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FOOD AND BEVERAGE CONSUMPTION AND HEALTH

COFFEE

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JOHN L. MASSEY
EDITOR

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Additional color graphics may be available in the e-book version of this book.

Library of Congress Cataloging-in-Publication Data

Library of Congress Control Number: 2016931591

ISBN: 978-1-63484-725-4 (eBook)

Published by Nova Science Publishers, Inc. † New York

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PREFACE

Coffee is among the most widely consumed beverages worldwide. Traditionally, high consumption of coffee has been considered to have negative health consequences due to the stimulant effects of caffeine. However, there is substantial evidence that coffee contains a range of bioactive compounds and antioxidants with potentially beneficial effects on human health. Chapter One presents a review of the works of research that have been developed in order to establish the functional potential of coffee, either raw or roasted and its byproducts. Chapter Two studies the extraction of bioactive compounds and antioxidant activities of coffee brews. Chapter Three focuses on two factors, aroma and geographic provenance, namely the geographic origin and the roasting procedure, and aims at providing a systematic assessment of the chemical compounds that are mainly responsible for the quality of coffee samples. Chapter Four focuses on the properties of spend coffee ground (SCG), based on its composition and structural organization, and the complex enzyme necessary for its conversion, highlighting its impact on biotechnology and bioenergy process. Chapter Five examines an optimal range of extraction conditions for extraction of polyphenols from espresso SCG by using response surface methodology and to investigate in vitro effects of obtained polyphenol-rich extracts on platelets activation and their aggregation with monocytes and neutrophiles. Chapter Six evaluates sensory acceptance and the effect of the expectations caused by information and the packaging characteristics of instant coffee. Chapter Seven overviews emerging evidence for the protective effects of coffee and its bioactive compounds on ocular health. Chapter Eight discusses coffee contaminated with Ochratoxin and genotoxicity. Chapter Nine studies health and health risks of the substances found in coffee.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 1

COFFEE AND ITS BY-PRODUCTS AS SOURCES OF BIOACTIVE COMPOUNDS

Adriana S. Franca and Leandro S. Oliveira
Universidade Federal de Minas Gerais
Belo Horizonte, MG, Brasil

ABSTRACT

Coffee is one of the most popular beverages consumed worldwide and the most important food commodity from an economic point of view. Coffee processing can be divided into two stages: primary processing, in which the coffee fruits are de-hulled and submitted to drying, the resulting product being the green coffee beans, the main product of international coffee trade; and secondary processing, which comprises the production of roasted and soluble coffee. Large amounts of solid wastes are generated during primary and secondary processing. These include coffee husks and pulp, parchment, spent coffee grounds, silverskin and others. These solid residues pose several problems in terms of adequate disposal, given the high amounts generated, environmental concerns and specific problems associated with each type of residue, and therefore there has been an increasing interest in the evaluation of their chemical profiles in order to propose alternative uses for them. The role of coffee consumption in preventing several diseases has been well established, in association with substances such as caffeine, chlorogenic acids and others, indicating that it could be viewed as a functional beverage. The solid wastes generated in coffee production also contain similar amounts of phenolic compounds and thus have also been recently considered as potential sources of antioxidants and other relevant bioactive chemicals. In view of the aforementioned, the objective of the present study is to present a review of the works of research that have been developed in order to establish the functional potential of coffee, either raw or roasted and its byproducts, e.g., solid wastes generated during primary and secondary processing.

1. INTRODUCTION

Coffee is one of the most widely consumed pharmacologically active beverages, and green coffee grounds can be viewed as one of the most important types of grain traded worldwide. Associations of coffee consumption and health issues has been quite a controversial subject, and over 8000 medical studies have been developed on this area on the past 40 years (Bae et al., 2014). Past studies have shown associations between coffee consumption and cardiovascular adverse effects as well as increased risk of myocardial infarction. Coffee consumption has been also associated with increased concentrations of serum total cholesterol and low-density lipoprotein cholesterol, in association with the diterpenes cafestol and kahweol (Bae et al., 2014). Nonetheless, such cholesterol-raising compounds are removed by paper filters during beverage preparation and therefore should not be a concern in the consumption of filtered coffee. Furthermore, harmful effects of coffee are usually associated with people who are sensitive to stimulants or to excessive consumption (George et al., 2008).

However, more recent and epidemiological and intervention studies have attributed many positive effects to moderate daily consumption of coffee (Bae et al., 2014). Several studies have demonstrated its inverse correlation with many diseases including Parkinson's disease (Higdon and Frei, 2006; Butt and Sultan, 2011), Alzheimer's disease (Butt and Sultan, 2011), several types of cancer (Higdon and Frei, 2006; Nkondjock, 2009), type 2 diabetes mellitus (Higdon and Frei, 2006), and depression (Lucas et al., 2011; Hall et al., 2015), among others. Coffee also shows protective effects on various systems including the skeletal system, the reproductive system, the nervous system and the cardiovascular system (George et al., 2008). Such health-promoting properties are attributed to coffee´s rich phytochemistry, including substances such as caffeine (the most widely used psychoactive substance) as well as many other biologically active compounds predominantly belonging to the polyphenol and alkaloid classes (Hall et al., 2015). These substances are present not only in coffee beans or in the associated beverage, but also in solid wastes generated during coffee processing, such as the peel and pulp of coffee fruit, spent coffee grounds and others.

In view of the aforementioned, recent studies have focused on the characterization of coffee and its by-products as sources of bioactive compounds. In the present chapter, we present an overview of such studies, in order to better establish the functional potential of coffee and its by-products. A brief overview of the major steps involving in coffee processing is presented as follows, in order to provide a better understanding on the types of by-products (solid wastes) that are generated. Then, a detailed discussion on coffee and each specific by-product, focusing on their chemical composition as well as the identification and recovery of biologically active substances is presented throughout the remainder of this chapter.

1.1. Coffee Processing Steps

A schematic view of the coffee fruit or coffee cherry is shown in Figure 1. Each fruit contains two coffee beans encapsulated by a thin closely fitting layer called silverskin, which in turn is encapsulated by another layer called parchment. The parchment is in direct contact with the fruit pulp, which is covered by the fruit skin or peel. The main product of

commercial interest constitutes the coffee beans or seeds, also referred to as green coffee. It represents 50–55% of the dry matter of the fruit, so the remaining material will constitute a wide range of byproducts during processing.

The general steps involved in the processing of coffee are divided into primary and secondary. Primary processing is constituted of post-harvesting operations, during which the coffee cherries are submitted to several steps until the green coffee beans are obtained. There are two major types: Dry and wet processing. Dry processing is the simplest method employed for primary coffee processing. Once the cherries are picked from the coffee trees, they are dried (10–11% moisture content) and the coffee beans are separated by removing the material covering the beans in a de-hulling machine. The solid by-products obtained during de-hulling are the so-called coffee husks, CH (outer skin + pulp + parchment). Wet processing, on the other hand, involves first de-hulling the fruits and then drying the coffee beans. For this type of processing, the outer skin and pulp are mechanically removed, thus generating the solid residue denominated coffee pulp, CP. The beans can either be fermented, in order to remove some of the remaining pulp material (pulped coffee), or be directly submitted to drying (de-hulled cherry coffee). Either way, after drying (~ 12% moisture content), the coffee beans are again de-hulled to remove the parchment. The resulting solid waste (parchment and some silverskin) is collectively termed parchment husks. A more detailed overview of both procedures is available in the literature (Franca and Oliveira, 2009; Kleinwächter et al., 2015).

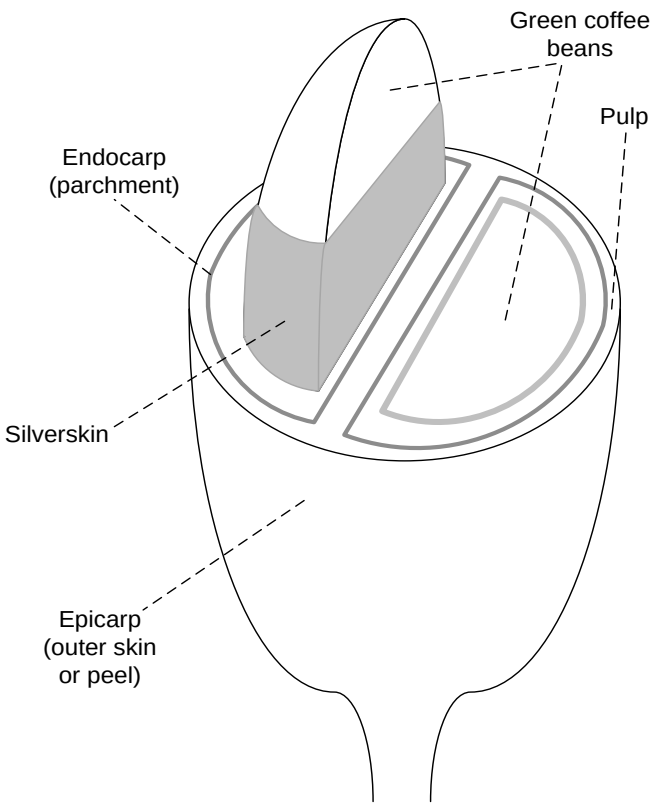

Figure 1. Schematic overview of the coffee fruit.

Roasting is a crucial step in coffee processing, because it causes several chemical, physical and structural transformations that will be ultimately responsible for the taste and smell of the beverage (Bertone et al., 2016). It is a time-temperature controlled heating that usually takes place at atmospheric conditions and employs hot air as the heating agent. The process can be characterized by three consecutive stages. During the first stage, characterized by a slow release of water and volatile substances, the beans are dried and their color changes from green to yellow. The actual pyrolysis reactions will take place during the second stage, with quantities of CO_2, water and volatile substances being released and the beans turning brown, due to sugar caramelization and Maillard reactions. Finally, a third stage of cooling is required in order to avoid burning the coffee (Dutra et al., 2001; Oliveira et al., 2005). Coffee silverskin, CS, the integument that covers the raw coffee bean, is detached and carried out by the heating air during this process, thus constituting another solid residue generated during coffee processing (Costa et al., 2014).

Beverage preparation (i.e., water extraction of the coffee solubles) is the final step necessary towards obtaining the final product of consumption. After roasting, the beans are ground in order to increase the specific extraction surface, i.e., to increase the interface between water and the solid in order to facilitate the transfer of soluble substances into the brew (Soares et al., 2015). There are several ways employed to prepare coffee brews, mostly associated to local traditions. They can be divided into three major methods, including infusion (filter or coffee drip and napoletana), pressure (espresso, press-pot or French press, and moka) and decoction (boiled, Turkish, vacuum, and percolation). Infusion can be described as a flow of hot water through roasted coffee, allowing a short contact time between solid and liquid. Decoction, on the other hand, is the process were the coffee is kept in contact with water, at an appropriate temperature, for a considerable amount of time, thus allowing for a more effective extraction of solubles in comparison to infusion (Soares et al., 2015). Pressure procedures are similar to infusion, but at higher pressures in order to improve extraction. Regardless of the type of process, a common characteristic is the generation of a large amount of solid residues, i.e., spent coffee grounds (SCG) that remain after extraction takes place.

Another processing step that generates a significant amount of solid resides or by-products is denominated secondary processing and corresponds to the production of soluble or instant coffee. During this type of processing, roasted and ground coffee beans are treated with pressurized hot water in order to extract the soluble substances. This extract is submitted to either spray or freeze-drying in order to obtain the solid final product (soluble or instant coffee, respectively). The insoluble residue (a slurry containing spent coffee grounds) is screw pressed, so the moisture content is reduced from 75-80% to approximately 50%. Large amounts of SCG are also generated at home or in commercial establishments in the conventional preparation of the coffee beverage as previously mentioned. A schematic simplified overview of the major coffee processing steps as well as the corresponding generated solid by-products is presented in Figure 2.

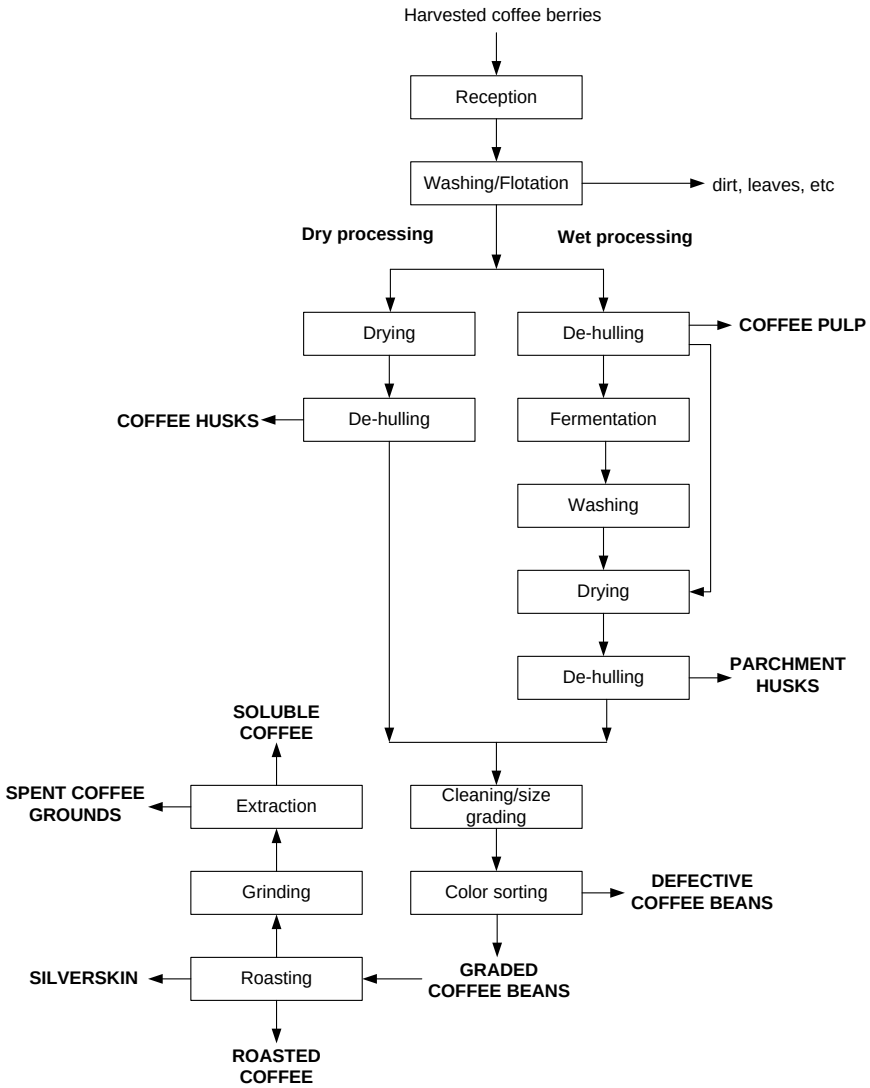

Figure 2. Schematic overview of the major coffee processing steps and the corresponding generated by-products.

2. COFFEE AS SOURCE OF BIOACTIVE COMPOUNDS

Coffee beans can be viewed as a complex food matrix, containing many substances that can interact within the human body, not only stimulating the neural system but also providing other desirable health effects (Stelmach et al., 2015). As previously mentioned, the health benefits are attributed to many substances such as caffeine (the most widely used psychoactive substance) as well as other biologically active compounds predominantly belonging to the polyphenol and alkaloid classes (Hall et al., 2015). Phenolic compounds are mainly found in coffee beans as chlorogenic acids (CGA). These CGA are water-soluble esters formed between quinic acid and one or two moieties of caffeic acid, a trans-cinnamic

acid. Reports indicate that there are at least 30 CGA in coffee beans, including caffeoylquinic acids (CQA), dicaffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids. Nonetheless, CGA levels are known to decrease significantly during roasting, being reduced from 5.8-8.0 g/100 g in green Arabica coffee down to 1.2-2.3 g/100 g after roasting (Wei and Tanokura, 2015). Another substance that has been reported not only as an important precursor of important flavor compounds (furans, pyrazine, alkyl-pyridines, and pyrroles), but also as a beneficial nutritional factor is trigonelline, a pyridine derivative (Ghule et al., 2012; Ilavenil et al., 2014; Makowska et al., 2014). Trigonelline levels are also reduced during roasting, with *N*-methylpyridinium and nicotinic acid (niacin or vitamin B_3) being the major nonvolatile decomposition products.

Given that both green and roasted coffee beans as well the beverage are rich sources of bioactive compounds, both their infusions have been evaluated in terms of health effects. Green coffee infusions are believed to accelerate metabolism and in that way they can be helpful e.g., in reducing weight and preventing or overcoming obesity. Physiological studies using mice indicate that a green coffee extract could be an effective fat absorption inhibitor and a suppressor of its metabolism in liver (Shimoda et al., 2006). It has also been reported to have an antihypertensive effect in rats (Suzuki et al., 2002) and to present potential use as a weight loss supplement (Igho et al., 2011). Recent studies have shown that moderate coffee consumption can be associated to the decreased risk of several chronic-degenerative diseases such as neurodegenerative disorders (Parkinson and Alzheimer), cirrhosis, asthma, and type 2 diabetes (Alves et al., 2009). An extensive overview on the effects of coffee on health was recently published (Preedy, 2015).

2.1. Green Coffee

Reported moisture contents of green coffee beans range from 8 to 13% for arabica and from 12-13% for robusta. The proximate composition of green arabica (A) and robusta (R) coffees (dry basis) is: protein levels ranging from 11 to 17% (A) and from 11 to 13% (R); lipid contents ranging from 9 to 18% (A) and from 9 to 18% (R); carbohydrate (by difference) ranging from 60 to 76% (A) and from 69 to 76% (R); and mineral levels ranging from 4 to 5% (A,R) (Franca and Oliveira, 2009). The reported variations are related to several factors including species/variety, origin, agricultural practices, growth and storage conditions and maturation degree (Clarke and Macrae, 1985; Franca and Oliveira, 2009).

Caffeine has been historically linked to most of the physiological effects of coffee and caffeine levels in green coffees vary mainly with respect to species. Robusta coffees have approximately twice the amount of caffeine found in arabica coffees, with average values ranging from 0.6 to 1.9% for arabica and approximately 2.2% for robusta (Macrae, 1985).

Phenolic compounds are mainly found in coffee beans as chlorogenic acids (CGA). CGAs represent a family of esters that are structural analogs of quinic acid (QA) carrying one or more cinnamate derivatives such as caffeic, ferulic, and *p*-coumaric acids (Narita and Inouye, 2015). There are at least 30 different types of CGA found in green coffee, including caffeoylquinic acids (CQA), dicaffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids. 5-CQA is the most abundant CGA found in green coffee, representing over 50% of total CGAs, followed by 3- and 4-CQAs. Several studies have associated the antioxidant activity and nutraceutical potential of green coffees to this class of substances

(Daglia et al., 2000; Sato et al., 2011; Suzuki et al., 2002; Gawlik-Dziki et al., 2014; Mikami and Yamazawa, 2015).

One of the earlier studies on antioxidant activity of green coffee was developed by Daglia and collaborators (2000), assessing antioxidant properties of both green and roasted coffee, as affected by species (*Coffea arabica* and *Coffea robusta*) and degree of roast. The levels of reducing substances (RS) of green coffee aqueous solutions were significantly higher for C. robusta (~11 g/100 g) in comparison to C. arabica (~5 g/100 g), whereas *in vitro* antioxidant activity was similar. The significant differences in RS values consistently were attributed to their different content of polyphenol compounds, particularly chlorogenic acids. All green coffee solutions showed an immediate, strong activity that increased with time of reaction (10 to 30 min). At the end of the monitoring period, all the green coffee solutions decreased the lipid peroxidation rate in a model system by at least 90%. The biological assay showed that green coffee presented very low protective activity (protection of microsomial lipid from peroxidation).

Naidu et al. (2008) also compared the antioxidant potential of *Coffea arabica* and *Coffea robusta* green beans. The extracts were prepared with solvent mixtures of isopropanol and water in different ratios. The total polyphenol content (based on Folin Cicateau essay) increased as the amount of water in the mixture was increased, with the best results (~32 g/100 g gallic acid equivalents) obtained from extraction with isopropanol 60/water 40 solution. DPPH-based free radical scavenging activity of the extracts ranged from 92 to 76% inhibition and from 88 to 78% inhibition for arabica and robusta coffees, respectively. Both hydroxyl radical scavenging activity and reducing capacity were also found to be significant for all extracts. Results indicated that extracts obtained from green coffee present potential antioxidant activity and could be used as nutraceuticals as well as preservatives in food formulations.

Ramalakshmi et al. (2008) developed another study on the antioxidant potential of green coffee beans. The study focused on triage (defective or low quality) coffee beans, i.e., beans that are separated from the good quality ones (color sorting) prior to commercialization in external markets (Franca and Oliveira, 2008). The physico-chemical characteristics of the coffee beans were evaluated. Reported values of total polyphenols and levels of chlorogenic acids were 4.50 ± 0.01 g/100 g gallic acid equivalents and 8.53 ± 0.01 g/100 g respectively. Other studies have reported higher levels of chlorogenic acids in low quality coffees in comparison to high quality ones (Farah et al., 2006; Franca and Oliveira, 2008). In order to evaluate the antioxidant potential of green coffee beans, extracts of beans were prepared using different solvents (hexane, chloroform, acetone and methanol). The extracts were evaluated through in vitro models of radical scavenging activity (α,α-diphenyl-β-picrylhydrazyl radical), antioxidant activity (β-carotene-linoleate model system), reducing power (iron reducing activity) and antioxidant capacity (phosphomolybdenum complex) in comparison to a synthetic antioxidant (butylated hydroxy anisole, BHA). The free radical scavenging activity of the extracts, based on the DPPH model system and measured by % inhibition, was in the following order: methanol (92.5%) > acetone (81%) > chloroform (25%) > hexane (8%). Performance of the methanol extract was similar to BHA. The antioxidant activity of green coffee extracts, based on the β-carotene-linoleate model was as follows: methanol (58.2%) > acetone (43.5%) > chloroform (28.2%) > hexane (14%), all significantly lower than BHA (90%). Given that the methanolic extract presented highest activity compared to the other solvents it was further analysed for its reducing power and antioxidant capacity

based on phosphomolybdenum method. Results for reducing power were in the following order: ascorbic acid > chlorogenic acid > BHA > methanol extract. The antioxidant capacity of the methanol extract was 1367 ± 54.17 μmol/g as equivalents to ascorbic acid, in comparison to 3587.9 ± 43.87 μmol/g for pure chlorogenic acid and 5098 ± 34.08 μmol/g for propyl gallate. In a subsequent study Ramalakshmi and co-workers (2008) further investigated the bioactivity of the methanolic extract of green low quality beans with reference to antioxidant (ORAC assay), anti-tumour (P388 cell assay) anti-inflammatory (J774A.1 cell assay) and anti-allergenic (RBL-2H3 cell degranulation assay) properties. The ORAC-based antioxidant capacity was significantly high (4416 μmol Trolox eq/g) in comparison to an earlier study (Wen et al., 2004) employing aqueous extract from coffee beans (40 μmol Trolox eq/100 g), confirming the importance of the employed extractant. Anti-tumor activity was observed, with P388 cell viability being reduced to 50.1 ± 3.6%, whereas anti-allergenic activity was not significant. These studies confirmed the potential of green coffee beans as source of antioxidants. It was particularly interesting as a proposition of an alternative use for low quality coffee beans.

Gawlik-Dziki and co-workers (2014) evaluated the antioxidant potential and capacity for inhibition of lipoxygenase of green coffee beans from different origins (Ethiopia, Kenya, Brazil and Colombia). The major antioxidant compounds identified in all the samples were 5-caffeoylquinic acid (5-CQA), 4-caffeoylquinic acid (4-CQA), 3-feruoylquinic acid (3-FQA) and 5-feruoylquinic acid (5-FQA). There were variations in total phenolics contents (TPC) in the following order: Kenya > Brazil > Colombia and Ethiopia. Evaluation of changes in TPC during simulated digestion and absorption was based on the Folin-Ciocalteau method. *In vitro* digestion did not increase the total content of phenolic compounds, with values being significantly lower than that in raw extracts. Nonetheless, phenolics released during simulated digestion were able to permeate the dialysis membrane, indicating high bioavailability. The highest values of bioaccessibility and bioavailability indices were observed from coffees from Kenya and Brazil, respectively. Raw extracts presented comparable lipoxygenase inhibition activities (EC_{50} ranging from 2.94 to 2.75 mg DW/mL), that were not affected by simulated digestion, however, did not significantly change this activity, which is confirmed by BAC (all values about 1). Unfortunately, lipoxygenase inhibitors from green coffee presented difficulty in permeating the dialysis membrane, indicating low bioavailability. Although green coffee beans possessed the ability to protect lipids against oxidation, regardless of origin, this activity was relatively low in the raw extracts. However, digestion *in vitro* released compounds able to protect lipids against oxidation. The authors concluded that green coffee beans are a potential source of bioaccessible and bioavailable compounds with multidirectional antioxidant activity.

In a subsequent study from the same research group, the potential of powdered green coffee beans (GCB) as a functional additive was evaluated (Dziki et al., 2015). Phenolics released during simulated digestion were highly bioavailable *in vitro.* Simulated digestion also released phytochemicals acting as chelating and reductive agents, free radical scavengers and lipid-preventers. The sensory properties of bread enriched with GCB were also evaluated. The color of both crust and crumb of the enriched bread was perceived as a little greener in comparison to the control bread, although this characteristic had only a slight negative influence on bread acceptability. Taste, aroma and overall acceptability presented high scores with substitution levels of 1–3%, whereas no significant differences were observed in texture characteristics regardless of the substitution levels. Overall results from sensory evaluation

indicated that a partial replacement of wheat flour in bread with up to 3 g/100 g ground green coffee powder was acceptable. Furthermore, a preliminary study showed that phenolic compounds from bread enriched with powdered green coffee beans were highly mastication-extractable, thus being favorable in terms of bioaccessibility and bioavailability. Bread enriched with GCB possessed higher antiradical activity than control samples. The results of this study indicated the potential of powdered green coffee beans in food supplementation.

The majority of studies that have been developed so far on the antioxidant potential of green coffee beans or their infusions have associated the significant antioxidant activity to the presence of phenolics, mainly chrologenic acids. Nonetheless, it should be pointed out that in case of natural products, health benefits are actually related to synergic effects of the whole matrix as opposed to individual substances or classes of compounds. Taking that into consideration, the recent study by Stelmach et al. (2015) evaluated the content of nutritionally important macro (Ca, Mg) and microelements (Fe, Mn, Cu) in infusions of green coffees, in order to access a possible correlation with antioxidant activity. Four methods were evaluated for preparation of the green coffee infusions: method A (10 min infusion with hot water), methods B and E (10 min heating until boiling, employing ground, B or whole, E coffee beans), method C (contacting the ground coffee beans with coffee water in a paper coffee filter) and method D (employing a household coffee maker). It was found that Ca and Mg were present in the highest concentrations in the infusions, with average concentrations of 6.49 and 12.4 $\mu g\ g^{-1}$, respectively. Concentrations of minor elements (Cu, Fe, Mn) were in the range from 0.04 to 0.13 $\mu g\ mL^{-1}$ in the prepared infusions. Infusion preparation was found to significantly influence the leachability of studied elements. The lowest rate of the extraction of elements was observed for method D (preparation using a coffee maker) whereas extractions were more effective employing methods B and C. Moderate positive correlation was observed between the antioxidant activity of green coffee infusions and their total content of phenolic compounds and Ca levels.

2.2. Roasted Coffee

There are extensive changes in the chemical composition with roasting, as a result of pyrolysis reactions. However, the proximate composition remains similar, since changes occur within a specific class of compounds. The proximate composition of roasted arabica (A) and robusta (R) coffees (dry basis) is: Protein levels ranging from 12 to 15% (A) and from 13 to 15% (R); lipid contents ranging from 15 to 20% (A) and from 11 to 16% (R); carbohydrate (by difference) ranging from 40 to 79% (A) and from 64 to 71% (R); and mineral levels ranging from 4 to 5% (A,R) (Franca and Oliveira, 2009). Slightly higher values and higher variations of lipids in roasted coffee in comparison to green coffee are attributed to the beans dry matter loss during roasting, which in turn varies with the degree of roast (Speer and Kölling-Speer, 2001; Franca and Oliveira, 2009).

Studies have shown that roasting can cause caffeine levels to be reduced down to 70% of the amount detected in green coffee (Franca et al., 2005a,b). Given that the solubility of this compound in water increases with temperature, the caffeine loss can be attributed to dragging, by the water vapor released during roasting (Franca and Oliveira, 2009). Another substance that is interesting from a nutrition point of view in roasted coffee is nicotinic acid (niacin or vitamin B3), the major non-volatile component resulting from demethylation of trigonelline

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during roasting. Niacin levels in roasted coffee range from 10 to 40 mg/100 g (Clarke and Macrae, 1985; Franca and Oliveira, 2009).

Green coffee beans are known to contain a wide variety of antioxidants, including chlorogenic acids, phenolic acids, polyphenols and alkaloids (Brezová et al., 2009), that are associated with their antioxidant potential as previously discussed. The content of antioxidants in green coffee varies according to the species (Daglia et al., 2000; Naidu et al., 2008) and origin (Chu et al., 2007). Such antioxidants will be partly decomposed during roasting and thus it is expected that the antioxidant activity associated to chlorogenic acids for example will decrease upon roasting. Nonetheless, roasting results in the generation of Maillard reaction products (melanoidins), which in turn presents significant antioxidant activities. Therefore, there is a significant amount of studies that evaluate the antioxidant potential of roasted coffee and the resulting beverages.

Some studies have focused on comparing the antioxidant potential of green and roasted coffees as well as the effect of roasting degree. However, results on the effect of roasting on antioxidant activity (AOA) are contradictory. Some studies report that AOA increases from light to medium roasts and then decrease in dark roasts (Del Castillo et al., 2005, Silveira-Duarte et al., 2005; Cämmerer and Kroh, 2006; Vignoli et al., 2014.) Others concluded that AOA increases with roasting (Nicoli et al., 1997; Borrelli et al., 2002; Sanchez-Gonzales et al., 2005; Stalmach et al., 2006;) whereas some claimed the opposite (Richelle et al., 2001). Daglia et al. (2000) compared the antioxidant properties of aqueous solutions obtained from green and roasted coffees with respect to variations among species (*Coffea arabica* and *Coffea robusta*) and the effect of degree of roast. The amount of reducing substances (RS) did not change significantly upon roasting in the case of arabica coffees, whereas, for robusta coffees, there was a slight decrease in RS after a light roasting followed by an increase for more intense roasting conditions. Thus, dark roasted coffee always showed higher RS values than green coffee. The same tendency was observed in terms of *in vitro* antioxidant activity, i.e., a slight decrease for light roasts followed by an increase for medium and dark roasts. This behavior can probably be attributed to the loss of polyphenolic compounds occurring in green coffee during light roasting and to the successive formation of other antioxidant compounds such as Maillard reaction products or pyrolysis products when more severe thermal conditions were applied. The biological assay showed that green coffee presented very low protective activity, PA (protection of microsomial lipid from peroxidation). Nonetheless, PA increased significantly with thermal treatment, so that all types of roasted coffee completely inhibited microsomial lipid peroxidation. Del Castillo and co-workers (2002) evaluated the effect of roasting on the antioxidant activity of coffee brews. Aqueous extracts obtained from green and roasted (light, medium and dark) coffees were evaluated. A progressive decrease in antioxidant activity with degree of roasting was observed with the simultaneous generation of high (HMM) and low molecular mass (LMM) compounds possessing antioxidant activity. Maximum antioxidant activity was observed for the medium-roasted coffee. The antioxidant activity of aqueous dilutions was higher in comparison to ethanolic dilutions, indicating that some components, making important contributions to the antioxidant activity of the aqueous dilutions, were not soluble in ethanol. Analysis of the gel filtration chromatography fractions showed that the LMM fraction made a greater contribution to total antioxidant activity than the HMM components.

Summa et al. (2007) evaluated the effect of roasting degree on in vitro radical scavenging capacity and acrylamide formation. The major motivation for this study was that previous

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works have shown an increase of the antioxidant activity of coffee with more intense roasts, in association to Maillard reaction products (MRP), mainly melanoidins. However, acrylamide, a substance of recognized toxicity, is also a MRP and its levels can also increase as roasting progressed. Thus, in this study both arabica and robusta coffees were roasted (236°C) over different time periods, in order to obtain very light, light, medium and dark roasts. It was observed that increase in roasting degree led to a decrease in acrylamide concentration as well as radical scavenging capacity, pointing towards lighter roasts as more interesting both in terms of antioxidant potential and toxicity. However, the effect of varying roasting temperature was not investigated. Sacchetti and co-workers (2009) evaluated the ABTS radical scavenging activity (RSA) and total phenolics content (TPC) of coffee brews obtained from different types of coffees submitted to different roasting conditions. Roasting temperatures and times ranged from 150 to 190^{o}C and from 2 to 10 min, respectively. Brews extracted from medium roasted coffees presented higher TPC and RSA values. Results indicate that roasting temperature plays an important role on the antioxidant potential. At higher roasting temperatures, the antioxidant activity tends to decrease due to thermal degradation of polyphenols, and this process is not counterbalanced by further MRP formation. These results are similar to those reported by Cämmerer and Kroh (2006) that reported a decrease in RSA with an increase in roasting temperature. Nonetheless, these authors tested several methods and concluded that results can differ significantly depending on the employed radical. They reported that 2,2,6,6- tetramethyl-1-piperidin-1-oxyl was the best radical marker for determination of antioxidant activity. A similar study was developed by Vignoli and co-workers (2014), in which the antioxidant activity of arabica and robusta coffee beans that were submitted to different roasting conditions (processing time varying from 7 to 10 min and temperatures varying from 215 to 225°C) was evaluated. They attributed the higher antioxidant capacity (AC) of coffees originating from light roasts to higher polyphenol content, and the higher AC of robusta vs. arabica to higher caffeine contents. The recent work developed by Kamiyama et al. (2015) tried to infer on the role of degradation products of chlorogenic acids on the antioxidant potential of roasted coffee. A sample of the major chlorogenic acid present in coffee, 5-caffeoylquinicacid (5-CQA) was heated at 250°C. They observed significant antioxidant activity (79.12 ± 2.49%) at the level of 10 μg/mL, whereas unheated 5-CQA showed only moderate antioxidant activity (44.41 ± 0.27%) at ten times this concentration. It was observed that heating produced significant amounts of pyrocatechol and 2-methoxy-4-vinylphenol, and their respective antioxidant activity levels were 77 and 99%, respectively. Although these results confirm that roasting degrades chlorogenic acids to form potent antioxidants, there is still a need to evaluate the interaction with other substances that are also present in green coffee and thus will affect the outcome of Maillard reactions.

Given that phenolic compounds can dissolve in several solvents including water, ethanol, and methanol, a few studies have focused on comparing the efficiency of different solvents or extraction procedures. Parra and co-workers (2007) evaluated the effects of different preparation methods (Italian or Mocha, Filter and Espresso) on the total polyphenol content (TPC) and antioxidant activities of brewed coffee. The order of TPC was Italian ≈ filter > espresso, with average values ranging from 2.1 to 3.2 g gallic acid eq./100 g dry matter. Total ferric reducing ability based on FRAP essay did not vary significantly among the three extraction procedures. In the case of scavenging activity based on ABTS the order was the same as observed for TPC. It was also reported that antioxidant activity increased

significantly (by 34%) after four hours of heating. The cause of this increase was attributed to the formation of Maillard products, due to the heat process. Chu et al. (2007) evaluated water/ethanol mixtures as extractants of catechin, rutin, ferulic acid, *o*-dihydroxybenzene, chlorogenic acid, caffeic acid, gallic acid, and protocatechuic acid from soluble coffee. When pure water was used as the extraction solvent, the antioxidant content determined in coffee was 188.0, 169.6, 57.7, 497.7, 8,638.9 and 114.8, μg/g, for catechin, rutin, ferulic acid, *o*-dihydroxybenzene, chlorogenic acid, and caffeic acid, respectively. Pure water provided better extraction for the previously cited phenolics, whereas pure ethanol was more effective for extraction of galic acid. These results are in agreement with previous findings from Del Castillo et al. (2005) that reported lower antioxidant activity of ethanolic in comparison to water solutions of green and roasted coffees.

Although many studies have established the antioxidant potential of roasted coffee, actual applications in food products are still scarce and quite recent. Lin and collaborators (2015) evaluated if roasted coffee could be employed to control oxidation in ground beef, as a replacement for commonly employed rosemary extracts. Salted ground beef samples were treated with rosemary extract (positive control) as well as light roasted, medium roasted or dark roasted coffees (0.1 g/100 g) and stored for 7 days at 4^{o}C. Antioxidant capacity was assessed by measuring thiobarbituric acid reactive substances. Dark roasted coffee showed the highest antioxidant capacity in salted beef after 7 days. The obtained results indicate that roasted coffee could act as an effective natural antioxidant in beef, extending its shelf-life, even in the presence of salt, which typically increases lipid oxidation levels.

3. Coffee By-Products as Source of Bioactive Compounds

3.1. Coffee Husks and Pulp

Coffee husks present moisture contents that range from 7 to 18%, with this wide range being attributed to variations in processing and storage conditions (Oliveira and Franca, 2015). Coffee pulps (wet processing residues) leave the dehuller with an average of 75% moisture, this value being reduced to approximately 13% after drying. The proximate composition (dry basis) is as follows: protein levels range from 8-11% and from 4 to 12% for CH and CP, respectively. Given that both residues comprise the hull and part of the pulp of the coffee fruits, carbohydrate contents are high, ranging from 58-85% in coffee husks and from 45-89% in coffee pulp. Lipid contents are low as expected, ranging from 0.5-3% and from 1-2% in husks and pulp, respectively. Mineral levels vary from 3-7% in CH and from 6-10% in CP (Franca and Oliveira, 2009).

Such residues are rich in organic matter and nutrients and contain compounds such as caffeine, tannins, and polyphenols. Caffeine is present in coffee husks and pulps at approximately 1% concentration (db). Average levels of tannins range from approximately 5% in coffee husks and from 1-9% in coffee pulp. Both caffeine and tannins are commonly viewed as anti-nutritional factors restricting the use of coffee husks and pulp in animal feed (Clifford and Ramirez-Martinez, 1991).

Prata and Oliveira (2007) evaluated the anthocyanin content and profile of fresh coffee husks, i.e., the residue obtained after dehulling coffee cherries during wet processing (CP).

Red Arabica coffee cherries of the cultivars Red Catuaí, Icatú, Mundo Novo, Acaiã do Cerrado e Rubi were evaluated. No significant differences in anthocyanin contents were observed among the five evaluated cultivars. Cyanidin 3-rutinoside was the major anthocyanin encountered in the extracts, followed by a small amount of cyanidin 3-glycoside. These results were confirmed in the recent study by Murthy et al. (2012). Anthocyanins were analyzed by high performance liquid chromatography with photodiode array detection and identified by electrospray ionization mass spectrometry. The anthocyanins from coffee pulp yielded 25 mg of monomeric anthocyanins/100 g of fresh pulp on a dry weight basis. The purified anthocyanin was identified as cyanindin-3-rutinoside and cyanidin-3-glucoside. The red color of coffee peels was attributed to the presence of cyanidin 3-rutinoside and confirmed by ^{1}H-NMR and ^{13}C-NMR. Furthermore, coffee anthocyanins showed multiple biological effects resulting in effective α-glucosidase and α-amylase inhibitory activities. Both studies indicate that, given the extensive amounts of coffee produced worldwide, CP could be viewed as an economical source of anthocyanins.

Tello and co-workers (2011) proposed the use of supercritical CO_2 for extraction of caffeine from coffee husks. They employed a continuous CO_2 flow unit and tested different pre-treatments and operational conditions. Higher extraction rates were obtained with increases in flow rate and/or operational times. The maximum extraction yield was 84% and, after water washing, the obtained caffeine was at least 94% pure. This study showed that coffee husks are a potential source for caffeine extraction.

A common agricultural practice in Brazil is the incorporation of coffee pulps or husks in organic substrates for the production of vegetables, fruit trees, or even in the coffee culture itself. These residues are employed not only as an organic amendment, but also as a way to control weeds. Thus, Silva and collaborators (2013) evaluated the allelopathic potential of ethanolic extracts obtained from dry and fresh Arabica coffee fruit peels, i.e., CH and CP. Lettuce, Malaysian cabbage and beggar's tick seeds and seedlings were employed as test subjects for the pre-emergence, post-emergence, and mitotic index of meristematic root cell tests. The extracts' contents of phenols, flavonoids and caffeine, in addition to their antioxidant activity were also determined. The mitotic index was reduced in comparison to the negative control. Seed germination and growth were significantly decreased as the extract concentration increased. A considerable quantity of phenols, flavonoids and caffeine was found in both types of e extracts. A progressively growing antioxidant activity of the extracts was observed as their concentrations increased. Given the obtained results, the authors suggest that the free radical scavenging activity, as well as the high polyphenol and caffeine contents of the extracts could be responsible for the mechanism that characterizes their allelopathic effect of the extracts and confirm the potential of coffee husks and pulps as inhibitors of germination and development of other species.

4.2. Coffee Silverskin

Coffee silverskin represents the solid residue generated in the production of roasted coffee, and this residue is collected by cyclone separation during roasting of coffee beans. Moisture contents have been reported to range from 5 to 7% (Narita and Inouye, 2014). The proximate composition (dry basis) is the following: protein, lipid and mineral levels are in the ranges of 16-19%, 1.6-3.3% and 7%, respectively. This type of residue has been recently

evaluated as a potential source of bioactive substances with antioxidant potential (Bresciani et al., 2014; Narita and Inouye, 2014).

CS has been reported to present high contents of dietary fiber (50–60%), divided into soluble (15%) and insoluble (85%) (Borrelli, et al., 2004; Pourfarzad et al., 2013; Narita and Inouye, 2014). Given that CS presents higher fiber contents in comparison to commonly employed fiber sources such as wheat and oat brans (29-42%), this coffee by-product has been pointed out as an alternate source of insoluble dietary fibers (Narita and Inouye, 2014).

Earlier studies have demonstrated that CS presents significant antioxidant potential (Borrelli, et al., 2004). A total amount of 1.1 mg/100 g of phenolic compounds (quantified by Folin−Ciocalteu) was detected, and chromatographic analysis indicated that chlorogenic and caffeic acids were the main components. The concentration of phenolic compounds in CS was found to be low in comparison to roasted beans. This was attributed to degradation of phenol compounds given the higher temperature experienced by the outer layer of the beans during roasting. Although a small amount of free phenol compounds was detected in CS, the antioxidative activity was significant, and attributed to the action of melanoidins. Experiments also indicated that CS presents potential prebiotic activity, inducing preferential growth of bifidobacteria rather than clostridia and *Bacteroides* spp.

CS extract was also reported to present high inhibitory effect against hyaluronidase (Furusawa et al., 2011). Hyaluronidase is a mucopolysaccharase related to inflammation by the histamine released from mast cells, and it has been shown to be effective in suppressing allergies and inflammation. An extract was prepared employing the soluble solids obtained from CS (aqueous extraction at 121^{o}C for 20 min, followed by spray drying). Acidic polysaccharides mainly composed of uronic acid were found to be the substances that were responsible for hyaluronidase inhibition by the prepared extract.

Narita and Inouye (2012) evaluated the antioxidant activity of CS extracts obtained by the treatment of CS with subcritical water (25–270°C). It was observed that antioxidant activity increased with increasing the extraction temperature. The maximum H-ORAC and DPPH values of the extracts were obtained at 270°C and were 2629 ± 193 and 379 ± 36 μmol TE per g of CS extract, respectively. The phenolic and protein contents of the CS extracts presented high correlation with the H-ORAC and DPPH values. Peptides produced by hydrolysing the protein in CS by subcritical water treatment were considered to be responsible for the high antioxidant capacity.

Pourfarzad and co-workers (2013) evaluated the potential of coffee silverskin as a source of dietary fiber in bread making. The optimum treatment of coffee silverskin with alkaline hydrogen peroxide that provided the best quality, shelf life, sensory and image properties of the prepared bread was obtained by response surface methodology. Contact time, solution portion and particle size were considered components of the chemical treatment. The best processing conditions obtained by the regression models were 1 h mixing time, 4.7:1 peroxide solution:CS volume ratio and 116.41 μm particle size. Results indicate that CS treated with alkaline hydrogen peroxide might be useful as an ingredient for reducing caloric density and increasing dietary fiber content of bread. Martinez-Saez et al. (2014) recently proposed another use of CS in food product development. They developed beverage formulations based on coffee silverskin aiming at body fat reduction and body weight control. Preparation was based on aqueous extraction of powdered coffee silverskin extracts. Tablets of a commercial supplement based on decaffeinated green coffee extract were employed for comparative analysis. Health benefits were evaluated *in vitro* and *in vivo* employing as animal model

Caenorhabditis elegans. In vitro antioxidant capacity and total amount of extractable phenolics were significantly greater in beverages prepared with robusta coffee silverskin extract in comparison to CS from arabica coffees. Nonetheless, the beverages made from CS extracts from both robusta and arabica presented total antioxidant capacities similar to those described in the literature for coffee beverage and coffee silverskin. The results obtained by quantification of fluorescence in each population of nematodes (wild type N2 *C. elegans*) indicated a clear dose–response effect on reducing accumulation of body fat. The most effective dose was obtained for the beverages containing the highest doses of CS extracts, 10 mg/mL. The corresponding values for body fat reduction were 21% and 24% for Arabica and Robusta coffee silverskin extracts, respectively. The response was similar to that obtained for pure caffeine and CGA. Therefore, it was concluded that the prepared beverages based on CS extracts contain physiologically active doses of these compounds and may have an effect in the prevention of obesity. Results from the sensory acceptance test indicated that 10% of the testing panel were prepared to consume the beverages as served, 85% would drink as long as other ingredient was added (sugar, milk, citrus, ice, etc.) whereas 5% declared they would not drink them. In general, results from the sensory analysis indicated a higher degree of acceptance for the beverages prepared using CS extracts from robusta in comparison to arabica coffees.

The phenolic composition, caffeine content and antioxidant capacity of CS was evaluated by Bresciani and collaborators (2014). Caffeoylquinic acids were the most relevant phenolics, with 5- and 3-CQA representing the ones in higher quantity (199 and 148 mg/100 g, respectively). The three feruloylquinic acids detected comprised 143 mg/100 g, representing 23% of the CGA. Two coumaroylquinic acids plus two caffeoylquinic acid lactones represented just a small amount of phenolics (only 3% of total hydroxycinnamates). Caffeine content in was 10 mg/g and total antioxidant capacity was 139 mmol Fe^{2+}/kg. This value is comparable to other food products already deemed as valuable sources of antioxidants such as dark chocolate, herbs and spices.

Costa et al. (2014) performed an optimization study on extraction of antioxidants from CS. The evaluated variables were solvent polarity, temperature and extraction time. Water and ethanol were selected as solvents because of their low toxicity and solvent polarity was varied by using different water/ethanol ratios. The extracts yield and composition varied significantly with the conditions used. The condition that provided the best combination of extraction yield and antioxidant capacity, according to a factorial experimental design, was the use of a hydroalcoholic solvent (50%:50%) at 40°C for 60 min. The extracts obtained under these conditions presented the following composition: total phenolics - 302.5 ± 7.1 mg GAE/L, tannins - 0.43 ± 0.06 mg TAE/L, and flavonoids - 83.0 ± 1.4 mg ECE/L. DPPH scavenging activity and ferric reducing antioxidant power were 326.0 ± 5.7 mg TE/L and 1791.9 ± 126.3 mg SFE/L), respectively.

4.3. Spent Coffee Grounds

Spent coffee grounds (SCG) are the residues resulting from water extraction of roasted and ground coffee during preparation of coffee beverage or preparation of industrial soluble coffee, i.e., are roasted and ground coffee depleted of its water-soluble compounds. These residues are largely available at households and commercial establishments and in even

higher amounts in the soluble coffee industry. SCG are inadequately disposed as waste in landfill sites or as fuel in combustion systems in the soluble coffee industry. These solid residues are highly pollutant because, when they are burned, large amounts of volatile organic compounds are generated (Franca and Oliveira, 2009). Aside from the environmental issues, care should be taken in choosing an adequate way of disposal, because SCG can be employed for adulteration of roasted and ground coffee, being rather difficult to detect as an adulterant (Reis et al., 2013ab). Hence, the main choice of the soluble coffee industry has been to use SCG as a boiler fuel. However, since the proximate composition of SCG is expected to be similar to that of roasted coffee beans, great interest has been put in recent years in the study of its composition and the possibilities of exploiting it for recovery of valuable compounds (Campos-Vega et al., 2015). The chemical composition of SCG presented in Table 1.

Table 1. Chemical composition roasted coffee and spent coffee grounds

	Spent Coffee Grounds Composition (g/100 g d.b.)[a]
Protein	10–17
Lipids	22–27
Minerals	0.1-1
Carbohydrate	45-89
Caffeine	0.07-0.5

[a] Compilation of data presented by Franca and Oliveira (2008; 2009), Delgado et al. (2008), Ballesteros et al. (2014) and Campos-Vega et al. (2015).

Lipid contents of SCG are higher than the contents in green and roasted coffee (9-15%). This is expected, since lipids are lightly extracted during beverage and soluble coffee preparation. Protein in green and roasted coffees was reported by Macrae (1985) to be in the range of 8.7 to 12.2% (w/w) after correction for caffeine and trigonelline-nitrogen. Average protein content in SCG was reported to be 13.6%, similar to that of green and roasted coffee, and the quality of SCG protein is such that it was deemed useful for formulation of food products with multiple human health benefits during liver diseases, oxidative stress and hypertension (Campos-Vega et al., 2015). Caffeine contents in green and roasted coffee were reported in the range of 0.9 to 2.9% for Arabica and Robusta and a sharp decrease in its content was observed for SCG, with the higher content being in SCG from espresso coffee, due to a shorter extraction time in this beverage preparation method when compared to those of other methods (Campos-Vega et al., 2015). Thus, based on the data presented in Table 1, it can be concluded that, even though caffeine is the most relevant bioactive compound in green and roasted coffee, it is rather insignificant in SCG. Trigonelline was not reported to be found in SCG for it is significantly degraded during roasting and the remaining non-degraded amount is expected to be completely extracted during beverage or soluble coffee preparation, due to its high solubility in water. The mineral contents of SCG (0.4-1.6%; Pujol et al., 2013) are expected to be lower than those of green and roasted coffee (4%; Clarke, 1985), given that most minerals are easily extracted with hot water.

Coffee beans are a rich source of polysaccharides (accounting for about 50% of the green beans' dry weight and about 40% of the roasted beans' dry weight) which are mainly comprised of galactomannans, type-II arabinogalactans and cellulose (Moreira et al., 2015).

Roasting increases arabinogalactan and galactomannan solubility by loosening the cell-wall structure as the beans swell and by polysaccharide depolymerization, and yet only a small amount (~30%) is extracted during beverage preparation (Oosterveld et al., 2003), and most of the polysaccharides remain bound to the SCG matrix (Mussatto et al., 2011a; Simões et al., 2013). Hence, SCG contain high amounts of monosaccharides polymerized into cellulose and hemicellulose structures. The monosaccharides composition are reported to be in the following ranges: 21-57% mannose, 14-30% galactose, 9-19% glucose, and 2-6% arabinose (Mussatto et al., 2011a; Simões et al., 2013; Ballesteros et al., 2014). The differences in the monosaccharides compositions were attributed to distinct varieties of coffee being processed.

Polysaccharides, such as those found in SCG, were deemed suitable for use as dietary fiber for they present immunostimulatory activity (Simões et al., 2013). Regarding fiber content, SCG are primarily comprised of 45.2% neutral detergent fiber, occurring as hemicellulose, cellulose, and lignin associated compounds, and 29.8% acid detergent fiber, consisting of cellulose and lignin (Vardon et al., 2013). Total Dietary Fiber (TDF) content was reported by Murthy and Naidu (2012) and Vilela (2015) to be in the range of 45 to 51 g/100 g, with 35 to 48 g/100 g as insoluble dietary fiber (IDF) and 2 to 8 g/100 g as soluble dietary fiber (SDF). TDF values for SCG are slightly lower than those of other coffee processing byproducts such as coffee silverskin (~62 g/100 g) (Borreli et al., 2002) and coffee husks and pulp (~66 g/100 g) (Melo, 2013). However, Vilela (2015) has demonstrated that TDF values can increase up to 70 g/100 g after treatment of SCG with hydrogen peroxide.

IDF are associated to porosity, low density, ability to increase faecal bulk and decrease intestinal transit (Elleuch et al., 2011). IDF corresponds to approximately 80 to 90% of the fibers present in SCG. SDF present the capacity to increase viscosity, to reduce the glycemic response and plasma cholesterol as well as prebiotic action (Elleuch et al., 2011). SDF contents in spent coffee grounds are low in comparison to other agricultural residues such as carrot peel and pomegranate bagasse (~9.8-19.9 g/100 g) (Chantaro et al., 2008; Elleuch et al., 2011; Viuda-Martos et al., 2012). However, SCG fibers were shown to exhibit antioxidant properties in levels similar to red wine products and were deemed by Murthy and Naidu (2012) antioxidant dietary fibers with potential as dietary supplement.

Phenolic compounds, the major substances associated to antioxidant activities of food sources, are mainly comprised of chlorogenic acids (CGA) in coffee beans. CGA are a class of water-soluble esters formed between quinic acid and one or two moieties of caffeic acid. CGA found in coffees include caffeoylquinic, dicaffeoylquinic, feruloylquinic, and *p*-coumaroylquinic acids, and the antioxidant activity and nutraceutical potential of green coffees have been mostly attributed to this class of compounds (Suzuki et al., 2002; Gawlik-Dziki et al., 2014; Mikami and Yamazawa, 2015). CGA contents in green Robusta and Arabica coffees have been reported to be in the respective ranges of 7 to 10% and 5 to 7.5% (Clifford, 1985), and have been shown to decrease by approximately 75% during roasting (Wei and Tanokura, 2015). Despite this drastic reduction in CGA concentration, it has also been shown that green and roasted coffees presented similar antioxidant activities, with a slight decrease for light roasts followed by an increase in darker roasts. This behavior is attributed to the loss of polyphenolic compounds (CGA) during light roasting and to the successive formation of other antioxidant compounds such as Maillard reaction products or pyrolysis products in dark roasts. Therefore, the antioxidant potential of spent coffee grounds is probably due to the combined effect of the remaining CGA as well as Maillard reaction products that were not extracted during beverage preparation. An evaluation of studies that

deal with the chemical composition of SCG with respect with substances associated with its antioxidant potential is presented as follows.

The recovery of phenolic compounds from SCG as well as and their antioxidant activity have been investigated by Mussatto and co-workers (2011b) with the use of water/methanol mixtures. A 90-minute extraction employing 60% methanol in a solvent/solid ratio of 40 mL/g of solid was demonstrated the most efficient for obtaining an extract with the highest content of phenolic compounds (16 mg gallic acid equivalents/g SCG) and the highest antioxidant activity (FRAP of 0.10 mM Fe(II)/g). Bravo and collaborators (2012) compared the concentration of several bioactive substances (3-, 4-, and 5-monocaffeoylquinic and 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids, caffeine, and browned compounds, including melanoidins), total phenolics and antioxidant capacity for SCG obtained from different types of coffeemakers: filter, espresso, plunger, and mocha. Except for the mocha coffeemaker SCG, all the others presented significant amounts of caffeoylquinic acids, ranging from 11.05 (espresso) to 13.24 mg/g (filter) for arabica and from 6.22 (filter) to 7.49 mg/g (espresso) for robusta, numbers representing 4 to 7 times higher than those of the respective coffee brews. Thus, it can be concluded that, although CGA are significantly water-soluble and are expected to be completely transferred to the beverage, the majority will remain on the solid matrix and contribute to the antioxidant activity of SCG. The antioxidant capacities of the SCG aqueous extracts represented 46.0–102.3% (filter), 59.2–85.6% (espresso), and <42% (plunger) of the antioxidant capacities of the respective coffee brews. Hence, SCG obtained from the most commonly employed types of coffeemakers was deemed a potential source of hydrophilic bioactive compounds. Bravo et al. (2013) further studied the effects of distinct extraction procedures on the antioxidant capacity of spent coffee grounds. The studied extraction methods were Soxhlet apparatus, solid-liquid extraction and a conventional coffee maker, and the extractants used were water, ethanol, and methanol, pure and mixed. Extraction in a coffee maker provided extracts with higher antioxidant capacity than the other extraction methods and the solid-liquid extraction method provided extracts with higher amounts of total phenolics and browned compounds than the other methods. The one-step coffee maker extraction method was deemed sufficient for obtaining extracts with high antioxidant capacities (approximately 80% extraction efficiency) and water was demonstrated to be the best solvent for such endeavor.

Zuorro and Lavecchia (2012) have focused their study on optimizing the extraction of phenolics from spent coffee grounds, studying the effects of temperature, time, liquid-to-solid ratio and ethanol/water concentration on their recovery. Recovery was up to 90% and total phenolics ranged from 17.75 to 21.56 mg GAE/g. In a further study, Zuorro (2015) employed response surface methodology in order to optimize the recovery of phenolics from SCG, evaluating the same parameters as in the previous study and obtaining a positive correlation for all the parameters with the extraction of phenolics, with temperature and liquid to solid ratio being the most influential. Xu and collaborators (2015) also performed an optimization study to maximize both the yield of phenolics and radical scavenging capacity of SCG extracts employing subcritical water as the extractant, resulting in caffeoylquinic acids being the major contributors to the antioxidant activities of the produced extracts. Under optimized conditions (179°C, 36 min, and 14.1 g/L) the total phenolic content of the extract was 86.23 mg GAE/g and scavenging activities presented values of 81.38 mmol TE/100 g for ABTS and 42.13 mmol TE/100 g for DPPH method.

Cruz et al. (2012) and Panusa et al. (2013) evaluated the potential of SCG from espresso machines as sources of bioactive compounds, both studies reporting high compositional variability, especially with regard to water-soluble components (such as caffeine, chlorogenic acids and minerals). High amounts of CGA (165-609 mg/100 g) and caffeine (453-1150 mg/100 g) were reported and a strong positive correlation between total soluble solids retained and the levels of caffeine, CGA and minerals was determined, confirming the significance of the extraction procedure on the antioxidant potential of SCG. Pujol et al. (2013) evaluated the parameters elemental analysis, mineral composition and ash content, summative composition, acidic functional groups, lipophilic extractives, total polyphenols, and condensed tannins for SCG obtained from a soluble coffee industry. The samples presented high carbon (>58%), low nitrogen (<2%), and low ash (<1%) contents, with lignin and polysaccharides present in similar amounts (20-26%), and total polyphenols and tannins with the highest values of 454 and 293 mg GAE/g, respectively.

The effect of ethanol concentration on the extraction of phenolics from spent coffee grounds was studied by Pavlović and collaborators (2013) employing Microwave-assisted extraction. The influence of ethanol concentration and time of microwave radiation was evaluated in terms of extraction yield, total polyphenol content, DPPH radical inhibition activity and FRAP ferric reducing ability. The best extraction conditions were 20% aqueous ethanol solution under 40 s of microwave radiation leading to a value of 398.95 mg GAE/g for total phenolics. The obtained IC_{50} value for the extract was 3.75 μg/mL, in the same range of commonly employed commercial antioxidants such as ascorbic acid (~6 μg/mL) and BHT (~3 μg/mL). Ranic et al. (2014) also performed a study on microwave-assisted extraction of SCG, employing low concentration ethanol in aqueous and response surface methodology in order to verify the effects of extraction time, liquid-to-solid ratio, and microwave power on total phenolics content and antioxidant activity. The highest concentration of phenolics were obtained for extraction time of 40 s, liquid-to-solid ratio of 6 mL/g and microwave power of 240 W, and antioxidant activity presented good correlation with the total polyphenol content.

Acevedo et al. (2013) studied solid–liquid extraction, supercritical extraction and direct saponification of oil from SCG to obtain the diterpenes kahweol and cafestol. These diterpenes have been associated to certain health effects, including anti-inflammatory and anticarcinogenic properties (Cavin et al., 2002; Kim et al., 2004; Speer and Kölling-Speer, 2006; Kotowski et al., 2015). The phenolic composition and antioxidant capacities of SCG were determined before and after oil extraction. Soxhlet extraction presented the highest oil amounts (26.4%). The fatty acid composition was determined to be similar to that of green and roasted coffee oil, regardless of the extraction procedure. The determined total phenolics was 255 and 273 mg GAE/g and free radical-scavenging activity was 83 and 201 μmol Trolox/g respectively for extracts of SCG and defatted SCG. The highest contents of diterpenes were determined for the extracts obtained by direct saponification: 214 mg/100 g SCG for kahweol and 466 mg/100 g SCG for cafestol.

A comparative study of composition and antimutagenic and antimicrobial activities of spent coffee grounds and their corresponding coffee brews was performed by Monente et al. (2015a). Both filter and espresso extraction methods were evaluated for arabica and robusta coffees. Similar contents of caffeine, 49 and 80 mg/g for arabica and robusta, respectively, were determined for both the SCG and their corresponding brews. Total CGA contents were slightly higher in the spent coffees, with values for the spent coffee/corresponding brew being 84/74 and 66/51 mg/g for arabica and robusta, respectively. All samples exhibited strong

protection activity against indirect acting mutagen 2-AF (≤92%), whereas the protection against the direct mutagen NPD was 12–35%. Spent coffee grounds extracts presented antimicrobial activity mostly against Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes*) and yeast (*Candida albicans*), with the growth inhibition of Gram-negative bacteria being attributed to the high content of melanoidins in the extracts. The antimutagenic and antimicrobial activity of spent coffee grounds suggest it can be deemed a potential ingredient for enhancing functional properties and extending the shelf life of food products. In a follow-up study, Monente et al. (2015b) evaluated the composition of free and bound phenolic acids in SCG. HPLC analysis of the SCG extracts were performed for alkaline, acid and saline treatments to quantify caffeoylquinic (3-CQA, 4-CQA, 5-CQA), dicaffeoylquinic (3,4-diCQA, 3,5-diCQA, 4,5-diCQA), caffeic, ferulic, *p*-coumaric, sinapic, and 4-hydroxybenzoic acids. The actual amount of phenolics present in SCG was determined to be over 2-fold higher when bound phenolics, deemed linked by noncovalent interactions to macromolecules such as melanoidins, were duly extracted and quantified. On the other hand, lower amounts of attached phenolics were determined for coffee brews (~20%). The most abundant of the phenolics were caffeoylquinic acids isomers, representing approximately 70% of the total CGAs.

4.4. Comparative Evaluation of Coffee By-Products

Studies by Murthy and Naidu (2012), Andrade et al. (2012) and Ballesteros et al. (2014) have dealt with a comparison of chemical compositions and antioxidant potentials of spent coffee grounds and other coffee processing residues, such as husks, pulp and silverskin. Murthy and Naidu (2012) employed a mixture of isopropanol and water (60:40 v/v) as solvents for the recovery of phenolic compounds from coffee pulp and husk, silverskin, and SCG. Chlorogenic acid content ranged from 2.3 g/100 g for SCG to 3.0 g/100 g for silverskin, and the determined values for total polyphenols were 22.19 ± 0.63, 21.71 ± 0.25, 20.12 ± 0.1, and 22 ± 0.2 μg/mL gallic acid equivalents, for coffee pulp, husks, silverskin and SCG, respectively. Hydroxyl radical scavenging activity ranged from 59, for coffee pulp, to 85%, for SCG and DPPH-based antioxidant activity ranged from 65% to 70%. Andrade et al. (2012) employed supercritical fluid extraction with CO_2 and with CO_2 and co-solvent (high pressure methods) to obtain extracts of spent coffee grounds and coffee husks extracts and further evaluate their chemical compositions and antioxidant activities. Low pressure methods, such as ultrasound and Soxhlet, were also evaluated, with the extracts obtained by low pressure extraction with ethanol presenting better results for the global extraction yield and total phenolics (TPC) than supercritical fluid extraction method. The highest TPC values were 151 (coffee husks, Sohxlet) and 588 mg of chlorogenic acid equivalent/g (spent coffee grounds, ultrasound). The highest antioxidant activity was presented by coffee husk extracts obtained by low pressure extraction.

Ballesteros et al. (2014) comparatively studied the chemical composition, functional properties, and structural characteristics of coffee silverskin (CS) and spent coffee grounds (SCG), concluding that both residues are sugar-rich lignocellulosic materials composed of high levels of insoluble, soluble, and total dietary fibers. SCG presented higher content of total dietary fiber (60.46% *w/w*) than CS (54.11% *w/w*), and the antioxidant potentials were similar for SCG and CS in terms of DPPH values, respectively 20 and 21 μmol TE/g.

However, SCG exhibited twice the antioxidant potential of CS when the FRAP method was employed: 0.102 vs. 0.045 mmol Fe(II)/g. The prebiotic, antimicrobial and antioxidant properties of both spent coffee grounds and coffee silverskin, pure and mixed with melanoidins extracted from SCG were assessed by Jiménez-Zamora et al. (2015). All residues were obtained from regular and torrefacto (sugar added) roasted coffees. Prebiotic activity was deemed relevant for both types of residue and the presence of coffee melanoidins were determined to interfere with such beneficial properties. On the other hand, antimicrobial activity was significantly increased with the addition of melanoidins. All the residues presented antioxidant potential and addition of melanoidins to the residues as well as addition of sugar during coffee roasting (torrefacto samples) increased the antioxidant and antimicrobial activity.

CONCLUSION

The coffee fruit as a whole present an abundance of bioactive compounds and, upon its processing (including green bean processing, roasting and soluble or instant coffee production), these bioactive compounds will be found in the major products, the green and roasted coffee beans, and in the respective residues of the processing steps. The main bioactive compounds of coffee and respective residues are caffeine, chlorogenic acids, trigonelline, coffee oil diterpenes, and polysaccharides comprising both insoluble and soluble dietary fibers. Caffeine, the major bioactive compound in coffee, is present in significant amounts in all the separable parts comprising the coffee fruit (beans, silverskin, parchment, and pulp and exocarp) and, consequently, will render all of them suitable for its recovery. The beans and the pulp and husks are the portions with the highest content, followed by the silverskin and the parchment. Even though it is reasonably soluble in water at the temperatures used in beverage preparation, caffeine will also be present in spent coffee grounds, although in different amounts depending on the method of extraction applied to the roasted coffee. The antioxidant capacities of coffee and its processing residues are mostly attributed to the polyphenols, and the majority of phenolic compounds present in all these products and byproducts is comprised of chlorogenic acids. Chlorogenic acids are present in both free and bound forms, with bound chlorogenic acids (mostly to melanoidins in spent coffee grounds) being determined to be present in contents two times higher than those of free CGA. Although positive correlations were found between polyphenol contents and antioxidant capacities for roasted coffees and their respective residues, Maillard reaction products were also determined to be significant contributors to the antioxidant capacity of roasted coffee and its byproducts. Green and roasted coffee residues were determined to be comprised of significant amounts of dietary fiber, mostly insoluble ones, with significant associated antioxidant activities. The polysaccharides galactomannans, type-II arabinogalactans and cellulose are the main constituents of the dietary fibers in both coffee and its residues.

There is an abundance of published works in recent years demonstrating the potential of coffee and its processing byproducts as a source of bioactive compounds. Based on the chemical composition data published so far, coffee byproducts may become a major target for

biorefineries, to be installed alongside with the processing units, for the recovery of valuable bioactive compounds.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support from the following Brazilian Government Agencies: CNPq and FAPEMIG.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 2

COFFEE BREWS PREPARATION: EXTRACTION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY

Josiane Alessandra Vignoli[1,*], Marcelo Caldeira Viegas[2], Denisley Gentil Bassoli[3] and Marta de Toledo Benassi[4,†]

[1]Departamento de Bioquímica e Biotecnologia, Centro de Ciências Exatas Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Londrina, Paraná, Brazil
[2]Companhia Iguaçu de Café Solúvel S.A, Research and Development Department, Cornélio Procópio-PR, Brazil
[3]Starbucks Coffee Company, Seattle, WA, US
[4]Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

ABSTRACT

Espresso, filter, and soluble coffee brews were prepared from arabica coffee. Folin-Ciocalteau, FRAP, ABTS, DPPH, and deoxyribose methods were used to measure the antioxidant activity. The bioactive compounds were measured using HPLC with diode array detection and GC-MS. The espresso brew had higher soluble solids content (3.41%) and differed from that in the soluble (2.66%) and filtered brews (2.04%). Considering the balance of the chemical composition in the brew and the volatiles, the composition of the filtered and espresso brews, which were prepared using the roasted coffee, were more similar to each other than the soluble brew, whose composition had been altered by the extraction process used in the soluble coffee production. The soluble and espresso brews were similar in melanoidin levels (25.82%; 26.89%, respectively). Lower concentrations of trigonelline, furfural, caffeine, 5-caffeoylquinic acid, maltol, guaiacols were detected in the soluble brew. All coffee brews had a similar capacity for the scavenging of

* Tel.: +55 43-33714270, Fax: +55 43-337140; E-mail address: javignoli@uel.br.
† Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Pr 445, Km 380, Campus Universitário, Cx. Postal 10011, 86057-970, Londrina, Paraná, Brazil. Email: martatb@uel.br.

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hydroxyl radicals by deoxyribose method (around 70%IA). The espresso and filtered brews presented however a greater antioxidant activity considering the Folin, FRAP, and ABTS results (30.0 g Trolox/100 g, 27,6 g Trolox/100 g, and 16,6 g gallic acid/100 g, respectively). Regarding the DPHH method, the espresso brew proved to be more efficient than the filtered and soluble brews, and the effect was attributed to concentration of the brew (high soluble solids concentration). The preparation method influenced the bioactive compounds extraction, however the production process strongly affected the composition and antioxidant activity of the coffee brews.

Keywords: espresso, filter, soluble, volatile compounds, caffeine, 5-caffeoylquinic acid

INTRODUCTION

The relationship between coffee and health has been extensively studied in recent years due to a strong interest in foods that promote physiological functions. Coffee is attractive for many reasons, including its antioxidant potential and pleasant flavor and aroma [1]. The two coffee species of the greatest economic importance are *Coffea arabica* and *Coffea canephora* var. robusta [2].

Seventy-five percent of soft drinks regularly consumed around the world are coffee-based; the brew types and consumption methods are strongly associated with different countries' social and cultural habits [3]. Consumption statistics for roasted and soluble coffee brews reveal significant variation among different markets. Canadians and Americans prefer roasted coffee by a large majority (95%), as do consumers in Western Europe, Africa, the Middle East, and Latin America (90%). In Eastern Europe and the Asia-Pacific, a smaller proportion of consumers (65% and 46%, respectively) prefer the roasted product. In contrast, consumers in the United Kingdom (90%) and Australasia (79%) prefer soluble coffee. Overall, soluble coffee is preferred in countries where tea is a traditional brew [4]. Regardless of the form of consumption, this brew is a source of antioxidants, particularly for consumers in countries such as Spain, Norway, and Italy [5]. Greater antioxidant activity (AA) has been found for soluble coffee and espresso than for red wine or green tea [5]. The antioxidant capacity of coffee is usually attributed to compounds such as caffeine, hydroxycinnamic acids, and products formed during the roasting process, such as melanoidins and volatile heterocyclic compounds (pyrroles, furans, and thiophenes) [6, 7].

The raw material and the process production, highlighting the roasting and extraction procedures (used only for soluble coffee) affects the final product's antioxidant activity. However, the literature points that the preparation procedure also plays an important role in the brew's AA. The compounds with antioxidant potential, such as caffeine [6], phenolic compounds [8, 9], or melanoidins [6, 10] are extracted in different amounts depending on the preparation method.

Coffee brews are often compared using products acquired from the market or under conditions that do not permit the control of all process parameters (e.g., raw material, roasting, and extraction procedures). Therefore, monitoring the preparation procedure of the brews does not ensure that the findings are uniquely attributable to this stage. This study investigated the influence of the preparation method on the AA of arabica coffee brews (i.e., filtered, espresso, and soluble) using FRAP (ferric reduced antioxidant power), ABTS (2,2'-Azino-bis(3 ethyl benzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl)

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Folin-Ciocalteau and Deoxyribose methods, relating with the chemical composition (volatile and non-volatile bioactive compounds) of the brews. The same samples (raw material) processed to prepare the roasted coffee brews (filtered and espresso) were extracted and lyophilized to produce the soluble coffee brew.

MATERIALS AND METHODS

Materials

Samples and Coffee Brew Preparation

The samples were provided by Companhia Iguaçu de Café Solúvel (Cornélio Procópio, Paraná State, Brazil) using a different raw materials and roasting degrees. *Coffea arabica* beans were roasted in a Raiar pilot roaster (São Paulo, SP, Brazil) until a medium roast degree (L* = 19.5). The same roasted beans were used for production of filter, espresso and soluble coffee brews.

For filter and espresso brews, roasted coffee was ground in the mill RA 337 (JR Araujo, São Paulo, SP, Brasil), for a particle size of approximately 1mm(16-mesh sieves).

For the production of soluble coffee product, the roasted coffee beans were granulated and fed into the extraction system. Pressure water heated to 180°C was fed into the first stage (the column containing the oldest coffee) following a sequence, percolating into the following stages, until reaching the newest coffee. The extract resulting from this process was lyophilized.

The filtered coffee brew was prepared as recommended by the Associação Brasileira da Indústria de Café (Brazilian Coffee Roasters Association, ABIC). Roasted and ground coffee (25 g) was placed in Mellita® filter paper and extracted with 250 mL of water at 90°C.

The espresso coffee brew was prepared as previously described [11]. Roasted and ground coffee (7.0 g) was loaded into a De-Longhi-BAR 41 (Italy) espresso machine; extraction was performed at 9 bar for 20 s. This procedure provided 50 mL of the brew with a dense layer of foam, in agreement with the aforementioned reference.

In the preparation of the soluble coffee brew, water at 90°C was used to dissolve 1.2 g of the lyophilized product to a 50 mL cup. This concentration was chosen based on the manufacturer's recommendation.

The preparation of the brews was performed in triplicate and they were used for the analysis of chemical composition and antioxidant activity.

Reagents and Equipment

The chromatographic grade compounds; caffeine, 5-caffeoylquinic acid (5-CQA), gallic acid, maltol, 2-furanmethyl acetate, 2,5-dimethyl pyrazine, 2,3-dimethyl pirazine, pyridine, 2,6-dimethyl-pyrazine, 4-ethyl-2-methoxy-phenol, 4-vinyl-2-methoxy-phenol, pyrazine, 2-methoxy-phenol (guaiacol), furfural, and hydroxymethylfurfural (HMF) were purchased from Sigma Aldrich (St Louis, USA). ABTS (2,2-azinobis-3 ethyl benzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma Chemical CO. (St Louis, USA). Trolox (6-hydroxy-2,5,7,8,-tetramethylchromane-2-carboxylic acid) and TPTZ (2,4,6-tri(2-pyridyl)-S-triazine) were obtained from Fluka/Sigma Aldrich (Vallensbaek

Strand, Denmark). Folin-Ciocalteau reagent, acetic acid (HPLC grade), and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Melanoidins were separated by dialysis using 12-14 kDa cutoff membranes (Spectra/Por, Irving, USA).

AA measurements were taken in a UV-Vis mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan) and bioactive compounds were determined by high performance liquid chromatography (HPLC). The HPLC apparatus consisted of a Dionex LC (Idstein, Germany) equipped with a P680 gradient pump, TCC-100 column oven, automated sample injector (model ASI-100) and photodiode array detector (model PDA-100). The system is operated by computer using Chromeleon version 6.6 software. Volatile compounds were identified using the system 6890N gas chromatograph coupled to a 5973 selective mass detector (Agilent, USA).

Antioxidant Activity Evaluation

ABTS Methodology (TEAC)

Antioxidant capacity was estimated in terms of radical scavenging activity using the procedure described by Vignoli et al. [12]. Briefly, ABTS radical cations ($ABTS^{+\bullet}$) were produced by reacting 7mM ABTS stock solution with 2.45mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16h before use. The $ABTS^{+\bullet}$ solution was diluted with 5mM phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730nm. After addition of 10μL of sample or trolox standard to 4mL of diluted $ABTS^{+\bullet}$ solution, absorbance were obtained after 6min using the UV–Vis spectrophotometer. Ethanol solutions containing known concentrations of trolox, a water-soluble analogue of vitamin E, were used for calibration. Results were expressed as g of trolox per 100 g of coffee (dry matter) and g de Trolox per cup 50mL.

FRAP Methodology

The reduction power was estimated according to the procedure described by Vignoli et al. [12]. The FRAP reagent was prepared by mixing 2.5mL of a 10mM TPTZ solution in 40mM HCl and 2.5mL of 20mM $FeCl_3 \cdot 6H_2O$ with 25mL 0.3mM acetate buffer pH 3.6. This solution was incubated at 37°C for 30min. Approximately 900μL of freshly prepared FRAP reagent, warmed at 37°C, was mixed with 90μL distilled water and either 10μL of test sample or standard or appropriate reagent blank to measure AA. After 30min at 37°C, absorbance at the maximum absorption wavelength (595nm) was taken. Ethanol solutions containing known trolox concentrations were used for calibration. Results were expressed as g of trolox per 100 g of coffee (dry matter) and g Trolox per cup of 50mL.

DPPH Methodology

The ability to scavenger the free radical DPPH was performed according to the procedure described by Vignoli et al. [12]. Briefly, solutions of coffee were prepared to obtain 2, 3, 4, 6, 10 and 15mg/mL. One millilitre of 100mM acetate buffer (pH 5.5), 1mL of ethanol and 0.5mL of 250μM ethanolic $DPPH^{\bullet}$ solution were mixed and 10μL of each sample of the above prepared solutions were added. After 10min, absorbance was measured at 517nm. A

positive control was prepared in the absence of coffee solutions, which indicates the maximum amount of free $DPPH^{\bullet}$ (considered 100% of free radicals in the solution), to calculate the hydrogen-donating ability (percent of inhibitory activity, IA%) of coffee. The blank was prepared from the reaction mixture without $DPPH^{\bullet}$ solution.

Folin–Ciocalteau Methodology

The reducing capacity of the coffee brews was determined using the Folin-Ciocalteau (FC) method adapted from Singleton et al. [13]. The sample (0.1mL) was diluted with deionised water to a volume of 7.5mL. Then, 300 µL of 0.9mol/L. Folin–Ciocalteau reagent and 1mL of 20% Na_2CO_3 solution were added and deionized water was added up to a final volume of 10mL. Solutions were maintained at room temperature for 60 min and total polyphenol content was determined at 765nm using the UV–Vis spectrophotometer. Gallic acid standard solutions were used for calibration. The results were expressed as g of gallic acid per 100g of coffee (dry matter) or g of gallic acid per a cup of 50mL.

Deoxyribose Assay

The scavenger activity of the coffee solutions, based on the inhibition of the deoxyribose degradation caused by the attack of hydroxyl radicals, was evaluated using the method of Daglia et al. [14]. In a final volume of 1.2 mL, the reaction mixture contained the following reagents at the final concentrations: $FeCl_3$ (25 µM) premixed with EDTA (100 µM) in KH_2PO_4/KOH buffer (pH 7.4), 2-deoxy-D-ribose (2.8 mM), H_2O_2 (2.8 mM), ascorbic acid (100 µM), and 12 µL coffee solutions (sample), or all the same volumes of KH_2PO_4/KOH buffer (control sample). Both samples were placed in a water bath at 37°C for 1 h and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid were added. The reaction mixtures were heated in a water bath at 80°C for 20 min, kept in ice for 5 min, and then centrifuged for 5 min at 3000 rpm to separate the particles. The absorbance of the samples' supernatant and of the control sample were obtained in the spectrophotometer at 532 nm against the relative solutions prepared as described but without ascorbic acid to correct for interference due to the brew color and thiobarbituric acid-reactive substances that might naturally occur in coffee solutions. The scavenger activity was expressed as IA % of deoxyribose degradation in the presence of the coffee brew (sample), relative to the control sample.

Determination of 5-CQA, Caffeine, Trigonelline, Furfural and HMF

A HPLC methodology adapted from Alves et al. [15] was used to simultaneous determination of caffeine, 5-CQA, trigonelline, furfural and HMF. After preparation of coffee brews, as previously described, the samples were filtered at 0.22 µm and directly injected into the chromatographic system. A 4.6 x 250 mm 5 µm particle Spherisorb ODS2 column (Waters, Taunton, USA) was used. Compounds were eluted with gradients containing 5% acetic acid (A) and acetonitrile (B), as follows: 0-5 min: 4% B; 5-10 min: 10% B; 10- 30 min: 10% B; 30-40 min: 0% B; 40-50 min: 4% B, at a flow rate of 0.7 mL/min. Trigonelline, caffeine, furfural and HMF were detected at 272 nm, while 5-CQA was detected at 320 nm. Quantification was carried out by external standardization using a 5-point calibration curve

with triplicate measurements. 5-CQA was evaluated over the range 5 to 30 µg/mL, caffeine from 10 to 50 µg/mL, trigonelline from 15 to 50µg/mL, hydroxymethyl-furfural from 0.5 to 8 µg/mL and furfural from 0.05 to 1 µg/mL. All results were expressed as g or mg of the bioactive compound per 100 g of soluble solids in the original coffee.

Volatile Compounds Determination

Volatile compounds usually studied for antioxidant activity (maltol, guaiacols, furfural, and HMF) were determined by a gas chromatograph and mass spectrometry detector (GC-MS). After preparation of the brews, aliquots of 10mL were transferred to vials, which were immediately sealed with silicone septa. The volatiles were extracted by SPME technique (Solid Phase Microextraction), where the fiber was exposed to the headspace of the sample at a constant temperature of 70°C. After 30 min of extraction, the fiber was inserted directly into the injector of the GC-MS. It used a polar capillary column HP - Innowax (60 m x 320 µm x 0.25 µm) (Agilent Technologies, USA). The injector operated in splitless mode with a constant temperature of 250 ºC. The helium carrier gas remained constant flow equal to 1.2 mL/min. The mass detector operated in the following conditions: ionization energy of 70 eV, interface temperature of 280°C, quadrupole temperature of 150°C, ion source temperature of 230°C. The data were analyzed using the MSD Chemstation software coupled with the library of mass spectra NIST/2002. The quantification was performed by external standardization. Analyses were performed in duplicate.

Determination of Melanoidins

The dialysis membrane separation process described by Bekedam et al. [16] was used with some modifications. The analyses were conducted in duplicate. The samples (soluble, filter and espresso brews) were transferred to a dialysis membrane with a cutoff of 12-14 KDa and placed in a beaker with 400 mL of distilled water under agitation. The water was changed every 8 h until it became colorless. The total volume of material retained by the membrane was determined and an aliquot was lyophilized. The amount of material with a molecular weight above 12-14 KDa, here considered to be melanoidin, was determined as a percentage of the original soluble solid mass. This fraction was expressed as g of melanoidins per 100 g of soluble solids in the brews.

Data Analysis

The results of antioxidant activity and composition of the brews were evaluated by one-way ANOVA, considering the preparation procedure as a source of variation, and compared by Tukey test ($p \leq 0.05$) in Statistica7.1[17].

RESULTS AND DISCUSSION

Chemical Composition and Antioxidant Activity

Regarding the preparation procedure, the amount of soluble solids in the espresso brew (3.41%) differed from that in the soluble (2.66%) and filtered brews (2.04%). The extraction yields for the espresso and filtered brews (24.0% and 20.4%, respectively) are within the ideal range. According to Lingle [18], coffee brews with extraction percentages below 16% are

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considered under extracted, while those with extraction percentages above 24% are considered over extracted. Although standard procedures were used to prepare each brew, consumers prepare brews in a variety of conditions.

Considering that different water/coffee proportions produce different yields, the calculations were based on the amount of soluble solids obtained by each procedure. The effects of the preparation procedures were thereby compared more directly in terms of each compound's extraction. The contents of bioactive compounds obtained by HPLC and GC (Table 1) are therefore presented with respect to the amount of soluble solids present (ng or g per 100 g of soluble solids).

Table 1. Contents of volatile and non-volatile bioactive compounds in the brews

		Filter	Soluble	Espresso
Non-volatile Compounds (g/100g soluble solids)	Trigoneline	2.34 ± 0.07^{a}	0.58 ± 0.01^{b}	2.32 ± 0.09^{a}
	Hidroximetilfurfural	0.02 ± 0.00^{b}	0.29 ± 0.00^{a}	0.02 ± 0.00^{b}
	Furfural	0.01 ± 0.00^{a}	0.00 ± 0.00^{b}	0.01 ± 0.00^{a}
	Caffeine	4.92 ± 0.09^{b}	2.65 ± 0.05^{c}	5.17 ± 0.10^{a}
	5-caffeoylquinic acid	1.96 ± 0.01^{a}	0.98 ± 0.02^{b}	2.01 ± 0.03^{a}
	Melanoidins	20.08 ± 0.54^{b}	25.82 ± 0.00^{a}	26.89 ± 0.20^{a}
Volatile Compounds (ng x 10^2/100g soluble solids)	Maltol	32.77 ± 7.03^{a}	18.85 ± 1.28^{b}	33.65 ± 1.90^{a}
	2-furanmethyl acetate	120.36 ± 12.79^{a}	0.26 ± 0.05^{b}	139.34 ± 20.05^{a}
	2,5-dimethyl pyrazine	8.70 ± 2.22^{a}	1.17 ± 0.16^{b}	9.16 ± 1.15^{a}
	2,3-dimethyl pirazine	2.48 ± 0.71^{a}	0.35 ± 0.04^{b}	2.78 ± 0.45^{a}
	Pyridine	18.79 ± 4.81^{a}	0.81 ± 0.12^{b}	24.75 ± 3.95^{a}
	2,6-dimethyl-pyrazine	6.74 ± 1.61^{a}	1.04 ± 0.18^{b}	7.04 ± 1.09^{a}
	4-ethyl-2-methoxy-phenol	19.28 ± 3.60^{a}	2.27 ± 0.33^{b}	25.01 ± 3.42^{a}
	4-vinyl-2-methoxy-phenol	100.70 ± 15.57^{b}	17.61 ± 2.07^{c}	139.85 ± 6.30^{a}
	Pyrazine	0.84 ± 0.14^{b}	0.00 ± 0.00^{c}	1.29 ± 0.34^{a}
	2-methoxy-phenol (guaiacol)	21.30 ± 2.52^{a}	4.70 ± 0.09^{b}	24.63 ± 4.34^{a}
	Furfural	38.47 ± 8.21^{b}	25.99 ± 1.95^{c}	51.51 ± 2.05^{a}
	Hidroximetilfurfural	2.83 ± 0.46^{a}	14.95 ± 1.37^{b}	0.99 ± 0.14^{c}

Mean of the three true replicates ± standard deviation; for melanoidins, two replicates. Different letters in the same row indicate a statistically significant difference at $p \leq 0.05$.

Considering the balance of the chemical composition in the brew and the volatiles, the composition of the filtered and espresso brews, which were prepared using the roasted coffee, were more similar to each other than the soluble brew, whose composition had been altered by the extraction process used in the soluble coffee production.

Regarding the brew composition, the filtered and espresso brews only differed in the greater extraction of caffeine and melanoidins, which was greater in the espresso preparation process. The contents of other compounds (trigonelline, 5-CQA, furfural, and HMF) did not differ between these two brews (Table 1).

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The soluble brew had the lowest concentration of caffeine and the greatest content of hidroximetilfural (HMF) (Table 1). Despite the possible degradation of the HMF in the roasting process [6, 19], the high extraction temperatures in the soluble coffee production led to the formation of the HMF present in the soluble brew.

Furfural, trigonelline, and 5-CQA were present in lower levels in the soluble brew (Table 1) than in the filtered and espresso brews prepared directly from roasted coffee. As has already been observed [6], the extraction process used in the production of soluble coffee was responsible for changes in 5-CQA content, which explains the differentiation of this brew. It is likely that other heat-sensible components have also exhibited the same behavior as chlorogenic acids, resulting in lower contents in the soluble brew.

With regard to the volatile fraction, HMF also had a greater concentration in soluble coffee. All of the volatile compounds in the soluble brew were present in lower concentrations than in the filtered and espresso brews, which generally had similar compositions of volatile components. Only 4-vinyl-2-methoxy-phenol had greater extraction in the espresso process (Table 1).

For melanoidins, compounds with high molecular weight, a lower concentration (20 g/100 g) was observed for the filtered brew in comparison to the others (approximately 26 g/100 g for the espresso and soluble brews) (Table 1). According to Leloup [20], the soluble coffee extraction procedure provides a greater release of these compounds due to the high temperatures and pressures used. It is likely that the same effect occurs in the preparation of the espresso brew (Table 1), in which the machine pressure could facilitate the release of melanoidins, achieving similar amounts to those found in soluble coffee.

López-Galilea et al. [3] compared the same product prepared by filtering and the espresso process and also reported greater amounts of caffeine and melanoidins for the espresso brew. However, they reported that the espresso displayed a greater concentration of 5-CQA and greater AA, neither of which was found in this study. These authors used a different water/solids ratio, which may explain the variation of the chemical composition and thus in the AA. Gloess et al. [21] investigated the influence of nine extraction methods in brewing coffee, and concluded that the different techniques used result indifferent chemical composition and sensory quality. In general, given that the results were expressed in soluble solids base, the chemical composition was influenced much more by the production process than by the preparation method. The espresso and filtered brews, prepared directly from the roasted and ground beans, preserved most of the thermolabile bioactive compounds for both the brew and the volatile fraction. The preparation process for espresso, however, allows for an even greater amount of compounds that could be favored by extraction under pressure such as melanoidins, which have high molecular weights, and 4-vinyl-2-methoxyphenol, which has a low solubility in water. The large difference exhibited by the soluble brew was attributed to the additional extraction stage that the soluble coffee undergoes. However, it should be stressed that the loss of thermolabile compounds also occurs in parallel to the increase of other bioactive compounds, such as melanoidins.

Table 2 presents the AA for the filter, espresso, and soluble brews. Given the lack of a universal method for measuring AA and the complexity of the raw material, various evaluation methods were used. For Folin, FRAP and ABTS methods, the AA was expressed as the concentration of the sample providing a better evaluation of the influence of preparation as the different brews are compared at the same scale. When the calculation was performed by cup, the solubilization influences the AA, masking the effect of the preparation

procedure. However, expressing AA this way allows the product to be evaluated as it would be consumed for the preparation conditions and the water/coffee proportion used here.

The greatest AA was found in a cup of espresso coffee, regardless of the evaluation method (Table 2). Due to the presence of bioactive compounds in a greater concentration, this brew was strongly favored in terms of the amount of antioxidants compounds in the cup. According to Svilaas et al. [22] the ingestion of 4 to 5 cups of (filtered) coffee per day is sufficient to supply nearly 64% of the total amount of antioxidants necessary in the diet.

Less variation was found among the brews, independent of the AA methodology applied, when the AA was expressed by concentration. Lower Folin, FRAP, and ABTS values were observed for the soluble brew than for the filtered and espresso brews (Table 2).

Table 2. Antioxidant activity of the soluble, filter and espresso brews evaluated by different methods

	Coffee brews		
Assays	Filter	Soluble	Espresso
ABTS			
g Trolox/100g	29.88 ± 2.53 [a]	24.10 ± 0.81 [b]	32.13 ± 1.41 [a]
g Trolox/cup	0.32 ± 0.04 [b]	0.29 ± 0.01 [b]	0.55 ± 0.00 [a]
FRAP			
g Trolox/100g	27.58 ± 1.09 [a]	22.60 ± 0.91 [b]	27.52 ± 1.30 [a]
g Trolox/cup	0.30 ± 0.04 [b]	0.27 ± 0.01 [b]	0.47 ± 0.01 [a]
Folin			
g gallic acid/100g	16.28 ± 0.41 [a]	13.45 ± 0.64 [b]	16.85 ± 0.51 [a]
g gallic acid/cup	0.17 ± 0.01 [b]	0.16 ± 0.01 [c]	0.29 ± 0.01 [a]
DPPH			
(% IA)	59.56 ± 3.11 [b]	53.76 ± 1.22 [b]	73.03 ± 3.10 [a]
Deoxyribose			
(% IA)	69.63 ± 0.38 [a]	72.35 ± 4.08 [a]	71.09 ± 2.24 [a]

Different letters in the same row indicate a statistically significant difference at $p \leq 0.05$. *IA DPPH: Inhibition of the activity of the free radical 2,2-diphenyl-1-picrylhydrazyl. **IA Deoxyribose: inhibition of activity of the hydroxyl radical. Cup = 50 mL.

Sacchetti et al. [23] evaluated the effect of the roasting degree on the radical scavenging activity of ABTS by the roasted filtered brew. Medium-roasted brews demonstrated increased scavenging activity of the radicals in comparison to green coffee due to an increase in the AA of the non-phenolic fraction. However, when dark-roasted brews were evaluated, this activity was reduced due to the loss of the phenolic fraction, which did not have its AA balanced by the increase of the AA of the non-phenolic fraction. This finding demonstrates the importance of the phenols in coffee AA. Perrone et al. [24] estimated that the contribution of free chlorogenic acids to the AA of coffee brews was higher than that of melanoidin-bound phenolic compounds. The latter contributed with 36%, and 47% of the AA of the brews measured by TEAC and FRAP, respectively.

It is interesting that the greater concentration of solids present in the espresso cup in comparison to the filtered coffee was not sufficient to cause an increase in the AA,

particularly in the FRAP and ABTS assays. Therefore, the concentration of the bioactive compounds of the filtered brew was sufficient to ensure the same AA as the espresso brew.

When AA was measured by the deoxyribose method, no difference was found among the brews. This method evaluates the scavenge of the hydroxyl radical (•OH), which is extremely reactive, may be produced under the physiological conditions of the human body and can react with proteins, DNA, unsaturated fatty acids, and most biological membranes [25]. It was possible to conclude that coffee possesses agents that scavenge this radical and that the soluble, espresso, and filtered coffee brews are all equally efficient in this function, despite having different compositions (Table 2).

Among the chemical compounds, caffeine has stood out as a potential scavenging agent of •OH radicals [26-27]. The caffeine concentrations in coffee brews are quite variable, but it is commonly assumed that one cup of filtered coffee provides approximately 100 mg of caffeine. Furthermore, it is thought that moderate consumption of coffee, equivalent to 3-4 cups/day, provides health benefits, providing 300-400 mg/day of caffeine [28-29]. Although espresso coffee and filtered coffee contain greater amounts of caffeine, these brews did not differ in terms of the degradation of deoxyribose, suggesting that other brew components are also involved in this function, as has been demonstrated for the phenolic and melanoidin compounds in coffee fractions [14, 24, 30]. In addition, the scavenging capacity against •OH was positively correlated to the content of browned compounds [31].

With regard to the inhibition of the activity of the DPHH radical, the espresso brew proved to be more efficient than the filtered and soluble brews (Table 2). It is likely that this effect can be attributed to the concentration of the brew, considering that filtered and soluble coffee have similar concentrations of soluble solids.

In general, the manufacturing process of soluble coffee, specifically the percolation stage, seems to cause a decrease in the antioxidant capacity measured by some methods (Folin, FRAP, and ABTS). For the filter and espresso brews, which were made from the same raw material, less difference in the AA were observed (Table 2).

However, it should be stressed that the soluble brew was prepared from *Coffea arabica*, with a medium-roasting degree, to avoid variation between the brews beyond the preparation method. This standard is quite common for filtered and espresso brews but not for soluble coffee, which is commonly produced from *Coffea canephora* species and with a darker-roasting degree. The preparation with the most common method would probably lead to different results, given that robusta coffee has greater AA than arabica coffee and that intense roasting process may lead to greater production of melanoidins, ultimately leading to an increase in the AA up until a certain point [9].

A comparison with the literature is difficult due to the variability in preparation and raw material, in addition to the method selected for evaluation, with a great divergence of the AA of the brews.

Parras et al. [25] evaluated the antioxidant capacity of espresso and filtered coffee brews against the lipoperoxyl and •OH radicals (deoxyribose) and did not observe a difference among the brews. Considering the capacity to react with H_2O_2, the filtered coffee was more efficient than espresso. In the same study, the protection of these brews was also tested with using Rancimat method, with the espresso brew providing greater protection than the filtered brew.

Results that differ from those of the present study were described by Sánchez-González et al. [8], who compared the AA of espresso, Italian, filtered, and soluble lyophilized brews

using the FRAP and ABTS methods. The authors found that the AA of the soluble brew was greater than that of the others, which did not differ from one another. However, the data do not consider the extraction yields of the brews.

CONCLUSION

The coffee brews displayed significant antioxidant activity, AA, which stands out for the large capacity for scavenging of the •OH radical, independent of the preparation method of the brews. Considering the balance of the chemical composition in the brew and the volatiles and the AA, the filtered and espresso brews, which were prepared from roasted coffee, had the most similar results. In contrast, the composition of the soluble brew was affected by the extraction process employed in production of the soluble product. The manufacturing process was mostly responsible for the AA of the brews and the preparation method was directly involved in the extraction of the bioactive compounds, which might favor the AA of the espresso brew in some situations due to the greater preservation of the volatile compounds and higher concentration of solids normally found in this brew.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundo de Apoio ao Ensino, à Pesquisa e à Extensão (FAEP/UEL).

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BIOGRAPHICAL SKETCHES

Josiane Alessandra Vignoli

Name: Josiane Alessandra Vignoli

Affiliation:
Universidade Estadual de Londrina (UEL) – Adjunct professor

Date of Birth:
December/01/1977

Education:
PhD and MSc in Food Science, BSc in Biological Science

Address:
Departamento de Bioquímica e Biotecnologia/Centro de Ciências Exatas/Universidade Estadual de Londrina - 86057970 - Londrina, PR - Brasil
Phone number: +55 (43) 33715471; Fax number: +55 (43) 33714080
URL: http://www.uel.br/cce/bioquimica
Contact e-mail: javignoli@uel.br; alternative e-mail: josivignoli@ yahoo.com.br

Research and Professional Experience:

Experience in Food Science and Technology, focusing on Chemistry and Biochemistry of Foods, acting mainly on the following subjects: coffee, antioxidant activity, HPLC.

Professor: Universidade Estadual de Londrina (2012 – current).

Professional Appointments:

Companhia Iguaçu de Café Solúvel S.A. (C. Procopio, BR) 2002...2012
Marubeni Corporation group (Tokyo-Japan)
Field: Production of soluble coffee
Positions: Development Analyst and Researcher
Areas: Basic and Applied Research and Product Development
Current Academic Advisories: 1 Master´s thesis
Scientific Journal Referee: Food Research International; Food Chemistry; Bioresource Technology; Journal of Pharmaceutical and Biomedical Analysis; International Research Journal of Microbiology.

Publications Last 3 Years:

Articles:

Martinez, Renata M., Pinho-Ribeiro, Felipe A., Steffen, Vinicius S., Caviglione, Carla V., **Vignoli, Josiane A.**, Baracat, Marcela M., Georgetti, Sandra R., Verri, Waldiceu A., Casagrande, Rubia. Hesperidin methyl chalcone inhibits oxidative stress and inflammation in a mouse model of ultraviolet B irradiation-induced skin damage. Journal of Photochemistry and Photobiology. B, Biology, v.148, p.145 - 153, 2015.

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Patents:

Santos, D. A., Silva, P. B., **Vignoli, J. A.**, Celligoi, M.A.P.C. Inventors; Universidade Estadual de Londrina (UEL), assignee. Obtenção Biotecnologica de Frutooligossacarídeos (FOS): Otimização de processos e aplicação em alimentos (Biotechnological Production of Fructooligosaccharides (FOS): Optimization of process and food application). Patent 2014: BR1020140088270.

Verri, W. A., Baracat, M. M., Medeiros, D. C., Casagrande, R., Georgetti, S. R., Freitas, O, Mizokami, S., **Vignoli, J.A.**, Celligoi, M.A.P.C. Inventors; Universidade Estadual de Londrina (UEL), assignee. Sistema microencapsulado contendo D-frutose-1,6 difosfato para melhora da eficácia analgésica e anti-inflamatória do fármaco (Microencapsulated system containing D-fructose-1,6 to improvement of analgesia and anti-inflammatory efficacy). Patent 2013: BR 1020130321524.

Marcelo Caldeira Viegas

Name: Marcelo Caldeira Viegas

Affiliation: Café Iguaçu

Date of Birth: 03/January/1972

Education: Chemical Engineer

Address: BR 369 (Rodovia Melo Peixoto), Km 88, Cornélio Procópio-PR, CEP 86300-000

Research and Professional Experience:

Experience in Chemical- and Biotechnological-Engineering Processes and Food Analysis with emphasis in the following topics: fluidized bed reactor; continuous fermentation of flocculent yeasts; ethanol production; simulation, optimization, statistical control and quality management of processes; experimental design and response surface analysis; development and validation of analytical methods [e.g., High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Flame Ionization (GC-FID)]; and quantification of aroma of instant coffee.

Publications Last 3 Years:

Weschenfelder, Thiago André; Lantin, Pedro; Viegas, Marcelo Caldeira; De Castilhos, Fernanda; Scheer, Agnes De Paula. Concentration of aroma compounds from an industrial solution of soluble coffee by pervaporation process. Journal of Food Engineering, v. 159, p. 57-65, 2015.

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Denisley Gentil Bassoli

Name: Denisley Gentil Bassoli

Affiliation: Starbucks Coffee Corporation (Seattle, WA

Date of Birth: April/19/1958

Education: PhD and MSc in Food Science, BSc in Chemical Engineering

Address: 6619 NE 1st street, Renton/WA, USA

Research and Professional Experience:

Denisley Bassoli, R and D Manager at Starbucks Coffee – Seattle - and formerly Quality Director at Café Iguaçu – Brazil - is an expert on the coffee field, with previous experience on the paint/varnish, glue and petrochemical industries.

Bachelor in Chemical Engineering post graduated on Food Science, respectively on green coffee quality (M.Sc.) and soluble coffee aroma (D.Sc.), he has been member of the international ISO committee for coffee (SC15-TC34) for more than fifteen years as Brazilian Delegate, presently an ANSI delegate, having participated and lectured on important seminars (ASIC, IAC, SBCTA meetings) as well as on universities and other academic and technical events worldwide. To date, on the Academia he has over 35 scientific publications and 240 citations on his articles, recently authoring a book chapter on soluble coffee.

He has developed his career in coffee since 1981, managing areas with over 70 team players on Quality Assurance and Quality control (raw materials, roasted and soluble coffees and packaging), Technical Assistance, Basic and Applied Research, Product Innovation and Development. For over 25 years tailoring products for specific markets/applications and defining products and packaging portfolio, he administered technical matters with B2B clients and suppliers accumulating vast international experience, constantly keeping a network of worldwide contacts.

He mastered the creation and implementation of the Research and Development area at Cafe Iguaçu, including its exclusive pilot plant and Research laboratory, heading among other developments a unique competitive technology generating annual savings of more than $12,5 million (yet at semi-industrial scale).

Managing multi-task international projects (Spain, UK, Romania, Germany) including budgets above US$ 90 million, he has managed areas including doctors, masters, experts, researchers and technicians (food, chemical, mechanic, electric, electronic, civil, safety and medical fields). He has as well validated suppliers (food services, packaging, plants) both nation and worldwide – Brazil, South Korea, Bulgaria, Argentina, Spain. Fully skilled on areas such as Technical Projects management and implementation, Customer Attendance and Satisfaction, Quality Assurance, Quality Control, Basic and Applied Research, Product and Packaging Development.

At Starbucks, he has been engaged in the Global Product and Process Solution Group, working on projects for qualifying plants, developing projects understanding innovation, optimization and development on coffee, teas, beverages, juices and waste/by-products.

Professional Appointments:

Starbucks Coffee Corporation (Seattle, WA), 2014...
- Field: Coffee, tea, juices, beverages, waste/by-products optimization
- Position: Research and Development - Technical Manager
- Area: Research and Development - Innovation

Companhia Iguaçu de Café Solúvel S.A. (C. Procopio, BR) 1981...2014

Marubeni Corporation group (Tokyo-Japan)
Field: Production of soluble coffee
Positions: Engineer, Section Head, Manager, Director
Areas: Technical Production, Project Management, Sales Engineering, Basic and Applied Research, Product and Process Development, Innovation, Quality Control and Assurance, Technical Assistance, Product Engineering, Market Intelligence.

Tintura S.A. – Industria de Tintas S.A. (Curitiba, BR) 1980...1981
PPG industries (Woodinville-WA)
Field: Manufacturing of resins, paints and varnishes
Positions – Trainee Engineer, Chemical Engineer
Areas: Quality Control, Research and Development

Honors:

National committees
ABICS - Technical representative of Café Iguaçu
ABICS - Member of the technical national committee
ABNT – ISO technical committee delegate
ANSI – ISO technical committee delegate

International committees
ISO - Technical delegate of United States of America at the ISO TC 34/SC 15
ISO – Technical delegate of Brazil at the ISO TC 34/SC 15

Academic records
- Universidade Estadual de Londrina
  Title: Doctor in Food Science – D.Sc. – 2006
  Thesis: Aromatic impact of the soluble coffee volatile components: an analytical and sensorial analysis.
- Universidade Estadual de Londrina
  Title: Master in Food Science – M.Sc. - 1990
  Dissertation: Quality evaluation of Brazilian green coffees: a multivariate analysis.
- Universidade Federal do Paraná
  Title: Chemical Engineer - C.E. - 1980

Publications Last 3 Years:

1. Teixeira, A.A., Teixeira, A. A. R., Reis, M., Bassoli, D. G., Palácios, H. A. Drying and moisture level influence in both sensory and physical quality of coffee. 40º Congresso Brasileiro de Pesquisas Cafeeiras, Serra Negra, Oct 2014.
2. Vignoli, J. A., Viegas, M. C., Bassoli, D. G., Benassi, M. T. Roasting process affects differently the bioactive compounds and the antioxidant activity of arabica and robusta coffees. Food Research International, 2013.
3. Bona, E.; Da Silva, R. S. S. F.; Borsato, D.; Bassoli, D. G. Self-organizing maps as a Chemometric tool for aromatic pattern recognition of soluble coffee. Acta Scientiarum. Technology, Maringá, v. 34, n. 1, p. 111-119, Jan.-Mar., 2012.

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Marta de Toledo Benassi

Name: Marta de Toledo Benassi

Affiliation: Universidade Estadual de Londrina (UEL) – Associate professor

Date of Birth: 10/11/1963

Education: Degree in Food Engineering (Universidade Estadual de Campinas,1985), Msc in Food Science (Universidade Estadual de Campinas, 1990) and Ph.D. in Food Science (Universidade Estadual de Campinas, 1997).

Address: Departamento de Ciência e Tecnologia de Alimentos/Centro de Ciências Agrárias/Universidade Estadual de Londrina
86057970 - Londrina, PR - Brasil
Phone number: +55 (43) 33715970; Fax number: +55 (43) 33714080
URL: http://www.uel.br/cca/dcta
Contact e-mail: martatb@uel.br; alternative e-mail: marta.benassi@ gmail.com

Research and Professional Experience:

Experience in Food Science and Technology, focusing on Chemistry and Biochemistry of Foods, acting mainly on the following subjects: coffee, antioxidant activity, HPLC, sensory analysis.

Professor: Universidade Estadual de Londrina (1998 – current), Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP (1991 – 1997).

Management and Administrative Positions (UEL): Deputy Head of Food Science and Technology Department (2006-2008, 2008-2010), Coordinator of Food Science Graduate Program (MS and PhD) (2013-current)

Academic Advisories concluded: 15 Master´s thesis, 4 Ph.D. thesis, 2 Post-doctoral supervisions.

Completed Research grants (last 10 years):

As project leader: 3 projects supported by the Brazilian National Council for Scientific and Technological Development (CNPq), 4 projects supported by Universidade Estadual de Londrina, 1 project supported by the Brazilian Agricultural Research Corporation (EMBRAPA)

As project team member: 4 projects supported by the Brazilian National Council for Scientific and Technological Development (CNPq), 1 project supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), 1 project supported by the São Paulo Research Foundation (FAPESP).

Scientific productivity grants funded by the Paraná State Funding Agency/Fundação Araucária (2009-2010) and funded by the Brazilian National Council for Scientific and Technological Development (CNPq) (2011-2013, 2014-current).

Member of Journal Editorial Board: Semina. Ciências Agrárias (2011-2014)

Bibliographic Production: 77 articles published in scientific journal; 2 book chapters; 165 articles published in events proceedings

Citation index (nov 2015):

Google Scholar (since 2010): 802 citations, h-index 15

ISI: 380 citations, h-index 10

Address to access CV: http://lattes.cnpq.br/7409756675845441

Research ID F-7213-2012

Professional Appointments:

Current Academic Advisories: 2 Master´s thesis, 2 Ph.D. thesis, 2 Post-doctoral supervisions.

Scientific Journal Referee: Journal of Sensory Studies; Food Research International; Journal of Food Composition and Analysis; Journal of Agricultural and Food Chemistry; Food Chemistry; Lebensmittel-Wissenschaft + Technologie

Ongoing Research grants:

As project leader: Assessment of composition, sensory characteristics and cup quality of *Coffea canephora* brews (2014-2017), supported by the Brazilian National Council for Scientific and Technological Development (CNPq)

As project team member: Brazilian pine (*Araucaria angustifolia*): assessment Brazilian pine: assessment of potential in the feed and in the development of new products (2012-2016), supported by the Brazilian Agricultural Research Corporation (EMBRAPA)

Scientific productivity grants funded by the Brazilian National Council for Scientific and Technological Development (CNPq).

Publications Last 3 Years:

Articles:

Sousa, J. M., Souza, E., Marques, G., **Benassi, M. T.**, Gullón, B., Pintado, M. M., Magnani, M. Sugar profile, physicochemical and sensory aspects of monofloral honeys produced by different stingless bee species in Brazilian semi-arid region. Lebensmittel-Wissenschaft + Technologie/Food Science + Technology, v.65, p.645 - 651, 2016. (http://dx.doi.org/10. 1016/j.lwt.2015.08.058)

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International Journal of Food Science and Technology, v.50, p.1413 - 1420, 2015. (http://dx.doi.org/10.1111/ijfs.12791)

Kobayashi, M. L., **Benassi, M. T.** Impact of packaging characteristics on consumer purchase intention: Instant coffee in refill packs and glass jars. Journal of Sensory Studies, v.30, p.1 - 12, 2015. (http://dx.doi.org/10.1111/joss.12142)

Rezende, N.V., **Benassi, Marta T.**, Vissotto, F.Z., Augusto, P.C., Grossmann, M. V. E. Mixture design applied for the partial replacement of fat with fibre in sucrose-free chocolates. Lebensmittel-Wissenschaft + Technologie/Food Science + Technology, v.62, p.598 - 604, 2015. (http://dx.doi.org/10.1016/j.lwt.2014.08.047)

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Reviewed by Dr Rafael Carlos Eloy Dias - Instituto Federal Catarinense, Joinville, Santa Catarina, Brazil.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 3

CONTROL OF COFFEE SAMPLES QUALITY: GEOGRAPHIC AND ROASTING FACTORS

Paulo R. A. B. de Toledo[1], Marcelo M. R. de Melo[2], Helena R. Pezza[1], Aline T. Toci[3], Leonardo Pezza[1] and Carlos M. Silva[2,*]

[1]Institute of Chemistry, São Paulo State University – UNESP, Araraquara, SP, Brazil
[2]CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal
[3]Latin American Institute of Science of Life and Nature, Federal University of Latin American Integration – UNILA, Foz do Iguaçú, PR, Brazil

ABSTRACT

The quality control of coffee and related products needs to consider several factors that are known to play important roles in the final organoleptic characteristics perceived by the consumers. The aroma exhibited by a coffee is determined by variables such as climate, soil, coffee species/variety, post-havest processing and the quality of the beans, as well as by the storage and roasting conditions. For instance, different degrees of roasting give rise to drinks with varying aromatic profiles, ranging from coffees with aromas rich in volatile acids and furans (responsible for fresh and floral notes), to coffees with aromas rich in compounds like pyrazines and pyridine (responsible for characteristic roasted and earthy notes). The geographic provenance of the coffee is also associated with characteristic compositional compounds, which can be used as markers to confirm or disclose the origin of a given sample.

This chapter focuses on two of these factors, namely the geographic origin (associated with climate and soil) and the roasting procedure, and aims at providing a systematic assessment of the chemical compounds that are mainly responsible for the quality of coffee samples. In this respect, multivariate statistical analysis can be employed with advantage to build discriminant models able to differentiate coffee

* Corresponding Author address: carlos.manuel@ua.pt.

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samples, as well as to identify the key chemical compounds suitable for such differentiation. Although more than 800 compounds have already been identified in coffee samples, the discriminant models can rely on only a few markers such as aldehydes, pyrazines, pyrroles, and furans. An evaluation is also made of the link between key compounds and the organoleptic characteristics perceived by the consumer. This approach provides reliable means of complementing typical quality control procedures that rely on sensorial evaluation (cup tests), because not all the odorant compounds present are useful for differentiating geographical origins and degrees of roasting, while some non-organoleptically active molecules are statistically decisive for an enhanced discrimination.

Keywords: aroma, coffee quality, discriminant analysis, geographic origin, multivariate statistics, roasting, volatile markers

1. INTRODUCTION

The subject of coffee quality is far from being a short-range challenge due to the wide scope it can encompass. To better address this issue, one may consider the official definition of quality provided by the International Organization for Standardization (ISO), saying it is "*the extent to which a group of intrinsic features (physical, sensorial, behavioral, temporal, ergonomic, functional, etc.) satisfies the requirements, where requirement means need or expectation, which may be explicit, generally implicit, or binding*" (NBR ISO 9001/2000).

If analyzed from a final consumer perspective, coffee quality is too linked to the experience of tasting this product under the form of a drink, containing water-soluble extractives, some of them with pronounced volatility and organolepsy. Hence, for these consumers, the quality of coffee is mostly played at the level of tasting and smelling the drink, explaining why the most widespread and perhaps pragmatic method to assess coffee quality is through sensorial experience panels. It is known that the concentration of aromatic compounds in roasted coffee can reach 1 g kg^{-1} (Flament and Bessière-Thomas, 2002; Toci and Farah, 2014), and their characterization has been extensively studied over the years.

From the perspective of traders, producers and intermediary entities, it is vital to control and actively optimize coffee samples' quality for the success of their activities. For this reason, such evaluation can benefit from going beyond sensorial tests, and may include also analytical/statistical methods based on measured physical and/or chemical indicators of these samples. In fact, the aroma of coffee is intrinsically related to the chemical composition of the beans and consequently its quality is directly affected by a wide range of parameters such as the plant species and variety, climate, soil, bean quality, post-harvest processing, type of roast, and storage parameters.

Owing to the acknowledged importance of keeping track of privileged characteristics of coffee samples' quality seals can be used for features such as geographic origin or processing methods. For instance, within the European Union, the certification known as "Protected Designation of Origin" (PDO) is devoted to identify territorial provenances that provide enhanced quality of given food products. Another important certification on quality control is the one known as "Protected Geographical Indication" (PGI), normally attributed to farms and/or food products whose quality, reputation or other characteristics are directly dependent

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on their origin. This obliges that the production, transformation and/or processing take place in a certain territorial area in order to maintain a given quality standard (Illy, 2005).

It is in this context that the current chapter addresses the topic of coffee quality control, with special emphasis on statistical methods that can be applied to chemical composition data in order to identify chemical markers for geographical origin and/or roasting degrees, with a particular emphasis on Discriminant Analysis. It is evidenced how this technique may be adopted for certifying the quality of coffee samples in complement to classical sensorial tests.

2. MULTIVARIATE STATISTICS TO MODEL COFFEE QUALITY

Multivariate statistics encompass a set a powerful tools that not only may consider the individual direct impact of the volatile chemical compounds present in coffee, but also takes into account eventual correlations between them. Two of the most popular multivariate analysis methods are Principal Component Analysis (PCA) and Discriminant Analysis (DA), but Partial Least Squares (PLS) regression may also be cited in this respect.

One particularity of PCA relies on the fact it is a fully automated method that identifies by itself the principal components without human specification of the groups (components) that should be sorted. In this sense, PCA is rather suitable for the analysis of multidimensional data where crossed correlation (redundancy) may be present (Jackson, 1991) and in cases where no grouping suggestion can be anticipated by the researcher. On the other hand, PLS can be used with advantage for complex systems such as coffee, particularly when seeking the construction of predictive models. However, this method is not devoted to the study of the underlying relationship between the variables. As a result PLS is of short suitability for screening out factors that have a negligible effect on the response (Tobias, 1995).

Examples of application of these statistical methods to coffee samples are listed in Table 1, where it can be perceived they have been adopted to achieve different goals: distinction of coffee samples of difference species/varieties (Zambonin et al., 2005; Korhoňová et al., 2009; Cheong et al., 2013; Özdestan et al., 2013), distinction of coffee samples with different geographic origins (Murota, 1992; Mayer et al., 1999; Freitas and Mosca, 2000; Costa Freitas et al., 2001; Akiyama et al., 2008; Risticevic et al., 2008; Fisk et al., 2012), distinction between defective and non defective coffee seeds (Agresti et al., 2008), influence of post-harvest processing (Gonzalez-Rios et al., 2007), evaluation of climatic effects on aromatic quality of roasted coffees (Bertrand et al., 2012), characterization of roasted coffees and coffee beverages (Bicchi et al., 1997; Maeztu et al., 2001), influence of roasting process and brewing procedure on coffee beverages (Lopéz-Galileia et al., 2006) and by roast degree (Akiyama et al., 2008).

Since for the purpose of quality control a deliberated grouping of samples may be sought (e.g., sorting by specific geographic regions and/or countries), DA has the advantage of simplifying the amount of data needed to answer that goal. This method is also able to reduce data redundancy (McLachlan, 2004) but, in the DA approach, the transformation process is said to be human guided and class dependent, which provides models that best matches the grouping expectations of the user.

Table 1. Scientific works using multivariate statistics to differentiate coffee samples

Differentiation target	Statistical method	Analytical Method	Reference
Aroma	PCA	HS-SMPE-GC-MS	Gonzalez-Rios (2007)
Aroma of expresso coffee	PCA	HS-GC-MS	Maeztu et al., (2001)
Asian coffee varieties	PCA	GC–MS/FID, HPLC	Cheong et al., (2013)
Climatic effects	PCA	HS-SMPE-GC-MS	Bertrand et al., (2012)
Defective seeds	PCA	HS-SMPE-GC-MS	Agresti et al., (2008)
Geographic origin	PCA	ICP- AES	Costa et al., (2010)
Geographic origin	PCA	Electronic sensor array/SPME-GC)	Costa Freitas et al., (2001)
Geographic origin	PCA, CA	P and T-GC	Costa Freitas et al., (2000)
Geographic origin	PCA	SPME ToF, MASE GC-MS	Risticevic et al., (2008) Fisk et al., (2012);
Geographic origin and cultivars	DA	HS GC-MS	Murota (1992)
Geographic origin and roast degree	PCA, DA	HS GC-MS	Akiyama et al., (2008)
Roasted coffees and coffee beverages	PCA	HS-SMPE, LS-SPME-GC-MS	Bicchi et al., (1997)
Roasting process and brewing procedure	PCA	HS-SMPE-GC-MS	Lopéz-Galileia (2006)
Specialty coffee varieties	PLS, DA	HS PTR-MS	Özdestan et al., (2013)
Species	PCA,CA	HS-SMPE-GC-MS	Korhonova et al., (2009), Zambonin et al., (2005)

AES = Atomic Emission Spectrometry, Cluster Analysis, CA = Cluster Analysis, DA = Discriminant Analysis, FID = Flame Ionization Detector, GC = Gas Chromatography, HS = Headspace, MS = Mass Spectrometry, HPLC = High-Performance Liquid Chromatography, ICP = Inductively Coupled Plasma, LS = liquid sampling, MASE = Membrane-Assisted Solvent Extraction, PCA = Principal Component Analysis, PLS = Partial Least Squares, PTR = Proton Transfer Reaction, P and T = Purge and Trap, SPME = Solid Phase Microextraction, ToF = Time-of-flight.

For the purpose of achieving a classification based on the chemical composition of coffee samples, DA models comprise linear equations combining the statistically relevant compounds (factors) unveiled through comparison of experimental data, as follows:

$$Y = \beta_0 + \beta_1 V_1 + \beta_2 V_2 + \beta_3 V_3 + \cdots + \beta_n V_n \quad (1)$$

where Y is the discriminant function, $\beta_0, \beta_1, \ldots, \beta_n$ are the linear discriminant coefficients, and $V_1, V_2, \ldots, V_n$ are the corresponding abundance ratios of the volatiles. These ratios imply that one of the volatile compounds is chosen as standard, and Toledo et al., (2016a) has shown that pyridine is particularly appropriate to be used as normalizing peak for the remaining volatile compounds.

In the following section, the application of DA approach to discriminate coffee samples regarding their geographical provenance and roasting degree is presented.

3. Discriminant Analysis as a Tool to Model Coffee Quality

3.1. Geographic Origin Models

Toledo et al. (2016a) applied DA approach to chromatographic results of 25 coffee samples from Central America, South America, Africa and Asia (see Figure 1), all submitted to Medium Roasting degree, with the aim of identifying the most relevant compounds to judge and confirm such origins, enabling thus a quality control procedure. In addition, a special DA was performed for Brazilian samples, which is justified in view of the fact this country holds the lion's share in terms of world production of this raw material.

The DA involved a database containing 73 compounds with the objective of identifying the best ones for a clear differentiation of samples origin, but at least for one of the samples only 17 compounds were reported in the original bibliographic source. In the whole, three discriminant functions relying only on 18 chemicals were proposed, being able to explain 100% of the variance through a tri-dimensional assignment of the data. These are identified in Table 2 together with the coefficients (β_i) of each discriminant function: Y_1, Y_2 and Y_3.

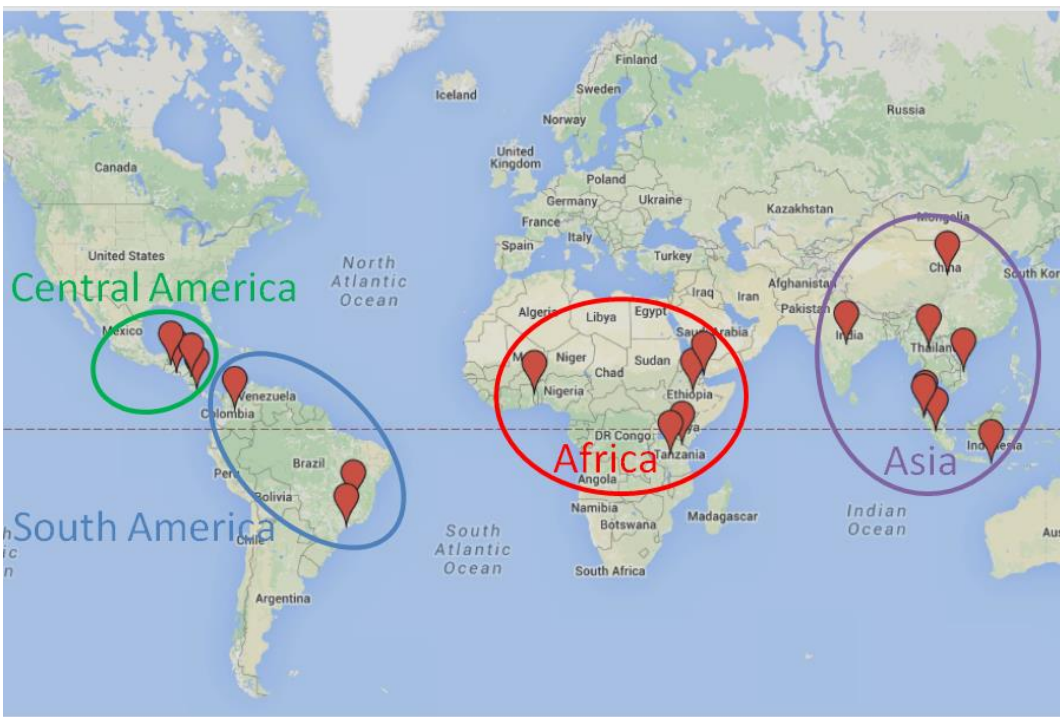

Figure 1. Geographic regions (colored circles) used for grouping coffee samples data (red icons) in the Discriminant Analysis (Toledo et al., 2016a).

The discrimination of coffee samples according to their geographical origin is graphed in Figure 2 using only Y_1 and Y_2, which jointly explain 97.3% of the data variance. For each geographical group considered, the centroids are plotted with filled marks while data points are graphed in their individual coordinates (non shaded symbols) and connected with a line to

the respective group centroid. To emphasize the areas belonging to the different groups, colored circles were drawn around each geographic group.

As far as the differentiation is concerned, Asia clearly outcasts from the remaining ones, while Africa, Central America, and South America share close positions in the graph, albeit without overlapping in this bi-dimensional representation. Nonetheless, while Y_1 function is enough to distinguish Asian coffee samples, the combination of Y_1 and Y_2 is necessary to clearly differentiate the two American classes and Africa.

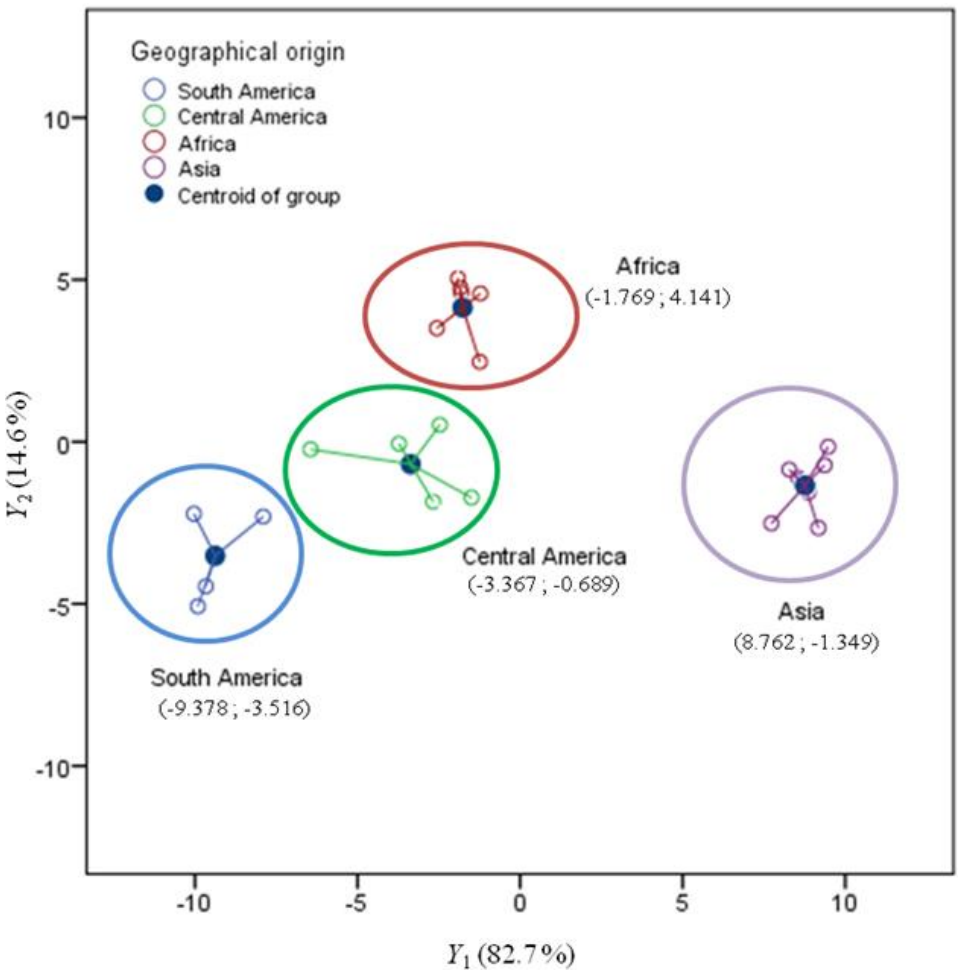

Figure 2. Discriminant scores and centroid values of DA for the "Four Geographic Regions." (Toledo et al., 2016a).

The dispersion of data points within a given group is a key parameter for assessing the performance of the differentiation achieved through DA. In this respect, attention should be paid to the Central America category, where the highest relative dispersion between samples was observed (see Figure 2), with the highest distance to the centroid being provided by a sample from El Salvador. With regard to the dispersion within Africa group, it is worth noting the two samples with greater deviation to the centroid belong to Togo and Yemen. With relation to Togo coffee, two aspects should be underlined: it is the unique sample that belongs

to the Atlantic coast of Africa (see Figure 1), and also it is one of Robusta coffee that was intentionally included in that study for evaluating if different coffee species could significantly disturb the model. The dispersion observed inside the Asia group (see Figure 2) may be interlinked with the physical distance between countries like China, Indonesia, India and Thailand, which imply differences on environmental conditions between their coffees. On the other hand, despite two Robusta samples were present in this category, no evidence of clear outcasting from the other samples was noticed. Hence, the eventual dispersion caused by the coffee species seems to be absorbed by the dispersion directly linked to geographic factors.

Table 2. Non-standardized coefficients($\boldsymbol{\beta_i}$) of the canonical discriminant functions for geographical origin of coffee samples. Data from Toledo et al. (2016a)

Compounds (V_i)	"Four Geographic Regions"			"Brazil *vs.* Others"	"Brazil *vs.* America"
	Y_1	Y_2	Y_3	Y_4	Y_5
(Constant)	-15.169	-1.024	-8.256	-3.429	-21.115
2-methylbutanal	-10.985	1.720	-3.751	-1.355	5.057
3-methylbutanal	-4.019	-18.606	0.230	6.952	13.738
2,3-butanedione	17.054	18.480	7.733	0.749	-28.552
2,3-pentanedione	-6.305	-0.294	-1.002	-0.200	2.368
2-methyl-1H-pyrrole	10.233	-3.429	4.809	6.357	-31.846
pyrazine	75.998	45.763	54.104	11.083	489.508
2-methylpyrazine	-3.867	-2.519	7.057	2.881	-5.038
2,5-dimethylpyrazine	76.283	6.368	11.547	-2.179	22.431
2,6-dimethylpyrazine	-19.994	-17.754	-17.163	-1.709	-
2-ethylpyrazine	-64.957	-0.781	16.457	7.192	-
2,3-dimethylpyrazine	-10.179	-4.642	2.662	-6.017	-
1-hydroxy-2-butanone	12.534	20.875	6.569	7.400	-
2-ethyl-6-methylpyrazine	-1.438	-1.666	-44.529	-1.914	-
2-ethyl-3-methylpyrazine	-12.705	19.236	7.666	8.803	-
furfural	28.312	22.991	-1.534	4.266	-
2-furfuryl-5-methylsulfide	199.682	44.156	45.226	-	-
5-methylfurfural	-22.335	-13.563	4.400	-4.208	-
pyrrole-2-carboxaldehyde	-7.785	-7.261	-6.501	-	-
2-methoxyphenol	-	-	-	-10.069	-
2-acetylpyrrole	-	-	-	4.193	-

With reference to South America, the dispersion observed in Figure 2 is caused by Brazilian samples. In light of its lion's share importance in the context of world coffee production, 35% (USDA, 2015), a DA was specifically applied to discriminate Brazilian coffee samples from all the other origins listed, as well as from the continental "neighbors" from America. For this all database was reprocessed again in order to fit only in two categories: "Brazil" and "Others." As a result, only one discriminant function was needed to differentiate Brazilian coffee samples from the samples with different provenances. The resulting single function is able to explain 100% of the data and rely on 18 compounds, which are given in Table 2 (see "Brazil *vs.* Others" column). In comparison to the discriminant

factors of the "Four Geographic Regions," the differentiation of "Brazil *vs*. Others" relies on the same compounds with exception of 2-methoxyphenol and 2-acetylpyrrole, that were replaced by two new molecules: 2-ethyl-3-methylpyrazine and pyrrole-2-carboxaldehyde. Hence, the reformulation of the model to specifically distinguish Brazilian coffee samples involved, in a greater extent, a refitting of the coefficients of the seminal model in spite of a substantial replacement of discriminant factors.

With regard to the discrimination of Brazilian coffee samples from others belonging to the same continent, the "Brazil *vs*. America" discriminant factors are also listed in Table2. Accordingly, a single function was also enough for effectively isolate the samples. Remarkably, the resulting discriminant model for this differentiation only needs 8 compounds: 2-methylbutanal*, 3-methylbutanal, 2,3-pentanedione*, 2,3-butanedione*, 1-methyl-1H-pyrrole, pyrazine, 2-methylpyrazine and 2,5-dimethylpyrazine*, four of them (marked with asterisk) being of impact on coffee aroma.

3.2. Roasting Degree Models

One of the most impacting treatments of coffee comprises the roasting of the beans. Although raw coffee exhibits a certain aroma, the desirable and expected aroma and color features are only obtained after roasting.

During roasting a decrease in the content of proteins, sucrose, phenolic acid esters, and so-called chlorogenic acids takes place. On the other hand important volatiles flavor active substances like pyrazines, aldehydes, pyrroles, pyridines (Strecker reaction), phenols, furans (thermal degradations) and important sulfur substances are generated (Vitzthum, 1999). The important sensory notes of roasted (pyrazines), rubbery (some thiols), woody (nonenal), phenolic (guaiacols), and "Cooked potato" (methanethiol) stem from them.

In this sense, the quality control of coffee roasting can be performed by analyzing and modeling the relative concentrations of the key volatiles generated during the process, which will naturally depend on roasting degree. Different methods for roasting raw coffee can be found in the literature, for example, "high temperatures and short periods of time" (HTST) and "low temperatures and low periods of time" (LTLT) (Eggers, 2005; Akiyama et al., 2008; Moon and Shibamoto, 2009). The degree of roast is assessed by measuring the reflectance of ground seeds, or simply by visual inspection of their color, or by the loss of mass. The Specialty Coffee Association of America established a colorimetric system for the classification of the degree of roast, called "AGTRON Roasting Classification," which comprise eight different degrees: Very Light, Light (American Roast), Moderately Light, Light Medium, Medium, Moderately Dark, Dark, and Very Dark (French Roast). On the basis of the available literature, and without loss of generality, an example of application of DA to Light, Medium, Dark, and French roasts is presented in the following.

Likewise the approach for modeling the geographic origins of coffee samples presented in Section 3.1, DA can be applied with advantage to screen samples of different roasting levels based on the aforementioned roasting methods/classification. In this case, the aim is to identify the most relevant volatile compounds to judge and confirm the roasting degrees of coffee samples, enabling thus a control tool for coffee samples. Accordingly, two discriminant functions relying on a total of 10 compounds (see Table 3) were built, explaining 99.4% of the variance through a bi-dimensional assignment of the data.

Table 3. Non-standardized coefficients(β_i) of the canonical discriminant functions for roasting degree of coffee samples. Data from Toledo et al. (2016b)

Compounds (V_i)	Y_6	Y_7
(Constant)	-10.305	-4.664
pyrazine	1.313	108.167
2-methylpyrazine	-16.195	2.492
2,5-dimethylpyrazine	19.100	5.613
2-ethylpyrazine	24.526	-24.077
2,3-dimethylpyrazine	-53.733	-37.723
dihydro-2-methyl-3(2H)-furanone	33.981	-1.656
5-methyl-2(5H)-furanone	1.900	1.287
furfural	-1.241	1.291
2,3-dimethyl-2-cyclopenten-1-one	8.586	106.019
acetic acid	1.586	1.708

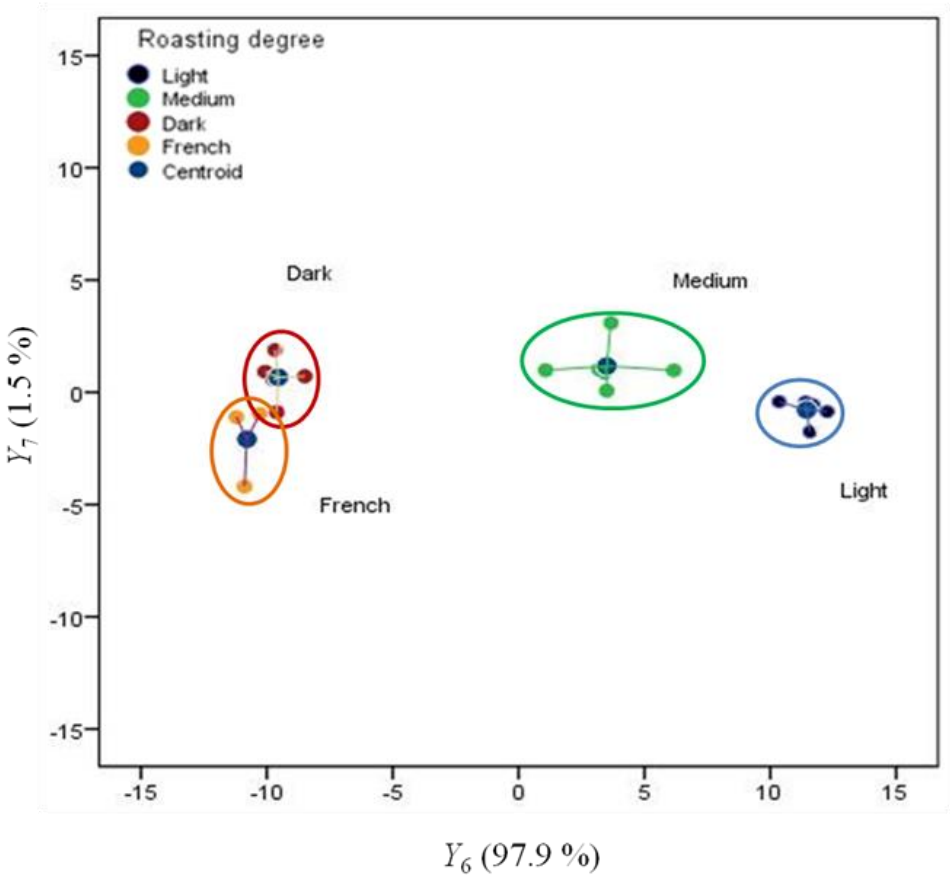

Figure 3. Discriminant scores and centroid values of DA for the four roasting methods (Toledo et al., 2016b).

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The graphical representation of the roasting discriminant functions is presented in Figure 3. For each roasting group, the centroids are plotted with filled marks, while data points are graphed in their individual coordinates and connected by a line to the respective group centroid. To emphasize the areas belonging to the different groups, colored circles are drawn around each roasting group. The coefficients of both discriminant functions, Y_6 and Y_7, are identified in Table 3, where it can be realized that half of the model compounds belong to the pyrazines family, which is known to confer sensorial notes related to roasted hazelnut and peanuts aromas. For instance, 2-methylpyrazine and 2,3-dimethylpyrazine are highly linked to intense roasting such as Dark and French. Moreover, these two methods exhibit a partial overlapping of their operating conditions, which may hinder their distinction. Consequently, while different, the DA results evidence that Dark and French roasting are very close to each other from a chemical composition point of view. On the other hand, results also show that Medium roasting is closer to Light than to Dark roasting, which creates a larger gap of possible coffee compositions in between Dark and Medium levels.

A worth noting point comprises the dispersion of data points inside each roasting group, which may be due to two aspects in addition to the experimental errors: (i) the usage of samples from different geographic origins (from places as far from each other as Sumatra and Nicaragua); (ii) the distinct roasting methods adopted to reach the desired roast degree, which impart fluctuations on the profile of volatiles compounds. Although considerable, the dispersions observed inside each roasting group are smaller in comparison to the differences directly linked to the roasting degrees (between groups), which noticeably affect the chemical markers in a much more prominent way.

3.3. Key Markers for Coffee Origin and Roasting Degree

More than 800 volatile compounds have been identified in roasted coffee so far (Illy, 2005). These can be divided into different classes, namely furans, pyrazines, ketones, pyrroles, phenols, hydrocarbons, acids and anhydrides, aldehydes, esters, alcohols, sulfur compounds, and others (Flament and Bessière-Thomas, 2002; Illy, 2005). Nonetheless, the desirable coffee aroma is produced by a delicate balance in the composition of volatiles, and it is believed that only about 5% of these compounds are actually odorous and capable of impacting coffee flavor (Yeretzian et al., 2003). Among these compounds, pyrazines stand out, followed by furans, aldehydes, ketones, phenols, and sulfur compounds, amongst others (Czerny et al., 1999; Maeztu et al., 2001; Sanz et al., 2002; Akiyama et al., 2005).

The utilization of DA technique for identifying key molecules to distinguish coffee samples on features such as roasting degree or geographic provenance allows a substantial reduction of the aforementioned list of volatiles to only 9-18 compounds, depending on the desired type of discrimination. Table 4 presents ten of the molecules that statistical modeling has shown to be influent to discriminate coffee samples. From these, 2-methylbutanal is perhaps the most versatile marker found, as it was considered important in all geographic discrimination tests discussed in this chapter (i.e., “Four Geographic Regions”, “Brazil *vs.* Others”, “Brazil *vs.* America”, “Roasting degree”). This molecule belongs to a group comprising chemical compounds with remarkable high odor activity values (OAV) (De Maria et al., 1999). 2-methylbutanal imparts buttery aromas to coffee samples, which are of positive contribution to their quality, a feature also found in compounds like 3-methylbutanal, 2,3-

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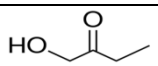
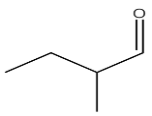
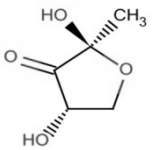
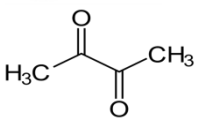
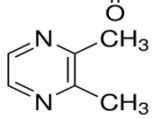
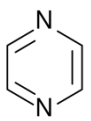
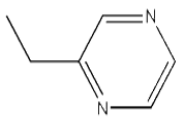
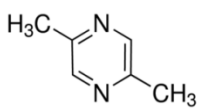
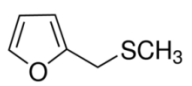
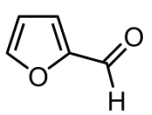
butanedione, and 2,3-pentanedione. Besides this aldehyde, 2-ethylpyrazine and 2,5-dimethylpyrazine are two worth noting cases of molecules that models consider relevant for both geographic origin and roasting degree assessments. From a sensorial perspective, the two compounds are linked to peanuts/roasted and hazelnut/roasted notes, respectively, which are also of positive contribution to coffee quality.

Table 4. Selected key chemical markers for differentiation of coffee samples regarding origin and roasting degree

Compound	Structure	Sensorial note	Marker scope
1-hydroxy-2-butanone		Toasted	"Four Geographic Regions" "Brazil *vs.* Others"
2-methylbutanal		Buttery	"Four Geographic Regions" "Brazil *vs.* Others" "Brazil *vs.* America" "Roasting degree"
dihydro-2-methyl-3(2H)-furanone		Coffee	"Roasting degree"
2,3-butanedione		Buttery	"Brazil *vs.* America"
2,3-dimethylpyrazine		Hazelnut/ Roasted	"Roasting degree"
pyrazine		Roasted	"Four Geographic Regions" "Brazil *vs.* Others" "Brazil *vs.* America"
2-ethylpyrazine		Peanuts/ Roasted	"Four Geographic Regions" "Roasting degree"
2,5-dimethylpyrazine		Hazelnut/ Roasted	"Four Geographic Regions" "Brazil *vs.* America" "Roasting degree"
2-furfuryl-5-methylsulfide		Sulfuraceous	"Four Geographic Regions
furfural		Almond/ Bitter	"Four Geographic Regions"

As a final remark, it should be emphasized that not all relevant compounds from a sensorial perspective (high OAV) were found statistically relevant to distinguish coffee

samples with different origins. Hence, one may anticipate that sensorial analysis may be inefficient to sort samples although being of more expedite implementation, because the DA approach requires time consuming analytical results. On the other hand, DA revealed a good accuracy and hints to a deep chemical understanding of what clearly differentiates coffee samples regarding their intrinsic origin and roasting features.

CONCLUSION

The quality control of coffee samples is a challenging task, for which multivariate statistics can be a powerful aid. It includes techniques such as Principal Component Analysis (PCA), Partial Least Squares (PLS), and Discriminant Analysis (DA). They can be used to model geographic origin and roasting degree of coffee samples based on their volatiles profiles.

The screening potential of DA was demonstrated through three models proposed to differentiate (i) samples from Brazil from others of the same continent, (ii) samples from Brazil from others of the world, and (iii) samples belonging to one of four geographic regions: Central America, South America, Africa and Asia. For this, a maximum number of 18 compounds was required to clearly distinguish the provenance of the various coffee samples.

With respect to the differentiation of coffee samples submitted to Light, Medium, Dark or French roastings, a DA model comprising two equations and 10 volatile molecules was enough to explain 99.4% of the variance exhibited by the experimental data. In the future, the presented DA tool can be extended with advantage to the eight roasting degrees of the AGTRON Roasting Classification, depending on the availability of a database.

While some chemical markers were specific for only one type of differentiation study addressed, 2-methylbutanal, 2-ethylpyrazine and 2,5-dimethylpyrazine were relevant for both geographic origin and roasting degree assessments. These compounds possess high odor activity values (OAV) and thus a great positive impact on coffee organoleptics.

In view of the successful application of the DA approach to databases of this size and variability, this essay provides compelling arguments for the development of DA-based tools with the purpose of controlling the quality of coffee in terms of their geographic and/or roasting features.

ACKNOWLEDGMENTS

The authors thank the Brazilian National Research Council (CNPq) and the Coordination for the Improvement of Higher Level Personnel (CAPES) for financial support. This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 4

SPENT COFFEE GROUND PROPERTIES AND APPLICATION IN BIOENERGY AND BIOPRODUCTS

Michel Brienzo,[1,2,*], María García-Aparicio,[2,3] and Johann Görgens[2]

[1]Laboratory of Biomass Characterization, Bioenergy Research Institute (IPBEN), Universidade Estadual Paulista-(UNESP), Rio Claro-SP, Brazil
[2]Department of Process Engineering and
[3]Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT

Coffee is one of the most popular beverages (almost 9 million tons of coffee was consumed in 2014) and the second largest commodity traded worldwide. Coffee beans have long been used for human consumption, in the last centuries, producing coffee brews and instant coffee. About 50% of the coffee produced is directed for the production of soluble coffee generating spent coffee grounds (SCG) as a byproduct that is rich in carbohydrates, protein, lipids and bioactive molecules. SCG has found application as compost (soil conditioner), animal feed (poultry and ruminants), mushroom production, a substrate for fermentation (enzymes) and energy (biogas, liquid biofuel and bioelectricity). SCG composition and structure depends on the coffee beans used in the coffee production, as well as the industrial extraction process. The instant coffee manufacturing uses several hot water extraction steps with different temperature and its severity influences in the soluble solids extraction from the roasted beans and the characteristics of the generated SCG. The industrial process impacts on the amount of SCG generated and soluble compounds extraction, in average 4 kg of wet SCG per kilogram of instant coffee produced. Moreover, the structure of the remained polysaccharides in SCG, cellulose, galactomannan and arabinogalactan, can be more recalcitrant to further use. The SCG still contain extractable compounds (flavor and oil) in a lignocellulosic matrix formed by cellulose, hemicelluloses and lignin. These structural compounds are organized in the cell wall as a recalcitrant structure to the biological conversion process. Moreover, SCG is resistant to degradation and may need a

* Corresponding author: mbrienzo@ipben.unesp.br, Tel +55 19 3531-8407.

pretreatment to make the material suitable for biotechnological conversion. The pretreatment disrupts the cellulose-hemicellulose-lignin structure, changing its properties and allowing a complex of cellulolytic and hemicellulolytic enzymes breaking down polysaccharides into fermentable sugars. The factors responsible for SCG recalcitrance is related to its chemical composition, structural and physicochemical properties that influence the pretreatment response end requires a complex pool of enzymes to its hydrolysis. This review will focus on discuss the recalcitrance properties of the SCG, based on its composition and structural organization, and the complex enzyme necessary for its conversion, highlighting its impact on biotechnology and bioenergy process.

Keywords: spent coffee ground, polysaccharides, recalcitrance, cellulosic ethanol, biodiesel, Bioenergy, enzymatic hydrolysis

INTRODUCTION

The use of fossil fuel for the production of energy and building blocks for the chemical industry has enabled the industrial development in the last centuries. The current economic system based on petrochemistry, however, is threatened by the increase in energy demand from emergent economies, the insecurity on both price and supply of fossil fuel, as well as the political and social interests in the reduction of greenhouse gas emissions generated from its consumption. In this context, most of the countries have started to look once again into biomass as a source of energy and products, owing to its renewable origin and wide distribution. The biorefinery is defined as the analogous of the fossil-oil refinery, a facility where the biomass is subjected to a refining process for the co-production of energy, chemical products and materials. The biorefinery is characterized by the integral use of all the fractions that compose the biomass as well as the sub-products generated in each conversion technology (Kamm and Kamm, 2007). In this way, the sub-products for one conversion technology would constitute the substrate for complementary transformations increasing the product portfolio as well as the revenue. Biotechnology plays an important role in the development of biorefinery technology platforms (Gobina, 2014). For instance, the use of biocatalyst either enzymes or whole microorganisms, will enhance process efficiency and reduce the environmental impact by reduction of the amount of waste generated (Alcalde et al., 2006; Heux et al., 2015).

The current development of biotechnology industry and environmental concerns requires a dual approach to both waste reduction and re-use, by conversion into a feedstock for production of high-value compounds (Murthy and Naidu, 2012a; Pfatzgraff et al., 2013; Kiran et al., 2015). The energy market resulted primarily from waste use in boilers and energy co-generation, as well as liquid fuels such as ethanol and biodiesel, with the potential for the production of high-value compounds by modern biotechnology (Heux et al., 2015). From the biotechnology point of view, a waste is considered as a potential feedstock for bioconversion, depending on its chemical, structural and physical properties. In this context, the coffee industry can be considered as a biorefinery and residues such as SCG are considered as potential feedstocks for production of bioenergy and/bioproducts by means of biotechnology (Murthy and Naidu, 2012a). Such an approach solves the problems with waste treatment and disposal and at the same time yields valuable products.

Coffee is one of the most consumed beverages in the world and it is the second largest commodity after petroleum. Arabica (*Coffea Arabica*) and Robusta (*Coffea Canephora*) are the two main coffee varieties that are worldwide traded. The global amount of coffee produced has been estimated in about 8.6 billion of Kg in the period 2014-2015 (International Coffee Organization, The Current State of the Global Coffee Trade, http://www.ico.org/monthly_coffee_trade_stats.asp). Arabica and Robusta represent, respectively, approximately 64% and 35% of the world's production while the non-commercial species account for just 1% (Rubayiza and Meurens, 2005). The high production together with the levels of consumption and the inefficiency of the coffee production process result on millions of metric tons of waste generated each year (Summers et al., 2014). The caffeine in these residues can exert detrimental effects on the environment. Liquid wastes are characterized by a high biological and chemical oxygen demand and, therefore, are subjected to an oxygenation process before being released to the environment. The solid wastes are typically burned, composted, ensilaged, or used as garden fertilizer.

The coffee solid waste accounts for more than 50% of the coffee berry (Campos-Vega et al., 2015). The coffee residue generation begins with the beans harvest and processing to remove the shell and mucilaginous part from the coffee bean. The coconut of the coffee berry is composed of epicarp (husk), mesocarp (pulp) and endocarp (parchment) (Acchar W, Dultra, 2015). To separate beans from the coconut, the coffee can be processed by dry, wet or semi-wet methods (Figure 1). Robusta coffee has a thinner pulp that allows for direct drying while Arabica is generally processed by the wet method. The removal of the coconut generates the green beans and wastes such as coffee pulp (CP) if wet processed (29% of the coffee berry) or coffee husk (CH) after the dry step (12% of the coffee berry) (Murthy and Naidu, 2012a). The green beans are further subjected to roasting and brewing, generating other types of waste such as coffee silverskin (CS, 4.2% of the coffee berry) (Ballesteros et al., 2014) and spent coffee ground (SCG), respectively. These residues are produced in a large amount, roughly husk is produced in a rate of 1:1 to grain (Rocha et al., 2006); and wet spent coffee ground of 4:1 (w/w) to instant coffee manufactured (Punnett, 1958).

These residues have a structure based on lignocellulosic material, composed of cellulose, hemicelluloses, lignin and extractable compounds of potential biotechnological value. However, the structure of lignocellulosic materials compounds is high organized in a recalcitrant structure resistant to microbial degradation, which limit some of the direct application such as animal feed as well as its biotechnological conversion. A pretreatment to disrupt this structure is a prerequisite to facilitate the conversion of the polysaccharides by means of enzymes and/or microorganisms (Sant'Anna et al., 2014).

About 50% of the coffee produced is directed for the production of soluble coffee generating mainly spent coffee grounds as a byproduct. SCG has a complex structure mainly rich in carbohydrates but also contain protein, lipids and bioactive molecules. As main components of the SCG residue are polysaccharides, which are from great interest as feedstock. The polysaccharides vary in content and diversity. Generally, cellulose and hemicelluloses appear in lignocellulosic materials and xylan is the main hemicelluloses class in grasses such as sugarcane bagasse (Brienzo et al., 2009). However, the SCG polysaccharides comprise sugars of mannose, galactose and arabinose (Chiyanzu et al., 2013), arranged mainly as galactomannan and arabinogalactan (Arya and Rao, 2007). Other components in the group of non-carbohydrates appear in a range of 45% (Chiyanzu et al.,

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2013), and include lignin (25%), ash (1%), protein (17%), and fat (2%) (Ballesteros et al., 2014). The organization and interaction or linkage between components in the SCG structure is not well known, including the protein interaction with the carbohydrates (Redgwell et al., 2005). Variations in the chemical composition of SCG provide several alternatives for application development, although the structural complexity requires a broad cocktail of enzymatic activities for its hydrolysis.

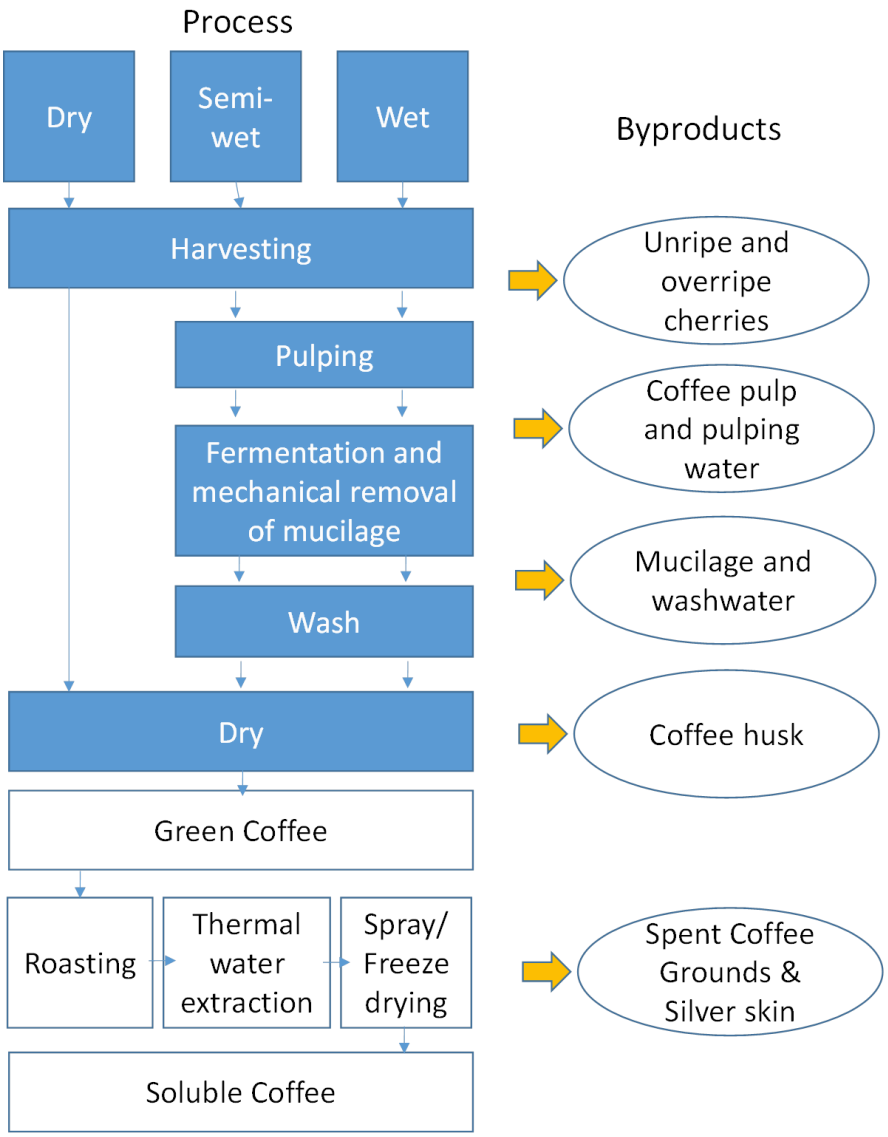

Figure 1. Layout of the processing steps for production of soluble coffee as well as the byproducts generated on each step.

There are two main concerns with SCG: I) environmental problems: the inappropriate disposal of material could generate problems of pollution, siltation of rivers and air pollution

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by inadequate burning; II) feedstock for compounds extraction or chemicals by fermentation: considering SCG diversity chemical composition several uses have been proposed to it uses, such as fuel generation (conversion of sugars into bioethanol, oil extraction for biodiesel, anaerobic digestion for biogas production); extraction of proteins, phenolic acids, polyphenols, fatty acids, terpenes and aroma compounds; ingredient for functional food (mannooligosaccharides); as solid fuel for direct burning or bricks preparation; as substrate material for fertilizer, cheap substrate for enzyme production and edible mushroom; fermentation of individuals sugars for production of alcohols, organic acids, and polyhydroxyalkanoates, among others.

Focusing on the SCG as a biomass feedstock for biotechnological applications, the following discussion will review the chemical composition and properties of the material that contribute to its recalcitrance, and related enzymes needed to the material conversion. According to material properties and characteristics, some application will be approach highlighting the field of biotechnology conversion into bioenergy and bioproducts.

SCG COMPOSITION AND PROPERTIES

SCG is a residue with fine particle size, high humidity (70-85%), organic load, and acidity. SCG is recognized as an important source of lignocellulosic materials. Glucan (cellulose) with hemicelluloses and lignin are the main components of the lignocellulosic biomass. The lignin content is low on coffee beans, approximately 5%, however, after the soluble coffee production, the SCG residue is enriched in lignin content (Table 1). During the instant coffee manufacture, the coffee beans are submitted to different processes and undergo several transformations: the beans are roasted, ground, extracted and the product is finally concentrated and dried. The green coffee beans are firstly subjected to a roasting process that increases the bean volume and the porosity of the beans leading to a polysaccharide fraction more extractable. The roasted beans are then grounded prior to the thermal water extraction. Soluble compounds from the roasted beans are efficiently extracted with compressed hot water in a series of five to eight columns, with temperatures from 100 to 180°C. The water passes through several columns at temperatures of 140-180°C, with some at higher pressure, to extract soluble compounds and polysaccharides. Flavor compounds are extracted in two or more columns with temperature of 100°C. The liquid phase extracted is cooled into about 5°C. About 20 to 30% of soluble compounds can be extracted from this process (Fritz, 1985). The resulting liquor containing water soluble solids is concentrated by evaporation and further dried by freeze or spray-drying to obtain the soluble coffee. The solid residue from soluble compounds extraction is the spent coffee ground (Figure 2). The SCG characteristics depend on the process for soluble compounds extraction and its severity. An industrial process with higher soluble compounds extraction yield generates a SCG with lower carbohydrate and flavor content than a low-yielding process. During the completion of the extraction process, the SCG becomes enriched in compounds that are more recalcitrant and cannot be extracted by hot water treatment.

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Table 1. Chemical composition of Green bean, Roasted and Spent Coffee Ground

Composition (%, dry base)							
Material	**Glucan**	**Hemi-cellulose***	**Lignin**	**Fat**	**Ashe**	**Protein**	**Reference**
Green bean	12.7	23.0	5.6	11.4	-	11.6	Arya and Rao, 2007
Roasted	13.2	24.0	5.8	11.9	-	3.1	Arya and Rao, 2007
	12.4	39.1	23.9	2.3	1.3	17.4	Ballesteros et al., 2014
	24.2	28.1	44.8	-	-	-	Chiyanzu et al., 2013
SCG	13.8	-	33.6	-	2.2	17.7	Caetano et al., 2014
	8.6	36.7	-	-	1.6	13.6	Mussato et al., 2011a
	47.3	19.0	29.3	-	1.2	-	Kelkar et al., 2015

(*) Hemicellulose expressed as sum of anhydro D-galactose, L-arabinose, D-xylose and D-mannose; (-) not determined; Glucan represents the total anhydro D-glucose.

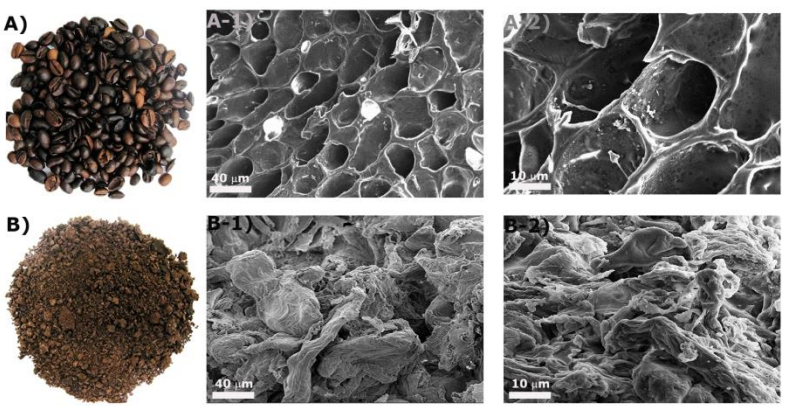

Figure 2. Roasted coffee beans (A), spent coffee ground (B), and scanning electron microscopy image (A-1 and 2, B-1 and 2, respectively 40 and 10 µm).

The coffee beans are composed mainly of polysaccharides and non-polysaccharides compounds such as proteins, lipids, aromatics, inorganic and lignin (Table 1). The polysaccharides extraction influences directly the soluble compounds yield and also the organoleptic properties of the coffee beverage (Smith, 1985). The role of the polysaccharides on the instant coffee production and on the SCG characteristics depends on their solubility. The beans are composed of polysaccharides such as cellulose, galactomannans, arabinogalactans and xylans (Redgwell et al., 2003). In brief, the SCG composition depends on the polysaccharides structure/solubility, the roasting step and water extraction process. Some of the polysaccharides can be completely extracted from the roasted beans while others are less affected, remaining in the SCG structure (Table 1). Polysaccharides such as

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galactomannans become more water-soluble with increases in the degree of roasting while cellulose fraction typically remains unextracted in SCG.

The polysaccharide content and diversity between Arabica and Robusta varieties have been the focus of several studies. Actually, there is no clear consensus among the reports regarding the type of polysaccharides, content, organization and properties in the coffee beans (Fisher et al., 2001). Arabica species have been reported to contain more polysaccharides compared to Robusta (Bradbury, 1990), reaching 48 and 55%, respectively (Clifford, 1986). Other studies have reported that these bean type similar total content of polysaccharides for these types of beans (Fisher et al., 2001). Differences were observed on the proportion and structure of the mannan. While a higher amount of mannan was determined for Arabica, the degree of polymerization of mannan in Robusta was superior (Fisher et al., 2001). However, other structural differences resulted in a higher solubilization of galactomannan and arabinogalactan from Robusta species (Fisher et al., 2001). The difficulty with literature comparisons is related to the method used for polysaccharide extraction. Hemicelluloses can be solubilized almost completely in alkaline and oxidative media (Brienzo et al., 2009). However, for selective extraction of specific polysaccharides, a sequence of pretreatments with different chemical and conditions (temperature and concentration) is required. This difficult limits the scope to determine the impact of coffee beans' origin and condition in the production process on the chemical composition of the SCG. The SCG final composition depends on the coffee bean composition, the roasting conditions and the industrial extraction process. These characteristics will influence the remaining polysaccharides in SCG with regards to hemicelluloses (mannan, galactan, arabinan) content and/or enriching the SCG with glucan and/or lignin (Table 1).

Thermal extraction also results in enrichment of the protein content of SCG compared to the coffee bean, with varying reported values of 7-17% (w/w) (Campos-Vega et al., 2015). The differences in the reported values could be due to different processing methods as well as an overestimation of protein since the SCG also has other compounds rich in nitrogen (Delgado et al., 2008). These non-protein nitrogenous compounds are caffeine (0.7-41 μg/mg SCG) and melanoidin resulted from Maillard reactions (16%). In terms of aminoacids composition, up to half of the composition corresponds to essential aminoacids. SCG is high in essential branched aminoacids (BCAA) such as Valine, Leucine and Isoleucine with a reduced content of aromatic aminoacids (AAA) like Tyrosine and Phenylalanine. These proportions of aminoacids have been shown beneficial for specific conditions such as liver disease, oxidative stress and hypertension.

Lipids of the coffee bean remain adhered to the SCG during instant coffee production. Values wide range depending on residue source such as espresso, filter coffee or industrial instant coffee and the extraction method applied (Campos-Vega et al., 2015). The lipids from SCG are composed mainly by glycerides (80-90%), including free fatty acids while the rest of lipids are terpenes (cafestol and kahweol), sterols (i.e., sitosterol, stigmasterol and campesterol) and tocopherols. The main fatty acids are linoleic, palmitic, stearic and oleic acids, with a small proportion of arachidic and linolenic acids. SCG contain a significant amount of phenolic compounds mainly chlorogenic acid (CGA) and its derivatives such as caffeoylquinic acids, feruloylquinic acids, p-coumaroylquinic acids and mixed diesters of caffeic and ferulic acids with quinic acid (Zuorro and Lavecchia, 2012). Values of 255 to 273 mg gallic acid equivalents (GAE)/g dm of SCG have been reported (Acevedo et al., 2013).

Based on the SCG final chemical composition and its importance for biotechnology and bioenergy conversion process, the following topics presents a discussion about the polysaccharides characteristics, properties and structure.

Glucan

Glucan is a class of a polysaccharide based on the structure formed by glucose units; in the plant cell wall glucose is primarily present in the cellulose polysaccharide. Cellulose is recognized as the most abundant renewable polysaccharide on nature, and is a linear homopolysaccharide composed of D-glucopyranose units linked by (1,4)-β linkages in the so-called glucosidic linkage. Cellulose is the main structural polysaccharide of the plant, responsible for fibre formation, which is associated with hemicelluloses and lignin, to form the plant cell wall. The cellulose chain is formed by an alternation of each glucose residues in 180°. Glucose is the monosaccharide of cellulose while the dimer (two glucose residues) named cellobiose is the repeating structure in the cellulose chain.

Cellulose chain has particular characteristics related to different ending groups. At one side of the chain there is a non-reducing end that is found as a closed ring sugar structure. On the other side, there is a reducing end with is formed by an aliphatic and a carbonyl group (Figure 3). The number of units forming the cellulose chain is defined as the degree of polymerization (DP). The DP ranges from 1,000 to 15,000 on native cellulose of plant depending on the origins. However, cellulose is high affected by pretreatment and the extraction process (Brienzo et al., 2015). Glucose on cellulose/glucan chain can form intra- and inter-molecular hydrogen bonds that confer chains stability and rigidity. These cellulose chain properties interact to form high organized and less organized regions, named crystalline and amorphous regions, respectively. The hydrogen bonds in cellulose influence several of the molecule properties, such as solubility (not soluble in most of the solvents, including water or alkali), reactivity and crystallinity.

In contrast to coffee arabinogalactans, glucan is less affected by the roasting process. Also, glucan is less vulnerable to extraction process than arabinogalactan and galactomannan. It could be expected that the amorphous fraction of the cellulose, could be modified or solubilized during the extraction process. However, the instant coffee production process hardly affects the glucan structure as it can be inferred by the enrichment of the cellulose content of the generated SCG when compared to roasted beans (Table 1). The increase on the glucan content is dependent on the extent of extraction of hemicelluloses, with cellulose mostly remaining intact in beans during the extraction process. Processes with higher severities (higher temperatures and longer process time) extract more hemicelluloses polysaccharides (mannan, galactan, arabinan and xylan). As a consequence of the severity, the SCG enrichment in glucan content is increased, resulting in a wide range of glucan contents for SCG from various processes (Table 1).

The glucan of SCG is resistant to other pretreatments. SCG submitted to steam explosion at relatively high severity (210°C for 15 min) showed higher resistance of glucan, as indicated by glucan enrichment of the pretreated solid from 46 to 56% (Chiyanzu et al., 2013). The SCG glucan is resistant to enzymatic hydrolysis, reaching relatively lower enzymatic hydrolysis glucose yield compared to other lignocellulosic byproducts such as sugarcane

bagasse, (Brienzo et al., 2015). SCG contains higher amount of non-extracted substance such as fats and lipids, compared to other types of lignocellulose, which contribute negatively to enzymatic action (Kwon et al., 2013). Microwave irradiation (1 kW, 2.45 GHz) of SCG (1:30 g:mL, 200°C/2 min) preferentially extracted hemicelluloses, while glucan content was enriched to 84% after several cycle (Passos et al., 2014). Dilute acid also preferentially extracted hemicelluloses from SCG, with a low amount of glucose solubilization (Mussato et al., 2011b). These examples of pretreatment and enzymatic hydrolysis approaches showed that SCG is a material more recalcitrant to biological conversion than other lignocellulosic materials, and it is necessary a pretreatment step to modify its structure and physicochemical properties for an efficient enzymatic hydrolysis and glucose release.

Hemicelluloses

Hemicelluloses are the second most abundant polysaccharides in lignocellulosic biomass, after cellulose. Hemicelluloses contain many different sugar monomers, besides β-D-glucose, such as β-D-xylose, β-D-mannose, α-D-galactose, α-L-rhamnose and α-L-arabinose. Hemicelluloses form amorphous structures with little strength compared to cellulose. Hemicelluloses are thus more reactive than cellulose, and can be hydrolysed by acid or solubilized by alkali, although it requires a more complex enzyme cocktail for its hydrolysis, compared to cellulose, due to its the complex and diverse composition. Certain types of hemicelluloses can have acidified sugars as side-groups to the polymeric structure, such as β-D-glucuronic, α-D-methylglucuronic and α-D-galacturonic acids, and acetic acid. Hemicelluloses of grasses are primarily comprised of xylan polysaccharides, reflected in its composition of xylose while softwoods hemicelluloses mostly contain mannans. The reported SCG compositions have been based on the main sugars such as D-glucose, D-mannose, L-arabinose, D-galactose and D-xylose (Jooste et al., 2013; Chyianzy et al., 2013). Based on these sugars, the SCG material has a diverse composition of hemicelluloses, with mannans (galactomannan and arabinogalactan) as the main type, proposed to be presented in a cellulose-mannan complex (Brabury, 2006). Minor components xylan (xyloglucan and arabinoxylan) can be present (Oosterveld et al., 2003). Moreover, according to the industrial instant coffee production, the hemicelluloses composition of SCG residue can vary. The main hemicelluloses characteristics are discussed in the following subsections.

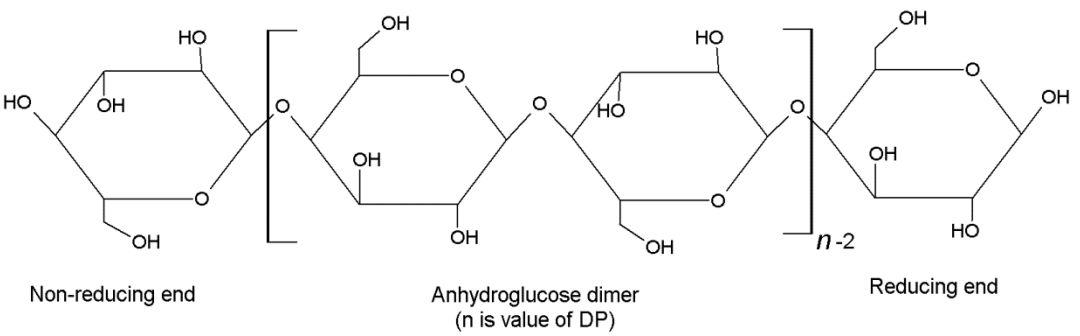

Figure 3. Cellulose structure.

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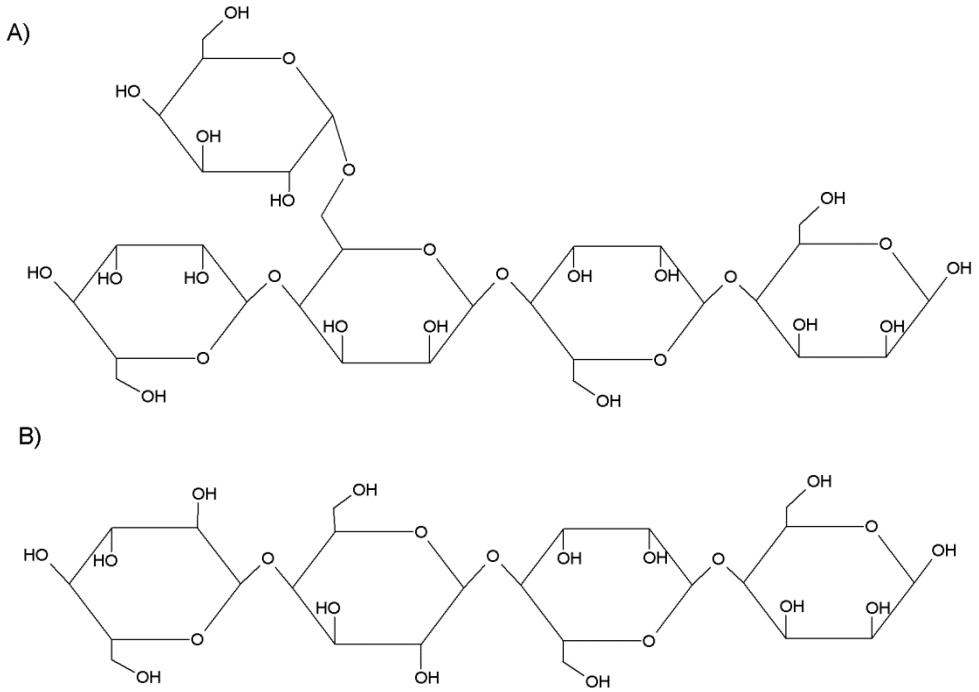

Figure 4. Hemicellulose structure, galactomannan (A) and glucomannan (B).

Mannan

Mannan is formed by a linear chain of mannose units linked by 1,4-β glycosidic bonds with an average degree of polymerization of 45 (Chauhan et al., 2014). The mannan polysaccharides include galactomannans (GMs) and galactoglucomannans (GGMs). The galactomannan polysaccharide backbone is formed of (1,4)-β-D-mannopyranose. The galactoglucomannan polysaccharide backbone is formed of (1,4)-β-D-mannopyranose and D-glucopyranose units in the main chain. As a substituent group linked at the carbon 6 position appears D-galacopyranose units, on both mannans polysaccharides (Figure 4). Mannan is normally found as branched, with galactose as substituent. The degree of substitution of mannan in coffee beans is an average of mannose:galactose of 23:1 (Navarini et al., 1999).

Considering extraction process for instant coffee production, the residual galactomannan in SCG could have a reduced substitution with side-groups. The mannose:galactose ratio is an important physicochemical property of this polysaccharide because of its influence on water solubility and solution viscosity. The mannan conformation is similar to cellulose and it is insoluble in water, and when present with low amounts of side-group substitutions, this polysaccharide tends to self-association forming a crystalline-like structure with low water solubility. However, GM that is highly branched with galactose residues (>30%) are soluble in water and found in storage tissue such as endosperm of seeds. GM from Robusta beans is more branched than Arabica coffee beans, what suggests a more crystalline organization for polysaccharides from Arabica beans. The properties of crystalline organization and extent of branching could explain the higher soluble solids extraction for Robusta beans than Arabica

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beans (Clifford, 1986). Considering these properties, the SCG from Arabica coffee beans could be more recalcitrant, what would imply a more severe pretreatment of the generated SCG to increase the polysaccharides accessibility and make it amenable to enzyme action or biotechnology conversion.

The extent of side group substitutions with D-galactose is typically 15% for both the GM and GGM (Enbrigerová et al., 2005). But, substitutions ratios for unbranched to branched mannoses between 14:1 and 30:1 have been reported for coffee beans (Fisher et al., 2001). Mannan is found in coffee beans around 50% of the polysaccharides from the cell wall. On SCG, the galactomannans also account around 50% of the polysaccharides (Chiyanzu et al., 2013). Together with glucan (cellulose), they remain in SCG as more resistant to extraction than other polysaccharides. SCG submitted to steam explosion at different severities (150 to 210°C, 10 to 15 min) showed the higher resistance of glucan to hydrolysis and extraction, which increased from 46 to 56%, while mannan decreased from 47 to 41% of total polysaccharides (Chiyanzu et al., 2013). The roasting process also enriches the beans in mannan content by approximately 15%, while also increasing the extractability of this polysaccharide during the thermal extraction (Simões et al., 2013). The glucan increases from 19 to 47% for SCG, and mannan suffers lower increment in percentage, from 38 to 39%, respectively to roasted beans and SCG (Jooste et al., 2013). Microwave irradiation (1 kW, 2.45 GHz) of SCG (1:30 g:mL, 200°C/2 min) extracted preferentially mannan (69%) after several cycles. On the contrary, the pretreated residue was enriched in glucan content reaching values of 84% (Passos et al., 2014).

The mannan polysaccharide content of SCG presents an interesting application, i.e., the production of mannooligosaccharides (MOS). MOS can be produced by physicochemical or enzymatic pretreatment of SCG (Chiyanzu et al., 2014). These compounds are of particular interest as food additives, based on the immunostimulatory properties of coffee mannan (Simões et al., 2009).

Arabinogalactan

Arabinogalactans are components of plant cell wall associated with pectin and are found in edible and non-edible plants. Arabinogalactans are water extractable and appear in a wide variety of plants, including coffee beans, soybeans, larch, tamarack and cereals. The association of these polysaccharides with the cell wall can be by physical interaction or covalent linkage, creating a polysaccharide network resistant to solubilization or degradation. Arabinogalactans also appear associated to proteins, forming a complex of arabinogalactan-proteins.

Arabinogalactan can present two different structural organizations, named as type I and II. Arabinogalactan type I is characterized as a linear (1,4)-β-galactopyranose backbone in short chains, with α-arabinofuranoside residues substituted at C3 positions by (1,5) links. Arabinogalactan type II has a (1,3)-β-galactopyranose backbone substituted at the C6 position by mono- and oligosaccharides of L-arabinosyl and D-galactosyl residues (Bradbury, 2001) (Figure 5). Arabinogalactan type II is frequently substituted with D-galactose, L-arabinofuranose, L-rhamnose, D-glucose and D-galactose, and can contain minor amounts of D-mannose, D-xylose and D-glucose.

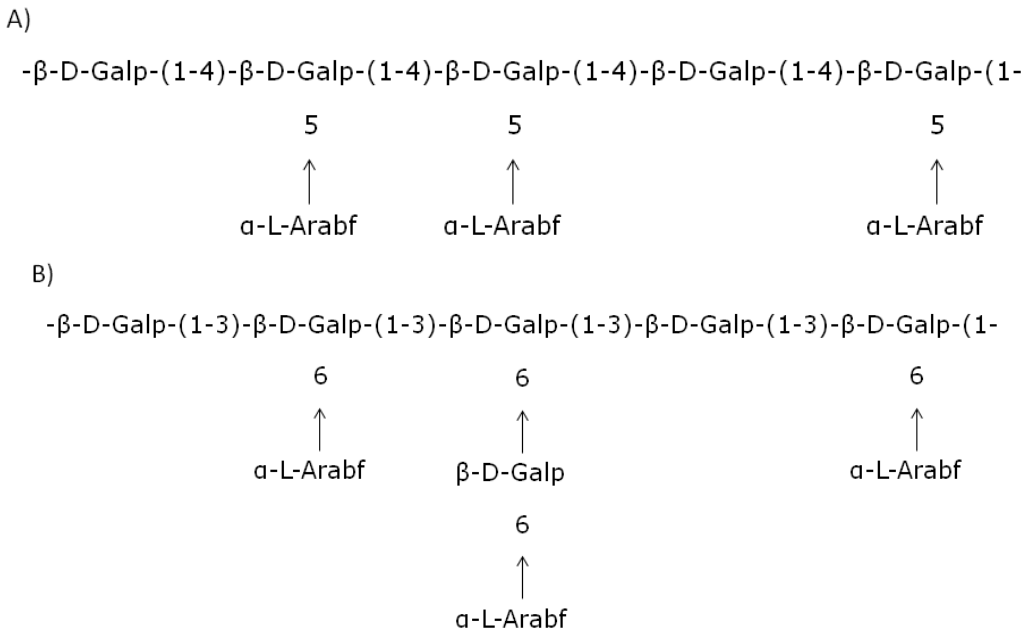

Figure 5. Structure of Arabinogalactan type I (A) and II (B).

Arabinogalactan type II is predominantly present in green Arabica coffee beans. Arabinogalactan common on the coffee bean (1,3-β-galactopyranose backbone) is substituted with L-arabinosyl and D-galactosyl residues. The arabinogalactan of coffee bean can contain up to 10% of glucuronosyl residues with (1,6) links, conferring a negative charge to the molecule. This molecule was found associated to protein in the cell wall. The arabinogalactan-protein complex of coffee beans accounted for approximately 2% of the protein in the isolated fraction of this polysaccharide (Redgwell et al., 2002a). The arabinogalactan is more easily degraded during coffee roasting, facilitating its solubilization into the coffee brew. In fact, roasting facilitates the extraction of this polysaccharide with sodium hydroxide solution (Oosterveld et al., 2003). Arabinogalactan is more reactive than galactomannan, which agrees with the lower amount of this polysaccharide found in SCG. The arabinogalactan content of the final SCG depends on the roasting and coffee extraction process conditions. Moreover, the arabinogalactans present in Robusta coffee beans are more substituted and has more side-chains than Arabica beans. Accordingly, arabinogalactan from Robusta is more easily solubilized than that from Arabica (Fischer et al., 2001), what in turn will result in a SCG with less arabinogalactan.

SCG AS BIOREFINERY FEEDSTOCK

The diverse chemical composition of SCG presents several potential applications. Carbohydrates can be used as a carbon source for microbial fermentation and production of organic acids, alcohols, biogas, enzymes or mushroom. The oil/lipids content of SCG can be used for biodiesel production while protein enriched material is useful for animal feed. The lignocellulosic characteristic of this material, including lignin content, allow its use as a

resource for steam/energy/bioelectricity generation by combustion. On the other hand, the diverse chemical composition also presents several challenges to such applications development with SCG. The extraction of specific components from SCG requires sophisticated technology or pretreatment to make the material amenable to biological processing or enzyme action. SCG as a lignocellulosic material has a natural recalcitrance due the plant cell wall organization. The carbohydrates are highly organized with lignin with a structural function on the plant. Considering that the coffee production process extracted the soluble solids more susceptible to water solubilization, the remaining SCG has a resistant structure.

Despite the SCG recalcitrance, two primary concerns motivate its use as a feedstock for various applications, i.e., environmental concerns with current disposal of the residues, and the potential for high-value products from SCG. The application of biotechnology can assist with both of these possible applications of SCG. The enzymatic degradation of the main components, polysaccharides, into monosaccharides is seen as a basis for the production of biofuels and bio-based chemicals. Enzyme applications on the coffee industry with particular emphasis on SCG are summarized below. The use of SCG for energy application or as raw material for bioproduct production highlighting biotechnology is also described next.

Application of Enzymes in the Coffee Industry

Enzymes are commonly used in many industrial applications, including plant cell wall degradation. Cellulases, hemicellulases and pectinases are commercially available enzymes used in several applications. The use of these enzymes in the food and beverage industry such as coffee industry has been driven due to an increasing interest in the use of natural ingredients for food processing and manufacturing for beverage production. Products generated in the enzyme-based process are of the same or even of better quality than those based on chemical process. Enzymes are highly specific and they normally require milder reaction conditions than traditional chemicals. These properties and their biodegradability reduce the amount of energy consumption and waste generated when compared to the traditional industrial production.

Enzymes are among the oldest and newest processing aids used in coffee treatment. Enzymes such as pectinases have been applied in the coffee industry, where these enzymes not only bring a more sustainable production alternative but also products with greater properties. The applications of enzymes for hydrolysis of polysaccharides in the coffee beans have been investigated. Pectinases enzymes used during the fermentation step of wet processing of coffee beans may assist in generating reducing sugar inside the bean that gives the roasted product its distinct flavor. Other enzymes such as proteases are useful for the release of flavor precursors, and polyphenoloxidase help to develop color.

The intensity of the pectin removal from the mucilage impacts economically the process owing to its influence on the quality of the end product. The external addition of microbial pectinases has become a general practice in coffee processing for a better control of the fermentation as well as reducing drastically the fermentation period (Murthy and Naidu, 2011). There are several commercial enzyme preparations that remove mucilage during coffee fermentation. BENEFACT was the first one in being commercialized, while others such as PECTOZYME, COFEPEC and ULTRAZYM have been developed more recently.

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All these enzymes are of food grade and produced by fungi. In spite of the advantages, enzymes application has not been widely adopted by the industry owing to the high cost. In order to reduce production cost, residues from the coffee industry can be used as a substrate for enzyme production.

The coffee extract generated during soluble coffee production is characterized by high viscosity provided by the mannans and galactomannans. Endomannananses hydrolyse unsubstituted mannan, galactomannan and glucomannan releasing mainly mannotriose, mannobiose and mannotetraose. Reduction of the viscosity of these extracts by means of endo-1,4-β-mannanases would simplify the posterior freeze-drying step as well as the cost involved (Chauhan et al., 2014). Other polysaccharide acting enzyme such as amylases have been evaluated in combination with protease and tannase for avoiding the foam formation during the carbonation and bottling process of beverages containing coffee extracts (US4105802 A). The application of mannanase in combination with arabinase and xylanase has proven to improve the shelve-live of liquid coffee extracts from automatic coffee dispensers. Pectinases and mannanases have been evaluated for the reduction of spent coffee ground formation at different enzyme dosages. Two commercial enzyme preparations, a pectinase (Rohapect B1L) and a mannanse (Galactomanannase ACH) resulted in a reduction of the spent coffee grounds to just 3.5% at concentrations that were economically feasible (0.1-0.3 mg/g substrate) (Delgado et al., 2008). Additional soluble coffee of 17% can be generated from SCG when enzymatically hydrolysed using recombinant mannanase (Jooste et al., 2013).

The polysaccharide solubilization from SCG by enzymatic action requires selection of the appropriate enzymes. Each enzyme acts on a specific component of its polysaccharide substrate, however considering the SCG substrate complexity in polysaccharides and its diversity, a pool enzymatic activities is likely to perform better than individual enzymes (Chiyanzu et al., 2013). Wild fungi have been isolated from SCG searching for potential enzymes, as well as commercial extract has been applied on SCG hydrolysis (Jooste et al., 2013). A combination between endoglucanase, polygalacturonase and mannanase in a cocktail of enzymes showed an advantage in the solubilisation of SCG components. Mannanase is required for the hydrolysis of mannan based polysaccharides (gluco-mannan, galacto-mannan and galacto-gluco-mannan) while a typical cellulase cocktail is comprised of three enzymes: endoglucanase, exoglucanase and β-glucosidase, all of which are required for complete hydrolysis of cellulose and glucose release. Several attempts have been made to apply enzyme processing with carbohydrate hydrolases so as to improve coffee quality and economics. The SU Patent 1597151 claims the production of high-quality soluble coffee and improved economics by extracting extra solubles from SCG by a combination of β-glucanase and pectinase. This extra solubles are then mixed with those obtained in the primary extraction of roasted ground coffee performed at mild conditions. The combination of wet milling and enzymes mannanase and cellulases has been applied to successfully obtain soluble coffee at optimal yield with reduced thermal degradation (EP 1745702-B1). The combination of pretreatment and enzymatic hydrolysis with mannanases and/or cellulases has also been proposed to improve the yield of soluble coffee extraction and/or treatment of the spent coffee grounds (US Patent 4,983,408; Kasai et al., 2006; Chiyanzu et al., 2013; Chiyanzu et al., 2014).

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Enzymes in SCG Biorefinery

Biorefinery technologies for the conversion of lignocellulosic residues into biofuels and bioproducts can be based on enzyme-based processes. There are numerous enzymes activities required for lignocellulose decomposition. Lignocellulosic materials are degraded in nature by oxidative and hydrolytic enzymes secreted by different bacteria and fungi. Given that lignocellulosic residues such as SCG are mainly made of polysaccharides, the main enzymes of interest on a biorefinery are those acting on carbohydrates, either cleaving, modifying or generating glycosidic bonds. For example, glycosides hydrolases are a varied group of enzymes that hydrolyse glucosidic bonds between a non-carbohydrate and carbohydrate moiety or between two or more carbohydrates. Precisely the core enzymes involved in lignocellulose degradation, generally called cellulases, belong to these types of enzymes. The main cellulolytic enzymes are endo-1,4-β-D-glucanases, exo-1,4-β-D-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases. The fungi *Trichoderma reesei* is the predominant industrial cellulolytic enzyme producer with the ability of secretion of enzyme for degradation of crystalline cellulose. Endo-glucanases reduce the degree of polymerization of cellulose by randomly hydrolyzing internal β-1,4-glucosidic bonds in the cellulose chain, mainly the amorphous regions. Exoglucanases (CBH) reduce the length of cellulose chains by releasing cellobiose units from both reducing and non-reducing ends. The cellooligosaccharides and cellobiose units released from endo-glucanase and exo-glucanase activity and then cleaved by β-glucosidases releasing glucose.

Other glycosidic hydrolases are required for the degradation of hemicellulose that, owing to its heterogeneous structure, will require a more diverse set of enzymes for its complete conversion to monomers. The main enzymes involved in the saccharification of linear mannans (pure mannan and glucomannan) are β-mannosidases and β-glucosidases (Yamabhai et al., 2016). Similarly to cellulose hydrolysis, the β-mannanases act inside the backbone mannan chain releasing short mannooligosaccharides (MOS). These MOS are the substrate of the β-mannosidases that release mannose from the non-reducing ends of the MOS and hydrolyse mannobiose into mannose. The β-glucosidases will act at the non-reducing end of the oligomers derived from the degradation of glucomannan and galactoglucomannan releasing 1,4-glucopyranose units. The removal of side groups such as galactose and acetyl groups from mannan requires additional enzymes such as α-galactosidases and acetyl mannan esterase, respectively. The degree of removal of these substituents will be determined by the substitution pattern that inhibits the exo-acting enzymes. Other enzymes such as polygalacturonidase, pectinase, xylanase and amylase could act positively on the material hydrolysis depending on the SCG chemical composition.

In addition to polysaccharide hydrolases, there are other enzymes involved in lignin degradation, or hydrolysis of the carbohydrates-lignin complex. Polysaccharide modifying enzymes as well as the accessory enzymes required in lignocellulose degradation are described in the Carbohydrate Active enZYme classification (CAZY) database (http://www.cazy.org/) that groups structurally related catalytic or carbohydrates domains into families. These families share similar 3D structure, mechanism of catalysis and catalytic residues but may differ on substrate specificity. Up to date, there are 135 families of glycoside hydrolases, 98 families of glycosyl transferases, 23 of polysaccharide lyases, 16 families of carbohydrate esterases and 13 of auxiliary activities.

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The complex structure of lignocellulose requires a pretreatment in order to open up the structure of cellulose and hemicellulose and make them accessible to the enzymes. Diverse type of pretreatments has been applied to lignocellulose to extract compounds and/or modify the physical and chemical characteristics of lignocellulosic biomass. Most pretreatment methods are based on either chemical and/or physical approaches (Alvira et al., 2010). Hydrothermal pretreatment and mechanical disruption of biomass partially removes or redistributes the lignin while altering the cellulose crystallinity for more efficient enzymatic hydrolysis. Mussatto et al. (2012) applied thermochemical pretreatment of SCG for the production of ethanol with a yield of 50.2%. A better ethanol yield of 85% was obtained by simultaneous saccharification and fermentation (SSF) with cellulases and pectinases of popping pretreated SCG (Choi et al., 2012). Chiyanzu et al. (2013) evaluated different conditions of steam explosion to increase enzyme digestibility of SCG for the production of extra soluble solids and mannooligosaccharides (2014) to be added to the instant coffee. Aromatic compounds were extracted from SCG by hydrothermal process to produce a distilled beverage (Sampaio et al., 2013).

POTENTIAL APPLICATIONS OF SCG

Steam and Electricity

The world is facing an increasing demand of energy, which is expected to become limited in future supply. On the one hand, the expected future scarcity of fossil fuels has motivated an increasing the demand and global interest for renewable energy (International Energy Agency, 2013). Furthermore, as a consequence of industrial development over the last century, an increase in greenhouse gas emissions and CO_2 levels have been observed, resulting in environmental modification and global warming (Nass et al., 2007). The combination of rising energy demand and environmental concerns has given worldwide attention to the biomass of plant origin, as one the renewable energy options. Biomass can be used production of steam and heat, electricity and liquid biofuels such as bioethanol and biodiesel. Ethanol is produced by sugars fermentation and biodiesel by triglyceride esterification from vegetable oils.

SCG is already burnt in the some coffee factories where it is generated (Silva et al., 1998), which avoids the transport and disposal costs, and environmental impacts due high moisture content. SCG is attractive as an energy source because of its high calorific value of around 25 MJ/kg, which is similar to coal and higher than wood (22 MJ/kg) and sugarcane bagasse (18MJ/kg) (Silva et al., 1998). The use of biomass as feedstock to steam and energy generation is an alternative to fossil fuels, and has already reached success in the coffee industry. Around 2.4 kg (dry base) of SCG produces the same amount of steam as 1 liter of heating oil. This SCG has about 50% of moisture, which impacts negatively on the combustion process, by increasing the requirement for fossil fuels, and on the burner capacity. The moisture content and its impact on combustion is reduced using dryers such as pneumatic, press and rotating dryers with internal tubes, prior to combustion (Silva et al., 1998).

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The thermochemical properties of SCG can be improved by increasing it energy density through conversion to biochar (charcoal). Charcoal is produced by heating to an appropriate temperature in an inert atmosphere, resulting in the decomposition of organic components. This process is named pyrolysis and has been applied to SCG (Tsai et al., 2012). The pyrolysis can be more advantageous than simple combustion, considering the large volume of material, its high moisture content, low density and low combustion efficiency. Alternatively, the SCG can be pelletized to be combusted in commercial and residential boilers. The combustion of SCG in boilers was shown to be efficient, but with low energy recovery when compared to sawdust (Limousy et al., 2013).

Liquid Biofuels: Bioethanol and Biodiesel

Of the biofuel options, ethanol has reached technical and economic feasibility for industrial production and has been produced from several decades in Brazil, the United States and other countries. Brazilian ethanol production is based on sugarcane cultivation for sugar extraction, and in United States the production is from corn starch. Generally, ethanol can be produced from juice extracted of sugarcane, sugar beet and sweet sorghum (sucrose-rich plant) and starch from grains and roots, such as corn and cassava. The sugars (from sucrose or starch) are fermented by microorganisms and distilled to produce the ethanol fuel. This ethanol produced from reserve polysaccharides is named as first generation bioethanol. This process generates a large amount of lignocellulosic residues (sugarcane bagasse and straw, corn cob, straw and stalk) that are rich in structural carbohydrates. The structural carbohydrates (cellulose and hemicellulose) contained in lignocellulosic biomass are suitable to produce ethanol, this approach is defined as second generation ethanol (2G ethanol, cellulosic ethanol). 2G ethanol can be produced using any lignocellulosic biomass, including SCG, forest and urban waste. However, the polysaccharides of the lignocellulosic biomass are of structural, and therefore not easily extracted like sucrose or starch.

Ethanol production from lignocellulosic biomass requires a combination of at least four-step processes: pretreatment, enzymatic hydrolysis, fermentation and distillation (Limayem and Ricke, 2012), with the option to perform hydrolysis and fermentation simultaneously. The potential of SCG as feedstock for ethanol production is due its carbohydrates content, i.e., cellulose and hemicellulose. Considering the polysaccharide types contained in SCG, two process options can be considered: (i) to use sugars from hemicellulose (5-carbon sugars, such as xylose and arabinose, and 6-carbon sugars such as mannose and galactose); (ii) to use sugars from cellulose (6-carbon sugars, glucose). For hemicellulose sugars recovery an acidic pretreatment can be used (Mussatto et al., 2012), while glucose recovery from cellulose is obtained with an enzymatic hydrolysis of a pretreated material (from the acid pretreated or steam exploded material) (Chiyanzu et al., 2013). The simultaneous fermentation of both the C5 and C6 sugars to ethanol is not a common property of microorganisms. One of the areas of research where more focus is directed is the development of robust fermentative strains with the ability of conferment both pentoses and hexoses. Examples of recent strain developments are CelluX™ and C5 FUEL™. CelluX™, from LEAF Technologies (France) a genetically modified strain of *Saccharomyces cerevisiae* with high tolerance to stresses during fermentation of cellulosic materials. C5 FUEL™, from Lallemand (Canada), is also a strain

of *S. cerevisiae* quite robust towards pretreatment inhibitory compounds besides having the ability of xylose fermentation.

The proposal of to use SCG as feedstock to ethanol production is recent and several improvements to this process are still required. Acidic pretreatment is effective for sugars recovery from hemicelluloses for the subsequent fermentation step. The fermentation of the C6 sugars in the hydrolysate by *S. cerevisiae* resulted in ethanol production of 11.7 g/L with efficiency of 50.2% (Mussatto et al., 2012). The authors also reported that other microorganism such as *Pichia stipitis* and *Kluyveromyces fragilis* performed less effective than *S. cerevisiae*. Acidic hydrolysate of SCG was fermented by *S. cerevisiae* and released 19 g/L of ethanol in 10h of process time (Rocha et al., 2014). The authors proposed first to extract the SCG oil for biodiesel production and residue from this process, oil-free SCG, submitted to the ethanol production process. A combined cooking followed by fermentation of SCG resulted in an overall ethanol yield of 018-0.22 L $kg^{-}1$ dry spent coffee grounds (Tehrani et al., 2015).

The SCG also contains from 7 to 20% (dry bases) of lipids with reduced content of caffeine and unsaponifiable lipids compared with the fresh coffee bean. As a consequence, these oils constitute a more appropriate raw material for biodiesel production than fresh coffee, resulting in a biodiesel of higher quality (Evans, 2014). However, the oil extraction from SCG at large scale requires technical improvement. Laboratory extraction of oil from SCG used methods such as soxhlet (time-consuming and a large amount of solvent), supercritical carbon dioxide (high pressure and temperature) and ultrasound-assisted extraction, which reduced process time and temperature, thus increasing the oil yield (Rocha et al., 2014). The authors reported ultrasound-assisted extraction of 12% of the oil from SCG, with a composition rich in palmitic and linoleic acids. Furthermore, they suggested SCG may be used as feedstock for biodiesel production, by a combination of ultrasound-assisted extraction with saponification, followed by acid hydrolysis and esterification with methanol (Rocha et al., 2014). The transesterification process for the production of biodiesel can be catalyzed by lipases enzymes at mild conditions of temperature and facilitate the downstream processing (Banerjee et al., 2013).

Considering the SCG composition and complexity, it has been suggested that biodiesel and ethanol may be produced in sequential steps (Rocha et al., 2014; Kwon et al., 2013). A first step of oil removal (e.g., soxhlet extraction with hexane during 6h), prior to the ethanol production process, provided benefits in terms of improved enzymatic hydrolysis (Kwon et al., 2013). The difficulties with enzyme action on oil-rich material could thus be avoided by oil extraction or a pretreatment to partially remove the oil before hydrolysis-fermentation. Such, an integrated process of biodiesel and ethanol production could be an interesting biorefinery concept for the use of multiple components of SCG.

Cultivation Media

Pigments and phenolic compounds are well known for their antioxidant activity and they have been commercially used as food colorants, animal feed supplements and, more recently, as nutraceuticals for cosmetic and pharmaceutical purposes. The toxicity of synthetic antioxidants coupled with the consumer's preference of natural and secure nutritional additives has prompted research towards natural sources as raw materials for antioxidants for

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food, cosmetics and pharmaceuticals. Solid-state fermentation (SSF) has become a suitable bioprocess for the management of agro-industrial residues as well as value addition. Utilization of low-cost industrial by-products as substrate for the production of enzymes is considered an effective way to reduce enzyme production costs as well as reduce waste disposal and treatment. SCG is an ideal substrate for SSF that can be used also for the production of secondary metabolites such as flavors, organic acid, pigments, etc. The structural components of these materials may be used as carbon, energy and nutrient sources for microbial growth.

Filamentous fungi are well adapted for bioconversion of solid substrates during SSF due to its tolerance to conditions of high osmotic pressure and low water activity. Fungal strains such as *Penicillium purpurogenum*, *Neurospora crassa* and *Mucor* have been proven to grow in SCG while producing significant amount of phenolic compounds that can be used in food and pharmaceutical industries (Machado et al., 2012). SCG can also be converted into carotenoids (30 mg/L) via fermentation by red-yeasts such as *Sporidiobolus roseus* (Petrik et al., 2014; Obruca et al., 2015).

SCG was a suitable substrate for the production of α-amylase by *N. crassa* when combined with other coffee solid residues (Murthy et al., 2009). SCG has been also evaluated for xylanase production, but other coffee residues such as coffee pulp were better suited for the production of this enzyme (Murthy and Naidu, 2012b). Coffee pulp, coffee waste waters, coffee husks and coffee mucilage have been proven good substrates for production of enzymes that can be applied in coffee processing such as pectinases (Murthy and Naidu, 2011) and treatment of coffee residues such as tannases and decaffeinases (Battestin and Macedo, 2007; da Luz et al., 2012; Gokulakrishnan et al., 2005).

Agriculture Application

A potential application of SCG has been studied is the conversion to compost for direct use in agriculture, where the SCG micronutrients collaborate positively. The studies have been carried on pot and field experiments on horticultural products. The SCG was directly composted in the soil, increasing the plant chlorophylls and carotenoids levels, and producing significant amounts of ascorbic acid and tocopherols. Increases in nutritional minerals, specifically potassium, phosphorous and sodium, was also observed. The SCG is attractive to compost because of the nitrogen content, with a C:N ratio of 20, similar to manure. The use of 5 to 30% of SCG for the cultivation of lettuce (model plant) resulted in healthy plants with green color intensity correlated to substrate percentage (Cruz et al., 2015). Conversion of SCG to valuable applications is preferred to disposal without any treatment and exposure to weather. Under such circumstances the SCG may start to ferment, also leading to spontaneous combustion (Silva et al., 1998).

Biomaterials

Apart from biotechnology application, SCG can be used as additive to bricks and composites improving the general characteristic of these materials in terms of mechanical,

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thermal and water uptake properties. The addition of 17% of SCG to bricks formulation mixture can reduce the thermal conductivity, although simultaneously decreasing porosity and consequently water absorption. The resulting bricks must be coated to avoid moisture absorption and cannot be used for decorative purposes. The advantage of SCG addition is to increase the compressive strength of the brick, improving suitability for structural purposes (Velasco et al., 2015). In the case of ceramics, addition of 10% of SCG improved mechanical strength, while the addition of 20% of SCG decreased the thermal conductivity by 70% (Fonseca et al., 2014).

Animal Feed

The protein content of SCG allows its application as an animal feed ingredient. The nutrients and bio-active compounds in SCG primarily motivated this application. The use of SCG added to feed is an old concept (Campbell et al., 1976), although still receiving recent research interest (Seo et al., 2015). The SCG has been studied and proposed for several kinds of animal feed, with different proportion recommended: ruminants (10-30%), pigs (10-24%), fish (13-30%) and chicken (2.5-5%) (Marcel et al., 2011). The suggested proportions were based on limiting the effect on the animal physiology and health, and providing good animal performance. However, increasing the amount of SCG added to animal feed can disturb the growth and animal health, due to the residual substances of caffeine, phenolic compounds such as chlorogenic acid, caffeic acid and tannins. These compounds are recognized as anti-nutritional factors and can limit protein availability while altering digestive enzymes and microorganisms in the gut. Alternatives to decrease the content of these components in SCG is ensiling, chemical extraction (alcohol) or microbial (specific microorganism fermentation) treatment. The use of microorganisms to decaffeinate the coffee waste has been proposed as a way to both reduce the toxicity of these residues as well as improve their potential application including animal feed (Summers et al., 2014). Some research suggested the processing of SCG with a low amount of sugarcane molasses to improve silage production (Bressani, 1991). The resulting silage is a fermented material used for animal feed (ruminants) or used as feedstock for anaerobic digesters. Ensiling has been proved as efficient for removal of the non-nutritional compounds of SCG (Moreau et al., 2003). Fermenting SCG with *Lactobacillus* spp improved the nutritional value, although increasing the cost of the final product (Table 2).

For successful application in agriculture, SCG from a decaffeinated process is more appropriate. There are some industrial processes and coffee machines that are efficient for the extraction of such compounds. SCG obtained from some coffee preparation using filter, espresso, plunger and mocha from coffeemaker showed different content of phenolic. SCG from mocha coffee maker showed traces of phenolic compounds, and higher content on the coffee brew. Caffeine was extracted in a higher amount from SCG obtained from filter preparation (Bravo et al., 2012).

Antioxidant

Residual compounds present in SCG include antioxidants that are of interest for different industrial applications such as nutritional foods and cosmetic additives. The antioxidant compounds can protect from free radical damage, thus reducing the risk of degenerative diseases. The components of interest in SCG are caffeine, chlorogenic acids and caffeic acid, which can be present in different quantities, depending on the coffee production process. Caffeine is recognized to promote energy metabolism. Chlorogenic acids are suggested to be involved in the prevention of atherosclerosis, diabetes and some types of cancer. Caffeic acid has antioxidant activity related to beneficial effects of anti-inflammatory, antimutagenic, antibacterial and anticarcinogenic properties. These effects are not totally clear, but caffeic acid is able to chelate iron ions, thus minimizing the Fenton-generated hydroxyl radicals and lipid peroxidation (Genero-Mattos et al., 2015).

Table 2. Chemical composition of SCG and Fermented SCG (*Lactobacillus* spp)

Component (g/kg)	SCG	Fermented SCG
Dry mass	550	925
Crude protein	138	141
Ether extract	136	157
Ash	20	18
Neutral detergent fiber	656	792
Acid detergent fiber	451	526
Lignin	142	146
Neutral detergent insoluble crude protein	105	114
Acid detergent insoluble crude protein	95	100
Non-fiber carbohydrate	155	6

Adapted: Seo et al., 2015.

Table 3. Total phenolic, flavonoid, chlorogenic and caffeic acid and antioxidant activity from extracts from SCG using water and ethanol/water mixture

	water	ethanol/water (3:2 v/v)
Phenolic (mg GAE/g)	19.6	28.2
Flavonoids (mg QE/g)	3.2	5.63
Antioxidant -EC_{50} (%,v/v)	2.3	1.4
Chlorogenic acid (mg CQAE/g)	6.67	5.97
Caffeic acid (mg/g)	11.2	11.5

Adapted: Panusa et al., 2013.

Solvents like ethanol or a mixture of ethanol-water have been shown efficient for phenolic extraction (Table 3). SCG from an espresso capsule showed a total phenolic content of 35.5 mg GAE/g, of which 55% could be extracted with water and 79% with ethanol/water in a proportion of 3:2 (Panusa et al., 2013). The ethanol/water mixture allowed extraction of more total phenolics and flavonoids than using only water while chlorogenic and caffeic acid were extracted at similar proportion for both solvents. The antioxidant activity, however, was

higher for extract using water (Panusa et al., 2013). Subcritical water is an extraction method alternative to solvent use. Subcritical water (5 MPa, 179°C, 36 min, solids loading of 14.1 g/L) (pressurized hot water) extraction of phenolic compounds of 86 mg GAE/g and antioxidant activity of 42 mmol TE/100g, using SCG from Arabica was reported (Xu et al., 2015).

Polyhydroxyalkanoates (PHAs)

SCG contains about 15% of oil, which can be extracted using hexane and used as a substrate to produce polyhydroxyalkanoates (PHAs) by microorganisms (Obruca et al., 2014a,b). PHAs are hydroxyacid polyesters and they have properties similar to fossil-fuel-derived polypropylene and polyethylene. The PHAs have as main advantage the possibility to be produced from wastes and it is biodegradable. Hexane extraction of SCG solubilized fatty acids such as palmitic (35.7%), linoleic (43.7%), oleic (9.4%), stearic (7.1%), arachidic (2.2%), α-linoleic (1.1%), eikosenoic (0.3%) and behenic (0.4%) (Obruca et al., 2014a). Supercritical fluid extraction with carbon dioxide showed similar fatty acids extraction content compared to soxhlet extraction (Melo et al., 2014). PHAs were also produced using glucan and hemicellulose hydrolysates (dilute acid and enzymatic hydrolysis). Bacteria *Burkholderia cepacia* has the ability to utilize hexoses and pentoses sugars. For utilization by this microbe, the sugar hydrolysate must be detoxified to remove the toxic compounds that may negatively impact on the growth of the microorganisms (Obruca et al., 2014b).

Mannooligosaccharides

Oligosaccharides that are not being digested are known to be beneficial to health and they are recognized as prebiotics. Not only they promote the growth of beneficial bacteria such as *Bifidobacterium* species, but also, they suppress the growth of harmful bacteria (*Bacteroides* and *Enterococcus* species) in the colon. An example of these probiotics are the mannooligosaccharides (MOS) derived from coffee mannan. MOS are classified as prebiotic compounds that are non-digestible carbohydrates, which stimulate the growth or activity of beneficial bacteria in the digestive gut. Oligosaccharides are used in animal and human feed to improve gastrointestinal health, growth performance and modulate the microbiota. MOS have been studied for its effect on digestibility and fermentability (Asano et al., 2003), intestinal bacterial consortium (Asano et al., 2001) and fecal microflora (Asano et al., 2004).

Coffee with increased MOS content present prebiotic effects in animals and humans. Coffee mannan is insoluble so mannooligosaccharides are not extracted during conventional coffee preparation methods, thus remains in the spent coffee grounds. However, they can be obtained a secondary high-temperature extraction of spent grounds during commercial soluble coffee production and then combined with the original extract. The elevated temperatures required for coffee solubilization, however, may lead to off flavors and add to capital costs. Endo-mannanase enzymes can breakdown the mannan polysaccharides, thus releasing MOS. However, mannan is associated to other polysaccharides as they protect themselves by hindering enzymatic action. The lignin present in SCG acts as an extra barrier to enzyme action. Various kinds of pretreatment can be applied for the production of MOS and/or to

improve the accessibility of polysaccharides to enzymes. MOS from SCG has been obtained at laboratory scale by using high-pressure steam at a high temperature (220°C for 8 minutes) (Asano et al., 2003). The production of MOS from SCG through combined physicochemical pretreatment and/or enzymatic hydrolysis has also been reported. Pretreatment such as dilute acid, hot water (hydrothermal) and steam explosion can release oligomers and monosaccharide from lignocellulosic materials, primarily from the hemicellulose, of which the majority is mannan. Moreover, the combination of steam pretreatment with subsequent enzymatic hydrolysis can increase the yield of mannooligosaccharides (MOS) from SCG (Chyianzu et al., 2014). Considering that the polysaccharides are present in a highly organized network and cell wall, it is reasonable to expect that different enzymes will be required for hydrolysis, in addition to a pretreatment exposing mannan to mannanase action. In fact, the use of an enzymatic cocktail containing several enzyme activities (mannanase, endoglucanase, exoglucanase, xylanase and pectinase) can provide an extensive hydrolysis of SCG polysaccharides (Jooste et al., 2013). The combination of mannanase with other enzymes such as cellulase has been proven more successful for MOS release from SCG (Chyianzu et al., 2014). SCG has been successfully pretreated with steam explosion, improving enzymatic hydrolysis for MOS release and the required enzyme dosage. Combined steam explosion and enzymatic hydrolysis with a cocktail of mannanase and cellulase could release 57% of the mannan to MOS, with a degree of polymerization of up to 6 (Chyianzu et al., 2014).

The MOS produced from SCG could be added to coffee products (Chyianzu et al., 2014). Supplementation of instant coffee with MOS as prebiotic compounds can contribute additional health benefits, generating a nutraceutical food. Considering the industrial point of view, such a practice will reduce the amount of SCG generated, and increase the coffee yield production.

CONCLUSION

Coffee bioproducts are among the most important waste generated from the industrial and agro-industrial process. The amount of SCG generated from instant coffee production poses environmental concerns if not properly stored and disposed. The SCG as feedstock represents a challenge to convert to an added-value product because of its complex structure, composition and carbohydrate and non-carbohydrate compounds organization. Furthermore, SCG is a highly heterogeneous material with properties that depend on the coffee origin, geographical distribution and the industrial processes of instant coffee extraction.

From the industrial and biotechnology point of view, SCG is of significant interest because of its diverse composition and thus different possible applications. The SCG waste can be used in the different field such as energy, food and pharmaceutical bioproducts production. Currently, the main industrial use of SCG is in steam and energy generation by the combustion process. Among of the several bioproducts that can be generated using SCG as feedstock, the most interesting is its conversion to oligosaccharides such as mannooligosaccharides. MOS are important as an ingredient of nutraceutical food and also can be added to instant coffee, improving the health benefits of the coffee products. This application could be interesting allowing the process of MOS production *in situ* with the

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instant coffee production industry, and avoiding costs of transport and disposal of SCG. This application could attend to both the waste management and environment conservation. Furthermore, considering SCG as feedstock, recent publications have shown an increasing potential of bioactive and polymers for biomaterials. The importance of energy and biofuel production rise attention of SCG as substrate to ethanol and biodiesel production. Biotechnological applications of SCG still require further research to develop technologies and industrially-feasible processes.

ACKNOWLEDGMENT

The authors are grateful to Cocam – Cia. De Café Solúvel e Derivados (Catanduva-SP, Brazil) for providing the spent coffee ground. The authors also thank Dr Celso Sant'Anna and Vânia Vieira from National Institute of Metrology, Quality and Technology for Electron Scanning Microscopy images.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 5

THE RE-USE POTENTIAL OF ESPRESSO SPENT COFFEE GROUNDS: OPTIMIZATION OF MICROWAVE-ASSISTED EXTRACTION OF POLYPHENOLS BY USE OF RESPONSE SURFACE METHODOLOGY AND THEIR EFFECTS ON PLATELET FUNCTION – PILOT STUDY

Marija Ranić[1,*], Aleksandra Konić-Ristić[1], Marija Glibetić[1] and Suzana Dimitrijević-Branković[2]

[1]Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, Belgrade, Serbia

[2]Faculty of Technology and Metallurgy, Department of Biochemical Engineering and Biotechnology, University of Belgrade, Belgrade, Serbia

ABSTRACT

A huge amount of espresso spent coffee grounds (SCG) are obtained as a waste product during the preparation of coffee beverages (cialdes, K-cups and in metallic filters). Although considered a waste and an ecological threat, SCGs could be an excellent source of bioactive components (mainly polyphenols) with outstanding antioxidative activity and potential to be used as raw material in fortified food production.

The antiplatelet action of dietary polyphenols represents an important part of their pleiotropic action, and underlying mechanisms of their shown beneficial effects on cardiovascular health. Accordingly, a reduction in platelet aggregation was accepted by EFSA as a beneficial effect of food and dietary constituents, in the context of maintaining cardiovascular health enabling claims related to foods or substances affecting disturbed platelet function. Thus, platelets are considered an emerging target for the prophylactic

* Corresponding author: Marija Ranić, PhD, Institute for Medical Research, Centre of Research Excellence in Nutrition and Metabolism, University of Belgrade, Tadeuša Košćuška 1, 11129 Belgrade, Serbia, Tel:+38111328-1564, Fax: +381112030-169, e-mail: marija.ranic@gmail.com.

effects of food bioactives in cardiovascular disease (CVD), with promising scientific evidence of their efficacy.

The overall objective of this study was to examine an optimal range of extraction conditions for the extraction of polyphenols from espresso SCGs by using response surface methodology and to investigate the *in vitro* effects of obtained polyphenol-rich extracts on platelet activation and their aggregation with monocytes and neutrophiles.

The obtained results indicated that espresso SCG extracts induce dose dependent inhibition of platelet activation, shown by the decrease in expression of P-selectin (8.7%) and GPIIb/IIIa (5.6%) and their aggregation with monocytes and neutrophiles (up to 20%). The sustainability of the coffee processing system can be substantially improved through the use of by-products by the adoption of new technologies that maximize process profitability. Shown antiplatelet effects of polyphenols, present in espresso coffee SCGs, indicate a favorable profile of bioactive compounds toward platelet function and thereby rationalize their use as functional food ingredients and nutraceuticals aiming to promote cardiovascular health.

Keywords: spent coffee grounds, polyphenols, platelets, response surface methodology, microwave assisted extraction, flow cytometry

INTRODUCTION

Currently available information from epidemiological research suggests that coffee consumption is associated with a lower risk of several chronic diseases including type 2 diabetes, cardiovascular disease (CVD) and cancer, as well as neurodegenerative conditions such as Parkinson disease (Ludwig et al., 2014). The beneficial effect of coffee consumption is, at least partly, attributed to the action of polyphenols, the antioxidant phytochemicals present in coffee (Vignoli et al., 2011; Cammerer and Kroh, 2006). Recent accumulating evidence suggests pleiotropic preventive effects of dietary polyphenols in CVD mediated by their action on multiple cellular and molecular targets (Kishimoto et al., 2013). Platelets are considered an emerging target for the prophylactic effects of food bioactives in CVD with the promising scientific evidence of their efficacy. Antiplatelet action of dietary polyphenols represents an important part of their pleiotropic action, and underlying mechanisms of their shown beneficial effects on CV health (Konic-Ristic et al., 2015). Accordingly, a reduction in platelet aggregation was accepted by EFSA as a beneficial effect of food and dietary constituents, in the context of maintaining CV health enabling claims related to foods or substances affecting disturbed platelet function (EFSA Panel, 2011). It was shown both in human and animal studies, *in vitro*, *in vivo* and *ex vivo* that coffee extracts decrease platelet aggregation, induced by different agonists (adenosine diphosphate (ADP), arachidonic acid or collagen) (Silverio Ados et al., 2013; Schumacher et al., 2011; Bhaskar et al., 2010) and also induces a significant increase in phenolic acid platelet concentration (Natela et al., 2008).

The industry of soluble coffee brings almost 50% of the world coffee production, with a proportional amount of spent coffee residues. Another 50% of residue refers to spent coffee, resulting from the preparation of various coffee beverages (Cruz at al., 2012). As a result of espresso coffee beverage preparation (cialdes, K-cups and in metallic filters), a huge amount of solid residue, known as spent coffee grounds (SCG), is dispensed as a waste product. It was estimated that only from the Province of Rome over 10,000T of espresso SCGs are

available per year (Zuorro at al., 2012). Evidence to date suggests the potential of SCG, as a source of natural phenolic antioxidants (Yen et al., 2005; Bravo et al., 2012; Cruz et al., 2012; Zuorro et al., 2012; Panusa et al., 2013; Ranic et al., 2014), particularly chlorogenic acids, whose beneficial effects on human health have received increasing attention (Panusa et al., 2013).

However, despite the large amount of evidence implicating SCGs as an important source of coffee bioactives with anti-oxidative activity and shown health effects, the exploitation of SCGs in the production of functional food and nutraceuticals stays mainly unreported. In this context, SCG re-use should be based on the bioactivity-guided optimization of the extraction process, the evaluation of their potential mechanisms, and the health effects of the final products.

The overall objective of this work was to identify the optimal range of extraction conditions and develop an effective and eco-friendly microwave assisted extraction (MAE) to optimize the extraction of phenolic compounds from samples of spent espresso coffee, using the response surface method (RSM). The optimization process was guided by a complex correlation between three factors: total phenolic content and antioxidant activity determined by two mechanistically different assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) decolorization assay and ferric reducing antioxidant power (FRAP) assay.

We hypothesized that espresso SCGs may exert potent anti-thrombotic activity. To test this hypothesis and to confirm the efficient optimization towards the extraction of biologically active components, we examined the effect of the espresso SCG extracts on platelet activation and platelet-leukocyte aggregates (PLA) formation *in vitro* using flow cytometry.

METHODS

Spent Coffee Preparation

A total number of samples “Doncafe - Espresso Aromatico – cialde” were donated by Strauss-Adriatic d.o.o. Doncafe Espresso Aromatico is a blend obtained from Arabica (around 40%) originated from Santos, Guatemala, Columbia, India and Honduras; Robusta (around 60%) originated from India and Vietnam (depending on the current offer on the market,while taking into accountsimilarity in composition). Espresso coffee was prepared on an Italian Frog Espresso Coffee Pod Machine (Power: 450W; Voltage: 230/120V) that uses cialda – pre-packaged espresso ground coffee in coffee pods, previously described (Ranic et al., 2014).

RSM Experimental Design

The RSM was employed to optimize the MAE of phenolic compounds from SCG samples and to determine the optimum extraction characteristic by employing the central composite design (CCD) (Cochran et al., 1992). Based on initial optimization the low concentration ethanol (20%) in aqueous solutions was employed as non-toxic extracting media (Pavlović et al., 2013). By using the RSM we investigated the effects of extraction time

(ET), liquid-to-solid ratio, (LSR) and microwave power (MWP) on total phenolic content (TPC) of the obtained extract.

The experimental domain was defined taking into account the results obtained in preliminary tests, as well as the operational limits of the instrument and all significant parameters in a typical MAE process were chosen as independent variables: ET (A=11.0-209.0s), LSR (B=4.76-13.24 ml/g) and MWP (C= 240 - 560W). The variables A and B have been coded at 5 levels; A:-α (11s), -1 (40 s), 0 (110 s), 1 (180 s), +α (209 s) and B: -α (4.76 ml/g), -1 (6 ml/g), 0 (9 ml/g), 1 (12 ml/g), +α (13.24 ml/g); while variable C was coded at 3 levels: -1 (240 W), 0 (400 W), 1 (560 W). The response variable were: total phenolic content (TPC; % (w/w) in extract), FRAP (mmolFe^{2+}/l) and DPPH (% of DPPH inhibition). The complete experimental design consists of 30 experiments performed in order to estimate the coefficients of the model and the results. All reactions were conducted in triplicates. Experimental runs were randomized in order to minimize the effects of unexpected variability in the observed responses. The data from CCD were analyzed by multiple regression to fit to a second-order polynomial regression model containing the coefficient of linear, quadratic, and two factor interaction effects.

Total Phenols and Antioxidant Capacity

The total phenolic content (TPC) of spent espresso coffee extracts was determined using Folin-Ciocalteau reagent as previously described (Ranic et al., 2014) and expressed as gallic acid equivalents (GAE). The correlation between polyphenol content and antioxidant activity was studied by employing a DPPH assay, based on the decolorization of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assay, based on interference with the reduction of ferric ion-TPTZ (2, 4, 6-tri (2-pyridyl) - 1, 3, 5-triazine) complex described (Ranic et al., 2014).

Antiplatelet Activity

The role of platelets in the pathogenesis of atherosclerosis and thrombosis is a result of their interactions with other platelets, monocytes, neutrophils and endothelial cells that occurs upon platelet activation. This effect is induced by various physiological and patho-physiological, endogenous and exogenous factors. Platelets interact with other cells via their surface antigens, P-selectin and GPIIb/IIIa (Ampofo et al., 2015). The activated form of GPIIb/IIIa, a receptor for fibrinogen, mediates platelet-platelet aggregation and is crucial for the effective hemostatic plug formation. On the other hand, P-selectin binds to its counter-receptor P-selectin glycoprotein ligand (PSGL)-1 on leukocytes (monocytes and neutrophils) and contributes to the pathogenesis of atherosclerosis.

Platelet surface P-selectin and GPIIb/IIIa expression and platelet–leukocyte aggregates were measured by full-blood flow cytometry according to a previously published protocol (Krueger et al., 2002; Barnard et al., 2003; Frelinger et al., 2006) with slight modifications for *in vitro* testing of the anti-platelet action of the investigated agents. Whole blood samples were obtained by venipuncture from healthy human donors, according to the guidelines for blood sampling in the analysis of platelet activation and aggregation (Krueger et al., 2002;

Barnard et al., 2003). Immediately after venipuncture, aliquots (360 μL) of whole blood were dissolved in HEPES–Tyrode's buffer (HTB) (1:10) and incubated with working solutions of investigated espresso SCG extracts for 10 min at 37°C. Incubation with DMSO is used as a negative control. The SCG extracts were standardized to a final concentration of 100, 50 and 25 μgGAE/ml (gallic acid equivalents) for the determination of platelet activation markers, of the anti-platelet action of investigated agents. Immediately after venipuncture, aliquots (45 μL) of treated whole blood samples (dissolved (1:10) in HTB) were stimulated with a platelet agonist (ADP, final conc. 250 μg mL−1), briefly prior to incubation with monoclonal antibodies (or isotope control) PAC1-FITC, CD61-PerCP, CD62-PE, for 20 min in the dark at room temperature. Subsequently, samples were fixed in CellFix solution (350 μL) for 15 min and analyzed.

For the determination of platelet-monocyte and platelet-neutrophil aggregates, the same procedure as for the determination of platelet surface P-selectin and GPIIb/IIIa expression was followed. The treated whole blood samples were stimulated with a platelet agonist (ADP, final conc. 250 μg mL−1) immediately prior to incubation with the monoclonal antibodies (or isotope control) CD61-FITC, CD11b-PE and CD14-PerCP for 15 min in the dark at room temperature. Post-incubation samples were treated with 1 mL of erythrocyte-lysing buffer (CellLysing solution) for 10 min, washed twice in HTB, fixed in fixing buffer for 15 min and analyzed.

Sample analysis was performed in a Becton Dickinson FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). The platelets were identified by their known light scattering properties and the expression of the pan-platelet marker CD61. Positive analysis regions for P-selectin and GPIIb/IIIa were set with appropriate non-specific controls. Monocytes and neutrophils were identified by light scatter properties and the differential expression of CD14 and CD11b, respectively. Staining with isotype-matched monoclonal antibodies was used as a negative control. The expression of platelet specific CD61 in the population of monocytes and neutrophils indicated the formation of platelet–leukocyte aggregates.

The antiplatelet effects of the monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated PAC1 (anti-GPIIb/IIIa antibody), FITC-conjugated anti-CD61, phycoerythrine (PE)-conjugated anti-CD62P, PE-conjugated anti-CD11b, peridinin chlorophyll protein (PerCP)-conjugated anti-CD61, PerCP-conjugated anti-CD14, and isotype controls, designed to measure the level of non-specific background signal caused by primary antibodies (IgG1-FITC, IgM-FITC, IgG1-PE, IgG2-PE, IgG1-PerCP and IgG2-PerCP) were used.

Statystical Analysis

The RSM model adequacy was determined by evaluating the lack of fit, coefficient of determination (R^2), and the Fisher test value (F-value) obtained by the analysis of variance (ANOVA) that was generated by the Design Expert 8 software (Stat-Ease, Inc., Minneapolis, MN, USA). The differences were considered significant if $p < 0.05$. Optimal independent variables (extraction conditions) for maximized responses were pre-established by superimposing the corresponding contour plots, by which two subsequent confirmatory experiments were carried out to validate their equations.

The Wilcoxon signed-rank test was employed to analyze the differences in platelet activation and aggregation markers, in samples pre-treated with investigated extracts compared to DMSO-treated samples. Differences were considered significant at $p < 0.05$. Data are shown as mean ± standard deviation. Statistical analyses were performed with SPSS software (SPSS, Chicago, IL, USA).

RESULTS

Optimization of the Total Polyphenol Content (TPC)

The full experimental and predicted datasets for 30 runs expressed as percent of TPC (%, w/w) in dry SCG extracts are given in the work of Ranic et al. (2014). The yield for TPC ranged from 18.83 up to 79.83% w/w. The maximum yield was recorded under following experimental conditions: 40s extraction time, 240 W MWP and 6-fold solvent to SCG ratio. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y_{TPC}=37.83 - 12.72*A - 3.80*B - 9.66*C + 5.66*A*C + 5.33*B*C - 4.07*B^2 - 11.90*C^2 \quad (1)$$

In Table 1, the analysis of variance (ANOVA) for the regression equation is shown. The linear and quadratic term were very significant ($p < 0.01$). The most significant interaction between independent variables is between A (ET) and C (MWP), and B (LSR) and C (MWP). The value of lack of fit (0,0679) indicating that the model fits the experimental data adequately ($p > 0.05$). For the model fitted, the coefficient of determination (R^2) for model (0.9182) was close to 1.0, which indicated that only 8.18% of total variation was not explained by the model.

Table 1. Analysis of variance (ANOVA) for the experimental results of the CCD

Source	TPC	DPPH	FRAP
P value Prob>F			
Model	<0.0001	< 0.0001	< 0.0001
A	< .0001	< 0.0001	< 0.0001
B	0.0040	0.9684	< 0.0001
C	<0.0001	< 0.0001	< 0.0001
AC	0.0007	< 0.0001	< 0.0001
BC	0.0013		
A^2		0.0117	0.0005
B^2	0.0087	0.0057	
C^2	<0.0001		< 0.0001
Lack of Fit	0.0679	0.1377	0.4664
R-Squared	0.918165	0.984501	0.943311
Adj R-Squared	0.892127	0.980458	0.928522
Pred R-Squared	0.844539	0.97341	0.90661
C.V. %	13.667171	3.710808	8.324233

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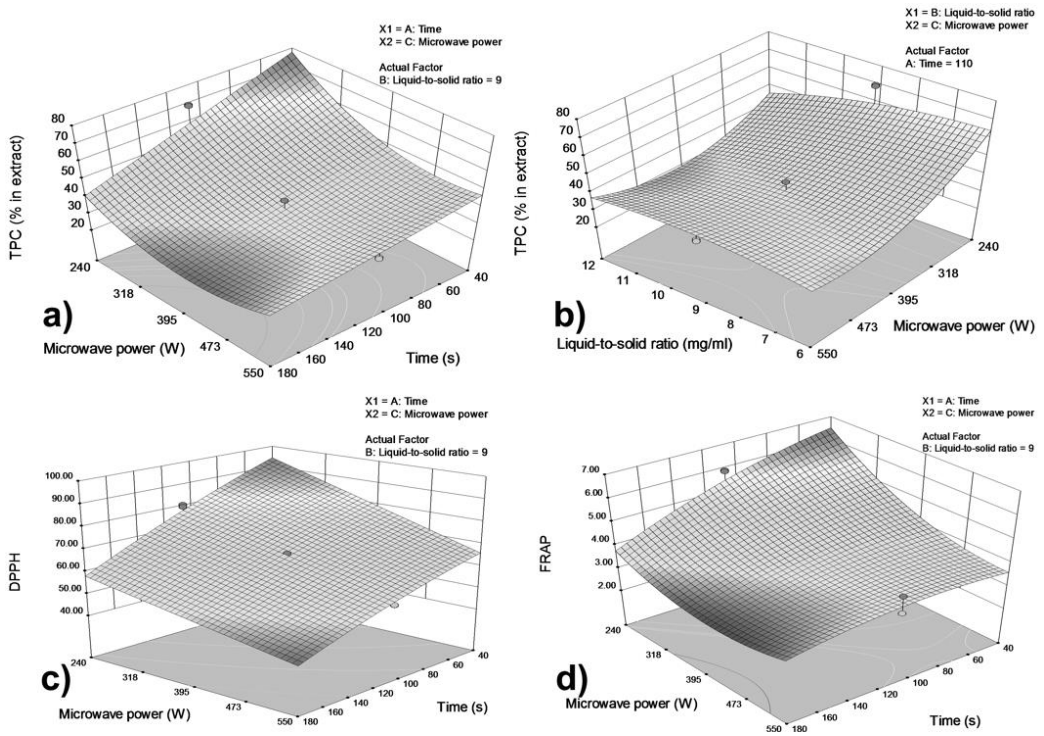

Figure 1. Response surface plots showing the combined effects of ET(A) and MWP(C) to TPC (a) and LSR(B) and MWP(C) to TPC (b); ET(A) and MWP(C) to DPPH (c); ET(A) and MWP(C) to FRAP (d). Adapted from Ranic et al., 2014, with permission from Elsevier.

The value of the adjusted determination coefficient (adj. R^2= 0.8921) also represented the satisfactory correlation between predicted and actual values. A coefficient of variance C.V. (%) of 13.667 indicated a satisfactory degree of precision and a good deal of reliability of the experimental values. Figure 1 shows the three-dimensional response surface plots (the graphical representation of the regression equation (1), called the response surface) of the TPC as affected by A and C (plot a), indicated as significant with a p value of 0.0007, and B and C (plot b), with a p value of 0.0013. The results suggest that shorter ET (40-110s) and lower range of MWP lead to higher TPC (plot a). The LSR lower than 6 mg/g with the use of 240-400W MWP also results in higher TPC (plot b).

Optimum Extraction Conditions Based on the DPPH Method

Full experimental and predicted datasets for 30 runs for the antioxidative activity of SCG extracts determined by the DPPH method are given in the work of Ranic et al.(2014). The antioxidative activity ranged from 36,56% up to 98.24%, under experimental conditions of 11s ET, 9 fold solvent to SCG ratio and 240 W, MWP. The response variable and the test variables are related by the second-order polynomial equation:

$$Y_{DPPH}=66.902 - 15.135*A + 0.019*B - 11.195*C + 3.031*A*C - 1.751A^2 - 1.948*B^2 \quad (2)$$

In Table 1, analysis of variance (ANOVA) for the regression equation is shown. The used model was found to be adequate for prediction within the range of experimental variables ($p < 0.0001$). The most significant interaction between independent variables is between A and C. The value of lack of fit (0.1377) indicated that the model fits the experimental data adequately ($p > 0.05$). The high obtained R^2 values (0.9845) indicated that the model equation could adequately predict the response; while the adjusted R^2 (0.9805) also showed a high significance for the model. The coefficient of variance C.V. (%) of 3.711 indicated a very high degree of precision and reliability of the experimental values.

Figure 1 (plot c) shows the three-dimensional response surface plots of the DPPH as affected by A and C indicated as significant with a p value <0.0001. Shorter MAE time (40-120 s) with the use of lower range of MWP (240-400W) results in higher % in neutralization of DPPH radicals.

It should be noticed that the samples in this study were adjusted to a concentration of 1mg/ml of extracts, so these findings also confirm the influence of investigated factors on TPC proportions in total solids of SCG extracts. The other researcher also noticed positive correlation of TPC and DPPH inhibition activity (Franco et al., 2008, Wang et al., 2011).

Optimum Extraction Conditions Based on the FRAP Activity

Full experimental and predicted datasets for 30 runs data for antioxidative activity of SCG extracts determined by the FRAP method are given in the work of Ranic et al. (2014). The antioxidative activity ranged from 2.620 mmol Fe^{2+}/l, up to 6.660 mmol Fe^{2+}/l, under experimental conditions of 11s ET, 9-fold solvent to SCG ratio and 240W MWP. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y_{FRAP} = 45.47 – 16.03*A – 0.22*B – 12.27*C – 2.81 * A *B + 2.46*A*C + 6.40*A^2 \quad (3)$$

In Table 1, analysis of variance (ANOVA) for the regression equation is shown. The used model is significant ($p < 0.0001$), with all significant parameters. The most significant interaction between independent variables is between A and C. The value of lack of fit (0,4664) implied the lack of fit that was not significant relative to the pure error. The relationship between the experimental and predicted values showed good fitness with a low dispersion. The high R^2 of the model (0.9433) indicated that only 5.67% of the total variations were not explained by the model and confirms that the model can adequately represent the true relationship between the parameters chosen. The coefficient of variance C.V.(%) of 8.324 indicated a high degree of precision and reliability of the experimental values. The FRAP value, as affected by the relation of A and C, is shown to be significant with a p value <0.0001. As evident in Figure 1 (plot d), the shorter MAE time (40-110 s) with the use of the lower range of MWP (240-300 W) are more effective in extracting antioxidative polyphenols from espresso SCG using MAE. This result is favorable since using of higher MWP with extended ET may lead to thermal degradation of polyphenol compounds (Chen et al., 2007).

The highest FRAP value in the range from 6.0 to 7.0 mmol $Fe^{2+/}$l, and the highest antioxidant capacity determined by DPPH (70% to 100%) correspond to a TPC range from

65% and 80% of dry SCG extract. A 9-fold solvent to SCG ratio, shorter MAE time (40-110 s), and a lower range of (240-400 W) gives the optimal DPPH and FRAP values. The minor deviations could be due to different solubilities of polyphenols showing antioxidative properties in different solvents (methanol for DPPH and water for FRAP method).

With 6 mg/g LSR, shorter ET (40-110 s), and a lower range of MWP (240-400 W), a polyphenol extract with high antioxidant activity can be achieved. The DPPH and the FRAP antioxidative activity showed good correlation with the total polyphenol content, with high correlation factors (0.841 and 0.859, respectively). The results presented in this study show that suggested models are efficient to predict the outcome variables (TPC, DPPH and FRAP) of espresso SCG using independent variables (ET, LSR, MWP). The results obtained from all models are highly correlated, which means that each model can be used to predict the outcome based on listed independent variables. Validation of the models showed no statistically significant differences between the results obtained by the experiment and those predicted by the models (Ranic et al., 2014).

The Impact of Espresso SCG Extracts on Platelet Activation and Their Aggregation with Monocytes and Neutrophiles *In Vitro*

The P-selectin and GPIIb/IIIa antigens, as markers of platelet activation, were evaluated in response to the platelet agonist ADP (0.5μM) *in vitro* in the whole blood of five healthy volunteers, according to the previously published method (Konic-Ristic et al., 2013). The results are presented as changes (%) of percentage of antigen-positive platelets in a pool of 20,000 analyzed platelets, in response to the agonist, and after treatment with different concentrations (25, 50 and 100 μgGAE/ml) of defeated extracts, compared to a vehicle-only negative control. The obtained results are presented in Figure 2.

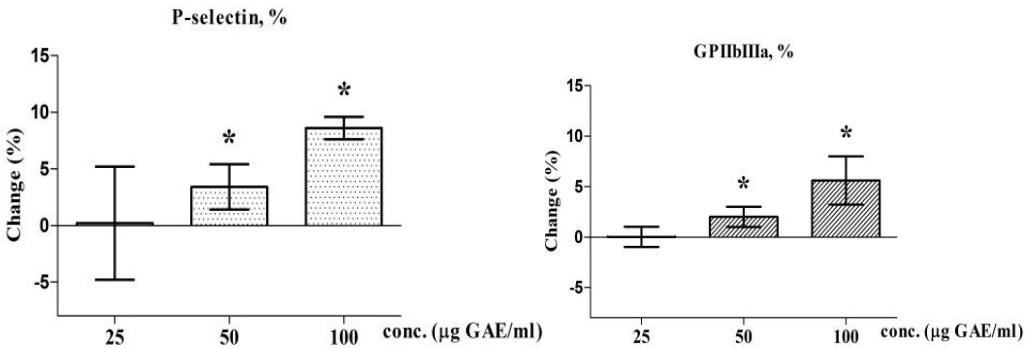

Figure 2. The impact of espresso SCG on the percentage of P-selectin and a GPIIb-IIIa positive platelets in the whole population of 20000 platelets of healthy subjects (n = 5) induced by the agonistic action of ADP (0.25 μM) *in vitro*. Results are expressed as change (%) compared to positive control (solvent pretreated, ADP induced cells) and represented as mean ± SEM. Significantly different from the control: * $p < 0.05$; (Wilcoxon signed rank test).

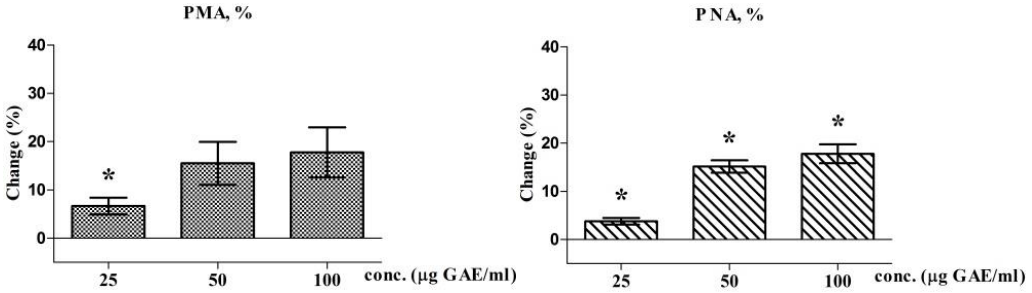

Figure 3. The impact of espresso SCG on percentage of platelets aggregates with monocytes (PMA) in the monocyte population (n = 1000) and platelets aggregates with neutrophils (PNA) in the population of neutrophils (n = 10000), in the whole blood of healthy subjects after in vitro exposure to ADP as agonists (0.25 µM). Results are expressed as percent change relative to control (solvent pretreated, ADP induced cells) and represented as mean ± SEM. Significantly different from the control:
* $p < 0.05$; (Wilcoxon signed rank test).

The effects of treatment with SCG extracts (25, 50 and 100 µgGAE/ml) extracts on the percentage of platelet-leukocyte aggregates in response to agonist were compared to a vehicle-only negative control, and expressed as change (%). The percentage of platelet-monocyte aggregates were measured in a pool of 1000 analyzed monocytes and the percentage of platelet-neutrophil aggregates in the pool of at least 10,000 neutrophils. The results were given in Figure 3.

There is no statistically significant difference in the effect on the individual activation markers (P-selectin and GPIIb/IIIa) at the same concentrations of polyphenols present in the espresso SCGs. A dose-dependent correlation of the effects of polyphenols was observed on both P-selectin and GPIIb/IIIa, with the inhibitory effects on P-selectin up to 8.7% and on GPIIb/IIIa up to 5.6% with the highest concentrations tested (100 µgGAE/ml).

The results indicate the pronounced inhibitory effect SCGs on platelet aggregation with monocytes, up to 20% with the highest concentration tested (100 µgGAE/ml).

The effect on platelet aggregation of neutrophils shows a moderate dose relationship ($r = 0.705$). The statistically significant effects on platelet aggregation with monocytes were observed for the lowest concentrations, due to the marked interindividual variation. However, significant decrease in the percentage of platelet-neutrophil aggregation was observed for all three concentrations tested (25, 50 and 100 mg GAE/ml), up to 20% with the highest concentration (100 µgGAE/ml).

Other factors putatively influencing the lack of the effects of higher concentrations of extracts on platelet-monocyte aggregation are components with pro-platelet activity that were known to be present in the coffee extract. In addition, unpublished results showed more pronounced effects of polyphenols present in SCGs compared to the effects of extracts from the correspondent beverage, which may indicate a more favorable profile of bioactive compounds or lower lever of unfavorable constituents. The physiological significance of the shown inhibition of platelet activation in the prevention of CVD are supported by the finding of previously performed animal studies that have shown that the presence of only 7% of activated platelets in the circulation, which were injected to mice 3 times daily during 4 weeks, and resulted in an increase of atherosclerotic lesions by 30% (Huo et al., 2003).

Our results confirm the existing scientific data suggested the potential of polyphenols to modulate platelet function as an important part of their pleiotropic beneficial effects on cardiovascular health. Although they have been used primarily for the assessment of biological activity of extracts, the shown effect of espresso SCG polyphenols on platelets confirm their beneficial effects on CVD and health in general, mediated among other mechanisms of pleiotropic effects, through their effects on blood cells. Other confirmed mechanisms of the beneficial effects of coffee polyphenols that favor their application include inhibitory effects on the enzymes responsible for the digestion of carbohydrates and fats in the gastro-intestinal tract (McDougall et al., 2008), inhibition of the absorption of glucose and other carbohydrates from the digestive tract (Williamson, 2013), specific inhibition of gluconeogenesis in liver, the increase in sensitivity to insulin (Akash et al., 2014), beneficial effect on blood vessel endothelium (Ochiai et al., 2014), and many others (Ludwig et al., 2014).

CONCLUSION

The accumulated body of knowledge generated since 1950 has been refined, (Campos-Vega, 2015) and the conclusion is an urgent need for practical and innovative ideas to use low cost SCGs and exploit their full potential. The use of the coffee agro-industry by-products for meaningful purposes would lead to an increase in overall industrial sustainability through the adoption of new technologies that maximize profitability from this process.

Espresso SCGs are widely available from restaurants, cafeterias and households at no-cost, demonstrating the great importance to the economic feasibility of its use. In addition, an environmental standard promotes a proper, responsible and safe waste management plan consistent with existing national regulations. This waste re-uptake not only benefits the environment, but also has the potential to eliminate a certain amount in annual disposal costs. In order to conceptualize the amount of espresso SCG waste, Keurig Green Mountain (the market leader for producing single-serving K-Cup coffee pods, launched in the 1990s), produced 8.3 billion K-Cups in 2013, enough to circle the Earth 10.5 times. According to a 2013 survey by the USA National Coffee Association (NCA, 2015), about 1 in 8 American households has a single-serving coffee brewer, which utilizes coffee pods. It is also worth mentioning that an ecological device for quick removal of the spent espresso SCGs (EP 1803380 A2) from the metallic filter already existed. This device allows the user to remove, with little effort, in just one quick step the used coffee grounds from the metallic filter, housed in the port filter of an espresso machine, after the preparation of a coffee beverage (Campos, 2012).

The obtained results in this study have shown that a great amount of polyphenol compounds with high anti-oxidative properties are present in SCGs. MAE along with RSM proved to be effective in estimating the effects of three independent variables (extraction time, liquid-to-solid ratio, and microwave power) on polyphenol extraction from SCGs. All process parameters have significant influence to extraction of polyphenols, and the results suggest that shorter extraction time (from 40 – 110 s), liquid-to-solid ratio from 6 to 9 ml/g with the use of 240 W microwave power results in the highest content of polyphenols.

Having in mind that the role of diet is essential in both the development and prevention of CVD, emphasizes an increasing need for researchers and the food industry to work together to develop healthier foods that will help reduce this health burden (Konic-Ristic et al., 2013). A focus on platelets, as targets for the prophylactic action of food bioactives against CVD, has been rationalized by the crucial role of platelets in the pathogenesis of CVD and observed correlation of disturbed platelet function with other CVD risk factors. Shown antiplatelet effects of polyphenols present in espresso SCGs indicate a favorable profile of bioactive compounds toward modulating platelet function and thereby rationalizing their use as a functional food ingredient and nutraceutical aiming to promote CVH. The obtained results for antiplatelet influence of coffee polyphenols, in the estimation of biologically active components extraction optimization, confirm the fact that bioactive components of coffee have a beneficial effect on platelet function. In addition, the more prominent effect of spent coffee extracts, compared to correspondent infusion, indicates a more favorable profile of bioactive compounds or the absence of components with pro-platelet activity and thereby rationalizes their use as functional ingredients.

The presented facts and results reported in this study could be of great significance for future exploitation of espresso SCGs as a valuable source of natural antioxidants in industrial scale-up, although a detailed cost-benefit analysis is needed to assess the economic practicability of the proposed approach. Shown antiplatelet activity provided a rational basis for the use of extracts of espresso SCG as functional food ingredients for CV health promotion even though the further research is needed on the bioavailability and pharmacokinetics of coffee components in order to elucidate the compounds responsible for those effects and mechanisms involved as well safety assessments of the applied doses performed (Mennen et al., 2005).

ACKNOWLEDGMENTS

This work was supported by the Projects TR31035 and III41030 financed by the Serbian Ministry of Education, Science and Technological Development.

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BIOGRAPHICAL SKETCH

Marija Ranić

Marija Ranić, PhD, Scientific Associate
Date of birth: 12.04.1968.
Institute for Medical Research, University of Belgrade

Centre of Research Excellence in Nutrition and Metabolism
Tadeuša Košćuška 1
11000 Belgrade, Serbia
Phone: +381 11 3281564
Fax: +381 11 2030169
e-mail: marija.ranic@gmail.com

Education

2015 PhD thesis defended "Coffee and spent coffee extracts antioxidative activity and influence on blood platelets activation", Faculty of Technology and Metallurgy, Biochemical Engineering and Biotechnology", University of Belgrade
1994 MSc. Faculty of Technology and Metallurgy, University of Belgrade

Working experience

2016 Scientific Associate at the Institute for Medical Research, Centre of Research Excellence in Nutrition and Metabolism
2010 Research Associate at the Institute for Medical Research, Centre of Research Excellence in Nutrition and Metabolism
1996 Junior Researcher at the Institute for Medical Research, Department of Nutrition and Metabolism
1994-1995 Working as a talent for scientific work at the Faculty of Technology and Mettalurgy, University of Belgrade, Department of Organic Chemistry

EC Projects

- BACCHUSS-FP7-Beneficial effects of dietary bioactive peptides and polyphenols on CVD health in humans, http://www.bacchus-fp7.eu, (2012-2016)
- CHANCE FP7 -Low Cost tecHnologies and traditional ingredients for the production of Affordable, Nutritionally correct, Convenient foods enhancing hEalth in population groups at risk of poverty,www.chancefood.eu, (2011-2014)
- EuroFIR –Nexus FP7-EuroFIR Food Platform: further integration, refinement and exploitation for its long-term self-sustainability www.eurofir.org,(2011-2013)
- BaSeFood FP7 – Sustainable exploitation of bioactive components from the Black Sea Area traditional foods,www.basefood-fp7.eu, (2009-2012)
- EURRECCA FP6- Harmonising nutrient recommendations across Europe with special focus on vulnerable groups and consumer understanding (NoE), www.eurreca.org, (2007-2012)
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Trainings and Workshops (last three years)

2013 MAITRE seminar: Communicating Food Science in Belgrade, 10-11 December, Belgrade, Serbia

2013 Workshop on Developing Knowledge-Sharing Partnerships in Europe and Central Asia, held between 4-6 December, in Budapest and Godollo, Hungary

2013 Food and Health Entrepreneurship Program (FHEP) one-week course open for international academic researchers and engineers working in the field of food science and nutrition to move ahead their research to the market, Barcelona, May 27-31, 2013

2012 Traditional Food International (TFI-2012); The Street Food Seminar, Cesena, Italy, October 4-5

In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 6

EFFECTS OF EXTRINSIC FACTORS ON THE ACCEPTANCE OF INSTANT COFFEE ENRICHED WITH NATURAL ANTIOXIDANTS FROM GREEN COFFEE

Marta de Toledo Benassi[1,*] and Marinês Paula Corso[2]
[1]Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[2]Departamento Acadêmico de Alimentos, Universidade Tecnológica Federal do Paraná, Medianeira, Paraná, Brazil

ABSTRACT

Consumers are becoming more concerned with their diet and the impact that food has on their health. An instant coffee that is enriched with bioactive compounds (polyphenols) from a green coffee could be an alternative product of interest. Such products are already available in the market of some countries, but not on the Brazilian market (the second largest coffee-consuming market in the world). There is only one global manufacturer of this type of instant coffee. Marketed in a restricted number of countries, the packaging of the product and its process are adapted to the demands of each place. In Brazil, medium- to dark-roasts processes are the most commonly used for coffee products; thus, it is important to study whether the presence of green coffee in the instant coffee product interferes with consumers' expectations. It is known that information provided to consumers and their experiences in relation to a product may raise expectations, which can affect their sensory perception. Therefore, the aim of this study was to evaluate sensory acceptance and the effect of the expectations caused by information and the packaging characteristics on the acceptance of instant coffee enriched with antioxidants from green coffee. Three instant coffee samples of the same brand, one conventional (A) and two enriched with antioxidants (B and C), with different package designs, were evaluated by 90 consumers. Overall liking was rated using a 10-cm hybrid hedonic scale in three sessions, under different informational conditions (blind test, expectation test and informed test). The samples were characterized regarding to contents of chlorogenic acid (5-CQA), caffeine, polyphenols (by HPLC) and browned

* Corresponding Author address, Email: martatb@uel.br.

compounds (by spectrophotometer). The colors of the brews were determined using a colorimeter. The samples received scores higher than 6.4 in the three sessions. In the blind evaluation, the acceptance score of one of the samples enriched with antioxidants (C) was not significantly different ($p > 0.05$) of the conventional sample. In the expectation evaluation and the informed evaluation, the scores for the three samples were not significantly different from each other ($p > 0.05$). In expectation test the mean values were higher ($p < 0.05$) than those obtained in the blind evaluation. In the informed evaluation, only for sample B the score increased regarding the blind test ($p < 0.05$). The concept of a coffee enriched with natural antioxidants from green coffee (expectation) as their correspondent brews were well accepted. The packages raised high expectations, and despite the occurrence of negative disconfirmation, the consumer assimilated the expectations raised by the extrinsic factors, thus increasing the product acceptance in the informed evaluation.

Keywords: functional food, package shape, enrichment claims, hedonic perception, expectation, consumers

INTRODUCTION

Instant coffee and its derived beverages, which are composed of complex mixes of natural substances, are produced using a roasting process. The roasting process causes changes in the chemical composition, conferring the characteristic flavor and aroma of coffee, but, consequently, alters the biological activities of green coffee, particularly by degrading phenolic compounds, such as chlorogenic acids, and inducing the development of Maillard reaction products (MRP). Instant coffee production from a roasted product requires extraction and drying processes that may cause the degradation of some bioactive compounds, but increased in others compounds contents due to the removal of non-soluble components can occur [1-5].

Besides being the largest coffee producer and exporter, Brazil is the second largest coffee-consuming market in the world [6]. Considering that both unroasted green coffee and roasted coffee show some positive functional features [1, 7-11], and that consumers are becoming more concerned with their diet and the impact that food has on their health, an instant coffee that is enriched with bioactive compounds from a green coffee could be an alternative product of interest. Such products are already available on the international market, but not on the Brazilian market. There is only one global manufacturer of this type of instant coffee, which is marketed restricted number of countries, so the packaging of the product and its process are adapted to the demands of each place. In Brazil, medium- to dark-roasts process are the most commonly used for coffee products [12-14]; thus, it is important to study whether the presence of green coffee in the instant coffee product interferes with consumers' expectations and influences the product's actual acceptance.

During conventional sensory tests, the samples are evaluated under blind conditions. However, as some extrinsic factors such as labels and packages are part of the identity of a commercial product, their influence on product acceptance must be considered [15]. In a previous study, Corso and Benassi [16] verified that the shape of the glass package, the color of the label and the picture on it had an impact on the intention of Brazilian consumers to purchase functional instant coffee. The acceptance of a food can be evaluated of three

conditions according to set of information provided to the consumers: blind evaluation, expectation evaluation (evaluation of non-sensory characteristics) and informed evaluation (evaluation of sensory and non-sensory characteristics) [17-20].

Previous information and experiences of the consumers, together with the product itself, the label, advertising and price, raise preliminary expectations that may be low, leading to product rejection, or may be high, contributing to their choosing the product [18]. After choosing the product, the consumer will try it and his or her expectation may be confirmed or disconfirmed [15, 17, 18]. When the product is better than expected, disconfirmation is positive. On the other hand, when the product is worse than expected, disconfirmation is negative [21, 22] and, in this case, the consumer will most likely not buy the product again [15]. Prediction models are created to explain the effect of the discrepancies between expectations and products acceptance. In the assimilation model, the differences between the consumers' expectation about a product and the characteristics they actually find in the product are assimilated, i.e., consumers change their evaluation in the direction of the expectation [17, 20-22]. In the contrast model, the product evaluation changes in the direction opposite to the expectation, increasing the discrepancy between the product evaluation and the expectation. For example, when the product does not meet the expectation, the consumer's acceptance tends to decrease, and, in the opposite, when the product is better than expected, the consumer's acceptance will be greater than when no expectation was raised [17, 21, 22].

Therefore, to offer in the Brazilian market an instant coffee enriched with antioxidants - chlorogenic acid - by the addition of green coffee, one should consider two hypotheses: 1) The addition of green beans can change the composition and, consequently, the sensory characteristics of the instant coffee brew, and it can influence on their acceptance. 2) Factors such as the information of the presence of green coffee beans in the product enriched with antoxidants and other characteristics of the label and packaging can influence sensory expectations and, consequently, the acceptance of the instant coffee brew.

Considering the sensory characteristics that chlorogenic acids and MRP provide to the coffee brew, the Brazilian habits in consuming coffee products with medium- to dark-roast degrees and the increasing in the demand for healthier products the aim of this study was to investigate the acceptance of commercial instant coffee products enriched with antioxidants - a mixture of green and roasted coffee extracts - as well as the effect of the extrinsic factors (informations and characteristics of the package) on the acceptance of the same products by Brazilian consumers. The color and compositional characteristics of the products were evaluated to complement the discussion.

METHODS

Materials

Three commercial instant coffee of the same brand (samples A, B, and C) were used. Sample A was a conventional non-enriched product that is highly commercialized in the Brazilian market (obtained by spray drying and agglomerated). Sample B was a granulated product obtained by freeze-drying, and sample C was an agglomerated product obtained by spray drying. Samples B and C were both instant coffees enriched with green coffee

antioxidants that are not available in the Brazilian market. As there is only one global manufacturer of this type of instant coffee, the packages for the enriched products had similar labels (claims, color and figures); however, one of the packages (B) had a format different from that of the traditional package for instant coffee. Table 1 shows the description of the packages and the different information about the product that was included on the respective labels.

Table 1. Description of the packages and labels for the instant coffees

Sample/Type/ Origin	Design		Type of information
	Shape (glass/lid) and color (lid)	Color and Images (label)	
A Agglomerated/ Spray dried Brazil	Flattened cylindrical, waisted glass container with round red plastic lid	Predominant color: red Image on front: cup of coffee	No claims
B Granulated/Freeze dried France	Hexagonal, waisted glass container with hexagonal green plastic lid	Predominant color: green Image on front: oval coffee bean, half green and half roasted	Claims of enrichment with natural antioxidants from green coffee (front and back). Percentage of green coffee (35%) (back)
C Agglomerated/ Spray dried Spain	Flattened cylindrical, waisted glass container with round green plastic lid		

The coffee brews were prepared using 1.4 g of instant coffee per 50 mL of water at 95ºC, as recommended by Kobayashi and Benassi [23]. The coffee was sweetened with sucrose to a final concentration of 9.5%, which is estimated to be the ideal concentration for sweetening [23, 24], both with Brazilian consumers. The standardization in sucrose and instant coffee concentration were applied in order to minimize these sources of variation. After preparation, the samples were stored in thermal bottles and maintained for a maximum of 2 h before being served at 70ºC.

Sample Characterization

The moisture content of the instant coffee (3.000 g) was determined using an infrared analyzer (MB200, Ohaus, Parsippany, NJ, USA) at 105ºC for 7 min. Measurements were conducted in triplicate. The results were used to calculate concentrations of components on a dry basis.

Color analysis was performed using a portable colorimeter (CR-400, Konica Minolta, Osaka, Japan) coupled to a CR-33 light-projection tube with 45/0 geometry and a D65 illuminant. The instant coffees were packed in a plastic recipient (CR-A50) for readings on the surface of the material. Samples of the coffee brews were packed in a plastic cell coupled to a cell support. The analyses were performed at room temperature as genuine duplicates, with triplicate measurements. L* (lightness), a* (red-green component) and b* (blue-yellow component) values were obtained and used to determine the hue angle (*h*) as follows: $h = \tan^{-1}(b/a)$.

An estimative of the polyphenol contents and the chlorogenic acid (5-CQA) and caffeine levels were determined using high performance liquid chromatography (HPLC). The chromatographic conditions were adapted from Marcucci, Benassi, Almeida and Nixdorf [14] and Vignoli, Bassoli and Benassi [5]. The instant coffees were dissolved in 5% acetic acid at a concentration of 0.5 mg mL^{-1} and filtered through a 0.22 μm membrane (Millipore, Brazil). The analyses were conducted using a Shimadzu HPLC (Kyoto, Japan) equipped with two pumps (model LC-10AD), a column oven (model CTO-20A), an on-line degasser, a UV/visible detector (model SPD-10A), and a Rheodyne injection valve with a 20-μL loop. The data were processed through a CBM-101 interface connected to a microcomputer. The chromatographic conditions used were: a Spherisorb ODS1 (250 x 4.6 mm, 5 μm) column (Waters, Ireland) coupled to a pre-column (C18, 5 μm), elution with a mobile phase of acetic acid/H_2O (5:95, v/v) (A) and acetonitrile (B) at a flow rate of 0.7 mL min^{-1}, using a gradient of 8% from 0-5 min and 15% from 5-35 min. Caffeine was detected at 272 nm and 5-CQA and other polyphenols were detected at 320 nm. The analyses were conducted in duplicate at 25ºC. The identification of 5-CQA and caffeine were based on comparison of the retention times and co-elution with authentic standards. Quantification was performed by external standardization, using calibration curves in the range of 1 to 31 μg mL^{-1} for 5-CQA and 5 to 40 μg mL^{-1} for caffeine (six concentrations in duplicate). The total polyphenol content was estimated from the sum of the areas of the compounds detected at 320 nm, based on Budryn et al. [4], using 5-CQA as a standard.

The content of browned compounds was estimated using the methodology described by Ludwig et al. [25] and adapted by Marcucci et al. [14]. The instant coffees were diluted directly in water at room temperature (25ºC) at the concentration of 0.57 mg mL^{-1}. The absorbance of the solutions was assessed at 420 nm using a Biochrom Libra S22 UV-VIS spectrophotometer (Cambridge, UK). The analyses were performed on genuine duplicate samples, with measurements in triplicate.

The color and composition results for the instant coffees and coffee brews were submitted to an analysis of variance (ANOVA), considering the sample as a source of variation, and the means test (Tukey test with significance set at 5%). The statistical analyses were performed using the Statistica 6.0 program [26].

Sensory Evaluation

Ninety regular coffee consumers between the ages of 18 and 65 were recruited at two universities. The participants were invited via a collective email sent to students, teachers and employees. A brief description about de survey was included, and it was specified that to

participate it was necessary the willingness to attend the three sessions and being a coffee consumer. Considering the absence of the product in the Brazilian market, the need to understand its concept, as well as the need to evaluate labels in other languages, participants with an educational level higher than the national average were selected. The participants came from counties of different sizes (Londrina, with 500 thousand inhabitants, and Medianeira, with 42 thousand inhabitants) located in Paraná state, a traditional coffee growing and coffee consuming state, where most of the instant coffee production plants in the country are installed. Before the evaluations, the participants answered a self-administered questionnaire on socio-demographic data and consumer habits regarding instant coffee and functional foods. The participants were informed that they would taste commercial instant coffee samples, but no information was provided on the objective of the test or the fact that some samples were enriched with antioxidants before the tests. The same participants took part in the three sensory evaluation sessions. Between different samples participants rinse their mouth with filtered water and were instructed to eat a water cracker, and at least a 2-min rest interval was timed before the presentation of the next sample. The participants were volunteers and received no reward.

The tests were performed in individual booths under white light, and in three sessions, with intervals of a minimum of two days between sessions. Time tests just before or after were avoided. The samples (coffee brews and package) were codified with three-digit random numbers. Approximately 30 mL of the brews were served at 70ºC [27] in styrofoam disposable open cups. For the evaluation of the packages, the information for products B and C, written in French and Spanish, respectively, were translated and presented to the consumers. The samples were presented monadically in all tests, following an experimental design of complete balanced and randomized blocks.

In the first session, the consumers evaluated only the coffee brews acceptance (B- Blind evaluation), i.e., they had no information about the products they evaluated. In the second session, the expectation regarding the acceptance of the products served in the previous session was evaluated based on observing only the packages (E-Expectation evaluation). This procedure allowed the consumer to evaluate the package attributes and the information on the type of coffee provided on the labels. Sensory evaluation of the consumer acceptance of the coffee brews with their respective packages present (I- Informed evaluation) was conducted in the third session. The consumers were asked to taste the coffee brews, considering that each originated from its respective package. A 10-cm hybrid hedonic scale anchored with the expressions “disliked a lot” (at the extreme left of the scale), “neither liked nor disliked” (at the center) and “liked a lot” (at the extreme right of the scale) [28] was used to evaluate global impression of samples in the three sessions. The sensory data were acquired using a paper sheet.

The mean hedonic scores for the three products were calculated for each session (B, E and I) and evaluated using a two-way ANOVA, considering the samples and participants as factors, and the Tukey test or the t-test, with significance set at 5%. To explore the impact of the package design and information on the hedonic scores, the differences among the scores obtained under different information regimens, I-E (degree of disconfirmation), E-B (degree of incongruence) and I-B (degree of response shift) were determined according to Schifferstein [19]. The significance of the differences among these values (I-E, E-B and I-B) for each product was evaluated at the 5% level of significance using the t-test. The presence of assimilation (α) was evaluated by a regression analysis based on the relationship between

the degree of response shift (I-B) and the degree of incongruence (E-B) [17, 20]. The bioactive compounds contents and color parameters for instant coffee samples were evaluated using a one-way ANOVA and the Tukey test, with significance set at 5%. The statistical analyses were conducted using the Statistica 6.0 program [26].

RESULTS AND DISCUSSION

The participants' socio-demographic profile and consumption habits are shown in Table 2. Most of the participants were under 45 years of age, with a prevalence of females (69%). This profile is adequate because in research on coffee consumers in Brazil, women are still the main people responsible for buying coffee for home (77%) as well as for its preparation, according to the *Brazilian Association* of the *Coffee* Industry (ABIC) research [29]. Despite this group's high degree of education (78% with higher education degrees), the participants had diversified family incomes. As for the consumption of functional foods, 97% said they do it at least occasionally. Among the types of coffee consumed, 89% said they consume instant coffee at least occasionally. In relation to the form and amount consumed, 52% of the participants reported consuming pure coffee (1 to 4 cups/day) and 51% reported consuming coffee with milk (1 to 2 cups/day). Overall, the group presented a high consumption level, considering that instant coffee is consumed only by 17% of the Brazilian consumers, with 40% of those consuming the product daily, 46% consuming it from 1 to 5 times a week and 14% consuming it occasionally [29].

Table 2. Participants' socio-demographic and consumption profile (*n* = 90)

Socio-demographic data	Percentage (%)	Consumption data	Percentage (%)
Age (years)		*Type of coffee consumed**	
18-25	42.2	Brewed/Filtrate	90.0
26-35	35.6	Soluble/Instant	76.7
36-45	14.4	Others	14.5
46-65	7.8	*Consumption of instant coffee (frequency)*	
Level of instruction			
Basic education/high school	22.2	Daily	30.0
Graduate	63.3	1 to 3 times a week	16.6
Post-graduate	14.4	Occasionally	42.2
Income (number of minimal Brazilian wages, R$)		Never	11.1
1-5	52.2	*Consumption of functional foods (frequency)*	
6-10	37.8		
>10	10.0	Daily	33.3
Occupation		1 to 3 times a week	37.8
Student	64.4	Occasionally	25.6
Teacher	12.2	Never	3.3
Another activity	23.3		

*The consumer could to mark more than one answer for this question.

The mean hedonic scores observed for the instant coffee samples evaluated under the three different informational conditions are shown in Table 3. Is worth noting, that the acceptance data were obtained under specific conditions of brews preparation (concentration of instant coffee and sugar), and that the results could be different if the conditions were changed. The samples received scores of over 6.4 (on a 10-cm scale) in the three sessions. In the blind evaluation (B), although the two enriched products reported the same percentage of green coffee addition, the acceptance score of one of the samples enriched with antioxidants (C) was not significantly different ($p > 0.05$) of the conventional sample (A), while sample B was less accepted ($p < 0.05$) than the others (Table 3). This result indicated that the addition of green coffee did not necessarily affect the acceptance of the instant coffee. The sensory acceptance difference observed was probably due to differences in the processing conditions (roasting/extraction/drying), and/or the raw material (coffee species/varieties) used in the blends [5, 14]. Indeed, according to the color and composition analyses (Table 4), coffee brew C is more similar to conventional sample A than to B. The L* and *h* color indices indicated that samples A and C had a less intense brown color than did B ($p < 0.05$) (Table 4). Browned compounds are measured to obtain an index of the MRP produced during the coffee roasting process, which contributes to the aroma and color of coffee brews [4, 30]. The results suggested that sample C exhibited a more intense degree of roasting, and thus was more similar to the coffee that Brazilians habitually consume [12-14]. Marcucci et al. [14] evaluated 33 instant coffees commercialized in Brazil, and reported that the absorbance at 420 nm ranged from 0.253 to 0.476, with a mean of 0.36. Sample B had a higher content of polyphenols and 5-CQA than did samples A and C (Table 4). In addition to providing the bioactivity of coffee, these compounds are also known to be responsible for color, aroma development and astringency of the beverage [3, 31]. Farah, Monteiro, Calado, Franca and Trugo [3] observed that the 5-CQA levels and the contents of other phenolic compounds showed a positive correlation with poor cup quality. Notably, sample B contained approximately 3 times more 5-CQA than that found in the regular instant coffees commercialized in Brazil (average 1.20 g/100 g) [14]. Sample B also had a higher caffeine content ($p < 0.05$) than did samples A and C (Table 4). Caffeine is one of compounds responsible for the bitter taste of coffee [3, 31]. The three samples had caffeine contents within the range found in conventional products commercialized in Brazil (2.32 to 4.08 g/ 100 g) [14].

In the expectation evaluation (E), the three samples showed no significant difference among themselves ($p > 0.05$) (Table 3) and obtained higher scores than those obtained in the blind evaluation ($p < 0.05$) (Tabel 6). The expectations raised by the samples containing green coffee (B and C) were as high as those raised by the conventional sample (A), which was interesting, considering the lower consumption of lyophilized coffee products and the higher intake of medium- to dark- roasts coffees by Brazilian consumers [12-14]. Instant coffee B (lyophilized) displayed a lighter and more yellowish-brown color ($p < 0.05$) than did products A and C (agglomerated) (Table 4). However, the color was within the range of variation found for the conventional lyophilized Brazilian products (L* = 34.6-43.7) [14].

In the last evaluation, when product acceptance was evaluated with the package available (I), only for sample B the score increased regarding the blind test ($p < 0.05$) in the direction the expectations (E) (Table 3 and 5).

Table 3. Mean values (M) and standard deviations (SE) for instant coffee acceptance (A: without enrichment, conventional, B: enriched with antioxidants, modern format and C: enriched with antioxidants, conventional format) obtained from groups that were provided different sets of information, and the results considered separately according to the gender

Sample	Group (n = 90)			Woman (n = 62)**			Men (n = 28)**		
	Blind evaluation (B)	Expectation evaluation (E) M(SE)*	Informed evaluation (I)	B	E M*	I	B	E M*	I
A	7.3 (1.6) [a]	7.9 (1.5) [a]	7.5 (1.7) [a]	7.3 [aA]	7.9 [aA]	7.8 [aA]	7.3 [aA]	7.8 [aA]	6.8 [aB]
B	6.4 (2.0) [b]	8.2 (1.8) [a]	7.5 (1.7) [a]	6.6 [bA]	8.2 [aA]	7.8 [aA]	5.9 [bA]	8.2 [aA]	6.9 [aB]
C	7.1 (1.8) [a]	7.7 (1.6) [a]	7.4 (1.6) [a]	7.3 [aA]	7.7 [aA]	7.7 [aA]	6.7 [abA]	7.8 [aA]	6.8 [aB]

*Values in each column bearing the same lower case are not significantly different (p > 0.05) from one another, according to the Tukey test for comparison of the mean values obtained using the 10-cm hedonic scale (0-dislike a lot, 10-like a lot). ** Values in each row, concerning the same evaluation session comparing male and female results, bearing the same upper case are not significantly different (p > 0.05), according to the t-test for related samples.

Table 4. Bioactive compound contents and color parameters for instant coffee samples (A: without enrichment, conventional, B: enriched with antioxidants, modern format and C: enriched with antioxidants, conventional format)

Sample	Color*				Bioactive compounds*			
	Instant coffee		Coffee brew		5-CQA (g/100 g)	Polyphenols (g/100 g)	Caffeine (g/100 g)	Browned compounds (A)
	L*	*h*	L*	*h*				
A	20.83 ± 0.55 [a]	49.5 ± 0.24 [a]	20.42 ± 0.10 [b]	25.3 ± 1.13 [b]	1.18 ± 0.01 [a]	4.24 ± 0.03 [a]	3.61 ± 0.03 [b]	0.371 ± 0.02 [b]
B	34.51 ± 1.41 [b]	58.7 ± 0.46 [c]	20.24 ± 0.05 [a]	21.7 ± 0.78 [a]	3.72 ± 0.02 [c]	7.89 ± 0.02 [c]	3.98 ± 0.02 [c]	0.306 ± 0.01 [a]
C	20.79 ± 0.77 [a]	50.8 ± 0.24 [b]	20.88 ± 0.05 [c]	28.9 ± 0.93 [c]	2.63 ± 0.01 [b]	6.14 ± 0.00 [b]	3.26 ± 0.03 [a]	0.372 ± 0.01 [b]

*Mean values (standard deviation) in each column bearing the same letters are not significantly different (p > 0.05), according to the Tukey test for comparison of the means.

Table 5. Effect of expectation on the acceptance of instant coffee samples (*n* = 90) (A: without enrichment, conventional, B: enriched with antioxidants, modern format and C: enriched with antioxidants, conventional format) that were tested with different sets of available information

Sample	(E-B)*		(I-B)*		(I-E)*	
	M	p	M	p	M	p
A	0.6	0.0015	0.2	0.2695	-0,4	0,0099
	Disconfirmation -					
B	1.8	0.0000	1.1	0.0000	-0.6	0.0010
	Disconfirmation -		Assimilation			
C	0.7	0.0078	0.3	0.1121	-0,3	0,1158
	Disconfirmation -					

*Tests: blind (B), expected (E) and informed (I). Differences between the mean values followed by $p \leq 0.05$ are considered significantly different from zero, according to the t-test for related samples.

In contrast to the results of the blind evaluation, in the informed evaluation, no differences regarding acceptance was observed ($p > 0.05$) (Table 3), showing the effect of the extrinsic factors of the product on sensorial perception. This performance could be due to the fact that conventional coffee products are healthy [1, 2, 4, 7, 11], and a significant portion of Brazilian consumers (over 50%) know the health benefits of coffee products [29]. Functional products that have healthy food as a functionality carrier are easily accepted by consumers [32, 33]. This fact is important, considering that, according to Jaeger, Axten, Wohlers and Sun-Waterhouse [34], several studies have reported that consumers are no longer willing to compromise sensorial quality even when considering functional foods.

Gender can play a significant role in the effect of expectation on food acceptance. Women are more concerned with eating healthy foods, whereas men are less critical and more traditional, and considering flavor characteristics as more important for food choices [35-37]. In the present study, a larger number of female consumers (69%) was used, and it was verified that while women consumed functional foods from between once a week to daily (79%) or at least occasionally (21%), 11% of the men reported having never consumed functional foods, 36% consumed them only occasionally and the others (53%) consumed them between once a week to daily. These two groups exhibited similar behaviors in the blind and expectation evaluations (Table 3), including providing similar scores ($p > 0.05$). However, when the samples were evaluated with their respective packages available (I), the female's average scores for all of the samples were higher than the male's average scores ($p < 0.05$), showing a greater effect of expectation and greater assimilation for the women, consistent with their greater interest in functional foods.

The differences among the scores received in each session were calculated for each sample (Table 5). Significantly positive differences ($p < 0.05$) between the (E) and (B) ratings were observed. The comparison demonstrated expectation-based negative disconfirmation for the three samples. Similarly, the differences among the mean scores obtained in the informed and blind evaluations (I-B) were calculated for each product. According to Lange, Rousseau and Issanchou [17] and Stefani, Romano and Cavicchi [20], a significant I-B difference shows the effect of information on product acceptance. In this case, there are two

possibilities, as follows: a) (I-B)/(E-B) < 0, revealing a contrast effect or; b) (I-B)/(E-B) > 0, revealing an assimilation effect. The (I-B) vs. (E-B) graphs for each product are presented in Figure 1. The regression equations revealed a positive slope for all of the samples, where the greater slope indicates a greater effect of the extrinsic factors on the hedonic perception, indicating the effects of assimilation on acceptance.

The I-B difference was significant ($p < 0.05$) only for sample B (Table 5), which used a glass package with a more modern or differentiated design (hexagonal shape, waisted). Therefore, the information on the enrichment with antioxidants/green colored lid/bean picture on the package did not significantly affect the hedonic perception of the product, because sample C was perceived similarly to the sample with no antioxidant enrichment (sample A), and both were from a package of the conventional format. However, the modern packaging format associated with the functional information had a significant positive impact on product acceptance.

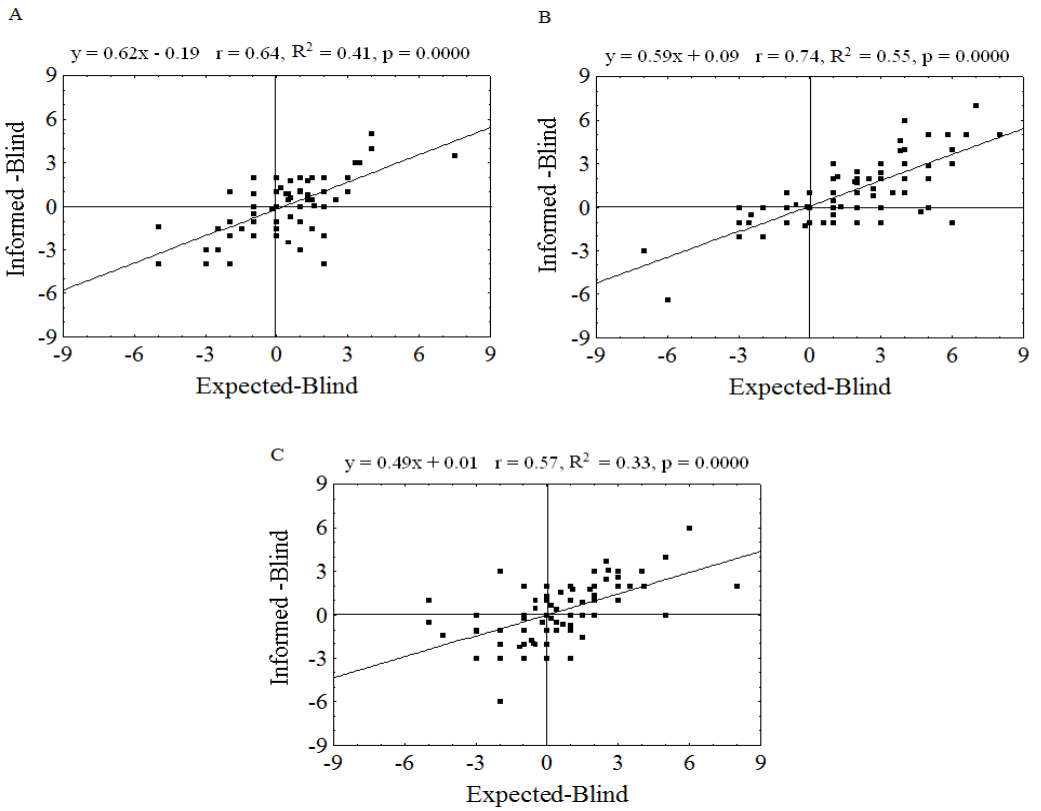

Figure 1. Expectation disconfirmation effect for the instant coffee samples (A: without enrichment, conventional, B: enriched with antioxidants, modern format and C: enriched with antioxidants, conventional format) (n = 90).

The difference between the informed and expected scores (I-E) was also calculated (Table 5). According to Lange et al. [17], when there is assimilation, significant effects for this difference show that assimilation was not complete, which can also be verified by the low proportion of variance explained by the model (R^2) (Figure 1B). Thus, although assimilation was significant in the acceptance of the sample B, both intrinsic and extrinsic factors,

particularly the package format, had an impact on the hedonic scores in the informed test. This behavior is similar to that observed by Lange et al. [17] in a study on the influence of extrinsic factors on the acceptance of fruit juice and to those obtained by Behrens, Villanueva and Silva [37] in a study of the effects of nutritional and health allegations on the acceptance of soymilk beverages.

It is noteworthy that in this study participated instant coffee consumers and also regular coffee drinkers who did not consume specifically instant coffee. This could be a limitation of the work considering that unfamiliar consumers of a product may follow a different pattern on the perception of extrinsic factors [18]. In fact, considering in the analysis only participants who do not consume instant coffee (11% of the participants, Table 2), it was observed that the assimilation of the product B was complete ((E-B) = 4.5, p = 0.0014, (I-B) = 2.3, p = 0.0135 and (E-I) = -1.2, p = 0.1130)), differently of results with the whole group (Table 5). However, when the analysis was performed with instant coffee consumers (89% of the participants, Table 2) it was observed the same pattern regarding the three samples than the behavior described for the whole group (Table 5). In the presented work, all consumers results were considered, since the product has a different concept (health-related), which may interest not only the regular consumer of instant coffee, but also new consumers. It is highlighted, however, that for these new consumers, the extrinsics factors may have more influence on product acceptance.

Figure 2 summarizes the information in each graph that is presented in Figure 1.

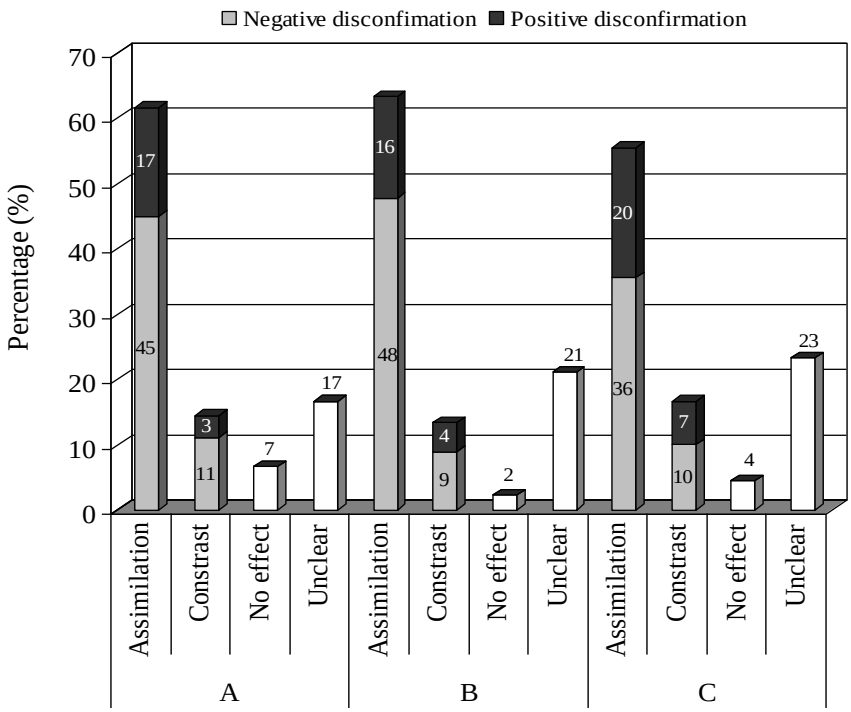

Figure 2. Observed proportion of consumers showing the effects of expectation raised by non-sensorial characteristics on the acceptance of the instant coffees (A: without enrichment, conventional, B: enriched with antioxidants, modern format and C: enriched with antioxidants, conventional format) (*n* = 90).

In examining the classification given by each individual, no effect was observed for a quarter of the group (2.2 to 6.7% of the judges) or the effect was unclear (16.7 to 23.3%), which does not follow the assimilation or contrast model. Most of the consumers demonstrated assimilation of the expectation, whether under negative (E > B and I > B) or positive disconfirmation (E < B and I < B). This behavior confirmed that assimilation effects are observed more frequently than are contrast effects [17,18], and that people tend to assimilate their sensory acceptance toward the expected acceptance after a negative disconfirmation [37-39]. Contrast (B < E and I < B or B > E and I > B) occurred on a smaller scale, ranging from 13.3 to 16.7%, a proportion similar to that observed by Behrens et al. [37] (which ranged from 3.6 to 14%).

CONCLUSION

The hedonic perception of an instant coffee product enriched with natural antioxidants formulated using green coffee was positively influenced by both the intrinsic sensorial characteristics as well as extrinsic characteristic, like the modern package format. The concept of an instant coffee enriched with natural antioxidants by the addition of green coffee was well accepted. Despite the existence of negative disconfirmation, the consumer assimilated the high expectation raised by the package (package appearance and/or information about the enrichment with antioxidants) and exhibited increased product acceptance in the informed evaluation, indicating the potential of the product in the Brazilian market.

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Reviewed by PhD. Valéria Paula Rodrigues Minim, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

BIOGRAPHICAL SKETCHES

Marta de Toledo Benassi

Name: Marta de Toledo Benassi

Affiliation: Universidade Estadual de Londrina (UEL)

Date of Birth: november/10/1963

Education:

BSc in Food Engineering (Universidade Estadual de Campinas,1985), MSc in Food Science (Universidade Estadual de Campinas, 1990) and Ph.D. in Food Science (Universidade Estadual de Campinas, 1997).

Address:

Departamento de Ciência e Tecnologia de Alimentos/Centro de Ciências Agrárias/Universidade Estadual de Londrina
86057970 - Londrina, PR - Brasil
Phone number: +55 (43) 33715970; Fax number: +55 (43) 33714080
URL: http://www.uel.br/cca/dcta
Contact e-mail: martatb@uel.br;
alternative e-mail: marta.benassi@gmail.com

Research and Professional Experience:

Marta de Toledo Benassi, professor at UEL, has experience in Food Science and Technology, focusing on Chemistry and Biochemistry of Foods. Her field of expertise covers the following subjects: coffee, antioxidant activity, HPLC, sensory analysis.

She advised 15 Master´s thesis and 4 Ph.D. thesis, and 2 Post-doctoral supervisions. She currently supervises 2 Master´s thesis, 2 Ph.D. thesis and 2 Post-doctoral researchers.

Regarding the research grants on the last 10 years, she was responsible for eight projects as research leader (3 projects supported by the Brazilian National Council for Scientific and Technological Development (CNPq), 4 projects supported by Universidade Estadual de Londrina, 1 project supported by the Brazilian Agricultural Research Corporation

(EMBRAPA)) and participated in six projects as a team member (4 projects supported by the Brazilian National Council for Scientific and Technological Development (CNPq), 1 project supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), 1 project supported by the São Paulo Research Foundation (FAPESP)). Regarding ongoing external funding, she is responsible for one project as research leader (Assessment of composition, sensory characteristics and cup quality of *Coffea canephora* brews (2014-2017), supported by CNPq) and participate in one project as team member (Brazilian pine (*Araucaria angustifolia*): assessment Brazilian pine: assessment of potential in the feed and in the development of new products (2012-2016), supported by EMBRAPA).

She was awarded with Scientific Productivity grants funded by the Paraná State Funding Agency/Fundação Araucária (2009-2010) and CNPq (2011-2013, 2014-current)

She is the author of 77 articles published in scientific journal; 2 book chapters; 165 articles published in events proceedings. She was also member of Editorial Board of the journal Semina. Ciências Agrárias (2011-2014) and acts as a reviewer for several scientific journal (Journal of Sensory Studies; Food Research International; Journal of Food Composition and Analysis; Journal of Agricultural and Food Chemistry; Food Chemistry; Lebensmittel-Wissenschaft + Technologie).

Regarding the scientific impact (until nov 2015), her publications received 1088 citations with h-index 17 at Google Scholar, and 381 citations and h-index 10 at Web of Science.

Address to access CV: http://lattes.cnpq.br/7409756675845441

Research ID F-7213-2012

Professional Appointments:

- Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP (1991 – 1997): professor

- Universidade Estadual de Londrina (1998 – current): Associate-professor.

Management and Administrative Positions: Deputy Head of Food Science and Technology Department (2006-2008, 2008-2010), Coordinator of Food Science Graduate Program (MS and PhD) (2013-current)

Publications Last 3 Years:

Articles:

Sousa, J. M., Souza, E., Marques, G., **Benassi, M. T.**, Gullón, B., Pintado, M. M., Magnani, M. Sugar profile, physicochemical and sensory aspects of monofloral honeys produced by different stingless bee species in Brazilian semi-arid region. Lebensmittel-Wissenschaft + Technologie/Food Science + Technology, v.65, p.645 - 651, 2016. (http://dx.doi.org/10.1016/j.lwt.2015.08.058).

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Marinês Paula Corso

Name: Marinês Paula Corso
Affiliation: Universidade Tecnológica Federal do Paraná (UTFPR) – Campus Medianeira
Date of Birth: August/09/1980

Education:

BSc in Food Technology (Universidade Tecnológica Federal do Paraná, 2001), MSc in Chemical Engineering (Universidade Estadual do Oeste do Paraná, 2008) and Ph.D. in Food Science (Universidade Estadual de Londrina, 2013).

Address:
Departamento Acadêmico de Alimentos/Universidade Tecnológica Federal do Paraná/Campus Medianeira
85884000 - Medianeira, PR - Brasil
Phone number: +55 (45) 32408109; Fax number: +55 (45) 32408101
URL: http://www.utfpr.edu.br/medianeira

Contact e-mail: corso@utfpr.edu.br;
alternative e-mail: marinescorso@yahoo.com.br

Research and Professional Experience:

Marinês Paula Corso, professor at UTFPR, has experience in Food Science and Technology, focusing on Technology of Foods. Her field of expertise covers the following subjects: coffee, meat products, antioxidant activity, sensory analysis and supercritical extraction.

She advised 27 bachelor's thesis and currently supervises 1 bachelor's thesis.

Regarding the research grants on the last 10 years, she was participated in four projects as a team member: 1 project supported by the Brazilian National Council for Scientific and Technological Development (CNPq) (2006-2007), 1 project supported by the Fund to Teaching, Research and Extension/UEL (2011-2013), 1 project supported by the Inter-American Development Bank (IDB) (2015-2015) and 1 project supported by the Paraná State Funding Agency/Fundação Araucária (2014-current).

She is the author of 9 articles published in scientific journal; 45 articles published in events proceedings. She acts as a reviewer for some scientific journal (Food Science and Technology-Campinas, CyTA - Journal of Food and Ciência Rural).

Regarding the scientific impact (until Dec 2015), her publications received 77 citations with h-index 3 at Google Scholar, and 39 citations and h-index 1 at Web of Science.

Address to access CV: http://lattes.cnpq.br/2613369929505068
Research ID P-5484-2015

Professional Appointments:

- Universidade Tecnológica Federal do Paraná – Campus Medianeira (2002 – current): Associate-professor.

Publications Last 3 Years:

Articles:

Corso, M.P., Benassi, M.T. Packaging attributes of antioxidant-rich instant coffee and their influence on the purchase intent. Beverages, v.1, p.273 - 291, 2015. (http://dx.doi.org/10.3390/beverages1040273).

Krummenauer, E.P., Paranhos, G.O., Silva, J.F., Silva-Buzanello, R.A., Kalschne, D.L., **Corso, M.P.**, Canan, C. Partial replacement of backfat by mozzarella cheese in Milano type salami. Revista Cultivando o Saber, v. 8, p. 143 - 161, 2015. (http://www.fag.edu.br/upload/revista/cultivando_ o_saber/55d1ee4e40f61.pdf).

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 7

COFFEE: EMERGING BENEFICIAL EFFECTS ON OCULAR HEALTH

Tae-Jin Kim[1,2,*], Holim Jang[1,3], Chang Yong Lee[3] and Sang Hoon Jung[1,2,*]

[1]Natural Products Research Center, Korea Institute of Science and Technology (KIST), Gangneung, Republic of Korea

[2]Department of Biological Chemistry, University of Science and Technology (UST), Daejeon, Republic of Korea

[3]Department of Food Science, Cornell University, Ithaca, NY, US

ABSTRACT

Coffee is among the most widely consumed beverages worldwide. Traditionally, high consumption of coffee has been considered to have negative health consequences due to the stimulant effects of caffeine. However, there is substantial evidence that coffee contains a range of bioactive compounds and antioxidants with potentially beneficial effects on human health. While much research has been devoted to understanding the relationship between coffee intake and the risk of diseases such as cancer, type 2 diabetes, and neurodegenerative disorders, the beneficial effects of coffee in protecting against retinal degeneration have been poorly elucidated. Highlighting recent investigations, this chapter overviews emerging evidence for the protective effects of coffee and its bioactive compounds on ocular health.

INTRODUCTION

In meeting the requirements of vision, the eye is necessarily a sophisticated organ. The human retina is a light-sensitive layer of tissue consisting of millions of photoreceptors that

* Corresponding author: Tae-Jin Kim, Ph.D. Phone: +82-33-650-3710, Fax: +82-33-650-3679, Email: tjkim@kist.re.kr.

* Corresponding author: Sang Hoon Jung, Ph.D. Phone: +82-33-650-3653, Fax: +82-33-650-3679, Email: shjung@kist.re.kr.

receive and convert light into neural signals (Curcio et al., 1990; Lamb, 2013). The retina is among the body's highest energy-consuming systems, demanding a constant and abundant blood oxygen supply (Wong-Riley, 2010) and making it more susceptible to oxidative stress-induced damage than other parts of the body.

Continuous oxidative stress is an important cause of many human diseases, including cancer, diabetes, and cardiovascular and neurodegenerative diseases (Uttara et al., 2009; Benz and Yau, 2008; Dhalla et al., 2000). This oxidative damage is widely implicated in the development of retinal diseases such as age-related macular degeneration (AMD), glaucoma, and retinopathy, all of which can lead to partial visual loss or to complete blindness (Winkler et al., 1999; Chrysostomou et al., 2013; Kowluru and Mishra, 2015). Although many therapeutic approaches have been developed for treatment of such disorders, there is at present no available treatment that can reverse the most common types of retinal degeneration.

There is evidence that the progression of certain diseases, including retinal degeneration, can be ameliorated by dietary treatment with defined antioxidant compounds such as vitamins, carotenoids, and phenolic phytochemicals (Butt and Sultan, 2011; Ludwig et al., 2014; Jang et al., 2015). Coffee is among the most frequently consumed drinks worldwide and contains more than 1,000 natural compounds that can affect human health through their physiological action. Beyond laboratory investigations, many epidemiological and clinical studies have shown that coffee intake is associated with health benefits in relation to chronic diseases such as type 2 diabetes, cancer, and Parkinson's and Alzheimer's disease (Nkondjock, 2009; Van Dam and Feskens, 2002; Ross et al., 2000). Current research has begun to unravel the association between coffee and ocular health, with particular regard to the prevention of retinal degeneration. The present chapter explores the association between oxidative stress and retinal diseases and reviews the beneficial effects of antioxidants in coffee on ocular health, including the latest evidence of the protective effects of coffee against retinal degeneration.

COFFEE

The word "coffee" is believed to have originated in the Kefa province of North Africa (now part of Ethiopia), where coffee was first discovered (Anthony et al., 2001). In the sixteenth century, coffee was introduced into North America and Europe, and in the seventeenth century, colonists from Europe began to cultivate coffee trees in Central and South America. Following production of the first modern soluble coffee by Nestle in Brazil during the 1930s, the popularity of coffee increased dramatically, and in crop year 2012/2013, global coffee production reached 145.1 million bags (ICO, 2014).

The coffee tree belongs to the Rubiaceae family, genus *Coffea*. While more than 80 species have been identified worldwide, *Coffea arabica* (commonly known as Arabica coffee) and *Coffea canephora* (Robusta coffee) account for the entire global market (ABIC, 2011; Farah, 2012). These two species differ in terms of their ideal growing climate, physical aspects, beverage characteristics, and chemical composition.

Table 1. Chemical composition of unroasted and roasted *C. Arabica* (Farah, 2012)

Compounds	Concentration (g/100g)	
	Unroasted	Roasted
Carbohydrates/fiber		
Sucrose	6.0-9.0	4.2
Reducing sugars	0.1	0.3
Polysaccharides	34-44	31-33
Lignin	3.0	3.0
Pectins	2.0	2.0
Nitrogenous		
Protein	10.0-11.0	7.5-10
Free amino acids	0.5	ND
Caffeine	0.9-1.3	1.1-1.3
Trigonelline	0.6-2.0	0.2-1.2
Nicotinic acid	ND	0.016-0.026
Lipids		
Coffee oil	15.0-17.0	17.0
Diterpene esters	0.5-1.2	0.9
Minerals	3.0-4.2	4.5
Acids and esters		
Chlorogenic acids	4.1-7.9	1.9-2.5
Aliphatic acids	1.0	1.6
Quinic acids	0.4	0.8
Melanoidins	ND	25

Coffee is composed primarily of water, carbohydrates, fiber, proteins, free amino acids, lipids, minerals, organic acids, chlorogenic acids, trigonelline, and caffeine. The chemical composition of coffee beans changes during the production process and especially during roasting (Table 1). During that roasting process, changes in the chemical composition of coffee beans are caused by the Maillard reaction, carbohydrate caramelization, and organic compound pyrolysis (Belitz et al., 2009), resulting in decreased amounts of carbohydrates, proteins, lipids, minerals, and free amino acids in the coffee beans.

Coffee contains a variety of polyphenols, which account for 10% of dry weight. The main phenolic component is chlorogenic acid (CGA), derived primarily from esterification of *trans*-cinnamic acids (e.g., caffeic, ferulic, and *p*-coumaric) with (-)-quinic acid, which causes the coffee to taste astringent, bitter, and acidic. The transformation of CGA during roasting and brewing is complex. Although levels are greatly reduced by high pressure and heat during processing, coffee is a significant dietary source of CGA; a single serving of espresso coffee supplies 24–423 mg of CGA (Crozier et al., 2012). Consumption of non-espresso coffee may provide 1–2 g of CGA each day, exceeding CGA intake from fruits and vegetables (Ludwig et al., 2014). Studies of colostomy patients indicate that about one-third of ingested CGA and 95% of caffeic acid are absorbed intestinally (Olthof et al., 2001); about two-thirds of

ingested CGA reaches the colon, where it is metabolized by microflora (Olthof et al., 2003). Coupled with available standards such as hydroxycinnamate sulfates and glucuronides, the use of high-performance liquid chromatography/mass spectrometry (HPLC/MS) has enabled elucidation of the CGA metabolic pathway (Figure 1).

THE EYE AND RETINA

As the organ of vision, the eye supports our perceptions of brightness and color on a photosensitive layer of cells (Jacobson and Marcus, 2011). The human eye is typically spherical in shape, with a diameter of about 25 mm and a volume of 6.5 mL, filled with a transparent gel-like substance known as the vitreous gel (Figure 2) (Atchison et al., 2000). To facilitate sight, the eye's complex structures must function in concert. The cornea is the outermost layer, covering the front of the eye; the iris is a thin, circular structure that controls the diameter and size of the pupil. Relaxing or tightening of the muscles around the iris changes the size of the pupil, regulating the amount of light that enters the eye. Additionally, the ciliary body releases a transparent liquid within the eye and contains the ciliary muscle, which changes the shape of the lens, allowing the formation of a focused image on the back wall of the eye (the retina). At the center of the retina, the macula is its most sensitive part, responsible for detailed central vision. The fovea is the centermost part of the macula.

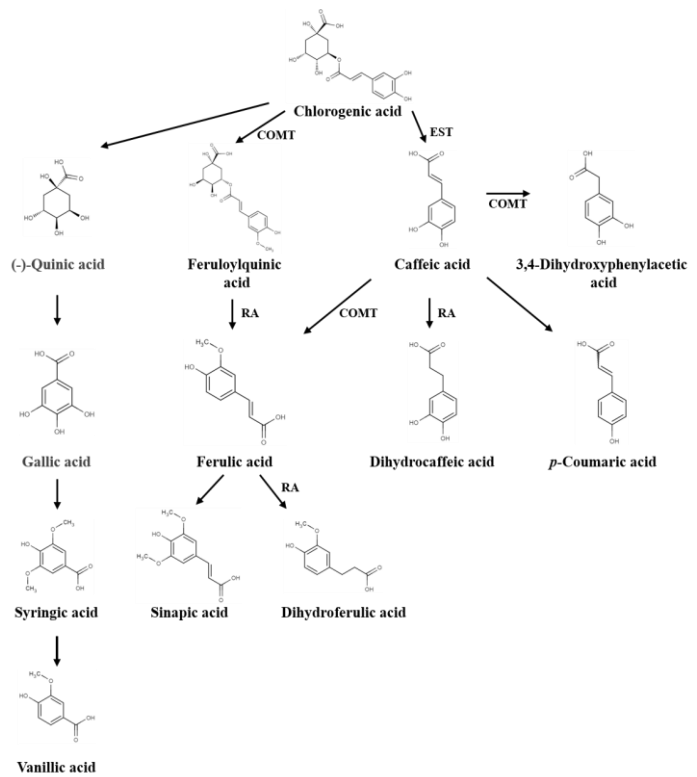

Figure 1. Proposed metabolism of chlorogenic acids in coffee (COMT: catechol-O-methyltransferase; EST: esterase; RA: reductase) (Adapted from Del Rio et al., 2010).

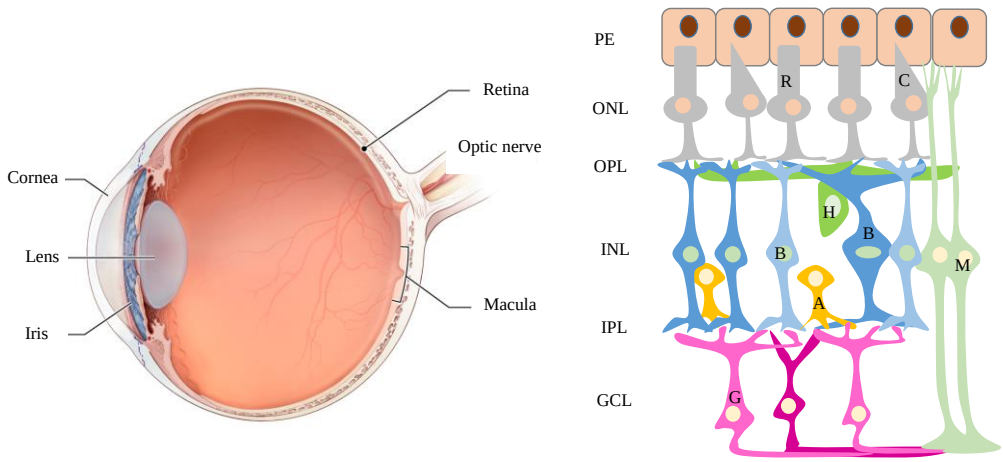

Figure 2. The structure of the eye and retina. The image on the left shows the structure of the eye, consisting of cornea, lens, iris, macula, and retina, and the optic nerve, which transmits visual information from the retina to the brain. The drawing on the right illustrates the cellular unit in the retina (A: amacrine cell; B: bipolar cell; C: cone; R: rod; H: horizontal cell; G: ganglion cell; M: Muller cell; PE: pigment epithelium; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer).

The retina is a layer of neural tissue, approximately 200 µm in thickness and containing several classes of specialized cell known as photoreceptors (Wässle, 2004), which are specialized neurons that transform light into electrical signals. When light enters the eye and reaches the retina, biochemical cascades occur in the photoreceptors; rod photoreceptors are highly sensitive and mediate dim-light vision, and cone photoreceptors are responsible for high-resolution and color vision. A photon of light activates a light-sensitive protein (rhodopsin), leading to photoisomerization of 11-cis-retinal to all-trans-retinal (Baehr et al., 2003). All-trans-retinal is reduced by NADPH-dependent all-trans-retinol dehydrogenase to all-trans-retinol. To sustain vision, the chromophore 11-cis-retinal must be properly recycled. In retinal pigment epithelium cells (RPE), all-trans-retinol is esterified and then converted to 11-cis-retinol that can be oxidized to 11-cis-retinal, which is then transferred back to the photoreceptor cells to regenerate rhodopsin. At the synaptic terminals of the rods and cones, signals are transferred to bipolar and horizontal cells (Wässle, 2004). Horizontal cells provide lateral interactions in the outer plexiform layer; bipolar cells transfer light signals to the inner plexiform layer, containing the dendrites of amacrine and ganglion cells. Amacrine cells provide a feedback synapse to the bipolar cell; ganglion cells collect the signals of bipolar and amacrine cells, and their axons transmit these signals to the brain through the optic nerve (Dowling, 1987) (Figure 2).

MAJOR TYPES OF RETINAL DEGENERATION

Retinal degeneration is deterioration of the retina caused by a number of factors that include genetic and environmental influences. Age-related macular degeneration (AMD) is a

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medical condition that affects older people, involving loss of the central field of vision and leading eventually to partial or complete blindness (Gehrs et al., 2006). AMD is characterized by the accumulation of drusen, extracellular deposits of lipids, cellular debris, and protein below the RPE basement membrane in the retina. Early AMD is marked by the initiation of drusen in the RPE, and advanced AMD takes two forms: geographic atrophy (or dry AMD) and choroidal neovascularization (or wet AMD). In the United States, an estimated 8 million people suffer from intermediate or advanced forms of AMD, making it the leading cause of vision loss in elderly individuals in developed countries (Ding et al., 2009; Buschini et al., 2011).

Diabetic retinopathy (DR) is the most common cause of vision loss among people with diabetes. DR begins with microaneurysms and progresses to exudative changes that lead to macular edema, ischemic changes, collateralization and dilatation of venules, and proliferative changes (Antonetti et al., 2012). DR causes impaired contrast sensitivity and loss in visual fields, resulting in difficulties with vision in daily life. Increased duration of diabetes and poor glucose control are major risk factors for retinopathy. Approximately 40% of diabetes patients over the age of 40 years have some retinopathy, and 8.2% of these patients have vision-threatening retinopathy (Bressler et al., 2011).

Glaucoma is a leading cause of irreversible vision loss worldwide, with predictions of 80 million sufferers by 2020 (Pizzarello et al., 2004). The condition is characterized by selective loss of retinal ganglion cells (RGCs) and their axons, which constitute the optic nerve. To begin, glaucoma patients typically present with symptoms of peripheral vision loss and may lose all vision in the absence of appropriate treatment. Intraocular pressure and an impaired ocular blood flow system are believed to be risk factors for glaucoma, which is not yet curable, and lost vision cannot be restored. Although medication and/or surgery is likely to halt further loss of vision, early diagnosis and treatment is likely to be the best protection against glaucoma, and dietary intake of antioxidants can help to lower the risk of retinal degeneration.

Retinitis pigmentosa (RP) is the most common inherited retinal disease. It is characterized by abnormalities of the photoreceptors or retinal pigment epithelium, resulting in the progressive dysfunction of photoreceptors and gradual visual loss, followed by complete blindness. In the US and Europe, the prevalence of RP is approximately 1:3000–4000 (Haim, 2002), but this varies slightly between studied populations. To date, more than 50 different genes or loci are known to be involved in the pathogenesis of RP. Its pathogenic variants can be determined by mode of inheritance (e.g., autosomal dominant: 15–25%; autosomal recessive: 5–20%; X-linked RP: 5–15%). The most frequent known causes are mutations in the *rhodopsin*, *USH2A* or *RPGR* genes (Abigail et al., 2013). There is no cure at present, but therapy with antioxidants (e.g., vitamin A, β-carotene) or dietary intake (e.g., docosahexaenoic acid (DHA), lutein/zeaxanthin) or other medications offer possible alternatives for slowing the progression of retinal degeneration caused by RP.

OXIDATIVE STRESS AND RETINAL DEGENERATION

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS). ROS can be free radicals, defined as oxygen species that have been elevated to a higher

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energy level or act as strong oxidizing agents. In pathophysiology, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($OH^·$), nitric oxide (NO), singlet oxygen ($^1O_2$), lipid peroxyl radicals ($LOO^·$), and peroxynitrites ($ONOO^·$) are major ROS (Stadtman and Berlett, 1997). These ROS are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes and from a variety of cytosolic enzyme systems, such as lipoxygenases, NADPH oxidase, and cytochrome P450. Exogenous sources, such as ultraviolet (UV) light, ionizing radiation, chemotherapeutics, inflammatory cytokines, and environmental toxins, can also trigger ROS production in the body (Sitte and von Zglinicki, 2003). Most of these ROS are present at low levels during normal physiological conditions as byproducts of normal aerobic metabolism or as secondary messengers in signal transduction pathways (Sharma et al., 2012). Excessive production of ROS is largely counteracted by an intricate antioxidant defense system, including enzymatic scavengers such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Finkel and Holbrook, 2000). In addition, nonenzymatic antioxidant defense systems are important for scavenging ROS. These include ascorbate, pyruvate, flavonoids, and carotenoids. However, when production overwhelms the defense system, ROS can cause severe damage to mitochondrial and cellular proteins, lipids, and nucleic acids (Uttara et al., 2009).

Retinal degeneration is attributed mainly to oxidative stress, which may be a consequence of attenuated antioxidant cell defense systems or augmented levels of ROS in the retina (Jarrett et al., 2008). The retina is vulnerable to and abundant in ROS; oxidative stress can cause severe damage to the retina through dysregulation of intracellular physiology, leading to ocular neurodegeneration. The retina is a high-metabolism tissue, with the highest oxygen consumption per unit weight of all human tissues (Yu and Cringle, 2001). Oxygen and nutrients are supplied by two separate circulatory systems: the retinal vasculature and the choroidal vasculature (Yu and Cringle, 2005). The retinal vasculature is supplied by the central retinal artery, which enters the retina with the optic nerve at the optic disc. Artery branches supply blood to the neurons and glial cells of the inner portions of the retina. Choroidal circulation is supplied by the long and short ciliary arteries and by the anterior ciliary arteries, which feed the large arteries in the outer portion of the choroid. These artery branches supply blood to meet the high metabolic demands of the photoreceptors (Newman, 2013). The high partial pressure of oxygen in the retina's photoreceptors promotes generation of ROS via the mitochondria, which may also cause damage to mitochondrial DNA (mtDNA) (Cui et al., 2012).

Importantly, the retina is also exposed to high-energy light. Each day, the human retina absorbs approximately 10^{12}–10^{15} photons (Hunter et al., 2012), which can cause irreversible damage to the retina, including immediate thermal injury by bright light and photochemical damage by exposure to light for an extended period of time (Nowak, 2013). Although radiation is partly absorbed by the cornea and lens, light from 400–760 nm reaches the retina. The shorter the wavelength, the greater the energy; within the visible light range, blue light (400–500 nm) has the highest energy and can cause damage to photoreceptors. According to one study, exposure of the retina to blue light *in vivo* leads to cell proliferation and mitotic alterations in RPE and choroidal cells as well as spots on the RPE cell layer, as in the early signs of AMD (Ham et al., 1978).

In addition, polyunsaturated fatty acids are abundant in the photoreceptor membrane. Docosahexaenoic acid (DHA) absorbed from the diet accounts for more than 30% of total fatty acids in the membrane (SanGiovanni and Chew, 2005). Because of the susceptibility of

unsaturated fatty acids to oxidation, photoreceptors are vulnerable to lipid peroxidation (Nowak, 2013). This may produce peroxides and radicals, which may in turn cause functional and structural damage to the cell layer, resulting in the degeneration of photoreceptors.

PYOTOCHEMICALS AND RETINAL DEGENERATION

Phytochemicals are nutrients and non-nutritive components that offer physiological benefits, including antioxidant, anti-inflammatory, anti-tumor, anti-viral, and immunomodulatory properties (Grusak, 2002; Surh, 2003). As the pathogenesis of retinal degeneration seems to involve a number of factors, phytochemicals with multiple pharmacological properties increase the likelihood of retarding progression of the disease. Accumulating evidence has shown that phytochemicals may be involved in the prevention and reversal of age-related eye diseases, including AMD, glaucoma, and diabetic retinopathy (A Omara et al., 2010; Rhone and Basu, 2008).

High dietary intake of omega-3 fatty acids and the macular pigments lutein and zeaxanthin is associated with lower risk of AMD (Group, 2013). The Age-Related Eye Disease Study (AREDS), a major clinical trial sponsored by the National Eye Institute, showed a beneficial effect of high doses of vitamins C, E, β-carotene, zinc, and copper in reducing the rate of progression to advanced AMD in patients with intermediate AMD or with one-sided late AMD (Chew et al., 2013). The AREDS-2 study reported that lutein and zeaxanthin may serve as substitutes for β-carotene because of the latter's potential association with increased incidence of lung cancer (Group, 2013). Resveratrol, originally isolated from the skin of grapes, has been shown to reduce symptoms of AMD; treatment with 50 or 100 μM resveratrol significantly reduces *in vitro* proliferation of RPE cells by 10% and 25%, respectively (King et al., 2005). There is also evidence that resveratrol protects human retinal pigment epithelium cells from acrolein-induced damage (Sheu et al., 2010). *Ginkgo biloba* extract has numerous properties that should in theory offer benefits in treating non-intraocular pressure-dependent mechanisms in glaucoma (Cybulska-Heinrich et al., 2012). The extract's multiple beneficial actions include increased ocular blood flow, antioxidant activity, platelet-activating factor inhibitory activity, nitric oxide inhibition, and neuroprotective activity. Based on electroretinogram measurements, epigallocatechin gallate (EGCG), a catechin-based flavonoid from green tea, confers neuroprotection for retinas injured by ischemia/reperfusion, indicating that EGCG supplementation may be beneficial in the treatment of glaucoma (Falsini et al., 2009). Mirtogenol is a combination of two herbal extracts: bilberry and French Maritime pine bark. Both have antioxidant properties and are linked to reduced risk for developing symptomatic glaucoma by controlling intraocular pressure and improving ocular blood flow (Steigerwalt et al., 2008). French Maritime pine bark extract has also been shown to strengthen capillaries in the retina, so contributing to protection against retinopathy (Jain et al., 2014). Grape seed extract can protect blood vessels and capillaries from ROS damage (Agarwal et al., 2004); research has shown that grape seed extract may be effective in protecting blood vessels and capillaries against symptoms of diabetic retinopathy (Zafra-Stone et al., 2007). The flavonoid quercetin may help to prevent

blood clots because it inhibits inflammation by reducing histamine formation (Guardia et al., 2001). Quercetin also mitigates formation of insulin-like growth factor, which is significant for the development of diabetes (Vessal et al., 2003). Quercetin also reduces high blood pressure, which can induce stress on the walls of retinal blood vessels (Edwards et al., 2007).

COFFEE AND NEURODEGENERATION

The brain and retina are the key areas of the central nervous system in which neuroprotection is pivotal in guarding neuronal functions against neurodegeneration. With its abundant bioactive compounds, coffee is thought to provide neuroprotection through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. Indeed, previous studies have reported that coffee is associated with the prevention of a number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and other neuropathies (Butt and Sultan, 2011; Ludwig et al., 2014). Moderate intake of caffeine in coffee can lead to decreased abnormal amyloid-β protein production by regulating Presenilin 1 (PS1) and β-secretase (BACE) (Arendash et al., 2006; Arendash and Cao, 2009). These two enzymes are known to play an important role in amyloid formation and their inhibition in AD conditions (Fujimoto et al., 2008). The most recent evidence also suggests that one component of coffee, eicosanoyl-5-hydroxytryptamide (EHT), inhibits hyperphosphorylation of tau protein and reduces intraneuronal amyloid-β accumulation in a sporadic AD model (Basurto-Islas et al., 2014). Coffee has been shown to have similar effects in the context of neuroprotection in PD models, apparently turning on the antioxidant defense system by regulating mRNA and enzymes such as CYP1A2, and Nrf2-ARE, all of which are actively involved in detoxification in noxious conditions (Popat et al., 2011; Higgins et al., 2008). Caffeine and antagonists for A2A adenosine receptor are likely to protect against dopaminergic neurotoxicity in PD models (Chen et al., 2001, Ikeda et al., 2002). In particular, interaction between caffeine and A2A receptors may rescue dopaminergic pathology in PD models by modulating the release and uptake of the neurotransmitter adenosine, with calcium signaling (Lim and Tan, 2012). Additionally, kahweol in coffee can induce an antioxidant enzyme heme oxygenase-1 (HO-1) expression in PD-related neurotoxin conditions by regulating the PI3K and p38/Nrf2 signaling cascades that may act on neuroprotection in PD conditions (Hwang and Jeong, 2008). More recently, in a useful study of well-known compounds of coffee, caffeic acid (CA) and CGA showed neuroprotective properties by inhibiting acethylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity, both of which are highly associated with acetylcholine and butyrylcholine breakdown in brain tissue (Oboh et al., 2013). As these enzymes are essential for the recycling of neurotransmitters, they may be connected to various neurodegenerative disorders if impaired. Other studies have shown that CGA in coffee exerts similar neuroprotective effects in scopolamine-induced amnesia in mice and ischemic stroke in rabbits (Kwon et al., 2010; Lapchak, 2007).

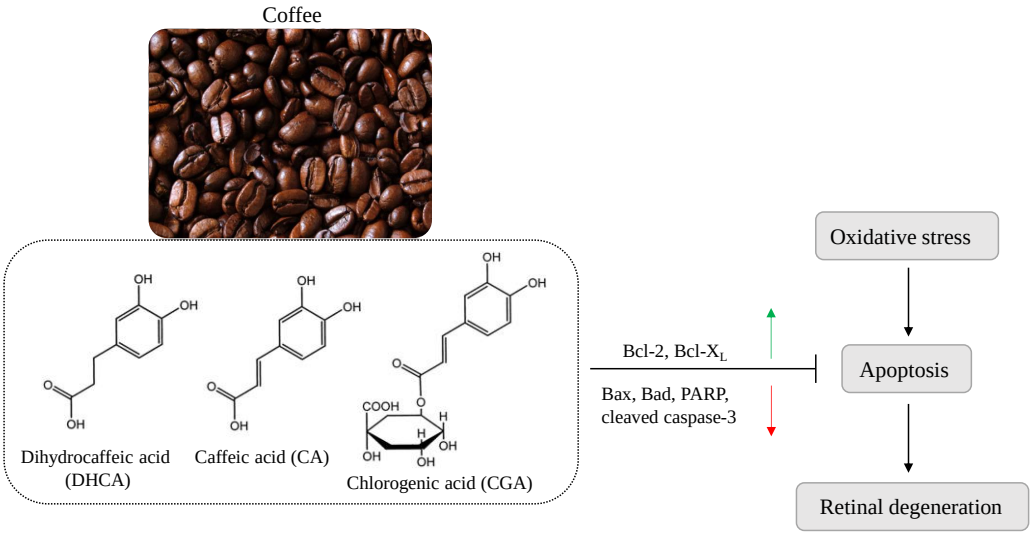

Figure 3. CGA and coffee metabolites (CA and DHCA) reaching the eye and their protective effects against oxidative stress-induced retinal degeneration.

COFFEE AND OCULAR HEALTH

As coffee contains many phytochemicals, it is thought to be a major source of dietary antioxidants. However, few studies have examined the association between coffee and ocular health, and most of these studies have focused on the effects of caffeine. Interestingly, one study showed that consumption of coffee may prevent dry eye syndrome (Moss et al., 2000). Moreover, according to a 5-year prospective cohort study, coffee consumption is not associated with the development of early age-related maculopathy (Tomany et al., 2001). The effects of caffeine on intraocular pressure, which can induce glaucoma, remain controversial. In particular, clinical studies on the relationship between coffee consumption and risk of glaucoma have yielded conflicting results (Jiwani et al., 2012; Pasquale et al., 2012). Caffeinated beverages (over 180 mg of caffeine) may cause ocular hypertension, but these have not been shown to have any clinical impact on glaucoma (Avisar et al., 2002; Jiwani et al., 2012).

Along with caffeine, CGA has been reported to exhibit several pharmacological effects, including antioxidant, antimicrobial, and hypoglycemic activities (Bassoli et al., 2008; Lou et al., 2011b; Wu et al., 2006). CGA may also have anti-angiogenic effects on neovascular age-related macular degeneration (Kim et al., 2010). *In vitro* study shows that CGA also plays a protective role in respect of lens opacity and high glucose-induced cytotoxicity in human lens epithelial cells, which suggests that CGA supplementation may offer a potential therapeutic approach for prevention of cataracts caused by diabetic complications (Kim et al., 2011).

COFFEE AND RETINAL DEGENERATION

Significant evidence has emerged of the beneficial effects of coffee extract and its bioactive compounds on retinal degeneration. In particular, one recent study has shown that CGA in coffee has the potential to reduce blood-retinal barrier (BRB) breakdown and vascular leakage by preserving tight junction proteins and low VEGF expression in a rat model of diabetic retinopathy (Shin et al., 2013). In RP patients, CGA supplementation may improve multifocal electroretinography, implying a beneficial effect on peripheral regions during retinal degeneration (Shin and Yu, 2014). There is also direct evidence that coffee and CGA offer protection against hypoxia-induced retinal damage (Jang et al., 2012). According to this report, CGA and coffee extract reduce RGC apoptosis caused by hypoxia through downregulation of apoptotic proteins such as Bax, Bad and cleaved caspase-3, both *in vitro* and *in vivo*. Another study has revealed that phenolic acid metabolites, including CGA, caffeic acid (CA), and dihydrocaffeic acid (DHCA) reach the eye following coffee ingestion, exerting protective effects against hypoxia and optic nerve crush-induced retinal damage (Jang et al., 2015). The evidence suggests that coffee metabolites upregulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL and downregulate pro-apoptotic proteins such as Bad, PARP and cleaved caspase-3. (Figure 3). These studies support the view that coffee consumption and its metabolites can reach the retina and protect against retinal degeneration, offering a therapeutic approach for prevention of human retinal diseases.

CONCLUSION

As the leading worldwide beverage, coffee's impact on human health is of great interest. The accumulating evidence supports the bioavailability of coffee to maintain ocular health by preventing retinal degeneration, as for instance in the case of excess ROS, which can be toxic to photoreceptors and retinal ganglion cells and may contribute to other types of degeneration by affecting ocular cells including glia and immune cells. In particular, chlorogenic acid (CGA), and caffeic acid (CA), and dihydrocaffeic acid (DHCA) can contribute to this protective role. In addition to these polyphenols, coffee contains many other bioactive phytochemicals that may have potential to protect the eye against retinal degeneration. Their potential remains largely unknown because of insufficient investigation to date, and further studies of the biofunctionality of coffee components are needed to elucidate the effects of coffee compounds and their biochemical and metabolic mechanisms on human ocular health. Along with this prospective data, future research may bring more promising evidence of how coffee and its constituent compounds can help to prevent retinal pathologies and maintain ocular health.

ACKNOWLEDGMENTS

This work was financially supported by an intramural grant (2E25980, 2Z04690) from the Korea Institute of Science and Technology. T.J.K and H.J contributed equally to this work.

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BIOGRAPHICAL SKETCHES

Sang Hoon Jung

Name: Sang Hoon Jung
Affiliation: Natural Products Research Center, Korea Institute of Science and Technology (KIST), Department of Biological Chemistry, University of Science and Technology
Date of Birth: September 7, 1975
Education: Seoul National University, Korea (Ph.D.)
Address: 679 Saimdang-ro, Gangneung 25451, Republic of Korea

Research and Professional Experience

- 2014-present: Chief for Natural Products Research Team for Aging Intervention, Convergence Research Center for Natural Products, Korea Institute of Science and Technology (KIST), Korea
- 2014-present: Principal Research Scientist, Korea Institute of Science and Technology (KIST), Korea
- 2013-present: Representative, The Korean Society of Pharmacognosy, Korea
- 2013-present: Associate Professor, University of Science and Technology (UST), Korea
- 2010-present: Editorial Board, Neural Regeneration Research
- 2011-2013: Senior Research Scientist, Functional Food Center, Korea Institute of Science and Technology (KIST), Korea
- 2006-2011: Senior Research Scientist, Natural Products Research Center, Korea Institute of Science and Technology (KIST), Korea
- 2004-2006: Research Scientist, Natural Products Research Center, Korea Institute of Science and Technology (KIST), Korea
- 2007-2008: Research Fellow, Nuffield Laboratory of Ophthalmology, University of Oxford, United Kingdom
- 2003-2004: Research Associate, Natural Products Research Institute, Seoul National University, Korea
- 1998-2000: Teaching Assistant, Natural Products Research Institute, Seoul National University, Korea

Professional Appointments

- Chief for Natural Products Research Team for Aging Intervention
- Associate Professor, University of Science and Technology (UST), Korea

Honors:

- 2014.12 Best Research Project, KIST
- 2013.12 Best Young Scientist in 2013, The Korean Society of Pharmacognosy
- 2012.2 Best Research Team, KIST

Publications Last Three Years:

30 SCI papers including

- Kim K., Kang S.W., Ahn H.R., Song Y., Yang S.J., Jung S.H.*, The leaves of persimmon (*Diospyros kaki* Thunb.) ameliorate *N*-methyl-*N*-nitrosourea (MNU)-induced retinal degeneration in mice. *J. Agric. Food Chem*., 63(35):7750-9 (2015)
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Tae-Jin Kim

Name: Tae-Jin Kim
Affiliation: Natural Products Research Center, Korea Institute of Science and Technology (KIST)
Date of Birth: September 7, 1977
Education: University of Illinois at Urbana-Champaign, IL, USA, (Ph.D.)
Address: 679 Saimdang-ro, Gangneung 25451, Republic of Korea

Research and Professional Experience:

- 2014-2015: Senior Fellow, Department of Bioengineering, Center for Cardiovascular Biology, Institute of Stem Cell and Regenerative Medicine, University of Washington at Seattle, WA, USA
- 2013-2014: Postdoctoral Research Associate, Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, IL, USA
- 2007-2013: Graduate Research Assistant, Neuroscience Program, University of Illinois at Urbana-Champaign, IL, USA
- 2006-2007: Research Fellow, Department of Biology, Kyungpook National University, Daegu, Republic of Korea
- 2004-2006: Graduate Research Assistant, Department of Biology, Kyungpook National University, Daegu, Republic of Korea

Professional Appointments:

- 2016-present, Adjunct Associate Professor, University of Science and Technology (UST), Republic of Korea
- 2015-present, Principal Investigator, Senior Research Scientist, Korea Institute of Science and Technology (KIST), Republic of Korea

Honors:

- 2013 BMES Fellow Award, BMES-CMBE conference, Hawaii, USA
- 2009-2010 Beckman Institute Graduate Fellowship, Beckman Institute for Advanced Science and Technology, USA
- 2006-2007 Korea Research Foundation Fellowship, Republic of Korea

Publications Last Three Years:

- Kim T.J., Joo C., Seong J., Vafabakhsh R., Botvinick E.L., Berns M.W., Palmer A.E., Wang N., Ha T., Sun J., Wang Y. Distinct mechanism regulating mechanical force-induced calcium signals at the plasma membrane and the ER in human MSCs. *eLife*, 4. doi: 10.7554/eLife.04876 (2015)
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Chang Yong Lee

Name: Chang Yong Lee
Affiliation: Department of Food Science, Cornell University.

Date of Birth: April 12, 1935
Education: Utah State University, Korea (Ph.D.)

Address: 349 Stocking Hall, Cornell University, Ithaca, NY 14853

Research and Professional Experience

- 1969-present: Assistant Professor, Associate Professor, Professor of Cornell University
- 2014-present: Distinguished Adjunct Professor of King Abdulaziz University, Saudi Arabia
- 2010-2013: International Visiting Scholar, Kyung-Hee University, Korea
- 2002-2008: Co-Director, Institute of Food Science, Cornell University
- 2002-2008: Department Chair, Food Science and Technology, Cornell University.
- 2002-2008: Faculty-in-Residence, Cornell University
- 2000-2001: Visiting Professor, Korea University, Korea
- 1991-1992: Visiting Professor, Ecole Nationale Superior des Industires Agricole et Alimentaire, France
- 1988-1991: Visiting Professor, Beijing Vegetable Research Center, China
- 1984 -1985: Visiting Scientist: Centre de Recherches D'Avignon, Institut National de la Recherche Agronomique (INRA), France

Professional Appointments

- 1992, Jury Member of Doctoral Thesis - University of Paris-VII and Paris XII, France
- 1987-1990, Board Member of Editors of the Journal of Food Science
- 1987-present, Editorial Board Member for the CRC Critical Reviews in Food Science and Nutrition
- 2004-2007, Advisory Board, Italian Journal of Food Science
- 2007-present, Editorial Advisory Board Member of International Journal of Molecular Sciences

Honors:

- 1990, Fellow, American Chemical Society-Division of Agriculture and Food Chemistry
- 1994, Platinum Award, ACS Division of Agriculture and Food Chemistry
- 1996, Fellow, Institute of Food Technologists
- 1998, Fellow, Korean Academy of Science and Technology
- 2001, Honor Award for Excellence, Secretary of USDA
- 2003, International Life Science Institute and Institute and Food Technologist Babcock-Hart Award
- 2005, Selected as one of the Highly Cited Researchers in 2004, ISI, Philadelphia
- 2006, Fellow, international Academy of Food Science and Technology

Publications:

Five representative papers from over 200 SCI papers published:

- Shallenberger, R. S., Acree, T. E. and Lee, C. Y. Sweet taste of D- and L-sugars and amino acids and the steric nature of their chemo-receptor site. *Nature*, 221:555-556 (1969)

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 8

COFFEE CONTAMINATED WITH OTA AND GENOTOXICITY

Daniel Lerda
PhD in Biochemistry, Genetic Molecular Laboratory, Qeen Fabiola University Clinic, School of Medicine, Córdoba Catholic University, Córdoba, Argentina

ABSTRACT

There is growing interest in the presence of OTA in coffee since it was detected in green coffee beans, roasted and infusions. Numerous studies show that the roasting process influences the destruction of OTA, although the results are quite contradictory, as some authors believe that no significant differences were detected below 12% in relation to OTA reduction roasting operation, while others have argued that OTA production around 80%, or even higher values is reduced.

Species *Allium cepa* is an efficient test organism to study the genotoxic effects induced by mycotoxins such as OTA. *A. cepa* were studied for genotoxic properties of roasted coffee beans and artificially contaminated with OTA. Genotoxicity tests in meristem cells indicated that the roasting process was not efficient enough for the degradation of OTA as the mutagenic and clastogenic effects were not reduced. This demonstrates that the compounds of OTA degraded possibly combined with compounds of *Coffee arabica* form toxic compounds for plant cells.

Keywords: coffe, roasted, OTA, *Allium cepa*, genotoxicity

INTRODUCTION

Ochratoxin (OTA), first detected in African corn samples [1], is considered a toxic secondary metabolite produced by species of filamentous fungi of the genera *Penicillium* and *Aspergillus*, with the ability to grow on a wide range of organic substrates.

OTA has demonstrated to have hepatotoxic, carcinogenic, immunosuppressive and teratogenic properties and was classified as carcinogenic to humans (Group 2B) [2, 3].

In humans, the main sources of dietary exposure is through cereals and in animals is through feed, but OTA levels can also be found in other foods. In cases of high consumption, we must consider green coffee beans, meat and its derivatives, grapes, wine, raisins and dried figs, chocolate, beans, beer and spices. Tea, herbal teas, olive oil and baby foods from cereals are sources of OTA. OTA levels in food are conditioned by agricultural practices, humidity and temperature during storage and transport. Also, it has been shown that a high activity of water favors the production of OTA in food [4]. In addition to OTA, there are other types of ochratoxin such as ochratoxin B (OTB) which is a less toxic, non-chlorinated derivative of OTA and ochratoxin C (OTC) ester of OTA with low toxic potential, both hydrolysis products from OTA and OTB respectively, which are characterized by their lack of toxicity [5].

OCHRATOXIN IN COFFEE

Ochratoxin A in coffee has aroused special interest since its first study in samples of green coffee beans [6] and especially in the detection of the toxin in samples of roasted coffee and coffee infusions [7, 8]. OTA in green coffee samples was usually found in concentrations between 0,2 and 62 ug kg-1 [9]. OTA in roasted and brewed coffee was reported by Tsubouchi, Yamamoto, Hisada, Sakabe and Udagawa [10]. Before these data, it was generally accepted that OTA was decomposed during roasting; however concentrations above 20 ug kg-1 have been reported in commercial roasted coffee [11]. Various reports concerning the impact of roasting on OTA content in coffee have shown an OTA reduction range from 0-12% to 90-100% [12]. Such variations may be related to different analytical conditions on the roasting process or heterogeneity in the distribution of the toxin [13].

Coffee is an important food in human consumption and despite technological advances, neither the roasting nor the preparation processes ensure a complete destruction of OTA, so it is necessary a proper hygiene control in the production of green coffee to preserve the health of the consumers, thus reducing their exposure to dietary intake of this toxin [14].

In literature, both analyses, Chromosome Aberrations (CA) and micronucleus (MN) in meristem cells of *Allium cepa*, have been reported as effective indicators of direct action on DNA. It is known that CA like fragments and loss of chromosomes can result in micronucleated cells, where both fragments and whole chromosomes cannot be incorporated into the nucleus during the cell cycle. Of all endpoint tests the MN is the most effective and simple indicator of cytological damage, the analysis of this parameter is the most efficient to evaluate environmental contaminants [15]. CA analysis also detects the genotoxic effects of various chemicals, allowing the evaluation of their clastogenic and aneugenic action.

COFFEE GENOTOXICITY

In the work done by Lerda et al. [16] random samples of green coffee beans (*Coffea arabica*) were used, coming from Brazil and sold in Córdoba, Argentina. These grains were artificially contaminated with OTA and then subjected to roasting. Subsequently, the green coffee beans not contaminated with OTA and the contaminated and roasted coffee beans were

analyzed for the content of OTA with the method of Nakajima et al. [17]. The conditions of the degree of roasting and OTA content are shown in Table 1.

Table 1. Condition, degree of roasting and OTA content in green and roasted coffee samples

Condition of roasting		Ochratoxin A (ng/Kg)[a]		degree of roasting
		Samples	Control[b]	
Temperature (ºC)	Time (min)			
230	0[c]	12 +/- 7,0	0	
	5	9 +/- 0,2	0_5	Moderate clear
	8	5 +/- 2,1	0_8	Dark
	12	2,2 +/- 1,0	0_{12}	Very dark

[a]The result is the average of SD +/- of four analysis.

[b]Control, coffee beans no contaminated with OTA and identified according to roasting time 0, 0_5, 0_8 and 0_{12}.

[c]Sample of non-roasted green coffee.

Table 2. Frequency and spectrum of cytological aberrations induced by OTA content in green and roasted coffee and non-contaminated roasted coffee in the *Allium cepa* Assay

						Frequency of aberrant Cell % based in		
			Abnormalities					
Ochratoxin A (ng/Kg)	Nº of cells	Cells in div.	Breaks	Bridges	binucleate cells	Total of aberrant cells	Total of cells	Nº of div. cells
0[a]	5100	385	-	-	-	-	-	-
0_5a	5400	344	-	-	-	-	-	-
0_8a	5200	360	-	-	-	-	-	-
0_{12}a	5350	381	1	3	2	6*	0.11	1.57
2.2	5200	390	-	-	-	-	-	-
5	5220	317	1	4	1	6*	0.11	1.89
9	5700	341	0	5	2	7*	0.12	2.05
12	5100	360	1	6	4	11*	0.22	3.05
Control								
Negative	5220	350	-	-	-	-	-	-
Control								
Positive[b]	5410	347	7	13	11	31	0.57	8.90

-no clastogenic effects are observed.

*Z = 8,01 significant to P < 0,05 compared to the positive and negative control.

[a]non-contaminated coffee beans.

[b]Ethyl methyl sulfoxide 0.2%.

Allium Cepa Assay

For this test pearl onion bulbs were used and green and roasted coffee beans were analyzed. Samples (OTA contaminated green coffee and roasted coffee) were added when the roots reached 2-3 cm in length.

Proliferative activity was evaluated and the samples were exposed to the Allium cepa roots to determine CA and MN. The CA were studied with concentrations of 2.2, 5, 9 and 12 ng/kg of contaminated and non-contaminated samples. OTA induces clastogenic effects in meristematic roots of Allium cepa. Data on the frequency of aberrant cells are shown in Table 2.

An increase in the frequency of aberrant cells was observed in the concentrations 5, 9 and 12 ng/kg. Aberrant cells were observed in the non-contaminated sample 0_{12}. The Irwin Fischer (Z) exact probability test was applied using the total number of aberrant cells, where the concentrations of 5, 9 and 12 ng/kg of OTA produced cell rupture, bridge and binucleated cells that differ from the positive control. Irwin- Fischer Z test was 8.33 (>1.90) and significant at $P < 0.05$. The detected CA included chromatid breaks, bridge and binucleated cells from other cell types.

The mutagenic effect in the MN assay was recorded by counting the *A. cepa* meristematic cells (Table 3).

At concentrations 5, 9 and 12 ng/kg an increase in the frequency of MN was observed when compared to the negative control. An increase in the frequency of MN was observed in the concentration 0_{12} of non-contaminated samples compared to the negative control. No increase of MN was observed in the concentration 2.2 ng/kg, nor in non-contaminated coffee beans 0^a, $0_5{}^a$ y $0_8{}^a$.

Table 3. Micronucleus (MN) frequency in meristematics cells *A. cepa* induced by OTA content in green and roasted coffee and non-contaminated roasted coffee

Ochratoxin A (ng/Kg)	MN
0^a	-
0_5a	-
0_8a	-
0_{12}a	1.80 +/- 1.02
2.2	-
5	1.30 +/- 1.03
9	2.10 +/- 1.10
12	3.40 +/- 1.01
Control	
Negative	0.03 +/- 0.07
Control	
Positive	4.55 +/- 1.40

[a]non-contaminated coffee beans.

[-]absence of micronuclei.

Allium cepa represents one of the most used organisms in biomonitoring studies. Different tests to identify the presence of potential genotoxic and mutagenic chemical compounds have been carried out with this plant.

Meristematic cells of *A. cepa* have several features that are suitable for cytogenetic studies, so it is recommended to assess chromosomal aberrations in environmental pollutants. The coffee bean, like seeds of any other plant, contains all the constituent materials of a plant cell, such as the cell wall: cellulose, hemicellulose, pectins, lignins, gums, proteins, minerals, pigments, fats, waxes and oils. Inside the cells, organelles, including the nucleus and mitochondria in the cytoplasm containing proteins, lipids, nucleic acids, carbohydrates, chlorophyll, carotenoids, and a large amount of enzymes and plant hormones. All these cell compounds are transformed in the roasting process, resulting in a number of identified and unidentified compounds that are consumed in the processed coffee. Some of the compounds are destroyed in the process resulting in new volatile and gaseous behaving substances. Cramer et al. [18] detected Ocratoxin A degradation products during coffee roasting. To this end different heating models were used and the reaction products were analyzed by HPLC-DAD and HPLC-MS/MS. Two products were identified, the isomerization to 14 -® Ochratoxin A and the decarboxylation to 14-decarboxy-Ochratoxin A. In the analyzed samples the first was formed in amounts up to 25.6% over the OTA and the second formed only in traces. For assessments of toxicity tests were performed in cultured human kidney epithelial cells and it was found that both compounds showed a lower cytotoxic effect and apoptosis that OTA.

In our study [16], we observed that the random sample of green coffee beans (*Coffea arabica*) artificially contaminated with OTA showed a concentration of 12 ng/kg, which is substantially reduced with roasting. Gopinandhan et al. [9] found, in green coffee, concentrations between 0.2 and 13.5 ng/kg.

At higher concentrations of OTA in green and roasted coffee, they showed chromosomal alterations and in the non-contaminated 0_{12} samples the most common abnormality were the Bridges. These probably occur due to the disruption or binding of chromosomes or chromatids or as a result of chromosome stickiness or it may be attributed to an unequal translocation or inversion of chromosome segments. The most frequent chromosomal aberration in the investigated samples was the bridge and this is an indicator of toxic effect on the genetic material, which can develop irreversible damage to the cells, including cell death. Cytological changes observed in chromosomal aberrations of *Allium cepa* showed that OTA can induce genotoxicity at chromosome level. The effectiveness of MN test for screening of mutagenicity has been well established. Micronuclei are chromosome fragments or chromosomes that were not incorporated into the nucleus of the daughter cells and appear in the cytoplasm. Micronuclei are a manifestation of chromosomal breakage and failure of normal function of the spindle. Probably MN formation is due to a clastogenic effect.

Genotoxicity tests in the meristematic cells of A. cepa indicated that the roasting process was not efficient enough in the degradation of OTA, because the clastogenic and mutagenic effects were not reduced, showing that possibly the OTA compounds that degraded may been linked to *coffea arabica* compounds forming compounds which are toxic for plant cells.

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In: Coffee: Production, Consumption and Health Benefits ISBN: 978-1-63484-714-8
Editor: John L. Massey © 2016 Nova Science Publishers, Inc.

Chapter 9

SUBSTANCES PRESENT IN COFFEE: HEALTH OR RISK?

Maurílio de Souza Cazarim*
School of Pharmaceutical Sciences of Ribeirao Preto,
University of São Paulo, Brazil

ABSTRACT

Coffee is one of the most popular beverages in the world with consumption of 6.7 million tons per year, approximately. This beverage made up of some substances that theirs clinical effects have been studied. Some studies highlight the caffeine as the most responsible for the coffee clinical effects of coffee. The Cafestol, kahweol and chlorogenics acid have been evidenced clinically as important as caffeine. There is evidence that cafestol and kahweol contribute to the worsening of cardiovascular disease due to the increasing in cholesterol levels. On the other hand, the cafestol provides a positive aspect in some pathologies. The chlorogenics acid provides a preventive action for some types of cancer. This substance shows an inhibitory effect in hyperplasia induction in hepatic cells. Otherwise, chlorogenic acid is a substance able to increase the release of gastrin, which can cause gastric discomforts. The caffeine has been studied in some diseases cases like type 2 diabetes, arrhythmias, cardiac arrest, nonfatal acute myocardial infarction, parkinson and alzheimer disease. However, the vulnerability and the clinical manifestations attributed to these substances depend of some attributes. In addition, their clinical effects touches the habit of consumption, frequency of coffee ingestion, kind of beverage preparation, sex of the individual and other important attributes.

Keywords: coffee, caffeine, food science, clinical pharmacology, risk factors

In the last two decades, the beneficial effects of the coffee on the liver have been the subject of growing interest. The evidence indicates that coffee consumption may reduce the risk of primary liver cancer development. Animal studies have demonstrated the inhibitory

* Corresponding author: Av. Bandeirantes, 3.900, Monte Alegre - Ribeirão Preto – SP, CEP: 14040-900, Brasil. Faculdade de Ciências Farmacêuticas, Campus USP/RP, Email: maurilio.jf@gmail.com.

effect of the coffee in the development of liver carcinomas [1-4]. Larsson and Wolk (2007) estimate that the ingestion of two cups of coffee a day is enough to reduce the risk of liver cancer, decreasing approximately 43% of these risks. However, these authors still argue that in people infected with hepatitis C a reasonable statistic is not observed, not allowing the association of a reduced risk of this cancer with coffee consumption. So, it seems reasonable to assume that the protective properties of coffee, considering the hepatic carcinoma, depends on variables ranging from the type of liver cancer and the clinical condition of the patient, to the personal characteristics and habits of life [3]. However, more consistent researchs is needed, since there are a lot of possible substances responsible for the inverse association between coffee and liver carcinoma [5].

The relation of coffee with cirrhosis is based mainly on studies in which such pathology is caused by alcohol abuse, regardless of the measure of this disease. From this perspective, Gelatti et al. (2005) have observed in different Caucasian populations the relationship between coffee consumption and the enzyme gamma-glutamyl transferase (GGT). The coffee was efficient to inhibit the induction of GGT, protecting the liver cells from damage induced by alcohol intake. Based on this fact, coffee was able to reduce the risk of cirrhosis. Data from literature reinforce the inverse association between coffee consumption and serum levels of liver enzymes, including the GGT, which is an indicator of cirrhosis risk, and the alanine aminotransferase, a marker of liver injury. Both enzymes are associated with the risk of chronic liver disease and the development of cirrhosis [3].

The coffee has also been the object of study taking into account the development of cardiovascular disease [1, 7-9]. This relationship can be assigned due to the association with elevated blood pressure, without the development of hypertension. On the other hand, this is not valid for people who have a predisposition to this clinical condition, including some risk factors [10].

The genetic influence of coffee on human body, considering the amount consumed, is an important factor due to the cardiovascular problems. Both the regular and irregular users may exhibit the same caffeine plasma levels. However, the irregular consumer presents an unusual increase in both systolic and diastolic blood pressure making use of caffeinated or decaffeinated coffee. In the regular user of caffeinated coffee is not observed a significant change in blood pressure, the heart rate and the cardiac frequency [11]. This habitual relationship of drinking coffee is based on the fact that people who consumed one or fewer cups of coffee per day, but not frequently, triggered infarction more easily. On the other hand, the regular drinking was not able to cause significant effects on blood pressure, which does not discard the significance for the infarction [1, 9, 12]. In some cases, most of non-fatal acute myocardial infarction takes place in the morning, although caffeine plasma concentration is lower during this period of the day. This can be explained by a natural increase in the blood pressure in the morning, which is maintained by the caffeine ingested in the breakfast, generating a hazardous situation for irregular consumers. The fact of having breakfast in the morning, associated with other risk factors, makes the irregular consumption essential for triggering nonfatal acute myocardial infarction [1].

Moreover, acute or chronic ingestion of coffee by both regular and irregular individuals strongly influences the blood lipid levels. This variable is influenced by gender, resulting an increased in triglyceride levels in men during acute ingestion. On the other hand, the chronic ingestion by women increases the high density lipoprotein (HDL) levels, which would be beneficial to the women health [9-13]. Moreover, the increase in HDL coffee is associated

with other cardiovascular benefits [14]. In cases of type 2 diabetes mellitus, there is an inverse relationship between coffee consumption and heart risks. The oxidative stress caused by hyperglycemia is a pathogenic factor of insulin resistance and/or a dysfunction of beta cells [14]. Some studies in several countries have found that high consumption of coffee is related to the low prevalence of hyperglycemia. Since the high blood glucose levels are relevant for cases of coronary heart disease, the antioxidant properties of coffee are able to minimize the oxidative stress, decreasing the total number of coronary and cardiovascular disease and cardiac arrests in patients with type 2 diabetes [13, 14].

In summary, despite the coffee present health benefits, it is not considered a functional food because it is responsible for generate disorders in the body [2]. Despite the progress of many researches over the years, the benefits and risks of coffee, considering the nutritional and clinical aspects, have been widely explored [15]. However, it is difficult to complete the studies because many factors work together with the substances present in the coffee. Moreover, the way it is prepared plays a significant influence [15]. Thus, many findings become contradictory, revealing a large discrepancy between the conclusions. However, there is the duality between the physiological actions of coffee substances, putting the caffeine as a fundamental icon to the studied effects of this beverage [16].

There are four substances that are responsible for the main clinical effects from the coffee, Cafestol, kahweol, Chlorogenic acid, caffeine. Its effects will often depend on the individual characteristics of coffee consumers, the coffee preparation mode, the type of grain used and also the drink intake habit.

CAFESTOL AND KAHWEOL

The cafestol and kahweol are substances present in unfiltered coffee showed that a tight relationship with increased levels of cholesterol in the blood [17] and the reduction of toxic effects of many carcinomas in animal models and in cell culture [6]. These two compounds are important to cardiovascular complications. The values of blood cholesterol levels depend on circulating lipids: the chylomicrons, triglycerides, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and HDL. These lipids are composed of a protein called apolipoprotein E (APOE), which varies from person to person. The genetic difference is caused by the alleles APOE ε2, APOE ε3, APOE ε4, which are located on the long arm of the chromosome 19. This heterogeneity of alleles leads the polymorphism of proteins. The most common allele is the APOE ε3 and its distribution frequency is around 60% in the whole population. People who have the APOE ε4 allele and make use of coffee on a controlled diet, they usually have high blood cholesterol levels, a fact that is directly related to an increased risk of coronary heart disease. However, people having the allele APOE ε2 show a significant decrease in cholesterol levels compared to people without this allele. Therefore, APOE ε2 is a protective factor against high cholesterol levels in front of the effects of coffee consumption. In individuals with APOE ε2 negative, the cafestol and kahweol contribute to the worsening of cardiovascular disease due to the increasing in cholesterol levels [17]. On the other hand, the cafestol provides a positive aspect in some pathologies. It acts via the induction of glutathione, acting as a cell protective factor, especially in liver cells [18].

CHLOROGENIC ACID

Chlorogenic acid belongs to a class of chemical compounds obtained from the esterification of uronic acid and quinic acid. It is present in the diet, even in an ordinary diet, coming to a daily intake of 0.5 to 1.0 g. This substance presents in vitro antioxidant activity, which refers to the protective action of the circulatory system against free radicals. Due to this fact, it provides a preventive action for certain types of cancer [19-21]. Tanaka et al. (2006) have demonstrated that this substance shows an inhibitory effect in hyperplasia induction in mice hepatic cells. However, other antioxidants present in coffee role in the a crucial effect by the protection against the development of hepatocarcinogenic cells of the man. Otherwise, chlorogenic acid is a substance able to increase the release of gastrin, which can cause gastric discomforts. An important example of this class of substances is the caffeic acid, which is well known and studied [19, 21].

CAFFEINE

Popularly, the clinical effects of coffee are assigned to caffeine, known as 1,3,7-trimethylxanthine. Really, many of the coffee properties can be attribute to this substance, which belongs to the xanthine class. Data from the U.S. Department of Agriculture Food Composition establish that in a cup of coffee (240 ml) there is, in average, 47mg of caffeine, approximately [22].

In this concentration, caffeine can show significant clinical effects on health. In the cardiovascular system, it may play a dual role. It may alter the neural and cardiac activity due to its relation with adenosine. Moreover, it also may dilate the blood vessels by action of nitric oxide. This xanthine acts as a non-selective antagonist of A1 and A2A adenosine receptors, a class of purinergic G protein coupled receptor. As a result, it blocks the action of adenosine on the central nervous system (CNS), decreasing the sedative and inhibitory effects of neuronal activity, as well as vasodilator, bronchoconstriction and immunosuppressive effects naturally caused by adenosine. Otherwise, there is a vasodilator function which takes place through the increased rates of endogenous nitric oxide, a substance which presents vasodilating properties. This effect is provided by the co-administration of caffeine and NG-monomethyl-L-arginine, which inhibits the enzyme that synthesizes nitric oxide. In this case, the results demonstrate that the rates of endogenous nitric oxide are still increased [18, 23].

Among the investigations of caffeine, it is well known that this substance is able to stimulate the CNS and cause vasoconstriction, one of the mechanisms by which it is used for the treatment of headache [23, 24]. Another way of CNS stimulation by caffeine involves an inhibition mechanism of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the CNS. On the other hand, this mechanism is not well established. With the stimulation of neural activity by these pathways there is also an increase of dopamine (DA) levels, which is a neurotransmitter responsible for the maintenance of wakefulness and for elevating the motor activity. The dependence by the caffeine, even in a lesser degree, is a controversial question defended by some authors as a possible causal relationship with DA, which is related to satisfaction and pleasure. In this case, caffeine induces the increase in the

norepinephrine concentration of in the synapses, which results in exacerbation of noradrenergic effects, such as tachycardia, increased blood pressure and bronchial dilatation [24-26]. This latter effect denotes the medical use of caffeine to treat respiratory depression. In this context, it is very important to point out that 3-8% of this substance is converted to theophylline, a bronchodilator [27-28].

The caffeine may exhibit a similar action to amphetamines in the CNS by a similar mechanism, but causing a less intense effect. When noradrenaline (NA) and/or dopamine (DA) are present in the synaptic cleft, the action of these substances are normally terminated with their reuptake in the axon terminal. However, the amphetamine blocks NA and DA reuptake, allowing a prolonged maintenance of NA and DA in the synaptic cleft [24-25, 29]. This stimulant effect allows the use of these substances to treat attention deficits and/or hyperactivity, as evidenced by studies [25, 26].

The similar effect of amphetamine and caffeine is is one of the causes of caffeine use as a weight loss agent, attributed to loss of appetite. There are two other considerations, the thermogenic action and lipolysis. The latter one is attributed to the blockage of the phosphodiesterase enzyme, which is responsible for converting the cyclic adenosine monophosphate (cAMP) messenger to adenosine monophosphate (AMP). With this block, the accumulation of cAMP leads to the activation of kinases proteins, causing a predominance of the phosphorylated form of the enzyme, which is normally the activated form. The accumulation of cAMP stimulates the lipolysis, as lipolysis is a way to produce energy. The lipolysis takes place mostly for fat oxidation in the muscle, promoting the release of fatty acids from peripheral tissues [22, 30]. This mechanism makes the caffein attractive by athletes/practitioners of physical exercises. Besides the fat loss, caffeine generates a less delayed effect that would be stimulation in cardiovascular in aerobic exercises and increased sensitivity of the medullary respiratory center to carbon dioxide. These effects are responsible for stimulating the central inspiratory impulse and improving the skeletal muscles contraction [27, 31-32].

Some of the gastric effects attributed to caffeine are related to the increased gastric secretion, because it modifies the release of gastrin. As a result, there is an increase in gastrin blood levels, providing an effect similar to chlorogenic acid. Caffeine may contribute with digestion. However, it can be a major aggravating factor in cases of diseases affecting the gastrointestinal tract. On the other hand, studies have shown that thermal treatment is capable of inactivating the effect of caffeine, among other substances, on gastrin [2, 20].

OTHERS CLINICAL EFFECTS ATTRIBUTE TO CAFFEINE

Some studies are targeting the caffeine effects on neuronal death caused by glutaminergic excitotoxic, because the inhibition of GABA, caused by caffeine, induces the decrease of glutamate concentrations, leading researchers to investigate this xanthine considering the clinical improvement on Alzheimer desease [29, 33-35]. In addition, Parkinson's patients treated with this xanthine demonstrated a decrease in daytime sleepiness and improvement of nocturnal sleep, besides movement’s recovery and tremors relief. Probably, due to motor

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problems associated with this disease are caused by a lack of dopamine. In general, dopamine production is inhibited by the action of adenosine. With the blockage of receptors adenosine, caffeine induces the production of dopamine, leading improvement of clinical symptoms of Parkinson disease [28, 36-38].

There are some studied benefits that approximate the activity of caffeine as an adjuvant for glycemic control in subjects with type 2 diabetes mellitus. Despite this xanthine promotes indirectly an adrenergic effect in the body, it would increase glucagon levels and, consequently, glycogenolysis, providing blood glucose. There are studies that identify this substance as effective by reducing peripheral resistance in skeletal muscle tissue in order to assist, even in discrete rates, the glycemic control. Studies demonstrated the decrease of cardiovascular complications in patients with type 2 diabetes, considering the reduction of the oxidative stress caused by hyperglycemia. However, this effect is also attributed to other substances [14, 30, 39].

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The excitation of the CNS and the activation of the adrenergic pathway induced by caffeine generate a cardiac stimulation which, in turn, tries to be controlled by the CNS, even if under effect of xanthine. Therefore, there is a series of ionic imbalances, causing changes in the cardiac fibers contraction. The most frequent alterations lead to flutter and atrial fibrillation. In some cases, severe ventricular arrhythmias are common. It is a dangerous condition to not healthy individuals, as it often causes ventricular tachycardia and cardiac arrest. Although caffeine is not considered a hypertension trigger, this xanthine may cause a sudden increase in blood pressure. This is a contributing factor for cardiac arrest and acute myocardial infarction, mainly associated with arrhythmic condition [18, 40-43].

The prominent role of caffeine on the risk of nonfatal acute myocardial infarction, among other roleseffects, is associated with genetic factors. The 1,3,7 trimethylxanthine is primarily metabolized by the liver enzyme Cytochrome P450 1A2 (CYP1A2), which is responsible for 95% of caffeine metabolism. Genetically, there is a diversification of alleles and individuals may present CYP1A2 * 1F or CYP1A2 * 1A alleles. People with the CYP1A2 * 1F allele have a low metabolism for caffeine. On the other hand, people presenting the CYP1A2 * 1A metabolize caffeine quickly. For those with slower metabolism of caffeine, the risk of myocardial infarction increases with eventual intakes of caffeinated coffee, because they are more susceptible to the noradrenergic effects of caffeine [44-45].

The effects of these four substances in coffee may be positive or negative for health. Additionally, the effects will depend on individual characteristics of each person, as well as the genetics (Frame 1).

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Frame 1. Coffee relevant substances and their clinical effects

Substances	Clinical effects in the health
Cafestol and kahweol	**POSITIVES:** • Decreased LDL levels in APOE ε2 individuals and, consequently, reduction of cardiovascular risk; • Protection of liver carcinomas via glutathione.
	NEGATIVES: • Increased cardiovascular risk in individuals APOE ε3 and 4.
Chlorogenic acid	**POSITIVES:** • Antioxidant capacity that decreases neoplasms aggravation.
	NEGATIVES: • Increased the risk of gastritis and gastric discomforts due to increase of gastrin levels.
Caffeine	**POSITIVES:** • Improvement migraine symptoms due to cerebral vasoconstriction; • Decreased cardiovascular risk in patients with type 2 diabetes mellitus due to help in the control of hyperglycemia and oxidative stress; • Promote the improvement of clinical condition of respiratory depression due to bronchial dilatation; • Control of hyperactivity and attention deficit by increasing the DA synapses; • Help in the physical performance due lipolysis of fat oxidation in skeletal muscle, thermogenic action, cardiac and CNS stimulation; • Preservation of senescent memory and positive results in clinical symptoms of Alzheimer's and Parkinson's disease.
	NEGATIVES: • Increased cardiovascular risks due to lead acute developement of arrhythmias and also sudden increase in BP in acute and unusual doses and in individuals with low metabolism for caffeine (CYP1A2*1F presence), which increases the risk of AMI and cardiac arrest; • Increased of gastrin levels, contributing to gastritis and gastrointestinal discomfort; • Worsening of urinary incontinence; • It can lead to slight degree of dependence; • Decreased appetite due to dopaminergic mechanisms.
Unspecified	• Increased triglycerides in men and HDL levels in women; • Gastric discomfort and gastritis risks due to the increase of gastrin secretion and gastric relaxation; • Inhibiting the induction of GTT providing hepatoprotection against the carcinomas.

Unspecified = clinical effects related to coffee and not attributed to a specific substance of the beverage; BP = blood pressure; GTT = Gamma-glutamyltransferase, AMI = acute myocardial infarction; Dopamine = DA; HDL = high density lipoprotein, LDL = low density lipoprotein, CNS = Central Nervous System.

The form of preparation of coffee contributed to the presence and concentration of these subtâncias in the beverage. Nevertheless, the manner of intake of the beverage is crucial to some clinical effects attributed to these substances in the coffee. In a way, there is a greater propensity for plasma elevation of triglycerides in men and for the increased cardiovascular risk associated with caffeine when coffee is ingested eventually. The usual coffee intake is associated with the elevation of HDL levels in women and specific for caffeine is associated with tolerance and cardiovascular risk reduction caused by this substance, in both sexes [11-13; 40-45].

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BIOGRAPHICAL SKETCH

Maurílio De Souza Cazarim

Pharmaceutical, master in pharmaceutical sciences - school of pharmaceutical sciences of ribeirão preto, university of são paulo, Brazil. Specialized in comprehensive care by residence program from school of medicine of ribeirão preto - usp (2013). Bachelor's at school of pharmacy in the federal university from juiz de fora (2010).

Name: Maurílio De Souza Cazarim

Affiliation: School of Pharmaceutical Sciences of Ribeirão Preto, University Of São Paulo, Brazil.

Date of Birth: 24 January, 1984.

Education: Graduated (Pharmacist)

Address: Cásper Líbero Avenue, N 235

Research and Professional Experience:

Has experience in collective health, focusing on public health, acting on the following subjects: health of the elderly, antibacterial agents, prescription medications, coffee and clinical pharmacology. Nowadays, works with clinical pharmacy and pharmacoeconomics like lines researches.

08/2010 - 12/2010 trainee activities, medquímica pharmaceutical industry ltda, Professional experience in supervision of good manufacturing practices and validation in quality assurance sector. Total hours: 656 hours.

03/2008 - 07/2010 trainee activities, health surveillance/grs - juiz de fora.

Health surveillance center of the regional health management - juiz de fora, minas gerais. Total hours: 760h.

03/2009 - 12/2009 training, faculty of pharmacy and biochemistry.

Monitoring project in the discipline: oriented fco043-activity v -attention pharmaceuticals in pharmacy dispensing manipulation and hospital. Ffb/ufjf. Responsible: ailson da luz a. Araujo. Scholarship. 432 hours.

07/2009 - 07/2009 continuing education, university of são paulo, ribeirão preto.

Extension Activities

Organization, preparation and execution of the activities of the culture and extension project "i at usp jr". Under the supervision of prof. Dra. Julieta ueta.

2008 - 2009 project participation activities, faculty of pharmacy and biochemistry.

Monitoring - pharmaceutical care in pharmacy dispensing manipulation and hospital.

2007 - 2009 project participation activities, faculty of pharmacy and biochemistry.

Dispensing system university hospital/ufjf: identification and prevention of errors

09/2008 - 12/2008 training, faculty of pharmacy and biochemistry.

Scholar discipline: oriented activity iv - pharmaceutical services. Under the supervision of prof. Dr. Ailson da luz a. Araujo. Total hours: 132 hours.

08/2008 - 12/2008 training, faculty of pharmacy and biochemistry.

Scholar discipline: oriented activity v - pharmaceutical care in pharmacy dispensing manipulation and hospital. Under the supervision of prof. Dr. Ailson da luz a. Araujo. Total hours: 132 hours.

05/2007 - 03/2008 trainee activities, faculty of pharmacy and biochemistry.

Trainee in quality control physical chemical drugs. Under the supervision of prof. Dra. Célia hitomi yamamoto. Total hours: 242 hours.

2008 - 2008 project participation activities, faculty of pharmacy and biochemistry.

Monitoring - pharmaceutical services

09/2007 - 09/2007 continuing education, faculty of pharmacy and biochemistry.

Extension activities

Pharmaceutical care service to community as part of the extensive discipline oriented activity v.

04/2007 - 07/2007 trainee activities, faculty of pharmacy and biochemistry.

Trainee activities

Pharmaceutical care internship in hospital pharmacy. University hospital/ufjf. Total hours: 150 hours.

2006 - 2007 project participation activities, faculty of pharmacy and biochemistry.

Research Projects

Acquisition of knowledge about the quality of medicines provided by the national health system and improvement of pharmaceutical care.

Professional Appointments: NA

Honors: Master of Sciences.

Publications Last Three Years:

Articles in Scientific Journals

[1] Maurílio de Souza Cazarim; Osvaldo de Freitas; Thaís Rodrigues Penaforte; Angela Achcar; Leonardo Régis Leira Pereira. Impact assessment of pharmaceutical care in the management of hypertension and coronary risk over seven years. Plos One. August, 2015 (in press).

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Chapter book:

Chapter book chapter "pharmacotherapy of Alzheimer". Title of book: guide diseases most prevalent; authors: Paul Obreli Neto, Camilo Molino Guidoni, André De Oliveira Baldoni. Publisher pharmabooks, publication forecast in first half of 2016 (in press).

Patent or Patent Application:

Patent filing: formulation containing microparticles with insulin. Purpose of the development: eye syndrome treatment of dry. Research project: "ophthalmic formulation containing insulin microparticles for treatment of dry eye syndrome" (thesis masters estael luzia rabbit wood cross, FCFRP/USP, 2014; guidance: Prof. Dr. Renata Lopez Fonseca Vianna). Involved: Prof. Dr. Renata Lopez Fonseca Vianna; Prof. Dr. Eduardo Rocha Melani; Estael Luzia cross wood rabbit; maurilio de souza cazarim.

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