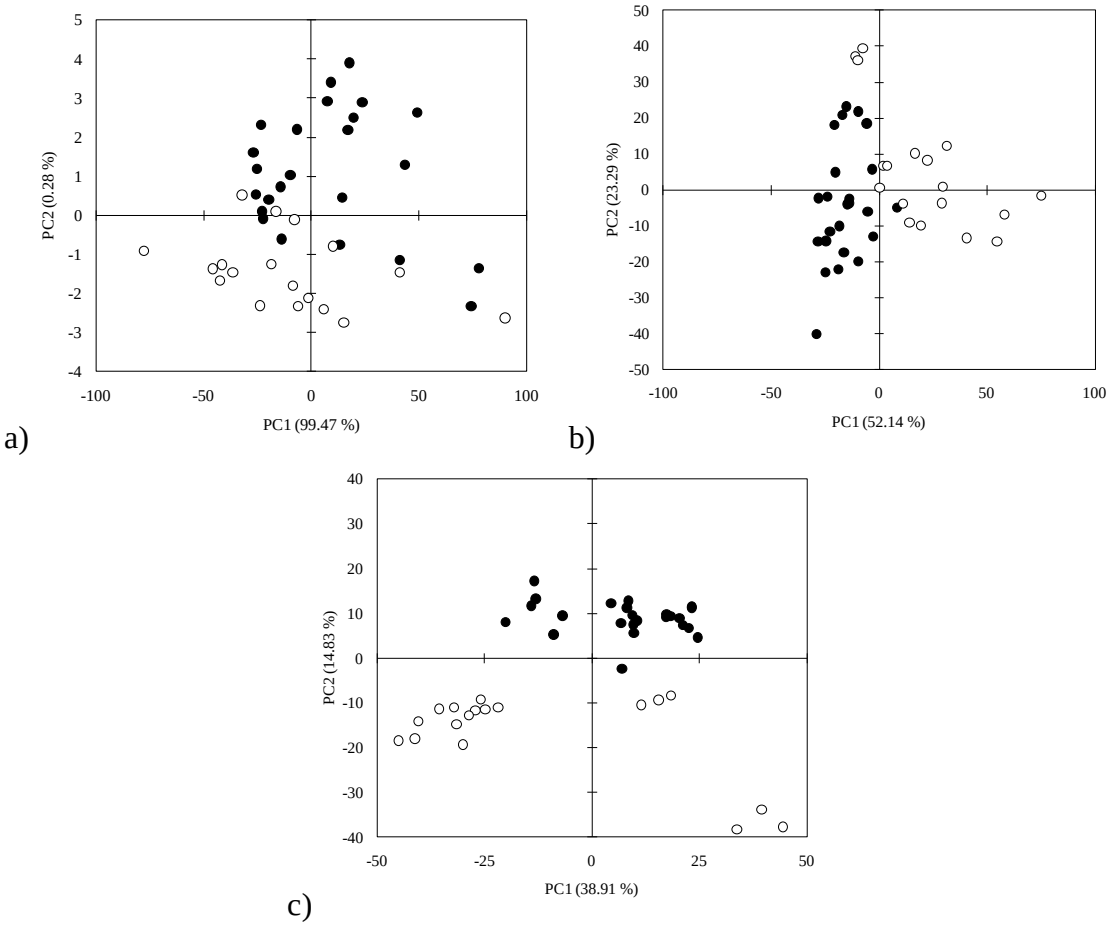

Figure 2. PCA scores scatter plots of (a) FTIR raw spectra, (b) FTIR normalized spectra and (c) first derivatives of FTIR spectra obtained for roasted coffee (•) and coffee husk (o) samples.

CONCLUSIONS

The feasibility of employing FTIR as a methodology for the identification of coffee husks as adulterants of commercial roasted and ground coffees was shown. PCA results showed that coffee husks and roasted coffee samples were separated into two distinct groups. However, there is some overlapping of the two groups, mainly in association with sample scattering. Analysis can be further improved by processing of the FTIR spectra. The preliminary results obtained in the present study confirm that FTIR analysis presents potential for the development of an analytical methodology for detection of adulteration in roasted and ground coffee. Further studies will be conducted employing other common contaminants such as barley, corn and spent coffee grounds.

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Defective Coffee Beans as Raw Materials For Adsorbent Production By Thermo-Chemical Activation

A.S. FRANCA[1,2], H.M. CLARK[2], L.S. OLIVEIRA[1,2]

[1]Dep. Engenharia Mecânica
[2]Programa de Pós-Graduação em Ciência de Alimentos
Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil. E-mail: adriana@demec.ufmg.br

SUMMARY

The objective of the present study was to evaluate the potential of defective coffee beans as raw materials for production of an activated carbon to be employed for adsorptive removal of phenylalanine from aqueous solutions. The adsorbent was obtained by H_3PO_4 activation followed by carbonization at 350 °C for 1 h. The experimental adsorption equilibrium data were fitted to both Langmuir and Freundlich adsorption models, with Langmuir providing the best fit. The adsorption kinetics was determined by fitting pseudo first and second-order kinetic models to the experimental data, with the second-order model providing the best description.

INTRODUCTION

Approximately 20% of the Brazilian coffee production consists of defective beans, which decrease beverage quality upon roasting. These low quality coffee beans are physically separated from the good quality ones prior to commercialization in international markets. However, since to coffee producers they represent an investment in growing, harvesting, and handling, these defective beans are commercialized in the internal market in Brazil, where the roasting industry uses them in blends with non defective beans. Thus, the quality of the roasted coffee consumed in Brazil is depreciated since, after separation from the exportable portion, defective beans may be representing more than 50% of the consumed coffee (Franca and Oliveira, 2008). In view of this situation, several studies are currently under development in order to find alternative uses for defective coffee beans. One of the possible alternatives is production of biodiesel using the oil extracted from defective coffee beans (Oliveira et al., 2008). This procedure, although feasible, generates a solid processing residue after removal of the oil by screw-pressing, for which there is still no profitable use (Nunes et al., 2009). A few recent studies have shown this type of residue can be employed as raw material in the production of adsorbents for wastewater treatment (Nunes et al., 2009; Franca et al., 2010).

Phenylketonuria (PKU) is a disease associated with a metabolism disorder in which the oxidation of Phenylalanine (Phe) is impaired due to the deficiency of the hydroxylase Phe enzyme. Untreated patients present mental retardation and their life expectancy is drastically reduced. PKU nutritional therapy is based on diets with protein ingestion restricted by protein hydrolysates containing a mixture of free amino acids or oligopeptides, where Phe contents are reduced to acceptable levels usually by adsorption with activated carbons (Lopes et al., 2005). However, its high cost makes the production the Phe-depleted hydrolysates almost cost-prohibitive in developing countries, and the use of low-cost adsorbents could drastically change this scenario (Oliveira and Franca, 2008). Although there are a few studies available

on Phe adsorption, the employed materials are expensive, including ion exchange resins, polymers and commercial activated carbons (Long et al., 2009; Silvério et al., 2008). Given the already established potential of defective coffee beans press cake in the production of adsorbents, the objective of this study was to verify the feasibility of employing defective coffee beans press cake as a raw material for production of an activated carbon to be employed for adsorption of phenylalanine.

MATERIALS AND METHODS

Materials

Defective coffee beans were acquired from Santo Antonio State Coffee (Santo Antônio do Amparo, MG, Brazil). The Phenylalanine (Phe) standard was purchased from Sigma-Aldrich (São Paulo, Brazil). Other reagents were purchased from VETEC (Rio de Janeiro, Brazil).

Adsorbent Preparation

Defective coffee beans were screw pressed (Ecirtec, Brazil) for oil removal. The coffee press cake (CC) was then submitted to treatment with phosphoric acid (H_3PO_4) with an impregnation ratio of 85% (3 min at 105 °C). After this period, the mixture was filtered in a paper filter. The acid-treated residue was then heat-treated in a muffle furnace under a stream of nitrogen at 0.3 L min^{-1}, initially at 170 °C for 30 min, and then the temperature was raised to 450 °C, and kept constant for 1 h. The carbonized material was cooled under nitrogen and subsequently was several times washed to remove excess acid with distilled water (500 mL). The wet solid material was dried at 105 °C for 4 h.

Adsorption Tests

Batch experiments of adsorption were performed using 250 mL Erlenmeyer flasks agitated on a shaker at 100 rpm for pre-determined time intervals (30 min to 48 hours), for different values of initial Phe concentration, ranging from 200 to 1500 mg L^{-1}, for initial solution pH of 2.5 and adsorbent concentration of 10 g L^{-1}. All tests were performed in two replicates and at room temperature (25 °C). After the specified time periods, 2 mL aliquots were taken from the Erlenmeyer flasks and the phenylalanine concentration was determined by UV-Vis spectrofotometry at 257 nm (1100 RS Spectrophotometer, Cole Parmer, Vernon Hills – EUA). The amount of phenylalanine adsorbed was determined by taking the difference between the initial concentration and the concentration of the solution at the time of sampling.

RESULTS AND DISCUSSION

The adsorption data presented in Figure 1 show that Phe adsorption presents a strong dependency on initial concentration. An increase in the initial Phe concentration led to an increase in the total amount adsorbed, which is attributed to the corresponding increase in driving force (concentration gradient). The total amount of Phe adsorbed increased from 13 to 50 mg g^{-1} as the initial concentration was increased from 200 to 1500 mg L^{-1}. Regardless of the initial Phe concentration, the adsorption process can be described by a two-stage kinetic behavior, with a rapid initial adsorption during the first two hours, followed by a much slower rate (92% average rate reduction) afterwards. The faster initial Phe adsorption indicates that adsorption occurs preferably on the surface of the adsorbent. The results displayed in Figure 1 also show that a contact time of 6 hours assured attainment of equilibrium conditions for all evaluated initial Phe concentrations.

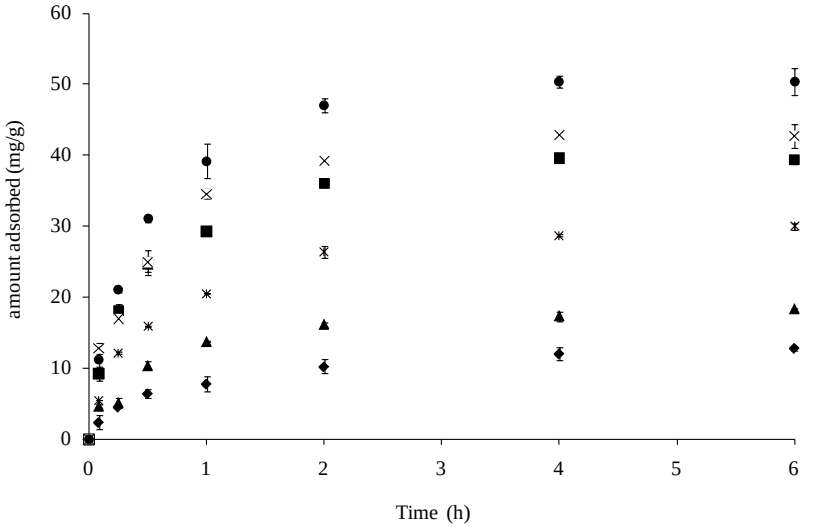

Figure 1. Effect of contact time on Phe adsorption (Initial Phe concentration: ◆ 200 mg.L-1, ▲ 300 mg.L-1, ✱ 500 mg.L-1, ■ 750 mg.L-1, ✕ 1000 mg.L-1, ● 1500 mg.L-1).

The controlling mechanism of Phe adsorption was further investigated by fitting pseudo first and second-order models to the experimental data (Ho, 2006). The kinetics models can be represented by the following equations:

Pseudo-first order: $$q_t = q_e\left(1 - e^{-k_1 t}\right) \quad [1]$$

Pseudo-second order: $$q_t = q_e\left(1 - \frac{1}{k_2 q_e t + 1}\right) \quad [2]$$

where q_e and q_t correspond to the amount of Phe adsorbed per unit mass of adsorbent (mg/g) at equilibrium and at time t, respectively, and k_n is the rate constant for n^{th} order adsorption (k_n units are min^{-1} for n=1 and g $mg^{-1}min^{-1}$ for n=2). The first-order model provided a somewhat satisfactory fit, with R^2 values ranging from 0.8943 to 0.9914, but q_e values were significantly underestimated (28 % average difference with respect to experimental values). An evaluation of the correlation coefficients ($0.9896 < R^2 < 0.9968$) and estimated q_e values (3 % average difference with respect to experimental values) indicates that the pseudo second-order model provided a better description of Phe adsorption by the prepared adsorbent.

Langmuir and Freundlich models were tested for equilibrium description at ambient temperature. Langmuir isotherm is based on a theoretical model assuming monolayer adsorption over an energetically and structurally homogeneous adsorbent surface, whereas Freundlich´s equation is an empirical model based on heterogeneous adsorption over independent sites. The corresponding equations are:

Langmuir: $$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \quad [3]$$

Freundlich: $$q_e = K_F C_e^{1/n} \quad [4]$$

where q_e (mg g^{-1}) and C_e (mg L^{-1}) correspond to the amount adsorbed per gram of adsorbent and to the solute concentration (mg L^{-1}) in the aqueous solution, respectively, after equilibrium was reached. q_{max} and K_L are constants related to the maximum adsorption capacity (mg g^{-1}) and the adsorption energy (L mg^{-1}), respectively. K_F is a constant that indicates the relative adsorption capacity ($mg^{1-(1/n)}L^{1/n}$ g^{-1}) and n is related to the intensity of adsorption. Phe adsorption equilibrium was better described by the Langmuir model (R^2 = 0.9928), indicating homogeneous and monolayer adsorption. Maximum Phe uptake capacity was 65.4 mg g^{-1}, a comparable value to other adsorbents reported in the literature (Long et al., 2009; Titus et al., 2003) for Phe adsorption at ambient temperature such as carbon aerogels (66.1 mg g^{-1}) and zeolites (41.3 mg g^{-1}). The results obtained in this study show that defective coffee beans press cake can be viewed as a potential candidate for the production of low cost adsorbents for phenylalanine removal, thus contributing for the implementation of sustainable development in both the coffee and biodiesel production chains

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Production of Adsorbents from Spent Coffee Grounds: Effect of Thermal/Chemical Treatments on Phenol Adsorption

L.S.OLIVEIRA[1], L.C.O. SILVA[2], A.S. FRANCA[1]

[1]Dep. Engenharia Mecânica [2]Dep. Química
Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil. E-mail: leandro@demec.ufmg.br

SUMMARY

The objective of the present study was to evaluate the potential of spent coffee grounds in the production of adsorbents for phenol removal. The following chemical activation agents were evaluated: NaOH, KOH, H_3PO_4, HNO_3, HCl, NH_3, H_2O_2 and HNO_3/H_2SO_4 mixture. Batch adsorption studies were conducted at 25 °C and the effect of varying the activating agent was investigated. The best results in terms of adsorption efficiency and capacity were obtained for the following activating agents: HNO_3 (98% removal efficiency, 10.0 mg/g adsorption capacity, 36% yield) and KOH (93% removal efficiency, 9.3 mg/g adsorption capacity, 84% yield). Results indicate that spent coffee grounds present excellent potential as adsorbents for wastewater treatment.

INTRODUCTION

The processing of coffee generates significant amounts of solid wastes, mainly coffee husks and spent coffee grounds (SCG) from soluble coffee production (Franca and Oliveira, 2009). This last residue amounts to 6 thousand tons in terms of yearly worldwide production and poses several problems in terms of its adequate disposal, given its limited use as animal feed or fuel source. Furthermore, it can be used for adulteration of roasted coffee, being practically impossible to detect in admixtures with roasted and ground coffee. There are a few studies available on the use of this solid residue for preparation of adsorbents, with applications in wastewater treatment for removal of heavy metals and dyes (Tokimoto et al., 2005; Franca et al., 2009; Hirata et al., 2002; Boonamnuayvitaya et al., 2004; Namane et al., 2005). SCG can either be directly used as an adsorbent (Tokimoto et al., 2005; Franca et al., 2009) or submitted to chemical and thermal treatments in order to increase adsorption capacity (Hirata et al., 2002; Boonamnuayvitaya et al., 2004; Namane et al., 2005). Thus, the objective of the present study was to evaluate the potential of spent coffee grounds, submitted to several types of thermal and chemical treatments, in the production of adsorbents for phenol removal.

MATERIALS AND METHODS

Adsorbent Preparation

Spent coffee grounds (90% particle diameter above 0.5 mm) were kindly donated by a local Soluble Coffee Industry (Café Solúvel Brasília, Varginha, MG, Brazil). The SCG were submitted to the following chemical activation procedures: PA - treatment with phosphoric acid (H_3PO_4) 12 mol L^{-1} (1 h at 100 °C); CA - treatment with chloridric acid (HCl) 12 mol L^{-1} (1 h at 100 °C); NA - treatment with nitric acid (HNO_3) 12 mol L^{-1} (1h at 100 °C); SH - treatment with sodium hydroxide (NaOH) 8 mol L^{-1} (3 h under sonication at 60 °C); PH -

treatment with potassium hydroxide (KOH) 8 mol L^{-1} (3 h under sonication at 60 °C); SN - treatment with a mixture of sulphuric acid (H_2SO_4) 18 mol L^{-1} and nitric acid (HNO_3) 16 mol L^{-1} (0.5 h at 25 °C); AP - treatment with a mixture of 25% ammonia (NH_3) and 25% hydrogen peroxide (H_2O_2) (1 h at 25 °C). After each treatment, the mixture was filtered in a paper filter and dried at 105 °C for 4 h.

Adsorption Tests

Batch experiments of adsorption were performed using 250 mL Erlenmeyer flasks agitated on a shaker at 100 rpm for pre-determined time intervals (15 min to 24 hours), initial solution pH and phenol concentration of 2.5 and 100 mg L^{-1}, respectively, and adsorbent concentration of 10 g L^{-1}. All tests were performed in two replicates and at room temperature (25 °C). After specified time periods, 2 mL aliquots were taken from the Erlenmeyer flasks and the phenol concentration was determined by UV-Vis spectrofotometry at 270 nm (1100 RS Spectrophotometer, Cole Parmer, Vernon Hills – EUA). Phenol removal percentage was calculated as:

$$(C_0 - C_t) \times 100 / C_0 \qquad [1]$$

where C_0 and C_t (mg L^{-1}) correspond to the liquid-phase concentrations of phenol at initial and sampling times, respectively.

RESULTS AND DISCUSSION

The data presented in Figure 1 show that the adsorption performance of the prepared activated carbon is significantly affected by the employed activating agent. Preliminary tests indicated that the amount of activating agent employed also affected adsorption performance. Three different concentrations were tested for each treatment and the results displayed in Figure 1 correspond to the best performance obtained for each specific agent. Among the evaluated activating agents, the more effective ones in terms of phenol removal were NA (nitric acid), PH (potassium hydroxide), SN (mixture of sulfuric and nitric acid) and PA (phosphoric acid). This can be attributed to the corresponding increase in the number of acidic surface groups provided by these treatments.

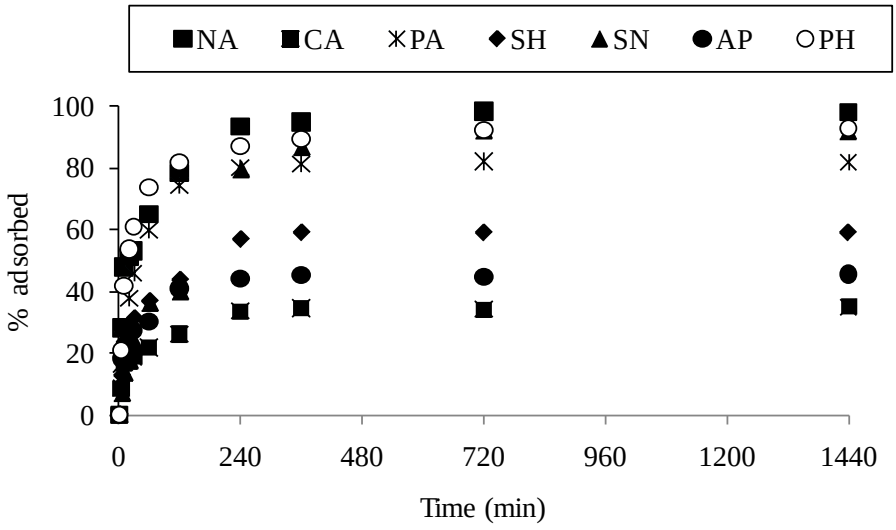

Figure 1. Effect of activating agent on phenol adsorption.

Data on the % mass yield of the prepared adsorbents associated with the chemical treatments that presented better performance are displayed in Table 1. Although the treatments with nitric acid and the mixture of sulfuric and nitric acid were satisfactory in terms of phenol removal, adsorbent yields were lower than those of the other treatments. Thus, phosphoric acid and potassium hydroxide were chosen as the more effective activation agents.

Table 1. Adsorbent production yield.

Chemical treatment	Adsorbent yield (% of initial mass)
NA	36
SN	40
PH	84
PA	58

The controlling adsorption mechanism was investigated by fitting pseudo first and second-order models (Ho, 2006) to the experimental data obtained for the selected treatments (PA and PH). The kinetics models can be represented by the following equations:

Pseudo-first order: $$q_t = q_e\left(1 - e^{-k_1 t}\right) \quad [2]$$

Pseudo-second order: $$q_t = q_e\left(1 - \frac{1}{k_2 q_e t + 1}\right) \quad [3]$$

where q_e and q_t correspond to the amount of phenol adsorbed per unit mass of adsorbent (mg/g) at equilibrium and at time t, respectively, and k_n is the rate constant for n^{th} order adsorption (k_n units are min^{-1} for n=1 and g $mg^{-1}min^{-1}$ for n=2). An evaluation of the data shown in Figure 2 indicates that the pseudo second-order model provided a better description of phenol adsorption by both adsorbents.

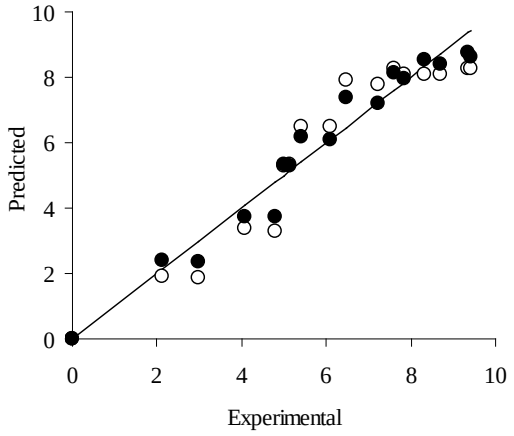

Figure 2. Comparison between experimental values and model predictions for adsorption capacity (o first order; ● second order).

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Taste Compounds Affecting Sensory Characteristics of Ready-to-Drink Chilled Espresso

T. MIYAI[1], M. AKIYAMA[1], T. MICHISHITA[2], T. KATAKURA[1], M. IKEDA[1], T. ARAKI[2], Y. SAGARA[3]

[1]Food Research & Development Institute, Morinaga Milk Industry Co., Ltd., 5-1-83 Higashihara, Zama, Kanagawa 252-8583, Japan
[2]Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[3]Food Kansei Communications Corp., Toa-mansion No. 103, 4-24-7 Sendagi, Bunkyo-ku, Tokyo 113-0022, Japan

SUMMARY

The main taste compounds affecting the sensory characteristics of ready-to-drink (RTD) chilled espresso beverages packed in plastic cups with an attached straw (chilled-cup espresso beverages) were investigated. First, non-volatile compounds of 18 espresso beverages, produced from beans from six countries of origin and prepared at three different degrees of roasting, were analyzed by high-performance liquid chromatography (HPLC). Principal component analysis (PCA) of the non-volatile compounds revealed the relationship between non-volatile compounds and manufacturing conditions. Second, sensory characteristics of the same beverages were evaluated by 13 expert panelists using 30 sensory descriptors. Fourteen of these sensory descriptors were selected for further analysis by analysis of variance (ANOVA) and correlation analysis. Factor analysis of the sensory scores of 14 descriptors extracted four perceptual factors to represent sensory characteristics -"bitter" (factor 1 (F1), proportion 23.8%); "light" (F2, 17.2%), "mocha flavor" (F3, 12.8%); and "mild" (F4, 12.7 %)- and identified the relationship between sensory characteristics and manufacturing conditions. Partial least squares (PLS) regression analysis of the HPLC data and perceptual factor scores revealed that some non-volatile compounds were highly correlated with the factors "bitter" (F1) and "light" (F2). Finally, triangle tests on espresso samples with or without the compounds selected by PLS regression analysis identified taste compounds that affect sensory characteristics. In summary, espresso brewed from dark-roasted coffee beans contained more quinic acids and pyroglutamic acids than that from light-roasted beans and these compounds increased the perceived quality of the descriptors "bitter" (F1) and "full-bodied" (F2). In contrast, espresso brewed from light-roasted beans was higher in citric acids, chlorogenic acids, trigonellines and formic acids, and these compounds affected the descriptors "green flavor" (F1) and "light" (F2).

INTRODUCTION

Ready-to-drink (RTD) chilled espresso beverages packed in plastic cups with an attached straw (chilled-cup espresso beverages) have gained popularity in the Japanese coffee beverage market. Previous studies aimed to improve the perceived quality of RTD chilled-cup espresso beverages by analyzing the relationship between instrumental measurements and sensory characteristics for the retronasal aroma of espresso. In the previous studies, we found that the sensory characteristics of the retronasal aroma from chilled espresso beverages were mainly affected by "sweet-caramel", "smoke-roast" and "acidic" odors. This information was based

on the artificial neural network model of data obtained by gas chromatography/olfactometry (GC/O, CharmAnalysis™) and sensory evaluation of the aroma obtained by retronasal aroma simulator (Akiyama et al., 2008; Michishita et al., in press). Although non-volatile compounds affecting the bitter taste of coffee have been reported (e.g. Frank et al., 2006), compounds affecting the perceived quality of overall flavor have not been identified.

The objectives of the present study were to correlate non-volatile compounds with sensory characteristics of chilled-cup espresso beverages derived from different manufacturing conditions (production countries and roasting degrees) and to specify the main taste compounds affecting the sensory characteristics.

MATERIALS AND METHODS

Coffee samples and brewing espresso

Arabica coffee beans from Brazil (B, no. 2/3), Ethiopia (E, mocha Sidamo grade 2), Guatemala (G, SHB), Colombia (C, supremo), Indonesia (I, Mandheling grade 1) and Tanzania (T, Kilimanjaro AA) were prepared at three degrees of roasting (Hunter L value 26 (L26), light roast; L23, medium roast; and L18, dark roast). Sub-samples (about 7.5 g) of beans from each origin and roasting degree were ground and espresso (about 59 g, Brix 2.8 and 73 °C) samples were brewed using ion-exchanged hot water at 15 atm pressure using an automatic espresso machine (Saeco Royal Professional, Nihon Saeco K. K., Tokyo, Japan). Freshly brewed espresso was diluted to the solute concentration of Brix 1.5 and kept at 10 °C.

High-performance liquid chromatography (HPLC)

Non-volatile compounds from 18 diluted espresso samples were separated with the following columns: saccharides, ERC-NH 1181 (6.0 × 250 mm) 3 μm (Erma Co., Ltd., Tokyo); organic acids, ULTRON PS-80H (8.0 × 300 mm) 10 μm (Shinwa Chemical Industries Ltd., Kyoto, Japan); trigonelline, chlorogenic acid and caffeine, Inertsil ODS-80A (4.6 × 250 mm) 5 μm (GL Science Inc., Tokyo).

Sensory evaluation

Thirteen expert panelists (2 females and 1 male in their 20s, 4 females and 6 males in their 30s) who have worked in quality control for a coffee roasting company evaluated the sensory qualities of 18 espresso samples. Each sample was presented in a white plastic cup and panelists tasted the sample using a straw. The perceived quality was described for “overall evaluation”, “flavor intensity” and 28 other terms objectively selected from 700 sensory descriptors for coffee based on similarity, frequency in use and qualitative as well as quantitative screenings (Ichiki et al., submitted for publication).

Partial Least Squares (PLS) regression analysis

The correlation between non-volatile compounds identified by HPLC and perceptual scores from sensory evaluation was evaluated by the coefficient of determination (R^2) value and the R^2 value for cross-validation (R^2val). The compounds correlated with the perceptual factors were selected from PLS weight plots.

Table 1. PC loadings of non-volatile compounds by PCA of HPLC data.

Non-volatile compound	PC1	PC2	PC3	PC4
Chlorogenic acid	0.98			
Other chlorogenic acid	0.97			
Xylose	0.97			
Fructose	0.94			
Ribose	0.94			
Trigonelline	0.93			
Sucrose	0.92			
Glucose	0.92			
Formic acid	0.90			
Arabinose	0.89			
Citric acid	0.81			
Malic acid	0.80			
Phytic acid	0.67			
Phosphoric acid	-0.49			
Glycolic acid	-0.68			
Propionic acid	-0.71			
Pyroglutamic acid	-0.86			
Quinic acid	-0.91			
Lactic acid	-0.96			
Acetic acid		0.79		
Galactose		0.57		
Caffeine			0.77	
Succinic acid				0.50
Variance	**14.4**	**2.3**	**1.6**	**1.3**
Proportion (%)	**62.8**	**10.2**	**7.2**	**5.7**

Triangle test

Eighteen panelists (selected from 2 females and 3 males in their 20s, 6 females and 10 males in their 30s, 6 males in their 40s, and 1 male in his 50s) who were good at evaluating five basic tastes completed triangle tests. Espresso samples (Brazil L23, Brix 1.5°, 10°C) were supplemented with compounds highly correlated with the perceptual factors from PLS weight plots. The amount of each spiked compound was the maximum found in the 18 diluted espresso samples. Controls were non-supplemented samples.

Statistical analysis

Data analyses were completed with JMP7 software package (SAS Institute Inc., Cary, NC, USA) except for PLS regression analysis using XLSTAT 2008 (Addinsoft Inc., Brooklyn, NY, USA).

RESULTS AND DISCUSSION

HPLC

Principal component analysis (PCA) of non-volatile compounds analyzed by HPLC extracted four principal components (PCs) (Table 1). PC1 scores decreased with increased roasting degree (L26 to L18) for beans of all origins (Figure 1). PCA demonstrated that non-volatile compound composition was related to bean origin and roasting degree.

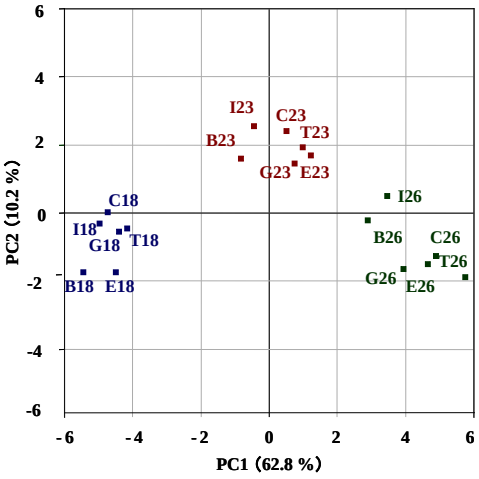

Figure 1. Scatter plots of PC1 and PC2 scores obtained by PCA of HPLC data (18 = dark roast, 23 = medium roast, 26 = light roast; letters refer to bean origin).

Sensory evaluation

Fourteen independent sensory descriptors (Table 2) were significantly different among 18 samples ($p < 0.05$; ANOVA and correlation analysis). Factor analysis of these sensory scores extracted four perceptual factors: F1 “bitter”, F2 “light”, F3 “mocha flavor”, and F4 “mild”. In addition, the F1 and F2 score plots (Figure 2) showed that the factors “bitter” (F1) and “full-bodied” (F2) were positively correlated with dark roasting (L18).

The PLS regression models between non-volatile compounds and perceptual factors (“bitter” (F1) and “light” (F2)) showed a high predictability (F1: $R^2 = 0.944$, $R^2val = 0.904$; F2: $R^2 = 0.859$, $R^2val = 0.726$). Also, PLS weight plots (Figures 3 and 4) segregated non-volatile compounds that correlated with F1 and F2.

Table 2. Factor loadings of sensory descriptors by factor analysis of sensory data.

Sensory descriptor	F1	F2	F3	F4
Bitter	-0.78			
Chocolatey	-0.61			
Berry-like	0.62			
Fruity	0.66			
Floral	0.73			
Green flavor	0.75			
Light		0.84		
Watery		0.72		
Full-bodied		-0.80		
Mocha flavor			0.82	
Intense flavor			0.62	
Mild				0.76
Balanced				0.71
Strong aftertaste				-0.54
Perceptual factor	**“Bitter”**	**“Light”**	**“Mocha flavour”**	**“Mild”**
Variance	**14.4**	**2.3**	**1.6**	**1.3**
Proportion (%)	**62.8**	**10.2**	**7.2**	**5.7**

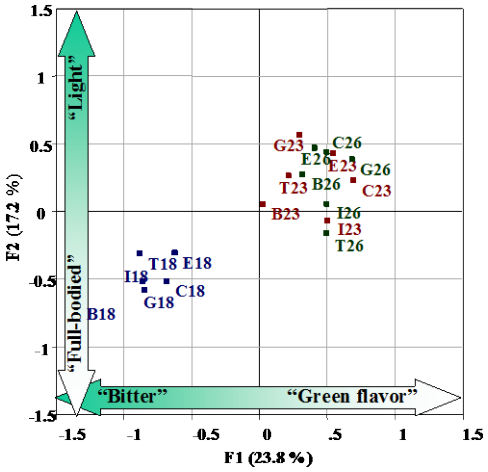

Figure 2. Scatter plots of F1 and F2 scores obtained by factor analysis of the sensory data (18 = dark roast, 23 = medium roast, 26 = light roast; letters refer to bean origin).

PLS regression analysis

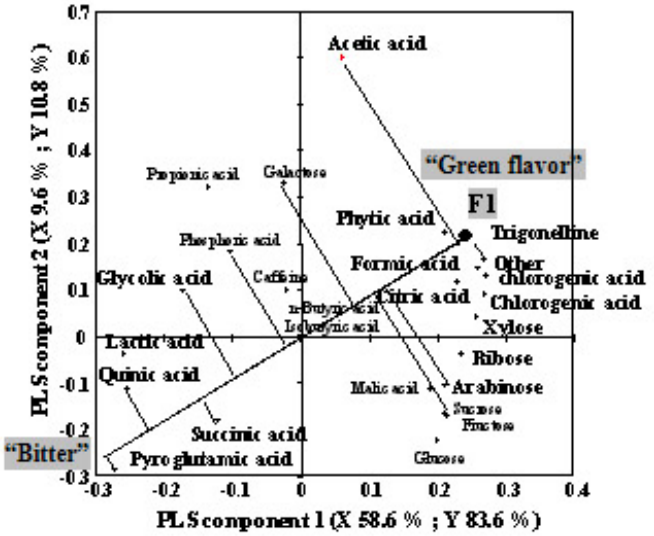

Figure 3. PLS weight plots of non-volatile compounds for F1. Boldface labels show principal compounds affecting F1.

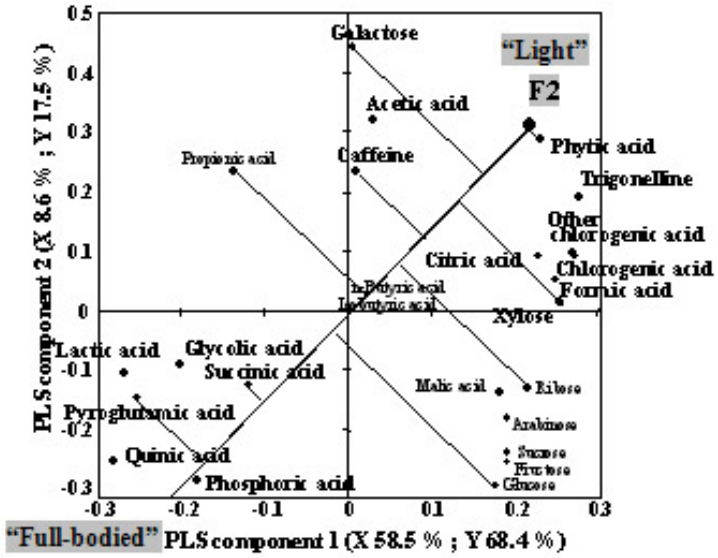

Figure 4. PLS weight plots of non-volatile compounds for F2. Boldface labels show principal compounds affecting F2.

Triangle test

Triangle tests showed significant differences in the sensory characteristics of non-spiked samples and those spiked with citric acid, quinic acid, chlorogenic acid, trigonelline, formic acid ($p < 0.001$) and pyroglutamic acid ($p < 0.05$). The results suggest that these non-volatile compounds greatly affect the flavor of chilled espresso beverages.

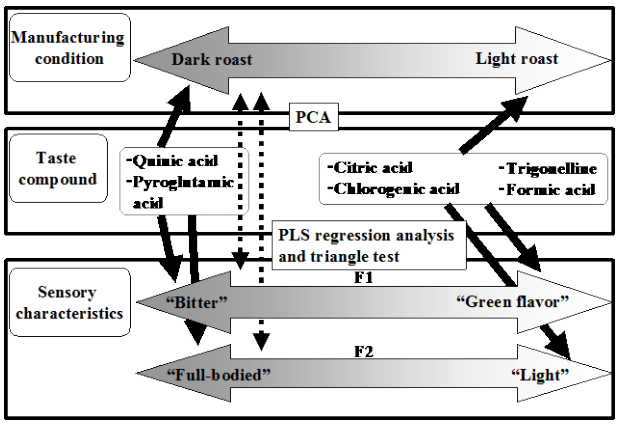

Figure 5. Relationship between manufacturing conditions, taste compounds, and sensory characteristics.

CONCLUSION

Espresso brewed from dark-roasted coffee beans contained more quinic acids and pyroglutamic acids than that brewed from light-roasted beans, and these compounds were found to greatly influence perceived quality ("bitter" (F1) and "full-bodied" (F2)). In contrast, espresso brewed from light-roasted beans was higher in citric acids, chlorogenic acids, trigonellines and formic acids, and these compounds affected the descriptors "green flavor" (F1) and "light" (F2). These data suggest that the above compounds are the main taste compounds affecting the sensory characteristics of RTD chilled-cup espresso beverages.

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Ethyl Formate As a Marker for the Fermented Off-Note in Coffee

C. LAMBOT[1], J. HUSSON[1], L. BEDON[1], S. MICHAUX[1], E. GOULOIS[1], C. LINDINGER[2], P. BROUN[1]

[1]Nestlé Centre R&D, Tours, France
[2]Nestlé Research Center, Vers-chez-Blanc, Switzerland

SUMMARY

An analytical method is developed for the determination of ethyl formate in roasted coffee. Coffee samples selected for this study are grown and processed in identical defined and reproducible conditions. After roasting of samples, a trained panel was able to detect samples having the fermented off-note among all samples. Ethyl formate is identified as a marker for fermented off-note in coffee (*Coffea canephora* and *Coffea arabica*) by means of gas chromatography combined with headspace solid phase microextraction (SPME-GC). A positive relation is therefore found between the sensory data and the chemical marker even for slightly fermented off-note samples identified by the sensory panel.

INTRODUCTION

The flavor is one of the amin quality criterion for coffee and the most important factor that contributes to consumer's likeness. Coffee flavor is the result of a complex balance of aromatic compounds mainly formed during the roasting process (Grosch, 1998). Some off-flavour compounds are negatively affecting coffee quality and can penalize the commercial value of coffee batches. These off-flavours are quite diverse and described as earthy, mouldy (Cantergiani et al., 2001), and Rio off-flavor (Spadone et al., 1990).

Recently, we identified ethyl formate as a quality marker for fermented off-note in Robusta coffee using online PTR-MS or gas chromatography combined with headspace solid phase microextraction (SPME-GC) (Lindinger et al., 2009). The application of untargeted chemometric methods on volatile compounds revealed the relation between individual compounds and the sensory attribute.

In the present study, we applied an optimised SPME-GC method for the determination of ethyl formate in roasted Arabica and Robusta coffee samples. The effect of roasting degree on the ethyl formate content was also investigated.

MATERIALS AND METHODS

Coffee samples

- Robusta samples: 12 samples from Guatemala, roasted at CTn 100.
- Arabica samples: 14 samples from Brazil, Ethiopia and Guatemala, roasted at CTn 125.

Sensory analysis

After roasting of samples at different roasting degrees depending on coffee species. Sensory analyses are performed by twelve trained panellists in a sensory panel room. The results obtained by the panellists were averaged. The panel detected slightly and well fermented off-notes among all the samples, results are shown in Table 1 and 2.

Chemicals

Ethyl formate (CAS 109-94-4) from Sigma-Aldrich.

Extraction method and SPME-GC analysis

2 g of ground coffee and 8ml of Vittel water and demineralised tap water (2:1) were mixed in a 20 ml Vial, the mixture was equilibrated at 50 °C for 30 min. The SPME fiber (carboxen/polydimethylsiloxane, from Sigma Aldrich) is preconditioned at 240 °C for 10 minutes. The fiber was then inserted into the headspace, extracting for 30 min at 50 °C with continued agitation. The fiber was desorbed in the GC injector for 10 min at 240 °C. The GC (Auto system XL, Perkin Elmer Technologies) was equipped with a 60 m x 0.32 mm id DB-WAX column with 0.5 µm film thickness. Results are shown as the means of extraction in two replicates expressed as ng/g ground coffee.

Table 1. Sensory evaluation and ethyl formate concentration in Arabica coffee samples.

Samples	Fermented note sensory panel scores	Ethyl Formate concentration ng/g
1	0	0,170
2	0	0,190
3	0	0,220
4	0	0,270
5	0	0,330
6	1,1	0,449
7	1,5	0,296
8	1,5	0,397
9	1,6	0,212
10	1.6	0.4463
11	1.9	0.5293
12	2.2	0.5036
13	2,5	0.2880
14	2.5	0.5618

Table 2. Sensory evaluation and ethyl formate concentration in Robusta coffee samples.

Samples	Fermented note sensory panel scores	Ethyl Formate concentration ng/g
1	0	0,009
2	0	0,047
3	0	0,009
4	0	0,332
5	0	0,047
6	0,26	0,530
7	0,34	0,571
8	0,5	0,602
9	0,65	0,368
10	1,51	0,663
11	1,66	0,613
12	2,38	0,684

RESULTS AND DISCUSSION

In our previous study, ethyl formate was identified as fermented off-note marker in Robusta coffee samples. In this work, ethyl formate was also detected in Arabica samples as well as in Robusta samples. The sensory panel was able to identify slightly and well fermented off-notes among different coffee samples. The volatile compounds of the fermented off-note and clean coffee samples were compared and investigated using gas chromatography combined with headspace solid phase microextraction. The double layer SPME fiber (carboxen/ polydimethylsiloxane) was used to collect coffee volatile compounds. This fiber showed better performance in terms of volatile compounds detection than that of the triple layer fiber (DVB/Carboxen/PDMS) used in our previous study. The chemical analyses confirmed the sensory panel results as shown in Table 1 and Table 2.

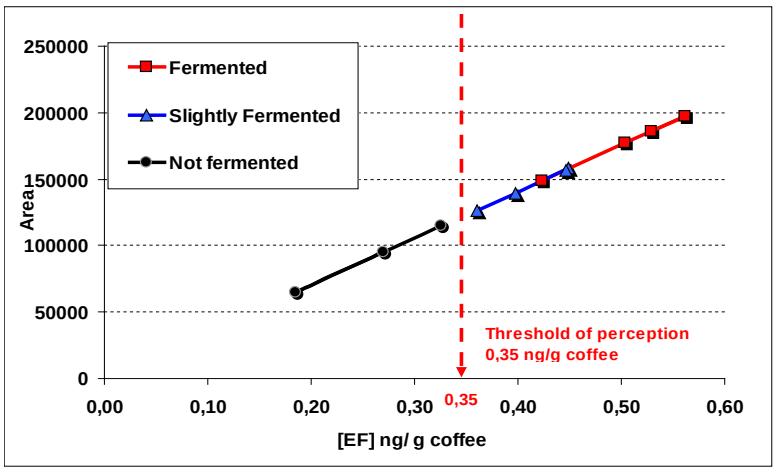

Figure 1. Fermented off-note attribute in relation with the concentration of Ethyl formate in Arabica coffee.

Arabica samples

The contents of ethyl formate quantified by SPME-GC from Arabica coffee samples are shown in Table 1 and Figure 1. The ethyl formate concentration ranged from 0.30 ng/g to 0.56 ng/g for fermented off-note coffee samples. Ethyl formate was also present in clean coffee samples with the concentrations below 0.3 ng/g.

Robusta Samples

The Ethyl formate content ranged from 0.4 ng/g to 0.9 ng/g in fermented off-note coffee samples **(**Table 2 and Figure 2**)**. It is worth mentioning that the ethyl formate concentration in fermented Robusta and Arabica samples is above 0.3 ng/g.

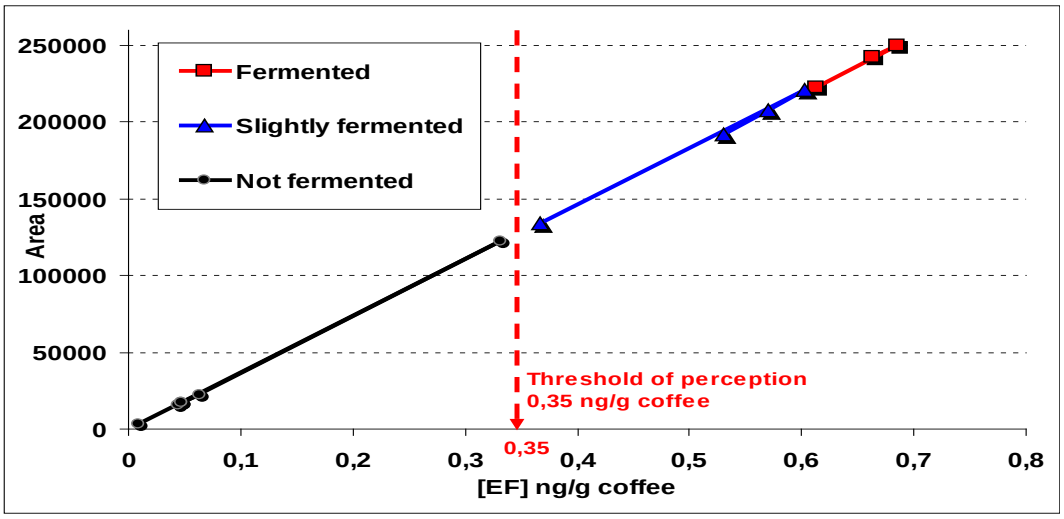

Figure 2. Fermented off-note attribute in relation with the concentration of Ethyl formate in Robusta coffee.

Roasting effect on ethyl formate concentration

One Arabica coffee sample with fermented off-note was roasted at different temperatures 'CTn': 171, 161, 149, 140, 126, 116, and 85. The ethyl formate amount is increasing with the increase of roasting degree up to CTn 126 **(**Figure 3**)**. The ethyl formate degradation was observed at higher roasting degree from CTn 116 and consequently its concentration went down to 0.24 ng/g **(**Figure 3**)**.

In conclusion, the presence of ethyl formate was confirmed in Arabica and Robusta coffee samples using SPME-GC method. Both coffee species samples exhibited similar sensory and chemical characteristics in terms of fermented off-note. The ethyl formate concentrations obtained by SPME-GC method are correlated to the fermented off-note attributes identified by a sensory panel. Changes in the content of ethyl formate were observed at different roasting degree with a decrease of ethyl formate concentration at high roasting degree. The combined SPME-GC methods can be an appropriate technique for routine quality control of fermented off-note marker in roasted Arabica and Robusta coffee. A potential on-line monitoring of coffee volatile compounds during roasting would be possible using combined SPME-GC method.

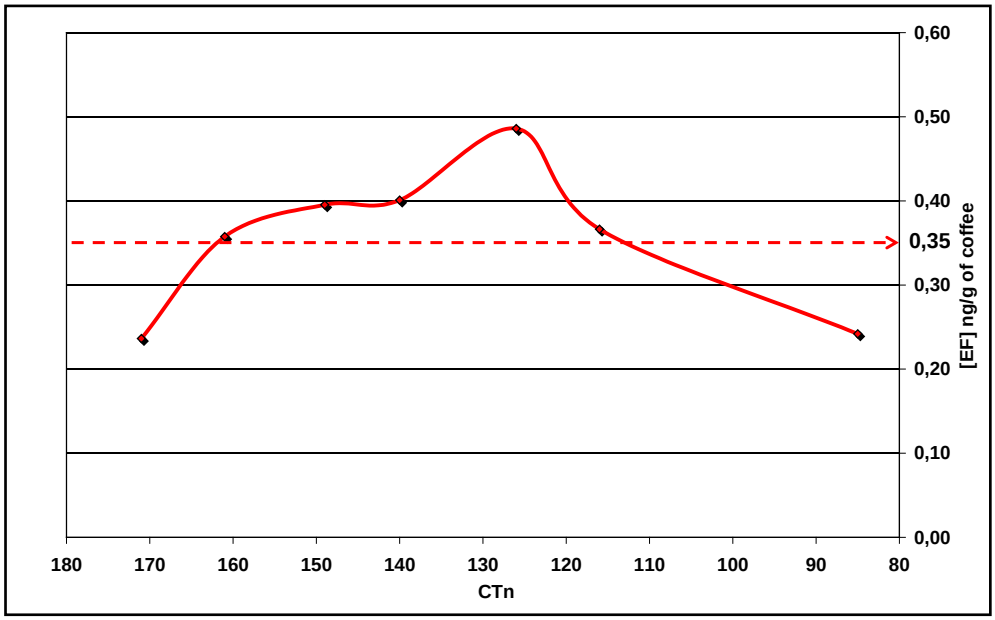

Figure 3. Ethyl formate concentration in relation with different roasting degrees.

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Effect of Roasting Variables in Antioxidant Properties of Coffee Beverages

M. NARANJO[1], A. TILANO[1], C. GAVIRIA[2], T. VELEZ[2], B. ROJANO[2]

[1]Centro de Investigación y Desarrollo, COLCAFE, Medellín, Colombia
[2]Universidad Nacional de Colombia, Medellín, Colombia

SUMMARY

Coffee, one of the most consumed beverages in the world, has antioxidant properties which are interesting in the human health field. These properties have been attributed to both phenolic compounds of green coffee which remain in roasted coffee and Maillard reaction products formed during coffee roasting. Colombian green coffee was roasted at industrial scale at two speeds changing the final roasting degree, moisture content and afterwards particle size obtained upon grinding. The effects of these modifications on antioxidant properties (ABTS, DPPH, FRAP and Total Phenols) and extraction yield of coffee beverages prepared in a commercial filter coffee maker were studied. Direct correlations were found between the studied variables in the coffee beverage and process and product conditions. With these correlations, individual and joint maximizations of studied variables were done, thus meeting the roasting degree, moisture content and particle size to achieve these maximized values. Colombian green coffee which is roasted at slow speed, with light roasting degree, high moisture content and then ground with a coarse particle size was the result of maximizing simultaneously all the parameters. This proposal was run industrially and the obtained values of antioxidant properties and extraction yield were properly adjusted to those described in the multiple maximization model. HPLC analysis was done to this final run finding chlorogenic, ellagic, ascorbic and caffeic acids.

INTRODUCTION

Colombian coffee, worldwide renown for its excellent flavor and quality, holds the third place in the world production after Brazil and Vietnam (United States, 2009). In different researches, interesting results about coffee antioxidant capacity according to the variety, origin, roasting color and preparation way have been found. In the different coffee types the antioxidant capacity depends on the preparation way and the roasting color (Del Castillo et al., 2002). It was found that medium roast coffee prepared in a filter coffee maker or coffee brewer has a greater antioxidant capacity than the same coffee prepared in an espresso machine (Calixto et al., 2004). It was also found that the antioxidant activity of a coffee beverage depends more on the roasting color than on the variety or type of green coffee due to the Maillard reaction products formation. (Nicoli et al., 1977; Saccheti et al., 2008; Cid et al., 1995).

The aim of this work is to evaluate the effects of roasting degree and speed, moisture and particle size of roast and ground Colombian coffee in antioxidant properties and extraction yield of final coffee beverages prepared in a commercial filter coffee machine. An analysis of mathematical models which describe antioxidant properties and yield behavior is done to see if there are some direct correlations with product and process modified variables.

MATERIALS AND METHODS

Green Coffee

The sample used came from the southwest region of the Department of Antioquia, Colombia.

Reagents

Free radical DPPH (1,1-diphenyl–2- picryl hydra-zyl), sodium acid phosphate, MeOH, ferric trichloride, 2,4,6-tri(2-pyridyl)triazine (TPTZ), 2,2-Azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), AAPH [2,20-Azo-bis(2- amidinopropane) dihydrochloride] used as a source of peroxyl radicals, Trolox (6-hydroxi- 2,5,8- tetramethylchroman-2-carboxylic acid), sodium fluoresceinate, ascorbic acid, Sigma-Aldrich (St. Louis, MO) were bought from Sigma Chemical Co. (St. Louis, MO), Folin-Ciocalteau was bought from Merck (Darmstadt, Germany). Water grade HPLC. Standards: Sigma Aldrich CAS 327-97-9 Chlorogenic acid 95%; Sigma Aldrich CAS 476-66-4 Ellagic acid (Chestnut Bark) 95%, Sigma Aldrich CAS 331-39-5 Caffeic acid and Sigma Aldrich CAS 50-81-7 L- Ascorbic acid.

Industrial Roasting

Batches of 160Kg of green coffee were roasted in a Probat RO1000 drum. Three roasting degrees and three moisture levels of coffee were studied. All this was done at two roasting speeds.

Roasted Coffee Grinding and Beverage Preparation

Roasted coffee was ground in a MAHLKONIG laboratory equipment. The beverage was prepared in an automatic coffee brewer using 60 gr of ground coffee and 1liter of water.

Color, Moisture, Particle Size and Extraction Yield Measurement.

For the color, ICONTEC NTC 2442 Standard was applied, using normal CIE (C.I.E., 1986) conditions and a Hunterlab D25 equipment. Moisture was measured according to ICONTEC NTC 2558 Standard. Particle size was measured according to ICONTEC NTC 2441 Standard using a Microtrac S3500 equipment. The extraction yield was measured according to ICONTEC NTC 4602-1 Standard. Some modifications made in COLCAFE ® were applied.

Evaluation of Beverage Antioxidant Properties

The method Brand-Williams, Cuvelier and Berset (1995) (Rojano et al., 2008) with some modifications (Re et al., 1999) was used to evaluate *DPPH*. Radical $ABTS^{\bullet+}$ was produced by an oxidation reaction of ABTS with potassium persulfate; The sample capacity was evaluated in order to trap radical *ABTS* by means of absorbance reading decrease after a 30 minute reaction (Benzie and Strain, 1996). The DPPH and ABTS results were expressed in µmol. Trolox / L of coffee beverage by means of a pattern curve using TROLOX® as antioxidant.

FRAP evaluation method was carried out according to Benzoe et al. (1996). The samples activities were expressed as: mg of ascorbic acid / L of coffee beverage. The Total Phenol determination was carried out by means of a Folin-Ciocalteu colorimetric method. The results were expressed as mg of gallic acid / L of coffee beverage.

Characterization of Roasted Coffee Sample with a greater antioxidant capacity

The beverage of the sample with better antioxidant capacity values was analyzed by HPLC using a column C18 and the identification was made with an UV-VIS spectrophotometer in a wave length range between 200 y 600 nm.

Statistic experiments Design

A random response surface design type Box-Behnken was performed. The ABTS, DPPH, FRAP, Total Phenols and Yield variables were double-analyzed for each run. Three factors were studied, two levels for each with central points. Color levels: L* (20 and 28); moisture levels: 1% and 4%; and particle levels (400 and 800) microns. Minitab 14 statistical pack was used.

RESULTS AND DISCUSSION

For both slow and fast roastings, the ABTS, DPPH and prepared beverage yield variables, present statistically significant quadratic models with color, moisture and particle size variation. The FRAP variable presented a statistically significant model only for slow roasting with color, moisture and particle size, although this model does not explain very well its variability. The total phenols did not have any model to represent them in their variation for a prepared beverage.

The variables studied with the models obtained were optimized, thus finding the values of color and moisture of roasted coffee and particle size of R&G coffee that allow meeting these optimum results (see Table 1).

Table 1. Studied variables maximized with the mathematic models obtained.

	Slow roasting speed				**Fast roasting speed**			
	Max value	**Color (L*)**	**Moisture (%)**	**Part. size (µm)**	**Max value**	**Color (L*)**	**Moisture (%)**	**Part. size (µm)**
ABTS (µmol Trolox/L)	32130	28	4	800	28360	28	3.9	800
DPPH (µmol Trolox/L)	19500	24.37	4	634	18820	20	4	800
Extraction Yield (%)	26.5	26.5	4	400	26.9	24	2.5	467
FRAP (mg ascorbic acid/L)	2907	28	4	800	-	-	-	-

A joint optimization of the variables studied by using the obtained models shows that, as much for a slow roasting as for a fast roasting, the roasting color should be light (L* between 26.0 y 28.0), moisture of roasted coffee should be 4% and particle size of ground coffee should be high, between 700 µm y 750 µm.

The results proposed by this optimization model coincide with some researches that suggest a roasted coffee with an L* value between 25 y 27 for Arabica coffee (Saccheti et al., 2008), or a coffee with medium roasting degree (Del Castillo et al., 2002). The light roasting degree is related to compound formations of Maillard reactions and the little degradation of the phenolic compounds of green coffee when it achieves to that roasting degree. The moisture high values could impact the beverage antioxidant capacity, thus possibly facilitating an adequate extraction of coffee compounds that have antioxidant properties. Big coffee particle sizes are related to the beverage high antioxidant capacity because an over extraction of compounds that are extracted with small particle sizes that could mask or decrease the beverage net antioxidant capacity.

After maximizing the models and finding the color, moisture and particle size to achieve a joint maximization of the antioxidant variables, these conditions were run at industrial level, using slow roasting and it was verified that the real values found are very close to those expected in accordance to the models. For ABTS and DPPH there were differences of 10%; for Total Phenols there were differences of 16%; for FRAP there were differences of 30% and for the Extraction Yield the difference was 10%.

The coffee beverage prepared under the proposal run at industrial level, presented important concentrations of the following organic acids (mg/L): ellagic 65,15; ascorbic 43,5; chlorogenic 31,85 and caffeic 18,78.

In aqueous coffee extracts with light roasting there is chlorogenic and caffeic acids, and the latter is formed by that roasting because green coffee before roasting it is not detected but it is lost when the roasting degree increases (Del Castillo et al., 2002). The chlorogenic and caffeic acids can contribute to prevent diabetes type 2 and cardiovascular decease. The ellagic acid which, was found in a greater concentration in the coffee beverage, is an antioxidant polyphenol, being typical of those fruits known by their high antioxidant activity such as strawberries, raspberries, blueberries and grapes. The ascorbic acid is worldwide well known as a good antioxidant and it is applied ad additive in a lot of food products to improve their preservation.

CONCLUSIONS

- The roasting color, moisture of roasted coffee and particle size of R&G coffee have effects in the antioxidant properties of the final beverage.
- The roasting speed can impact the antioxidant capacity of the final beverage due to the chemical reactions dynamic that may occur during the process, thus forming a larger or smaller number of compounds with high or low antioxidant capacity.
- When roasting coffee at industrial level at a slow speed, a color of L* between 26 y 28, a moisture of 4% and ground up to achieving a particle size close to 750 µm, a brewed beverage with antioxidant properties high values and an acceptable extraction yield is obtained.
- The coffee beverage with antioxidant properties high values run at industrial level, presents important concentrations of organic acids that have been found en coffee beverages and other beverages which are renown by their high antioxidant capacity.
- The statistic model found with the response surface analysis could be validated at industrial scale with optimum results.

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Differentiation of Brazilian Arabica Crude Coffees Using Diffuse Reflectance near Infrared Spectroscopy and Chemometrics

J.S. RIBEIRO, T.J.G. SALVA

Agronomic Institute of Campinas-Coffee Center, P.O. Box 28, 13012-970, Campinas, SP, Brazil

SUMMARY

In this work near infrared spectroscopy of ground crude coffees and principal component analysis were used to differentiate 65 Brazilian Arabica coffee samples according to the overall quality of their beverage. Moreover, the figures of merit sensitivity and specificity (or selectivity) were used to interpret the sensory attribute studied. The results indicated 6 important wavelength ranges (63 variables) for the sample differentiation. PC1 described 76.53% of the selected data. Analysis of figures of merit revealed sensibility values 100% for excellent overall quality coffees and 99% for very bad overall quality beverages. The respective specificity values were 93.4% and 64.6%.

INTRODUCTION

Nowadays, the use of near infrared spectroscopy (NIRS) to differentiate and discriminate species, blends, roasting degrees had been successfully applied in coffee science (Ribeiro et al., 2009; Alessandrini et al., 2008).

Essentially, in the near-infrared (NIR) region vibrational bands of organic compounds are generally related to overtones and combination bands involving C-H, O-H and N-H vibrations. However, a precise band attribution in the NIR region is difficult due to the overlapping of the several possible combination and to the overtone bands. This is the main reason why NIR analyses were so limited a few decades ago, before the introduction of chemometric tools for data analyses (Geladi and Kowalski, 1986).

The scenario nowadays is greatly improved, and NIRS coupled with chemometric methods has become a very useful tool in many fields in which a large number of variables is involved (Ribeiro et al., 2010).

The results presented herein refer to the exploitation of the NIRS technique and chemometrics tools for the differentiation of coffee samples according the overall quality of the beverage.

MATERIALS AND METHODS

Sixty-five preselected Arabica green coffee samples from different origins were used in this study. According to the sensory analysis carried out by experts, the beverage overall quality of 33 samples was excellent (*class A*). The beverage overall quality of the remaining 32 samples was very bad (*class B*).

Diffuse reflectance spectra of all the ground green coffee samples were obtained using a near-infrared spectrophotometer (Foss NIRSystems 6500, Raamsdonksveer, Netherlands) equipped

with a reflectance detector and sample transport module. Each spectrum was profiled from 256 scans in the 1100 to 2500 nm range at 2 nm resolution. Three different aliquots of the 65 samples were analysed resulting 195 spectra.

The original spectroscopic data 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.) (Wise et al., 2004).

Principal component analysis was the exploratory method used. More information about PCA can be found in Ferreira et al. (Ferreira et al., 1999).

The figures of merit sensitivity, expressing the percentage of objects belonging to the category which are correctly identified by the mathematical model, and specificity, expressing the percentage of objects foreign to the category which are classified as foreign were also calculated (Meléndez et al., 1999).

For the calculation of these figures of merit, principal component analysis is carried out for two or more predetermined classes of samples (*A, B,…, Z*) and the error matrix is calculated in accordance with Equation 1:

$$\mathbf{E} = \mathbf{X} - \mathbf{S}_k \mathbf{P}_k^{\mathrm{T}} \qquad [1]$$

Where: *k* is the appropriate number of principal components (PC) used to describe the data set and $\mathbf{S}_k$ (*I*x*k*) and $\mathbf{P}_k$ (*J*x*k*) are, respectively, the score and loading matrices truncated in *k* and **E** (*I*x*J*) is the residual matrix. Following these considerations, if two classes are predetermined it would be possible to obtain the residual matrices for both classes and the standard deviation of each *i* object, inside its respective classes. For the class *A*, for example, the standard deviation, s_i^A is calculated according Equation 2:

$$s_i^A = \sqrt{\frac{\sum_{j=1}^{J} \left(e_{ij}^A\right)^2}{\left(J-k_A\right)}} \qquad [2]$$

The classification of the object in one of the classes (*A* or *B*) could be visualized in a Coomans graphic, in which axles correspond to the standard deviation s_i^A and s_i^B. It would be possible because both parameters represent the distance of the object to the hiperplane defined by k_A and k_B principal components of the classes *A* and *B*, respectively.

Three pre-treatments were applied to the original data matrix (195x700) in this study: normalization by norm one (unit area), Savitzky-Golay smoothing (Savitzky and Golay, 1964), with a window size of 5 points and first derivative.

RESULTS AND DISCUSSION

In order to have insight into which infrared wavelength ranges could be involved in the discrimination of the Arabica coffee samples according to their overall quality, the pretreated spectra were overlapped. Then, a visual inspection was carried out and 6 wavelength ranges

were selected. The important wavelength ranges selected (63 variables) to the differentiation are indicated in Figure 1.

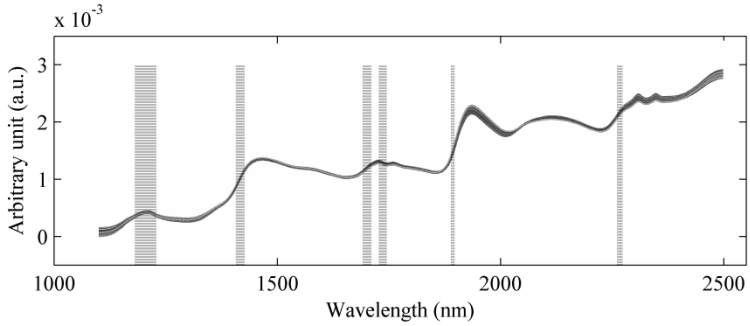

Figure 1. NIR spectra of coffee samples and the regions selected for PCA analysis.

PCA applied to the pretreated and selected data matrix (195 x 63) showed two clusters (Figure 2) in PC1 scores; one of them, at left, consisting of excellent beverage overall quality beans and the other, at the right side, consisting of very bad beverage overall quality coffees.

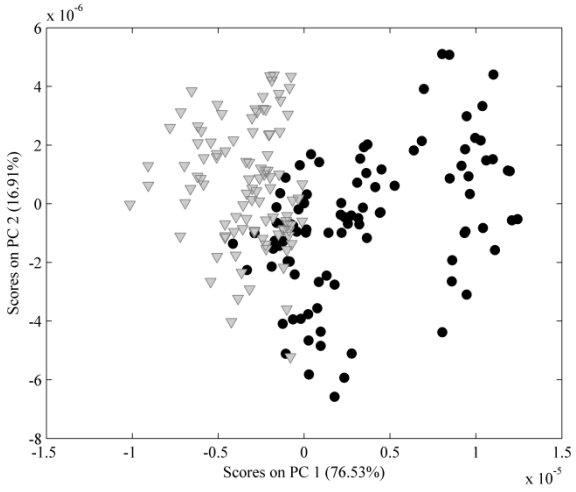

Figure 2. PC1 x PC2 scores plot. (∆) Excelent beverage overall quality samples; (●) very bad beverage overall quality samples.

The loading of PC1 in Figure 3 indicated the importance of the 6 spectral regions for the differentiation. The regions 2, 3 and 6 with negative values contributed significantly to the discrimination of the excelent beverage overall quality.

Regarding figures of merit, the sensibilities of the classes were 100% and 99% for *class A* (Excellent overall quality) and *class B* (very bad overall quality), respectively. The specificity for *class A* was 93.4% and for *class B* was 64.6%.

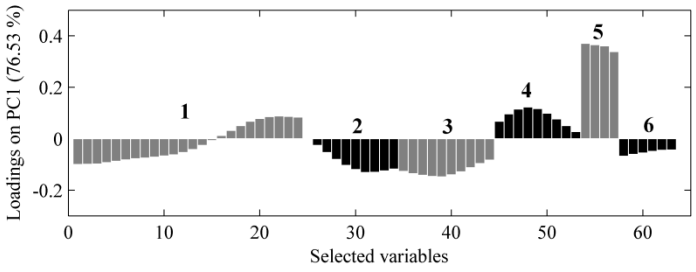

Figure 3. Loadings on PC1 for the selected regions.

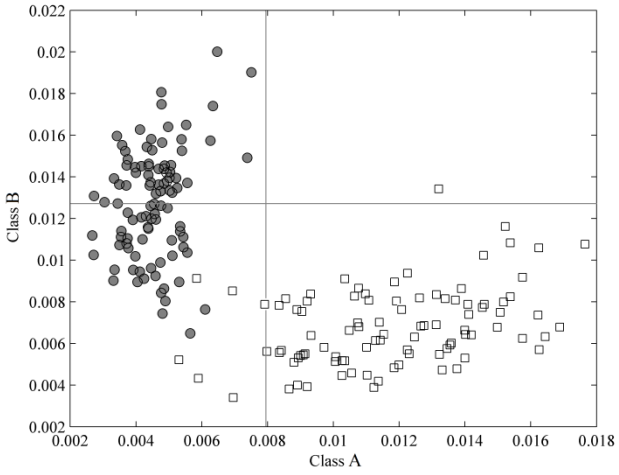

Figure 4. Coomans graphic for the two sample classes *A* and *B*.

CONCLUSIONS

Sixty seven crude coffee samples were well differentiated according the overall quality of their beverage using PCA analyses of the diffuse reflectance near infrared spectra data. Six wavelength ranges were used to differentiate the classes. The sensibility of the two classes was higher than 99% and the specificity of *class A* was higher than 93.4%.

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Chemical Characterization of *Espresso* Coffee Foam: Influence of the Extraction Method

L. NAVARINI[1], V. LONZARICH[2], S. COLOMBAN[2], D. RIVETTI[2], E. DE ANGELIS[2], N. D'AMELIO[3], F. SUGGI LIVERANI[1]

[1]illycaffè S.p.A., Via Flavia 110 – 34147 Trieste, Italy
[2]illycaffè S.p.A., Aromalab, AREA Science Park, Padriciano 99 – 34149 Trieste, Italy
[3]Bracco Imaging-CRB Trieste and CBM, Cluster in Biomedicine, AREA Science Park, S.S. 14, km 163.5, Basovizza, 34149 Trieste, Italy

SUMMARY

Espresso coffee extraction is the most common brewing method in Italy and it is increasingly becoming very popular in many other countries around the world, where more than 120 million cups of espresso are consumed every day. Differently from other brewing techniques, conditions normally used to brew espresso coffee enhance several surface tension-related phenomena such as foam formation and stabilisation. In spite of the relevant role played by foam in assessing the beverage quality, no systematic chemical and physical studies have been devoted to better understand this complex system. A few studies have been aimed at individuating coffee surface-active compounds and at characterizing espresso interfacial properties, but as far as we know, no chemical characterization of espresso foam has been reported. Moreover, nowadays, in addition to the traditional espresso brewing method, new espresso extraction techniques have been developed to satisfy several consumers' needs mostly oriented to practical and sensorial aspects. *Iperespresso* (formerly Hyper Espresso) coffee extraction is a recently introduced innovative brewing method which permits to prepare a beverage able to offer a different sensory experience if compared with that of traditional espresso coffee. A relevant role in differentiating traditional and iperespresso extraction methods is played by the foam generated during brewing.

This study reports on the chemical characterization of traditional espresso coffee foam. High resolution ^{1}H-NMR, chromatographic and other analytical techniques have been used to compare collapsed foam liquid with the corresponding bulk beverage in terms of important chemical compounds and total lipids content. The comparison between traditional espresso and *iperespresso* coffee extraction methods as evinced by chemical characterization of the collapsed foam liquids and the corresponding beverages has been also performed. Chemical composition of espresso coffee foam is different from that of the corresponding beverage and this difference is remarkably affected by the extraction method. The possible influence of the chemical composition on espresso coffee foam sensory properties is discussed.

INTRODUCTION

It is well known by professional *espresso* coffee cup tasters, that the flavour of the beverage is somehow different from that of its *crema*. The foam layer is generally perceived as slightly more bitter and astringent than beverage. Due to the important role played by foam as a visual attribute and preparation quality marker, more attention has been paid in investigating foam promoting and foam stabilizing chemical compounds rather than possible responsible for aroma and taste difference from beverage (D'Agostina et al., 2004; Piazza et al., 2008). As

far as we know, only one preliminary sensory analysis revealing the bitterness of some foaming fractions has been reported so far (D'Agostina et al., 2004). Moreover, due to the presence of tiny solid coffee particles in the foam, the sensory mismatch between foam and beverage has usually been related to such characteristic.

According to several reported data, the liquid resulting from collapse of beer foam is more enriched in some proteins, hop resin and iron than the residual liquid (Kishimoto, 1963). Iso-α-acids, the major source of hop-derived bitter flavour in beers, are concentrated in beer foam. For example, when lager (50 L) containing 25-26 ppm iso-α-acids was foamed, the collapsed foam (2 L) contained 93-120 ppm iso-α-acids (Bishop et al., 1974). Various workers have found that the isocohumulones were concentrated to a lesser extent than their less polar isohumulone and isoadhumulone counterparts, and one report has indicated a seven-fold concentration of *trans*-isoadhumulone in beer foam relative to the remaining liquid beer (Baxter and Hughes, 2001). Some transfer of bitter substances from the bulk of the liquid into the foam on the beer, has been indicated as the origin of the perceived bitterness of beer heads (Ono et al., 1983). Such an enrichment of constituents by the foaming phenomenon is ascribed to the adsorption of these constituents at the gas/liquid interface of foam.

The so-called "foam fractionation" is a method, based on adsorptive bubble separation, commonly used to separate surface-active substances (Backleh-Sohrt et al., 2005). Gases are introduced, under proper conditions, into aqueous extracts, where certain substances tend to adsorb on the hydrophobic surface of gas bubble and, therefore, enrich the formed foam (Backleh-Sohrt et al., 2005).

In spite of the obvious differences between *espresso* coffee and beer, possible enrichment phenomena in the *espresso* foam cannot be excluded a priori. This hypothesis largely stimulated the present investigation aimed at comparing the chemical composition of collapsed foam and the corresponding *espresso* coffee beverage. Several coffee compounds have been selected and their content determined for the first time in the collapsed *espresso* coffee foam. Traditional *espresso* brewing method as well as the recently introduced *iperespresso* coffee extraction method have been explored and compared in terms of collapsed foam/beverage chemical composition. Chemical composition of *espresso* coffee foam, under well defined preparation conditions, is different from that of the corresponding beverage and this difference is remarkably affected by the extraction method. Moreover, the gas phase producing the *espresso* coffee foam, seems to be involved in the transfer of substances from the bulk of the beverage to the foam on the *espresso* coffee.

MATERIALS AND METHODS

Different lots of medium roasting degree coffee beans (total weight loss: 16%; 100% *Coffea arabica* L. blend) were used. Three lots have been industrially processed to obtain *iperespresso* capsules whereas six lots have been used to prepare traditional *espresso* coffee. Three lots have been used to obtain both *iperespresso* capsules as well as to prepare traditional *espresso* coffee. In the case of traditional *espresso*, roasted coffee beans were used both within 24 hours from roasting process (≤ 24 h) and after degassing (deg) and in all cases they were ground using a professional coffee grinder (mod. MC99, Faema S.p.A., Italy) immediately before *espresso* preparation. *C. arabica* from different geographical origins (Colombia, Tanzania, India, Brazil, Guatemala and Nicaragua) were also used. Each sample (100 g) was roasted with a Probat laboratory roaster to a medium roasting degree and it was used within 24 hours from roasting.

Three different coffee machines have been used: a professional *espresso* coffee machine (mod. 2EE, La Marzocco s.r.l., Italy), a professional *iperespresso* coffee machine (mod. X2, illycaffè S.p.A., Italy) and a home *iperespresso* coffee machine (mod. X7, illycaffè S.p.A., Italy) . Traditional *espresso* coffee has been prepared under strictly controlled conditions as far as coffee dose (6.5 ± 1.5 g), water temperature (90 ± 5 °C), pressure (9 ± 2 bar), brewing time (30 ± 5 s) and cup volume (23 - 27 mL) are concerned.

Compaction has been performed by using a dynamometric tamper (Macap s.r.l., Italy) which ensures 23 ± 1 kg of constant dynamic pressure on the coffee portion.

iperespresso coffee has been prepared under the following conditions: coffee dose = 6.7 ± 0.2 g, water temperature = 93 ± 3 °C, pressure = 12 ± 2 bar, beverage flow = 1.2 ± 0.2 mL/s. Tap water (total hardness 19 French degrees, °F) has been treated by ion exchange softening up to a total hardness of 4 °F.

Espresso coffee foam was separated from the corresponding bulk beverage by using a separating funnel. The brews were percolated directly into the separating funnel and separation was performed 30 seconds after stopping the percolation. To determine the oil content, an average of 20 cups of *iperespresso* and 40 cups of traditional *espresso* were prepared and separated, in order to obtain an adequate amount of sample. Collapsed foam and the corresponding bulk beverage were freeze-dried (freeze-dryer apparatus Christ 100-200). Soxhlet apparatus was used to extract oil from freeze-dried samples. Extraction was carried out with *n*-pentane (Sigma) for 4h at 50 °C. The extract was then paper filtered (40 ashless Whatman) and dried over anhydrous sodium sulphate. The solvent was removed by a rotatory evaporator (Buchi, Rotavapor R114) and the residue was dried up to constant weight to obtain the coffee oil.

For caffeine, trigonelline and chlorogenic acids (3-, 4-, 5-caffeoylquinic acids) analyses, foam and the corresponding bulk beverage were separated, diluted and analysed by HPLC system (1100 HPLC system Agilent, Germany), consisting of degasser, quaternary pump, column thermostat and diode array detector (DAD) operating at 254 nm, 272 nm and 324 nm. A Gemini C18 column, 5 μm 250 x 4.60 mm (Phenomenex, USA) and gradient elution (methanol and 1% phosphoric acid) were used. Every sample was analysed in triplicate and an average standard deviation of 5% on analytical determination was obtained. Student t-test was performed where applicable.

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. To 500-600 μL of sample, 60-80 μL of D_2O and 2 μL TSP 0.1 M were added directly into the NMR tube. For caffeine quantitative determination, integration of aromatic as well as of N-methyl protons was used. Integral values were converted in concentrations using the area of internal TSP as a reference but also comparing spectra with the same number of scans (16) and taking into account different receiver gain values.

RESULTS AND DISCUSSION

Traditional *espresso* extraction method

In order to ascertain possible enrichment of chemical compounds in the collapsed foam, caffeine, trigonelline, mono-caffeoylquinic acid isomers and total lipids have been selected due to their sensory relevance. As shown in Table 1, *C. arabica* blend used within 24 hours

from industrial roasting or after degassing led to a collapsed foam more rich in caffeine, trigonelline and chlorogenic acids than the corresponding bulk beverage. However, the difference is statistically significant ($p<0.01$) for all the examined compounds only in the case of roasted coffee beans used within 24 hours from roasting.

Table 1. Traditional *espresso* from *C. arabica* blend used within 24 hours from roasting and after degassing: comparison between foam and corresponding bulk beverage.

Compound	$Foam_{\leq 24h}$ mg/mL	$Beverage_{\leq 24h}$ mg/mL	$Foam_{deg}$ mg/mL	$Beverage_{deg}$ mg/mL
Caffeine	3.83 ± 0.16	2.88 ± 0.39	3.03 ± 0.31	2.77 ± 0,45
Trigonelline	2.36 ± 0.22	1.69 ± 0.24	2.04 ± 0.35	1.82 ± 0.22
3-CQA	0.85 ± 0.11	0.61 ± 0.05	0.60 ± 0.02	0.54 ± 0.06
5-CQA	1.81 ± 0.29	1.29 ± 0.05	1.32 ± 0.04	1.19 ± 0.07
4-CQA	1.05 ± 0.14	0.76 ± 0.05	0.76 ± 0.01	0.69 ± 0.05

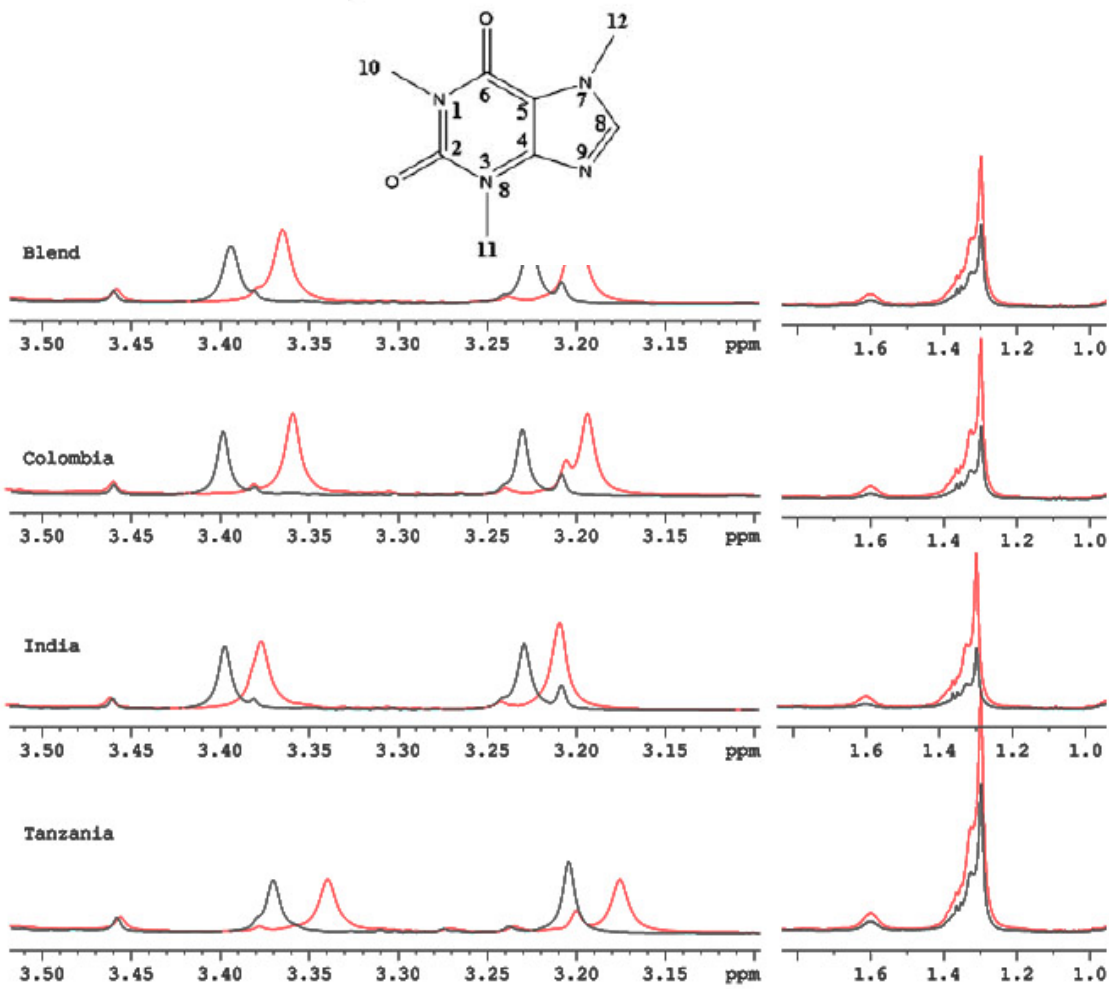

Figure 1. ^{1}H-NMR spectra of traditional *espresso* coffee (red: foam; black: corresponding bulk beverage). Caffeine N-methyl protons H10 (3.20-3.25 ppm) and H11 (3.40-3.35 ppm) and lipids (1.6-1.0 ppm) regions are reported. Caffeine molecular structure and atom numbering are reported to assist the interpretation of the spectra.

Table 2. Traditional *espresso* from *C. arabica* from different geographical origins used within 24 hours from laboratory roasting: comparison between collapsed foam and corresponding bulk beverage.

Origin	Caffeine (mg/mL)		Trigonelline (mg/mL)		3-CQA (mg/mL)		5-CQA (mg/mL)		4-CQA (mg/mL)	
	Foam	***Beverage***	***Foam***	***Beverage***	***Foam***	***Beverage***	***Foam***	***Beverage***	***Foam***	***Beverage***
Colombia	4.21	2.58	2.62	1.56	1.15	0.72	2.66	1.68	1.48	1.06
Tanzania	3.06	2.15	3.49	2.83	1.57	1.10	3.60	2.53	1.97	1.36
India	4.16	2.92	2.36	1.72	0.90	0.64	1.91	1.39	1.10	0.80
Brazil	3.70	2.87	2.61	1.96	1.07	0.80	2.53	1.89	1.37	1.01
Guatemala	3.76	2.67	2.14	1.41	0.92	0.62	2.10	1.41	1.17	0.78
Nicaragua	3.82	2.57	2.33	1.42	1.01	0.64	2.47	1.50	1.30	0.81

Table 3. Comparison between caffeine concentration determined by HPLC and by ^{1}H-NMR. *7 replicates showing more caffeine in beverage than in the corresponding foam.

Sample	HPLC (mM)		NMR (mM)	
	Foam	***Beverage***	***Foam***	***Beverage***
$Blend_{\leq 24h}$	19.72 ± 0.82	14.83 ± 2.01	21.00	12.1
$Blend_{iper}^{*}$	15.31 ± 0.95	16.15 ± 2.58	12.45	14.80
Colombia	21.65	13.26	25.80	12.90
Tanzania	15.76	11.07	20.10	11.90
India	21.41	15.05	26.60	14.30

Table 4. Total lipids content in foam and corresponding beverage prepared by using the same *C. arabica* blend.

Extraction Method	Foam (% w/w freeze dried)	Beverage (% w/w freeze dried)
Traditional$_{\leq 24h}$	20.1 ± 3.2	3.7 ± 0.1
Traditional$_{deg}$	9.0 ± 1.0	3.6 ± 0.2
iperespresso	16.7 ± 1.3	6.3 ± 0.8

In all cases, no preferential enrichment of one species seems to occur, being close to 40% and to 10% for all compounds in the case of roasted coffee beans used within 24 hours from roasting and after degassing, respectively. Experimental data of *espresso* coffee prepared by using *C. arabica* from different geographical origins are close to those of the blend used within 24 hours from industrial roasting, as shown in Table 2. This result is confirmed by NMR data reported in Figure 1. The shift of the caffeine N-methyl protons of blend and selected pure origins, in passing from bulk beverage and corresponding collapsed foam, is due to different caffeine concentration or larger fraction bound to chlorogenic acids (D'Amelio et al., 2009). The comparison between caffeine concentration determined by HPLC and by NMR is reported in Table 3. In Figure 1, an increase in the intensity of the peaks attributable to lipids in passing from beverage to collapsed foam is also clearly evident (particularly evident for the signal at 1.29 ppm where many methylene moiety from different lipids overlap). Assuming that a relevant contribution to this peak derives from total lipids, the chemical investigation has been extended to this class of compounds. By freeze-drying collapsed foam and corresponding bulk beverage, the coffee lipids have been Soxhlet extracted from the resulting freeze-dried material and then gravimetrically quantified. As shown in Table 4, collapsed foam is richer in total lipids than the corresponding bulk beverage, however, as observed for the other selected compounds, the blend used within 24 hours from roasting led to a collapsed foam characterized by a higher content of total lipids.

In both cases, the total lipids content from the bulk beverages is not affected by coffee beans aging and it is in excellent agreement with the typical content reported for traditional *espresso* regular preparation (Illy and Viani, 2005).

Iperespresso extraction method

The chemical characterization of the collapsed foam and the corresponding bulk beverage has been replicated on *iperespresso* coffee preparation. In Table 5, the comparison between the foam and the corresponding bulk beverage is reported for caffeine, trigonelline and chlorogenic acids.. Also in this case, the average concentration of selected compounds is higher in the collapsed foam but the differences are not statistically significant, as in the case of traditional preparation with the blend used after degassing. However, in respect to traditional preparation, among the whole set of experimental data (17 replicates), several times (7 replicates) the beverage resulted more rich in caffeine and in the other compounds than the corresponding collapsed foam.

Table 5. *Iperespresso* from *C. arabica* blend: comparison between collapsed foam and corresponding bulk beverage.

Compound	$Foam_{HE}$ mg/mL	$Beverage_{HE}$ mg/mL
Caffeine	3.21 ± 0.42	3.00 ± 0.44
Trigonelline	1.99 ± 0.33	1.83 ± 0.30
3-CQA	0.67 ± 0.11	0.62 ± 0.10
5-CQA	1.42 ± 0.27	1.29 ± 0.21
4-CQA	0.90 ± 0.17	0.83 ± 0.20

This is clearly shown in Figure 2, where the NMR spectra of 2 of these 7 replicates *iperespresso* coffee brews are compared with the same blend (≤24h) traditional preparation of Figure 1. In Table 3 the average caffeine concentration of *iperespresso* preparations from NMR spectra is compared with the HPLC data obtained on the same samples (7 replicates showing more caffeine in beverage than in collapsed foam).

iperespresso beverages are richer in total lipids (see Table 4), suggesting that this extraction technique is more efficient than traditional one in emulsifying the coffee oil. As shown in Table 4, the total lipids content in *iperespresso* collapsed foam is intermediate between those of the two collapsed foam from traditional *espresso* even if the capsules have been filled with ground coffee blend after degassing.

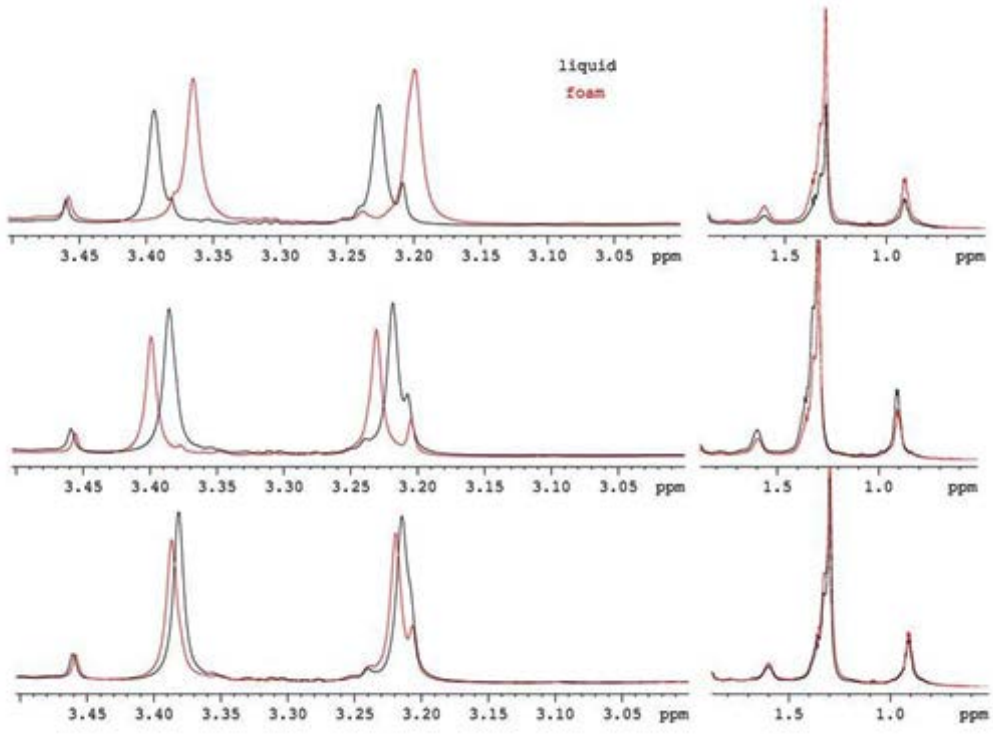

Figure 2. ^{1}H-NMR spectra of *espresso* coffee (red: foam; black: corresponding bulk beverage). Upper: $blend_{\leq 24h}$ of Figure 1; intermediate and lower: two different samples of *iperespresso* brew showing more caffeine in the beverage.

CONCLUSIONS

For the first time, selected *espresso* coffee compounds have been quantitatively determined in collapsed foam and in the corresponding bulk beverage. A remarkable enrichment of caffeine, trigonelline, mono-caffeoylquinic acid isomers and total lipids in collapsed foam has been observed in *C. arabica* both blend and unblended ingredients from different geographical origins only if used in the traditional extraction method within 24 hours from roasting.

Roasted coffee beans aging, strongly influences the enrichment of total lipids in collapsed foam, whereas, the total lipids content in the corresponding bulk beverage is unaffected. It is conceivable that the progressive carbon dioxide release after roasting (Shimoni and Labuza, 2000) could be at the basis of the differences observed in the traditional extraction method. It is well known that carbon dioxide quickly effervesce in the cup, and bubble up to build a layer of froth. Depending on the total amount of carbon dioxide available for bubbling up, and then on the time between roasting and use, chemical compounds can be adsorbed on the surface of gas bubbles or on the surface of oil droplets dragged upwards by the gas phase for longer (high CO_2 content) or shorter times (low CO_2 content). The prolonged effervescence normally observed after traditional *espresso* preparation of freshly roasted coffee could cause not only an abundant foam layer but also the enrichment of selected compounds in the foam.

In the case of *iperespresso* method, the enrichment of caffeine, trigonelline and chlorogenic acid isomers in the collapsed foam is similar to that of the traditional method when coffee is used after degassing, but the enrichment in total lipids is close to that of traditional method when coffee is used within 24 hours from roasting. In other words, it seems that in this extraction method, carbon dioxide effervescence and oil droplets creaming are unrelated phenomena. Moreover, in comparison with traditional *espresso* brew, *iperespresso* beverage is richer in total lipids. From a sensory point of view, the combination between higher concentration of taste-contributing compounds and of liposoluble aroma compounds into the foam of a traditional preparation from freshly roasted coffee (used within 24 h from roasting) may lead to organoleptic properties quite different in respect to those of the corresponding bulk beverage. However, by using the *iperespresso* method, the non statistically significant difference together with a reduced gap of total lipids concentration between collapsed foam and corresponding bulk beverage produce a very limited sensory mismatch, if any. Further studies are necessary to investigate this aspect.

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Oil Bodies Ultrastructure in Green Coffee Seeds of Different Geographical Origin

P. CRISAFULLI[1], F. SILIZIO[2], A. PALLAVICINI[1], L. NAVARINI[2]

[1]University of Trieste, Department of Life Sciences, via Giorgieri, 10 – Trieste – Italy
[2]illycaffé S.p.A., via Flavia, 110 – Trieste - Italy

SUMMARY

Lipids in the coffee endosperm cells can be in the form of oil bodies functioning as an energy reserve, mobilized during germination. Lipids of mature coffee endosperm observed by electron microscope are in the form of bodies isolated in the cytoplasm of parenchimal cells and form masses close to the cell walls (Dentan, 1985). Oil bodies are frequently spherical, sometimes may have more irregular shapes as pressed by other cellular structures (Huang, 1992). They originate from the endoplasmic reticulum and consist of triacylglycerols surrounded by a phospholipids monolayer. Proteins, called oleosins, are partially submerged in this monolayer, preventing the fusion between oil bodies (Simkin et al., 2006).

Portions of a 2-3 mm of *Coffea arabica* L. seeds from different geographical origins were fixed in paraformaldehyde and glutaraldehyde in cacodylate buffer (pH 7.8) for 2 hours; subsequently washed in the same buffer, postfixed in osmium tetroxide and then dehydrated with increasing ethanol series. Samples were thus included in the LR White resin, treated with the contrasting stain and then cut with an ultramicrotome for the observation by TEM.

In the present study, oil bodies, thanks to their osmiophilic nature, have been easily observed and morphologically characterized. Oil bodies sizes have been determined and a comparison intra- and inter- geographical origin has been performed. In some cases, the portion of protein matrix surrounding oil bodies has been observed.

INTRODUCTION

In the seeds, oil bodies represent an important energy reserve to face the germination step. Lipids of mature coffee endosperm are in the form of bodies isolated in the cytoplasm of parenchimal cells and form masses close to the cell walls (Dentan, 1985). Generally, these bodies are frequently spherical, sometimes may have more irregular shapes as pressed by other cellular structures (Huang, 1992). The development of these oil bodies has been the subject of considerable controversy over the years, but current evidence points clearly to their origin in the endoplasmic reticulum. They consist of triacylglycerols surrounded by a phospholipids monolayer (Bewley and Black, 1994). Proteins called oleosins, are structurally incorporated into the membrane of the body during its ontogeny. The main roles of these structures are that to stabilize the phospholipid membrane and to provide the surface with a net negative charge, preventing the aggregation of these organelles. Oleosins represent also an important binding site for lipases during the triacylglycerol mobilization in postgerminative phase.

MATERIALS AND METHODS

Portions of 2-3 mm of *Coffea arabica* seed from three different geographical origin (India, Colombia, Tanzania) were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.8) for 2 hours. They are subsequently washed in the same buffer for three times, postfixed in a buffer solution of osmium tetroxide 1% for 45 minutes. After washing with the buffer, samples are dehydrated with increasing ethanol series (25%, 50%, 70%, 90% and 100%). Samples were thus included in LR White™ Hard Acrilic Resin and polymerised at 60 °C for 24 hours, using gelatine capsules. They were cut with an ultramicrotome (Leica ultracut with diamond knife) to obtain thin (>100 nm) and ultrathin (<100 nm) sections for the observation by a standard optical microscope (Leica Leitz DMRXE) and a Trasmission Electron Microscope (TEM Philips EM 208). The thin sections were stained with a Toluidin Blue solution and the ultrathin are treated with the standard contrasting stain (20 minutes in uranyl acetate 4% and 3 minutes in lead citrate).

The oil content was determined by gravimetry after oil isolation by means of Soxhlet extraction (Speer and Kolling-Speer, 2005). In particular, 60 g of coffee sample was extracted with 650 mL of *n*-pentane for 4 h, siphoning five times per hour. The extract was paper filtered and dried over anhydrous sodium sulphate. The solvent was removed and the residue was dried up to constant weight to obtain the coffee oil.

Measures of the major oil bodies diameter were made on the images obtained by TEM at the same magnification, using the program TESI Imaging µImage.

RESULTS AND DISCUSSION

Seeds of these three different geographical origin have different percentage oil content, less in the case of India (11.06%), more for Tanzania (15.62%) (Table 1). Colombian seeds represent the intermediate situation. Oil bodies are always to the cell periphery and they appear as an ellipsoid osmiophilic structures. The morphology is not different, but their size is surprising. Generally, oil bodies from most seeds are 0.2-2.5 µm in diameter; this range provides a maximal surface area for lipase binding during germination without sacrificing excessive oleosins and phospholipids. The average size is species dependent and likely affected by nutritional and environmental factors (Huang, 1992). Observing the ultrathin sections at the same magnification, the coffee oil bodies looks like smaller and more homogenous in indian samples (Figure 1, India C, D) than colombian and tanzanian samples (Figure 1, Colombia C, D; Tanzania C, D). This situation is supported by measure of oil bodies diameter (Table 1), underestimated because most of the oil bodies are viewed in different plan and they are pressed between them. In any case, coffee oil bodies are in the standard range of size diameter and this size is related to ratio triacylglycerols vs oleosins with different percentage of proteins and phospholipids (Huang, 1992). According to Huang's data, this ratio should be halved in indian oil bodies. This situation could be translated in a different strategy to have a rapid oil mobilization during seedling growth (Huang, 1992).

The difference between the oil content of colombian and tanzanian samples could be caused by differences in the number of oil bodies rather than their size.

Table 1. Percentage of oil content of India, Colombia and Tanzania arabica seeds vs. oil bodies major diameter approximate (µm, mean and standard deviation, minimum and maximum values of random 100 oil bodies).

Geographical origin	% oil content	Oil bodies diameter – mean (µm)	Oil bodies diameter – min (µm)	Oil bodies diameter – max (µm)
India	11.06	0.39±0.11	0.14	0.69
Colombia	13.35	0.92±0.29	0.47	2.31
Tanzania	15.62	0.82±0.28	0.45	1.93

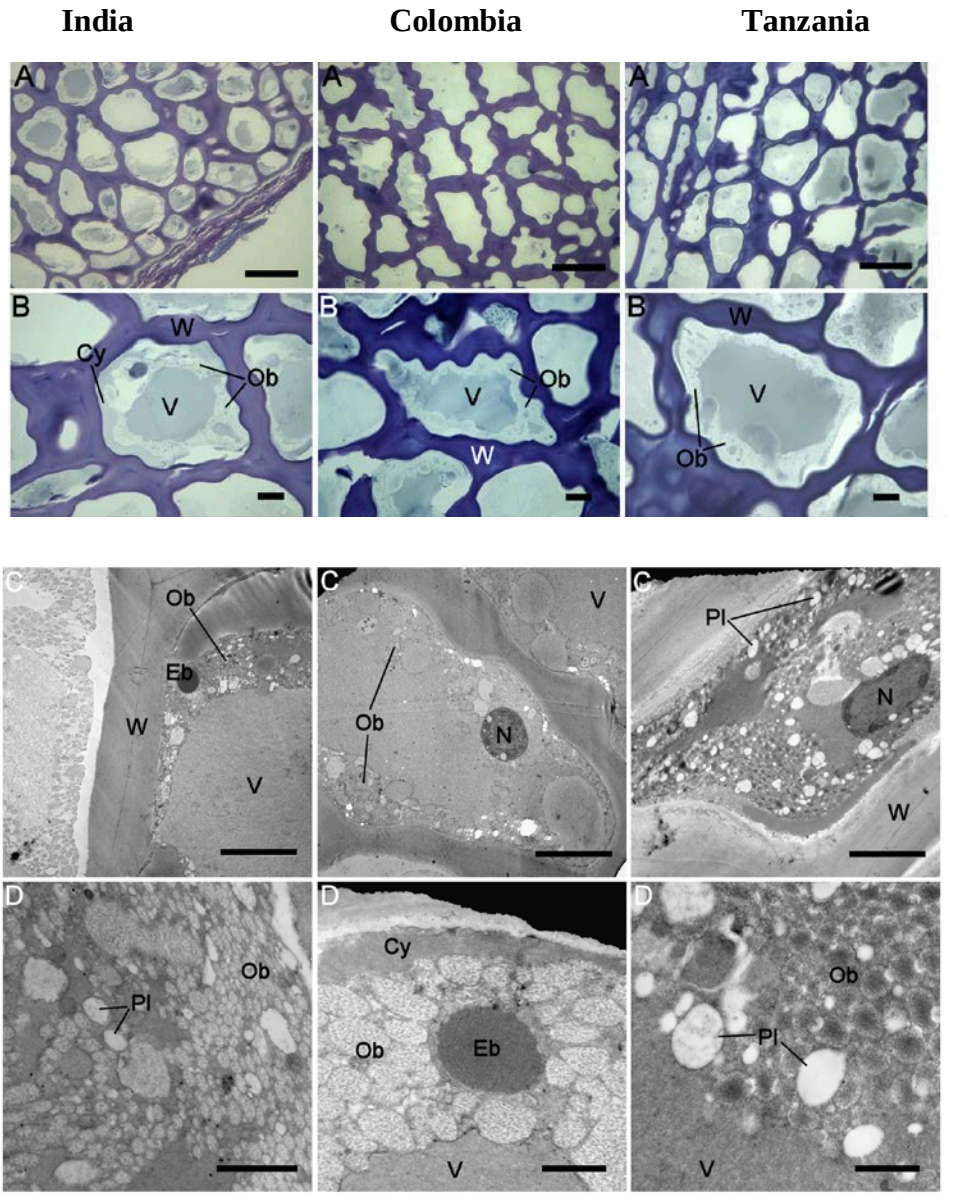

Figure 1. Samples of India, Colombia and Tanzania arabica seeds. A, B: thin sections of endosperm cells; C, D: ultrathin sections; Ob: oil body; Eb: electrondense body; V: vacuol; W: cell wall; Cy: cytoplasm; N: nucleus; Pl: polysaccharides. Bars: A, 50 µm; B, 10 µm; C, 5 µm; D, 1µm (for India, 2 µm).

In conclusion, the microscopic observation confirm partially the chemical results and shows the real distribution of oil bodies inside the endosperm cells.

In the future it could be interesting to deepen the different strategy to mobilize lipids inside different coffee seeds and to study the consequences for the plant growth or during industrial processing (Borém et al., 2008).

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Comparison among Different Discrimination Tests: The Case of *Espresso* Coffee

M. BARNABÀ, N. UMECH, L. NAVARINI, F. SUGGI LIVERANI

illycaffè SpA - Via Flavia 110, 34147 Trieste, Italy

SUMMARY

Discriminative tests are widely used in sensory analysis because they are relatively easy to manage, results are simple to be interpreted and recruited panellists require only a limited skill. Judges just have to recognise the odd sample (or odd samples) from among the whole set of samples presented. Ability to discriminate differences would be inferred from consistent correct choices above the level expected by chance.

For data analysis, only statistical tables derived from the binomial distribution are necessary in order to conclude that two samples are different or not with a certain level of significance. Replications are often needed to produce a sufficient number of total evaluations. Sensory analysts have thus the opportunity to choose among several different tests on the base of level of significance and the power required and on the base of number of judges available. Once significance and power of tests are fixed, both the number of answers required and the minimum number of correct responses required to conclude that a perceptible difference exists, depend by the kind of test. Number of answers required is in inverse proportion to probability to guess the odd sample by chance.

In this study we investigate the sensitivity of different tests by comparing results coming from a triangle test and from a "2 out 5" test performed on two different *espresso* coffee preparations. Both kind of test have been replicated in order to investigate the effect of repeated replicas on the corresponding discrimination capability.

A total of five triangular tests and three "2 out 5" tests have been repeated with a fixed time interval between each other. A total of 36 judges participated to each test.

Results show that discrimination tests comparable from a mathematical point of view do not exhibit the same results, moreover when sample differences are subtle the choice of test may lead to a different conclusion on similarity of products.

INTRODUCTION

One of the main topics in Sensory Analysis is to demonstrate if two samples are perceptibly different or not as well as the opposite case: to determine if two samples are sufficiently similar to be used interchangeably.

For this purpose, since the '50s several tests have been developed in order to support sensory scientists in their efforts (Harrison and Elder, 1950; Helm and Trolle, 1946; Lockhart, 1951; Pfaffmann, 1954; Hopkins, 1954); such tests have been called discriminative tests and they count several methods such as A-not A, 2-AFC, 3-AFC, duo-trio, "2 out 5" and others. One of the most used is the triangle test. In this test, respondents have just to choose one sample as

"different" among a set of three presented, even if they don't perceive any difference: for this reason this family of tests is called "forced choice methods".

Discriminative tests are based on an assumption known as the null hypothesis (Ho); the null hypothesis for the triangle test states that the long-run probability (Pt) of making correct selection when there is no perceptible difference between the samples is one in three (Ho: Pt=1/3). The alternative hypothesis is that the probability that the population will make the correct decision when it perceives a difference will be larger than one in three (Ha: Pt>1/3). This is known as one-sided alternative hypothesis.

The alpha–risk is the long-term probability of rejecting a null hypothesis when it is true. Usually one should set its value before the experiment, and it indicates the upper acceptance limit of committing a so-called Type I error (that is the rejecting the null-hypothesis when it is true).

The beta-risk is the long-term probability of accepting the null hypothesis when it is false and thus of committing a so-called Type II error, that is the chance of missing a true difference.
The power of a test is, by contrast, the statistical estimation of the probability that a test will reject a false null hypothesis, and its value is given by 1-beta.

The proper selection of alpha and beta depends on the specific target of the test: when a sensory analyst has to put in evidence if any sensory difference occurs between two products, his target is to minimize the Type I error and consequently he sets alpha, usually at 5% or 10% (test for difference) (Ennis, 1993). On the other hand, in a similarity test, it is necessary to establish whether two products can be used interchangeably and to do this we have to minimize the Type II error by setting a proper value of beta. Moreover, testing for similarity needs to take in account also the maximum allowable proportion of distinguishers, Pd (proportion of distinguishers).

When a sensory analyst has to perform an experiment in which one or more discrimination tests are used, he has to set for each test the levels of alpha, beta (and consequently the power of the test) and Pd, the maximum allowable proportion of distinguishers.

Once the type of test is selected, the researcher can easily determine the number of responses required and the critical number of them resorting to tables (presented for the first time by Roessler et al., in the 1978) present in specialized books or in the ISO standard (2004).

These tables are based on the binomial distribution, which allows sensory specialist to define whether the result of the study was just due to chance or whether respondents actually perceived a difference between the samples.

In the following formula P represents the probability of success (of making a correct decision, p)

$$P(y) = \frac{n!}{y!(n-y)!} \cdot p^{y}(1-p)^{n-y}$$

where *n* is the total number of judgements, *y* the total number of correct judgements and *p* the probability of making the correct judgement by chance.

The kind of test has a strong influence on the number of assessors required: in Table 1 is shown a comparison between number of responses required for a certain set of values of alpha, beta and Pd in case of triangle test (p=1/3) and two out five test (p=1/10).

Table 1. Parameters chosen for this study and number of assessors needed for each kind of test.

Prameter	Value	Number of responses required (critical number of responses)	
α	0.05	Triangle test 36 (18)	2 out 5 test 12 (4)
β	0.2		
Pd	0.3		

In order to reduce the number of responses it would seem quite simple to opt for the "2 out 5" test, which allows to save time in sample preparation and sensory evaluation.

This method is statistically very efficient because the chances of correctly guessing two out of five samples are 1 in 10, as compared with 1 in 3 for the triangle test. Conversely, the test is strongly affected by sensory fatigue and by memory effects: for these facts specialized books (Meilgaard et al., 1999; Lawless and Heymann, 1999) and also ISO standard suggest to use it mainly in visual, auditory, and in tactile applications, and not in flavour testing.

Sensory experts from industry usually have a limited number of panellists available, less than the number required in case of triangle test according to Table 1.

In these situations a common way to solve the problem is to resort to replications. Unfortunately, using this expedient the sensory specialist can not be assured that all judgements were made independently; moreover effects like fatigue might influence the performance of the panel when it evaluates the last sets of samples (Bi and Ennis, 1999; Brockhoff and Schlich, 1998; Kunert and Meyners, 1999; Kunert, 2001; Brockhoff, 2003).

In the present work we investigate the sensitivity of the final result (accepting or rejecting the null hypothesis) to experimental setup by using the *espresso* coffee as case study. The choice of *espresso* is challenging because its strong aroma and its textural properties should emphasize effects such as sensory fatigue and carryover (Navarini et al., 2004; Catherine and Zata, 2010).

We decided to compare the triangle test with the "2 out 5" test with levels of alpha, beta and Pd as displayed in Table 1. In order to study the fatigue effect on panel performance (Bi et al., 2000), we decide to replicate the triangle test five times and the "2 out 5" test three times, so at the end of each test the panellists tasted the same total number of samples.

A rigid protocol has been followed in samples preparation and presentation.

Two different situations have been explored: one in which samples are similar (*barely perceivable difference test*, BPDT), and one in which, using different preparation, their differences are amplified (*well perceivable difference test*, WPDT).

In order to ensure the right number of responses, 36 judges participated at each session of triangular test and 12 at each session of “2 out 5” test.

MATERIALS AND METHODS

Sensory evaluation

Sensory tests were performed in 4 different sessions. In each of two sessions dedicated to the triangular test, judges had to perform each of five consecutive tests within 3 minutes from receiving the samples.

A timer was displayed on the monitor placed in front of judge; on this monitor was also displayed the presentation order of samples with their code. From one set to the following one, judges had the opportunity to clean their mouth with rinsing water; no puffed rice or other mouth-cleanser was given to the judges. The judges only received the usual information normally given before running a triangle test. Special attention was dedicated to fix and to control the time given to evaluate each set. The timer in front of the judges started when the first set was served and re-started from zero at each following presentation; in this way, the total duration of the test was strictly determined in 15 minutes for each judge.

The “2 out 5” tests followed the same protocol, with the only change in the time between each set of samples; in this case time was fixed in 5 minutes, in order to have the same total duration of the triangle test.

Coffee used in the *barely perceivable difference test* (BPDT) and in the *well perceivable difference test* (WPDT) were both 100% *C. arabica* roasted and ground coffee present on the market and belonging to the same production batches, differing only in roasting degrees (medium, called A and medium-dark, called B). In the BPDT each sample was prepared using 7.0 ± 0.1 g of powder with a professional “La Marzocco” *espresso* machine model 2EE. 25.0 ± 1.0 g of beverage were tasted.

In the WPDT test samples were prepared using 6.5 ± 0.1 g of A and 7.5 ± 0.1 g of B in order to make their discrimination easier; moreover, 30.0 ± 1.0 g, and 20.0 ± 1.0 g of beverage prepared by using A and B, respectively, were tasted.

Immediately after preparation the samples were collected in a Dewar; a total of 10 cups of each sample (A and B) were prepared per judge per session. The Dewar was used to ensure the maximum homogeneity of samples presented during the test and to avoid any bias given by the *crema*. The temperature of presented samples was determined: the range from the first set of samples to the last one was less than 5 °C.

Coffee was poured into plastic cups immediately before presentation to judges; cups were coded with a 3 digit number, different in each replicate and in each session.

Evaluation forms were created using FIZZ NETWORK software (version 2.31G, BIOSYSTEMES, Couternon, France). Panellists evaluated samples in a dedicated room built in compliance with the ISO standard (2007) (ISO 8589: Sensory analysis -- General guidance for the design of test rooms).

Data analysis

Data were elaborated using FIZZ CALCULATION software (version 2.31C, BIOSYSTEMES, Couternon, France) and XLSTAT (XLSTAT version 2010, ADDINSOFT, NY, USA); Microsoft Office EXCEL 2003 was also used.

RESULTS AND DISCUSSION

Raw data for BPDT and calculated values of significance level. are reported in Table 2.

Table 2. Results for triangular BPDT, statistically significant results shown in bold.

Test	Number of total responses	Correct responses	Significance level	
Triangle test 1	36	12	0.140	
Triangle test 2	36	18	0.026	< 0.05
Triangle test 3	36	15	0.017	
Triangle test 4	36	19	0.010	< 0.01
Triangle test 5	36	17	0.053	!

Table 2 shows that, surprisingly, results are not consistent during the whole experiment: in the first replicate the number of correct answers is the lowest of the entire experiment. In the second session the number of correct identifications reaches the critical number (fixed at 18 using parameters set of Table 1). The number of correct answers in the third test is below the critical number whereas in the fourth test it is above 18. In the fifth test the significance level is very close to the target value of 0.05.

According to the parameters reported in Table 1 only one session of triangle test with 36 assessors should be needed: on this base, in the present case, the samples are not different. In our study however, we found that further session immediately following the first one led to different results, so the question is: are samples different?

The same samples compared by using the “2 out 5” test led to the results reported in Table 3. Also in this case the first test has a number of correct responses below the critical level; the second one is consistent with the first one but in the last test the number of correct answers exceeds the critical level.

Table 3. Results for 2 out 5 BPDT, in bold results statistically significant.

Test	Number of total responses	Correct responses	Significance level	
2 out 5 test 1	12	1	0.377	
2 out 5 test 1	12	2	0.230	
2 out 5 test 1	12	5	0.004	< 0.05

By using the “2 out 5” test we observe, like in the triangular test case, an increase in the number of correct answers during replications.

The second kind of test still did not answer univocally to the question: are these samples perceptibly different?

There are two possible ways to explain the present data

1. results are different because judges learn from the first test: they learn what are differences between samples, for this reason they perform better in the following tests
2. the first test is affected by the fact that judges do not have tested any coffee samples before, so the impact of the first sip of coffee might influence the overall perception of that sample and by consequence influence the perception of the entire first set of samples (O_Mahony et al., 1988; Dacremont et al., 2000).

In order to reduce the influence of the first test, which weights for about 20 % on the final score, we developed a method which tries to take into account performance of different judges in a "judge by judge computation", instead of conventional "replication by replication computation". The method is based on the generation of 240 virtual sessions starting from our real dataset. In each session 7 judges perform 5 triangular tests each. Data of each judge are randomly chosen from results of judges in the real dataset.

By grouping all sessions with the same number of correct responses and then by calculating the relative frequency of each of these groups it is possible to evaluate the probability of correct responses for each group as shown in Figure 1.

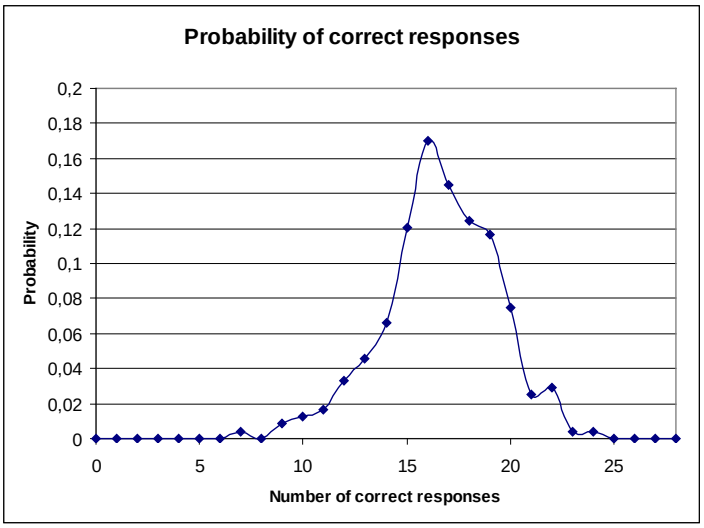

Figure 1. Probability to obtain a certain number of correct answers in a triangle test.

In Figure 2 is represented the cumulative relative probability of these frequencies: it is interesting to note that about 30% of cases (generated starting from real data) lead to accept the null hypothesis, concluding that samples are not perceptibly different. In about 70% of cases the conclusion is that samples are different with $p<0,05$.

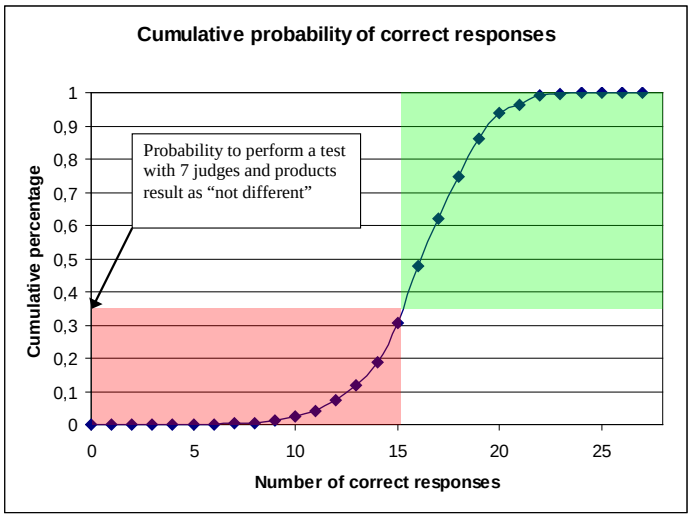

Figure 2. Cumulative probability of correct responses for triangle test. In red area in which samples are considered as "not different": about 30% of possible cases.

Figure 3 reports the results obtained by applying the same principle to the dataset of the "2 out 5" test. In this case we generated 240 virtual sessions performed by 4 judges who replicate a "2 out 5" test three times. Like in the triangle test case, scores of each virtual judge are taken from those of real judges. It is easy to see that the percentage of cases in which the null hypothesis is accepted is higher than in triangular test: 70% vs. 30%. "2 out 5" test in this case, is less sensitive than the triangular test, even if they are comparable from a statistical point of view.

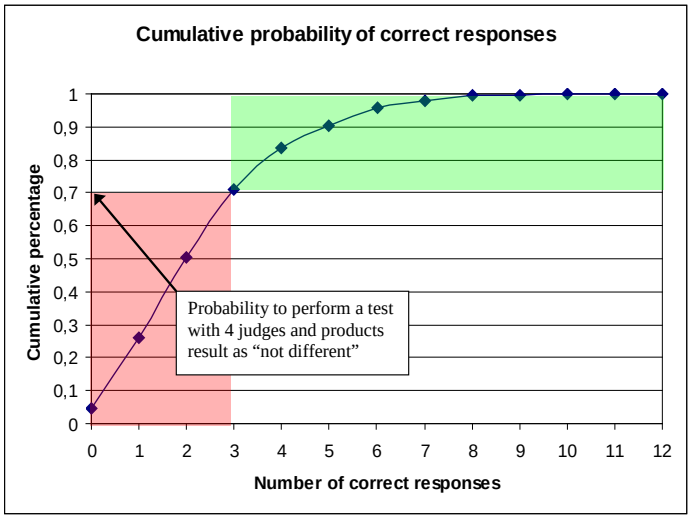

Figure 3. Cumulative probability of correct responses for "2 out 5" test. In red area in which samples are considered as "not different": about 70% of possible cases.

Raw data for WPDT and calculated values of significance level of triangle test and "2 out 5" test are reported in Tables 4 and 5, respectively. In all cases the first test is not different from the following ones.

This behaviour can be explained by considering that samples were quite easy to be differentiated and moreover by the fact that differences between samples were so large to cover the effect of the first sip of coffee on the first sample.

Table 4. Results for triangle test with WPDT samples. In all replicates samples are discriminated.

Test	Number of total responses	Correct responses	Significance level	
Triangle test 1	36	23	0.0001	< 0.001
Triangle test 2	36	26	0.0000	< 0.001
Triangle test 3	36	26	0.0000	< 0.001
Triangle test 4	36	23	0.0001	< 0.001
Triangle test 5	36	26	0.0000	< 0.001

In this situation results coming from triangle test and from "2 out 5" test are comparable; there is no fatigue effect and judges maintain their performance during the whole series of tests.

Table 5. results for two out five test with WPDT samples. Each replica shows a number of correct identifications of the odd sample above the critical number.

Test	Number of total responses	Correct responses	Significance level	
2 out 5 test 1	12	7	0.000	< 0.001
2 out 5 test 1	12	8	0.000	< 0.001
2 out 5 test 1	12	7	0.000	< 0.001

These results suggest that in case of *espresso* coffee and using a panel constituted by trained judges it is possible to perform more than one discriminative test in each session. In our study 15 samples seem to be not too many; in both cases (*well and barely perceivable differences*) performances are maintained during the whole experiment.

CONCLUSION

In this study we investigate sensitivity and discrimination ability of two widely used discriminative tests, the triangle one (probably the most used discriminative test) and the "2 out 5" test. The first one is widely used because its simplicity in test preparation, in sensory evaluation and data analysis; the second one is suggested only for tactile or visual tests because it is very challenging for judges in the case of taste and flavour. In order to compare these tests the experimental conditions have been selected to ensure the same statistical power.

The use of replication in discriminative test has also been explored since it permits to save time and/or to optimize the number of panelists required in the case of scarce availability of judges.

The first session (36 judges for the triangle test and 12 for the “2 out 5” test) shows consistent results for both kinds of test, in both BPDT and WPDT. However, by analyzing replications it can be observed that (when differences between samples are subtle), the first replication is different compared to the following ones. A possible explanation is that judges did not taste anything before session and probably they need a “warm-up sample” before starting test.

In the case of barely different samples, results coming from triangular test and “2 out 5” test are not comparable. In particular, in the “2 out 5” test, the probability to conclude that samples are not differentiable on sensory bases is higher. When samples are well recognizable, triangular test and “2 out 5” test show the same results, even when five and three subsequent replications are respectively performed.

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Evaluation of *Coffea arabica* and *Coffea canephora* Beverages Using Electronic Tongue (ET)

E.S.M. SANTOS[1], L.G. PATERNO[2], F.J. FONSECA[2], L.H.C. MATTOSO[3], D.D.G.C. FREITAS[4], R. DELIZA[5,6]

[1]Federal Rural University of Rio de Janeiro, Seropédica – RJ, Brazil
[2]Department of Electronic Systems Engineering – EPUSP, São Paulo – SP, Brazil
[3]Embrapa Agricultural Instrumentation, São Carlos – SP, Brazil
[4]Embrapa Food Technology, Rio de Janeiro – RJ, Brazil
[5]Embrapa Labex Europe, [6]Inra, UMR CSGA, Dijon, France

SUMMARY

Sensory analysis carried out by trained assessors has been used in the assessment of coffee beverage in order to get sensory profile and avoid weaknesses inherent of the traditional classification system named of the "cup proof". However, it is desirable to find instrumental measures that correlate with sensory ones, and use the instrument as a routine analysis of a laboratory quality control. The electronic tongue (ET) may be an option. It consists of an innovative tool which allows assessing coffee beverage, enabling agility, and precision, high sensitivity at low cost. Therefore, the ET can be an effective alternative for analysis of coffee beverage. The aim of the present study was to evaluate blends of Arabica and Conilon coffee and their pure beverages by instrumental method of ET. The Arabica and Conilon beans were roasted, ground and samples were prepared at the Laboratory of Sensory and Instrumental Analysis of Embrapa Food Technology (Rio de Janeiro – Brazil), using the following proportions of Conilon: 0 (100% Arabica), 10, 20, 40, 60, 80 and 100%. The ET analyses were performed by the Molecular Electronics Group, USP, São Paulo - Brazil. The beverages were prepared with boiled mineral water at a concentration of 1% (1 g of ground coffee in 100 ml of mineral water), and were analyzed by ET which was compound of 10 polymeric sensors (films of conductive polymers) at 25 °C. The data were obtained in the form of a matrix and processed by Principal Component Analysis (PCA). The results showed that the ET discriminated extracts of coffee according to the proportion of Arabica and Conilon beans. ET could well discriminate the samples 100%, 80% Conilon and samples with higher proportions of Arabica. The breakdown of the sample with 100% Arabica was perfectly observed, however, despite this discrimination, the blends with 90, 60 and 40% Arabica were similar to each other, indicating that from 40% Arabica in the blend ET considered the samples as equals. The ET allowed in a short time to discriminate between two different varieties of coffee (Arabica and Conilon). Therefore, further studies are recommended to achieve better results and provide an appropriate instrumental method for the evaluation of blends of Arabica and Conilon.

INTRODUCTION

Sensory analysis carried out by trained panels has been used for the assessment of coffee beverages to get the product sensory profile, avoiding the disadvantages of the traditional classification system named the “cup proof” (Santos, 2010; Stone and Sidel, 2004). Despite its recognized usefulness, it is desirable to develop instrumental measures that correlate with

sensory ones, in such a way that they can be used as an instrument in the routine analysis of a quality control laboratory.

The electronic tongue (ET) is an innovative tool consisting of multiple sensor units produced with thin films deposited onto interdigitated electrodes, and can be used for the coffee analysis allowing to assess its quality with precision and high sensitivity at low cost. It allows the coffee classification by scores and by comparing with standards. ET is an important tool, since it can assist the experts in the tasting, making possible continuous and more precise measurements, with no loss of sensitivity after long exposure times to the product (Cabral, 2006). Many studies have been conducted using the ET to differentiate types of drinks and beverages with the same flavour, such as wine, coffee, beer, tea, milk, juice, mineral water and others (Deisingh et al., 2004; Riul et al., 2003). The ET was used to evaluate different mineral waters, which the goal was to distinguish between natural water and that prepared artificially. The ET was able to distinguish the two types, as well as differentiate the samples of each type of water (Legin et al., 2002). Therefore, the ET can be a useful alternative to the coffee industry. The aim of the present study was to evaluate coffee beverages made from the Arabica and Conilon beans using the ET.

MATERIALS AND METHODS

The Arabica and Conilon beans were roasted and ground and the samples were prepared at Embrapa Food Technology Sensory and Instrumental Analysis Lab. (Rio de Janeiro – Brazil), using the following proportions of Conilon: 0 (100% Arabica), 10, 20, 40, 60, 80 and 100%.

Samples were packed in aluminum foil bags and sent to the Molecular Electronics Group, Department of Electronic Systems Engineering, at the University of São Paulo, SP – Brazil. The beverages were prepared using 1g of ground coffee in 100 ml of boiling mineral water. Water was added to each individual sample in a beaker, kept for 5 minutes and then filtered through regular coffee filter paper. Subsequently each sample was analyzed by the ET which was composed by 10 polymeric sensors (films of conductive polymers). The samples temperature was kept constant at 25 °C with the aid of a thermostat bath. Samples were analyzed in triplicate. The data were obtained in a matrix format and processed by Principal Component Analysis (PCA) using the software MatLab®. Figure 1 illustrates part of the the experimental setup of the ET.

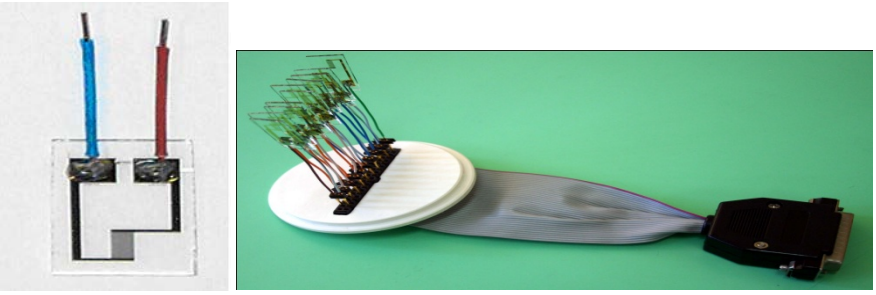

Figure 1. ET system: a) interdigitated sensor; b) electronic tongue with 10 sensors.

RESULTS AND DISCUSSION

Figures 2, 3 and 4 show the results. In Figure 2 three clusters of samples can be noted. One of the clusters is that containing exclusively water samples. The sensors respond to water in a constant way, regardless the measurement is performed before or after coffee analysis. The

observed signal reset proves that the ET system provides reliable information. Despite of some clustering, it is possible to find a straight correlation of PC1 and Conilon content, which increases from left to right. The correlation is high so that detection of pure Conilon sample is performed unequivocally by the ET.

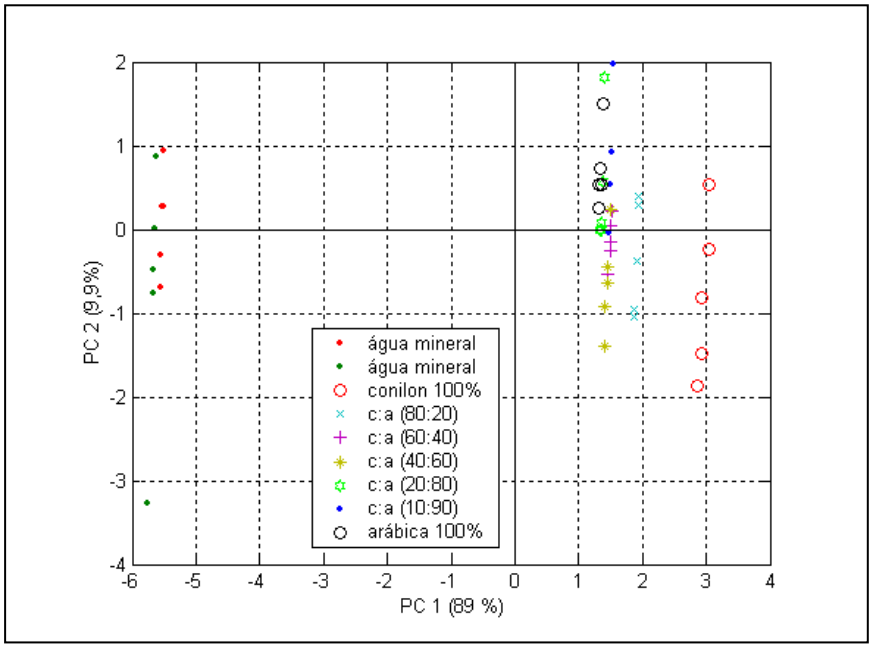

Figure 2. PCA for the mineral water samples and seven different coffee blends measured with the ET at 1 kHz, 50mV, 25 °C.

When water data is removed, the composition of coffee samples variations could be more easily observed. According to Figure 3, the ET distinguished three main groups. Group 1 (in the right), composed by pure Conilon samples; Group 2 (Conilon 80%); Group 3, composed by different blends of Conilon and Arabica beans.

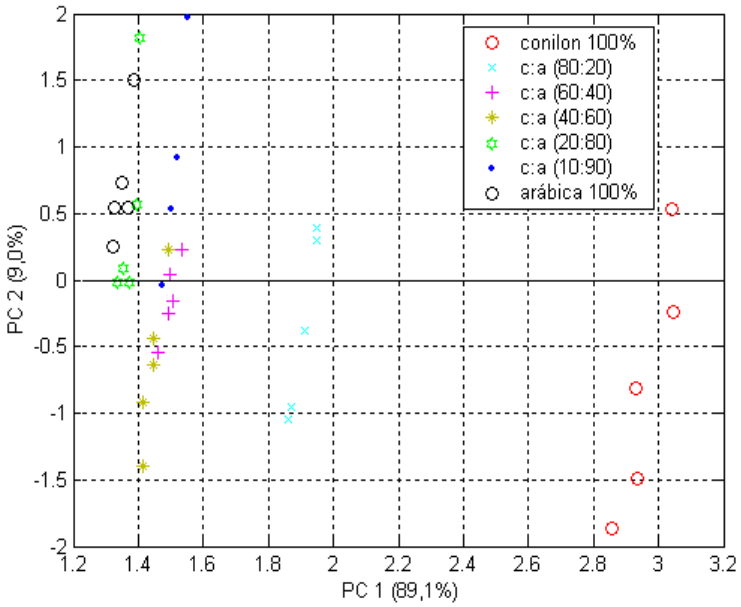

Figure 3. PCA of the seven coffee blends measured with the ET at 1 kHz, 50mV, 25 °C.

If pure Conilon data are removed, clustering in Group 3 is decreased, and discrimination of samples of intermediary composition is slightly improved, as shown in Figure 4. However, blends with 10, 40 and 60% Conilon were still very similar and discrimination was difficult to be performed. The use of sensory attribute references used by the trained panel might be a useful alternative for calibrating the ET, which might help achieve better results. Further studies are recommended to improve this instrumental method for the evaluation of blends of Arabica and Conilon.

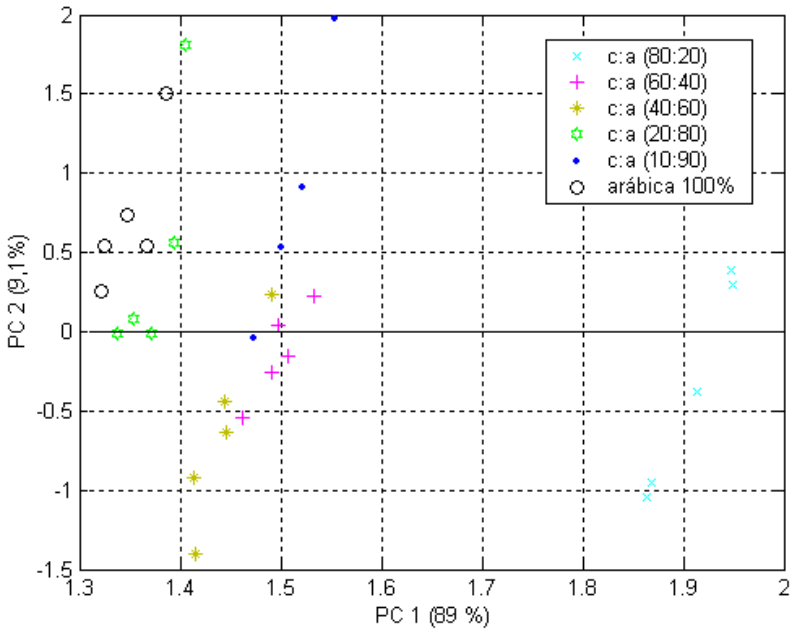

Figure 4. PCA of the six samples of different coffee blends measured with the ET at 1 kHz, 50mV, 25 °C.

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Fast Method for the Quantification of 16-O-Methylcafestol in Roasted Coffee

S. BUCHMANN, G. KÜCHENMEISTER, I. KÖLLING-SPEER, K. SPEER

Technische Universität Dresden, Food Chemistry, Germany

SUMMARY

16-O-methylcafestol as a component of coffee oil can be used for the determination of admixtures of Robusta coffee added to Arabica coffee. In Germany, DIN Method 10779 is used for the analysis of the 16-O-methylcafestol content. Due to the lipid extraction with the steps saponification and extraction of the unsaponifiable matter, this valid method is very time-consuming and expensive. With the new quick method developed here, the esters of the 16-O-methylcafestol were analysed directly without saponification. The triglycerides and the 16-O-methylcafestol esters were separated by solid phase extraction with aminopropyl modified silica gel. The total 16-O-methylcafestol content was calculated via the content of 16-O-methylcafestol palmitate.

INTRODUCTION

In roasted commercial coffees, admixtures of Robusta coffee added to Arabica coffee can be distinguished by the presence of 16-O-methylcafestol (16-OMC) in Robusta coffees but not in Arabica coffees (Montag et al., 1991). 16-OMC (Figure 1), as a component of the lipid fraction, is mainly esterified with different fatty acids. For the determination of the 16-OMC content, in Germany, the DIN Method 10779 is applied (1999). After lipid extraction and saponification, the unsaponifiable matter is analysed by RP-HPLC with DAD (Figure 3, left). The validated method offers reliable data, however, it is very time-consuming (about three days) and, therefore, also expensive.

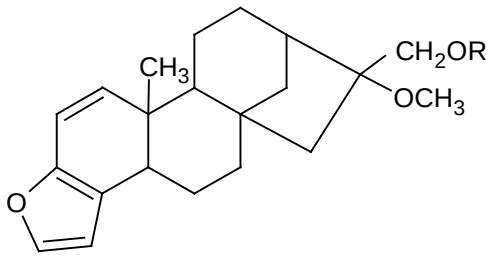

Figure 1. 16-O-Methylcafestol (R=H; R=fatty acids).

A further possibility for determining the 16-OMC content is the direct determination via the individual 16-OMC esters. Therefore, the interfering components of the lipid fraction, in particular the triglycerides, were cut off by means of gel permeation chromatography (GPC) (Figure 3, centre). The obtained fraction with the diterpene esters was separated using solid phase extraction with silica gel in the fraction containing the cafestol/kahweol esters and the other fraction containing the 16-OMC esters (Speer, 1991; Kurzrock, 1997). In Figure 2, an HPLC chromatogram of the 16-OMC fraction is shown.

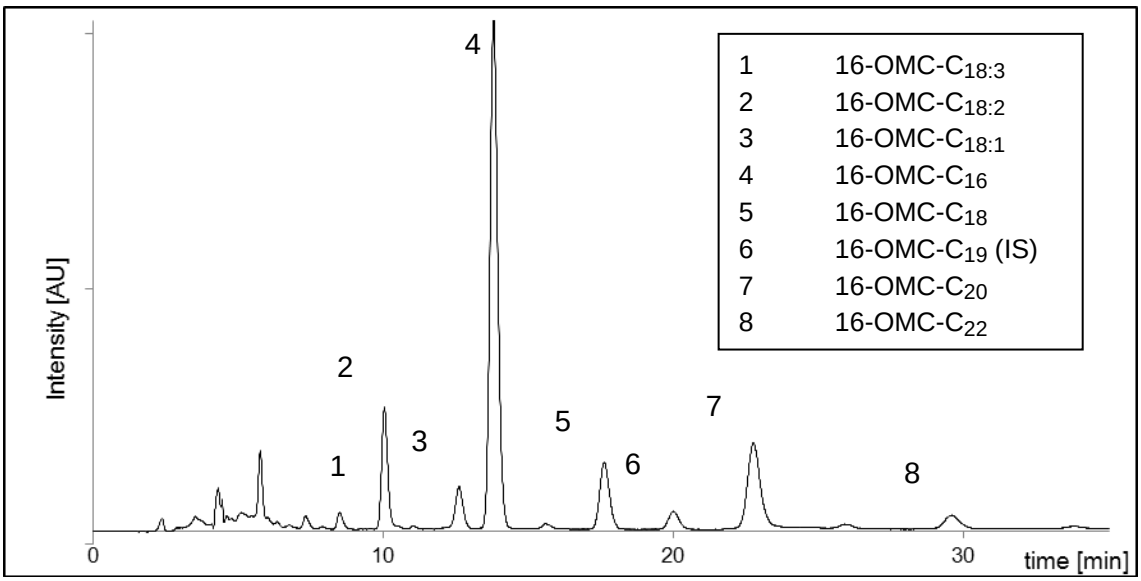

Figure 2. Chromatogram of the 16-O-methylcafestol ester fraction of a Robusta coffee (λ = 220 nm).

The analysis of the 16-OMC esters by means of GPC is not as time-consuming as DIN Method 10779, but the solvent consumption of the GPC is enormous and, so, more expensive. Therefore, the aim of this study was to develop a new quick and cost-efficient method for the quantification of the 16-OMC esters.

MATERIALS AND METHOD

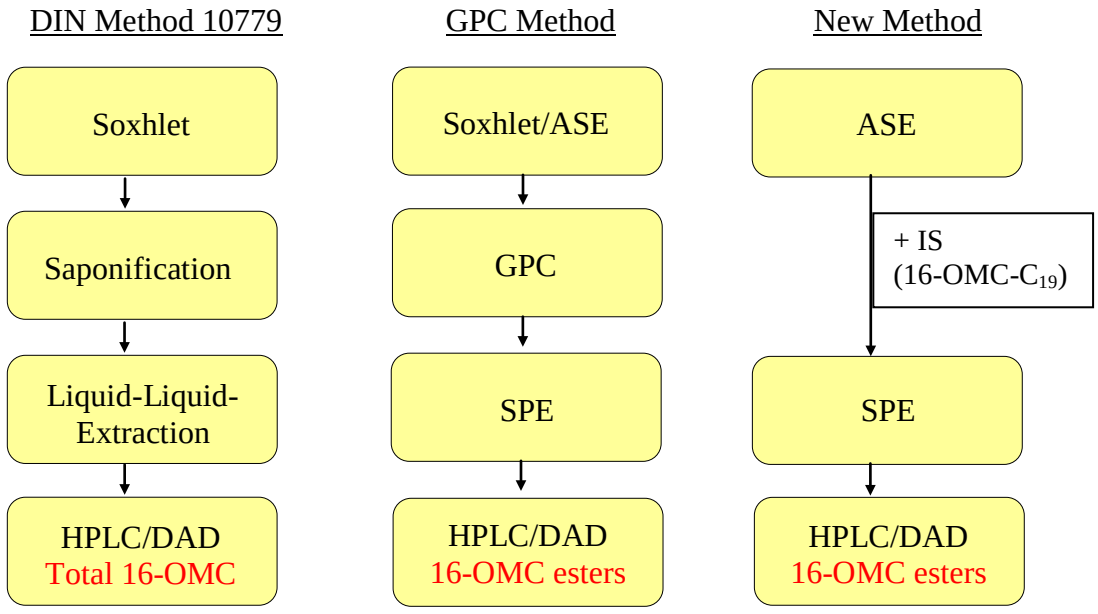

Figure 3. DIN Method 10779 (left), GPC method (centre) and new method (right).

The coffee oil is extracted from ground roasted coffee by means of accelerated solvent extraction (ASE) with tert.-butyl methyl ether. An aliquot of the solution is applied to an activated aminopropyl modified silica gel column (SPE). The 16-OMC esters are eluted with a mixture of n-hexane/toluene. The separation of these esters is carried out by RP-HPLC

equipped with DAD. As internal standard, 16-OMC nonadecanate synthesized (16-OMC-C_{19}) is used and added prior to the SPE (Figure 3, right). With the newly developed method, the triglyceride content in the 16-OMC ester fraction averages 10% and, hence, does not interfere with the analysis.

RESULTS AND DISCUSSION

Using DIN Method 10779, Robusta parts of up to 2% can be detected in Arabica coffee mixtures, a requirement, which is also postulated for the new method. In order to estimate the performance of the newly developed method, coffee blends with Robusta parts of 2%, 5%, 10%, 25% and 50% were prepared and analysed with the new method (Figure 4).

In the 50/50 admixture, most of the fourteen 16-OMC esters were detectable; in the blends with a Robusta content lower than 10%, only the 16-O-methylcafestol palmitate (16-OMC-C_{16}) was evaluable. This compound was even quantifiable in the 2% Robusta blend.

Studies concerning the amounts of the 16-OMC esters in numerous Robusta coffees of different proveniences show that the 16-OMC palmitate content is about 50% of all the 16-OMC esters (Table 1) and that, furthermore, this proportional distribution for the 16-OMC esters is unaffected by roasting (Speer et al., 1994). In all the analysed samples, the proportional distribution could be approved. In Table 1, the percentage distribution of the quantitatively most important 16-OMC esters is presented.

Table 1. Distribution of the 16-O-methylcafestol esters in coffee beans (Kurzrock and Speer, 2001).

Esterified fatty acid	
C_{16}	48%
C_{18}	5%
$C_{18:1}$	15%
$C_{18:2}$	12%
$C_{18:3}$	1%
C_{20}	15%
C_{22}	4%

Therefore, the 16-OMC content can be calculated from the 16-OMC palmitate content, even more so as the peak areas of the 16-OMC palmitate in the chromatograms were nearly linear across the 2% to 50% range of the Robusta admixtures.

Employing the 16-OMC nonadecanate as internal standard proved to be helpful.

The limit of quantitation was determined with 0.02 g 16-OMC palmitate/kg. Assuming a mean total 16-OMC content of 1.7 g/kg (free and esterified) analysed with the DIN Method 10779 for numerous Robusta coffees, an admixture of 2% Robusta was still detectable.

Compared to the other methods described for the quantification of the 16-OMC content, the newly developed method allows for a reduction in solvent usage and labour costs. Furthermore, it requires only four hours for the analysis of one coffee sample.

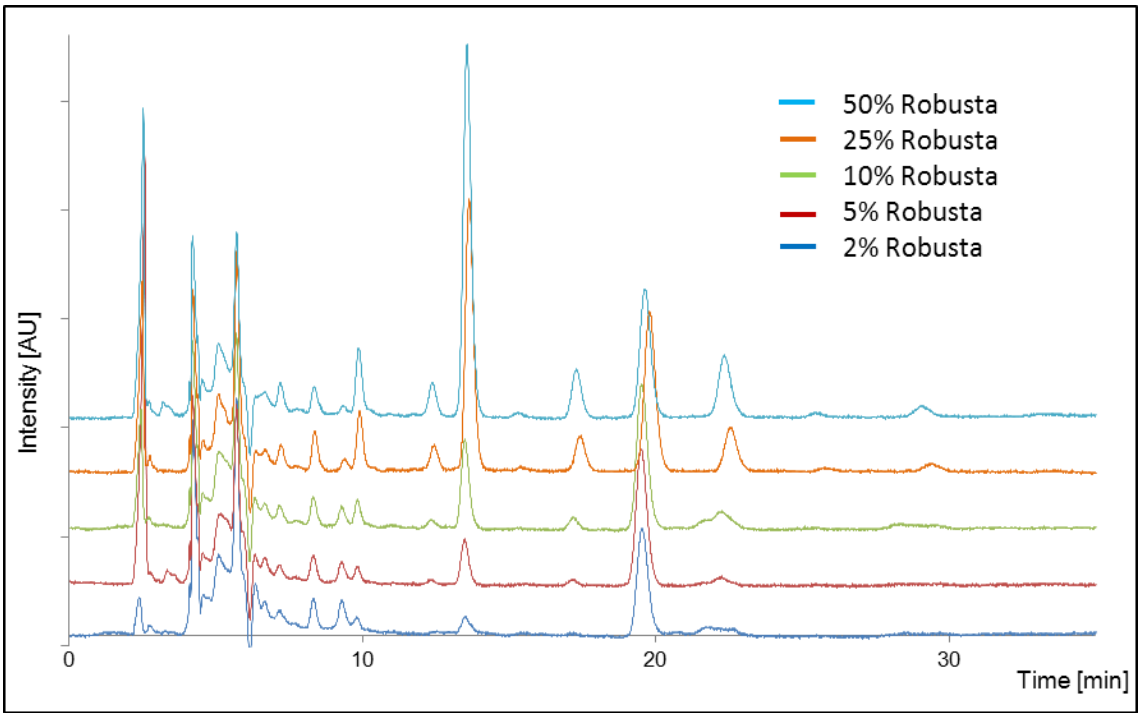

Figure 4. Chromatograms of Arabica blends with Robusta admixtures (λ = 220 nm).

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Coffee Aroma – Influence of the Brewing Method and the Used Coffee

A. ZAHM AND K. SPEER

Technische Universität Dresden, Food Chemistry, Germany

SUMMARY

Twenty-one flavour compounds, of coffee identified as potent odorants were analysed with HS/SPME-GC/MS in brews prepared from roasted Arabica coffees. The volatile profile of the coffee powder and the brew are very similar. The analysis showed a clear difference between espresso beverage and all other beverages brewed by different methods. Furthermore, an increasing coffee/water-ratio caused higher amounts of volatiles in the coffee brew. Compared to the brewing method, the impact of the coffee/water-ratio on the amount of volatiles is less important.

INTRODUCTION

In Germany coffee is one of the most popular beverages next to spring water and beer. In addition to the stimulatory effects, freshly brewed coffee is appreciated for its pleasing overall aroma and its typical taste. More than 800 odorous constituents have so far been identified in numerous previous studies on ground and brewed coffee (Semmelroch and Grosch, 1995; Czerny et al., 1996; Bicchi and Panero, 1997; Sanz et al., 2002). Sensory studies revealed that only about 30 odorants have great impact on the aroma of the brew (Mayer et al., 2000).

Among the analytical techniques used to analyse and separate volatile fractions of coffee brews, GC/MS has been established as one of the most important. Auto-sampling coupled with solid phase micro-extraction (SPME) has been shown to be an excellent sampling method, allowing the simultaneous extraction and concentration of the analytes from sample matrices such as ground coffee and brewed coffee (Risticevica and Pawliszyna, 2008).

MATERIALS AND METHODS

Preparation of coffee brews

Commercially roasted Arabica coffee was ground with an industrial centrifugal mill (VTA6S, Stahwert Mühlenbau, Germany). The particle size distribution was determined employing a laser diffraction particle size analyzer (LS 13320, Beckman Coulter).

To ensure comparable conditions, the coffee powder with a medium particle size and de-ionized water was used to prepare the coffee brews. Although knowing that espresso is brewed with a coffee/water-ratio of about 150 g per liter, the ratio was kept at 50 g per liter for all coffee brews to compare the different brewing methods.

Turkish coffee was brewed with cold water and boiled three times before it was decanted. A vending machine (Maas SL1000) was used to prepare a typical espresso and a fresh brew coffee.

Coffee brews with filtration were prepared in several ways: by using a paper filter or by using a French Press (Bodum®-Plunger).

An aliquot of the brewed coffees (5 ml) was immediately added to a sealed 20 ml headspace-vial.

SPME

A CombiPAL SPME autosampler equipped with an agitator and fiber conditioning station was utilized to allow a completely automated SPME procedure. The autosampler was employed in combination with a GC/MS (Trace GC Ultra/ DSQ II, Thermo).

The SPME triple phase divinylbenzene/carboxen/polydimethylsiloxane (Supelco, USA) was employed for the extraction of volatiles. The incubation and extraction procedures were completed by utilizing the sample temperature and the agitation speed of 60 °C and 500 rpm, respectively. The employed incubation and extraction times were 20 min whereas the thermal desorption of analytes in the GC injector port was enabled for 5 min at 250 °C. After each extraction-desorption cycle, the fiber was cleaned in the fiber conditioning station at 270 °C for 5 min.

RESULTS AND DISCUSSION

In Figure 1, a chromatogram of a coffee brew and the used coffee powder is compared. It was possible to extract and detect a great number of volatile compounds. According with literature (Semmelroch and Grosch, 1995; Sanz et al., 2002; Mayer et al., 2000) we focused on 21 volatiles, which have an impact on the aroma of the coffee brew (Table 1).

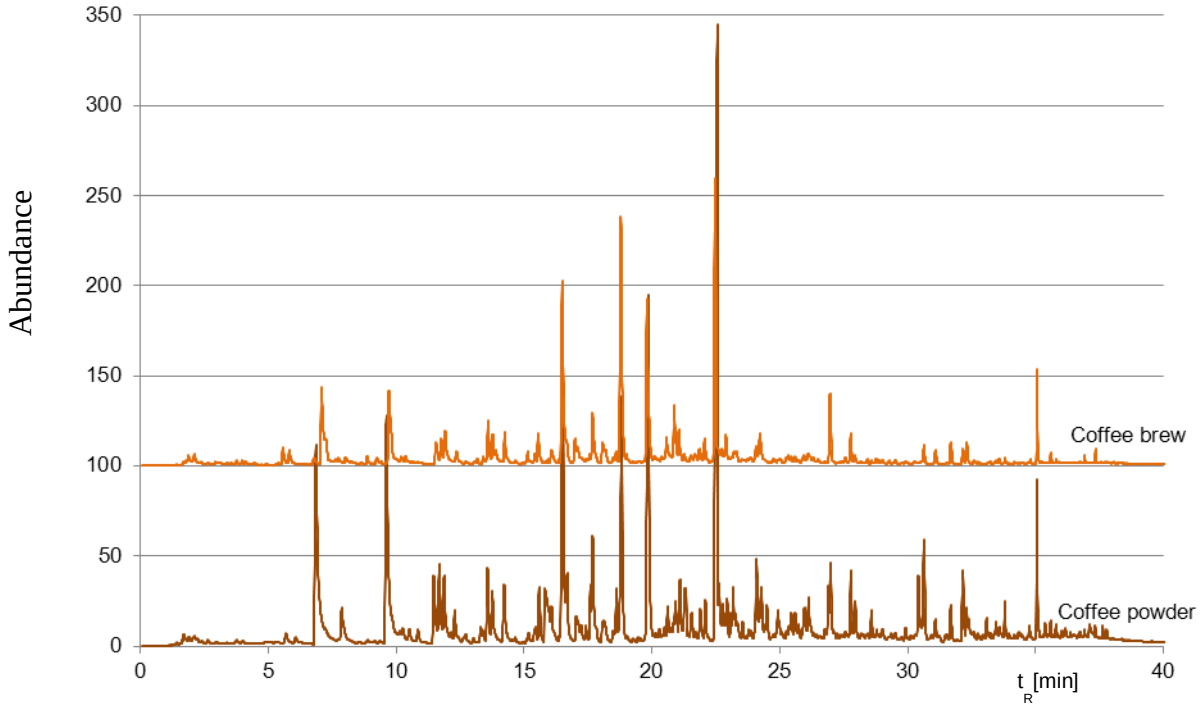

Figure 1. HS-SPME-GC/MS chromatogram of coffee powder and coffee brew.

Table 1. Volatiles with an impact on the aroma of coffee brew (Semmelroch and Grosch, 1995; Sanz et al., 2002; Mayer et al., 2000).

Peak no.	Compound	CAS no.	Odour description
1	3-Methyl-butanal	590-86-3	malty
2	2,3-Pentanedione	600-14-6	buttery
3	Pyridin	110-86-1	fishy
4	Methylpyrazin	109-08-0	roasty
5	Nonanal	124-19-6	fruity
6	2-Ethyl-3,5-dimethyl-pyrazin	27043-05-6	earthy
7	2,3-Diethyl-5-methyl-pyrazin	18138-04-0	earthy
8	Furfural	98-01-1	smoke, green
9	1-(2-Furanyl)-ethanone	80145-44-4	cooked vegetable
10	2-Furanmethanolacetat	623-17-6	ethereal-floral
11	Linanool	78-70-6	fruity
12	5-Methyl-2-furancarboxaldehyde	620-02-0	spicy
13	2-Furanmethanolpropanoate	623-19-8	green banana
14	N-methyl-2-formylpyrrol	1192-58-1	
15	2-Furanmethanol	98-00-0	ether like
16	β-Damascenone	23696-85-7	boiled apple like
17	1-(2-Furanylmethyl)-1H-pyrrole	1438-94-4	bready
18	2-Methoxyphenol	90-05-1	phenolic, burnt
19	4-Ethyl-2-methoxy-phenol	2785-89-9	phenolic
20	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	spicy
21	p-Vinylguaiacol	7786-61-0	rotten off-flavour

Comparing the 21 volatiles in the coffee powder, the obtained brew revealed some inherent differences. In Figure 2, a diagram with the relative area of some selected volatiles is presented. While some volatiles such as 2-ethyl-3,5-dimethyl-pyrazin and p-vinylguaiacol barely influenced the content of methylpyrazin, 2-furanmethanol and 2-furanmethanolacetat are changed during brewing. One reason for this may be the different water solubility of the volatiles.

The chromatograms of the different brewing methods were all very similar. In Figure 3, the influence of the brewing method on the total area of all 21 volatiles is presented. The espresso coffee showed a nearly doubled peak area compared to all other brewing methods. However, there could no difference observed between boiled and paper filtered coffees.

Furthermore the profile of the evaluated 21 volatiles showed no difference between the different brewing methods.

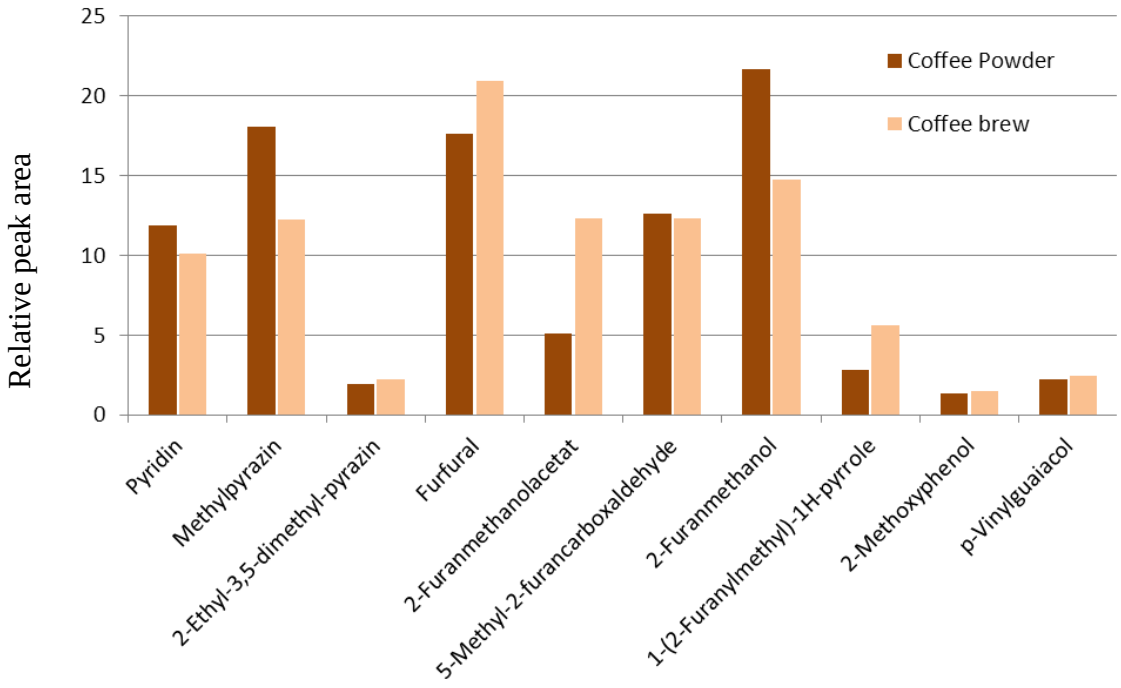

Figure 2. Comparison of selected volatiles in coffee powder and coffee brew.

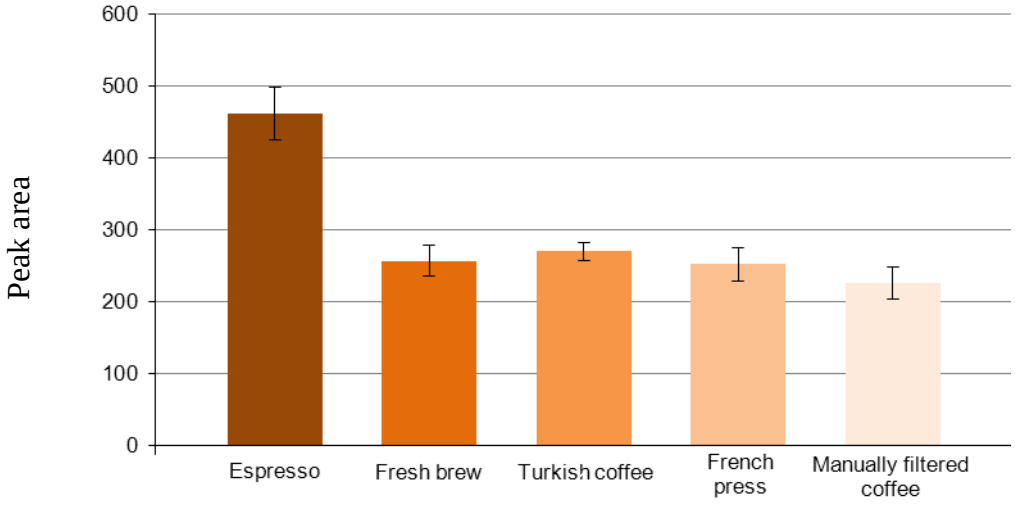

Figure 3. Impact of the brewing method on the volatiles.

To study the influence of the coffee/water-ratio different espresso coffees were prepared. The recommended ratio for espresso coffee is between 50 and 150 g per liter. Due to limitations of the espresso machine we used the following steps 6.5/7.5/8.5 g coffee and 40/65/90 mL water. The impact of the coffee was studied at 65 ml of water. Otherwise, the coffee powder was kept at 7.5 g to evaluate the influence of the volume of the water.

With an increasing amount of coffee the sum of the evaluated volatiles increased by about 20%. The same effect was observed while increasing the amount of water up to 90 mL. A

further increase above 120 mL caused a decrease in the sum of volatiles due to dilution effects.

In Figure 4, two different commercially Arabica coffee powders and the prepared brews are compared. As expected, the volatile profile of the coffee brew also depends on the coffee powder used.

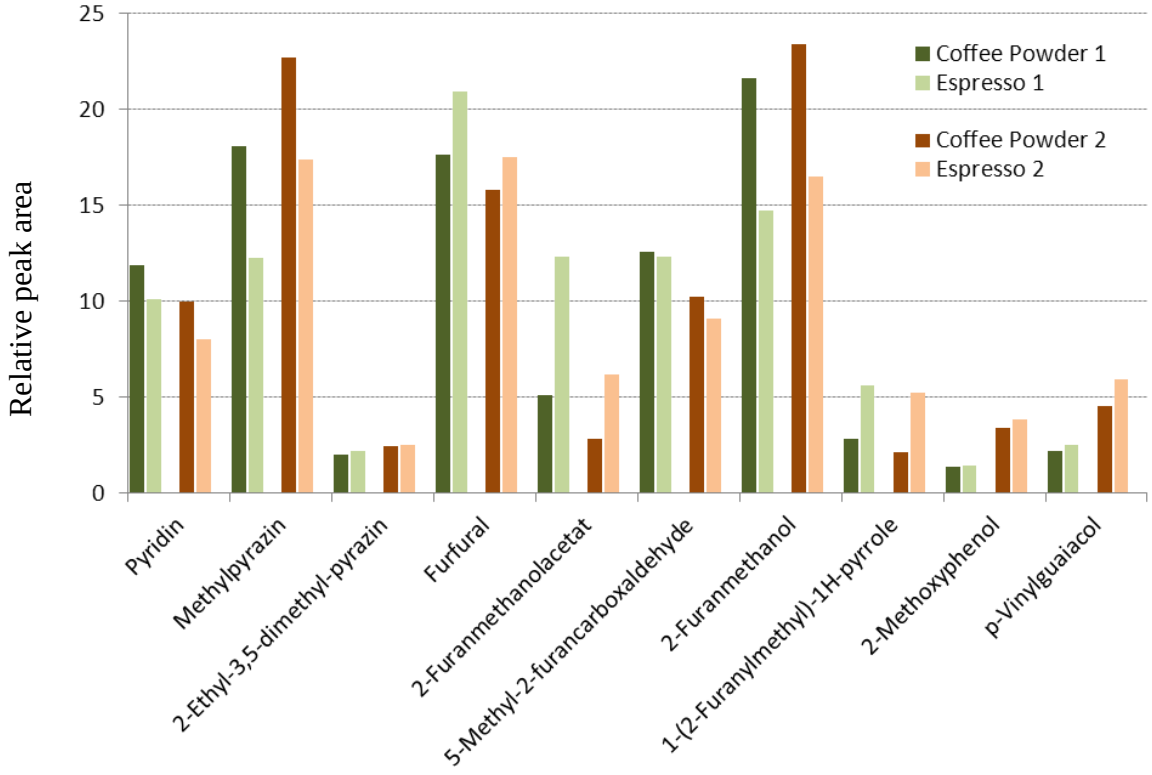

Figure 4. Impact of different coffee powder on the volatiles in the brew.

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Identification of C-5-HT Degradation Products Using Pyrolysis-GC/MS

A. ZAHM[1], J. PETERS[2], K. SPEER[1]

[1]Technische Universität Dresden, Food Chemistry, Germany
[2]Technische Universität Dresden, Institute of Plant and Wood Chemistry, Germany

SUMMARY

Carboxylic acid-5-hydroxytryptamides (C-5-HT) are part of the coffee wax on the surface of coffee beans. During the roasting process, a decomposition of the C-5-HT was observed (Hinkel, 2009). The Pyrolysis-GC/MS is a capable method for identifying further degradation products of the C-5-HT. In contrast to the literature, 5-hydroxyindole, 3-methyl-5-hydroxyindol, 3-ethyl-5-hydroxyindol, and serotonin were identified as degradation products. The further numerous degradation products assumed by Viani and Horman could not confirmed.

In addition, the homologue rows of the alkanes and alkenes, the fatty acid amide, the fatty acid nitrile as well as the fatty acid were identified as general degradation products of the C-5-HT.

INTRODUCTION

Coffee beans contain 0.2-0.3% coffee wax, which is located on the surface of the bean. The main components of this waxy layer are the so called carboxylic acid-5-hydroxytryptamides (C-5-HT) (Wurziger and Harms, 1968). During the roasting process, the C-5-HT levels are reduced to approximately 70% (Hinkel, 2009). Numerous potential degradation products, for example, indan and indol were assumed by Viani and Horman (1975). Recently, we introduced the three main degradation products on the basis of heating experiments: serotonin, hydroxyindole, and 3-methyl-5-hydroxyindole (Zahm and Speer, 2009). In roasted coffee, the concentration of these 3 components is not enough to reach the calculated concentration caused by the degradation of the C-5-HT. Consequently, some degradation products are still unidentified.

MATERIALS AND METHODS

According to Hinkel, octadecanoic acid-5-HT (C_{18}-5-HT) was synthesised and analysed by HPLC-DAD, GC-MS and NMR to ensure high purity (Hinkel, 2009; Hinkel and Speer, 2009).

Pyrolysis-GC/MS experiments were accomplished using a general GC by Curie Point Pyrolyzer and MS detector. Curie Point Pyrolyzers work by ferromagnetic heating of a wire to its Curie point. The specific blend of metals comprising the wire dictates its Curie point and, therefore, the final sample temperature and extent of pyrolysis. In this study, pyrolysis temperatures from 358 °C up to 700 °C were used.

RESULTS AND DISCUSSION

In Figure 1, the Pyr-GC/MS chromatogram of C_{18}-5-HT shows homologue rows of the alkanes and alkenes (C6 - C17) and the recently introduced degradation products 5-hydroxyindole (1), 5-hydroxyskatol (2), 3-ethyl-5-hydroxyindol (3), and serotonin (4). Furthermore, octadecanenitrile (5), octadecanoic acid (6), and octadecanamide (7) were detected.

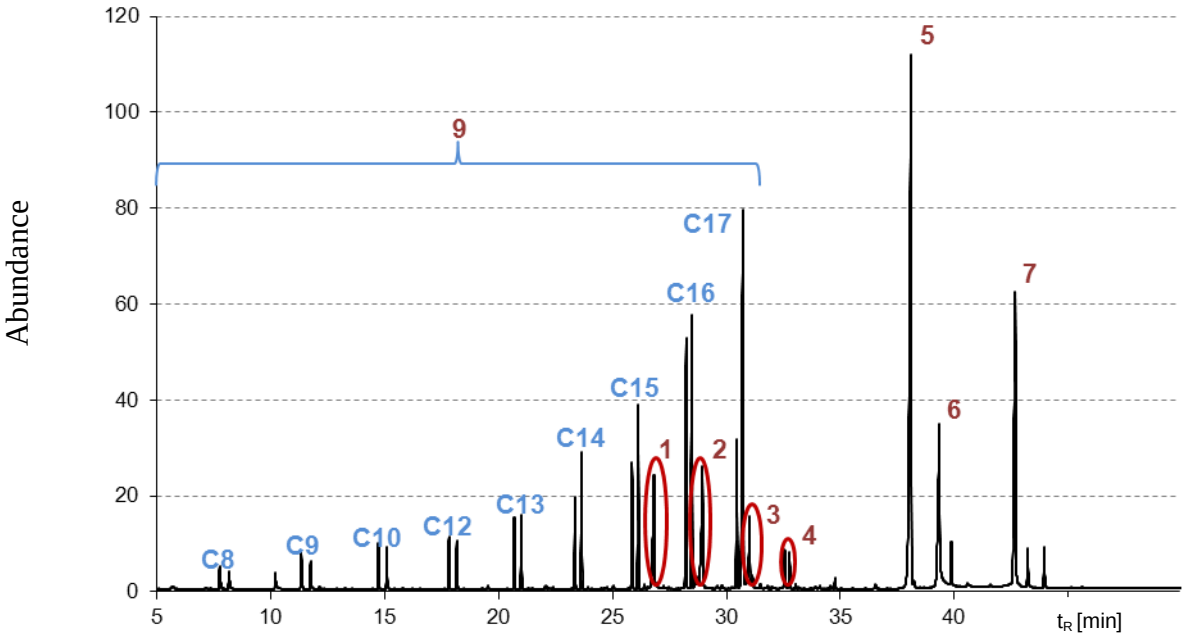

Figure 1. Pyr-GC/MS chromatogram of C_{18}-5-HT.

In addition, C_{21}-5-HT was analyzed with Pyr-GC/MS. According to C_{18}-5-HT, henicosanamide, henicosanenitrile and henicosanoic acid were identified as degradation products of C_{21}-5-HT. Furthermore, the homologue rows of the alkanes and alkenes (C6-C20) were detected. Consequently, amide, nitrile, and fatty acid are potential degradation products of C-5-HT.

Employing different pyrolysis temperatures offers the possibility of better understanding the degradation of the C-5-HT.

With a pyrolysis temperature of 358 °C, obviously neither alkanes nor alkenes occur. Only fatty acid and a small amount of fatty acid amide are shown (Figure 2). Increasing the pyrolysis temperature to 500 °C causes a higher amount of fatty acid and the formation of 3-ethyl-5-hydroxyindole. Next to the other already mentioned degradation products, the homologue rows of the alkanes and alkenes appear at 740 °C.

The temperatures applied at Pyr-GC/MS are incommensurable to those applied during the roasting of coffee. Due to this fact, an experiment under roasting conditions was carried out. A small amount of C_{18}-5-HT was sealed in a 20 mL headspace vial and heated at 200°C for 15 min. Afterwards, the degradation products were identified using HS-SPME-GC/MS (Figure 3). In accordance with the Pyr-GC/MS, the homologue rows of the alkanes and alkenes as well as 5-hydroxyindole, 3-methyl-5-hydroxyindol, and 3-ethyl-5-indol were detected. Furthermore, the residue from roasting was dissolved in ethyl acetate and analysed

with GC/MS. Next to the C_{18}-5-HT (4), the fatty acid nitrile (1), the fatty acid amide (2) and the fatty acid (3) were detected (Figure 3).

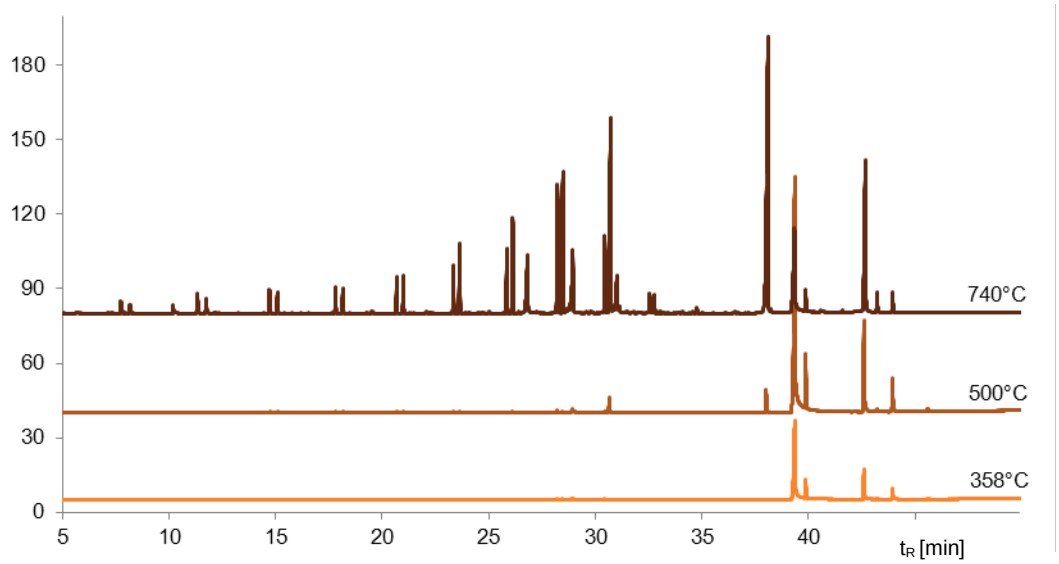

Figure 2. Pyr-GC/MS chromatogram of C_{18}-5HT at different pyrolysis temperatures.

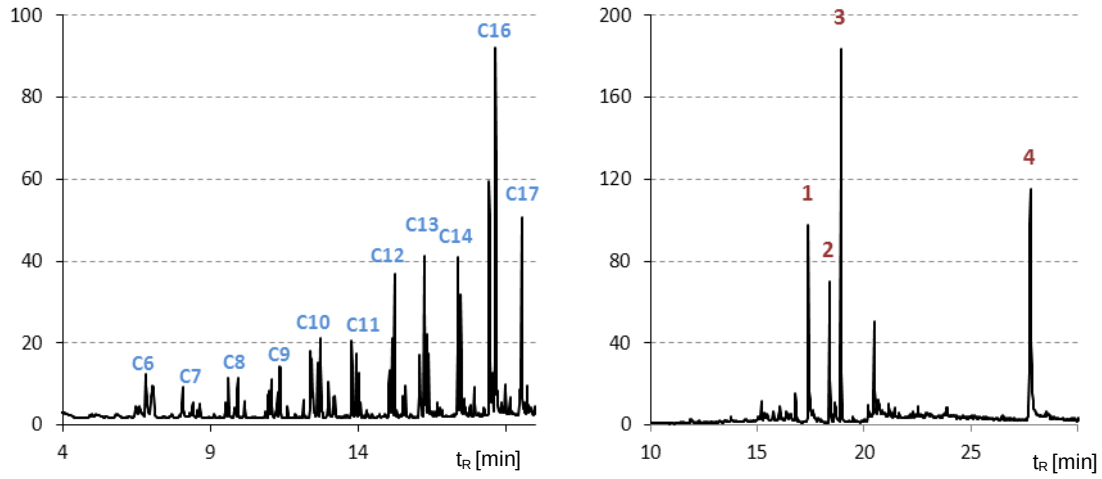

Figure 3. GC-HS-SPME- (left) and GC-MS-Chromatogram (right) of C18-5-HT.

These results enable drawing a possible pathway of degradation of the C-5-HT. First, the C-5-HT is degraded to the corresponding fatty acid nitrile, the fatty acid, and the fatty acid amide, respectively. Along with higher temperatures, the fatty acid degrades to alkanes and alkenes. Serotonin easily breaks down to 3-ethyl-5-hydroxyindole, hydroxyskatole, and, finally, to hydroxyindole. To which extent this pathway is transferable to the roasting of coffee will have to be investigated in further studies.

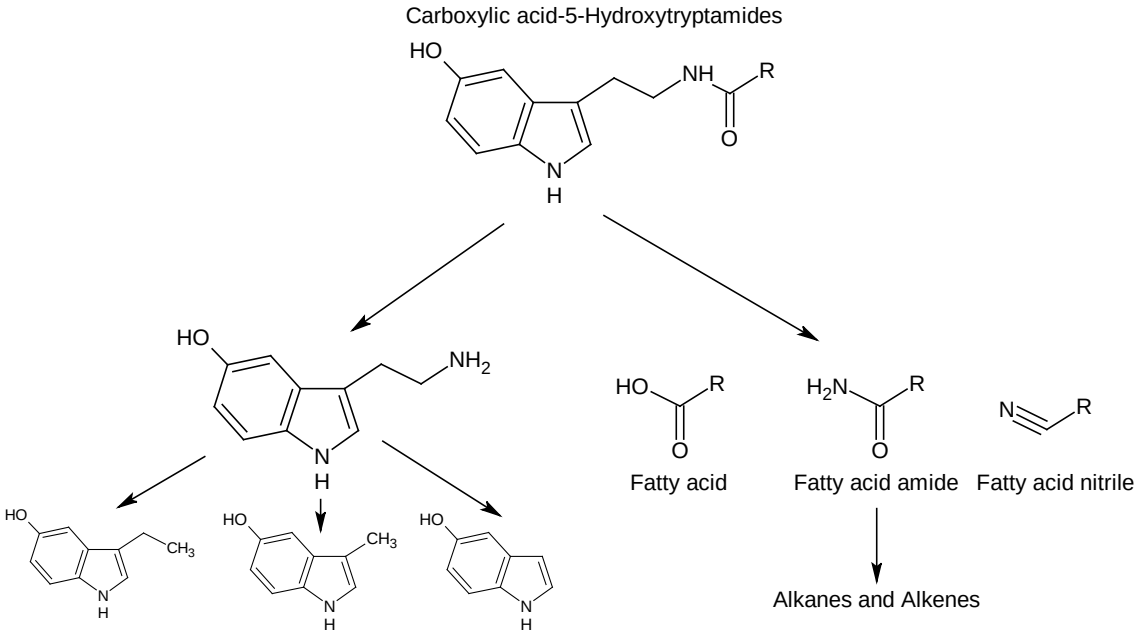

Figure 4: Proposed pathway of C-5-HT degradation

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Serotonin and 5-Hydroxyindole in Green and Roasted Coffee

A. ZAHM, R. RONCZKA, K. SPEER

Technische Universität Dresden, Food Chemistry, Germany

SUMMARY

The well known group of C-5-HT located in the outer waxy layer of the coffee beans are decomposed to serotonin, 5-HI, and 5-HS during roasting.

20 green Arabica and Robusta coffees from Asia and South America were studied. The analysed Arabica coffees showed serotonin levels between 22 and 32 mg/kg whereas Robusta coffees reached amounts of up to 40 mg/kg. The difference between Arabica and Robusta varieties may be caused by different post-harvesting processes. However, neither 5-HI nor 5-HS were detected in green coffee samples.

The thermal load during the roasting process led to a degradation of serotonin and to the formation of 5-HI and 5-HS. Furthermore, a significant increase of the serotonin content at mild roasting conditions perhaps caused by the decomposition of the C-5-HT, was observed.

INTRODUCTION

Coffee beans contain 0.2-0.3% coffee wax, which is located on the surface of the bean. The main components of this waxy layer are the so called carboxylic acid-5-hydroxytryptamides (C-5-HT) (Wurziger and Harms, 1968). During the roasting process, a decomposition of the C-5-HT was observed (Hinkel, 2009). The main degradation products were identified as serotonin (5-hydroxytryptamine), 5-hydroxyindole (5-HI), and 5-hydroxyskatole (5-HS) (Zahm and Speer, 2009).

Serotonin is a major neurotransmitter that is involved in numerous functions of the mammalian central nervous system which can cause unnatural or toxic effects when consumed in large quantities. During the roasting process, both 5-HI and 5-HS were generated as tryptophan metabolites. Especially 5-HI and its analogue oxoindol (2-indolinone) are known to significantly decrease neuronal excitability and to cause convulsions in experiments with rodents (Mannaioni, 2003).

MATERIALS AND METHODS

Frozen green and roasted coffee beans were ground (Grindomix 200, Retsch) and sieved (630 µm test sieve, Retsch). The obtained powder was spiked with internal standard (5-methoxytrytptamin) and extracted with methanol-water 50/50 (v/v) using an accelerated solvent extraction system (ASE 200, Dionex). The subsequent HPLC analysis was carried out with fluorescence detection and LC-MS/MS.

The presence of the matrix may affect the behaviour of the analytes. Especially during the extraction of roasted coffee, many melanoids and other roasting products are extracted. Due to this fact, the extract was diluted and then cleaned up by means of solid phase extraction

(SPE). The SPE was carried out by using C18-cartridges. The conditioning step was accomplished with methanol and water. The anlytes were eluted with acetonitril and water. To increase the repeatability, the Rapid Trace system (Caliper) for automated SPE was used.

For all coffee samples, the diterpene content and the Cafestol/Dehydrocafestol ratio was analysed according to DIN 10779.

The results reported in this paper are the average of two determinations carried out on different samples in which the coefficients of variation were less than 5%. The evaluation and presentation of the data was accomplished by using Origin® 8.0. To highlight the different results, boxplots were used. The box includes 50% of all the values. Inside the box, the median is presented. The whisker enframes 95% of all measured data.

RESULTS AND DISCUSSION

Green coffee

In contrast to the published data by Casal et al. (2004), the analysed green coffees showed serotonin levels up to 40 mg/kg. As distinguished from the green Robusta coffees, the analysed Arabica coffees showed a significant lower serotonin level (Figure 2). The reason could be the post-harvesting processing. Usually, Robusta coffee cherries are dried on patios or on racks whereas Arabica coffee cherries are mostly passed through washer-separators before the removal of the pulp. Hereby, it is possible that a considerable amount of serotonin is extracted. In all green coffee samples neither 5-HI nor 5-HS were detected.

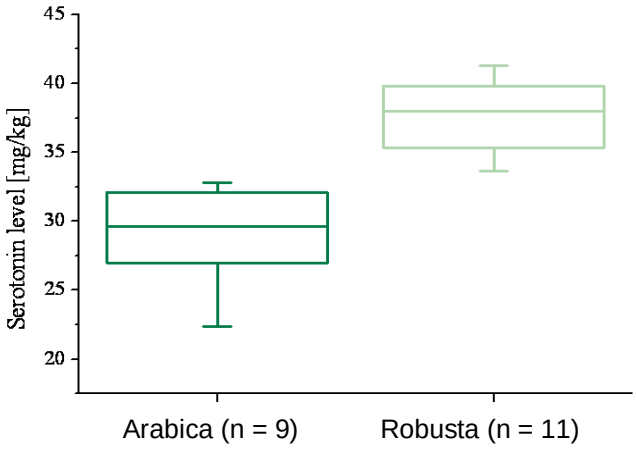

Figure 1. Serotonin level in green Robusta and Arabica coffee.

Roasted coffee

To study the impact of the roasting process on the level of the degradation products, numerous commercially available roasted coffees were analysed. The roasting degree is obviously influenced by the roasting temperature and the roasting time. *Kölling-Speer et al.* reported that the cafestol/dehydrocafestol-ratio (C/DHC-ratio) is linear to the roasting taste and is a suitable measure of the roasting grade of coffees (Kölling-Speer et al., 1998). Ratios below 20 can be assumed as darkly roasted while values above 35 indicate lightly roasted coffees. In Table 1, the roasting degree, the number of coffees at each roasting degree, and the C/DHC-ratio is shown.

Table 1. number of coffees at each roasting degree.

Roasting degree	C/DHC-ratio	Number of coffees
light	>35	5
medium light	35 - 25	9
medium dark	25 - 15	7
dark	<15	5

In Figure 3, the levels of serotonin are presented in reference to the C/DHC-ratio. In general, the serotonin level decreases with an increasing thermal load during roasting. Only light roasted coffee (C/DHC-ratio >35) showed significant lower serotonin levels than medium roasted coffee (C/DHC-ratio 25-35). The decomposition of the C-5-HT may possibly have caused this increase.

The determined serotonin levels in roasted coffee were in accordance with published data (Kölling-Speer et al., 1998; Cirilo et al., 2003). However, an increasing serotonin level from green to roasted coffee discussed by Casal et al. was not observed.

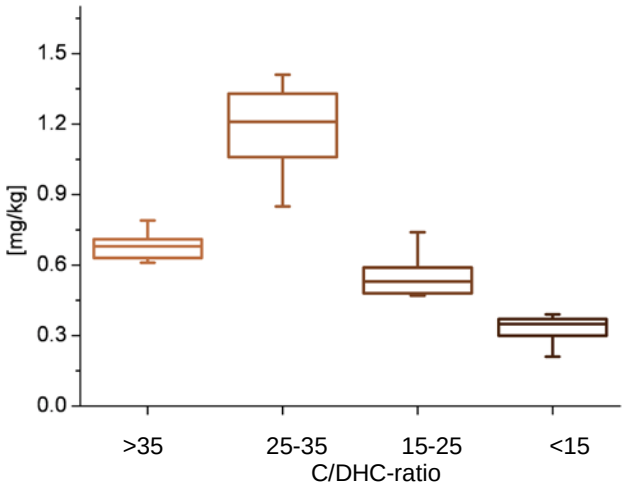

Figure 2. Impact of the roasting degree on the serotonin level.

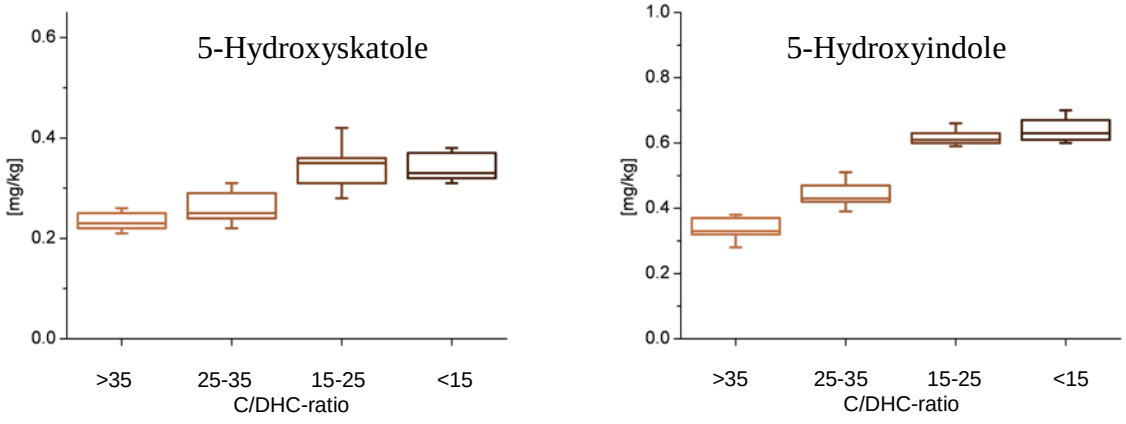

Figure 3. Impact of the roasting degree on the 5-HI and 5-HS level.

The contents of 5-HS and 5-HI in different roasted coffees are presented in Figure 4. In contrast to the serotonin level, the content of 5-HI and 5-HS increased almost linearly with increasing roasting degree, indicating a thermal decomposition of serotonin or other tryptophan metabolites.

The content of serotonin, 5-HS, and 5-HI also depends on the contents of C-5-HT and serotonin in the green coffee. To eliminate the natural difference in the serotonin level among the different roasted coffees, the serotonin/5-HI and serotonin/5-HS-ratio was taken into consideration. Exemplarily, the serotonin/5-HS ratio is shown in Figure 5. Both ratios did not give a linear correlation to the C/DHC-ratio. Consequently, it can be concluded that the serotonin level during roasting is not only influenced by its degradation but rather by the degradation of C-5-HT.

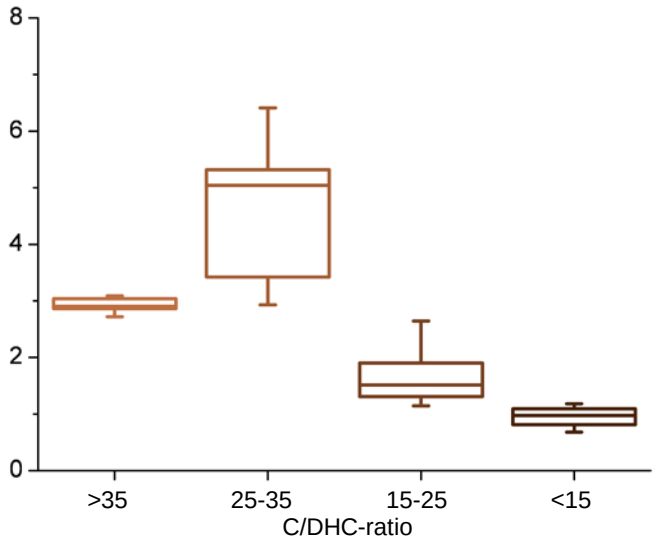

Figure 4. Impact of the roasting degree on the Serotonin/5-HDS-ratio.

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Chemical Stability and Consumer Acceptance during Storage of a Cold Instant Soy Coffee Beverage

I. FELBERG[1], R. DELIZA[2], A. FARAH [3], V. CALADO [4], J.P.L. SILVA[1], A.T. TOCI [3], S.C. FREITAS[1], C. M.DONANGELO[3]

[1]Embrapa Agroindústria de Alimentos, RJ, Brazil. E-mail: ilana@ctaa.embrapa.br
[2]Embrapa Labex Europe, Montpellier, France
[3]Instituto de Química, Universidade Federal do Rio de Janeiro, RJ, Brazil
[4]Escola de Química, Universidade Federal do Rio de Janeiro, RJ, Brazil

SUMMARY

Considering the current consumer trend for healthier alternatives in food products and the possibility to combine in a functional beverage the health benefits of two important brazilian commodities, we elaborated an instant soy-coffee based beverage. The present work was addressed to study the chemical stability, microbiological safety and consumer acceptability, of this instant soy-coffee beverage stored during 32 weeks at 25 °C. For this purpose, the instant beverage powder containing 10% of soymilk powder, 2% of instant coffee and 13% of sugar was packed in flexible aluminum bags, heat sealed and stored at 25 °C. The study design was planed so that samples with different storage times could be analyzed at the same occasion. Every 8 weeks along the 32 weeks of storage, three packages of the instant beverage were transferred from the storage place to a freezer at –18 °C and kept until the end of the study. All samples were considered safe according to Brazilian Regulatory Policies, and no significant changes were observed on the chemical composition during storage. Protein content ranged from 20.22 to 18.88 g/100 g, oil content from 12.28 to 11.49 g/100 g, ash from 3.10 to 3.02 g/100 g, caffeine from 0.39 to 0.38 g/100 g, and total chlorogenic acids from 0.32 to 0.26 g/100 g for the samples with 0 and 32 weeks of storage, respectively. Trigonelline content was 0.08 g/100 g for all samples. Similarly, no changes were detected by sensory analysis. The hedonic mean scores varied from 5.5 for sample with 0 weeks of storage to 4.9 for sample stored for 32 weeks. However, a tendency for a decrease in the concentration of some of the evaluated compounds as well in the hedonic scores was observed and should be investigated in studies evaluating longer storage time.

INTRODUCTION

Brazil is the first coffee producer, and the second largest coffee consumer market in the world (ABIC, 2010). Althoug Brazil is the second largest producer of soybeans (IBGE, 2010), and Brazilian market for soy beverages has grown in 30% in the last years (Abreu et al., 2007), soy and soy products are not commonly used in the Brazilian diet. Several epidemiological studies have shown that a regular diet including soy products or habitual coffee consumption can reduce the risk of several chronic diseases (Barnes et al., 2006; Farah and Donangelo, 2006). Coffee is very much appreciated among Brazilian people for its pleasant taste and aroma and, as a consequence, it has been used to improve the flavour of other food products like soymilk (Felberg et al., 2010). However, during storage, changes in non-volatile and volatile compounds caused by oxidation and degradation may negatively affect the flavor of the beverage, and decrease its acceptance (Manzocco and Lagazio, 2009). Thus the objective of

this study was to evaluate the chemical stability, microbiological safety and consumer acceptance, of an instant soy-coffee beverage stored for 32 weeks.

MATERIALS AND METHODS

The instant beverage powder containing 10% soymilk powder, 2% instant coffee and 13% sugar was packed in flexible aluminum bags, heat sealed and stored at 25 ºC in storage room under controled temperature. The study design was planed so that samples with different storage times could be sensory and chemically analyzed at the same occasion. Every 8 weeks along the 32 weeks of storage, three packages of the instant beverage were transferred from the storage place to a freezer at –18 °C. Samples were evaluated for the presence of *Salmonella sp., Bacillus cereus and* coliforms according to APHA (2001). The proximate composition was carried out according to AOAC (2005). Caffeine, trigonelline and total chlorogenic acids (CGA) were analyzed according to Farah et al. (2006). For sensory evaluation beverages were obtained by mixing the instant powder with mineral water 24h prior to the test and kept refrigerated at 5ºC. One hundred and twelve individuals participated in the consumer test. Samples were evaluated for overall acceptability using a 9-point structured hedonic scale (1= *dislike extremely*; 9=*like extremely*) (Stone and Sidel, 1993). Acceptance and chemical data were analyzed by ANOVA and Fisher test (LSD) ($p<0.05$).

RESULTS AND DISCUSSION

All samples were considered safe (Table 1) according to Brazilian Regulatory Policies (ANVISA RDC n° 12/2001) for *Salmonella* (absence in 25 g), *Bacillus cereus* ($<1.0x10^1$CFU/g), and detection and enumeration of coliforms (MPN <3).

Table 1. Microbiological data from the instant soy-coffe beverages during storage.

	Storage time (weeks)					
	0	**8**	**16**	**24**	**28**	**32**
Coliformes a 45 °C (MPN/g)	< 3	< 3	< 3	< 3	< 3	< 3
Salmonella sp. (Absent in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus(CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10

**MPN/g (most probable number per gram); CFU/g (colony-forming units per gram).*

Samples were chemically stable during the storage study. No significant changes were observed on the chemical composition on 0, 16 and 32 weeks of storage, respectively, (Table 2). However, a tendency for a decrease (19%) in the concentration of CGA was detected from the 28th week to the 32nd week of storage (Figure 1).

Table 3 presents the mean acceptability scores during storage. The analysis of variance of acceptance data revealed that samples were not significantly different in terms of acceptability ($p \leq 0.05$) with a range of mean scores from 5.5 for sample with 0 weeks of storage to 4.9 for sample stored for 32 weeks.

However, a tendency for a decrease in the hedonic scores was observed from the 28th week to the 32nd week of storage and should be investigated in studies evaluating longer storage time.

Table 2. Chemical composition of soy-coffee instant beverage during storage.

	Mean (g/100 g dry matter)					
	Storage time (weeks)					
	0		16		32	
Protein	20,22	± 0,18	19,17	± 0,11	18,88	± 1,78
Carbohydrate	65,56	± 0,61	65,03	± 0,93	69,07	± 1,35
Ash	3,10	± 0,26	3,31	± 0,07	3,02	± 0,22
Lipids	12,28	± 0,72	13,31	± 0,42	11,49	± 0,39
Total solids	98,86	± 0,04	98,80	± 0,29	97,98	± 0,16
Fructose	0,28	± 0,02	0,24	± 0,02	0,23	± 0,04
Glucose	ND		ND		ND	
Sucrose	45,76	± 0,45	44,42	± 0,96	45,60	± 0,76
Raffinose	0,72	± 0,01	0,74	± 0,01	0,74	± 0,02
Stachyose	1,41	± 0,03	1,37	± 0,08	1,45	± 0,03
Caffeine	0,39	± 0,04	0,40	± 0,06	0,38	± 0,04
Trigonelline	0,08	± 0,00	0,09	± 0,01	0,08	± 0,00

ND-Non Detetected.

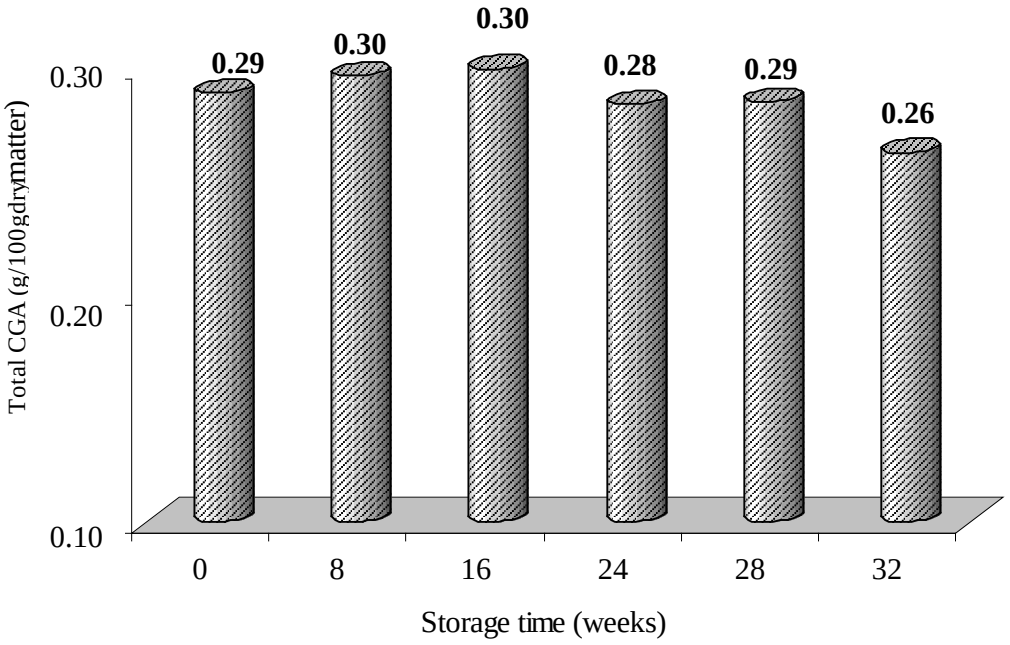

Figure 1. Total chlorogenic acids (CGA) from soy-coffee instant beverage during 32 weeks of storage (% dry matter).

Table 3. Mean acceptability scores* of soy-coffee beverage during storage.

	Storage time (weeks)					
	0	**8**	**16**	**24**	**28**	**32**
Acceptability scores (mean)[§]	5.5[a]	5.3[a]	5.1[a]	5.2[a]	4.9[a]	4.9[a]

**values that are followed by different letters within rows are significantly different by Fisher´s test (p< 0.05). § 1 = dislike extremely, 5= neither like nor dislike, 9 = like extremely.*

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Soluble Coffee Classification through Rapid Scanning Methodologies

I.D. FISK[1], A.VIRDIE[1], J.S. KENNY[1], F. ULLRICH[2]

[1]Kraft Foods UK R&D Ltd. Ruscote Avenue, Banbury, Oxfordshire, OX16 2QU, UK
[2]Kraft Foods R&D Inc. Unterbiberger Str. 15 81737 Munich, Germany

SUMMARY

This work was conducted to study the predictive ability of a range of rapid scanning methodologies to estimate raw material composition and processing variability for freeze-dried soluble coffee. To this purpose a series of soluble coffee samples were evaluated by a range of techniques (Chemsensor, Raman, FT-IR, NIR) and the resulting individual spectra evaluated for their predictive ability.

Preliminary screening studies removed Raman and FT-IR due to their restricted sampling area which required an impractical number of sample replicates to compensate for inherent product in-homogeneity.

Chemsensor using direct MS injection and NIR offered reliable product discrimination and classification and were successfully used to development effective predictive models for the generation of raw material descriptors and product classification.

INTRODUCTION

Historically a series of rapid scanning methodologies such as e-nose and NIR have been used to effectively describe food materials, food composition, origin and /or authenticity (He, 2007; Hall, 2007; Barbaste, 2002; Liu, 2009). Literature examples using roast and ground coffee have used PLS techniques and component concentration modelling to describe chemical profiles which can be used to describe bean phenotype and genotype. During the soluble process raw ingredient subtleties such as aroma profile and caffeine content lose their resolving power due to process variability and the relative dilution of carbohydrates.

Effective rapid scanning predictive models for soluble coffee has so far not become a mainstream technique due to product and process variability, the large numbers of samples required to develop long term databases and the lack of transparency between producers.

Technical approaches for screening soluble coffee can be separated into two key fractions, the aroma fraction, often separately controlled using aroma management systems and the bulk chemistry fraction, a carbohydrate and melanoidin rich fraction that is dominated by raw material composition, thermal processing and processing yield levels.

In this proof of principle study >100 samples of varying origin, roasting parameters, and solubilisation protocols were evaluated by a range of rapid scanning techniques for their ability to provide robust datasets that could then be used to develop predictive tools for the generation of raw material descriptors and product classification.

MATERIALS AND METHODS

NIR

Full wavelength scans (400-2400 nm) of each sample (80 rep analysis on a ¼ cup powder analysis unit) was carried out on a Foss Rapid Content Analyser XDS, near infra-red, and resulting spectrums analysed via WinISI, 4.20.1.1.12480, resulting spectrum are then exported to The Unscrambler (CAMO) and predictive models generated after transformation and normalisation algorithms are applied.

Chemsensor

Freeze dried soluble coffee (5 g) is stored in a 30mL vial (Teflon septum) allowed to equilibrate for 30 min and incubated at 80 °C (20 min), 2.5 mL of headspace is removed (MPS2, Gerstel) and directly injected into a Agilent 5975C inert XL MSD triple axis detector (helium, 2.5 mL/min, split 30:1, injection temperature 280^{o}C, MSD full scan m/z 50- m/z150) the total cumulative sum of each mass unit over the sample detection time is them exported into pirouette 4.0 (Infometrics, Bothell) and predictive models generated after transformation and normalisation algorithms are applied.

RESULTS AND DISCUSSION

Bulk Chemistry

NIR, Raman and FT-IR were evaluated for their ability to produce robust datasets that reliably represented and discriminated key products across the soluble coffee landscape.

Raman and FT-IR measure samples with small sampling areas and the high levels of product inhomogeneity made the large number of replicate sample points impractical for a rapid screening method, these methods were therefore excluded from future work due to the natural product variability between individual grains of freeze-dried soluble coffee.

NIR offered a larger sampling area and the ability to measure a number of replicate points within a sample within a short period of time (1 min, 80 sampling points) this offered the greatest potential for predictive modelling, NIR cannot be easily automated and current setup allow the analysis of ~200 samples per work day per machine.

Example results

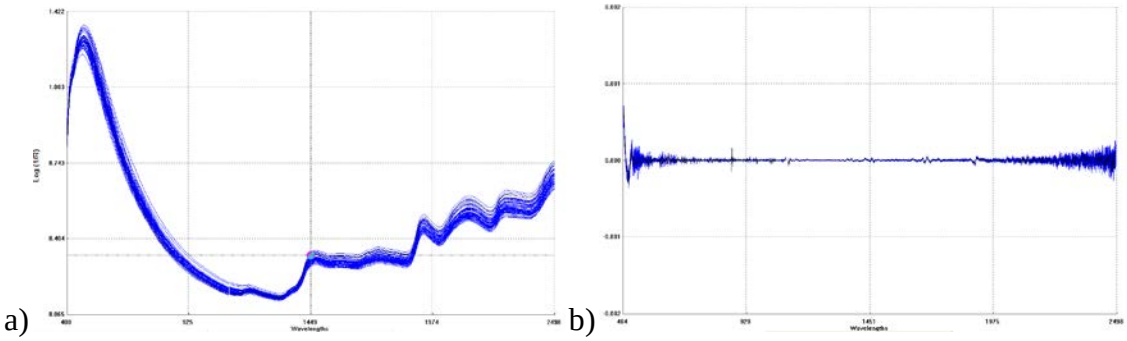

Figure 1. a) Spectral absorbance plot of exemplar sample coffees, and b) resultant trace after spectral transformation and normalisation.

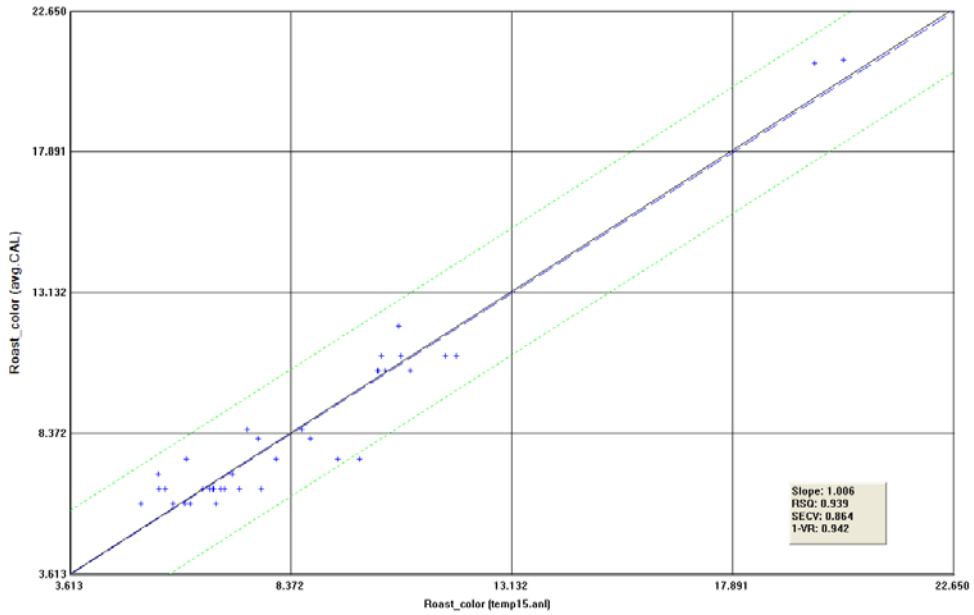

Figure 2. Predictive models generated from NIR traces to predict roast colour of roast coffee raw material.

Effective and reproducible predictive models can be generated that calculate raw material descriptors (see Figure 2 for roast colour). These models are generated on two sample sets, firstly an independent prediction set and secondly an independent validation set (shown in Figure 2).

Aroma Chemistry

Chemsensor was the method of choice (directly compared to traditional GC-MS brew aroma composition) for rapid scanning evaluation of soluble coffee aroma profile.

Chemsensor operates rapid direct injection of the fixed aroma into the MS allowing the production of a mass frequency plot (relative abundance of each mass unit). Testing takes 3 minutes per sample and can be fully automated allowing the analysis of 480 samples per 24 hour period, per analytical machine.

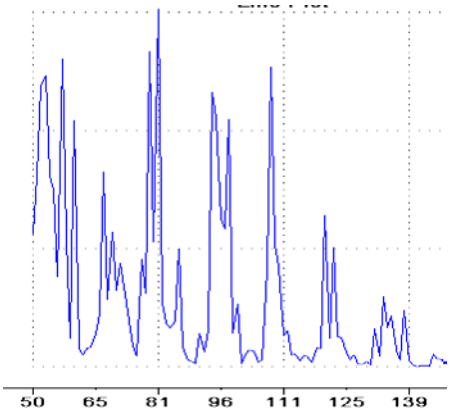

Figure 3. Mass frequency plot of m/z of 50-150 for soluble coffee.

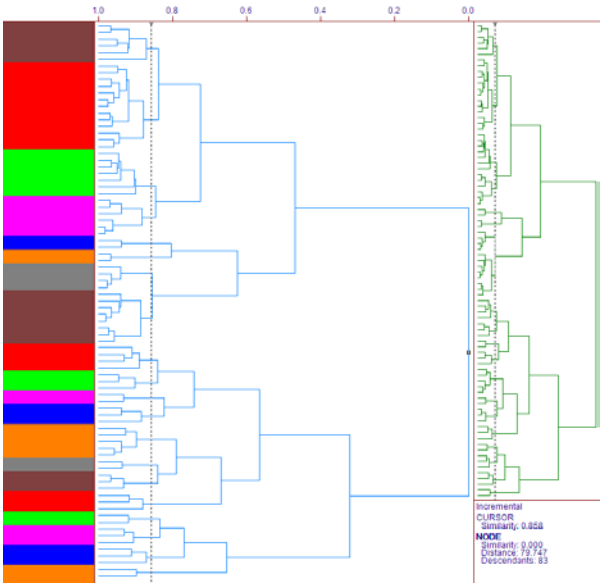

Figure 4. Hierarchical cluster analysis after normalisation and transformation (sample descriptors have been removed for confidentiality reasons).

Effective and robust hierarchical cluster analysis and multi-dimensional principle component analysis based on aroma chemistry can be used in conjunction with outputs from the predictive models generated through the NIR technique, when used in conjunction they offer a significant tool to understanding changes in product and process within supplier chains and over time to evaluate and track product quality and enable product optimisation through six sigma or comparable process evaluation tools

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Contents of Chlorogenic Acids, Caffeine Trigoneline and Sucrose in Brazilian Defective Seeds

A.T. TOCI[1] AND A. FARAH[1,2]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[2]Instituto de Nutrição, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.
E-mail: afarah@iq.ufrj.br

SUMMARY

In this study, we evaluated the contents of caffeine, trigonelline, chlorogenic acids and sucrose in Brazilian defective and healthy coffee seeds. Our results indicate that the contents of caffeine and trigonelline in defective beans were associated to the degree of maturation of the fruits, while the contents of chlorogenic acids and sucrose seemed to be also influenced by other factors such as inappropriate storage.

INTRODUCTION

Although Brazil is the first world coffee producer and exporter, the harvesting methods practiced in the country generate a considerable amount of defective seeds. The major defective seeds generated by harvesting of fruits at different maturation stages or by the action of microorganisms and oxidation are black, immature and sour seeds. These seeds are inappropriate for exportation and after sorting they are usually sold for lower prices to the Brazilian coffee industry to be included in commercial blends. Because defective seeds may dramatically decrease cup quality and possibly cause health implications, especially in the case of sour and black seeds, there is a general concern about the amount of these seeds used in the commercial blends. Therefore, there has been a growing effort aiming to characterize the chemical composition of such seeds in order to identify large amounts of them in coffee blends - especially in the case of marker compounds (Toci and Farah, 2008), or to create other purposes for them. However, to date, only a few studies evaluating the chemical composition of Brazilian defective seeds have been performed.

Therefore the aim of this study was to evaluate the contents of caffeine, trigonelline, chlorogenic acids (CGA) and sucrose in Brazilian defective beans, comparing them with those from the respective healthy seeds.

MATERIAL AND METHODS

Samples

Three groups of raw immature, sour, black and healthy (control) coffee seeds were harvested in different farms of Guaxupé, (South of Minas Gerais, Brazil) and kindly supplied by COOXUPE. Groups 1 and 2 contained one control and the respective black, immature and sour defective seeds. The third group contained one control for each type of defect. Samples were roasted in a spouted bed roaster (IRoast, Gurnee, IL, USA) at 210 °C, for 6 min, to give a light-medium degree and for 15 min, to give a dark degree, according to the Roast color Classification System (AGTRON – SCAA, USA, 1995).

Analyses

CGA (caffeoylquinic acids-CQA and feruloylquinic acids-FQA), caffeine, trigonelline, and sucrose, were determined in raw and roasted seeds, according to Farah et al.(2006) and Toci et al. (2006). Results were treated by factorial ANOVA analysis and Fisher LSD tests, being considered significant when $p \leq 0,05$ (Statistica®, version 8.0, USA).

RESULTS AND DISCUSSION

The contents of CGA in the raw control samples ranged from 4.34 to 6.42 g/100g, in agreement with the literature (Toci and Farah, 2008; Farah et al., 2006; Toci et al., 2006; Farah et al., , 2009). Regarding the defective seeds, as previously observed by Farah et al. (2009), CGA contents in immature (3.52 to 5.48 g/ 100 g) and sour seeds (2.70 to 5.25 g/100 g) were higher than those in black seeds (0.27 to 2.50 g/100 g). According to Farah et al (2009) and Farah and Donangelo (2006) the content of CGA tend to gradually decrease as the coffee fruits ripen. In the present study, despite the lower levels observed in black seeds as expected, the immature seeds showed lower contents than the control seeds which were expected to be in an advanced stage of maturation and therefore, to contain less CGA than the immature seeds. This reveals an additional influence of factors other than maturation degree on the contents of CGA in defective beans as, for example, inappropriate storage conditions of seeds. It is noteworthy to comment that immature seeds may also be at different maturation stages, and therefore present different colours, ranging from dark to light green, as the fruits ripen. In conformity to this, raw immature seeds in groups 2 and 3 presented a dark green colour and higher CGA content (4.64 and 5.48 g/100 g, respectively), compared to seeds from group 1 which were lighter and presented lower CGA content (3.52 g/100 g).

The present results as well as previous results from the same group (Toci and Farah, 2008) indicate that total CGA is related to enzymatic browning, which may be identified by the brown colour and or presence of small black dots in the seeds. The low content of CGA observed in the raw black defective seeds is probably related to natural enzymatic browning caused by the action of polyphenol oxidases and peroxidases that are naturally activated by cell damage and ageing (Farah et al., 2006; Farah and Donangelo, 2006; Eskin, 1990). Additionally, the action of microorganisms can also activate enzymes and promote browning (Garay et al., 1987; Dentan, 1987). In the present study, it was also observed that, the raw sour seeds from groups 1 and 2, that showed signs of microorganisms contamination and enzymatic browning (black dots), contained lower levels of CGA (3.02 and 4.61 g/100 g, respectively) comparing to group 3 (5.25 g/100 g). Microbiological analyses should be conducted in order to confirm the hypothesis of seeds deterioration due to microorganisms growth.

As well known and expected, roasting caused a decrease in CGA content. In the light-medium roasting degree, the percent loss in control seeds ranged from 73 to 85%, with average of (80%), while losses in immature seeds ranged from 57 to 62% with average of 59% and in black seeds ranged from 11 to 49% with average losses of 35%. The sour defective seeds showed the highest variability in CGA loss among the samples (from 11 to 78%), with average of 49%.

Such large differences in CGA loss observed among the types of defective seeds are most probably caused by differences in the cell wall structure, as previously suggested (Dentan, 1987). In the dark roasting degree, total losses of CGA were similar in all samples, averaging 93% (Table 1).

Table 1. Contents in g/100 g of chlorogenic acids (CGA), trigonelline, sucrose and caffeine from raw and roast healthy and defective coffee seeds roasted to light-medium and dark color degrees.

Analytes	Roast degree	Group 1								Group 2								Group 3											
		Control		Black		Immature		Sour		Control		Black		Immature		Sour		Control A		Black A		Control B		Immature B		Control C		Sour C	
CGA	raw	4,88	a	0,48	b1	3,52	c2	3,02	c2	4,34	a	0,27	b1	4,64	a2	4,61	a2	6,42	a	2,50	b1	5,97	a	5,48	c2	6,28	a	5,25	a2
	medium	0,98	a	0,26	b1	1,49	a2	2,70	c2	1,03	a	0,24	a1	1,97	a2	1,89	a2	1,42	a	1,27	a1	0,84	a	2,05	a1	1,26	a	1,16	a1
	dark	0,13	a	0,04	b1	0,16	a1	0,18	a2	0,14	a	0,03	a1	0,08	a1	0,08	a2	0,14	a	0,26	b1	0,05	a	0,11	a2	0,07	a	0,05	a3
Trigone lline	raw	1,23	a	0,35	b1	1,00	a2	1,17	a3	1,12	a	0,30	b1	1,04	a2	0,43	b1	1,05	a	0,62	b1	0,96	a	1,15	a2	1,02	a	0,82	c3
	medium	0,57	a	0,31	b1	0,67	a2	0,47	a2	0,41	a	0,13	b1	0,25	c2	0,22	b1	0,30	a	0,27	a1	0,19	a	0,38	a1	0,26	a	0,22	a1
	dark	0,04	a	0,03	a1	0,03	a1	0,06	a1	0,03	a	0,01	a1	0,05	a2	0,02	a1	0,08	a	0,05	a1	0,04	a	0,07	a1	0,05	a	0,04	a1
Sucrose	raw	6,40	a	1,99	b1	7,00	a2	1,74	b1	6,48	a	0,36	b1	4,52	a2	6,53	a2	6,61	a	0,58	b1	7,16	a	3,75	c2	6,02	a	4,31	c2
	medium	nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd	
	dark	nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd		nd	
Caffeine	raw	1,04	a	0,63	a1	0,94	a1	1,03	a2	1,20	a	0,42	b1	1,38	a2	0,85	a2	1,04	a	0,94	a1	0,96	a	1,20	a1	1,08	a	0,94	a1
	medium	0,92	a	0,63	a1	1,31	a1	1,39	a1	1,22	a	0,42	b1	1,40	b1	1,18	b1	1,16	a	1,07	a1	1,11	a	1,35	a1	1,12	a	1,10	a1
	dark	1,42	a	0,63	a1	1,12	a1	1,29	a1	1,13	a	0,42	a1	1,29	a1	0,84	a1	0,95	a	1,20	b1	1,04	a	1,10	a1	0,91	a	0,84	a2

a, b, c: Statistical difference between control and defects in the same group. 1, 2, 3: Statistical difference between defects in the sama group.

The caffeine contents observed in the raw control samples (0.96 to 1.20 g/100g) are in agreement with the literature (Toci and Farah, 2008; Farah er al., 2006; Clarke and Macrae, 1985). The highest caffeine contents were observed in immature and control seeds (0.94 to 1.38 g/100 g). These contents were followed by those from sour seeds (0.85 to 1.03 g/100 g) and black seeds (0.42 to 0.94 g/100 g). The lower contents of caffeine found in black and sour seeds are in agreement with Farah (2004).

Caffeine is heat stable, and the differences in composition observed in the roasted samples result basically from the degradation of other components from the matrix, in addition to a small loss of the compound itself by sublimation. This loss may be observed in a few roasted samples (Table 1). The black seeds showed the lowest loss of caffeine. This supports the hypothesis of difference in the cell wall among defective seeds.

Trigonelline contents in the raw control seeds ranged from 0.96 to 1.23 g/100 g (Table 1), which is in agreement with the literature (Toci et al., 2006; Clarke and Macrae, 1985). The black seeds showed the lowest levels of trigonelline (0.30 to 0.62 g/100 g), as observed with CGA contents. These values are in agreement with studies evaluating defective seeds (Toci and Farah, 2008; Mazzafera, 1999). In the immature seeds, the contents of trigonelline (1.00 to 1.15 g/100 g) were not statistically different from those in controls (0.96 to 1.23 g/100 g) (Table 1).

Losses in trigonelline content during roasting were quite variable. In the light-medium degree, the lowest losses were observed in black seeds (10-12%), while in the controls seeds losses ranged from 26-61%. In the dark roasting seeds, the losses ranged from 73-96% (Table 1).

The sucrose contents in the raw control seeds (6.02 to 7.16 g/100g) are in agreement with the literature (Toci et al., 2006; Toci and Farah, 2008) (Table 1). Once again, the lowest contents were found in the black seeds (0.36 to 1.99 g/100g), followed by sour (0.43 to 1.17 g/100 g) and immature (3.75 to 7.00 g/100 g). Mazzafera (1999) also found lower sucrose contents in immature seeds compared to controls. With the methodology applied in the present work, sucrose was not detected in samples roasted.

CONCLUSION

All analytes showed a relationship with the degree of maturation of the fruit, where the highest contents were found in the controls and immature seeds, followed by sour and finally black seeds. However, the levels of trigonelline and chlorogenic acid, in addition to being influenced by fruit maturation, appear to be also influenced by also other factors such as inappropriate storage.

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Climate Change, Harvesting Systems and Sustainability

C.H.J. BRANDO

P&A Marketing International

SUMMARY

Climate irregularities in coffee producing areas are a reality now. Irregular rainfall patterns affect uniformity of flowering, cherry maturation, harvesting and its costs, especially if selective harvesting remains as the preferred technique for Arabica coffees. Selective coffee harvesting works against economic sustainability and perpetuates poverty in coffee growing areas. Coffee pickers will be progressively less able to pay for their basic needs, let alone satisfy their aspirations if they remain in the countryside, with adverse impacts on their economic sustainability.

MANUSCRIPT

Whereas the debate about global warming will only be resolved in decades, when reliable long-term data will be available, the fact that rainfall in coffee producing areas is becoming more erratic is a reality now. Irregular rainfall patterns affect the uniformity of flowering, coffee cherry maturation, the way harvesting is performed and its costs, especially if selective harvesting remains as the preferred technique for Arabica coffees.

The objectives of harvesting include the collection of all ripe cherries available (in order to maximize quality), with as few unripe, partially ripe and over-ripe cherries as possible. Traditionally this has been done with the use of selective harvesting.

Nonetheless, it is interesting to note that poor selective harvesting is becoming standard in many coffee producing countries around the world. The percentage of unripe cherries is growing markedly due not only to climate changes, but also as a result of scarcity of labor and escalating production costs. It is already true that in some places between 5 and 15% of the harvested cherries are unripe i.e., green cherries. This phenomenon generates the need to sort out cherries after harvesting, something that was not a main concern for growers in the past. Cherries have to be either manually or mechanically sorted to avoid cherries with different degrees of ripeness to be processed together. If cherries with different degrees of ripeness are indeed processed together, serious quality losses can take place, with the quality of the resulting coffee certainly being affected.

Reality is showing that the use of “pure” selective harvesting in coffee is no longer doable. To be sustainable, harvesting has to be done at a competitive cost, which is not the case of selective harvesting anymore. Selective coffee harvesting works against economic sustainability and perpetuates poverty in coffee growing areas.

The equation is simple. A person can only selectively pick a given weight of coffee per day. As a result pickers' real salary, discounted for inflation, will remain basically the same indefinitely. But development raises the cost of living and aspirations all the time. In addition salaries in rural areas do not increase as fast as in cities. The outcome is that coffee pickers

will be progressively less able to pay for their basic needs, let alone satisfy their growing aspirations if they remain in the countryside, with adverse impacts on their economic sustainability.

Is there a way out? Fortunately yes, with technology and techniques already available. However their implementation is usually hindered by tradition, misconceptions about quality losses, and other arguments that do not resist a sound technical and economic analysis.

First, selective picking is not the only way to obtain high quality coffee. Top quality coffee derives from fully ripe cherries irrespectively of the way they are obtained: hand picked selectively or as part of a mixture of cherries to be separated later. A lot of what is today referred to as "selective" hand picking is not so because the mixed product that is picked requires manual sorting.

Second, coffee volumes picked by hand may be increased by 2 to 5 to 20 times using available techniques applicable to any terrain and variety, shaded or not, to most if not all conditions. If manual stripping is employed, which is already the case of natural Arabicas and most Robustas, volumes picked can be increased up to 5 times; stripping with hand held harvesters can increase volumes picked by 20 times; whereas mechanical harvesting with self-propelled machines, which has been successfully done in some areas of Brasil, may increase volumes picked by 500 times! The degree of selectivity with either method will not be much worse than what is observed today.

Third, mechanical handling and harvesting of coffee have evolved greatly in recent years. They can now cope with steep slopes, narrow spaces between coffee trees, all Arabica varieties, and shaded coffee. The selectivity of the equipment is increasing and its costs are falling; hand-held harvesters may now be owned by the pickers themselves.

Fourth, modern post-harvesting processing machinery can sort not only unripe cherries, identified visually, but also not-fully-ripe cherries that cannot be separated by hand in response to visual inspection. Even with perfect selective hand harvesting, modern wet milling equipment can ensure higher coffee quality because it discards cherries that are only partially ripe and cause astringency in the cup.

One concern is that if selectivity falls, less quality coffee will be produced. This can be solved by producing more coffee altogether. More coffee has to be grown to satisfy quality markets; additionally, more markets have to be created for the lower quality coffees produced.

Another concern regards the fear that mechanical sorting of cherries may cause indiscriminate increase in the percentage of unwanted cherries that are harvested. This may be avoided by using incentive schemes for pickers to harvest as many ripe cherries as possible.

Still another concern is that what is proposed above will create unemployment in coffee areas. Shortages of labor to pick coffee already seem to affect many coffee producing countries, including Brazil, Mexico, Colombia and even populous India. It is vital that jobs for the displaced pickers are created in rural communities and towns in order to avoid their migration to capital cities and regional centers where most of them are obliged to live in peripheral areas and/or slums, adding to the numbers of poverty, violence and drugs. It is also important to have capacity building programs directed at this type of worker, so ex-pickers can eventually find adequate jobs. With this proposal, labor that remains in coffee will have higher incomes

and offer better opportunities to their children. Others who leave will seek better wages than they had before, with or without government help.

Overall, the likelihood is that all will be better off — growers and labor — if proper conditions are created to facilitate the transition from an archaic to a modern coffee harvesting system.

Coffee Processing Water Minimization Options

J.K. MBURU

Coffee Research Foundation, P.O. Box 4 – 00232 Ruiru, Kenya. Tel: +254 067 25081/2.
E-mail: mburu.karanja@crf.co.ke. OR josephkaranja.m@gmail.com

SUMMARY

The environmental pollution by the coffee processing effluent in Kenya, can be alleviated by a concerted application of processing water minimization and treatment options. Currently, the only water minimization attributes are featured mainly where the circulation of pulping, pre-grading and the corresponding transport water is practiced. The overall water consumption in such a system can be 3.56 l/kg cherry processed, though it is generally higher and variable than this due to other inherent factors among which the attitude of the operators impacts prominently. For this reason, the operators need to appreciate the importance of such an endeavour instead of resorting to it only after a pollution crisis has manifested itself in with respect to the ecosystems proximate to the respective primary coffee processing plant. Besides, further gains can also be achieved by strictly processing coffee with the specified amount of water. However, the most recent and more viable complementary intervention has been identified in the recycling of the final grading water for pulping, pre-grading, transport and intermediate washing operations. However, being a new front in this regard, studies were set up to establish the effect of such a processing regime on coffee quality.

The results showed that not only did recycling of grading water contribute appreciably in reducing the processing water use to 1.87 l/kg of cherry but improved the overall coffee quality as well. This was more so for the water sourced from the initial stages of final grading. Other possibilities reportedly bearing potential for the same purpose like use of rotary grading sieves, pulp screw conveyance press, mechanical mucilage removal (identified with a water use of 0.5lt/kg cherry), reduced cross sections of the channel for transporting coffee and use of centrifugal pumps to wash and transport coffee among others are also discussed in comparison with this new dimension.

INTRODUCTION

The wet coffee factories can erratically and enormously pollute their corresponding processing water supply (rivers) to the extent of contaminating the quality of coffee. As such, the subject of processing water minimization dates back to the 1952 when primary coffee processing factories used to be permitted to abstract the equivalent of $91m^3$ of water/ton of clean coffee and return 80% of this to the river in a clean state (Holme, 1961). Later on however, it became necessary to identify other complementary measures for reducing water usage in coffee processing operations to $22.5m^3$ per ton of clean coffee and retain all of it on land (Aagaard, 1961). For that purpose, the option of water re-circulation was perceived as a viable intervention because it was not objectionable to separate the coffee bean with water containing the same by-products freshly removed from the beans. In pursuance to that, water re-circulation coffee factories (Aagaard, 1961; Holme, 1961) were designed from a water saving point of view among other factors. The new designs were also facilitated by the intention to use pumps, which was a prior idea muted and considered an economic

proposition (Holme, 1961) because with small capital expenditure, it was possible, by means of a suitable re-circulation system to, actually reduce the processing water requirements as required. In a nutshell therefore, the revised system involved re-circulation of pulping, pre-grading, intra-transport, intermediate washing, final washing and final grading water (Wallis, 1968) as well as rapid stirring and agitation of parchment (Holme, 1961) to remove mucilage and conveyance of coffee from grading channel by a pump to the drying tables all of which had a net water use of 12.32 m^3.

In view of that, the current water abstraction limits would suffice greatly although the actual usage in practice has been quoted to generally vary from 59-105 m^3 (Mburu et al., 1994) and 24-114 m^3 (Finney, 1990) per ton of dry parchment depending on the equipment used, degree of water re-circulation during processing, factory layout, efficiency of the operation and mode of supply cum availability of water to the factory (Mburu et al., 1994; Zuluanga, 1987). This concern was addressed in a recent study by Wood et al. (2000) who consequently recommended the recycling of the final grading water to the pulping and intermediate washing of coffee in order to effectively reduce processing water even further. Incidentally, the final grading and soaking of parchment account for the largest amount of processing water (1644 m^3/tone clean coffee) used. Therefore, to achieve such an impressive water use reduction, water movement within the system; needs to be modified only slightly to re-circulation flow instead of the linear flow of the traditional process. However, it was deemed necessary, to assess the coffee quality implications arising from the adoption of such a practice in Kenya first to pave way for the proposed system. The ensuing experiments were therefore intended to verify the suitability of recycling the final grading water as such without affecting the quality of coffee.

However, while alternative water minimization options were being sought for within the current coffee processing system, the aspect of mechanical mucilage removal plus adoption of rotary grading sieve and coffee pulp screw conveyance press were gradually gaining recognition as its potential substitute. For instance, recent studies conducted in Rwanda comparing different coffee processing systems merited these newly introduced processing machines equipped with mechanical mucilage removers over the traditional disc system with respect to their minimal water requirement, shorter processing time, elimination of the capital intensive fermentation as well as soaking tanks and grading channels. Besides that, it was also confirmed that the new systems could produce coffee whose quality attributes was comparable to that from the disc system if not better (Anon., 2008)). It was therefore deemed necessary to consider the applicability of these machines towards easing off the prevail coffee processing constraints. This aspect is also discussed in this paper in relation to the predominantly used disc coffee pulping systems in Kenya.

MATERIALS AND METHODS

Experiment trials were set up at the central and regional coffee research foundation stations (Table 1) from 2005 to 2008.

First, it was ensured that, coffee was processed using the final grading ***water, which had not*** been re-circulated during the grading process. In preparation for the trials, adequate water was collected when the parchment (P) grades (i.e. P3/Pl, P2 and P1) were being discharged during the final grading of parchment coffee. It was preserved under suitable conditions to pulp coffee with it in the afternoon. After the coffee harvest had been sorted in a coffee factory, 422 kg cherry was weighed from which handfuls of cherry were randomly drawn from different locations of the coffee cherry mass into 2 separate bags to make up 1 kg each.

These samples were transferred to the laboratory in perforated clear polythene papers for further analysis. The remaining 420 kg cherry was divided into 3*120 kg and a 60 kg lot which were pulped separately with different types of water (Table 2). Table 2 further shows how the samples were subdivided and the corresponding prescribed treatments with the final grading water. After pulping, every subsample was then committed to the subsequent dry fermentation in separate plastic basins.

Table 1. Experimental sites.

CRF stations	Latitude	Longitude	Elevation (m)
Mariene	0° 00’	37° 39’E	1631
Kisii	0° 41’S	34° 47’E	1700
Koru	0° 07’S	35° 16’E	1554
Ruiru	1° 05’S	36° 54’E	1623
Kitale	0° 59’E	35° 01’E	1982

Table 2. Experimental design.

Initial (kg)	Subdivided lots (kg)	Exp units (kg*Reps)	Processing water	Pulping	1st wash	Final washing and grading
420	60	20*3	F_{rw}	F_{rw}	F_{rw}	F_{rw}, All the samples
	120	20*3	G_{P3}/G_{Plw}	G_{P3}/P_{lw}	Frw	
		20*3			$G_{P3}/_{Plw}$	
	120	20*3	G_{P2w}	G_{P2w}	F_{rw}	
		20*3			G_{P2w}	
	120	20*3	G_{P1w}	G_{P1w}	F_{rw}	
		20*3			G_{P1w}	

Key

G_i - Water samples sourced during the discharge of different parchment grades (P_i) from the final grading channel respectively where i =1 – 3,
G_iP – Coffee samples only pulped with Pi water where i = 1,2 and 3,
G_iPW – Coffee samples pulped and intermediate washed with Pi water where i = 1, 2 and 3,
FrPW – Coffee samples processed fully with fresh water only (Control),
Reps – Replicates,

On the 2nd day fresh grading water was drawn as on the previous day. Three (3) subsamples pulped with P_1 water the previous day were for instance intermediate washed with the same type of water and the other 3 with fresh water. This was repeated accordingly for the other subsamples pulped with the corresponding grading water. The subsamples pulped with fresh water were washed with the same type of water. Any defects were then sorted out from all the subsamples and dry fermentation continued for another night.

Come the 3rd day, all the samples were washed with fresh water after which the samples were committed to drying in separate coffee trays. Drying then continued as normally practiced up to fully dry status. The experimental trials were repeated 2 times per year.

RESULTS AND DISCUSSION

The results from the water minimization trails in Kisii (Table 3) show that the Colour of the processed parchment did not change with the type of water used for pulping and washing. However, only F_rPW produced significantly better raw quality over G_1P while the other pulping washing combinations yielded similar quality outputs. On the other hand, F_rPW produced acid score/rating distinctly inferior to G_2P and G_2PW (i.e. both grade 2 water treatments). The other pulping, washing combinations with grading water produced similar acidity levels to F_1Pw. As for the body, F_rPW yielded a higher status over all the other pulping and washing combinations except G_3P that gave similar body rating. Otherwise, in terms of overall quality, F_rPW and G_3P showed superiority over all the other combinations responsible for parchment pulped and washed with grading water.

Table 3. Quality of coffee pulped and washed with grading water at Kisii.

Treatment	Reps	Colour (Raw)	Quality (Raw)	Acidity	Body	Flavour	Overall Quality
G_1P	3	2.56a	4.33a	1.56ab	1.56a	4.33a	4.47a
G_2P	3	2.28a	4.11ab	1.89a	1.78a	4.22ab	4.49a
G_3P	3	2.22a	4.00ab	1.44ab	1.44ab	4.11ab	4.31bc
G_1PW	3	2.61a	4.22ab	1.56ab	1.56a	4.22ab	4.36ab
G_2PW	3	2.17a	4.11ab	1.78a	1.56a	4.22ab	4.49a
G_3PW	3	2.11a	4.00ab	1.56ab	1.67a	4.22ab	4.36ab
F_rPW	3	2.00a	3.78b	1.11b	1.00b	4.00b	4.20c

Notes: 1. Means within a column not sharing a letter are significantly different at $P \leq 0.05$. 2. The small the figure, the better the quality attributes in Tables 1-3.

The results from the trials in Koru (Table 4) indicated that the raw colour of the beans subjected to G_2PW was superior over all other combinations while F_rPW turned out the weakest colour value. The other combinations showed no significant differences among them. However, the control F_rPW produced poorer raw quality over all other combinations while the same treatment yielded poorer acidity and body than G_1P but was comparatively similar to the other combinations. Pulping and washing coffee with grade 1 water (G_1PW) realized superior flavour over all the other combinations. Finally, the control or F_rPW yielded significantly poorer quality than G_1P and G_1PW. All other combinations gave similar quality to F_1PW

Table 4. Quality of coffee pulped and washed with grading water at Koru.

Treatment	Reps	Colour (Raw)	Quality (Raw)	Acidity	Body	Flavour	Overall Quality
G_1 P	3	2.00b	4.00b	1.33b	1.11b	4.11ab	4.20b
G_2 P	3	2.00b	4.00b	1.56ab	1.56ab	4.00b	4.27ab
G_3 P	3	2.00b	4.00b	1.56ab	1.56ab	4.00b	4.36ab
G_1 PW	3	1.78bc	4.00b	1.33ab	1.44ab	3.67c	4.16b
G_2 PW	3	1.67c	4.11ab	1.56ab	1.67ab	4.11ab	4.33ab
G_3PW	3	2.00b	4.11ab	1.78ab	1.67ab	4.11ab	4.38ab
F_rPW	3	2.33a	4.22a	2.00a	2.00a	4.33a	4.47a

Means within a column not sharing a letter are significantly different at P≤0.05.

Table 5. Quality of coffee pulped and washed with washing/grading/soaking water at Kitale.

Treatment	Reps	Colour (Raw)	Quality (Raw)	Acidity	Body	Flavour	Overall Quality
GP	3	1.67c	4.11ab	1.56ab	1.67ab	4.11ab	4.33ab
SP	3	2.00b	4.00b	1.56ab	1.56ab	4.00b	4.36ab
I_1P	3	2.00b	4.00b	1.33b	1.11b	4.11ab	4.20b
GPW	3	2.00b	4.11ab	1.78ab	1.67ab	4.11ab	4.38ab
SPW	3	1.78bc	4.00b	1.33ab	1.44ab	3.67c	4.16b
I_1PW	3	2.00b	4.00b	1.56ab	1.56ab	4.00b	4.27ab
F_rPW	3	2.33a	4.22a	2.00a	2.00a	4.33a	4.47a

Means within a column not sharing a letter are significantly different at P≤0.05.

Key

I_1 – First wash water equivalent to water from intermediate washing,
S - Soaking water,
G – Grading water
I_1P – Samples pulped with first wash water
I_1PW - Samples pulped and intermediate washed with first wash water
SP – Samples pulped with soaking water
SPW – Samples pulped and intermediate washed with soaking water
GP – samples pulped with grading water
GPW – samples Pulped and intermediate washed with grading water

At the Kitale site (Table 5) where coffee was pulped and intermediate washed with Intermediate washing water i.e. first washinmg water (I_1), grading (G) and soaking (S) water, the best raw colour value was realized by GP which was closely followed by SPW while F_rPW produced the poorest raw colour. F_1PW yielded the worst raw quality closely followed by GPW and GP. However, better raw quality was realized by SP, I_1P , SPW and I_1P W.

Besides that, only the I_1P produced better acidity and body values than F_rPW though similar to the other combinations. Pulping and washing with soaking water (SPW) recorded significantly better flavour while all the other treatments had significantly inferior results. Finally, I_1P and SPW gave better overall quality than F_rPW.

In other studies where the suitability of grading water for pulping and intermediate washing was assessed following the criteria shown in table 6, G_3PW and G_2P had significantly different aroma and fragrance respectively from G_1P. Actually, G_1P produced more distinctly poorer fragrance than G_3PW while G_1P had inferior aroma to G_2P. According to these results, body and acidity were not affected by the use of various processing water categories. Otherwise, significantly superior overall quality (preference) results were recorded from fresh water (F_rPW) treatments over both grade 1 pulping and intermediate washing and pulping only with grade 3 water treatments.

Table 6. Coffee quality assessment method applicable to table 7 and 8.

Rating	Fragrance	Aroma	Body	Acidity	Preference
5	Very strong	Very strong	Very strong	Very strong	Excellent
4	Strong	Strong	Strong	Strong	Very good
3	Moderately strong	Moderately strong	Moderately strong	Moderately strong	Good
2	Moderately weak	Moderately weak	Moderately weak	Moderately weak	Bad
1	Weak	Weak	Weak	Weak	Very bad
0	None	None	None	None	Undrinkable

Table 7. Quality of coffee pulped and washed with grading water at Mariene.

Treatments	Reps	Fragrance	Aroma	Body	Acidity	Preference
G_1P	3	2.83a	2.83a	3.00a	2.3333a	3.00b
G_2P	3	3.00ab	3.23b	3.00a	2.3333a	2.90ab
G_3P	3	3.00ab	3.00ab	3.00a	2.1667a	3.00b
G_1PW	3	3.00ab	3.00ab	3.03a	2.5333a	3.03b
G_2PW	3	3.00ab	2.80ab	3.00a	2.2000a	2.93ab
G_3PW	3	3.10b	3.10ab	2.93a	2.5333a	2.93ab
F_rPW	3	2.93ab	2.97ab	2.77a	2.2667a	2.43a

Means within a column not sharing a letter are significantly different at $P \leq 0.05$.

From similar studies at Rukera CRF coffee farm (Table 5, only G_1P had significantly inferior aroma than G_3P and G_3PW while G_2P turned out distinct inferior preference than all the other treatments. As for fragrance, body and acidity, no variations occurred with all the combinations of processing water use.

Table 8. Quality of coffee pulped and washed with grading water at Ruiru.

Treatments	Reps	Fragrance	Aroma	Body	Acidity	Preference
G_1P	3	2.67a	2.60a	2.83a	1.93a	2.83b
G_2P	3	2.87a	3.00ab	3.10a	2.17a	2.30a
G_3P	3	2.93a	3.13b	3.00a	2.37a	3.10b
G_1PW	3	2.93a	2.93ab	3.00a	2.37a	3.00b
G_2PW	3	3.00a	2.93ab	3.00a	2.63a	3.10b
G_3PW	3	3.00a	3.10b	2.83a	1.93a	3.03b
F_rPW	3	3.00a	3.00ab	3.10a	2.37a	3.10b

Means within a column not sharing a letter are significantly different at $P \leq 0.05$.

Generally the results verified that, grading and soaking water can be used for pulping and intermediate washing of coffee without compromising coffee quality. Another aspect observed in the field was that, use of grading water and in particular grade 3 water as such enhanced the fermentation of parchment coffee. Finally, the raw appearance of parchment coffee arising from grade 3 treatments were better than for coffee from all the other treatments.

CONCLUSION

As has been demonstrated, processing water use can be minimized from the current 22.5 m^3 to 11.7 m^3 per ton clean coffee or 1.7m^3/ton cherry (Appendix 1). However, since the available water minimization options can only be actuated indirectly and are also rather involving to control, processing water requirements still remains higher than recommended. As such, stringent implementation measures will have to be put in place to ensure that the proposed revisions in water usage succeeds.

At the same time, water resources endowment in the coffee growing regions has dwindled remarkably. In response, these areas have become sensitive to any environmental pollution threat all the more. Coffee growing has also expanded to new areas with rather limited water resources. In view of these consequences among others, the newly developed improved coffee processing concept encompassing minimal water requirements for pulping, mechanical removal of mucilage, rotary grading sieves/screens and coffee pulper conveyance via a screw press appear to offer a more reliable option with respect to minimized water requirements. These equipment would eliminate the high and erratic volume water used by the disc pulping system through mechanically removing the mucilage after rasp pulping the cherries. Furthermore, since mechanically demucilaged coffee can also be placed directly on the drying tables, the costly fermentation and washing steps are consequently not required. That implies additional savings in capital investment which other goes with fermentation tanks, water channels, and water tanks. At the moment, some of the new technologies have been installed and are already in operation in Kenya. At the same time, the trend of replacement of any of the existing disc pulping systems with the new technology wherever and whenever time is due as well as in new installations is bound to continue. All the same, it is necessary to complement their adoption by subjecting them to simultaneous technical performance appraisals under local conditions.

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Appendix 1. Coffee processing water consumption, litres per ton of coffee cherry.

Operation	Non re-circulation system	Recirculation system	Recycling grading, soaking water	
Pulping	4,000	533	533	Use recycled grading and soaking to satisfy this demand
Intermediate washing	400	400	400	
Transport from one stage to another	800	800	800	
Transport to final washing channel	533	533	533	Supply fresh water to meet this demand plus 89 litres in excess and recycle after use.
Final washing and grading	3,200	800	800	
Soaking heavy coffee	311	311	311	
Cleaning factory	222	222	222	
Total requirement	9,467	3,600	1733	Equal to requirement for pulping, Intermediate washing and transport.
Water consumption/kg cherry	9.47	3.60	1.73	

Assessing the Performance of Ecological Wet Processing Plants under Tanzanian Conditions

G.P. MARO, J.M. TERI, I.K. MUSHI, W. KIMARO

Tanzania Coffee Research Institute, P.O.Box 3004, Moshi, Tanzania
E-mail: godsteven.maro@tacri.org

SUMMARY

An assessment of different primary wet processing technologies was conducted in two TaCRI sub-stations (Lyamungu in Hai district and Mbimba in Mbozi). New ecological wet pulpers were tested against the traditional disc pulpers. The design used was RCBD with 4 replicates and 7 treatments, six of which involved the use of the ecological pulpers with varying soaking hours, and one control, using the conventional approach with the traditional disc pulper. The efficiency of the pulpers was measured in terms of cherry-parchment ratio, and the percentage of unpulped, broken, squeezed or nipped cherries. Cupping was done and the cup quality scores compared.

Cherry-parchment ratios were not significantly different at 5% level, and ranged from 4.9 to 5.4. An increase in weight of 2% or more was noted with the eco-pulpers and under-water soaking; and it was speculated that the soaking activity of water could be responsible for the change in weight. As for quality, the traditional disc pulper gave the best cup, followed by under-water soaking for 6 hours. The difference between these was not significant at 5% level, implying that the eco-pulping technology can be adopted without compromising cup quality. To get the best cup with the eco-pulpers, under-water soaking of 6 hours is recommended.

The new eco-pulpers are suitable for reducing water usage, as an adaptive strategy to climate change whereby water is becoming an increasingly scarce resource. This will be our next phase of research.

INTRODUCTION

Arabica coffee (*Coffea arabica*) requires wet processing in order to attain top quality (Smith et al., 2009; De Freitas et al., 2008; Duarte et al., 2008). Wet processing method involves removal of coffee bean mucilage (mesocarp) by either fermentation or mechanical abrasion (Strawn, 2010). Fermentation of coffee allows degradation of mucilage so that it becomes readily separable from the parchment by washing (Kulaba, 1979; Wrigley, 1988). Proper fermentation and washing are known to improve coffee quality significantly. It is however believed that fermentation leads to dry matter loss due to diffusion of water-soluble chemical components.

Kusserow (2004) describes a rather new technology called “Ecological wet processing”, developed in the 1980s. It involves the use of ecological pulping machines fitted with demucilagers, the latter removing mucilage from parchment by abrasive action (mechanical removal). Coffee fermentation is therefore avoided (Kamau, 1978). Then the coffee is soaked under water for some hours before drying. The importance of under-water soaking after

mucilage removal is that it enhances final coffee quality in terms of acidity and body of the coffee (Carvalho and Chalfoun, 1989; Sunalini, 1989).

The "Penagos" ecological processing machines (Kusserow, 2004) are said to have many attributes, including economical use of water, high pulping efficiency, shorter processing time and better cup quality resulting from minimized risks related to the fermentation process. On the other hand, farmers' adoption of any new technology goes with a bit of skepticism. They are not sure of the reality of these attributes, and whether adoption of this technology will not compromise the quality of Tanzanian coffee.

Therefore, a study was conducted to compare these two types of technologies, in terms of mechanical efficiency and change in coffee quality if any due to mechanical removal of mucilage, and to establish the optimum soaking time with the ecological pulpers.

MATERIALS AND METHODS

Two ecological wet pulpers (Penagos UCBE 500, model 181 C) were purchased and installed at Lyamungu and Mbimba stations. Seven treatments, six of which involved the use of the new pulpers, and one control, using the conventional approach with the traditional disc pulper, were replicated four times. The following are the details of the treatments.

Table 1.

Treatment	Code	Treatment Description	Type of pulper
T1	PWD	Pulp, wash and dry	Ecological
T2	PS3D	Pulp, wash, soak 3hr, wash and dry	Ecological
T3	PS6D	Pulp, wash, soak 6hr, wash and dry	Ecological
T4	PS12D	Pulp, wash, soak 12hr, wash and dry	Ecological
T5	PS18D	Pulp, wash, soak 18hr, wash and dry	Ecological
T6	PS24D	Pulp, wash, soak 24hr, wash and dry	Ecological
T7 (contr)	PFWD	Pulp, ferment 24hr, wash and dry	Disc type

For each treatment and block, 15 kg of uniformly ripe cherries (about 9,000 cherries) were used. These were weighed before pulping, after skin drying and after final drying. The efficiency of the pulpers was measured in terms of cherry-parchment ratio, bean weight after drying, cherries that escaped unpulped, and the percentage of broken, squeezed or nipped cherries.

Samples were prepared and sent for cup tasting (each location to its nearest cupper), and the cup quality scores compared. Classes were assigned for raw, roast and liquor (acidity, body and flavour) for each sample. The observed defects (including pulper nipping) were scored separately, and the values added to the average cup quality scores to get the overall quality scores. These were exposed to statistical analysis of variance of blocks and treatments under GenStat software. The best cup was provided by the treatment which recorded the lowest mean score, quality decreasing with increasing score number.

RESULTS AND DISCUSSION

Cherry parchment ratio

The ratio of cherry to parchment obtained under different processing regimes is given in Figure 1. The overall mean was 5.48:1 which agrees well with the standard cherry-parchment ratio of 5:1 (Wrigley, 1988). The lowest ratio of 4.9:1 was recorded by the eco-pulper with 6 hours soaking under water, while the highest ratio of 5.68:1 was observed in the eco-pulper without soaking. The difference, however, was not significant at 5% level.

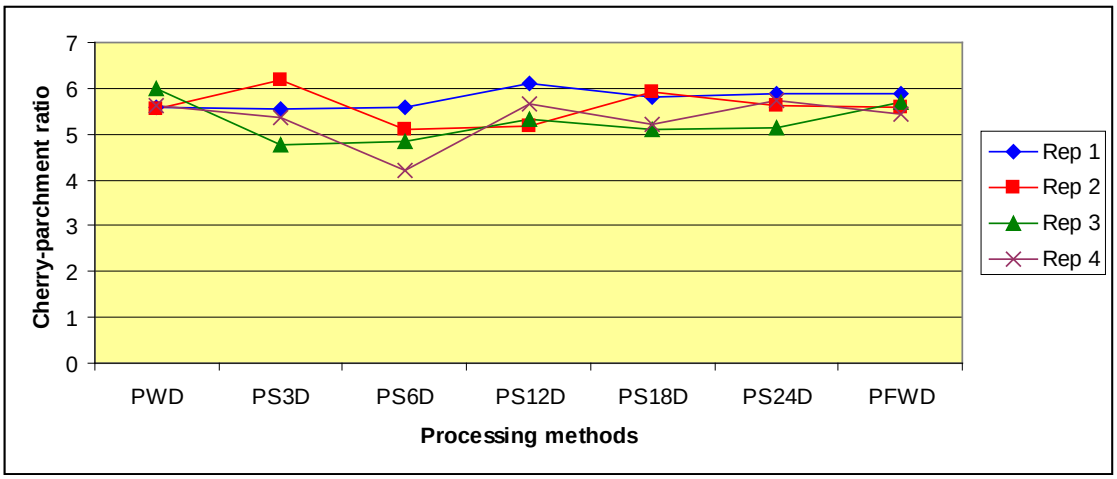

Figure 1. Variation in cherry parchment ratios from different techniques.

Using the formula ((µWp - µWd)/µWd)*100 with p and d representing Penagos and Disc pulpers respectively, the unsoaked treatment PWD had a slight loss in weight of 0.6% compared to the control. All other treatments registered an increase in weight ranging from 2.5-15% with the highest gain reached by 6 hours under water soaking. This supports the argument that the new pulpers increase the weight of dry parchment by at least 2% (Kamau, 1978). The loss in weight by the unsoaked treatment suggests that it is the soaking water that increases the weight rather than the machine. On the other hand, one would not have expected PWD and PFWD to have more or less equal weights. In this case, the only advantage of the new pulpers would be the saving of waiting time for fermentation, which normally takes 24 hours at the minimum.

Mechanical efficiency of the pulpers

The efficiency of pulpers was measured in terms of unpulped cherries and damaged ones. Unpulped cherry was observed irregularly over blocks and treatments, but was generally well under 1% with the new pulper, but went as far as 3% with the old disc pulper. This is in line with the figures provided by Kusserow (2004). On the other hand, the percentage cases of damaged/nipped beans were lowest with the disc pulper, but were inexplicably variable with the different treatments involving the eco-pulper, bearing in mind that nipping is most likely caused by the abrasive action of the demucilager, and not the soaking time. This therefore calls for further research.

Coffee quality from raw green beans to the cup

Qualities for raw green beans and roasted ones did not follow any particular trend, and this is not surprising because those are the attributes of the coffee itself and the way it was managed in the field. Raw beans were of medium and mixed sizes with greenish colour, while roasted beans were ordinary with normal to brownish centre cut. They were therefore variably classified as FAQ to FAQ minus.

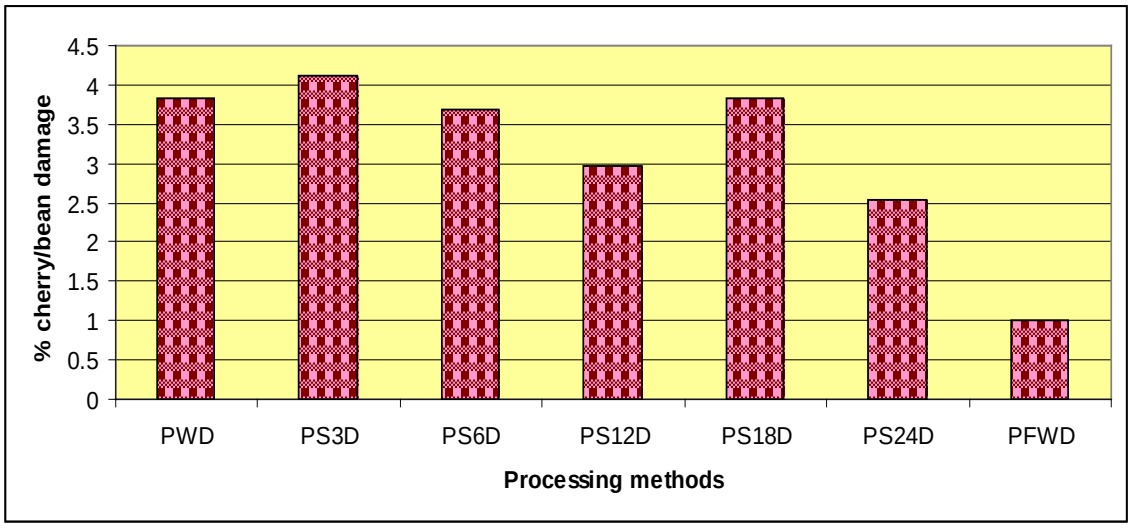

Figure 2. Variation in percent cherry damage by different techniques.

The interest narrowed down to the organoleptic characteristic of the cup. By using the Tukay's means separation technique at 5% level, data from Mbimba showed significant variation between the disc pulper and all the other treatments in terms of acidity and body, implying that thorough under-water fermentation improved the acidity and body of the cup. Flavour, on the other hand, did not show any significant variation between the control and either the unsoaked or 6 hours soaked treatments. The best cup was produced in the traditional method with a disc pulper and under water fermentation, followed by pulping with the new pulper and soaking under water for 6 hours. This suggestion is also supported by Anon (2010) who recommended that wet demucilaged coffee should be left in bulk overnight to finish up fermentation, so as to build up quality characteristics like acidity and aroma. The lack of a significant variation between the 6 hours soaking and the control suggests that the new pulpers can be adopted without seriously compromising coffee quality.

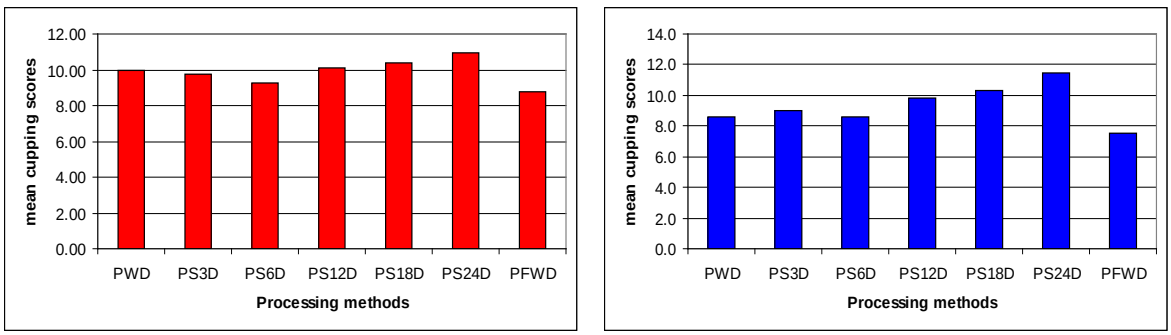

Figure 3. Comparative cup quality results Mbimba (left) and Lyamungu (right).

The results followed the same trend at Lyamungu, except that this time the unsoaked treatment PWD matched in mean scores with the 6 hours soaking PS6D. For both experiments, as the soaking time was increasing from the 6 hours the cup quality tended to deteriorate, with the worst case represented by 24 hours under water soaking. This observation seems to contrast the findings of Mburu (1997) that parchment can be soaked under water for up to 7 days without any adverse effect on the cup quality. The significant variation between PS24D and the control is difficult to explain because fermentation took the same 24 hours. There is therefore a need for further research on the role of water in fermentation versus soaking after mechanical removal of mucilage.

A similar work was conducted recently in Rwanda (SPREAD, 2009), which included more details such as processing cost attributes (time, fuel and water). In our experiment, however, such details could not be included, as the new pulpers had been installed as stand-alone experimental units. The collection of such data would require a fully operational pulper where a ton of cherry can be processed and the cost attributes recorded. It would also need specialized gadgets such as water flow meters to measure the amount of water that enters the system. Because the water footprint is becoming more and more important at consumer level (Chapagain and Hoekstra, 2007), while water itself is becoming a more and more scarce resource (Verplancke, 2003) partly due to climate change (Agrawala et al., 2003; Maro and Teri, 2008), a research investment into this is justifiable.

Importance of this study to the Tanzanian coffee industry

Tanzania is a smallholder coffee country, where smallholders contribute over 90% of the total export volumes (Carr et al., 2003, Hella et al., 2005). These are characteristically resource-poor farmers who practise subsistence agriculture, and can barely afford to own a hand pulper (Nkonoki, 2006). With the traditional backyard pulping, fermentation, washing and drying, it becomes very difficult to monitor the quality of the coffee; hence the current efforts by TaCRI and other stakeholders to mobilize growers into groups that can share a common processing facility (CPU). The idea of central pulpers was also supported by Agrisystems (1998) who cited a possible premium gain of USD 200 to 700 per ton.

TaCRI has put a lot of emphasis on coffee quality, which forms an important agenda for dissemination through its extensive coverage of substations and stakeholder links. The type of pulpers used for this work is already quite popular among coffee growers in the country. TCB statistics place the number of CPUs countrywide around 400 pieces. However, actual counts from Ruvuma region alone gave a total of 163 CPUs, out of which 14 are run by smallholder farmer groups. Extrapolating this to a total of 10 major coffee regions the number is likely to be roughly over a thousand. It is therefore clear that studies such as this will provide the farmers with the required knowledge on how to get the best out of their investment on eco-pulpers.

CONCLUSION

This study compared the performance of Penagos brand eco-pulpers under Tanzanian condition, against the traditional disc pulper. The results show that an increase in weight of 2% or more is possible with the eco-pulpers and up to 6 hours under-water soaking. As for quality, there was no significant difference between the various soaking regimes and the control. The traditional disc pulper gave the best cup, followed closely by the 6 hours soaking. This implies that the new eco-pulpers can be adopted without seriously compromising the cup

quality. A tentative recommendation to the farmer groups which have eco-pulpers is to adopt 6 hours under-water soaking after pulping.

We are planning to extend the work in the near future to cover water utilization, labour and the processing time required. Also, we have installed a much bigger eco-pulper at Maruku, Kagera, which we plan to assess for processing of "Gourmet" Robusta.

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Flavor Profiles of Robusta Coffee Processed by Wet Hulling and Pulped Natural Method

YUSIANTO, SURIP MAWARDI

Indonesian Coffee and Cocoa Research Institute, Jl. PB Sudirman no. 90, Jember, East Java, Indonesia. Email: otnaisuy@yahoo.com.

SUMMARY

Wet hulling process had been applied on Arabica coffee at Sumatra. Pulped Natural Process had been applied on Arabica at Flores. Experiment of wet hulling process and pulped natural process had been conducted at Indonesia Coffee and Cocoa Research Institute in 2009. Operating sequence of wet hulling process was coffee cherry pulping, fermentation of depulped coffee for 12 hours, washing, sun drying of coffee parchment for 4 hours, hulling of semi dry parchment, sun drying of wet green coffee until moisture content less than 12%. Procedure of pulped natural process was pulping of coffee cherry and directly sun drying of the depulped coffee. The experiment controls were full wash and natural processing. The green coffee samples were graded to get the first quality according to Indonesian National Standard before cupping. The coffee samples were cupped by some cuppers from Indonesian Coffee and Cocoa Research Institute (ICCRI). The experiment showed that the fastest sun drying is wet hulling method, 5 (five) to 8 (eight) days. Sun drying of parchment of full wash method was 10 days, parchment of pulped natural processing method was 13 days, and coffee cherry of natural processing was more than 14 days. Green coffee of natural processing is covered with brown silver skin similar to pulped natural. But, some green coffee of wet hulling method is no silver skin similar to full wash processing. Green coffee color of full wash processing is green, but that's of wet hulling is grey-bluish. The best coffee flavor was produced by wet hulling method, although similar to full wash coffee. Pulped natural has lower flavor with strong harsh and astringent. Natural processing method produced green coffee with some off-flavors; those are fruity, winy, sour, fermented, harsh, cereals, woody, and earthy.

INTRODUCTION

Processing of coffee is the method converting the raw coffee cherry to be green coffee. The cherry has the fruit or pulp removed leaving the seed or bean which is then dried. While all green coffee is processed, the method that is used varies and can have a significant effect on the flavor of roasted and brewed coffee. Coffee processing is generally divided in two method, those are Wet Process and Dry Process. But, in the coffee processing evolution, the methods are differentiated to be more then two, dependent on the coffee type/variety, equipment availability, climate, and water resources. Generally, Arabica coffee is processed by Full Wash method (dry hulling), but Robusta coffee is processed by natural method. In the wet area, natural process of Robusta coffee need very long time drying. In this condition, Robusta coffee cherry will be splitted with special machine (Kneuzer), not pulper machine. This process is very common in East Java and some district in South Sumatera. In Large estate, where pulper and water available, Robusta coffee cherry will be pulped, washed, and dried, sun drying and/or mechanical drying. In some producing country, Robusta coffee

cherry is pulped, and the parchment mucilage is removed by special machine without or minimum water (demucilager). The clean parchment is dried (Marsh et al., 2006).

Generally Arabica coffee is processed by Full Wash (dry hulling), but there re some different processing method had been applied. In Indonesia, especially North Sumatera, Aceh and Sulawesi, Arabica coffee is processed by Wet Hulling (Full wash) method or semi dry. Semi dry is a hybrid process used in Indonesia and Brazil. In Indonesia, the process is also called "wet hulled", "semi-washed" or "Giling Basah". Literally translated from Indonesian, Giling Basah means "Wet grinding" (SCAA., 2008). Wet hulling could be called full-washed, wet hulled. It is essentially the same as full-washed coffee, with one extra critical step of wet-hulling. The wet hulling process is pulping coffee cherry, parchment fermentation 12-24 hours, washing, and sun drying 1-2 days. After horn skin of the parchment had been dried, the parchment is hulled to get Wet Green Coffee (***Kopi Labu***) with moisture content 30-40% (Marsh, 2009). This process is to speed up drying, because the heavy rainfall in North Sumatera, Aceh and South Sulawesi. Thus, the initially concept of wet-hulling was purely a physical process to speed drying. No one had an idea of the flavor and character ramifications of removing the parchment coating from a wet coffee bean (Marsh, 2009). The Wet Hulling (Full Wash) method produces coffee with special flavors, which is not found by other process method. In Flores, low rainfall and water shortage, Arabica coffee cherry is pulped and dried directly, without fermentation and washing (Pulped Natural/Descacado). If clean and good condition, this process may be produce special flavor, those are caramels, honey or dark chocolate flavors. The methods, Wet Hulling (Full Wash) and Pulped Natural, is not yet applied in Robusta coffee. This experiment is conducted to evaluate Wet hulling (full wash) and pulped natural on kopi Robusta, especially the influence on flavor profile. The Experiment had been conducted at Indonesia Coffee and Cocoa Research Institute in 2009.

MATERIALS AND METHOD

A material of the experiment is fresh red Robusta cherry from experiment garden of Indonesian Coffee and Cocoa Research Institute. The experiment design is randomized complete bloc by 3 replications. The used treatments are P0 (Natural Process), P1 (Full washed dry hulling), P2 (Full washed Wet Hulling) and P3 (Descacado Process or Pulped Natural Process). P0 (Natural Process), fresh coffee cherry is directly sun dried on the plastic mat. P1 (Full Washed Dry Hulling), fresh coffee cherry is pulped, fermented over night in plastic sack, washed, and dried on plastic mat. P2 (Full Washed Wet Hulling), fresh coffee cherry is pulped, fermented over night in plastic sack, washed, and dried on plastic mat for 1-2 days. The semi dried parchment was hulled to get "Wet green coffee". The "wet green coffee' is sundried on the plastic mat until the moisture content < 12%. P3 (Descacado Or Pulp Natural)), fresh coffee cherry is pulped, and dried on plastic mat. The experiment controls were P1 (full wash dry hulling) and P0 (natural processing). The green coffee samples were graded to get the first quality according to Indonesian National Standard before cupping. The green coffee was roasted in medium level for cupping test by some cuppers from Indonesian Coffee and Cocoa Research Institute (ICCRI).

RESULT AND DISCUSSION

Drying Profile

The experiment showed that the fastest sun drying is wet hulling method, 5 (five) to 8 (eight) days. Sun drying of parchment of full wash method was 10 days, parchment of pulped natural processing method was 13 days, and coffee cherry of natural processing was more than 14

days (Figure 1). Treatment of P2 (Full Washed Wet Hulling) produce"naked wet green coffee" in 2-3 days after washing. Wet green cofee is drying faster than parchment or cherry, because of no skin resistance to get energi for evaporation and no skin resistance to evaporate mosture. The naked green coffee had been moisture content less than 12% in 5-8 days sundrying period. In Arabica, the parchment removed, the typical 12 day drying process can be reduced to 3 to 4 days (Marsh, 2009).

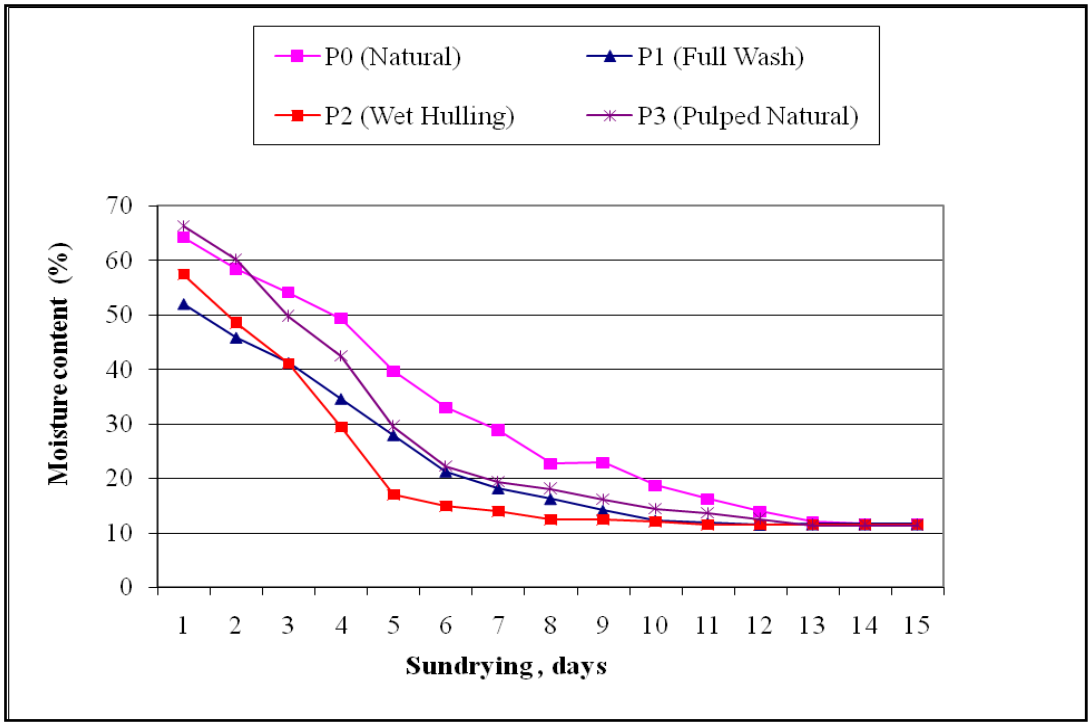

Figure 1. Moisture decreasing of Robusta coffee during sun drying variated by some processing method.

Parchment of full wash process (P1) need more than 10 days sundrying time to get green coffee with moisture content less than 12%. The pulped coffee of pulped natural process (P3) need more than 13 days sun drying to get coffee beans with moisture content less than 12%, because of more resistence materials. Natural coffee need more than 14 days of sundrying to get dried coffee, and very difficult to get moisture content less than 12%.

Robusta green coffee of natural processing (P0) is similar to pulped natural/descacado process (P3), those are contain brown silver skin. But, some green coffee of (full wash) wet hulling (P2) method is no silver skin similar to full wash process (dry hulling, P1). Green coffee color of full wash processing is green, but that's of wet hulling is grey-bluish. The grey-bluish color is found too in wet hulled Arabica coffee. There are some wet hulled Arabica use "Blue" as part of the trade mark, those are "Blue Flores Organic", and "Blue Java".

Green Coffee Performance

Figure 2. Robusta grean coffee performance variated by some processing method.

P0 = Natural Process. P1 = Full washed dry hulling. P2 = Full washed Wet Hulling. P3 = Descacado Process or Pulped Natural Process.

Table 1. Size distribution of Robusta green coffee variated by some processing method (%).

Processing Treatments	> 7,5 mm	7.0-7.5 mm	6.5 -7.0 mm	6.0-6.5 mm	5.5-6.0 mm	5.0-5.5 mm	<5,0 mm
P1 = Full washed dry hulling	11.84 a	22.53 a	21.54 a	23.03 a	10.95 a	8.30 a	1.80 a
P2 = Full washed Wet Hulling	16.98 a	21.08 a	18.74 a	20.10 a	8.56 a	9.38 a	5.16 a
P3 = Descacado Process or Pulped Natural Process	14.29 a	22.37 a	8.42 b	37.67 a	6.97 a	7.11 a	3.17 a

Note: Data in a column with same letter are not significantly different according to Duncan Multiple Range Test 5%.

Green coffee of natural process (P0) has highest of bulk density and apprent swelling of the roasted beans, similar to full washed coffee (P1). Full washed Wet Hulling (P2) produce coffee with highest yield roasting and bulk density of roasted beans, but lowest apparent swelling of roasted beans. On the other hand, pulped natural (descacado process, P3) produce green coffee with lowest bulk density. Recent studies in Myanmar and Lao have shown that pulper or demucilager units are a cost efficient and an effective way to consistently produce high quality coffee without the need for fermentation and washing (FAO, 2008).

The best coffee flavor was produced by wet hulling method (P2), although similar to full wash coffee (P1). Wet hulling (P2) produce coffee with winy and harsh characters, but full wash (P1) produce clean flavors. The flavor profiles of these processing methods are presented in Figure 4. In Arabica coffee, wet hulling produce specific flavors. Sumatran coffee, those mostly processed by wet hulling method, has unique flavor, identity, character which is hard to find anywhere else in the world. Words used to describe it are spicy flavor, complex, earthy body and low acidity (Marsh, 2009).

Table 2. Bulk density of green and roasted coffee, yield and apparent swelling of roasted beans variated by some processing method (%).

Processing Treatments	Bulk Density of Green Coffee	Bulk Density of Roasted Coffee	Roasting Yield (%)	Apparent Swelling (%)
P0 = Natural	0,75 a	0,40 a	87,50 ab	62,82 b
P1 = Full washed dry hulling	0,74 a	0,41 a	89,18 ab	59,44 b
P2 = Full washed Wet Hulling	0,71 ab	0,47 b	89,58 b	37,60 a
P3 = Descacado or Pulped Natural	0,69 ab	0,42 ab	86,25 a	40,20 a

Note: Data in a column with same letter are not significantly different according to Duncan Multiple Range Test 5%.

Table 3. The flavor profiles of Robusta coffee variated by some processing method.

Specific Flavor Attributes	Processing Treatments			
	P0 = Natural	P1 = Full washed dry hulling	P2 = Full washed Wet Hulling	P3 = Descacado or Pulped Natural
Fragrance	6.89	6.75	6.79	6.70
Aroma	6.70	6.70	7.08	6.43
Flavor	6.14	6.60	7.02	6.55
Body	6.49	6.78	7.38	6.79
Bitterness	6.48	6.15	6.20	6.95
Astringency	4.31	3.78	4.65	6.08
Aftertaste	6.19	6.74	6.92	6.46
Clean Cup	6.75	7.87	7.63	6.70
Balance	6.08	6.78	6.99	6.21
Overall	5.70	6.82	6.95	6.15

Notes : Quality scale (SCAA, 2009)

6.00 - < 7.00 Good	*7.00 - < 8.00 Very Good*	*8.00 - < 9.00 Excellent*	*9.00-10 Outstanding*

Table 4. Spesific characters of Robusta coffee produced by some processing methods.

Spesific Characters / Off Flavor	Processing methods			
	P0 = Natural	P1 = Full washed dry hulling	P2 = Full washed Wet Hulling	P3 = Descacado or Pulped Natural
Fruity	4,13	0,00	0,00	0,00
Winy	3,92	0,00	1,00	0,00
Sour	3,75	0,00	0,00	0,00
Fermented	4,50	0,00	0,00	0,00
Harsh	0,75	0,00	1,44	2,17
Cereals	1,25	0,00	0,00	0,00
Woody	2,00	2,00	2,00	0,00
Earthy	0,75	0,00	0,00	0,00
Smoky	0,50	0,00	0,00	1,00

Notes: level range of spesific characters / off flavors are 0.00 (none) – 10 (outstanding).

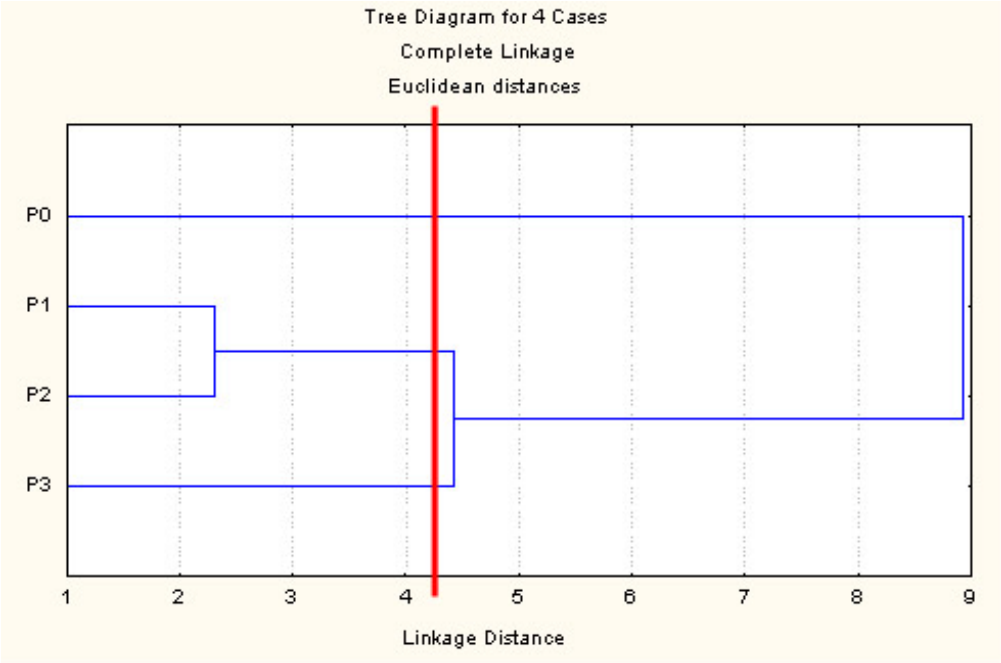

Figure 3. Clustering of processing methods basic on the flavors profile.

Pulped natural (P3) has lower flavor with strong harsh and astringent. Natural processing method (P0) produced green coffee with some off-flavors; those are fruity, winy, sour, fermented, harsh, cereals, woody, and earthy. In Lao, natural processing method produces unclean coffee with fermented and fruity off-flavor. This is the low cost, traditional system resulting in a low quality coffee, and is not recommended. But, no significant cup quality differences were detected between demulage process and full wash process, and it is the recommended process (Marsh et al., 2006). Basic on the flavor profile, there are 3 processing clusters, those are cluster of P3 = Descacado Process or Pulped Natural Process, cluster of P1

= Full washed dry hulling and P2 = Full washed Wet Hulling, and cluster of P0 = Natural Process. Flavor profile distance of P1 = Full washed dry hulling and P2 = Full washed Wet Hulling are very close. Euclidian distances among processing methods basic on the flavors profile are presented on Table 5.

Table 5. Euclidian distances among processing methods basic on the flavors profile.

Processing methods	P0 = Natural	P1 = Full washed dry hulling	P2 = Full washed Wet Hulling	P3 = Descacado or Pulped Natural
P0 = Natural	0.00	8.58	8.27	8.90
P1 = Full washed dry hulling	8.58	0.00	2.16	4.24
P2 = Full washed Wet Hulling	8.27	2.16	0.00	3.53
P3 = Descacado or Pulped Natural	8.90	4.24	3.53	0.00

The flavor profiles of these processing methods are presented in Figure 1.

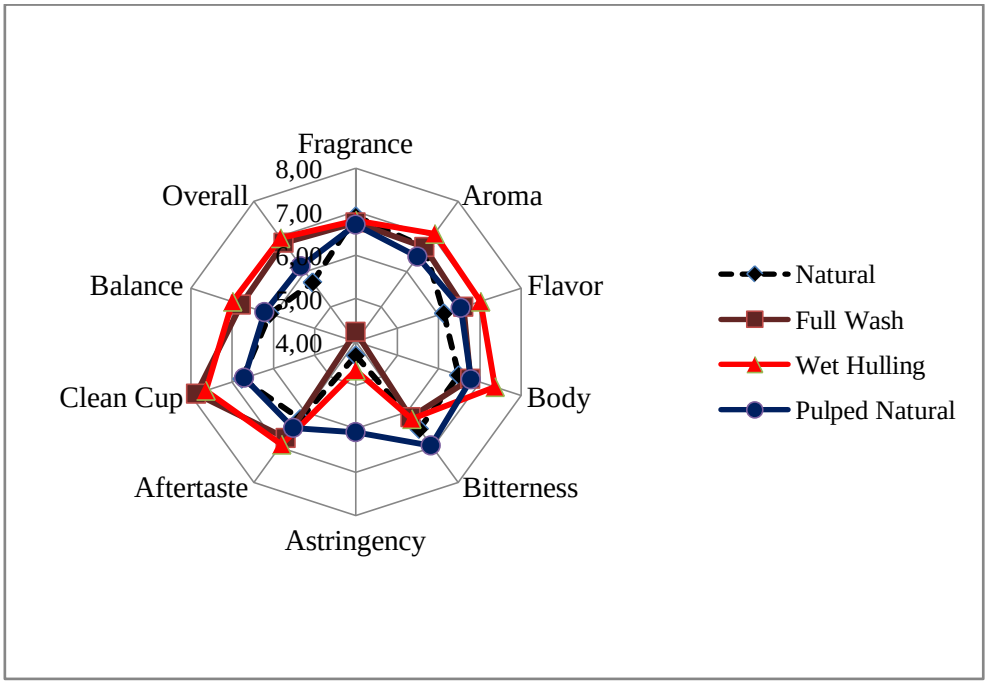

Figure 4. The flavor profiles of Robusta coffee by some processing methods.

CONCLUSIONS

- The experiment showed that the fastest sun drying is wet hulling method, 5 (five) to 8 (eight) days. Sun drying of parchment of full wash method was 10 days, parchment of pulped natural processing method was 13 days, and coffee cherry of natural processing was more than 14 days.
- Green coffee of natural processing is covered with brown silver skin similar to pulped natural. But, some green coffee of wet hulling method is no silver skin similar to full

wash processing. Green coffee color of full wash processing is green, but that's of wet hulling is grey-bluish.

- The best coffee flavor was produced by wet hulling method, although similar to full wash coffee.
- Pulped natural has lower flavor with strong harsh and astringent. Natural processing method produced green coffee with some off-flavors; those are fruity, winy, sour, fermented, harsh, cereals, woody, and earthy.

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Single Coffee Bean Roast Gas Measurements with Microprobe Single Photon Ionization Time-of-Flight Mass Spectrometry

R. HERTZ[1], R. ZIMMERMANN[1,2,3*]

[1]Joint Mass Spectrometry Centre, Chair of Analytical Chemistry, Institute of Chemistry, University of Rostock, D-18057 Rostock, Germany
[2]Joint Mass Spectrometry Centre, Cooperation Group Analysis of Complex Molecular Mixtures, Institute of Ecological Chemistry, Helmholtz Zentrum München – German Research Centre for Environment and Health, D-85764 Neuherberg, Germany
[3]BlfA- Bavarian Institute of Applied Environmental Research and Technology GmbH, Environmental Chemistry, D-86167 Augsburg, Germany
*E-mail: ralf.zimmermann@uni-rostock.de

SUMMARY

A new sampling device coupled to single photon ionisation time-of-flight mass spectrometer (SPI-TOFMS) for on-line analysis of roasting gases originating from individual coffee beans were developed and firstly appliedt. The roasting gases were sampled either outside or inside the coffee bean during roasting at approx. 250 °C and mass spectra were recorded in real-time. During this process, differences in the chemical signatures as function of roasting time and sampling point were observed. The formation profiles of selected volatile organic compounds (VOCs, e.g. *m/z* 44 – acetaldehyde, *m/z* 79 – pyridine, *m/z* 98 – furfuryl alcohol, *m/z* 150 vinylguaiacol, *m/z* 194 caffeine and *m/z* 256 hexadecanoic acid) over a roasting time of 15 min are presented. The experiments outside the bean are comparable to results of previous works with PI-TOFMS. On the other hand, the PI-MS results of the sampling within the bean show sharp intensity peaks associated with the “popping” or “cracking”of the beans due to the pressure of the formed pyrolysis gases.

INTRODUCTION

Coffee is one of the foods with the richest flavour; roasted coffee comprises 0.1% volatile organic compounds (VOC) per dry weight. During the roasting process at high temperatures over 200 °C, these are formed from precursors which are already present in raw coffee. Since 1960, the number of identified flavour compounds increased; nowadays 850 compounds are identified in roasted coffee (300 in green coffee – only in traces) (Flament, 2002) 500 of them are produced by the predominant Strecker and Maillard reactions. There are several reviews on the chemistry of coffee roasting and the formation of the flavour compounds (Flament, 2002; Grosch, 1998; Mayer et al., 2000; Nijssen, 1996; Vitzthum, 1999). Considering the complexity of the chemical and physical processes that occur while roasting, it is not surprising that knowledge about these processes is fragmentary and sometimes speculative. To ensure a constantly good roast quality, the roaster still uses a set of indicators, his senses and his empirical experience for process controlling. Up to now, some (automated) on-line process control methods for coffee roasting are still in progress. Indeed, a rational control of roast quality requires a real-time monitoring of properties relevant for the coffee flavour throughout the whole roasting process. Ideally, the development of sensory evaluation should be characterized by formation and release of several coffee-flavour compounds in real time. One possible method to realise that is the photon ionization time-of-flight mass spectrometry

(PI-TOFMS), which allows the detection of VOC in complex matrices without the detection of disruptive primarily inorganic gases such as CO_2, CO, N_2 and H_2O with a high-time resolution, with high sensitivity, and with chemical selectivity. The direct inlet of gases into a mass spectrometer coupled to photon ionization allows ionization without fragmentation (i.e. soft ionisation) of a wide range of organic compounds with single photon ionization (SPI), or a selective ionization of aromatic compounds with resonance-enhanced multiphoton ionization (REMPI). By PI-MS relative simple mass spectra without non-deconvolutable, overlapping fragment peaks are obtained. In combination with the high-time resolution of time-of-flight mass spectrometry a fast chemical profiling can be achieved (Dorfner, 2004; Dorfner et al., 2003; 2004; Zimmermann et al., 1996). A considerably better chemical description of the roasting process is achieved, if compared to other on-line analysis methods, such as IR-spectroscopy, gas sensors or sensor arrays, ("electronic nose") (Pearce, 1997; Gardner and Bartlett, 1992). In particular for research and pilot plant application PI-MS has a large potential, in the future even application for process control are possible.

MATERIALS AND METHODS

For the analysis of volatile organic compounds in the roast gases of coffee, a PI-TOFMS coupled to a microprobe (µ-probe) capillary sampling device was used (Figure 1D). The instrumental setup has been described in detail elsewhere (Adam et al., 2009; Mitschke et al., 2005; Mühlberger et al., 2004).

The µ-probe was initially constructed for a study dealing with analysis of tobacco smoke inside the coal of a cigarette. The heated and deactivated sampling capillary allows the detection of high masses of VOCs without plugging the µ-probe and the transfer line to the inlet system of the mass spectrometer. For the roasting experiments outside of individual coffee beans (Figure 1A), the bean was placed in a small glass flask, which then was connected to the µ-probe sampling capillary. In roasting experiments conducted for sampling within individual coffee beans (Figure 1B), holes were drilled approximately 5 mm deep in the beans by a Ø 1 mm drill. Then the capillary of the µ-probe was inserted directly in the bean and the hole was sealed by inorganic glue based on zirconium oxide. A blow drier (Figure 1 C) was taken for simulating the roasting process and a mantle thermocouple was used to control the temperature (T = 250 °C) inside the glass or on the surface of the bean during the simulated roasting process, respectively. For all experiments, green Arabica coffee beans from Bolivia in organic quality were used. In these experiments, VUV laser photons with 118 nm were generated for SPI (Dorfner et al., 2004; Mühlberger et al., 2004; Zimmermann et al., 2000). One single photon (E_{phot} = 10.5 eV) ionizes the molecule directly if its ionization energy is lower than 10.5 eV. The mostly fragmentation-free formed ions were extracted into the flight tube of the reflectron time-of-flight mass spectrometer (Kaesdorf Instuments). For SPI the mass range was set from 10 to 320 *m/z*. Ten mass spectra per second were recorded, and data processing was carried out with a LabView-based (National Instruments) software written for this purpose.

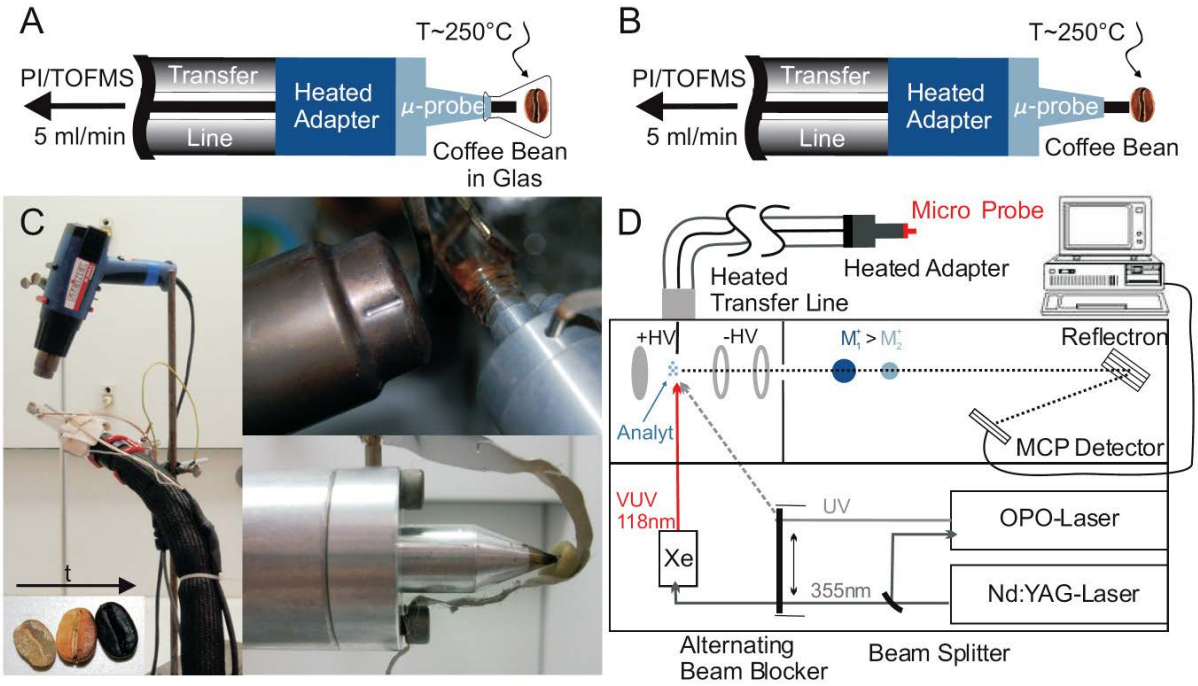

Figure 1. A and B show the microprobe sampling of the roast gases produced by a single coffee bean (A outside/ B within a single bean). C shows some photos of the sampling system and three coffee beans with different roast degrees. D is a schematic figure of the single-photon ionization time-of-flight mass spectrometer (SPI-TOFMS).

RESULTS AND DISCUSSION

Coffee beans are natural products, so every single bean exhibits individual properties. In this work, however, as a proof of concept-study, only single measurements are presented. These measurements depict a somewhat typical behaviour, reflecting the full experimental data set which is currently under analysis. To get an overview of all formed detectable VOCs in the roasting gases of a single bean, the mass spectra of the first 15 min roasting time were averaged (9000 mass spectra recorded with 10 Hz). The resulting spectra are presented in Figure 2. At the top the SPI mass spectrum from the experiment outside the single bean is shown, while at the bottom the SPI-mass spectrum from the measurement of the roasting gases evolved inside an individual bean is depicted.

Additionally, 3D-graphs of the comprehensive SPI-TOFMS on-line monitoring of the whole roasting process of both sampling techniques are shown. Furthermore, unlike in past works of our workgroup (Dorfner, 2004; Dorfner et al., 2003; 2004; Zimmermann et al., 1996), it is possible to detect additional to heterocyclic compounds, pyrroles, pyrazines as well as ketones (Dorfner, 2004). Furthermore several compounds with masses higher than *m/z* 200 have been acquired. In detail the mass spectre in Figure 2 show the detection of homologue series of fatty acids, which can be assigned to: *m/z* 256 hexadecanoic acid, *m/z* 284 octadecanoic acid, *m/z* 298 nonadecanoic acid and *m/z* 312 eicosanoic acid and also of unsaturated fatty acids, e.g. *m/z* 280 linoleic acid.

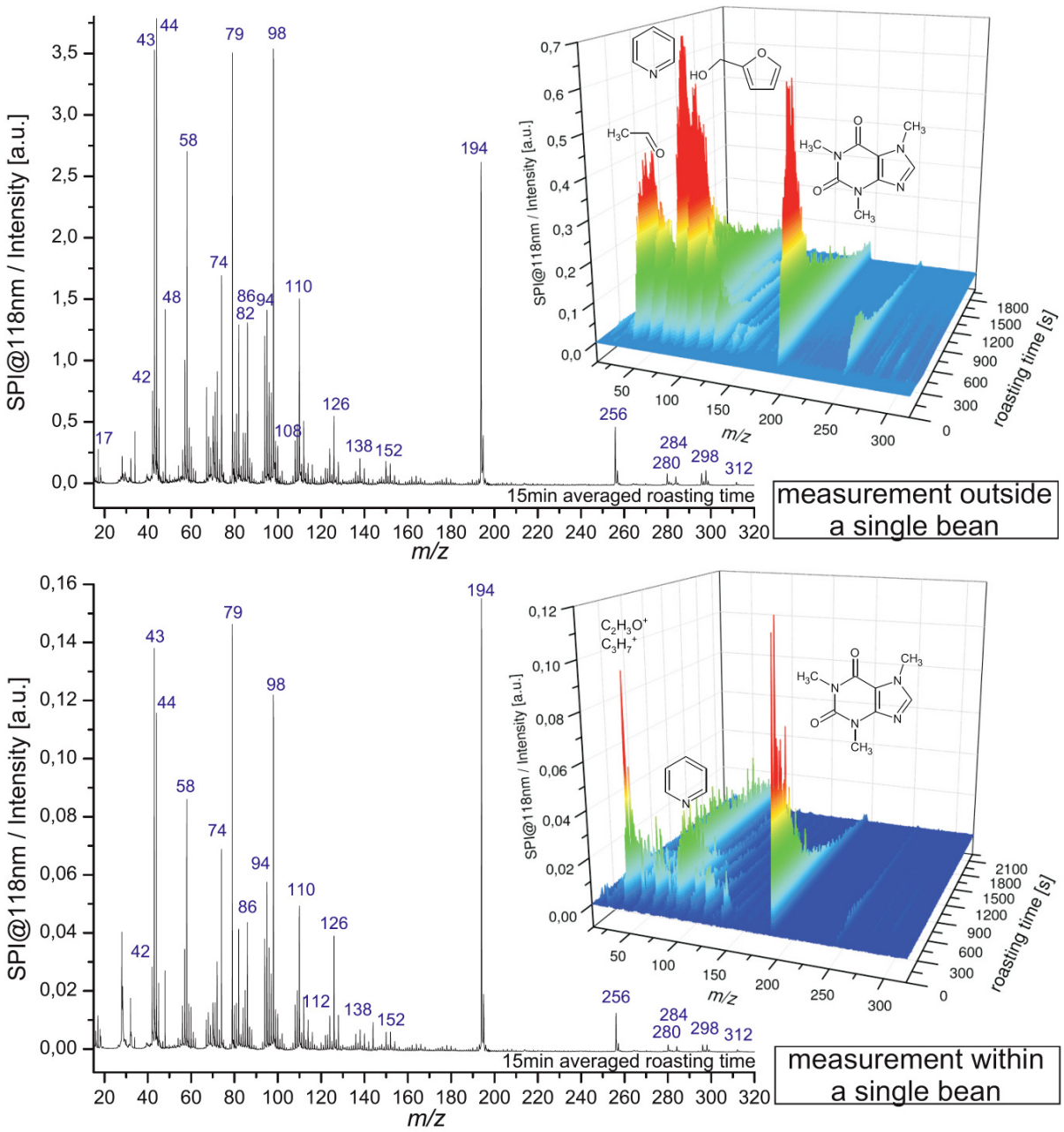

Figure 2. shows the mass spectra, which were averaged over 15 min roasting time for the single bean experiment outside (top) and within (bottom) a coffee bean. Furthermore the time resolved 3D-graphs show the changes of chemical compounds which are formed during the roasting process.

Comparing both sampling methods, it is remarkable that in both cases the same compounds are observed (e.g. *m/z* 43, 44, 58, 79, 98, 110 and 194). In Table 1, the assigned compounds for the marked signals in the mass spectra are given. Another important observation is that the intensities of the formed substances are clearly lower for the experiments inside a coffee bean. Especially in the 3D-graphs, it is evident that the intensities of both measurement techniques are completely different, e.g. the measurement within the coffee bean shows caffeine (*m/z* 194) as most dominant signal, whereas the result of the experiment outside the bean resulted in particularly intense peaks for the compounds pyridine (*m/z* 79), furfuryl alcohol (*m/z* 98) and acetaldehyde (*m/z* 44).

Table 1. Assigned compounds in coffee roasting gases measured with 118 nm.

m/z	**Compound**	*m/z*	**Compound**
17	Ammonia	112	2-furancarboxylic acid, methyl cyclo-pentadion,
42	$C_3H_6^+$ (propene)		methyl furfuryl alcohol
43	$C_3H_7^+$, $C_2H_3O^+$	126	(iso)maltol, dimethyl cyclopentadion
44	Acetaldehyde	138	1-(2-furanyl)-3-butanone
48	methane thiole		dihydroxy benzaldehyde
58	acetone, glyoxal, propanal	150	4-vinylguaiacol,
74	propionic acid, methyl acetate,		C_3-alkylated pyrazine
79	Pyridine	152	4-ethylguaiacol, vanillin
82	2-methylfuran	194	Caffeine
86	methyl butanal, 3-methyl-2-butan,	256	hexadecanoic acid
	2,3-butandione,	280	linoleic acid
94	phenol, methyl pyrazine,	284	octadecanoic acid
98	furfuryl alcohol	298	nonadecanoic acid
110	catchol, hydrochinone, methyl furfural	312	eicosanoic acid

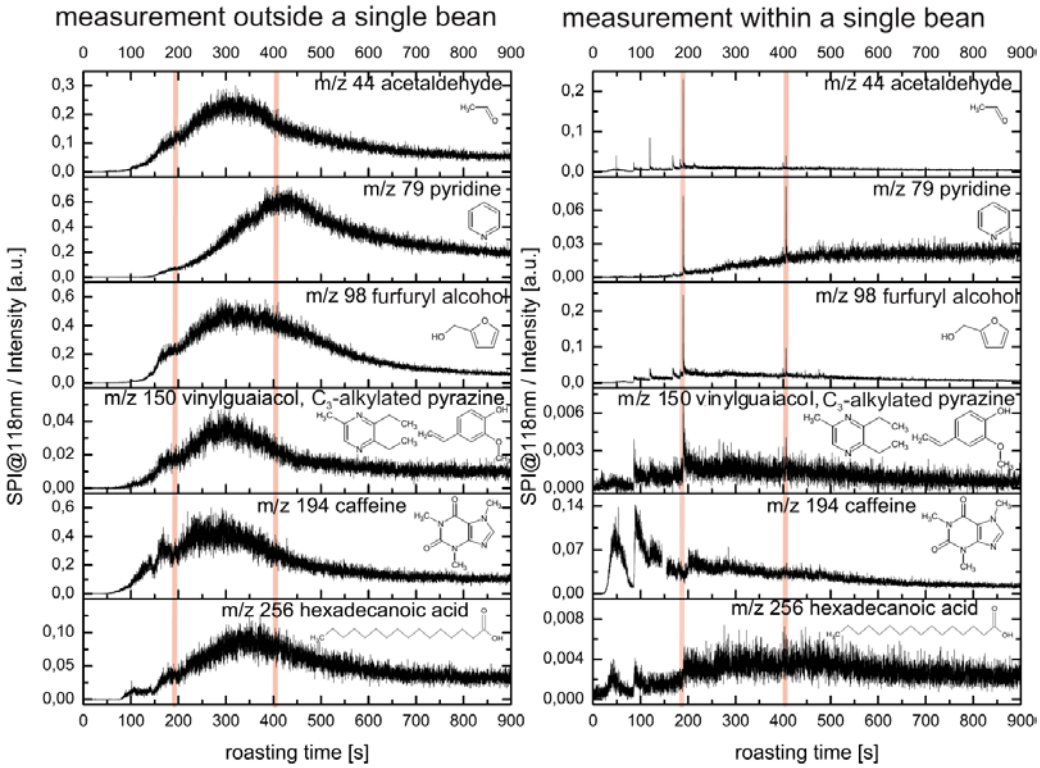

Figure 3. shows the time-resolved formation profiles of selected VOCs for both sampling variation; left – sampling outside a single bean and right – sampling within a single bean.

In Figure 3, selected time-resolved formation profiles are presented. For both sampling methods, *m/z* 44 acetaldehyde, *m/z* 79 pyridine, *m/z* 98 furfuryl alcohols, *m/z* 150 vinylguaiacol/ C_3-alkylated pyrazine, *m/z* 194 caffeine and *m/z* 256 hexadecanoic acid were chosen. The formation profiles of the compounds measured outside of the individual coffee bean increase slowly at the beginning of the roasting process. Subsequently the signals form a maximum and afterwards decrease again. The duration of the phases with increasing ion intensities as well as the positions of the intensity maxima are different for the respective considered VOCs. In this context, *m/z* 150 and *m/z* 194 have the earliest temporal maxima followed by *m/z* 44, *m/z* 98, subsequent from *m/z* 256 and, last, *m/z* 79; these correlate directly with the chemical formation of the molecules. Compared to the sampling within an individual coffee bean, there are different behaviours observed. Some substances, e.g. *m/z* 44, 79, 98 and 150 show sharp "popping" or "cracking" peaks while others show an early intense maxima like *m/z* 194. Additionally, the detection of formed VOCs occurs earlier, possibly because the products of the mild pyrolysis are trapped within the bean during the onset of the roasting reactions.

In conclusion soft photo-ionisation mass spectrometry in combination with a µ-probe sampling is a promising analytical method to sample and profile the chemical signatures of pyrolysis gases within a coffee.

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Case Study of Biogas Production from Plant-Based Materials and Animal Manure Resources Available in the Coffee Farm

S. MULATO AND E. SUHARYANTO

Indonesian Coffee and Cocoa Research Institute (ICCRI), Jl. P.B. Sudirman No.90, Jember 6811, East Java. Phone/Fax: 0062.331.758191. www.iccri.net.
E-mail: mulatosri@yahoo.com

SUMMARY

One of the proposed alternatives to overcome environmental and energy issues faced by Indonesian coffee farm is the application of co-digestion technology to convert coffee farm and coffee processing wastes to produce biogas. At a small scale trial, twin pilot scaled bio-digesters were designed and tested in the ICCRI Experimental Station. The digester was designed using an in-ground rectangular type reactor made from concrete materials with total volume of substrate 4 m^3 and 2 m^3 of gas holder per digester. The daily co-digestion input was fixed at 75 kg per 12 to 13% solid content. At the first four months prior to the coffee harvesting season, 1/1 (by weight) water diluted-fresh cow dung substrate was fed into the digesters, to initiate the anaerobic digestion process. The following 5 months duration, the biogas digester was fed fully with a neutralized coffee factory waste. At the last three months period, when the coffee harvesting was over, the digesters were reloaded again with a fresh cattle dung substrate as a single input. After a 45 days retention time, the co-digestion cattle manure-coffee waste produced 0, 55 m^3 biogas per day per m^3 digester volume. The digester was then scaled up to 30 m^3 using in-ground circular type digester. The daily input of the digester was 1 ton and the biogas production was increasing up to 40 m^3 per day. A simple household biogas stove could boil 5 liters of water and cook 500 g of rice within 17 and 25 minutes which consumed about 150 and 190 liters of gas respectively. While, a small scale industrial stove was able to roast 5 kg coffee beans within 20 minutes which required 220 liters of biogas. The digester was also able to reduce significantly the organic pollutants of the coffee factory waste as indicated by its low COD/BOD values of the slurry out from the digester. This biogas technology is intended to help coffee farmers to conserve their energy need for the daily cooking as well as running the household industry. The additional benefit is that the slurry has a potential use for being recycled as organic fertilizer to coffee plants.

INTRODUCTION

Coffee farm is facing with relatively similar environmental and energy issues (De Matos et al., 2001; Von Enden et al., 2002; Deepa et al., 2002; Ntiamoah and Afrane, 2008). In fact, there are several scientific reviews describing that coffee farm residues can be treated in the bio-digester to produce useful biogas and thereby reduce the pollutants (Lane, 1983; Chacon, 1984). Murthy et al. (2004) underlined a technical solution for treatment of coffee effluents using large scale lagoon type digesters. The results showed that the digester provided not only a solution to waste disposal, but also an alternative fuel for electricity generation. Working differently, Bruno et al. (2008) evaluated the efficiency of two stages up flow anaerobic sludge blanquet (UASB) reactors, in a bench scale, treating a liquid effluent from the coffee pulping. The result showed that the recovery of methane gas varied from 69 to 89%.

As perennial crops, coffee harvesting season take place intermittently. Hence, during off season, the digester should be kept running by co-digesting various feed stocks (Phalla, 2004). These can be any biologically degradable residues available in the coffee farm, leaves and trunks of the coffee tree as well as from the shade tree. Some advantages of applying co-digestion inputs are to maintain closed natural resources cycles, to increase gas yield and to enhance additional income for coffee farmers from converting any waste materials into added value products (Heinz-Peter Mang, 2009). The current intensive coffee production is to apply an integrated farming system (Richard, 2006). The integration of crop and animal production system gives sustainability supply of biodegradable feed stocks. The animals help in efficient recycling of organic crop residues (Channabasavanna et al., 2009). There is marked complementary in resource use in this system, with inputs from one sector being supplied to others (Van Der Vossen, 2005; Zhanserikova, 2008).

The paper is focused to review the practical experiences gained from a serial pilot trials of biogas production using a co-digestion of plant-based and animal manure resources which are available in the coffee farm. The biogas is utilized mainly for household cooking, lighting and processing of roasted coffee.

MATERIALS AND METHODS

The amount of residues was calculated based on the cropped area, planting density, the type of farm management and the crops variety (Lim, 1986a; 1986b). Animal-based residue was estimated by multiplying number of animals and the quantity of waste. While, factory waste materials consist of two groups, i.e., solid and liquid in states. Solid waste wastimated using a mass balance method. This involved measuring the crop and product ratio (CPR). The total factory waste is estimated by multiplying the CPR value and the total crops production per hectare per year (Upreti et al., 1991). The coffee processing was done based on a fully washed processing procedure (Figure 1**)**. Liquid wastewas estimated by measuring loading rates of coffee processing in each of the two waste waters outlet per unit time. The loading rates were calculated by multiplying the measured concentration or average concentration for triplicate analyses by the average flow rate over the 24-hours sampling period. The digester design was based on the results gained from a laboratory scale digester tested by ICCRI and the experiences collected from various researchers working at the subject. An in-ground circular type digester was finally to be chosen for the field test and was constructed by using brick masonry materials. The gas holder made from steel plates was installed on the top of the tank.

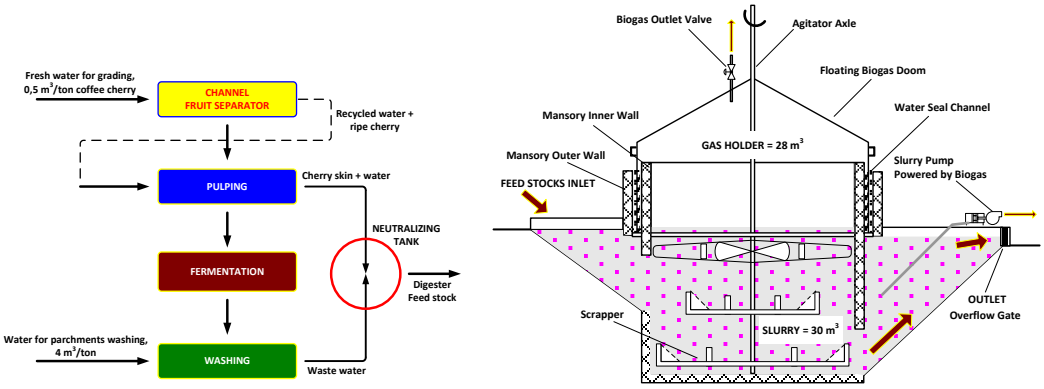

Figure 1. Coffe processing wastes routes and a digester design.

The feeding mechanisms during the whole trials were depicted in the Figure 2:

- Sequence-1: Three months prior to the coffee harvesting season, a single fresh cow dung feed stock (after diluted with water at 1/1 by weight proportion) was loaded into the digester to initiate the anaerobic digestion process. The feeding rate was 1 ton/day.
- Sequence-2: When, the coffee harvesting started, the effluents from the factory were initially neutralized with commercial lime in the equalization tank to reduce their acidity till their pH values achieve 6.5 to 7. The feeding rate was 1,2 ton/day. This was a little higher feeding than that of in the previous sequence. The reason was to obtain a homogenous mixture of two substrates with the final total solid content (TS) from 12 to 13%.
- Sequence-3: When, the coffee harvesting season has been averred, the mixture of coffee leaves chips, trunks of coffee trees chips, cow dung and water at a certain proportion was used as a feed stock. The feeding rate was 1,8 ton/day. As the plant based materials were solid in state and contained high value of C/N, closed to 90, the higher feeding rate was used to reduce the value of C/N about 30 – 35 and the TS value ranged from 12 to 13%. The digester was operated naturally under ambient tropical conditions.

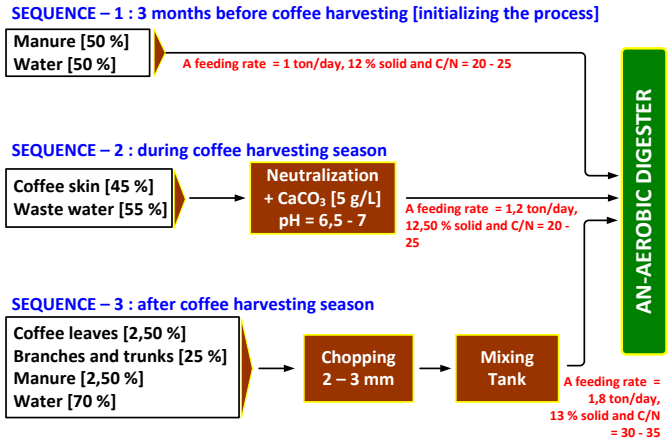

Figure 2. A schematic diagram of the feeding sequences.

Gas pressure was measured with a pressure gauge made of a 10 cm^2 section of plastic tube attached (in a U-shape) to a graduated rectangular plank and an analog flow meter. Ambient pressure, temperature and relative humidity were monitored by a sling thermometer and a barometer. Biogas produced by the digester was tested in a various applications such as for household cooking and lighting and for agro processing such as roasting of coffee. A simple biogas burner for cooking and heating was designed to match with the available cooking devices in the rural household area. The performance of stove for cooking was determined by calculating the heat gained by the water subjected for boiling and amount of fuel consumed during this process. The time taken for the various tasks was measured by a digital data logger.

RESULTS AND DISCUSSIONS

Farm residues

Biogas feed stocks out of coffee farm were derived from the pruning process of main crop and shade trees. With the average main crop population was 1,100 trees per hectare, the amount of plant based residue from the pruning process ranges from 7 to 9 tons/hectare/year. The integration of 3 cows in the coffee farm produced an animal-based material to an average 11 ton/hectare/year which is very useful for co-digestion process. Table 1 summarizes the amount on-farm residual estimation in coffee farm and Table 2 shows the average value of C and Nitrogen ratio (C/N) each type of residual waste. Based on their C/N value, the plant-based and animal based residues can be mixed, hence, it is suitable as feed stocks for bio-digester.

Table 1. Biomass residue potential of coffee and cocoa farm (ton/hectare/year).

Residual Type	Coffee	C/N value
Plant-based		
Main crop trees	12 - 15	1/110 – 125
Shade trees	7 - 9	1/150 – 200
Animal-based		
3 Cattle	10 - 12	1/25 – 30
3 Goat	0,5 - 1,0	1/20 – 25

Coffee Processing Wastes

Table 2. Mass balance of coffee processing (in 1 hectare during coffee season).

	Input	Output	
		Main product	**Waste**
Coffee cherries, kg	1.000		
Water, L (4 L/kg cherries)	4.000		
Total, kg	**5.000**		
Coffee bean, kg		190	
Husk, kg			50
Fruit skin, kg			410
Pulp, kg			164
Water loss and evaporated, kg			186
Waste water, kg			4.000
Total, kg		**5.000**	

In case of coffee farm, the amount of water used in coffee processing was quite large and depends strongly on the type of processing. As shown in Figure 1, the amount of waste materials derived from coffee processing can be seen in Table 2. The waste estimation was based on the daily average of coffee processing of 1 ton coffee cherry.

Waste water of coffee processing contained high levels of pollutants. There was a significant change in physical, chemical and biological properties of water before and after the processing (Table 3).

Table 3. Raw and waste water characteristic.

Quality	pH	BOD (mg/l)	COD (mg/l)	SS (mg/l)	TS (mg/l)
Influent raw water	6,8	250	500	500	800
Waste water	3,8	12.000	18.000	3.600	12.000

The main component in the waste water was organic matter, stemming from pulping and mucilage removal. The waste water consisted of fermenting sugars from both pulp and mucilage components such as, proteins, sugars, pectin, and polysaccharide carbohydrates. The majority of organic material in the wastewater caused high COD value as high as 18,000 mg/L. The BOD coming from biodegradable organic material reached a value of 12,000 mg/L. Other components in pulping water were acids causing the waste water were very acidic. The total suspended solids in the effluents were also high.

After pulping, the beans were fermented in 12 to 36 hours to break down microbiologically the remaining mucilage on the beans surface. The fermentation of the sugars (disaccharide carbohydrates) into ethanol and CO_2 leaded to acid conditions in the washing water. The ethanol was converted in acetic acids after reaction with oxygen, lowering the pH to levels of around 3.8 (Von Enden and Calvert, 2002). The combination of waste water from pulping and washing process was suitable as a bio-digester feed stock. In order to optimize the anaerobic processing of the wastewater pH values was adjusted at a value between 6.5 and 7.5 from initially 4, which was highly acidic by adding a commercial lime solution into a wastewater pond.

Biogas Production

The digester was operated naturally under ambient tropical conditions at a temperature range between 28 and 32 °C. Thus, a number of mesophilic anaerobic bacteria were assumed to grow well in its optimum condition promoting a higher biogas production. The gas production rate for the total solid (TS) concentration level of 12% was detected 3 weeks after the first input was loaded. The total biogas production was steadily increased from 2 to 6 m^3 per day (Figure 3). Biogas production continued remarkably to 16 to 18 m^3 per day till the coffee harvesting season was commenced.

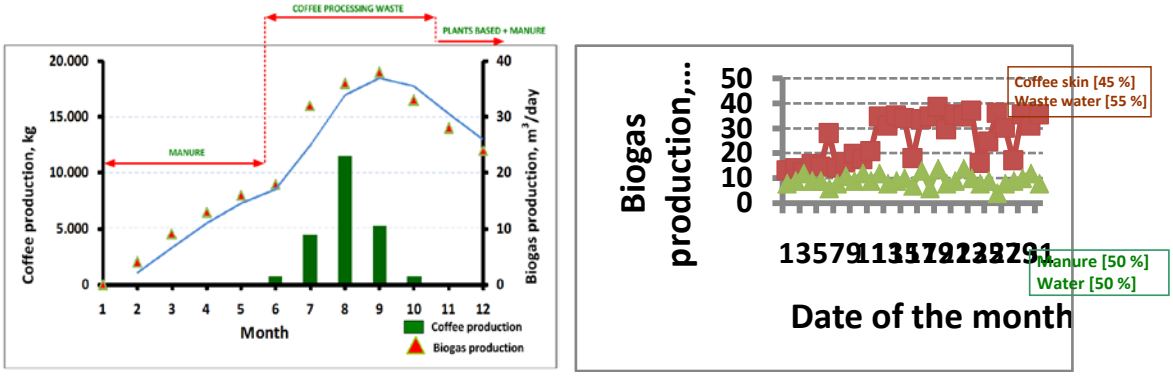

Figure 3. A relationship between and the composition of digester feed stock (left side) and daily biogas production profiles at two different feed stocks (right side).

At the beginning of coffee harvesting season, the coffee processing factory started producing wastes that afterward are used to be a subsequence feed stock. Temporaray, during the coffee harvesting, the cow manure feed stock was not use any more. One of the main characteristics related to the composition of the coffee waste water substrate was a high value of chemical oxygen demand (COD), which was directly proportional to the total amount of organic compounds contained in the substrate (Calvert, 1997). Thus, it was presumably that coffee waste water might produce more biogas yield.

The coffee waste water after being neutralized by lime produced a seething solid mass of micro-organisms and will float out more solids on the surface of the water. That solid was a highly methane-enriched biogas raw material (Rathinavelu and Graziosi, 2005). No nutrient limitations and no substrate inhibition as reported by Field and Lettinga (1987), had been observed during the tests. The anaerobic degradability was higher between 65 to 70%. As a result, the total biogas production can reach a maximum value approaching to 40 m^3 per day, 20 days after the co-digestion input of coffee processing wastes were loaded. The trend of high biogas output was recorded higher than that of using cow manure feed stock. When, the coffee harvesting season was ended. In case of using plant based materials chips as co-digesting material, the digester needed more time to achieve a steady biogas production and hence, produce less amount of biogas compared that of using coffee waste materials. Plant based material contained more than 35% cellulose and hemi-cellulose particularly branches and trunks of coffee trees. As a result, the digestion of cattle manure and coffee waste materials with their high carbohydrate and protein contents faster than cocoa waste materials. Consequently after 45 days retention time, the maximum attainable biogas production from co-digestion planted based materials, cattle manure was decreasing to a relatively lower rate below 33 m^3 per day (Figure 3).

Biogas Application

Biogas has a calorific value of approximately 23.5 MJ/m^3. In principle, it can be used in the same way as any other combustible gas such as LPG or LNG for cooking and lighting. A biogas pressure of between 30 and 60 mm H_2O column was able to flow within a simple stove for providing heat to boil water and to cook rice, noodle and vegetables (Table 4).

Table 4. Various biogas applications for supporting household activities.

Utilization	Capacity	Time (min)	Demand (Liter)
Boiling water	1 liter	8	70
Boiling water	2 liters	14	130
Boiling water	5 liters	17	150
Cooking rice	500 g	25	190
Cooking noodle	300 g	17	150
Electricity	450 W	60	425
Lighting lantern	60 W	60	1250

A small scale industrial used of biogas was demonstrated for coffee roasting, coffee grinding, producing electricity and water pumping (Table 5). The biogas stove was able provide a sufficient heat source to roast the coffee beans within less than 20 minutes. The specific flavor and aroma of the coffee has been developed, when the beans temperature reached about 200 ^{o}C and the beans color turned a darker brown produces.

Table 5. Various biogas applications for SME scale of coffee industry.

Utilization	Capacity	Time (min)	Demand (Liter)
Roasting coffee	5 kg	18	220
Electricity	1.000 W	60	1.060
Grinding coffee	1.500 W	60	1.560
Pumping water	6 m^3/hr	60	1.240

Environmental And Economic Benefits

Conversion of coffee farms residues to biogas could significantly decrease the potency of environmental problems associated with wastes. The field trials showed that the pollution indicator of the coffee processing effluents from the digester could be reduced significantly to an allowable level for direct disposal (Figure 4).

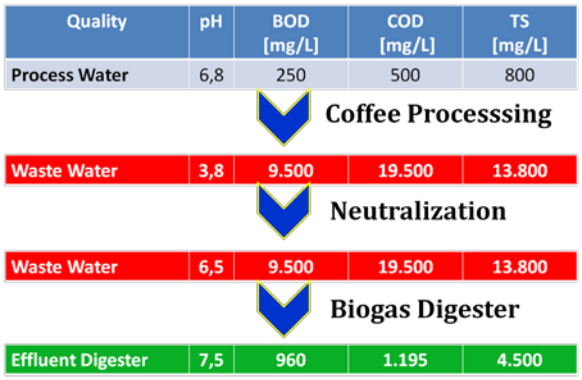

Figure 4. The pollutant level of wastes at every level of processing.

A field observation showed that a farmer family (1 parent and 2 children) consumes 1,50 to 2,0 liters of kerosene for daily cooking. This amount of kerosene can be replaced by 1,10 to 1,20 m^3 of biogas. Recently, the kerosene price in the coffe farm areas was Rp 8.500,-/liter (Us $ 1).

CONCLUSIONS

A 30 m^3 cylindrical in-ground biogas digester has been installed and tested at the ICCRI Experimental Station as a field observation and demonstration for treating coffee farm residues and coffee processing wastes. The results showed that,

1. A simple co-digestion technology application is a promising solution to convert coffee farm and processing waste to biogas and compost and hence minimizes environmental problems.
2. The daily biogas production ranged from 25 to 40 m^3 and was able to provide a sufficient energy supply for household and SME scale coffee industry.
3. By adopting this technology, farmers may take up alternative-income generating activities (energy saving cost and selling compost) so that they can take care their coffee farm more intensive and focus.

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Identification of Variety and Its Suitable Cherries Processing Method for Improving Specialty Arabica Coffees from Dry Climate Area at Flores Island of Indonesia

S. MAWARDI[1], YUSIANTO, A. MARS[2]

[1]Indonesian Coffee and Cocoa Research Institute, Jl. PB Sudirman no. 90, Jember, East Java, Indonesia. E-mail: surip.mawardi@gmail.com
[2]Private Coffee Consultant, Australia

SUMMARY

Flores island is one of an important specialty coffee origins from Indonesia. The island lays on the Lesser Sunda islands chain of eastern Indonesia which is characterized by a relatively dry agro-ecological climate.

A study to identify Arabica coffee varieties grown in the area and suitable processing for each variety to improve specialty coffee quality in Flores was conducted during the 2009 harvest season. Variety identification was conducted by making survey at the Arabica coffee farms in Ngada Bajawa highland. Three processing methods consisted of wet process by dry hulling (WPDH) or 'full-washed', wet process by wet hulling (WPWH) or 'wet-hulled' and decascado or pulped natural (PN) were applied to three dominant varieties. Quality here was observed by sensory analysis to mean cup quality. Cup taste profiles were judged by 44 domestic and international panelists by evaluating fragrance and aroma, flavor, acidity, body, after taste, balance and preference (overall).

The result showed that the three dominant Arabica coffee varieties being grown by smallholder farmers in Flores were 'Juria' (Typica type), S 795 and Hybrid of Timor (HdT) progeny. Based on cup profile the most preferred variety under Flores conditions was S 795, which was sequentially followed by HdT and Juria. S 795 presented an excellent cup profile with nice balance and strong sweet tones. In general, there was no significant effect on cup taste profile when the three different processing methods when applied to S 795. However, WPDH resulted better flavor, acidity and after taste than that of WPWH and PN. In contrast WPWH showed better body, balance and preference than that of WPDH and PN. PN only showed better fragrance and aroma than that of WPDH and WPWH. Therefore to have excellent cup profile it is suggested to apply WPDH and/or WPWH on S 795 under Flores condition.

HdT is also promising to be grown in Flores, especially for less fertile soils. Since the variety still presents morphological heterogeneity, breeding measures must be carried out mainly through selection to identify the best progeny. The best processing method to be applied in this variety was WPWH, which resulted in a better performance according to all taste components compared with that of WPDH and PN. This research emphasizes the continued importance of local conditions in determining recommendations for quality improvement based on both planting materials and processing methods.

INTRODUCTION

Indonesia produces a number of specialty coffees having distinct and unique taste profiles from different geographic origins.

Consumption of specialty coffee is growing such as in the USA which 17 % of the adult population consumed a gourmet beverage on a daily basis in 2008 compared with 14% in 2007 (NCA-USA, 2009). Specialty coffee (including espresso, cappuccino, latte, and ice blend) consumption in Canada also increase from 6 % in 2007 to be 7 % in 2008 (Elliot, 2008).

Taste and flavor requirements vary considerably with market segments. For instance, in northern Europe there is a general preference for acidity, light body and pleasant flavor of the cup, while in the south the coffee should taste sweet with a full body and strong flavor (Barel and Jacquet, 1994 cit. Van der Vossen, 2009).

Result from a study in Honduras showed that high altitudes and annual rainfall of less 1500 mm were favorable factors for the sensory quality, and an optimum roasting time must be sought for each type of coffee, hence for each *terroir* (Decazy et al., 2006).

Flores Island is considered as dry area and it also consists of a number highland area suitable to grow Arabica coffee, including Ngada Bajawa highland at the slope of Inerie volcano. This study was aimed to identify varieties of coffee Arabica suitable for Flores highland condition dealing with taste profile.

MATERIAL AND METHOD

This study was carried out at Arabica coffee growing area of Ngada Bajawa highland in the island of Flores during harvesting season of 2009. The area is characterized by fertile volcanic soil and dry climate.

Variety survey was conducted over 5 villages representing 14 villages by making morphological identification of different varieties grown by the farmers. Variety proportion of each variety was estimated by making consultation with the local peoples. Three dominant varieties were chose for processing trial in order to identify suitable method for such variety.

Three processing methods consisted of wet process by dry hulling (WPDH) or ‘full-washed’, wet process by wet hulling (WPWH) or ‘wet-hulled’ and *decascado* or pulped natural (PN) were applied to three dominant varieties. Quality here was observed by making sensory analysis to mean cup quality. Cup taste profiles were judged by 44 domestic and international panelists by evaluating fragrance and aroma, flavor, acidity, body, after taste, balance and preference (overall).

RESULT AND DISCUSSION

Variety identification

There were 7 Arabica coffee varieties identified in Ngada Bajawa highland as mentioned in Table 1.

Table 1. Arabica coffee variety grown by farmers at Ngada Bajawa highland of Flores island.

No.	Identified variety	Estimated proportion at village of (%)					Average
		Susu	Bomari	Ubedolumolo	Mangulewa	Were 1	
1	Andungsari 1	0	0	1	0	0	0.2
2	Catimor (unidentified)	3	2	2	1	0	1.6
3	Juria	5	10	15	15	20	13.0
4	Kartika 1	5	5	10	7	5	6.4
5	Kartika 2	2	3	2	2	0	1.8
6	S 795	55	45	40	40	35	43.0
7	Timor Timur (Hybrid of Timor)	30	35	30	35	40	34.0

Table 1 shows three dominant Arabica coffee varieties grown by the farmers are S 795 (43.0%), HdT (34%), and Juria (13%). S 795 is a progeny of natural hybrid of *C. Arabica var. Typica* and *C. liberica* selected in India. HdT is a progeny of natural hybrid of *C. Arabica var. Typica* and *C. canephora var. Robusta* found in Timor Leste (Van der Vossen, 2009). Juria is a local name for *C. Arabica var. Typica.* The three dominant varieties were harvested for cup profile identification by using different processing methods.

Preference of panelists to each variety

Preference most of the panelists to cup taste profile of such variety is presented in Figure 1. The highest percentage of prefer most of the panelists was belonged to variety S795 (48%), followed by Juria (27%) and HdT (26%). In contrast, the highest percentage of prefer least of the panelists was belonged to Juria (40%), followed by HdT (46%) and S 795 (24%). Mid preference was belonged to HdT.

However, the often heard assumption, that it is an interspecific hybrid and therefore should have many characteristics intermediate between Arabica and Robusta coffees including lower cup quality than a pure Arabica, does not appear to hold. The fully washed arabica coffees from Timor-Leste, in particular those grown at higher altitudes, are considered speciality arabicas comparable to some of the best mild Arabica coffees. Apparently, the coffee trade has been unaware of the fact that practically all these coffees are produced by HdT trees (Van der Vossen, 2009).

Typica variety is assumed by many people having excellent flavor, but in this study the taste panelists provided preference least for the variety. Taste deterioration could be mainly caused by defoliation due to coffee leaf rust (*Hemileia vastatrix*) attack. Defoliation caused over bearing so that the coffee beans were not fully filled. This situation will bother on the formation of taste precursors such as chlorogenic acic, caffeoilquinic acid, trigonellin, etc.

Agwanda et al. (2003) mentioned that the success of a new variety of Arabica coffee (*Coffea arabica* L.) depends to an important extent on its liquor and bean qualities. These families

were best differentiated for bean sizes in the site where moisture supply was optimal throughout berry expansion and filling stages, whereas discrimination on the basis of liquor traits were best observed in the site where moderate moisture stress occurred during bean filling stage.

Trigonelline and 3,4-dicaffeoilquinic acid and, to a lesser extent, caffeine, showed association with good cup quality, for both green and light roasted coffee. The fact that similar correlations between cup quality and chemical attributes were observed that chemical analysis of green beans may be used as an additional tool for evaluating coffee quality (Farah et al., 2006).

Genetic analysis indicated that caffeine content in seeds was quantitatively inherited and controlled by genes with additive effects. The estimates of broad-sense heritability of caffeine content in seeds were high for both generations (Priolli et al., 2008).

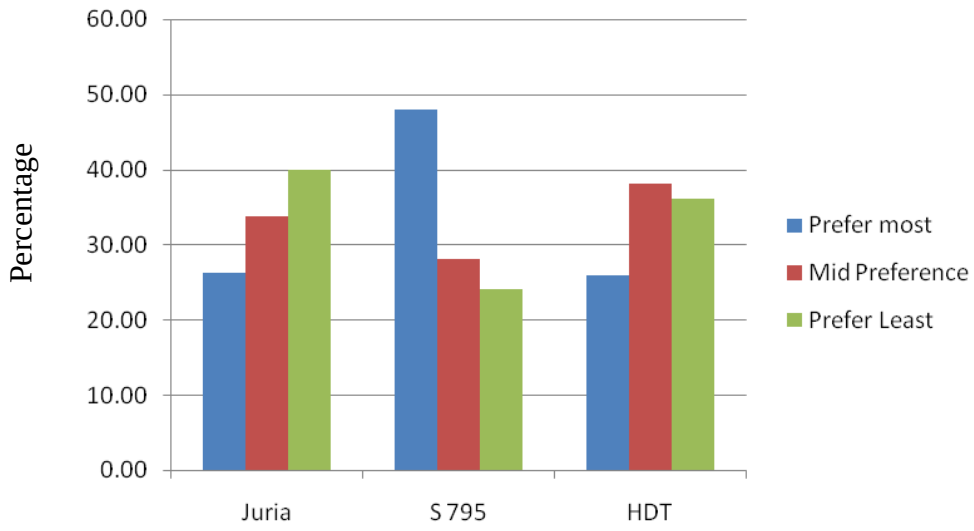

Figure 1. Preference of panelists to cup profile performed by each variety.

Cup profile of each variety

Cup profile of each variety processed by three different methods as mentioned in Figure 2. Under Ngada Bajawa highland of Flores island condition, S 795 variety performed the best cup profile compare to HdT and Juria varieties. Variety of S 795 performed excellent balance of fragrance and aroma, flavor, acidity, and body. An excellent sweetness characteristic was also detected on the variety.

Cup taste profile of HdT was slightly better than that of Juria. HdT performed better flavor, acidity, balance and aftertaste that that of Juria, however fragrance and aroma as well as body of the two varieties was similar.

Result of cluster analysis of cup profile based on three different varieties and three different processing methods as mentioned at Figure 3. There three groups of the treatments namely Group 1 (SFWPD, SFPN and SFWP), Group 2 (HFWPW), and Group 3 (JFWPW, HFPN, JFWPD, HFWPD, and JFPN).

Different processing methods have not affected on cup profile of S 795 under Flores condition. HdT was suitable to be processed by using wet hulling method. Dry hulling and pulped natural processing methods on HdT provided similar cup quality to Juria. As observed on S 795, the different processing methods application on Juria variety has not performed different cup profile as well.

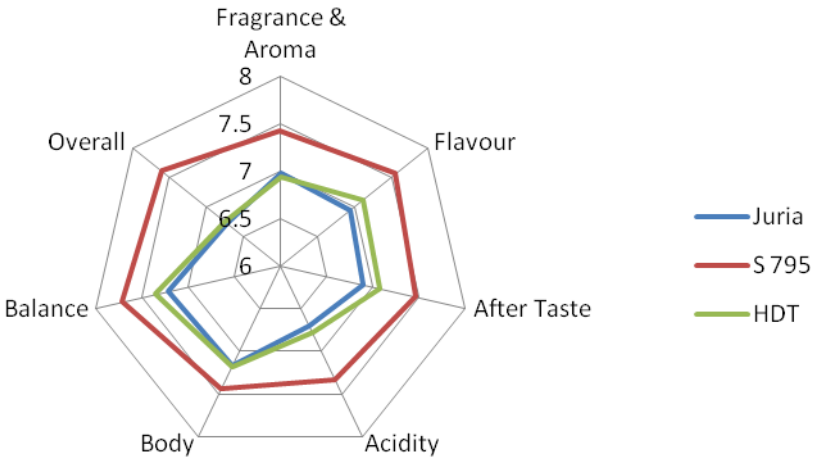

Figure 2. Average of cup profile on each variety processed by three different methods.

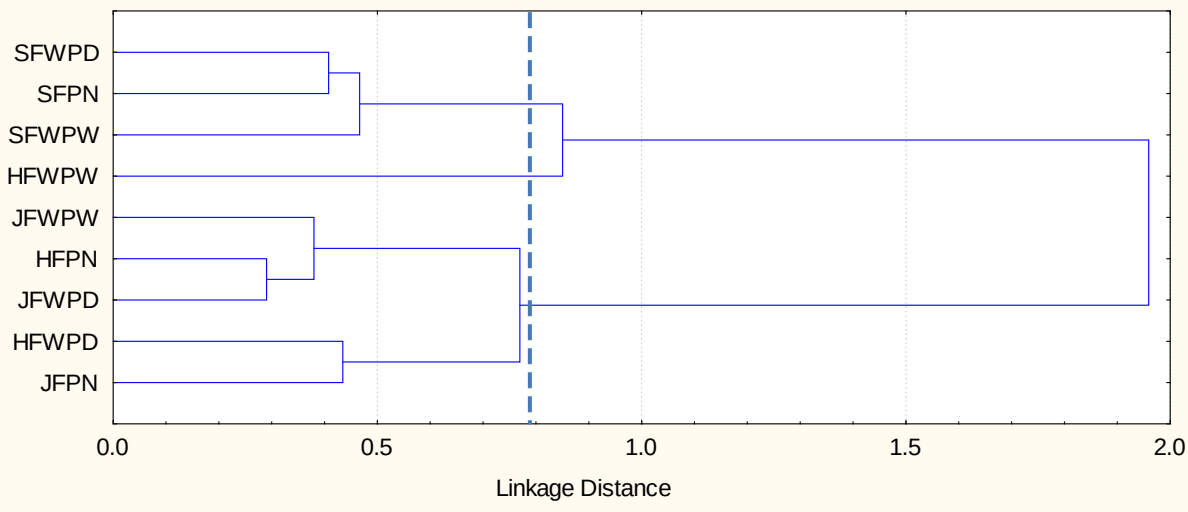

Figure 3. Cup profile similarity of three different varieties processed by three different methods. Note: SF – S 795 Flores, HF – Timor Hybrid Flores, JF – Juria Flores, WP – Wet Processed, PN – Pulped Natural (decascado), W – Wet hulling, D – Dry hulling.

Individual effect of each processing methods on S 795 variety is mentioned on Figure 4. Dry hulling method provided better flavor, acidity and after taste than that of the other two methods. In contrast, better body, balance and overall evaluation were performed by wet hulling. Pulped natural processing only performed better fragrance and aroma.

Wet hulling performed the best cup profile on HdT variety than that of the two methods, it followed by dry hulling (Figure 5). HdT apparently is not suitable to processed by pulped natural (*descacado*) under Flores condition.

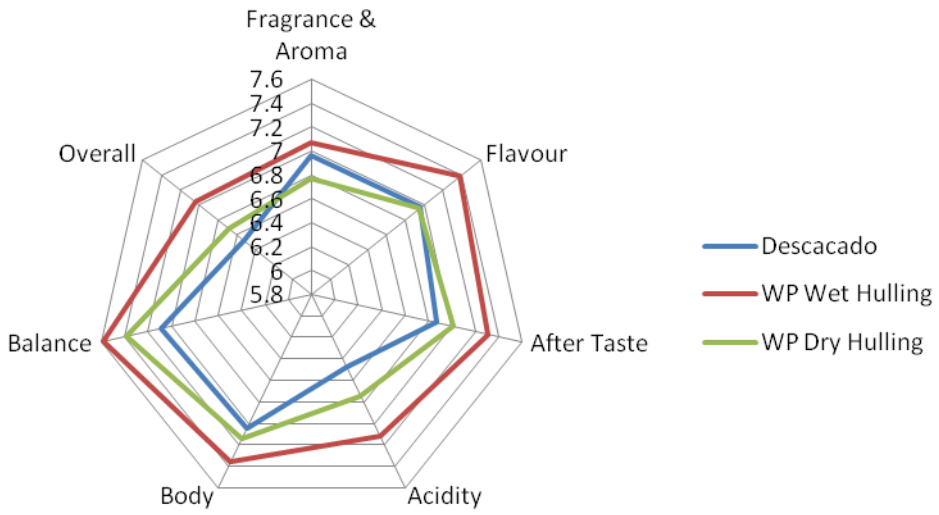

Figure 4. Cup profile performed by S 795 under three different processing methods.

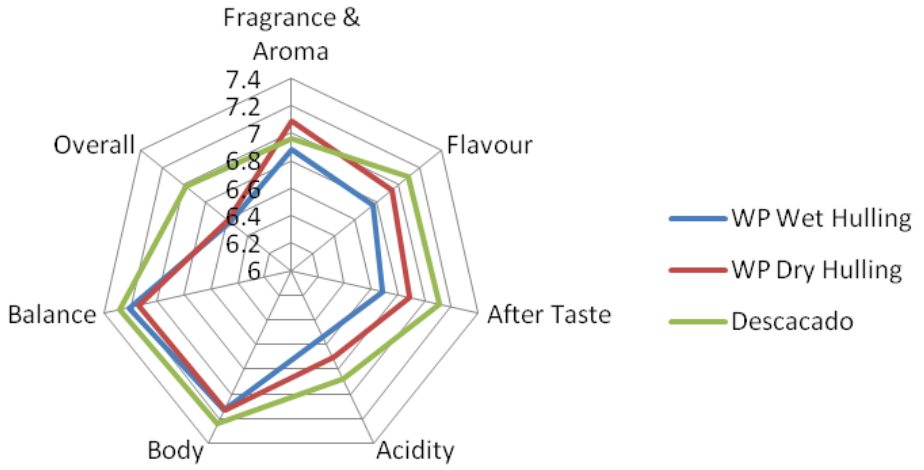

Figure 5. Cup profile performed by Timor Hybrid under three different processing methods.

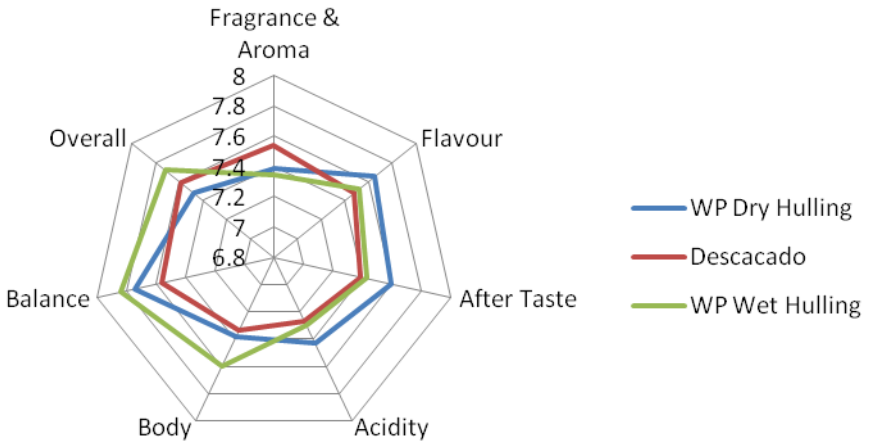

Figure 6. Cup profile performed by Juria (Typica) under three different processing methods.

Under Flores condition, pulped natural method is most suitable for Juria (Typica) variety. It performed the cup profile than that of two other methods, followed by dry hulling. Wet hulling method apparently was not suitable for Juria variety (Figure 6).

Considering variety traits in relation to growing area (*terroir*) is essential in producing specialty coffee. Sera (2001) mentioned that late cultivars should be avoided in cold regions to reduce irregular maturity and, similarly, early cultivars should not be planted in hot areas to reduce incomplete chemical maturity. Earlier or later cultivars should be also used according to the rainfall pattern in the region to avoid excessive moisture at harvest. Late cultivars should be avoided in areas prone to frosts which affect unripe fruits.

In addition, Avelino et al. (2005) mentioned that a positive relation was found between altitude and taster preferences in both *terroirs*. A negative relation was also found between yield and beverage acidity. In both *terroirs* the caffeine, trigonelline, fat, sucrose and chlorogenic acid contents were not well correlated with the sensory characteristics.

CONCLUSION

There were three dominant Arabica coffee varieties grown by smallholder farmers in Flores namely 'Juria' (*Typica* type), S 795 and Hybrid of Timor (HdT) progeny. Based on cup profile the most panelists preferred S 795. The variety performed an excellent cup profile with nice balance and strong sweet tones. In general, there was no significant effect on cup taste profile when the three different processing methods to be applied on S 795.

The best processing method to be applied on HdT variety was Wet Processed Wet Hulling, which resulted better performance of all taste components compared to Wet Processed Dry Hulling and Pulp Natural.

Local variety of Juria performed lower cup profile than that of S 795 and HdT. Pulped Natural processing performed best cup profile than that of other processing methods.

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Cup Profile Similarity and Uniqueness of Specialty Arabica Coffees from Different Origins in Indonesia

YUSIANTO, SURIP MAWARDI, SULISTYOWATI, CAHYA ISMAYADI

Indonesian Coffee and Cocoa Research Institute, Jl. PB Sudirman no. 90, Jember, Indonesia.
E-mail: otnaisuy@yahoo.com

SUMMARY

Recently Indonesia produces about 1.25 million bags of Arabica coffee, which is mostly to be marketed at specialty segment using geographic name. Specialty coffee will play more important role in the coming years. A preliminary study to evaluate cup taste profile similarity and uniqueness of specialty Arabica coffees from different origins in Indonesia was conducted at the Sensorial Analysis Laboratory of the Indonesian Coffee and Cocoa Research Institute (ICCRI) in 2008 and 2009 by involving specialists from ICCRI as well as domestic and overseas coffee industries. The coffee samples were collected from different Arabica coffee growing areas consisted of 138 samples and 51 samples in 2008 and 2009, respectively. Cupping evaluations were carried out over grade 1 of green coffee 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. Data collected were analyzed by using multivariate statistics. The result showed that specialty coffees from Indonesia having different cup profile. The coffees can be divided into three groups namely Group A (dominated by coffee from Sulawesi, Group B (dominated by coffee from Java and Bali), and Group C (dominated by coffee from Sumatra). Group a performed significantly different taste to the other two groups for all taste components. However, there were similarity on components of flavor and sweetness on Group B and Group C. Members of Group A were Toraja coffee and Sumatra coffee pea berry (sub group 1), Sumatra coffee jumbo bean (sub group 2) as well as Kalosi coffee and Blue Java coffee (sub group 3). Main unique characteristics observed by this group were mild, floral, complex flavor, chocolaty and fruity in addition to nice balance. Members of Group B were Bali Kintamani coffee, Java Estate coffee and Flores Bajawa coffee (sub group 1), Sumatra Mankuraja coffee (sub group 2) as well as Java Sumbing coffee (sub group 3). Main unique characteristics observed by this group were clean, fresh, spicy, rich acidity and lemony. Members of Group C were Sumatra Lintong and Java Preanger (sub group 1), Sumatra Mandheling and Sumatra Sidikalang (sub group 2), as well as Sumatra Siborong-borong, Sumatra Gayo coffee and Blue Flores organic (sub group 3). Main unique characteristics observed by this group were complex flavor, rich body, good balance and fresh.

INTRODUCTION

Recently world coffee market is segmented based on product characteristic, namely commercial and specialty. A study on specialty coffee consumption trend in the USA showed that the consumption of specialty coffee of adult peoples increased 3% from 14% in 2007 to be 17% in 2008 (NCA., 2008). Trend on specialty coffee consumption also observed in the other coffee consuming countries (Mawardi, 2009). The Coffee character is derived from

complex interactions between the four key factors of, those are Coffee Variety, Growing Environment, Farm Management, and Processing chain (Marsh, 2009).

Indonesia is well known as Robusta coffee producer, but during last 5 years the production of Arabica coffee increases gradually. By 2006 the country exported Arabica coffee more than 1 million bag (60,000 ton) or equivalent to about 15% of the total export annually (Association of the Indonesian Coffee Exporters, 2007). The government already set Arabica development as national priority program in order to reach about 30% of the total export. The target will be reached by intensification on farming practices and enlargement on suitable area.

The Arabica coffee is mostly to be marketed at specialty segment using geographic name. Production area of Arabica coffee is Aceh, North Sumatra, East Java, South Celebes (Sulawesi selatan), Flores, and some in Bengkulu, West Java, Central Java, and Western Papua. About 55,000 tons of the Arabica Coffee was produced in the two Northern Sumatran provinces and exported though the port of Belawan / Medan. This coffee is known as "*Sumatran Coffee*" (Aceh and North Sumatra). The proportion of coffee originating from the Provinces of North Sumatra and the Province of Aceh is unclear. Coffee flows from the production areas to the export warehouses of Medan in many ways. Most stakeholders consider Aceh produces more than North Sumatra and a reasonable estimate is: Aceh, 30,000 tons, North Sumatra, 25,000 tons. Thus, over 85% of the Arabica produced in Indonesia is from Northern Sumatra (Marsh, 2009). Specialty coffee will play more important role in the coming years.

A preliminary study to evaluate cup taste profile similarity and uniqueness of specialty Arabica coffees from different origins in Indonesia was conducted at the Sensorial Analysis Laboratory of the Indonesian Coffee and Cocoa Research Institute (ICCRI) in 2008 and 2009 by involving specialists from ICCRI as well as domestic and overseas coffee industries.

MATERIALS AND METHOD

The coffee samples were collected from different Arabica coffee growing areas consisted of 138 samples and 51 samples in 2008 and 2009, respectively. Cupping evaluations were carried out over grade 1 of green coffee 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 (SCAA, 2009). Data collected were analyzed by using multivariate statistics.

RESULT AND DISCUSSION

The result showed that specialty coffees from Indonesia having different cup profile. The coffees can be divided into three groups namely Group A (dominated by coffee from Sulawesi, Group B (dominated by coffee from Java and Bali), and Group C (dominated by coffee from Sumatra). Group a performed significantly different taste to the other two groups for all taste components. However, there were similarity on components of flavor and sweetness on Group B and Group C.

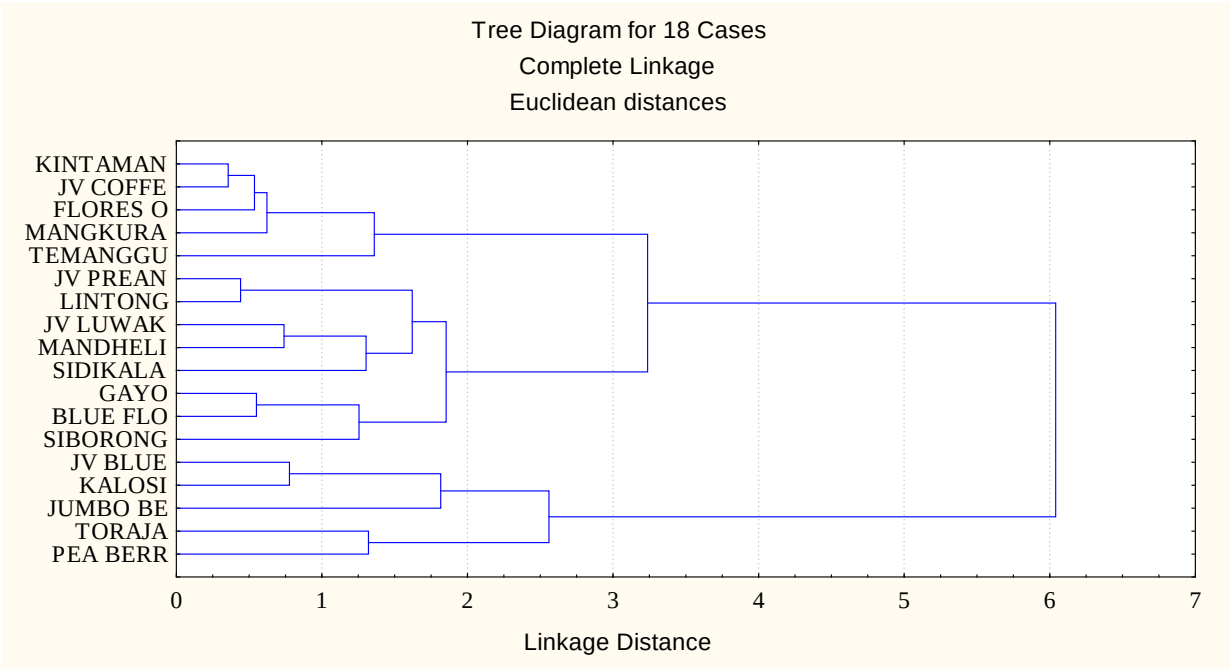

Figure 1. Clustering of Indonesian Arabica Specialty coffee basic on the flavor profile.

Notes :

Coffee codes	***Description***	***Coffee codes***	***Description***
KINTAMAN	*Bali Kintamani Coffee*	*FLORES O*	*Flores Organic Bajawa Coffee*
MANGKURA	*Mangkuraja Coffee*	*KALOSI*	*Kalosi Coffee*
JV PREAN	*Java Preanger*	*TORAJA*	*Toraja Coffee*
TEMANGGU	*Java Sumbing Coffee*	*LINTONG*	*Sumatran Lintong*
JV LUWAK	*Java Luwak Coffee*	*MANDHELI*	*Sumatran Mandheling*
JV BLUE	*Blue Java*	*PEA BERR*	*Sumatran Pea berry*
JV COFFE	*Java Coffee*	*JUMBO BE*	*Sumatran Jumbo Bean*
GAYO	*Gayo Mountain coffee*	*SIBORONG*	*Sumatran Siborong Borong*
BLUE FLO	*Blue Flores Organic*	*SIDIKALA*	*Sumatran Sidikalang*

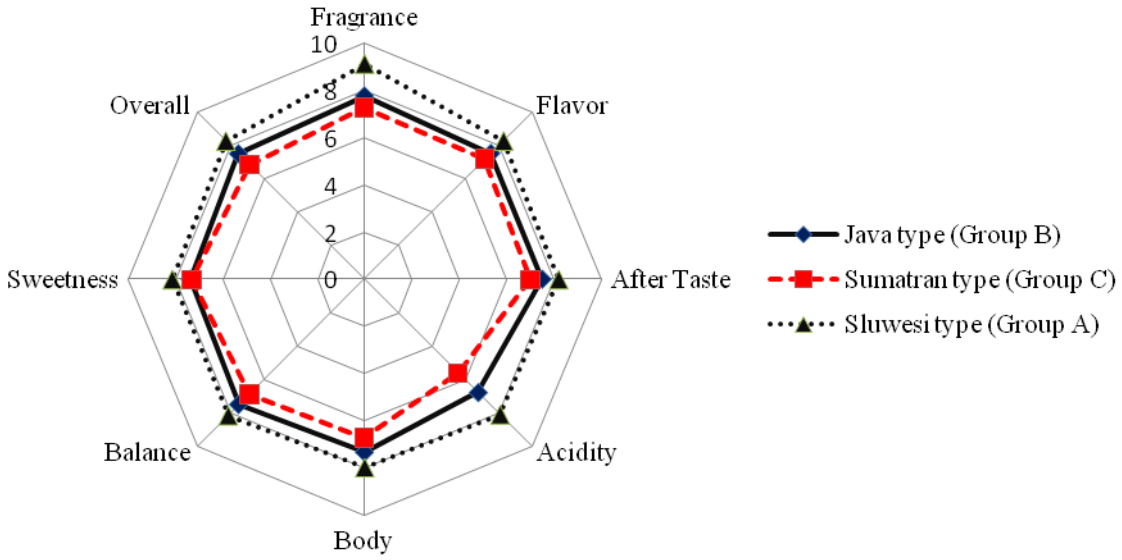

Figure 2. Flavor profile of Indonesian Specialty Coffee.

Table 1. Flavor profile of Group A of Indonesian Specialty Coffee (Sulawesi Type).

Sulawesi type	Fragrance and Aroma	Flavor	After Taste	Acidity	Body	Balance	Sweetness	Overall
Subgroup 1								
Kalosi coffee	8.58	8.22	8.11	7.92	7.99	8.15	8.17	8.30
Blue java	8.80	7.98	8.00	7.98	7.84	8.02	7.56	8.02
Subgroup 2								
Sumatran jumbo bean	10.00	8.33	8.00	8.00	7.00	7.75	8.00	8.00
Subgroup 3								
Toraja coffee	8.88	8.76	8.65	8.58	8.53	8.76	8.48	8.87
Sumatran Pea berry	9.43	8.23	8.28	8.03	8.48	8.13	8.48	8.31

Table 2. Uniqueness and specific character of group a (Sulawesi type) of Indonesian Specialty Coffee.

Sulawesi Type	Uniqueness and Specific Character
Subgroup 1	
Kalosi Coffee	Clean clear. Smooth, floral, complex, nice acidity, sweet, fruity, salty. Slightly earthy, and herbal.
Blue Java	Full aromatic, chocolaty, astringent, floral, harsh.
Subgroup 2	
Sumatran Jumbo Bean	Sweet chocolaty, fresh, fruity, floral, high acid, Sumatran
Subgroup 3	
Toraja Coffee	Complex flavor, fresh bright, chocolaty, clean, sweet, nice herbal, spicy, excellent acidity, good balance. Astringent, grassy.
Sumatran Pea berry	Complex, rich. Herbal, floral mild, fruity, fresh, chunky. Dark chocolate aftertaste. Light green.

Table 3. Flavor profile of Group B of Indonesian Specialty Coffee (Java Type).

Java Type	Fragrance and Aroma	Flavor	After Taste	Acidity	Body	Balance	Sweetness	Overall
Sub Group 1								
Bali Kintamani Coffee	7.79	7.58	7.47	6.48	7.18	7.40	7.50	7.45
Java Coffee	7.80	7.56	7.55	6.43	7.19	7.40	7.16	7.42
Flores Bajawa Coffee Organic	8.03	7.58	7.52	6.63	7.31	7.51	7.12	7.62
Average	7.87	7.57	7.51	6.51	7.23	7.43	7.26	7.50
Sub Group 2								
Mangkuraja Coffee	7.67	7.41	7.36	6.84	7.41	7.57	7.38	7.53
Sub Group 3								
Java Sumbing Coffee	7.54	7.58	7.58	7.58	7.58	7.67	7.58	7.58

Table 4. Uniqueness and Specific Character of Group B of Indonesian Specialty Coffee (Java Type).

Java Type	Uniqueness and specific character
Sub Group 1	
Bali Kintamani Coffee	Very clean rich, sweet, apricot, Zen fruit, bright acidity, clear lemon, dark chocolate, spicy, herbal.
Java Coffee	Clean, full aroma, sweet, gentile, rich, fresh vegetable, chocolaty, medium body, potato, smooth, ginger aroma
Flores Bajawa Coffee Organic	Full aroma coffee floral, nice sweetness, fruity, clean, very balanced, mild buttery, very good acidity, after taste, citrus. Potato flavors, mild aroma, herbal, caramel, chocolate, very good complex, good balance.
Sub Group 2	
Mangkuraja Coffee	Very good aroma, complex and rich flavor, floral, sweet, good body and balance. But sours, winy, green, vegetable, potato, some earthy and harsh.
Sub Group 3	
Java Sumbing Coffee	Citric acid, lack body, grassy, aromatic, floral, very nice balance

Table 5. Flavor profile of Group C of Indonesian Specialty Coffee (Sumatra Type).

Sumatra Type	Fragrance aroma	Flavor	After Taste	Acidity	Body	Balance	Sweetness	Overall
Subgroup 1								
Java Preanger	7.09	7.20	7.07	6.48	6.65	6.99	7.09	6.99
Sumatra Lintong	7.25	7.33	7.00	6.17	6.50	7.00	7.25	7.00
Subgroup 2								
Java Luwak Coffee	7.23	7.16	7.14	5.36	7.03	6.82	7.41	6.76
Sumatra Mandheling	7.50	7.25	7.17	5.00	6.83	7.17	7.50	7.17
Sumatra Sidikalang	7.58	7.50	7.33	5.67	6.50	7.50	7.58	7.50
Subgroup 3								
Sumatra Siborong Borong	7.58	7.17	7.00	5.33	6.33	6.33	7.58	6.33
Gayo Mountain Coffee (Semi Washed)	7.05	6.95	6.62	5.38	6.68	6.58	7.03	6.57
Blue Flores Organic	6.93	7.11	6.91	5.46	7.07	6.55	6.88	6.55

Table 6. Uniqueness and Specific Character of Group C of Indonesian Specialty Coffee (Sumatra Type).

Sumatra Type	**Uniqueness and specific character**
Subgroup 1	
Java Preanger	Winy, green, grassy, some earthy flavor.
Sumatran Lintong	Clean, astringent.
Subgroup 2	
Java Luwak Coffee	Floral, sweet, mild, spicy, complex, light earthy.
Sumatran Mandheling	Complex flavor, good balance
Sumatran Sidikalang	Clean, fresh, high acidity, good balance. Green, grassy.
Subgroup 3	
Sumatran Siborong Borong	Clean, winy, fruity, balance
Gayo Mountain Coffee	Strong, very good and strange fragrance, complex flavor, clean, sweet, good balance, mild clean, fruity, balance, strong body, salty, nice, syrupy, high sweetness.
Blue Flores Organic	Complex and excellent flavor, spicy, good balance, fresh, clean

Sulawesi type

Members of Group A were Toraja coffee and Sumatra Coffee Pea Berry (sub group 1), Sumatra Coffee Jumbo Bean (sub group 2) as well as Kalosi Coffee and Blue Java Coffee (sub group 3). Main unique characteristics observed by this group were mild, floral, complex flavor, chocolaty and fruity in addition to nice balance.

Arabica Coffee from Sulawesi is mostly produced from Enrekang, Tana Toraja and Mamasa districts. The high level area may produce Arabica Coffee with very strong fragrance, aroma and flavor. The most cultivated variety is S-line type. The unique character is a distinct floral and flowery. Other coffee origin may be produce Sulawesi characters are Sumatra Jumbo Bean, Sumatra Peaberry, and Blue Java. The most popular of Sulawesi Coffee is Toraja Coffee. Reputation of Toraja coffee was initiated at an advertisement of Kimura Coffee Co. In 1934 as follow: "In Celebes, an island of the Dutch East Indies there is a coffee which is produced in a very low quantity. Its aroma and flavor is recognized as the best in the world, and it is also said the quality is better than that of the famous coffee of Mandheling from Sumatra. Cup taste characteristics: excellent harmonic taste blend of acidity and bitterness (Mawardi, 2009).

Java Type

Members of Group B were Bali Kintamani Coffee, Java Estate Coffee and Flores Bajawa Coffee (sub group 1), Sumatra Mankuraja Coffee (sub group 2) as well as Java Sumbing Coffee (sub group 3). Main unique characteristics observed by this group were clean, fresh, spicy, rich acidity and lemony.

Java coffee is full washed coffee, that's produced by Government Estate in East java. In the world market, Java Coffee has well known trade mark; those are Blawan, Jampit, Kayu Mas, and Pancur. Java Coffee trade mark had been registered in Directorate General for Intellectual Property Rights, Ministry of Law and Human Rights, Indonesia by Law Office Badrulzaman, Riza & Dewi. In USA, Java Coffee had been registered in Patent and Trade Mark Office (PTO) US Department of Commerce by Lawyer Collard & Roe, P.C. In 1997, Java Coffee had received honor "Taste of The Harvest" on SCAA Annual Conference & Exhibition IX, April 1997 (PT. Perkebunan Nusantara XII, 2006). Uniqueness of traditional "Java Coffee" is Clean, full aroma, nutty, light body, balance, smooth, sweet, light body, fresh vegetable, Chocolaty and spicy tone (Mawardi, 2009). Uniqueness of "New Java coffee", that's produced by small holder, is full aromatic, clean, low body, winy, floral, fruity, complex flavor, balance, chocolaty.

The Kintamani Bali coffee beans size is generally larger than the Arabica coffee beans in average from other origin in Indonesia. The main taste profile of Kintamani Bali Arabica coffee is: (1) a medium to high acidity, (2) good to very good aroma quality and intensity, (3) a fruity taste (often lemony), (4) a medium body, a not too high bitterness, (5) a very light astringency and (6) a clean cup, free from defects. This profile is almost similar to Java coffee, when the Java normally provides spicy note. However, Kintamani Bali coffee taste profile is different from Sumatra (Mandheling) coffee, which normally performs complex aroma and flavor as well as strong body (almost syrupy) in addition to lower acidity However, by 2006 Kintamani Bali Arabica coffee in one the most expensive coffee in Indonesia. In 2008 the price at farmer gate was about 310 cents USD per kg of unsorted green coffee (Mawardi, 2009).

The pilot project has provided fruitfully results so that a certificate of GI protection for Kintamani Bali Arabica coffee has bees issued by the Directorate General for Intellectual Property Rights, Ministry of Law and Human Rights, on 5 December 2008. The name of the product is 'Kintamani Bali kopi Arabica' (Mawardi, 2009).

Flores-Bajawa Coffee is produced in Ngada highland, Flores Island, Eastern Sunda Lesser, low rainfall. The best Flores-Bajawa Coffee is produced by Full wash processing method in some Central processing Units. The varieties in Flores are Typica/Juria, Hibrido De Timor, S-Line (S795), Andongsari 1, Kartika, and Caturra Yellow. S 795 is the prefferest variety. Typica variety is not so preffered, because of the leaf rust desease and low production.

Sumatra type

Members of Group C were Sumatra Lintong and Java Preanger (subgroup 1), Sumatra Mandheling and Sumatra Sidikalang (subgroup 2), as well as Sumatra Siborong-borong, Sumatra Gayo coffee and Blue Flores organic (subgroup 3). Sumatran type is mostly produced by Wet Hulling Process ("*Giling Basah"*). In this process, coffee cherries are mechanically pulped, then fermentation for up to a day, and washing. The parchment coffee is partially dried in the sun until 30% to 35% moisture content, and hulling. At the green bean stage, "Wet Hulling" coffee has a distinctive bluish color. Main unique characteristics observed in this group were complex flavor, rich body, good balance and fresh. Northern Sumatera and Sulawesi appear to be the only two places in the world that use this process (Marsh, 2009). Sumatran coffee is exported out of Medan under a range of names that indicate regions of production, like Lintong, Sidikalang, Mandheling (North Sumatra) Gayo, Takengon, Gajah Mountain, Tawar Laut (Aceh,) (Marsh, 2009). Varieties of North Sumatra are Hibrido de Timor, S-line, and Catimor type. Lintong coffee is produced in Slope area between Pinapah and Sihab Mountain, south of Toba Lake, North Sumatra, and high rainfall. The specific characters are Complex flavor and aroma, full body, low to medium acidity, good balance. Mandheling coffee is produced in Northern Sumatra highland, medium to high rainfall.

Gayo Mountain Coffee is produced in Gayo Highland (Aceh Tengah/Takengon). The cultivated varieties are Catimor type, Hibrido de Timor, Bor Bor (the second generation of Hibrido de Timor), S-line (S 288, S 795). Bergendal, Typica type, is the old variety, but now is only a few. The coffee processing is Full wash wet hulling (Mawardi, 2009). The uniqueness flavor of Gayo Mountain Coffee are strong fragrance, complex flavor, high acidity, high body, high balance mild, fruity, salty, nice, syrupy, high sweetness, rich (Mandheling type). The extra characters are herbal, green, and grassy (Mawardi et al., 2008).

Java Preanger and Blue Flores Organic have Sumatra flavor characters, because of the varieties and processing similarity (Wet Hulling). Java Luwak Coffee have Flavor Profile similar to Sumatra, those are high body, low acidity, and some what earthy. 'Kopi Luwak' is produced by involving native animal behavior "luwak" (*Paradoxorus hermaproditus*). The coffee beans in Luwak ingestion is between 3-4 hours, and dropped as wet parchment. The coffee parchment should be washed and dried before hulled (Mawardi, 2009).

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A Novel Approach to Triphenyltetrazolium Staining As a Quality Control Tool for Green Coffee: Correlation with Aging, Geographical Origin and Sensory Analysis

L. DEL TERRA, M. BARNABÀ, F. SILIZIO, L. NAVARINI

illycaffè SpA - Via Flavia 110, 34147 Trieste, Italy

SUMMARY

TTC (2,3,5-triphenyltetrazolium chloride) is widely used in the seed industry as a staining agent to evaluate the viability of seeds. This molecule is easily absorbed in plant tissues, where the presence of active dehydrogenases reduces it to the insoluble red dye triphenylformazan. In this way the live tissues are stained in bright red while the dead tissue remains unstained, providing an easy and effective way to evaluate seed viability.

In the coffee industry there is usually no need for such a test, because the beans are not meant for germination, being instead destined to the destructive process of roasting and grinding. Moreover, considering that the embryo constitutes only a minimal part of the weight of the seed, its viability was not usually considered a relevant parameter for the quality of the beverage. However, recent advances in the study of the physiology of the coffee bean suggest that the metabolism of the seed remains active for most of the post-harvest processing. This can have an influence on the aging behaviour of green coffee during storage, as it has often been observed that different treatments and origins have different “shelf lives”, especially with regard to the development of “woody” and “stale” off-flavours. This led to the speculation that a correlation could be found between the viability of the coffee endosperm tissue (which constitutes the vast majority of the bean) and the aging behaviour.

The present work investigated the subject using TTC staining coupled to an image analysis software, in order to measure the percentage of stained tissue in a given seed sample. The output is a quantitative index (not correlated to the germinative capability of the seeds) which was used to monitor the storage of 40 coffee lots from 5 geographical origins (Ethiopia, India, Costa Rica, El Salvador and Guatemala) over the period of one year. At each time-point of the experimental campaign, the lots underwent TTC staining and parallel sensory analysis by a panel of trained judges, in order to monitor the insurgence in the cup of aging-related defects.

The TTC index gradually decreased in all the samples during the course of the campaign, reflecting the general loss of viability of the stored green coffee. However, different origins showed different staining behaviours, as well as different speeds in developing cup defects. These results suggest that in the coffee industry, TTC staining could be a useful tool for monitoring the aging behaviour of green coffee lots and the insurgence of storage-related defectiveness.

INTRODUCTION

After harvesting, the coffee seed undergoes a series of treatments, whose purpose is to prepare it for shipment and storage without compromising its quality, which is largely defined by the absence or presence of cup defects. The origin of the most common of these off-flavours, such

as “woody”, “fermented” and “stinker”, can be traced back to incorrect post-harvest processing, drying and/or storage. In particular, it is well known that during prolonged storage, green coffee tends to develop a woody taste (Kurzrock et al., 2004). The main goal of coffee post-harvest processing is the removal of water from the seed, to prevent fermentation and mould development. Green coffee is considered ready for storage when it reaches a water content of about 11±1% w/w (Teixeira et al., 2005).

From a physiological standpoint, seeds are classified in three categories according to their drying behaviour (Roberts, 1973). “Orthodox” seeds can survive for extremely long periods at very low water content (around 5% w/w) and subzero temperatures. Conversely, “recalcitrant” seeds do not withstand desiccation and survive for a short time only if kept imbibited. The coffee seed is classified as having an “intermediate” drying behaviour (Ellis et al., 1990), as it can be maintained for some months at 10-12% w.c. but it doesn’t tolerate extreme drying and/or freezing temperatures. It is well known that coffee seeds rapidly lose germinative potential during the first few months after harvest, and it has been suggested that the development of defectiveness may be related to the loss of viability during storage. In recent years, various studies have attempted to correlate the storage-related decline in quality with loss of viability and with various metabolic parameters of the seed, such as oxidative stress, lipid hydrolysis, and amino acid or carbohydrate levels (Dussert et al., 2006; Selmar et al., 2008).

Viability is usually measured in terms of germinative capability and, apart from germination tests, a standard method for checking the viability of a seed sample is the tetrazolium test (International Seed Testing Association, 1999). This assay is based on the colourless salt 2,3,5-triphenyltetrazolium chloride which is easily absorbed into plant tissue where, in the presence of active dehydrogenases (evidence of biological activity in a live tissue), it is transformed into the non-diffusible red dye 1,3,5-triphenylformazane. In this way it is possible to distinguish by visual analysis the red-stained live tissue from the unstained, dead parts of the seeds. This method usually focuses on the embryo, which must obviously be alive for the seed to germinate. The coffee seed, however, is largely made up of endosperm, with the embryo taking up only a negligible amount of volume and weight. Moreover, the embryo is the most delicate part of the seed, while the endosperm is a very tough reserve tissue. It is not unimaginable, therefore, that during processing and storage the embryo can be the first tissue to lose viability, while the endosperm cells remain alive.

This is an important consideration when we take into account the commercial use of coffee: the roast&ground product is made up almost exclusively of endosperm tissue, and therefore seed viability, measured in terms of embryo germination, may not be the most accurate measure of quality.

Similar considerations were made by Schafer et al. (2002) regarding hazelnuts, whose processing is in some ways comparable to coffee (the seed is harvested, dried and roasted). He devised a variant of the TTC method to measure the percentage of live tissue in a given hazelnut sample, independent of germinative capability.

The present work proposes an adaptation of Schafer’s protocol to monitor green coffee aging through TTC staining coupled to electronic image analysis and sensory evaluation.

MATERIALS AND METHODS

Selection of samples

Green coffee (*Coffea arabica* L.) was sampled from freshly harvested, wet-processed commercial lots, which were preliminarly evaluated by a panel of trained judges and selected for the absence of aroma defects. 8 lots were chosen from each of 5 origins (Costa Rica, El Salvador, Ethiopia, Guatemala and India) for a total of 40 lots. The green coffee was stored for 1 year at room temperature in the dark (the room was conditioned but there was no strict control of temperature and humidity, to simulate the conditions of a conventional warehouse), and sampled at regular intervals (0, 4, 8, 12 months). At each time-point, the samples were evaluated with TTC staining coupled to cup-tasting.

TTC staining and image acquisition

At each time-point of the campaign, 3 replicates of 100 seeds from each sample were tested. The seeds were pre-imbibited overnight in water at room temperature, to facilitate cutting and absorption of the dye. The following day, after thorough washing in water, each seed was cut in two: one half was discarded, while the other was soaked overnight in tetrazolium staining solution according to Schafer et al. (2002) (1% w/v 2,3,5-triphenyltetrazolium chloride in phosphate buffer 75 mM, pH 7.5 – all reagents were purchased from Sigma).

After staining, the seed halves were accurately washed to remove excess dye and then placed on a square 120x120mm Petri dish, with the cut plane downward. The plate, covered with a black box to provide a homogenously dark background, was subsequently scanned with a flat-bed scanner (RGB colour image, resolution 300 dpi) and the resulting image saved in .psd (Photoshop) format.

Image analysis

The images were processed with Photoshop 7.0 software (Adobe Systems Inc., San Jose, California) adaptating the method of Schafer et al. (2002). A specifically designed set of actions was used to automate the image analysis process, giving maximum objectivity and reproducibility.

First, the brightness and contrast of the image were normalized, adjusting the levels of the RGB channels so that the background was uniformly black (RGB levels <10). This reduced to a minimum the inevitable differences in exposure between the images, and allowed reliable comparison between different scansions. The black background was selected ("magic wand" tool, tolerance 100, anti-alias on) and deleted. From this point onwards, the image contained only pixels corresponding to seed tissue. 4 working copies of the image were created; from the first one, the green channel was selected, a threshold of 128 was applied and the image inverted; this channel was multiplied with the source image. The 3rd copy was converted to "lab colour mode", and its lightness channel was subtracted from the red channel of the 2nd copy. After applying a threshold of 70, this red channel was added to the source image. From the 4th copy the red channel was selected and, after applying a threshold of 255, multiplied with the source image. Finally, the red channel histogram was visualized, the "255" frequency was selected, and the corresponding pixel count was annotated.

The whole procedure was recorded as a macro and run by a key press, so that the full analysis of a sample required less than a minute.

Sensory evaluation

The starting evaluation at Time=0 was performed by the Quality Control of illycaffè SpA, that found the commercial samples free of defects. Subsequently, the sensory analysis at 4, 8 and 12 months of storage were performed by an *ad hoc* sensory panel, as described below.

Coffee batches of 100 g were prepared to a medium roast using a laboratory roaster. Samples were evaluated in *espresso* preparation using a professional "La Marzocco" model 2EE espresso machine; each cup was prepared from 7.0 ± 0.1 g of ground coffee; particle size was adjusted in order to obtain 25.0 ml of beverage in 25.0 s of percolation. One origin per day was tested, for a total of 8 coffee samples per session; samples were presented to judges in random order, each sample was presented in a white plastic glass coded with a random 3-digit number.

Sensory analysis was conducted with a panel of judges expert in evaluation of coffee; judges had to evaluate the presence of off-flavour in samples and score it in a discrete 9-point scale in which 1=none and 9=very intense. Evaluation forms were created using FIZZ FORMS software (version 2.30B, BIOSYSTEMES, Couternon, France). A minimum of 7 judges were present at each session. Panelists evaluated samples in a dedicated room built in a compliance with the ISO standard (ISO 8589: Sensory analysis -- General guidance for the design of test rooms).

Data analysis

Data were elaborated using software XLSTAT (XLSTAT version 2010, ADDINSOFT, NY, USA) and FIZZ. ANOVA (analysis of variance) was performed for both image and sensory data; the explanatory variables were always considered as qualitative. In both sensory and image data a Type III Sum of Squares analysis was computed to estimate the influence that removing an explanatory variable has on the fitting of the model, all other variables being retained, as regards the sum of the squares of the errors (SSE), the mean of the squares of the errors (MSE), Fisher's F, or the probability associated with Fisher's F. The lower the probability, the larger the contribution of the variable to the model, all the other variables already being in the model.

In the analysis of sensory data, the judge effect was neglected because the panel was not the same during the whole experiment. Judge performances were evaluated session by session by analyzing the correlation index between the scores of each panelist and the mean of scores of the panel. In case of poor correlation (<0) the judge was excluded from the final dataset used for calculation.

As criterion for multiple comparison the Tukey HSD (HSD: Honestly Significant Difference) test was chosen. Because the sensory analysis at Time=0 was performed by a different panel (see above), the statistical calculations take into account only sensory data coming from the sessions at 4, 8 and 12 months of storage.

RESULTS AND DISCUSSION

The electronic image analysis yielded a quantitative measure of the percentage of stained endosperm tissue in each sample of 100 green coffee seeds. The final result was a two-color, black and white image in which the parts of the seeds stained in bright red corresponded to the

white pixels, and every other colour (including pink and dark red) was changed to black (Figure 1).

From this step, the "Histogram" function readily gave a count of the white pixels (Red channel, level 255) over the total number of pixels in the selection. This number, multiplied by 100, was named the "TTC index" and used as the raw data for subsequent statistical analysis. Note that the TTC index is not in any way correlated to the viability and germinative capability of the embryos, but is simply the measure of the proportion of viable endosperm tissue in the seed. Theoretically, the TTC index should vary between 0 (completely dead seeds) and 100 (fully viable tissue). In practice, the actual values obtained ranged between 2 and 80, because of the settings of the electronic analysis: it was preferred to use a conservative approach to avoid oversaturation of the colours; in this way it was impossible to obtain values close to 100, but was maximized the sensitivity at medium-low staining intensities.

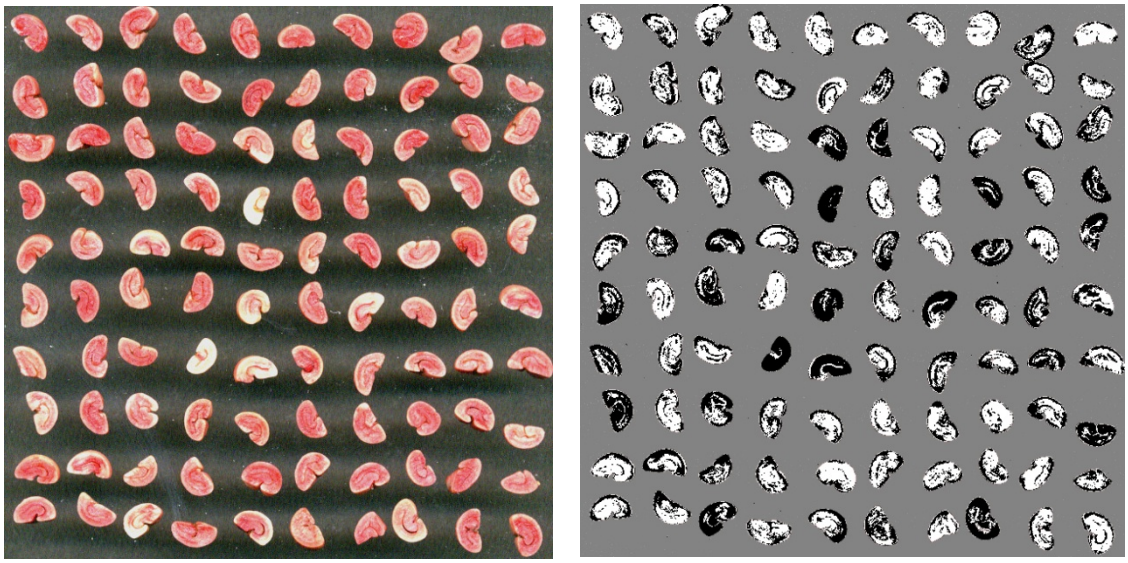

Figure 1. example of TTC staining of coffee seeds and subsequent image analysis. Left: unprocessed image, as downloaded from the scanner. Right: the same image after processing. Note how the red-stained tissue is converted to white and every other colour to black (background is represented here in grey for graphical purposes, but was deleted during actual image analysis).

At each time-point, all the data from the same origin were pooled in a single group; statistics were not elaborated on individual lots because of the high variability of the data. Table 1 reports the results of ANOVA for staining data: both factors (Origin and Storage Time) have a significant effect as well as the interaction between Origin and Storage Time. This interaction is well explaned in Figure 2 where it is easy to see the different behaviour of Guatemala in comparison to the other origins: its TTC index decreases more rapidly and this fact leads to an Origin*Storage Time interaction.

Table 1. ANOVA of TTC staining data.

Type III Sum of Squares analysis					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr>F**
Storage Time	3	31540.366	10513.455	99.280	<0.0001
Origin	4	47558.042	11889.510	112.274	<0.0001
Storage Time* Origin	12	2268.632	189.053	1.785	<0.048

As expected, the TTC index of all origins showed a gradual decrease, which was more marked in the first four months of the campaign (Figure 2). The Ethiopian lots showed the highest TTC index at all time-points, consistently being the samples with the most intense staining. Among the other origins, the Tuckey HSD test couldn't distinguish between Costa Rica, Guatemala and India, while El Salvador had a slightly lower TTC index throughout the campaign (Table 2).

It is interesting to note that, apart from Ethiopia, no origin showed a very high TTC index at the beginning of storage: this could reflect local differences in post-harvest treatments that affect the vitality of the seeds even immediately after harvest.

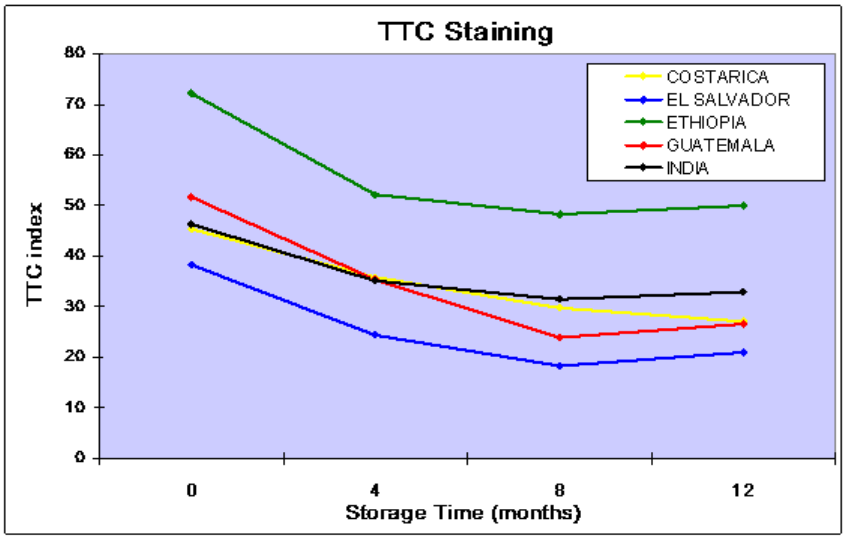

Figure 2. TTC index graph of staining data during the campaign.

Table 2. statistical analysis (Tuckey HSD test) of staining data during the campaign.

Category	**Mean**	**Groups**		
Etiopia	55.713	A		
India	36.501		B	
Costarica	34.451		B	
Guatemala	34.372		B	
El Salvador	25.509			C

ANOVA of sensory data leads to Table 3 which shows all factors to have a significant effect on the defectiveness of the samples. Interaction between Origin and Storage Time derives from the "anomalous" behaviour of Indian samples: it is strange that a sample reported as "from slight to medium defected" at time=8 months, after 4 further months of storage shows a better sensory profile with a decreased defectiveness. This fact could be explained with the changing in panel composition and with the intrinsic variability of sensory judgements.

Table 3. ANOVA of sensory data.

Type III Sum of Squares analysis					
Source	**DF**	**Sum of squares**	**Mean squares**	**F**	**Pr>F**
Storage Time	2	113.643	56.822	20.021	<0.0001
Origin	4	75.982	18.995	6.693	<0.0001
Storage Time* Origin	8	116.197	14.525	5.118	<0.0001

The sensory analysis (Figure 3) revealed that after 4 months of storage all origins had developed a barely perceptible defectiveness (between 1 and 2.5 on a 1-9 scale, where 1="no defect", 2="barely perceptible defect", 3="perceptible defect", and so on up to 9="unacceptable"). Ethiopian samples were clearly the less defective ones, as even after 12 months their average defectiveness was still less than 2. Conversely, all other origins developed some perceptible defect, which was more intense for Costa Rica, Guatemala and India and slightly less intense in the case of El Salvador (Table 4).

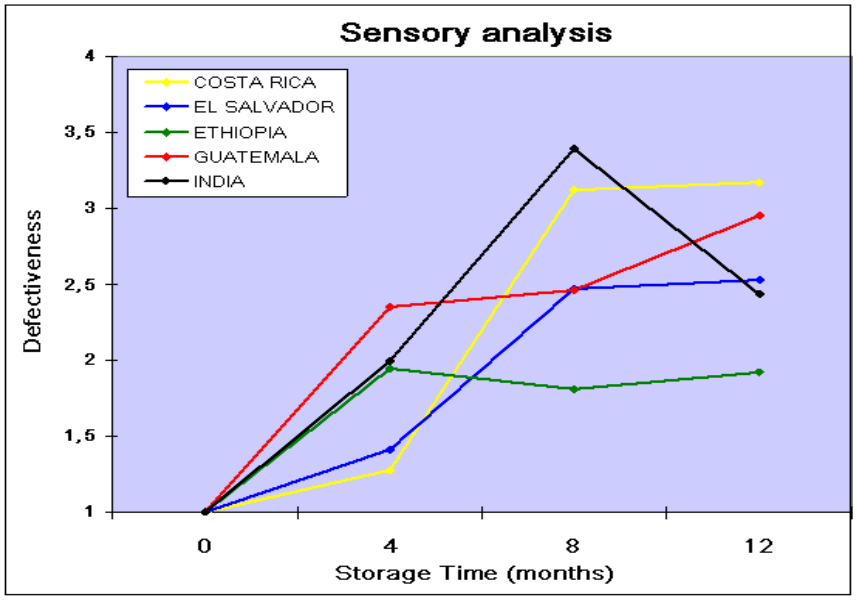

Figure 3. Defectiveness graph of sensory data during the campaign.

Table 4. statistical analysis (Tuckey HSD test) of sensory data during the campaign.

Category	Mean	Groups		
Costa Rica	2.775	A		
India	2.703	A	B	
Guatemala	2.655	A	B	
El Salvador	2.264		B	C
Ethiopia	1.879			C

CONCLUSION

From these analysis, some general conclusions can be drawn. Pooling together all data from every origin, we put in evidence the general decrease in TTC index and the corresponding development of storage-related cup defects (Figure 4). The temporal pattern of these aging dynamics is also strikingly similar, with the biggest changes happening in the first 8 months of storage, and little difference between 8 and 12 months. In this respect, the TTC index offers a reliable picture of aging behaviour.

When the different geographical origins are considered individually, Ethiopian samples clearly stand out as the coffee with the best aging performance, developing only barely perceptible off-flavours and consistently exibiting the highest TTC index (the only origin above 50). The other origins do not show such a clear-cut correlation between staining and sensory analysis. This is maybe due to the relatively short duration of the campaign, which didn't allow the coffee to develop significant off-flavours: the highest defectiveness only reached a value of 3.5 in a 1-9 scale; this obviously increased data noise and made it difficult to discriminate among the various origins.

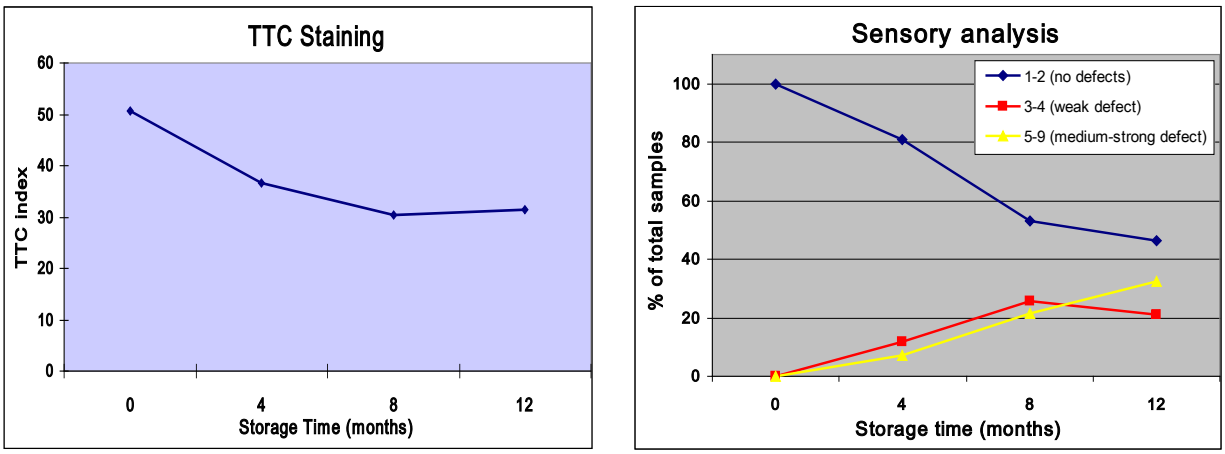

Figure 4: general aging behaviour of all green coffee lots during the campaign. Left: TTC staining. Right: sensory analysis. The sensory evaluation votes were grouped in the following categories: "1+2"="not defective", "3+4"="weakly defective", "5 to 9"= "defective".

Possible further developments of the technique may include a longer storage period and/or the testing of additional samples with a high TTC value, to substantiate the data of Ethiopian

coffee, which seem to suggest that a TTC index ≥50 corresponds to an absence of cup defects and a prolonged storage life.

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Effects of Soaking Parchment on Sensory and Some Biochemical Components of *Coffea arabica* L. Produced in Kenya

C.W. KATHURIMA

Coffee Research Foundation (CRF), P.O Box 4, Ruiru, 00232, Kenya

SUMMARY

Soaking stage has been conveniently used, as one of the prime options of increasing the capacity of a coffee factory. The objective of this study was to assess the beverage quality and the levels of caffeine, trigonelline and fat of coffees soaked for different durations up to a maximum of seven days. Seven sensory descriptors fragrance and aroma, flavour, aftertaste, acidity, body, balance and overall were assessed by a panel of seven professional judges and rated on a 10-point scale. Fat was extracted by soxhlet method using pet ether. Caffeine and trigonelline were extracted from green coffee powder by refluxing in distilled water and analysed by HPLC. Coffee subjected to different soaking periods up to seven days did not show any significant differences in fragrance and aroma, aftertaste, body, balance and overall at $P \leq 0.05$. Significant differences were observed in the flavour and acidity ($p \leq 0.05$). The levels of caffeine, trigonelline and fat did not differ significantly in coffee subjected to the different soaking durations at $P \leq 0.05$.

INTRODUCTION

Coffee is among the main export crops since its introduction by missionaries in the early 1900s' (Mwangi, 1983) and has remained an important commodity in Kenyan economy. Coffee processing transforms fresh coffee cherries into green beans of 10 to 12% moisture ready for export or for roasting (International Trade Centre, 2002). Kenya coffee is mainly fully washed with a small proportion of naturals, commonly known as buni. The unit operations involved in wet processing are described in details by Mburu (2004). Soaking in wet coffee processing is the complete immersion of the parchment under water. Studies in Kenya have shown that soaking of parchment coffee after fermentation improves the coffee quality both in colour and taste (Wootton, 1963). The soaking stage which has been conveniently used, though to a limited extent, to hold parchment in order to overcome temporary congestion at the drying stage has been under investigation as one of the prime options of increasing the capacity of a coffee factory (Mburu, 1997). Cup quality is based on the characterization of a large number of factors which are related to the biochemical compounds appearing during roasting, which arise from a smaller number of biochemical compounds present in green beans (Clifford, 1985; Macrae, 1985). Their presence or through modification could have a favourable or an unfavourable effect on the coffee cup quality. The objective of this study was to assess the beverage quality and the levels of some green bean biochemical components of coffees soaked for different durations up to a maximum of seven days.

MATERIALS AND METHODS

Processing

Cherry samples were picked from CRF-Jacaranda farm and pulped using a disk-pulper during the 2009/10 main season. The samples were fermented overnight and pre-washed in the following day and a sample drawn for skin drying (Day 0). The rest of the parchment was soaked in clean water. In the days that followed a sample was drawn from the soaked coffee and taken to dry an exercise which was repeated for 7 days. Soaking water was changed everyday. The parchment was dried to attain a moisture content of 10.5-11%.

Roasting and sensory evaluation

Roasting of green coffee was done to attain a medium roast using a Probat laboratory roaster within 24 h of evaluation and coffee allowed to rest for at least eight hours. The sensory evaluation procedure described by Lingle (2001) was followed. Seven sensory variables were assessed by a trained panel of seven and rated on a 10-point scale. For the attributes fragrance/aroma, flavour, aftertaste and balance, 1= very poor and 10 = outstanding while for acidity 1= very flat and 10 = outstanding and body 1= very thin and 10 = very bright.

Biochemical analysis

Caffeine and trigonelline were extracted from green coffee powder by refluxing in distilled water. The extract was filtered through a micro-filter 0.45µm and injected into the HPLC. Caffeine and trigonelline were analysed using Shimpack X R –ODS II 30mm idx100 mm at a wave length of 278 nm and 266 nm respectively. Fat was analysed as outlined in the AOAC (1995).

RESULTS

Table 1 shows the mean sensory ratings of the coffee subjected to different durations of soaking up to seven days. Analysis of variance on the sensory data showed that coffee subjected to different soaking periods up to seven days did not show any significant differences in fragrance and aroma, aftertaste, body, balance and overall at $P \leq 0.05$. However significant differences were noted in flavour and acidity at the same level of significance. The levels of caffeine, trigonelline and fat did not differ significantly in coffee subjected to the different soaking durations at $P \leq 0.05$ (Table 2).

Table 1. Mean of sensory attributes evaluated in coffee subjected to different soaking durations.

Soaking duration in days	Sensory characteristics						
	Fragrance	Flavour	Aftertaste	Acidity	Body	Balance	Overall
0	7.43	7.45ab	7.54	7.50ab	7.48	7.48	7.45
1	7.41	7.53ab	7.53	7.64a	7.50	7.48	7.54
2	7.41	7.57ab	7.54	7.59a	7.59	7.54	7.57
3	7.43	7.45ab	7.43	7.50ab	7.50	7.43	7.50
4	7.43	7.64a	7.59	7.63a	7.52	7.52	7.55
5	7.43	7.50ab	7.57	7.54ab	7.54	7.52	7.54
6	7.45	7.48ab	7.52	7.66a	7.55	7.48	7.55
7	7.43	7.38b	7.41	7.38b	7.46	7.39	7.43
F-test	NS	**	NS	***	NS	NS	NS
CV%	2.51	2.33	2.31	2.22	2.16	1.91	2.48

Means followed by the same suffix in the same column are not significantly different at P = 0.05.

Table 2. Mean biochemical components (caffeine, trigonelline and, fat % Dwb) of coffee subjected to different soaking durations.

Soaking duration in days	Caffeine	Trigonelline	Fat
0	1.22±0.16	1.00±0.01	16.67±0.45
1	1.05±0.21	1.10±0.05	16.91±1.27
2	1.20±0.09	1.11±0.05	16.27±0.72
3	1.23±0.29	1.06±0.05	15.88±0.19
4	1.36±0.06	1.08±0.02	16.93±0.86
5	1.27±0.21	1.08±0.02	16.18±1.70
6	1.15±0.30	1.08±0.02	16.56±1.22
7	1.20±0.05	1.04±0.06	16.81±0.94

DISCUSSION

Sensory evaluation revealed no significant differences in the coffee subjected to different durations of soaking up to seven days except in flavour and acidity. These findings are in agreement with the report of Mburu (1997) that coffee soaked for less than 7 days showed a general improvement in the overall quality. A study by Northmore (1965) showed that some bean components diffused out during the underwater soaking and associated them with improved colour of raw beans obtained during soaking. Coffees distinctive flavour is certainly the principle reason for its acceptability and enjoyment throughout the world. Trigonelline and fat are constituents which have been reported to contribute to flavour development in

coffee (Ky et al., 2001; Clifford, 1985). There is no data to support relationship between caffeine and coffee quality but the stimulating effect of coffee has been accredited to the caffeine content hence making it an important constituent of coffee. This study therefore showed that apart from maintaining the organoleptic quality, soaking parchment for up to seven days did not have a significant effect on the levels of caffeine, trigonelline and fat.

ACKNOWLEDGEMENTS

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Development of a Grinder for the Special Requirements of Ground Coffee Used in Capsules or As Coffee Powder Admixture to Other Foodstuff

T. KOZIOROWSKI, T. SCHNEIDER, H. BAUMEISTER, C. BECKMANN, U. MÖLLER-WILLENBERG

PROBAT-WERKE, Reeser Strasse 94, 46446 Emmerich, Germany

SUMMARY

PROBAT has successfully developed a grinder with individual gap sizes and adjustable rates of revolutions of the rolls as well as variable direction of rotation of the grinding rolls to each other. This flexibility offers a high individuality of grinding combination.

INTRODUCTION

Current requirements with regard to the coffee grinding result arise from the special application areas of the individual portion processing (pads, pods, capsules) and the admixture of coffee powder to other foodstuff.

Especially for the capsule market the grain distribution and small fines (dust) are of significance for the throughput behavior of the coffee drink and thus for the aroma in the cup. In contrast, only dust contents are to be generated in the course of the coffee powder production which does not involve any sensory influence on the tongue when the product is consumed.

RESULTS AND DISCUSSION

To specifically meet the requirements of the market, a new grinder for fractionizing coffee was developed. A three-stage grinding was selected which renders possible a 0.01 mm accuracy via a gap measurement of the individual grinding gaps and where the rate of revolutions of each individual roll is arbitrary within a range of 200-2000 min^{-1}.

The trials carried out showed that the changes in the rate of revolutions have a considerable influence on the grain size and grain distribution. Also the direction of rotation of the grinding rolls to each other has an influence on the grinding result.

A special fineness of x_{50} = 30 µm of the ground coffee could be achieved in addition through the selection of a special roll surface geometry.

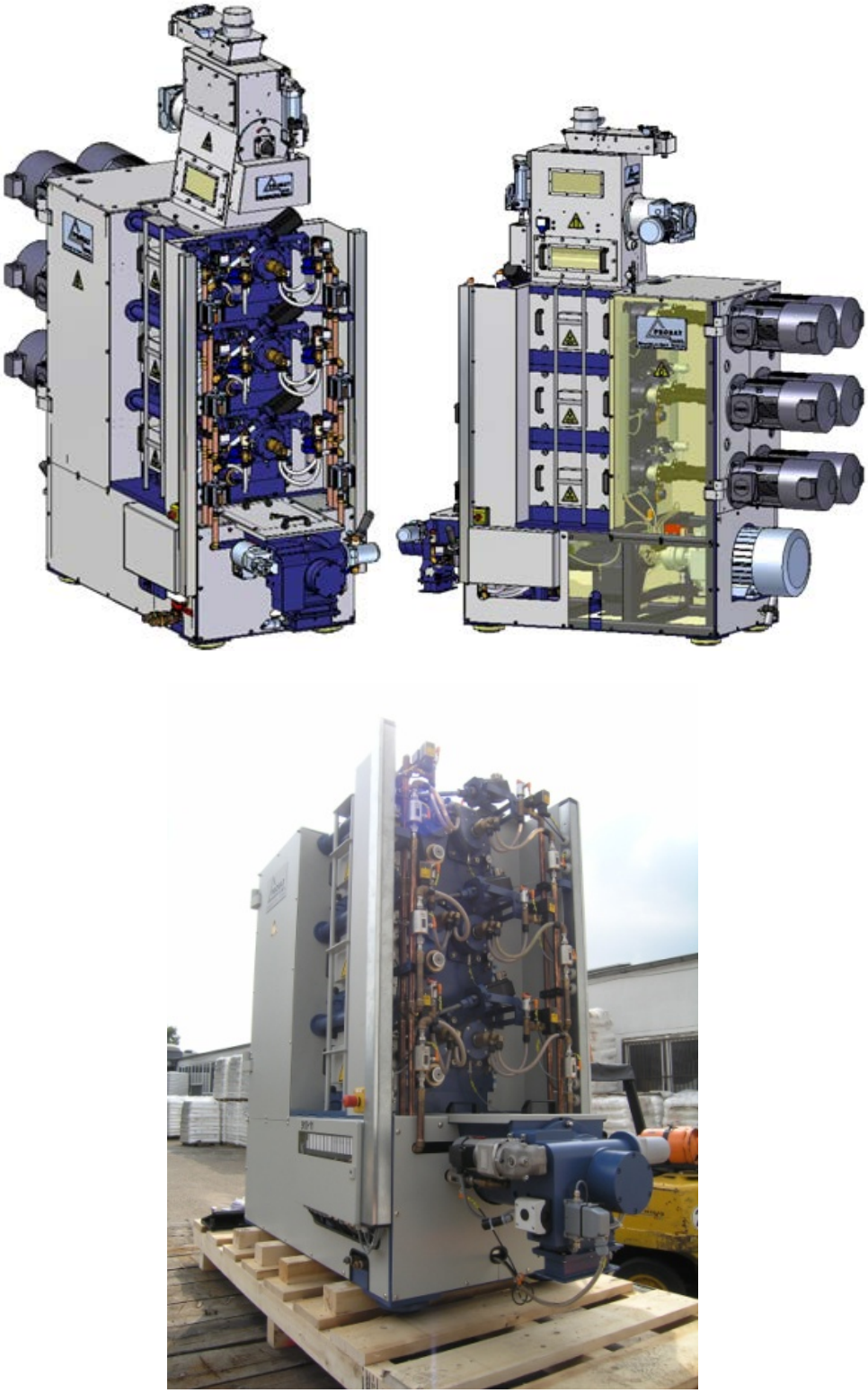

Figure 1. The PROBAT roll mill with 3 stages.

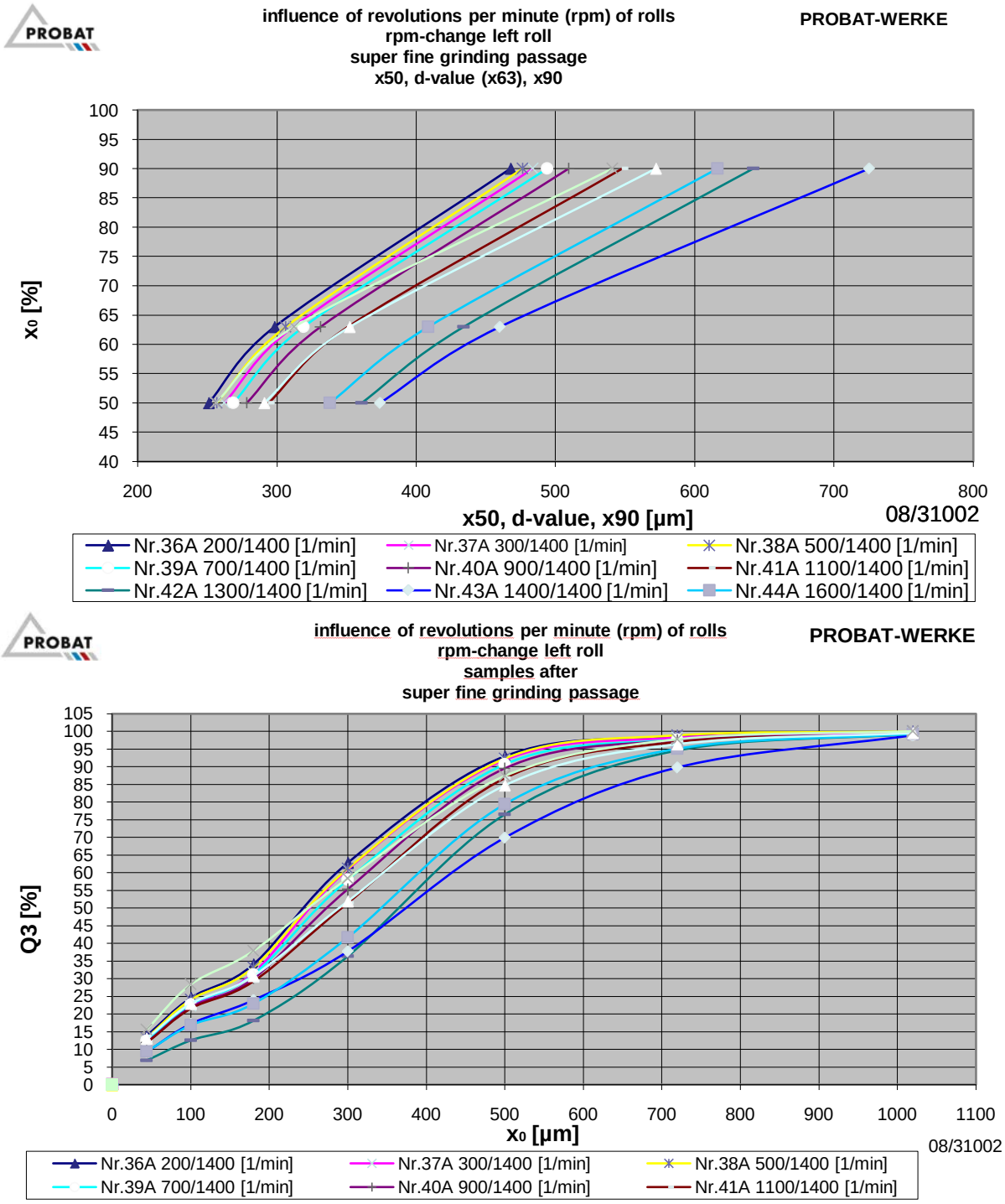

Figure 2. The influence of revolutions per minute (rpm) of rolls.

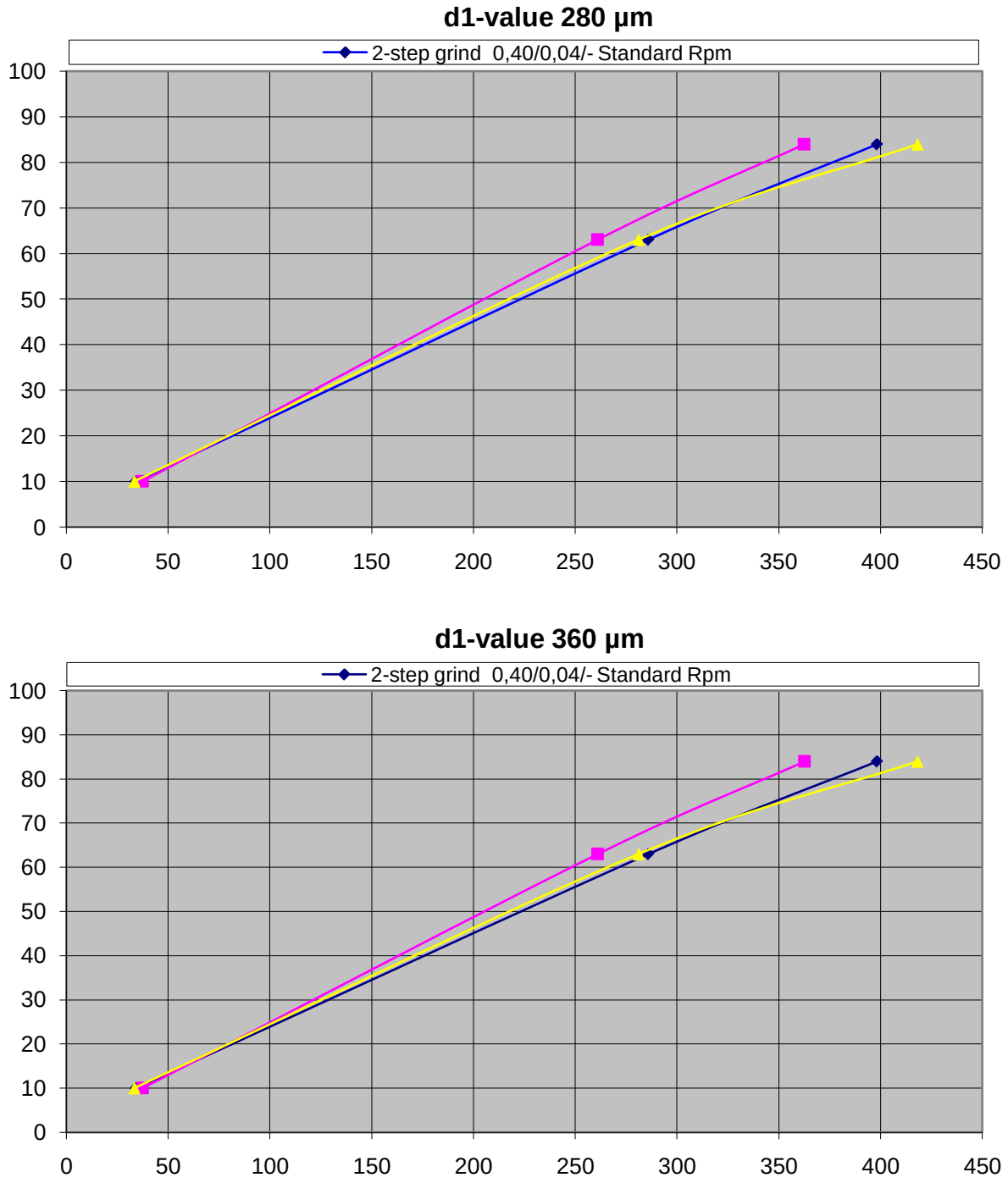

Figure 3. d1-values with 2 step grind and standard revolutions of the rolls.

CONCLUSION

It could be proved that influence can be gained on a constant grain size and the requested distribution of the fines via special rate of revolutions combinations and senses of rotation. Extremely fine degrees of grinding can also be produced so that coffee powder can be used as an aromatic admixture to various foodstuff.

Optimizing of Single Column Reactor for Robusta Coffee Beans Decaffeination

SUKRISNO WIDYOTOMO[1], HADI K. PURWADARIA[2], A.M. SYARIEF[2], SRI MULATO[1]

[1]Indonesian Coffee and Cocoa Research Institute
[2]Dept. of Agric. Engineering, Faculty of Agric. Technology, Bogor Agric.University

SUMMARY

Indonesian Coffee and Cocoa Research Institute in collaboration with Bogor Agricultural University have succeeded developing the single column reactor for decaffeination process of coffee bean. Decaffeination process with steaming and dissolving which was done together and integrating in a single column reactor would ease the handling and controlling the process. This paper is discussing the optimizing of decaffeination process in a single column reactor using ethyl acetate solvent at 10% of concentration. Optimum process condition was determined based on time, decaffeination temperature, as well as energy needed to do the process till obtain maximum 0.3% of caffeine substance in coffee bean. The research result showed that decaffeination process was initiated with 3 hours steaming and coffee bean with size criteria of A_1 and A_4 were dissolved at 90-100 °C along 10 and 8 hours, respectively. While the coffee bean with A_2 and A_3 classifications were dissolved at temperature of 60-70 °C along 12 hours. The taste of the dissolving result of low caffeine coffee bean was better relative than low caffeine coffee resulted from water solvent. Economical analysis of low caffeine coffee products with limited scale showed that production cost of low caffeine coffee powder was as much as Rp 54,960.00 per kg.

INTRODUCTION

Grounded coffee could be said decaffeinated if it has caffeine substance between 0.1-0.3 percent (Charley and Weaver, 1998). Decaffeination process can be done with soaking or combination of steaming and dissolving consecutively in a reactor separately. In food industry, decaffeination process could be conducted using water, organic, and inorganic solvent (Toledo, 1999). Long term researches of low caffeine coffee production in a single column reactor have been done by Indonesian Coffee and Cocoa Research Institute in collaboration with Bogor Agricultural University (Purwadaria et al., 2008). Ethyl acetate is one of organic compound that could be used as caffeine solvent of coffee bean (Sivertz and Desroiser, 1979). Patent regulation caused the methods and process characteristic, as well as end quality product resulted could not be published for public. At initial step, it has been done decaffeination process characterization of coffee bean using 10% ethyl acetate solvent in a single column reactor (Widyotomo et al., 2009). This research were objected to determine the optimal condition of Robusta coffee bean decaffeination process in a single column reactor with 10% ethyl acetate ($C_4H_8O_2$) solvent.

MATERIAL AND METHODS

Main material research used was Robusta coffee bean resulted from dry process method with water content of 13-14% (wet basis), and 10% concentration ethyl acetate as solvent. Main

machine used was single column reactor. Robusta coffee bean were classified into 4 groups based on size. A_1 is coffee bean with diameter > 7.5 mm, A_2 is coffee bean in between 6.5 to 7.5 mm, A_3 is coffee bean with diameter in between 5.5 to 6.5 mm, and A_4 is coffee bean with diameter < 5.5 mm. Coffee bean was steamed in a single column reactor with saturated water media as long as 1-4 hours. Hot water media replaced with 10% concentration of ethyl acetate solvent. Caffeine dissolving process was done in temperature and time range of 50-100 °C and 2-12 hours, respectively. Heat source used in steaming process was stove with bio-fuel (protos), and 650 Watt electrical stove for facilitating the dissolving process. As comparison, it was used low caffeine instant grounded coffee that distributed in the market.

Optimum steaming time was determined based on achievement of coffee bean maximum enlarging that was notified with water content changing insignificantly (Sri Mulato et al., 2004). During steaming process, coffee bean water content was determined by using gravimetric method (Brooker et al., 1974). Caffeine substance was analyzed using HPLC, and GC MS. Taste test of decaffeinated grounded coffee was done with mixing the 100 g grounded coffee with steamed water. Steaming efficiency ($Eff._{steaming}$) was computed using following formula from Green (1984). Energy for pumping (Q_{pump}) was calculated based on fitted power with assumption of no power loss due to friction, etc. Energy needed to move the pump was a 0.25 HP. Initial economical analysis was done based on data's collected from the result of single column reactor testing for coffee bean decaffeination processing with limited scale of ethyl acetate solvent.

RESULTS AND DISCUSSION

Research results of coffee bean that steaming process showed that coffee bean water content that initially between 13-14% increased to be 38-42% during 3.5 hours of steaming process (Figure 1). Water molecule inserted into coffee bean by diffusion way and then emerged the cell wall inside the bean networks. Water molecule was trapped inside the cells, so the water content of coffee bean increased. At this condition, coffee bean has experienced rewetting process. The highest decreasing rate of caffeine was reached after first 2 hours of decaffeination process, and tended to slow at each next hour (Figure 2). Burning process to develop heat energy with *protos* resulted relatively low efficiency when it was compared to electrical energy. Research results showed that the lowest and highest steaming efficiency were 13.28% and 15.61%, respectively based on 3.5 hours steaming process using *protos* heat source at coffee bean heating treatment of A_3 dan A_1. While, when it was using electric heater, the lowest and highest efficiency were 49.3% and 62.75% at heating treatment of coffee bean with size A1 and A_2, respectively. Research result showed that to reach saturated water content, it needed 2 hours. The same case also reported by Toledo (1999) that continued heating could not add bean volume.

Table 1 indicated that some taste parameter values relatively higher than decaffeination of Robusta coffee bean with water solvent (Sri Mulato et al., 2004). Figure 3 (A) showed that pure low caffeine coffee with ethyl acetate solvent had a better taste value than with water solvent. Pour taste were related with dissolved non-volatile compound; while aroma were related to volatile compound (Lingle, 2001). Instant coffee was a one of coffee product diversifications which was able to increase the added value and improve the Indonesia domestic consumption. Figure 3 (B) shows that taste value of 3 in 1 low caffeine instant coffee was better than 3 in 1 instant coffee in the market.

Economical analysis was conducted in limited production scales (pilot plan) with material capacity of 24 Kg/day. Process steps of low caffeine coffee production covered decaffeination

process in single column reactor, mechanical drying, roasting, and grinding. Analysis results showed that based price of low caffeine grounded coffee was as much as Rp 54,960.00 per kg.

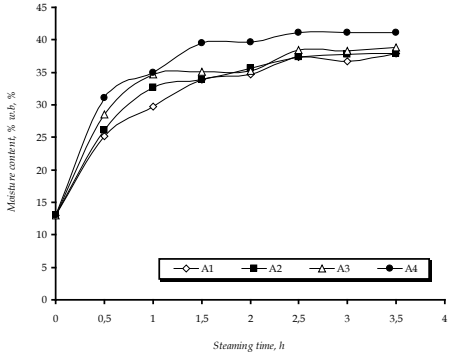

Figure 1. The changes of coffee bean water content during steaming process. Notes: A_1 > 7,5 mm; 6,5 mm < A_2 < 7,5 mm; 5,5 mm< A_3 < 6,5 mm; and A_4 < 5,5 mm

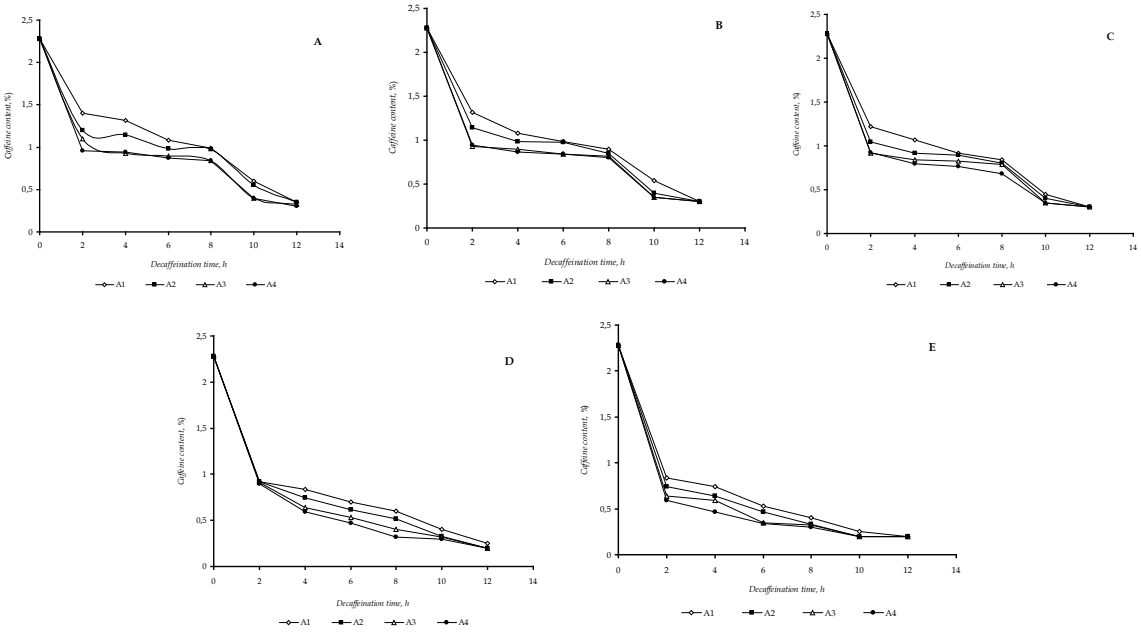

Figure 2. Caffeine reducing rate of solvent temperature treatments at 50-60 °C (A), 60-70 °C (B), 70-80 °C (C), 80-90 °C (D), and 90-100 °C (E). Notes: A_1 > 7,5 mm; 6,5 mm < A_2 < 7,5 mm; 5,5 mm< A_3 < 6,5 mm; and A_4 < 5,5 mm

Table 1. Operational condition of single column reactor with 10% ethyl acetate as solvent.

Solvent temp, °C	Time, hours	Beans size code	Caffeine, %	Chlorogenic acid, %	Trigonellin, %	pH solvent	Taste parameters				Energy consumption	
							Aroma	*Flavor*	*Bitt.*	*Body*	Electric, kJ	Bio-fuel, kJ
50-60	12	A_4	0.30	1.30	0.30	5.11	2.3	2.3	2.3	2.4	123,325.2	225,238.9
60-70	12	A_1	0.30	1.54	0.3	5.22	2.4	2.4	2.4	2.3	132,841.2	230,220.9
60-70	**12**	**A_2**	**0.30**	**1.30**	**0.22**	**5.15**	**2.3**	**2.3**	**2.3**	**2.3**	**132,301.2**	**232,996.4**
60-70	**12**	**A_3**	**0.30**	**0.93**	**0.22**	**5.10**	**2.2**	**2.2**	**2.2**	**2.2**	**132,301.2**	**231,479.4**
60-70	12	A_4	0.30	0.75	0.22	5.00	2.2	2.2	2.2	2.3	132,409.2	231,979.4
70-80	12	A_1	0.30	0.80	0.21	5.03	2.2	2.2	2.2	2.2	139,978.2	237,357.9
70-80	12	A_2	0.30	0.75	0.21	5.07	2.2	2.2	2.2	2.2	139,438.2	240,133.4
70-80	12	A_3	0.30	0.70	0.21	4.97	2.1	2	2	2	139,438.2	238,616.4
70-80	12	A_4	0.30	0.70	0.21	4.85	2.0	1.9	2	2	139,546.2	239,116.4
80-90	12	A_1	0.25	0.70	0.21	4.68	2.1	2.1	2.1	2.1	144,736.2	242,115.9
80-90	10	A_2	0.33*)	0.80	0.22	4.66	2.3	2.1	2.2	2.2	121,595.7	219,576.5
80-90	10	A_3	0.32*)	0.80	0.21	4.60	2.3	2.1	2.1	2.1	121,595.7	218,059.5
80-90	10	A_4	0.30	0.70	0.21	4.50	2.0	1.8	1.9	2	121,703.7	218,559.5
90-100	**10**	**A_1**	**0.25**	**0.75**	**0.21**	**4.64**	**2.3**	**2.4**	**2.1**	**2.3**	**128,083.2**	**222,748.5**
90-100	8	A_2	0.33*)	0.80	0.25	4.68	2.5	2.2	2.3	2.3	103,753.2	199,019.6
90-100	8	A_3	0.32*)	0.80	0.21	4.60	2.6	2.3	2.2	2.3	103,753.2	197,502.6
90-100	**8**	**A_4**	**0.30**	**0.70**	**0.21**	**4.50**	**2.1**	**2**	**1.8**	**2.2**	**103,861.2**	**198,002.6**

*Note: *) caffeine substance are still > 0,3%.*

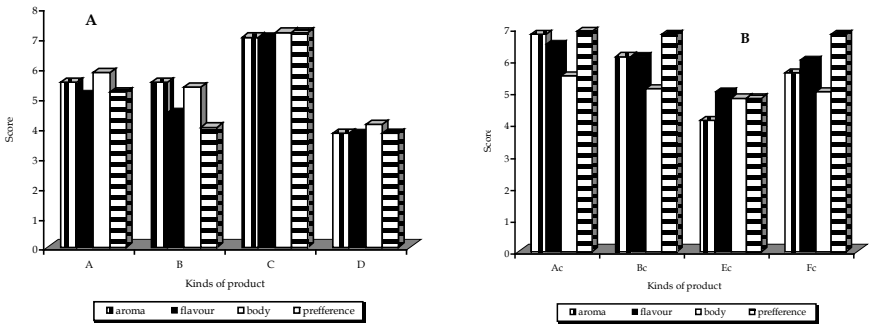

Figure 3. Value of some taste parameter of low caffeine coffee (A= pure, and B= instant 3 in 1). Notes: A = ethyl acetate, A4, 8 hours, 90-100 °C; B = ethyl acetate, A1, 10 hours, 90-100 °C; C = commercial 1; D = water, A4, 100 °C; Ac = ethyl acetate, A4, 8 hours, 90-100 °C; Bc = ethyl acetate, A1, 10 hours, 90-100 °C; Ec = commercial 1 (Comic); and Fc = commercial 2

CONCLUSION

Optimizing of single column reactor for caffeine extraction of Robusta coffee beans have been done, and resulted as follows: 1) Coffee decaffeination process was initiated with steaming process during 3 hours, and coffee bean with size criteria of A_1 and A_4 were dissolving at temperature of 90-100 °C along 10 dan 8 hours, respectively. While, A_2 and A_3 classification of coffee bean were dissolving at solvent temperature of 60-70 °C during 12 hours, and 2) Economical analysis of limited scale of low caffeine coffee production process showed that cost production of low caffeine coffee powder was as much as Rp 54,960.00/ kg.

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Development a Portable-Direct Digital Display of Coffee Beans Moisture Tester

SRI MULATO, EDY SUHARYANTO

Indonesian Coffee and Cocoa Research Institute. E-mail: mulatosri@yahoo.com

SUMMARY

It is crucial to have a portable-direct digital display of coffee beans moisture tester to maintain consistent processing, to examine and adjust quality and to ensure equitable transactions. A locally-made digital coffee moisture tester has therefore been developed and tested by ICCRI to provide an affordable, appropriate and quick coffee bean moisture tester for the benefit of farmers and their related coffee business in the village. The moisture meter was designed based on the principle of dielectric constant variations due to change in moisture of bean. A moisture sensor was made from a co-axial stainless steel [SS] tube as an outer capacitor and a cylindrical SS solid rod as an inner one. The sample was placed in between both capacitors and acted as the dielectric medium. High frequency square wave oscillator working in 100 MHz excited the sensor. Changes of moisture content affected the dielectric constant of the coffee bean, which in turn made variation in capacitance. The variance output across the sensor was fed to a signal electronic circuit to convert it into frequency variation which directly displayed on a LCD panel. A serial test was performed using a set of coffee beans samples with varying moisture (3 to 25%). The result showed that correlation curve between the resultants output frequency [Y] against the varying moisture content of samples [X] followed the Linier Model Equation of Y = 1712, 66557+56, 04099 X [R^2 = 0, 80073]. The tester was then calibrated by measuring the same samples using the standard oven method as a reference. The calibration curve followed a linier equation of T_{ester} =0, 20039+0, 98363 O_{ven} [R^2= 0, 99266]. Beyond the moisture range of 11% < X > 18%, the digital tester did not give an accurate reading. For practical purposes, the digital tester was also compared with the commercially available tester in the market to measure the similar varying moisture samples. It was concluded that the digital tester has proved working satisfactorily for practical drying, storage and marketing tests with an accuracy of ± 1%. Portability of the meter was very good. The unit was light, durable and self-contained in a plastic case.

INTRODUCTION

The amount of moisture in a product can greatly affect perceived quality and is also very important when making transactions based on weight. Excessively high moisture content of the bean may cause quality deterioration and may inflict a financial loss both for farmers and traders due to the differential in price paid for beans of different moisture content. Thus, the final moisture content of the beans before being traded should not exceed 12% [w/w] and should be measured accuratelly and quickly. This is best done with a rapid portable moisture meter which allows testing to be done at the site of trading. This meter is intended to replace the subjective coffee moisture testing done by village traders during transaction.

METHODOLOGY

A locally-made digital coffee moisture tester has therefore been developed and tested by ICCRI to provide an affordable, appropriate and quick coffee bean moisture tester for the benefit of farmers and their related coffee business in the village. The moisture meter was designed based on the principle of dielectric constant variations due to change in moisture of bean. A moisture sensor was made from a co-axial stainless steel [SS] tube as an outer capacitor and a cylindrical SS solid rod as an inner one. The sample was placed in between both capacitors and acted as the dielectric medium. High frequency square wave oscillator working in 100 MHz excited the sensor. Changes of moisture content affected the dielectric constant of the coffee bean, which in turn made variation in capacitance. The variance output across the sensor was fed to a signal electronic circuit to convert it into frequency variation which directly displayed on a LCD panel (Figure 1).

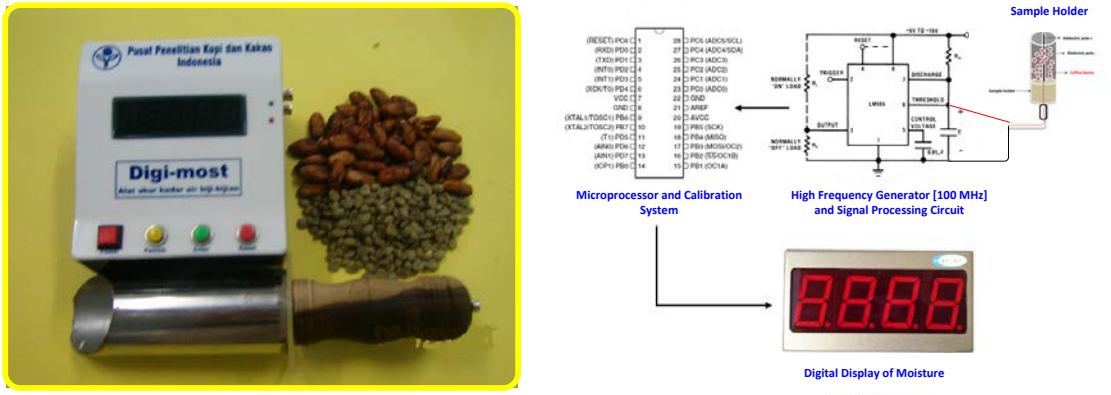

Figure 1. Functional Block Diagram of the capacitance-based moisture meter.

RESULTS AND DISCUSSION

A serial test was performed using a set of coffee beans samples with varying moisture (3 to 25%). The effect of dimensions of the coffee bean, along with surface characteristics and the temperature of ambient air during the measurement determine the output frequency of the measurement (Figure 2).

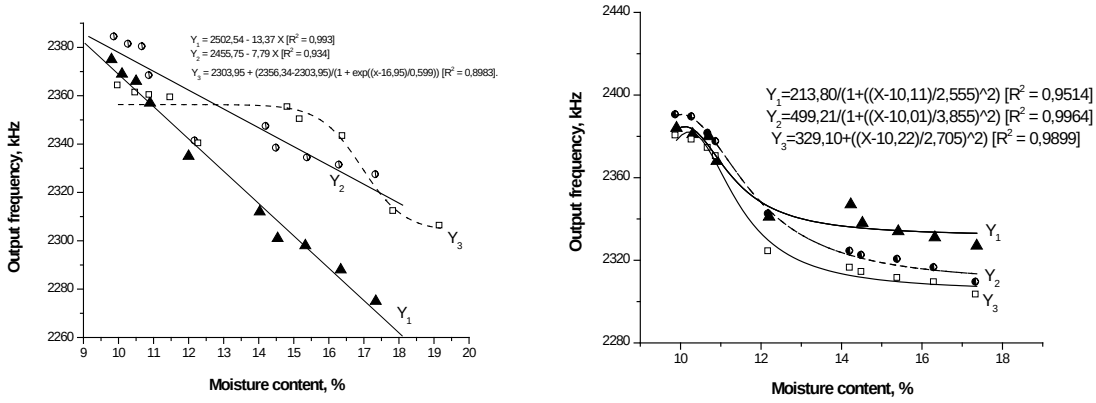

Figure 2. Frequency output vs bean size [left] and frequency output vs air temperature.

The correlation curve between the resultants output frequency [Y] against the varying moisture content of samples [X] followed the Linier Model Equation of Y_1 = 2502,54 - 13,37

X [R^2 = 0,993] for small size of coffee bean, Y_2 = 2455,75 - 7,79 X [R^2 = 0,934] for medium size of coffee bean and the Sigmoidal-Boltmanz Model Equation of Y_3 = 2303,95 + (2356,34-2303,95)/(1 + exp((x-16,95)/0,599)) [R^2 = 0,8983] respectively. The linear equations showed larger R^2 value. This value indicated that the meter responded positively to small changes in moisture. This linear frequency-moisture relationship was found on other agricultural products such as shelled, yellow-dent field corn and soybean (Nelson, 1978; Afonso et al., 2001; Berbet, et al., 2004).

For all size of the beans, the output frequency of coffee bean sample decreased with decreasing temperature of measurement, except at moisture content level more than 14% [Y1 indoor measurement at ambient air 20 °C, Y indoor measurement at ambient air 27 °C and Y3 outdooor measurement at ambient air 35 °C]. The coffee bean sample absorbed more the field power at lower temperature indicating the presence of thermally activated mechanisms of water molecul. These mechanisms probably are interracial polarization, where the external electric field causes ionic charge carriers to accumulate at internal discontinuities in the bean sample.Temperature corrections should be made when using a capacitive-type meter on specimens that are warmer than 30 °C or cooler than 25 °C.

Figure 3 shows that the calibration curve followed a linier equation of T_{ester} = 0,20039 + 0,98363 O_{ven} [R^2= 0,99266]. Beyond the moisture range of 11% < X > 18%, the digital tester did not give an accurate reading. For practical purposes, the digital tester was also compared with the commercially available tester in the market to measure the similar varying moisture samples.

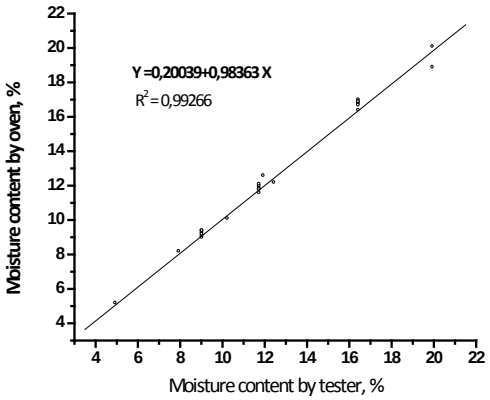

Figure 3. Moisture value of Robusta coffee bean measured by the meter vs the oven standard.

CONCLUSION

It was concluded that the digital tester has proved working satisfactorily for practical drying, storage and marketing tests with an accuracy of ± 1%. Portability of the meter was very good. The unit was light, durable and self-contained in a plastic case.

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Coffea arabica Beans Microstructural Changes Induced by Roasting: an X-Ray Microtomographic Investigation

P. FRISULLO[1], M.A. DEL NOBILE[1], M. BARNABA[2], L. NAVARINI[2], F. SUGGI LIVERANI[2]

[1]Dept. of Food Science, University of Foggia, Via Napoli, 25 - 71100 Foggia, IT
[2]Illycaffè S.p.A., Via Flavia 110 - 34147 Trieste, IT

SUMMARY

Roasting is probably the most important process in the coffee technology. Thanks to this thermal process, in facts, the not particularly appealing smell of green coffee beans is transformed into the well known and highly appreciated roasted coffee aroma. The thermally induced coffee aroma development is accompanied by several physical changes ranging from those of colour to those of bean volume and from those of density to those of texture. Concerning the latter, for instance, it is well known that during roasting, coffee beans loose their strength and toughness becoming brittle and fragile.

The porosity increase can summarize the overall effects of roasting process on coffee beans. Both macropore and micropore systems of roasted coffee beans have been studied, albeit not extensively.

Information about the 3-D structure of foods can be obtained using various imaging techniques. Although a wide variety of imaging techniques exist, they are mostly invasive as they require sample preparation (hence, formation of artifacts) or are restricted to certain types of food products. X-ray microtomography (μCT) is a non-invasive technique that has several advantages over other methods, including the ability to image low moisture materials. μCT uses the differences in X-ray attenuation arising, mainly, from differences in density within the specimen.

The aim of this study is to assess the capability of X-ray microtomography as a new useful tool in characterizing microstructure of *Coffea arabica* beans at different roasting degree: from green to very dark one.

Several parameters such as density, total pore volume and total number of pores have been calculated and their changes upon roasting have been discussed.

INTRODUCTION

Coffee bean is a very complex system which during roasting changes its physical and chemical properties. It is well known that during roasting, coffee beans lose their strength and toughness becoming brittle and fragile (Pittia, et al., 2007).

Moreover, the complex chemical reactions and chemical-physical changes that take place during roasting, such as de-hydration, non-enzymatic browning and pyrolysis, dramatically alter the cell structure. This structural alteration is mainly due to the high internal pressure generated by the large amount of gases released as a consequence of the thermal treatment,

and it results in a large increase of bean volume and in microstructural changes of both the cell wall and the cytoplasm of the green bean. The porosity increase can summarize the overall effects of roasting process on coffee beans.

Information about the 3-D structure foods can be obtained using a wide variety of imaging techniques. However, these techniques are mostly invasive as they require sample preparation (hence, formation of artifacts), and therefore they are restricted to certain types of food products. µCT, on the other hand, is a non-invasive technique that has several advantages over other methods, including the ability to image low moisture materials. Given the enormous success of µCT in different fields such as medical applications, material science and chemical engineering, it is not surprising that in recent years much attention has been focused on extending this imaging technique to food science, as a useful technique to aid in the study of food microstructure.

X-ray microtomography has been successfully used to observe the stability of gas bubbles in dough during the bread making process (Whitworth and Alava, 1999), the microstructure of foams (Lim and Barigou, 2004), three-dimensional quantitative analysis of breadcrumb (Falcone et al., 2005), the study of bread porous structure (Falcone et al., 2004) and ice crystals within frozen foods (Mousavi et al., 2005). Recently this technique has also been used to study the bubble size distribution in wheat flour dough (Bellido et al., 2006), the effect of far-infrared radiation assisted drying on microstructure of banana slices (Léonard et al., 2007), three-dimensional pore space quantification of apple tissue (Mendoza et al., 2007), the role of sugar and fat in sugar-snap cookies (Pareyt et al., 2009), the study of processed meat microstructure and the assessment of intramuscular fat level and distribution in beef muscles (Frisullo et al., 2009).

The aim of this study is to demonstrate the capability of X-ray microtomography as a useful tool for the characterization of microstructure of coffee Arabica beans at different roasting degree: from green to very dark one.

MATERIALS AND METHODS

Raw materials

Coffea arabica var. S-795 from India, crop 2008-2009, has been used. Green coffee beans have been roasted with a Probat model BR74 (220 °C, 100 g starting sample) and sampled during the roasting process at 2, 3, 4 and 5 minutes (t0 – t5, for the sake of brevity). The roasting degree has been determined gravimetrically and expressed in terms of total weight loss (6.8, 10.7, 13.9, and 21.1% w/w, respectively) it corresponded to very light, light, medium and dark, respectively.

Five coffee beans at each roasting time were chosen for the experiment. Each coffee bean was weighed on a digital precision balance (± 0.1 mg) (Gibertini Europe, Italy) to calculate the density of the sample.

Tomographic analysis

Each sample was placed vertically on a metal plate using modelling clay, which is a material irrelevant to X-ray. The samples were imaged under the same conditions, using the Skyscan 1172 high-resolution desktop X-ray microtomography system (Skyscan, Belgium). A sample was placed in a cylindrical tube; the source and the detector were fixed, while the sample was

rotated during measurement. Power settings of 29 kVp and 175 µA were used. A CCD camera with 4000 x 2096 pixels was used to record the transmission of the conical X-ray beam through all samples. The distance source-object-camera was adjusted to produce images with a pixel size of 2.8 µm. Four-frame averaging, a rotation step of 0.60° and an exposure time of 589 ms were chosen to minimize the noise, covering a view of 180°. Smoothing and beam-hardening correction steps were applied to suppress noise and beam hardening artifacts, respectively. Beam hardening correction was only moderately applied, set to 25% within NRecon and a fast ring artifacts reduction (set to 25 within NRecon) was also applied. Once initial parameters were set, the acquisition step was completely automated and did not require operator assistance. Scan time, on average, required 70 minutes. A set of flat cross section images, was obtained for each sample after tomographical reconstruction by the reconstruction software NRecon (Skyscan). Three-dimensional reconstructions of samples were created by effectively stacking all 2D tomographs, a total of 420 slice images with a slice spacing of 0.069 mm.

For image processing and analysis, the skyscan software, CT-Analyser (CTAn) was used. Image segmentation was first carried out on the smoothed 8-bit grey-scale images obtained from the reconstruction step, using CTAn (Skyscan) software. Segmentation is the process of converting the grey-scale images into black and white images by assigning the value 1 to all pixels whose intensity was below a given grey tone value and 0 to all the others. For this, an automatic threshold based on the entropy of the histogram (Sahoo et al., 1988) was calculated for each image. The lower grey threshold (0) and upper grey threshold (60) values were identified; each sample was processed under the same conditions.

For data analysis, prior to 3D reconstruction, a component-labelling algorithm, available within CTAn, was used to isolate the largest 3D connected structures. All reconstructions where created in CTAn (Skyscan) using an adaptive rendering (locality 10 and tolerance 0.25) algorithm and saved as P3G surface model (SkyScan model format). P3G models were then imported into CT vol software (Skyscan) for visualization.

Statistical analysis

All parameters obtained were submitted to one-way analysis of variance (ANOVA) and Duncan's test ($p<0.05$) through the statistical package Statistica for Windows (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

In Figure 1, which shows grey-scale reconstructed cross-section images of green and dark roasted coffee beans, the contrast is produced by a variation of density and a change in composition of the sample and is based exclusively on the detection of an amplitude variation of X-rays transmitted through the sample itself. The obtained image is a 3-D map of the spatial distribution of the X-rays in which the brighter regions correspond to the higher level of attenuation, i.e. higher density region. It can be assumed from Figure 1 that the darkest areas represent the holes (air) as it has a lower absorption coefficient with respect to structure. Figure 1 shows just one of the several cross-sections constituting the 3-D image reconstruction of each sample.

By performing image analysis on 3-D reconstructions, it is possible to obtain several parameters useful to characterize the bean micro-structure. Figure 2, for instance, shows the average total volume of the holes (pores) present in the coffee bean samples at the five

different roasting times. The values reported in the graph does not take into account the large central hole of the coffee bean, as this structure is only air it is therefore excluded in this micro-structural study. It can be noted from this graph that as the roasting time increases, the total pore volume increases, with a significant rise at roasting time t3 due to the rupture of bonds in the internal bean structure, as expected. Statistical analysis, performed on these values confirmed that there are significant differences of the total volume between roasting times t0 and t3 and roasting times t3 and t5, although there are no statistical differences between the total volumes at roasting time t4 and t5.

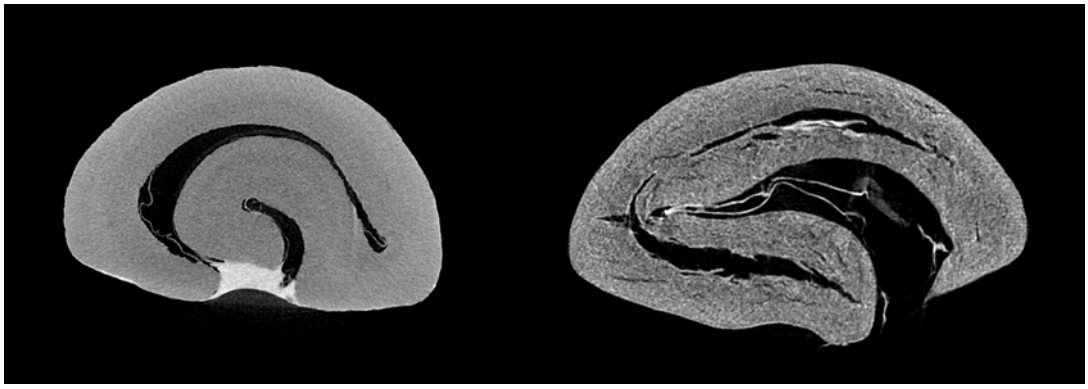

Figure 1. Reconstructed cross-section of coffee bean at roasting time 0 (left) and after 5 minutes of roasting (right).

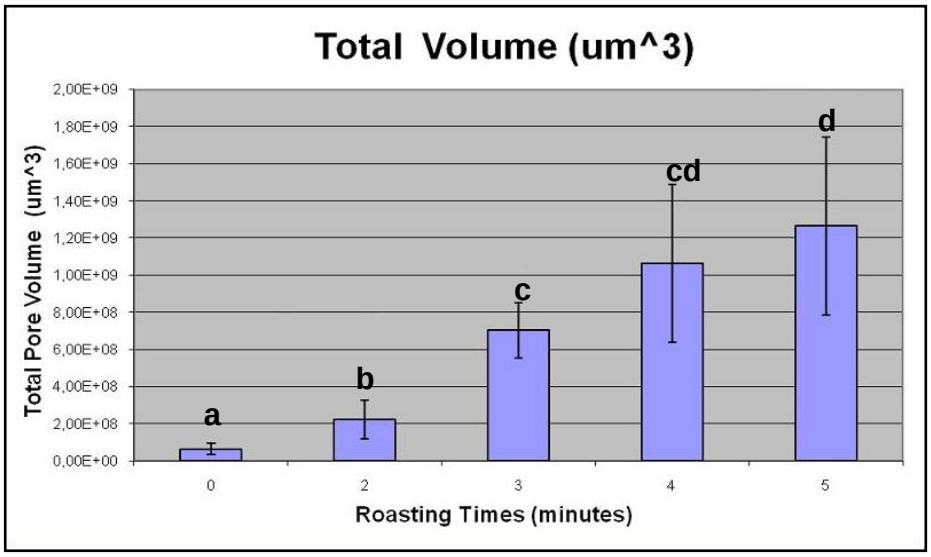

Figure 2. Total volume of bean holes at different roasting times. Samples with the same letter are not statistically different. Error bar shows the standard deviation.

Figure 3 shows a porosity-related parameter: the total number of pores present in the samples of coffee examined at different roasting times. Also in this case the large central hole has not been taken into the account. The graph shows that at roasting time t2 there is a significant increase due to a remarkable change of the internal structure induced by thermal process. After roasting time t2 the increase of the total number of holes is gradual and statistical analysis performed on the results confirmed that although there is a statistical difference between the total number of holes at roasting times t2 and t5, there are no statistical differences among the values for roasting time t3 and t4.

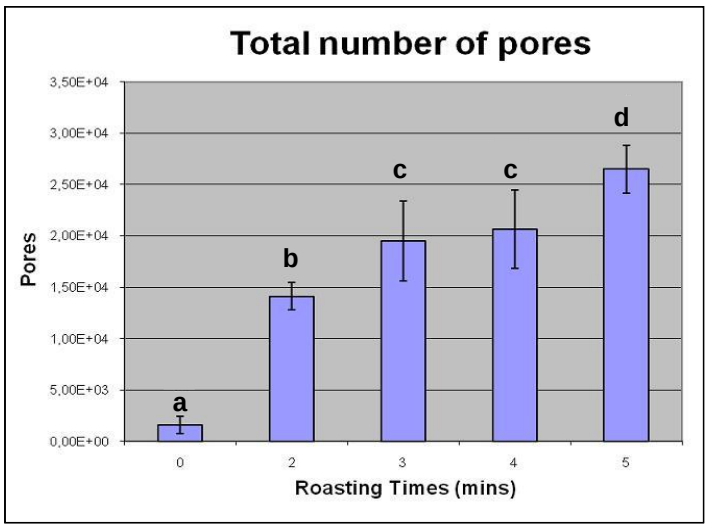

Figure 3. Number of bean holesat different roasting times.

Another useful parameter which can be determined by µCT data is the bean density. The average bean density at the different roasting times is shown in Figure 4. There is a significant decrease in the bean density as effect of the thermally induced increase in pore volume and simultaneous weight loss. Both absolute values and trend are in full agreement with literature data (Dutra et al., 2001).

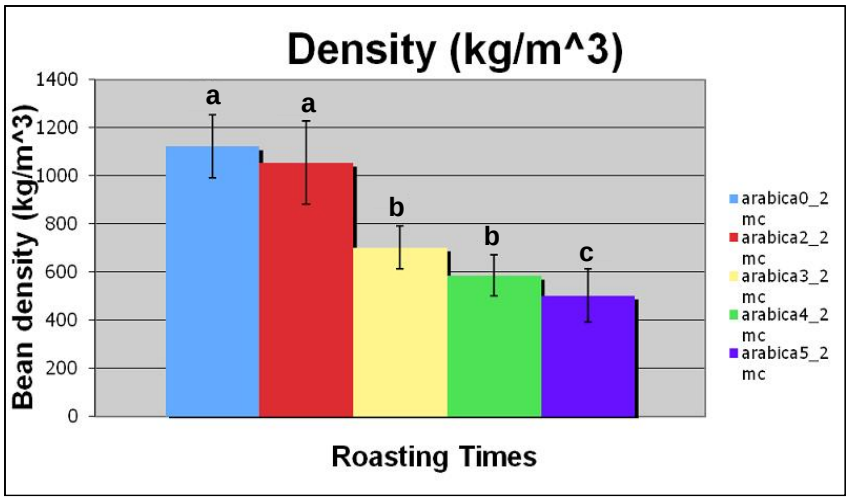

Figure 4. Bean density for different roasting times.

CONCLUSION

X-ray microtomography allows to obtain high resolution 3-D images of coffee bean samples. Several parameters useful to characterize the bean micro-structure can be determined. Calculation from the 3-D microstructure are not always achievable from 2-D image analysis even by using statistical techniques, and this represent an advantage in respect to classical microscopy techniques. When used to follow the coffee roasting process, this technique is able to quantify the structural alteration of the microstructure caused by the high internal pressure generated by the large amount of gases released as a consequence of the thermal treatment. In view of the present work, X-ray microtomography can be proposed as a useful and sophisticated tool for coffee solid state studies.

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Characteristics of Coffee Extraction Process in a Single Column Reactor

Y. HANANTO[1], S. MULATO[2], H.K. PURWADARIA[1]

[1]Department of Mechanical and Biosystem Engineering, IPB – Bogor Agricultural University, Bogor, West Java, Indonesia
[2]ICCRI, Jember, East Java, Indonesia

SUMMARY

The objective of this study was to determine the characteristics of extraction process of decaffeinated coffee powder in a single column reactor to produce thick coffee extract as raw material for instant coffee. The results indicated that extraction was recommended to use fine coffee powder, ratio of water to powder at 4: 1, and 90 °C extraction temperature in 30 minutes process time, and another 30 minute draining previous to collecting the extract.

INTRODUCTION

Indonesia produced coffee bean as high as 698 thousand tons in 2008 (Ditjenbun, 2008) involving 1.84 million farmer households. However, the consumption of coffee is still low with the average of 0.5 kg/capita/year, especially if the figure is compared to the consumption in the European countries such as Norway, Denmark, The Netherlands, Austria, and Germany that can reach 8.11 kg/capita/year (USDA, 2000). ICCRI has developed diversification in the form of various coffee drinks, one out of them is instant coffee (Mulato et al., 2005). Instant coffee in a small sachet that can be practically found at any peddler on the street, and can be purchased at reasonable price by low income people is increasingly popular nowadays. Unfortunately, coffee is considered as having controversial effects for human health. A solution for people who has to limit the coffee drink due to the health problem is by consuming low caffeine instant coffee. Studies had been done in the decaffeination of coffee powder (Almada, 2009; Mulato et al., 2001).

The objective of this study was to determine the characteristics of extraction process of decaffeinated coffee powder in a single column reactor to produce thick coffee extract as raw material for instant coffee.

MATERIAL AND METHODS

Robusta coffee bean from the plantation of ICCRI, Jember, East Java, Indonesia was decaffeinated using water as the solute at 100 °C, and later dried to 13% moisture content dry basis. The coffee bean was roasted at 110-140 °C while rotated at 16-20 RPM, ground to coffee powder and extracted in a single column reactor designed by ICCRI (Figure 1). Previous to extraction process, the coffee powder was separated by a series of screen diameters into coarse powder halted by 2.0 mm diameter screen, medium powder halted by 1.5 mm diameter screen, and fine powder halted by 1.0 mm diameter screen.

Treatments were applied at three stages, firstly in observation of various extraction temperatures 70, 80, and 90 °C, secondly in observation of various ratios of water as solute to

coffee powder (w/w) 4:1, 5:1, and 6:1, thirdly in observation of various size of powder coarse, medium, and fine. The extraction process held in 30 minutes, and then the powder cake drained for another 30 minutes before the extract was collected. Three replications were carried out for each stages, and data was then analyzed statistically.

Figure 1. Single column reactor for extraction of coffee powder designed by ICCRI.

RESULTS AND DISCUSSION

The specification of various sizes of decaffeinated coffee powder was indicated by the distribution of grain through a series of mesh screen: 100, 140, 200, and 230 (Figure 2). It was revealed that fine coffee powder had larger portion of grain smaller than 230 mesh, while coarse powder had larger portion of grain bigger than 100 mesh, and medium coffee powder was in between. The solubility test indicated that the fine powder had the highest level of 25.2 %, the medium 20.0%, and the coarse 19.1%. It should be noted that SNI (Indonesian National Standard) required a solubility of 20-36% for coffee powder, but no standard established yet for decaffeinated one.

The extraction temperature influenced the specific density of coffee extract significantly (Figure 3) with the temperature of 90 °C yielded to the highest specific density of 1013 $kg.m^{-3}$ compared to temperatures of 70 and 80 °C, but not significantly to the viscosity. The linear regression (Figure 4) of the relationship of coffee extract specific density as a function of extraction temperature (in °C) was $\rho = 1000 + 0.1429\ T$ ($R^2 = 0.958$). The ratio of water as solute to coffee powder also influenced the specific density significantly with the ratio of water over coffee powder 4:1 yielded to the highest specific density compared to others.

The size of coffee powder influenced significantly the extract viscosity but not the specific density. Fine powder provided the highest extract viscosity of 0.0031 Pa.s compared to medium and coarse powder which yielded to 0.0028, and 0.0026 Pa.s, respectively.

The caffeine content in the extract solution decreased from 0.31 % in the decaffeinated coffee powder as the raw material to 0.27%.

Extract coffee solution met the requirement for input material to manufacture low caffeine coffee instant. The extraction process was recommended to use fine coffee powder, ratio of water to powder at 4: 1, and 90 °C extraction temperature in 30 minutes process time, and another 30 minute draining previous to collecting the extract.

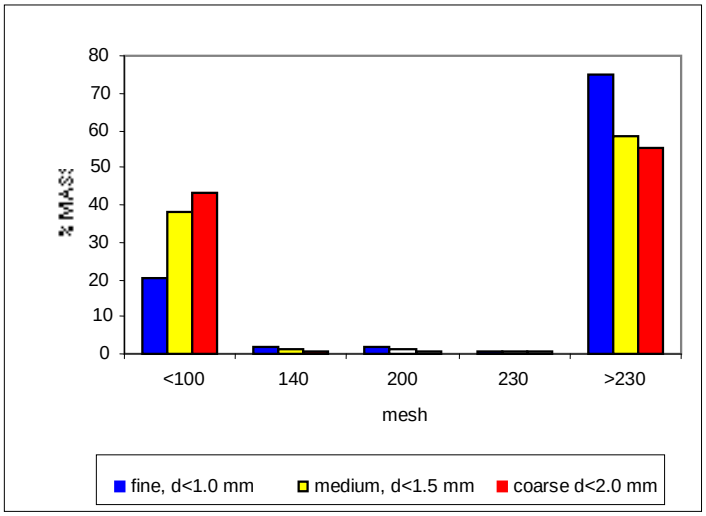

Figure 2. Percentage of grain distribution in various sizes of coffee powder.

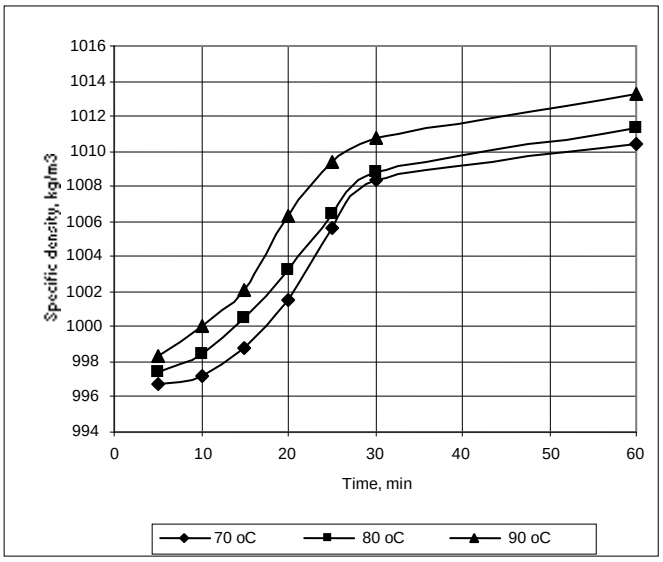

Figure 3. Development of coffee extract specific density along extraction process at various temperatures.

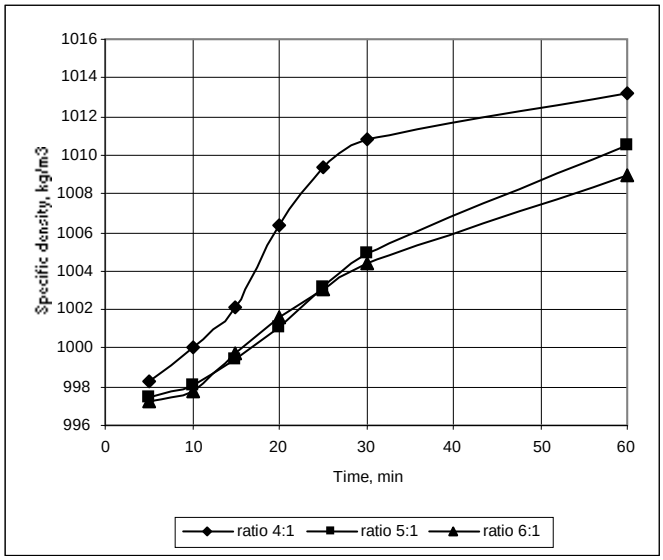

Figure 4. Development of coffee extract specific density along extraction process at various water ratios to coffee powder (w/w).

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Characteristics of Crystallization Process for Low Caffeine Instant Ginseng Coffee

R. TIRTAGUNA [1], S. MULATO [2], H.K. PURWADARIA [1]

[1]Department of Mechanical and Biosystem Engineering, IPB – Bogor Agricultural University, Bogor, West Java, Indonesia
[2]ICCRI, Jember, East Java, Indonesia

SUMMARY

This study aimed to determine the ginseng concentration and evaporation temperature of the crystallization process of low caffeine ginseng coffee solution to produce instant coffee. The results indicated that crystallization process of low caffeine ginseng coffee solution with 7% ginseng concentration (w/w) at evaporation temperature ranged from 70 to 80 °C produced instant coffee at 52.3% yield in 55 minutes, with a level of moisture content 0.94 % (dry basis), and brightness 50.32 L. The coffee drink had a solubility of 132 g coffee/100 g water at brewing temperature of 75 C, and an organoleptic score of 3.7 at the rank of 1-5 compared to 4.1 score of a commercial decaffeinated instant coffee.

INTRODUCTION

Production of coffee bean in Indonesia reached 698 thousand tons in 2008, involving about 1.84 million farmer households (Ditjenbun, 2008). Since the consumption of coffee in Indonesia is still relatively low, i.e., 0.5 kg/capita/year, efforts have been done to produce diversified coffee drinks including low caffeine instant coffee for people with health problem. Other recent trend is to add herbal such as Brazilian ginseng (*Pfaffia paniculata*) into the low caffeine coffee drink product to raise issue of coffee as health drink. Ginseng has been up hailed as substance to stand carcinogenic problems (Matsuzaki, 2003).

The process of decaffeination of coffee has been studied (Katz, 1997), and developed using either water (Mulato et al., 2001), or waste product for the extracting solvent such as cocoa pulp (Purwadaria et al., 2007). For small scale industry, a simple process to produce low caffeine instant ginseng coffee is by crystallization of thick extract from decaffeinated coffee powder, added by sugar and ginseng.

This study aimed to determine the ginseng concentration and evaporation temperature of the crystallization process of low caffeine ginseng coffee solution to produce instant coffee. The crystallization process used an agglomeration type which is simpler than the jet agglomeration for instant food (Schuchman, 1995).

MATERIALS AND METHODS

Materials used in this experiment were thick Robusta coffee extract with low caffeine of 0.27%, and six month old Brazilian ginseng (*Pfaffia paniculata*) roots from the orchard of ICCRI, Jember, East Java, Indonesia. The low caffeine thick coffee extract was made from fine Robusta coffee powder extracted in a single column reactor using water at ratio of water

over coffee powder 5:1 (v/v) and 90-100 °C extraction temperature. Ginseng roots was extracted in an expeller and mixed with water to make into thin solution.

Coffee extract was then added by ginseng solution, and granulated sugar, and heated into boiling point in a pilot scale crystallizer designed by ICCRI (Figure 1). After boiling point was reached, the evaporation process was maintained at a selected of temperature range for a period of time, and mixing was introduced to enhance the effect of crystallization. Preliminary experiment was carried out to determine the boiling point, the time to reach the boiling point, the resident time for evaporation temperature, and the RPM of mixer.

Complete Randomized Experimental Design was applied with two factorials, i.e., evaporation temperature at four levels 50-60 °C, 60-70 °C, 70-80 °C, 80-90 °C, and ginseng concentration (v/v) at also four levels 1%, 3%, 5%, and 7%. The experiments was replicated three times.

RESULTS AND DISCUSSION

Boiling point was found at 93-94 C after heating for 30-34 minutes, the resident time for evaporation was 20-23 minutes, while the RPM for mixing was 80. The results of statistical analysis showed that the treatments did not give influence on the yield and moisture content of instant coffee which, respectively, ranged from 50.9 to 52.3%, and from 0.90 to 1.08%.

The brightness of instant coffee was significantly influenced by the treatments as illustrated in Figure 2. When the instant coffee was made into coffee drinks, the solubility was influenced only by the ginseng concentration at various brewing temperatures as described in Figure 3, and the organoleptic score were influenced by both the evaporation temperature and ginseng concentration listed in Table 1.

Crystallization process of low caffeine ginseng coffee solution with 7% ginseng concentration (w/w) at evaporation temperature ranged from 70 to 80 °C produced instant coffee at 52.3% yield in 55 minutes, with a level of moisture content 0.94% (dry basis), and brightness 50.32 L. The coffee drink had a solubility of 132 g coffee/100 g water at brewing temperature of 75 °C, and an organoleptic score of 3.7 at the rank of 1-5 compared to 4.1 score of a commercial decaffeinated instant coffee.

Figure 1. Pilot scale crystallizer designed by ICCRI.

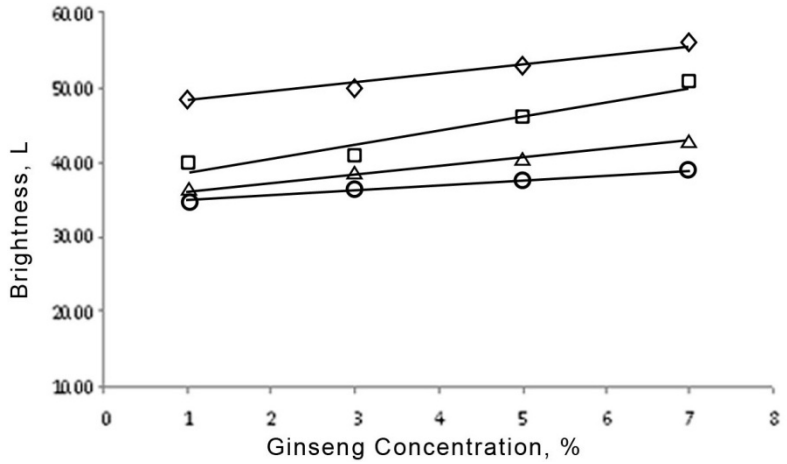

Figure 2. Brightness of instant coffee as a function of ginseng concentration at various evaporation temperatures.

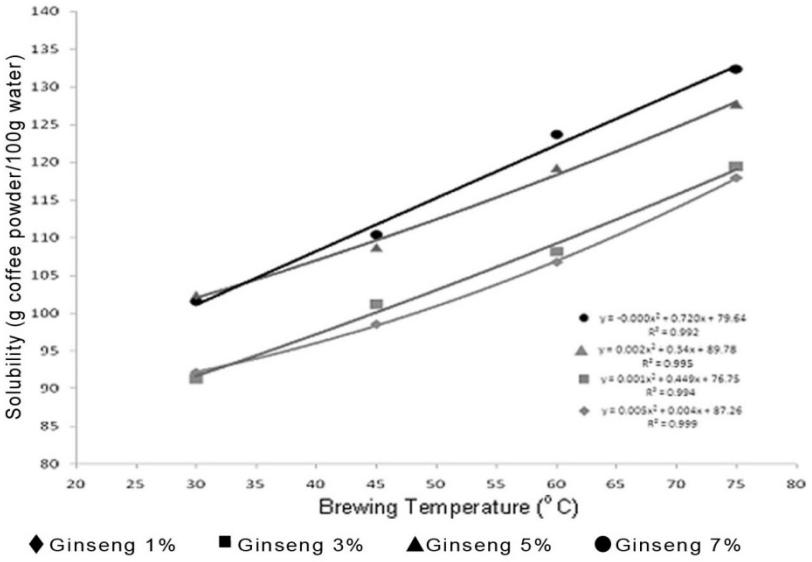

Figure 3. Solubility of instant ginseng coffee at various brewing temperatures and ginseng concentrations.

Table 1. Organoleptic score of brewed coffee from 12 expert panelists.

Evaporation Temperature, °C	Ginseng Concentration, %			
	2	3	5	7
80-90	3.2	3.4	3.5	3.3
70-80	3.4	3.1	3.5	3.7
60-70	3.5	3.6	3.3	3.6
50-60	3.0	3.1	3.6	3.4

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Chorogenic Acids and Lactones in Coffees Decaffeinated by Water and Supercritical CO_2 and Roasted in a Pilot Plant Scale Fluidized Bed Roaster

A. FARAH[1,2]; D. PERRONE[1]; J.FERNANDES[3], J. SILANES[3]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, RJ, Brasil
[2]Instituto de Nutrição, Universidade Federal do Rio de Janeiro, RJ, Brasil.
E.mail: afarah@iq.ufrj.br
[3]Kraft Foods, Coffee RD&Q Centre, Ruscote Avenue, Banbury, OX162QU, United Kingdom

SUMMARY

In the present study, we compared the effect of decaffeination using either water or supercritical CO_2 as a medium for caffeine extraction, on chlorogenic acids (CGA) and their 1,5-γ-quinolactones (CGL) contents in green and roasted Arabica and Robusta coffees. Ten CGA compounds and five CGL were evaluated. Coffee samples decaffeinated by CO_2 presented higher contents of CGA and CGL compared to the water method. However, since decaffeination is a complex process and involves various steps, the fact that the samples used in the present study were decaffeinated in different industrial plants, using different protocols, should be considered and investigated in future studies.

INTRODUCTION

The world coffee market is in continuous expansion. This includes the decaffeinated coffee market branch, which is growing especially for people who are health concerned and/or sensitive to the stimulating effects of caffeine. However, the caffeine extraction may imply in losses of additional beneficial compounds such as chlorogenic acids (CGA) and, consequently, their 1,5-γ-quinolactones (CGL) in roasted coffee. Nowadays, especially in Europe and the US, there is a preference for natural decaffeination methods that use water or supercritical CO_2 as opposed to methods employing organic solvents for caffeine removal. We have previously reported average losses of 3-6% in CGA and average increments of 6-18% in CGL contents in water decaffeinated roasted coffees (Farah et al., 2006). In the present study, we compared the effect of decaffeination using either water or supercritical CO_2 as a medium for caffeine extraction, on CGA and CGL levels of green and roasted Arabica and Robusta coffees.

MATERIALS AND METHODS

Three Arabica coffee cultivars from Colombia, Ethiopia and Indonesia were water decaffeinated, and one Arabica blend and one Robusta sample from Colombia and Indonesia, respectively, were decaffeinated by CO_2. Regular and decaffeinated samples were roasted in a pilot plant scale fluidized bed roaster operating at maximum temperatures for the product at the end of roast of 247-275 °C, and inlet air temperatures of 318-360 °C for 250 sec to reach three roasting degrees: moderately light (#75), medium (#55) and dark (#35), according to the standards of the Specialty Cup Association of America. Cooling and moisture adjustments were performed in a separate chamber, with addition of water.

Analyses of CGA and CGL were performed by HPLC and LC-MS, according to Farah et al. (2006). Eleven CGA compounds and four CGL were quantified: 3-caffeoylquinic acid; 4-caffeoylquinic acid; 5-caffeoylquinic acid; 3-feruloylquinic acid. 4-feruloylquinic acid; 5-feruloylquinic acid; 5-coumaroylquinic acid; 3,4-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 4,5-dicaffeoylquinic acid and caffeoylferuloylquinic acid; 3-caffeoylquinic lactone; 4-caffeoylquinic lactone, 3+4-feruloylquinic lactone; and 3,4-dicaffeoylquinic lactone.

RESULTS AND DISCUSSION

Water decaffeination produced a 21% average decrease in the content of total CGA in green Arabica coffee. Average losses in the roasted samples ranged from 8.5 to 13.7% for the three roasting degrees. Losses of 13.8-23.6% were also observed in the content of lactones in the roasted water decaffeinated samples compared to the regular ones (Table 1 and Figure 1). These losses are higher than those observed by Farah et al. (2006), possibly due to the use of different protocols for decaffeination, different samples used in both studies and in the case of roasted samples, because of the use of different roasting parameters.

CO_2 decaffeination produced an increase of 1.2% in the CGA content in the green Arabica sample and increases from 1 to 20.5% in the samples with different roasting degrees. CGL contents in the Arabica sample varied with the different roasting degrees from an increase of 8.4% to a decrease of 3.7%, compared to the regular sample. In the green Robusta coffee decaffeinated by CO_2, CGA content decreased in 3.9%, while increases of 58.0-92.0% were observed in the roasted samples, compared to the regular ones. Increases of 46.7-92.8% were observed in the CGL content of different roasting degrees compared to the regular Robusta samples.

Higher losses than those obtained by both methods in the present study have been observed in Arabica and Robusta samples decaffeinated with dichloromethane (Toci et al., 2006).

The present results suggest that the decaffeination method using CO_2 is more appropriate, compared to the water method, when higher levels of CGA and CGL are desired, probably due to CO_2 specificity towards caffeine compared to water. Water, on the other hand, may be able to wash out other components from the matrix, including not only CGA but also carbohydrates (Farah et al., 2006), which are major components in green coffee. This will therefore cause a relative increase in the contents of less water soluble substances, as it was apparently the case in Farah et al. (2006), where lower losses of CGA in water decaffeinated coffee samples were observed compared to those in the present study. Because of the lack of selectivity of water, therefore, it is very hard to predict the exact losses and relative gains in coffee components's concentrations.

The fact that the samples used in the present study were decaffeinated in different industrial plants and using different protocols should be considered here and future studies should eliminate this bias in order to be able to compare the use of both mediums for caffeine extraction. It is also clear that Robusta beans present a different behavior compared to Arabica ones, due to differences in cell wall constitution and chemical composition (Farah et al., 2006), and therefore more samples of Robusta coffee should be examined after extraction by both methods for the sake of comparison.

Table 1. Contents of chlorogenic acid and lactones in Arabica and Robusta coffees decaffeinated by water and supercritical CO_2 (mean of two replicates ± SD, expressed as mg/100 g db).

Sample	CQA	FQA	diCQA	CFQA	CQL	FQL	diCQL
C. arabica Colombia							
Regular							
Green	5807.7±37.9	312.5±3.6	788.3±6.2	45.8±3.0	6.7±0.4	2.2±1.2	0.6±0.1
Moderately Light	2291.9±5.1	151.7±2.4	159.2±0.7	16.1±0.0	294.3±1.4	30.9±0.3	3.1±0.1
Medium	1690.4±12.7	131.3±15.8	102.0±1.0	12.4±0.0	279.6±1.5	31.8±3.3	2.2±0.1
Dark	807.9±4.0	103.0±2.8	37.1±0.9	7.8±0.6	219.0±0.9	31.5±0.9	1.0±0.2
Water decaffeinated							
Green	4806.0±18.1	332.8±2.3	407.8±9.9	24.2±0.6	5.4±0.7	1.6±0.2	0.2±0.0
Moderately Light	1975.7±11.8	170.5±1.8	98.4±0.5	9.0±0.1	230.8±1.5	26.5±0.2	1.5±0.2
Medium	1341.6±3.1	137.4±0.4	57.1±0.6	5.5±0.8	232.3±0.7	30.0±0.4	1.1±0.1
Dark	811.4±5.9	104.9±2.1	29.8±0.1	3.3±0.1	192.5±0.6	28.8±0.5	0.6±0.0
C. arabica Ethiopia							
Regular							
Green	6537.3±120.8	172.8±7.5	677.5±6.8	40.3±1.1	2.2±0.3	1.9±0.2	0.4±0.1
Moderately Light	2711.7±15.5	162.1±25.5	158.8±2.1	23.5±1.3	344.9±3.7	30.6±2.4	2.8±0.3
Medium	1813.6±2.1	126.5±2.7	90.9±0.4	17.6±0.1	328.0±2.4	31.3±0.6	2.0±0.1
Dark	738.7±6.1	87.3±3.9	28.6±0.6	16.0±0.5	170.9±5.6	25.3±0.3	0.8±0.1
Water decaffeinated							
Green	5153.7±95.0	326.0±10.9	448.8±3.2	18.9±0.1	3.3±0.1	0.7±0.1	0.5±0.0
Moderately Light	2048.7±6.5	155.9±0.4	106.9±0.8	9.1±0.0	249.7±1.9	26.5±0.1	1.5±0.1
Medium	1403.4±7.1	127.6±1.3	62.2±0.6	6.6±0.3	242.9±1.0	28.6±0.2	1.1±0.1
Dark	582.0±1.7	80.4±0.3	20.0±0.1	5.3±0.5	157.8±0.9	23.0±0.1	0.3±0.0

C. arabica Indonesia							
Regular							
Green	6296.4±24.0	334.3±19.6	708.6±25.6	67.5±4.5	7.3±0.8	0.9±0.2	0.8±0.0
Moderately Light	2013.8±8.9	149.9±3.3	111.9±1.1	21.2±1.3	308.8±1.2	33.7±0.4	2.5±0.2
Medium	1406.6±26.5	145.4±11.0	60.6±7.4	17.4±5.9	256.4±25.3	33.6±2.3	1.3±0.3
Dark	739.1±2.6	107.2±4.2	28.9±0.3	12.3±0.7	194.7±0.6	31.1±0.9	0.6±0.1
Water decaffeinated							
Green	5075.6±34.1	362.7±1.7	379.0±3.2	26.9±0.4	5.9±0.1	0.7±0.0	0.5±0.0
Moderately Light	2071.1±8.1	186.7±2.2	92.3±1.9	11.5±0.8	236.5±2.1	29.0±0.2	1.4±0.1
Medium	1435.9±24.7	153.9±4.7	61.3±6.4	9.2±6.6	259.9±26.6	36.0±4.9	1.1±0.4
Dark	707.6±5.3	99.8±2.9	23.3±0.4	6.1±0.9	173.5±1.1	27.9±0.2	0.4±0.0
C. arabica Colombia							
Regular							
Green	5554.9±171.9	289.4±6.9	760.6±11.5	40.5±0.1	3.4±0.7	0.8±0.1	0.5±0.0
Moderately Light	2259.5±4.4	136.5±1.6	150.4±0.8	17.2±0.5	291.3±2.0	28.4±0.2	3.5±0.3
Medium	1652.4±4.1	123.1±3.8	96.6±1.8	16.0±0.0	297.2±0.9	31.7±0.4	2.8±0.1
Dark	658.0±3.1	95.5±0.3	27.0±0.1	14.5±0.0	195.1±0.6	30.7±0.1	0.6±0.0
CO_2 decaffeinated							
Green	5582.4±40.3	750.6±2.6	776.8±20.6	57.3±4.2	4.1±0.3	0.8±0.1	0.3±0.1
Moderately Light	2228.9±13.6	396.0±1.2	159.4±2.0	20.9±0.1	279.2±0.5	35.3±0.0	2.8±0.0
Medium	1480.2±7.2	288.9±1.9	86.4±0.8	16.5±1.6	279.1±1.5	38.6±0.3	1.8±0.1
Dark	791.2±4.9	189.2±0.4	36.4±0.2	12.9±0.3	209.6±1.7	35.2±0.2	0.8±0.0

C. canephora Indonesia							
Regular							
Green	6135.4±138.4	546.5±10.3	1453.0±49.4	211.7±32.2	6.0±3.2	0.5±0.1	0.4±0.2
Moderately Light	1124.9±5.3	185.6±17.9	87.1±0.7	55.6±1.0	209.3±1.7	51.6±5.8	2.7±0.5
Medium	665.1±13.9	145.9±6.7	40.7±1.0	41.0±1.4	150.4±2.5	46.6±2.5	1.1±0.2
Dark	273.6±4.2	80.2±1.0	12.5±0.4	18.1±1.0	79.9±1.6	29.6±0.6	0.3±0.0
CO_2 decaffeinated							
Green	5910.8±31.6	385.5±6.9	1192.9±13.9	312.0±7.1	10.5±0.6	0.5±0.0	0.6±0.0
Moderately Light	1687.4±9.3	197.5±0.7	170.5±1.8	78.6±0.0	288.1±1.3	93.3±0.5	4.3±0.4
Medium	1008.2±6.7	162.6±19.9	79.2±1.3	48.1±0.7	209.4±1.5	79.6±0.1	2.8±0.1
Dark	502.8±3.6	120.8±1.5	28.7±1.1	27.2±0.7	145.3±2.0	65.6±1.2	0.8±0.1

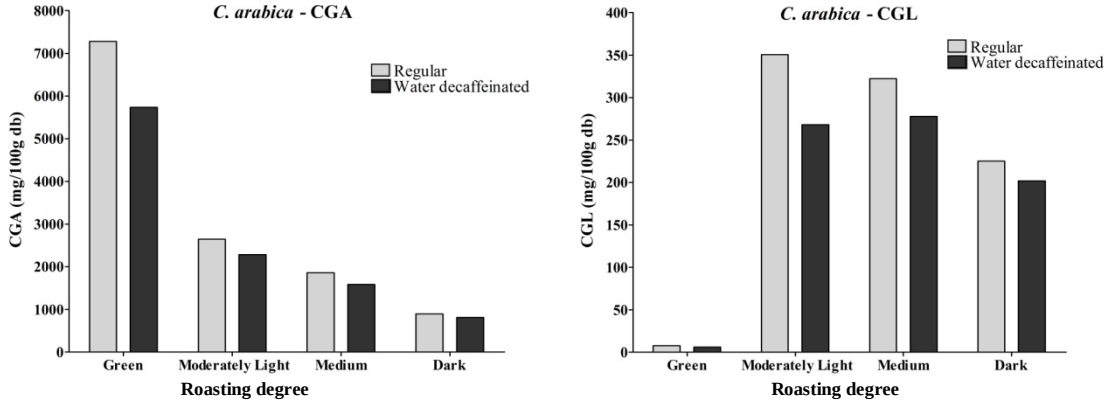

Figure 1. Average total content of chlorogenic acids (CGA) and lactones (CGL) in regular and water decaffeinated green and roasted Arabica coffees (*n*=3).

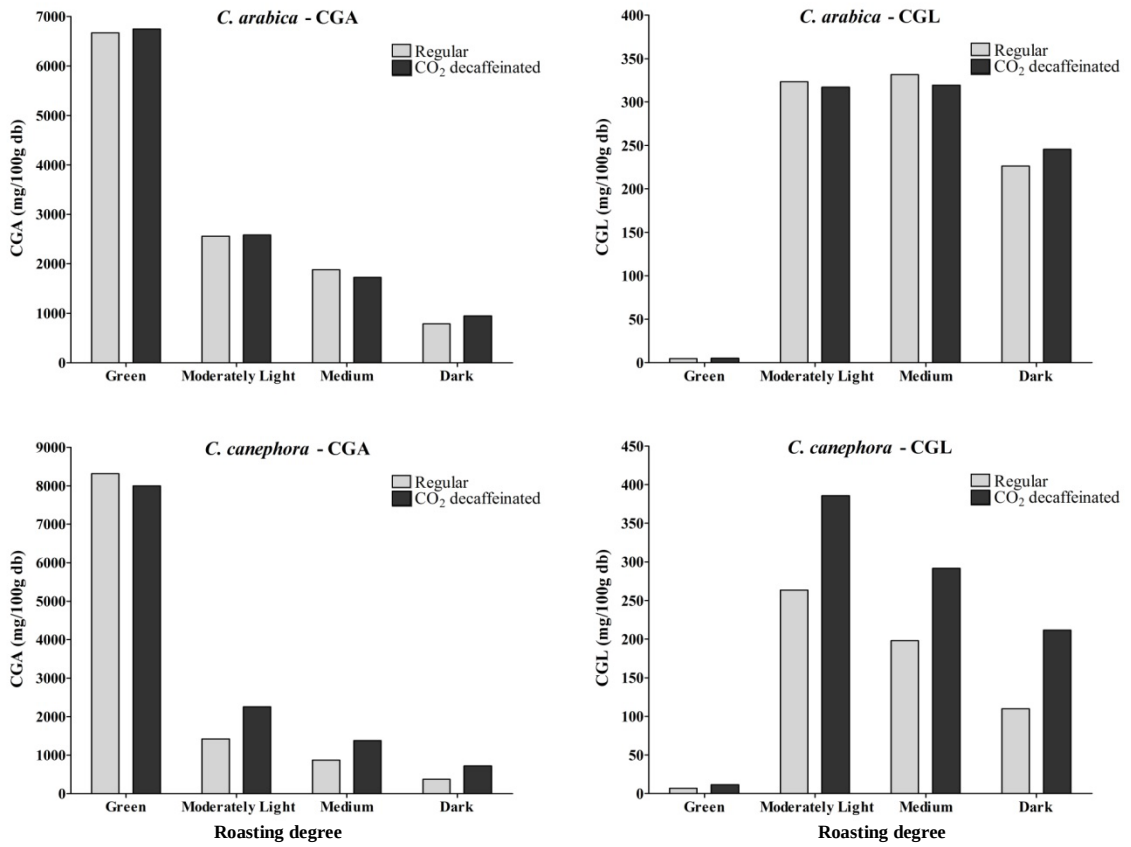

Figure 2. Total content of chlorogenic acids and lactones in regular and supercritical CO_2 decaffeinated green and roasted Arabica (*n*=1) and Robusta (*n*=1) coffees.

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Study of the Impact of Extrusion Cooking Conditions on the Atmospheric Extractability of Roast Coffee Solids

F. GUILMINEAU, C. SALDANHA DO CARMO

Kraft Foods, Global Coffee Technology Centre, Ruscote Avenue, Banbury - OX16 2QU, UK

SUMMARY

Extrusion cooking is a well developed technology which allows the combined application of heat, mechanical shear and pressure forces to the extruded material. When applied to ground green coffee, this process provides conditions for thermal reactions at high pressure and humidity which are very different from conventional roasting. This was notably shown to lead to roast coffee with increased atmospheric extractability in water and modified flavour (Mahlmann, 1973).

The present study is designed to identify the optimum extrusion conditions in order to maximise the atmospheric extraction yield of robusta coffee and describe the sensory characteristics of the obtained extract. The impact of the barrel temperature, product initial moisture and screw rotation speed is studied using a pilot scale single screw extruder. The physico-chemical characteristics of the obtained roast coffee as well as the chemical composition of the obtained atmospheric coffee extract were determined, with particular regards to the molecular weight distribution and composition of the extracted carbohydrates.

Both the initial moisture of the coffee and the barrel temperature are found to have a significant impact on the atmospheric brew yield, whereas the effect of the screw rotation speed is not significant. The maximum total soluble solids extraction yield achieved is 30% (w/w dry basis), which is 20% higher than the maximum yield obtained with conventionally roasted coffee. This is shown to be driven by an increased extractability of mannose polymers with relatively high molecular weights. Those compounds are also shown to be part of (or associated with) nitrogen containing molecules assumed to be melanoidins. The discussion of those results aims to provide explanations for the increased solubility of coffee mannans after extrusion cooking, as well as the observed disconnect between flavour and colour intensity when compared to conventionally roasted coffee.

MATERIALS AND METHODS

Extrusion and roasting

Green robusta coffee from Eastern Africa was ground using a toothed disk grinder (Condux mill) to a d_{50} of about 1 mm and fed into a lab scale single screw extruder (model KE19, Brabender) at a constant rate of 1.5 kg/h using a vibratory feed system. The extruded product was recovered in a tray and allowed to cool at room temperature.

Conventional roasting of the same green coffee was performed on a Rotary Fluidized Bed roaster (model RFB-10, Neuhaus-Neotec) using batches of 2 kg of beans and adjusting the air inlet temperature so as to achieve the desired roast colour after 200 seconds.

Water extraction of roasted/extruded ground coffee

The roasted and extruded coffees was ground to a target d_{50} of 780 µm using a Perlen Mill, and 200 g of boiling deionised water were poured in a conical flask on exactly 10 g of the ground coffee solids. This preparation was placed in a water bath at 85-90 °C under agitation (115 rpm) during 15 minutes, then cooled down in an icy water for 5-6 min, and filtered on standard filter paper (Grade 315, 25 µm) to recover the extract. The method has presented good repeatability and reproducibility.

Physico-chemical characterisation

The roast colour was measured using an LK100 colour meter (Hach Lange) and moisture contents of the green, extruded and roast coffee were measured by gravimetry after drying in an oven. The solids content of the water extract was measured by refractometry, calibrated against gravimetric measurements. A sample is hydrolysed with acid for total carbohydrates determination and with water for free carbohydrates determination. The sugars are then separated using high pressure anion exchange chromatography and detected using a DIONEX ED 40 pulsed amperometric detector.

Sensory evaluation

A panel of 12 assessors was trained for Quantitative Descriptive Analysis of coffee brew flavour profiles, using attributes pre-defined as part of the training to suit the sample set analysed.

RESULTS AND DISCUSSION

Impact of extrusion conditions on the degree of roast and extraction yield

A central composite experimental design was used in order to study the combined effect of the extruder screw rotation speed (rpm), the extruder barrel temperature (°C) and the initial product moisture (% w/w) on the extruded product's characteristics. Statistical analysis (ANOVA) of the results revealed that the screw rotation speed had no significant effect on the roast colour or brew yield of the obtained product. This can probably be attributed to product slip as a consequence of oil expression during extrusion: the accumulation of coffee oil at the feed-end of the barrel was observed during extrusion. Both product colour and brew yield were significantly impacted by the extrusion conditions (Figure 1).

It is found that the barrel temperature is the main factor driving the product colour, with a rapid darkening of the product taking place at barrel temperatures above 230 °C. The initial product moisture has in comparison a relatively low influence on the product colour. A decreasing initial moisture level leads to a darkening of the obtained product at all temperatures observed, reflecting the increased level of pyrolysis taking place in the product at reduced moisture level. This is also reflected in the extratability of the roasted coffee solids. Hence, the maximum extraction yield correponds to the darkest product obtained, which is in conditions where pyrolysis reactions are favoured, at low product moisture and high temperature. The maximum brew yield of about 30% (dry solid basis) measured on extruded coffee is significantly higher to that measured on conventionally roasted coffee at the same roast colour (i.e about 26% yield at 8 La). This indicates that the extreme physical conditions present in an extruder in terms of pressure and shear forces enhance the solubility of certain

green coffee solids in hot water. Those solids are thought to be derived from the structural carbohydrates that constitute the thick cell walls of the coffee beans.

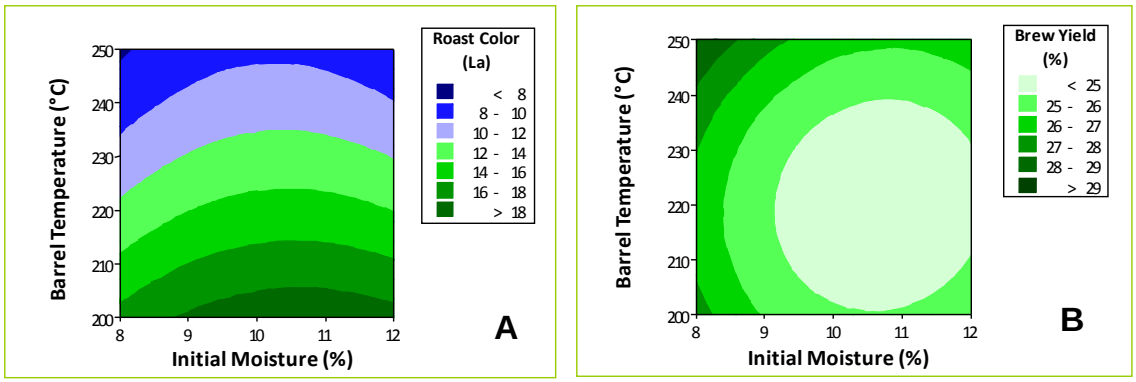

Figure 1. Contour plots for product roast colour (A - La) and brew yield (B - %) as a function of barrel temperature (°C) and initial feed moisture (%). The extruder screw rotation speed is constant at 55 rpm.

MW distribution of Extruded coffee extract at Light and dark roast colour

Water extracts of extruded coffee samples were prepared using standard conditions and fractionated into high, medium and low molecular weight su-fractions by ethanol precipitation. The relative amount of key types of sugars present in each of the sub-fractions after hydrolysis gives an indication of the types and degree of branching of the polymeric carbohydrates extracted in the brew (Figure 2).

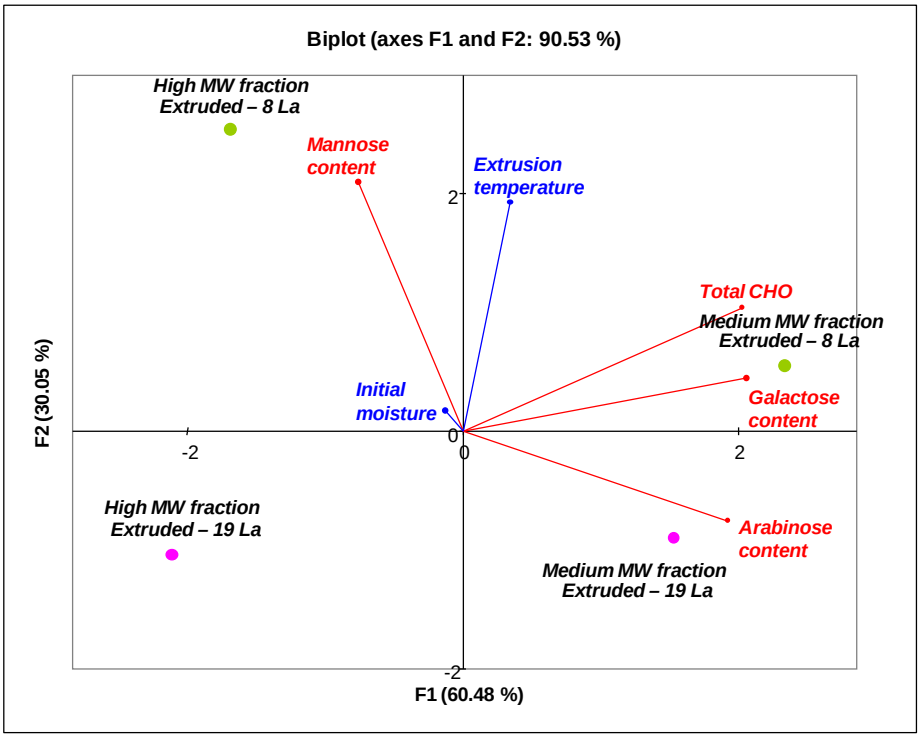

Figure 2. Principal Component Analysis of the High (50% ethanol precipitation) and Medium (75% ethanol precipitation) Molecular Weight (MW) fractions of the brew extracted from light (19 La) and dark (8 La) extruded robusta coffee.

In samples with a light roast colour, the medium molecular weight fractions is dominated by arabinose and galactose, reflecting the relatively high solubility of medium size arabinogalactans compared to larger structures, confirming observations made by Nunes and Coimbra (2001) on conventionally roasted coffee. However as the temperature of the barrel is increased andextruded product gets darker, the proportion or arabinose decreases in favour or galacotose and mannose, indicating an increased solubilisation of medium size galactomannanes, also observed in conventional roasting by Oosterveld et al. (2003). The High MW fraction is largely dominated by mannose and the amount of mannose present in this fraction is positively correlated to the product colour and barrel temperature. This indicates that the increase in brew yield observed at high barrel temperature is mainly driven by the increased solubilisation of high and medium molecular weight compounds containing mannose and galactose, probably structural galactomannanes which are known to have a pour solubility in lightly roasted coffee.

Sensory profile of extruded coffee

The differences in flavour profile between the water extract of extruded and conventionally roasted robusta coffee can be suitably discribed along a single axis on a PCA map (Figure 3), opposing the typical flavour characteristics of dark roasted robusta coffee (e.g. rubbery, smoky) to those of under-roasted robusta coffee (e.g. pop-corn, grainy).

The most noticeable sensorial feature of an extract of extruded coffee is the low intensity of roasted flavour notes even in products with a colour that would suggest a high level of browning (12 La is considered a medium roast in conventional roasting).

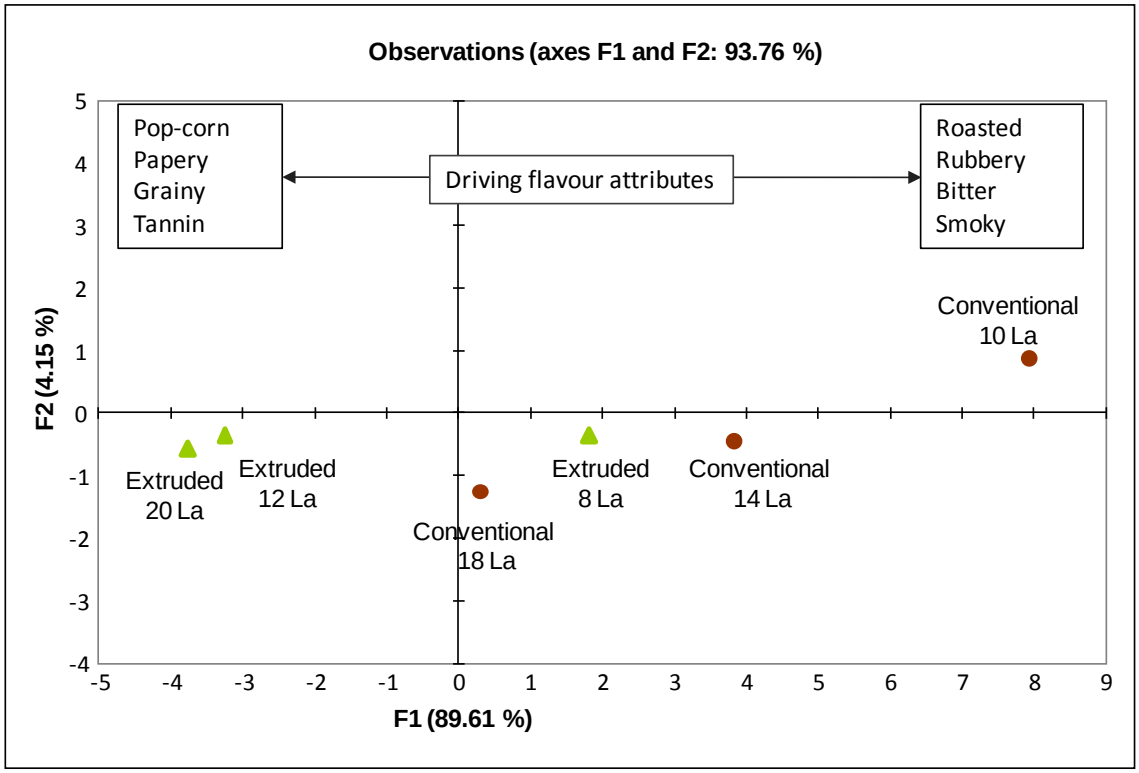

Figure 3. Principle Component Analysis representation of the flavour profile of extruded and conventionally roasted robusta coffee at various degrees of roasting (in lange, La).

The roast flavour intensity of a dark extruded robusta (8 La) is comparable to that of a very lightly conventionally roasted robusta (18 La). Although browning of the product takes place during extrusion cooking, the generation of typical roast coffee aroma compounds is greatly reduced. These results highlight the de-coupling of browning and aroma generating reactions in extrusion roasting, due to the differences between the conditions met by ground green coffee in an extruder barrel and those met by a whole bean in an hot air roaster, notably in terms of moisture and heating time.

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KENCO Coffee, 100% Committed to Sustainability: How Innovation Enables the Commercialisation of Sustainable Coffee in the Mainstream Retail UK Market

F. GUILMINEAU, J. MELROSE, M. HETT, J-A. CLARK

Kraft Foods, Global Coffee Technology Centre, Ruscote Avenue, Banbury - OX16 2QU, UK

SUMMARY

Coffee sold as certified coffee represents only about 6% of all coffee produced, and coffee products claiming to be sustainable are considered as a niche within the market. With the increasing consumer awareness of the global need to consume in a more responsible way, the demand for sustainable products is increasing, requiring a shift from the current niche into the mainstream. Kenco is a major coffee brand in the UK which has taken a multidimensional approach to improving the sustainability of their instant coffee product range. Initiatives have allowed significant improvement on all three key aspects of the product's footprint: the agricultural production of the coffee, the processing of the product, and the packaging. The instant coffee product has been redesigned to be produced solely from Rainforest Alliance certified coffee while retaining sensory equivalency to the former design. Investments made in the factory on technology and training of the employees have allowed some significant reductions in the energy and water used as well as the amount of waste being sent to landfill. Finally, the design and introduction of an innovative "Eco-refill" packaging format provides a huge reduction in packaging weight, energy consumption, and the possibility for the consumer to up-cycle the empty packaging in a creative way.

The work presented on those three aspects of the product illustrates how an innovative technology driven approach can enable the broadening of a sustainable coffee offering into the mainstream market.

SUSTAINABLE COFFEE IS CURRENTLY NICHE AND COULD BE INTRODUCED INTO MAINSTREAM

The certification of farms producing coffee has grown in the recent years and is projected to grow some more in an attempt to meet consumer demand (Giovannucci, 2008). However the coffee purchased as certified coffee, and therefore sold making sustainability claims is estimated to represent only aout 6% of worldwide production in 2008 (Figure 1).

These products in the coffee sector are therefore still considered as niche and most mainstream retail coffee products do not make any sustainability claims in relation to the agricultural production of the raw material used to make them.

SUSTAINABILITY IN THE MAINSTREAM WORKS

Consumer facing communication highlighting the importance of ethics and the environment has proliferated in the past few years. However, even though consumers know it is important, most of them have reservations about buying from brands that communicate about

sustainability. Many worry about having to compromise on quality (i.e. perception that sustainable products are lower quality) or price (i.e. sustainable products are more expensive). Kenco broke through this barrier by talking about sustainability in a way that really connected with consumers in their world, transforming the brand and business in the process. The key to the success in repositioning the brand lies in the respect of consumers' priorities and the clarity of the connection between consumers' world and the issues highlighted in the communication.

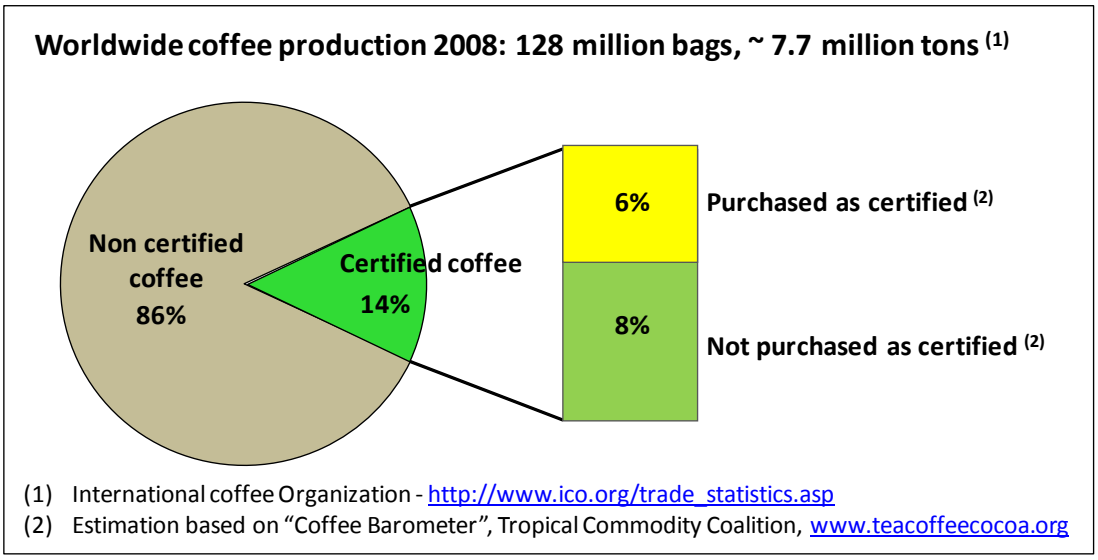

Figure 1. Estimated proportion of certified coffee in total coffee production and estimation of the ratio sold as certified.

Over the course of two advertising campaigns, the perception of Kenco was transformed and 1.2 million new households bought into the brand[1]. Business results on the UK market were monitored over a 19 months period following the start of the Kenco product re-positioning campaigns (various sources: Nielsen, TNS and IPSOS, 2010). An increase of 44.6% in value sales was measured, as well as a 2.8 point increase in value share, a 4.9 point increase in household penetration, and a 2.8 point increase in top-of-mind brand awareness.

These campaigns were leaning on actual changes to the product design in terms of the raw material used, the packaging format, and backed up by improvements in the factory to reduce the environmental impact of the manufacturing process.

Raw material

The product has been redesigned to be produced solely from Rainforest Alliance certified coffee while retaining sensory equivalency to the former design. Rainforest alliance coffee is produced following the Sustainable Agricultural Standard[2]. This standard for sustainable agriculture is particularly focused on protecting wildlife, wild lands, workers' rights and local communities. Considerable efforts have been made in recent years to collect quatitative data on the environmental impact of certification, notabley by the Committee On Sustainability Assessment (COSA, see Giovannucci et al. (2008) and this work is still in progress.

[1] Sources: TNS and Office for National Statistics, 2010
[2] http://www.rainforest-alliance.org/agriculture/documents/sust_ag_standard_july2010.pdf

Processing

Reducing the energy requirement of instant coffee manufacturing mostly consists in recovering as much as possible of the energy used rather than rejecting it into the environment as waste. A typical example of that is the recovery of thermal energy using plate heat exchangers to recover heat from a product stream needing cooling, and transfer it to a product stream needing heating. Judicious use of this principle has for example lead to a 35% decrease in the thermal energy rejected to the environment from cooling towers over the past 2 years in the factory where the Kenco product is manufactured. This was also complemented by investing in Mechanical Vapour Recompression evaporators, which uses 1/4 of the energy required for the same operation compared to the previous generation of evaporators.

The water usage in that same factory has also been decreased by 15% over the past 2 years. This was achieved partly by reducing the amount of water evaporated into the atmosphere for cooling, and also by recycling some of the process water to feed into the liquid ring vaccum pumps, rather than using fresh water.

The vast majority of the waste material from the production site is spent ground coffee. On average, about 95% of the spent grounds are burnt on site in a special boiler for energy recovery, thereby greatly reducing the need to send waste offsite for composting, and enabling the production of part of the plant's energy requirements. The emissions from that special boiler are very tightly controlled to minimise their environmental impact.

Packaging reduction

The idea to develop a refill packaging built on a piece of research carried out as part of the Waste and Resource Action Program (WRAP) in the UK (Ross, 2008), and which suggests that the increase in environmental awareness in the UK may create an opportunity to promote the environmental advantages of refillable packaging by creating a 'feel good' factor.

Figure 2. Illustration of the use of packaging to improve sustainability credential of a soluble coffee product.

As pointed out by INCPEN[3], although recycling has the potential to minimise environmental impact, it is more important to aim for minimum use of materials and energy. One of the most effective ways to reduce material and energy use is to combine two, three or more layers of different materials to provide the same level of protection as one thicker, single-material. There may not be any environmental benefit in recycling the multilayer packaging but it can be the most resource efficient option by reducing material and energy use in production and distribution, reducing the number of lorries needed for transport and value, as energy, can be recovered after use.

LCA is currently undertaken to quantify the environmental impact of packaging options in terms of carbon footprint, primary energy demand and pollution in the end of life.

CONCLUSIONS

The market is ready for mainstream coffee products with sustainability credential, the key to success remaining the clarity of the connection between consumers' world and the issues highlighted in the communication. Kenco in the UK is an example of how this can be achieved successfully using a combination of technology driven initiatives.

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[3] Source: INCPEN - the industry council for packaging and the environment. "Packaging in the supply chain" research facts, 23rd November 2006

Expression Analysis and Nucleic Polymorphism of Candidate Genes for Drought Tolerance in Coffee

L.P. FREIRE[1], N.G. VIEIRA[1], F. VINECKY[1], G.S.C. ALVES[1], T. LEROY[2], D. POT[2], S. ELBELT[1,2], T. MARQUES[1,2], G.C. RODRIGUES[3], P. MARRACCINI[1,2], A.C. ANDRADE[1]

[1]Embrapa Recursos Genéticos e Biotecnologia (LGM-NTBio), Brasilia, DF, BR
[2]CIRAD UMR DAP, Montpellier, FR
[3]Embrapa Cerrados, Planaltina, DF, BR

SUMMARY

Aiming at the establishment of a breeding program based on marker-assisted selection for drought tolerance on coffee, the present work had as goals (i) the validation of candidate genes (CG) previously identified by differential gene expression in leaves of tolerant and susceptible clones of *Coffea canephora* grown in greenhouse under different water conditions and also in leaves of *Coffea arabica* under field conditions and (ii) the analysis of nucleic polymorphism of some CG. Gene expression experiments were performed in young plants of Iapar59 (drought tolerant) and Rubi (drought sensible) cultivars of *Coffea arabica* grown in the experimental station of Embrapa Cerrados (Planaltina DF), which is characterized by a regular dry season. Plants were grown under two water treatments: T1 a non limited watering treatment (irrigated during the dry season) and T2 a limited watering treatment (non irrigated during the dry season). Leaf samplings were performed along the two years of the project and used to analyze CG expression by quantitative real-time PCR (qPCR). In parallel, corresponding gene sequences of some CG were also amplified from several coffee (*C. arabica* and *C. canephora*) accessions believed to cover these species' genetic diversity for drought tolerance.

INTRODUCTION

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, or affecting flowering or bean development in case of moderate stress (DaMatta and Ramalho, 2006). In relation to coffee genetic diversity, several works reported the identification of plants of *C. canephora* var. Conilon susceptible or tolerant to drought (Ferrão et al., 2000; Fonseca et al., 2004) which were analyzed at the physiological level (DaMatta et al., 2003; Lima et al., 2002; Pinheiro et al., 2004; 2005) and also used to identify candidate genes underlying stress responses (Andrade et al., 2006; Marraccini et al., 2009; Vinecky et al., 2008). Even narrow, a genetic diversity for drought tolerance also exist in the species *C. arabica* (Beining et al., 2008). 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 meristem tissues from Iapar59 (I59, drought tolerant) and Rubi (R, drought sensible) cultivars of *Coffea arabica* grown under field-grown with (I) or without (NI) irrigation were extracted and used to generate cDNA that were further sequenced. The preliminary results of this study are presented here.

MATERIALS AND METHODS

Plant materials

Field trials were conducted using the 2 years old plants of cultivars Iapar59 (I59) and Rubi of *Coffea arabica*, the former being considered more tolerant to drought than the latter (M.A.G. Ferrão, personal communication), grown in field condition. at the experimental station of the Embrapa Cerrados center (Planaltina-DF, Brazil, 15°35'43"S - 47°43'52"O).

Field experiment

For both cultivars, plants were cultivated with (I) and without (NI) irrigation during the dry season. Under the irrigated (I) condition, water was supplied by sprinklers (1,5 m height) organized in the field to perform uniform irrigation. Water soil moisture was controlled using PR2 profile probes (Delta-T Devices Ltd, Cambridge-UK). Our main points of analysis corresponded to P1 (06/2008), P2 (08/2008), P4 (05/2009) and P5 (08/2009) with P2 and P5 being analysis points performed during the dry seasons (Figure 1).

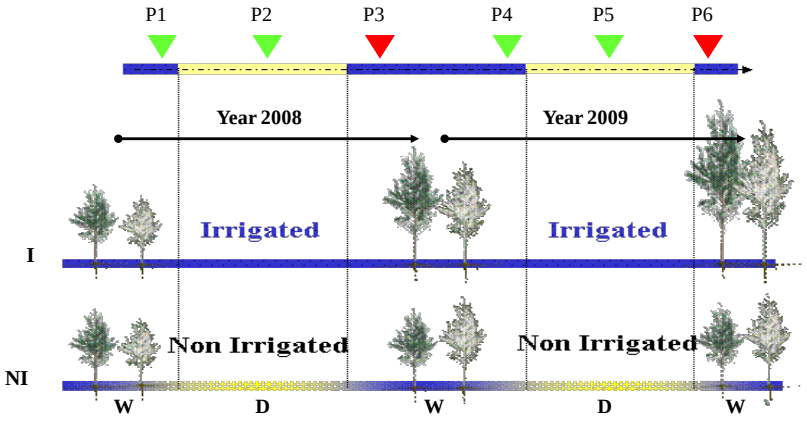

Figure 1. Schematic representation of water treatments applied to coffee plants. I and NI treatments were respectively irrigated and non-irrigated during the dry (D: may to September). During the wet season (W: October to April), irrigation was suspended. P1 to P6 corresponded to points of analysis of the project. Leaves analyzed in this work corresponded to those harvested at green points.

Sampling procedures

For P2 and P5 analysis points, the water stress was evaluated by measurements of predawn leaf water potential (Ψpd) with a Scholander-type pressure chamber using fully expanded leaves corresponding to those of the third node of plagiotropic branches usually localized in the third upper part of the plants. For qPCR analyses, the same leaves were day collected (between 10:00 to 12:00 am) in the field by freezing in liquid nitrogen and further conserved in the temperature of -80 °C before the extraction of the total RNA.

Sequence accession and primer design

Primer pairs used for qPCR analysis were designed using the "Primer Express 3.0" program (Applied Biosystems) and the candidate gene sequences (contigs) produced by the Brazilian Coffee genome project (https://alanine.cenargen.embrapa.br/cafEST) (Vieira et al., 2006).

RNA extraction and qPCR experiments

Total RNAs were extracted as described previously (Geromel et al., 2006), treated with DNaseI-RNase-free (Promega) and 1µg was reverse-transcribed with the ImPromII enzyme according to the recommendations of the furnisher (Promega). Synthesized single-strand cDNA were diluted (1/25 to 1/100) and tested by qPCR using CG primer pairs preliminary tested for their specificity and efficiency. The qPCR was performed with 1µl of cDNA in a final volume of 10µl with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a FAST7500 apparatus (Applied Biosystems). For each sample, CG expression levels were standardized (internal control) to the expression of *G3PDH* gene (Barsalobres-Cavallari et al., 2009) coding for the glyceraldehyde-3-phosphate dehydrogenase enzyme. Data were treated by SDS 2.0.1 program (Applied Biosystems). Expression levels were calculated with the ΔC_T (C_T [*CG*] - C_T [*G3PDH*]) method and using I59-I as calibrator. Expression levels were then expressed in relative quantification by calculating the values of $2^{-\Delta\Delta C_T}$.

DNA sequences

DREBA genomic sequences were amplified by conventional PCR reactions using the DREB1A-F (5'-GTTGAATTAACTCCTCACTGTCCACTA-3') and DREB1A-R (5'-CCAAAAACT GCAGTACGGAATAGA-3') primers, 6.25 ng of DNAg and High Fidelity Platinum *Taq* DNA Polymerase (Invitrogen). PCR conditions were 94 °C-30 sec., 55 °C-30sec. and 72 °C-4 min during 40 cycles followed by a final extension of 10 min. at 72 °C. PCR products were purified by treatment with "Wizard SV Gel and PCR Clean-Up System" (Promega), then cloned into the pCRII-TOPO vector (Invitrogen) before being sequenced with universal primers.

Sequence analysis

After sequencing, *DREBA* DNA sequences were treated the "Sequencing analysis, v5.2" program (Applied Biosystems) and analyzed with DNAStar package (LaserGene).

RESULTS

Evaluation of field water stress

For this study, the main points of interest are those corresponding to the dry season (P2 in 2008 and P5 in 2009) when plants suffered water stress in NI conditions and were compared to those irrigated used as a control. The water stress of NI-plants was evaluated by measuring predawn leaf water potential (Ψpd) (Table 1). For both, lower Ψpd were measured in leaves of NI plants in 2008 than in 2009, showing that the water stress was higher in the former. This analysis also revealed that in NI condition, Ψpd values the I59 cultivar always appeared twice lesser than in Rubi, therefore suggesting drought tolerance of I59 by comparison to Rubi. As a control, it is worth noting that Ψpd of I (irrigated) plant always ranged from -0.3 to -0.1 MPa, demonstrating unstressed conditions.

Effects of water stress on CG expression

The qPCR experiments were used to analyze in leaves of *C. arabica*, expression profiles of several candidate genes (CG) previously identified to show differential expression with water stress in the leaves of clone 14 (drought-tolerant) and 22 (drought-susceptible) of *C. canephora* var. conillon (Andrade et al., 2006; Marraccini et al., 2009; Vinecky et al., 2008).

Our attention was focused on the study of *RD29*, *DREB* (both encoding for trans-acting factors controlling water stress gene expression) and *M6PR* (responsible of mannitol synthesis). These results are presented in Figure 2.

Table 1. Predawn leaf water potential (Ψpd) measured during the dry seasons of 2008 and 2009 in leaves of I59 and Rubi cultivars grown under I and NI conditions. Values are expressed in mega-Pascal (MPa). Values of SD are also indicated.

	2008		2009	
	I	**NI**	**I**	**NI**
I59	-0,38 ± 0,10	-0,80 ± 0,12	-0,12 ± 0,00	-0,59 ± 0,03
Rubi	-0,22 ± 0,07	-1,88 ± 0,36	-0,11 ± 0,00	-1,20 ± 0,16

Analysis of RD29 gene expression

In 2008, levels of *RD29* gene expression appeared similar in leaves of I59 and Rubi cultivars cultivated under unstressed conditions (P1 and P2I) showed differential expression. However, differential gene expression was clearly observed between the two cultivars during P2NI point of harvest, with higher (x4) *RD29* gene expression observed in Rubi than in I59. In that sense, these results confirmed those observed in *C. canephora* which showed higher *RD29* gene expression in leaves of clone 22 than in those of the clone 14 (Marraccini et al., 2009). It is worth noting that *RD29* presented very low level of expression during the P5 point of analysis (data not shown). This could be explained by the lower water stress applied to the plant in 2009 by comparison to 2008.

Analysis of M6PR gene expression

In 2008, *M6PR* gene also showed differential expression during P2 point analysis. For both cultivars, the results presented clearly showed higher expression of this gene in NI leaves than in those of irrigated (P2I) plants. Under NI conditions, it is also interesting to note that *M6PR* gene expression appeared more than two fold higher in I59 than in Rubi. Even with a lesser amplitude, identical results were also observed in 2009 (data not shown). The results presented here also confirmed those observed in *C. canephora* showing water-stress induction of *M6PR* gene with higher expression in leaves of drought-tolerant clone 14 than expression levels of the same gene in leaves of drought-susceptible clone 22 (Vinecky et al., 2008).

Analysis of DREBA gene expression

Expression profiles of *DREBA* gene showed obtained in 2009 also showed differential expression with water stress. With the exception of Rubi P5I, levels of *DREBA* expression always appeared low and similar for both cultivars under unstressed conditions like during I and NI conditions of P4 (end of wet season, Figure 1) and P5I. By comparison to P5I, levels of *DREBA* expression under NI increased eight fold in leaves of I59 and around four fold in those of Rubi. These expression profiles were not observed during the P2 point of analysis (2008) that presented more severe water stress conditions (Table 1). The results of *DREBA* gene expression in *C. arabica* differed from those previously obtained in *C. canephora* that showed a strong water-stress induction of *DREBA* in the leaves of clone 14 but very low expression in the leaves of clone 22 (Marraccini et al., 2009).

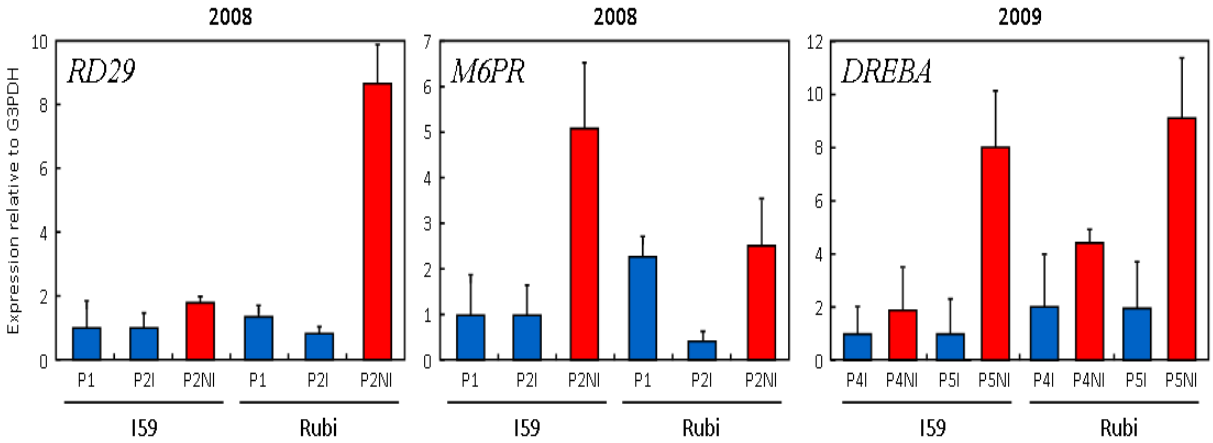

Figure 2. Expression profiles of *RD29*, *M6PR* and *DREBA* candidate genes in leaves of Iapar59 (I59) and Rubi cultivars of *C. arabica*. Points of analysis corresponded to P1-P2 (in 2008) and P4-P5 (in 2009). Cultivars and treatments are indicated. Results (in triplicate) of qPCR are expressed in relative quantification standardized with expression of *G3PDH* gene. For each gene and treatment, expression of I59I was used as internal calibrator.

Analysis of DREBA genetic diversity

Due to the importance of the *DREBA* gene in the control molecular mechanisms involved in the control of gene expression by abiotic stress (Yamaguchi-Shinozaki and Shinozaki, 2006), and even if variations of *DREBA* gene expression are mainly controlled transcriptionaly, a genetic study of *DREBA* was initaited by analyzing its corresponding genomic sequences in genotypes of *C. arabica* and *C. canephora* covering the *Coffea* diversity. Using specific primers designed at the 5' and 3' extremities of the *DREBA* cDNA, PCR products (879bp) were then cloned and sequenced. For each genotypes, DNA alignments showed the absence of introns and the presence of seven single nucleotidic polymorphisms (SNP), four of them within the coding and the 3 others in the 3' transcribed unstranslated region.

Figure 3. Schematic representation of the DREBA gene structure. SNP identified by DNA sequencing are indicated in red (when changing amino acid), green (without changing amino acid) or in yellow (when localized in the 3' UTR region). Positions of the DREB1A-F and DREB1A-R primers are also indicated.

Despite the great conservation of the *DREBA* gene, sequence analyses obtained from *Coffea* genotypes permitted to identify the existence of 7 haplotypes (allelic forms) for this gene. According the origin of *C. arabica* (Lashermes et al., 1999), the D haplotype characterizing *C. eugenioides*, was also present in all genotypes analyzed of *C. arabica*, but absent in all accessions of *C. canephora*. It is worth noting that the drought-tolerant clone 14 of *C. canephora* was homozygotous for the haplotype A while the drought-susceptible clone 22 was heterozygotous, with the presence of 3 haplotypes (A, B and C). Interestingly, the haplotype A was also found in the Iapar59 cultivar of *C. arabica* as well as in SG1 (in the

homozygous form) accessions of *C. canephora*. Further work is required to see if the presence of this haplotype could be linked with drought tolerance in coffee.

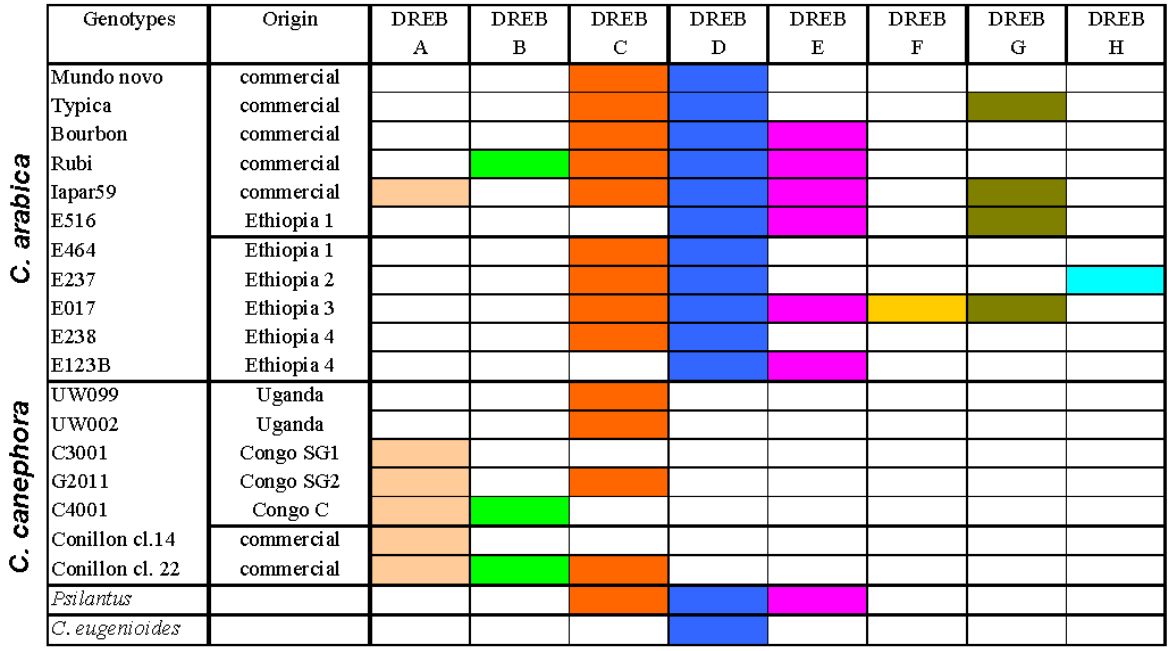

Figure 4. Genetic diversity of *DREBA* candidate gene. Haplotypes of this gene are noted from A to H. Coloured boxes were used to show the presence of haplotypes found in the coffee genotypes studied. For both species (*C. arabica* and *C. canephora*), genotypes are classified in "commercial" and "wild" classes.

To complete this analysis, work is ongoing to study the genetic diversity of the promoter region of this gene and also from other candidate genes [see G.S.C Alves et al., in the same issue]. Such analysis, that should be speed-up with the forthcoming information of coffee genome sequencing initiatives, will now open the way to the identification of molecular markers for drought tolerance that could be further used to accelerate coffee breeding programs dedicated for the creation of new cultivars better adapted to water stress.

ACKNOWLEDGMENTS

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MOCCADB - An Integrative Database for Functional, Comparative and Diversity Studies in the Rubiaceae Family

V. PONCET[1], O. PLECHAKOVA[1], F. BENEDET[2], R. GUYOT[1], M. COUDERC[1], P. DE BLOCK[3], P. HAMON[1], C. CAMPA[1], A. DE KOCHKO[1], S. HAMON[1], C. TRANCHANT-DUBREUIL[1]

[1]UMR DIAPC, IRD, 911 avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France
[2]CIRAD TA C 37/D, Campus International de Baillarguet 34398 Montpellier Cedex 5, France
[3]National Botanic Garden of Belgium, Domein van Bouchout, 1860 Meise, Belgique

SUMMARY

In the past few years, genomics information has been rapidly accumulating on Rubiaceae species and especially on those belonging to the *Coffea* genus. An increasing number of expressed sequence tag (EST) data and genomic- or EST-derived microsatellite markers have been generated, together with Conserved Ortholog Set (COS) markers. This considerably facilitates comparative genomics or genetic map based studies through the common use of ortholog loci across different species. Similar genomic information is available for tomato or potato, members of the Solanaceae family. Since both Rubiaceae and Solanaceae belong to the Euasterids I clade, integration of information on genetic markers would be possible and should lead to more efficient analyses and discovery of key loci involved in important traits such as fruit development, quality or adaptation. Our goal was to develop a comprehensive web data source for researchers working on *Coffea* genus, the Rubiaceae family or related species.

MoccaDB (http://moccadb.mpl.ird.fr/) (Plechakova et al., 2009) is an interactive online database that manage information about EST-SSR and SSR markers. Markers were checked for redundancy, in vitro tested for cross-amplification and diversity over up to 38 Rubiaceae species. MoccaDB includes Cmap and BLAST tools and links to other related databases (e.g., SGN, NCBI).

We believe that MoccaDB will be extremely useful for all researchers working in the areas of comparative and functional genomics and molecular evolution, in general, and population analysis and association mapping of Rubiaceae and Solanaceae species, in particular.

INTRODUCTION

Accumulation of available genetic markers directly contributes to advances in marker-assisted genetic studies with a wide range of applications such as identification of individual genes and/or quantitative trait loci (QTL), or exploration of the genetic diversity and population structure with regard to natural variations. The recent and rapid accumulation of sequence resources, mainly from crop species, ensures an improvement of the genetics approach in combination with the comparative genomics. The extension of these genome resources to their close relatives as well as to more distant genera greatly facilitates the elucidation of evolutionary histories. This elucidation involves the discovery and study of key orthologous loci, phylogeny reconstruction and a variety of other biological questions.

The Rubiaceae family is the fourth largest family of flowering plants, most of the genomic information has been generated from the major economic crop species of the *Coffea* genus: *C. arabica* and *C. canephora*. They are thus used as molecular models for the Rubiaceae. Integrative information of genomic and genetic knowledge acquired for these plants can be further extended to other *Coffea* species but also to other economically important Rubiaceae genera.

Among PCR-amplified markers, microsatellites (or simple sequence repeat, SSR) are commonly used in large-scale genomic studies owing to their ubiquitous distribution in both protein-coding and non-coding regions and the high degree of length polymorphism among individuals. Due to the increase in *Coffea* genomics resources (Table 1), SSR were obtained from both *C. arabica* and *C. canephora* (de Kochko et al., 2010).

Table 1. Available *Coffea* ESTs resources, September 2010 (Source: http://www.ncbi.nlm.nih.gov).

Species	Number of EST sequences
C. arabica	56,231
C. canephora	43,619

The use of markers directly targeting expressed genes important for each specific trait would be beneficial to these studies.

Previous publications (Poncet et al., 2007) and the present study have revealed that coffee EST-SSR and anonymous SSR markers show a high level of transferability across distantly related species, thereby providing additional markers for orphan Rubiaceae species.

Although the genomic data available on coffee plants are rapidly increasing, they are often isolated and scattered and rarely available online. In the present study, an effort has been made to create a centralized access to both published and original new data on evolutionarily conserved and validated markers. MoccaDB is an integrated comprehensive information system which will be useful for the research community working on plant genetics and evolution of coffee tree related organisms.

MATERIALS AND METHODS

Data Source

The current version of MoccaDB provides information regarding *Coffea* EST and genomic SSR markers retrieved from 11 published studies as well as original data (Table 2).

Maps, transferability and diversity

Marker mapping data were retrieved from a published inter-specific *Coffea* linkage map (Lefebvre-Pautigny et al., 2010).

The high transferability of SSR markers at evolutionarily conserved (orthologous) loci within the Coffea genus has been previously reported by different authors. For example, the

percentage of transferability of SSR markers developed on C. *arabica* genomic DNA ranged from 72.7% for *C. liberica Hiern* to 86.4% for *C. pseudozanguebariae Bridson*.

Table 2. Microsatellite markers, Sequences sources and original data.

Reference	**Sequence type**	**Sequence origin (*Coffea*)**	**No of markers**
Poncet et al., 2007	Genomic	*C. canephora*	213
Crouzillat et al., public data	EST	*C. canephora*	132
Poncet et al., 2006	EST	*C. canephora*	99
Poncet et al., 2004	Genomic	*C. arabica*	77
Moncada et al., 2004	Genomic	*C. arabica*	34
Coulibaly et al., 2003	Genomic	*C. arabica*	17
Combes et al., 2000	Genomic	*C. arabica*	10
Geromel et al., 2006	EST	*C. canephora*	10
Aggarwal et al., 2007	EST	*C. sp.*	9
Baruah et al., 2003	Genomic	*C. arabica*	9
Bhat et al., 2005	EST	*C. canephora x C. congensis*	9
Lashermes et al., public data	Genomic	*C. arabica*	9
Leroy et al., 2005	Genomic	*C. canephora*	8
Total			**638**

Our previously published and newly designed EST-SSR markers, at a total of 99, were tested for amplification on a panel consisting of up to 21 Rubiaceae species belonging to the Cinchonoideae and Rubioideae sub-families. A new set of EST-SSR markers, provided by Crouzillat et al. (Table 2), was also tested on the following Coffea species: *C. canephora*, *C. heterocalyx Stoff.*, and *C. pseudozanguebariae*. Only those showing a good and specific PCR amplification with an easy scoring of allele sizes were retained.

Database and web application

MoccaDB is an online MySQL-PHP driven relational database. It integrates bio-informatics tools such as BLAST and CMap. Figure 1 shows an overview of the MoccaDB structure and interactions with the bioinformatics tools and external data sources.

RESULTS

An easy access to markers and genomics data

An user-friendly web interface has been developed to facilitate data retrieval and to provided specific features:

1. Multiple options for data search (markers, DNA sequences, maps and diversity) by using the corresponding multi-option query forms.

2. The data can be viewed with a different degree of details, either as an overview (a list of search results), or as a detailed result page for a selected marker, sequence or map, with information on marker transferability, diversity and mapping.
3. Complete description of the markers, going from in vitro PCR amplification conditions, SSR and functional annotation of original DNA sequences and marker location on genetic maps, to cross amplification and diversity data;
4. The experimental conditions, sequences and other relevant data are easily downloadable in different formats (excel, fasta).
5. Access is provided to integrated bioinformatics tools (CMap, BLAST), as well as to external hyperlinks to various public data sources (NCBI GenBank and Pubmed, SOL Genomics Network)

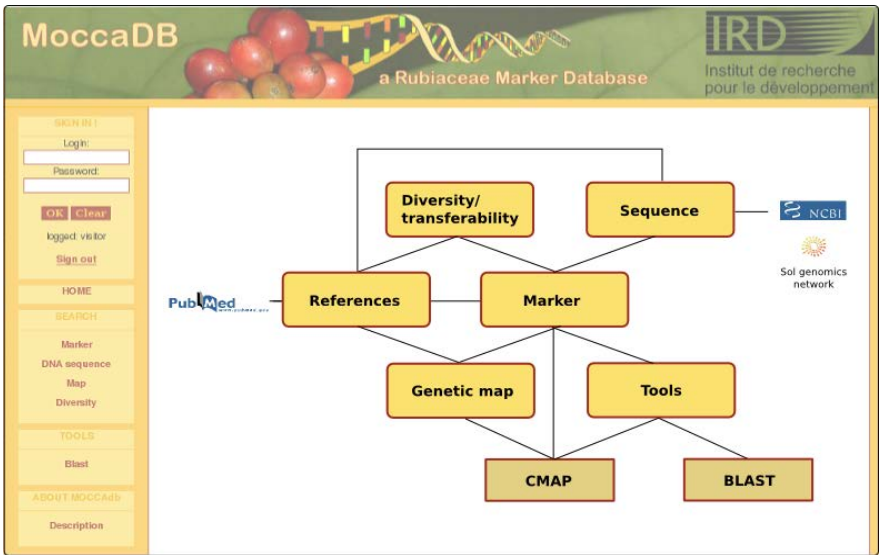


Figure 1. Overview of the MoccaDB application. MoccaDB integrates different data types, which are interconnected and linked to external resources and bioinformatic tools (CMap and BLAST). From (Plechakova et al., 2009).

Transferable markers and polymorphism status

In its current release, MoccaDB gives access to a maximum of available transferability-associated information for tested markers: transferability status, amplification quality, information on the polymorphism (number and size of alleles, polymorphism information content (PIC) value).

In particular, out of the *C. canephora* and *C. arabica* markers screened for cross-amplification and polymorphism, a minimum of 83% amplified alleles from any wild *Coffea* species independently from its genetic relationship to both cultivated species (Figure 3). More coffee markers were transferable to wild relatives from the Cinchonoideae subfamily, but only a small fraction, up to 12%, was transferable to distantly related genera from the Rubioideae subfamily (Table 3).

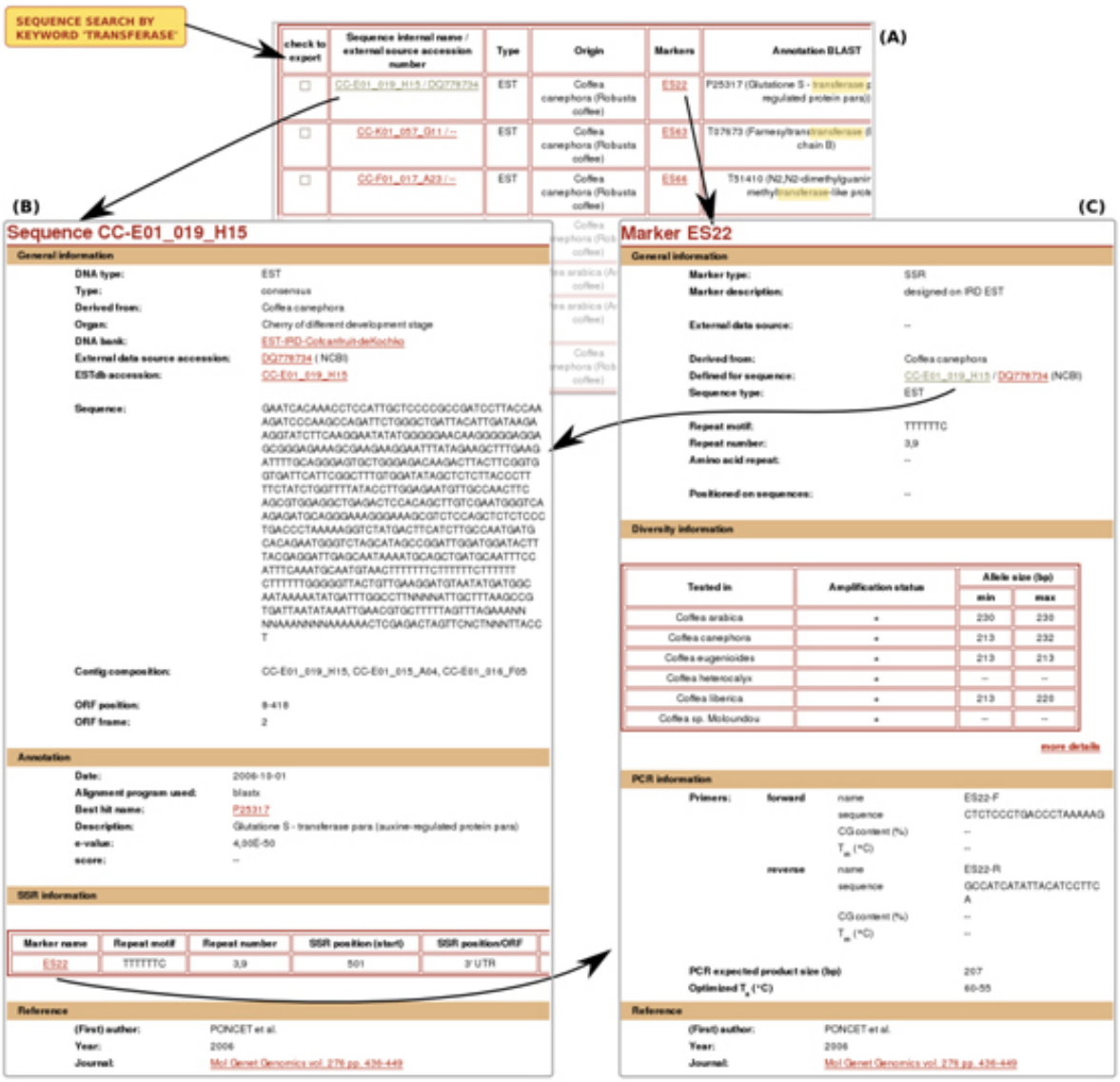
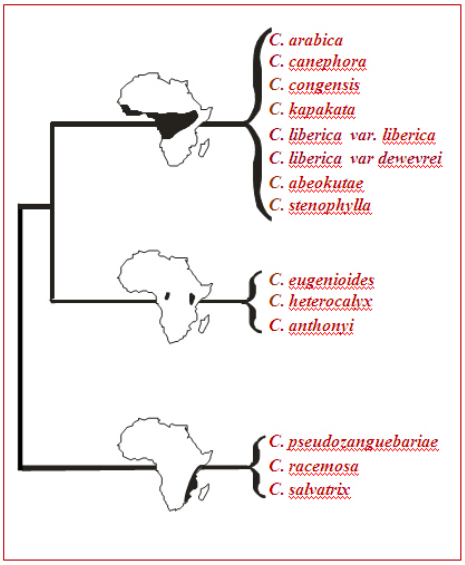


Figure 2. Screenshots of sequence/putative function MoccaDB result pages. Sequences can be searched by name, putative annotation. The search can be restricted using different criteria such as sequence origin or marker type. The group of screenshots shows an example of sequence search using 'transferase' as a keyword to find out sequences that have been "putatively" annotated with this term. (A) The result page displays sequences and related data resulting from searching for the annotation term 'transferase'. The tabular text summary lists all the sequences found, each line in the table contains the sequence name and related information (sequence type and origin, marker name and BLAST annotation). The marker name and sequence name are respectively linked to the marker detail page (C) and to the sequence detail page (B). The user can select sequences and export them in FASTA format. The sequence detail page (B) displays all the associated information for that sequence which includes general information (e.g.: sequence type, DNA bank), annotation information, marker information and publications related to that sequence. Hyperlinks give access to associated data within MoccaDB such as markers, DNA bank or link to external resources such as SOL or NCBI. From (Plechakova et al., 2009).



	Amplification		
***Coffea* species tested**	**All *Coffea* markers**	***C. canephora* markers**	***C. arabica* markers**
C. arabica L.	120/120 (100%)	59/59	49/49
C. canephora Pierre ex A.Froehner	216/234 (92%)	189/207	15/15
C. congensis A.Froehner	18/18 (100%)		9/9
C. kapakata (A.Chev.) Bridson	17/18 (94%)		8/9
C. liberica Hiern var. *liberica*	90/93 (97%)	73/75	9/9
C.liberica Hiern var. *dewevrei* (De Wild. & T.Durand) Lebrun	90/90 (100%)	72/72	9/9
C. liberica Hiern var. *liberica* (*C. abeokutae*)	18/18 (100%)		9/9
C. stenophylla G.Don	17/18 (94%)		8/9
C. eugenioides S.Moore	90/93 (97%)	72/75	9/9
C. heterocalyx Stoff	172/207 (83%)	172/207	
C. anthonyi Stoff. & F.Anthony *(C. sp. Moloundou)*	71/75 (95%)		
C. pseudozanguebariae Bridson	178/205 (87%)	178/205	
C. racemosa Lour	18/18 (100%)		9/9
C. salvatrix Swynn. & Philipson	15/18 (83%)		7/9

Figure 3. Schematic phylogenetic tree adapted from (Plechakova et al., 2009) and number of successfully amplified/tested markers (percentage) observed for each species. The information was extracted from MoccaDB database. Names of *Coffea* species follow (Davis et al., 2006; Stoffelen et al., 2009).

CONCLUSION AND PERSPECTIVES

In MoccaDB, a large amount of information is centralized and freely accessible to all users. The database currently houses SSR markers from both transcribed and non-transcribed regions of the genome. Markers whose polymorphism is due to single-nucleotide polymorphism (SNPs), insertion/deletion (indels) or transposable elements are in the process of being added. Whole genome sequencing, genetic, physical and comparative maps are being developed. MoccaDB will be extended to include all these new types of data.

Data obtained, stored and organized in this data bank could benefit to wide comparative genomics studies including those on Rubiaceae species.

Table 3. Transferability to Rubiaceae species: efficiency of cross amplification of *Coffea* markers in other Rubiaceae genuera (Nb species tested when over 1).

Genus	% amplification	Nb of tested markers
Cinchonoideae		
Coffea (14 sp.)	94%	Up to 207 C, up to 49 A
Psilanthus (4 sp.)	82%	9 A
Tricalysia (2 sp.)	68%	25 C
Bertiera	56%	25 C
Pavetta	48%	25 C
Coptosperma	40%	25 C
Leptactina	36%	25 C
Tarenna	36%	25 C
Paracephaelis	32%	25 C
Genipa	24%	25 C
Chiococca	16%	25 C
Ixora	16%	25 C
Uncaria	4%	25 C
Rubioideae		
Oldenlandia (2 sp.)	12%	25 C
Psychotria (4 sp.)	10%	25 C
Spermacoce (2 sp.)	6%	25 C

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A Coffee High Density Genetic Map for Quantitative Trait Loci Analysis on Agronomical, Technological and Biochemical Characteristics in Robusta and Arabica.

D. CROUZILLAT[1], M. RIGOREAU[1], F. LEFEBVRE-PAUTIGNY[2], PRIYONO[3], P. BROUN[1], C. LAMBOT[1]

[1]Nestlé Centre R&D, Tours, France
[2]Nespresso, Lausanne, Switzerland
[3]ICCRI, Jember, Indonesia

SUMMARY

The development and availability of a reference genetic map in coffee is an important step towards the management of key quantitative trait loci (QTLs). In this regards, a genetic mapping population from *Coffea canephora* (2n = 2x = 22), growing in two locations in Indonesia, was initiated using two Indonesian elite clones BP409 and Q121. These two Robusta clones are highly heterozygous and allow the development of a consensus genetic map including more than 1200 loci covering approximately 1400 cM. Different molecular markers such as RFLPs, SSRs and SNPs were used for the establishment of this Robusta reference genetic map. An important part of the loci mapped are coming from ESTs from which COSII (conserved orthologous set) allow the characterization of synteny relationship between coffee and tomato genomes.

The transposition of the reference genetic map to other Robusta progenies growing in Ecuador, Indonesia and in Thailand has allowed the determination of numerous QTLs for the agronomic and biochemical traits of interest. The use of a common set of SSRs in all the Robusta genetic maps studied gave the opportunity to compare the QTL data across the different genetic backgrounds and the environments.

The construction of an Arabica genetic map is more difficult due to the tetraploid status of *Coffea arabica* (2n=4x=44) and also due to the limited genetic diversity of this species at least on the cultivated germplasm. A F_2 mapping population, obtained from the cross of two wild accessions, was used in conjunction with SSR markers already mapped in the Robusta reference map. The first results indicated a reduced level of polymorphism especially in the SSRs coming from ESTs but the remaining level of diversity detected in the genomic SSRs appear to be sufficient for the establishment of an Arabica map draft. Up to now, 277 SSRs have been successfully mapped covering 1860 cM. The total genetic map size is estimated to be close to 3700 cM suggesting that the recombination rate could be higher in Arabica than Robusta genome. These results also indicated that the number of SSRs required to cover the entire Arabica genetic map will be around 600 markers.

A first QTL comparison study has been performed based on the Arabica draft map in conjunction with the different Robusta maps on shared agronomic, technologic and biochemical quantitative traits. The first results clearly indicate some major differences in the genetic control of these key characters for both Arabica and Robusta coffee species.

INTRODUCTION

Two species *C. canephora* and *C. arabica*, are widely cultivated for the production of the coffee beverage. The former is diploid and allogamous, the latter, allotetraploid and preferentially autogamous. Despite of its economic importance, coffee suffers of a lack of investments in terms of science resource. Moreover, coffee is a perennial plant which makes genetic studies more difficult and time consuming. While some genomic information is publicly available for coffee such as EST database (Lin et al., 2005; Poncet et al., 2006), it lags far behind what is available for many other agricultural species. As a result, coffee researchers have only limited access to the plethora of genomic and genetic resources available for most major crop species. Comparative genomics provides the opportunity to leverage genetic/genomic information from one species to another via comparative genetic maps (Wu et al., 2006; 2009a; 2009b). However, the value of comparative genomic information is inversely proportional to the evolutionary distance of the species being compared. The model species most closely related to coffee, for which significant genetics and genomic resources exist, is tomato.

Recently, comparative genetic maps have been generated for coffee and tomato based on COSII markers **(**Lefebvre-Pautigny et al., 2010), it might allow coffee researchers to access to the large set of genetic and genomic data currently available for tomato.

In the same time, this genetic map established on Robusta, was used to add a high number of SSR and SNP markers from various origins such as ESTs and genomic sequences. This high density reference map was then used to transpose molecular markers from Robusta to Arabica genetic map. Due to a lower genetic diversity in the Arabica species compared to Robusta the map construction is more difficult and requires high polymorphic markers such as microsatellites.

The primary objective of the current study aims at construct a high density Robusta map including both COSII and ESTs or genomic sequences (Poncet et al., 2007) that can be used for both synteny and genetic or genomic research in coffee. The second target is the establishment of an Arabica genetic map using previous mapped SSRs in order to create a global coffee genetic frame work. A third and important goal is the study of the QTLs detection and management for key traits on these two major coffee species.

MATERIAL AND METHOD

Plant Material

The cross pollinated Robusta population: BP409 x Q121 from ICCRI includes a total of 96 progenies is used to establish the Robusta reference genetic map. These progenies are growing in two different locations in Indonesia.

The F_2 Arabica population of 138 plants, obtained from a cross between two wild accessions belonging to public collection, is used to construct the Arabica genetic map.

Phenotypic evaluation

The field evaluations were done by ICCRI for the Robusta progeny and by Nestlé Ecuador for the Arabica population. All Arabica and Robusta green beans were extensively characterised for chemical composition, including: Caffeine, trigonelline, chlorogenic acids, sucrose,

proteins, lipids and citric acid. The biochemical composition was predicted by Near Infrared (NIR) spectroscopy using calibrations previously developed for green coffee. NIR reflectance spectra were collected on green coffee samples using a FT-NIR instrument (Antaris II, Thermo-Fischer). Spectrum were collected using a cup of 12 cm diameter, at a resolution of 8 cm-1 and 80 scans are necessary to do one complete round. For each sample, the predictive values were calculated as an average of four replications.

Genetic mapping and QTL detection

The genetic maps were performed on the basis of the reference genetic map established by Crouzillat et al. (in preparation). The two genetic maps were constructed by using JoinMap® software **(**Van Ooijen, 2006**)** with Kosambi's mapping function **(**Kosambi, 1944).

The software MapQTL5® (Van Ooijen, 2004**)** was used for QTLs detection on the genetic maps. In a first step, the non-parametric Kruskal-Wallis (KW) test was applied to detect individually significant association ($\alpha = 0.05$) between markers and traits. In a second step, interval mapping (IM) analysis was used to detect QTL. A threshold of LOD values of 4 and 3.8 for CP and F_2 populations, respectively, have been defined and used to declare the presence of a QTL. A QTL is validated when both LOD and K tests are significant. The genetic linkage groups carrying QTLs were represented using MapChart software (Van Ooijen, 2006**)**.

RESULT AND DISCUSSION

This Robusta genetic reference map includes mainly PCR based markers such as SSRs and SNPs. Among these markers a set of 369 COSII were used to study the syntenic relationship between Coffee and Tomato genomes (Lefebvre-Pautigny et al., 2010). Up to now, the Robusta reference map comprises 1200 loci covering approximately 1400 cM on the eleven haploid linkage groups (Figure 1). Due to the high level of polymorphism and heterozygosity observed in the two parents BP409 and Q121 Indonesian clones it was easy to obtain such high density mapping.

Unfortunately, the level of polymorphism detected in Arabica is quite low especially in the cultivated clones (Alemayehu et al., 2010) and the establishment of a core genetic map is difficult due to the limited access to wild Ethiopian Arabica germplasm with a higher level of polymorphism. However, Pearl et al., (2004) elaborated the first draft of the Arabica map using Catimor and Mokka clones with 288 AFLP markers covering 1802 cM on 31 linkage groups. For this study we used two wild accessions from which a F_1 hybrid plant was selfed to give the F_2 population. Moreover, we used only co-dominant markers such as SSRs for the mapping study in order to increase the mapping efficiency due to the higher level of polymorphism linked to microsatellites.

Up to now, our arabica map 277 SSR loci have been map covering 1860 cM on 61 linkage groups (Figure 2). A set of 70 SSRs are mapped on both the Robusta reference map and the Arabica genetic map allowing to perform a map comparison analysis. The main conclusion is that the two maps are collinear but the Arabica map exhibits a larger size with a final size estimated about 3700 cM. However, the differences observed for the recombination ratio are located specifically in different areas of the genetic map. Due to the co-dominant status of the SSRs it is possible, in some cases, to determine the species origin of the segregating alleles in Arabica using genotypes representative of the genetic diversity from the two putative diploids species at the origin of Arabica. There is no significant difference between these two

homologous genetic maps. This Arabica genetic map draft covers approximately 50% of the genome.

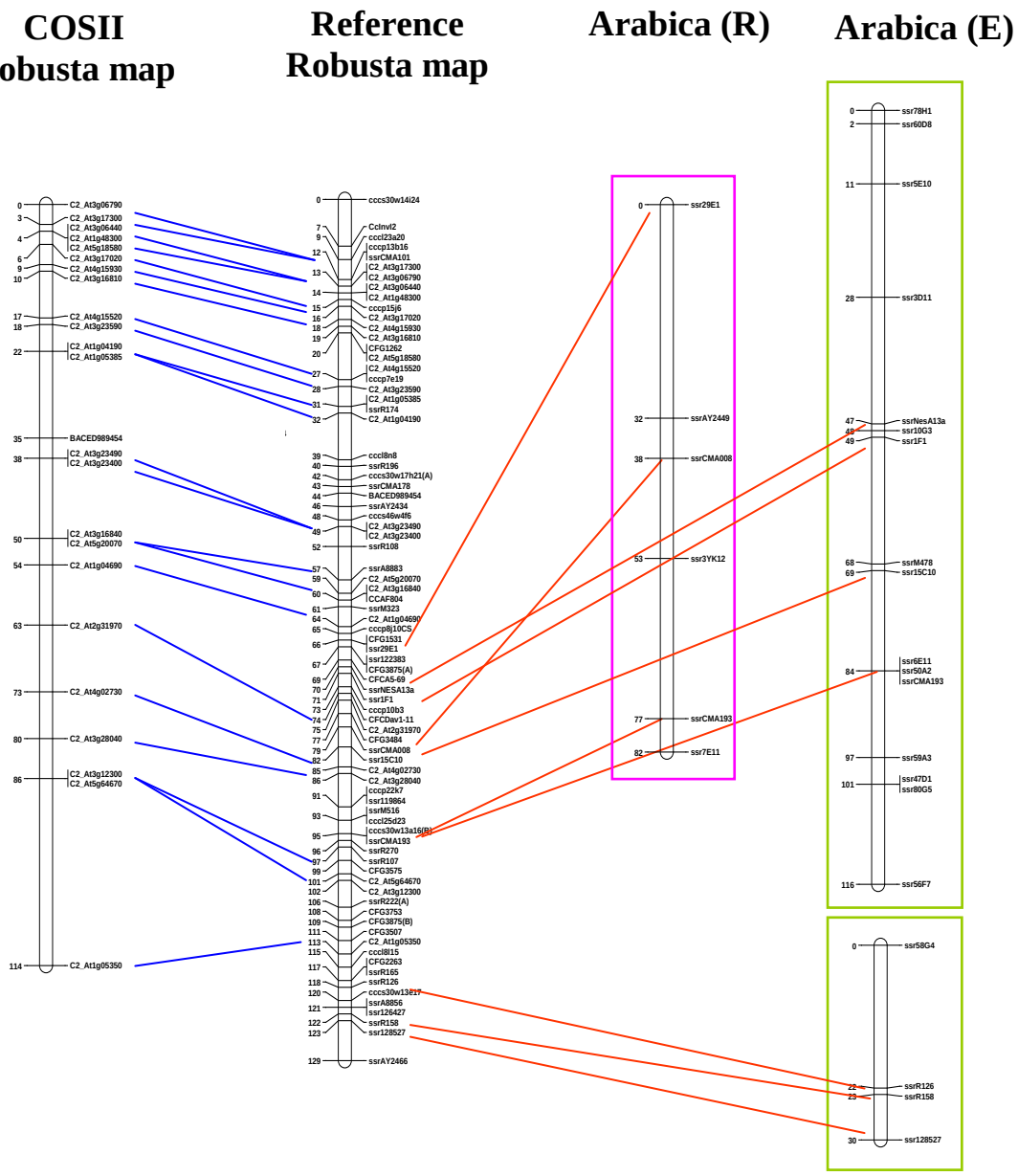
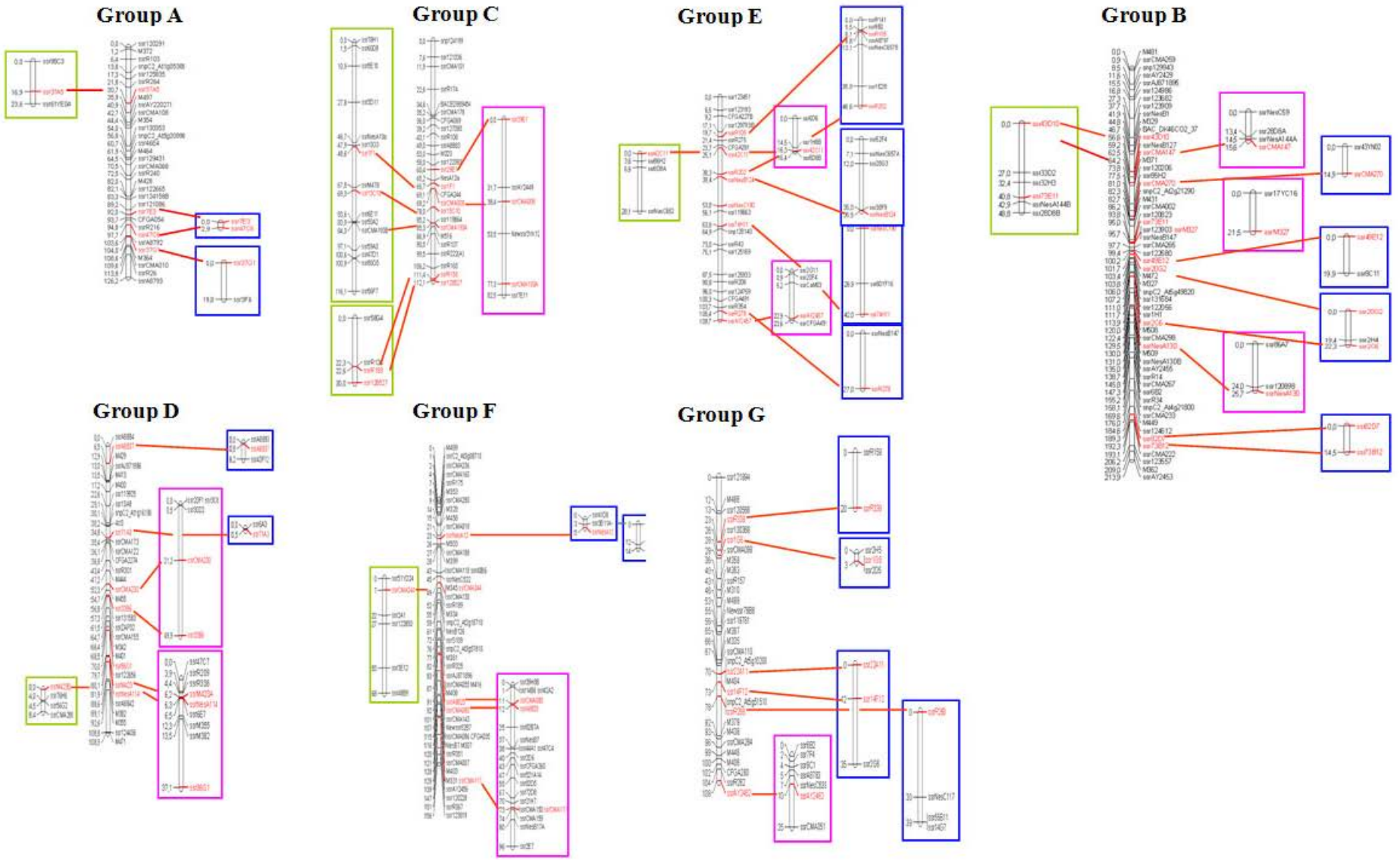


Figure 1. Marker transposition across Robusta and Arabica showing relationships on linkage group C between COSII, reference Robusta and Arabica genetic maps. The two homologous Arabica linkage groups C, originating from the two diploid coffee species, are coded as R and E according to the putative origins from *C. canephora* (Robusta) and *C. eugenoïdes*, respectively.


Group A
Group C
Group E
Group B
Group D
Group F
Group G

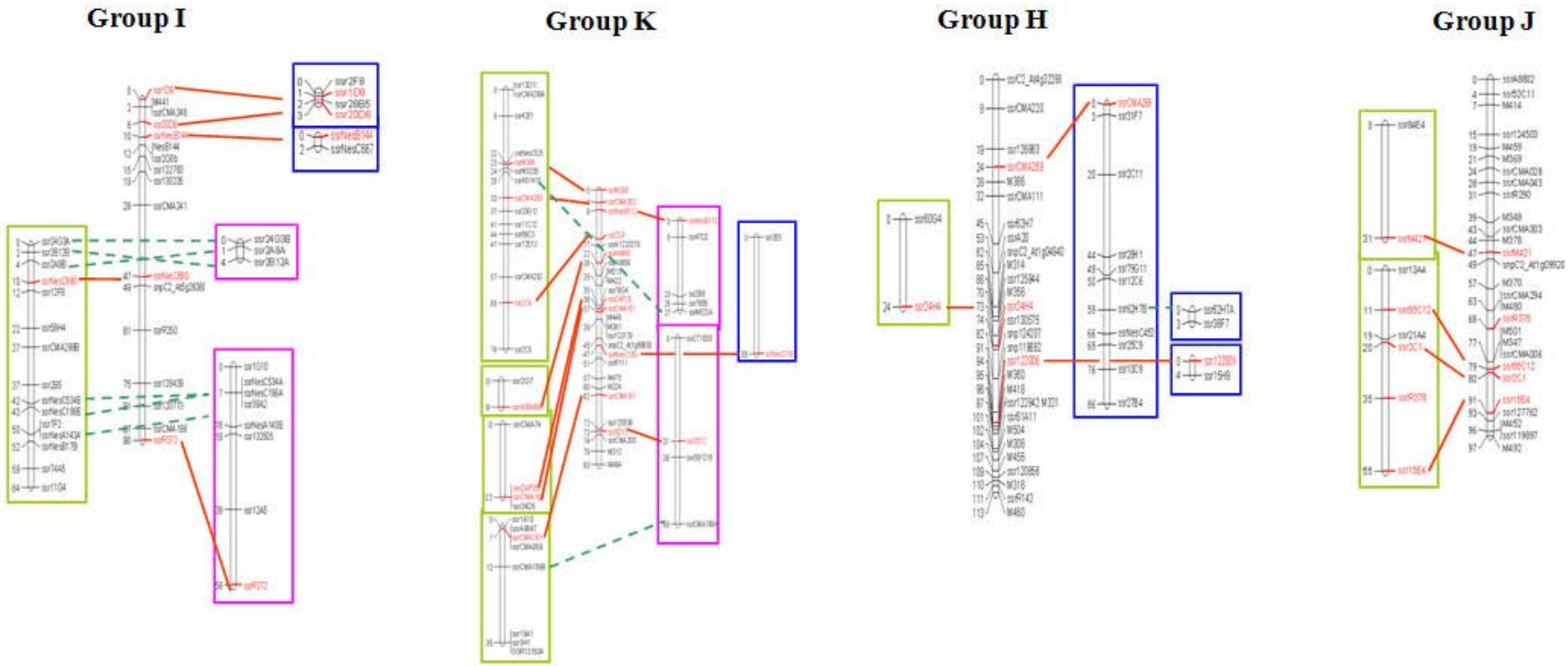


Figure 2. Robusta and Arabica map comparison. Each Arabica linkage group from putative Robusta origin is on the right of the reference map with pink colour, the putative *C. eugenoïdes* origin is shown on the left in green colour and the Arabica linkage group not yet determined are in blue. The shared loci between Arabica and Robusta maps are linked by a red line. The loci segregating on both Arabica homologous linkage groups are indicated by a green dot line.

A comparative QTL study started on agronomic, technologic and biochemical using different Robusta progenies and the Arabica population. The first results indicated that a main part of the QTLs detected are specific to a coffee species (Arabica versus Robusta) and/or from a given growing location (Indonesia versus Ecuador) as illustrates in Table 1.

Table 1. QTL analysis on bean density and FQA (Feruloyl quinic acid) in green beans from Robusta and Arabica progenies. For each QTL the linkage group, the growing location of the progenies, the LOD score and the variance percentage explained is indicated.

Trait	Coffee species	Linkage group	Location	LOD score	Variance
Bean density	Robusta	D	Indonesia	3.5	12%
		H		4.4	14%
	Arabica	K	Ecuador	4	14%
FQA content	Robusta	A	Indonesia	5.5	25%
		J		5.4	24%
	Arabica	D	Ecuador	5.1	26%

Even if the Arabica genetic map is not completely achieved the use of a share set of coffee genetic maps linked together by SSR markers will allow us to establish a genetic frame work to perform a comparison study on key traits of interest for coffee breeding. The influence of the environment on these traits will be assessed in Ecuador where both Robusta and Arabica progenies are growing.

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Functional Markers in Assisted Selection – Study of an Expressed SSR Locus Associated with Leaf-Miner Resistance

J.V.D. MOLINO[1], M. SILVESTRINI[1], J.C. MARTINATI[1], O. GUERREIRO-FILHO[1], M.P. MALUF[2]

[1]Coffee Center "Alcides Carvalho" – Institute Agronomic (IAC) Campinas, Brazil
[2]Coffee Unit – Embrapa Brasília, Brazil

SUMMARY

The development of novel coffee cultivars has been delayed by limitations on early selection of desirable agronomic traits. Currently, molecular markers have been used as an alternative and efficient selection tool by breeding programs of several plant species. In coffee, identification of microsatellites markers (SSR) that co-segregate with resistance to leaf-miner represents a significant step for an efficient assisted selection. In this study, expressed ssr markers were identified by directed searches on the Brazilian Coffee ESTs Database and evaluated regarding their expression pattern. Expression of 35 EST- SSR loci was evaluated in susceptible and resistant genotypes infected with the leaf-miner, through RT-PCR. One locus, containing a CCA motif, exhibited differential expression pattern in susceptible infected leaves. The locus was cloned and sequenced from both susceptible and resistant genotypes. Sequence analyses allowed the identification of 3 alleles, considering the motif copy number of CCA, but with several other polymorphisms outside the motif. However, none of these alleles are co-related with resistance trait. On the other hand, cloning and sequencing of expressed SSR alleles demonstrated that one allele, identified at genomic level and containing a 7-CCA motif repetition, is not expressed in susceptible leaves, of both control and infected plants. Resistant plants exhibited normal expression of the 7-CCA allele in all evaluated samples. *In silico* analyses indicated that the SSR is located in the 5'-UTR sequence, a region associated with mRNA stability that is not part of the coding region. These analyses suggest that the SSR motif may have a role in regulation of gene expression, which then leads to the development of a functional polymorphism. The occurrence of this type of polymorphism may have an impact on how markers can be used for assisted-selection, once in such cases not only the presence/absence of the allele associated with a desired trait must be evaluated, but its expression pattern may be considered as well.

INTRODUCTION

The leaf-miner, *Leucoptera coffeella* (Guérin-Méneville, 1842) (Lepidoptera-Lyonetiidae) represents a major treat in *Coffea arabica* plantations in all brazilian productive regions. The insect overcomes a complete metamorphosis, including egg, larvae, chrysalis and adult phases (Souza et al., 1998). The lesions caused by the insect feeding in infested leaves are called "mines or galleries", have irregular boarders and color changing from yellow to brown (Guerreiro-Filho, 1994).

All *C. arabica* germplasm evaluated so far is highly susceptible to the leaf-miner. Therefore, breeding strategies aiming the selection of resistant cultivars initiate with interspecific hybrids, using *Coffea* diploid species as donors of resistance genes. In the breeding program developed by the IAC (Institute Agronomic, Campinas, Brazil) resistance is been transferred

from the species *C. racemosa*. Selection of resistant progenies is performed by evaluating individual plants with controlled infestation according to methodology described in Guerreiro-Filho, (1994). Resistance response is specified by the type of reaction observed in infested leaves.

One of the major limitations for the development of leaf-miner resistant cultivars is the difficulty of early selection for this trait. Both the long life-cycle of *Coffea* species and also the impossibility to obtain homozigote plants bearing resistance genes represent major difficulties to be overcome. In this case, modern breeding tools that allow for an early selection are under development.

Molecular markers have been largely used as breeding tools in assisted-selection for different cultivated plant species. In coffee, several studies related to the identification of molecular markers are underway. At IAC, microsatellites, or ssr, present in expressed sequences (EST) and associated with leaf-miner resistance are under investigation. For this the genetic resources available in the Brazilian Coffee Genome Database (Vieira et al., 2006) are used to identify sequences containing SSR motifs. In a preliminary study, Pinto et al. (2007) evaluated the diversity of 35 EST-SSR loci in a hybrid population of *C. arabica* X *C. racemosa* (F_2BC_5) segregating to leaf-miner resistance. Despite the fact that parental species are genetically distant (Silvestrini et al., 2008), no polymorphisms associated with the resistance trait were observed. However, *in silico* analyses of the putative EST-SSR transcripts indicated that ssr motifs are located both in coding regions and in no translated regions, the 5'- and 3'- UTR. As UTR have been associated with gene expression regulation, and also the presence of ssr motifs in coding regions may interfere with protein structure, these data suggests that ssr motifs may play a role in gene expression and/or function. Therefore, ssr may be associated with the development of functional polymorphisms.

In this context, this study represents an alternative approach aiming to identify possible functional polymorphisms associated with the presence of ssr motifs in selected loci. Also, these polymorphisms were investigated in leaf-miner resistant segregating population in order to establish possible relationship between functional polymorphisms and resistance response.

MATERIALS AND METHODS

Plant material - The segregating population used in this study represents an advanced generation of an inter-specific hybrid between the susceptible species *C. arabica* and the resistant species *C. racemosa*. The population consists of 136 plants, derived from open pollination of the accession H14954-46 C1351 EP473. This accession is resistant to leaf-miner and corresponds to the F2RC5 generation (Guerreiro-Filho, 1994). For analyses plants were grouped in bulks of 5 plants each. Expression analyses were initially performed in 6 different bulks, 3 of resistant and 3 of susceptible plants.

Seedlings from both susceptible and resistant plants were infested with leaf-miner, according to methodology described before Guerreiro-Filho (1994). Leaves were collected at different times during insect development, and corresponding to: T0, non-inoculated; T1, oviposition; T2, larvae eclosion; T3, initial lesion; T4, late lesion. After collection, leaves were immediately frozen and kept at -80 °C until used.

Search of EST-SSR loci - Contigs containing ssr motifs were previously selected by Pinto et al. (2007). A total of 10 EST-SSR loci were selected for this study, and included those contigs in which SSR motifs are located at 5`- and 3`- UTR.

Expression analyses of EST-SSR loci- Total RNA was extracted from collected leaves using a commercial Trizol based protocol (Invitrogen). Purified RNAs were quantified using a spectrophotometer before cDNA synthesis. For this synthesis the commercial kit First-Strand (Invitrogen) was used, and the reactions conditions were those specified by the manufacturer. EST-SSR expression was evaluated by semi-quantitative PCR, using selected specific primers (Pinto et al., 2007). Actin transcripts were used as the endogenous control to calibrate initial amounts of all samples cDNA. Amplified products were first separated through 1% agarose gel electrophoresis to verify amplification pattern, and afterwards were also separated through 5% polyacrilamide gel electrophoresis (Creste et al., 2007) to verify allele polymorphisms.

SSR_CCA sequence analyses – both genomic and cDNA amplified sequences corresponding to the SSR_CAA were cloned and sequenced, according to conventional protocols (Sambrook nad Fritsch, 2001). The same plant bulks were used for amplification of genomic and expressed sequences. Resulting sequences containing the CCA motif were aligned and compared using ClustalW (Higgins et al., 1994) e MEGA version 3.1 (Kumar et al., 2004) softwares. Number of CCA repeats, presence of SNPs, in/del and other sequence arrangements were analyzed using those applicatives.

RESULTS AND DISCUSSION

In this study the expression of 10 EST-SSR loci was investigated in plants infected with the leaf-miner. The purpose of this evaluation was to verify if the presence of ssr motifs in regulatory regions of transcripts could interfere with gene expression. Therefore, the strategy here was to amplify selected EST-SSR sequences through semi-quantitative RT-PCR from both resistant and susceptible leaves along insect development. In the analyses, the presence/absence of amplified fragments and the color intensity upon gel staining were compared. Almost all evaluated loci exhibited a regular expression pattern in resistant and susceptible leaves. Only three loci exhibited differential expression patterns. Two of them exhibited an apparent reduction of transcript accumulation in susceptible leaves, at T3 and T1 infection stages (Figure 1). The third locus exhibited a differential expression in all infection stages of susceptible leaves. In this case, expression of one allele was not observed (Figure 2). These preliminary results may indicate that the presence of ssr-motifs can somehow affect expression of the host sequence.

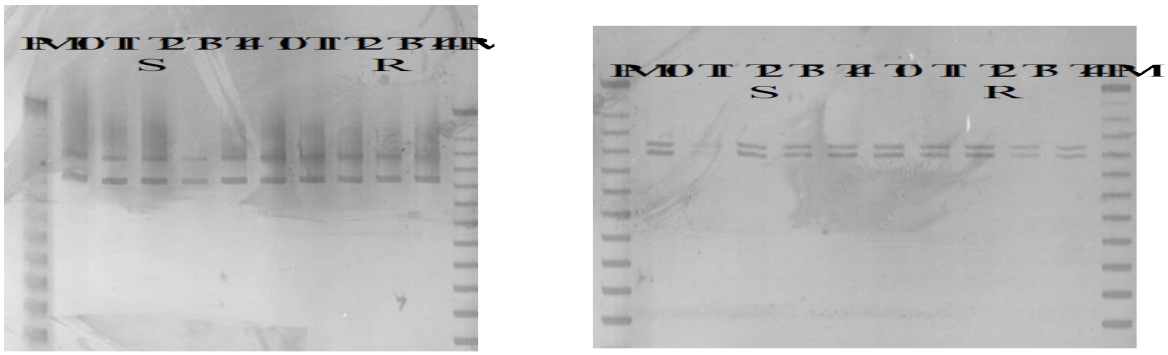

Figure 1. Expression pattern of EST-SSR amplified transcripts in *C. arabica* resistant and susceptible plants along leaf-miner development (see Material and Methods for details). PM corresponds to a 10 bp ladder.

In order to identify possible polymorphisms in the EST-SSR sequence, possibly associated with the lack of expression in susceptible plants, both genomic and expressed fragments were

cloned and sequenced. A total of 40 clones from susceptible plants and 47 from resistant plants were analyzed. In all evaluated sequences the identified ssr motif is a CCA repetition. Genomic sequences indicated the occurrence of three different alleles, with 7 (CCA_7), 17 (CCA_17) and 18 (CCA_18) copies of the motif. Besides these, several SNPs and in/del mutations were also observed outside the motif region. However, these were present in both resistant and susceptible plants, and therefore are not polymorphisms suitable as markers in assisted-selection.

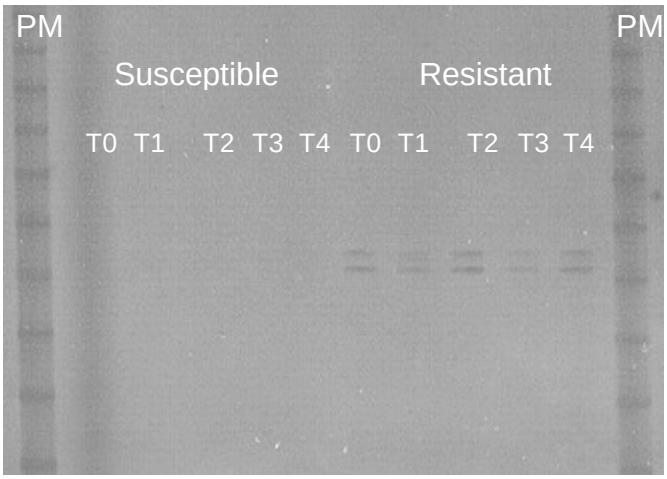

Figure 2. Expression pattern of SRR_CAA amplified transcripts in *C. arabica* resistant and susceptible plants along leaf-miner development (see Material and Methods for details). PM corresponds to a 10 bp ladder.

Results of expressed SRR_CAA sequences showed that all genomic alleles were detected, indicating that the corresponding genes are active in coffee plants. However, in this analysis two types of polymorphism were observed: first, the allele SSR_CCA18 was not expressed in all plants, either resistant or susceptible; and second, the allele SSR_CCA7 was not expressed at all in susceptible plants. Polymorphisms that are common to all genotypes, regardless resistance response type, may represent natural variability still present among evaluated progenies, as the analyses were performed in bulked samples. However, the absence of SSR_CCA7 expression in all evaluated bulks suggests that this allele is probably linked to the susceptible response in this population. At this point, this expression pattern of SSR_CCA7 allele is under evaluation in genotypes from different breeding populations in order to validate the suitability of this marker for assisted-selection. Also, as comparisons of expressed and genomic sequences do not indicate a mutation that could explain how the presence of SSR motif is associated with the differential expression of the locus, further studies are required to fully understand the regulatory steps involved in this polymorphism. Not only the SSR motif may be involved, but the interaction with other gene, not yet identified, could also explain the observed phenotype. *In silico* analysis indicated that the ssr motif is located in the 5'-UTR of a putative splicing factor transcript. Once splicing factors are not direct defense-related genes, there is a probability that this transcript may be associated with regulatory steps associated with defense response.

On the other hand, this type of polymorphism is very interesting for breeding programs of *C. arabica*, a species with restricted genetic variability. The results here indicate that not only genomic polymorphisms, which may be detected by conventional methodologies, are involved in the development of agronomic traits, but also the differential regulation of specific alleles may contribute to this development. In this case, those markers associated

directly with gene expression and/or gene function are called functional markers. However, as these functional polymorphisms are not easily detected and require specific methodologies for evaluation and characterization, breeding programs of *Coffea* species should include more multidisciplinary approaches in order to understand genetic control of desired traits, in order to incorporate these in new coffee cultivars.

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Sequencing the Coffee Genome: Overall Strategy and Progress Made in the Frame of ICGN

P. LASHERMES[1], X. ARGOUT[2], D. CROUZILLAT[3], PRIYONO[4], M. YEPES[5], P. WINCKER[6]

[1]IRD, Montpellier, France
[2]CIRAD, Montpellier, France
[3]Nestlé R&D, Tours, France
[4]ICCRI, Jember, Indonesia
[5]Cornell University, Geneva, New York, USA
[6]Génoscope-CEA, Evry, France

SUMMARY

The International Coffee Genomics Network (ICGN) is a worldwide network of scientists. ICGN's ultimate goal is to sequence the coffee genome and decipher through international partnerships the genetic and molecular bases of important biological traits in coffee tree species that are relevant to growers, processors, and consumers. As part of ICGN efforts, an international high density reference genetic map for *Coffea canephora* Pierre is being constructed in collaboration with R&D Nestlé Center and the Indonesian Coffee and Cocoa Research Institute. Furthermore, with funding from the French Agency ANR (Agence Nationale de la Recherche), several institutes (Genoscope-CEA, IRD and CIRAD) are combining their scientific resources and expertise to sequence, assemble, and annotate the entire genome of *C. canephora*. Several others ICGN members are planning to join these efforts particularly for mapping and genome sequencing and annotation. The *C. canephora* genome consists of 11 chromosomes, is about 710 Mb in size, and is being sequenced *de novo* with deep coverage using different sequencing platforms to obtain a reference genome for *Coffea*. The overall sequencing strategy and progress of the project are described.

INTRODUCTION

The International Coffee Genome Network (ICGN) is a worldwide network of scientists from universities, research institutes and industry within the coffee producing and coffee consuming countries (http://www.coffeegenome.org/). Our collaborative network is focused on building the foundation for advancing agricultural research for coffee by developing genomic tools and resources to further our understanding of the coffee genome at the molecular, biochemical, and physiological levels.

ICGN includes more than 100 individual and Institutional members networking scientific groups around the world in Africa, America, Europe, & Asia (http://www.coffeegenome.org/about/members.php). ICGN membership is opened to any individual, laboratory, or institution that can contribute to this effort in genomics resource development, sequencing and genome assembly, annotation, biological scientific expertise, or funding. ICGN is committed to advancing coffee genomic research through international partnerships for sustainable coffee production worldwide.

IMPORTANCE OF SEQUENCING THE COFFEE GENOME

Significant advances in our understanding of the coffee genome and its biology must be achieved in the next decades to increase quality, yield and protect the crop from major losses caused by insect pests, diseases and abiotic stress related to climatic change. Sequencing the coffee genome will help decipher the genetic and molecular bases of important biological traits in coffee that are relevant to growers, processors, and consumers. This knowledge is fundamental to allow efficient use and conservation of coffee genetic resources. Although considerable diversity exists in diploid *Coffea* species, its use in conventional coffee breeding programs has been very limited. *Coffea arabica* is characterized by a very low genetic diversity, which is attributable to its allotetraploid origin, reproductive biology, and evolution. The narrow genetic base of cultivated *C. arabica* has created a bottleneck for coffee breeding and limits cultivar improvement. Similarly, the considerable genetic diversity observed in *C. canephora* is still largely unexploited in the cultivated varieties. In the future, the ability to capture and manipulate genetic diversity and effectively utilize germplasm in traditional coffee breeding programs will be vital for sustainable coffee production.

DEVELOPMENT OF A HIGH-DENSITY GENETIC MAP FOR *COFFEA CANEPHORA*

An identified common objective is the establishment of a high-density genetic map. Ideally for genome assembly, this reference coffee genetic map would need to have 2 sequence-based markers per 1 million bp. To reach this objective, it was decided to take advantage of the initial important effort done by Nestle Tours Centre in collaboration with the Indonesian ICCRI institute. Those groups have already developed a map based on a cross between two highly heterozygous genotypes, a Congolese group genotype (BP409) and a Congolese-Guinean hybrid parent (Q121). The segregating population is composed of 93 F1 individuals. ICGN members will continue to saturate the map by mapping approximately another 1,000 sequence based markers such as SSRs and SNPs into the Indonesian population.

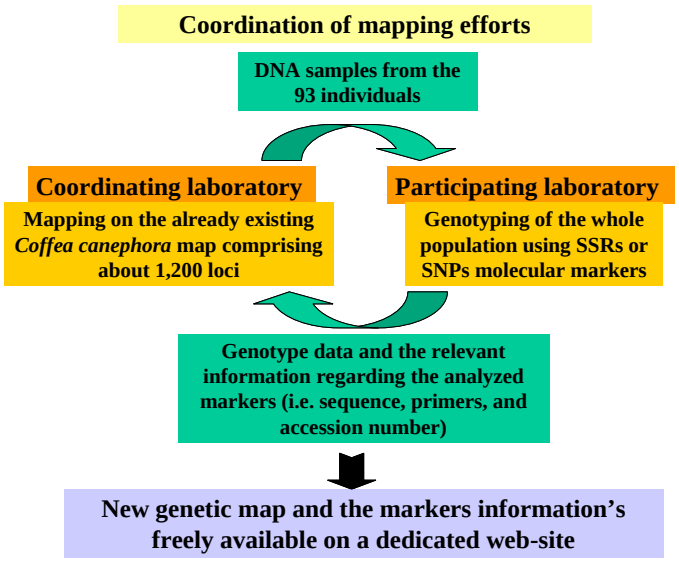

Figure 1. Organisation of the ICGN genetic mapping initiative.

A three-steps strategy has been adopted (Figure 1): 1) Upon request, DNA samples from the 93 individual segregating plants of the population BP409 X Q121 and the two parental clones is sent to the participating ICGN members; 2) After genotyping of the whole population, the

participating ICGN members must send back the genotype data and the relevant information regarding the analyzed markers (i.e. sequence, primers, sequence accession number); and 3) The additional sequence-characterized markers is mapped on the already existing *C. canephora* map to produce a high density reference map. Both, the high density genetic map as well as the marker information's will be freely available on a dedicated web-site (e.g. SOL web site).

ESTABLISHMENT AND ANNOTATION OF A REFERENCE GENOME SEQUENCE FOR COFFEE-TREES

Several institutes are combining their scientific resources and expertise to establish a reference genome sequence for coffee. *C. canephora* was chosen initially for this purpose because it is a diploid species (about 710 Mb in size). Also, *C. canephora* is one of the ancestral progenitors of the widely cultivated, *C. arabica* a recent allotetraploid species formed of the merge of the diploid species *C. canephora* and *C. eugenioides*. The accession DH200-94, a doubled haploid genotype was selected because of its homozygous status to facilitate genome assembly. *De novo* sequencing with deep coverage is being performed using both Roche pyrosequencing (454) and Illumina technologies. Direct whole genome shotgun sequencing and paired end sequencing of large insert libraries are underway; two 8kb and 20 kb insert libraries have been constructed. Furthermore, clones from two *C. canephora* BAC libraries were BAC-end sequenced using Sanger technology.

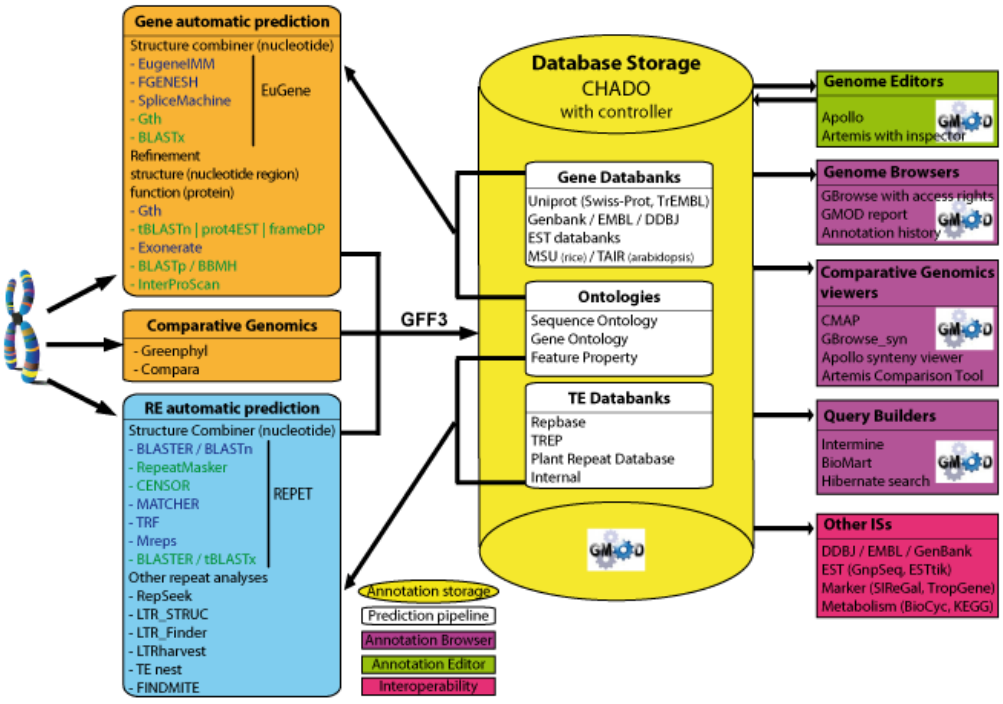

Figure 2. GNPannot, Community system for structural and functional annotation.

In addition to the publicly available ESTs, more transcriptome sequencing for *C. canephora* was done via 454 sequencing to facilitate genome annotation. All available evidences (cDNA sequences, the uniprot database, *ab initio* predictions) will be used for automatic annotation. The whole genome automatic predictions will then be integrated into a community annotation system (CAS) such as GnpAnnot (Figure 2) for gene expert annotation and genome analysis.

PERSPECTIVES

A future goal of the coffee ICGN community will be to establish the complete genome sequence of the allotetraploid *C. arabica* using the diploid progenitor species *C. canephora* and *C. eugenioides* as frameworks. *De novo* sequencing of *C. eugenioides* is expected to start by the end of 2010 with funding from the InterAmerican Development Bank allocated to the Colombian National Research Center and Cornell University. Furthermore and to ensure a full benefit of the generated resources by the coffee sector, funding for long term maintenance of the databases, and for the development of friendly end-user tools as well as training courses will be necessary.

Overview of the Functional Virulent Genome of the Coffee Leaf Rust Pathogen *Hemileia vastatrix*

P. TALHINHAS[1*], H.G. AZINHEIRA[1], A. LOUREIRO[1], D. BATISTA[1], B. VIEIRA[2], F. PINA-MARTINS[2], E. TISSERANT[3], A.-S. PETITOT[4], O.S. PAULO[2], S. DUPLESSIS[3], M.C. SILVA[1], D. FERNANDEZ[4]

[1]Centro de Investigação das Ferrugens do Cafeeiro/Instituto de Investigação Científica Tropical, Oeiras, Portugal. *E-mail: ptalhinhas@iict.pt
[2]Computational Biology and Population Genetics Group, Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal
[3]Institut National de la Recherche Agronomique, UMR 1136 "Interactions Arbres Micro-organismes" Nancy, France
[4]Institut de Recherche pour le Développement, UMR186 "Résistance des Plantes aux Bioagresseurs", Montpellier, France

SUMMARY

Coffee plants are seriously affected by leaf rust (*Hemileia vastatrix*) and loss of resistance to emerging races is a current threat. A deep knowledge of the mechanisms of pathogenicity is necessary for better understanding the plant resistance mechanisms, particularly because the *Coffea* spp. – *H. vastatrix* interaction follows the gene for gene theory, enabling the inference of virulence genes in the pathogen and the identification of susceptibility genes in the plant from resistance/susceptibility phenotypes. In the absence of *H. vastatrix* genome sequence information, transcriptomic analysis is the most effective gene discovery strategy, prompting gene family identification, the establishment of phylogenetic relationships and the development of PCR based molecular markers. High-throughput sequencing of cDNA transcripts by 454 pyrosequencing is capable of producing millions of bases per run, enabling large-scale expression analysis.

The objective of this work is the characterisation and comparison of *H. vastratrix* transcriptomes at three key differentiation/infection stages (germinated uredospores, appressoria and intercellular hyphae with haustoria) for an isolate from race XIV (containing the virulence genes $v_{2,3,4,5}$). For such, cDNA was obtained from germinated uredospores and appressoria produced *in vitro*, as well as from intercellular hyphae with haustoria (infected coffee leaves 21 days after inoculation) and subjected to GS-Flex Titanium 454 sequencing (each library comprising over 500000 reads of 300-600bp).

Prior to annotation, for the *in vivo* sample and in the absence of genome sequencing information for both organisms, *H. vastatrix* transcripts were separated from *Coffea* sp. transcripts by the analysis of codon usage in ESTs (programme EST3), of GC content, and of blastn homology score against angiosperms and basidiomycetes genomes, as well as of blastx homology score against coffee and Pucciniales EST libraries. The three *H. vastatrix* EST collections (germinating uredospores, appressoria and intercellular hyphae with haustoria) were annotated using international databases (nr, Swissprot, GO, KOG), aiming to attribute a putative gene function. A particular attention was given to the comparison with the Pucciniales genome sequences (*Melampsora larici-populina* and *Puccinia* spp.) and Pucciniales EST databases, in order to identify homologues of genes with relevant function in

pathogenicity. The libraries representing the three *H. vastatrix* differentiation/infection stages were compared among them, in order to identify genes differentially expressed. Genes identified were further studied by RT-qPCR expression analysis in a detailed time-course of *H. vastatrix* differentiation/infection (Vieira et al., 2010). The identification of *H. vastatrix* genes involved in pathogenicity will lead to a better understanding of the molecular basis of coffee rust gene for gene interaction.

INTRODUCTION

Coffee plants are seriously affected by leaf rust (*Hemileia vastatrix*) causing yield losses of up to 35% in *Coffea arabica* if no control measures are employed. The financial cost and environmental impact of chemical control of the disease make the cultivation of resistant varieties a more sustainable strategy of disease control. Nine different resistance genes have been characterised in *Coffea* spp., which govern the resistance against at least 45 physiological races of the pathogen (Rodrigues et al., 1975; Várzea and Marques, 2005), following the gene for gene model. However, loss of resistance in some cultivars shows the appearance of new races (Várzea and Marques, 2005), highlighting the dynamic nature of plant and pathogen co-evolution. Although detailed studies have been conducted on the cytology, biochemistry and molecular biology of coffee resistance to rust (e.g., Fernandez et al., 2004; Silva et al., 2006; 2008; Diniz et al., 2010), very little is known on the molecular mechanisms governing the fungal infection process. Information available for other rust (Pucciniales) pathosystems can be applied to the study of molecular mechanisms of the *H. vastatrix* infection process, namely profiting from knowledge of *in planta*-induced genes (Hahn and Mendgen, 1997), haustoria-secreted proteins (Catanzariti et al., 2006), rust transferred proteins (Kemen et al., 2005) and several others (Jakupovic et al., 2006; Puthoff et al., 2008), as well as the genome sequences of *Melampsora larici-populina* and *Puccinia* spp. Moreover, the relative taxonomic apartness of *H. vastatrix* from the other rust fungi (*Hemileia* is one of the most ancient rust lineages; (Wingfield et al., 2004)) and its unique features (such as haustorial invasion of stomata subsidiary cells, before further tissue colonisation, and induction of hypersensitive reaction in resistant hosts as early as at the appressorial stage (Silva et al., 2006)), highlight the importance of studies specifically aimed at coffee leaf rust transcriptomics. In the absence of *H. vastatrix* genome sequence information, transcriptomic analysis is the most effective gene discovery strategy, prompting gene family identification, the establishment of phylogenetic relationships and the development of PCR based molecular markers. Recently, large-scale expression analyses became feasible due to the development of high-throughput sequencing of cDNA transcripts by 454 pyrosequencing.

The objective of this work is the characterisation and comparison of *H. vastratrix* transcriptomes at three key stages of the differentiation and infection of the pathogen (germinating uredospores, differentiation of appressoria and generalised colonisation of leaves by intercellular hyphae with haustoria) as well as the identification of putative secreted proteins, aided by information available for other rust fungi and other fungal pathogens.

MATERIALS AND METHODS

Biological material and cDNA synthesis

Hemileia vastatrix isolate 178a (race XIV) was used for all experiments. For the germinating uredospores sample (U), spores were spread in sterile distilled water in petri dishes and incubated for 18 h at 24 °C under darkness. For the appressoria sample (A), spores were spread over oil-collodion membranes (Azinheira, 2005) in petri dishes, sprayed with water

and incubated for 24 h at 24 ºC and 100% relative humidity, under darkness. For the intercellular hyphae with haustoria sample (H), leaves of *Coffea arabica* H147/1 plants (susceptible to race XIV) were inoculated as previously described (Silva et al., 1999) and collected 21 days after inoculation, just before sporulation.

In vitro fungal material (samples U and A) and infected leaves (sample H) were frozen in liquid N_2 and stored at -80 ºC. RNA was extracted using the RNeasy Plant minikit (Qiagen) and cDNA was produced using the SMART PCR cDNA Synthesis (Clontech) technology.

Pyrosequencing and assembly of 454 reads

For each sample, up to 20 µg cDNA were sent for 454-pyrosequencing on a Genome Sequencer FLX System using long-read GS FLX Titanium chemistry (Roche; www.454.com) at the Genoscope (Centre National de Séquençage, Evry, France; www.genoscope.cns.fr). Sequences were assembled into contigs using the Newbler 2.3 (Roche) assembler. Singletons were excluded from the three libraries.

Bioinformatic analysis of fungal transcripts

Because sample H contains fungal and plant material, transcripts from both origins were separated prior to bioinformatic analysis. This was carried out both by sequence homology and trinucleotide frequency approaches. Sequence homology involved searches against plant (dicotyledons) or fungal (basidiomycetes) nucleotide sequence databases and against *Coffea* spp. and Pucciniales EST databases. Species-specific trinucleotide frequencies were considered using the EST3 programme (Emmersen et al., 2007), trained with *Coffea* spp. and Pucciniales EST (Fernandez et al., 2010). In total, six different prediction methods were used to determine plant or fungal origin of the contig sequences, with contigs being classified in function of the confidence of these predictions as: plant or fungus; likely plant or fungus; dubious plant or fungus; not resolved; not determined.

Contigs assigned to *H. vastatrix* were compared to the NCBI non-redundant nucleotide (nr) and swissprot databases, to the *M. larici-populina* (http://genome.jgi-psf.org/Mellp1) and *Puccinia* spp. (http://www.broadinstitute.org/annotation/genome/puccinia_group) genome sequences, to the Pathogen-Host Interactions database (www.phi-base.org (Winnenburg et al., 2007); mostly ascomycetes) and to a Pucciniales EST database (retrieved from GenBank at January 2010) containing 155280 sequences from *Cronartium* sp., *Melampsora* spp., *Phakopsora* sp., *Puccinia* spp. and *Uromyces* spp. Putative gene function was attributed using the euKaryotic Orthologous Group (KOG) classification.

Contigs from the three libraries were compared using a best reciprocal hit approach using BioEdit 7.0.4.1 (Hall, 1999). Pairs of contigs with an e-value lower than e^{-30} were considered as representing the same transcript and assembled.

Prediction of secreted proteins was carried out using the Secretome Prediction Pipeline (FungEffector; http://mycor.nancy.inra.fr/FungEffector/resources), based on SignalP, TargetP and TMHMM programmes (www.cbs.dtu.dk).

RESULTS AND DISCUSSION

Sequencing, assembling and fungus/plant differentiation

A total of 807943 sequences were originated by 454 pyrosequencing for the three libraries, containing about $2.3x10^8$ bp, with an average of 284.4 bp per sequence (Table 1). These sequences were assembled into a total of 31308 contigs, with an average length of 609.1 bp. The longest contig has 4589 bp and 12% of contigs are longer than 1000 bp, indicating that the 454 approach is useful to generate large transcript sequences. Average number of reads per contig was 15.4, while the average of maximum coverage depth per contig was 10.1. About 11% of contigs had a maximum coverage depth over 20, representing transcripts with a medium to high rate of expression.

Table 1. Descriptive statistics for 454-sequences and assembled contigs for germinating uredospores (U), appressoria (A) and intercellular hyphae with haustoria (H) libraries.

Library	U	A	H
Number of bases	66687862	44949909	118113878
Number of sequences	269192	186605	352146
Mean size of reads	247.7	240.9	335.4
Number of contigs	4426	3757	23125
Mean size of contigs	665.1	624.4	537.8
Size of contigs*	176/538/1266/3794	187/526/1122/4463	193/410/1051/4589
Mean number of reads per contig	25.7	21.6	12.41
Reads per contig*	4/9/51/962	4/9/45/986	2/5/28/421
Mean coverage depth	9.73	7.76	11.19
Coverage depth*	3/5/18/210	2/5/14/169	3/7/23/186

**Values are 10/50/90/100 percentiles.*

From the 23125 contigs assembled for library H, 6943 were predicted has being of fungal origin, 14060 has plant, 1407 were not resolved (similar likelihood of being plant or fungus) and 715 were not determined (no prediction method was able to determine their origin). It is worth noting that fungal transcripts represent only about 30% of the contigs, even if the biological sample was comprised of leaves heavily colonized by the fungus. The 14060 plant contigs were not further analysed in the context of this work, but can include interesting data regarding late plant responses and senescence. Only one third of fungal contigs was predicted with high confidence, unlike for plant contigs, where over 71% were predicted with high confidence. This is probably due to a closer evolutionary proximity between *C. arabica* and plant sequences (including *Coffea* spp.) available in nucleotide and EST sequence databases, as opposed to the higher evolutionary distance between *H. vastatrix* and the nucleotide and EST sequences available in databases for Pucciniales (where *H. vastatrix* is barely represented).

Comparison among U, A and H libraries

Comparison among the libraries (Figure 1) reveals that at least 1032 transcripts are common to the three libraries, representing only 9% of the total number of assembled transcripts. Another 15% of contigs are shared among any two libraries. The vast majority of contigs (76%) were considered unique to each library, although this number is likely to decrease as further details for the *H. vastatrix* genome are revealed. Still, important differences arise among the three libraries. While 73% of H contigs are unique to this library, only 47 and 41% of U and A contigs are unique to each of these libraries, indicating a higher proportion of genes in common among these early stages of fungal development (germination of uredospores and formation of appressoria).

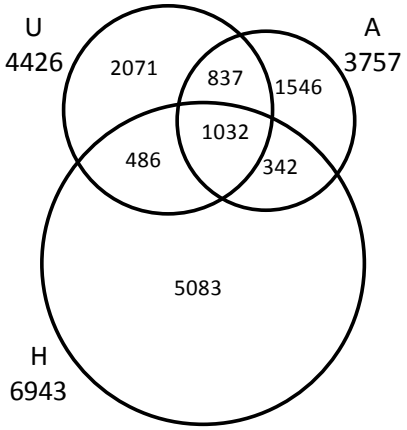

Figure 1. Number of contigs identified in the three libraries (U, germinating uredospores; A, appressoria; H, intercellular hyphae with haustoria) and their interceptions.

Database homology searches

Comparison of contig sequences to the nr NCBI database yielded 7560 hits (57% of all contigs). A similar number of hits was obtained against *M. larici-populina* and *Puccinia* spp. genome sequence databases (50% of all contigs). An important proportion of contigs (41%) presented homology to all three databases.

At least 380 transcripts presented homology in PHI base to genes from other organisms where they are reported to cause reduction or loss of pathogenicity when mutated, either in animal (e.g., *Candida* sp. and *Cryptococcus* sp.) or plant (*Colletotrichum* spp., *Magnaporthe* sp. and *Ustilago* sp.) hosts. Many of these transcripts are highly expressed in U and A and encode transcripts putatively involved in ion transport, signalling, adhesion and energy production and conversion (superoxide dismutases, G-protein alpha subunit, collagen related proteins and isocitrate lyases). These could represent important genes for fungal pathogenicity, conserved across ascomycetes and basidiomycetes.

On the other hand, 17% of contigs (representing over 3300 transcripts) had no homology to any of the databases queried, and could correspond to *H. vastatrix* specific genes. Among these, transcripts that are found in the three libraries represent 8% of the total, in a similar proportion to that found for all transcripts (Figure 1). However, a higher proportion of transcripts specific to the library U and a lower proportion of those specific to library H were found among these no-homology transcripts as compared to the overall transcriptome.

Intermediately, many transcripts present high homology to genes from other rusts, being of particular relevance those genes involved in the pathogenic process, either in signalling (MAP kinases, rust transferred proteins, haustorially-expressed secreted proteins and plasma membrane ATPases), cell wall decomposition (pectate lyases, pectinesterases, glucanases, chitinases and chitin deacetylases) and nutrient transport (amino acid and carbohydrate transporters).

Putative gene function attributed according to the KOG classification showed that more than half of the transcripts are categorised into "Translation, ribosomal structure and biogenesis", "Posttranslational modification, protein turnover, chaperones", "Energy production and conversion", "General function prediction only", "Intracellular trafficking, secretion and vesicular transport" and "Lipid transport and metabolism". However, comparisons among the three datasets indicated a shift in the putative function of transcripts detected, suggesting higher frequency of transcripts involved in "Translation, ribosomal structure and biogenesis", "Lipid transport and metabolism" and "Energy production and conversion" in appressoria, and higher frequency of transcripts involved in "Signal transduction mechanisms" and "Intracellular trafficking, secretion and vesicular transport" both in germinated uredospores and in intercellular hyphae with haustoria. Genes involved in "Replication, recombination and repair" and in "Cell cycle control, cell division, chromosome partitioning" are more frequently found in the *in vivo* sample, announcing the onset of the production of sporogenic hyphae.

The comparison of *H. vastatrix* transcripts to *Cronartium quercuum* f. sp. *fusiforme* EST databases (Warren and Covert, 2004; Baker et al., 2006) enabled the identification of homologues to genes involved in *in planta* stress response (some of these are similar to genes required for pathogenicity in *Magnaporthe grisea*, *Botrytis cinerea* and *Cryptococcus neoformans*; PHI base) or specific to germinating rust spores.

A similar comparison to a *Phakopsora pachyrhizi* EST database (Posada-Buitrago and Frederick, 2005) showed that most conserved genes are those involved in basic cellular functions, while most genes involved in more specialised functions in *P. pachyrhizi* do not match any of the *H. vastatrix* transcripts, with few but noteworthy exceptions that will be further investigated.

Comparison with a *Puccinia coronata* f. sp. *lolii* EST database associated with SSRs (Dracatos et al., 2006) shows homology to several *H. vastatrix* contigs, which may prompt further studies regarding the potential use of these loci as markers for genetic diversity.

In *P. graminis*, two class III chitin synthases (Broeker et al., 2006) and several homologous to plant induced rust protein 8 from *Uromyces fabae* (Broeker et al., 2006) had homology to *H. vastatrix* transcripts.

Similarly, several transcripts show high homology to genes known to be up regulated in haustoria in *U. vicia-fabae* infected faba bean leaves (Vieira et al., 2010).

Prediction of secreted proteins

Prediction of secreted proteins enabled the identification of 570 *H. vastatrix* candidate transcripts. From these, 25% were found in the three libraries, but 20% were restricted to the U library, frequently with high levels of expression (maximum coverage depth above 20),

suggesting the presence of several putative secreted proteins specific to germinating uredospores, which may be involved in host surface recognition, interpretation of topographic signals or detection of stomata (for the differentiation of appressoria). More than one third of secreted protein candidates had no homologies to databases, representing potential *H. vastatrix*-specific genes, while another 16% presented homology to ESTs from other rust fungi.

CONCLUSIONS

With ca. 11000 putative genes identified, this work represents the first insight into the functional virulent genome of *H. vastatrix*, placing the research on molecular genetics of this pathogen in closer proximity to that of other rust fungi, such as species of *Melampsora*, *Phakopsora*, *Puccinia* or *Uromyces*. This will enable comparison of *H. vastatrix* genome to other rusts, with potential phylogenetic relevance, but especially it will be the start point for several studies addressing the expression profiles of specific genes. An example of this is the gene expression study conducted on 11 genes, involved in signalling, nutrient transport, carbohydrate metabolism and maintenance of the biotrophic interaction, by RT-qPCR along the infection process (Vieira et al., 2010).

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Molecular Genetic Diversity of Arabica Coffee (*Coffea arabica* L.) Using ssr Markers

T.N. ALEMAYEHU[1], V. PETIARD[2], P. BROUN[3], D. CROUZILLAT[3]

[1]Jimma University Jimma, Ethiopia.
[2]Nature Source Genetic, Ithaca, US.
[3]Nestlé Centre R&D Tours, France

SUMMARY

Arabica coffee (*Coffea arabica* L.), the world most important commercial coffee species, has its centre of origin and diversity in south-western Ethiopian highlands. Its populations exist as wild state and under production systems. So far little is known about the molecular genetic diversity of Ethiopian Arabica coffees which in turn has limited its utilization in the improvement programs. Thus, generating genetic diversity information has a significant value in the future efforts of Arabica coffee' genetic resources conservation and sustainable utilization. Hence, in this study the genetic diversity of Arabica coffee collections of different geographical origin and historical backgrounds were studied using 32 microsatellite (SSRs) markers. The result indicated high genetic variability reserve with a lot of specificity in Ethiopian Arabica coffees. More than 90% of the total alleles were detected in Ethiopian Arabica coffees. Of the total alleles detected in Ethiopian Arabica coffees, about 83.7% and 46.4% were polymorphic and specific, respectively. The cultivated cultivars contained only 53.6% of the total alleles detected in the current study indicating the genetic diversity bottleneck due to early human impacts. In the cluster analysis, based on the detected polymorphic alleles, the Ethiopian Arabica coffees with larger within population genetic distances were clearly separated from the cultivated cultivars obtained from other countries. The result suggests the potential application of SSRs in genetic diversity study of Arabica coffee according to its geographical origin and the possibility of high potential to use Ethiopian Arabica coffees' gene pool in the breeding programs.

INTRODUCTION

Of the 103 species in the genus *Coffea*, *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) are the two most important commercial species with *C. arabica* considered as a high quality coffee and contributes more than 70 percent of the world coffee production Geographically, most of the coffee species are originated from tropical African countries: Ethiopia for the tetraploid *Coffea arabica*, Central and West African countries for other coffee species (Berthaud and Charrier, 1998). However, the Arabica coffee in major producing areas such as Latin and Central America, countries are believed to have a narrow genetic basis due to the few seeds or plants used for dissemination and the successive genetic reductions due to human impacts (Anthony et al., 2002).

The genetic diversity of *Coffea arabica* is less polymorphic as compared to its diploid relative species. However, the populations in its place of origin, particularly south-western Ethiopia, have a lot of genetic variability for many agronomic characters. These populations exist in different forms: as wild coffee that are inaccessible and non-used, forest or semi-forest coffee and garden (landraces) coffees.

Coffee genetic diversity can be assessed using different techniques that range from the traditional morphological technique to the modern DNA-based molecular markers. Today, a number of DNA-based techniques are in use in different coffee genetic studies. These include the conventional PCR-based method such as microsatellite markers (Dufour et al., 2001; Anthony et al., 2002; Moncada and McCouch, 2004). Microsatellites are highly polymorphic and reproducible, locus-specific and "co-dominant". Because of this, SSRs are the markers of choice for many genetic studies.

In the present study we used SSRs to study the genetic diversity of Arabica coffee collections with different geographical origins and historical backgrounds.

MATERIALS AND METHODS

Plant material

A total of 133 genotypes of *C. arabica* were used. It includes 78 Ethiopian Arabica accessions and 55 cultivated genotypes. Of the total 78 Ethiopian Arabica accessions, 54 accessions were obtained from Ethiopian Institute of Agricultural Research. The remaining of Ethiopian accessions (24 genotypes) and the cultivated genotypes were obtained from Nestlé.

DNA extraction and PCR Amplification

Total DNA was isolated from frozen leaves following QIAGEN DNeasy plant procedure. Thirty two SSRs are used to assess the overall genetic diversity.

PCR is performed in 22 µl reaction volume containing 9 µl of AmpliTaq Gold® (Applied Biosystems), 9 µl of deionised water, 1 µL of forward primer (10nM) and 0.5 µL of reverse primer (20 nM), 2.5 µl (20 ng) of DNA. PCR is carried out using the following program: 10 min initial denaturation step at 94 °C, followed by 35 cycles at 94 °C for ½ min, annealing at 50 °C for ½ min and extension at 72 °C for 1 min with a final extension step at 72 °C for 7 min.

Data scoring and analysis

The amplified PCR products were separated and detected by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). All the alleles were used to calculate the following genetic parameters: allelic richness (A), percent of polymorphic alleles (rP), average number of alleles per SSR locus. The genetic diversity distribution was also studied with principal component analysis (PCA) using NCSS software (Hintze, 2006).

RESULT AND DISCUSSION

A total of 209 alleles are detected for 32 SSRs on 133 Arabica accessions. Out of 209 alleles, 200 (96%) alleles are polymorphic for the overall samples (Table 1). The allele number per SSR ranges from two to fourteen with an average of 6.5 alleles for the overall Arabica accessions. The allele number in Ethiopian Arabica ranged from two to 12 with an average of 5.9 alleles per SSR locus while it ranged from one to eight with an average of 3.5 in cultivated group.

As compared to the previous studies, higher values are obtained in our analysis for the different genetic parameters analysed. Anthony et al. (2002) reported an average number of

4.7 alleles per SSR using only six SSRs in Arabica coffee collections containing four Typica, five Bourbon and ten sub-spontaneous derived accessions. Using 34 SSRs, Moncada and McCouch (2004) reported an average of 2.5 and 1.9 alleles per SSR in 11 wild Arabica genotypes and 12 cultivated Arabica, respectively, with the number of alleles ranging from one to eight. Maluf et al. *(*2005) also reported an average number of 2.87 alleles in 28 cultivated Arabica lines using 23 SSRs.

Table 1. Genetic parameters on the genetic diversity of the Ethiopian and cultivated Arabica accessions. A: Total allelic richness, Ar: Range of allele per locus, An: Average number of alleles per locus, pA: Total number of polymorphic alleles, npA: Number of non-polymorphic alleles, rP: Rate of polymorphic alleles, PrA: Private alleles, H: Observed heterozygosity.

	A	Ar	An	pA	npA	rP (%)	PrA	H (%)
Ethiopian	189	2-12	5.9	175	14	93	97	7.6
Cultivars	112	1-8	3.5	89	23	80	20*	1.9
Overall	209	2-14	6.5	200	9	96	N.A.	5.7

*Note: *11 out of the 20 alleles are coming from Robusta introgressions, only 9 alleles (4%) are specific to Arabica cultivars.*

The present study has confirmed the genetic diversity of Arabica in Ethiopia that can used as potential source in breeding programs. Of the total 209 alleles, 189 (90.4%) alleles were detected in Ethiopian accessions, while only 112 (53.6%) alleles were detected in cultivated group. From the 189 alleles detected in Ethiopian accessions, 175 were polymorphic, while only 90 alleles were polymorphic in the cultivated group.

About 46.3% (97 alleles) of the total alleles were specific to Ethiopian accessions while only 9.6% (20 alleles) were specific to the cultivated group. Eleven of these 20 specific alleles found in the cultivated group were detected in Catimor, Sarchimor, and other introgressed genotypes. When the Robusta introgressed alleles were not taken into consideration, only 101 (48.3% of the total alleles) alleles were observed in the cultivated varieties.

This result is in agreement with the early history of Arabica coffee distribution when the commercial cultivars have undergone successive genetic reductions (Anthony et al., 2002). Historical data indicated that the Arabica coffee populations in major producing countries were derived from few plants or seeds taken from Ethiopia. This could be the main factor to explain the low allelic diversity of the commercial varieties. In this study, about 46.4 % of the total allelic diversity of Arabica coffee was not detected in Arabica cultivars.

Diversity study of the overall analysed Arabica is shown using a principal component analysis **(**Figure 1**).** The first and second components covered 36.9 % of the total variance. The genetic analysis revealed two separated clusters: one cluster for Ethiopian accessions and the other for cultivated group.

In conclusion, the results indicated that the Arabica accessions collected from its centre of origin, Ethiopia, are genetically more diverse and rich with a lot of specific alleles than commercial cultivars which have undergone genetic diversity bottleneck due to early human impacts. The potential of SSRs to clearly differentiate coffee genotypes from different origins suggests the possibility to use these SSRs in quality control (DNA-based traceability) for

Ethiopian premium specialty coffees. The information from this study can be used in the future endeavours to utilise coffee genetic resources in sustainable ways, which can be conservation in its wild natural habitat or field gene banks, development of core collection and improved varieties. Other applications such as Arabica coffee genetic map development and QTL detection to be used in marker assisted selection are also in progress.

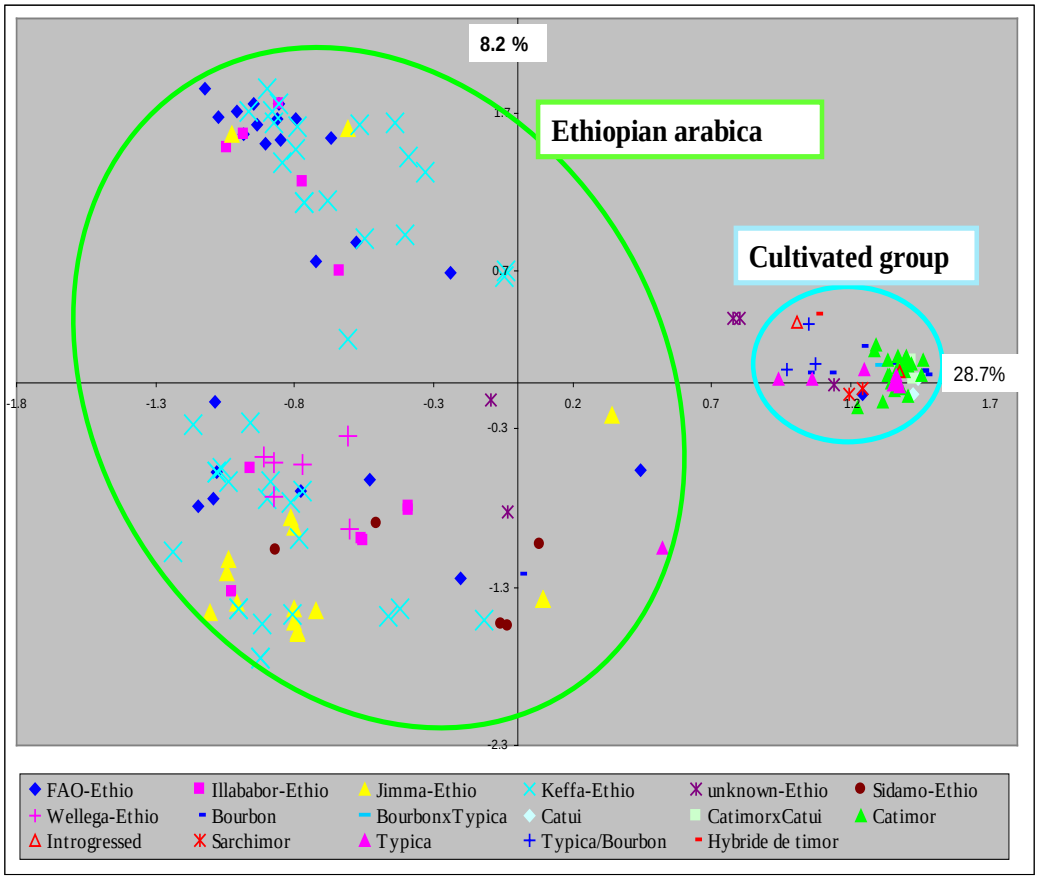

Figure 1. Diversity of Arabica coffee accessions based on PCA components: the first and second axes represent 28.7% and 8.2% of the total variance.

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The Effect of Low Temperature on Metabolism of Membrane Lipids in *Coffea* spp. and Associated Gene Expression

P. BATISTA-SANTOS[1], L.F. GOULÃO[1], A.S. FORTUNATO[1], I.P. PAIS[2], P.S. CAMPOS[2], F. PARTELLI[3], A.I. RIBEIRO[1], J.C. RAMALHO[1,*]

[1] Centro de Ecofisiologia, Bioquímica e Biotecnologia Vegetal/Instituto de Investigação Científica Tropical, Quinta do Marquês 2784-505 Oeiras, Portugal.
*E-mail: cochichor@iict.pt
[2]Inst. Nac. Inv. Agrária/INRB, I.P., Quinta do Marquês, 2784-505 Oeiras, Portugal
[3]Univ. Federal de Goiás/Esc. Agron. Eng. Alimentos. Campus II Samambaia. CP 131, 74001-970, Goiânia - GO, Brazil

SUMMARY

Cold has negative effects on plant growth and development, and thus on yield and productivity rates of crops. Membrane lipid metabolism plays a decisive role in the mechanism of cold-tolerance in many plants. In fact, changes in fatty acid (FA) unsaturation and in the lipid class balance are amongst the first responses to cold exposure. In this way, in the present work, it was analyzed the lipid composition of chloroplast membranes and the transcriptional changes in genes involved in lipid metabolism, in order to evaluate their role in the photosynthetic acclimation to low temperatures in coffee. The less cold sensitive Icatu showed higher unsaturation degree of MGDG and of PG. In the later lipid class, Icatu presented also a lower impact on 16:1*t* and C16:0 upon cold, two important fatty acids for the maintenance of photochemical efficiency of the photosynthetic apparatus. This genotype also showed lower PA (a stress metabolite) content under cold and rewarming conditions. Fatty acid desaturase genes (*caFAD1* and *caFAD*2) presented up-regulation only for Apoatã upon chilling or rewarming. Lipoxygenase gene (*caLOX*) was also up-regulated only upon rewarming in Apoatã, while phospholipase gene (*caPLIP*) presented only some fluctuations in both genotypes. This analysis allowed the detection of some changes in chloroplast lipid membranes that could contribute to photosynthetic cold acclimation. Expression studies need to go further in order to better integrate with physiological/biochemical data.

INTRODUCTION

Exposure to low positive temperatures limits coffee geographical distribution, having strong negative effects on plant growth and productivity (Ramalho et al., 2003; Partelli et al., 2009). Cold affects all photosynthetic components, from stomatal conductance to gene expression, along with structural impacts on photosystems and composition of pigment and membrane lipids (Ramalho et al., 2003; Campos et al., 2003; Silva et al., 2004), with implications on crop viability in potentially less adequate regions. Also, cellular membranes are a main target of environmental stresses, playing a major role in the acclimation to environmental adverse conditions, including exposure to low temperatures (Leshem, 1992; Routaboul et al., 2000).

Previous work showed that coffee plants have some ability to cold acclimate, related to a better protection against oxidative stress (Ramalho et al., 2003; Fortunato et al., 2010) and to the maintenance of membrane stability (Campos et al., 2003). Two coffee genotypes with contrasting cold sensitivities, *C. canephora* cv. Apoatã (highly sensitive) and Icatu (*C.*

canephora x *C. arabica*) (moderately tolerant), were used to study the role of changes in lipid components of chloroplast membranes, as well in the expression of lipid metabolism related genes, in the cold acclimation process in coffee plants.

MATERIAL AND METHODS

Plant Material and Growth Conditions

The experiments were carried out as previously described (Ramalho et al., 2003), using 1.5 years old plants from the genotypes Icatu (IAC 2944 - *C. canephora* x *C. arabica*) and *C. canephora* cv. Apoatã (IAC 2258). Potted plants were submitted successively to: 1) a gradual temperature decrease (0.5 °C day^{-1}) from 25/20 °C to 13/8 °C (day/night), to allow the expression of acclimation ability; 2) a 3 day chilling cycle (3x13/4 °C), where the plants were subjected to 4 °C during the night and in the first 4 h of the morning (with light) and 13 °C, throughout the rest of the diurnal period; 3) a 7 days rewarming period (25/20 °C), to plant recover. Photoperiod was set to 12 h, RH to 65-70% and irradiance to *ca*. 750-850 μmol m^{-2} s^{-1}.

Quantification of chloroplast membrane lipids

Chloroplast membranes were obtained from 3 g of leaf tissue, following (Ramalho et al., 1999), and the lipid components were obtained through a gas-liquid chromatography analysis previously optimized for *Coffea* spp. (Campos et al., 2003).

Gene Expression Studies

Based on coffee cDNA sequences from ESTs NCBI data base, specific primers were designed (data not shown) in order to perform the mRNA expression studies by real time PCR as described in Batista-Santos et al. (2011 accepted).

Statistical analysis

A two-way ANOVA ($P < 0.05$) was applied to evaluate differences between temperatures and between genotypes, followed by a Tukey test for mean comparison (95% confidence level).

RESULTS AND DISCUSSION

Lipid analysis

Temperature-induced changes in membrane fluidity are strongly influenced by the lipid molecular species composition, length of the acyl chains and unsaturation degree of FAs (Harwood, 1998), and were suggested to be the first signal in the stress perception (Siegenthaler and Trémolières, 1998). A gradual reduction of temperature is known to promote a higher unsaturation of glycerolipid FAs which will allow maintenance of appropriate membrane fluidity, optimal photochemical and electron-transport reactions, ultra-structural integrity and thermal stability of the photosynthetic apparatus (Routaboul et al., 2000; Öquist, 1982; Gombos and Murata, 1998).

The analysis of total fatty acids (TFA), as well as that of the distribution of lipid classes (data not shown) did not distinguish Apoatã and Icatu in terms of cold sensitivity since both genotypes showed active lipid synthesis (Figure 1). However the analysis of FAs within each lipid class revealed some fine tuned responses.

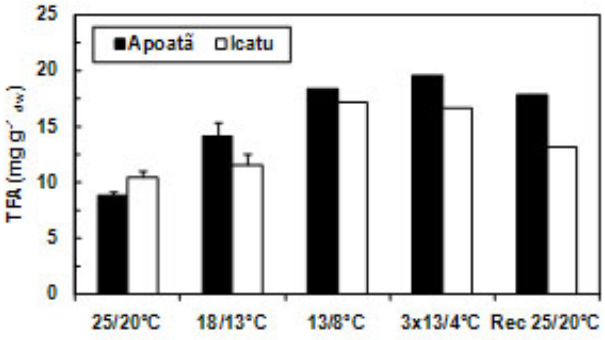

Figure 1. Changes in total fatty acids (TFA) contents of chloroplast membrane lipids from the studied genotypes, under the imposed experimental conditions. Each value represent the mean + SE (n=3).

The FA composition of 2 key classes for photosynthetic apparatus functioning, monogalactosyldiacylglycerol (MGDG) (Figure 2) and phosphatidylglycerol (PG) (Figure 3) revealed changes along cold imposition.

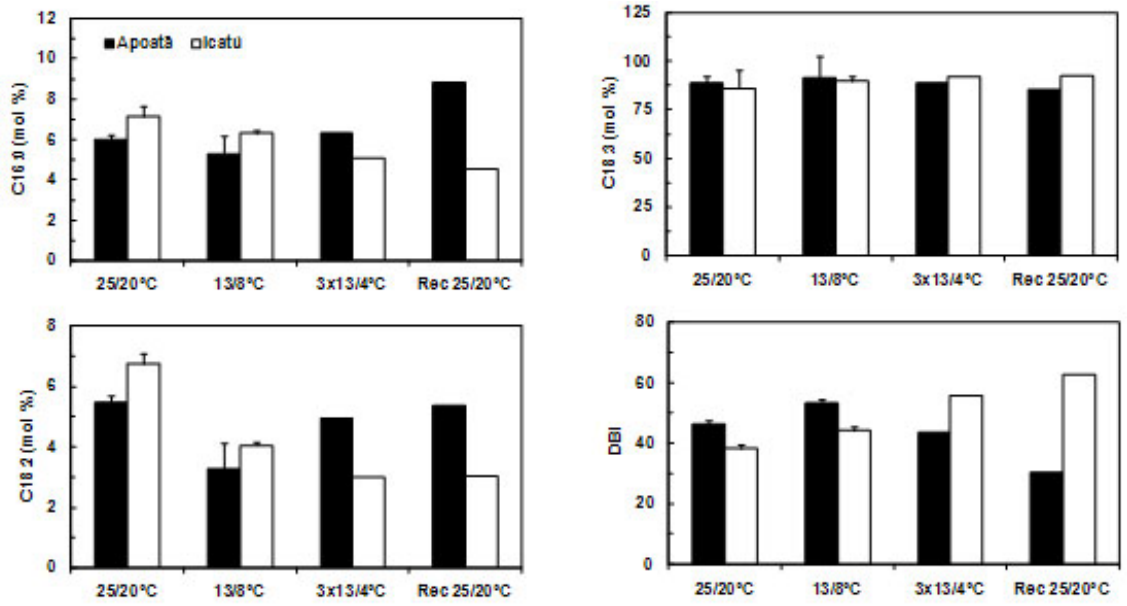

Figure 2. Fatty acid composition (mol %) and unsaturation degree (DBI) of galactolipid MGDG of chloroplast membranes from the studied genotypes under the imposed experimental conditions. Each value represent the mean ± SE (n = 3).

A sustained cold-induced rise in the unsaturation degree of MGDG was observed only in Icatu (Figure 2). On the other hand, PG's unsaturation degree displayed a striking difference between genotypes and with cold exposure (Figure 3). Icatu showed higher PG unsaturation degree under control conditions and, despite some decreases associated with initial cold imposition (13/18 °C), was always higher than Apoatã.

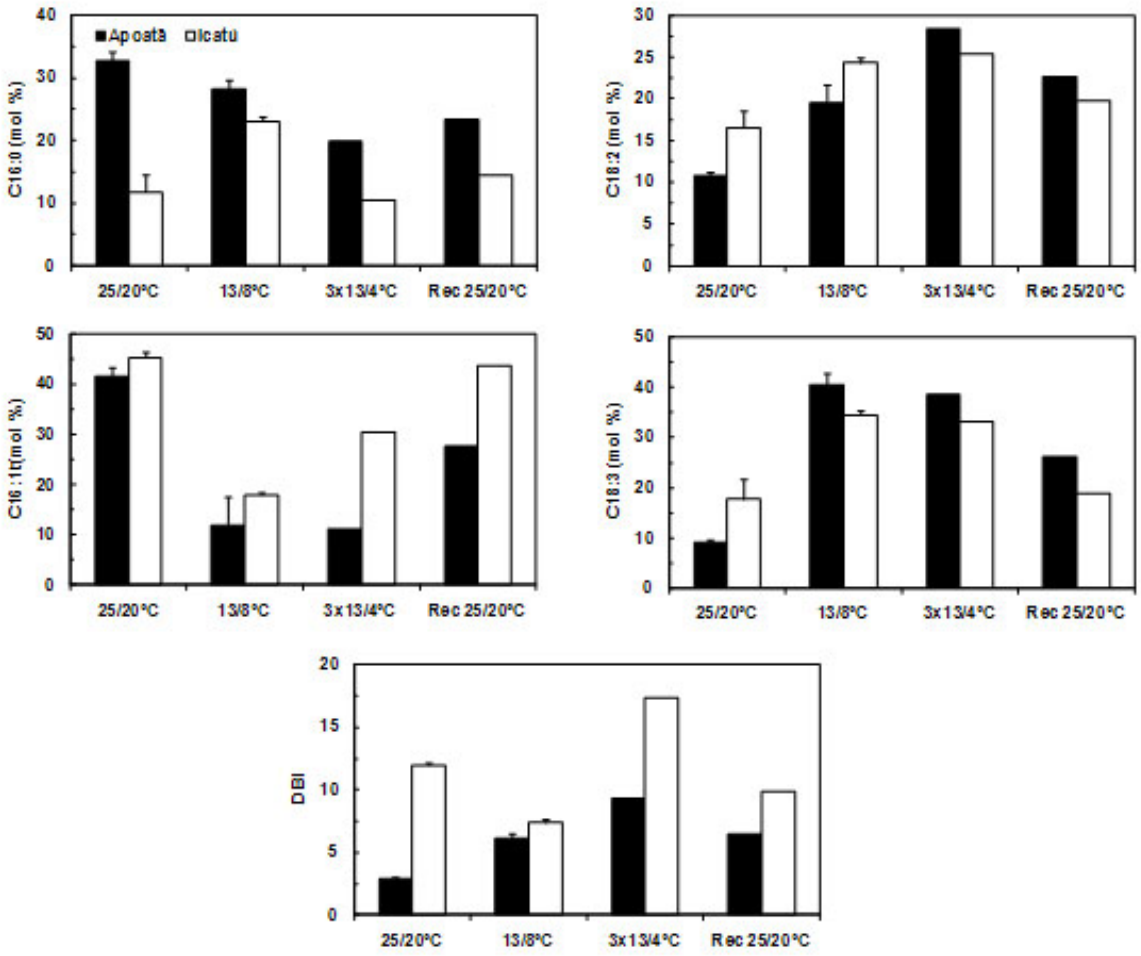

Figure 3. Fatty acid composition (mol %) and unsaturation degree (DBI) of phospholipid PG of chloroplast membranes from the studied genotypes under the imposed experimental conditions. Each value represent the mean ± SE (n = 3).

Regarding specific FAs, namely to C16:1*t* (that exists only on PG), smaller reductions were measured in Icatu during chilling, and a complete recovery was observed with the return to control conditions. On the other hand, C16:0 did not decrease in Icatu, contrary to what was observed in Apoatã. Since both C16:1*t* and C16:0 are involved in the functioning and damage repair of photosystem (PS) II (Dubertret et al., 2000; Siegenthaler, 1998; Vijayan et al., 1998), the maintenance of PSII efficiency will be favored in Icatu.

Both genotypes, but specially Apoatã, showed an increase in phosphatidic acid (PA) content at 13/8 ºC, decreasing thereafter, throughout chilling imposition (3x13/4 ºC) and subsequent rewarming period (Rec. 25/20 ºC) (Figure 4). Since PA might be considered a stress metabolite, the higher increase and absolute contents in Apoatã suggests a significantly higher degradation level than in Icatu.

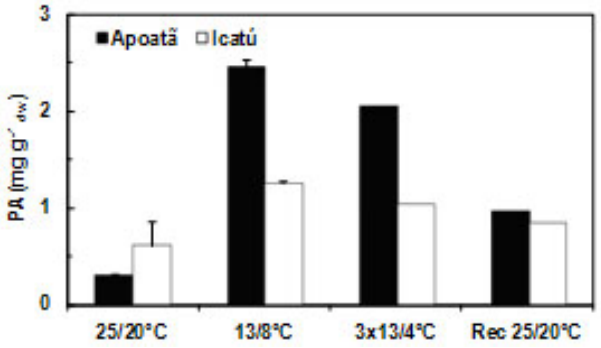

Figure 4. PA content (mg g^{-1}_{dw}) in the chloroplast membrane lipids from the studied genotypes under the imposed experimental conditions. Each value represent the mean ± SE (n = 3).

Gene expression studies

In this work, the gene expression of two different fatty acid desaturases (FADs) showed significant reductions in Icatu, while in Apoatã the expression level was maintained (*caFAD1*) or increased (*caFAD2*) upon chilling exposure (Figure 5). Since both genotypes showed an increase of unsaturation degree (data not shown) it is suggested that Icatu is able to increase DBI through a different way than that of Apoatã. Plants from several species, including rice (Shimada et al., 2000), tobacco (Zhang et al., 2005) or tomato (Dominguez et al., 2010), transformed to overexpress specific ω-3 FAD genes, showed increased 18:3/18:2 ratios in leaves accompanied with enhanced tolerance to chilling temperatures. However, differential transcription of ω-3 FAD (Iba, 2002; Martz et al., 2006; Tang et al., 2007) and ω-6 FAD (Kargiotidou et al., 2008; Teixeira et al., 2009) genes was observed in several species in response to cold imposition, with up-regulation of some isoforms, while others are not affected or down-regulated during chilling conditions. For example, in *Betula pendula* an increase in the expression of two genes encoding ω-3 FADs was observed with a synchronous increase in 18:3 in glycerolipids during exposure to low temperatures but a third isoform was down regulated (Martz et al., 2006). Similarly, in *Gossypium hirsutum* or *Portulaca oleracea*, the expression of some ω-6 FAD genes was induced by low temperatures, while other genes proved to be unaffected (Kargiotidou et al., 2008; Teixeira et al., 2009).

In addition to the direct effect on the molecular organization of membrane lipids, loss of integrity of the membrane via lipid peroxidation, protein oxidation or inhibition of key enzymatic activities is boosted by oxidative processes due to increased levels of ROS that are induced by chilling stress (Alonso et al., 1997; Mittler, 2002). Lipid peroxidation is a common symptom associated with low-temperature stress (Saruyama and Tanida, 1995), in which phospholipid catabolism is associated to a rise in phospholipase D (PLD) and lipoxygenases (LOX) gene transcription and enzymatic activities.

LOX are key enzymes in triggering lipid peroxidation of the plasma membrane, causing a decrease in the lipid desaturation level and membrane fluidity (Lee et al., 2005), and was observed that chilling injuries are accompanied by increases in LOX activity in some plants (Mao et al., 2007). LOX may also cause damage to membrane lipids by direct attack from free radicals formed due to its activity (Vick BA, Zimmerman, 1987; Griffiths et al., 1999; Zhao et al., 2010). In this work, the *caLOX* transcripts decreased under chilling in both genotypes (as well as in 13/8 °C in Apoatã), but a significant expression increase (more than 6-fold) was detected in the cold-sensitive Apoatã upon rewarming. The later could implicate degradation

of membrane lipids, which is consistent with the strong leaf shed observed in this genotype during the rewarming period (Batista-Santos et al., 2011 accepted).

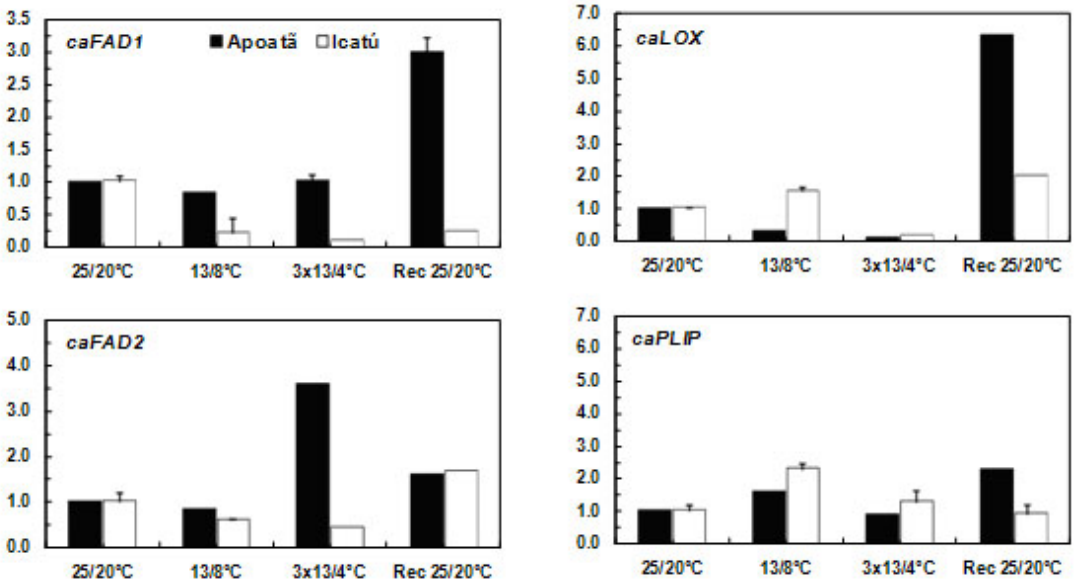

Figure 5. Real-Time PCR gene expression studies of *caFAD1*, *caFAD2*, *caLOX* and *caPLIP* in the studied genotypes, under the imposed experimental conditions. Each value represent the mean ± SE (n = 6-9), from 3 independent biological assays.

Some LOXs are able to act on substituted FA substrates, while others require free FAs and a previous action by lipases (Ford-Hutchinson et al., 1994). Most plant FAs are esterified to a glycerol backbone in the form of glycerolipids and lipases are thought to cleave these phospholipids into free FAs, which are then used as LOX substrates (Ferrie et al., 1994). Furthermore, phospholipases, especially PLD, hydrolyze phospholipids to PA during stress, which may serve as substrate for LOX activity and involve loss of cell-membrane integrity (Ryu SB, Wang, 1996; Wang et al., 2006; Bargmann et al., 2009). The contrasting genotypes showed only some *caPLIP* expression changes during cold imposition. However, upon rewarming, Apoatã showed a 2-fold increase in this gene expression, while Icatu showed values close to the control. These results suggest that the cold induced PA increase observed in Apoatã would result from degradation "pathways", other than that involving *caLOX* and *caPLIP* gene expression, namely through direct reactive oxygen species action due to the presence of a weak antioxidant system (Fortunato et al., 2010).

In conclusion, Icatu showed qualitative lipid adjustments allowing this genotype to better cope with cold conditions and to recover after that. These results sustain the better acclimation ability previous reported in Icatu (Ramalho et al., 2003; Campos et al., 2003; Batista-Santos et al., 2011 accepted) and could constitute useful tools to the management, breeding and selection of adequate *Coffea* spp. Genotypes.,

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Coffee Control Quality on Geographic and Genetic Origins Determined By DNA Traceability

E. MOREL[1], M. RIGOREAU[1], A. RODRIGUEZ[2], C. LAMBOT[1], P. BROUN[1], D. CROUZILLAT[1]

[1]Nestlé Centre R&D Tours, France
[2]Nestlé Nespresso, Lausanne, Switzerland

SUMMARY

Cup tasting profiles of Espresso products are strongly influenced by the raw material quality. This quality is depending on many factors including the environment, harvesting and post-harvesting conditions, and the coffee variety. All the factors could lead to the product differentiation either by their own or their combination. Coffee market is mainly organised by geographical origins and post-harvest categories, giving poor attention to the variety. Nevertheless, some exceptions could be mentioned as Maragogype and Blue Mountain varieties which are available as specific source of raw material. The difficulty to distinguish varieties by traditional methods represents a major constraint for the establishment of a traceability system for the supply chain. The DNA analyses could afford an efficient system to trace the raw material and assess its quality in relation to the variety concern. DNA traceability is based on the use of polymorphic markers such as microsatellites. These specific molecular markers should be initially identified for the differentiation of the selected varieties. This phase is delicate considering the lack of diversity in the cultivated Arabica varieties. It is therefore essential to have access to large database of polymorphic markers identified in Arabica germplasm. An appropriate method of sampling combined to statistical analyses of results is leading to the acceptance or rejection of coffee supply chain from field to factory gates. Even, after roasting and grinding, there is sufficient quantity and quality DNA to perform the analysis. Efforts are now allocated to develop a routine test to be integrated in industrialized process. The test should be able to deliver results in a short period of time with high reliability and at a low cost. Automated PCR markers look to be well adapted, combining speed and accuracy.

INTRODUCTION

The Arabica coffee species is originating from Ethiopia where the diversity centre is located. The wild Arabica types offer a substantial rate of genetic diversity but the cultivated varieties, for their part, are extremely homogeneous (Alemayehu et al., 2010). This low genetic diversity within these cultivars is due to a few number of Arabica plants propagated by human in major producing countries such as Latin and Central America.

The commercial world coffee production is based on two species, *Coffea arabica* (70%) and *Coffea canephora* (30%). The best cup quality is associated with the species *C. arabica*. The quality may varied in the coffee batches selected by Nespresso according to several factors such as environment, harvest, post-harvest processes and the Arabica varieties used (Lambot et al., 2010). Moreover, Nespresso capsules are usually made using blends of several Arabica varieties or from different geographic origins. This feature is found in the range of “Pure Origine” from Nespresso “Grand Crus”. For example, “Dulsão do Brasil”, which is made with

red and yellow bourbon from Brazil. Two specific field locations provide the source of the green coffee beans. It is important for Nespresso to ensure the origin of these products because it is a guarantee of quality to its customers. DNA traceability has been established to assess and sustain the source of these coffee batches.

Usually, DNA markers such as microsatellites (SSRs) were used to assess the genetic diversity in *C. arabica* in order to determine the genetic profiles of the Arabica varieties. This PCR based technology can be run in quality control especially for variety authentication. Due to the sensitivity and specificity of the method the quality control test is becoming also a reality in processed foods, even for a complex food matrix such as ground coffee. Despite that the factory processes degrade DNA in coffee beans during the roasting and grinding steps, there is enough DNA to perform a quality control assay. Use of a new DNA technology such as HRM (High Resolution Melting) coupled with SSR marker will give access to the genetic characterization and differentiation of Arabica varieties with a high level of reliability using fast and cheap assays.

MATERIALS AND METHODS

Reference Material

Green coffee samples (beans) and ground coffee (capsules of Grand Crus) used in this study were provided by Nespresso.

DNA Extraction of green coffee seeds

90 green beans per sample are individually and finely cut, pieces of seeds are collected in collection microtubes. The DNA extraction was performed with the Qiagen kit (DNeasy 96 Plant) following the recommendations of the supplier.

DNA extraction from Capsules

For each capsule, a sample of 3g of ground coffee is placed in a Falcon tube of 50mL. DNA extraction was performed according to Meyer and Jaccaud (1997).

Selection of SSRs markers

The microsatellite markers have been selected according to their rate of polymorphism in *C. arabica* cultivated varieties.

PCR was performed in a 25 µL volume containing 20 ng of genomic DNA; 0.04 µM of primer forward (labelled with fluorochrome), 0.4µ M of primer reverse; 1X of AmpliTaq Gold® at 0.05 U/µL and QSP to 20 µL water. The reaction mixture was initially denatured at 94 °C (10 min), followed by 40 cycles of amplification at 94 °C (30 sec), 50 °C (30 sec), 72 °C (30 sec) and a final extension at 72 °C for 7 min.

Microsatellite analysis on 3500 xL Genetic Analyzer

Amplified DNA products were separated on the sequencer using capillary electrophoresis according to their sizes and to the fluorescent labelling. Experimental data were analysed using GeneMapper® software (Applied Biosystems).

High Resolution Melting (HRM): amplification and analysis

HRM is a post PCR method able to detect the genetic variations (SNPs) in PCR amplicons.

PCR reaction consisted of 1X LightCycler® 480 High Resolution Melting Master (Roche); supplemented with 3 mM $MgCl_2$, 0.3 µM for each primer, qsp water to 7.5 µL and 5 µL of genomic DNA. The cycling program for all tested microsatellites consisted of: 10 minutes initial denaturation followed by 45 cycles at 94 °C (10 sec), annealing at 60 °C (15 sec) and extension at 72 °C (25 sec). The amplification cycles were followed by the high resolution melting steps.

RESULTS AND DISCUSSION

Microsatellite analysis

The establishment of a core database based on the genetic diversity detected in the *C. arabica* cultivars has allowed collecting the necessary information for DNA traceability on Bourbon varieties. Thanks to this database, it is possible to differentiate and characterized the green coffee at the origin of the "Dulsão do Brasil" Nespresso capsules. Microsatellite analysis gave reliable results for detecting the presence of Arabica adulterants (Figure 1). From green beans (field) to Nespresso capsule (factory), it is possible to determine the reference profile of the specific Arabica variety used all along the supply chain (Figure 2).

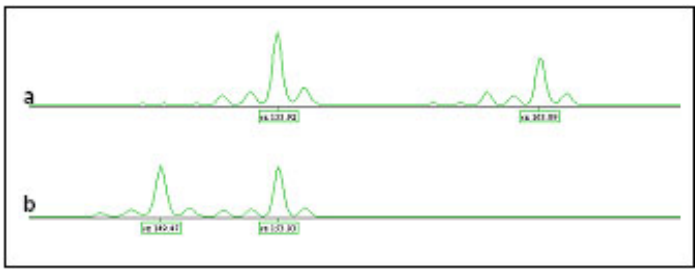

Figure 1. Example of microsatellite profile obtained with a selected SSR marker on Bourbon variety and one adulterant Arabica variety. a. Control (Bourbon variety selected); b. Adulterant.

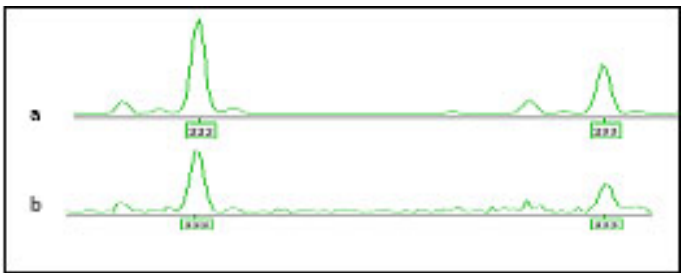

Figure 2. Routine quality control assay on green coffee seeds and Nespresso capsules. a. Control (Bourbon variety selected); b. Capsules of Dulsão Do Brasil.

HRM study

HRM allows us to analyse genetic diversity by highlighting genomic variations (SNPs, SSRs) in PCR amplicons (Mackay et al., 2008). As illustrated in Figures 3 and 4, HRM was also

able to differentiate the Arabica control from the adulterants tested. All HRM profiles were confirmed by SSR genotyping using capillary electrophoresis technology. In some case, the primers selected can also identify a third type of genetic profile link to allo-pollination. In this last case, the accuracy of the HRM method allows to assess the true adulterant rate for the Arabica batches analysed.

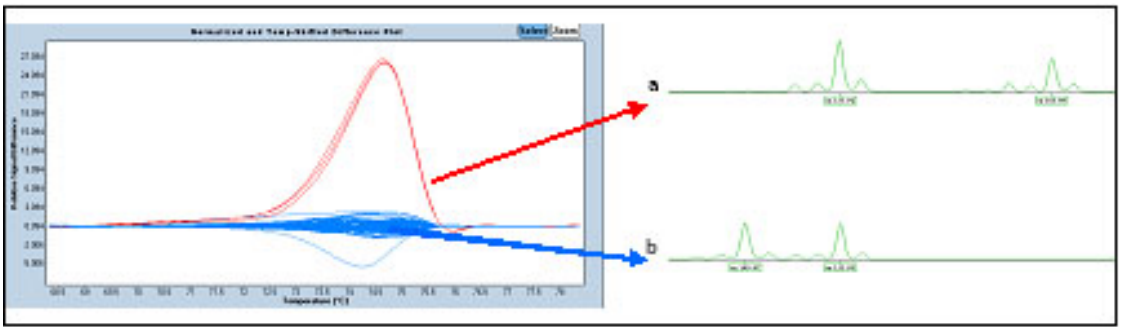

Figure 3. HRM test illustrating the routine DNA traceability assay on green coffee seeds with a specific SSR marker. a. Reference control: true to type of Arabica Bourbon; b. Adulterant.

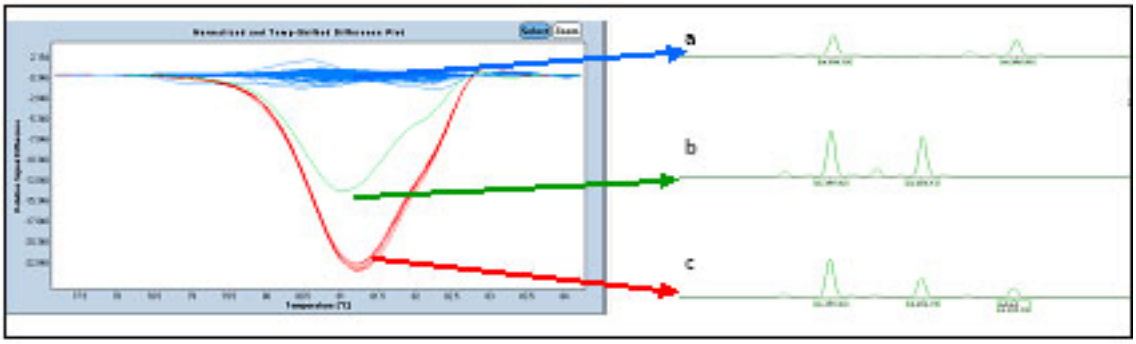

Figure 4. HRM test illustrating the routine DNA traceability assay on green coffee seeds with a specific SSR marker. a. Reference control: true to type Arabica Bourbon; b. Adulterant; c. Allo-pollination.

Our current challenge is to trace and control the genetic or geographic origin in raw materials. Now, with the specific markers it is possible to verify the genetic origin claimed by a supplier. HRM is a sensitive, robust technique that could significantly reduce the time and cost of screening for mutations in a traceability process.

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Coffee Seeds Gene Expression Analysis in Different Post-Harvest Processes

M. MODONUT[1], L. DEL TERRA[2], M. BARNABA[3], G. GRAZIOSI[1], A. PALLAVICINI[1]

[1]University of Trieste, Trieste, ITALY
[2]illycaffe SpA, Trieste, ITALY

SUMMARY

Coffee processing must begin immediately after the fruit is harvested, to prevent pulp fermentation and quality loss. The coffee beans are usually prepared for roasting through one of three processes: wet, semi-dry and dry. It is well known that these treatments can alter the beverage quality not only due to sugar fermentation, but also because of transient occurrence of germination events, which are mainly related to the quick decrease in water content of the seed during desiccation.

Taking advantage of the *C. arabica* expressed sequence database CoffeeDNA.net, we were able to select 16 different genes for a targeted gene-expression analysis. The transcripts selected are involved in many different physiological mechanisms: desiccation stress, germination, caffeine and chlorogenic acid synthesis, hormone biosynthesis, fatty acid synthesis and degradation, glycolysis and gluconeogenesis, sugar and starch metabolism. By means of quantitative real-time PCR assays, we monitored the expression of the 16 aforementioned genes in various samples. In the first experiment, green coffee samples from two different Brasilian regions in the Bahia state were subjected to three different post-harvest treatments; the seeds were then sent to our laboratory for RNA extraction. In a second experiment, RNA was extracted from coffee seeds obtained from a single batch, sampled at successive times during the drying process. Analyzing these two sets of samples, it was thus possible to elucidate new aspects of the modulation of gene expression during post-harvest treatment.

Seeds from the two Brasilian locations showed significant quantitative and qualitative differences in gene expression. In detail, one of the locations presented a markedly reduced variability between the three different post-harvest treatments. Considering that the genetic base was the same for all samples and that the two locations presented similar geo-climatic characteristics, these data indicate a predominating influence of the environmental factors, including human activities, in the post-harvest processes. The second set of samples revealed that the seeds maintain a detectable level of metabolic activity even at low water contents. In fact, while the expression level of some transcripts showed, as expected, a gradual decrease with the progress of desiccation, other transcripts indicate a modulation of metabolic activity until the last stages of the drying process (16-14% water content). These results lend further support to the hypothesis that the post-harvest processing is not simply a removal of water from an inert material, but instead a complex interaction between human manipulation and the physiology of the coffee seed.

INTRODUCTION

When defining the quality of green coffee, some of the most important aspects to be considered are post-harvest treatments, environmental factors (comprehensive of geographical location, soil, climate and farming techniques), and water content. All of these factors can affect positively or negatively the quality of the final product.

We studied the effect of post-harvest processing on gene expression in samples of green coffee beans through real-time quantitative PCR of 16 genes, possibly related to organoleptic qualities of the coffee drink. During post-harvest processing, coffee bean initiate the germination process, as indicated by the activity of *β-tubulin* and *isocitrate lyase* (Bytof et al., 2007), while the enzyme *glutamate decarboxylase* (GAD) is activated in water stress situations (Bytof et al., 2005). These three genes were chosen as markers of stress and germination. Joët et al. (2009) have shown that in different post-harvest treatments, the content of caffeine and chlorogenic acids does not change. According to this paper, only the maturation process of the fruit affects the final quantity. In the synthesis of caffeine an important role is played by the enzyme *caffeine synthase*, which takes part in the final two reactions of the biosynthetic pathway. In the biosynthesis of chlorogenic acids, two enzymes have been considered: *p-coumaroyl-3 hydroxylase* that acts in three different reactions and *shikimate hydroxy transferase*. *Steroid 5-α reductase* is involved in the biosynthesis of steroid phytohormones (brassinosteroids). These chemical compounds act as molecular signals and allow the regulation of plant growth (leaf and flower formation, growth and ripening of fruits). *Geranyl transferase* is an enzyme of the biosynthesis of abscisic acid and is involved in various processes in plants like blocking cell division (da Silva et al., 2004), and that is why it plays a key role in seed dormancy, in environmental stress (heavy loss of water or low ambient temperature) and plant pathogen response. The *fatty acid synthase* intervenes in the biosynthesis of fatty acids that occurs in plastids starting from acetyl-coenzyme A. Fatty acids are usually accumulated in the form of triacylglycerols. The cycle of β-oxidation occurs in plastids through the action of several enzymes, including *enoyl CoA hydratase* and involves the breakdown of fatty acids into acetyl-CoA which is then converted to glucose in gluconeogenesis and the glyoxylate cycle. These compounds have an important role in seed germination because they provide immediate energy necessary for the growth of the germ before photosynthetic activity begins. The *aldolase* is an enzyme of the glycolysis pathway that catalyzes the degradation of D-fructose 1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The enzyme *pyruvate kinase* catalyzes the last reaction of glycolysis, converting ADP and phosphoenolpyruvate into ATP and pyruvate. Sugars play an important role not only as a source of carbon and energy, but as signal molecules as well. Of the several enzymes involved in the metabolism and transport of sugars, 3 were considered in this work: *sucrose synthase*, *invertase* and a *hexose transporter protein*. We also included in the analysis the *starch synthase*, which participates in the elongation of the starch chain by incorporating sucrose molecules.

This selection allowed us to monitor a limited number of genes that, if obviously not exhaustive, was representative of a number of pathways of primary and secondary metabolism, directly and indirectly related to the quality of the coffee beverage.

MATERIALS AND METHODS

Two sets of green coffee samples were used. The first set ("wet-dry" samples) consisted of *Coffea arabica* cv. Catuai harvested in 2008 from two different Brasilian farms (Barra do

Choca and Inhobim) and processed with 3 different post-harvest treatments (wet, semi-dry and dry), for a total of six samples named Ba-W, Ba-S, Ba-D, In-W, In-S and In-D.
The second experimental set ("water content" samples) was made up of a single lot of green coffee *(Coffea arabica* cv. Mundo Novo, brasilian harvest 2005) sampled at 4 successive times during desiccation, thus resulting in four samples with final water contents of 16.9%, 14.1%, 11.8 and 7.2%.

After selection of the genes of interest (see introduction), primers for real-time PCR were designed. All primer sequences of the reference genes were designed on sequences of *C. canephora* present in the SOL Genomics Network (Solanaceae Genome Project http://solgenomics.net/index.pl). Reference genes were identified by geNorm™ software, and in each experiment two reference genes were simultaneously used: Rpl7 and GAPDH.

The sequences of interest (public and from our database http://www.coffeedna.net) were aligned with the program SeqMan (DNaStar). Real-time PCR primers were designed on the most conserved regions using the online software Primer3 (http://frodo.wi.mit.edu).

Quantitative Real-time PCR was performed on a Bio-Rad CFX96™ Real-Time PCR Detection System, using IQ SYBRGreen Supermix™. Raw fluorescence data were used to calculate the efficiency of the real-time PCR, in order to identify the best conditions for each primer pair. The analysis was done with LinReg PCR program (Ramakers et al., 2003; Rujiter et al., 2009) (version 11.0, download: http://LinRegPCR.HFRC.nl).

The relative changes in expression were reported as the ratio of the target gene on the reference, in agreement with the mathematical model proposed by Pfaffel (2001) and using the Bio-Rad CFX Manager software version 1.1.

All data obtained from the real-time reactions were analyzed with the statistical software package Xlstat. The normalized data were analyzed with multivariate ANOVA. To assess the interaction of environment and treatment with the expression levels of each gene, a Tukey comparison test was used with a confidence interval of 95%. PCA (Principal Component Analysis) was performed to obtain a graphical representation of the overall correlation between genes, samples and treatments, using the Pearson correlation matrix.

RESULTS AND DISCUSSION

Gene expression analysis in "wet-dry" samples

Isocitrate lyase and *β-tubulin* genes are associated with the onset of germination events, while GAD is associated with levels of stress sustained by the seeds during harvesting, processing and storage. GAD presented differences among the two locations, with Inhobim constantly showing the higher expression. Barra do Choca was characterized by increased expression of *isocitrate lyase* in washed samples (Ba-W vs. Ba-S, p-value 0.030; Ba-W vs Ba-D, p-value 0.045), while expression levels were stable in the 3 treatments of Inhobim samples. In Barra do Choca, β-tubulin had similar expression values between dry and semi-dry methods, with a decrease in the wet treatment (Ba-D vs Ba-W, p-value 0.010; Ba-D vs. Ba-S, p-value 0.011); in Inhobim β-tubulin showed higher expression in the semi-dry method (In-S vs. In-D, p-value 0.015).

Caffeine synthase had the highest expression in samples of washed coffee from Inhobim, with a significant difference between locations (p- value 0,001), while in dry and semi-dry samples there were no important differences between locations.

The enzymes involved in the biosynthesis of chlorogenic acids were more expressed in dry and semi-dry samples, with important differences concerning the location of growth. In detail, *p-coumaryl hydroxylase* showed about 4 times higher expression in washed samples from In than from Ba, while semi-dry samples had an opposite behaviour, and in dry samples a certain stability was maintained. The *shikimate hydroxy transferase* presented significant differences between the two locations, with Barra do Choca showing higher expression levels in semi-dry and dry treatments (Figure 1).

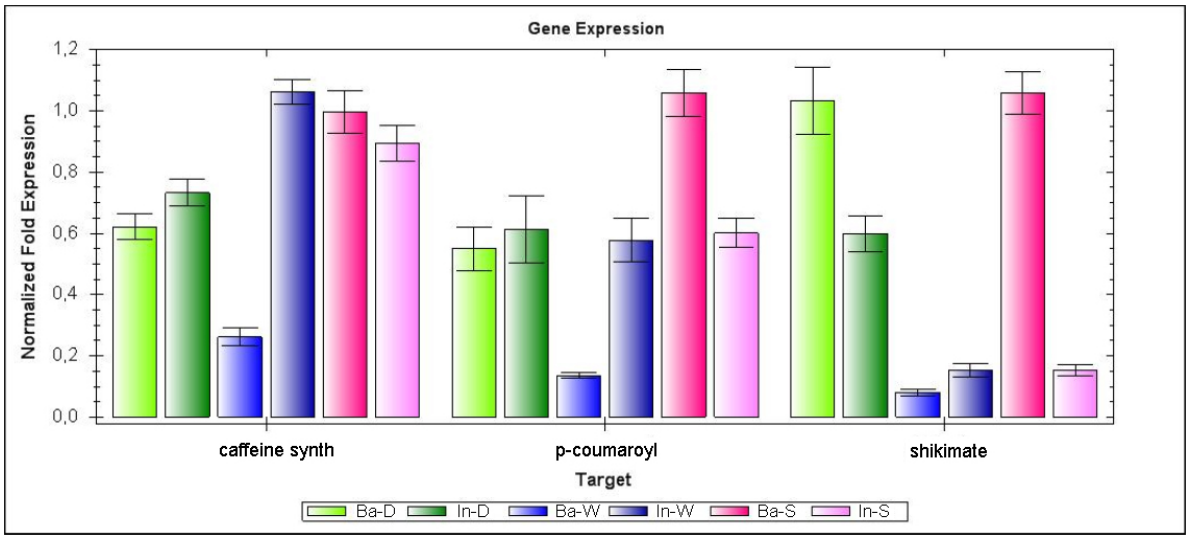

Figure 1. Expression levels of genes involved in biosynthesis of caffeine and chlorogenic acids.

While the *steroid 5-α reductase* showed significant differences only in the washed samples, *geranyl transferase* was significantly different in all treatments: in samples coming from Ba the level of expression was respectively 1.86 and 1.75 times greater in dry and semi-dry samples, while in wet samples the level of gene expression was 2.4 times greater in In than in Ba.

Analyzing the results of genes involved in fatty acid metabolism, we found that *fatty acid synthase* was more expressed in semi-dry samples than in the two other treatments, and *enoyl CoA hydratase* was more expressed in semi-dry and dry samples, with slight but significant expression differences related to the location of harvesting.

As for genes involved in glycolysis and gluconeogenesis, it was possible to show that *pyruvate kinase* was expressed in greater quantities in dry samples, while *aldolase* expression was slightly higher in washed samples. The analysis of each area shows that the levels of expression had no significant differences in the three post-harvest methods in the location Inhobim, while the location Barra do Choca showed more variability.

Gene expression analysis reveals how the environmental factors have an important influence on the level of expression of enzymes related to sugar metabolism. The *sucrose synthase* in Ba crops had higher expression in dry treatment, followed by semi-dry and dry (Ba-N vs. Ba-

S, p-value 0.006; Ba-N vs. Ba-L, p-value <0.0001). The same enzyme showed the highest expression in the In wet samples. *Starch synthase* and *invertase* were more expressed in washed samples, while the *hexose transporter protein* showed no clear trend, with different behaviors strongly dependent on harvest location.

Gene expression analysis in "water content" samples

Real-time PCR has been used to assess whether the amount of water remaining in the seed can influence gene expression related to the quality of the coffee beverage. For each gene, the expression levels were normalized on the value of the 16.9% w.c. sample, that was taken as the first time-point and arbitrarily set at 1. This allowed to monitor the relative quantity of mRNA during the other three time-points of the drying phase.

Isocitrate lyase and *GAD* showed higher expression levels in the samples 14.1% w.c., compared to the reference sample (16.9%, p-value 0.015). Expression of *β-tubulin* remained constant in the samples.

Caffeine synthase showed a progressive decrease in expression from 16.9% down to 7.2% w.c., indicating a possible degradation of the transcript as water is removed from the seed. The enzymes involved in the biosynthesis of chlorogenic acids showed the highest expression in the 14.1% w.c. samples, which then decreased with decreasing percentage of remaining water. The *geranyl transferase*, in relation to the control showed equal or lower expression in all other samples, indicating a probable dormant fase in the seeds and/or degradation of the transcript.

Steroid 5-α reductase followed the trend already seen in other genes, with an increased expression in the 14.1% w.c. sample, which then declines with the reduction of residual water. *Fatty acid synthase* also showed the highest expression in seeds at 14.1% w.c., which then returned to the expression levels of the control sample (14.1% vs 11.8%, p-value 0.041; 14.1% vs. 7.2%, p-value 0.035). *Enoyl CoA hydratase* showed, however, a constant expression in all samples.

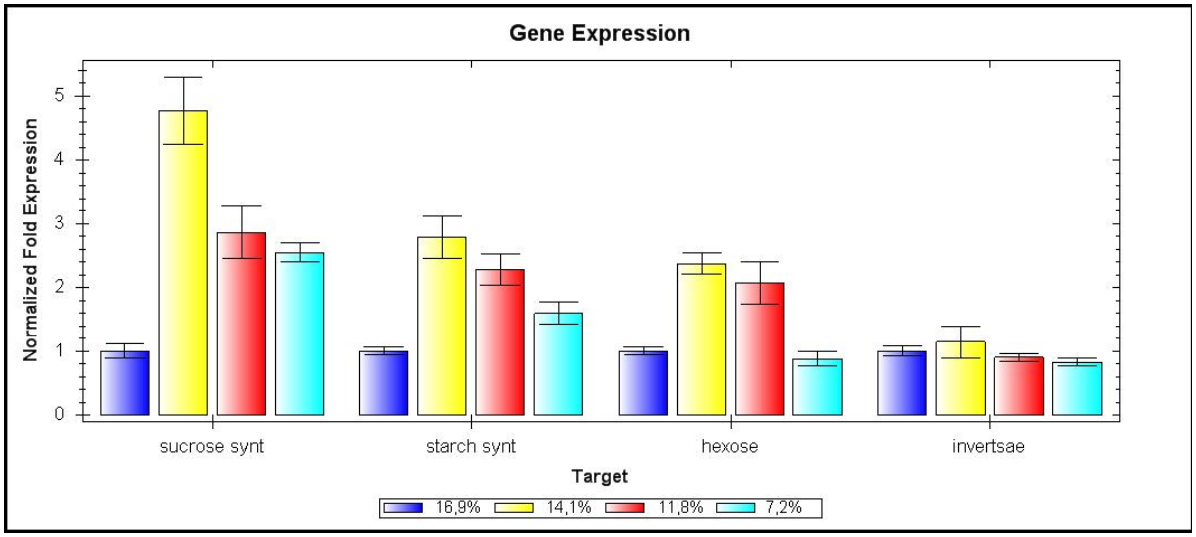

Figure 2. Expression levels of genes involved in metabolism of sucrose, starch, and transport of sugars.

Pyruvate kinase and *aldolase* showed the highest expression levels in 14.1% w.c. samples, and then transcript levels remained constant (16.9% vs 14.1%, p-value 0.048). As for genes involved in sugar metabolism, they all followed the trend of maximum expression in seeds with 14.1% of water, with a subsequent decrease with decreasing percentage of water (Figure 2).

CONCLUSIONS

Samples treated with different post-harvest techniques showed significant differences, especially those that had been collected in the location of Barra do Choca, while Inhobim expression profiles were more consistent in all 3 treatments. This is probably due to environmental factors such as the climatic conditions in different locations, as the operator was the same person as well as the duration of the various processing steps, the method of storage and shipment of the samples.

Subsequently the genetic, geographic (Barra do Choca vs. Inhobim) and processing (wet vs. dry vs. semi-dry methods) variables were analyzed by PCA (Principal Component Analysis), which allowed to confirm that the locations are a determinant factor of gene expression values: within the location Inhobim there were not significant differences between the three post-harvest treatments, while in the samples from Barra do Choca the treatment affected the expression levels of analyzed transcripts.

These samples were also analyzed by the sensory point of view. The analysis indicated that dry-processed coffee presented relevant quality problems (“fermented” off-flavour), while in semi-dry and washed coffee no appreciable defects were detected. Harvest location had no significant effect on the sensory profile of the beverage.

It was not possible to create a common profile of gene expression values between the samples grown at Barra do Choca and those grown at Inhobim, as each gene behaved independently.

There is instead a significant trend followed by most of the transcripts in the "water content" samples: seeds with 14.1% of residual water showed, in most cases (11 genes out of 16), gene expression values higher than the reference sample at 16.9% w.c. As the residual water further decreased, the value of expression levels decreased accordingly.

It is relevant mentioning that the standard water content for proper storage of green coffee is 11±1% (international standards ISO 1446:2001). The detection of metabolic activity at water contents close to that value, can assume considerable relevance in understanding the evolution of coffee quality during shipment and storage.

ACKNOWLEDGEMENTS

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Screening of SSR Markers for Diversity Studies in *Coffea arabica*

M.A. DOS SANTOS[1], R.V. FERREIRA[1], T. SERA[1], D. POT[2], L.F. PEREIRA[1,3], P. CHARMETANT[1,2]

[1]IAPAR, Caixa Postal 481, CEP 86047-902, Londrina, PR, Brasil.
E-mail: mariaaparecida.ciapsan@gmail.com
[2]Cirad, UMR DAP, F34398 Montpellier Cedex 5, France
[3]Embrapa-IAPAR

SUMMARY

The characterization of genetic diversity of a collection of *Coffea arabica* from Ethiopian origin has been performed with SSR markers. Initially 245 SSR markers were used for screening 21 genotypes of *Coffea* including 13 *C. arabica* accessions from Ethiopia collection and cultivars 'IAPAR59', 'Typica' and 'Bourbon', one genotype of *C. canephora*, and five hybrid genotypes that were used to derive mapping populations. The objective of this work was to discriminate SSR markers showing a significant level of polymorphism for futures studies of diversity and mapping projects. 19% of the markers showed useful polymorphism and will be used for further studies on the structure of the variability.

INTRODUCTION

One of the bottlenecks for Coffee breeding programs in *Coffea arabica* is the limited genetic diversity of commercial cultivars. This is a challenge for the introduction and breeding of new traits. However, a wider diversity can be found in *C. arabica* germplasm from the primary center of origin, Ethiopia (Hendre, 2008). Besides the low diversity level, the long reproduction cycle, the lack of efficient selection tools, linkage maps, and difference in ploidy levels between species increase the challenge of the improvement of *C. arabica* cultivars. The use of DNA marker technology offer possibilities to overcome some of these limitations by providing informative genetic markers and marker assisted breeding (Baruah et al., 2003).

Among the molecular markers, microsatellites are most desirable for germplasm characterization and crop improvement due to their high-information content, codominant nature, sensitivity and ease to analyze with minimal quantities of test samples (Baruah et al., 2003). More than 500 SSR including EST-SSR markers have been reported for coffee, (Combes et al., 2000, Rovelli et al., 2000, Baruatt et al., 2003, Bhat et al., 2005, Aggarwal et al., 2007, Poncet et al., 2007, and Hendre et al., 2008). However, these studies did not generate enough information to identify informative markers for fine studies of the structure of *C. arabica*'s diversity.

AIMS

To contribute identifying SSR markers that show a significant level of polymorphism for analyzing diversity and its structure and possibly contribute to futures studies of association and mapping.

MATERIAL AND METHODS

In 1964-1965 spontaneous and sub spontaneous *C. arabica* genotypes were sampled in Ethiopia, the primary center of origin (FAO, 1968); 130 accessions from this survey are currently cultivated at IAPAR (Londrina, Paraná, Brazil). Thirteen *C. arabica* genotypes from Ethiopia collection, cultivars 'IAPAR 59', 'Typica' and 'Bourbon', one genotype of *C. canephora*, and five genotypes of hybrid origin where used in this study.

245 SSR markers designed by University of Trieste (2010) and by Ird-Cirad were used. The PCR products were analyzed by electrophoresis on a 10% polyacrylamide gel using a system of vertical electrophoresis. Gel images were annotated manually, assigning allele sizes to each individual on the basis of visual analyses. The polymorphism information content (PIC) values were calculated according to Weir (1990); a dissimilarity matrix was computed using the DARwin 5 software (Perrier et al., 2003; Perrier and Jacquemond-Collet, 2006).

Table 1. Allelic diversity of SSR markers as revealed across 13 *Coffea arabica* genotypes.

Marker name*	No. of alleles	PIC		Marker name*	No. of alleles	PIC
M755	4	0.73		M270	2	0.45
M260	5	0.67		M411	2	0.44
M258	4	0.62		M318	2	0.43
M414	4	0.62		M754	2	0.43
M509	4	0.59		M314	2	0.43
M501	5	0.58		Cma0109	2	0.41
M514	3	0.57		M363	2	0.36
M350	3	0.56		Cma0103	3	0.33
M355	3	0.54		M465	2	0.32
Cma0117	3	0.54		Cma0113	3	0.29
M503	3	0.51		Cma0166	2	0.28
M377	2	0.50		Cma0168	2	0.26
Cma0118	2	0.50		Cma0179	2	0.26
Cma0199	2	0.50		M512	2	0.2
M766	2	0.50		M462	2	0.17
416	2	0.50		M454	2	0.14
Cma0105	2	0.49		Cma0127	2	0.14
M772	3	0.47		M356	2	0.14
Cma209	2	0.47		M344	2	0.14
M254	2	0.47		M488	2	0.14
M327	2	0.47		M394	2	0.07
Cma0198	2	0.47		M365	2	0.07
Cma200	2	0.47		M382	2	0.07
M395	2	0.45				

** Cmaxxx : University of Trieste (free web access), Mxxx designed by CIRAD-IRD.*

RESULTS AND CONCLUSION

Forty seven markers (19%) showed allelic diversity among the 13 *C. arabica* genotypes from Ethiopia (Table 1). Of these, 11 had more than 2 alleles and they will be used for diversity studies of the collection.

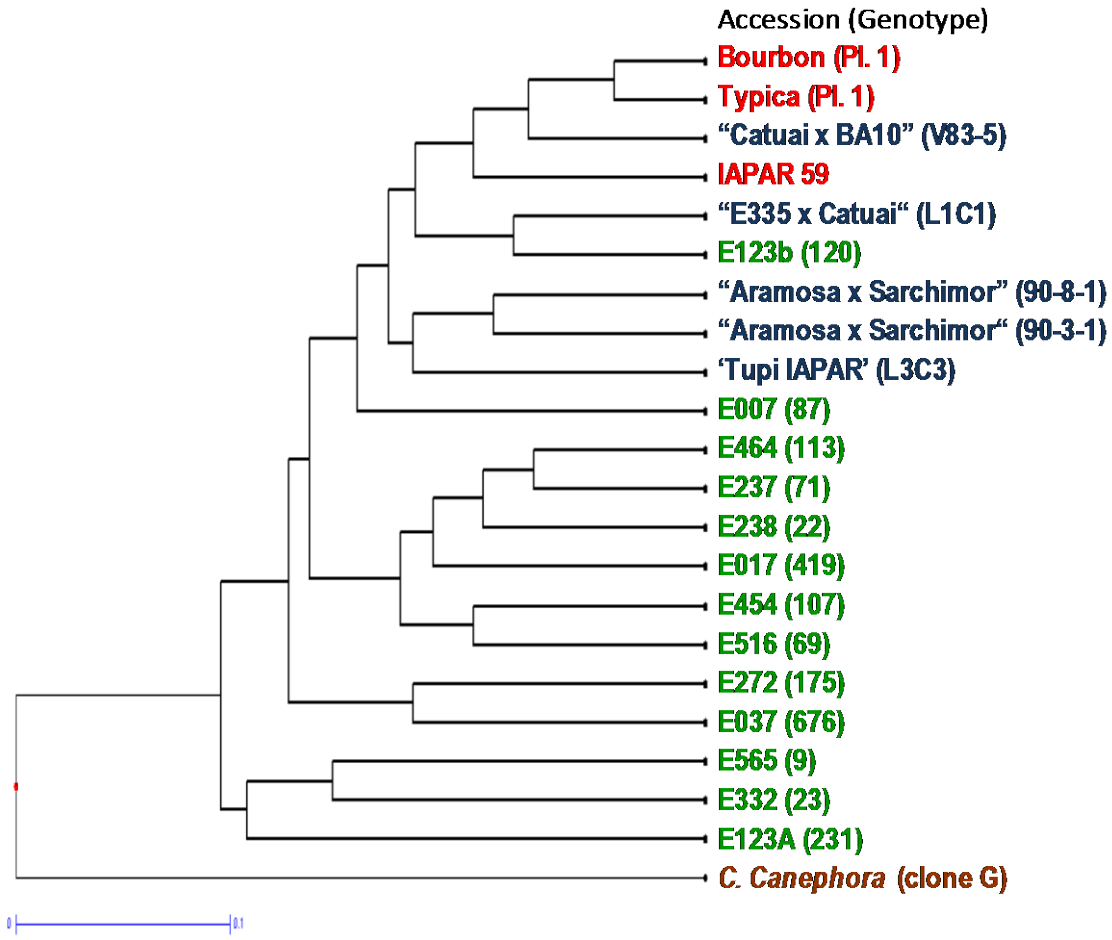

Figure 1. Genetic similarity among 13 wild *C. arabica* (green), 3 arabica cultivars (red), one *Coffea canephora* genotype (brown), and 5 hybrid genotypes (blue), for 53 SSR markers, as determined by average simple matching coefficients (SMC).

The genetic similarity between genotypes of the panel shows the divergence between wild and cultivated accessions of *C. arabica* (Figure 1). Genotypes from hybrid origin appear much closer to cultivars than to *C. canephora*, due to backcrossing. More markers should be screened to be used in mapping studies, as only 16 markers, in the best case, differentiate two genitors. The most polymorphic markers will be used to describe the structure of the variability within the Ethiopian collection, in relation with phenotypic structure. Variability within accessions will also be considered, as it may reveal some variability at the original site of seed collection; it may also be due to successive replanting without selfings.

Figure 2. Phenotypic variability: flowering.

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Quality Upgrading in Specialty Coffee Chains and Smallholder Livelihoods in Eastern Indonesia: Opportunities and Challenges

J. NEILSON[1], B. ARIFIN[2], Y. FUJITA[1], D.F.S. HARTATRI[3]

[1]The University of Sydney, Australia
[2]University of Lampung, Indonesia
[3]Indonesian Coffee and Cocoa Research Institute (ICCRI), Jember, Indonesia

SUMMARY

A popular rural development strategy in recent years has been the value-chain approach, where improved linkages between farmers in marginalized rural communities and expanding market opportunities form the foundations for development assistance. The direct involvement of international buyers can facilitate knowledge transfer and skills upgrading for producers, placing them on potentially dynamic learning trajectories that enable improved competitiveness in a global market. The growing international demand for specialty coffees, and the increasing willingness of global coffee buyers to engage with rural producers in countries such as Indonesia, appears to offer one such opportunity. However, the capacity for Indonesian smallholders to benefit from these opportunities is determined by the ways in which coffee production is embedded within the socio-institutional and agro-ecological aspects of farmers' lives. Value-chain interventions that aim to upgrade the quality and consistency of coffee at the farm-level require locally tailored approaches that take into account the basis of farmers' livelihood strategies and resource constraints. This paper questions rural development strategies that assume enhanced farmer integration with specialty coffee chains will inevitably result in improved livelihood outcomes without further institutional supports.

INTRODUCTION

The growth of international specialty coffee markets in recent decades has increased the demand for high-quality coffee production at origin. This enhanced demand offers opportunities for smallholders to engage in product upgrading and potentially increase the farm-gate price of their coffee. Eastern Indonesia has begun participating in relatively high-priced commodity production in recent decades. The volume of Arabica exports from Sulawesi alone surged five-fold between the mid-1980s and the mid 1990s, due to strong demand from the USA and Japan following deregulation of the ICO export quota system. This study examines smallholder farmer engagement in specialty coffee production across the islands of Sulawesi and Flores. The study integrates global value chain analysis with a livelihoods approach to address the critical linkages between quality upgrading in the value chain and farm livelihood strategies, asking the question whether or not quality upgrading directly contributes to improved livelihoods. Do quality upgrading initiatives generate spatially variable outcomes? If so, can we determine the institutional conditions under which quality upgrading is likely to confer benefits upon farmers' livelihoods?

The global trade in specialty coffees has changed remarkably over the last decade. Global coffee buyers are now actively seeking to develop new relationships with coffee farmers across Indonesia through quality improvement programs, price premiums and transparent

supply chains. A key motivation for this engagement is the construction of marketing narratives for the benefit of consumers (West, 2010). This engagement is somewhat problematic and a challenge for international roasting firms.

On its website, an Australian coffee roaster from specialty coffee company describing his effort to build relationship with local producers in Bali.

"Being at Kintamani, observing the negotiations with farmers, being part of making improvements in harvesting and understanding each others' needs was all about give and take. Trading at origin – not just buying direct trade – is a philosophy of coffee quality and a long term commitment. While it's easy to talk the talk in the industry, managing a relationship with coffee farmers is far more complicated. This is a tough job and always a work in progress." (Five Senses Coffee, 'Back to our Roots)

The findings presented in this paper address the complexity of farmers' livelihood strategies across different coffee origins coffee in Eastern Indonesia. The study should be of interest to industry actors wishing to engage with farmers in eastern Indonesia, as well as regional governments seeking to facilitate rural development through improved market engagement.

According to an increasing number of international development agencies, enhanced integration with global markets is seen to be a key ingredient for achieving broader rural development in underdeveloped regions. Value chain approaches have thus been embraced by international donors and, to a lesser extent, national governments. This embrace has occurred alongside (albeit in a somewhat parallel process) a vigorous debate in the academic literature on the implications for firms and individuals in developing countries following integration with the global economy. These implications have been explored through an increasingly voluminous number of studies employing what has come to be known as a Global Value Chain (GVC) approach (Gereffi et al., 2005; Gibbon et al., 2008; Humphrey and Schmitz, 2002; Kaplinsky, 2000). A key set of insights generated by the GVC approach has been the importance of chain 'governance' structures – the parameters usually set down by powerful lead firms under which other actors in the chain must conform – in dictating upgrading possibilities for developing country actors (Gibbon, 2001). This body of literature has tended to align itself with the traditions of critical political economy. Riisgard et al. (2010), for example, criticise the 'win-win managerial solutions' posed by some development agencies which ignore the 'asymmetrical power relations' that characterise many global agri-food chains. The same terminology – value chains – has thus been employed by two competing perspectives on the processes of rural development.

This paper seeks a middle ground by contributing to the conceptual framework presented by Bolwig et al. (2010), which attempts to integrate the 'vertical' aspects of chains (most notably governance structures) with 'horizontal' aspects (especially livelihood strategies and poverty alleviation pathways). To this end, we extend the model set out earlier by Neilson and Pritchard (2009) that presents a fuller account of the role played by 'institutional contexts' in shaping both GVC structures and upgrading potentials. A key finding from this particular coffee-informed case-study is that distinct livelihood strategies affect both the willingness of farmers to participate in value chain upgrading as well as their potential to gain tangible benefits from enhanced value chain integration.

METHODOLOGY

The research findings presented here were generated through a combination of household surveys and interviews with various value chain stakeholders located on the Indonesian islands of Sulawesi and Flores. Household livelihood surveys were conducted during 2009 across the six case-study districts of Enrekang, Toraja and North Toraja (on Sulawesi) and Manggarai, East Manggarai and Ngada (on Flores). A total of 803 respondents were involved in the survey. These respondents were randomly selected from the three most important coffee-growing sub-districts within each District, based on official production data obtained from the District-level Estate Crop Development Agencies (generally Dinas Perkebunan). The surveys obtained data related to agricultural and non-agricultural household income sources, on-farm coffee management practices, post-harvest handling, product marketing and institutional support structures. The field surveys were facilitated by local staff of Dinas Perkebunan in four of the six districts and by a local NGO in two of the districts. Unless otherwise stated, all data presented in this paper is primary data taken from this household survey. This quantitative survey was complemented by value chain interviews with producer organizations (farmer groups and cooperatives), village collectors, regional traders, processors and exporters in Indonesia, along with importers and roasters in Australia.

BACKGROUND TO THE INDONESIAN COFFEE INDUSTRY

According to the International Coffee Organisation (ICO), Indonesian coffee production exceeded that of Columbia in 2008, making Indonesia the world's third largest volume producer after Brazil and Vietnam. The majority of coffee produced and exported from Indonesia is of the Robusta variety, and is currently of little interest to international specialty buyers. Much of this production of low-value Robusta coffee takes place in southern Sumatra, and is exported via the Panjang port in Lampung. Indonesia, however, is also the largest Arabica producer in the Asia-Pacific region and is a well-known producer of specialty origins such as *Aceh-Gayo*, *Mandheling*, *Java* and *Toraja-Kalosi* (Figure 1). Approximately 80 percent of Indonesia's Arabica coffee is produced by smallholders, while the remainder comes from large estates and state-owned plantations, the latter of which are located exclusively in East Java (Directorate General of Estate Crops, 2009). Most of these smallholders engage in low-input agriculture, sometimes integrated into traditional swidden systems at the forest frontier, with low per hectare productivity.

Sulawesi is already well-regarded as a quality coffee origin by international buyers, with Arabica exports routinely attaining significant price premiums above the New York Terminal. The total volume of Arabica exports from Sulawesi, however, is relatively low, with data from the Makassar Port indicating exports of between 3000 and 4000 tonnes annually between 2002 and 2007 (Marsh and Neilson, 2007).

Flores is a less well-known origin, with total annual Arabica production estimated at about 2500 tonnes (Neilson, 2008). Flores coffee is exported predominately through the Surabaya port in East Java. A number of quality-improvement programs have been initiated by both government and industry in Flores over the last five years. As a result, a small portion of the island's production is now marketed as a specialty coffee, although the majority is processed using rudimentary techniques and sold as a standard commercial coffee.

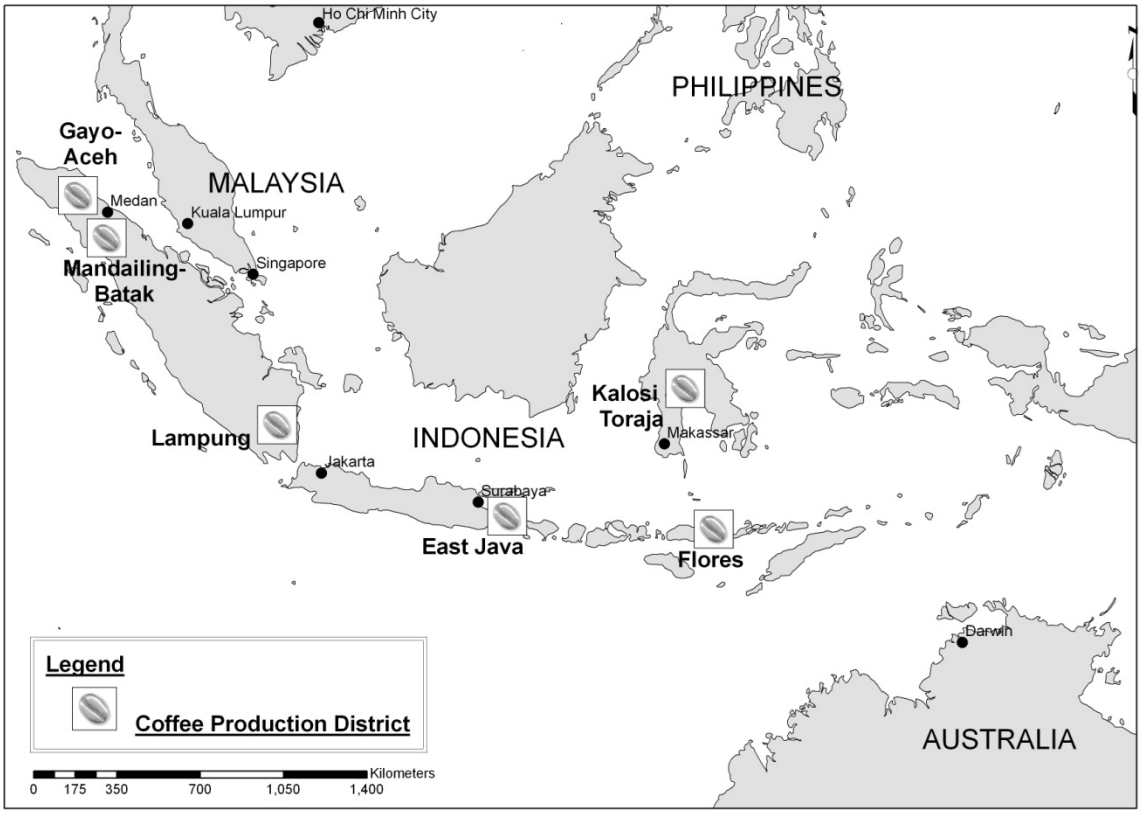

Figure 1. Map of major coffee-producing districts in Indonesia. Source (Neilson, 2008).

Farmers on both islands are connected to the global market through a network of institutions consisting of marketing chains, international development assistance, government support programs and local social institutions. The alignment of these structures collectively determines the farm-gate price of coffee. The Toraja districts of Sulawesi already have relatively 'short' value chains with the local presence of two large foreign-owned processing mills. Government intervention in the Toraja districts is minimal. In contrast, the role of government in supporting coffee production has been much more pronounced in the case of Flores, where international buyers have been less influential.

COFFEE-BASED LIVELIHOODS IN EASTERN INDONESIA

The implicit assumption behind many value chain interventions for rural development is that farmers are reliant solely on the chosen product for their livelihood. Across the different sites of Eastern Indonesia, however, coffee constitutes one element within a complex, and highly varied, strategy that farmers employ to secure their livelihoods (Figure 2). While reliance on coffee for cash-income is higher in the Flores Districts than in Sulawesi, farmers in these districts are also intensively engaged in primary food production. The way coffee is inserted within varied livelihood strategies will inevitably determine the effectiveness of any initiatives to upgrade farmers through the value chain. The dominant livelihood strategies employed by farmers across three case-study districts across Sulawesi and Flores highlight this variability.

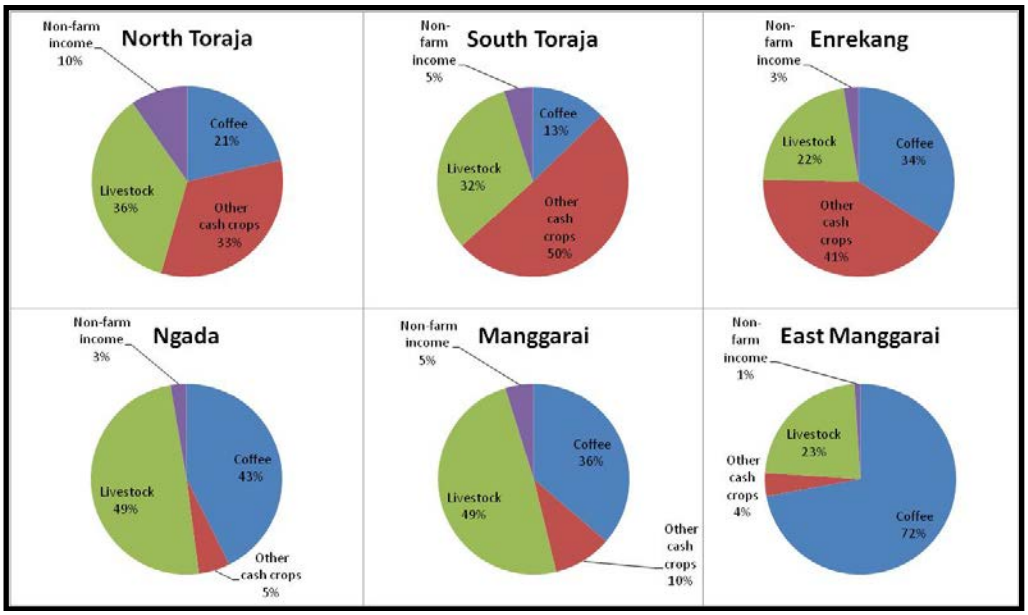

Figure 2. Sources of household income across the six case-study districts in Sulawesi and Flores.

Remittances and traditional farm systems: North Toraja

Of the six case-study districts, farmers in North Toraja received the highest price for their coffee (Table 1). The farmers in this district, however, receive less than 21% of their total income from coffee (Figure 1), prioritizing instead pig and buffalo rearing (primarily for local sale and ceremonial consumption), rice farming (associated with high social prestige - 56% of households were fully self-sufficient in rice), and remittances from émigré family members (37% of households received regular remittances). While North Toraja shows the highest degree of dependence on remittances, these transfers were an important income source for rural families across all districts (Table 2). North Toraja is also unique in that no households held formal land certificates and land transactions are extremely rare. Access to land, including for coffee cultivation, is determined primarily by traditional inheritance customs and ceremonial participation (Neilson, 2004). The livelihood strategy in North Toraja can therefore be characterized by strong embeddedness within traditional cultural and agricultural practices, but with an increasing reliance on migration and remittances for wealth accumulation. Despite the strong demand for coffee produced in this region, and the fact that few other locally-produced commodities are traded out of the region, coffee is a relatively minor element within household-level strategies.

Table 1. Average Farm-gate prices in 2009 (Rp/ kg GBE).

District	South Toraja	North Toraja	Enrekang	Ngada	Manggarai	East Manggarai
Average farm-gate price	23,819	25,434	24,005	17,309	16,632	17,141

Note: Approximate exchange rate in 2009 1 USD = Rp10,000.

Table 2. Percentage of households receiving remittances from family members.

South Toraja (n=65)	North Toraja (n=135)	Enrekang (n=199)	Ngada (n=207)	Manggarai (n=97)	East Manggarai (n=100)	Average
14%	37%	13%	26%	33%	17%	23%

Intensive agricultural cultivation: Enrekang

Enrekang is located directly to the south of the Toraja Districts in Sulawesi. Livelihood strategies here, however, are distinct from those in North Toraja. Less reliant on both local production of rice and remittance incomes, farmers here have instead prioritized commercial agricultural production as a key element of their broader livelihood strategy. 41% of total income in Enrekang is obtained from agricultural crops other than coffee (Figure 1), with substantial cash incomes generated from vegetable crops and fruit trees (e.g. red onion, salak, tomato). Farmers in Enrekang apply external inputs (synthetic fertilizers and agrochemicals) to their coffee plots at rates far-exceeding those in North Toraja (Table 3), suggesting a far-greater willingness to invest capital in coffee production. As a result, per hectare yields in Enrekang are double those in North Toraja. In a relative sense, the farm systems in Enrekang are highly commercialized, and farmers will allocate household resources across the farm system to those activities where a financial return is most likely.

Table 3. Intensive cultivation in Enrekang (use of synthetic fertilizers and herbicides).

	South Toraja (n=65)	North Toraja (n=135)	Enrekang (n=199)	Ngada (n=207)	Manggarai (n=97)	East Manggarai (n=100)
Urea	0%	1%	89%	0%	0%	0%
ZA	80%	24%	26%	0%	0%	0%
KCl	42%	7%	30%	0%	2%	0%
SP36	0%	0%	71%	0%	1%	0%
NPK	15%	10%	27%	0%	13%	0%
Herbicides	54%	33%	92%	0%	5%	3%

Prioritising food security: Ngada Districtc of Flores

Conditions on the island of Flores are distinct again from both the commercial orientation of Enrekang and the remittance-based economy of North Toraja. Farmers in the Ngada District of Flores are mostly concerned with self-sufficiency of staple food crops, both rice and corn, and the rearing of livestock (cattle). These farmers have few other sources of cash income (Table 4) and 70% of the farmers surveys in Flores were self-sufficient in corn production. Average corn production in Ngada was reported by farmers to be 184 kg per household per year, compared to 56 kg / year and 26 kg / year in the Manggarai and East Manggarai Districts respectively. The recent history of regional food shortages in Bajawa seems to have encouraged a conservative livelihood strategy emphasizing food security (e.g. corn, rice production and livestock). Farmers in Ngada may be reluctant to increase allocation of household resources to coffee farming and quality improvement at the expense of food production in the absence of improved institutional supports for food security.

Table 4. Income sources amongst coffee farmers in Ngada.

Sources of livelihood	Ngada	Average of all 6 districts
Coffee	42%	33%
Other cash crops	5%	29%
Livestock	50%	33%
Non-farm income	3%	5%
	100%	100%

VALUE CHAIN UPGRADING IN EASTERN INDONESIA

At least three types of upgrading are identified in the value chain literature: product, process and functional upgrading (Schmitz, 2006). According to this framework, process upgrading is where improvements are made to the production process to generate outputs more efficiently, usually through technology improvements (eg. mechanisation). Product upgrading is where suppliers move into higher value product lines to achieve increased unit values (eg. organic or specialty production). Functional upgrading is where suppliers acquire new functions in the chain such as engaging in downstream processing of raw materials. To different extents, all three types of upgrading are evident in Eastern Indonesia.

The dominant industry development policy adopted by the government of Indonesia (frequently implemented at the District-level) has been to assist farmer organisations engage in functional upgrading, generally through the provision of small-scale processing equipment (such as hulling machines, graders, and even roasting machines). This development approach reflects a belief in agro-industrialisation as a poverty alleviation pathway and assumes that downstream processing will deliver 'value-added' to rural communities. Downstream processing, however, does not always lead to overall 'value-adding'. Coffee farmers in the Sulawesi districts generally sell wet parchment to traders, whereas farmers in Flores sell green beans. As presented in Table 1, farm-gate prices in Sulawesi are noticeably higher due, to a large part, to the ability of centralised mills to manage quality effectively.

Table 5. Institutional supports for coffee farmers (as reflected by farmer responses).

	S. Toraja	N. Toraja	Enrekang	Ngada	Manggarai	E. Manggarai	Average
Participation in govt. extension	34%	34%	74%	57%	45%	22%	44 %
Participation in farmer groups	26%	27%	66%	65%	51%	41%	46%

Product upgrading, essentially through quality improvement and product certification, has been driven primarily by international buyers and, to a lesser degree, by the Government of Indonesia. Consumer demands for product traceability have clearly been a primary driver of upstream coordination in the Indonesian coffee industry (Neilson, 2008). Large processing mills, with significant foreign ownership, are located in the Toraja Districts of Sulawesi and have been responsible for a campaign of quality improvement over number of years (Neilson, 2005). In contrast, government agencies have played a limited role in facilitating coffee development in the Toraja region (as indicated by levels of government extension presented in

Table 5). Furthermore, Table 5 suggests the participation in farmer groups does not necessary correlate with product upgrading or help farmers to negotiate for a higher market price. Farmers in Toraja largely manage farm production on an autonomous basis with close linkage to international coffee buyers, yet they are the most advantaged in terms of gaining high return for their coffee.

CONCLUSIONS

The case of coffee production in Toraja strongly suggests the critical role that can be played by international buyers in facilitating quality improvement and product upgrading, leading to substantially increased farm-gate prices in these districts. The increasing interest from specialty coffee buyers to engage with Indonesian farmers through value chain integration, therefore, offers significant opportunities for quality improvement and enhanced farm-gate prices for coffee. Coffee farmers in Eastern Indonesia, however, employ highly diverse livelihood strategies within which cash income from coffee is frequently a minor contributor. These farmers effectively participate in a range of distinct value chains. The value chain development approach tends to ignore this diversity of farmers' livelihoods and their strategies, and frames rural development issues in terms of a single-commodity logic. This contradiction may help explain the apparent unwillingness of farmers to engage in upgrading initiatives that buyers and development agencies claim to be in their (the farmers') interest, but fails to consider the constraints faced by farmers themselves.

From a policy perspective, there are implications from this analysis for the appropriate role to be performed by public-sector support institutions. It is unrealistic to expect private sector buyers of a single commodity to deliver broad-ranging rural development for communities reliant on a range of livelihood sources. However, sectoral development initiatives instigated by the government of Indonesia have all too often failed to align strategically with the upgrading opportunities presented by enhanced value chain integration. There are many obvious synergies that could be developed that would allow a greater overall impact on rural development without requiring a greater overall investment of public resources. The focus of government interventions should no longer be simply in those areas of market failure or in the provision of public goods. Instead, governments (both local and national) should be looking at those areas of rural development not being provided through value chains.

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Evaluation of Organic Coffee Farming Potential from Perspectives of Production and Natural Resources Management in Yayu District, Southwestern Ethiopia

TESFAYE AYANO[1], BOBE BEDADI[2], KETEMA BELETE[2]

[1]Gudina Tumsa Foundation P.O. BOX 4003, Addis Ababa, Ethiopia
[2]Haramaya University

SUMMARY

Southwestern coffee growing agro-ecology of Ethiopia is the center of origin and diversity of Arabica coffee. Presence of resistant materials, conservation of natural forest and soil, growing coffee under shade trees, using organic inputs and integrated cropping are the fundamentals of organic and sustainable coffee production systems. This study therefore, evaluated the production potential of coffee in Yayu District from production systems and natural resources conservation perspectives through detailed surveying. Coffee production systems were shade grown and bird friendly. The inputs utilized for coffee cultivation were animal and plant sources. No synthetic inputs were employed in coffee farms in the area since 15 years of cultivation. Slashing and hand weeding, inter-row cultivation were the common weed control methods. Resistant cultivars and cultural practices were used to prevent coffee diseases. To date, 100% of the coffee farmers in the area are producing organic coffee. Also more than 90% of the farmers conserved the vegetation with coffee. About 57% of coffee farm owners applied different conservation practices to conserve soil. Therefore, the area has got high potential for organic coffee production. However, the price of such organically produced coffee is very low and unfair. Thus, certification for organically produced coffee in the area is recommended.

INTRODUCTION

Arabica coffee has its centre of origin in Southwestern and Southeastern part of Ethiopia, where it occurs naturally in the undergrowth of Afromontane rainforests between 550 and 2,600 masl (Warkafes and Kassu, 2000). Coffee is the most important export crop contributing 41% of the Ethiopian's foreign currency income and 25% of the population depends directly or indirectly on coffee for their livelihood (FAO and WFP, 2006).

Organic farms are mostly mixed farms, integrating animal husbandry with crop production, diverse rotations, intercrops and green cover crops, and maintaining soil fertility by cultivating nitrogen fixing legumes plants, and consisting of a higher diversity of domesticated species than conventional farms (FAO, 2002).

Farming systems and agronomic management involves natural resources conservation which largely affects organic coffee production system. Thus, assessment of the production systems and conservation mechanisms put a base for potential of organic coffee production under sustainable way in the region. Furthermore, there is very limited research study, quantitative and qualitative scientific information documented on organic coffee farming and farming potential in coffee growing regions of Ethiopia. Therefore, the study was carried out to assess organic coffee farming potential in Yayu District.

MATERIALS AND METHODS

The study was conducted in Yayu District, Oromia Regional State of Southwestern Ethiopia. The altitude of the district ranges 1160-2581 masl while that of the study area covers 1336 to 2070 masl. Total land of the district was 84626.005 ha; coffee land under production was 25%. The mean annual maximum and minimum temperatures during the study period were 28.00 °C and 14.16 °C respectively. The annual rainfall in 2007 was 1633 mm.. The mean annual rainfall averaged over 20 years is 1087 mm**.**

There are 17 coffee growing PAs (Peasant Associations) in Yayu District. Three of the 17 PAs were selected for the survey to represent the three agro-ecologies: low (<1500 masl), medium (1500-1800) masl, and highlands (>1800 masl). The total respondents in the three PAs were 90. The survey was conducted by using random sampling design. Data were collected through personal interview of 30 respondents using formatted questionnaire. The primary criterion for sample PAs selection was identification of known coffee growing PAs in the district. Agricultural development agents and regional agricultural bureau expert were involved in identification and selection processes of representative PAs. Personal observations along the transit walks and secondary data from the district agricultural bureau were also incorporated. The collected data were analyzed by SPSS Computer Software Version 14.

RESULTS AND DISCUSSION

Socio- economic Condition and Awareness of Farmers on Organic Coffee

Twenty eight percent of the coffee producing farmers are illiterate. Females take part in production systems specially in harvesting processes. More than 50% of household head indicated that organic coffee production systems created additional workload on females particularly transporting bulky organic inputs to coffee farm site. Low price of organically produced coffee, lack of coffee traders' preference to well prepared pure red cherry to mixed ones created negative impact on household income.

The majority of the farmers recognized organic farming as using organic inputs coffee for production. On the other hand, large number of farmers understood as using shade tree for coffee, slashing weeds and cultivating soil under coffee tree using uprooting hoes and the combinations. Major coffee producing farmers (61%) acquired organic production information from their ancestors and some from local agricultural bureau. To date, 100% of the coffee farmers interviewed are producing organic coffee in Southwestern Ethiopia and more than 90% of the households planned to keep on producing organic coffee. About 87% of the farmers witnessed that organic way of production increased the quality of coffee.

Inputs for Coffee Production in the District

The farmers indicated that major organic inputs are of animal and plant origin or the combination of the two sources. Organic coffee production in Southwestern part of the country is 100% free of inorganic fertilizers, pesticides, herbicides, fungicides as investigated by the researcher and from documented data of the district. Coffee producing farmers of the region have not used inorganic inputs and inorganic chemical treatment in production systems since 15 years.

Even if use of herbicides saves time and labor for controlling coffee weed, it is costly and reduces coffee age as the farmers suggested. Almost all farmers informed the negative impact of inorganic fertilization, reduction in the consistency of the yield, age of the coffee tree, natural coffee quality and cherry weight. Experienced farmers suggested that adding animal manures to coffee trees reduce the thickness of coffee pulp in return increased the weight of coffee bean.

More than 90% of the farmers reported that mulch is important to increase soil fertility and yield of coffee. Greater than 70% of the farmers indicated that mulch conserves soil moisture and about 68% of the respondent pointed out that it suppresses weeds, which in turn 60% of the farmers indicated mulching reduces weeding labor. Farmers approved that mulching improves yield and quality; increases fruit bearing branches and increases stem girth. Mulching further prolongs the age of coffee, makes bean filling complete and conserves soil moisture for coffee in dry seasons. Organic coffee farmers in the area use animal manures, FYM, crop residues, grass, tree foliage, and slashed weeds as a main sources of mulch under coffee (Table 1).

Table 1. Application percentage of organic inputs used by coffee growing farmers in Yayu District.

Organic inputs used	Coverage (%)	Source of inputs
Animal manures	67	Cattle, sheep, goat, horse, mule, and donkey
Farm yard manure (FYM)	68	Cattle, sheep, goat, horse, mule, and donkey with plant sources
Household wastes	59	Ash and associated house hold wastes
Leaves and twigs	69	Shade tree and shrubs on coffee farm
Mulches	94	Weed mulch, grass mulch, tree foliage and few coffee husk
Compost	60	Broad leaved shade tree, weeds and animal sources
Crop residues	50	Cereals and pulse crops
FYM+ Crop residue	37	Animal and crop sources

Weeds, Pests and Diseases Control in Organic Coffee Production

Organic coffee farmers in Southwestern part of Ethiopia control weeds in coffee by hand weeding, inter-row cultivation by turning up the soil and slashing grown weeds using bushman knife among these more than 50% of the farmers use slashing and hand weeding. From the experiences of organic farmers, repeated slashing of weeds in coffee increases the weight of coffee fruit bean as the slashed weed acts as mulch for the coffee tree. Farmers consider three times proper slashing of weeds in coffee per the season as an optimum practice. Considering time of slashing weeds in the open space of coffee is important to reduce wash of fertile soil during heavy rain. Hence, some farmers prefer to slash weeds heavily at the beginning and end of rainy season to keep fertile soil on the farm.

Coffee berry disease (CBD) is a major disease in heavy rain receiving highland areas of Southwestern Ethiopia. Farmers in the Yayu District controlled CBD by various methods. The dominating CBD control method by the farmers (80%) was using resistant cultivars. Generally, to the capacity of this study and from secondary data of the district, as well as from indirect examination by detail questioner interview; no coffee farm owner was found applying

fungicide to control CBD since 15 years. Therefore, in this regard, coffee production systems in this district were free from fungicide inputs.

Soil and Forest Conservation in Yayu District

As result of sloppy and undulating land topography of the region, 54.4% of the coffee farm is vulnerable to soil erosion and 44.4% of coffee farm sites are free from erosion hazards. Consequently, about 57% of coffee farm owners applied different conservation practices to conserve fertile top soil, to protect coffee root exposure to the sun, to protect land degradation, to promote and sustain yield of coffee plant and to provide long-term opportunity of organic coffee production. More than 90% of the farmers used contour ploughing, planting trees and more than 80% of them used furrow making to control soil erosion. Soil conservation is an alternative practice to decrease the need of synthetic fertilizers and improve the quantity of main cations (K, Ca, and Mg) (Snoeck and Vaast, 2004). Vetiver grass (*Vetiveria zezanioides*) terracing is an important soil conservation aspect, which was observed in the district.

About 91% the farmers conserve the natural forest as they strongly associate coffee with forest and forest with coffee. In addition, more than 75% of the farmers who conserved forest, use coffee farm as a source of firewood whereas more than 25% of the farmers who did not conserve forest used it as a source of firewood. Thus, growing coffee with shade is a strong strategy to conserve natural forest in the nation.

Soil Fertility Management, Farming Systems and Constraints in Yayu District

Rotation of coffee associated crops is a common cultural practice used by farmers to improve and maintain soil fertility. From the farmers' point of view, fertility potential of soils on which various crop rotations practiced were 75%, 21% and 1.4% fertile, slightly fertile and poor in fertility respectively. However, farmlands, which lack crop rotation, were 36%, 57%, and 7% fertile, slightly fertile and poor in fertility respectively. Farmers in the study area also use shade trees to improve the fertility of the soil particularly leguminous like *Albizia* spp., *Acacia* spp., and *Millia ferruginea.* More than 75% of the farmers use shade tree leaves and cut of twigs as a form of green manure for organic coffee production. Farmers in the district use animal source input to improve the fertility of the soil.

The farming system of Yayu District is well diversified and 92.2% of the population was involved in livestock rearing, crop production, coffee growing along with bee culture. Thus, 96% of the farmers confirmed that the farming system of the region is very convenient for organic coffee production as the animal and crop products are the sources of bulk inputs for the system. Some of the major constraints faced coffee farmers in organic coffee production involves labor intensive production, shortage of cover crops and suitable crops to intercrop in coffee for fertilization, and shortage of animal manure due to death of animal by diseases at lowland areas. Unfair and low price paid for organically produced coffee in the region is also a common set back to organic coffee production in the study area.

Coffee Shade Tree Management and Contribution to Organic Coffee Farming

More than 50% of the farmers use native leguminous along with non-legume native shade trees on their coffee farm. The leguminous shade trees are small leaved like *Albizia* spp. and *Acacia species* and non-leguminous shade tree and 40% of coffee growing farmers used native leguminous shade for their coffee. From farmers experience *Albizia* spp. and *Acacia*

spp. shade trees are very useful to improve yield of coffee, quality of coffee, to protect some coffee diseases better than other types of shade trees. The great contribution of *Albizia* spp. and *Acacia* spp. for coffee quality, yield and diseases is due to their proper screening of light (25%-75% of sunlight transmitted) Yacob et al. (1996). Farmers also suggested that coffee produced under *Albizia* spp. shade trees is sweet and full of oil during roasting. Ninety three percent of coffee growing farmers in the district conserve trees on coffee farm to provide shade. Coffee producers keep suitable balance of shade level for coffee by thinning of main stem and branches. More than 90% of the farmers manage the shade trees to increase the yield of coffee and 60% manage for the sake of improving coffee quality. To protect coffee branch dieback and to control pest and disease were also sounding reasons for the farmers to manage shade trees. Organic coffee farmers all agree that birds are numerous in diversity on shaded coffee. Coffee growing in the district was bird friendly, as the producers did not apply synthetic inputs that discomfort birds and chase them away from the area.

Organic Coffee Production Management in the District

Most of the farmers in the district manage coffee in terms of keeping proper coffee density, select suitable coffee cultivars, self-raise of coffee seedlings or collect wild seedlings from nearby forest and semi-forest. Experienced farmers used disease resistance, good yielding, number of primary branches and bean size as criteria of the best coffee cultivars selection. About 80% of the farmers select suitable coffee cultivars and raise coffee seedlings by their own or collect wild seedlings from near forest and semi-forest. Above 90% of farmers, apply row planting with proper spacing for convenience of slashing weeds, harvesting cherry and preventing diseases as result of overlap of coffee branches. They also manage their coffee by maintenance pruning, stumping old coffee and control the height of coffee.

Organic Coffee Harvesting and Processing Methods

Coffee harvesting system was carried out in the district by manual selective picking method. Selective red cherry picking as the farmers reported is advantageous; it is heavier and sweeter than green stripped, over ripped and dry cherries dropped to the ground. Selective red cherry picking is also important for the health of mother tree than branch wounding green stripping method. All organic coffee farmers process coffee cherry by dry processing method. Eighty eight percent of the farmers practiced dry processing on local bed made from wood and straw of teff. Six percent of the farmers used combination of raised local bed and raised wire mesh.

CONCLUSION

Cropping system was well diversified and suitable for organic coffee production. Organic inputs, weed, and disease, pest control systems were generally eco-friendly, harmonious with the producers and wild life. Coffee growing materials were from wildlings and self-growing seedlings. Coffee management purposes were to increase yield, quality and protect pests and diseases. Conservation and management of natural forests, soils and shade on coffee farms were common in the district which showed the potential of coffee farm sites for organic coffee production. However, the price of such organically produced coffee is very low and unfair. Therefore, organic coffee certification is recommend.

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Sustainable Coffee Farming Framework: a Chain View

G. CELI[1,2], D. LIVERMAN[2,3]

[1]illycaffè S.p.A., Via Flavia 110 – 34147 Trieste, Italy
[2]Oxford University Centre for the Environment, University of Oxford - South Parks Road, Oxford, United Kingdom
[3]Institute of the Environment, University of Arizona - N Park Ave, Tucson, AZ, USA

SUMMARY

This study has adapted the Sustainable Livelihood Framework (SLF) to comprehend and compare the sustainability of different green coffee farming systems. The SLF is a people-centred framework designed for a better understanding of the complexities of poverty. The framework presents the main influencing factors for the people's livelihood and it emphasises their multiple interactions. The SLF has been largely pursued during the last decade by the UK Department for International Development, the Food and Agriculture Organization (F.A.O), the International Fund for Agricultural Development (I.F.A.D.), the United Nations Development Programme (U.N.D.P.), the World Food Programme and several NGOs.

The resulting framework, called Sustainable Coffee Farming Framework (SCFF), has been applied in its completeness to gain knowledge on how the supply chains and the external environment are influencing the coffee farmer and consequently the sustainability of coffee farming. The SCFF is made of the following components: a) the vulnerability context; b) the coffee farmer assets; c) the transforming structures and processes; d) the coffee farming strategies and e) the coffee farming sustainability outcomes.

An in-depth multi-case research analysis has been carried on in three very different farming systems: 1) Brazilian full-sun coffee farming system; 2) Guatemalan shaded coffee farming system and 3) Indian shaded multi-cropping system. Questionnaires have been submitted and farming data have been gathered on the field for all the three cases.

This study highlights the mechanisms and interactions that are influencing the sustainability of each coffee farming system.

The SCFF can be used as a supporting tool to invest in the improvement of the farmer's capital assets for a more sustainable coffee farming (resilience to the supply chain and environmental influences). It has therefore the potential to become a practical governance tool for sustainable supply chain management in the coffee sector.

INTRODUCTION

Sustainability is somehow a misused and abused term. A milestone definition has been given in 1987 by the report of the World Conference on Environment and Development (WCDE), also known as the Brundtland Commission. The report of the Brundtland Commission interprets sustainable development as the present economical exploitation of any kind of resource in a way that the same resource will be equally available for the future generations. After that, the definition of sustainable development has been expanded to include the ideas of

fairness and interdependence, not only between generations but also between the countries and peoples of the globe (Brown et al., 1995). It is therefore clear that, in global businesses, the adoption of fair practices strongly contributes to the long term sustainable development of the businesses themselves. Sustainability recognizes also the interdependence of ecological, social and economic systems, the three pillars of sustainability (Hutchins and Sutherland, 2008)

Because of this complexity, sustainable supply chains decision-makers are often limited in what they can know (bounded rationality) and thus rational calculations cannot guarantee optimal solutions (Matos and Hall, 2007).

In light of the current scenario of climate change and global population growth, the concept of sustainable agriculture, meant as the ability of agro-ecosystems to remain productive in the long term (van der Werf and Petit, 2002), has become more and more important. Sustainable agriculture has been defined by Lewandowski et al. (1999) as the management and utilization of the agricultural ecosystem in a way that maintains its biological diversity, productivity, regeneration capacity, vitality and ability to function, so that it can fulfil, today and in the future, significant ecological, economic and social functions at the local, national and global levels and does not harm other ecosystems.

There is a wide range of different Arabica coffee agro-ecosystems varying from full-sun coffee mono-culture agro-ecosystems to shade-grown coffee mono-culture or poly-culture agro-ecosystems. The farmer's decision about the agricultural strategies to adopt, including the choice of the coffee agro-ecosystem type, is the result of the continuous and complex interactions of a number of internal and external factors. The arising question is whether the currently managed coffee agro-ecosystem is the most sustainable one for that specific reality at that specific time or differently there are better alternatives, changes or improvements to be possibly done. The sustainability of an agro-ecosystem should be there is the need to improve the methodology to understand measure and compare the sustainability of coffee farming systems, their dynamics and mechanisms in response to different external and internal factors. The objective of this research is the design of a methodological framework to become a living tool useful for decision-makers in the private and public sectors to invest and work towards more sustainable coffee supplies.

The starting point of this research has been the analysis of the existing frameworks useful for this research objective. The analysis has found that the Sustainable Livelihood Framework (SLF) is the more appropriate framework to understand the three-dimensional, dynamic and complex sustainability concept. The SLF is the flexible analysis component of the Sustainable Livelihood Approach created by the UK Institute of Development Studies. It is a non-linear people-centred analysis to derive a set of guidelines and principles in order to address and fight poverty. The Framework is composed of the following parts (see Figure 1): 1) The Vulnerability context; 2) The Livelihood assets; 3) The Transforming structures and processes; 4) The Livelihood strategies; 5) The Livelihood outcomes.

The framework has been widely used to design multiple entry points for investment strategies or policies for sustainable development aimed at poverty reduction or resource management improvement in the long term (Brock, 2000; Nicol, 2000; Scoones, 2000; Allison and Horemans, 2006). The framework has been used to assess the impact of fair trade initiatives on small farmers (Utting, 2008). This framework has never been used for understanding the sustainability of farming with a supply chain/business view. In the following paragraph, the case of the global Arabica coffee supply chain is presented.

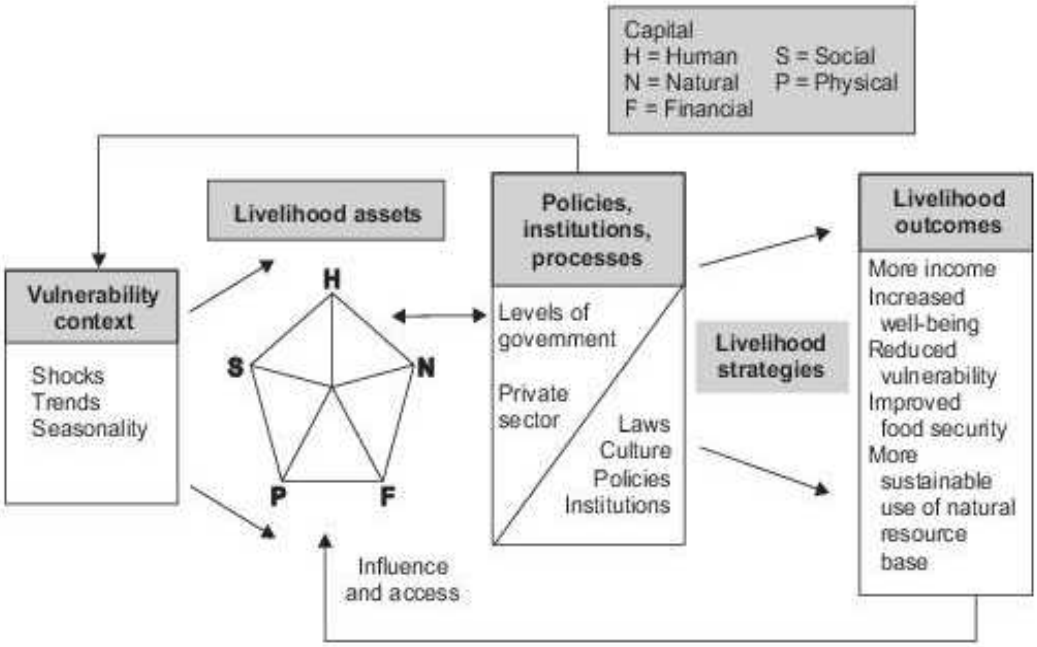

Figure 1. The Sustainable Livelihood Framework.

The research has then adapted the design of the SLF to deeply understand the sustainability of different coffee farming supplies. The newly designed framework, called Sustainable Coffee Farming Framework (SCFF), has been tested using the case study methodology in three different coffee agro-ecosystems in three different coffee producing countries: 1) Brazilian full-sun coffee farming; 2) Guatemalan partially shaded coffee farming and 3) Indian highly shaded multi-cropping system.

The SCFF has underlined differences among the farming systems in terms of outcome indicators and emphasized the main interacting factors. The SCFF analysis has been used as a decision-making tool to find the most important areas to invest towards improving the sustainability performance of coffee farming.

MATERIALS AND METHODS

Each part of the SLF and the interactions among the parts have been adapted to coffee farming and coffee supply chains using an analysis of the existing scientific literature, a review of the recent news articles and collecting experts' interviews. Afterwards the newly designed sustainable coffee farming framework (SCFF) has been tested with three case studies. Three medium-big case-farms (more than 150 hectares) representing three different farming strategies have been selected: full-sun coffee farming in Brazil, partially shaded coffee farming in Guatemala and highly shaded multi-cropping system in India. The three cases represent different degrees of farming intensity: from high intensity coffee farming in Brazil, to medium intensity coffee farming in Guatemala to low intensity farming in India. The farming intensity can be defined by the intensity of land use (planting density, shading and crop specialization), the degree of agricultural technology adoption and the amount of chemical inputs use for crop production.

The three selected case-farms are located in three well-know producing areas: Cerrado Mineiro (Brazil), Fraijanes (Guatemala) and Coorg (India).

The research has considered more interesting to test the new framework using medium-big farms as they are more exposed to global trade.

The three case-farms have also been selected for the high quality profile in terms of data record and in terms of agronomical knowledge in order to facilitate the data collection and to test the best-in-class for each farming model. In each case-farm a one hectare experimental area has been chosen for field data collection. The selection criteria of the experimental areas have been: a) presence of coffee plants producing for at least three years and b) a maximum slope of 20%.

The available, common and relevant assets for each category have been defined using interviews with experts and reviews of the literature. The existing transforming structures have been determined by the analysis of the global coffee supply chain actors using various expertises. The transforming processes relevant for the sustainability of the coffee farming have been analyzed through the literature review. In each selected farm-case questionnaires have been submitted and one-to-one interviews have been conducted with the farm owners and farm managers at the end of the coffee season 2009/2010. The questionnaire has been directed to understand the farmer's capital assets as well as the transforming structures and processes each coffee farming business is exposed to. The vulnerability context has been structured through the PESTEL analysis using experts' interviews and latest news media review. The coffee farming strategies have been defined by the degree of farming intensity: crop specialization or diversification, shading intensity, planting density as well as chemical and technological input use. The coffee farming outcomes have been analyzed through a set of twelve specifically designed strategic indicators: 4 environmental indicators, 4 economic indicators and 4 social indicators. The 12 strategic indicators have been identified from selected principles and criteria following the MESMIS 6 steps framework (Lopez-Ridaura *et al.*, 2002; Van Cauwenbergh *et al.*, 2007). The indicators have been structured as multi-dimensional index, named AGROECO*SPA (Agro-Ecosystem Sustainability Performance Assessment), to measure and visualize the sustainability performance.

RESULTS AND DISCUSSION

The Sustainable Coffee Farming Framework – SCFF – is shown in Figure 2.

The global vulnerability context of the coffee production has been analyzed taking into consideration the most important contextual features. In Table 1 the relevant vulnerability context issues are reported.

The available coffee farmer assets, which are also common to all coffee growers around the world, are listed in Table 2.

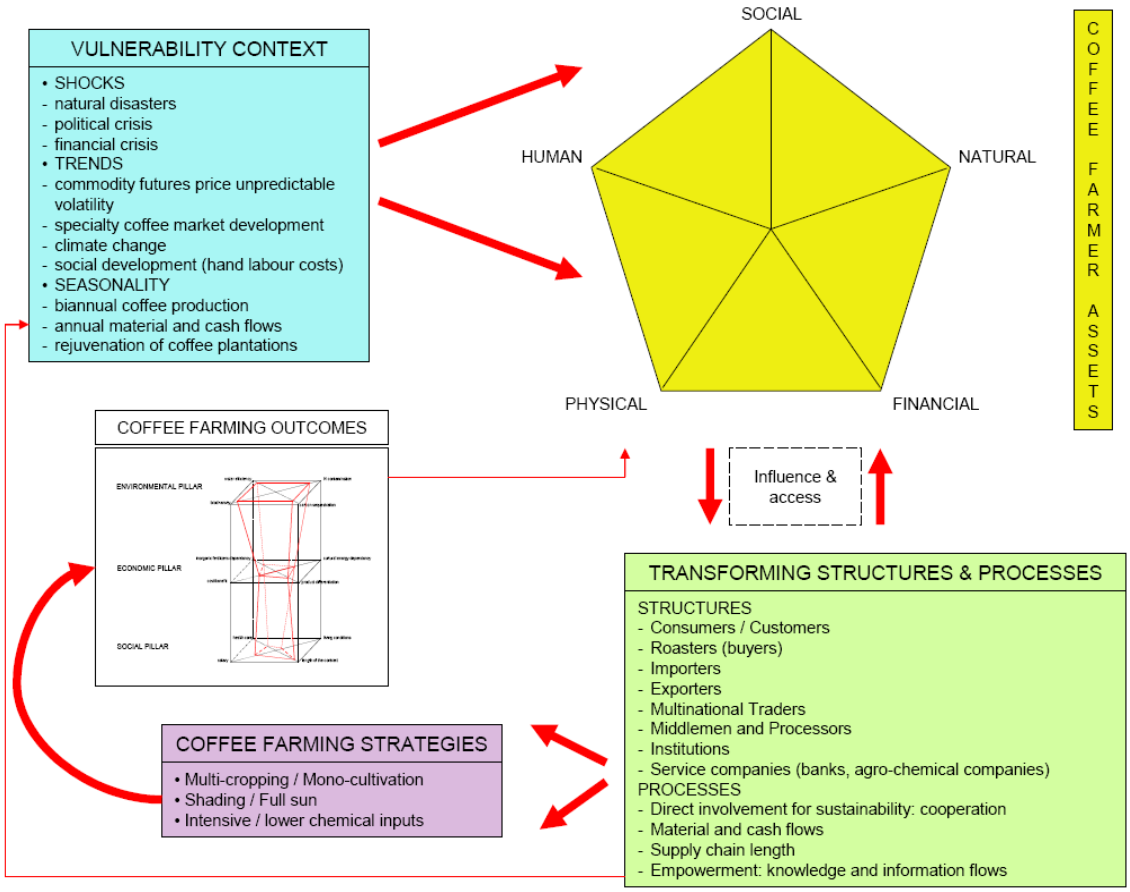

Figure 2. Sustainable Coffee Farming Framework (SCFF).

Table 1. PESTEL analysis of the relevant vulnerability context issues.

Factors	***SHOCK***	***TREND***	***SEASONALITY***
Political	Political crisis	No relevant issues	No relevant issues
Economic	Financial crisis	Commodity futures price volatility Specialty coffee market development	Annual material and cash flows
Social	No relevant issues	Social develop. / generational change	No relevant issues
Technological	No relevant issues	Agricultural technology / crop shift	Biannual production
Environmental	Natural disasters	Climate change	No relevant issues
Legal	Legislation change	No relevant issues	No relevant issues

Table 2. List of available and common coffee farmer capital assets.

Social	***Natural***	***Financial***	***Physical***	***Human***
Efficiency of national/regional coffee organization	Water access	Credit availability	Farm roads and vehicles	Green culture
Membership to cooperative/association	Soil fertility	Savings level	Energy availability	Knowledge of sustainable farming
Contacts with other coffee growers	Land adaptability	Sales time	Equipments (machineries and milling)	Availability of professional labour
Direct contacts with exporters/roasters		Income differentiation	Warehouse	Availability of hand labour
		Hedging tools	Communication	

The transforming structures are all the possible existing coffee supply chain actors. The transforming processes can exert negative or positive pressures on the coffee farmers. The relevant processes influencing the sustainability performance of coffee farming are delineated and described in the above Table 3. The interactions among the vulnerability context, the capital assets and the transforming processes are very important and complex parts of the framework. In order to comprehend the interactions, these are resumed and explained in Tables 4-5.

The sustainability of the transforming processes can heavily influence the sustainability of farming strategies. Strategies are oriented by the farmers mainly to satisfy the economic viability and to attend the social standards required by the national labour law, whereas only some of them tend to satisfy a virtuous environmental protection too. This can lead to more marked differences among farms in the environmental system respect to the economic system. This does not mean that there cannot be evident differences for the economic or social outcomes among different-strategy farms. Nevertheless, this might be caused more by lacking assets, negative processes, unintentional wrong strategies, side effects or by a combination of these. There is an asymmetry between the environmental system and the economy (Málovics et al., 2008). The suitability of the chosen farming strategy can be assessed with the measurement of the coffee farming outcomes. As mentioned before, the sustainability level of the outcomes can be measured through key sustainability performance indicators.

The results of the framework tests, obtained with the three cases during the year 2009, are given in the paragraphs below.

High farming intensity: the Brazilian case

The pentagon in Figure 3 is the result of the analysis of the Brazilian farmer's capital assets.

Table 3. List of relevant and influencing transforming processes for coffee farming.

PROCESS	*Literature references*	*DESCRIPTION*
Cooperation	Beherens et al., 2006	Cooperation means direct involvement. The dedication for sustainability and the monitoring of the sustainable coffee supply chain are fundamental for sustainable resources exchange
Material and Cash flows	Hinterberger et al., 1997 Hutchins and Sutherland, 2008	These are the main tangible flows along the supply chain. Coffee is a renewable resource that must be used according to its regeneration rate. To generate a cash return, the farmer must use not only renewable resources, but also the non-renewable resources and the assimilative capacity of the ecosphere. The resources are called natural capital. The devaluation (or depreciation) of natural capital is incompatible with non-declining well-being.
Knowledge and Information flows	Gereffi, 1999 Bitzer et al., 2008 Seuring and Müller, 2008	These are very important intangible flows. Participation in global commodity chains is a necessary step for industrial upgrading. Knowledge can become a powerful competitive weapon. Vertical partnerships present in the coffee supply chain aim at promoting sustainable production, building producer capacity, stabilizing producer environment and creating market access. Partnerships based on knowledge and information flows can actually empower other chain actors next to the lead firms (or focal companies). Nevertheless, most of the existing partnerships have used the word “empower” in the meaning of “enable”. The producer empowerment can be reached through deep producer market standards knowledge and the ability to meet them spontaneously.
Supply chain length	Vachon and Mao Z, 2008	It represents the distance between the customers (markets) and the suppliers. The proximity between these two actors is a characteristic of supply chain strength. Sustainability improves as supply chain strength increases and therefore as supply chain becomes shorter.

Table 4. Relationships between the transforming processes and the capital assets.

PROCESS	***Interaction with the capital assets***
Cooperation	Positive correlation with the human, social and financial assets. The human assets are improved as a consequence of a better monitoring of sustainable coffee farming. The social assets are stimulated by the possible vertical and horizontal contacts resulting from the direct involvement. The financial assets are improved as a consequence of a better cost analysis.
Material and Cash flows	Positive correlation with the financial and natural assets. The natural assets are positively correlated with a cash flow rewarding the real value of the materials used for production. The financial assets are improved as a consequence of a long term sustainable financial balance.
Knowledge and Information flows	A clearly positive correlation with the human and social assets.
Supply chain length	Positive correlation with the financial (less dispersed value) and social assets (stronger networking).

Table 5. Relationships between the transforming processes and the vulnerability context.

PROCESS	***Interaction with the vulnerability context***
Cooperation	It reduces the risk and impact of financial crisis, market price volatility, climate change and social growth. It facilitates the specialty sector participation. It is positively correlated with biannual production and plant rejuvenation.
Material and Cash flows	They reduce the impact and the risk of a financial crisis.
Knowledge and Information flows	They have a beneficial effect on all the trends and seasonality.
Supply chain length	It allows to catch the opportunity of a growing specialty coffee market and to reduce the negative impact of the gaps between the annual material and cash flows.

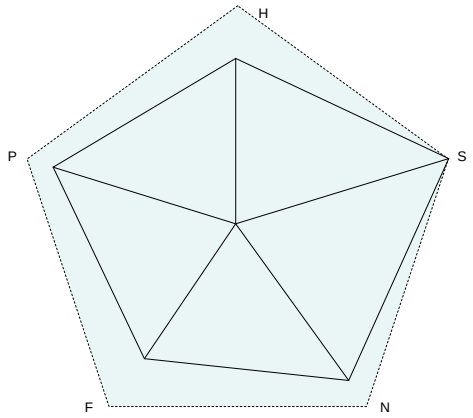

Figure 3. The Pentagon Capital Assets of the Brazilian Coffee Farmer.

In Table 6 the results of the analysis are given.

Table 6. Analysis of the transforming processes: Brazilian case-study.

Cooperation	Supply chains 1 and 3 involve the farmer into training programmes and stimulate him towards sustainability. Supply chain 2 is not working in cooperation with the farmer nor is it attempting to stimulate the sustainability of coffee farming. 70% of the coffee is sold by the farmer in structures promoting human, social and financial capitals.
Material and Cash flows	Supply Chain 1 is directly stimulating the environmental management improvement and the economic viability with a higher and stable price for better natural resources exploitation. Supply Chain 3 is compensating the natural resources use with a competitive and long term price, stimulating the economic viability but not the environmental management. Supply Chain 2 is not compensating the resource exploitation and is not stimulating a better environmental management nor the economic viability of coffee farming. Supply Chain 3 is also improving the market volatility. 70% of the coffee is sold by the farmer in structures promoting the financial asset. 40% of the coffee is sold by the farmer in a structure stimulating a better environmental management. 30% of the coffee is promoting the market volatility tend.
Knowledge and Information flows	Information is practiced by all the supply chains. Knowledge is spread only by Supply Chain 3.
Supply chain length	Supply chain 1 and 3 have 3 actors after the farmer and before the consumer. Supply chain 2 has got 4 actors before the consumer.

The centre of the framework is the analysis of the farmer's assets and their interactions with the other components of the framework (Figure 4): vulnerability context and transforming processes. The financial asset is the weakest. The external environment is not stimulating the substantial reduction of the financial asset in the long-term. Moreover, the farmer owns the key financial assets (hedging tools and specialty sector participation) to contrast the negative pressure of the external environment and to benefit from the positive pressure. Nevertheless, the farmer is concentrated to continuously foster the financial asset mainly by altering two other assets: the human (reducing labour) and the natural assets (exploring natural resources). Some of the transforming structures are working to improve the knowledge and the information along the supply chain (human capital). Only 40% of the product is sold directly under the influence of an environmental code; therefore, protecting the natural asset from a possible abuse. None of the supply chains is working towards the promotion of an environmental culture change.

The Brazilian coffee farmer's strategy is the consequences of the objective of improving and preserving mainly the financial capital. The risk that arises from this analysis is a possible natural and human resources decay due to their unsustainable management. This is a consequence of the scarce importance given by the farmer to preserving the human and natural assets.

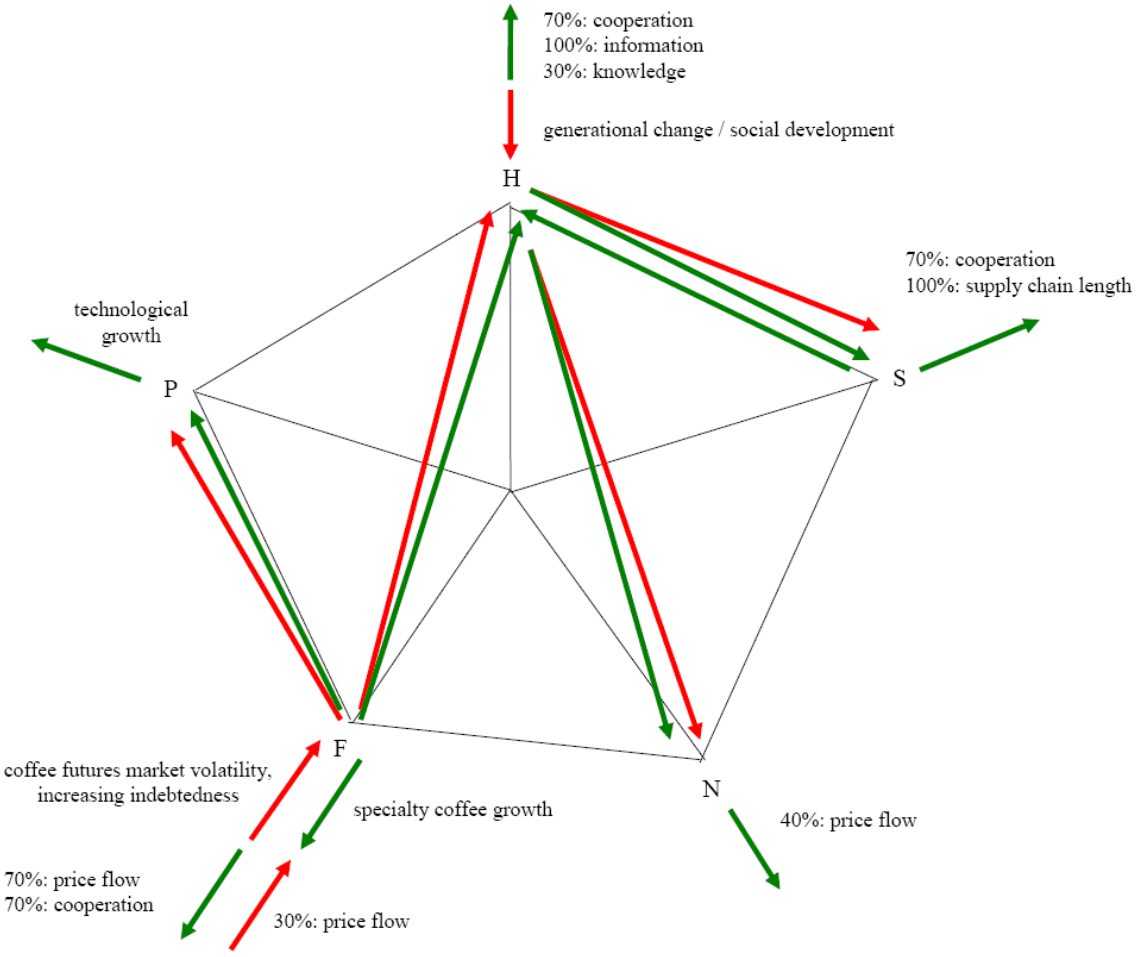

Figure 4. The Brazilian farmer's assets and their interactions.

Medium farming intensity: the Guatemalan case

Figure 5 shows the pentagon of the Guatemalan farmer's capital assets.

In Table 7 the results of the analysis of the transforming structures are given.

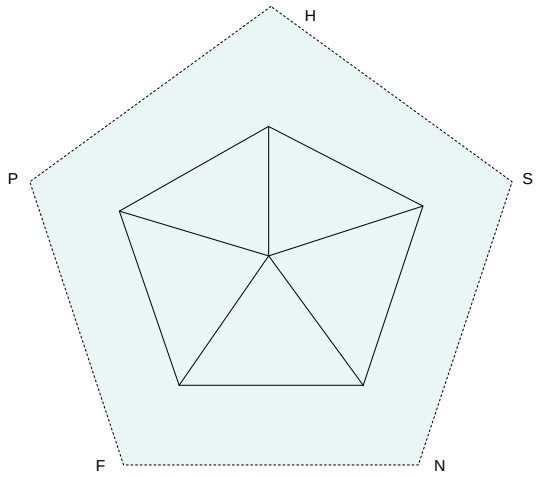

Figure 5. The Pentagon Capital Assets of the Guatemalan Coffee Farmer.

Table 7. Analysis of the transforming processes: Guatemalan case study.

Cooperation	Supply chain 1 stimulates the farmer's knowledge. Supply chain 3 is cooperating with the farmer to stimulate the sustainability of coffee farming. Supply chains 2 and 4 are not involved into improving the sustainability of the coffee farming. 60% of the coffee is sold by the farmer in structures promoting human and social capitals.
Material and Cash flows	Supply Chain 3 compensates the natural resources use with a competitive and long term stable price, stimulating the economic viability but not the environmental management. Supply Chain 1, 2 and 4 are not compensating with the price stability the right resource exploitation in the long term and are not stimulating a better environmental management or the economic viability of coffee farming. Supply Chain 2 is also improving the market volatility (trend). 30% of the coffee is sold by the farmer in structures promoting the financial asset. 30% of the coffee sales promote the market volatility.
Knowledge and Information flows	Information is practiced by supply chains 1, 2 and 3. Knowledge is spread only by the National Coffee Association, not by the supply chains.
Supply chain length	Supply chains 1 and 3 have 3 actors after the farmer and before the consumer. Supply chains 2 and 4 have 4 actors after the farmer and before the consumer.

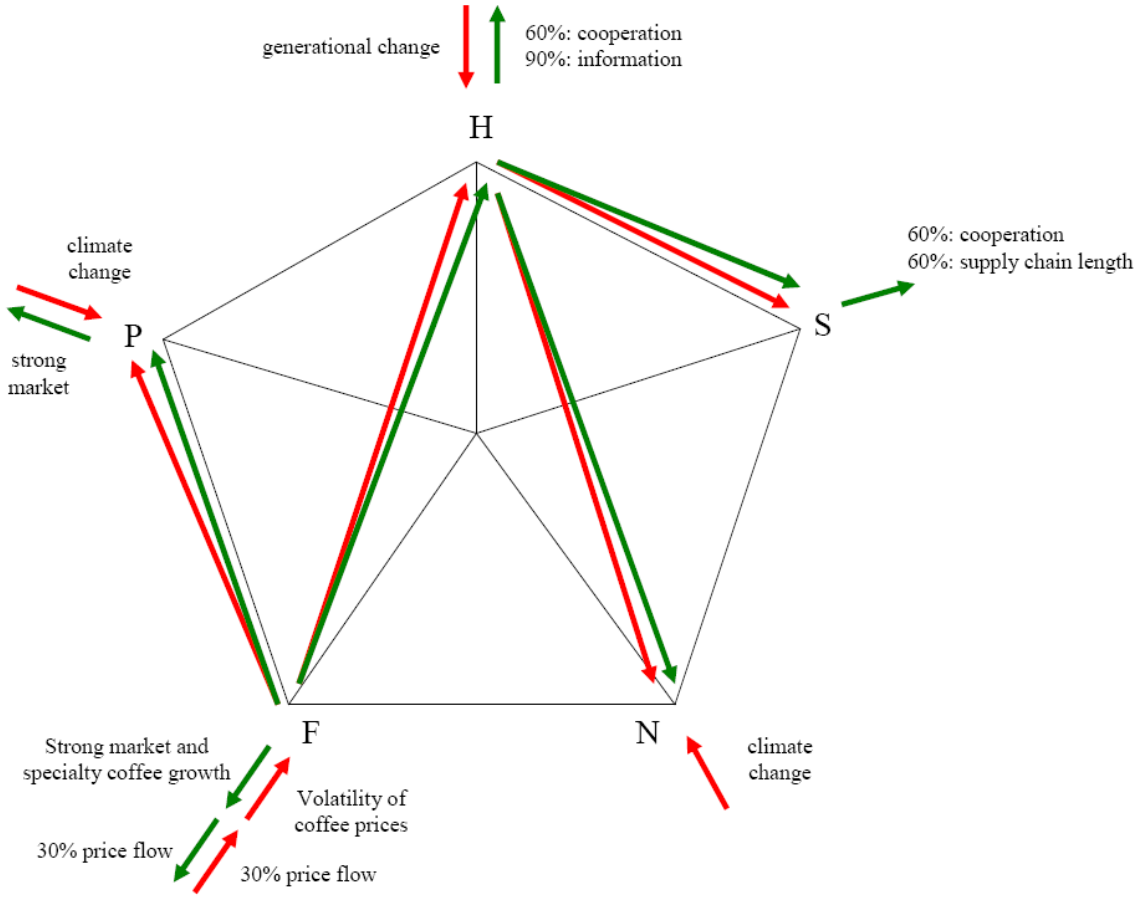

Figure 6. The Guatemalan farmer's assets and their interactions.

Figure 6 illustrates the analysis of the farmer's assets and their interactions with the other components of the framework. The Guatemalan coffee farmer does not have markedly stronger assets. The vulnerability context is not significantly influencing the financial asset. Indeed, the volatility of coffee prices is well balanced by a growth in the specialty coffee sector and by a temporary strong market. Nevertheless, the climate change could reduce the natural and physical assets. The farmer does have a particular key asset to contrast the negative pressure of climate change, the green culture asset. The farmer has chosen to shade the coffee trees and this is a good climate change mitigation strategy. The difficult generational change poses a threat on the human assets. The transforming processes are just slightly enriching the human and social assets through cooperation and information sharing. Nevertheless, none of the supply chain's transforming structures is investing in knowledge.

The Guatemalan coffee farmer's strategy is the consequences of a mix of green culture and orientation to maximise the financial capital. The risk that arises from this analysis is that the difficult generational change could reduce the key asset of green culture in the future, therefore reducing the resilience to climate change effects.

Medium-low farming intensity: the Indian case

The Indian farmer's capital assets are represented in Figure 7

Table 8 resumes the analysis of the transforming structures.

The negative pressure for the increasing hand labour scarcity is eroding the farmer's human assets. These assets are not resilient to this negative pressure. The pressed human assets are not supported by strong social assets. The financial assets are under pressure for the increasing commodity market volatility and for the noted trend of shifting from Arabica coffee to Robusta coffee cultivation, posing the farmer even more exposed to the price volatility. Represented in Figure 8 is the analysis of the farmer's assets and their interactions with the other components of the framework: vulnerability context and transforming processes.

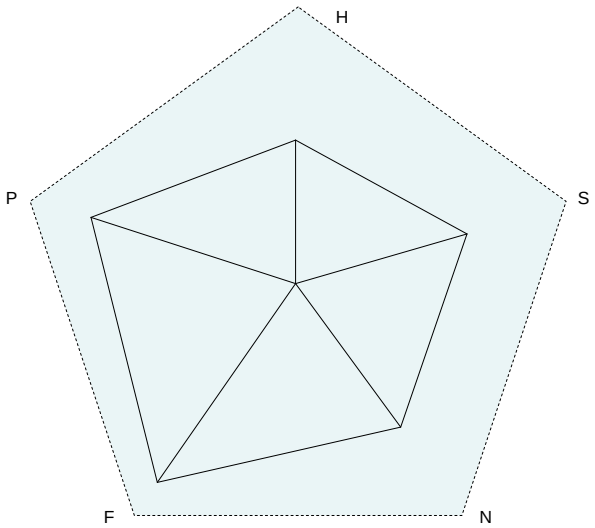

Figure 7. The Pentagon Capital Assets of the Indian Coffee Farmer.

Table 8. Analysis of the transforming processes: Indian case study.

Cooperation	Supply chains 1 and 3 involve the farmer into training programmes to stimulate him towards sustainability. Supply chain 3 is also working directly in cooperation with the farmer attempting to stimulate the sustainability of coffee farming. Supply chain 2 does not stimulate the sustainability of the coffee farming. 50% of the coffee is sold by the farmer in structures promoting human and social capitals.
Material and Cash flows	None of the supply chains is compensating the natural resources use with a competitive and long term stable price. Supply Chain 3 is indeed compensating with the price stability the economic viability of coffee farming. Supply Chain 2 is also improving the market volatility. 30% of the coffee is sold by the farmer in structures promoting the financial asset. 50% of the coffee is promoting the market volatility tend
Knowledge and Information flows	Information is practiced by supply chains 1, 2 and 3. Knowledge is spread only by Supply Chain 3.
Supply chain length	Supply chains 1 and 3 have 3 actors after the farmer and before the consumer. Supply chain 2 has 4 actors after the farmer and before the consumer.

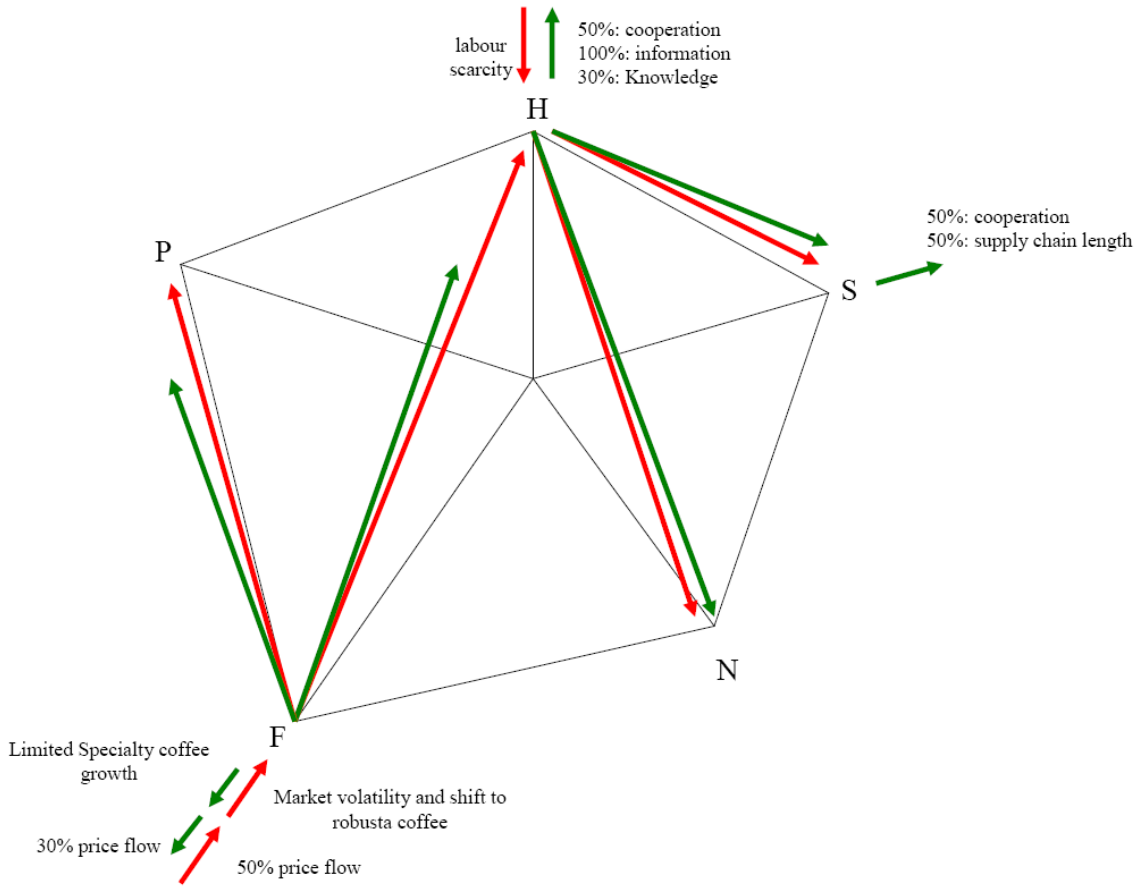

Figure 8. The Indian farmer's assets and their interactions.

The Indian coffee farming strategy is a direct consequence of the human assets of green culture and product differentiation. This has positively contributed up to now to strengthening the financial assets. Nevertheless, a marked and continuous negative pressure on the financial assets could stimulate the reduction of the human and physical assets too. In the long term we could also assist to an increasing risk of erosion of the natural assets, which are not positively stimulated by the vulnerability context and the transforming processes.

CONCLUSION

In conclusion, the Sustainable Coffee Farming Framework has proven to be a useful sustainable coffee supply chain management tool. The analysis of the three case studies has helped to discover the most important risk areas for a sustainable development of the coffee farming business. This is the starting point to select the high-priority areas of investment for improving the sustainability performance of coffee farming. In Brazil, it has been found that the risk of eroding the human and the natural assets poses a threat on the sustainability of the coffee farming. It is therefore recommendable to invest in improving the human assets directly or indirectly through the social assets. The focus should be the promotion of a more advanced and progressive environmental and social culture among the younger generations too. In Guatemala the phenomenon of the difficult generational change is not supported by investments to stimulate the younger generations to take over the family coffee business and adapt it to the changing context in a sustainable way. This is possible taking advantage of all the opportunities given by the developed specialty coffee market. Younger generations and businesses development are therefore the top priority targets of investment in Guatemala.

Finally, in India, it is recommendable to invest in helping the human and social capitals, particularly directing the investments to improving the hand labour availability, attracting it to the coffee estates. At the same time, it would be recommendable to invest into developing the physical assets, particularly into finding solutions for possible temporary hand labour shortages. It would also be positive to reduce the pressure over the financial assets by improving the market for specialty coffees in India.

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Performance of *Coffea arabica* F1 Hybrids in Comparison with American Pure Line Varieties

B. BERTRAND[1*], E. ALPIZAR [2], L. LLARA[3], R. SANTACREO[4], M. HIDALGO[5], J.M. QUIJANO[6], P. CHARMETANT[1], C. MONTAGNON[1], F. GEORGET[1], H. ETIENNE[1]

[1]Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD). Département des Systèmes Biologiques, (UMR-RPB, CIRAD-IRD-UM2), 911 Avenue Agropolis, BP 64501, 34394 Montpellier, France.
*E-mail benoit.bertrand@cirad.fr
[2]Café Total, AP 129-4050, Alajuela, Costa Rica
[3]ECOM Coffee Group – Nicaragua. Rotonda Jean Paul Geani, 300 m abajo. Edificio San Marino, Managua, Nicaragua
[4]Instituto Hondureño del Café (IHCAFE), AP 3174, Tegucigalpa, Honduras
[5]Instituto del Café de Costa Rica (ICAFE), AP 37-1000, San José, Costa Rica
[6]Fundación Salvadoreña para la investigación del Café (PROCAFE), AP 23, Santa Tecla, El Salvador

SUMMARY

Arabica F1 hybrids deriving from crosses between wild Sudan-Ethiopian and American varieties and propagated in somatic embryo form have been created in Central America. These new hybrids considerably increased the genetic diversity of coffee in the region. Based on 15 trials we assessed whether using hybrids constitutes substantial genetic progress in productivity terms in agroforestry and full-sun systems. The new germplasm was grown in the same conditions as the best American varieties (homozygous pure lines). Results showed the yield of hybrids was superior to the yield of American varieties and displayed better earliness. Also, the hybrids displayed better stability over environments than did the American varieties. In the agroforestry system, a comparison of the mean yields revealed that hybrids yielded 58% more than American varieties. In the full-sun system, the yield increase was 34%. Introducing hybrids in the coffee-based agroforestry systems would considerably increase productivity. The conditions for a large-scale dissemination of those new hybrid varieties, that represent a major innovation for the Arabica culture, was analysed.

INTRODUCTION

Arabica coffee is the main source of export earnings for numerous countries. Production is dominated by Latin America, which accounts for 80% of the world volume of Arabica exports. In Central America, coffee was traditionally grown in agroforestry system (AS) under forest trees or planted trees. Through several concurrent technical advances in the 1950s and 1960s, a switch was made to an unshaded intensive system with abundant use of fertilizers (notably 150 to 300 units of N/hectare) and pesticides. However, in many countries (Andean America, Honduras, El Salvador, Nicaragua, Guatemala and the Chiapas region of Mexico), the majority of the producers have not abandoned the agroforestry systems (Figure 1).

A **B**

Figure 1. Illustration of the *C. arabica* network trials. A/ full-sun system, B/ Agroforestry system.

The *Coffea arabica* is an autogamous species. Producers in Latin America currently have the choice between two types of homozygous lines propagated by seed: American traditional varieties (Bourbon, Typica, Caturra, Catuai) and varieties 'Catimors'. These latter recently distributed are varieties derived from the Hybrid of Timor, which comes from a natural cross between *C. arabica* (4n=2X=44) and *C. canephora* (2n=2X=22) (Bettencourt, 1973; Moreno, 1989). In the text we'll refer to this material propagated by seeds as American varieties or lines.

Some F1 hybrids clones propagated in *in vitro* conditions through somatic embryogenesis have been available to producers since 1997 throughout Central America (Etienne-Barry et al., 1999; Etienne 2005). These new hybrids deriving from crosses between wild Sudan-Ethiopian and American traditional varieties or 'Catimors', considerably increased the narrow genetic base characterizing cultivated coffee trees in Latin America. We refer to this material as 'hybrids', keeping in mind their inter-origin genetic background. In controlled trials conducted in Costa Rica and Nicaragua in three environments, the heterosis found in hybrid populations relative to parental performance was 20% to 50% (Bertrand et al., 2005). In sensory evaluations comparing the hybrids with traditional lines under various edapho-climatic conditions, the hybrids appeared equivalent to or better than traditional lines (Bertrand et al., 2006).

The aim of the present work is to test if introducing new coffee hybrid varieties in the coffee-based AS of Central America would increase cropping system productivity and therefore contribute to the profitability of the Agroforestry systems. We analyzed the productivity performance of these new hybrids in Agroforestry (shade) and unshaded (full-sun) cropping systems in comparison with the best American pure line varieties distributed in Central America.

MATERIALS AND METHODS

Plant material

Two types of varieties were tested in the network:

- Thirteen lines (i.e; American varieties), namely 'Caturra', 'Pacas', 'Catuai', and 'Bourbon' as traditional varieties and 'CR95' (also called 'Lempira'), 'Catisic', 'Iapar59', T5296, T17931, T17933, T18121, T18138, and T18141 as 'Catimors'.

- Twenty-one *C. arabica* F1 hybrids: crosses of American varieties with Ethiopian origins (‘ET6’, ‘ET15’, ET25, ‘E41’, ‘E416’, ‘E531’, ‘Anfilo’, ‘Rume Sudan’,).

The hybrids were obtained through micropropagation using a somatic embryogenesis procedure (see Figure 2) previously described (Etienne, 2005).

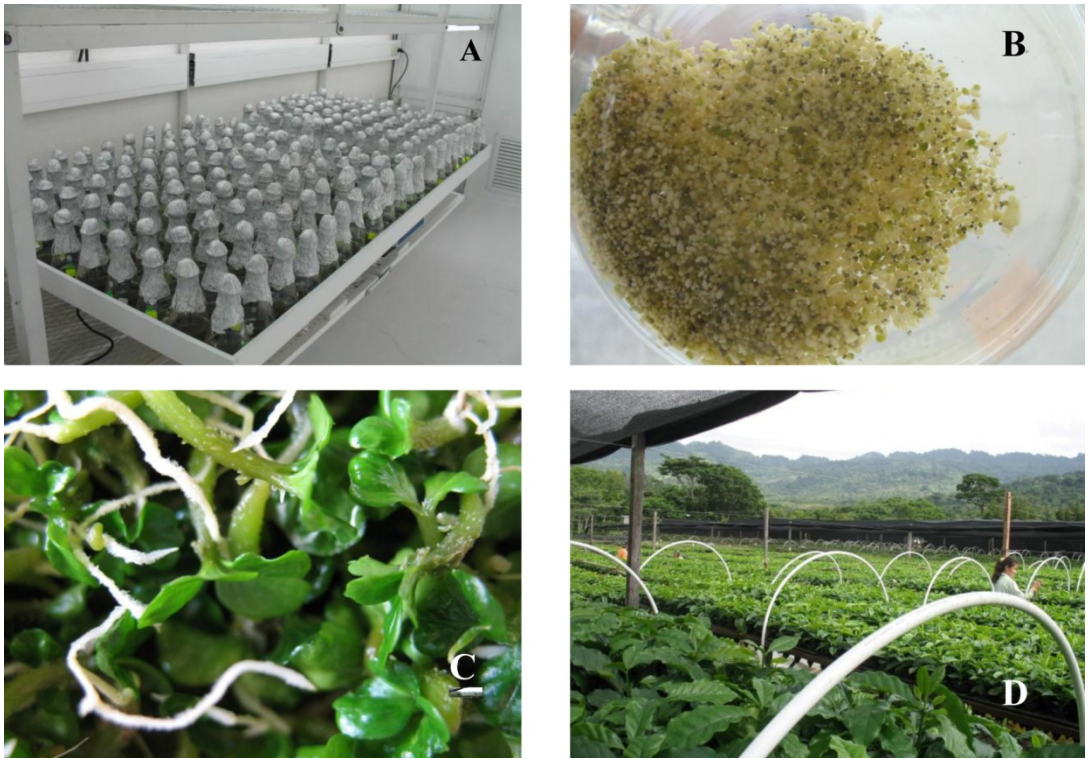

Figure 2. Illustration of the coffee micropropagation process trough somatic embryogenesis in industrial conditions (Nicaragua, CIRAD-ECOM project). A and B/ Multiplication of embryogenic suspensions in Erlenmeyer flasks; C/ Mass regeneration in temporary immersion bioreactors of directly acclimatizable pre-germinated somatic embryos; D/ Plant hardening in nursery before field transfer.

Field trials

Observations were carried out from 2000 to 2006 in a network of 15 trials (i.e. locations) established in 1999, 2000, and 2001 in three countries of Central America. A randomized block designs was used in each trial. Inside each block, the varieties were set out in unit plots of ten trees. The main characteristics of the trials and the replication of the genotypes in the 15 trials are summarized in Table 1 and Table 2, respectively.

Table 1. Characteristics of the trials. Elevation (m.a.s.l), planting year; country; region; T = planting density (number of trees/ha); number of varieties tested per type, with TC= traditional cultivars, LdHdT = lines derived from the Hybrid of Timor, F1 = clones of F1 hybrids; Cropping system and shading (%) with FS= full-sun, AS= Agroforestry system; Type of soils (FAO, classification 1979); Taver, mean of average daily temperatures (°C); R, annual rainfall (mm.y^{-1}); Nprod =Number of production years observed.

Elevation (m.a.s.l)	Planting Year	Country	Region	T (trees/ha)	Number of varieties tested per type	Cropping system shading	Soils (FAO, 1979)	R (mm.y^{-1})	Taver (°C)	Nprod
750	2001	Honduras	La Fé	4000	TC=1, F1 =12, LHdT=1	AS, 40%	Luvisols	3300	25	4
800	2000	Costa Rica	Palmira	5850	TC=1, F1 =10, LHdT=1	AS, 20%	Inceptisol	4000	24	3
820	2000	El Salvador	San Antonio	4000	TC=1, F1 =6, LHdT=1	AS, 40%	Luvisols	1800	24	7
880	2001	El Salvador	San José	4000	TC=2, F1 =8	AS, 30%	Andisols	2300	24	6
1000	2000	Costa Rica	Coto Brus	5000	TC=1, F1 =8	AS, 20%	Andisols	3500	23	4
1060	2001	El Salvador	San Jorge	4000	TC=1, F1 =8	AS, 40%	Nitisols	1900	22	6
1100	1999	Honduras	Linderos	4000	TC=1, F1 =7, LHdT=1	AS, 50%	Luvisols	2500	22	5
1180	1999	Costa Rica	Barva de Heredia	5000	TC=2, F1 =5, LHdT=7	FS, 0%	Andisol	2370	20.5	7
1185	2000	Costa Rica	Barva de Heredia	5000	TC=2, F1 =13	FS, 0%	Andisol	2370	20.5	4
1260	2000	El Salvador	Usulután, Los Pirineos	4000	TC=1, F1 =6, LHdT=1	AS, 50%	Nitisols	2100	20	4
1340	2000	El Salvador	Ahuachapán, El Milenio	4000	TC=2, F1 =4	AS, 30%	Luvisols	1850	20	7
1400	2001	Costa Rica	Tarrazu	5000	TC=2, F1 =12	FS, 0%	Ultisol	2200	21	5
1420	1999	Honduras	Marcala	4000	TC=1, F1 =7, LHdT=1	AS, 30%	Luvisols	1800	20	5
1425	1999	Costa Rica	Sabanilla de Alajuela	5000	TC=2, F1 =1, LHdT=7	FS, 0%	Andisols	2100	20	6
1580	1999	Costa Rica	Santa María de Dota	5000	TC=2, F1 =2, LHdT=7	FS, 0%	Ultisol	2400	21	7

Table 2. Repartition of the varieties (hybrids and lines) in the *C. arabica* multisite trial. Type of variety, Cultivars, Number of the trial (1-15) and elevation (750- 1580) in m.a.s.l. X= presence in the trial.

		Trial number and elevation in m.a.s.l														
Type of variety	**Cultivars**	**1 (750)**	**2 (800)**	**3 (820)**	**4 (880)**	**5 (1000)**	**6 (1060)**	**7 (1100)**	**8 (1180)**	**9 (1185)**	**10 (1260)**	**11 (1340)**	**12 (1400)**	**13 (1420)**	**14 (1425)**	**15 (1580)**
Hybrid	Centroamérica		X	X	X		X	X	X				X	X		X
Hybrid	L02A11		X			X					X	X	X			
Hybrid	L02A30		X	X	X	X				X	X	X	X			
Hybrid	L03A07		X			X	X			X			X			
Hybrid	L03A15		X			X	X			X			X			
Hybrid	L03A17		X		X	X				X	X	X	X			
Hybrid	L04A05						X									
Hybrid	L04A05						X			X			X			
Hybrid	L04A20	X					X	X		X			X	X		
Hybrid	L04A34	X						X	X					X	X	
Hybrid	L04A42	X			X			X		X				X		
Hybrid	L05A26	X	X	X	X	X	X	X	X				X	X		
Hybrid	L05A27						X			X						
Hybrid	L09A22									X			X			
Hybrid	L10A25						X			X						
Hybrid	L11A26	X					X	X		X			X	X		
Hybrid	L12A05								X							
Hybrid	L13A12		X	X		X				X						
Hybrid	L13A22		X	X	X	X					X	X				
Hybrid	L14A08								X							X
Hybrid	L22A08		X	X	X					X			X			
Line	Catisic			X												
Line	Catuai		X		X	X			X	X			X		X	X
Line	Caturra			X	X		X		X	X	X	X	X		X	X
Line	CR95	X	X					X	X					X	X	X
Line	IAPAR59								X						X	X
Line	T17931								X						X	X
Line	T17933								X						X	X
Line	T18121								X						X	X
Line	T18138								X						X	X
Line	T18141								X						X	X
Line	T5296	X														
Line	Tekisic		X		X		X				X	X				

Table 3. Comparison of F1 hybrids with lines in the 15 trials over the first production cycle before coppicing. For each trial, are indicated : the elevation (m.a.s.l), the mean annual production of green coffee (Y in g and expressed for one tree) for the mean of the F1 hybrids (*Yhybrids*) and for the mean of the lines (*Ylines*), D(%) that represents ($D = \frac{Yhybrids - Ylines}{Ylines} x100$) and PrY the associated probability to this difference, *Ehybrids* and *Elines* the earliness of yields respectively for the mean of the F1 hybrids and for the mean of the lines and PrE the associated probability to this difference, cvY%hybrids and cvY%lines the coefficients of variation for the harvests respectively for the mean of the F1 hybrids and for the mean of the lines and PrcvY% the associated probability to this difference. NS = not significant at P=0.05.

Elevation (m.a.s.l)	***Yhybrids* (g)**	***Ylines* (g)**	**D (%)**	**PrY**	***Ehybrids***	***Elines***	**PrE**	***cvY% hybrids***	***cvY% lines***	**Pr cvY%**
750	631	450	40	0.04	33.4	22.6	NS	44.6	52.6	NS
800	613	530	16	0.0315	22.3	16.0	0.01	48.4	61.8	0.05
820	250	152	64	0.0001	0.6	0.0	0.02	70.0	94.0	0.001
880	409	180	127	0.0001	2.0	1.5	NS	32.7	59.8	0.000
1000	737	472	56	0.0001	21.8	7.6	0.001	62.4	89.3	0.005
1060	412	372	11	0.0004	1.7	0.3	0.04	50.2	56.3	NS
1100	357	300	19	NS	20.0	9	NS	39.6	54.5	NS
1180	890	718	24	0.0001	1.9	0.0	0.001	52.9	51.6	NS
1185	486	328	48	0.0001	16.8	12.1	0.01	38.4	46.1	NS
1260	243	140	73	0.037	5.5	0.0	0.00	29.7	57.5	0.006
1340	323	146	121	0.0003	5.0	2.5	NS	50.5	73.3	0.0003
1400	621	318	95	0.001	16.5	10.5	0.001	56.1	86.6	0.0001
1420	602	380	58	0024	15.85	11.50	NS	42.4	46.2	NS
1425	855	794	8	0.049	11.0	10.5	NS	49.5	55.7	NS
1580	729	672	8	0.022	1.8	0.7	0.01	68.2	80.3	0.02

The trials were set up on farms of various sizes (5 to 150 ha). The elevation ranged from 750 to 1,580 m a.s.l. Unlike many multi-site trial networks, we considered that the comparison of a new germplasm with traditional varieties should not be accompanied by a change in producers' cultural practices. Consequently, we decided to leave plot managers "to their own devices" according to their experience and resources. In Costa Rica, the trials were mostly managed in an unshaded intensive system, apart from two low-altitude trials managed with slight shade from *Erythrina poeppigina* at 150 to 200 trees/ha. In El Salvador and Honduras, the trials were managed in AS with shade provided by *Inga edulis* (200 to 300 trees/ha), sometimes with a few tall native trees such as *Cordia alliodora*, *Juglans steyermarkii* and *Quercus costaricensis*. The shade levels varied between trials and within each plot depending on the season. They were estimated visually at between 20% and 50%. The planting density of coffee trees ranged between 4,000 and 5,800 trees ha^{-1}. In Costa Rica, the plants received [250-350], [200-300], [20-50] kg ha^{-1} $year^{-1}$ of N, K_2O, P_2O_5, respectively, along with 2 to 4 applications of copper hydroxide or triazole per year against leaf diseases (coffee leaf rust and brown eye spot). In Honduras and El Salvador, the plants received [50-100], [50-100], [0-20] kg ha^{-1} $year^{-1}$ of N, K_2O, P_2O_5, respectively, along with zero or two applications of copper hydroxide per year against leaf disease (coffee leaf rust).

Traits observed

The traits observed were average production over the first production cycle before pruning coppicing. The number of harvests during the first production cycle varied depending on the elevation above sea level, the degree of intensification, and the edapho-climatic conditions. Table 1 shows the number of production years observed for each trial. These harvests were evaluated in grams of fresh berries and then expressed in grams of green coffee per tree by considering that the weight of green coffee amounted to 20% of the fresh berry weight. To facilitate comparisons between trials, we calculated Y (yield), which was calculated as the ratio of the sum of the harvests (CUM) to the number of harvests. Y is expressed in gram of green coffee per tree and per year. Two other variables were calculated: the earliness variable (E), which represented the % of the first harvest (Y1) over CUM, and the coefficient of variation for the harvests (cvY%), calculated as the ratio between the standard deviation of CUM and Y.

Data analysis

The SAS System for Windows V9.1 was used for all statistical analyses. Within-location and multi-location analyses of variance were carried out over the 15 trials. For each trial we compared the lines to the hybrids by an ANOVA followed by a Newman and Keul's test at $P \leq 0.05$ for Y, E, and cvY%. A two-way ANOVA (referred as multi-location analysis) where trials (locations) and types of varieties (*i.e*. hybrids *vs* lines) were considered as fixed effects was performed to study the interactions between the type of variety and the locations. For this two-way ANOVA, Yield (Y), earliness (E) and production stability (cvY%) were studied by considering the. The hybrids' stability was compared to the lines' stability by a joint regression analysis on locations (Eberhart and Russel, 1966).

In a second analysis, we estimated Y according to the cropping system. We used a nested model as follows:

$$Y_{ijk} = \mu + v_i + S_k + B_{j|k} + a_l + v_i*a_l + E_{ijkl},$$

where Y= yield, μ was the overall mean, v_i represented the fixed effect of the type of variety i (*i.e* mean of hybrids *vs* means of lines), S_k represented the random effect of location S_k, $B_{j|k}$ was the random effect due to the jth block within location k, a_l represented the fixed effect of the cropping system (*i.e*. agroforestry system vs full-sunlight system), $v*a_{il}$ represented the random interaction between the type of variety i and cropping system l, and E_{ijkl} represented the random error associated with a particular observation.

RESULTS

Hybrids compared to lines

For each trial, means of lines and hybrids were compared through an analysis of variance focusing on yield, earliness, and production stability. Hybrids had a significantly greater yield than lines at 14 of the 15 trial sites (Table 3). The differences in yield ranged from 8% to 127% (Table 3). At low elevations (750 to 880 m.a.s.l), the differences were not uniform, with extremes ranging from 16% to 127% and a median of 52% for this group of 4 trials. At moderate elevation (1,000 to 1,340 m.a.s.l), which was the case for most of the coffee areas in Central America, the superiority of the mean of the hybrids was substantial: it ranged between 11% and 121%, with a median of 48%. Lastly, at high altitude (≥ 1,400 m.a.s.l), the differences in yield went from 8% to 95% with a median of 33%. The two-way ANOVA revealed a highly significant effect of location and of type of varieties, respectively (F= 69.7, P<0.0001 and F= 87.5 and P < 0.0001) and a low but significant interaction between the two (F=2.65, P <0.01). There thus was an interaction between type of varieties and locations, even though the mean square for the interaction (1.33) was smaller than that for the locations and the type of varieties (respectively 34.7 and 44.1). This significant interaction is due to the different rate of response of hybrids to lines at each site as showed in the Figure 3. The analysis of interaction by regression slope showed that mean of the hybrids displayed a non significant regression coefficient (regression coefficient R= -0.04 ± 0.02) and that the mean of the displayed a significant regression coefficient (R= -1.03 ± 0.09).

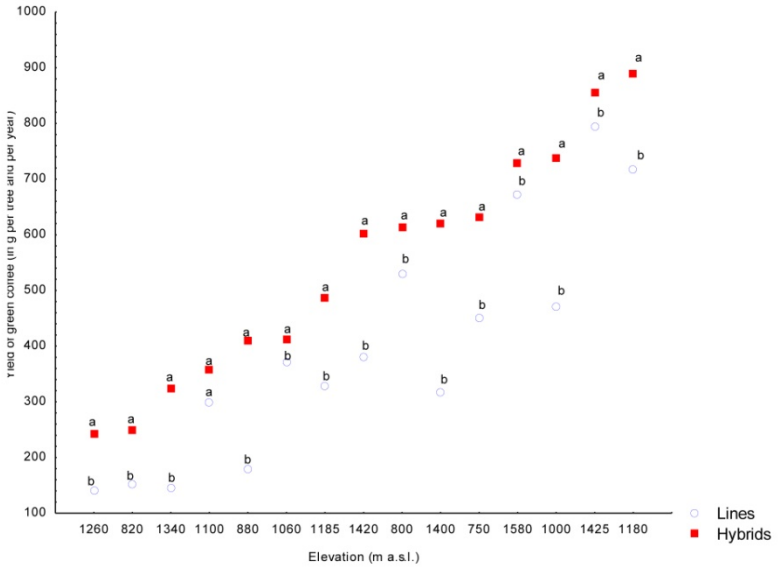

Figure 3. Comparison of the yield mean of the lines and yield mean of the hybrids in the 15 trials; (X axis: elevations in m.a.s.l, Y axis= Yield in green coffee). Two different letters indicated significant differences at P<0.05 between hybrids and lines at the same trial (i.e. elevation).

We measured earliness as an index representing the percentage of the first harvest (Y1) over the cumulation of harvests for the production cycle. The percentages were highly variable from one trial to the next, ranging from 0.6% to 33.4% for the mean of hybrids and from 0% to 22.6% for the mean of lines (Table 3). These differences were statistically significant in only 9 trials out of the 15 in the network. At first year production (data not shown), the mean of hybrids yielded significantly more than the mean yield of the lines at all the locations.

As regards production stability over the production cycle, the coefficients of variation (cvY%) for the mean of the hybrids ranged from 29% to 70% whereas those for the mean of the lines ranged from 46% to 94% (Table 3). The coefficients of variation for the hybrids were always lower than those for the lines. Nevertheless, the differences were significant at only 8 locations. Overall, it appeared that the mean of the hybrids were more stable than the mean of the lines, i.e. the production differences between one year and the next were less marked for the hybrids.

Comparison of hybrids and lines according to the cropping system

A mixed-effect analysis of variance was used to estimate the yield differences between types of varieties according to the two farming systems. The interaction between farming systems and type of variety was highly significant (F=77.4, P <0.0001). In the AS, the mean of hybrids produced 460 $g.y^{-1}$ of green coffee per tree as opposed to 290 $g.y^{-1}$ per tree for the mean of the lines (Figure 4). The difference in favor of the mean of the hybrids was highly significant (P<0.0001) and the increase in yield amounted to 58%. In the full-sun system, the mean of the hybrids gave yields estimated at 754 $g.y^{-1}$ as opposed to 562 $g.y^{-1}$ for the mean of the lines (Figure 4). The difference in favor of the hybrids amounted 34% and was highly significant.

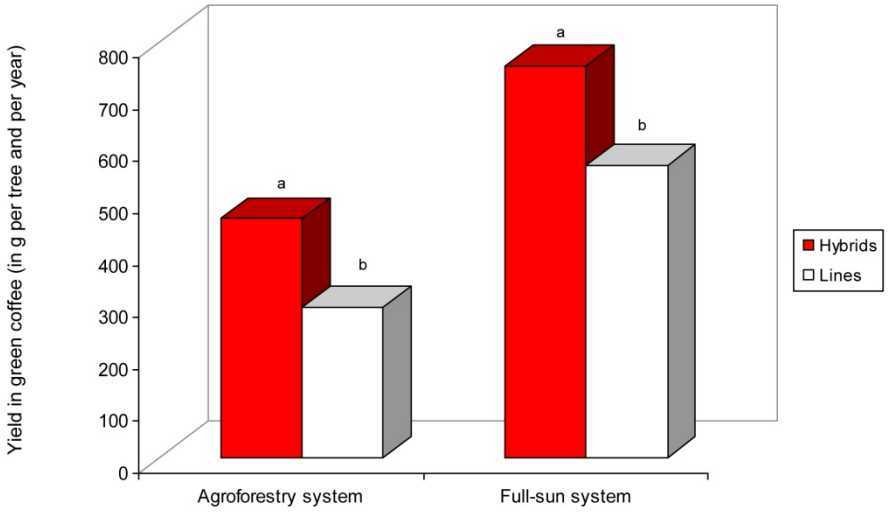

Figure 4. Comparison of the yield mean of the lines and yield mean of the hybrids according to the cropping system. Two different letters indicated significant differences at P<0.05 between the cropping systems.

DISCUSSION

The large-scale adoption of the Agroforestry systems depends on the economic benefits that they provided to producers in various socio-economic and ecological contexts. The present article shows that introducing new intraspecific hybrid varieties in the coffee-based AS of

Central America would considerably increase cropping system productivity, while considerably broadening the narrow genetic base of cultivated arabica by introducing genetic diversity from wild Ethiopian progenitors.

This network of trials tried also to represent the diversity of the cropping systems in Central America, since it consist of ten trials in agroforestry systems and five in full-sun systems. In the latter case, agronomic practices were relatively uniform, particularly fertilizer applications and pesticides treatments. On the other hand, the agroforestry systems ranged from very low-input agriculture to intensive coffee growing. As emphasized previously, we left plot managers "to their own devices" depending on their experience and their resources. The result was a network of trials with contrasting cropping systems and yields. For example, in El Salvador, the ‘San Antonio’ trial at 820 m a.s.l. represented a low-intensity coffee plantation in an AS where there was no use of agrochemichals, which probably explains the low yields. Conversely, at 1,420 m a.s.l., the Sabanilla de Alajuela trial was on a farm that practiced intensive coffee growing, which explains the high yields. The network of trials had variable production cycle lengths. That variability was linked to soil fertility, temperatures and farmer choices. In the hot, wet, low elevation climate at Palmira, after three years of high yields, the trees needed coppicing. In the temperate climate at Santa Maria de Dota (1,580 m a.s.l), coffee was produced during 7 years before coppicing. In the very low-intensity, hence low-yield AS at San Antonio or at Ahuachapán in El Salvador, it also took 7 years before the trees needed coppicing according to the producer's criteria.

The network set up therefore made it possible to compare varieties in contrasting situations representative of the edapho-climatic conditions and agricultural practices of Central America. Under these conditions differences in yields between the hybrids and lines ranged from 8% to 127%, with a median of 48 % in favor of hybrids. This first result tallies with those obtained in small controlled trials in Latin America and Africa by Walyaro (1983), and Cilas *et al*. (1998), where hybrids produced between 10% and 200% more than lines.

A few tendencies were also seen that confirmed previous observations. Firstly, hybrids start producing earlier, and they appear to be more stable over the production cycle and more stable across different environments. Part of the earliness can be explained by heterosis but also it may have been due to the propagation method used for these hybrids. Indeed, somatic embryogenesis causes some secondary effects yet to be elucidated and poorly measured but which seem to play a role in tissue rejuvenation (Hackett et al., 1993; Perrin et al., 1997). Working with the same genotype, we found that, for nursery coffee plants exhibiting the same height, plants derived from somatic embryos were significantly more vigorous than plants derived from seeds (Menéndez-Yuffa et al., 2010).

As regards stability, most authors agree that hybrids are more stable than lines (Lefort-Buson, 1986; Gallais 1990). In general, heterozygotes are more capable than homozygotes of exploiting an environment that is variable over time and in space. We found this homeostasis in the coffee hybrids, which led to lower yield variations over the cycle than did the lines. That greater homeostasis in the hybrids probably played an important role in yield stability. As we have already highlighted (Bertrand et al., 2006), hybrids with greater vegetative vigor provide higher leaf to fruit ratios and a better carbohydrate supply to berries than traditional varieties, which results in better quality beverage. We therefore conclude that heterosis conferred to the hybrids a true advantage over the lines in terms of productivity, but also in terms of yield stability. Moreover, this difference in productivity was not achieved by increasing inputs. The comparison of the mean of the hybrids and the mean of the lines according to cropping system showed that the yield increase in the agroforestry system

amounted to 58%. That increase, which seems considerable, amounts to 170 g of green coffee per tree. In the full-sun system, the relative increase was less (34%) but the difference in green coffee was 190 g. Consequently, the returns on investment with both systems are highly similar.

The success of large-scale dissemination of a hybrid variety depends on the control over the reproductive system. Somatic embryogenesis has been perfectly mastered technically in Nicaragua. The remaining question concerns economic risks associated with the adoption of this innovation. Investing in hybrids vitroplants is expensive. The additional cost per tree is currently 0.5 to 0.6 USD, i.e. an investment of 2500 to 3000 USD/ha. However, at this price, we believe that hybrid varieties would be more cost effective. A recent study of ECOM-INCAE (data not shown) showed that renovation with hybrid plants is better than renovation with traditional varieties in Agroforestry systems. After 6 years, the difference in net present value between hybrids and traditional varieties was found to be more than 5000 USD/ha.

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An Example of Successful Technology Transfer: *Coffea arabica* Propagation by Somatic Embryogenesis

F. GEORGET[1], B. BERTRAND[1], E. MALO[2], C. MONTAGNON[1], E. ALPIZAR[2], E. DECHAMP[1], I. JOURDAN[1], H. ETIENNE[1]*

[1] Centre de Coopération Internationale en Recherche Agronomique pour le Développement–Département des Systèmes Biologiques (CIRAD-BIOS).
UMR-RPB, 911 Avenue Agropolis, BP. 64501, 34394 Montpellier, France
[2] ECOM, Exportadora Atlantic S.A. ECOM-Nicaragua. Centro ejecutivo San Marino A-101, P.O. Box: 6011 Managua, Nicaragua. *E-mail: herve.etienne@cirad.fr

SUMMARY

Of all the possible micropropagation techniques, it is widely accepted that vegetative propagation by somatic embryogenesis is by far the most promising for rapid, large-scale dissemination of elite individuals. Yet, to date, examples of somatic embryogenesis processes applied on an industrial scale are very few and far between. There are many complications. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or are related to the quality of regenerated somatic embryos, the incidence of somaclonal variations and, more generally, a lack of reproducibility and efficiency at certain stages of the process, leading to production costs that prove prohibitive. Research on coffee somatic embryogenesis began at the end of the 1970s at various institutes, including CIRAD. Between 1995 and 2001, CIRAD moved the technique forward from a research laboratory scale to a technique enabling industrial dissemination of extremely promising *Coffea arabica* F1 hybrids. Over that period, two technological innovations made technology transfer economically feasible: mass production of somatic embryos in temporary immersion bioreactors and the possibility of sowing them directly in the nursery. At the same time, reassuring data were obtained on the genetic conformity of regenerated plants (somaclonal variation frequency < 3%). In 2002, in partnership with the ECOM group, CIRAD decided to transfer the somatic embryogenesis method on an industrial scale for the dissemination in Central America of four Arabica hybrid clones, selected for agroforestry-based farming systems. This article describes the different stages and the difficulties overcome up to successful technological transfer in 2010, and one of the very first examples of somatic embryogenesis technology applied on a commercial scale.

INTRODUCTION

Somatic embryogenesis, a long-awaited technology!

Somatic embryogenesis enables rapid and massive vegetative propagation of elite genotypes by doing away with lengthy and costly pedigree selection processes. Still, to date, examples of somatic embryogenesis processes applied on an industrial scale are few and far between. Nonetheless, a few recent examples can be mentioned, such as loblolly pine (*Pinus taeda*), cocoa tree, oil palm or coffee tree, for which annual production now exceeds one to several million plants. Yet, this vegetative propagation technique is widely accepted as being by far the most promising for capturing genetic gain as quickly as possible through rapid and large-scale dissemination of elite individuals. This is all the more true with woody species for

which biological cycles are long. In the 1980s, there was great enthusiasm for developing this technology and expectations were running high, which explains why research was undertaken on a very large number of species, without any immediate justification in some cases. There are many complications in developing this technology. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or they are related to the mediocre quality of regenerated somatic embryos, the incidence of somaclonal variations, and more generally a lack of reproducibility and efficiency at some stages, leading to production costs that prove prohibitive.

Quickest possible dissemination of genetic progress in the Arabica species

Research on coffee somatic embryogenesis began in the early 1980s at various institutes, including CIRAD, without any clear objective. At the beginning of the 1990s, CIRAD, in partnership with the Central American research network, PROMECAFE, set out to create *Coffea arabica* intraspecific F1 hybrids, by crossing the varieties traditionally grown in Latin America with wild individuals originating from Ethiopia and Kenya. The resulting hybrids proved to be extremely promising as they displayed a high level of heterosis, producing an average 40% more than the best cultivated varieties, with some of them producing coffee exhibiting better sensory qualities than those of the reference varieties (Bertrand et al., 2005). The co-breeders of these new varieties soon found the need for a somatic embryogenesis process capable of massively propagating Arabica F1 hybrid clones. However, moving from a technique developed in a research laboratory to an industrial process enabling the annual production of several million plants is a major leap forward. The co-breeders decided to go ahead and fund this first change of scale, which took place under CIRAD management at CATIE, a regional research centre in Costa Rica.

RESULTS

Situation prior to technological transfer (1995-1996): identification of points for improvement

Several limitations were identified that were an obstacle to technological transfer of the somatic embryogenesis process developed by CIRAD thus far, in its current state. First of all, production costs. With the development of a software package capable of estimating a range of production costs under different culturing conditions, the verdict was announced: 1.5-2 USD/plant, whereas a conventional seedling cost 0.25-0.35 USD! There was a further handicap, because the planting densities practised with Arabica in Latin America were between 6 and 8,000 trees/hectare given the dwarfism of the varieties used, making it possible to intensify production. The additional cost of planting *in vitro* plantlets needed to be limited, even though significant added value was expected with hybrid material. The software also proved very useful for precisely identifying the stages in the process responsible for the high cost of production; it involved some later stages, including germination and the development of weanable plantlets, i.e. possessing at least two pairs of leaves to withstand the shock of acclimatization to *ex vitro* conditions. This *in vitro* growth period was classically labour-intensive for subculturing and manufacturing nutrient media, but it also took up a great deal of space in the culture rooms. The second limitation was a risk that had so far been overlooked, namely that the somatic embryogenesis process would lead to a high frequency of somaclonal variations, “photocopy errors”, which are somewhat undesirable since “variant” plants do not display all the agricultural qualities of the selected "mother-plant". Somaclonal variations were a recurring problem in *in vitro* cultures, particularly with somatic embryogenesis systems, which used relatively high concentrations of auxins, such as 2,4-D

and IAA to induce the formation and multiplication of embryogenic cells. These growth regulators have often been shown to be implicated in the induction of somaclonal variations.

Technological innovations and reassuring information on genetic conformity (1996-2001)

Over this period, two technical innovations made technological transfer economically feasible: i) mass production of pregerminated somatic embryos in temporary immersion bioreactors (Albarran et al., 2005) and ii) the possibility of sowing them directly on horticultural substrate to achieve the regeneration of photoautotrophic plantlets in the nursery (Barry-Etienne et al., 1999; 2002; 2002). These two technological leaps made it possible to transfer most of the late stages (germination, embryo conversion into plants) from the laboratory to the nursery and thereby considerably reduce production costs. The cost price per plantlet was thus estimated at 0.5 USD. It was wagered that costs could be reduced further by moving on to industrial production conditions.

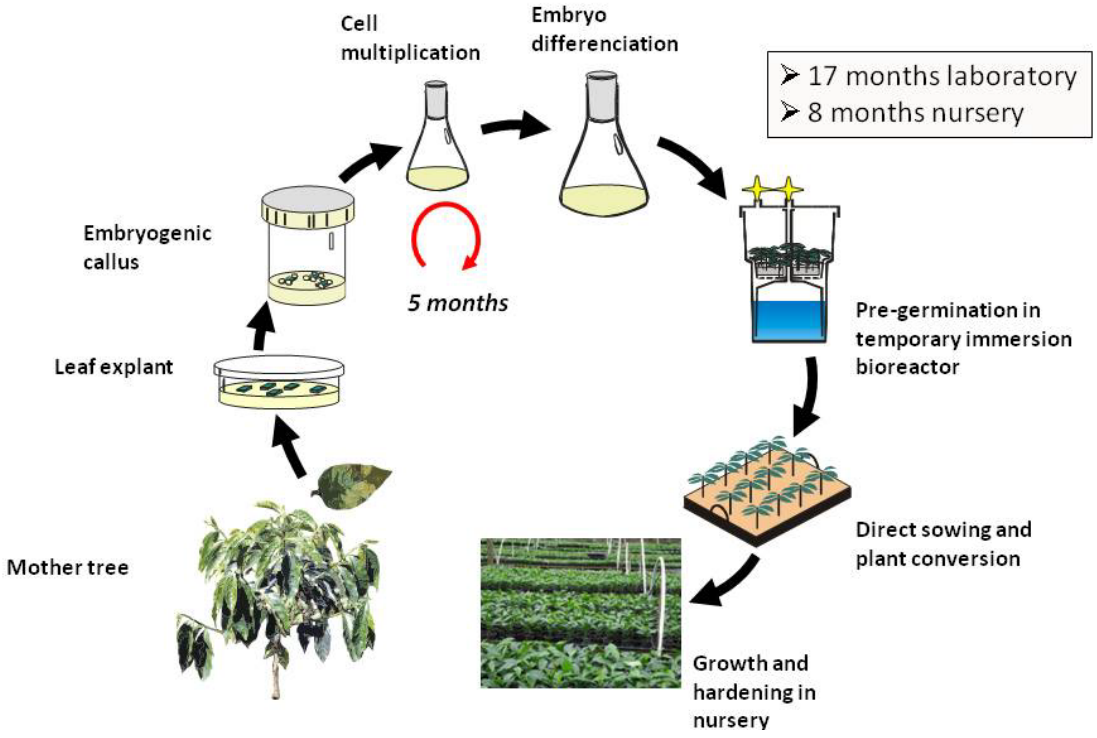

Figure 1. Schematic representation of the coffee somatic embryogenesis process transferred at the industrial scale.

Establishing the partnership (2003)

At the same time, some reassuring data were obtained on the genetic conformity of regenerated plants. Firstly, the frequency of somaclonal variations in the field proved relatively low, since it was less than 3%. Secondly, the only variations observed over five years were qualitative, i.e. easily identifiable on a phenotypic level, and not quantitative. For example, the quantity of coffee produced or the amount of a given biochemical contained in the beans were not modified (Etienne and Bertrand, 2001). Seven types of phenotypic variants were thus described: the Angustifolia (narrow leaves), Variegata (variegated leaf colouring) and Dwarf variants were the most frequent (Etienne and Bertrand, 2003). In addition, multiplication conditions were specified for embryogenic material in cell suspensions, whereby the regeneration of somaclonal variants could be controlled. In 2001, the process

(Figure 1) was therefore considered transferrable to the industrial level, particularly as it had functioned on all nineteen of the F1 hybrids tested.

In 1999-2000, CIRAD decided to go all the way in commercially developing this somatic embryogenesis process for large-scale multiplication of F1 hybrids, but also endeavoured to acquire useful experience on the other tropical species for which application of this technology was being considered. It sought a partner interested in technological transfer for *C. arabica*. A contract was signed with the ECOM group in 2003. The Swiss group, which is a trader of quality coffees and well established in Latin America, notably in Mexico and Central America, proved to be greatly interested, as it was keen to secure its top-of-the-range coffee supplies in that zone. At the time, agronomy trials involving F1 hybrid clones were revealing their remarkable adaptation to agroforestry conditions and confirming the excellence of certain clones in sensory terms (Bertrand et al., 2010). The ECOM group was logically very interested, as the majority of coffee plantations in Latin America are managed as agroforests. The adoption of F1 hybrids might make it possible to increase the quantity of coffee produced and its quality. The partners chose Nicaragua as the technological transfer site to disseminate Arabica hybrids throughout Central America.

However, both partners were aware of the difficulty of such a technological transfer, though probably for different reasons. CIRAD focused on technical difficulties linked to the actual technological transfer itself, and the major change of scale to be achieved (increasing from an annual production of 50,000 plants to several million). For its part, ECOM's main concern was to be able to sell *in vitro* plantlets, as it was justifiably worried about the dual particularity of this new planting material: F1 hybrid and *in vitro* plantlet. Indeed, at that time, there were no known examples in coffee tree of a breeding programme having led to the commercial distribution of F1hybrids or *in vitro* plantlets. The market had to be created from scratch and there was likely to be a lot of hesitation on the part of coffee growers.

Construction of infrastructures and first adjustments (2004-2006)

The choice was made to construct a small operational 300 m^2 laboratory (Figure 2), so as not to increase production costs with unavoidable expenses (fluids, work surfaces, etc.). It was also decided to locate it on the same site as a large coffee processing factory (‘beneficio’) at Sebaco, a small town 100 km away from the capital Managua, so that the many producers bringing their “despulpated coffee” to be processed could also discover the hybrid material and familiarize themselves with this new *in vitro* propagation method. A collection of "mother-plants" (horticultural cuttings or graftings of selected hybrids) was set up near the laboratory. It was to provide the basic material required for *in vitro* propagation. Six to eight copies of each selected tree were maintained there in the vegetative state and in excellent phytosanitary condition to encourage plant reactivity once placed under *in vitro* conditions. The acclimatization structures were also installed near the laboratory because acclimatization, which is a very delicate stage of the process, calls for meticulous preparation and monitoring, following on from the work carried out *in vitro*. The acclimatization, hardening and development nurseries were installed in one farm of the ECOM group (‘La Cumplida’), located near Matagalpa in the coffee growing zone at 30 km from the laboratory.

Many problems were encountered over this period, preventing routine production. Firstly, on a technical level, the water used proved to be extremely hard and heavy limescale deposits covered the heating elements of the autoclaves, the stills and the bioreactors, but also the leaves of the mother-plants. There were numerous power-cuts which considerably hindered laboratory operations. High contamination levels were recorded during the wet season. Access

to locally unavailable manufactured products proved to be a complication. The impossibility of finding staff trained in *in vitro* culture was a major difficulty and meant that the personnel had to be fully trained in the different tasks involved in production operations.

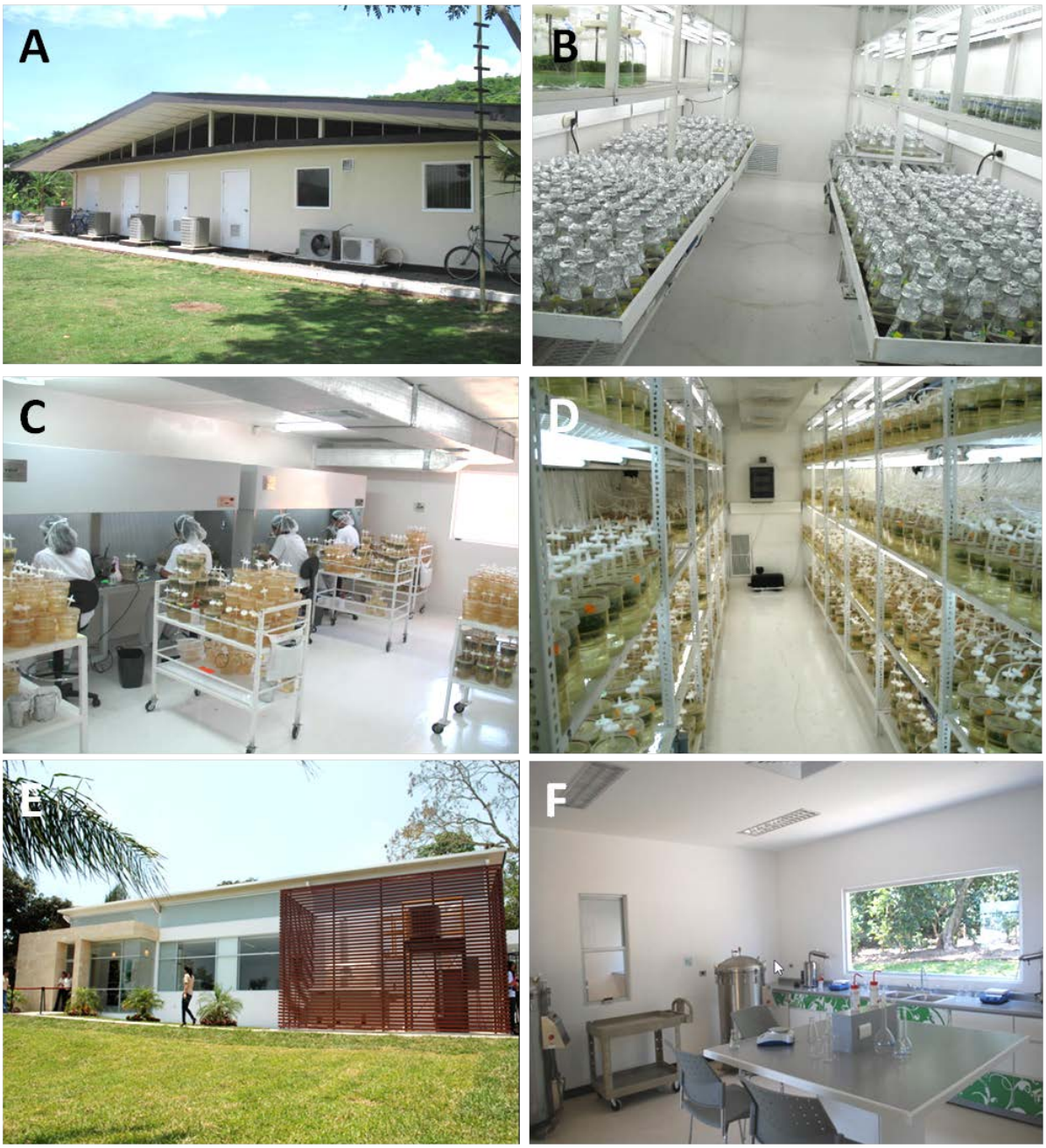

Figure 2. Coffee *in vitro* propagation laboratories of Sebacco, Nicaragua (A, view; B, culture room for cell suspension; C, sub-culturing room; D, culture room for bioreactors) and Jalapa, Mexico (E, view; F, room for nutritive media preparation).

Industrial production and change of scale (2007-2010)

By 2007, most of the technical problems mentioned above had been ironed out and a team of 25 people, eleven of whom were working in the laboratory, had been trained and organized. Several people were trained for each specialized job (medium preparation, autoclaving, cell suspensions, reporting, acclimatization, etc.), to cover for anyone leaving their post.

Production started at the beginning of 2007 and rose steadily over 3 years: 30,000 plants sold in 2007, 280,000 in 2008, 650,000 in 2009, and 1,000,000 so far in 2010 with a forecast of 1,500,000 plants by the end of 2011. Eventually, the production target for this laboratory is 5 million plants, without any modifications or additional facilities. As we shall see later, it will be possible to achieve this change of scale by technically optimizing the process. Over this period, around ten F1 hybrids were cloned. This production has been used to establish a network of several hundred thousand plants under agroforestry conditions in Meso-America and Mexico. The first pre-industrial output provided an opportunity to test each stage of the somatic embryogenesis process, identify trouble spots and implement major optimizations. This experience is detailed below, stage by stage.

Industrial feasibility of the different stages of the process

The different stages of the somatic embryogenesis process are diagrammatically represented in Figure 1. Cloning as a whole, from the culturing of leaf fragments to the production of plants transferrable to the field, takes 2 years, with 8 months in the nursery. Commercial production was launched once the genetic conformity of all the mother-plants had been checked by microsatellite molecular markers (SSR). Three trees did not conform to the expected genotype and were discarded.

- *Embryogenic tissue production*. This stage does not raise any problems in Arabica, apart from the fact that it is relatively long (8-10 months). All the explants react by producing a primary scar callus (Figure 3A) and between 10 and 40% of them, depending on the genotype, produce a secondary embryogenic callus (Figure 3B). These frequencies are enough for large-scale production, particularly as the embryogenic tissues are subsequently multiplied in the form of suspended cell aggregates. A genotype effect exists but it is easily taken into account by adapting the quantities of leaf explants used.

- *Multiplication of embryogenic tissues and embryo differentiation*. These two stages are carried out in a stirred liquid medium in Erlenmeyer flasks (Figure 2B), which drastically reduces manpower and laboratory space requirements. For example, 4 million embryos are produced annually on 4 m^2 of stirring tables. This does not raise any problems industrially, but it requires major technical know-how in relation to the other stages, particularly for initially establishing the suspensions. These stages also enable important synchronization of plant material development, which will subsequently persist and help to reduce the work involved in sorting acclimatizable embryos. Consequently, each of these stages corresponds to a single development stage, i.e. embryogenic aggregates, then fully developed embryos at the torpedo stage at the end of the differentiation stage (Figure 3C).

- *Pre-germination of somatic embryos in bioreactors*. The scaling-up at this stage has been successfully achieved over the last 3 years; 4 million pre-germinated embryos were produced in 2010 in one-litre RITA® temporary immersion bioreactors (Teisson and Alvard 1995) (Figures 2C, D). These bioreactors had to be harvested 2 to 3 times to collect all the pre-germinated embryos (Figure 3E) capable of continuing their development into nursery plantlets. However, we found that the volume of this bioreactor (Figure 3D) was too small for industrial production and prevented any further change of scale by negatively affecting several production parameters. Tests with larger bioreactors (3 litres) revealed several advantages. The reduction in the total number of bioreactors led to greater efficiency, i.e. a larger number of acclimatizable

embryos produced for the same amount of work involved. Fewer bioreactors also mean less investment and less cleaning work for the different constituent parts. In addition, the embryo stirring achieved in a larger volume is much more effective and makes for better synchronization during the initial germination stages. Thanks to this optimization all the embryos suitable for transfer to the nursery can be harvested in one go.

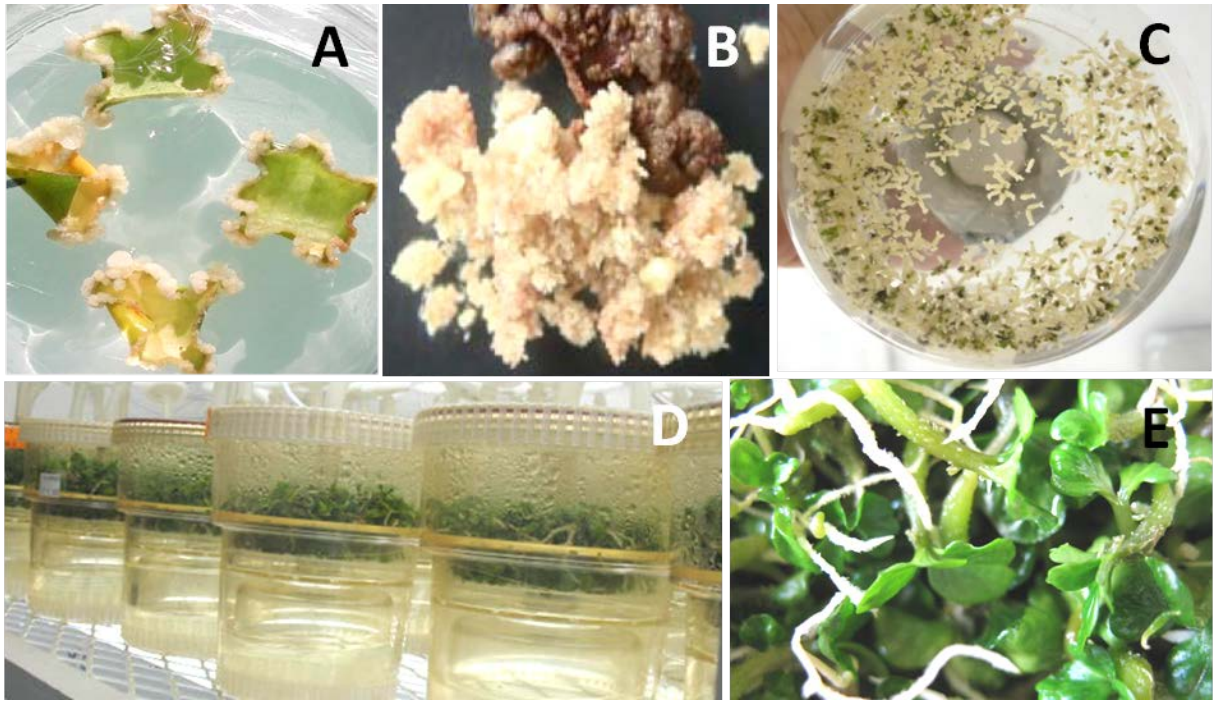

Figure 3. Development stages of coffee tissues during the *in vitro* steps of the somatic embryogenesis process. A, 1 month-leaf explants; B, embryogenic callus regenerated 8 months after *in vitro* introduction; C, somatic embryo differentiation in Erlenmeyer flasks; D, embryo pre-germination in 1 L-RITA® temporary-immersion bioreactors; E, pre-germinated embryos ready for nursery transfer.

- *Direct sowing of pre-germinated embryos on horticultural substrate and conversion into plantlets*. This is the trickiest stage of the process during which embryos have to be left to adapt to non-sterile *ex vitro* conditions, which are more subject to variations in temperature and relative humidity than under laboratory conditions. The transfer is made under conditions of saturated relative humidity in plastic tunnels (Figure 4A). Somatic embryos are grown in the tunnels at high density in an inert, peat-based substrate (Figure 4B). At the moment, this stage is an industrial bottleneck, as only 60% of embryos regenerate plantlets (Figure 4C), on average, for all 11 genotypes propagated. Quite a strong genotype effect is found between the propagated hybrid clones. The average time taken for conversion into plants after sowing is relatively long (22 to 24 weeks) compared to seedlings (14 to 15 weeks after seed sowing). Conversion into plants is asynchronous and two to three successive harvests are needed. These observations illustrate the possible major room for improvement at this stage.

Figure 4 . Nurseries of somatic embryo-derived coffee plants in Nicaragua. A and B, acclimatization tunnels; C, plantlets obtained by direct sowing of pre-germinated embryos; D and F, transfer of plantlets in 'tubetes' and hardening (E); G and H, growth nurseries (farm 'La Cumplida', Matagalpa, Nicaragua).

- *Growth in the nursery*. Plantlets with two to three pairs of leaves are transferred to more traditional nursery conditions where they are "hardened" to outside conditions by gradually reducing relative humidity and increasing light intensity (Figures 4D, E, G, H). This stage is well mastered and raises no problems. Although the initial growth of plantlets derived from the somatic embryos of *in vitro* plantlets is slower and more heterogeneous than that of their seedling counterparts, they catch up by the end of the nursery phase (Figure 4H) and prove to be even more vigorous than seedlings (Menéndez-Yuffá et al., 2010). Losses are minimal, amounting to around 9% of plants, which are discarded in quality checks at the end of the nursery stage. The rejects mostly consist of plants with horticultural defects (lack of vigour, curved stem, etc.); a few plants displaying early symptoms of somaclonal variations (Angustifolia (Figure 5A) and Variegata (Figure 5B) are also discarded, but they only amount to 0.3% of total production. The genotype effect is limited or nonexistent during the

plant growth stage. The main difficulty for a change of scale at this stage was the choice of containers, as if they were too big, very large volumes of horticultural substrate were needed and they took up a great deal of room in the nursery. Moreover, plant transportation to producers, then planting out, were subsequently complicated, especially in the predominantly mountainous zones of Central America. A small 200 ml container, called a 'tubete', was chosen (Figure 4F), given the laboratory's remit to disseminate *in vitro* plantlets in an inert substrate, free of nematodes and diseases, throughout the Central American zone (Figure 5D).

Figure 5. A, 'variegata' somaclonal variant; B, 'angustifolia' somaclonal variant; C, Arabica F1 hybrid clone in the field; D, transport of hybrid vitroplants to coffee growers; E, Arabica F1 hybrid clones in agroforestry systems.

And what of the genetic conformity of plants derived from somatic embryogenesis?

One of the expectations from this technological transfer was to obtain information about somaclonal variations. We have so far only identified morphological variations (qualitative) in *Coffea arabica* and we have demonstrated that phenotypically normal plants grow and produce normally (Etienne and Bertrand, 2001; 2003). Some variants can also be detected and discarded early in the nursery; such is the case with Angustifolia and Variegata variants. As we have seen, these variants only amount to 0.3% of total nursery production. The other phenotypic variants can only be detected at the mature stage, one or two years after planting. The first massive observations carried out on 100,000 plants established in 2008 in commercial plots in Nicaragua revealed a frequency of around 1%, which is commercially perfectly acceptable. The cases seen have mostly involved dwarf variants (84%) that were not detected in nursery.

Apart from sorting in the nursery, various strategies have been introduced upstream to limit the occurrence of this problem. Firstly, the multiplication time for suspended embryogenic tissues has been limited, as we previously showed the frequency of variations to be affected by that parameter (Etienne and Bertrand, 2003). Likewise, multiplication is carried out in the presence of a reduced levels of auxin (2,4-D). Another strategy has consisted in diluting the risk of encountering a high frequency of somaclonal variations by grouping together plants regenerated from different cell lines at the end of the nursery phase. Of course, in order to do this, it was necessary to introduce a system of traceability beforehand for production batches derived from independent cell lines.

CONCLUSIONS ON TECHNOLOGICAL TRANSFER

We acquired some important experience during this technological transfer and we found that it was unavoidable to move forward by trial and error, as it was so impossible to foresee all the possible problems, particularly in a developing country. We learned from this experience that, in the event of problems, it is necessary to analyse and intervene rapidly, so as to avoid production losses that can quickly become dramatic, and that in order to do that, it is necessary to establish faultless cohesion and communication beforehand within the team established with the partner. This technology transfer brought together partners from two different worlds, industry/private and research/public. To achieve an efficient partnership based on balanced complementarity, we had to train a joint, multidisciplinary team, which took a while. Be that as it may, this action in partnership is exemplary as it demonstrates that public/private synergy can effectively work for a sustainable agricultural policy under certain conditions. Indeed, F1 hybrids are set to serve as a catalyst for the return of farmers to agroforestry (Figure 5E), after they turned away from it in the 1980s for productivity reasons, in favour of the not so ecological practices of the green revolution.

The technological transfer of somatic embryogenesis is now complete for the Arabica species and demonstrates the feasibility of mass propagation by somatic embryogenesis. A substantial change of scale has been possible for each stage of the process, enabling a continuous flow of production from the laboratory to the nursery, and thereby a precise identification of the strengths and weaknesses of the technical process adopted. The commercial operation was launched in 2008 and the landmark million plants sold to Central American producers was reached in 2010. In addition, F1 hybrids have confirmed their superiority over traditional lines (Figure 5C) and are generating such enthusiasm that demand now outstrips production capacity (2 million plants for Nicaragua alone). The aim of the partnership is therefore to respond to demand as quickly as possible through a major change of scale at the production unit in Nicaragua (5 to 6 million plants within 4 years), but also by setting up other units in the region. Indeed, a model is now available for the laboratory aspects and for the nursery aspects, which can be "photocopied" to other sites. Another production unit was established in May 2010 in the state of Veracruz, Mexico (Figures 2E, F), to propagate other hybrids; in addition, several industrial nurseries have been established in Mexico, Guatemala and El Salvador.

Lastly, the demonstration that it is feasible to propagate *C. arabica* on a large scale by somatic embryogenesis renews the range of possibilities for this species in the field of genetic selection. Indeed, the success of this technological transfer now means we can consider introducing new varieties from hybrid or mutant materials, but also the dissemination of GM varieties by somatic embryogenesis, since efficient genetic modification methods are also available for this species (Ribas et al., 2010).

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The Impact of Participatory Village Based Training for Increased Coffee Productivity, Production and Income in Tanzania

J.M. MAGESA, F.B. SWAI, I.K. MUSHI, N.M. NGH'OMA, S. MDEMU, J.M. TERI

Tanzania Coffee Research Institute (TaCRI), Lyamungu, P.O. Box 3004, Moshi, Tanzania.
E-mail: tacriced@kicheko.com

SUMMARY

Empowering farmers to become leaders (farmer to farmer extension) of their fellow farmers was conducted through participatory village based training (PVBT) to create awareness and diffusion of technologies to coffee growers. The major focus of PVBT was on sustainable methods of improved seedlings multiplication of coffee varieties, coffee agronomy and primary processing. Farmers have been empowered by conducting structured training courses in their villages, during which, organized farmer groups were trained to become leaders in technology promotion and dissemination. Additionally, farmers' field days, execution or exchange visits of selected farmers and farmer groups were conducted to farmers' fields where there have been outstanding impacts as a result of participatory village based training. This has resulted into increased National coffee production to 68,000 tons from 47,000 tons, improved coffee quality classes from 14-9 to 9-4 and livelihood of coffee growers in areas where farmers have become leaders in technology promotion and dissemination.

This paper summarizes the impacts of participatory village based training focusing on empowering farmers to become leaders in technology dissemination for sustainable coffee production and increased livelihoods of coffee growers in Tanzania.

INTRODUCTION

Many developing countries see the agricultural extension system as an essential tool for promoting agricultural production and rural development. The system was getting weak for long time due to inadequate funds resulted in the lack of mobility leading to insufficient field orientations (Wolf, 1995). However, continued support from governments and donor agencies in the area of sustainable development, in particular, has allowed participatory extension initiatives to grow in some parts of the world (Cassara, 1995; Rolling, 1995).

Participatory approaches put much emphasis on participation of local at the same time encouraging farmers to share their skills and knowledge to promote innovation and creativity (Esber and Sthapit, 2000). Increasing farmers' participation in technology dissemination of research outputs is important for sustainable technology dissemination (Wolf, 1995). Participatory research leads to the empowerment of rural communities through knowledge and creativity and hence contributing to their own development (Arthur et al., 2005). It enhances the relevance and use of technologies by all the beneficiaries, and it increases the likelihood that behavioral change interventions will be adopted in a community in cost – efficient ways.

Recently, a number of participatory approaches have been developed and applied in different developmental projects (Esber and Sthapit, 2000; Rolling, 1995), with some approaches

suggesting the need to support farmer networking and empowering through participation to reinforce individual learning, centered with a process that is facilitated by professionals.

Farmers' participation in technology dissemination is becoming a facilitation structure rather than a linear transfer of technology extension model and is gaining acceptance within developmental and public sector institutions (Killough, 2010; Fara, 2006). Instead of trying to "sell" predefined packages, participatory extension will increasingly focus on building capacity among rural people to identify and take the advantage of technical and economic skills.

(Killough, 2010; Van Veldhuizen et al., 2003) have outlined key characteristics of participatory approach which promotes farmers' capacity to adapt to change and equal partnership between farmers and professionals who can all learn and contribute their knowledge and skills. In this system, an approach of farmer to farmer extension "use of farmer promoters" is used and it requires considering farmers first as the key leaders of the technologies being disseminated (Killough, 2010; Van Veldhuizen, 2003). It involves the empowering or capacity building of farmers to become leaders in technologies dissemination as opposed to the purely mechanistic which puts much efforts of participation as a means within the technology development flow.

Farmers' empowerment plays a key role in improving agricultural productivity and efforts as it aims at developing systems that foster greater farmer knowledge, control of resources, and institutional participation; allowing producers to become active partners in agricultural productivity initiatives which include but not limited to access to technologies, enabling them to learn and turning the communities into knowledge centers for dissemination.

This paper summarizes the impacts of participatory village based training with main focus on empowering farmers to become leaders in technology dissemination for sustainable coffee productivity, production and quality improvement for increased income and livelihoods of coffee growers in Tanzania.

THE MAIN COMPONENTS OF THE APPROACH

Participatory Methods

(White, 1996) has classified participation techniques that involve the use of farmers in dissemination of research findings. The techniques contribute to empowerment of the participants who could be farmers or professionals from research institutes or developmental organizations. This paper will focus on participatory techniques that attempts to put grassroots-level communities at the center of technology development and dissemination through:

Village based training of farmers

It is now generally recognized that working with farmer groups rather than individual farmers is more conducive to effective dissemination and adoption of technologies extension (Esber and Sthapit, 2000; Fara, 2006). Farmer groups are empowered through training (Figure 1a) on coffee agronomy, primary processing and multiplication of improved seedlings of coffee varieties through participatory approach (Figure 1a) with the end users becoming the leaders in technology dissemination. 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.

Figure 1. Village based training of farmers (a); Participatory approaches during of farmers (b).

Training of extension officers

It has been reported that the task of encouraging farmers to participate in research and development makes the extension officers feel appreciated by farmers and vise versa (Reij and Water-Bayer, 2001). Intensive training courses have been offered to village and district extension officers to become trainers of farmers in village while encouraging the use of participatory approach. Discussions and exchange of ideas on good agricultural practices and hybrid seedlings multiplication are conducted within and between group members to build knowledge and confidence on participation and technology dissemination. Collection of ideas on the same aspect is followed by decision making by all participants.

Exchange and study visits

These form part of training course whereby farmer groups or selected farmer promoters conduct field visits to their fellow farmers within or outside their villages, districts, regions or zones. They focus in areas with impacts such as successful groups on clonal seedlings multiplication or rehabilitated coffee farms. Farmers and farmer promoters appreciate these approaches as ways of gaining new experience, knowledge and techniques, which they informally experiment at home with their fellow farmers.

Farmers' field days

These are set as learning and promotional tools for other farmers who have not yet adopted the new technologies. Such events which provide opportunities for training are conducted in villages where various technologies on good agricultural practices and seedlings multiplication of improved coffee varieties that are being promoted by means of demonstration plots. The same methods have been practiced on maize seed production by farmers (Coorter, 2000; Temu et al., 2006). The main leaders during the field days are farmers themselves who take the responsibilities leadership by explaining the importance of the technologies to their fellow farmers. Within a demonstration plots crop husbandry practices can be used as observation units for discussion. After a field visit, a wrap up session is conducted for feedback on the observed problems.

Identifying farmer promoters

These are selected from a group of trainees (farmers) who show outstanding performance in technology adoption after the training courses. Selection is participatory which is done by farmers themselves from members of the groups. Farmer promoters can also be picked from a group of retired extension officers with good knowledge in coffee production. Both of them work closely observing and documenting new things that farmers are developing in the filed and bring the feedback to the researchers for further action. The same classical method that can be used to study the performance of the technology being disseminated through participants' observation and documentation has been reported (Esber and Sthapit, 2000). Furthermore, new ideas can also be collected and disseminated through field visits, training and other forms of farmer to farmer extension.

The following are the guidelines for a farmer to be called a farmer promoter

- He must first be trained by researchers or extension officers trained by TaCRI
- He/she must own a coffee fields that is maintained as a demonstration plots for other farmers
- He must be able to adopt diversification options e.g coffee banana intercropping pattern, bee and livestock keeping
- Be able to prepare monthly village training programme and report progress to researchers
- He must be able to demonstrate good attributes of being an extension agent, be accepted by the village leaders in his areas and adjacent areas
- He must be committed to members of the groups

a b

Figure 2. Training of farmer promoters by extension officer (a), Farmer promoters taking the lead in field layout (b).

Follow-up on technology dissemination

Technical follow up by extension officers is important for successful monitoring the dissemination of technologies throughout the process. Regular backstopping trips of farmer groups and village extension officers are being done by researchers in collaboration with farmer promoters, village and district extension officers to monitor the adoption of the technologies by coffee growers. Meetings with farmer groups and special seminars for farmer promoters have been conducted to share lessons learnt and identify new training needs to

specific farmer groups or individuals coffee growers. Each farmer promoter or member of the group is given chance to explain and contributions are invited from his fellow farmers; and at the end conclusion and way forward is reached.

Increased coffee production

Average annual production of coffee in Tanzania over the past 30 years has been 47,000 tons per year (TCB, 2010). However, since the release of hybrid coffee varieties and subsequent packaging appropriate technologies for dissemination to coffee growers by Tanzania Coffee Research Institute (TaCRI), outstanding yield of up to 68,000 tons (Figure 3) has been realized in the year 2008/09, which is National record in the country. This is the results of adoption of the technologies (good agricultural practices including planting of high yielding hybrid coffee varieties) by coffee growers.

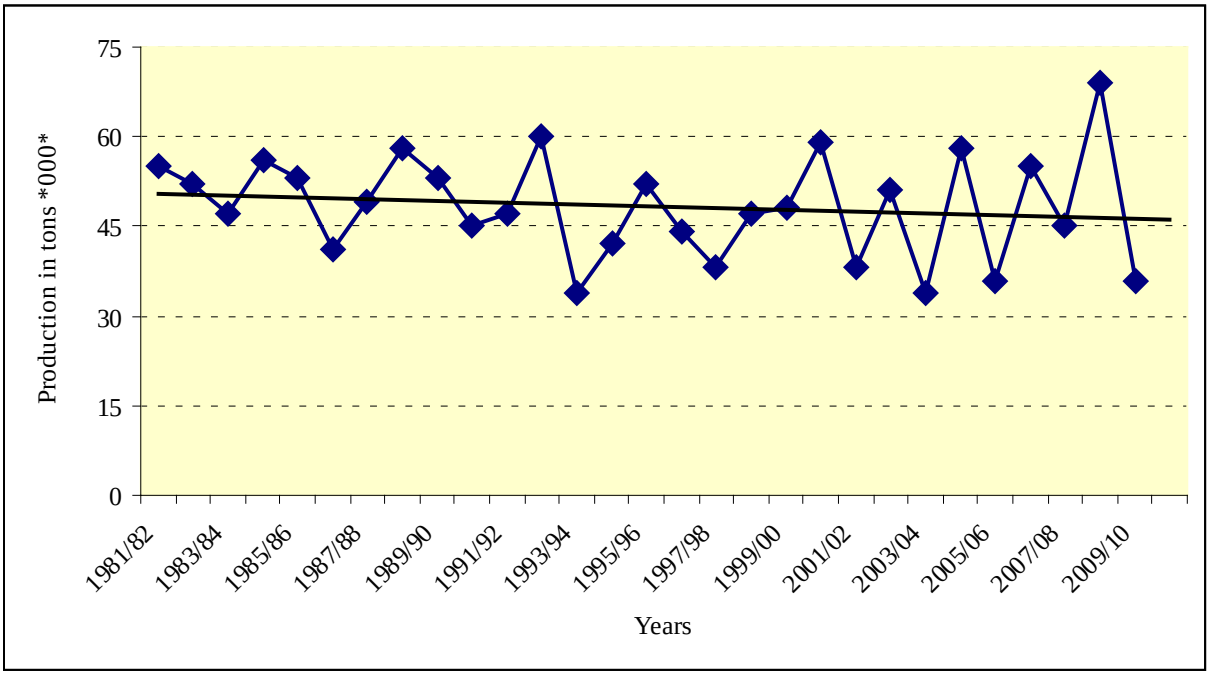

Figure 3. Coffee production trend in Tanzania from 1981-2008/09.

Capacity building to farmer groups

Through participatory approach, TaCRI has already empowered over 800 farmer groups and 212,213 farmers (80% males and 20% females) scattered in 33 coffee growing districts in the country (Figure 4). Farmers are now conducting seedlings multiplication activities in collaboration with TaCRI, farmer promoters, district and extension.

Through participatory approaches, farmer groups have been empowered with the technologies of seedlings multiplication by clonal propagation and grafting methods. Seedlings multiplication has been on exponential increase (Figure 5). Since 2003, a total of 32,268,991 seedlings have been distributed to various coffee growers where a large part is multiplied by farmer groups. In realizing the contribution of small scale producers who contributes about 90% of the coffee that is produced in the country, TaCRI has been putting much emphasis on empowering small scale coffee growers to take up the role of seedlings multiplication through farmer groups; and it is possibly that Tanzania is the first country worldwide in empowering

farmer groups to adopt the technologies of hybrid seedlings multiplication by clonal propagation and grafting methods.

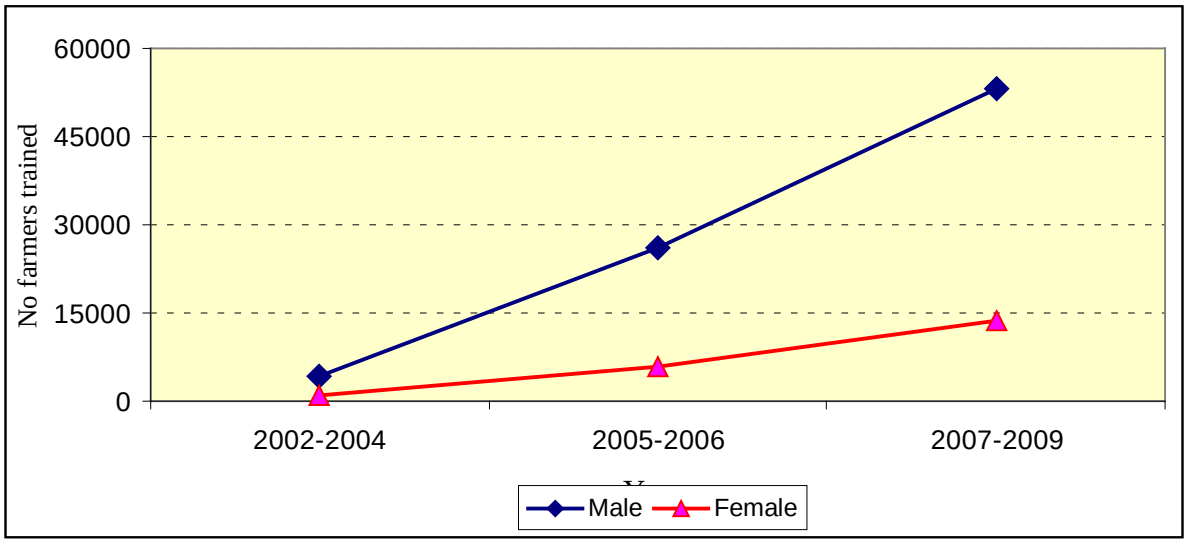

Figure 4. Number of farmers trained across coffee growing districts in Tanzania.

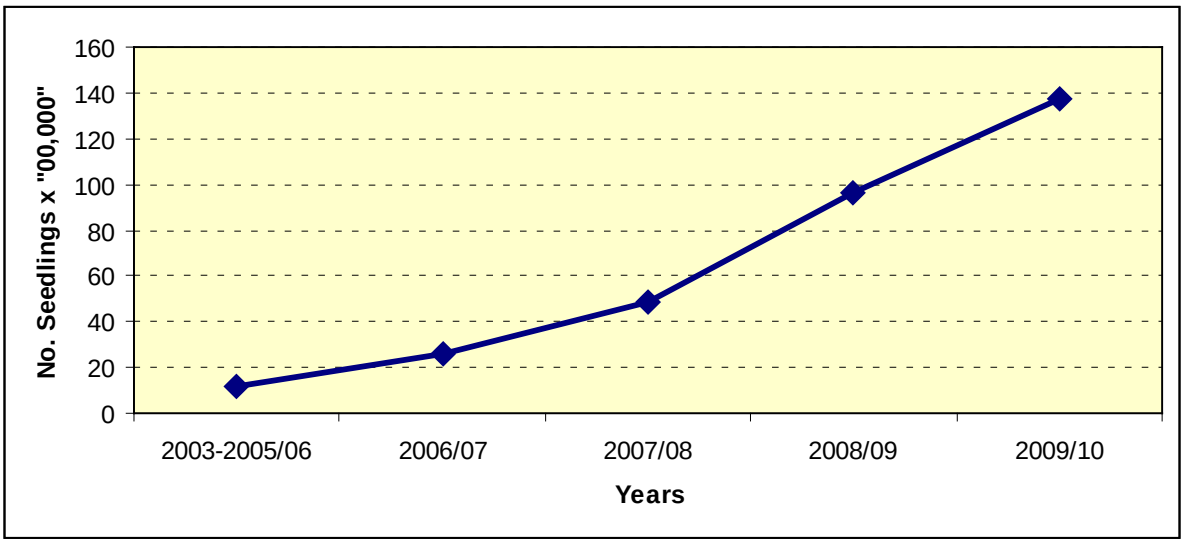

Figure 5. Progress on multiplication and distribution of seedlings of improved coffee varieties, 2003-2009/10.

Change in livelihoods

There are changes in terms of farmers' income as the result of increased adoption of the new technologies through participatory village based training. Improvements in social services have been noticed where by roads, houses, primary and secondary schools have been constructed in some districts where there is high adoption of technologies. Also levies collection in coffee growing district councils have gone up (Figure 6).

Quality improvement

Quality is among the aspects that farmers are being taught during participatory village based training. Much stress has been on adopting quality improvement practices i.e the 10 commandments to improve quality. Furthermore, farmers have also been organized into groups and processing their coffee is done through central pulpery units (CPUs). This has resulted into increased coffee grades (Table 1).

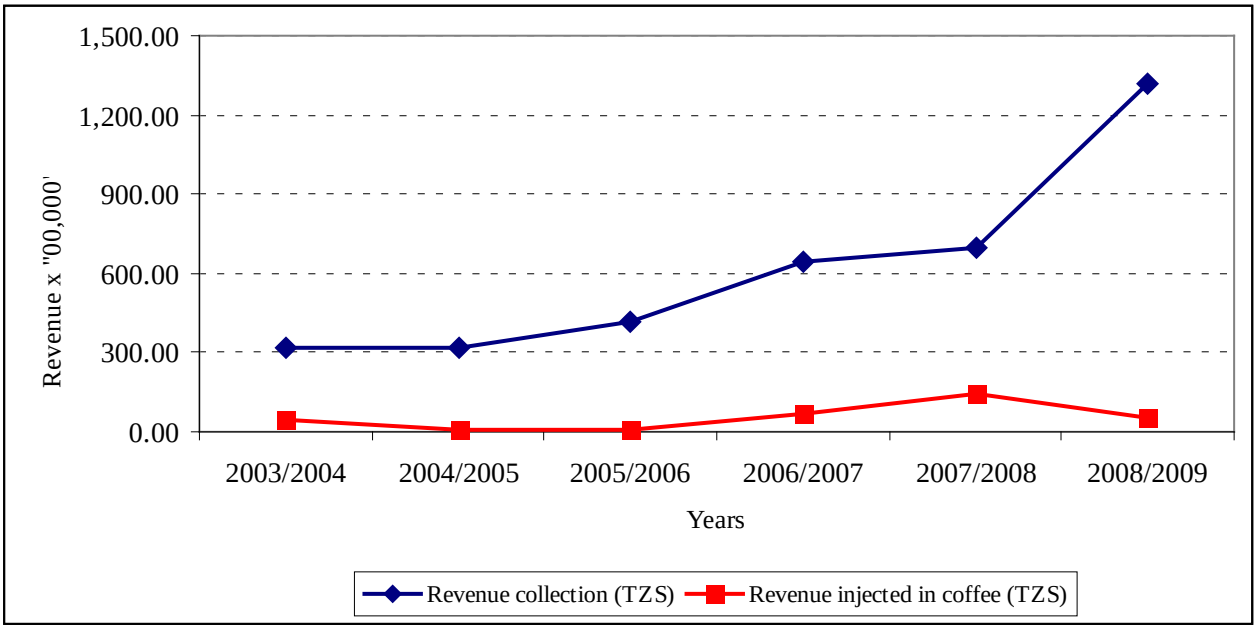

Figure 6. Total revenue collection from coffee in Mbinga district, southern part of Tanzania.

Table 1. Improvement in quality classes in some of the coffee growing districts in Tanzania.

Year	**2001/02**	**2002/03**	**2003/04**	**2004/05**	**2005/06**	**2006/07**	**2007/08**
Classes	17-9	9-7	9-6	8-5	7-5	7-4	7-4
Groups	0	22	13	33	64	206	317
Price (TZS)	290/-	500/-	750/-	800-1300/-	2,000/-	2,300/-	2400/-

Key: 17 = Low Quality; 1= High Quality

CONCLUSION

From this paper has summarized the importance of participatory village based training (PVBT) as a step forward in to rejuvenate the coffee industry in Tanzania. PVB have created farmers' leaderships and ownership of technologies that are being disseminated. Through these approaches, a good number of technologies have been disseminated successful with outstanding impacts on coffee productivity and livelihood of coffee growers. Therefore, an investment in technology dissemination also involves investments in growth and livelihoods of the community.

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Integrated Control of Coffee Berry Borer (*Hypothenemus hampei*) on Arabica Coffee in South Sulawesi, Indonesia

S. WIRYADIPUTRA[1], R. JAHUDDIN[2], I.L. MAINSESE[2], G.I. ODUOR[3], S.S. SASTROUTOMO[4]

[1]Indonesian Coffee and Cocoa Research Institute (ICCRI), Jl. P.B. Sudirman 90, Jember, Indonesia. E-mail: soekadar@yahoo.com
[2]Provincial Estate Services of South Sulawesi
[3]CABI Africa, Kenya
[4]CABI Southeast and East Asia, Malaysia

SUMMARY

Coffee berry borer is one of the main pests which may reduce coffee production in Indonesia. A trial of coffee berry borer (CBB) control was conducted in Arabica coffee plantation in South Sulawesi from June, 2009. Five treatments were evaluated on coffee of over 10yrs owned by farmers at four locations in Enrekang and Tana Toraja Districts. The treatments were sanitation, application of *Beauveria bassiana* (Bb), integration of sanitation and Bb, trapping and untreated control. Sanitation was done by removing all berries left on the coffee trees after harvesting period and all berries falling on the ground. Bb was applied at the dosage of 100 g pure spore per ha and sprayed every 2 months, and trapping was practiced using Hypotan (CBB attractant produced by ICCRI) with a density of 10 traps per 0.25 ha coffee plantation. Observation conducted at the initial infestation of the coffee berries on February 2010 revealed that trapping using Hypotan was the best method in reducing CBB infestations on Arabica coffee in Enrekang and Tana Toraja areas. It could reduce the CBB infestation by 80.0% compared to untreated plot. Observation on the coffee berries on the trees indicated that the average infestation of CBB on untreated plot reached of 21.77%, whereas on the trapping plot was only 4.36%. On the plots treated with sanitation, Bb and combination of both, the CBB infestation were 11.71%, 11.93% and 9.79%, respectively. In general, the levels of CBB infestation on the treated plots were significantly lower than those of untreated plot. Applications of Bb also significantly increased the level of Bb infection on the damaged berries and reached around 12.0% on treated plot. Contamination of Bb application also occurred on the plots without Bb spraying, especially on treatments of sanitation and untreated control. This presumably, due to the distance among treatment plots were not far enough, except for the trapping plot.

INTRODUCTION

South Sulawesi is well known for production of high quality arabica coffee called Toraja coffee. Coffee produced from this area belongs to the special coffee which attracts a premium price in the world coffee market. The main areas of Arabica coffee production is Enrekang and Tana Toraja districts, where around 28,570 ton green coffee are produced per year by 48,496 smallholder households. Constraints to coffee production from this area include infestation by pests and diseases, inferior of planting material, poor maintenance, and limited technology transfer to the farmers. In the case of pest and diseases, coffee berry borer (CBB) is the main pest causing the highest financial losses.

Management of CBB in Indonesia has been practiced using several measures, especially sanitation method and implementation of biocontrol agent using the entomopathogenic fungus *Beauveria bassiana*. Recently, trapping was developed as a safe control method to manage sustainable coffee production. However, sanitation method is difficult to practice in wet areas like in Sumatra due to the fruiting season of coffee occurring continuously over the year. Application of *B. bassiana* (Bb) also faces on several constraints, especially in mass production of the fungus and virulence instability of the fungus under field conditions. Trial conducted by Wiryadiputra (1996) revealed that *B. bassiana* fungus very effective in controlling CBB in Enrekang and Tana Toraja district of South Sulawesi. Application of *B. bassiana* at a dose of 2.5 kg of solid culture on maize medium per hectare could suppress CBB infestation by 74.3% (in Enrekang) and 82.1% (in Tana Toraja), and effect that was not significantly different from that of endosulfan application. *B. bassiana* has been used extensively in controlling of CBB on the production of organic coffee in South America (Furst and Bergleiter, 2008). Trapping of adult CBB has been tried on robusta coffee smallholders in Lampung province (Indonesia). The result showed that use of Brocap traps over a period of four months suppressed CBB infestation by between 22.10% and 72.62% and increased production by 19.1% (Wiryadiputra et al., 2008).

Technology transfer to coffee farmers in South Sulawesi will enable farmers to practice integrated pest management (IPM). This will increase production and productivity of their coffee and in turn will enhance farmers' income and livelihoods. There is the need to identify sustainable cost effective CBB management technologies which conserve the natural resource base. The objective of the trial was to choose the efficient control method which will be practiced by the farmers to solve the CBB problems on their coffee gardens.

MATERIALS AND METHODS

Trials were conducted on over ten-year old coffee at four sites in South Sulawesi; the two sites in Enrekang district were located at the farms of Mr. Kasim, at Batukede village, Masale subdistrict with elevation of 1327 m asl., and the other of Mr. Faisal at Pana village, Alla subdistrict with elevation of 1098 m asl. In Tana Toraja district, trials were conducted at Mr. Abba's coffee garden, in Gandang Batu village, Gandang Batu Silanan subdistrict with elevation of 1023 m asl. and at Mr. Johanes Tanga's coffee garden, in Benteng Ambesso village, Gandang Batu Silanan subdistrict with elevation of 1325 m asl. The trial consisted of five treatments, i.e; Sanitation, application of *B. bassiana* , integration of sanitation and application of *B. bassiana* , trapping and untreated (control). Each treatment was applied to a plot consisting of 49 coffee trees separated by at least five rows of coffee trees (15 m) except for trapping plot that was separated by minimal 200 m from the other treatment plots.

Sanitation was done by removing all coffee berries about 5.0 mm in diameter left on the tree and on the ground after peak harvesting period, every two months until the next fruiting season. . Application of *B. bassiana* was conducted by spraying using knapsack sprayer with a dose of 100 g pure spore of the *B. bassiana* strain Bb-725 produced by ICCRI, and with standard solution of 400 liters of water per ha. Viability of the *B. bassiana* spore was between 80% and 90%. Spraying of *B. bassiana* was conducted every two months. Trapping was done by installation of mineral bottle (volume 1500 ml) trap with two holes (4 cm x 6 cm) on both sides with attractant substance (contains10 ml of Hypotan, produced by ICCRI) inside. Ten traps were installed evenly at an area of 0.25 ha of coffee garden. Coffee production was observed until third harvest against fresh coffee berries and parchment coffee.

Observation of CBB infestation was conducted every two months for a period of one year from June 2009. Observation was done by counting of percentage of perforated berries on one branch per sample tree on 20 sample trees per plot. Similar observation was also conducted on the berries on the ground by counting the berries on the plot of (50x50) cm^2 under coffee tree samples. Adult CBB captured was observed every two weeks. Collected data were analyzed using SAS-9.1 software to identify the differences among treatments using Duncan's Multiple Range Test at 5.0% significance level. Data from the 4 sites were averaged.

RESULTS AND DISCUSSION

CBB infestation

At initial observation, CBB infestation in four trials ranged between 9.7% and 26.8%, the lowest average was in the sanitation treatment and the highest was in trapping treatment (Figure 1)., There were no significant difference between treatments at this observation. After second observation in August 2009, all treatments were lower compared to the untreated plot but still not significantly different compared with control plot.

Average CBB infestation on the berries on the ground was higher compared with on the tree, ranging between 13.5% and 33.5% at initial observation and not significantly different among treatments (Figure 2). On the last observation, especially on April and June 2010, treatments with *B. bassiana* , integration *B. bassiana* and sanitation and trapping were significantly different compared with untreated plot in suppressing CBB infestation both on tree and ground berries. But for sanitation treatment was not significant to suppress CBB infestation compared with untreated plot.

Integration of several measures to control of CBB has been recommended by several researchers. Dufour (2008) declared that implementation of triple-action of integrated pest management by implementation of meticulous agronomic control of the coffee plantation, strict branch stripping and trapping had shown that it is possible to reduce CBB infestation by over 90% compared to control plots in shaded coffee plantations. The advantages of this technique are numerous: efficient basis for control, no risk of contaminating the environment; it is a preventive strategy that is simple to apply, it is compatible with biological control and it does not affect biodiversity. On this trial, trapping was most effective control for CBB infestation, but in these trials the trap density was higher than recommended in Latin America with 24 Brocap® traps per hectare as mentioned by Delabare (2001).

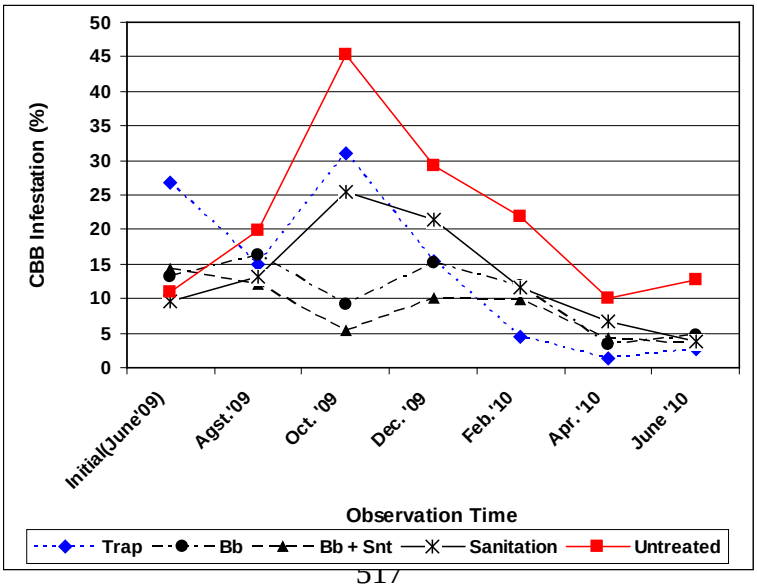

Figure 1. Effect of several control methods on infestation of the coffee berries on the tree.

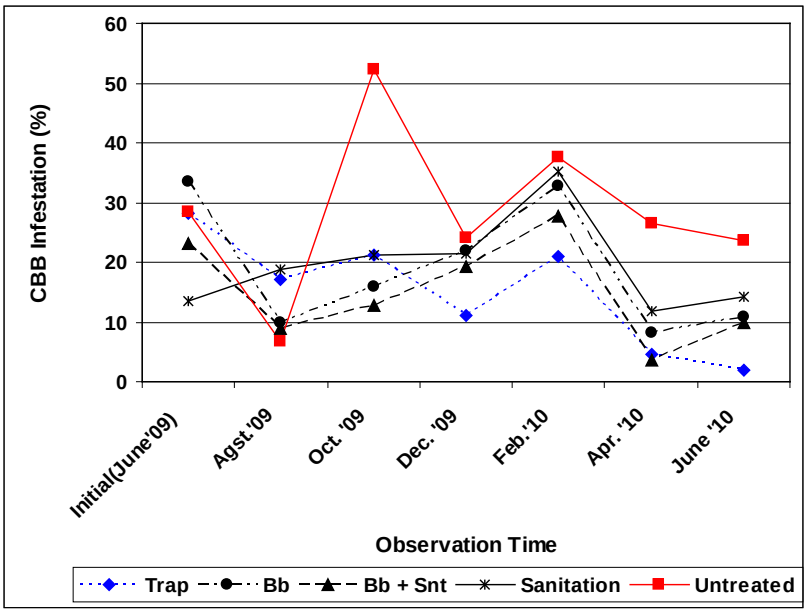

Figure 2. Effect of several control methods on CBB infestation of the coffee berries on the ground.

B. bassiana infection on CBB adults on perforated berries was assessed by counting of percentage of the berries with *B. bassiana* per sample branch to total perforated berries. On observation in February 2010, the *B. bassiana* infection level on the berries in coffee tree and on the ground as shown in Figure 3. *B. bassiana* infection was found not only on the plot treated with *Beauveria* (plot Bb and Bb+Sn) but also on the other plots untreated with *B. bassiana* , i.e; untreated (control), sanitation and trap. The highest infection of *B. bassiana* occurred on the treatment of *B. bassiana* on the berries in coffee tree and then followed by integration treatment between *B. bassiana* and sanitation. Although, no *B. bassiana* spraying on treatment of sanitation, trap and untreated plots, there are apparently natural infection of this fungus in the trial locations, in spite of in the low level of infection. On the assessment of the berries on the ground, there were also found *B. bassiana* infection, but in lower level compared with coffee berries on the tree. In this case, *B. bassiana* treatment plot also has the highest level of infection and followed by integration treatment Bb + Sn and sanitation treatment.

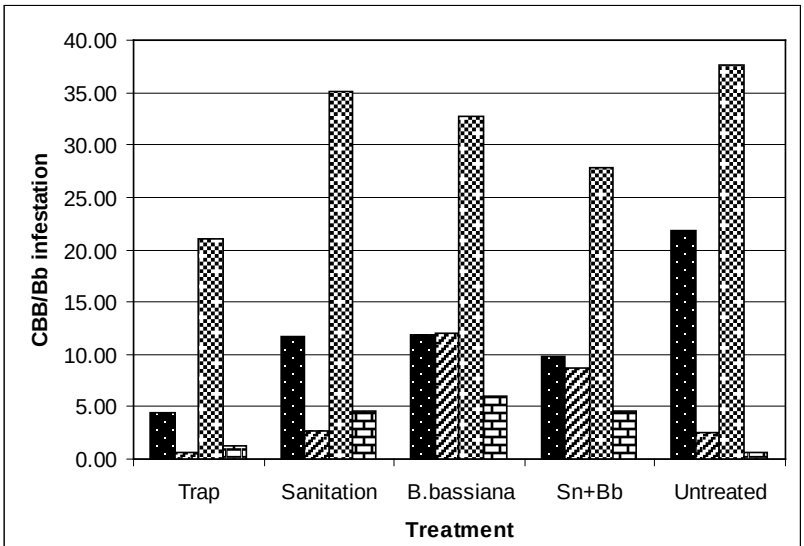

Figure 3. Effect of several control methods on infestation of CBB and infection of Beauveria bassiana on coffee berries on the tree and on the ground.

Beauveria application has been implemented in production of organic coffee in Latin America, and was the main biological control applied, where57% of the coffee growers control the CBB by biological control (Fürst and Bergleiter, 2008) *Beauveria* trial against of CBB has also been conducted by De La Rosa *et al*. (2000) in Mexico. On their trial, maximum average percentage mycosis varied according to altitude and strain of *Beauveria bassiana*. At 450 m asl mycosis was 14.3%, at 880 m asl mycosis was 40.6% and at 1,100 m asl was 33.9%. At lower altitude the best strains was Bb25, and at middle and higher altitudes was Bb26. Wiryadiputra (1996) made two trials on the effectiveness of *B. bassiana* in Enrekang and Tana Toraja, and the results revealed that infection level of the *B. bassiana* reached of 33.05% at Enrekang and 33.91% in Tana Toraja. The lower infection level of *B. bassiana* in this trial is probably due to the strain of the fungus.

Trapping of CBB adult.

Results of trapping treatment on CBB infestation were very promising. Despite the high CBB infestation at the beginning of the trial at 3 of the 4 sites, the population of the pest dropped drastically on month 7 and remained so for the rest of the trial period (Figure 4). At the end of the trial, traps suppressed the CBB infestation by 78.2% compared to the untreated plot. The effectiveness of the trap in controlling of CBB has been reported by Dufour et al. (2004). Brocap trap validation under field conditions in commercial coffee plantation in El Salvador resulted in reductions in infestations levels by up to 80.0%. The same validation conducted in Indonesia in small holder Robusta coffee resulted in reduction of CBB infestation levels by up to 72.62% and increase of green coffee production by 19.1% (Wiryadiputra et al., 2008).

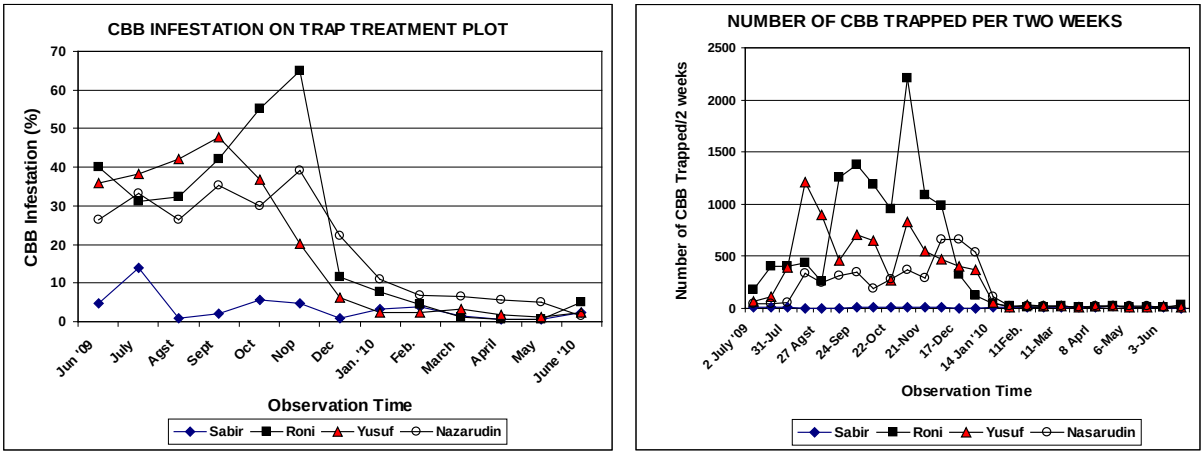

Figure 4. CBB infestation on the tree (left) and CBB captured (right) on trapping plot treatment in the trial of CBB control in South Sulawesi.

Effect of the trials on coffee production

The term "production or yield increase" in this paper means compared with the untreated control plot, i.e. production increase is a decrease in the yield losses. For these trials, the highest of yield increase was observed on the treatment of trapping and reached up to 66.5 % of parchment coffee compared with untreated plot (Figure 5).

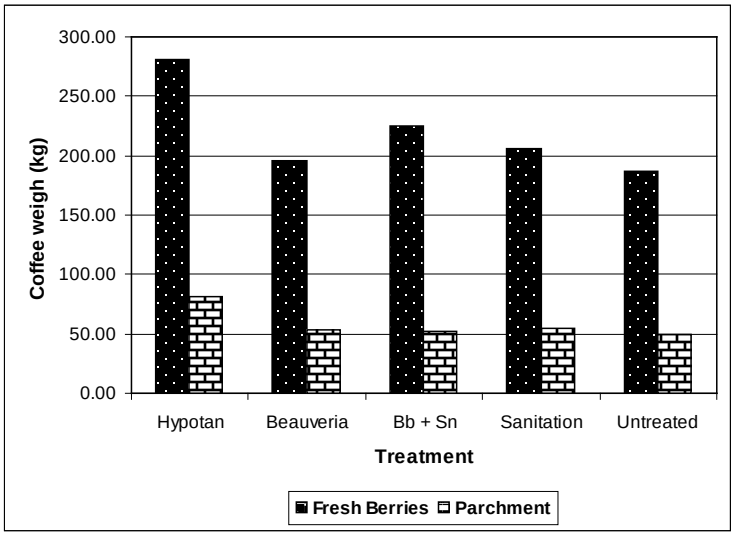

Figure 5. Effect of several control methods on coffee yield on the trials of CBB control in South Sulawesi.

Figure 5 shows yield increase of the parchment coffee as percentage from untreated plot for each treatment as 66.5, 9.4, 12.4 and 6.3% for trapping, was 66.5 *B. Bassiana*, was 9.4sanitation4and integration *B. bassiana* , respectively. sanitation was 6.3The average yield of Arabica coffee in Enrekang and Tana Toraja in 2008 was 554.5 kg/ha/year green coffees (Anonym, 2009). Combining this yield with the results from Figure 5, the yield increase for each treatment would be 368.74 kg for trapping, 68.81 kg for sanitation, 34.77 kg for *B. bassiana* , and 23.38 kg for integration of sanitation and *B. bassiana* . From these results and based on a cost-benefit analysis, only trapping would be economically feasible and gives high profit for control of CBB (Table 1).

Table 1. Benefit-cost analysis for several control methods tried for controlling of CBB in Arabica coffee in South Sulawesi, Indonesia.

Treatment	Yield increase (kg green coffee/ha/yr)	Revenue (USD)*)	Cost (USD)	Profit (USD)
Trapping	368.74	1024.28	354.02	670.26
Sanitation (Sn)	68.81	191.15	280.00	-88.85
Beauveria bassiana(Bb)	34.77	96.59	276.61	-180.02
Sn + Bb	23.38	64.94	556.61	-491.67

*Notes: *)Coffee price in the harvesting season of 2010 on the farmer's level about Rp.25.000,-/kg green coffee or 2.78 USD with rate of exchange of 1 USD = Rp.9000.*

CONCLUSION

Trapping was the most effective in controlling of CBB on Arabica coffee in Enrekang and Tana Toraja areas of South Sulawesi. Observation at the mid and at the end of the trial revealed that the treatment could suppress the CBB infestation on the berries in the tree of up to 80.6% and 78.2% compared with untreated, respectively. On the berries on the ground, however, could suppress the infestation of up to 71.0% and 91.9%, respectively. Trapping also gives the highest profit to the farmer due to the highest increase of the coffee yield.

ACKNOWLEDGEMENTS

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Nematodes: an Emerging Constraint to Coffee Production in Kenya

E.K. GICHURU[1], J.N. MAUNDU[2], H.M. MUGO[1], J.N. MBURU[1], K.T.K. GITONGA[1], J.W. KIMENJU[2], G.K. MUTUA[2]

[1]Coffee Research Foundation, P. O. Box 4-00232, Ruiru, Kenya
[2]University of Nairobi, Department of Plant Sciences and Crop Protection, P. O. Box 30197-00100, Nairobi, Kenya

SUMMARY

Coffee is the third most important agricultural commodity in Kenya but the production has declined from a peak of 130,000 tonnes to the current 50-60,000 tonnes. Among the constraints limiting coffee production are neglect, poor agronomic practices, pests and diseases coupled with low prices. Frequent detection of galls on coffee roots has raised concerns of nematodes in coffee production systems. This study aimed at determining the occurrence of nematodes associated with coffee in Kenya and the role of field management practices on the abundance of nematodes. Results showed that nematodes belonging to thirty (30) genera were recovered from coffee agro-ecosystems. Plant parasitic nematodes were the most prevalent with 64% frequency of occurrence followed by bacterial feeders at 24%. Among the plant parasites, nematodes belonging to the genera *Tylenchulus*, *Meloidogyne* and *Pratylenchus* were the most dominant across all the coffee growing areas. Amongst the agroecological zones, coffee farms in the coffee-tea zones (UM1) harboured the highest numbers of plant parasitic nematodes, followed by UM2 and least in the marginal coffee growing zones (UM3). Similarly, farms that were relatively well managed harboured a lighter load of plant parasitic nematodes compared to neglected farms. This study has demonstrated the prevalence of plant parasitic nematodes and justifies further work towards their management.

INTRODUCTION

Coffee (*Coffea arabica* L.) is among the leading commodity crops for many developing countries, contributing over US$ 10-11 billion annually (Alpizar et al., 2007). Commercial coffee production is mainly on large plantations as well as small-holder farms averaging less than 0.5 ha. The crop is grown especially for berries containing several substances that are of physiological importance such as caffeine, chlorogenic acid, carbohydrates and trigonellin (Campos and Villain, 2005). The coffee beans are roasted, ground and usually diffused in hot water to give a popular beverage.

In Kenya, coffee is the third most important agricultural commodity after horticulture and tea earning the country about KES 10 billion in 2008/09 coffee year (MoA, 2009) . However, in the last two decades, coffee production has declined from a peak of 130,000 tonnes of bean to the current 50,000 tonnes. The principal factors limiting coffee production include unreliable rainfall, poor agronomic practices, pests and diseases. In Kenya, the major diseases of economic importance are coffee berry disease, coffee leaf rust and *Fusarium* bark and root diseases. Although the occurrence of nematodes in coffee production systems in Kenya has been observed in the past, they have often been ignored or misdiagnosed due to their

microscopic size and symptoms confounded by either malnutrition and root infections by other pests. However, recent observations in the field have increasingly reported specific symptoms of nematode infestation based mainly on the easily observable galls induced by root knot nematodes (*Meloidogyne* species). This has added impetus to the concerns that nematodes are increasingly becoming more important especially due to the dynamics of coffee production systems that favour their build-up over time.

Plant parasitic nematodes are considered to be major pathogens of coffee worldwide, causing yield losses estimated at 15 percent. Other losses attributed to nematodes in coffee include; destruction of seedlings in nurseries, yield loss, unemployment in traditional coffee-producing areas, decreased profit margins due to increased costs of production. Root knot nematodes (*Meloidogyne* spp.) are particularly damaging to coffee plantations, with at least 15 species shown to be afflicting all major coffee producing countries. In Colombia, *M. exigua* and *M. Javanica* have caused an estimated loss of US$800 million/year on coffee. In Costa Rica, the attacks of *M. exigua* cause general weakening of the trees with an estimated drop in yields ranging from 10 to 45% (Campos and Villain, 2005; Alpizar et al., 2007). Soil texture has been shown to play a vital role in nematode activities with reports indicating that sandy soils are most favourable for these pathogens (Bertrand et al., 2001). In addition, poor agronomic practices have led to depletion of organic matter thus aggravating damage caused by *M. exigua* on coffee (Etienne et al., 2000). This study was conducted with the aim of evaluating the occurrence and distribution of nematodes in the main coffee growing areas of Kenya.

METHODOLOGY

To assess the distribution of plant parasitic nematodes, a survey was undertaken in the three coffee agro-ecological zones, namely: coffee-tea zone (Upper Midlands 1; UM1); main coffee zone (Upper Midlands 2; UM2) and the marginal coffee zone (Upper Midlands 3; UM3). Anthropogenic factors were visually evaluated by ranking the management practices in coffee farms into high, medium, low and neglected farms. A total of 200 samples were collected. At each sampling point, soil was collected at depth of 5-15cm where the feeder roots of coffee are predominant, with a minimum of six sampling points per farm laid out along a zigzag pattern. The soil collected was composited, placed in a sampling bag, and kept in cool box awaiting delivery to the laboratory. Observable symptoms of nematode infestation were recorded during the sampling. In the laboratory, nematodes were extracted using a combination of centrifugal floatation and the modified Baermann techniques as described by Hooper et al. (2005). The nematodes were fixed, mounted on slides and identified to genus level. All data collected was log transformed (Log(x+1)) and subjected to analysis of variance (ANOVA) using Genstat computer software package (Lawes Agricultural Trust Rothamsted Experimental Station 2006, version 9). Means, when significantly different, were separated using the Fisher' protected LSD test at 5% probability level.

RESULTS AND DISCUSSIONS

Nematodes belonging to thirty (30) genera were found to be associated with coffee (Table 1). Among these, nineteen (19) genera are known to contain plant parasitic nematodes. Nematodes classified as plant parasitic dominated the trophic guild accounting for 64% followed by bacteria feeding nematodes (24%). Among the plant parasitic group, nematodes belonging to the genera *Tylenchulus*, *Meloidogyne* spp., *Pratylenchus* spp. were the most dominant across all the coffee growing areas investigated. Citrus nematodes (*Tylenchulus* spp.) have been shown to be responsible for slow decline, loss of feeder roots, poor vigour and yield reductions of up to 30% in citrus and grapevines (Rahman et al., 2008). In

Colombia, Costa Rica and Brazil, root knot nematodes (*Meloidogyne* species) which show manifestation of galls are the leading nematodes responsible for the slow decline disease of coffee with an estimated drop in yields ranging from 10 to 45% (Campos and Villain, 2005; Alpizar et al., 2007). Lesion nematodes (*Pratylenchus* spp.) are other important nematodes that are associated with coffee all over the world. In this study, it was apparent that many genera and species of nematodes occurred in association with coffee, including potentially damaging nematodes of significance to crop production in Kenyan. Any claim of presence of nematodes on coffee without nematode densities is inadmissible especially due to the non-specific above ground symptoms caused by nematodes (Figure 2). The abundance of plant parasitic nematodes associated with coffee was variable ($P \leq 0.05$) among the three coffee growing zones (Figure 2). Amongst the regions, coffee farms in the coffee-tea zones (UM1) harboured the highest numbers of plant parasitic nematodes, followed by main coffee zones (UM2) and least in the marginal coffee growing zones (UM3). Moisture is one of the abiotic factors known to have profound effects on the distribution of nematodes. Agro-ecological zone UM1 has the highest soil moisture content favouring build up of nematode numbers compared to moisture limited agro-ecozone UM3.

The level of crop husbandry was found to significantly impact on the abundance of nematodes (Figure 3). Farms that were ranked to be highly managed recorded the least numbers of nematodes while those ranked as neglected had the highest numbers of plant parasitic nematodes. Farms that were ranked as highly managed were characterized by weeding and frequent application of manure, which is known to have nematicidal properties. The absence of weeding in the neglected farms could be responsible for the high numbers of nematodes given that some of the weeds offer alternative food sources for the pests. Weeds have for long been recognized for their ability to maintain nematode populations targeted for suppression by various management strategies (Thomas et al., 2005). Besides serving as alternative hosts, certain weeds can protect nematodes from pesticides and the environment, contribute to changes in future nematode biotic potential, or exert indirect effects through competition with crops or by the effects of weed control strategies on nematode populations.

Figure 1. A coffee bush uniquely showing symptoms of poor nutrition (yellowing) due to infection by nematodes (left) and galled coffee feeder roots (right).

Table 1. Mean population densities plant parasitic nematodes associated with coffee in the main production zones in Kenya.

Genera	Rank Abundance	Abundance	Trophic group
Tylenchulus	1	166.6	Plant parasitic
Meloidogyne	2	161.4	Plant parasitic
Tylenchorhynchus	3	159.5	Plant parasitic
Pratylenchus	4	158.0	Plant parasitic
Tylenchus	5	156.4	Plant parasitic
Rotylenchus	6	147.7	Plant parasitic
Hemicyclophora	7	138.5	Plant parasitic
Ucephalobus	8	138.5	Bacterial feeder
Acrobeles	9	138.3	Bacterial feeder
Mononchus	10	136.7	Predator
Rhabditis	11	135.8	Bacterial feeder
Hoplolaimus	12	134.0	Plant parasitic
Aphelenchus	13	132.9	Fungal feeder
Chromadora	14	132.6	Bacterial feeder
Scutellonema	15	130.1	Plant parasitic
Prodorylaimus	16	127.8	Predator
Helicotylenchus	17	125.0	Plant parasitic
Hemicriconema	18	115.8	Plant parasitic
Iotonchus	19	114.5	Predator
Xiphinema	20	113.9	Plant parasitic
Criconema	21	109.5	Plant parasitic
Cephalobus	22	105.7	Bacterial feeder
Aphelenchoides	23	103.5	Plant parasitic
Plectus	24	101.4	Bacterial feeder
Bunonema	25	93.0	Plant parasitic
Trichodorus	26	90.8	Plant parasitic
Paratrichodorus	27	90.4	Plant parasitic
Radopholus	28	89.8	Plant parasitic
Longidorus	29	87.3	Plant parasitic
Alaimus	30	61.1	Bacterial feeder

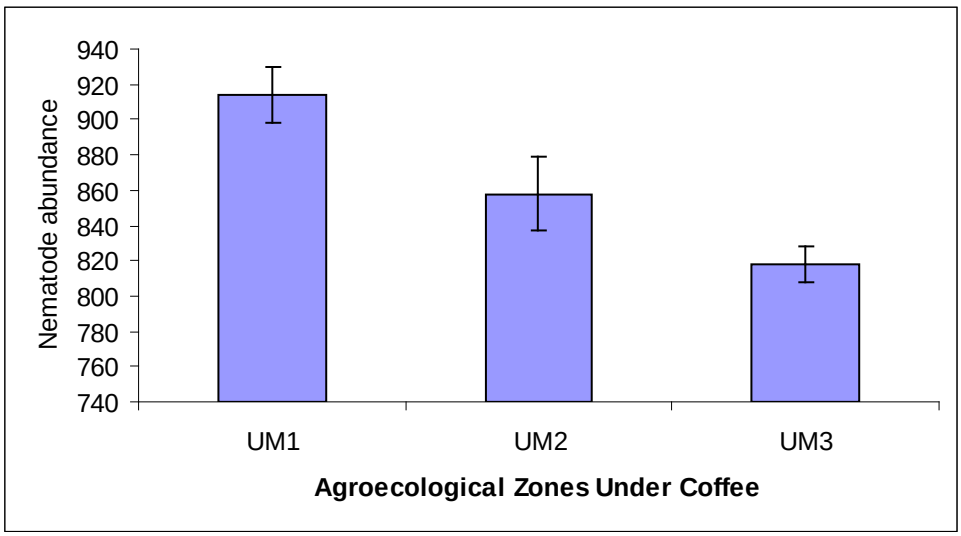

Figure 2. Influence of agroecological zonation on abundance of plant parasitic nematodes.

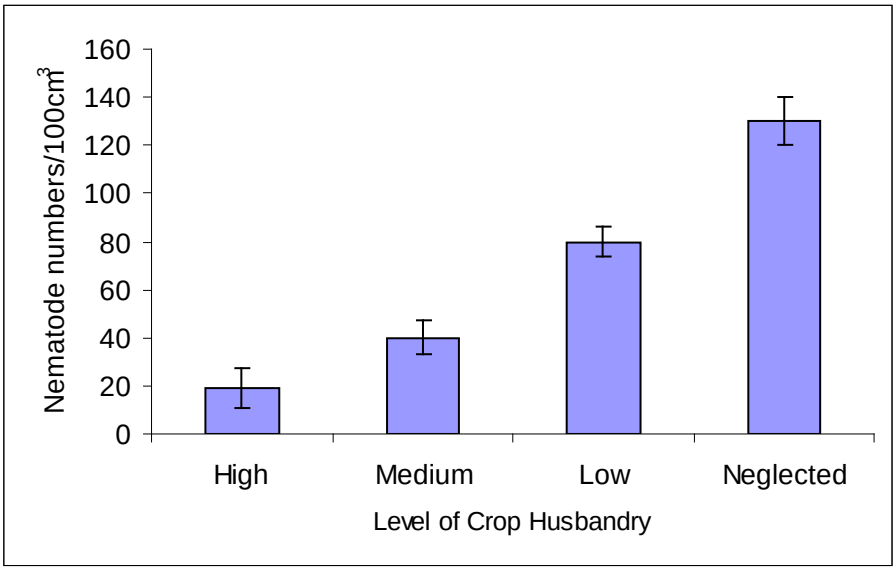

Figure 3. Influence of levels of crop husbandry on the abundance of nematodes in coffee.

CONCLUSION AND RECOMMENDATIONS

The results of this study clearly demonstrated the existence of high populations of plant parasitic nematodes associated with coffee in Kenya. In addition, sedentary stages of the *Meloidogyne* species were isolated from the galled roots. It can be concluded that nematodes do affect coffee in Kenya. This is an area that has not been widely studied and opens a new frontier of studies and concern that with time, it could become a major constraint to coffee production in addition to the other pests and diseases. It is also import to note that overtime, coffee farming system has been changing from the predominant mono-cropping to intercropping due to changes in socio-economic factors. This is expected to affect the below ground ecosystems including nematode populations. It is recommended that detailed studies are undertaken to study the plant parasitic nematode populations that are a threat to coffee in the country, predisposing factors, possible disease complexes with other pathogens such as *Fusarium* and management methods. Studies in this direction are underway in a

multidisciplinary national partnership in Kenya that aims at understanding the effects of cropping systems, soil fertility and nutrition on the populations of nematodes and crop tolerance to the nematodes. In addition, locally available coffee varieties including Robusta coffee are to be evaluated for genetic tolerance to the pathogen. Other possible interventions such as biological, selected chemical and integrated pest management strategies need to be evaluated.

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Population Structure of *Colletotrichum kahawae*, the Causal Agent of Coffe Berry Disease (CBD): Insights from a Multi-Locus Approach

D.N. SILVA[1,2]*, P. TALHINHAS[1], V. VÁRZEA[1], A. LOUREIRO[1], M.C. SILVA[1], O.S. PAULO[2], D. BATISTA[1]

[1]Centro de Investigação das Ferrugens do Cafeeiro (CIFC)/ Instituto de Investigação Científica Tropical (IICT), Oeiras, Portugal
[2]Computational Biology and Population Genomics group, Centro de Biologia Ambiental (CBA), Faculdade de Ciências da Universidade de Lisboa (FCUL), Lisboa, Portugal
*E-mail: diogo_nuno_silva@hotmail.com

SUMMARY

The pathogenic fungus *Colletotrichum kahawae* is the causal agent of the economically devastating Coffee Berry Disease, hitherto restricted to the African continent. The disease was first reported in 1922, Kenya, where the causal fungus was thought to have emerged from mild parasitic forms of the closely related group species *C. gloeosporioides*, and from there it would have spread to most *C. arabica* plantations on the African continent. Understanding the historical and ongoing patterns of population genetic structure as well as the evolutionary history of plant pathogens is vital to answer important questions about their origin and dispersal. Such knowledge can then be used to implement better plant breeding strategies. Previous population genetic studies have shown *C. kahawae* as a nearly clonal population and were unable to unravel any genetic structure of the pathogen's populations, which has impaired possible evolutionary or demographic inferences. Therefore, most of our current knowledge about its origin and dispersal is based on historical data which can be biased and misleading. In order to provide a more accurate view of these events, we developed and tested a new set of gene markers from *β-tub2*, *ITS* and *MAT1-2-1* genes, to detect patterns of population genetic structure and dispersal in a phylogeographical analysis using more than fifty isolates of *C. kahawae* from nine geographical locations. To allow further inferences about the evolutionary history and origin of *C. kahawae*, an additional sampling of *C. gloeosporioides* and other closely related taxa from coffee hosts was assembled. The results confirm the low genetic variability previously detected in *C. kahawae*, albeit revealing a slight but consistent genetic structure of the sampled populations which seems to be correlated with their geographical location. The combined information from the multi-locus analysis was able to discriminate the isolates in three divergent but clonal geographical groups: Angola, Cameroon and East Africa. Interestingly, when the obtained structure was framed in a larger sample containing diverse isolates of *C. gloeosporioides*, the phylogenetic analysis revealed that populations from Angola and Cameroon are ancestral and that East African populations, such as Kenya's, are derived. Continuance of this work will ensure a more comprehensive understanding of the population dynamics of *C. kahawae* and its relation with *C. gloeosporioides*, which may hopefully contribute to the expansion of our knowledge on the evolutionary history of these species.

INTRODUCTION

The importance of coffee for man cannot be overstated for several economical and social reasons (Vega et al., 2003). As a consequence, the production of this commodity can be quite sensitive to disturbances from extrinsic factors on the crops, such as fungal diseases. Moreover, most of the current cultivated coffee varieties worldwide result from a recent domestication period which, despite allowing the creation of highly productive and profitable varieties, has greatly narrowed their genetic variability (Anthony et al., 2002). In practical terms, this homogenizing event is known to foment the emergence and spread of plant pathogens, as it facilitates their adaptation and transmission between infected and uninfected plants (Stukenbrock and McDonald, 2008). Therefore, successful disease control strategies should integrate as much information as possible regarding these evolutionary and demographic aspects of pathogens, addressing the signatures left on their population structure, since this will be critical in assessing their present and future harmful potential.

Coffee Berry Disease (CBD), caused by the hemibiotrophic fungus *Colletotrichum kahawae* Waller & Bridge, is an emergent disease on coffee crops, specifically on the most relevant commercial species, *Coffea arabica* L. (Arabica coffee). It severely affects crops and, when no control measures are applied, it may cause up to 70-80% yield losses (Silva et al., 2006). This disease is relatively recent, as it was first reported in 1922 in western Kenya, where it led to the abandonment of coffee plantations in some regions (McDonald, 1926). Despite the little attention received during its early stages of emergence, African coffee growers soon witnessed a swift spread of CBD throughout most of the African continent (Gordon, 1988). Within a period of roughly 50 years, the presence of the disease had been documented in nearly every region where Arabica coffee is grown, from Ethiopia to Zimbabwe (Firman and Waller, 1977). Nonetheless, the causal agent of CBD is currently restricted to the African continent, more frequently at high altitudes and cool and humid climates, which are also the most suitable habitats for *C. arabica* (Hindorf et al., 1997). Still, an eventual dispersal from Africa to the coffee plantations of Latin America and Asia is cause for great concern and has motivated a growing interest on the evolutionary potential of *C. kahawae* (Sreenivasaprasad et al., 1993).

Several studies have attempted to unveil the genetic diversity and population structure of *C. kahawae*, mostly driven by the information that these factors may provide on the pathogenic potential of the fungus and to take a glimpse on how its populations have evolved and dispersed through temporal and geographical scales. However, these aims have been challenged by the astonishing lack of genetic variability of the pathogen. Molecular techniques such as Restriction Fragment Length Polymorphism (RFLP) of mitochondrial (mtDNA) and ribossomal DNA (rDNA) (Sreenivasaprasad et al., 1993; Bridge et al., 2008), Random Amplification of Polymorphic DNA (RAPD) (Derso and Waller, 2003), Variable Number of Tandem Repeats (VNTR) (Sreenivasaprasad et al., 1993; Bridge et al., 2008), and sequencing of the ITS locus (Sreenivasaprasad et al., 1993; Manuel et al., 2009) have revealed the entire species as a single clonal unit, i.e. in these studies almost no genetic variability was found. Only with the Amplified Fragment Length Polymorphism (AFLP) technique, some variation could be unraveled, though with a very limited sampling (Bridge et al., 2008). In contrast, vegetative compatibility techniques have detected small differences with some indication of geographical specialization (Várzea et al., 2002; Bella Manga et al., 1997) and small but significant differences in pathogenicity, mainly due to aggressiveness, were found (Omondi et al., 1997; Várzea et al., 1993). Nonetheless, no global and consistent structuring has been clearly revealed yet.

The overall genetic uniformity of *C. kahawae* has been attributed to its putative asexual nature and presumably recent evolution from the closely related group-species, *C. gloeosporioides* Penz & Sacc. (Sreenivasaprasad et al., 1993). Although this species complex is a common saprophyte on many hosts, the populations from coffee hosts have been regarded as the most likely candidates behind the emergence of *C. kahawae* (Bridge et al., 2008).

In this work, we made use of a multi-locus sequencing approach with the main objective of reassessing the genetic variability and structure of *C. kahawae* populations at CIFC/IICT collection from across most of its area of occurrence. Secondarily, an opportunity was taken to evaluate the phylogenetic relationships of these populations with *C. gloeosporioides* populations from coffee hosts worldwide.

MATERIALS AND METHODS

Isolates and DNA extraction

A sample of 52 *C. kahawae* and 15 *C. gloeosporioides sensu lato* isolates from the collection maintained at CIFC/IICT were analyzed. Isolates of *C. kahawae* were collected from *Coffea arabica* and spanned 9 distinct countries: Angola (14), Cameroon (7), Burundi (1), Ethiopia (4), Malawi (3), Kenya (9), Rwanda (1), Tanzania (11) and Zimbabwe (2). *C. gloeosporioides sensu lato* isolates were also gathered from *Coffea* spp. hosts from South America, Africa and Asia, except for 3 isolates, which were phylogenetically equivalent to the recently typified *C. gloeosporioides sensu stricto* (Cannon et al., 2008) and were included for purposes of phylogenetic reference. Three samples of *C. fragariae* were included as outgroup taxa to root phylogenetic trees, as recent studies show this species as the most basal lineage of the *C. gloeosporioides* complex (Cai et al., 2009). The isolates were cultured by the procedure described by Loureiro (2008). DNA was extracted using the DNeasy Plant Mini Kit following the manufacturer's instructions.

Molecular data

For this study, three nuclear regions were amplified and sequenced to generate a total of 1910bp of sequence data: the internal transcribed spacer (ITS) region of the ribossomal DNA, *β-tubulin* 2 and *MAT1-2-1* from the mating-type (MAT) locus. Although the MAT locus is usually an inappropriate phylogenetic marker for many fungi, due to the presence of highly dissimilar idiomorphs (MAT1-1 and MAT1-2) at the same locus, all *Colletotrichum* species sampled to date present only a single MAT1-2 copy, making it a suitable marker for this group. PCR amplification generated amplicons of ~500bp for ITS, ~580 for *β-tubulin* 2, and ~843bp for *MAT1-2-1*, which were purified and used as templates for sequencing reactions performed with the BigDye version 3.1 chemistry (Applied Biosystems) on an ABI prism 310 automated sequencer. Amplicons were sequenced in both directions. Multiple sequence alignments were performed in MAFFT v6.717b (Katoh et al., 2009) using the L-INS-i method, followed by manual refinement in BioEdit v7.0.5.1. The Concatenator program Pina-Martins and Paulo, 2008) was used to create a concatenated matrix from the three individual datasets.

Phylogenetic analysis

Phylogenetic trees were constructed from the separate and combined datasets of the three nuclear regions, using the Maximum Likelihood (ML) method. ModelTest (Posada and Crandall, 1998) was used to select the best fit model of nucleotide evolution, under the

Akaike Information Criterion (AIC), for each gene dataset. The ML analysis was run in PAUP* v4.0d99 (Swofford) with heuristic searches of 100 replicates with random sequence addition and Tree-Bisection-Reconection (TBR) branch swapping. Nonparametric bootstraping was also conducted using 100 pseudoreplicates with 10 random additions and TBR branch swapping. For the concatenated dataset, a Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist et al., 2003), which allowed for the different data partitions to be modeled separately using the evolutionary models and informative priors that best described the data. The optimal model selected under the AIC, as implemented in ModelTest, was specified as prior for each gene. Bayesian posterior probabilities were generated with $1x10^7$ generations sampled every 1000 generations. The analysis was run three times, with one cold and three incrementally heated Metropolis-coupled Monte Carlo Markov chains, starting from random trees. $1x10^6$ generations were discarded as a burn-in. Trees were combined and summarized on a 50% majority-rule consensus tree.

RESULTS

The results obtained confirmed the extremely low genetic variability of *C. kahawae* populations throughout most of its range (Nucleotide diversity, Pi: 0.00097; Segregating sites: 3). However, the multi-locus dataset employed in this study was able to reveal three divergent but clonal haplotype groups: Angola, Cameroon and East African groups, with the later including isolates from the remaining seven east African countries (Figure 1a; 1b).

Table 1. Summary statistics for the individual and combined datasets used in this study.

	Sequenced region			
Parameter	**ITS**	***β-tubulin* 2**	***MAT1-2-1***	**Combined**
Nucleotide characters (bd)	489	578	843	1910
Indels	2	30	0	32
Total characters	487	548	843	1878
Parsimony informative	11	70	138	219
% Parsimony informative	**2.25%**	**12.11%**	**16.37%**	**11.47%**
Variable, uninformative	0	4	5	9
Variable in *C. kahawae*	**0**	**2**	**1**	**3**
Model*	TrNef+I	TIM+G	TrN+G	

****** *Best fit evolutionary model under the AIC using the program ModeTest.*

This structuring was provided by intronic (neutral) polymorphisms on *β-tubulin* 2 and a non-synonymous mutation on *MAT1-2-1*, that resulted in the replacement of a serine residue, on the Angola's population, for a proline residue on the derived populations of Cameroon and East Africa (Table 1). Despite small, these differences were consistent across the individual datasets (data not shown) and when combined, revealed a clear population structure that seems correlated with geographical location (Figure 1b). Moreover, migrants could not be detected within the sampling studied, i.e. there was no mixing of different haplotypes in the same geographic location.

The phylogenetic reconstruction using the combined 1910bp dataset provided a good resolution for the relationships between *C. kahawae* and *C. gloeosporioides* and within *C. gloeosporioides sensu lato* (Figure 1a). Regarding the *C. kahawae – C. gloeosporioides* relationship, this phylogenetic framing revealed an ancestral position for Angola's population of *C. kahawae*, with Cameroon and East African as derived populations, supported by both bootstrap and posterior probabilities values. For the segregating sites within *C. kahawae*, the Angola's population shares the same nucleotide sequence at all sites with the ancestral state inferred from the *C. gloeosporioides* sample, thus representing the most ancestral haplotype (Table 2). The Cameroon haplotype has only one derived mutation, while the East African haplotype has derived mutations in all the segregating sites. Furthermore, *C. kahawae* seems to be a very well diverged lineage from the *C. gloeosporioides* isolates from coffee hosts, as revealed by the basal split of these two groups (Figure 1a). In fact, *C. gloeosporioides* isolates from coffee appear to be more closely related to *C. gloeosporioides sensu stricto* representative isolates, found on hosts like *Citrus* sp. and *Olea* sp., than to *C. kahawae*.

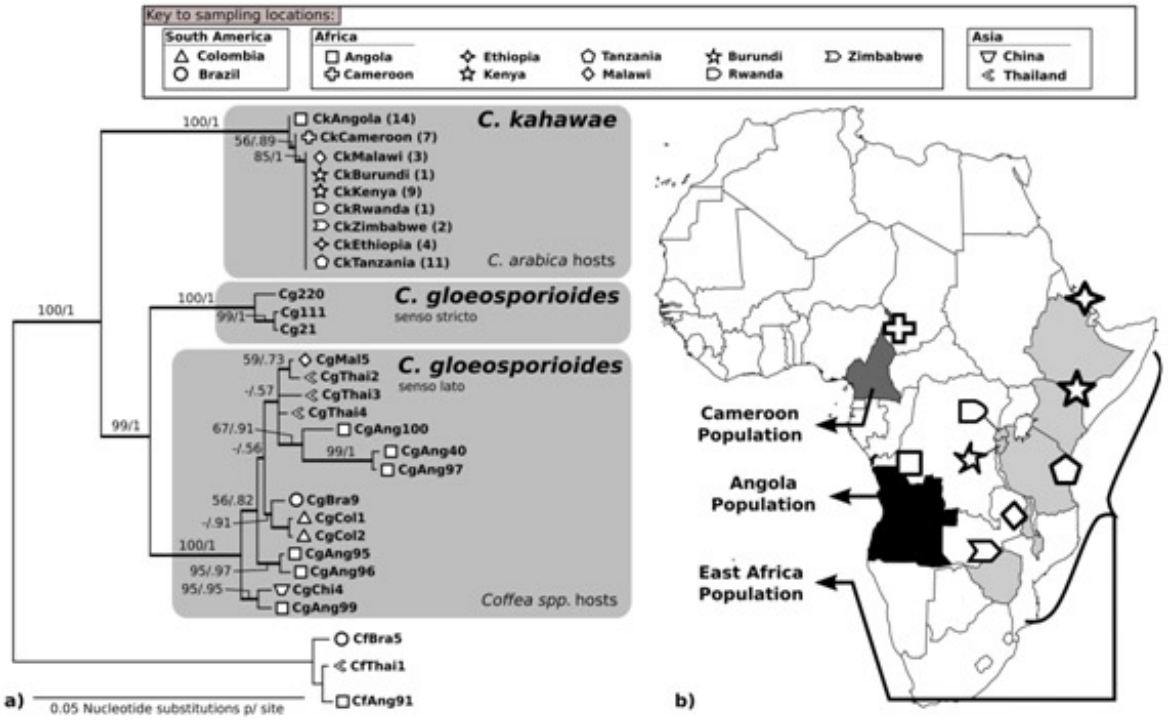

Figure 1. a) 50% majority rule Bayesian ML tree with the concatenated three-gene (1910bp) dataset. Numbers above branches represent ML bootstrap and posterior probability values, respectively. The tree was rooted using *C. fragariae* as outgroup taxa. Symbols next to taxa represent geographic location. The key of the sampling locations is presented on top. In the *C. kahawae* clade, due to clonality within the same geographic origin, only one representative is shown with the total sampled isolates in brackets; b) Geographic representation of the three divergent populations of *C. kahawae*, with respective location key.

Table 2. Sites segregating in the *C. kahawae* sample, shown for *MAT1-2-1* and *β-tubulin* 2 genes.

	MAT1-2-1	***β-tubulin* 2**	
Site position	934	1774	1776
Coding Status*	R	I	I
Inferred Ancestral state**	**T**	**T**	**T**
Angola Population	-	-	-
Cameroon Population	**C**	-	-
East Africa Population	**C**	**A**	**C**

**Coding status: R, aminoacid replacement, I, Intronic; **Ancestral state inferred from the total C. gloeosporiodies sample; - Identity to the inferred ancestral state.*

DISCUSSION

To our knowledge, this is the first report of a consistent and unambiguous population structure of *C. kahawae*, revealed by polymorphisms in nuclear gene markers. These have shown the existence of three divergent populations within the species, very well correlated with their geographical distribution: Angola, Cameroon and East Africa. Our results confirmed previous indications of some geographic structuring, particularly between east and west African populations (Bridge et al., 2008; Bella Manga et al., 1997), and were able to resolve the western populations even further. Despite the usefulness of this information for breeders and plant pathologists interested resistance to CBD, who should integrated these populations whenever possible for more comprehensive tests, this division does not necessarily correlate with pathogenicity. Of the three segregating sites, only one resulted in an aminoacid replacement on the *MAT1-2-1* gene, which may directly influence the fitness of the individuals, but only indirectly their pathogenic potential. This mating-type gene is a known transcription factor involved in sexual development and has been reported in association with virulence in some fungi, such as *Cryptococcus neoformans* (Kwon-Chung et al., 1992).

Furthermore, this unexpectedly organized population structure reveals no evidence of present migration between the geographical locations of each haplotype, or the migration rate is too low to be detected. This might be explained by seldom sequential colonization events with subsequent geographic isolation. Arabica coffee growing areas in Angola, Cameroon and East African are separated by extensive lowland areas, which are not suitable for the pathogen nor the host, thus representing a potential effective barrier to migrants flow (Hindorf et al., 1997). In contrast, the geographic trench of the Great Rift Valley that spans most of the eastern African locations sampled and the higher concentration of Arabica coffee plantations in this region (ICO), provides effective pathways for the pathogen's dispersal. Overall, the amount and distribution of the genetic variation in *C. kahawae* is consistent with a recent emergence and clonal reproduction, which may lower its evolutionary potential (McDonald and Linde, 2002).

In this phylogenetic analysis, some unexpected results were obtained. Based on our dataset and as shown in Table 2, the *C. kahawae* population from Angola seems to represent the ancestral population of the species in our sampling. Assuming this inference, the most likely pathway of dissemination would have been a subsequent colonization of the Cameroon region

and from there, of the east African area. On the other hand, both *C. gloeosporioides* sampled from coffee hosts and the representatives of the type strain from other hosts, formed a monophyletic group and were more closely related to each other than to *C. kahawae*, which would not be expected if it had emerged from them in the near past. The lineage that originated *C. kahawae* seems to have diverged sometime before. Perhaps this finding is only evident here due to the use of much more informative markers than the ITS alone as previous studies have applied (Sreenivasaprasad et al., 1993), which in this study was also insufficiently variable to reconstruct the main groups and topology of the concatenated tree (data not shown).

These results represent an improvement on our ability to discriminate populations within *C. kahawae* and led to a different hypothesis about its origin and dispersal. However, this subject certainly requires a more comprehensive analysis with a broader sampling of both *C. kahawae* and *C. gloeosporioides* isolates from coffee and other hosts as well as more informative markers, before solid conclusion can be taken.

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The Regional Coffee Wilt Programme: Where Do We Go from Here?

N. PHIRI[1], P. BAKER[2], M. RUTHERFORD[2], J. FLOOD[2], P. MUSOLI[3], K. MBUYI[4], D. KILAMBO[5], G. ADUGNA[6], G. HAKIZA[3], F. PINARD[7], G. ODUOR[1]

[1]CABI, African Regional Centre, Nairobi, Kenya
[2]CABI, Bakeham Lane, Egham, UK
[3]Coffee Research Centre, National Agriculture Research Organisation, Uganda
[4]University of Kishansa, DRC
[5]Tanzania Coffee Research Institute, Tanzania
[6]Ethiopia Institute of Agriculture Research, Ethiopia
[7]CIRAD, Nairobi, Kenya

SUMMARY

Coffee Wilt Disease (CWD, *Gibberella xylarioides*) is a serious fungal disease that has caused losses of about $1billion in the Democratic Republic of the Congo, Ethiopia, Tanzania and Uganda since it re-emerged in the 1980s and 1990s. From 2000 to 2007, a regional programme, funded by the Common Fund for Commodities (CFC), European Union (EU), Department for International Development (DFID), and national contributions from affected countries, with scientific collaboration by CIRAD, UCL and CABI, studied many aspects of the disease including its distribution, spread, severity, taxonomy and control. Principal findings of the Regional Programme include: i) CWD is widespread in Uganda and Ethiopia, restricted in Tanzania, and spreading in DRC. It is found too in wild forest coffee, giving rise to concern that it may weaken the genetic base of both Robusta and Arabica genomes. Measures are needed to collect and conserve this material; ii) Two fungal strains exist, one infecting Robusta and the other Arabica. The strain of the current Robusta disease outbreak is very similar to a strain isolated from DRC in 1960. The Arabica strain does not infect Robusta coffee and vice versa. The Arabica and Robusta strains are likely to be co-evolved pathogens, evolving with their respective hosts, close to their respective centres of origin. iii) CWD transmission from infected wood to adjacent uninfected seedlings was confirmed; hence leaving infected wood near uninfected trees in the field is an infection pathway. Healthy seedlings become infected by CWD when potted in soil from around infected trees. Soil can be highly infective for at least three months; at least one year without coffee is advisable to avoid re-infection; iv) Many farmers weed by machete and hoe, which may spread CWD through frequent wounds to the base of the stem. Farmers can remove diseased coffee wood from plots and may sell them as fuel – a major route of transmission that must be stopped by increased training and quarantine inspections. The programme therefore intensified training and created awareness to help in stopping this other modes of transmission for CWD; v) No Arabica tested cultivars displayed mortality less than 20%, suggesting that Ethiopian Arabica CWD could present a serious threat to production in other countries if it spread, as has happened with Robusta CWD. On the other hand, a breeding programme in Uganda has screened thousands of Robusta plants for resistance to CWD. The initial screening produced over 1,500 lines potentially resistant to the disease. Further screening and agronomic trials have reduced this to seven final candidates for release to farmers, hopefully in 2010. Screening studies in Tanzania have resulted in identification of six Robusta clones with resistance to CWD. If CWD is to be effectively controlled, a distinct, long term and proactive

strategy is needed to suppress it in the future. The paper will cover a number of suggestions as to what needs to happen next.

INTRODUCTION

During the last decade of the 20th century, an almost forgotten African fungal pathogen called coffee wilt disease (CWD) or tracheomycosis, resurged to become the principal production constraint for Robusta coffee in Uganda and the DRC. Over the same period, a similar disease of Arabica coffee, present since the 1950s in Ethiopia, became a growing problem.

Why this happened is by turns both a mystery and a tragedy – a mystery because we understand so little about the origins of the disease; a tragedy because such a major outbreak, which has cost hundreds of millions of dollars in lost earnings, could have been avoided.

CWD is of special significance because, unlike other major diseases such as Coffee Leaf Rust (CLR) and Coffee Berry Disease (CBD), it will kill the tree. The first signs of it are a yellowing of the leaves, which then wilt and develop brown necrotic lesions. The leaves then curl, dry up and fall off. This process may start on one part of the tree but eventually it spreads to the rest of the plant. The period between infection by CWD and death of the coffee tree varies from weeks in young plants to eight months in trees more than ten years old, although most affected trees die two to three months after initial symptoms are observed.

In most cases the symptoms start on one side of the coffee stem where the vascular bundles become blocked by a combination of fungal colonization and host responses. These symptoms are confirmed by scraping the diseased stem with a knife. A blue black stain is characteristic of an infected coffee stem.

Once a tree is infected there is no remedy other than to uproot the tree and burn it in situ to reduce the chances of spreading the infection. No new tree should be planted in the same place for at least six months because the soil retains viable spores of the disease.

Thus the arrival of the disease suddenly changes the Robusta species from being, as the name suggests, a strong tree capable of withstanding attack from several diseases – a good bet for smallholder farmers with few inputs – into one that easily succumbs. It can turn coffee from being a source of ready seasonal cash for poor farming families into a liability that represents wasted time and effort.

Many smallholder farmers, if they control coffee diseases at all, are used to dealing with problems on a ‘just-in-time’ basis rather than a ‘just-in-case’ approach. But by the time the farmer realizes that his plot is infected with CWD, it is too late to take remedial action to save any infected trees and other apparently healthy trees in the plot may already be infected as well. This makes the conventional approach to controlling coffee diseases – spraying on appearance of early symptoms – wholly inappropriate in the case of CWD. For this disease then, an entirely different, proactive approach to disease management is abruptly required.

Re-emergence: in the 1970s, a farmer in north east DRC, observed a wilt-like disease of coffee in abandoned plantations around Aketi, about 76 km from Isiro (Kalonji-Mbuyi et al. 2009). Later, the disease was observed on coffee plants during a survey conducted in 1974 and1975 in a number of INERA fields at Yangambi (Kalonji-Mbuyi, 1975). An analysis of the samples collected by Kalonji recovered a strain of *Fusarium xylarioides*, the asexual form of CWD.

Throughout 1980 and 1981, reports of the ONC indicated the presence of a CWD source near the town of Isiro in Haut-Uélé region, Oriental Province. These reports indicated that the disease attacked only Robusta coffee plantations and that both young as well as old established plantation were affected.

Outbreak: the disease then became widespread in Haut-Uélé plantations, progressively spreading to all the surrounding territories. More plantations were abandoned and smallholders became increasingly distressed as they saw their only source of income disappear. The existence of a very marked and decreasing gradient of infection moving away from the triangle formed by Isiro territories, Wamba and by Mungbere in the valley of Nepoko led to the conclusion that this area was the primary source of the infection. An assessment of the CWD situation in Eastern DRC confirmed confirmed the presence of CWD (Flood, 1996). In 1997, assessments indicated that the incidence exceeded 50% on coffee in Haut-Uélé district (Kalonji-Mbuyi & Onyembe, 1996). More recently, CWD has infected plantations in the province of Equator through the district of Mongala on the border with the Oriental province (Kalonji-Mbuji, unpublished, 2007). This paper covers a series of projects that were developed towards the end of the last century, with funding from the CFC, EU and DFID, to improve understanding of the disease and to help find lasting remedies for it. Although a lot was achieved under the RCWP, the uncomfortable fact is that, despite the best efforts of researchers in 10 or more countries, CWD is still a problem which needs further efforts.

The Regional Coffee Wilt Programme (RCWP)

Coffee Wilt Disease (CWD, *Gibberella xylarioides*) is a serious fungal disease that has caused losses of about $1billion in the Democratic Republic of the Congo, Ethiopia, Tanzania and Uganda since it re-emerged in the 1980s and 1990s. From 2000 to 2007, a Regional Coffee Wilt Programme (RCWP) (Figure 1), funded by the Common Fund for Commodities (CFC), the European Union (EU), the Department for International Development (DFID), and governments of participating countries from affected countries, with scientific collaboration by CIRAD, UCL, CABI, and National Coffee Research Institutes of Uganda (Coffee Research Institute, now Coffee Research Centre (COREC)), Tanzania (Tanzania Coffee Research Institute (TaCRI)), Ethiopia (Jima Agriculture Research Centre of the Ethiopia Institute of Agriculture Research (EIAR)), Democratic Republic of Congo (Organisation Nationale du Café (ONC), University of Kinshasa (UNIKIN), Institut National pour l'Etude et la Recherche Agronomiques (INERA), Rwanda (Institut des Sciences Agronomiques du Rwanda (ISAR)) studied many aspects of the disease including its distribution, spread, severity, taxonomy and control. Coffee institutes of Cameroon (Institute Of Agricultural Research for Development (IRAD)) and Cote d'Ivoire (Centre National de Recherche Agronomique (CNRA)) participated in the biological surveys. This paper will mention some of these achievements, however the paper will discuss in more detail the way forward after RCWP and suggestions.

This paper will also draw some general conclusions and lessons learned about the efficacy of the response to the problem and provide suggestions for the next steps after the RCWP.

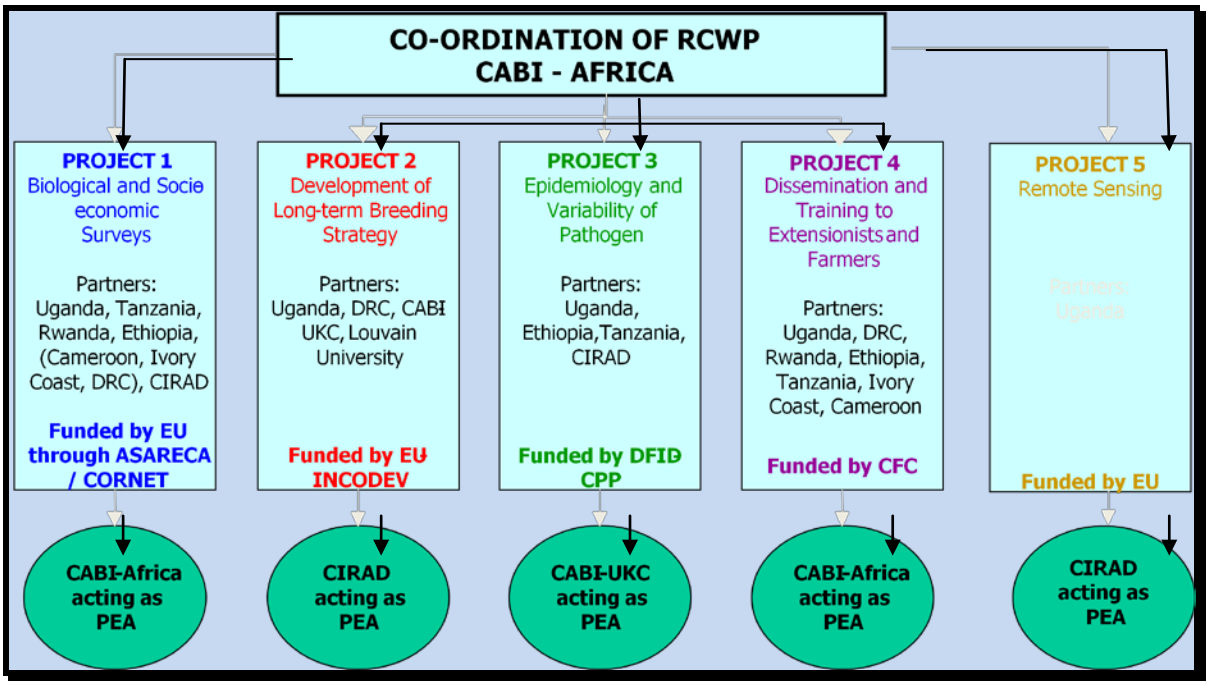

Figure 1. Projects of the Regional Coffee Wilt Programme: relationship between projects, partners and funding sources.

KEY FINDINGS OF THE REGIONAL PROGRAMME

A total of five projects were implemented under the RCWP, which included the surveys to establish the distribution and severity of CWD in Cameroon, Cote d'Ivoire, DRC, Ethiopia, Rwanda, Tanzania and Uganda. A range of the findings were achieved by the Regional Coffee Wilt Programme which ran from 2000 to 2007, however, it is not possible to document these in detail in a paper, especially that the emphasis of the paper is to provide suggestions on the way forward in order to further contain this deadly disease. The RCWP carried out a wide range of activities and represents a major advance in our understanding of CWD. However much further work is required to bring this disease under control and eventually eradicate it. A summary of principal findings is presented below:

The surveys

Coffee Wilt Disease (CWD) is present in four African countries: DRC, Uganda, Tanzania and Ethiopia and absent from the other countries surveyed (Rwanda, Cote d'Ivoire, Cameroon) (Table 1). The CWD epidemic in DRC and Uganda represents the most severe natural disaster ever to befall African coffee, with total losses to small farm incomes exceeding USD$1 billion. The current outbreak of CWD started in DRC where it was first detected in the 1970s. An interesting finding of the surveys is that CWD is also found in wild forest coffee, giving rise to concern that it may weaken the genetic base of the Arabica genome in Ethiopia, where the species evolved. Additionally, the disease is particularly prevalent in plantation coffee, which may be linked to the intensive agronomic methods employed there. Below are a summary from the surveys:

- Through extensive field surveys in Ethiopia, Uganda, DRC, Rwanda and Tanzania, a very wide range of farming practices was found; in most cases coffee was in a poor

state, with very old trees (>50% over 15 years), poor soil fertility and consequent very low yields.

- Many farmers weed by machete and hoeing, both of which may tend to spread CWD; Ethiopian farmers especially control weeds by machete that also causes frequent cuts to the base of the main stem.
- Farmers are highly diversified, nine or more crops per farm is common. At the time of surveys (2001 to 2003), across the countries, most farmers thought CWD was getting worse and that coffee is becoming less important to them. Some farmers diversified to other enterprises except for Ethiopia.
- Farmers clearly found it difficult to understand the nature of the disease and their use of diseased coffee wood for a range of purposes confirmed that many did not understand how the disease is spread.

Table 1. Status of infestation in surveyed countries as of 2007 (no CWD observed in Rwanda, Cote d'Ivoire and Cameroon).

Country	Robusta infected?	Arabica infected?	% infected farms	% trees infected
DRC	yes	no	27	18
Uganda	yes	no	90	45
Tanzania	yes	no	2.2	0.7
Ethiopia	no	yes	28	3

Molecular studies

Powerful molecular identification methods were used to differentiate between the different strains of CWD, which confirm the complexity of the disease. It is likely that CWD was never completely eradicated in DRC after the historical (mid 20th century) outbreak. Arabica, although present, is not attacked by the disease in these three countries. In summary the following are findings from molecular studies:

- The CWD found on Arabica in Ethiopia since 1957 is a different strain which does not attack Robusta and does not interbreed with the Robusta CWD strain.
- **T**he strain of the disease currently affecting Robusta coffee in DRC, Uganda and Tanzania is identical with a strain isolated from DRC in 1960.
- There is no detectable genetic variation in the strain present in DRC, Uganda and Tanzania, making it very likely that the disease spread from a small initial outbreak in DRC, perhaps a single farm.
- The CWD found on Arabica in Ethiopia since 1957 is a different strain which does not attack Robusta and does not interbreed with the Robusta strain.
- A collection of more than 300 purified (monosporic) anamorphic (asexual form) and teleomorphic (sexual form) strains of CWD was assembled throughout the research programme. The collection will serve as a reference library for future studies.

Robusta genotype discoveries

Robusta materials were collected from Ugandan forest sites of Kibale and Itwara and genetic diversity analysed using SSR marker technology. The materials were compared with

cultivated genotypes from Kalangala Islands on Lake Victoria as well as *nganda* and *erecta* phenotypes. Genetic analysis revealed significant differences between the groups collected, and the Ugandan genotypes were found to be sufficiently different from Guinean and Congolese types to represent new, genetically distinct diversity groups within the *C. canephora* genome. The variability inherent in the Ugandan Robusta genome therefore represents a very valuable resource for future breeding programmes. However, in Uganda this valuable genetic resource is under threat from deforestation and CWD itself – it is urgent that steps are taken to collect and conserve this material in safe sites.

Screening tests

Various methods were evaluated to establish a simple, reliable and cost-effective way of evaluating resistance of coffee germplasm to CWD so that mass screening activities could be accomplished. Two methods were found suitable for screening for resistance: dipping of roots into a suspension of spores for about 20 minutes, and scraping the stem of seedlings with an infected scalpel. Standard concentrations and exposure times were established. Spore concentrations as low as 13 spores/mL were sufficient to cause seedling mortality. Collections of wild Robusta from Kilangala and Itwara forests showed a high level of resistance to CWD. Furthermore, in Uganda a very wide difference in susceptibility to field grown clones was found, from 0 to 96% mortality. However, in Tanzanian Robusta material, the highest level of resistance was found in the Maruku germplasm collection. Furthermore, studies at the University of Kinshasa, revealed a range of resistance amongst the tested material with several genotypes showing substantial levels of resistance with levels of mortality less than 10%, five months after inoculation. None of the tested Ethiopian Arabica cultivars displayed a repeatable low mortality (less than 20%), suggesting that presently there are no Arabica genotypes currently available that are highly resistant to CWD. One CWD strain from the historical outbreak (DSMZ62457) was found to cause at least some mortality of seedlings of three *Coffea* species: *C. canephora*, *C. Arabica* and *C. liberica*. However, no currently extant CWD strain exhibits this lack of specificity. It is therefore concluded that the Ethiopian Arabica CWD would present a serious threat to Arabica production in other countries if it spread, as has happened with Robusta CWD.

Breeding a resistant variety

A breeding programme in Uganda resulted in screening of thousands of Robusta plants for resistance to CWD. The initial screening produced over 1,500 lines potentially resistant to the disease. Further screening and agronomic trials have reduced this to seven clones which have been officially released in Uganda.

Field studies

Coffee wilt disease transmission through wounding of coffee with an infected machete was studied in the field and screen house. Some evidence of transmission in the field was seen but further tests are needed as well as improvement in experimental procedure to develop a standard technique that can reliably deliver a dose of infective inoculum. However, its transmission from infected wood to adjacent uninfected seedlings was confirmed in screen house trials, suggesting that leaving infected wood near uninfected trees in the field is a significant infection pathway. In addition, its transmission from infected soil to healthy seedlings potted in that soil was also confirmed, with high infectivity lasting at least three months and declining subsequently, providing evidence that the furrow period after uprooting infected coffee bushes could be much shorter than the previous recommended two year

period. Preliminary results of screening insects as possible CWD vectors resulted in no evidence that they carry the disease, although they were found to be carrying spores of other Fusarium diseases. In related results, other crop and weed plants did not harbour CWD. The pattern of spread of CWD through Robusta plots did not give clear indication of a general spread of infection across fields, which might be expected if the disease was water or wind-borne. There was evidence however that once established in a plot, the subsequent spread of infection is from tree to neighbouring tree, since the disease gradually spreads outwards from initial foci of single-tree infections. Statistical analysis suggests that an infected tree can cause infection to trees up to 10 m distance, i.e. three rows away. Hence eradication of isolated trees in a healthy plot should also include neighbouring trees to stand a good chance of halting an infection.

On-farm trials

In Ethiopia, DRC, Tanzania and Uganda, on-farm participatory trials and classical research station trials were carried out using a number of possible techniques to prevent CWD infection. Through pre-initiation workshops, farmers were consulted about the treatments to be applied and some of their suggestions incorporated into the experiments. The most salient result was that ways to limit the use of machetes or slashers for weeding seemed to have the most effect on lowering incidence of CWD. Fungicide stem applications and herbicide sprays also showed promise in some cases, but may be too expensive for many of the poorest farmers.

Training

A major effort was undertaken in CWD affected countries to train a range of coffee stakeholders, especially extension staff, trainers of trainers, farmers and scientists. A total of 2,578 extensionists were trained as trainers and farmer field school (FFS) facilitators. Training techniques involved class room sessions, participatory group discussions, outdoor on-farm practical sessions (hands-on discovery – based learning), group building exercises, experience sharing by coffee extension staff, and working with farmers. Extension workers in selected CWD hotspot areas and those located in the vicinity of on-farm trials were specifically trained on FFS group extension methodology, in addition to technical training on CWD identification and management. Based on the training of trainers work, slightly more than 1 million farmers were trained by extensionists from 2002-2007 in DRC, Ethiopia, Rwanda, Tanzania and Uganda.

Dissemination

A total of 487,700 items of information materials about CWD were produced during the course of the RCWP, and disseminated throughout coffee regions of Ethiopia, Uganda, Tanzania, DRC and Rwanda. Materials included leaflets, posters, calendars, T-shirts, coasters, pens, and exercise books. Printed materials were produced in a total of 13 languages from the five countries. Over 250 radio broadcasts were made in 5 countries reaching a potential audience of tens of millions. By the end of the project, over 30 scientific papers and conference presentations were prepared. A CWD book was produced which gives the recent scientific information on CWD.

WHERE DO WE GO FROM HERE: WHAT NEXT?

If CWD is to be effectively controlled, a distinct, long term and proactive strategy is needed to suppress it in the future. Three broad recommendations for the management of the CWD are: a) Establishing an international task force to regularly visit coffee countries to carry out farm surveys, collect plant material for subsequent analysis, conduct training and interview plant protection personnel, so that any information of an emerging threat reaches decision-makers quickly and can be acted upon in a timely fashion at minimal cost. This should include countries that are currently CWD-free. We are confident that if such a facility had been available since the 1970s it would have detected the disease at a much earlier date and control measures could have been instigated before it became the billion dollar problem that it has now become; b) Carry out regular socio-economic surveys to continuously evaluate the status of African coffee; and c) Studies of Fusaria on wild coffees and other species to learn more about the origins of the disease. More specific action points to achieve the above include the following.

Quarantine and surveillance

All efforts should be made to ensure that the Arabica CWD does not spread south to Kenya or any remnant wild coffee in the highlands of Sudan, and the Robusta CWD north to CAR or West to Cameroon and beyond. In order to achieve this, the following are recommended: a) regular surveys should be instigated in coffee zones most likely to be invaded to look for signs of the disease; b) An international workshop should be convened to discuss quarantine services in the region with a view to major upgrading of facilities, training and how this can be funded. This would require soliciting relevant authorities and policy makers; c) a contingency plan is needed for special emergencies such as regional unrest or natural disasters, when large populations suddenly move across borders bringing plant material with them. E.g. a rapid reaction team to organize incineration of plant materials brought with refugees when they cross borders.

The Coffee Wilt in Ethiopia

As indicated above, the CWD situation in Ethiopia has a different history and origin to that of Robusta in DRC, Tanzania and Uganda. It is recommended therefore that**:** a) a new and detailed survey to update our knowledge on the status of CWD in Ethiopia, to know how farmers are coping, how much diseased wood is being moved around (especially south towards Kenya) as well as an assessment of quarantine activities at the Ethiopia-Kenya border. Much more needs to be understood about the great range of severity of the disease in Ethiopia and how much of this is related to natural resistance to the infection and how much to agronomic or other factors. A comprehensive breeding programme needs to be initiated to tap on the existing broad genetic base in Ethiopia for the eradication of CWD; b) a workshop with national and international experts to discuss CWD in Ethiopia with the aim to review options and prepare a research, control and/or eradication strategy for Arabica CWD. The disease, if possible, should be eliminated before it escapes Ethiopia to other Arabica coffee producing countries.

Robusta conservation and breeding

The collections made in Uganda in forest areas (currently under threat) reveal a major source of genetic variation of wild Robusta not only for purposes of breeding for resistance to CWD, but also for other breeding traits. As a recommendation: a) Further collections of Robusta

materials from forest areas in Uganda should be made and conserved in Uganda and other countries in order to maximize the probability that this material is permanently available for future breeding activities; b) A conservation plan should be drawn up and funds sought to implement a plan to project Ugandan Robusta diversity – the plan could be developed jointly with other experts in the field conservation, in a form of a meeting or workshop; c) Other Robusta gene pools need to be exploited for generating a range of resistant genotypes.

The Tanzanian experience

CWD was found in Tanzania in 1996 but the subsequent percentage of farms infected and the severity of infection found on farms are both substantially less than in Uganda and DRC. Additionally it has not spread from the relatively small infected zone in the north-west corner of the country – indeed Robusta production has increased in recent years in Tanzania, unlike Uganda and DRC. The reasons for the different experience of Tanzania include great determination by coffee experts and government authorities in uprooting and burning infected bushes. This involved communities coming together and forming clubs for the same purpose, i.e. assisting each other in uprooting and burning infected bushes. Members went to the extent of uprooting diseased bushes for their elderly, sickly or disabled members in the community. Numbers of uprooted bushes were recorded, and this coupled with other strategies seemed to have contributed to the slowdown of CWD It is recommended other countries **learn from the** CWD situation in Tanzania, in particular from a detailed account of the various monitoring and eradication activities carried out in recent years (even beyond the RCWP).

Breeding/selection for resistance

Uganda officially released seven clones, which have resistance to CWD as well as other good agronomic properties. This is a major achievement and requires support to make the resistant clones available to farmers. Tanzania selected six CWD resistant clones from a range of collections at Maruku Robusta collections and fresh collections from the farmers' fields. In DRC studies at the University of Kinshasa screened several genotypes which showed substantial levels of resistance. However, in both countries the number of resistant clones is relatively small and it is uncertain how long they might remain resistant. Efforts need to continue therefore to find more resistant materials to continuously develop resistant lines. It is therefore recommended funds are required to support long term breeding activities in Uganda, DRC and Tanzania in order to be able to continue to produce new lines to broaden the genetic basis of resistance to the disease and closely monitor the field performance of the new clones.

Coffee wilt disease management on the farm

Several interesting results were obtained on possible ways to prevent or slow the spread of the disease at the farm level including mulching, cover crops, and reduced use of cutting implements. However most farmers only have very limited resources and much further work is required to determine which the most cost-effective strategy to employ is. The strategy may vary according to local conditions. It is therefore recommended that multi-site, multi-year on-station and on-farm evaluations of the various and new options to prevent infection in all affected countries should be a continuous process in the affected countries. These could be part of a bigger plan to revitalize African smallholder coffee (see coffee revitalization below).

Molecular taxonomy and pathology

It is suggested that the Arabica and Robusta strains of CWD are in fact separate diseases that arose independently, most likely from undetected disease forms on wild coffee species or even non-coffee species. It is possible that a new outbreak could occur spontaneously at any time in the future. It is therefore recommended that all African coffee zones be regularly monitored to detect new disease events so that they can be quickly contained. It is therefore recommended that a major international effort to develop: a) a rapid test to detect the presence of CWD which can distinguish it from other *Fusarium* species; b) more research to discover genetic markers for CWD resistance in the coffee genome; c) more research to study the genetic variation of the CWD disease complex; d) substantial upgrading of disease recognition and research facilities in Africa.

Project development & management and institutional capacity building

The programme of activities recounted in this paper took much too long to develop and get underway – from the initial surveys in 1996; it took until 2000 to get activities going in the field. A recommendation is for urgent matters such as project development, approval and start-up activities all need to be streamlined. We suggest that a special fund is required that could be sourced to initiate activities quickly without the time lags that are customary in getting a major international project started. It is important therefore that a much greater investment in research and extension is required in all countries. It is important to create a cadre of scientists that can carry out long term field and laboratory studies in all countries, coupled with support to regional centres of excellence, together with a basic level expertise in detection, identification and monitoring activities in all countries, backed by a reliable source of long-term funding.

An African CWD strategy

Coffee wilt disease is now endemic in Africa and a regional decision needs to be made about whether countries will have to live with it or whether it is feasible to eradicate it. At present we are at a half-way house, the research programme reported here has made strenuous efforts to study the disease but the funding is now insufficient to maintain the intensity of activities required to ensure that control can ultimately be achieved in all corners of affected countries. The present efforts by affected countries will not be enough to ensure that the disease is eradicated or reduced to an insignificant level. A further major international and regional effort is therefore required – to prevent spread of CWD to more countries, and consolidate the breeding programmes in affected and countries at risk of CWD. Doing nothing is not a cost-effective strategy. The authors therefore recommend an international conference to review progress and discuss what needs to be done next – essentially the work reported here could be regarded as Phase 1 of the project, Phase 2 needs to develop and roll-out wide-scale control programmes as well as expanded research to follow up on potentially fruitful lines of investigation. Phase 3 could be that to eradicate the disease or arrive at a situation where the problem is maintained under tight control. This could cover multiplication and dissemination of planting material of the resistant clones in Tanzania and Uganda, where possible sharing the resistant varieties with their affected neighbor, DRC. In addition, the phase 2 could also intensify further screening of existing germplasm and those from other countries. This would cover Uganda, Tanzania, DRC (for Robusta) and Ethiopia (for Arabica).

Revitalization

The socio-economic surveys carried out during this project (2001-2003) confirmed that coffee in many places is in very poor shape, characterised by ageing farmers with little knowledge of modern production techniques carrying out only the most rustic management of their coffee with extremely low yields – often only 10% of the yields that Vietnamese or Brazilian farmers can produce. The large number of other crops that they grow attests to the very diversified nature of their farming strategy and suggests a fundamental lack of confidence in coffee to provide anything more than some occasional ready cash. It is important though to mention the fact that a lot happened in training and creating awareness among farmers not only in CWD, but improved coffee farming methods as well from 2003 to 2007, but a follow-up socioeconomic survey was not carried out to come up with the current status. The authors therefore conclude that much of the African coffee that had been studied in this project is fundamentally uncompetitive, and the increasingly erratic climate in East and Central Africa will only add to these problems. Globally however, African coffee has much to offer the world because of its genetic resources and its very low carbon footprint (perhaps less than a quarter of Brazilian or Colombian production). I.e. it is likely that the coffee industry could most easily reduce its carbon footprint by modestly increasing production in Africa. If the coffee industry is to continue to promote its sustainable credentials, it must make a major effort to resuscitate African coffee. The continent that has given coffee to the world and received no royalties in return, the continent that has least contributed to climate change but is becoming most affected by it – African coffee surely cannot be allowed to decline any further. The economic, social and environmental costs are just too high and the public image of coffee will suffer accordingly as this tragedy becomes more apparent in the coming years. It can be summarized that a concerted effort to control CWD that ignores the many other pressing problems of African coffee will not have a major effect in reviving it, since such poorly productive coffee is unlikely to prosper in a world of mounting food insecurity, declining availability of good quality land and an increasingly erratic climate. Farmers will not want to commit to such a crop when the price of food rises and they can turn the land to more productive crops. The principal reasons that we can see for a major revitalization effort are: *Economic* - Coffee originated in Africa where it harbours the vast majority of genetic material that will be needed to face future threats; *Socio-economic* - African coffee brings cash to diverse rural communities where it sustains tens or hundreds of millions of the rural poor. Many African countries are unlikely to be able to develop industrially in the short term – so a new paradigm must return towards increased support for sustainable rural livelihoods, of which coffee is an important element; *Environmental* - coffee production, even when intensive is relatively benign to the environment, alternatives are often less so; *Environmental* - the decline of coffee in Africa inevitably means increasing exploitation of natural resources in Latin America and Asia – African coffee has a low carbon footprint so that moderately increasing production intensity in Africa is the best way to control and even lower the global GHG footprint of coffee. It is recommendation that a major programme of activities to revitalize African coffee is required. This would look at all aspects of coffee growing, and develop a range of activities to develop sustainable coffee strategies for long term revival of the crop.

CONCLUSION

CWD is now a touchstone for African coffee. The resurgence of this disease reveals deficiencies in support structures that need to be remedied if the industry can continue to lay claims to be a modern and sustainable enterprise. Coffee farming has always needed a minimum level of institutional support to flourish, and sufficient flexibility to respond to

urgent problems such as this disease. Such support has weakened over the past two decades and a phenomenon such as CWD is the almost inevitable result of this weakness. The African coffee industry must re-establish an adequate support infrastructure or otherwise expect to continue to degenerate. It is difficult to escape the conclusion that African coffee is going to have to change rapidly – with a completely new approach to growing, commercializing, researching and monitoring coffee. As things stand, there are just too many problems to expect current levels of support to get control of them all. It is, however, unthinkable that we should continue to allow this to happen.

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Progress in Breeding for Resistance to Coffee Wilt Disease (Tracheomycosis) in Tanzania

D.L. KILAMBO[1], N.L. NG'HOMA[2], D.J. MTENGA[1], J.M. TERI[1], N. PHIRI[3]

[1]Tanzania Coffee Research Institute (TaCRI) Lyamungu P.O.Box 3004, Moshi Tanzania
[2]Tanzania Coffee Research Institute (TaCRI) Maruku P.O.Box 127, Bukoba Tanzania
[3]CABI International Africa Regional Centre, P.O.Box 633-00621, Nairobi Kenya

SUMMARY

Coffee wilt disease (CWD) caused by *Gibberella xylarioides* Heim & Saccas, is a serious production problems of Robusta coffee in Eastern and Central African countries. In Tanzania CWD was reported in 1997 in Misenyi District, Kagera region and since its appearance the disease has demonstrated its ability to spread rapidly to new areas and cause serious losses on Robusta coffee. CWD is a threat to Robusta coffee industry and livelihoods of more than 90,000 families who depend on the crop in Kagera region. Current recommendation to manage spreading of the disease is by uprooting, burning, and use of copper based fungicides for stem painting to prevent landing of *G. xylarioides* spores. These approaches limits effective control of CWD as they are both expensive, and use of copper based fungicides may lead to soils copper toxicity. Use of resistant varieties is the cheapest and reliable method for the management of CWD. Search for CWD resistance clones was initiated in April 2004, a total of 875 breeding lines from a collection of *Coffea canephora* germplasm were artificially inoculated with spore of *G. xylarioides* at a concentration of 1.3×10^6, using root dipping procedures. Out of 875 breeding lines, 201 were found to completely resist CWD. In 2006, the 201 completely resistant genotypes were planted in clonal mother garden to raise planting materials for field evaluations. In addition to CWD resistant evaluation, production and cup taste of the 875 lines were assessed continuously between 2001 and 2008. Six CWD resistant Robusta lines were selected out of 201 which were also resistant to leaf rust, productivity range from 0.5 to 3.0 kg of clean coffee per tree and cup taste described as "clean/smooth cup of natural Robusta", were selected for multi-locational evaluation resided in CWD hot spot areas in Kagera region. Eighteen month results have shown that the varieties are still resistant despite being established in CWD hot spot areas. The six breeding lines under evaluation are expected to be released for commercial use in the near future.

INTRODUCTION

In Tanzania coffee is an important cash crop that contributes about $ 117 million to export earning annually (Anon, 2009), and provides employment to 420,000 families. Out of these about 90,000 families are from Kagera region. The major varieties of coffees grown in Tanzania constitute *Coffea arabica* and *Coffea canephora*. *Coffea arabica* is grown in the northern and southern highlands regions while *Coffea canephora* is grown in the western part, mainly Kagera region.

Since its appearance in Tanzania in 1997, coffee wilt disease (CWD) has clearly demonstrated its ability to spread rapidly to almost all Robusta growing areas in Kagera Region (Figure 1). Losses of Robusta trees can be equated to yield produced which is approximately 162,400 kg

of clean coffee lost due to killings of 54,200 from CWD. It is estimated that the disease has been causing a financial loss of approximately US $ 316,200 for over 10 years.

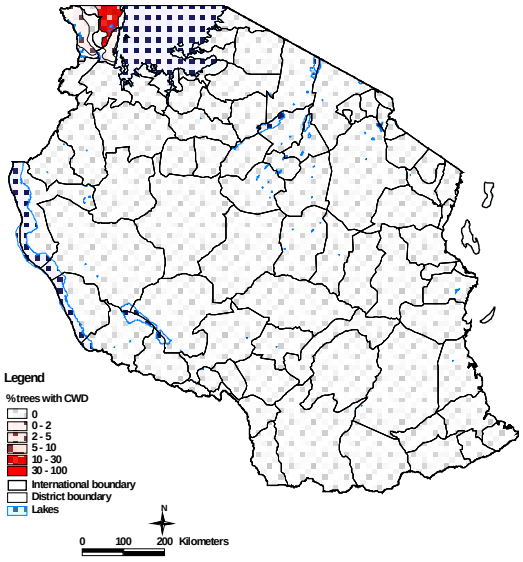

Figure 1. Map of Tanzania showing areas infected by *Gibberella xylarioides.*

The disease attacks plants at all stages of growth and all infected plants are killed (Figure 2). Symptoms include wilting, defoliation and blue black discolouration of the vascular tissues. Coffee berries on diseased plants are mummified and remain attached to branches.

Figure 2. Robusta tree infected by *Gibberella xylarioides*.

Current control method of CWD in Tanzania include; eradication of diseased trees and protection by stem painting as a preventive measures. However these methods are expensive, impractical to implement and do not offer effective control. Host resistance is the only viable control measure. Search for CWD resistance was initiated in April 2004 whereby 875 lines were screened for resistance under screen house conditions. Out of 875 lines 201 were found to resist CWD. Six clones were selected for multi-locations. Progress for their performance is being highlighted in this report.

MATERIALS AND METHODS

Source of clonal seedlings

Shoots were harvested from Robusta germplasm of Maruku, cuttings prepared and raised in propagation boxes. After three month rooted clones were potted ready for artificial inoculation by the spore suspension of *G. xylarioides.*

Pathogenicity test of the *Giberrella xylarioides* isolates

Fourteen isolates of *G. xylarioides* tested for the pathogenicity are listed in Table 1. Susceptible seedlings of MS1 and MS2 each 10 clonal seedlings were root dipped into spore suspension of *G. xylarioides* at 1.3 x 10^6 spores per ml. Days to initial wilt symptoms and dead plants were recorded to determine the level of pathogenicity.

Host-resistance assessment by artificial inoculation of selected clones

Artificial inoculation procedure of using root dip technique was used to assess host-resistance of selected clones. Three to six month old seedlings were removed from the potted soil and their roots cleaned with tap water, then immersed in the standard conidia suspension (1.3 x 10^6 spores per ml) of isolate 2004/1 and removed instantly (Hakiza et al., 2004; Girma, 2004). Seedlings were then carefully re-potted with fresh soil.

Assessment of the inoculated clones

The inoculated seedlings were monitored and data recorded on survived plants (plants with no signs of wilt) for nine months (about 270 days). Percent survivors were calculated per each set of clonal variety. Survivors of the clonal seedlings are presented.

Productivity of the varieties

Yields were recorded from the mother trees of each variety. Ripe cherries were harvested, dried and then processed by removing the husk and parchment skin. Clean coffee of each variety was then recorded from 2000 to 2004 and average determined per tree.

Bean sizes and beverage assessment

Samples for liquoring were harvested from the mother trees sent to liquorers. Bean size percentage was determined by selecting beans in sizes AA, A, B, and PB per sample of 100 g, weighed separately and then calculating percentage of each size per sample.

CWD assessment in multilocational trials

Eight varieties were established in multilocational trials in CWD hot spot areas in November 2008. One site was established in each of the Districts of Karagwe (Omkagandu), Bukoba (Kiilima), Misenyi (Byametemba) and Muleba (Katoke). Performance to assess CWD resistance of the varieties started from the month of establishment in November 2008. Scales used to assess clones were; 1=nil and 2=presence of CWD symptoms.

RESULTS AND DISCUSSION

Pathogenicity studies

Results on the level of pathogenicity of the 14 *G. xylarioides* isolates are presented in Table 1. Mean percentage of dead plants ranged 90-100 % per isolate. Based on seedling death rates there is no significant differences in the level of pathogenicity among *G. xylarioides* isolates collected in Kagera. Isolate 2004/1 was selected to screen the clones because of its stability in culture media.

Table 1. Pathogenicity test results of *Giberella xylarioides* on MS 1 and MS 2.

CWD Isolate acc. No		Location collected			Number of dead seedlings	
TaCRI	**CABI UK**	**District**	**Coordinates**	**Altitude**	**MS 1**	**MS 2**
2004/10	T 1	Muleba	S 01°45.901''; E 31°35.491''	1547 m	9	9
2004/13	T 2a	Muleba	S 01°46.827"; E 31°34.541"	1545 m	10	9
2004/07	T 3a	Muleba	S 01°49.702"; E 31°41.137"	1395 m	9	10
2004/08	T 4	Muleba	S 01°43.159": E 31°38.078"	1510 m	10	9
2004/02	T 5a	Muleba	S 01°41.172"; E 31°37.731"	1287 m	10	9
2004/06	T 8a	Bukoba	S 01°00.595"; E 31°46.582"	1189 m	10	10
2004/01		*Bukoba	S 01°14.836"; E 31°50.682"	1200 m	10	9
2004/12	T 9a	Bukoba	S 01°01.612"; E 31°32.758"	1256 m	10	9
2004/14	T 12a	Karagwe	S 01°18.600"; E 30°47.205"	1424 m	9	9
2004/03	T 13a	Karagwe	S 01°26.166"; E 30°52.801"	1317 m	10	9
2004/05	T 14a	Karagwe	S 01°17.308"; E 30°53.896"	1659 m	9	9
2004/09	T 15a	Karagwe	S 01°15.309"; E 30°57.347"	1354 m	9	9
2004/09	T 15b	Karagwe	S 01°15.309"; E 30°57.347"	1354 m	9	9
2004/09	T 15c	Karagwe	S 01°15.309"; E 30°57.347"	1354 m	10	10
Mean SE ± C.V L.S.D					9.57 0.14 5.30 0.30	9.21 0.11 4.50 0.23

Robusta clones survived CWD inoculation test

Results of Robusta clones survived after artificial inoculation of *G. xylarioides* using root dip technique are presented in Figure 3. Seedlings of commercial variety MS1 were completely whipped out at 270 day. Clones ML2, KR23, BK27, 1/62 and 13/61 survived at 100% at 270 day. However proportional seedlings of clones NG10, NG20 and MR10 indicated that they are good sources of resistance to CWD (Figure 3). Artificial inoculation of *G. xylarioides* enabled segregation of resistant and susceptible accessions of *C. canephora* in Uganda (Musoli et al., 2000). Genotypes which are completely resistant to CWD if they are combined with other attributes such as leaf rust resistance, productivity and cup taste, it will facilitate progress in breeding programme to release promising varieties to coffee growers.

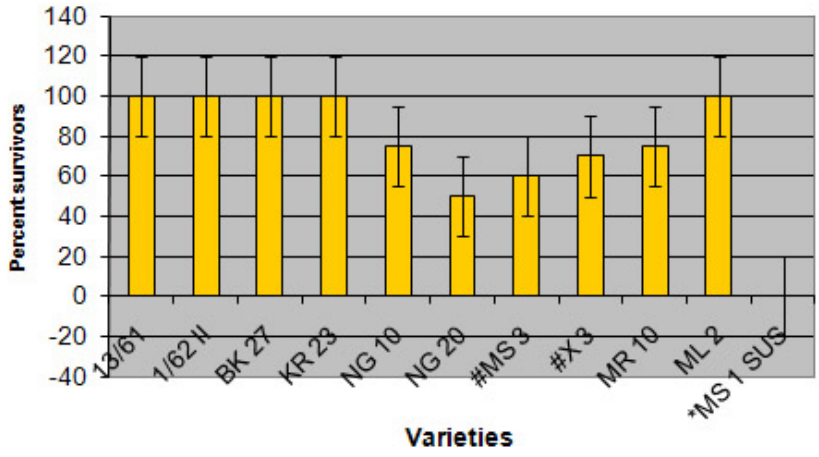

Figure 3. Robusta varieties survived artificial inoculation with G. xylarioides after 270 days. Key: ≤50 % survivors considered good sources for CWD resistance # and/ or *Commercial Robusta varieties.

Productivity of clones

Results on the productivity of the varieties per tree are presented in Figure 4.

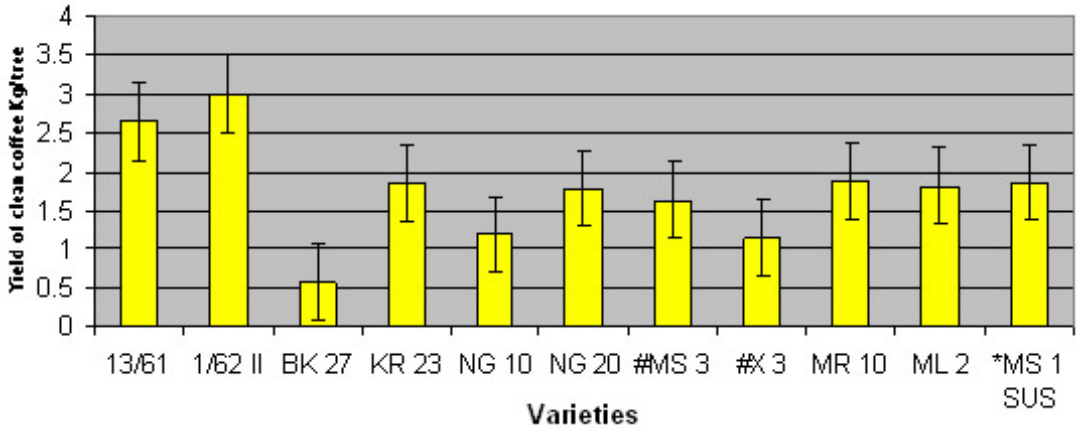

Figure 4. Mean productivity of Robusta varieties tested for their resistance to CWD for five years. Key: # and/ or *commercial Robusta clones.

Data collected on-station showed that clones 13/61, 1/62, KR23, NG20, NG10, MR10 and ML2 produced more than 1.5 kg of clean coffee per tree which is double or triple to what farmers realize in their fields. Clone MS1 a susceptible variety to CWD also produces more than 1.5 kg of clean coffee but on the other hand is susceptible to CWD.

Assessment on Productivity of selected clones

Summary of results on berry clusters counted from two years old plants of selected clones established in trial sites are summarized in Figure 5. With the exception of selected clone KR23, the rest of selected clones produce higher number of berry clusters than MS1. This is a reflection of higher productivity of selected clones.

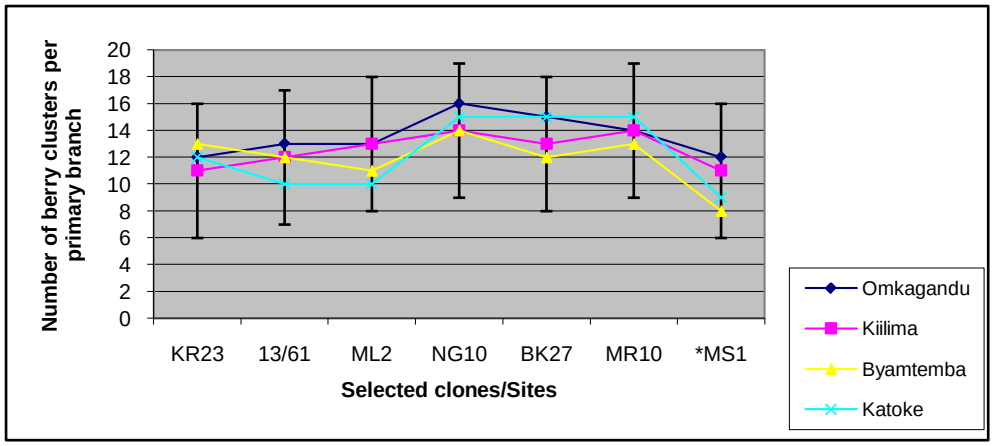

Figure 5. Performance of selected Robusta clones on the number of berry clusters per primary branches.

Beverage assessment

Generally Robusta coffee is known to produce a bitter taste. Test results of the varieties to beverage assessment and their bean sizes are summarized in Table 2 to 4.

Table 2. Summary of the bean sizes and beverage assessment of four selected Robusta clones 2007.

S. no	Variety	Description of the cup taste	Comments
1	KR 23	Good acidity, good body, smooth after taste, less characteristic bitter Robusta taste.	Good Robusta Flavour
2	NG 10	Light acidity, Good body	FAIR Robusta flavour
3	MR 10	Good body, fair acidity, very common, greenish	About Fair Robusta Flavour
4	ML 2	Lacking acidity, full body	Poor to Fair Robusta flavour
5	*MS1SUS	Light medium acidity, Light medium body	About Fair Robusta Flavour

Beverage assessment of the tested samples of the clones indicated that the clones have beverage qualities suitable for the market (Table 2 and 3). The description ranged from poor to fair, about fair and fair Robusta flavour. Typical natural Robusta coffee is the description of some of the samples (Table 4). Moschetto et al. (1996) and Ky et al. (2001a) described presence of diversity in the cup taste within genetic groups of *C. canephora* in terms of aroma, acidity, body and bitterness; to range from excellent to average cup taste.

CWD assessment in multilocational trials

Usually seedlings of a susceptible variety to CWD begin to get infection 3 month after establishment in diseased areas. Varieties under evaluation were established in CWD hot spot areas in multilocational trials in November 2008. By August 2010 different reactions to CWD infection was expected. Table 5 shows that among the tested clones, clones KR23 and NG10 had plants with CWD infection at Kiilima. MS1 the check variety was infected in almost all

the sites. As there are some plants of selected clones still surviving, it shows that careful selection of Robusta clones done in the germplasm would perform successfully under field conditions (Musoli et al., 2008).

Table 3. Summary of the bean sizes and beverage assessment of four selected Robusta clones 2009.

S. no	Variety	Bean sizes			Description of the cup taste	Comments
		Screen 16	Screen 14	Screen 13		
1	KR 23	96	2	2	Some acidity, good body, smooth after taste, less bitter, no taint, FAIR Robusta flavour	Good candidate
2	NG 10	92	5	3	Light acidity, Light body, not common and not harsh, about FAIR Robusta flavour	Average candidate
3	MR 10	98	2	0	Good body, fair acidity, about FAIR Robusta flavour	Good candidate
4	ML 2	94	5	1	Lacking acidity, full body, Poor to Fair Robusta flavour	Average candidate
5	*MS1SUS	88	10	2	Light acidity, Light body, Poor to Fair Robusta flavour	Average

Table 4. Summary of the beverage assessment of four selected Robusta clones 2010.

Sample name	Description	Remarks
MR10	Typical natural Robusta coffee	Neutral cup
NG10	Natural Robusta coffee	Clean cup
BK27	Natural Robusta coffee	Clean/smooth cup
13/61	Smooth cup. Balanced cup, nice aroma like mild arabica	Clean cup
KR23	Fair Robusta flavour	Clean cup
ML2	Clean cup, Typical natural Robusta coffee	Clean cup
*MS1	Bitterness, unusually Robusta acid	Average cup

*Key: *Control*

Table 5. Performance of the clones to CWD resistance in the four multilocational trial sites.

Varieties	Resistance performance of Robusta clones to CWD resistance			
	Omkagandu-Karagwe	Kiilima-Bukoba	Byamtemba-Misenyi	Katoke-Muleba
13/61	1	1	1	1
BK 27	1	1	1	1
KR 23	1	2	1	1
NG 10	1	2	1	1
MS 3	1	1	1	1
X 3	1	1	1	1
MR 10	1	1	1	1
ML 2	1	1	1	1
*SUS MS1	2	2	2	2

Key: CWD scale 1=nil symptoms, 2=presence of symptoms

CONCLUSION

The study shows that there are good clones of *C. canephora* consistently indicating resistance to CWD from the screen house to the field. The clones have also higher productivity and of accepted beverage. The clones are 13/61, BK27, MR10 and ML2. Also the pathogenicity test performed indicated that there is no pathogenic variation among *G. xylarioides* strains isolated from diseased Robusta coffee in Tanzania. It is expected to evaluate more accessions within Robusta germplasm to identify useful clones for breeding advancement.

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Nematode Diversity after Forest Conversion into Coffee-Based Agroecosystems

I. GEDE SWIBAWA[1], K. HAIRIAH[2], D. SUPRAYOGO[2]

[1]University of Lampung, Lampung
[2]University of Brawijaya, Malang, Indonesia
Email: igswibawa@yahoo.com

SUMMARY

Forest conversion into agricultural land use systems lead to the decline of vegetation diversity and land canopy cover, subsequently it followed by changes in soil environment due to less litter input and lower canopy cover. The changes in the soil environment include soil moisture, food availability for soil biota, and below-ground biodiversity. Nematodes are sensitive soil biota; they may be affected by the soil environmental changes. Simplification of soil food web and low soil biodiversity can enhance the population density of plant parasitic nematodes. This research was conducted to answer four questions i.e.: (1) How did agricultural land use systems (LUS) affect the community of soil nematodes?; (2) Could the shade-tree species of coffee in agroforestry system reduce the abundance of plant parasitic nematodes?; (3) Do plant parasitic nematodes abundance link to changes in soil moisture or litter input? (4) How could plant parasitic nematodes (pest) be controlled in the coffee field? The landscape level survey was conducted in CSM-BGBD (Conservation and Sustainable Management of Belowground Biodiversity) Benchmark Sumberjaya, West Lampung (Indonesia), from January to February 2004, while the plot level survey at the same site and greenhouse experiments were conducted in February 2007 to February 2008. The results showed that the diversity and abundance of soil nematodes in Sumberjaya landscape were high, with a total of 103 nematode genera and a total number ranged 190-636 individuals/300 cc of soil. Forest conversion into agricultural coffee-based system decreased the diversity and abundance of free-living nematodes, but increased the abundance of plant parasitic nematodes. Nematode diversity decreased from 61 genera in the forest to around 33 genera in young monoculture coffee system (1-3 year-old), while the relative abundance of plant parasitic nematodes increased from 16% (in forest) to around 80% in the young monoculture coffee system. Those data also supported by the higher ratio of plant parasitic or free-living nematode over maturity index (PPI/MI) in young monoculture coffee (around 9.8) than that in the agroforestry coffee (PPI/MI = 3.0). The abundance of plant parasitic nematodes in agroforestry coffee was influenced by shade-tree species. Planting banana as shade tree of coffee increased abundance of plant parasitic nematode especially *Radopholus*, but provided direct yield to the farmer. Using *Gliricidia* as shade-tree in coffee-based agroforestry system reduced abundance of plant parasitic nematode from 708 individuals/300 cc of soil (as found in 3 years old monoculture coffee to 82 individuals/300 cc of soil in 3 years old of coffee in agroforestry system). The density of shading indirectly affected the abundance of the plant parasitic nematodes through variation of soil water content. Shading level of 40% and soil water content of 70% (relative to field capacity) facilitated optimal development of plant parasitic nematodes. On the other hand, increasing litter input can suppress the plant parasitic nematodes. The agroforestry system with higher tree species diversity can reduce population density of weed, while maintaining thick surface litter enhance the population of predator and free-living nematodes and suppressed plant parasitic nematodes.

INTRODUCTION

Lampung province is one of the centers of coffee production in Indonesia. Most of coffee fields in Lampung especially in West Lampung are built by forest conversion. About 70% of forest area in Sumberjaya (West Lampung, Indonesia) was converted to coffee garden in year of 1970-2000 (Verbist et al., 2004).

Forest conversion into agricultural land use system often followed by the decline of environmental quality. Forest conversion to coffee-based agroforestry leads to rapid loss of the litter layer and a decrease in the rate of litter fall, increase soil moisture, reduced food for ecosystem engineers such as earthworms and reduced termite diversity (Hairiah et al., 2006; Dewi et al., 2006; Aini et al., 2006; Susilo and Aini, 2005). With time, however, a new litter layer is created which potentially form forest-like conditions at the soil surface. The forest conversion into coffee system can change community composition of soil nematode because the biota is very sensitive to soil environmental change. Coffee system with poor soil organic matter can reduce abundance of free-living nematode group and promote the increase in population density of plant parasitic nematode. A dominant plant parasitic nematode species may change their status into severe pest that threat the coffee production. The plant parasitic pest problem usually occurs on crop grown on infertile soil or crop with nutrient deficiency (Desaeger et al., 2004).

The objectives of this research are to evaluate the effect of forest conversion into coffee garden on soil nematode community and to study the link between the changes in soil environmental factors and plant parasitic nematode abundant on coffee-based agroecosystem.

MATERIALS AND METHODS

This research is a part of activity of *Conservation and Sustainable Management of Below-Ground Biodiversity (CSM-BGBD) Project, Indonesia Universitas Lampung*. Two steps of activities performed were inventory (survey) of nematodes diversity of various land use types, and a semi-controlled as well as controlled (greenhouse) experiments. Inventory studies were conducted during rainy season in 2004 and 2007, while the semi- and controlled- experiments were conducted in 2007-2008. The study was located in Way Besai catchment area, Sumberjaya, West Lampung (Indonesia) bounded by S 4°64' - 5°10' and E 104°15' - 104°20', where coffee gardens are commonly practiced. The site is part of Bukit Barisan Mountain with altitude 600-1718 m above sea level, and slope 16-45%, with *Oxic Dystrudept* soil type, dominated by clay fraction with pH H_2O 4.0-5.0. A mean annual rainfall of 2614 mm, an average daily air temperature of 21.2 °C, and relative humidity in the range of 81-89% (Dariah et al., 2004).

Five land-use systems (LUS) were compared: natural forest, disturbed forest, shrub or grassland, coffee-based agroforestry, and sun (monoculture) coffee. Sampling at landscape level was done according to CSM-BGBD standard sampling method; twelve soil cores were collected at 0-20 cm depth in each sampling point (Huising et al., 2008).

At plot scale nematode community survey was conducted in five types of smalholder offee-based systems i.e.: (1) monoculture coffee one years old, (2) monoculture coffee 3 years old; (3) coffee shading with *Gliricidia* tree, 3 years old, (4) agroforestry coffee system contain banana tree > 5 years old, and (5) agroforestry coffee system contain avocado tree > 5 years old. Soil core at 0-30 cm depth was collected from every quadrat 20 m x 10 m sampling unit wich were selected randomly in the coffee field. Nematodes were extracted from each

collected soil sample and identified according to CSM-BGBD method (Cares and Huang, 2008).

To study the effect of shading intensity on plant parasitic nematode, a semi-field experiment was conducted in Sumberjaya, West Lampung. The treatment was performed on Arabica coffee nursery 1 m x 1 m. Treatments were arranged in a factorial experimental block design. The first treatment was shading density and the second factor was fertilizer input. Nematode community and soil water content were indentified and measured from each plots. Two greenhouse experiments were done in Universitas Lampung. The objective of the first experiment was to study the effect of cropping pattern and application of inorganic N, P and K fertilizer of coffee seed on plant parasitic nematode population, while the second experiment is to study the effect of several qualities of litter and inorganic (N, P and K) inputs of coffee seed on plant parasitic nematode abundant. Factorial complete, randomized design (CRD) with three replications was applied on first experiments. A three month old seed of *Coffea arabica* var. kartika-1 was planted on pot 30 l capacity filed by sterile soil. The first factor is cropping pattern consisting of five levels i.e. (1) monoculture system of coffee, (2) simple mixed coffee system (coffee + banana), (3) simple mixed coffee system (coffee + *Gliricidia*), (4) complex mixed coffee systems (coffee + *Gliricidia* + avocado), and (5) complex mixed coffee systems (coffee + *Gliricidia* + avocado + mahogany). The second factor is application of inorganic N, P, and K fertilizer that consists of three levels i.e. (1) control, (2) one rate of recommendation dosage of 5 month old coffee seed and (3) two rate of recommendation dosage. Arabica coffee nursery in the pots as a experimental units for the second experiment is designed by factorial complete, randomized design (CRD) with three replications. Experiental unit was 25 l capacity of pot filled unsterilized mix of soil + sand (2:1) and inoculated with *Radopholus*. The first factor was litter inputs that consist of six levels i.e.: (1) without litter (control), (2) input single *Gliricidia* leaf litter, (3) input single coffee leaf litter, (4) input single avocado leaf litter, (5) input mix of coffee +*Gliricidia* leaf litter, and (6) input mix of coffee + *Gliricida* + avocado leaf litters. The second factor is inorganic (N, P, and K) fertilizer application consisting of two levels i.e.: (1) without fertilizer (control) and (2) application of inorganic (N, P and K) fertilizer at 1 rate of recommendation dosage.

RESULTS AND DISCUSIONS

Based on laboratory isolation and extraction of soil collecting from five land-use systems (LUS) showed that 103 nematode genera of 29 families were found from West Lampung Area. Natural forest conversion into coffee systems in the area tends to decrease nematode diversity. Nematode diversity in natural forest and agroforestry system is higher than that in young monoculture coffee or shrub systems. Generic number of nematodes in forest and agroforestry coffee systems was in a range of 53 to 61, whlie in monoculture young coffee was between 31 to 35 genera (Table 1). The high soil nematode diversity can be explained by high diversity of vegetation in the natural forest and in coffee-based agroforestry systems with higher litter thickness on soil surface which important for maintaining soil moisture (Hairiah et al., 2006).

Diversity of soil nematode community can be indicated by Shannon and Simpson's diversity indices. Both of Shannon and Simpson's diversity indices for nematode community on forest and agroforestry coffee systems were higher than that in young monoculture coffee. The highest of the two indices occurred on shrub systems. Nematode community diversity index in shrub is also higher than that ini forest in New Zeeland (Yeates, 1996). High Shannon index indicated high species (or genera) diversity (Krebs, 1985), while low Simpson's index

indicated community is dominated by certain species (Pielou, 1977). The result of this survey show that young coffee monoculture has low nematode diversity, but some genera of nematode community are dominant, as was indicated by low Shannon and Simpson's diversity indices of young coffee system.

Table 1. Characteristic of nematode community on several land use systems in West Lampung.

Nematode Community Characteristic	Land Use Systems					
	I	II	III	IV	V	VI
Diversities						
Genera number**	61	53	45	36	31	55
Shannon Index	1.98	1.79	2.14	1.25	0.85	1.71
Simpson's Index	4.56	5.53	6.25	3.0	1.79	5.18
Abundance						
Total of individual (indiv/300 cc of soil)	260	267	637	407	674	241
Plant Parasitic (%)	18,6	13,8	59,2	75	87	70,5
Bacterian Feeders (%)	70.6	59.6	34.8	21	7	18.5
Fungal Feeders (%)	1.2	1.0	3.4	2.0	4.0	4.0
Predators (%)	1.8	3.6	1.8	1.0	1.0	4.0
Omnivores (%)	1.0	3.6	2.6	1.0	1.0	3.0

*Notes: ** = commulative number from all sampling point, I = Natural forest, II = disturbed forest III = Shrub, IV = Young monoculture coffee (1 yr), V = Young coffee monoculture (3 yrs) VI = Agroforestry of coffee (> 5 yrs).*

Forest conversion into coffee system enhances plant parasitic nematode abundance. Relative abundance of plant parasitic nematodes in forest (16%) increased to around 80% in the young monoculture coffee. That was contrasted with free-living nematodes; relative abundance of bacterial feeding nematodes in forest decreased from 70% to 7% on young coffee 3 years old (Table 1). Agroforestry coffee system has similar environmental characteristic with forest and they have ability to suppress plant parasitic nematode population. Plot scale survey result showed that *Helicotylenchus* was dominant in plant parasitic nematode community of young coffee systems. *Helicotylenchus* population in agroforestry coffee systems was only 40 individual/300 cc of soil, while the population in young monoculture coffee 3 years old, was as much as 515.6 individual/300 cc of soil (Table 2). Open area with high density of weed might be important factor for the high nematode population on young coffee system. Correlation analyzes showed that plant parasitic nematode *Helicotylenchus* population was positively related with weed biomass (r = 92).

Maturity indices are sensitive to indicate agroecosystem condition (Bongers, 1990). In this study we calculated the plant parasitic maturity index (PPI) to evaluate the suitability of agroecosystem to plant parasitic, while evaluation on intensity of disturbance in agroecosystem was done by calculating free-living maturity index (MI). Calculation on ratio of PPI/MI index indicated soil fertilization that supported plant parasitic nematodes (Bongers, 1998). Based on the result of plot scale survey, MI was significantly different ($P < 0.01$)

among coffee systems. The highest PPI value (2.5) was found in young monoculture coffee 1 year old. It means that young monoculture coffee was more favorable to plant parasitic than others. The young coffee was favorable to plant parasitic nematode can also be indicated by the high value of PPI/MI ratio (9.8). In contrast, coffee-based agroforestry system was not favorable to plant parasitic nematodes; the value of PPI/MI of this coffee system was only 3.1. Free-living nematode maturity index (MI) was also siginificantly affected ($P<0.001$) by type of coffee systems. MI value of young (3 years) monoculture coffee field (0.34) was lower than that of agroforestry coffee system (0.87). That indicated, that environmental disturbance intensity in young coffee was higher than that in agroforestry coffee system.

Table 2. Population density of various plant parasitic nematodes (individual/300 cc of soil) on several types of coffee-based systems in West Lampung.

Plant parasitic nematodes	Coffee Based Systems				
	I	II	III	IV	V
Paralongidorus	3.10 b	1.2 b	1.2 b	2.1 b	10.9 a
Criconemella	81.4 a	5.8 b	17.1 b	6.6 b	7.4 b
Ditylenchus	2.10 a	0.1 c	1.0 ab	0.1 bc	0.1 c
Helicotylenchus	68.9 b	516 a	20.9 b	62.0 b	21.5 b
Tylenchus	4.70 bc	2.4 bc	1. c	9.9 b	25.2 a
Meloidogyne (JI2)	1.50 b	7.0 b	0.5 b	5.9 b	23.3 a
Others genera[@]	18.2 a	8.6 bc	7.4 c	18.9 a	16.8 ab
All Genera	358.4 a	708.4 a	81.8 c	184.6 b	219.8 b

Notes: [@] = others genera pooled ; I = young monoculture coffee 1 yr, II = young monoculture coffee 3 yr, III = Gliricidia shade coffee 3 yr, IV= agroforestry coffee with banana > 5 yr, V= agroforestry coffee with avocado > 5 yr; data at a row folowed by same letter not different by LSD test at 5% siginificantly level.

Tree species used for shading coffee affected the population of plant parasitic nematode on coffee systems. Result of survey at plot scale showed that using *Gliricidia* as shade tree of coffee reduced plant parasitic population density. The population of plant parasitic nematode in young coffee (3 years), 708 individual/300 cc of soil, was significantly higher than in coffee shade with *Gliricidia* systems 3 year old (82 individual/300 cc of soil) (Table 2). Further study in greenhouse indicated that intercropping banana and coffee increased population of plant parasitic nematode *Radopholus*. The nematode *Radopholus* population in simple mixed coffee with banana cropping pattern, around 328.4 individual/300 cc of soil was higher than that in others coffee cropping pattern (95-136 individual per 300 of soil) (Table 3).

The application of inorganic (N, P and K) fertilizer to coffee also increased the plant parasitic nematode *Radopholus* population. Coffee applied with twice rate of recommendation dosage of the fertilizer N,P and K increased *Radopholus* population from 90 to 198 individual/300 cc of soil (Table 3).

Table 3. Population density of plant parasitic *Radopholus* sp. in various planting patterns coffee applied by different strategy of fertilizer application.

Treatments	Population density (indiv/300 cc of soil)
Cropping Patterns of Coffee*	
Monoculture	97.5 b
Simple mix (coffee + banana)	328.4 a
Simple mix (coffee + *Gliricidia*)	95.0 b
Complex mix (coffee + *Gliricidia* + avocado)	88.5 b
Complex mix (coffee + *Gliricidia* + avocado + mahogany)	136.9 b
LSD 0.05	4,02
Aplication of (N, P and K) Fertilizer*	
Control	89.6 b
1 x of recommendation dosage	139.1 ab
2 x of recommendation dosage	197.6 a
LSD 0.05	2.04

*Notes: Data were √ (x+1) transformation, ** = very significant, * = significant, number followed same letter not different of LSD test at 5% significantly level.*

Table 4. Relative abundance of *Radopholus* and feeding groups of nematode on coffee seed inoculated by *Radopholus* and treated with several kinds of litter input.

Leaf litter inputs	Relative abundance of nematode (%)					
	Rado.	Plt. Par.	Bact.	Fungi.	Pre#	Omni
Without litter (control)	20.0 a	15.3	38.3 bc	21.3 b	4.4 bc	1.4
Gliricidia	6.3 c	6.7	49.3 a	32.3 a	1.3 c	4.1
Coffee	13.3 ab	8.0	41.5 ab	26.0 ab	4.1 bc	2.6
Avocado	17.7 ab	7.7	29.7 c	11.3 c	25.3 a	4.1
Coffee + *Gliricidia*	17.0 ab	9.7	38.3 bc	21.7 b	8.7 b	1.4
Coffee+ *Gliricidia* + avocado	12.0 bc	5.0	41.3 ab	31.7 a	9.3 b	0.0
LSD 0.05	6.90	ns	8.9	8.9	1.5	ns

***Notes**: # = data transformed to √ (x+1), ns = not significant, data in a colom followed by same letter not significantly different at 5% levelt by LSD test; Rado = Radopholus, Plt. Par. = plant parasitic other than Radopholus, Bact = bacterial feeders, Fungi = fungal feeders, Pre = predators, Omni = omnivores.*

Litter input produced by shading tree of coffee can control population density of plant parasitic nematodes. Green house experiment to test the effect of litter input on coffee seedling indicated that application leaf litter of *Gliricidia* not only suppresed plant parasitic nematode *Radopholus* but also increased free-living nematode abundance such as bacterial

and fungal feeders (Table 4). Higher litter input also increased abundance of nematode predator. Compared with untreated soil, application of avocado leaf to soil resulted in relatively higher abundance of nematode predators (25% as versus 4%).

The negative effect of coffee shading tree on plant parasitic nematode abundance may indirectly, throughout soil water content. Water film on soil particles is a critical factors for nematode activities. Semi-field experiment result showed that the highest plant parasitic nematode abundance in coffee nursery (500 individual/300 cc of soil) is at 40% shading density. Increasing soil water content of coffee nursery was positively correlated with shading intensity. Optimum soil water content for plant parasitic nematode in 70% to field capacity. Plant parasitic nematodes prefer soil moisture condition around field capacity level (Wallace, 1971).

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The Raise of Eco-Label Coffee: a Comparative Study of Their Effects on Costa Rican and Kenyan Cooperative Coffee Sector

J.F. LE COQ[1,3], F. PINARD[1,2], E. KIRUMBA[2,4], G. SOTO[3]

[1]CIRAD – UPR ARENA, UPR 31. Av. Agropolis, 34398 Monptpellier, France
[2]World Agroforestry Center (ICRAF), PO BOX 30677-00100 Nairobi, Kenya
[3]CATIE, Aptdo 739-3000 Heredia, Costa Rica
[4]IFRA, Laikipia road, Kileleshwa, BP 58480 – 00200 Nairobi, Kenya

SUMMARY

Since the last coffee crisis of early 2000s, development of specialty coffee, and especially eco-friendly labelled coffee, has been raising rapidly. Although effect on producers and global relationship has been documented, the dynamic of certification adoption and its comprehensive effects on coffee sectors in producing countries are still poorly documented. The poster presents a comparative study of the dynamic of development of Eco-labels in two high quality Arabica coffee producing countries, Costa Rica and Kenya. Using available secondary data and original data's derives from stakeholders comprehensive interviews, we show that the different pace and magnitude of the adoption of the certification schemes between this two countries depended on pre-existing institutional environment, governance and balance of power in the commodity chain and that the development of certification schemes tended to modify both structure and functioning of the commodity chains and contribute to gradual evolution of balance of power.

INTRODUCTION

Since the last coffee crisis of early 2000s, national coffee sectors all over the world has tended to develop response to sustain coffee production. One of the current responses is the development of specialty coffee, and especially eco-friendly labelled coffee such as Organic, Fair Trade, UTZ certified, Rainforest Alliance, Coffee Practices, Nespresso AAA or 4C. Since the late 90s, specialty coffee experimented a very rapid growth (Giovannucci, 2008). Many studies deal with the impact of certification at producers' level (Kilian et al., 2006; Ponte, 2004), analyzed the role of certification in the evolution of actors relationship in global chains at international level (Ponte, 2004; Ruben et al., 2006) or outlined the contrast of governance structures, environmental and social standards, and market positions of certified coffee (Raynolds, et al., 2007). Study showed that participation to certified coffee system does not ensure a better economic performance, but it may facilitate coordination between roasters/traders and some growers, which may lead to upgrading opportunities (Muradian and Pelupessy, 2005). Nevertheless the dynamic of development of certified coffee production and its comprehensive effects on cooperative coffee sectors in producing countries are still poorly documented.

In this study, we analyse and compare the dynamic of development of certification schemes in two fine quality Arabica coffee producing countries: Costa Rica and Kenya, and identify explicative some factors of differential development and effects of certification schemes at sector level, with a focus on cooperative sector.

MATERIAL AND METHOD

We choose to compare two producing countries, Costa Rica and Kenya, with recognized high quality arabican producing countries. For each country, we gather and analysed available statistical data on certification. We complemented this information with comprehensive interviews of the main actors involved in coffee certification in each country, including direct actors such as cooperatives leaders and traders, and indirect actors such as national institutions, promoters of certification, and auditors.

RESULTS AND DISCUSSION

Contrasted pace and magnitude of certification adoption

The analysis of the dynamic of certification showed that the different pace and magnitude of the adoption of the certification schemes between this two countries (Figures 1 and 2). In Costa Rica, the first coffee certification of cooperatives and associations took place in the 90s with organic and fair trade certification. Further, in early 2000s, cooperative and associations adopted rapidly certifications in UTZ certified, Rainforest Alliance, Cafe Practice. Nowadays, all the cooperative of Costa Rica have at least one certification, and most of them have multiple certification. The most common is the fair trade certification and the cafe practice certification. In Kenya, certifications development shows a very different situation. The first cooperative was certified in 2005. Nowadays, only 11 cooperatives are certified, which represent less than 2% of total cooperative number. Except from organic certification which is particularly difficult to develop due to coffee wilt disease, all the other certification is nowadays present in Kenya. Fair trade and Utz certified are the more common certifications.

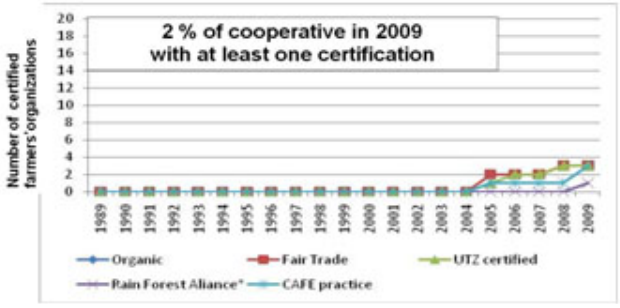

Figure 1. Evolution of the number of certified Farmers' Organizations in Kenya (1989-2009). Source: compilation of secondary data and interviews of the authors.

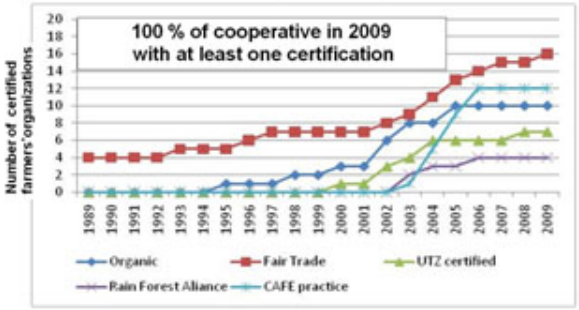

Figure 2. Evolution of the number of certified Farmers' Organizations in Kenya (1989-2009). Source: compilation of secondary data and interviews of the authors.

Contrasted dynamics of certification promotion process

The analysis of the process of certification promotion show different patterns (Figure 3 and Figure 4). In Kenya, the promotion of certification is done by the international traders according to a Top-Dow approach (Figure 3). International traders generally negotiate with the chair man of the cooperative to obtain his agreement to initiate the certification process in its cooperative. Then, international traders provide technical assistance to help farmers and cooperatives to comply with norms requisites. They also finance the necessary investments to achieve the certification standards and the cost of auditing. In Costa Rica, the cooperative adopted a proactive strategy upon certification as they effectively decide of the certification they wanted to develop (Figure 4). They decided to be certified to develop new market opportunities, to diversify their outlet, or to maintain their relationship with their coffee buyers. The cooperatives and association promote the certification to their members. Sometimes with the support of cooperation agencies, they organize training information and technical support to their members to help them complying with norms requisites. They organize the audit and assume its costs. They may also facilitate financial support to help farmers to make the necessary investments at farm level. Some cooperatives of Costa Rica developed specific trading consortium to facilitate access to certification, such as Coocafe consortium created in 1989 to facilitate access to fair trade certification or Suscof consortium created in 1998 to facilitate access to UTZ certified certification.

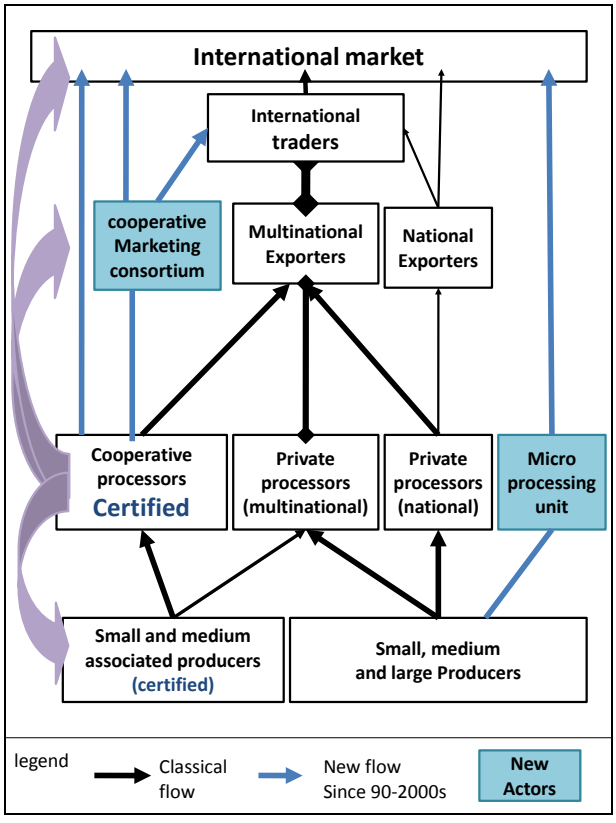

Figure 3. Certification process and evolution of value chain in Kenya. Source: based on secondary data and interviews 2007-2009.

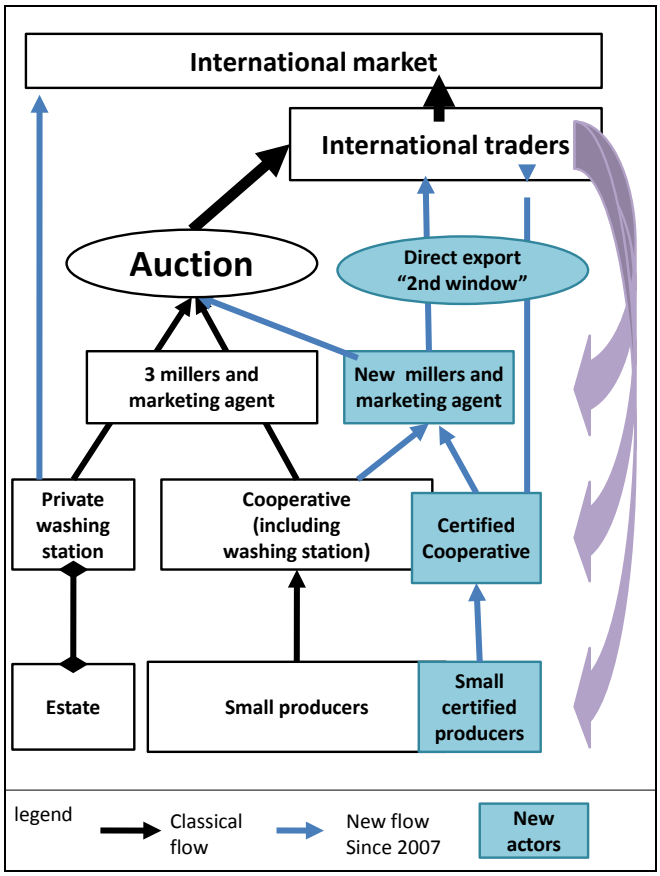

Figure 4. Certification process and evolution of value chain in Kenya. Source: based on secondary data and interviews 2007-2009.

Effects of certification in the value chain

The effect of the development of certification in value chain show a contrasted pattern between Kenya and Costa Rica, resulting from the existing balance of power in the commodity chain.

In Kenya, the certification development appears as a component of the strategy of vertical integration carried out by international traders since the reform of the coffee act in 2007. According to this reform, direct transactions between traders and cooperatives ("second windows") became possible outside the traditional auction system (Figure 3). Moreover, the milling and marketing function that was a quasi monopole of 3 enterprises, was opened to new actors. With the creation of the "second windows" and the "opening" milling and marketing functions inside the countries, international traders could integrate their supply chain downward reaching directly the cooperative level. With this new regulation, the investment done by international trader to promote certified production at cooperative and farm level can then be better secured since they can buy directly the product to the cooperative; whereas with the traditional auction system, they had no control on the traceability of the certified coffee production they promoted (as it could be mixed by the marketing agent) and may suffer high risk that the certified coffee they promoted was bought by other traders during the auction. Despite the new possibility granted by the opening of the second window, the Kenyan cooperative no have yet developed lots of direct selling. As they never had direct access to market with traditional auction system, they have no experience of

marketing activities. Thus they are poorly informed of evolution of market demand (and the raise of demand in Ecolabelled coffee). For these cooperative, certification is an opportunity to access to investment capacities, to get a better valorisation of their products and to enter in a process of improving their management practices and quality control.

In Costa Rica, certification contributes to the strategy of upgrading developed by cooperative consisting in improving their products quality, and diversifying and developing direct access to international market (Figure 4). With the existing institutional framework of regulation of Costa Rican coffee sector, cooperative have no institutional impediments to develop marketing and export activities. Following the crisis of coffee, the cooperatives entered, with the support of public institutions, in a process of quality management improvement including development of trademark, classification and differentiation of coffee, organoleptic quality tasting. Along with quality management improvement, they also oriented their strategy in developing marketing and exports functions individually or collectively through marketing consortium either specific to one Eco-labelled products, such as Coocafé for Fair trade, or Suscof, for UTZ certified, or no specific such as Cafecoop created in 2001 by 6 cooperatives to promote a wide range of qualities and Eco-labelled products. Nowadays the majority of the cooperative is member of one or several consortium in order to enlarge its marketing opportunities. Aside from this dynamic, but with the same objective of capture more added values, some producers, in very specific areas, even invested in micro processing unit to develop very high specific high quality coffee.

CONCLUSION

Whereas in Costa Rica certification development was rapid and pro-actively used by cooperative to strengthen an upgrading strategy in an enabling institutional environment, the certification in Kenya is still poorly developed and is pro-actively used by international traders to strengthen their strategy of downstream integration enabled by the reform of the coffee act allowing direct transaction with cooperatives since 2007. Thus, the contrasted pace and the magnitude of dynamic of certification is rooted in the existing institutional environment. The development of certification development is contributing to the change of structure and functioning of the commodity chain and to a gradual change of balance of power between stakeholders by offering new marketing and learning process opportunities.

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CAFNET email: jflecoq@cirad.fr

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Comparative Analysis of Coffee Eco-Certification Initiatives

F. PINARD[1,2], E. KIRUMBA[2,3]

[1]CIRAD – UPR 31. Av. Agropolis, 34398 Monptpellier, France
[2]World Agroforestry Center (ICRAF), PO BOX 30677-00100 Nairobi, Kenya
[3]IFRA, Laikipia road, Kileleshwa, BP 58480 – 00200 Nairobi, Kenya

SUMMARY

The major coffee eco certification/verification schemes that are currently operational include: Fair trade, C.A.F.E practices, Nespresso AAA, Organic, Rainforest Alliance, Utz Certified, Common Code for the Coffee Community (4C) and Bird Friendly. The various certification schemes focus on the three pillars of sustainability namely, social and economic justice, and environmental responsibility. However, due to marked similarities between them, there has been apparent confusion among industry players and producer organizations about the unique aspects of each standard and what they would offer if taken up, thereby obscuring choice making. As such, this study seeks to offer clarity and a constructive critique of the different schemes with a view to providing holistic information that would enable sound decision-making across the supply chain. To begin with, a comprehensive review of all standards was done, focusing on 'critical' and 'required' criteria, after which data was analyzed by use of Principal Component Analysis. Results on Critical Criteria, the mandatory one, indicate that in general, all standards focus on Environment, Agriculture and Social criteria but with various level of expectation. Only Fairtrade and, to a lesser extend, 4C labels give some importance to the Economic domain. The analysis performed on the Required Criteria didn't lead to any results, indicating the lack of labels' specificity based on these criteria

INTRODUCTION

Coffee certification started some 20 years ago with Fairtrade and organic initiatives. Since then, new labels have emerged, being now more than eight. Nowadays, certification has become the paradigm for producers who want to address the increasing demand from the specialty market, and for consumers looking for quality, of any kind (social, environment, test, etc.) (Raynolds et al., 2007, Giovannucci et al., 2008).

Theoretically, labels should convey a specific philosophy that, once translated into objective criteria, regulates the production sector. Some of these messages are clear enough and well understood by everyone. But others seem to lack specificity, may overlap, and generate confusion among the stakeholders. Such situation is of concern as it could generate disinterest in the label concept (Potts et al., 2003).

In an attempt to bring clarification for a better understanding, this work aims to characterize and to compare the major labels.

MATERIAL AND METHODS

This work focuses on 8 major coffee standards: UTZ Certified, Rainforest Alliance (RFA), Fairtrade (FT), Organic (ORG), Nespresso AAA (NES), CAFE Practices, 4C and Bird

Friendly. From their official guidelines, as per Nov 2009, the specifications of each standard were listed exhaustively. They were organized in 2 main class: the "**Critical Criteria (CC)**", mandatory, and the "**Required Criteria (RC)**" that allow some kind of adjustment and negotiation. Classes were further divided into domains (Environment-Agriculture, Social, Economic) and categories, and then numbered per category. Data analysis was performed using Principle Component Analysis.

RESULTS

Critical Criteria (CC)

Labels greatly differ in regard to the number of CC they define: UTZ and FT impose the highest number, followed by RFA, 4C and ORG. The CC number is reduced for BIRD and CAFÉ, null for NES. A global trend is the higher demand for Environment-Agriculture and Social domains than for Economic domain (Figure 1).

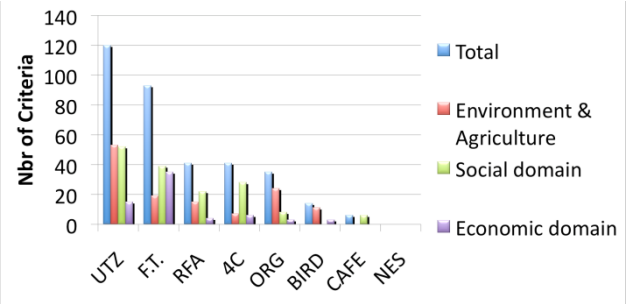

Figure 1. Standards critical criteria.

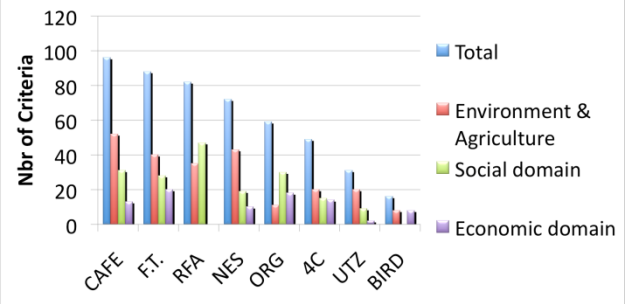

Figure 2. Standards required criteria.

On the 2 first axis of the Principal Component Analysis (Figure 3), 3 groups of criteria could be distinguished:

- Group 1 stands alone at the top of the 2nd axis; it is mainly represented by economic criteria.
- Group 2 represents a subset of environment criteria, independent from Group 1.
- Group 3 is the most numerous group and cannot be separated from group 2. It is heterogeneous, but its main 'trend' is environment and social. All the criteria from group 3 are strongly correlated on the first axis.

From this organization, it can be assumed that axis 1 represents an "Environment + Social" dimension, while axis 2 is more an "Economic" axis.

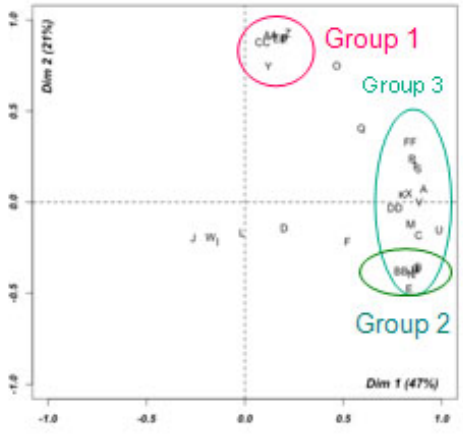

Figure 3. Variable (CC)* map.

Figure 4 shows the relative position of standards on the same pair of axis.

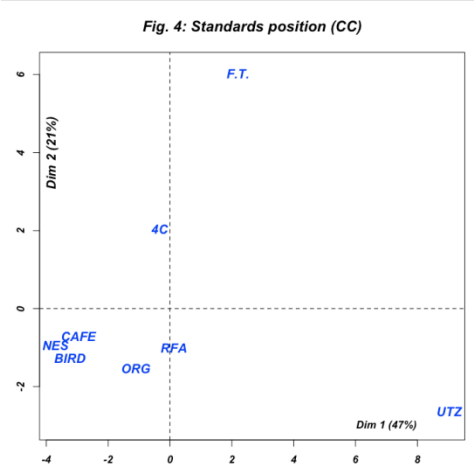

Figure 4. Standards position (CC).

- Utz appears quite demanding in the environment and social domains, with less expectation on economic criteria, as it stands quite far on the left side of the graph.
- All other standards express lower demand in terms of social and environment criteria: Organic, RFA and 4C having less expectations, CAFE, Bird Friendly and Nespresso, even lesser.
- The position of Fairtrade on the 2nd axis, and to a lesser extend 4C, reveals the importance given by these labels to economic criteria.

Required Criteria (RC)

RC are more numerous than CC (Fig. 2), but the demand from labels still greatly varies with a magnitude from 1 (BIRD) to 10 (CAFÉ, FT). Again, the main expectations from labels are for environment, Agriculture and, to a lesser extend Social domains. Less emphasis is still given to Economic criteria.

PCA on RC revealed the absence of pattern of the variable distribution. Variable associations were detected, but in a way that no specific meaning could be given to the association. This situation did not permit to detect any label specificity.

CONCLUSION

From our results, it appeared that coffee labels specificity could only be deducted from Critical Criteria. All labels could be ranked on a scale that combined Environmental-Agriculture and Social categories, with a high level of correlation. In so doing, labels appeared to differ only by the number of criteria they impose, from little (BIRD, NES, CAFÉ) to medium (4C, FT, RFA, ORG), then large (UTZ). Using such quantitative scale, the qualitative specificity of ORG is not expressed, the prohibition of inorganic compounds being just one among the other criteria for Environment-Agriculture.

Using an economic scale as a second dimension was useful to reveal the specificity of FT and 4C, that opposes the 'non-specificity' of the other standards in these domains.

Surprisingly, our results indicate the total absence of specificity related to Required Criteria. This could suggest that when they do not 'impose', all labels recommend more or less the same set of criteria, representing a combination from the 3 domains.

As such, it would be still premature to conclude on the level of label specificity although some differences could be revealed. Our comparative analysis was conducted at category level, not criteria level, and therefore does not allow a comparison in terms of similarities and distance. More work on data coding is still required and planned for this purpose.

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CAFNET email: f.pinard@cgiar.org; fabrice.pinard@cirad.fr

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The CAFNET/Coffee-Flux Project: Evaluating Water, Carbon and Sediment Ecosystem Services in a Coffee Agroforestry Watershed of Costa Rica

O. ROUPSARD[1,2], F. GÓMEZ-DELGADO[1,5], F. CHARBONNIER[1], L. BENEGAS[2], S. TAUGOURDEAU[1], R. KINOSHITA[2], R. MOUSSA[3], E. DREYER[3], A. LACOINTE[3], B. RAPIDEL[1,2], A. PEREZ[2], A. ROBELO[4], A. BARQUERO[4], C. RIVERA WILSON[2], M.N.V. NAVARRO[1], C. JOURDAN[1], G. LE MAIRE[1], P. THALER[1], J-M. BONNEFOND[3], J-M. HARMAND[1], P. VAAST[1]

[1]Cirad, Persyst, FR
[2]CATIE, DID, CR
[3]INRA, FR
[4]Aquiares farm, CR
[5]ICE, CR

SUMMARY

"Coffee-Flux" is a sub-project of CAFNET (EuropAid/121998/C/G): "Connecting, enhancing and sustaining environmental services and market values of coffee agroforestry in Central America, East Africa and India". It was launched in December 2008 in Costa Rica by Cirad, CATIE, PCP and the Aquiares farm. The aim of Coffee-Flux is to assess the water, sediment and carbon Environmental Services (ES) at the scale of a coffee agroforestry coffee watershed. Experimentation, modelling and remote-sensing are combined. Coffee-Flux is a contributor to FLUXNET (http://daac.ornl.gov/FLUXNET/). The platform is wide open to new projects, scientists and of course to students.

INTRODUCTION

Land use affects water and carbon balance and erosion processes, which are still poorly documented at plot and basin scale in coffee agroforestry systems. Also, anthropogenic management (tree planting, roads, terraces, etc) might substantially impact the provision of ES through infiltration, surface runoff and sediment genesis or affect the balance between C capture and C losses.

A comprehensive experimental display allows to assess water, sediment and C balance for small basins (≈ 1 km^2) and can be scaled up to region using modeling and remote-sensing (Taugourdeau et al., 2010, see next ASIC Poster). The evaluation of Environmental services (ES) requires balanced representation of agronomical, hydrological and ecophysiological processes and a tight coupling between experimental and modelling approaches.

Such a display was installed in 2008 in Costa Rica on a coffee agroforestry system with a low density of erythrins (Figure 1) and is expected to run as an observatory.

Figure 1. Arabica (Caturra) coffee agroforestry watershed in the Aquiares farm (Costa Rica), below *Erythrina poeppigiana* shade tree.

MATERIALS AND METHODS

Site and infrastructure description

A 1 km^2 coffee watershed, homogeneously shaded with tall *Erythrina poeppigiana* was selected in Aquiares, one of the largest coffee farms of the country, "Rainforest" certified, located on the slopes of the Turrialba volcano, ranging from 1,020 up to 1,280 m.a.s.l., strongly influenced by the climatic conditions of the Caribbean hillside, and without strong dry spell. The watershed is instrumented (Figure 2) with automatic flumes, pluviometers, soil moisture probes, piezometers, turbidimeters, sapflow and eddy-covariance tower (for H_2O and CO_2 gas fluxes).

Figure 2. The Coffee-Flux experimental display.

RESULTS AND DISCUSSION

Hydrological service

Coffee-Flux is monitoring and modelling the water balance partitioning (rainfall, interception, superficial runoff, infiltration, sapflow, soil water balance, evapo-transpiration, aquifer fluctuations and total streamflow), cf. Figure 3 and Figure 4.

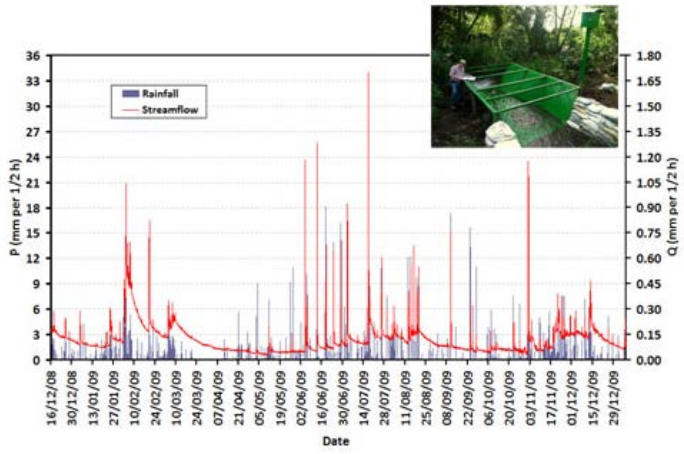

Figure 3. The red line is the time-course of streamflow at the outlet of the watershed (automatic flume), showing a large contribution of the baseline (aquifer responding in terms of discharge and recharge) + episodic and rather low contribution of superficial runoff (peaks after rainfall events, in blue). This behaviour is typical of watershed with large infiltration capacity, low superficial runoff and probably low laminar sediment transport. (Gómez-Delgado et al., HESS 2010).

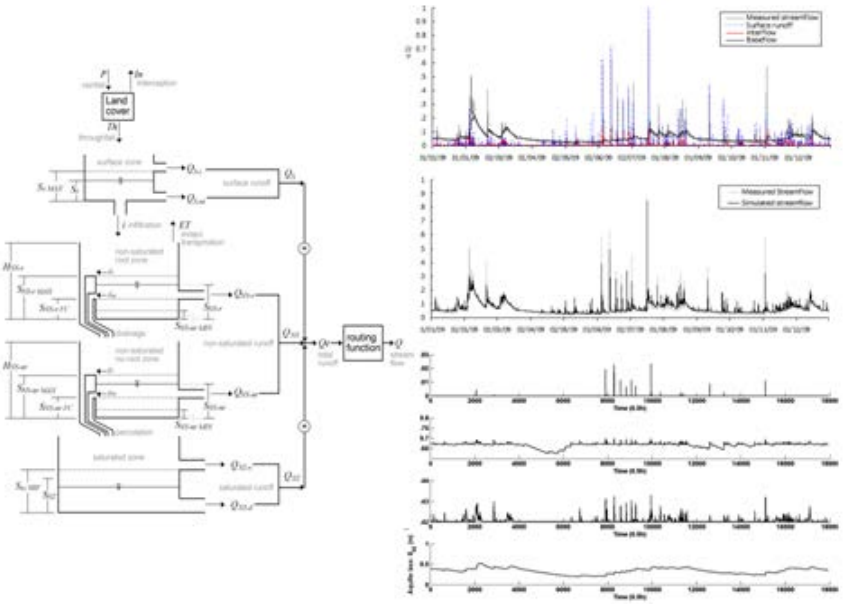

Figure 4. Structure and simulations of the lumped Hydro-SVAT model for the water-balance partitioning of the Coffee-Flux watershed (Gómez-Delgado et al., HESS 2010).

Sediment service

Sediment yield from coffee plots was very low in this rapidly infiltering soil. It was even lower in shaded plots. The basin sediment yield was monitored (Figure 5) and totalized less than 1 t ha^{-1} yr^{-1}.

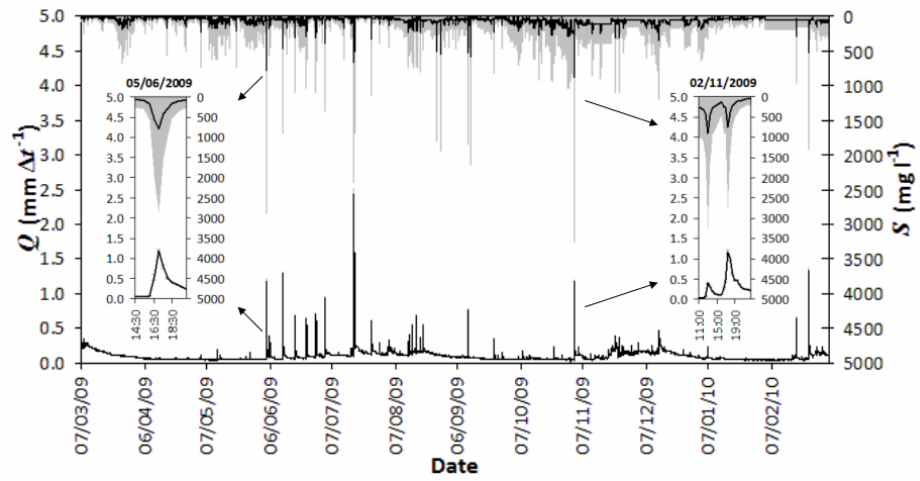

Figure 5. Streamflow Q and suspended sediment concentration S (black lines, measured with automatic OBS-3 probe (photo) and calibrated) in the experimental basin for the one-year measurement period. A 95% confidence interval (grey region) is displayed around the estimated values. Average annual sediment yield is < 1 t ha^{-1}.

Carbon service

Coffee-Flux is monitoring the Net Primary Productivity (NPP: tree + coffee growth and mortality) above and below-ground (minirhizotrons), the Gross Primary Productivity or (GPP = ecosystem photoynthesis), the ecosystem + soil respiration and the Net Ecosystem Exchange (NEE) (Figure 6) which is the ecosystem C balance, using combined eddy covariance and growth + litter monitoring.

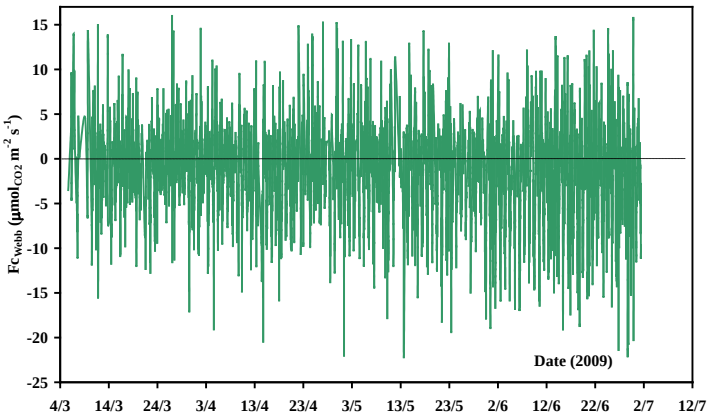

Figure 6. Time-course of carbon net ecosystem exchange, and its partitioning into night C emissions (positive values) and C uptake (photosynthesis + respiration, negative values). One cycle is one day.

CONCLUSIONS

The philosophy is to concentrate several investigations on one specific site and for a few years, to share a useful common experimental database, to develop modelling and to publish results in highly-ranked scientific journals. Coffee-Flux is a platform with infrastructure, easy access from CATIE and good security conditions, ready to welcome complementary scientific

investigations and collaborations. The project is wide open to complementary projects, scientists and of course to students.

ACKNOWLEDGMENTS

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Climate Change - Impact on Coffee Pests

P.K. VINOD KUMAR

Coffee Board of India, Central Coffee Research Institute, Coffee Research Station - 577 117 Chikmagalur, Karnataka, India. E-mail: i2vinod03@gmail.com

HISTORICAL ASPECTS OF CLIMATE CHANGE

Earth's climate has changed many times during the planet's history. Events have occurred ranging from the ice age to long periods of warmth. This has had a telling effect on the flora and fauna with many species becoming extinct and new ones arriving.

Historically, natural factors such as volcanic eruptions, changes in the earth's orbit and the amount of energy released from the sun have modified earth's climate. From the late 18th century, human activities associated with the industrial revolution have changed the composition of the atmosphere, which has in turn influenced the climate (IPCC, 2007). According to the US National Oceanic and Atmospheric Administration there are ten indicators for climate change. Seven indicators are expected to increase while three are expected to decrease. The seven indicators that are expected to increase are troposphere temperature, humidity, temperature over oceans, sea surface temperature, sea level, temperature over land and the ocean heat content. The three indicators that are expected to decrease are glaciers, snow cover and sea ice. Scientists in 48 countries concur that the past decade was the warmest.

Human activities contribute to climate change by increasing the anthropogenic greenhouse gases, aerosols and cloudiness. The four principal greenhouse gases that increase due to human activity are carbon dioxide, methane, nitrous oxide and the halocarbons – a group of gases containing fluorine, chlorine and bromine. The gases gradually accumulate over time and result in increased concentrations.

Very significant increase has occurred during the industrial era. For example the concentration of carbon dioxide which ranged from 180 to 300 ppm during the last 650,000 years was 379 ppm during 2005 (see Figure 1). Similarly, the concentration of methane was 1774 ppb during 2005 while the range during the last 650,000 years was 320 to 790 ppb. For nitrous oxide the pre industrial value was 270 ppb while it was 319 during 20005 (IPCC 2007).

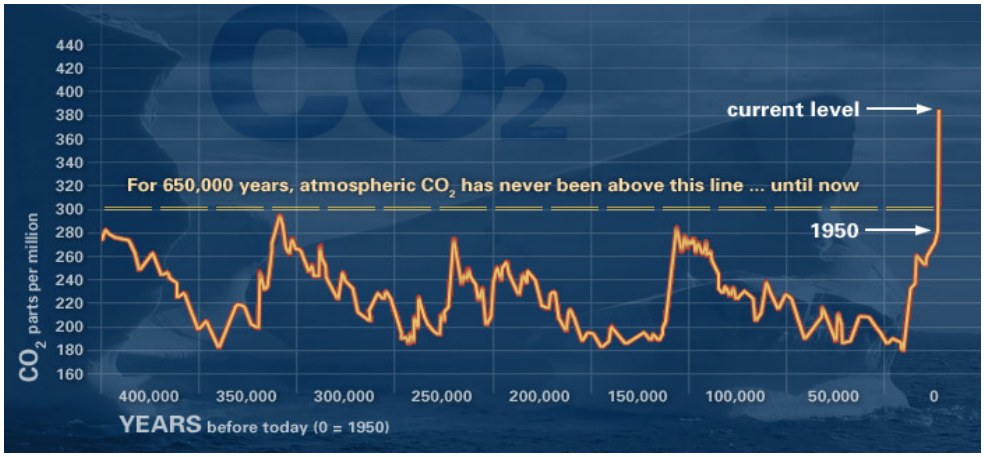

Figure 1. This graph, based on the comparison of atmospheric samples contained in ice cores and more recent direct measurements, provides evidence that atmospheric CO_2 has increased since the Industrial Revolution. (Source: National Oceanic and Atmospheric Administration).

Warming and cooling effects on the planet are described in terms of radiative forcing – the rate of change of energy in the system, measured as power per unit area. The Radiative forcing components are shown in Figure 2.

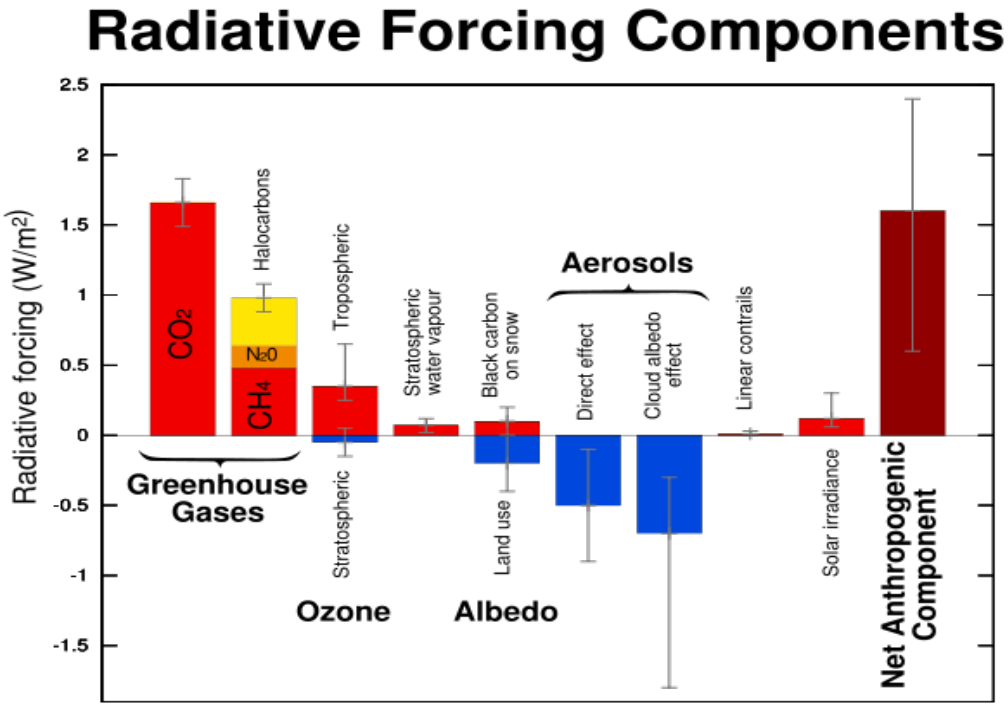

Figure 2. Radiative forcing components as estimated by the IPCC 2007 (all values are shown as a change from pre-industrial conditions).

EFFECTS OF CLIMATE CHANGE

So what is the effect of climate change? Climate change results in less frequent cold days, nights and frost events; with more frequent hot days, nights and heat waves. Eleven of the

twelve years between 1995 and 2006 were among the top twelve warmest years. Since, 1961 the ocean is absorbing more than 80% of the heat added to the climate system. There is increase in wind intensity, decline of permafrost coverage and increase in both drought and heavy precipitation events. Ocean warming causes seawater to expand which contributes to the sea level rice. Sea surface temperature increase correlates with increase in hurricane intensity in the North Atlantic. Increase in hurricane intensity is larger that climate models predict and there is more that 60% likelihood that hurricane intensity would increase during the 21st century.

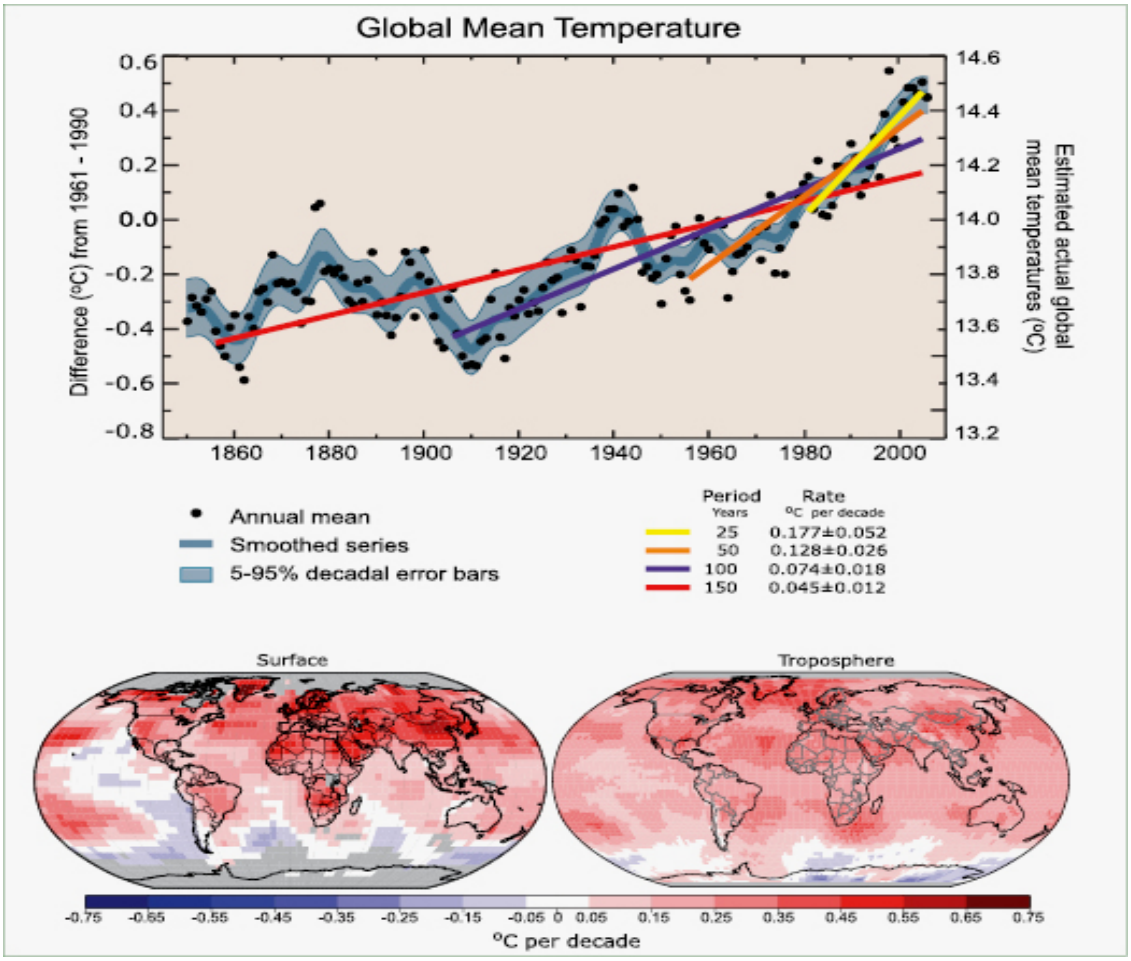

Figure 3. (Top) Annual global mean observed temperatures1 (black dots) along with simple fits to the data. The left hand axis shows anomalies relative to the 1961 to 1990 average and the right hand axis shows the estimated actual temperature (°C). Linear trend fits to the last 25 (yellow), 50 (orange), 100 (purple) and 150 years (red) are shown, and correspond to 1981 to 2005, 1956 to 2005, 1906 to 2005, and 1856 to 2005, respectively. Note that for shorter recent periods, the slope is greater, indicating accelerated warming. The blue curve is a smoothed depiction to capture the decadal variations. To give an idea of whether the fluctuations are meaningful, decadal 5% to 95% (light grey) error ranges about that line are given (accordingly, annual values do exceed those limits). (Bottom) Patterns of linear global temperature trends from 1979 to 2005 estimated at the surface (left), and for the troposphere (right) from the surface to about 10 km altitude, from satellite records. Grey areas indicate incomplete data. Note the more spatially uniform warming in the satellite tropospheric record while the surface temperature changes more clearly relate to land and ocean. (Courtesy: IPCC 2007).

According the IPCC report, due to increase in temperature the hydrological cycle is expected to become more intense leading to 'very wet' and 'very dry' areas. More people would be exposed to extreme droughts. The El Nino and the La Nina effect on agriculture are well known. The trends in annual precipitation from 1901 to 2005, available in the IPCC 2007 report, suggest that the annual mean precipitation can vary from + 30 to -30% percent in most of the coffee growing countries (Figure 4).

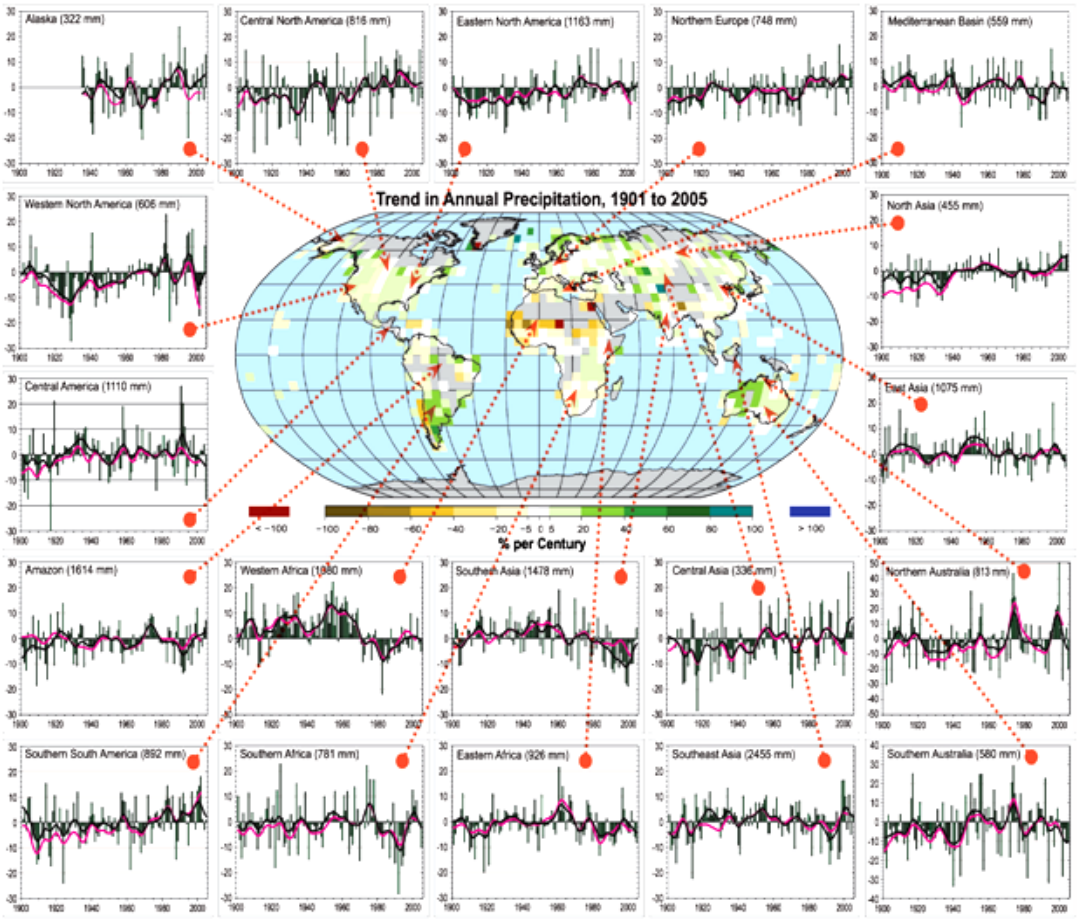

Figure 4. Precipitation for 1900 to 2005. The central map shows the annual mean trends (% per century). Areas in grey have insufficient data to produce reliable trends. The surrounding time series of annual precipitation displayed (% of mean, with the mean given at top for 1961 to 1990) are for the named regions as indicated by the red arrows. The range of change is +30 to –30% except for the two Australian panels (Courtesy: IPCC 2007).

EFFECT OF CLIMATE CHANGE ON COFFEE

Climate change is believed to be one among the numerous factors that may affect global coffee production. The International Coffee Organization thinks that it would be the most important; particularly considering the large number of small holder coffee farms whose capacity to implement means and methods to mitigate climate change effect may be low.

Why is coffee sensitive to climate change? It is because of its environmental requirements. Arabica coffee evolved in the cool shady environment of the Ethiopian highlands, where there is a single dry season coinciding with winter months. The optimum temperature range is 15 to 24^0C with a precipitation of 1500 to 2000 mm per year (Mitchell, 1988). Robusta is slightly

hardier, as it evolved in lowland equatorial Africa, but grows well in areas with abundant rainfall of about 2500 mm per year. For Robusta the rainfall should be well distributed as the plants are shallow rooted. The optimum temperature range for Robusta is 24 to 30^0C, but it is less tolerant to very high or very low temperature. Climate disturbances have led to fluctuations in yields in almost all the coffee growing countries. Global warming is expected to result in the actual shifts on where and how coffee would be produced. Dr. Peter Baker of CAB International (www.cabi.org) is of the opinion that if there is a 3 °C increase in temperature by the end of this century, the lower altitude limit for growing good quality Arabica coffee may go up by 15 feet per year. This may affect millions of producers as well as the all participants in the value chain of industry the end user, the coffee consumer.

But, it is very difficult to pin point how climate change would affect the coffee industry. Both Arabica and robust coffee growers would be affected that is the prediction. Rising temperature is expected to make some areas less suitable or completely unsuitable for coffee cultivation, incidences of pest and disease may increase and quality may suffer. Growers may have to depend more on irrigation, putting pressure on water resources. Overall, the production cost is expected to increase.

Increase in temperature will force coffee to ripen faster than normal, impacting the inherent quality. Increase in temperature coupled with low rain fall or erratic distribution will affect flowering and fruit set.

WHAT IS HAPPENING IN COFFEE GROWING COUNTRIES?

In Brazil, coffee cultivation is becoming viable in areas considered prone to frosts. But high temperatures will also lead to reduction of overall acreage with suitable climatic conditions. In Colombia, the cost of production is likely to increase due to increased incidence of pest and diseases and water requirement may increase forcing growers to depend more on irrigation. In Costa Rica the coffee area may be pushed upwards to as high as 2000 meters. Nicaragua is already facing extreme variation in coffee production due to the El Nino and La Nina cycles. In Guatemala, Honduras and Mexico, temperature has risen between 0.2 to 1 °C and in some cases rainfall has fallen by up to 15% (Castellanos et al., 2003). India is facing the problem of erratic rainfall distribution in the last few years. Rainfall in some areas is changing the growing conditions. Erratic rainfall distribution and increase in temperature are favoring proliferation of coffee berry borer, coffee white stem borer and coffee leaf rust. For Kenya, the area under coffee is expected to remain unchanged but coffee is expected to migrate upwards. Increase in temperature is expected to influence pest and disease spread and affect the quality. In Mexico, temperature rise is causing proliferation of pests - coffee trees growing at 1200 meters altitude, previously considered to be out of risk, are now being affected by coffee berry borer. Crop appears to ripen earlier in Peru, and growers are reporting that high altitude coffee plants are maturing earlier. Erratic rainfall distribution is having a negative influence on the coffee farm. An Oxfam report suggests that if temperature rises by 2 °C or more, most of Uganda is likely to become unsuitable for growing coffee (www.thecoffeeguide.org).

IMPACT OF CLIMATE CHANGE ON PATHOGENS AND INSECTS

Increased carbon dioxide levels can influence both the host and the pathogen in several ways. High carbon dioxide rates may increase the growth rates of plants resulting in dense canopies which, with higher humidity, may favor, especially leaf and twig infecting pathogens. This may be a positive for infection by the coffee leaf rust pathogen. In high carbon dioxide

regimes, lower decomposition rates are expected, assisting the pathogens to persist in plant residues, leading to higher inoculums and faster disease epidemics. Higher CO_2 may also lead to greater spore production. The positive sign is that increased CO_2 can bring about physiological changes to the host plant which can increase host resistance to pathogens (Coakley et al., 1999). Moisture can also impact both the host and the pathogen. There are several models predicting the effect of moisture on disease manifestation; almost all the models are based on leaf water, relative humidity and rainfall distribution pattern. This has serious implications with regard to coffee leaf rust disease progression. More frequent and extreme precipitation events could lead to more and longer periods favorable to the pathogen.

In the case of insects, temperature is the single most important environmental factor, which influences the behavior, distribution, development, survival and reproduction. According to Bale et al., 2002, temperature alone overwhelms the effect of other environmental factors. Yamamara and Kirtani 1998, estimate that a 2 °C increase in temperature could lead to insects experiencing one to five additional life cycles per season. Moisture and carbon dioxide effect could also be potentially important consideration in a global climate change setting (Hamilton et al., 2005; Coviella and Trumble, 1999; Hunter, 2001). Some crop pests are "stop and go" in relation to temperature as they develop rapidly during periods of suitable temperature. These types of insects will accelerate development with increase in temperature and could have more generations and inflict more damage to the crop. The environmental factors that influence the pest population may also influence their natural enemy complex, thus setting up completely new dynamics which may be positive or negative in terms of crop damage. Impact of carbon dioxide on insects is presumed to be indirect, mostly due to changes in the host. Carbon dioxide enrichment studies predict more damage from insects; studies on Soybean seem to indicate this trend (Hamilton et al., 2005). Precipitation could have effect on insects, mainly due to wash out effect or as hindrance to flight, but this is less studied. Increased precipitation can bring about favorable situation for entomopathogenic fungi to thrive and put appreciable pressure on the pest population.

SPECIFIC STUDIES ON COFFEE PESTS

Not many studies have been conducted specifically on the effect of climate change on coffee pests. Rainfall distribution patterns have influenced the coffee pests year to year in all coffee growing countries. Jaramillo *et al.*, 2009 determined the thermal tolerance of coffee berry borer, *Hypothenemus hampei*, under eight temperature regimes. According to them, egg to adult development occurred between 20-30 °C and the lower and upper thresholds for development were 14.9 and 32 °C. The possible effect of climate change was inferred using data from Colombia, Tanzania and Ethiopia. Analysis of 32 years climate date in Jimma, Ethiopia revealed that prior to 1984 it was too cold for *H hampei* to complete one generation per year, but thereafter 1-2 generation could be completed. The authors also developed a model which indicates that for every 1 °C rise in the thermal optimum, the maximum intrinsic rate of increase will increase by 8.5%. The implications of the study are that the berry borer can easily adapt to temperature variations in coffee growing areas. It may change its distribution pattern, thereby becoming a menace in the sup-tropical coffee growing areas. Rising temperatures will increase population leading to more damage to the crop. Recent data from Uganda and Indonesia show that the coffee berry borer has already expanded its altitudinal distribution ranges and it now attacking coffee plantations at sites as high as 1864 meters.

Change in rainfall distribution pattern is also causing huge problems in terms of berry borer spread and development. This is mainly due to change in crop phenology. Change in rainfall

pattern in the year 2009 led to a sudden increase in the incidence and spread of coffee berry borer in India. Reports from higher altitudes confirm that the berry borer is causing damage as never before.

Ghini et al., 2008 studied the potential impact of climate change in Brazil on the spatial distribution of coffee nematode, *Meliodogyne incognita* and the leaf miner, *Leucoptera coffeella* using the geographical information system. The further scenarios focused on the decades, 2020's, 2050's and 2080's as per the scenarios, A2 and B2 from GC models of IPCC. The assignment by the authors indicated the increased infestation of nematode and leaf miner due the greater number of generations per month that occurred under the climatologically normal from 1961-1990.

SITUATION IN INDIA

India has to deal with several pests as both arabica and robusta coffee varieties are cultivated commercially. The notable pests are coffee white stem borer, *Xylotrechus quadripes* (Coleoptera: Cerambycidae) on arabica and the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae) both on arabica and robusta, though causing less damage on arabica due to the crop maturing earlier than robusta. There are other pests like mealy bugs *Planococcus* sp., the twig borer, *Xylosandrus compactus* and the green scale, *Coccus viridis*, which can cause considerable damage given favorable conditions. Then there is the hidden threat of the root lesion nematode, *Pratylenchus coffea* on arabica coffee.

For the last few decades the weather parameters have not shone extreme variations but during the last couple of years the extremes are becoming more frequent. The mean monthly minimum temperature has shown an increasing tendency and the cold days and nights are becoming less frequent (Figure 5). The mean maximum temperatures are also showing an increase (Figure 6).

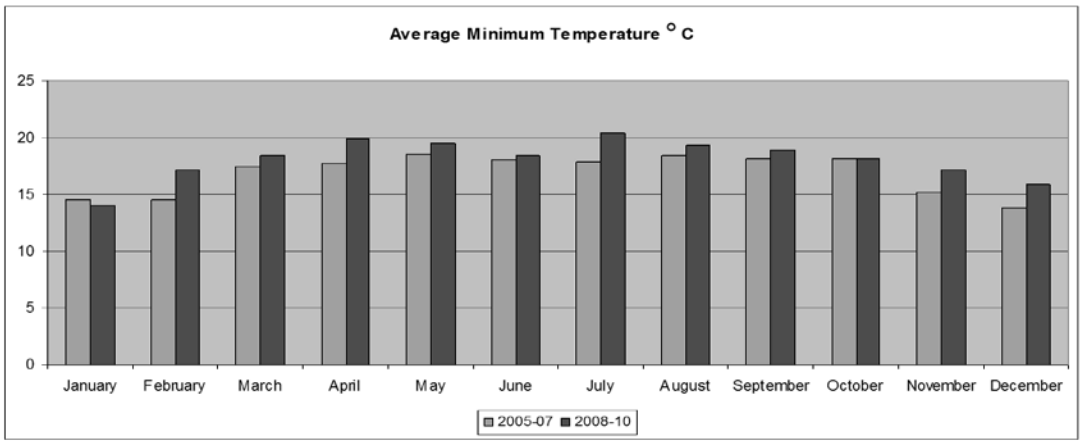

Figure 5. Change in monthly average minimum temperature.

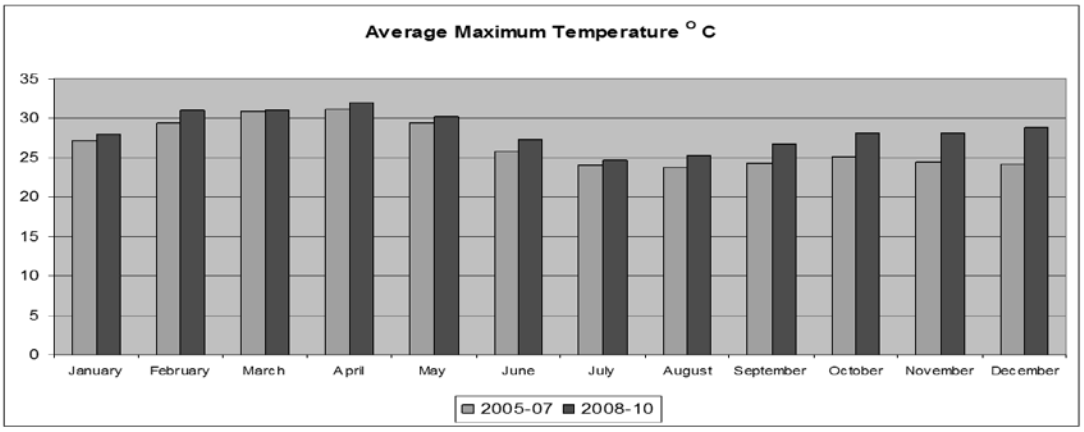

Figure 6. Change in monthly average maximum temperature.

The rainfall pattern is also showing variation in the last three years (Figure 7 and 8) and due to this the incidence of the coffee berry borer is increasing. The rainfall distribution is particularly important to coffee growers in India because untimely rains induce flowering and also impact on the harvesting operation. This leads to multiple fruit stages facilitating berry borer increase and more left over and fallen fruits due to rains during the harvest period. A better distribution of rainfall, on the other hand, assists in keeping the activity of the stem borer low. Rainfall during the flight periods is known to negatively impact their population size. The coffee stem borer is the major problem for Arabica coffee cultivation in Nepal. The flight period of this pest is worth monitoring in Indonesia and Nepal to ascertain the impact of rainfall and temperature.

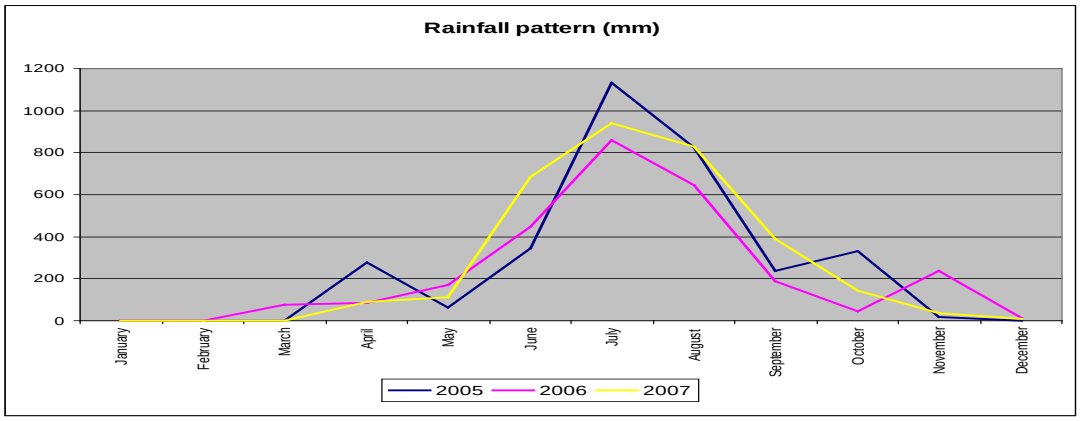

Figure 7. Rainfall pattern during 2005, 2006 and 2007.

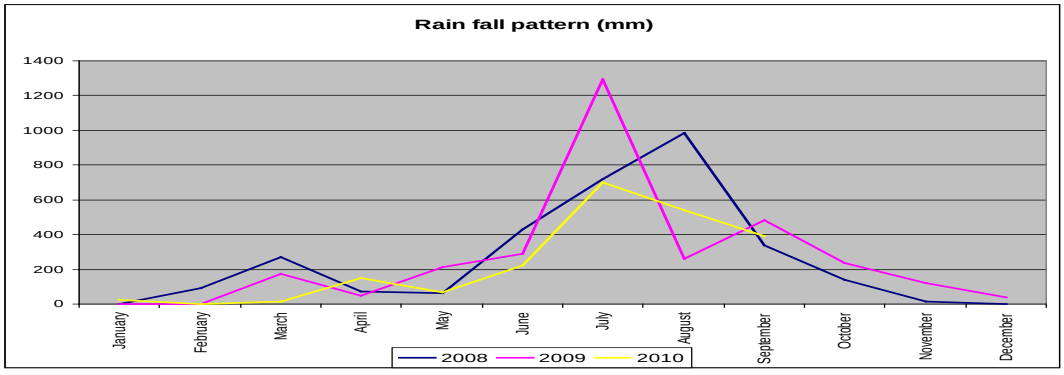

Figure 8. Rainfall pattern during 2008, 2009 and 2010.

THE WAY FORWARD

Since the impact of climate change on coffee pests have not been critically studied in all the coffee growing countries, it is appropriate to monitor the pest population in relation to weather parameters in all countries. It is also important to map the likely climate change in regions within the country also. To mitigate the effect of climate change, both short term and long term strategies would be required like improved farming practice and efficient on farm processing; different production models like shade grown coffees would also have to be thought about. It would be a challenge to the coffee breeders to develop drought and pest and disease tolerant varieties. In very extreme cases, it is possible that the growers may have to diversify out of coffee and or shift production to more suitable areas.

It is important to get together and develop sustainable strategies in place as climate change is here to stay.

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The Socio-Economic Impact of Climate Change on Mesoamerican Coffee Production

P. LÄDERACH[1], O. OVALLE[1], M. BACA[2], A. EITZINGER[1], T. OBERTHÜR[3]

[1]International Center for Tropical Agriculture (CIAT), Managua, Nicaragua
[2]Centro Internacional de Agricultura Tropical y Enseñanza (CATIE), Managua, Nicaragua
[3]International Plant Nutrition Institute (IPNI) Southeast Asia Program, c/o The World Fish Center, Jalan Batu Maung, Batu Maung, Bayan Lepas, Penang 11960, Malaysia

SUMMARY

According to the fourth assessment report (AR4) of the Intergovernmental Panel on Climate Change (IPCC), is Mesoamerica one of the regions that will suffer severe impacts from a progressively changing climate. Coffee production is the mainstay of thousands of families and the major contributor to the agricultural GDP of Mesoamerican countries. As a result of climate change, the suitability of traditional coffee growing regions will decrease drastically. A large number of farmers will not be able to produce coffee in the future, while other farms will become marginally suitable with decreased coffee quality and increased pest and disease pressure. Using the IPCC's A2a emission scenario (business as usual) and twenty downscaled global circulation models (GCM) in combination with MAXENT, we map the change in suitability of coffee in Mesoamerica. Combining the spatial modeling with socio-economic impact analysis we quantify the impact of changing climate on socio-economic indicators at local, regional and national scale. The analysis shows that the vulnerability to climate change varies a great deal in space depending on the geographical location. The predictions confirm drastic reduction in the overall suitability of the current coffee growing area, and subsequently in yield, quality, the number of producers and their incomes. We propose concrete adaptation strategies for producers with different profiles of vulnerability and draw general conclusions to inform policy makers at regional and national level.

INTRODUCTION

The global climate has changed over the past century and is predicted to continue changing throughout the twenty-first century. Global circulation models (GCMs) all point in the direction of higher mean temperatures and changes in precipitation regimes. Both indicate that there will be a drastic shift in current land use and crop suitability, in addition to general increases in vulnerability to climate variability. Climate change will affect agricultural productivity, farm incomes and food security (Busuioc et al., 2007; Lobell et al., 2008). The predicted changes in future climates have the potential to expose agricultural systems to conditions and extremes not experienced before. Some of the climate changes could have beneficial effects while others are likely to be detrimental. Whilst attention needs to be paid to adapting annual cropping systems to future changes in climate more urgent action is required to address these issues as they apply to high-value perennial cropping systems such as coffee. Coffee systems are characterized by requiring longer-lead times for both farmers and business partners to make changes. In these cases, decisions made today come to fruition in 8-15 years, by which time the climate is likely to have changed.

Decreases in the suitability of current coffee-producing areas will likely have large impacts on national economies: for example, coffee is the largest contributor to agricultural GDP in Central America. In Nicaragua, coffee produces 14.3% of agricultural GDP (MAG-FOR, 2006). In Mexico, coffee production is considered as a strategic activity, since it is grown on 664,800 ha, providing livelihoods to 700,000 families (ICO, 2005). Because of their limited livelihood resources, smallholder farmers are most vulnerable to climate change; it is therefore essential for them to understand its likely impacts and develop strategies to adapt. Understanding the implications of these changes is also critically important to all stakeholders in the value chain. To ensure the livelihoods of millions of smallholder farmers and the related rural industries, it is crucial to identify adaptation pathways for these production systems or identify opportunities for them to diversify into other high-value crops. In this paper we firstly quantify the impact of climate change on the suitability of land to produce coffee in Central America, by quantifying the impact on yield and income at the municipality level in Nicaragua. Secondly, we assess the sensitivity of coffee farmers to climate change and their adaptive capacity. We conclude by seeking mitigation and adaptation strategies at both the community and national levels.

METHODOLOGY

Vulnerability analysis

Vulnerability is the degree of susceptibility and incapability of a system to confront adverse effects of climate change (IPCC, 2001). Vulnerability consists of three components, the exposure, the sensitivity and the adaptive capacity. Exposure is the degree to which a system is exposed to variations in climate; the sensitivity is the degree to which a system is positively or negatively affected by climate-related stimulus and the adaptive capacity is the ability of a system to adapt to climate change (Equation 1). We assessed the exposure of smallholder coffee farmers using data from GCMs as input to models of crop suitability. We analyzed farmers' sensitivity and adaptive capacity using the DFID sustainable livelihoods approach (DFID, 2009) in combination with expert knowledge.

$$\text{Vulnerability} = \int \text{Exposure} + \text{Sensitivity} - \text{Adaptive capacity} \quad [1]$$

Evidence data and sampling design

To predict the areas that will be suitable to grow coffee in the future, we first need the geographical coordinates (evidence data) of areas where coffee is currently produced. In Nicaragua we used 4919 coordinates of the current coffee-growing areas mapped by Valerio-Hernádez (2002), in El Salvador 975 coordinates, 705 supplied by PROCAFE and 271 from Läderach et al., (2009), in Guatemala 5515 coordinates, 4800 supplied by ANACAFE and 715 from Läderach et al. (2008) and in Mexico 6702 coordinates, 6629 supplied by Más Café and 73 for Veracruz by Perez-Portilla et al. (2005). The sampling design selected 150 producer households for interview at random within the categories of their exposure of coffee and their 30 most important companion crops to climate change and their ranking within the indices of poverty at the municipality level.

Historical climate data collation

We obtained historical climate data from the WorldClim database (Hijmans et al., 2005a; http://www.worldclim.org/). WorldClim data were generated at a 30 arc-second spatial resolution (1 km) through an interpolation algorithm using long-term average monthly climate

data from weather stations. Variables are monthly total precipitation, and mean monthly minimum and maximum temperatures. Hijmans et al. (2005) used data from stations for which there were long-standing records, calculating means of the 1960-1990 period. The WorldClim database also includes 19 bioclimatic variables that are derived from monthly temperature and rainfall values to generate more biologically meaningful variables (Busby, 1991). They represent annual trends (e.g., mean annual temperature, annual precipitation), seasonality (e.g., annual range in temperature and precipitation), and extreme or limiting environmental factors (e.g., temperature of the coldest and warmest month, and precipitation during the wettest and driest quarters) and are often used in ecological niche modelling.

Future climate

The Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report (AR4) was based on the results of 21 global climate models (GCMs), data of which are available through the IPCC website (www.ipcc-data.org.ch). The spatial resolution of the GCM results is, however, inappropriate for analyzing the impacts on agriculture as in almost all cases the grid cells are 1 arc-degree (more than 100 km a side at the equator). This is especially a problem in heterogeneous landscapes such as coffee growing areas. We therefore used statistical downscaling to increase resolution by using interpolation and explicit knowledge of the distribution of current climates at a fine-scale, which includes the high-resolution SRTM elevation data. We produced 30-arc-second (1-km) resolution surfaces of the mean monthly maximum and minimum temperature and monthly precipitation. In all cases, we used the IPCC scenario SRES-A2a ("business as usual"). Specifically, the centroid of each GCM grid cell was calculated and the anomaly in climate was assigned to that point. The statistical downscaling was then applied by interpolating between the points to the desired resolution using the same spline interpolation method used to produce the WorldClim dataset for current climates (Hijmans et al., 2005a). The anomaly for the higher-resolution was then added to the current distribution of climate (derived from WorldClim) to produce a surface of future climate.

Suitability prediction

Maximum entropy (MAXENT) is an accurate ecological niche model (Elith et al., 2006; Hijmans and Graham, 2006), which we selected for the analyses. It is a general-purpose method for making predictions or inferences from incomplete information (Phillips et al., 2006). The model estimates a target probability distribution by finding the probability distribution of maximum entropy, subject to a set of constraints that represent (one's) incomplete information about the target distribution. We assessed future suitability predictions for each of the GCM models using MAXENT (Phillips et al., 2006) and two measurements of uncertainty for each location: (1) the agreement among models calculated as the percentage of models predicting changes in the same direction as the average of all models; and (2) the coefficient of variation (CV) among models, in which the prediction for any variable that was significantly different from the mean of the other models according to Tukey's (1977) outlier test was eliminated. We applied the logistic function to the distribution estimated in MAXENT, which gave us probabilities between 0 and 1 of site suitability under the climate change scenario. It was interesting that the models that were eliminated differed from one country to another.

Sensitivity and adaptive capacity

There are five assets that DFID (2009) identified as important for smallholder farmers to create new resources: human, social, financial, natural and physical assets. We assessed these assets by identifying 18 relevant indicators through workshops with farmers, expert consultation and literature review (Table 1).

Table 1. Eighteen identified indicators across five assets. The numbers in parentheses are the numbers of questions asked in each indicator.

Human asset	**Social asset**	**Physical asset**
- Access to formal and informal education (11) -Level of knowledge of farming system management (8) - Health and feeding (4)	- Organization (6) - Take decisions / work distribution (2)	- Access routes (3) - Transport of products (2) - Quality of accommodation (3)
Natural asset	**Financial asset**	
- Access and availability of water (7) - Contamination (6) - Conservation (4) - Soil conditions and fertility (6)	- Credit access (6) - Variability in production (2) - Price variability (1) - Variability in annual revenue and income diversification (3) - Access to markets (4) - Access to alternative technology (2)	

Each indicator consisted of verifiable and comparable attributes that reflect the indicator. We assessed the attributes by asking specific questions within each of the different indicators in interviews. For example, one of the seven questions of the indicator access and availability of water was, “What is the quality of your drinkable water?” The possible answers cover the categories from low to high sensitivity with the answers ranging from very bad quality to very good quality.

RESULTS AND DISCUSSION

According to the MAXENT model there will be a general pattern of decrease in the area suitable for coffee and a decrease in overall suitability within these areas across Mesoamerica (Figure 1). The areas that in 2050 will still be suitable for coffee production are mainly areas that currently show particularly high suitability.

With progressive climate change, areas at higher altitudes become more suitable for producing coffee (Figure 2). The optimum coffee-producing zone in Nicaragua is currently at 800-1400 masl elevaton; by 2050 the optimum elevation will increase to 1200-1600 masl. Between today and 2050 areas at altitudes around 500-1500 masl will suffer the greatest decrease in suitability, while areas above 1500 masl will have the greatest increase in suitability. As the altitude of areas suitable for coffee increases there will be less and less land area available (Figure 2 green line). In general, physical assets of households in Nicaragua are sensitive to the quality of postharvest infrastructure, their financial assets are sensitive to variability in coffee production and hence the extent to which they depend on coffee for income, and their human assets are sensitive to migration (Figure 3). Households have low adaptive capacity in their post-harvest facilities. They are unable to process the harvest in wet

weather, so they are forced to sell it unprocessed, and they have little knowledge on how to manage pests and diseases during uncommon weather events. With regard to their financial assets, they have poor access to new technologies and poorly developed linkages to markets.

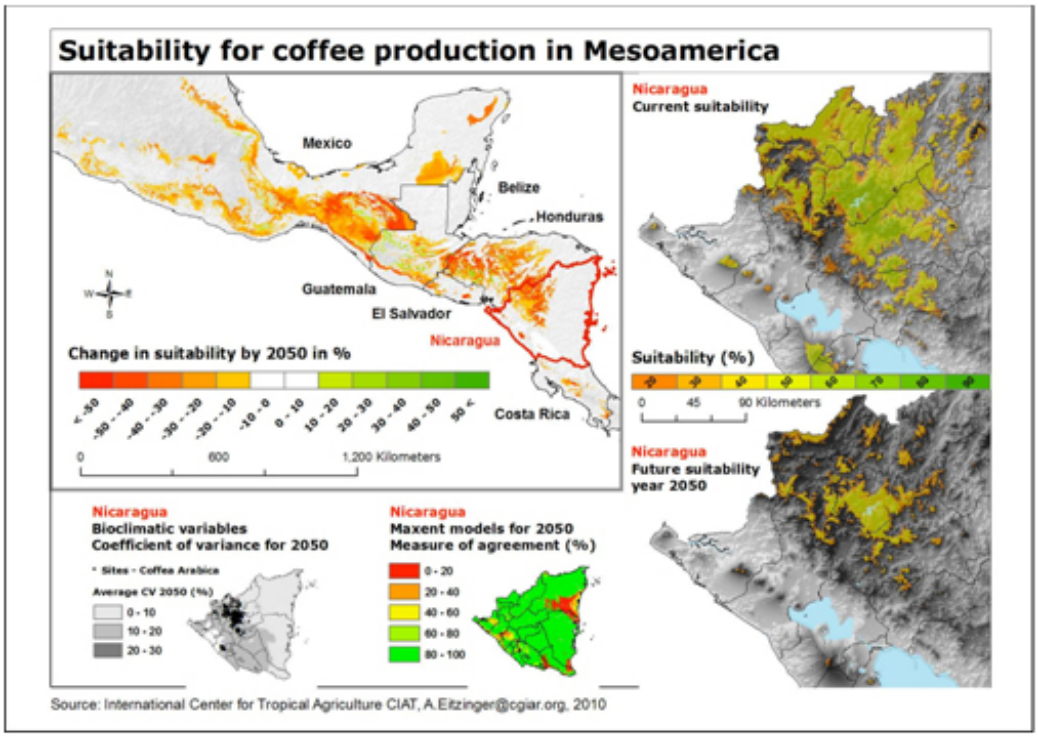

Figure 1. MAXENT predictions of change in suitability for coffee production in the Mesoamerican coffeeproducing area (Mesoamerica map) and MAXENT predictions of current and future (2050) suitability for coffee production in Nicaragua. The insets show the coefficient of variation and measure of agreement for the study area. The points are the coffee farms sampled.

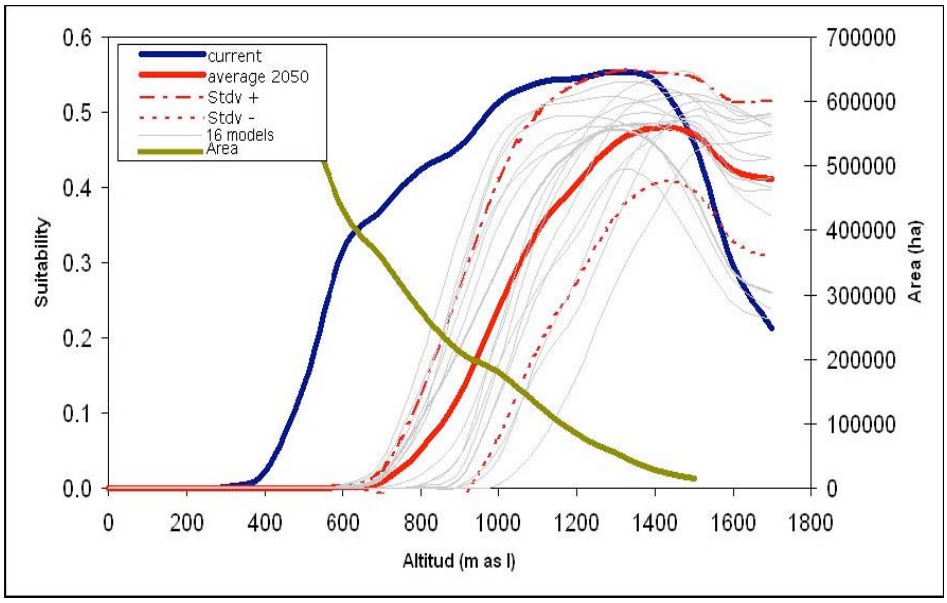

Figure 2. Relation between current and future (2050) coffee suitability as estimated by 16 global circulation models, the area of land, and altitude in Nicaragua.

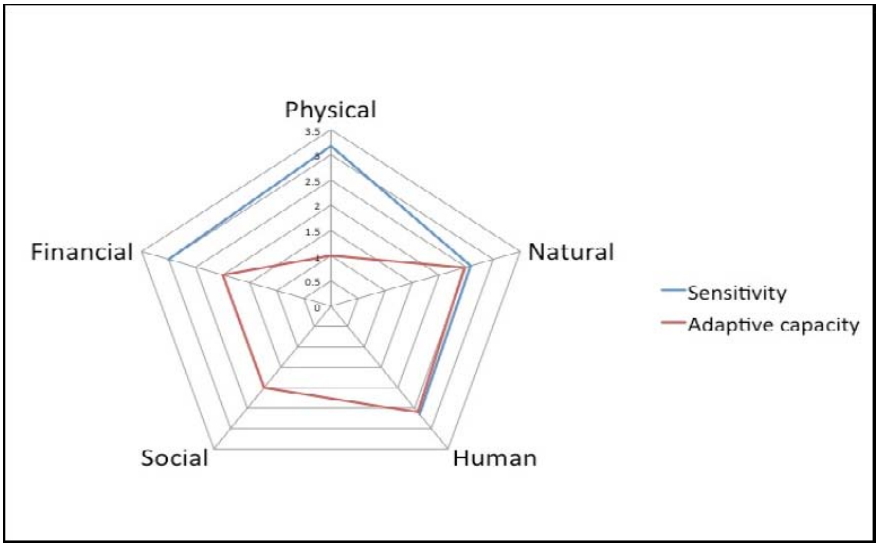

Figure 3. The average adaptive capacity and sensitivity of Nicaraguan coffee farmers according to five livelihood assets.

In general, the first step in adaptation is to reduce the vulnerability of coffee farmers to climate change. In this regard, use of technical "no regret" measures that strengthen the resilience of the system (e.g. sound agronomy, sustainable management of natural resources) will be beneficial to growers and their livelihoods and may as well minimize the effects of climate change. In areas that will become unsuitable to grow coffee, farmers will need to identify alternative crops. In areas that will remain suitable for coffee but with some reductions in suitability, agronomic management might be adapted to buffer the impacts of climate change. Drought resistant varieties, irrigation, and shade cover are all useful practices that can be implemented; shade cover can decrease average temperatures by up to 4 °C (Vaast et al., 2006). Areas where coffee is not grown today but which in the future will become suitable for coffee need strategic investments to develop coffee production. Account needs to be taken of environmental viability, since higher altitudes are often forest reserves that provide environmental services to the lowland population and to agriculture. More specifically, however, it is apparent that coffee-growing municipalities in Nicaragua have very distinct and site-specific vulnerability profiles and require site-specific adaptation strategies. Matagalpa, for example, is characterized by high exposure (coffee suitability decreases drastically), high sensitivity (high variability in yields), and low adaptive capacity (poor access to credit, poor knowledge on pest and disease management and low diversification). The adaptation strategies developed in participatory workshops in Matagalpa focus on diversification, capacity building, strengthening of the organizations and on the enforcement of environmental laws and development policies for the coffee sector.

Madriz on the other hand is characterized by low exposure (coffee will still grow in 2050), high sensitivity (bad roads, high variability in yield) and low adaptive capacity (poor organizational structure, few benefits provided by the organization and high contamination of natural resources). The emphasis of their adaptation strategy is on the conservation of natural resources, capacity building, diversification and awareness of governments.

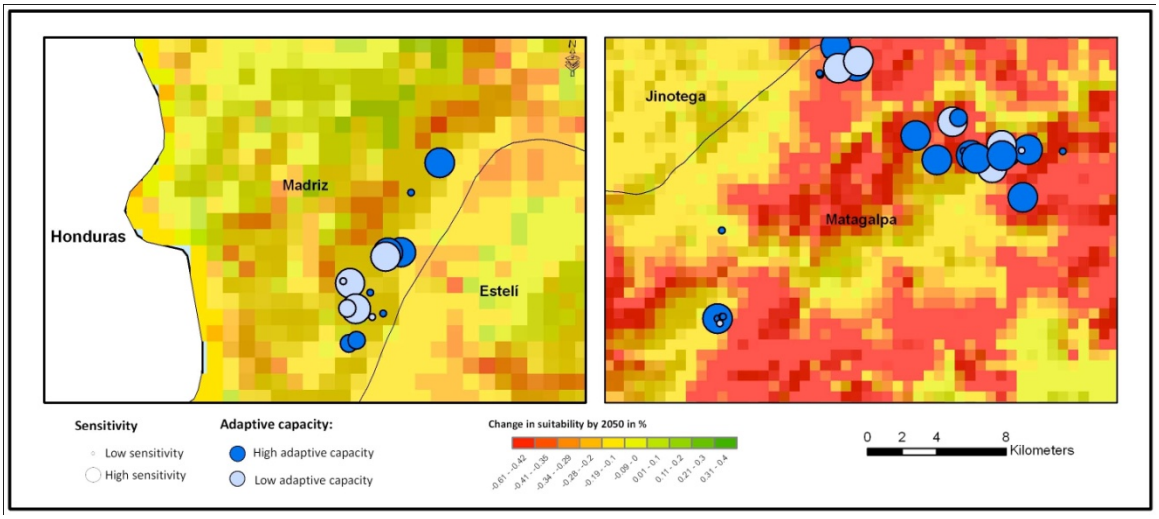

Figure 4. Specific vulnerability profiles of farmers in Madriz (left) and farmers in Matagalpa (right) according their exposure (colour of map), sensitivity (size of dots) and adaptive capacity (colour of dots).

CONCLUSIONS

The consensus of the twenty downscaled IPCC GCM models for the future implies that threat is real and due to the long lead-time of perennial crops such as coffee, action is needed urgently. Variability in vulnerability requires a robust process to site specifically determine impacts and derive site-specific adaptation strategies. Adaptation needs a portfolio of options building on existing risk management strategies. Upgrading the portfolio is likely to include product (i.e. higher quality, symbolic quality), process (i.e. new genetic materials), channel (supplying different market segments), and increasingly inter-sectorial (e.g. strategies based on different crops or ecosystem services). The complexity requires innovative partnerships between the private and the public sector.

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Climate Change: Potential Impact on Eastern Africa Coffees

J.D. WALYARO

Coffee Consultancy Services, P.O. Box 65092 Nairobi, 00618 Kenya.
E-mail: djawalyaro@yahoo.co.uk

SUMMARY

The Eastern Africa sub-region is the origin of Arabica coffee, *Coffea arabica*, and also represents, together with the lowland forests of Western and Central Africa, the primary centre of genetic diversity for *Coffea canephora* (Robusta coffee being the main sub-type). As would be expected, coffee production is the main-stay of economies of most countries in the sub-region where it also represents a major source of cash income for millions of smallholder families not to mention the large number of workers directly employed in the industry. The sub-region furthermore, produces some of the finest coffees in the world. Production of this coffee however, may face the greatest challenge from climate change given that both Arabica and Robusta coffee have rather exacting agro-ecological requirements.

Due to the impact of climate change, coffee cultivation in Ethiopia for example would have to shift from the lowermost optimal cultivation areas now at 1000 m to 1800 m within the next 70 or so years while the optimal coffee area in Oromya, the most important coffee growing region in the country, could be reduced by as much as 30%; not to mention the threat to the already dwindling coffee genetic resources in the country. In Kenya, the rising temperatures and drastic changes in participation patterns could severely limit coffee growing in the medium and low potential areas. For Tanzania however, different zones could experience negative or positive impacts depending on location, with Arabica coffee in the northern and south western sectors remaining unaffected or even recording some improvement. The situation regarding Robusta coffee in Uganda is rather alarming; effects of an overall change of say 2 °C on the ecological suitability of this coffee would severely reduce the total area suitable for its cultivation to less than a tenth of the present area.

Climate change may also lead to a resurgence of certain pests and diseases on coffee. For example, rising temperatures will increase infestation by the Coffee berry borer (*Hypothenemus hampeii*) particularly where coffee grows unshaded and the cropping is continuous throughout the year. Among coffee diseases, Coffee rust (*Hemileia vastatrix*) epidemics will certainly become more severe with increased temperature and rainfall intensity.

A number of adaptation and mitigation strategies for climate change are proposed; perhaps the most important among these being conservation of major forest ecosystems plus intensive but well planned reforestation programmes to rehabilitate and restore the seriously depleted forest covers. Alternative conservation measures could further enhance preservation of coffee genetic resources still remaining in the sub-region. Specific adaptation measures at the farm level should involve promoting sustainable land management practices aimed at halting soil degradation and restoring soil fertility. Coffee varieties with key attributes such as durable host resistance to the present and emerging coffee diseases and better adaptation to a range of adverse growing conditions need to be developed to enhance sustainability of coffee production in the changing environment.

INTRODUCTION

The Eastern Africa sub-region is the home of Arabica coffee *Coffea arabica* L. whose primary centres of origin are the highlands of South Western Ethiopia, the Boma plateau of Sudan and around Marsabit mountain in Kenya (Davis et al., 2006). The sub-region, especially the area around the Lake Victoria crescent in Uganda and Tanzania, together with the lowland forests of Western and Central Africa, also represent the centre of maximum genetic diversity of *Coffea canephora* Pierr ex Froehner (Robusta being the main sub-type). Coffee production is the mainstay of economies of most countries in the sub-region and even for countries that are not so heavily dependent on coffee, it is still among the top agricultural export commodities. The vast majority of coffee growers in the sub-region are smallholder farmers and coffee growing represents not only the way of life but the major source of cash income for millions of such smallholder families as well as an equally large number of workers who directly or indirectly depend on the industry. Though the sub-region produces only modest quantities of coffee compared to major producers like Brazil, Vietnam and Indonesia, it is the source of such fine coffees as Harrar, Sidama and Yirgachefe of Ethiopia, Kenya AA Coffee, Ugandan Gold premium Robusta and Bugisu Arabica, Tanzania Kilimanjaro/Moshi and Bourborn Arabica of Rwanda. The production of this unique coffee however, may face the greatest challenge as a result of global warming given that both Arabica and Robusta Coffee have rather restrictive agro-ecological requirements and that in many areas where they are presently cultivated, the growing environments are approaching their thermal limits (National Geographic, 2010).

It is generally agreed that there are a number of factors that are responsible for climate change. Among these however, human activities or anthropogenic factors are largely responsible for increased gas emissions that have the greatest influence on global warming. According to the assessment report by IPCC (2005) warming during the past 100 years was only 0.74 °C with most of it occurring during the past 50 years. For the next 20 years, projected warming is estimated at 0.20 °C per decade and if green house emissions continue at the present levels, the world average temperature would rise a whopping 3 °C this century. Some of the predicted impacts in the tropics include changes in rainfall patterns, increased intensity and severity of floods and droughts, upward shifts in the range of plant and animal species, reduction in arable land and increased water shortage. Even with moderate increases of 1-2 °C in local temperature, crop yields are expected to decrease sharply. Given this scenario and the narrow agro-ecological margins within which Arabica and Robusta coffee thrive, it is easy to appreciate the potential enormity of climate change effects on the survival of coffee within the sub-region. The following is the projected scenario in several countries in the sub-region.

CLIMATE CHANGE IMPACTS

Though Ethiopia is the largest coffee producer in Africa with estimated average production of around 330,000 tonnes in 2007/2008, the rainfall trend for example in the central highlands (Abuhay Takele and Mesfin) has become extremely erratic in terms of commencement, stoppage, intensity and distribution, in addition to a general decreasing trend. With regard to temperature, there has been an increased warming trend over the last 50 years with the annual minimum temperature increasing 0.25 °C every 10 years and average maximum temperature increasing by 0.10 °C every decade. Over the recent years there has been also a dramatic increase in the frequency of floods and droughts. An investigation involving three global models predicts further increased frequency of droughts under climate change, a decrease in

precipitation by 1-2% in 2030 and 2050 and a warming trend of 1-2 °C within the same period.

As a result of these changes, coffee cultivation may be greatly affected particularly by the rising temperature and to some extent the rainfall pattern and intensity. Coffee cultivation is predicted (Wolfgramm et al., 2008) to shift to higher elevations as most of the current areas become unsuitable for coffee to the extent that within the next 70 or so years the lowermost optimal cultivation area will shift from 1000 masl to 1800 masl. Furthermore, it is postulated that the optimal cultivation area for coffee in Oromya, one of the most important coffee growing region in Ethiopia, could be reduced by as much as one third by the end of the century. The coffee genetic resources of Ethiopia on the other hand, comprising the maximum genetic diversity found among the forest coffees of South Western highlands and the rich taxonomy of landraces in Eastern Ethiopia, are becoming highly endangered (Labouisse et al., 2008). Apart from deforestation and encroachment by agricultural activities, climate change is responsible in a significant way for the rapid loss of these most valuable resources. Predicted climate change scenario in Ethiopia is depicted in Figure 1.

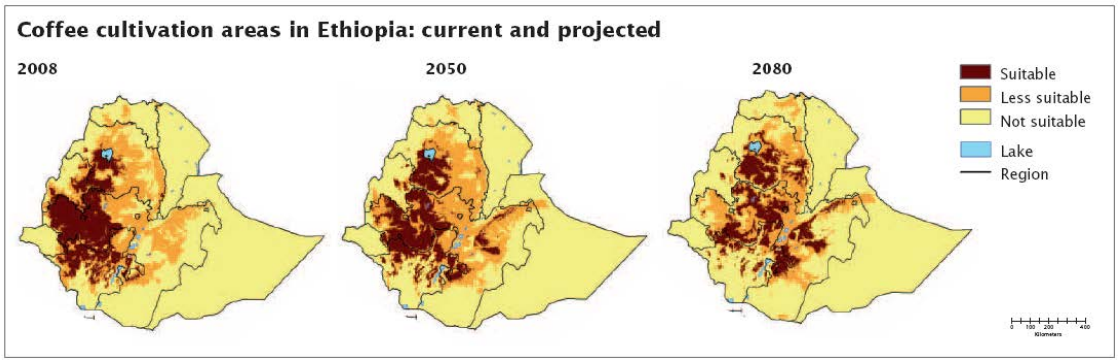

Figure 1. Projected change of coffee cultivation areas in Ethiopia over the next 70 years. Data: Worldclim and IFPRI. Map algebra: Michael Rüegsegger 2008. More details: www.cde.unibe.ch, search entry "Michael Ruegsegger".

Uganda, the second largest coffee producer after Ethiopia has continued to record considerable yearly fluctuations in output over the recent years partly due to biennial bearing but mostly due to erratic weather conditions. For example, 2008 was the year with the highest production at 234,000 tons; this declined the following year to about 160,000 tons due to severe drought however, because of favourable rains this season, yield is projected at just over 180,000 tons. Such changes in coffee output reflect early signs associated with climate change that comprise periods of heavy downpours causing floods and landslides and often out of season, interspersed with prolonged droughts. There is in fact recent scientific evidence by UNEP and NEMA (2009) which indicates, among its findings, that the melting of the icecap on the Ruwenzoris due to climate warming is already causing such increased water flow in the Semliki that it is eroding its banks and silting Lake Albert.

Many coffee growers in Uganda claim that climate change is already affecting not only their harvests but also the quality of their coffee. Future predictions of Robusta coffee cultivation in Uganda are quite alarming as global warming could cause growing conditions in the current areas to change drastically in terms of sustaining coffee production. The average temperature in Uganda coffee growing areas is about 25 °C (Nsangi, 2008) while the optimum temperature range for Robusta coffee is between 22-26 °C (ITC, 2010) Furthermore, Robusta is more sensitive to extreme climate than even Arabica coffee. As indicated in Figure 2, if

average temperature were to rise by 2 °C, which is a likely scenario, the total area suitable for optimal growing of coffee would be reduced to only 10% of the present area. The rest would be too hot to sustain optimal growth of Robusta coffee. Of more concern is that this could happen within the next 40 or so years.

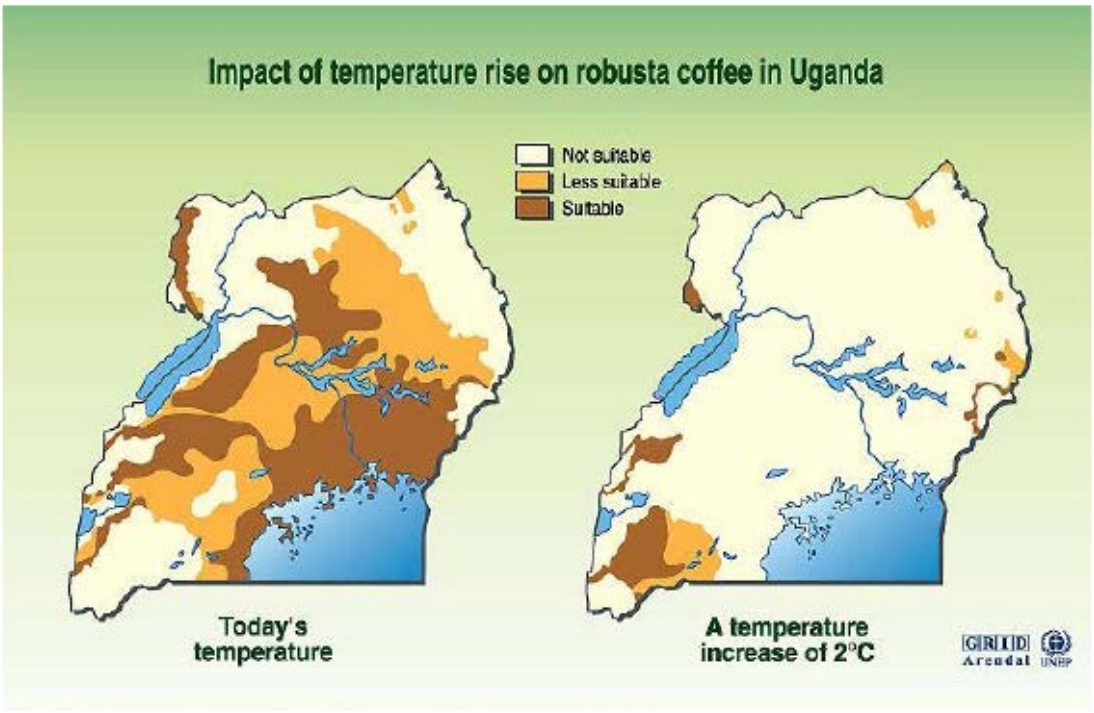

Figure 2. Impact of temperature rise on robusta coffee in Uganda.

Of the coffee growing countries in the sub-region, Kenya is the most water deficit and therefore perhaps the most vulnerable to climate change. The impacts of unmitigated climate change may have the most far reaching consequences not only on coffee production but the entire agricultural sector. Coffee production has plummeted from a high of over 120,000 tons in 1985/86 to a low average of between 40,000 to 50,000 tons per annum. While this decline may be due to a number of other reasons, the fluctuations in output over recent years reflect unpredictable rainfall patterns in terms of seasonality, alternating with periods of severe droughts. In 2007 for example, production dropped to 41,000 tons due to abnormal rains but was followed by a high of almost 58,000 tons in 2008 and then a decline to 42,000 tons in 2009.

Recent predictions (Fitzinger, 2010) indicate that total rainfall in coffee areas will continue to show extreme variability but may increase by about 12.5% while yearly average temperatures will rise by 2.3 °C by 2050. Like Ethiopia, areas suitable for coffee growing may migrate to higher elevations from 1400-1600 masl to 1600-1800 masl by 2050. Only 30% to 40% of the present areas will remain suitable for coffee growing. Increased temperatures will also cause substantial reduction in coffee quality unless adaptive strategies are effected. While coffee in lower altitudes between 1000 to 1400 masl could almost be obliterated, that in areas up to 2000masl will experience better growing conditions. The above picture however, will be site specific as there will be areas that fare much better than others. The major impact of climate change in Kenya therefore, would be a severe reduction in coffee growing in the low and medium potential areas of the country.

In Tanzania, the fourth largest coffee producer in Africa, the effects of climate change are already being felt in terms of rising temperatures, erratic rainfall and prolonged droughts Coffee yield in recent years has fluctuated with peaks of 68,000 tons to lows of 34,500 tons. A comprehensive analysis of climate trends (Agrawal et al., 2003) indicates that the country will also experience significant impacts of climate change. For example, while changes in rainfall maybe uncertain, average temperature increases countrywide of 1.3 °C to 2.2 °C may be expected by 2050 and 2100 respectively. However, unlike the situation in Ethiopia, Kenya and Uganda, agriculture and water resources sectors in Tanzania will experience both negative and positive impacts. Average annual rainfall is expected to increase by 10% in 2100 on doubling of CO_2 but there will be considerable variation both in intensity and distribution. Under this scenario, the major cash crops in Tanzania i.e. coffee and cotton may experience some increases in yield (Tanzania Initial National Communication, 2003). For example, depending on the actual change in precipitation, the increases in coffee yield may range from just below 20% in an area like Lyamungu in the North under bimodal rainfall to about 15% in unimodal rainfall areas like Mbozi.

Apart from the predicted disruptive effects described above, climate change may also lead to serious resurgence of certain pests and diseases of coffee. A recent ICIPE study (Jaramillo et al., 2009) confirms that rising temperature will cause an upsurge of infestation by the Coffee berry borer (*Hypothenemus hampeii*), one of the world's most dreaded coffee insect pests. Studies found that egg to adult development occurred between 20-30 °C, and the fastest development of the insect was between 27-30 °C. The number of pest generations per year in Kenya and Colombia, 3.1 and 3.4 respectively were positively correlated with the warming tolerance. Before 1984 when temperatures were fairly cold in Jimma, Ethiopia, *H. hampei* could hardly complete 1 generation per year, however, because of climate change in the same area, it now completes 1-2 generations per year. Infestation appears worse where coffee grows in the open and where flowering and therefore cropping is continuous throughout the year. Apart from Coffee Berry Borer, there are also indications that global warming would influence other coffee pests such as the Leaf miner (*Leueoptera coffeeilla*) and some nematode species.

Among coffee diseases, global warming would significantly increase pressure from Coffee leaf rust (*Hemileia vastatrix*). The fungus is favoured by warm and humid climate and normally rainfall of 7.5mm or above is required to trigger an outbreak. It is these warm and humid climate conditions that led to perhaps one of the most serious epidemics of any coffee disease, when rust completely decimated coffee plantations in South and South East Asia.

STRATEGIES TO ENHANCE SUSTAINABILITY

Perhaps the most important among mitigation strategies to climate change in the sub-region, is enhanced conservation of major forest ecosystems, which are the major water reservoirs and habitats of biodiversity among which are the endangered coffee gene pools of the sub-region. At the same time, massive but well planned reforestation programmes must be undertaken urgently to rehabilitate and restore the seriously depleted forest covers so common in most countries in the sub-region. Where possible, there should be continued expansion of forest cover on most degraded and non arable land including steep slopes and water catchments in every country. Forests, apart from being some of the most important carbon sinks due to their high biomass are also the most efficient in conserving soil organic carbon thus providing additional carbon sequestration.

As regards preservation of coffee genetic resources still remaining in the sub-region, alternative complementary conservation measures could mitigate to some extent, climate change impacts. Genetic resources held *in-situ* consisting mainly of protected genetic reserves e.g. biospheres etc which contain wild coffee germplasm, and on-farm conserved semi-cultivated germplasm and coffee landraces (Dulloo et al., 2001) are the most vulnerable. Effective conservation will require concerted efforts of all stakeholders including national Governments, local communities and various organizations to protect such habitats. Financial support from donor communities and well wishers would be crucial to such efforts. For *ex-situ* conservation, the options for protection of field genebanks are the same as adaptation strategies that are outlined below for promoting sustainable land management practices. However, even now, maintenance of these field collections is becoming a heavy burden to the various national institutions. It is because of this that the more innovative methods for *ex-situ* conservation may be the ideal solution for long-term conservation. The most promising option for now, is cryopreservation (Dussert et al., 2007). As more recent results indicate, the IRD protocol as was used for cryopreservation of *C. arabica* seed at CATIE (Costa Rica), appears to be the most cost effective long-term conservation strategy that can now be routinely applied for coffee genetic resources.

Specific adaptation measures to minimise effect of climate change at the farm level should involve promoting various sustainable land management practices. Increased use of shade is probably among the most effective of such adaptation strategies in coffee. Shade decreases air temperatures as much as 2-6 °C (ITC, 2010), it also decreases wind speed thus conserving relative humidity and moderating extreme weather changes and also provides conditions unfavourable for Coffee berry borer infestation. Moreover, shade trees themselves are an additional carbon sink and though shade entails some reduction in yield, it improves coffee quality thus compensating for the yield loss. Other important sustainable land management practices include, (i) use of mulch to reduce evaporation and soil temperature, prevent erosion and improve soil fertility; (ii) terracing/ contouring, trapping and conserving runoff water and, (iii) adapting high density planting. Apart from making the soil more resilient and increasing productivity, these other interventions also mitigate climate change through carbon sequestration.

Conservation of head water forests or water towers that increase infiltration of rainwater is vital for sustaining dry season flows, an important source of water especially in water deficit countries. Such water can be used for supplemental irrigation in coffee and for other purposes. To illustrate the importance of conserving such forests, it has been documented (Wolfgramm et al., 2008) that forests and moor lands of Mount Kenya between 1985-1999 contributed up to 65% of dry season flow while glaciers accounted for only 8%. Finally, coffee processing both on-farm and final processing can be improved greatly to counter effects of climate change through use of technologies that are already available for example, use of eco-friendly pulpers to economize on water use, installation of recirculation systems and proper disposal of factory effluent for example, in biogas production and, conversion of milling by-products into fuel or charcoal briquettes etc.

Among the most important long-term options for ensuring sustainability of coffee production in the sub-region is developing new varieties of both Arabica and Robusta coffee that are able to withstand adverse growing conditions emanating from climate change. Among the key attributes of such varieties would be durable host resistance to the present and emerging major coffee diseases, drought tolerance, tolerance to high temperatures that exceed the range of present optimum temperatures for Arabica and Robusta coffee and better adaptation to high density plantings. In addition, it may be worth investigating the feasibility of breeding types

with coffee quality attributes that are least sensitive to temperature variation i.e. types that perform relatively better in terms of quality regardless of whether they are being grown in cooler or warmer environments.

Finally, providing and improving access to reliable climate data, mapping potential climate change impacts in different coffee areas in each country as well as strengthening institutions that can provide technical and financial support are all extremely vital in the implementation of most of the suggested mitigation and adaptation options against the impact of climate change on coffee in the sub-region.

CONCLUSION

Climate change is a real potential threat to sustainable coffee production in the Eastern Africa sub-region. Though the effects will vary with geographic location and altitude, predictions indicate drastic changes in suitability of coffee growing areas ranging from migration of such areas to higher altitudes to almost complete obliteration of suitable growing areas in some countries. Climate change will also affect both yield and quality of coffee and may increase certain coffee pests and diseases. It is therefore essential that sound adaptation and mitigation strategies are put in place to save the coffee industry. Such measures must include, enhanced conservation of major forest ecosystems, implementation of appropriate agronomic measures to halt soil degradation and exploitation of modern genetic tools to develop more resilient coffee varieties. At the same time, improving framework conditions for adaptation and institutional capacity building, including providing financial support will be vital in minimising the potential effect of climate change.

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An Assessment of Land Degradation in Kilimanjaro Region, Tanzania, and Its Implications to Coffee Sustainability

G.P. MARO[1]*, A. KITALYI[2], M. NYABENGE[3], J.M. TERI[1]

[1]Tanzania Coffee Research Institute, P.O.Box 3004, Moshi, Tanzania
[2]International Centre for Research on Agroforestry, Tanzania, c/o Mikocheni ARI, P.O.Box 6226, Dar es Salaam, Tanzania
[3]International Centre for Research on Agroforestry (GIS unit), Regional Headquarters, Nairobi, Kenya
*E-mail: godsteven.maro@tacri.org, marogp2002@yahoo.com

SUMMARY

A study was conducted in Kilimanjaro region, Tanzania, to map its vulnerability to land degradation using GIS modelling approach. The Revised Universal Soil Loss Equation (RUSLE) model was adopted, with spatial data processed under weighted overlay approach. 13 representative locations were surveyed for bio-physical indicators of land degradation (erosion and decline in soil quality). Soil losses were calculated from field observation of rills, gullies, root exposure, solution notches and build-up against barriers. Fertility decline was determined from inverse ratings of soil analytical data with coffee as a reference crop. The final ratings were used to validate the GIS model ratings, and other ratings empirically assigned on basis of soil types.

A regional vulnerability map was developed from the model, together with maps of 3 selected watersheds. It was noted that the more vulnerable areas are located within the coffee belt, and are mainly those whose land use was changed from coffee to less shade-loving crops like vegetables and spices. The bio-physical assessment confirmed the existence of the problem at varying magnitude, being more marked in the Kikafu/Rau watershed. Soil loss was 7 times here compared to Butu/Ngofi, while it was apparently non-existent in Vunta. Validation of the vulnerability ratings showed high positive variation by 25%, whereas low positive and negative variation was in 32% and 13% respectively. Ratings assigned according to soil types showed closer similarity to field observation than the GIS model.

The high vulnerability to land degradation in the coffee areas, as revealed in this work, poses a major threat to the coffee agroforestry system, which is predominant in the region, and also to the sustainability of the famous Kilimanjaro Coffee. Concluding discussion compares this development scenario with global debate on importance of shaded coffee for sustaining ecosystems services. Suggestions for sustainable land management practices and areas for further research are given.

INTRODUCTION

Kilimanjaro Region is located in the northern part of Tanzania, between latitudes 3°39’ and 4°37’ South, and longitudes 36°52’ and 38°25’ East. It has an area of 13,209 sq. km, divided into 6 districts Siha, Hai, Moshi (Rural and Municipal), Rombo, Mwanga and Same. According to KRP (2009) it is the third most densely populated region with an average of 104

people.km^{-2} ranging from 650 people.km^{-2} in the upper belt to about 50 people.km^{-2} in lower belt. The population is estimated at 1,602,530 people.

Coffee production in Kilimanjaro is both historical and traditional. The region was the first in Tanzania to grow coffee as a commercial crop. It was established by German missionaries in 1896, and had been adopted by both estates and smallholder farmers by 1910 (Robinson, 1964, Wrigley, 1988). The Kilimanjaro coffee is a special mild Arabica, sold mainly to Germany and Japan (Ikeno, 2007). It is of such high quality that it attracts blending with lesser-quality coffees (Fitzsimmons, 2007), so there is always a demand that the supply cannot meet.

Coffee production has declined irregularly from 19,859 tons clean coffee in 1981/82 to a shocking minimum of 3134 tons in 2005/06. Many reasons for this have been suggested, including the coffee price slump (Envirocare, 2004), change in cropping patterns (Maghimbi, 2007), climate change (Agrawala et al., 2003; Orindi et al., 2006 and Maro and Teri, 2008), land fragmentation (Chimilila et al., 2008) and land degradation. The latter is defined (Stocking and Murnaghan, 2000) as the reduction or loss of the biological or economic productivity and complexity of land resulting from land uses or from other human activities and habitation patterns.

It is important to study the contribution of various processes of land degradation within Kilimanjaro region in order to single out the factors of declining productivity caused by land mismanagement, which can be mitigated through good policies and practices. The objectives of this work were therefore to map the vulnerability of Kilimanjaro Region (and selected watersheds) to land degradation using GIS modeling approach, to perform bio-physical field assessment in selected locations within the watersheds so as to check and validate the findings of GIS modeling against ground-level truth, and to advise on ways to manage the land more sustainably, with special focus on the coffee land in Kilimanjaro.

METHODOLOGY

GIS mapping of vulnerability to land degradation

The basic vector map layers (boundaries, rivers, road networks, altitude) were obtained from ARI Mlingano GIS Centre. They were projected to the UTM projection and then rasterized to have uniform grid sizes of 20m. Land degradation was taken to mean the loss of land quality as a result of human induced soil erosion. The Revised Universal Soil Loss Equation (RUSLE) model was adopted (RUSLE, 2003). The slope map was generated from the DEM with the resulting slope units as percentages, while land cover map was from NDVI. The other RUSLE data input and output requirements were adopted and different layers converted to GIS modeling format and processed under weighted overlay model in ArcGIS 9.3.

Data reclassification was done to introduce the influence of the various degradation factors as suggested by Waruru et al. (2003). The reclassified layers were combined and given equal weights according to RUSLE requirements.

Bio-physical assessment of selected watersheds:

Selection of watersheds for biophysical assessment was guided by the information from the GIS model. A total of 3 watersheds were selected; to represent the slopes of Mt. Kilimanjaro and Pare Mountains. Five locations in the Kikafu watershed (Moshi/Hai) including four along

a northeast-southwest transect and a 5th located in the lowland out of the coffee zone were selected. In the Butu/Ngofi watershed (Mwanga), two locations were randomly selected in the upland and two in the lowland. In the Vunta Hedaru watershed (Same), four locations were randomly selected.

In each location, a focal profile was opened and described according to FAO (1990). Bulk and undisturbed samples were taken and subjected to physical and fertility analysis respectively by using procedures outlined in NSS (1990). From this focal point, an area of estimated minimum radius 50 metres was surveyed and assessed for land degradation indicators (rills, gullies, pedestals, tree mounds, build-up against barriers, etc), with guidance from Stocking and Murnaghan (2000).

Ratings of soil erosion (by water) were adopted from Stocking and Murnaghan (2000). Soil fertility decline was rated according to the soil fertility requirement of coffee (Sys et al., 1993). Rating scores were assigned separately, and added to get total fertility ratings which were finally inverted. Topsoil depth, overall profile depth, textural classes, porosity (Brady and Weil, 1999) and subsoil rooting intensity were rated separately and means calculated and re-rated by inversion. In all cases, final ratings ranged from 0 (no effect) to 3 (severe effect).

Model validation

Model validation was done by normalizing the ratings based on field observation in the 13 selected locations by assigning zero values, and assessing the positive or negative variation of the model from the normal (Maro, 2004). Vulnerability ratings based on soil types (Stocking and Murnaghan, 2000) were concurrently assessed.

RESULTS AND DISCUSSION

The results of GIS modeling were land degradation maps for the Kilimanjaro Region (Figure 1a), plus 5 watersheds of Kikafu and Rau (Figure 1b), Butu and Ngofi (Figure 1c), and Vunta (Figure 2) produced with 3 levels of vulnerability (low, medium and high) to land degradation.

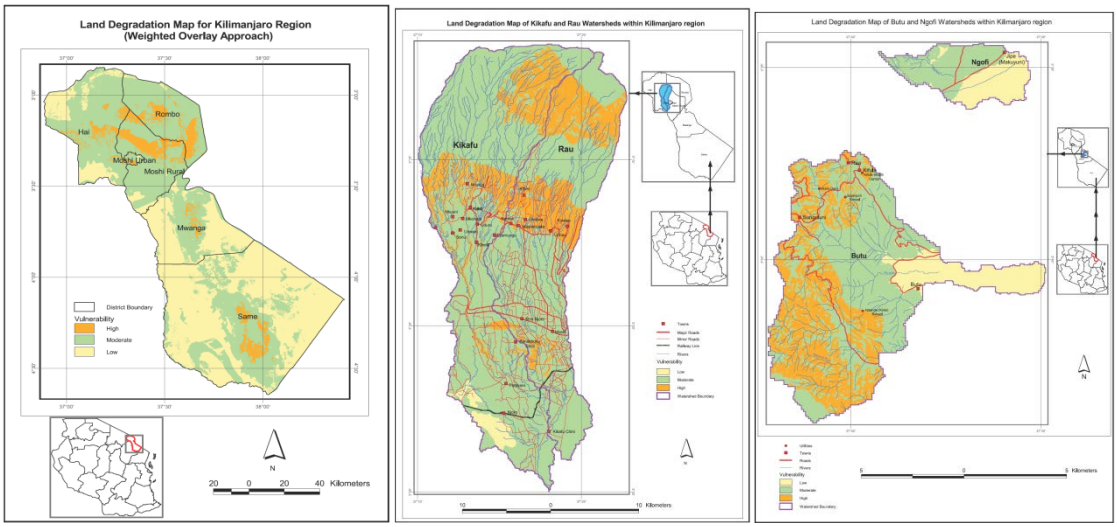

Figure 1. Vulnerability map for Kilimanjaro region (left), Kikafu/Rau (centre) and Butu/Ngofi (right).

Site selection for biophysical assessment was based on the ratings given in the resulting GIS maps (Figures 1 and 2a). The 13 sites were georeferenced and input as point themes in the SOTER soils shapefile of Kilimanjaro region to define the soil types as in Figure 2b.

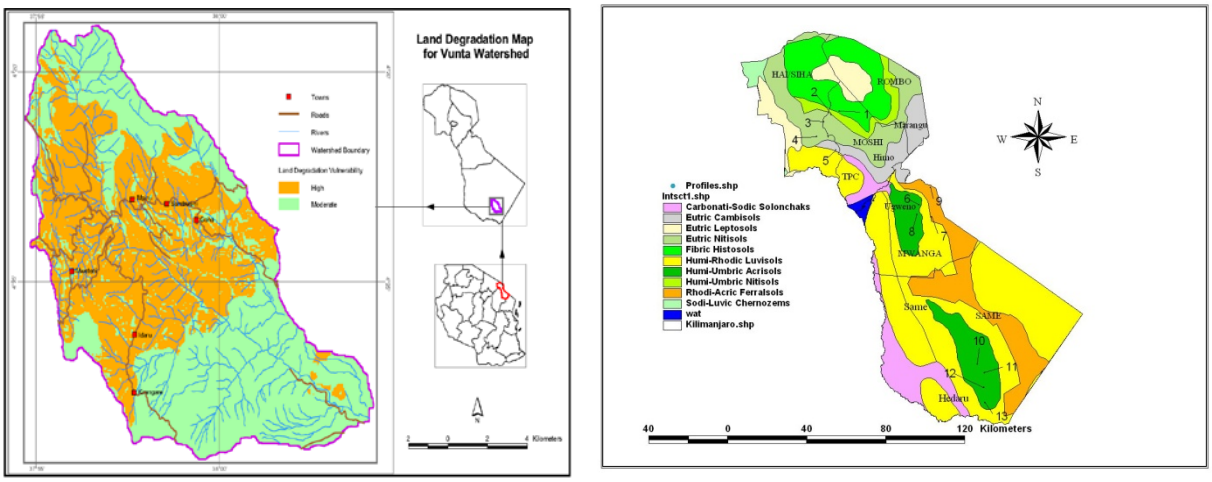

Figure 2. The vulnerability map of Vunta watershed (a), soil map of Kilimanjaro (b).

Table 1. Summary of erosion and nutrient depletion susceptibility per village.

Site	Village	WRB code	Resilience to erosion	Erosion rating	Resilience to fertility decline	Nutrient depletion rating	Coffee	Other crops
1	Omarini	HSfi	2	x	2	1	1	x
2	Umbwe	NTeu	2	0	1	1	1	x
3	Narumu	NTeu	2	2	1	2	0	2
4	Kwasadala	NTeu	2	3	1	2	0	1
5	Shiri	CMeu	3	1	2	2	0	1
6	Mringo	ACum	1	3	2	1	1	1
7	Butu	LVro	2	1	1	1	0	1
8	Kiriki	ACum	1	2	2	2	0	2
9	Jipe	FRac	2	2	2	3	0	1
10	Hotolwe	ACum	1	1	2	2	0	2
11	Mpinji	ACum	1	1	2	1	0	1
12	Idaru	ACum	1	1	2	2	3	1
13	Vunta Papa	ACum	1	2	2	2	0	1

NB: x = not apparent; 0 = none; 1 = slight; 2 = moderate; 3 = severe/ most prominent
Key adapted from Stocking and Murnaghan, 2000.

The status of soil degradation in the selected watersheds is given in Table 1, together with resilience ratings assigned to respective soil types (Stocking and Murnaghan, 2000). Coffee was seen in only 4 sites and is in poor condition, partly attributed to the land being too

fragmented to be economical (Maghimbi, 2007). Other crops (and particularly, annual crops) are grown everywhere, even in very steep slopes. Some unfamiliar crops such as ginger were found in open table terraces in Same district.

Soil erosion was appreciated as a big problem, with 2 sites severely affected, 4 moderately affected, and 4 slightly affected. Nutrient depletion seems to be most serious at Jipe, characterized by Ferralsols of low CEC (Van Ranst, 1997). As far as soil loss is concerned, Vunta watershed registered none. Comparing the remaining watersheds, the Kikafu-Rau watershed registered soil loss about 7 times that registered from Butu-Ngofi. One of the major contributing factors was seen to be indiscriminate logging, particularly at Hotolwe and Mpinji villages.

Comparing the total loss associated with the individual processes, gullies result into largest amounts of soil loss (69%), followed by root exposure and build-up against barriers (23 and 6% respectively). Tree mounds, rills and solution notches were the lowest (Figure 3).

Validation of the vulnerability ratings given in the maps showed high positive variation in 3 sites out of 13 (which is 23%), whereas low positive and negative variation was in 5 (38.5%) and 2 (15.4%) sites respectively. On the other hand, validation of ratings assigned according to soil types (Stocking and Murnaghan, 2000) showed low positive and negative variation in 4 and 2 sites respectively. The former showed similarity in 3 sites while the latter showed similarity in 7 sites (Figure 4). At the scope represented in this work, soil type proved to give a slightly better estimation of land degradation vulnerability than GIS modeling.

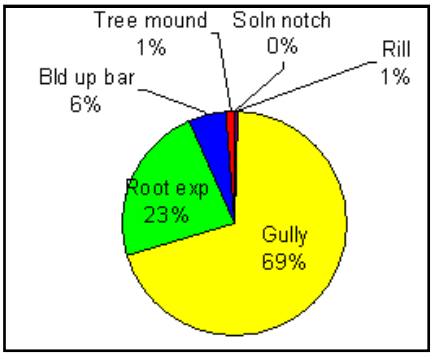

Figure 3. Contribution of various processes to the total soil loss.

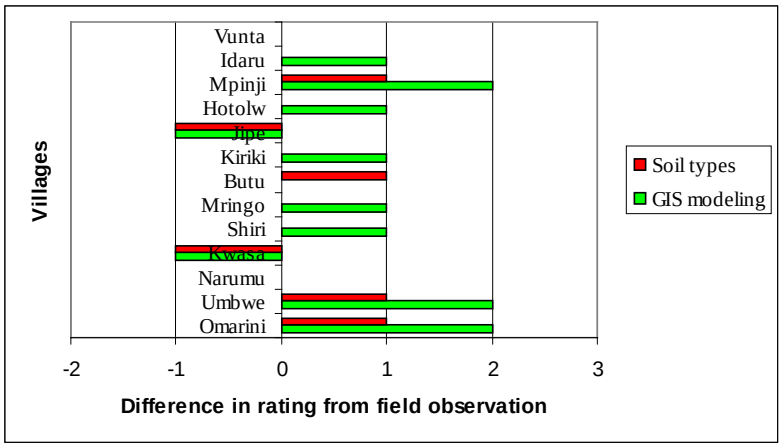

Figure 4. Comparison of GIS modelling and soil types against field observation.

The findings of this work fuel the already existing debates on coffee economics and feasibility, and on shaded coffee culture. Economics dictate that, if coffee does not make ends meet, you either move out of it altogether (like in parts of Machame – Hai and Vunta – Same, the latter opting for ginger, in open table terraces) or look for complementary ventures. For the sake of sustaining both the quality Kilimanjaro coffee and the environment, this paper recommends the latter option. While banana intercropping, livestock-keeping, bee-keeping and mushroom growing are the recommended diversification options, TaCRI has addressed the issue of land crisis and coffee economics by developing "compact" coffee varieties that require less spacing.

Shaded coffee production is traditional among smallholder coffee farmers in Kilimanjaro. Advantages of shade culture in coffee have been well described by Wrigley (1988). Shade culture for coffee smallholders also provides an appropriate canopy to significantly sequester carbon (Soto, 2010). Incentives to shaded culture among smallholder coffee farmers include certified market niches such as Utz Kapeh, Rainforest Alliance, Fair Trade and Organic (Saes et al., 2003; Mendez and Bacon, 2006; Consumer International, 2008). Other possible incentives are the Carbon Credits, whereby a farmer is paid for the contribution of his/her trees in mitigating the effects of climate change (Cacho et al., 2003; Montagnini and Nair, 2004; Polzot, 2004). The role of coffee in carbon sequestration also needs to be determined so that coffee can be considered for carbon credits (Dengu, 2010).

CONCLUSION

This study demonstrated that it is possible to map the vulnerability of defined locations to land degradation using GIS modeling approach. On the other hand, from the validation process, it proved the common understanding that modeling is a crude representation of reality, in this case over-estimating the problem. So care should be exercised when interpreting and using models in decision making.

From the bio-physical field assessment, the more vulnerable areas are located within the coffee belt, and are mainly those whose land use was changed from coffee to less shade-loving crops like vegetables and spices as the result of land shortage and coffee price slump. To improve coffee sustainability in Kilimanjaro, an institutional approach is needed to enable re-consolidation of coffee land, and enforce strict laws against land mismanagement. People should be convinced to turn back to coffee, not only as a stable economic venture, but also an environmentally friendly one. They should replant coffee where it has been removed, and also rejuvenate old unproductive trees by stumping. Sustainable land management practices such as shade coffee, mulching, rain water harvesting and well-guided fertilizer interventions are recommended, concurrently with adoption of new compact coffee varieties developed by TaCRI, which require less spacing. Income diversification endeavours in smallholder coffee farms (such as livestock, apiary and mushroom growing) should be encouraged.

It was also proved that land degradation contributes substantially to the change of microclimate in the Mt. Kilimanjaro catchment. As such, global climate change should not be taken as a sole cause of the problem. Further research is needed to distinguish between global and local contributing factors.

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Reducing N-NO_3^- Leached on Coffee Based Agroforestry System by Impede Nitrification

S. KURNIAWAN[1], K. HAIRIAH[1], WIDIANTO[1], D. SUPRAYOGO[1], R. WANDRI[2]

[1]University of Brawijaya, Faculty of Agriculture, Department of Soil Science, Malang – Indonesia
[2]Graduate Soil and Water Management-Brawijaya University.
E-mail: kurniawan_syahrul@yahoo.com

SUMMARY

N-NO_3^- loss by leached in coffee based agroforestry system can reduced by inhibiting nitrification process through controlling of litter quality input. To this purpose, various litter input (single or mixed) from shade trees applied on coffee based agroforestry system shaded *Gliricidia sepium*, which gave on a single application compared to the same amount of litter provided in small 5-daily increments ('spoon-feeding') application. The actual concentration of NH_4^+ and NO_3^- in the soil were measured from an extract of soil samples collected at 0-10 cm, 10-20 cm, 20-40 cm, and 40-80 cm depth on 1st, 2nd, 4th, 8th, and 12th weeks after application (WAA). Application of a mixed litter of coffee + *Gliricidia* + timber trees (*Maesopsis*, mahogany and teak) can cause the immobilization of N-NH_4^+ and N-NO_3^-. These immobilizations can occur either as single or spoon-feeding application. It is suggested that small amounts (ranging from 30-50 kg N ha^{-1}) of inorganic-N fertilizer may be needed at the same time of litter application to avoid N deficiency in multistrata agroforestry systems. The WaNuLCAS simulation explains that efficiency use of soil mineral N in coffee-based agroforestry multistrata from litter decomposition is better than in coffee monoculture.

INTRODUCTION

Forest conversion into coffee based agroforestry system or other land use type leads to rapidly changes of the soil surface microenvironment and affects the activity of soil organisms, nutrient cycling and other soil processes. At plot level, land use change leads to declining soil fertility, indicated by compacted soil, low soil organic matter content, and high nutrient losses (N-NO_3^-) to deeper layer due to (temporary) excess in availability in the soil, beyond what plants currently need. N-NO_3^- concentration in several agroforestry coffee (i.e coffee shaded by *Paraserianthes falcataria*, coffee shaded by *Gliricidia sepium*, coffee monoculture and complex agroforestry coffee) ranging from 7.2 to 12 mg kg^{-1}. It was higher than N-NO_3 concentration on forest 4.6 mg kg^{-1} (Purwanto et al., 2006). On the other research showed that nitrate concentration in the soil solution and consequently less leaching under hedgerow intercropping with *Leucaena leucochepala* and annual food crops than in the agricultural control treatments in southern Benin (Horst, 1995). Several mechanism may contribute to reduce N-NO_3^- leached under agroforestry system compared with agricultural monocrop through increased litter, mulch, and root (Lehman and Scroth, 2003). This research is focused on the influence of litter input from many type of shade trees on N-NH_4^+ and N-NO_3^- released.

The types of shade trees in coffee based agroforestry system influence litter input (quantity and quality) and nitrification potential (NH_4^+ release). Commonly, high litter quality from

leguminosae family will be mineralized rapidly and release $N\text{-}NH_4^+$ into soil solution. On the contrary, low litter quality input such as avocado (*Persea americana*) and durian (*Durio zibethinus*) cause $N\text{-}NH_4^+$ immobilization at early weeks decomposition process (Purwanto et al., 2006). Therefore, the keys of controlling nitrification process can be done by manipulate decomposition and maintain low concentration of $N\text{-}NH_4^+$. Of interest is also the possibility of mixing different litter types, such as recalcitrant materials, which provide a long-lasting surface mulch, with high-quality materials, which decompose and release nutrient rapidly (Scroth, 2003).

In semi-controlled field experiment, research about organic matter decomposition and nitrogen mineralization was conducted by a number of researchers (Handayanto, 1994), but the litter had given in single in the early research. In the mean time, in the natural condition, litter input occur by litter fall except pruning and branching. Commonly, litter input from litter fall is very low, but it was occur continuously. As an impact, the potential $N\text{-}NO_3^-$ leached from the litter fall is lower than organic matter input from pruning. This research is aimed to reduce $N\text{-}NO_3^-$ leached for the best management practices in coffee based agroforestry system by impede nitrification process at closed natural condition.

MATERIALS AND METHODS

Semi-controlled experiment conducted on 2006 in farmer owned-land particularly on coffee shaded by *Gliricidia sepium* in Sumberjaya-West Lampung (5°01' – 5°02' LS and 104°26' – 104°27'). Based on topography map, the study site is located between 600-1718 m above sea level, has a mean annual rainfall of 2614 mm, an average daily air temperature of 21.2 °C, and relative humidity in the range of 81-89% (Agus et al., 2002). The soil type in the area mostly classified as Oxic dystrudept which specify has clay texture, total pore about 69%, particle density 0.9 g cm^{-3}, and soil pH between 4.0 and 5.0 (Dariah et al., 2004).

Two factors were arranged according to randomized block design i.e type of organic materials in combination with time of application. The first factor consisted of six organic matter input: (1) *Coffea robusta* (C), (2) *Gliricidia sepium* (G), (3) mix of C+G (ratio of 1 : 1), (4) C + *G* + LCC (*Arachis pintooi*) with ratio of application of 1:1:1.5, (5) mix of *C+G* + fruit trees (durian, *Durio zibethinus;* avocado, *Persea americana*, belinjo, *Gnetum gnemon*, candle nuts, *Aleurites moluccana)* with ratio of application of 1:1:1.5:1.5:1:1, and 6) mix of *C+G* + timber trees (african trees, *Maesopsis eminnii*; mahogany, *Swietenia mahogany;* teak, *Tectona grandis*) with ratio of 1:1:1:1.5:1.5. The results were compared to control treatment: (1) Without or no organic matter input and 2) no organic matter input but with Urea application.
The dose of organic matter input was equivalent to 200 kg N ha^{-1} (Purwanto et al., 2006). Amount of applied organic matter for each treatment was given proportionally according to its N content of each material. The second factor were time of application i.e. a single application of litter compared to the same amount of litter provided in small 5-daily increments ('spoon-feeding') application.

Methods for analyze of lignin and polyphenol in litter have been described in detail by Anderson and Ingram (1993) (Scroth, 2003). Lignin was analyzed via acid detergent fibre (ADF) (Anderson and Ingram, 1993). ADF is prepared from plant material by boiling with a sulphuric acid solution of cetyltrimethyl ammonium bromide (CTAB) under controlled conditions. Lignin is removed by oxidation with a buffered permanganate solution (Anderson and Ingram, 1993). Furthermore, Total soluble polyphenolics are analysed by the Folin-Denis method, and include hydrolysable tannins and condensed tannins, as well as non-tannin polyphenolics (Anderson and Ingram, 1993).

Data of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentration reported here based on 12 weeks of measurements i.e. prior application, 2 week, 4 week, 8 week, and 12 week after application (WAA). Composite soil samples were collected from four soil depths: 0-10 cm, 10-20 cm, 20-40 cm, and 40-80 cm. Soil samples were taken in the morning to minimize N loss through volatilization, it placed in cool box during transportation to the laboratory. Five gram of soil sample was extracted with 20 ml KCl 2 M, subsequently concentration of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ in soil solution was determined by flow injection analysis (FIA) (Anderson and Ingram, 1993).

Organic matter input with different quality will affect N mineralization rate, which can be calculated using an equation $Y = a + \boldsymbol{k}\ t$, where Y = mineralization rate, t = time, and $\boldsymbol{k}$ = the value of mineralization constant (Anderson and Ingram, 1993).

The effect of different of organic matter quality and time application were analyzed using Genstat Discovery Edition. Amount of $N\text{-}NO_3^-$ leached to deeper layer was estimated using WaNuLCAS (Water, Nutrient, and Light Capture in Agroforestry System) model developed by Van Noordwijk and Lusiana (1999). The input data needed by the model were daily rainfall (mm), soil texture (percentage of clay, silt, and sand), soil bulk density, soil C-organic, and annually litter input. Four scenarios of land management were applied to reduce $N\text{-}NO_3^-$ leached i.e. increasing the diversity of shade trees to divers quality of litter input, regulating plant distance, and applying pruning regime.

RESULTS AND DISCUSSION

Quality of applied litter in the experiment

To classified litter used in this experiment, laboratory analysis was done to quantify lignin, polyphenolic, total C and total N (Table 1). The quality of litter used in this experiment is lower than that found in the field under multistrata and shaded coffee systems reported by Hairiah et al. (2006) with ratio (Lignin + Polyphenol) to N was about 18. A (Lignin + Polyphenol) to N ratio > 10 is classified (Vanlauwe et al., 1996) as 'low quality'. Based on this criteria, leaf litter of coffee, mix CGF, and mix CGT have been classified as 'low quality' (Table 1). In the Century model of soil carbon dynamics that has been widely used and tested across temperate and tropical agro-ecosystems, about 45 to 55% of litter inputs would be allocated to the metabolic pool on the basis of the (Lignin + Polyphenol) to N ratios of four land cover types.

Net $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ formation in upper layer (0-10 cm)

A single application of *Gliricidia* litter (high quality) or coffee litter (low quality) led to immobilization of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ for 8 WAA. In contrast, the spoon-feeding application reduced the immobilization of $N\text{-}NH_4^+$ by 90% and increased the $N\text{-}NO_3^-$ concentration in soil solution (a positive change of 4 mg kg^{-1} instead of an immobilization of 25 mg kg^{-1}) (Figure 1).

Table 1. Chemical properties of litter used for the experiment.

No	Litter type	Code	Litter quality						Criteria
			Lignin (L)	Polyphenol (P) (%)	Total C	Total N	C/N	(L+P)/N	
			----------%						
1	Coffee	C	28.40	8.92	35.62	2.98	12	12.52	Low
2	*Gliricidia*	G	11.36	19.94	38.16	4.14	9	7.56	High
3	Coffee + *Gliricidia*	CG	16.60	11.46	41.34	3.71	11	7.56	High
4	C+G+ *Arachis pintooi*	CGA	14.76	15.95	37.52	3.23	12	9.51	High
5	C +G+ Fruit tree	CGF	19.50	17.60	39.43	3.34	12	11.11	Low
6	C +G+ Timber tree	CGT	20.70	12.43	40.07	2.84	14	11.66	Low

Table 2. The effect of different litter quality input on the constant of N mineralization rate for 12 week after application.

Litter input	Equation	R^2	K week^{-1}	1/k, week
Single application				
Control (K)	y = 0.14x + 0.32	0.27	0.14	7.3
Coffee (C)	y = 0.11x + 0.35	0.23	0.11	9.1
Gliricidia (G)	y = 0.21x + 0.29	0.35	0.21	4.7
Coffee + *Gliricidia* (CG)	y = 0.13x + 0.27	0.29	0.13	7.4
C+G+ *Arachis pintooi* (CGA)	y = 0.17x + 0.11	0.16	0.17	5.9
C +G+ Fruit tree (CGF)	y = 0.33x + 0.13	0.76	0.33	3.0
C +G+ Timber tree (CGT)	y = 0.15x + 0.25	0.30	0.15	6.7
Spoon feeding				
Control (K)	y = 0.06x - 0.87	0.07	0.06	16.2
Coffee (C)	y = 0.11x + 0.41	0.38	0.11	9.4
Gliricidia (G)	y = 0.09x - 0.16	0.58	0.09	11.3
Coffee + *Gliricidia* (CG)	y = 0.01x + 0.05	0.10	0.01	80.6
C+G+ *Arachis pintooi* (CGA)	y = 0.17x + 0.11	0.34	0.17	5.9
C +G+ Fruit tree (CGF)	y = 0.23x - 1.31	0.54	0.23	4.3
C +G+ Timber tree (CGT)	y = 0.05x - 1.10	0.04	0.05	19.0

Table 3. Coefficient correlation all of parameters.

Parameter	**Lignin (%)**	**Polyphenol (%)**	**C/N**	**L/N**	**P/N**	**(L+P)/N (%)**	**NH_4^+ (g m^{-2})**	**NO_3^- (g m^{-2})**	**N Mineral (g m^{-2})**
Lignin (%)	1	- 0.78*	0.58*	0.98**	- 0.54	0.86**	- 0.50	- 0.39	- 0.60*
Polyphenol (%)	- 0.78*	1	- 0.52	- 0.77**	- 0.87**	- 0.47	0.39	0.41	0.54
C/N	0.58*	- 0.52	1	0.68*	- 0.04	0.75**	- 0.05	-0.71**	- 0.53
L/N	0.98**	- 0.77**	0.68*	1	- 0.47	0.91**	- 0.44	- 0.46	- 0.61*
P/N	- 0.54	- 0.87**	- 0.04	- 0.47	1	- 0.07	0.48	0.07	0.36
(L+P)/N	0.86**	- 0.47	0.75**	0.91**	- 0.07	1	- 0.28	- 0.49	- 0.52
NH_4^+ (g m^{-2})	- 0.50	0.39	- 0.05	- 0.44	0.48	- 0.28	1	0.099	0.63*
NO_3^- (g m^{-2})	- 0.39	0.41	-0.71**	- 0.46	0.07	- 0.49	0.10	1	0.72*
N Mineral (g m^{-2})	- 0.60*	0.54	- 0.53	-0.61*	0.36	- 0.52	0.63*	0.72*	1

*Keterangan: * = significantly different on 5%; ** = significantly different on 1%; L = Lignin, P = polyphenol; N = Nitrogen.*

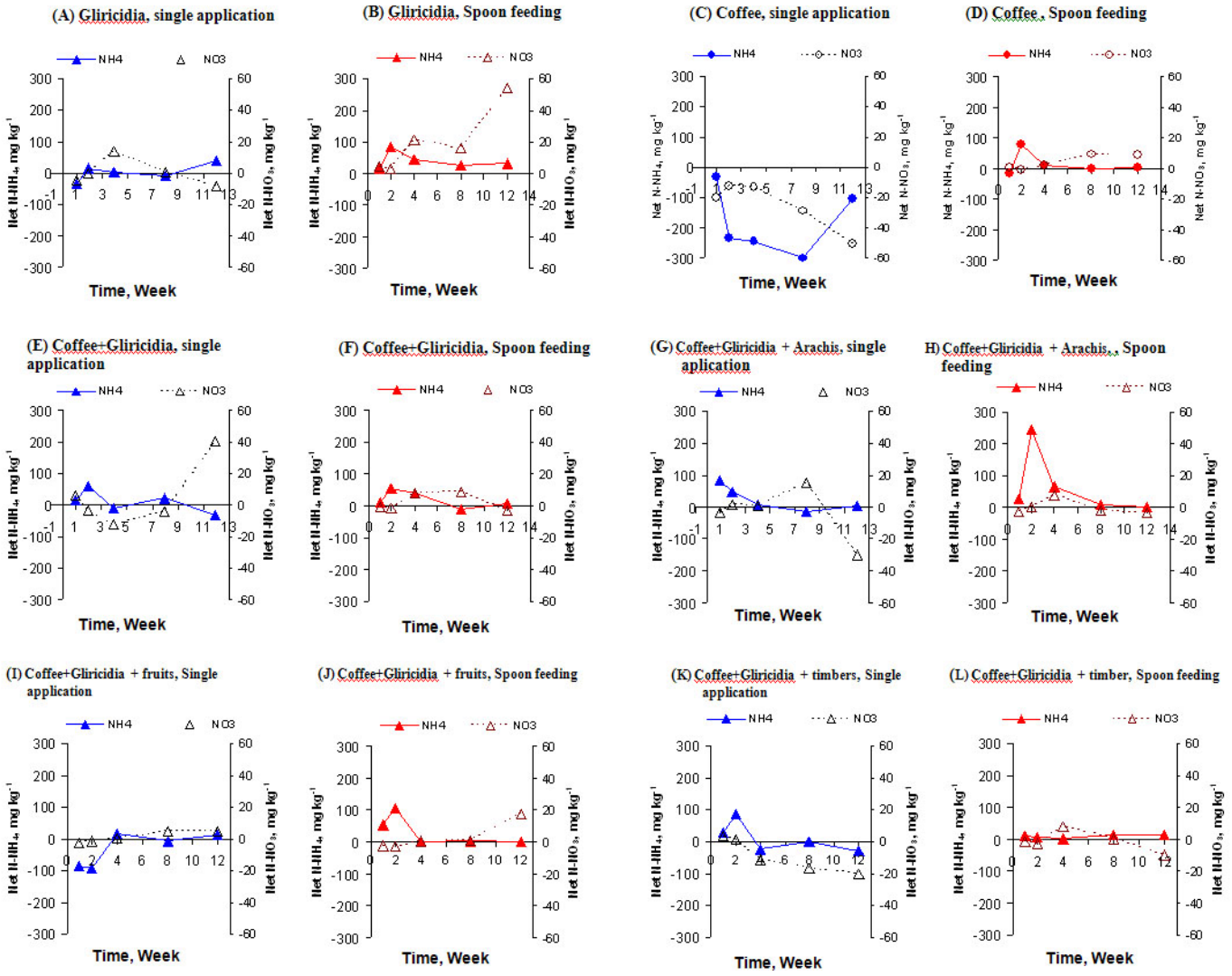

Figure 1. Net N-NH_4^+ and Net N-NO_3^- of various treatments in 0-10 cm soil depth: (A&B) Gliricidia (G); (C&D) Coffee (C); (E&F) Coffee + Gliricidia (CG); (G&H) Coffee + Gliricidia + Arachis (CGA); (I&J) Coffee + Gliricidia + Fruit (CGF); (K&L) Coffee + Gliricidia + Timber (CGT).

Application of mixed litter of coffee + *Gliricidia* changed the mineralization pattern of N-NH_4^+ and N-NO_3^-. It tended to reduce initial immobilization of N-NH_4^+ and also reduced the later release of N-NO_3^-, compared to application of a single source litter. Application of mixed litter of coffee + *Gliricidia* + fruit trees (durian, avocado, candlenut, belinjo) led to immobilization of N-NH_4^+ when applied in a single dose; but when it was applied several times net mineralization of N-NH_4^+ and N-NO_3^- was recorded at 2 WAA. Application of a mixed litter of coffee + *Gliricidia* + timber (*Maesopsis*, mahogany and teak) resulted in immobilization of N-NH_4^+ and N-NO_3^-, either as single or spoon-feeding application. This finding suggested that small amounts of fertilizer (ranging from 30-50 kg N ha^{-1}) may be needed in the growing season to avoid N deficiency in multistrata agroforestry systems.

The mineralization rate

The results showed that *k* value from low quality of coffee litter with single application is the same as found in spoon feeding application with *k* value approximately 9.25, however, when it mixed with high quality of *Gliricidia* litter increase *k* value to 81 (spoon feeding application) and 7.4 (for single application). The mixture of low quality of litter CGF (*Coffea* + *Gliricidia* + fruit trees) was the slowest to release N as showed by the lowest of *k* value, 3.0 for at single application and 4.3 for spoon feeding application. While the application of the litter of CGT (*Coffea* + *Gliricidia* + timber tree), N mineralization was faster than mix application of CGT with *k* value about 6.7 and 19 for single and spoon feeding application (Table 2).

Effect of litter quality on nitrogen mineralization

The coefficient correlation (r) of lignin, L/N ratio, C/N ratio, (L+P)/N ratio with N mineral were -0.60; -0.61; -0.53; -0.52 (Table 3). The two research that was conducted previously showed that (L+P)/N ratio has significantly correlate with nitrogen release Purwanto et al., (2006) and Handayanto (1994). Results of regression analysis in this experiment, however, showed that higher (L+P)/N ratio did not affect significantly on N-mineral concentration.

Estimating Mineral N leached using WaNuLCAS

The WaNuLCAS simulation indicated that the mineral N content at top soil of shaded coffee based systems was greater than the coffee monoculture (Figure 2A). The spoon-feeding application of litter showed greater mineral N content at top soil than in single application of litter. On the other hand, the mineral N leaching on the shaded coffee base systems except on C+G was lower than the coffee monoculture. The model predicted that coffee-based agroforestry multistrata can reduce leached N-NO_3^- up to 50 % compare to monoculture coffee system (Figure 2B). This simulation explain that efficiency use of soil mineral N in coffee-based agroforestry multistrata from litter decomposition especially using the low quality litter or mix high and low quality litter is better than in coffee monoculture. Suprayogo (2000) found that a deep tree root system spreading as a “safety-net” under the crops is needed to help intercept leached or leaching of mineral-N so as to improve nutrient use efficiency of the cropping systems (Suprayogo, 2000). However, the effectiveness of agroforestry systems in reducing mineral-N leaching depends on the performance of the root systems of the tree species in N uptake. The peltophroum hedgerow intercropping system with deep rooting system and low litter quality reduced significantly the cumulative amount of leached mineral-N in comparison with the gliricidia hedgerow intercropping system (reduced by 31%).

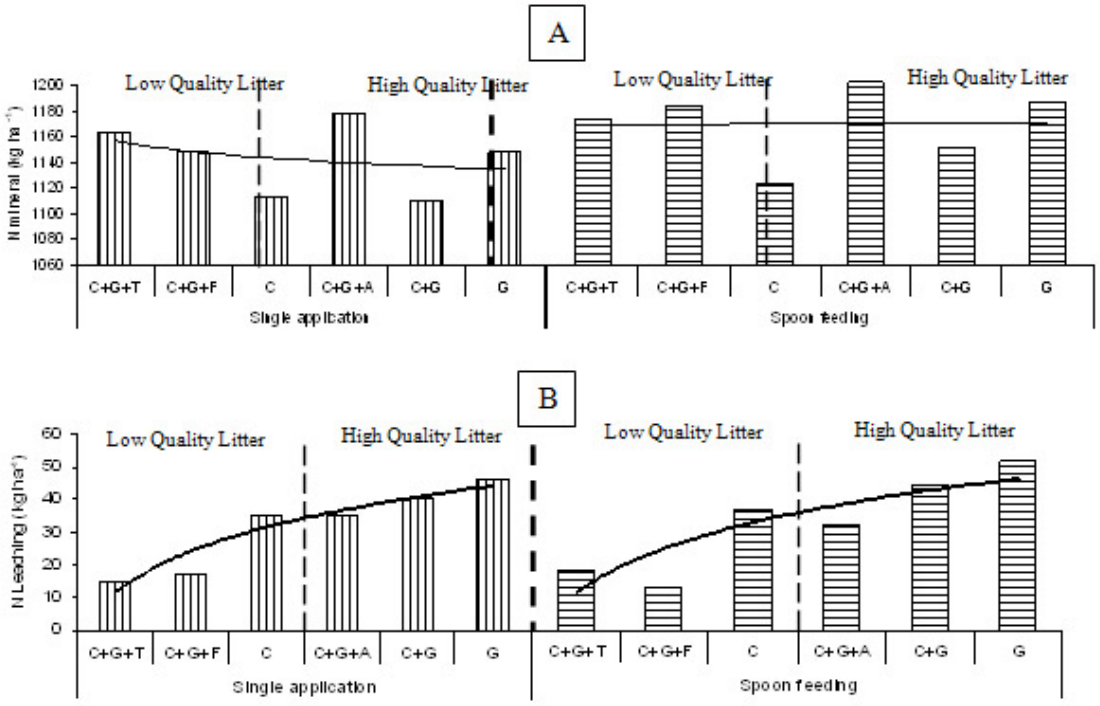

Figure 2. Results of simulation using WaNuLCas on 20 days after litter application: A) Total N mineral in upper layer (0-10 cm) and B) Total N mineral leached in sub layer (20-80 cm).

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Carbon Stock Assessment for a Forest-to-Coffee Conversion Landscape in Kalikonto Watershed (East Java, Indonesia): Scale up from Plot to Landscape Level

K. HAIRIAH[1], S. KURNIAWAN[1], F.K. AINI[1], N.D. LESTARI[1], I.D. LESTARININGSIH[1], WIDIANTO[1], T. ZULKARNAEN[2], M. VAN NOORDWIJK[2]

[1]University of Brawijaya, Faculty of Agriculture, Malang, Indonesia
[2]World Agroforestry Centre, ICRAF Southeast Asia, Bogor, Indonesia

SUMMARY

The impacts of a change in land use from natural forest to tree-based agricultural system on net sequestration of CO_2, or release to the atmosphere of CO_2, can be rapidly estimated by measuring the change of carbon (C) stocks for a period of time using RACSA (Rapid Carbon Stock Appraisal). Aim of this study was to assess the changes of aboveground C stock at landscape level after forest conversion to various types of land use systems. The activity was started with analysis of land use maps of 1990 and 2005 of the Kalikonto sub-watershed (Malang regency, East Java).The data showed that remaining natural forest was reduced by 33%, but the total area of annual crop area and of settlements increased by 45% and 18%, respectively; while area of tree plantation and agroforestry were reduced about 10%.

Measurements of aboveground C stock at plot level were made in June –December 2008 in up-stream parts of the Kalikonto sub-watershed covering a range of land use system (LUS). The eight LUS most commonly found in the study area were natural forest, bamboo forest, 3 types of plantation i.e. pine (*Pinus mercusii*), mahogany (*Swietenia mahogany*) and dammar (*Agathis philippensis)*; multistrata shaded coffee with fruit and timber trees, as well as nitrogen-fixing shade trees; single shade coffee (using *Gliricidia sepium* as shade tree); annual cropping systems (vegetable and food crops). The natural forest in Kalikonto area has been severely disturbed as shown by a low aboveground C stock of about 136 Mg ha^{-1}. Aboveground C stock in coffee-based agroforestry systems was lower, at about 44 Mg C ha^{-1}. The time averaged C stock of tree plantations (pinus, mahogany, and damar mostly aged 25-40 years) was estimated to be 85 Mg C ha^{-1}. The estimated time averaged-C stock (above-ground) in annual crops was only 2 Mg ha^{-1}. Extrapolation of C stock at plot level to watershed level were done by multiplying the area of each land cover with its time-averaged aboveground C stock. Within 15 years, C lost for the whole watershed (23810 ha) was estimated to be 27750 Mg yr^{-1} or equivalent to a yearly C loss of 1.17 Mg ha^{-1}. Carbon lost from natural forest was about 0.92 Mg ha^{-1} yr^{-1}, tree plantations lost 0.15 Mg ha^{-1} yr^{-1}. Carbon lost from coffee-based agroforestry systems was relatively small, about 0.03 Mg ha^{-1} yr^{-1}. Planting more diverse of shade trees in coffee-based agroforestry system may increase the potential of coffee garden as C sequester in the landscape.

INTRODUCTION

Trees and other plants growing inside or outside forest sequester CO_2 by removing it from the atmosphere through photosynthesis and incorporating into plants tissues. The residence time of carbon stored in plant tissues varies among plant species, carbon released again to atmosphere mostly by burning and decomposition (IPCC, 2006). Improving carbon stock in

landscape can be achieved by moving from lower-biomass land use systems such as annual crops and grasslands to tree-based systems. The reforestation program also contributes to the sequestration of carbon, although the rate of carbon used and stored by plants are varies depending on plants species, location and land management such as tree diversity and its population density (Mutuo et al., 2005).

Agroforestry systems including shaded coffee systems can effectively sequestered carbon rather than in open-grown (monoculture) system. Result of study in Togo showed that carbon stock of shade coffee system (coffee- *Albizia*) was about 81 Mg ha^{-1}, four times higher than in open-grown system (23 Mg ha^{-1}) (Dossa et al., 2008). Another study in West Lampung (Indonesia) showed that aboveground carbon stock in multistrata coffee system (shaded coffee with fruit and timber trees, as well as nitrogen-fixing shade trees) was about 34 Mg ha^{-1}, compared to 23 Mg ha^{-1} in simple shaded coffee system (mainly *Gliricidia* as shade trees) and only 7 Mg ha^{-1} in open-grown coffee system (Van Noordwijk et al., 2002). Under natural forest, the average C stock was estimated about 195 Mg ha^{-1}. However, study on potential of coffee-based agroforestry system as carbon sequester at landscape level after forest conversion is very limited. Aim of this study is to estimate the changes of carbon stock at landscape level after forest conversion to coffee-based agroforestry system and various types of land use systems using RACSA (Rapid Carbon Stock Appraisal).

MATERIALS AND METHODS

Measurements of aboveground C stock at plot level were made in June-December 2008 in up-stream parts of the Kalikonto sub-watershed is located at Ngantang and Pujon sub-district (East Java, Indonesia) covering a range of land use system (LUS). The eight LUS most commonly found in the study area were secondary forest, bamboo forest, three types of plantation i.e. pine (*Pinus mercusii*), mahogany (*Swietenia mahogany*) and 'damar' (*Agatis philippensis*); multistrata-shaded coffee with fruit and timber trees, as well as nitrogen-fixing shade trees (mostly *Gliricidia sepium*); single (*Gliricidia*) shade coffee; annual cropping systems (vegetable and food crops). Tree diversity varies among agricultural system. In agroforestry multistrata-shaded coffee consist of 51% timber trees (mahogany, Paraserianthes or Maesopsis), 29% fruit trees (mostly durian, avocado, lansium, rambutan, jackfruit) and the rest was non-woody species (banana and papaya). Species composition in single shade coffee agroforestry system was slightly lower i.e. 40-60% was timber, 34% was fruit trees and the rest was non-woody species. In timber plantation system (pine, mahogany and agathis), other plants species were also found such as coffee, durian *Gliricidia*, papaya and banana although the population density was low in a range of 10 to 20% of total tree.

Land cover change analysis was conducted on landsat images using post classification comparison methods (Widayati et al., 2005) where information of changes is derived from land cover maps 1990 and 2005. The carbon stock of each land use systems was assessed according to RaCSA (Rapid Carbon Stock Appraisal) methods developed by ICRAF (2005) which similar to methods developed by Winrock (McDicken, 1997). The main carbon pools are above- and below ground biomass of tree and understorey, necromass and soil organic matter. All tree measurement, plant and soil samples were done on a plot of 40 x 5 m^2. When a big tree > 30 cm diameter are present in the sampling plot an additional sample of 20 * 100 m^2 was made. Estimation of aboveground tree biomass was done non-destructively by measuring tree diameter at breast height (dbh) and using allometric biomass regression equations (Palm et al., 2001; Hairiah et al., 2007) as shown in Table 1. Root biomass was estimated using default assumptions of shoot:root ratio of trees in tropical forest of 4:1 (Mokany et al., 2006). All

biomass and necromass data are converted into C by multiplying it with plant C content is 0.46 (Hairiah et al., 2007).

Table 1. Allometric regressions for estimating tree biomass of common trees grown in agroforestry and plantation system.

Tree species	Equations	Source
Branched tree	Y = 0.11 ρ D2.62	Ketterings et al., 2001
Pruned Coffee	Y = 0.281 D2.06	Arifin, 2001
Banana	Y = 0.030 D2.13	Arifin, 2001
Bamboo	Y = 0.131 D2.28	Priyadarsini, 1999
Paraserianthes	Y = 0.0272 D2.831	Sugiharto et al., 2002
Pinus mercusii	Y = 0.0417 D2.6576	Waterloo, 1995

Note: Y = aboveground biomass, kg/tree; D = dbh= tree diameter, cm; H = tree height, cm; ρ = wood density, g m-3 can be accessed at: http://www.worldagroforestry.org/sea/Products/AFDbases/AF/index.asp.

Understorey were measured in ten 0.25 m^2 quadrat samples, total fresh weight was measured and subsamples were collected for determining dry matter content. Diameter and length of dead wood was measured within sampling plot and converted to volume on the basis of a cylindrical form; three apparent density classes were used. Surface litter (including wood < 5 cm diameter) of each land use was measured by taking litter samples down to the surface of the mineral soil in ten 0.25 m^2 samples and sub samples were taken for dry matter content.

Soil samples were collected (composite from 10 sample points) for the 0-5, 5-10, 10-20 cm depth zone below the litter layer, for analysis of texture (sand, silt, clay), pH (1N KCl), pH(H2O), C_{org} (Walkey and Black), N_{tot} (Kjeldahl).

For estimation of carbon stock at landscape level, the time-averaged C stock of tree-based system was derived from the life cycle of the system (Samson and Scholes, 2000). The CO_2 emissions can be estimated by combining data on the vector of time-averaged C stocks for all land use classes used with the matrix of land cover change (Widayati et al., 2005; Hairiah et al., 2010).

RESULTS AND DISCUSSION

Land cover changes

Land cover change analysis was conducted based on landsat images using post classification comparison methods where information of changes is derived from land cover maps of the Kalikonto sub-watershed (East Java, Indonesia) of 1990 and 2005. The data showed that within 15 years (period 1990- 2005), area of natural forest decreased 33% from 7269.93 ha in 1990 to 4852.26 ha in 2005; annual forest conversion rate was about 2.2% (Figure 1). On the other hand, total area of annual crop area (mostly vegetable, maize and upland rice) and of settlements increased by 45% and 18%, respectively; while area of tree plantation and agroforestry (coffee based) were reduced about 10%.

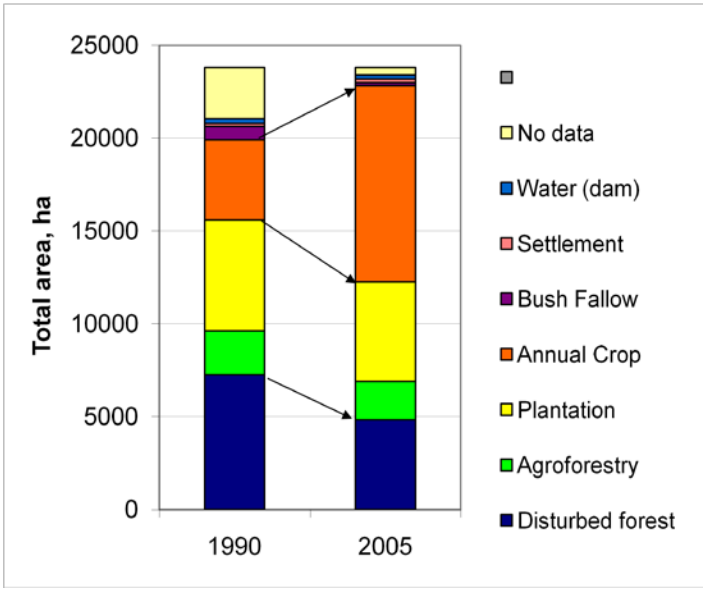

Figure 1. Land cover changes in Kalikonto sub-watershed based on analysis of land cover maps 1990 and 2005.

Carbon stock of each land use system

The natural forest in Kalikonto area has been severely disturbed shown by a low total C stock of about 161 Mg ha^{-1}. The total C stock in coffee-based agroforestry systems was lower, ranged from 99 to 111 Mg C ha^{-1} (Figure 2 and Table 2).

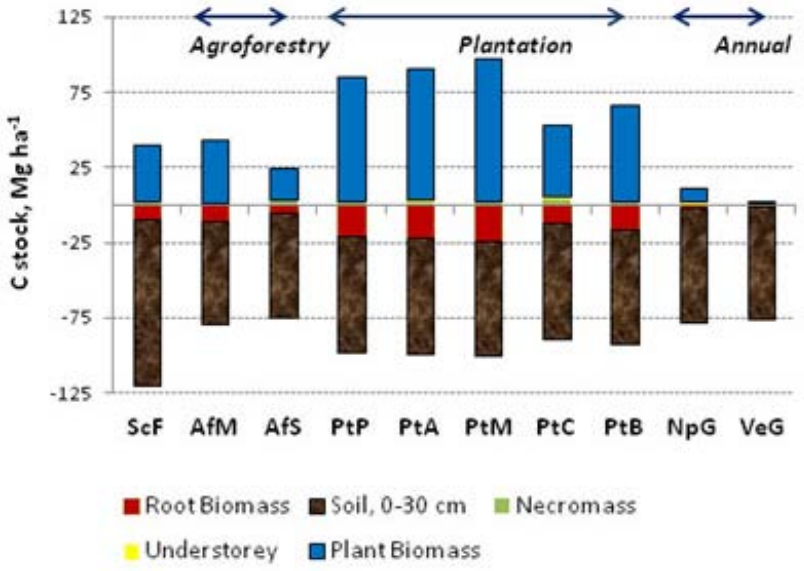

Figure 2. Total C stock of different C componens of various land use tipes (ScF = secondary forest, AfM_Mult = Multistrata coffee-based Agroforestry; AfS = simple coffee agroforestry, PtP = plantation pinus, PtA= plantation Agathis, PtM = plantation mahogany, PtC = plantation clove and PtB= plantation bamboo), NpG = napier grass and VeG = annual crops mainly vegetable.

For tree plantations system (pinus, mahogany, and damar mostly aged 25-40 years) the C stock was ranging from 159 to 198 Mg C ha^{-1} (Table 2). In coffee agroforestry system in west Lampung, shade trees contributed about 40% of total C stock (Hairiah et al., 2006). This

contribution can be higher by increasing tree diversity and its population density (Mutuo et al., 2005).

The time-averaged C stock was calculated to reflect the dynamics of C that is present in a certain land use systems over its life span, it is depends on rate of C accumulation, the minimum and maximum of C stored by the systems, and the time required to reach the maximum value and the rotation time. The time averaged C stock of tree plantations (pinus, mahogany, and damar mostly aged 25-40 years) was calculated based on equation developed in Figure 3. In plantation system the time averaged C stock was estimated to be 139 Mg C ha^{-1} (Table 1), agroforestry was to be 111 Mg ha^{-1}, while annual crops was only 1.5 Mg ha^{-1}. The soil in Kalikonto (mostly Andisol and Inceptisol) contribute C stock about 40-70% of total C stock of each land use, which is higher than earlier C stock soil data of from Ultisol (Sumatra) around 10-20% only.

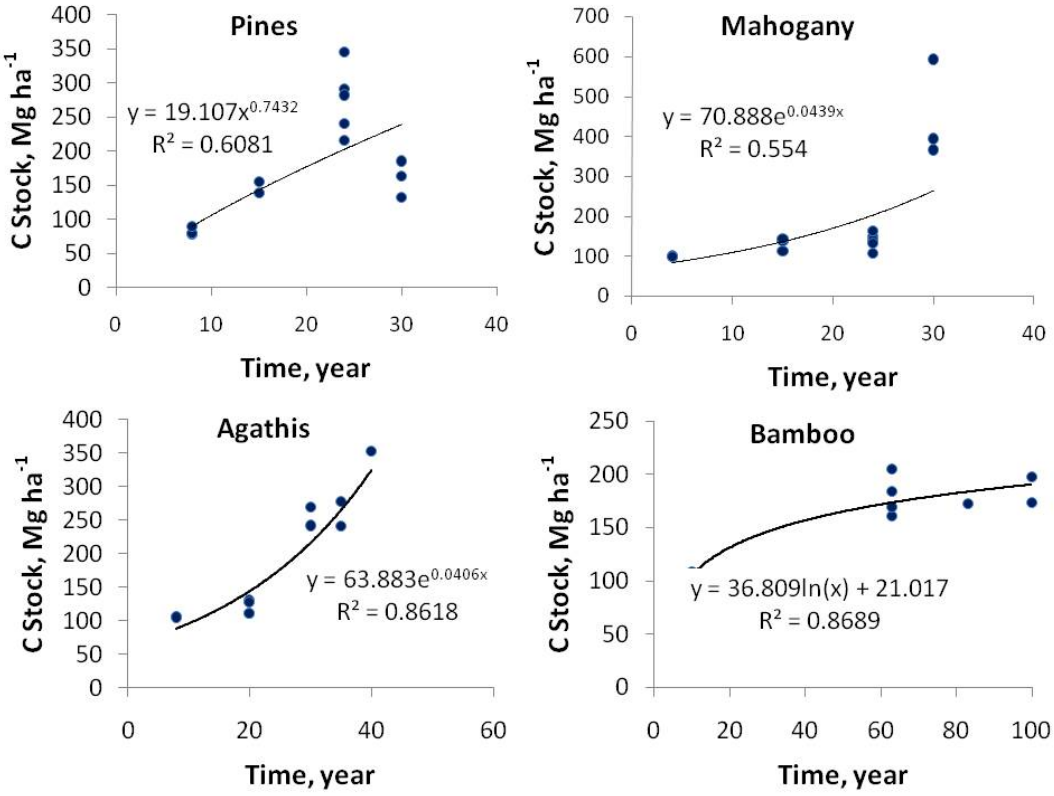

Figure 3. Relationship between total C stock and age of trees in monoculture system, the time averaged C stock of each species was estimated using its equation.

Estimation the changes of C stock at landscape level

The geographic distribution of forest conversion and thus C stock reduction was mainly occurred in the area of high forest conversion in up-north of Pujon district covering 5 villages i.e. Pandesari, Wiyurejo, Madiredo, Tawangsari and Ngabab village (Figure 4). While in the southern part includes 3 vilages from Pujon district i.e. Pujon, Sukomulyo, and Bendosari, and 3 villages in Ngantang District i.e. Purworejo, Sidodadi, and Banjarejo.

Table 2. Carbon stock of various components of different land use types in Kalikonto sub-watershed.

Land cover	LUS	Plant density per ha	Above ground	Estimated Root	Understorey	Necromass	Soil, 0-30 cm	Total C stock	Max. Age,	Time Avg. C Stock,
			---------- Mg ha^{-1}----------						year	Mg ha^{-1}
Forest	Degraded Forest	2248	38.4	9.60	0.15	2.15	111	161	50	161
Agroforestry	AF_Multistrata	3970	42.1	10.5	0.14	1.29	69	123	30	111
	AF_Simple	4018	21.4	5.3	0.91	2.33	69	99	30	
Plantation	Pinus, 24yr	795	82.6	20.7	1.22	1.59	77	183	30	144
	Agathis		87.5	21.9	2.67	1.34	77	190	40	146
	Mahogany	963	95.2	23.8	0.69	1.54	77	198	50	212
	Clove		47.3	11.8	1.53	4.15	77	142	35	70
	Bamboo	3188	63.9	16.0	0.40	2.20	77	159	15	121
Grass-land	Napier grass, 4 months	-	15.0	3.7	4.41	1.02	76	100	0.25	11
	Napier grass, 1 month	-	0.9	0.2	0.21	0.53	76	78		
Annual crop	Vegetables	-	1.8	0.4	0.68	0.55	76	79	0.25	1.5

Napier grass = Pennisetum purpureum (Ind: Rumput Gajah).

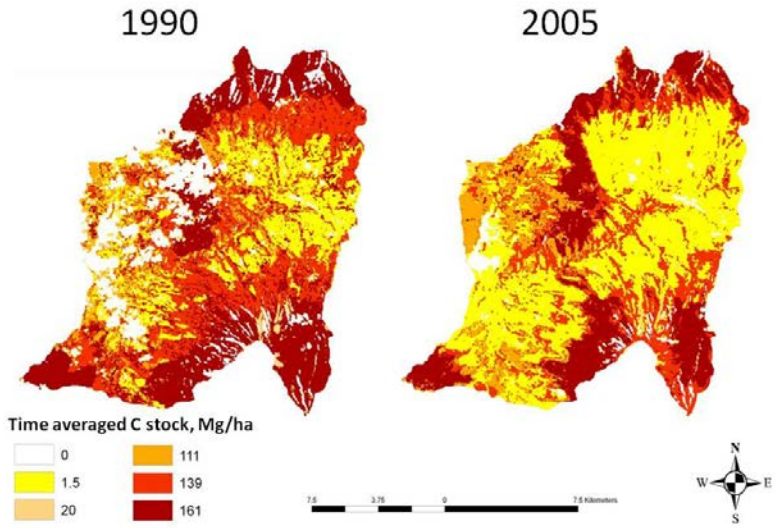

Figure 4. Distribution of carbon density in Kalikonto sub-watershed in 1990 and 2005.

Extrapolation of C stock at plot level to watershed level were done by multiplying the area of each land cover with its time-averaged aboveground C stock (see Table 2). Within 15 years, C lost for the whole watershed (23810 ha) was estimated to be 25,924 Mg yr^{-1} or equivalent to a yearly C loss of 1.48 Mg ha^{-1}. Carbon lost from natural forest was about 1.09 Mg ha^{-1} yr^{-1}, tree plantations lost 0.25 Mg ha^{-1} yr^{-1}. Carbon lost from coffee-based agroforestry systems was relatively small, about 0.05 Mg ha^{-1} yr^{-1}. Increasing the area of annual crops in 2005 lead to a small gaining of C stock in the landscape was about 0.03 Mg ha^{-1} yr^{-1} but the C lost from the landscape exceeded this gain. Planting more trees (damar, pinus, mahogany) in the landscape through the Reforestation Program of the Forest Estate (PERHUTANI) in the 1990-2005 period was not able to reduced the C lost from the landscape, planting more trees in the landscape through agroforestry and plantation may compensate the lost of C through forest conversion.

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