FACULTAD DE FARMACIA

Contribution of Phenolics and Maillard Reaction Products to the Antioxidant Capacity of Coffee Brews

Contribución de los compuestos fenólicos y productos de la reacción de Maillard a la capacidad antioxidante de bebida de café

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Pamplona, Junio de 2013

Facultad de Farmacia

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Memoria presentada por Dña. Iziar Amaia Ludwig Sanz-Orrio para aspirar al grado de Doctor por la Universidad de Navarra.

El presente trabajo ha sido realizado bajo la dirección de la Dra. Mª de la Concepción Cid Canda y la co-dirección de la Dra. Mª Paz de Peña Fariza en el Departamento de Ciencias de la Alimentación y Fisiología y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 10 Junio de 2013

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La directora del Departamento, Dra. Diana Ansorena Artieda, CERTIFICA que el presente trabajo de investigación ha sido realizado por la Licenciada Dña. Iziar Amaia Ludwig Sanz-Orrio, en el Departamento de Ciencias de la Alimentación y Fisiología de la Facultad de Farmacia de la Universidad de Navarra.

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Los datos presentados en este trabajo son el resultado de las investigaciones llevadas a cabo dentro del proyecto “Evaluación de la actividad funcional *in vitro* de extractos bioactivos de café y sus subproductos” del que es investigador principal la Dra. Mª de la Concepción Cid Canda, y financiado por el Ministerio de Economía y Competitividad de España (AGL2009-12052)

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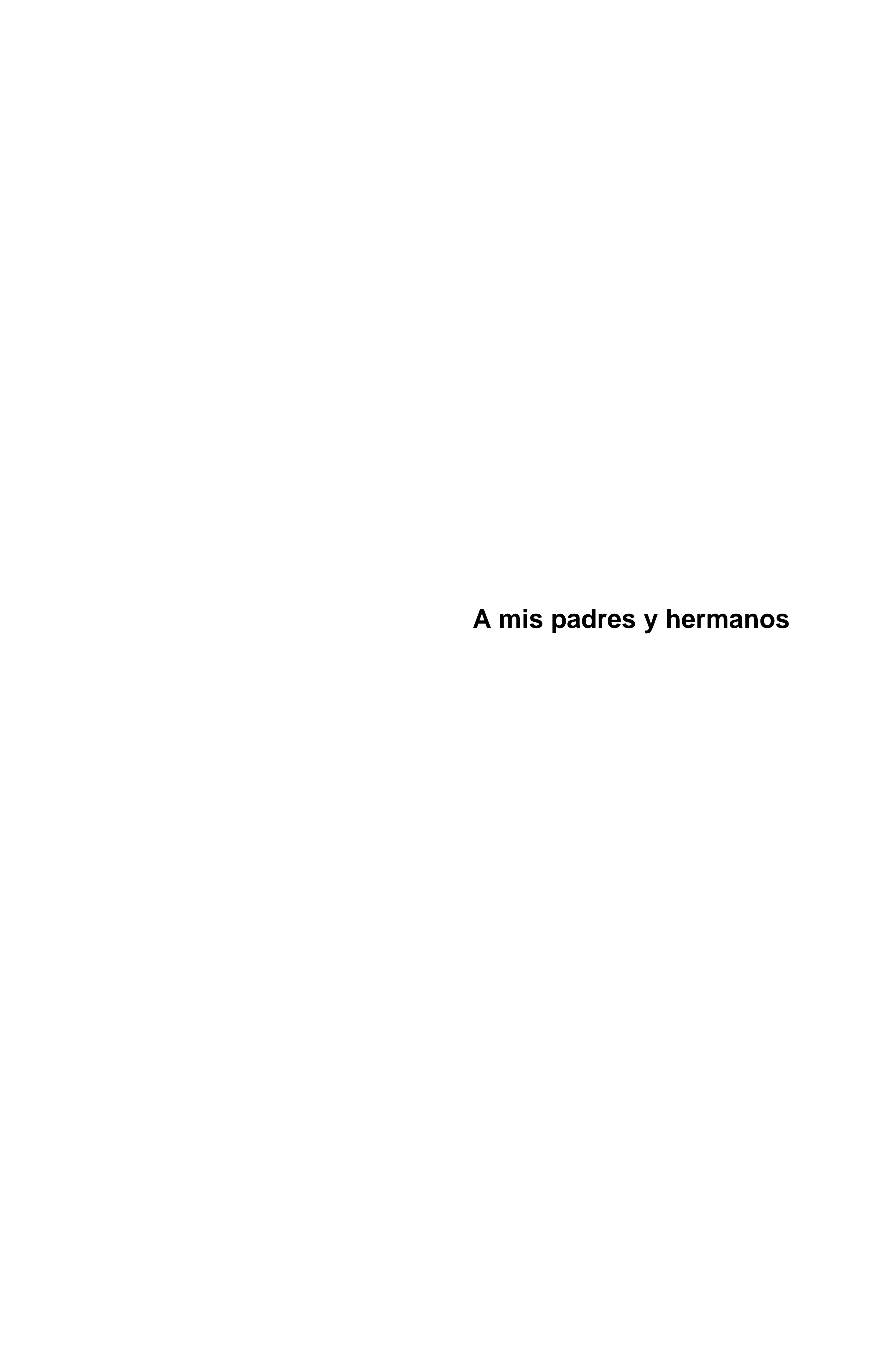

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ABSTRACT

Coffee is one of the most consumed beverages in the world and a rich source of antioxidants. The amounts of these antioxidants are influenced by several technological factors. Besides, antioxidants identified in coffee (chlorogenic acids, volatile and non-volatile Maillard reaction products) may contribute to the overall antioxidant capacity in different proportions. Therefore the aim of this research was to evaluate the actual contribution to the overall antioxidant capacity of coffee brews by the most relevant coffee compounds (phenolics and Maillard reaction products). To achieve this aim, radical scavenging assays were applied to assess overall antioxidant capacity. Qualitative and quantitative analysis of coffee components were carried out by chromatographic methods (HPLC-PDA, HPLC-PDA-MS and GC-MS). Model systems and the on-line ABTS-HPLC technique were used for the assessment of the contribution of coffee compounds to the overall antioxidant capacity.

Firstly, the influence of: variety, roasting degree and roasting technique (conventional and torrefacto), on antioxidant compounds (phenolics and Maillard reaction products) and overall antioxidant capacity of coffee was studied. Results showed that among technological factors, torrefacto roast increased radical scavenging capacity but had no influence on the amounts and composition of phenolic compounds, which were more affected by the roasting degree and variety.

Secondly, the extractability of these compounds in the most commonly used coffeemakers (espresso and filter) and the contribution of coffee antioxidants to the overall antioxidant capacity of the coffee brews were assessed. Brewing time and conditions have a great impact on the extraction of antioxidants. High water pressure during the brewing process increases the extraction speed. Nevertheless parameters like turbulence during extraction and longer contact time favour the extraction efficacy. Volatile Maillard reaction products contribute very little to the antioxidant capacity of coffee compared to high molecular weight Maillard reaction products (melanoidins) and phenolic compounds. Among 22 identified coffee phenolics, caffeoylquinic acids showed the highest contribution to the overall antioxidant capacity, whereas feruloylquinic acids and their lactones did not have any radical scavenging capacity. Moreover, the antioxidant capacity of the chlorogenic acids was highly dependent on their chemical structure. However, antioxidant capacity of chlorogenic acids accounted only for 7-16 % of the overall capacity of coffee.

Additionally, to go deeper into the fate of coffee antioxidants in the human body (after ingestion/during passage through the gastrointestinal tract), *in vitro* faecal fermentation was carried out to study the influence of the colonic microbiota on these compounds. *In vitro* faecal fermentation of espresso coffee showed that chlorogenic acids are rapidly and extensively metabolized by the human colonic microflora, leading to the formation of up to 11 degradation products, which are better absorbed in the colon than their parent compounds.

ABREVIATIONS

ABTS	2,2'-Azino-bis(3-ethylbenzothiazonile-6-sulfonic acid) diammonium salt
CFQA	Caffeoylferuloylquinic acid
CGA	Chlorogenic acid
CQA	Caffeoylquinic acid
CQL	Caffeoylquinic acid lactone/Caffeoylquinide
dB	Decibel
DiCQA	Dicaffeoylquinic acid
DiFQA	Diferuloylquinic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESR	Electron spin resonance
FC	Folin-Ciocalteau
FCQA	Feruloylcaffeoylquinic acid
FQA	Feruloylquinic acid
FQL	Feruloylquinc acid lactone/feruloylquinide
GA	Gallic acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
MR	Maillard reaction
MRP	Maillard reaction product
MS	Mass spectrometry
mV	Millivolt
*p*CoQA	*p*-Coumaroylquinic acid
PDA	Photodiode array
SD	Standard deviation
TEAC	Trolox equivalent antioxidant capacity
TEMPO	2,2,6,6-Tetramethyl-1-piperidin-1-oxyl

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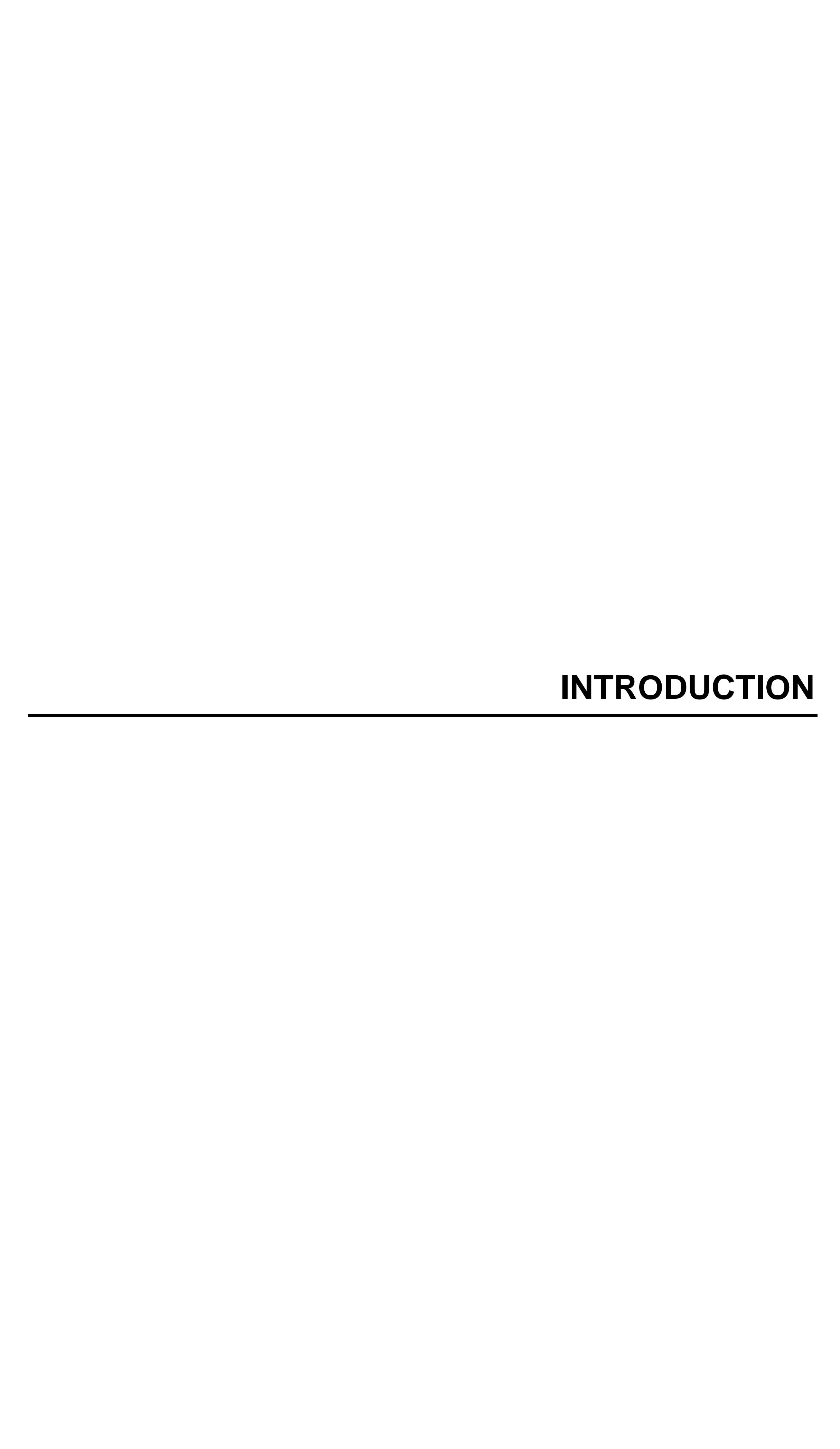

INTRODUCTION

Coffee is the most traded commodity in the world after oil. Although native to Africa (Ethiopia, Abyssinia), nowadays it is cultivated in regions between the Tropic of Cancer and the Tropic of Capricorn, from 23.5 degrees above the equator to 23.5 degrees below it, also known as the coffee belt (Illy & Viani, 2005). The generic name *Coffea* covers approximately 70 species, but only two of them are economically important: *Coffea arabica*, commonly known as Arabica coffee, which accounts for three quarters of world production, and *Coffea canephora* var. Robusta, commonly known as Robusta coffee, with one quarter of world production. Differences between these two species include: ideal growing climate, physical aspects, chemical composition, and characteristics of the brew made with the ground roasted seeds. In general, Arabica coffee brew is appreciated for its superior cup quality and aroma, whereas Robusta brew possesses a more aggressive flavour but contains higher amounts of soluble solids, antioxidants, and caffeine (Farah, 2012).

Green coffee composition is dominated by carbohydrates (~60 % dry matter), including soluble and insoluble polysaccharides (cellulose, arabinogalactane, and galactomannane), disaccharides (sucrose) and monosaccharides (glucose, galactose, arabinose, fructose, mannose, manitol, xylose, and ribose) (Mazzafera, 1999; Hu et al., 2001). The lipid content of green coffee account for 8-18 % of its dry matter. The coffee lipid fraction consists to 75 % of triglyceride, other lipids are sterols (stigmasterol, sitosterol), fatty acids (linoleic, linolenic, oleic, palmitic, stearic, araquidic, lignoceric and behenic), and pentacyclic diterpenes (methylcafestol, cafestol, kawheol) (Kurzrock & Speer, 2001). Proteins, peptides, and free amino acids account for 9-16% of the green coffee dry matter. The main amino acids, both protein-bound and free, are asparagine, glutamic acid, alanine, aspartic acid and lysine (Hu et al., 2001). Several other N-compounds are present in coffee, such as caffeine, trigonelline and nicotinic acid. The caffeine concentration in Robusta coffee is approximately twice that of Arabica.

Green coffee contains also a large amount and variety of polyphenols. The main components of the phenolic fraction of green coffee are the chlorogenic acids (CGAs), which include different groups of compounds and related isomers formed by esterification of one molecule of quinic acid and one to three molecules of *trans*-cinnamic acids. The main groups of CGA include caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), *p*-coumaroylquinic acids (*p*CoQAs) and mixed diesters of caffeoyl-feruloyl-quinic acids (CFQAs) (Clifford, 1999; Clifford et al., 2003) (**Figure 1**). In addition, numerous minor CGAs were recently found in coffee including isomers of dimethoxycinnamoylquinic acids, dimethoxy-hydroxycinnamoylquinic acids, dihydroxy-methoxycinnamoylquinic acids, diferuloylquinic acids, di-*p*-coumaroylquinic acids, and mixed diesters of the above reported hydroxycinnamic and methoxycinnamic acids (Stalmach et al., 2011). CQAs are by far the most abundant CGAs in coffee (Clifford & Wight, 1976). In addition, at least three free cinnamic acids were also identified in green coffee, namely caffeic acid, ferulic cid, and 3,4-dimethoxycinnamic acid. Phenolic compounds different from CGAs and related compounds, such as cinnamoyl-amino acids (Clifford & Knight, 2004; Alonso-Salces et al., 2009), cinammoyl-glycosides (Clifford,

1985), anthocyanidins (Mazza & Miniati, 1993), and lignans (Milder et al., 2005) have also been identified in green coffee in minor amounts. Green Robusta coffees usually contain greater amounts of any CGA isomer than Arabica coffees (Stalmach et al., 2011).

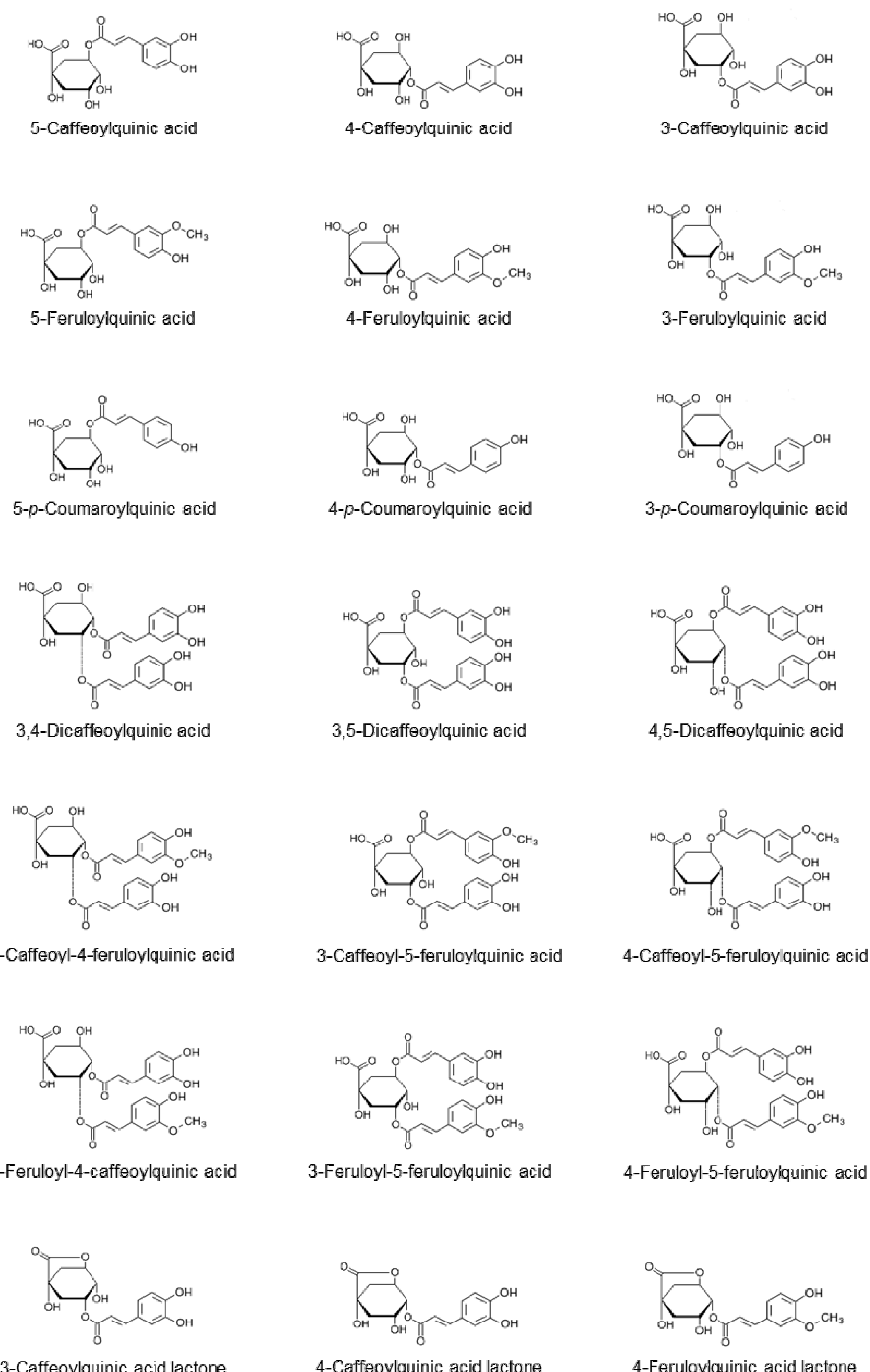

Figure 1. Main chlorogenic acids in coffee.

Roasting process

Whereas green coffee has a mild green, bean-like aroma, the desirable aroma associated with coffee beverages develops during roasting. Hence the roasting process is applied to green coffee beans to form the pleasant aroma, taste, and brown colour of brewed coffee. During roasting, the beans are heated to 180-250 °C, and roasting time can range from 2 to 25 min, depending on the roasting technique and desired roasting degree (light, medium, dark) (Parliment, 2000; Belitz et al., 2009). Three major phases are distinguished during the roasting process: drying, pyrolysis and cooling. During the drying phase most of the free water evaporates keeping the bean temperature at 100 °C. Temperature raise indicates the second phase. At 170-200 °C pyrolytic reactions drastically modify the chemical composition of the bean with the formation of hundreds of substances which give coffee its characteristic aroma and taste. Release of volatile products (CO, CO_2) leads to volume increase of 50-80 %. At the end of roasting the beans then burst due to increase in internal pressure and the popping of the beans indicates that roasting must be stopped by cooling (*quenching*) using either air or water to avoid over-roasting (Sivetz & Desrosier, 1979; Rodrigues et al., 2003). Also the physical parameters of coffee change considerably. The bean colour changes to cinnamon brown, an 11-20 % loss of weight occurs, and the specific gravity falls from 1.2 to 0.6 due to volume increase. The formerly tough beans become mellow and brittle (Illy & Viani, 2005; Belitz et al., 2009). Roasting leads to profound changes in the chemical composition of coffee according to the transformation of naturally occurring substances in green coffee and the generation of compounds deriving from the Maillard reaction, carbohydrate caramelization, and pyrolysis of organic compounds (Belitz et al., 2009). Caffeine is quite thermostable and decreases only slightly during roasting.

Phenolic compounds suffer great changes during the heat processing of coffee due to their thermal instability (Trugo & Macrae, 1984). During the drying phase, when there is still adequate water content, isomerization of CGAs occurs, which leads amongst others to a marked increase in the levels of 4- and 3-CQAs in roasted coffee (Stalmach et al., 2006). Additional some of the CGAs are hydrolysed to cinnamic acids and quinic acid. Later in roasting part of the CGAs lactonises giving rise to several isomers of caffeoylquinic, feruloylquinic, and dicaffeoylquinic acid lactons (Farah et al., 2005). The cinnamates, originally present in green coffee or produced by hydrolysis, may be decarboxylated and transformed into low molecular weight compounds (Trugo & Macrae, 1984; Clifford, 2000; Farah et al., 2005). Decomposition of CGAs has a great impact on the quality attributes of roasted coffee and is directly related to flavor development (Dorfner et al., 2003).

The Maillard reaction (MR) involves the condensation of the carbonyl group of the reducing sugars with the amino group of amino acids and proteins. It consists of a complex network of chemical reactions during which a myriad of products are formed, commonly known as Maillard reaction products (MRP). These compounds have different chemical compositions and, consequently, different properties (i.e., nutrient or contaminant binding, antioxidant, prooxidant, mutagenic, and antimutagenic) (Kato et al., 1987; Jenq et al., 1994; Friedman, 1996; Manzocco

et al., 2000). The polymeric brown coloured final products of the MR are called melanoidins, and result from cyclization, dehydration, retroaldolization, rearrangements, isomerization, and condensation of MRP. Phenolic compounds also participate in the Maillard reaction and are at least partly incorporated in coffee melanoidins through non-covalent or covalent bounds. Melanoidins are one of the major components of coffee, accounting for up to 25 % of dry mater (Belitz et al., 2009). Beside colour development, Maillard reaction plays a key role in aroma generation. Pyrazines, resulting from degradation of unstable MRP, are potent odorants in roasted coffee. Other main classes of aromatic compounds formed during MR are aldehydes, ketones, pyridines, oxazoles, thiazoles, and thiophenes. In addition to MR, volatile compounds in roasted coffee are also formed via caramelization and degradation of carbohydrates (mainly aldehydes and volatile acids), and denaturation of proteins (thiophenes, thiols, etc.) (Flament, 2001; Illy & Viani, 2005).
In contrast to the above described conventional coffee roasting, torrefacto is a roasting process in which sugar is added to coffee, normally Robusta. This roasting technique is used in several Southern European and South American countries where some segments of the population prefer coffees with a dark brown, intense aroma and a strong taste with a tendency to bitterness. This kind of roasting process was initially used to mask negative sensorial attributes in Robusta coffees. Nowadays, torrefacto roasted coffee is usually blended with conventional roasted coffee (Arabica or Robusta) to be commercialized. The addition of sugar at the end of the torrefacto roasting process might intensify the development of Maillard reactions and, consequently, increase the antioxidant capacity of coffee (Nicoli et al., 1997; López-Galilea et al., 2006; Lopez Galilea et al., 2008). However, torrefacto samples analysed in these previous works were blends of conventional and torrefacto roasted coffees from different varieties, and unknown origins, percentages and roasting degrees. Thus, differences in the antioxidant capacity reported so far for torrefacto coffee might well be a consequence of other paramenters (coffee variety, origin, and roasting degree) rather than the addition of sugar during roasting.

Coffee brew and antioxidants

The antioxidant capacity of coffee can be influenced by several factors, such as the variety and origin of the coffee (Parras et al., 2007), the roasting degree (Nicoli et al., 1997), the type of roast (natural or torrefacto) and the blend (López-Galilea et al., 2006; Lopez Galilea et al., 2008). Besides, the brewing process is essential to the antioxidant composition and health properties of a coffee brew, because the contact of water with roasted coffee grounds is the crucial step for extraction of coffee compounds. The preparation of a cup of coffee can be made by several extraction methods. Filter coffee (drip filter) is the most widely used coffee brew obtained by infusion method, whereas espresso coffee is the most appreciated coffee brew produced by pressure method. In drip filtration methods, water at 92-96 °C flows through a hardly compressed ground coffee bed and the extract drips from the brewing chamber into the pot. Turbulence in the brewing chamber prevents water from becoming saturated (Lingle, 1996). In pressure methods, water at approximately 9 bars and 88-92 °C is forced to go through coffee

grounds compacted in a small brewing chamber (coffee cake). Also rapid brewing time and fine particle size are necessary (Lingle, 1996). Many chemical species identified in roasted coffee, including antioxidants, exhibit different extraction rates that may also be influenced by the choice of brewing technique and conditions (Peters, 1991; Petracco, 2001; Petracco, 2005). In each case, the technical conditions applied, such as the coffee/water ratio, water temperature, and water pressure, contribute to the different chemical composition of coffee brews. In this regard, Andueza et al. (2007) observed increased extraction of caffeine and 5-CQA in espresso coffee brews prepared with high coffee/water rations. The extraction of these two compounds also increased with temperature and pressure but only up to 92 °C and 9 bar, while further increase of these parameters led to lower amounts of caffeine and 5-CQA (Andueza et al., 2002; Andueza et al.; 2003). Taking all these facts into account, the knowledge of the extraction behaviour of coffee antioxidants may help to increase their amounts in the beverage and thus improve the health benefits associated with coffee consumption.

Several studies have indicated potential health benefits associated with coffee consumption (Higdon & Frei, 2006; Nkondjock, 2009; Bøhn et al., 2012; Williamson & Stalmach, 2012). These benefits might be ascribed in part to the antioxidant capacity reported for coffee brews (Richelle et al., 2001; Parras et al., 2007). In fact, chronic diseases, such as cancer, cardiovascular, inflammatory, and neurogenerative pathologies are associated with oxidative stress (Beal, 1995; Aruoma, 1999; Dorea & da Costa, 2005), and coffee brew has been proposed as an important source of antioxidants in human diet (Pulido et al., 2003; Svilaas et al., 2004).

The antioxidant capacity of coffee brew is attributed to both antioxidants originally present in coffee beans, like phenolic compounds, and roasting-induced antioxidants, like melanoidins and other Maillard reaction products (MRP) (Del Castillo et al., 2002; Borrelli et al., 2002; Crozier et al., 2009).

Coffee is one of the richest sources of chlorogenic acids (Clifford, 1985), which are considered to be powerful antioxidants. Several authors have studied their antioxidant activity using pure reference standards. *In vitro* CQAs scavenge radicals (Rice-Evans et al., 1996; Foley et al., 1999), increase the resistance of LDL to lipid peroxidation (Nardini et al., 1995; Abu Amsha et al., 1996) and inhibit DNA damage (Shibata et al., 1999; Kasai et al., 2000), and *in vivo* have anticarcinogenic effects in animal models (Huang et al., 1988; Tanaka et al., 1993). Antioxidant capacity of caffeic and ferulic acid, hydrolysis products of CGAs formed during the roasting, has also been demonstrated *in vitro* (Nardini et al., 1995; Daglia et al., 2000) and *in vivo* (Nardini et al., 1997).

However, due to the coffee roasting process, natural phenolic compounds can be lost by progressive thermal degradation. Nevertheless, the loss of antioxidant capacity due to the degradation of phenolics has been found to be minimized by the formation of melanoidins and other Maillard reaction products (Nicoli et al., 1997; Daglia et al., 2000). Beside its contribution to flavor and colour, one of the important functional properties of melanoidins is its antioxidant

activity (Delgado-Andrade & Morales, 2005; Caemmerer & Kroh, 2006; López-Galilea et al., 2006; Rufián-Henares & Morales, 2007), which may be enhanced due to the at least partly incorporation of CGAs into their structure (Bekedam et al., 2008; Nunes & Coimbra, 2010). The simultaneous presence of phenolic hydroxyl groups, reductones, enaminols, and other potential active scavenging groups, which are typical of MRP, could explain the strong antioxidant action of these polymers. These compounds were found to break the radical chain reactions by donation of a hydrogen atom (Eichner, 1981; Yen & Hsieh, 1995), to reduce hydroperoxide to non-radical products and to act as effective metal chelating agents (Gomyo & Horikoshi, 1976; Eichner, 1981; Asakura et al., 1990; Takenaka et al., 2005). Thus, a simultaneous antioxidant action by antioxidants having different mechanisms can be hypothesized for these high-molecular weight compounds.

Furthermore, several volatile heterocyclic compounds present in coffee, which are major flavour components formed by the Maillard reaction (Shibamoto, 1983; Flament, 2001), such as pyrroles, furans, thiophenes, thiazoles and imidazoles have been suggested as antioxidant compounds. Fuster et al. (2000) and Yanagimoto et al. (2002) analyzed one by one the inhibitory effect of isolated volatile compounds towards hexanal oxidation and reported considerable antioxidant capacity for some pyrrols, furans and thiophenes. Later Yanagimoto et al. (2004) analyzed the antioxidant activity of chromatographic fractions obtained from a dichloromethane extract of coffee brew. This study suggested that some volatile compounds were responsible for the antioxidant activity exhibited by dichloromethane fractions. Nevertheless, contradictory findings were reported by other authors. More recently (Lopez-Galilea et al. (2008) studied the correlation between total antioxidant capacity of coffee and some selected constituents, including heterocyclic volatile compounds, and found that these latter were negatively correlated with radical quenching activity. However, in this latter study, those coffees that exhibited higher antioxidant activity showed lower amounts of volatile compounds due to botanic variety and roasting process.

Several other minor components are found to act as antioxidants in coffee, such as methylpyridinium ion, generated upon the decarboxylation of trigonelline during roasting. This compound was found to be a powerful coffee-derived inductor of the chemopreventive gluthation-S-transferase in Caco-2-cells (Stadler et al., 2002; Somoza et al., 2003). Also caffeine, beside its tonifying qualities, which activate nervous system, enhancing perception and reduce fatigue, seems to contribute to the overall antioxidant capacity of coffee. In fact, Devasagayam et al. (1996) studied caffeine as a protector agent of cell membranes against oxidative damage. Lee (2000) suggested as a source of caffeine's antioxidant capacity its degradation into the metabolites methylxantine and methyluric acid. Nevertheless, its potential antioxidant activity may be overshadowed by phenolic compounds and MRP.

However, it should be noted that the health effects of coffee antioxidants *in vivo* are not necessarily a consequence of their antioxidant activity. To exert activity *in vivo*, coffee antioxidants must be bioavailable. Although there is still a discussion about the bioavailability of

melanoidins, it is clear that at least they may act as prebiotics or even antimicrobials depending on their nature and concentration (Borrelli & Fogliano, 2005; Rufian-Henares & de la Cueva, 2009). In the case of chlorogenic acids, several studies have indicated that the large intestine is the most likely site for CGAs absorption (Spencer et al., 1999; Olthof et al., 2001; Stalmach et al., 2010). As they pass along the gastrointestinal tract some of them may be metabolised and it is their metabolites rather than the parent compounds which predominate in the circulatory system (Stalmach et al., 2009; Stalmach et al., 2010).

In a study using an *in vitro* model with rat small intestine, very little absorption of 5-CQA was observed (Spencer et al., 1999), pointing towards the large intestine as the most likely site for CQA absorption and metabolism. The important involvement of the colon is also supported by studies on humans with an ileostomy. Levels of ingested CGAs recovered in ileal fluid indicate that about one-third is absorbed in the stomach and/or small intestine (Olthof et al., 2001; Stalmach et al., 2010). In subjects with an intact functioning colon the remaining two-thirds will reach the large intestine, where the gut microbiota will mediate breakdown to a wide range of low-molecular catabolites. Because the fate of (poly)phenols is very much dependent upon any structural changes that occur during the passage through the gastrointestinal tract (Selma et al., 2009; Williamson & Clifford, 2010), the identification and quantification of microbial catabolites produced in the large intestine are of importance in the context of overall bioavailability and potential health benefits of dietary (poly)phenolics.

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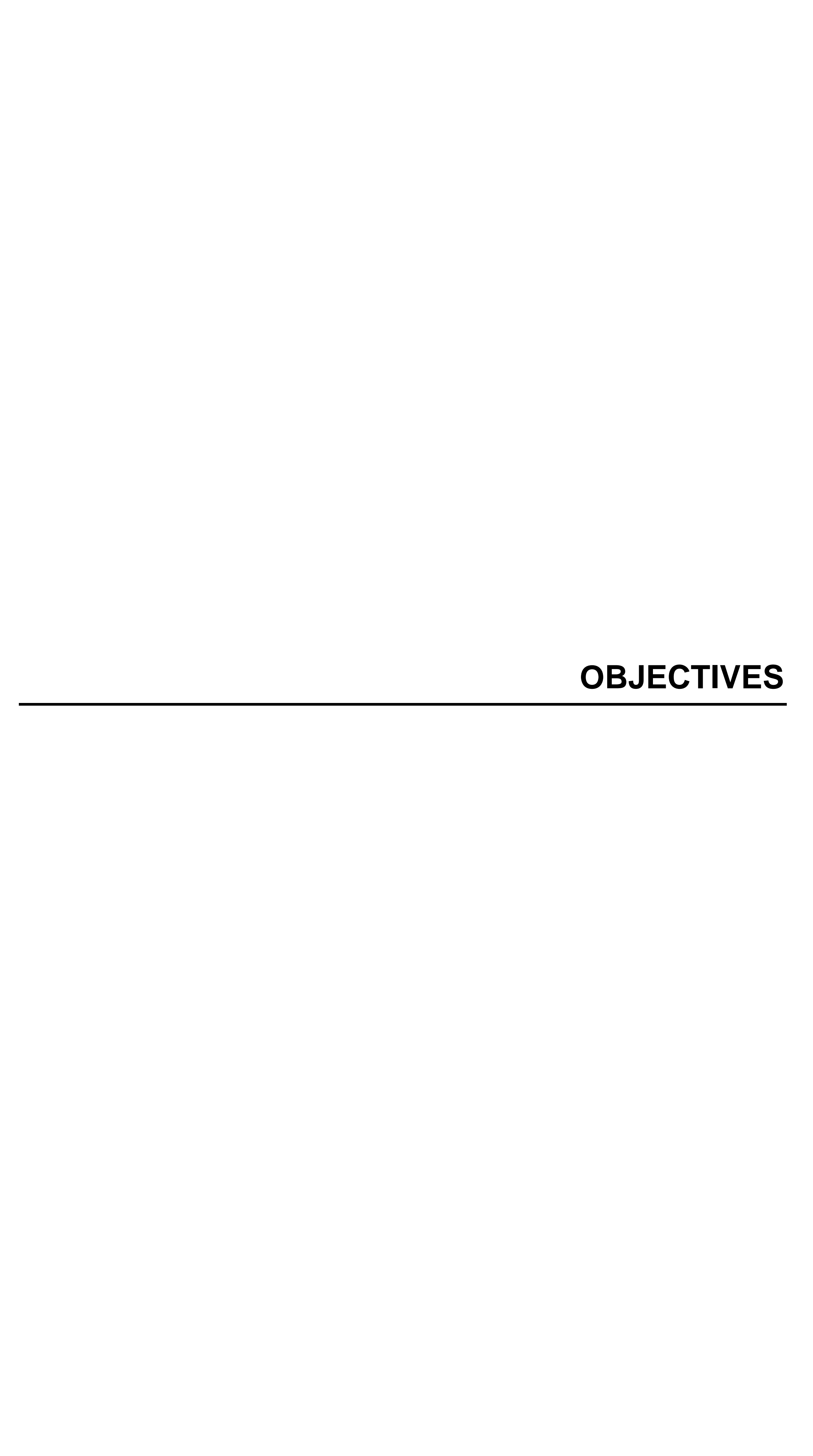


OBJECTIVES

Coffee has been proposed as one of the main sources of antioxidants in the diet. Roasting and brewing processes are two necessary steps before having a good cup of coffee. Roasting causes loss of natural antioxidants, mainly polyphenols, but induces formation of Maillard reaction products, such as melanoidins and volatiles, some of which also act as antioxidants. Previous studies at the University of Navarra showed that commercial Torrefacto coffee blends have a higher antioxidant capacity, but other factors, like coffee origin and roasting degree play an important role. Also, coffee antioxidant extraction might be modulated by the brewing process. Despite technological factors, various coffee antioxidants (chlorogenic acids, volatile and non-volatile Maillard reaction products) may contribute in different proportions to the overall antioxidant capacity. Last, but not least, to be of benefit to human health, coffee antioxidants and/or their metabolites must be present at a gastrointestinal level.

For all these reasons, the general aim of this PhD thesis was **to evaluate the actual contribution to the overall antioxidant capacity of coffee brews by the most relevant coffee compounds (phenolics and Maillard reaction products) and to study their colonic catabolism.**

To achieve this aim, the following objectives were established:

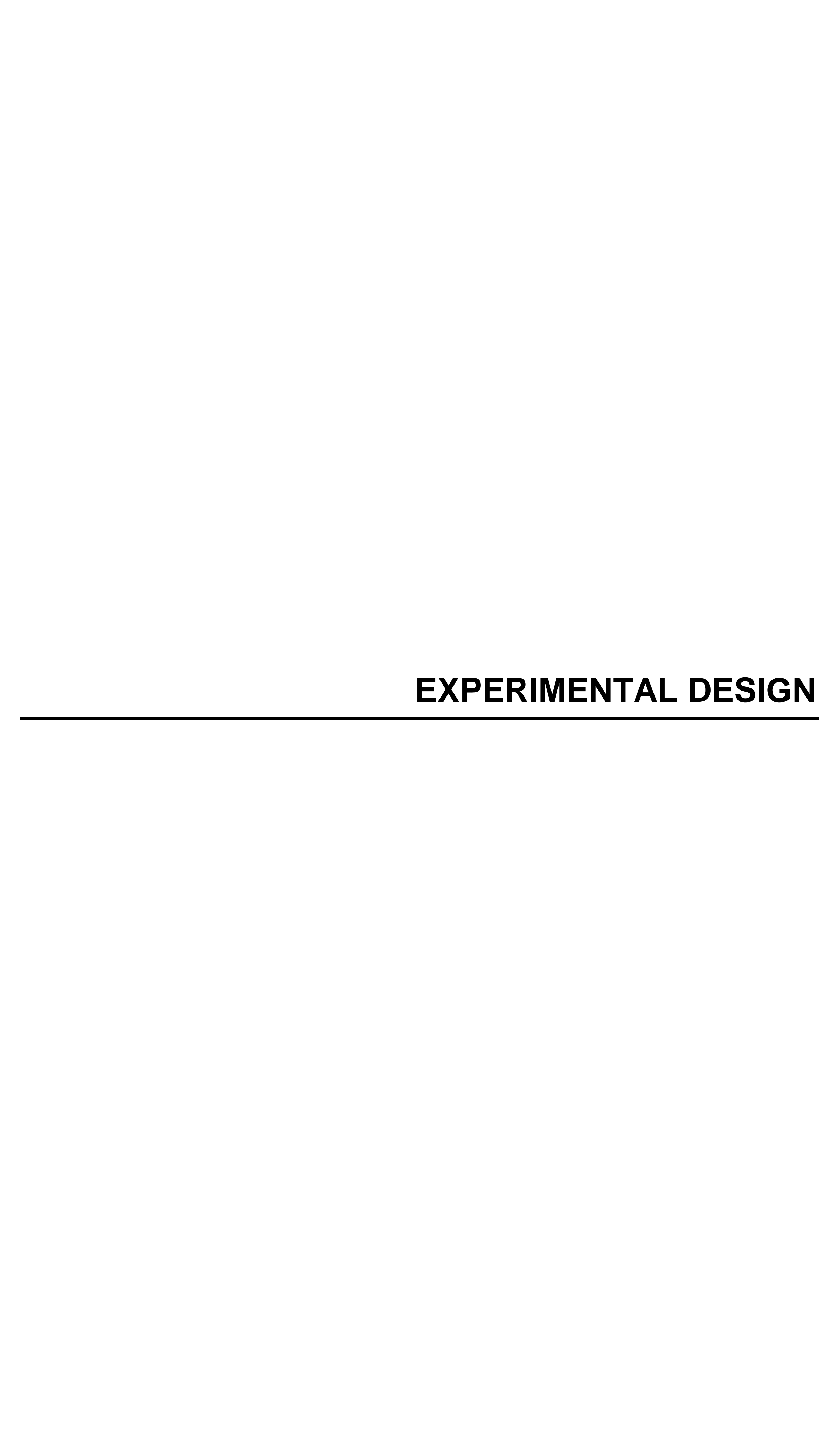
1. To study the influence of roasting techniques (conventional and torrefacto) and degree of roast on the main coffee antioxidant compounds (phenolics and Maillard reaction products) and overall antioxidant capacity. (Paper 1)
2. To study the extractability of the main coffee antioxidant compounds during the most common brewing processes (espresso and filter coffeemakers) (Paper 2).
3. To evaluate the contribution of volatile Maillard reaction products, generated during roasting, to the antioxidant capacity of coffee. (Paper 3, submitted).
4. To evaluate the contribution of phenolic and non-phenolic compounds to the antioxidant capacity of coffee brews (espresso and filter). (Paper 4, in preparation).
5. To study the fate of coffee phenolics at colon level as influenced by the metabolism of human colonic microbiota (Paper 5).

El café se ha propuesto como una de las principales fuentes de antioxidantes en la dieta. La obtención de una buena taza de café implica que previamente ha sido sometido a un proceso de tueste y a la extracción del café molido con agua en una cafetera. Durante el tueste, parte de los antioxidantes naturales (compuestos polifenólicos) se degradan, pero se forman productos de la reacción de Maillard, como las melanoidinas y muchos compuestos volátiles, que también presentan actividad antioxidante. Estudios previos de la Universidad de Navarra mostraron que cafés comerciales con mezcla de torrefacto presentan mayor capacidad antioxidante que los de tueste natural, sin embargo otros factores como el origen del café y el grado de tueste también influyen en las propiedades antioxidantes. Además, la extracción de los compuestos antioxidantes del café podría ser modulada por el proceso de preparación de la bebida de café. Aparte de los factores tecnológicos, los diferentes compuestos antioxidantes del café (ácidos clorogénicos, compuestos volátiles y no volátiles producto de la reacción de Maillard) pueden contribuir en diferente grado a la capacidad antioxidante global de la bebida de café. Por último, y no por ello menos importante, es preciso considerar que para poder ejercer sus potenciales cualidades saludables en el hombre, los compuestos antioxidantes del café y/o sus metabolitos deben estar al menos presentes a nivel gastrointestinal.

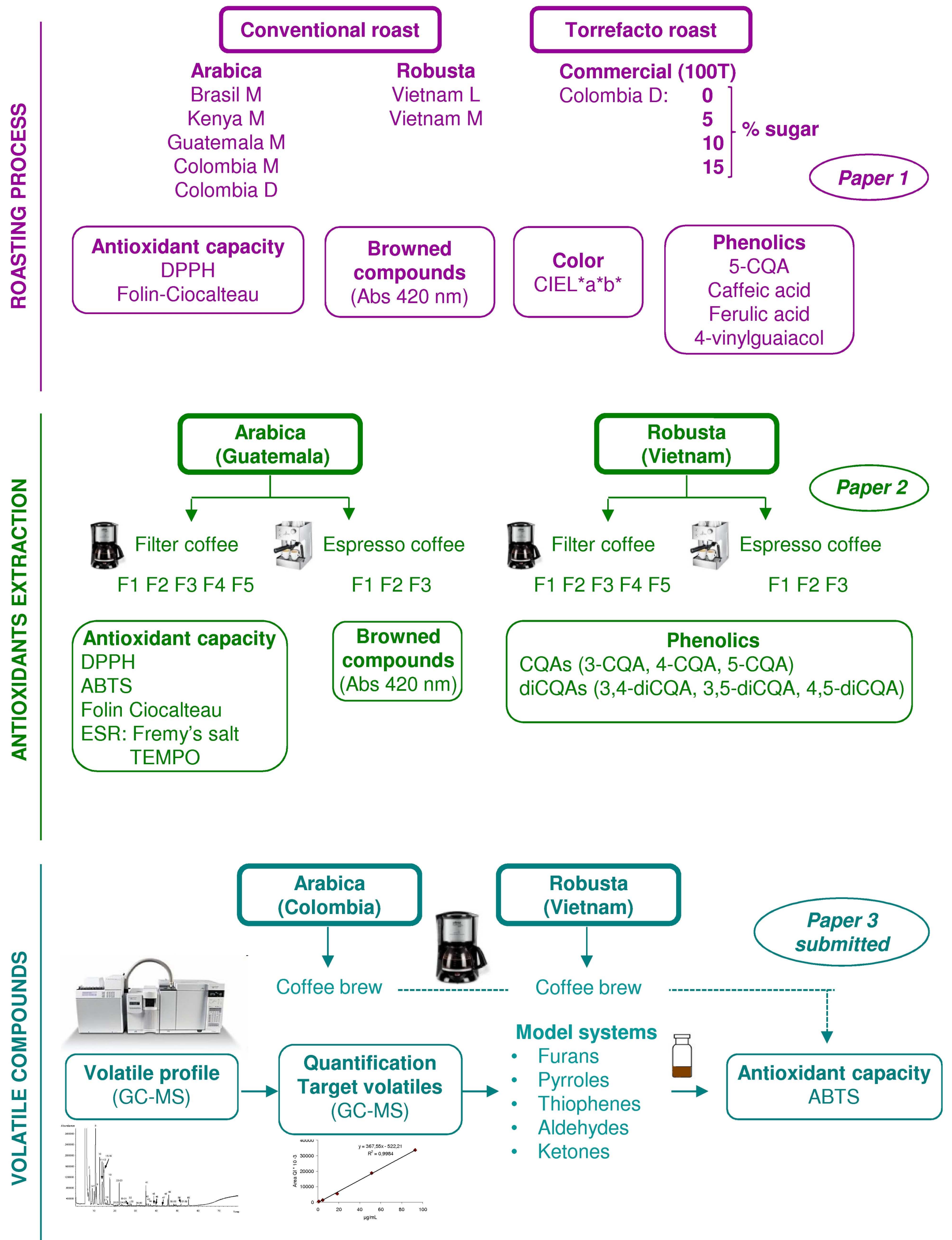
Por estas razones, el objetivo general de la presente tesis doctoral es **evaluar la contribución de los compuestos más relevantes del café (compuestos fenólicos y productos de la reacción de Maillard) a la capacidad antioxidante global de la bebida de café y estudiar las reacciones catabólicas por la microbiota intestinal.**

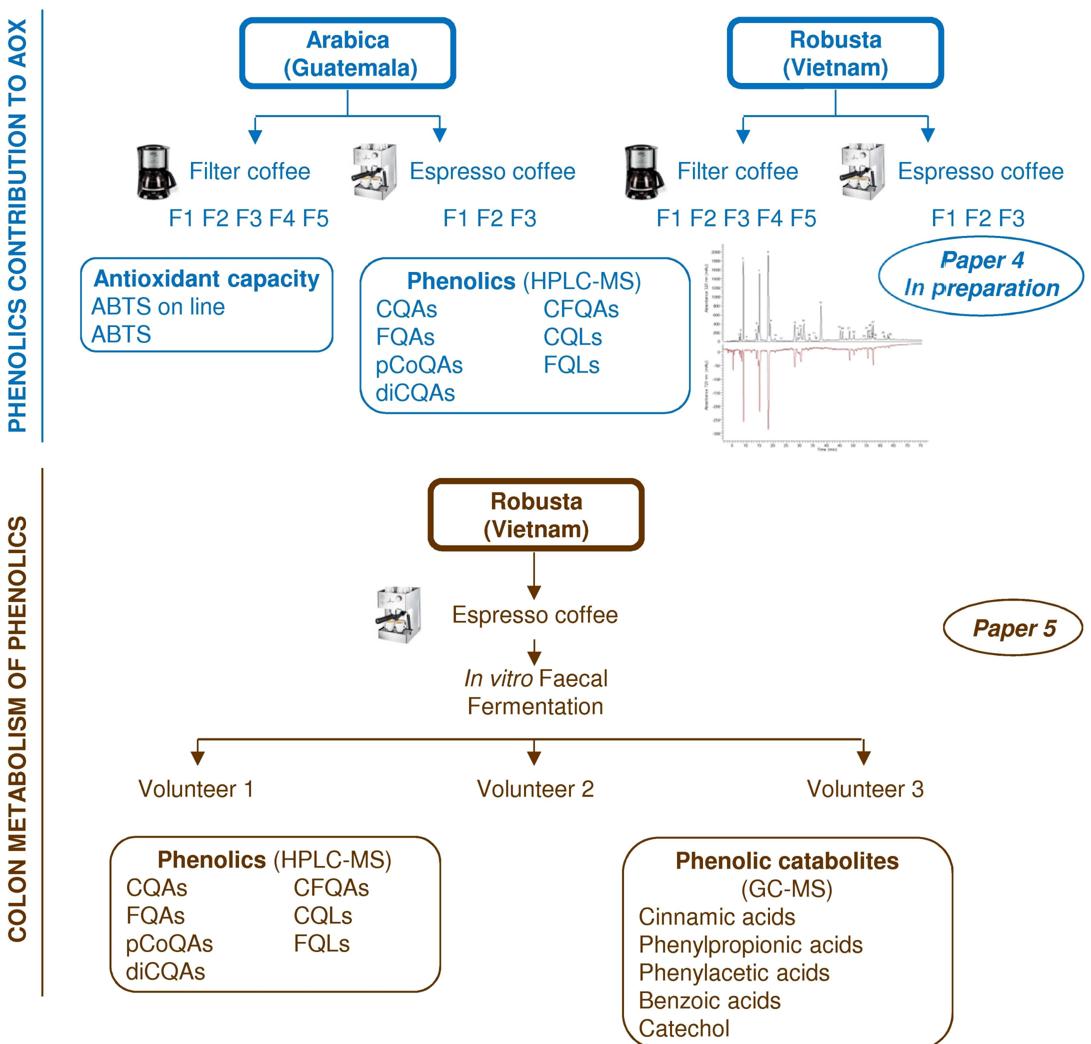
Para lograr este objetivo general se plantearon los siguientes objetivos parciales:

1. Estudio de la influencia del tipo de tueste (natural y torrefacto) y del grado de tueste sobre los compuestos antioxidantes del café (fenólicos y productos de la reacción de Maillard) y la capacidad antioxidante global. (Publicación 1).
2. Estudio de la extracción de los principales compuestos antioxidantes durante la preparación de la bebida de café mediante los sistemas de extracción más habitualmente utilizados (cafeteras expreso y de filtro) (Publicación 2).
3. Evaluación de la contribución de los compuestos volátiles, resultantes de la reacción de Maillard y generados durante el tueste, sobre la capacidad antioxidante de café. (Publicación 3, enviada).
4. Evaluación de la contribución de los compuestos fenólicos y no-fenólicos a la capacidad antioxidante de la bebida de café (expreso y de filtro) (Publicación 4, en preparación).
5. Estudio de la influencia del metabolismo de la microbiota intestinal humana sobre los compuestos fenólicos a nivel del colon.(Publicación 5).



EXPERIMENTAL DESIGN


ROASTING PROCESS
Conventional roast
Torrefacto roast
Arabica
Brasil M
Kenya M
Guatemala M
Colombia M
Colombia D
Robusta
Vietnam L
Vietnam M
Commercial (100T)
Colombia D: 0 5 10 15
% sugar
Paper 1
Antioxidant capacity
DPPH
Folin-Ciocalteau
Browned compounds
(Abs 420 nm)
Color
CIEL*a*b*
Phenolics
5-CQA
Caffeic acid
Ferulic acid
4-vinylguaiacol
ANTIOXIDANTS EXTRACTION
Arabica (Guatemala)
Robusta (Vietnam)
Paper 2
Filter coffee
Espresso coffee
F1 F2 F3 F4 F5
F1 F2 F3
Antioxidant capacity
DPPH
ABTS
Folin Ciocalteau
ESR: Fremy's salt
TEMPO
Browned compounds
(Abs 420 nm)
Phenolics
CQAs (3-CQA, 4-CQA, 5-CQA)
diCQAs (3,4-diCQA, 3,5-diCQA, 4,5-diCQA)
VOLATILE COMPOUNDS
Arabica (Colombia)
Robusta (Vietnam)
Paper 3 submitted
Coffee brew
Volatile profile
(GC-MS)
Quantification
Target volatiles
(GC-MS)
Model systems
Furans
Pyrroles
Thiophenes
Aldehydes
Ketones
Antioxidant capacity
ABTS


PHENOLICS CONTRIBUTION TO AOX
Arabica (Guatemala)
Robusta (Vietnam)
Filter coffee
Espresso coffee
F1 F2 F3 F4 F5
F1 F2 F3
Antioxidant capacity
ABTS on line
ABTS
Phenolics (HPLC-MS)
CQAs
CFQAs
FQAs
CQLs
pCoQAs
FQLs
diCQAs
Paper 4
In preparation
COLON METABOLISM OF PHENOLICS
Robusta (Vietnam)
Espresso coffee
In vitro Faecal Fermentation
Paper 5
Volunteer 1
Volunteer 2
Volunteer 3
Phenolics (HPLC-MS)
CQAs
CFQAs
FQAs
CQLs
pCoQAs
FQLs
diCQAs
Phenolic catabolites (GC-MS)
Cinnamic acids
Phenylpropionic acids
Phenylacetic acids
Benzoic acids
Catechol

RESULTS

Objective 1

Study of the influence of roasting techniques (conventional and torrefacto) and degree of roast on the main coffee antioxidant compounds (phenolics and Maillard reaction products) and overall antioxidant capacity.

Estudio de la influencia del tipo de tueste (natural y torrefacto) y del grado de tueste sobre los compuestos antioxidantes del café (fenólicos y productos de la reacción de Maillard) y la capacidad antioxidante global.

Paper 1:

Effect of sugar addition (torrefacto) during roasting process on antioxidant capacity and phenolics of coffee

Iziar A. Ludwig, Jimena Bravo, M. Paz De Peña, Concepción Cid

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ABSTRACT

The addition of sugar during roasting (torrefacto) has been proposed as a technique to increase the antioxidant capacity. However, other factors such as roasting degree and coffee origin also play a key role. Two batches of Colombian green coffee were roasted adding increased amounts of sucrose (0-15 g per 100 g of coffee) to reach the same roasting degree than a commercial Colombian coffee. Moreover, seven conventional roasted coffees from different origins (Colombia, Brazil, Kenya, Guatemala and Vietnam) and roasting degrees (Dark, Medium and Light), and one 100% Torrefacto roasted coffee were analyzed. Although the addition of sugar during roasting increased the DPPH quenching activity, phenolic compounds (5-caffeoylquinic, caffeic and ferulic acids, and 4-vinylguaiacol) were hardly affected by torrefacto roasting process, showing that Maillard and other roasting reactions products, such as browned-colored compounds including melanoidins (Abs 420nm), have an important role as antioxidants. Principal Component Analysis (PCA) showed that roasting degree also plays a key role on overall antioxidant activity. Moreover, the Absorbance at 420nm has been proposed as a good marker of torrefacto roasting process, whereas the roasting degree might be better characterized by L* values.

KEYWORDS: Coffee, roasting, antioxidant, phenolic compounds, Maillard Reaction Products

1. INTRODUCTION

During last few years, roasted coffee has been proposed as one of the main source of antioxidants in the diet (Svilaas et al., 2004; Pulido, Hernandez-Garcia, & Saura-Calixto, 2003). The roasting of coffee is a complex process where the loss of antioxidant activity due to natural antioxidants – mainly represented by polyphenols – by progressive thermal degradation has been found to be minimized by the formation of Maillard reaction products (MRPs) (Nicoli, Anese, Manzocco, & Lerici, 1997).

Torrefacto is a roasting process in which sugar is added to coffee, normally Robusta. This roasting technique is used in several countries of Southern Europe and South America where some segments of the population prefer coffees with a dark brown, intense aroma and a strong taste with a tendency to bitterness. This kind of roasting process was initially used to mask negative sensorial attributes in Robusta coffees. Nowadays, Torrefacto roasted coffee is usually blended with conventional roasted coffee (Arabica or Robusta) to be commercialized. The addition of sugar at the end of the torrefacto roasting process might intensify the development of Maillard reactions and, consequently, increase the antioxidant capacity of coffee (Lopez-Galilea, Andueza, di Leonardo, de Peña, & Cid, 2006; Lopez-Galilea, de Peña, & Cid, 2008; Andueza, Cid, & Nicoli, 2004). However, the analyzed samples in these works were commercial coffees in which Arabica and Robusta coffees from different unknown origins, percentages and roasting degrees were blended.

Nicoli et al. (1997) reported that dark-medium roasted coffee had the highest antioxidant capacity showing that roasting degree is a key factor. But, the origin and the variety of coffee (Arabica and Robusta) with different amounts of phenolics in green coffee also can play an important role. Consequently, the different antioxidant capacity of commercial Torrefacto roasted coffee blends previously studied by our research

group (Lopez-Galilea et al., 2006; Lopez-Galilea et al., 2008) can not be attributed only to Torrefacto roasting process. Thus, the influence of the sugar addition during torrefacto roasting process on the antioxidant capacity of coffee should be deeper studied controlling the other parameters. So that, the aim of this work was to know whether the addition of increased amounts of sugar to coffee during roasting process (torrefacto) could be a key factor to increase the antioxidant capacity, and to know its influence on the most relevant coffee antioxidant compounds (phenolic compounds and melanoidins). And secondly, whether the addition of sugar during roasting had higher or lower influence than the roasting degree and the origin of coffee.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents. The methanol (spectrophotometric grade), Folin-Ciocalteau reagent and sodium carbonate were obtained from Panreac (Barcelona, Spain). Gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) 5-caffeoylquinic acid (5-CQA), caffeic acid, ferulic acid, 4-vinylguaiacol, were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile, HPLC, grade was provided by Scharlau (Barcelona, Spain).

2.2 Coffee samples. Seven conventional roasted vacuum-packed coffee samples from different origins (5 Arabica coffees from Colombia, Brazil, Kenya and Guatemala and 2 Robusta coffees from Vietnam), and one commercial 100% Torrefacto roasted coffee (T100 Light) were selected. Roasted coffee samples were classified into 3 roasting degrees according to the L* color parameter results: Dark (L*<23), Medium (L*23-26) and Light (L*>26) following similar criteria of other authors (Nicoli et al., 1997; Vignoli, Bassoli, & Benassi, 2011). Colombia Dark and T100 Light coffee samples of the same brand were purchased in a local market. Colombia Medium, Brazil Medium,

Kenya Medium, Guatemala Medium, Vietnam Medium and Vietnam Light roasted coffee samples and green coffee beans (variety *Coffea arabica*, from Colombia) were supplied by two roasting companies.

2.3 Coffee roasting process. Two batches (I and II) of Colombian green coffee beans were roasted adding increased amounts of sucrose (0, 5, 10 and 15 g per 100 g of coffee) to reach the same roasting degree (L* 19-23, Dark) than the selected commercial Colombian coffee sample (Colombia Dark). The amount of added sugar must not exceed 15g/100g coffee beans as regulated by law in Spain (Real Decreto 1231/1988). Roasting process was developed following the time and temperature conditions presented in Figure 1. Sucrose was dissolved in the minimum volume of water and homogenously spread out to the coffee beans at 21 min of roasting. During the roasting process, pan surface and air temperatures were controlled. Each batch of coffee was roasted in duplicate. At the end of the process, coffee samples were controlled by the L* value (19-23, Dark) and weight loss (18-19 g per 100 g). Weight loss was calculated by the difference between green and roasted coffee weights and expressed as g per 100 g. After 4 hours of degassing, 60 g of roasted coffee were packed in plastic bags (type *160*300 PA/PE 90 µm*, Vaessen-Schoemaker Industrial S.A.U., Barcelona, Spain*)* and sealed under vacuum (Ramon Serie VP Mod.450, Barcelona, Spain). Samples were named with the amount of added sugar followed by the roasting degree and the batch number (0 Dark I, 5 Dark I, 10 Dark I, 15 Dark I, 0 Dark II, 5 Dark II, 10 Dark II and 15 Dark II). All coffee samples were stored in darkness and at 4 ºC up to the coffee analysis (<1 month after roasting or purchasing).

2.4 Sample preparation. Coffee packages were opened immediately before the preparation of the coffee extracts in order to avoid oxidative damage. Sixty g of roasted coffee beans were ground in a Moulinex coffee grinder (model Super Junior "s", Paris,

France) for 30 seconds. Coffee extracts were obtained by solid-liquid extraction, using deionized water at 100 ºC. The ratio between coffee and water was 10/100 (g/mL). The extraction time was 10 min. The extracts were immediately cooled with cold running water and filtered through Whatman No. 1 filter paper.

2.5 Color analysis. Color analysis was carried out on ground roasted coffees by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in an 1 cm Petri plate, and the color measured was expressed in L*, a* and b* CIELab scale parameters.

2.6 Browned compounds (Abs 420 nm). Fifty microliters of coffee extract were diluted up to 2 mL with deionized water. Browned compounds were quantified by measuring the absorbance of the sample at 420 nm after exactly 1 min, in a 3 mL capacity cuvette (1 cm length) with a spectrophotometer Lambda 25 UV-VIS (Perkin-Elmer Instruments, Madrid, Spain) connected to a thermostatically controlled chamber (25 ºC) and equipped with UV Win- Lab software (Perkin Elmer).

2.7 Antioxidant capacity by DPPH assay. The antioxidant capacity was measured using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) decolourization assay (Brand-Williams, Cuvelier, & Berset, 1995) A 6.1×10^{-5} mol/L $DPPH^{\bullet}$ methanolic solution was prepared immediately before use. The $DPPH^{\bullet}$ solution was adjusted with methanol to an absorbance of 0.7 (±0.02) at 515 nm in a 3 mL capacity cuvette (1 cm length) at 25 ºC (Lambda 25 UV, VIS spectrophotometer, Perkin Elmer Instruments). Coffee extracts (50 µL) were added to $DPPH^{\bullet}$ solution (1.95 mL). After mixing, the absorbance was measured at 515 nm after exactly 1 min, and then every minute for 18 min at 25 ºC. Calibration was performed with Trolox solution (a water-soluble vitamin E analogue) and Total antioxidant capacity was expressed as µmol Trolox per g of ground coffee.

2.8 Folin-Ciocalteau (FC) assay. The Folin-Ciocalteau reducing capacity of coffee was performed according to the Singleton´s method (Singleton, Orthofer, & Lamuela-Raventos, 1999). For every coffee sample extract, 1:10 dilutions with demineralized water were prepared and 500 μL of Folin-Ciocalteau reagent were added to 100 μL of the coffee sample solution. After 2 min 1.5 mL a 7.5 g/100g sodium carbonate solution was added. Next, the sample was incubated in darkness at room temperature for 90 min. The absorbance of the sample was measured at 765 nm in a spectrophotometer (Lambda 25 UV, VIS spectrophotometer, Perkin Elmer Instruments). Gallic acid (GA) was used as reference, and the results were expressed as g GA per 100 g of ground coffee.

2.9 5-Caffeoylquinic acid (5-CQA). A 500 μL amount of the coffee brew was diluted up to 50 mL with milliQ water. HPLC analysis was carried out with an analytical HPLC unit model 1100 (Agilent Technologies, Palo Alto, CA), equipped with a binary pump and an automated sample injector. A Hypersil-ODS column (5 μm particle size, 250 mm × 4.6 mm) was used. The chromatographic separation was achieved at 25 ºC by using a gradient solvent system with acetonitrile/water adjusted to pH 3.0 with a phosphoric acid solution according to the method described by Perez-Martinez, Sopelana, De Peña, & Cid (2008). The wavelength of detection was 325 nm.

2.10 Hydroxycinnamic acids (caffeic acid and ferulic acid) and 4-vinylguaiacol. The extraction, clean-up and HPLC analysis of these three compounds were performed simultaneously, according to the method developed in our department (Alvarez-Vidaurre, Perez-Martinez, De Peña, & Cid, 2005). The HPLC analysis was carried out with the same equipment and column described above. The chromatographic separation was achieved at 25 ºC by using a gradient solvent system with acetonitrile/water adjusted to pH 2.5 with a phosphoric acid solution according to the procedure published

by Perez-Martinez et al. (2008). The wavelengths of detection were 314 nm for caffeic acid, 325 nm for ferulic acid and 210 nm for 4-vinylguaiacol.

2.11 Statistical analysis. Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. Analysis of variance (ANOVA) was applied to the parameters. Tukey test was applied as test *a posteriori* with a level of significance of 95%. Correlations among variables were assessed by means of the Pearson Correlation test. Principal component analysis (PCA) was applied to the analytical data (based on the Pearson correlation matrix) to observe differences among coffees samples. Principal components (PC) with eigenvalues greater than 1 were selected. All statistical analyses were performed using the SPSS v.15.0 software package.

3. RESULTS AND DISCUSSION

3.1 Influence of torrefacto roasting process on coffee color

Brown color development is one of the most visual changes in heat-treated foods, such as coffee, cereal, cookies, etc. during processing. In the present work, the color of ground roasted coffees was measured by means of the CIELab parameters (L* or lightness, and a* and b* as the chromaticity parameters) and the Absorbance at 420nm (Table 1). Although Torrefacto coffee samples were roasted to reach the same roasting degree that Colombia Dark, a slight tendency to increase L* value with the addition of sugar can be observed in the lab roasted coffee samples, except in 15 Dark II. However, this increase was not significant and, in fact, no significant correlation ($p>0.05$) between L* value and the amount of added sugar has been found. These results are in agreement with those reported previously by Lopez-Galilea et al. (2006) who observed a similar L* increase with the amount of torrefacto roasted coffee in commercial blends, but only

in two of the three analyzed brands. Consequently, L* value is clearly related with roasting degree, but not with the torrefacto roasting process.

In Table 1, it can also be observed that the Light roasted coffees showed significantly higher a* (+red) and b* (+yellow) values than Medium and Dark roasted coffees. In fact, significant correlations (0.786 and 0.912, $p<0.05$) between chromaticity parameters (a* and b*, respectively) and lightness (L*) have been found. Other authors also obtained similar results and correlations in conventional roasted coffees and in conventional/torrefacto coffee blends (Lopez-Galilea et al., 2006; Summa, de la Calle, Brohee, Stadler, & Anklam, 2007). However, no significant correlations have been found between any of the chromaticity parameters (a* or b*) and the amount of added sugar during roasting. Thus, the CIELab parameters seem to be independent of the type of roasting process (conventional or torrefacto), maybe because torrefacto roasting process only induces the formation of an external caramel coating and hardly affects the interior of the coffee beans.

The absorbance at 420nm has been commonly used to characterize melanoidins, which are mainly originated by Maillard Reactions during roasting process of coffee and other heat-treated foods (Morales, 2005; Nunes & Coimbra, 2007). However, it has been reported that melanoidins accounted for only 65 % of color potency of the high molecular weight fraction obtained from light roasted coffee, and for only 39 % from dark roasted coffee (Nunes & Coimbra, 2007). Many other brown-colored products appear to be sugar (retro)aldolization/dehydratation and carbohydrate condensation products, which may or may not be attached to proteins or other structures of amino nitrogen in a similar way to the Maillard Reactions (Rizzi, 1997; Hofmann, 1999). So that, hypothetically, the addition of sugar to coffee during Torrefacto roasting process might induce a higher formation of brown-colored Maillard Reactions and

caramelization products that are water soluble and can be measured by the Absorbance at 420nm. In Table 1, it can be observed that those coffees with sugar added during roasting (torrefacto) showed significantly ($p<0.05$) higher Absorbance at 420nm than those roasted conventionally with the same roasting degree (Torrefacto lab roasted coffees versus 0 Dark and Colombia Dark, or T100 Light versus Vietnam Light). Moreover, commercial torrefacto coffee (T100 Light) showed similar results ($p>0.05$) to those coffees roasted with 15g sugar per 100g coffee. In fact, highly significant ($p<0.001$) and excellent correlation (0.876) between the Absorbance at 420nm and the amount of sugar added during roasting process has been found showing that this parameter might be proposed as a marker of torrefacto roasting process. The highest Absorbances at 420nm in Torrefacto coffees also explain that caramelization products in torrefacto roasted coffee are mainly water soluble.

Principal Component Analysis (PCA) has been applied to evaluate at a glance the influence of the roasting type (conventional or torrefacto) and roasting degree (Dark, Medium and Light) on the antioxidant activity and color of coffee samples. Figure 2 shows the bidimensional representation of all the variables and coffee samples according to the two selected Principal Components (PC). PC1 (65.2% of the total variance) was mainly characterized by the CIELab color parameters (L*, a* and b*) and the Folin-Ciocalteau reducing capacity. It could be observed that PC1 distributed all the coffee samples according to the roasting degree, being the dark samples on the left half-part of the graphic, but independently of the origin, variety and type of roasting process (conventional or torrefacto). PC2 (19.2% of the total variance) was mainly and positively characterized by the Absorbance at 420nm. So that, those coffees roasted with sugar addition (torrefacto) were mapped in the top half-part of the graphic. Roasting degree also exerts influence on brown compounds formation because dark

roasted coffees showed significantly ($p<0.05$) higher Absorbances at 420nm than medium and light conventional coffees (Table 1). However, this influence was much lower than that induced by torrefacto roast because (1) there were no significant differences between medium and light conventional coffees in agreement with other authors (del Castillo, Ames, & Gordon, 2002), and (2) PC2 can not discriminate among different roasting degree coffees. In conclusion, the Absorbance at 420nm might be proposed as a good marker of torrefacto roasting process, whereas the roasting degree might be better characterized by L* values.

3.2 Influence of torrefacto roasting process on phenolic compounds and antioxidant capacity. The antioxidant capacity of coffee was evaluated by two colorimetric assays, the chain-breaking activity by DPPH· radical quenching assay and the Folin-Ciocalteau assay.

Figure 3 shows the antioxidant capacity, measured by the DPPH quenching assay, of the Torrefacto roasted coffees in comparison with conventional roasted coffees of different origins (Colombia, Brazil, Kenya, Guatemala, and Vietnam) and different roasting degrees (Dark, Medium and Light). Commercial conventional roasted coffees showed lower DPPH results than lab-roasted torrefacto roasted coffees. This might be explained by a longer storage (from roasting to purchase) under less controlled conditions (room temperature) in commercial samples, because during storage the antioxidant capacity decreases due to the presence of residual oxygen, and other radicals or pro-oxidant compounds formed during the roasting process (Manzocco, Calligaris, & Nicoli, 2002).

According to the DPPH quenching activity, conventional roasted coffees of different origins and the same roasting degree (Medium) can be ranked in increasing order as Brazil < Vietnam < Colombia < Guatemala < Kenya. However, roasting degree seems to influence conventional roasted coffees in different way depending on the origin or

variety because DPPH increased with a higher roasting degree (Dark vs Medium) for Colombia coffee (Arabica), but decreased for Vietnam one (Robusta) (Medium vs Light). This could be due to the fact that although Robusta coffee has higher amounts of phenolic compounds than Arabica ones, roasting induces a higher loss of these antioxidant compounds in Robusta coffees (Clifford, 1997; Perrone, Donangelo, Donangelo, & Farah, 2010). In fact, only moderate correlation between DPPH and L* values (-0.483, $p<0.001$) has been found.

Focusing into the influence of the sugar addition during roasting process, correlation results show a clear and significant (0.701, $p<0.001$) tendency to increase the DPPH antioxidant capacity with the amount of sugar added during roasting process. Also T100L coffee exhibited higher DPPH results than the commercial conventional roasted coffees. A significant and good correlation between DPPH and Absorbance at 420nm, proposed as a good marker of torrefacto roasting process, has been found (0.721, $p<0.001$). Moreover, DPPH quenching activity contributed partially to the PC2 in Principal Component Analysis (Figure 2) that mapped those coffees roasted with sugar addition (torrefacto) in the top half-part of the graphic. These findings are in agreement with those obtained in commercial torrefacto roasted coffee blends (Lopez-Galilea et al., 2006; Lopez-Galilea, de Peña & Cid, 2007). The higher DPPH quenching activity can be attributed mainly to the formation of Maillard Reactions and caramelization antioxidant products ((Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001), but the influence of torrefacto roasting on phenolic compounds should be deeper studied in those coffees roasted with increasing amounts of sugar addition.

Figure 4 shows the FC results of the Torrefacto roasted coffees in comparison with conventional roasted coffees of different origins (Colombia, Brazil, Kenya, Guatemala, and Vietnam) and different roasting degrees (Dark, Medium and Light). The Folin

Ciocalteau method is traditionally used to measure phenolic compounds, but several authors have reported that this method also evaluates other reducing nonphenolic compounds, such as melanoidins, proteins and thiols, and thus should be seen as a measure of total antioxidant capacity rather than phenolic content (Perez-Martinez, Caemmerer, De Peña, Cid, & Kroh, 2010; Caemmerer & Kroh, 2006; Everette, Bryant, Green, Abbey, Wangila, & Walker, 2010). A decrease of the antioxidant capacity measured by Folin-Ciocalteau technique with the increase of roasting degree can be observed. A highly significant ($p<0.001$) and good correlation (0.785) with L* values has been found. Moreover, FC values also contributed to the PC1 in Principal Component Analysis (Figure 2) together with the CIELab parameters and, then, to the distribution of coffees according to the roasting degree. Similar patterns were reported by other authors (Bekedam, Loots, Schols, Van Boekel, & Smit, 2008; Sacchetti, Di Mattia, Pittia, & Mastrocola, 2009) in conventional roasted coffees, mainly due to a higher degradation of chlorogenic acids, the most abundant phenolic compounds in coffee. Loss of phenolic compounds during roasting is very well known and losses of 8-10% for every 1% loss of dry matter (Clifford, 1997; Clifford, 1999; Clifford, 2000) up to 95% of the chlorogenic acid content in green coffee with drastic roasting conditions (Trugo & Macrae, 1984) were reported.

Higher FC reducing capacities were found in those coffees roasted with sugar (torrefacto) in comparison with their respective conventional roasted ones (0 Dark I and 0 Dark II) (Figure 4), but only were statistically significant in the batch II. For that reason, 5-caffeoylquinic, caffeic and ferulic acids, and 4-vinylguaiacol were quantified in lab-roasted coffees by HPLC analyses (Table 2). Little differences among lab-roasted coffees in the four phenolic compounds (3.48-6.34 mg 5-CQA, 4.03-5.16 μg caffeic acid, 37.22-49.15 μg ferulic acid, and 6.60-7.87 μg 4-vinylguaiacol per g of coffee)

were observed. These differences, most of them statistically non-significant ($p<0.05$), seem to be due to the normal variations during roasting process, but not to the addition of sugar during torrefacto roasting process. The most abundant chlorogenic acid in coffee, 5-caffeoylquinic acid, and caffeic acid that is a hydroxycinnamic acid partially originated by hydrolysis of caffeoylquinic and dicaffeoylquinic acids during roasting process, were found in similar amounts than in commercial Colombian coffees (4.3 mg/g for 5-CQA and 5.5 µg/g for caffeic acid) (Lopez-Galilea et al., 2008). However, in commercial torrefacto coffee blends in the latter study (Lopez-Galilea et al., 2008) ferulic acid was found in lower amounts (12.8-19.3 µg/g), whereas 4-vinylguaiacol was higher (30.6-57.6 µg/g). Ferulic acid is a hydroxycinnamic acid mainly derived from the roasting degradation of feruloylquinic acids (FQAs) that only account for a 5-13% of total chlorogenic acids in green coffee (Farah, Monteiro, Calado, Franca, & Trugo, 2006). And 4-vinylguaiacol is a degradation product of ferulic acid. So that, both compounds might be present in different amounts depending on the initial content of feruloylquinic acids in green coffee and roasting process conditions.

According to the results of Table 2, the addition of sugar during torrefacto roasting process did not reduce the decrease of the main phenolic compounds caused by heat treatment as it was suggested by the Folin-Ciocalteau technique if it is used as a measurement of the total phenolic compounds. This discrepancy in the results might be explained by the formation of other reducing nonphenolic compounds that react with the Folin Ciocalteau reagent during torrefacto roasting process, but not by a protective effect of torrefacto roasting against the degradation of phenolic compounds. This agrees with the results of Lopez-Galilea et al. (2008) who do not find any correlations between phenolic compounds and torrefacto roast in commercial coffee blends. Thus, it could be said that torrefacto roasting process hardly affects the final content of phenolic

compounds that seems to be more influenced by other factors such as roasting degree, the variety of coffee, etc. (Farah et al., 2006).

In conclusion, although the addition of sugar during roasting increases the antioxidant properties of coffee measured as radical quenching capacity, roasting degree and other factors, such as coffee variety, origin or also storage conditions, influence overall antioxidant activity as well. Moreover, in this study, the Absorbance at 420 nm has been proposed as a good marker of torrefacto roasting process, whereas the roasting degree might be better characterized by L* values.

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Table 1: CIELab color parameters (L*, a* and b*) and browned compounds (Abs 420nm) of ground roasted coffee.

Coffee samples[1]	L* [2]	a* [2]	b* [2]	Abs_{420} [2]
Lab roasted coffees				
Arabica				
0 Dark I	19.40 ± 0.50 [ab]	11.80 ± 0.29 [c]	13.16 ± 0.35 [cd]	0.457 ± 0.012 [c]
5 Dark I	20.23 ± 0.11 [ab]	11.95 ± 0.04 [c]	13.90 ± 0.16 [e]	0.499 ± 0.023 [de]
10 Dark I	20.56 ± 0.11 [b]	11.88 ± 0.14 [c]	13.30 ± 0.20 [cde]	0.535 ± 0.021 [f]
15 Dark I	22.45 ± 0.34 [c]	11.80 ± 0.28 [c]	15.51 ± 0.31 [fg]	0.574 ± 0.020 [g]
0 Dark II	19.24 ± 0.81 [a]	11.73 ± 0.14 [bc]	13.77 ± 0.44 [de]	0.458 ± 0.017 [c]
5 Dark II	20.10 ± 0.19 [ab]	11.84 ± 0.27 [c]	13.04 ± 0.30 [bc]	0.483 ± 0.014 [cd]
10 Dark II	20.57 ± 0.26 [b]	11.90 ± 0.14 [c]	13.48 ± 0.19 [cde]	0.518 ± 0.015 [ef]
15 Dark II	19.94 ± 0.05 [ab]	11.37 ± 0.20 [b]	12.45 ± 0.14 [b]	0.575 ± 0.021 [g]
Commercial coffees				
Arabica				
Colombia Dark	19.38 ± 0.42 [ab]	9.86 ± 0.02 [a]	10.12 ± 0.08 [a]	0.464 ± 0.017 [cd]
Colombia Medium	24.82 ± 0.81 [d]	12.70 ± 0.13 [d]	15.11 ± 0.40 [f]	0.352 ± 0.002 [a]
Brasil Medium	25.70 ± 0.13 [d]	12.88 ± 0.08 [d]	16.51 ± 0.07 [h]	0.356 ± 0.018 [a]
Kenya Medium	25.20 ± 0.69 [d]	12.62 ± 0.18 [d]	15.83 ± 0.34 [g]	0.352 ± 0.024 [a]
Guatemala Medium	24.92 ± 0.01 [d]	12.69 ± 0.02 [d]	15.73 ± 0.03 [fg]	0.421 ± 0.022 [b]
Robusta				
Vietnam Medium	25.40 ± 0.71 [d]	11.84 ± 0.36 [c]	15.65 ± 0.56 [fg]	0.379 ± 0.015 [a]
Vietnam Light	30.93 ± 0.64 [f]	13.49 ± 0.18 [e]	21.53 ± 0.39 [i]	0.354 ± 0.006 [a]
T100 Light	28.33 ± 0.27 [e]	13.34 ± 0.24 [e]	21.61 ± 0.48 [i]	0.599 ± 0.010 [g]

[1] 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees. The origin of coffee is indicated with the name of the country. T100 is commercial 100% Torrefacto roasted coffee.

[2] All values are shown as means ± standard deviations (n=6). Different letters in the same column indicate significant differences ($p < 0.05$) among different roasted coffees.

Table 2. 5-CQA, Caffeic acid, Ferulic acid and 4-Vinylguaicol (4VG) amounts of ground roasted coffees.

Coffee samples[1]	5-CQA (mg/g)[2]	Caffeic acid (μg/g)[2]	Ferulic acid (μg/g)[2]	4VG (μg/g)[2]
Lab roasted coffees				
Arabica				
0 Dark I	4.44 ± 0.06 [cd]	4.03 ± 0.12 [a]	37.22 ± 1.18 [a]	7.46 ± 0.15 [cd]
5 Dark I	4.74 ± 0.03 [d]	4.91 ± 0.19 [bc]	46.30 ± 0.46 [cd]	7.67 ± 0.32 [d]
10 Dark I	3.96 ± 0.04 [b]	4.46 ± 0.25 [b]	42.43 ± 1.91 [b]	7.37 ± 0.31 [bcd]
15 Dark I	4.23 ± 0.13 [bc]	4.90 ± 0.21 [bc]	44.44 ± 3.11 [bc]	7.83 ± 0.44 [d]
0 Dark II	6.34 ± 0.07 [f]	5.16 ± 0.23 [c]	49.15 ± 0.96 [d]	7.87 ± 0.14 [d]
5 Dark II	5.83 ± 0.22 [e]	4.86 ± 0.17 [bc]	45.33 ± 0.53 [bc]	6.94 ± 0.14 [abc]
10 Dark II	4.54 ± 0.03 [cd]	4.81 ± 0.06 [bc]	44.25 ± 2.36 [bc]	6.90 ± 0.09 [a]
15 Dark II	3.48 ± 0.08 [a]	4.55 ± 0.18 [b]	38.59 ± 1.09 [a]	6.60 ± 0.13 [ab]

[1] 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees.

[2] All values are shown as means ± standard deviations (n=6). Different letters in the same column indicate significant differences ($p < 0.05$) among different roasted coffees.

FIGURE CAPTION

Figure 1. Time and temperature conditions of coffee roasting process and control of pan surface (bold line) and air (dotted line) temperatures.

Figure 2. Principal Component Analysis of ground roasted coffee. 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees. The origin of coffee is indicated with the name of the country. T100 is commercial 100% Torrefacto roasted coffee.

Figure 3. DPPH antioxidant capacity (μmol Trolox/g) of ground roasted coffee. 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees. The origin of coffee is indicated with the name of the country. T100 is commercial 100% Torrefacto roasted coffee. All values are shown as means ± standard deviations ($n = 6$). Different letters indicate significant differences ($p < 0.05$) among different roasted coffees.

Figure 4. Folin-Ciocalteau reducing capacity (g Gallic acid/100g) of ground roasted coffee. 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees. The origin of coffee is indicated with the name of the country. T100 is commercial 100% Torrefacto roasted coffee. All values are shown as means ± standard deviations ($n = 6$). Different letters indicate significant differences ($p < 0.05$) among different roasted coffees.

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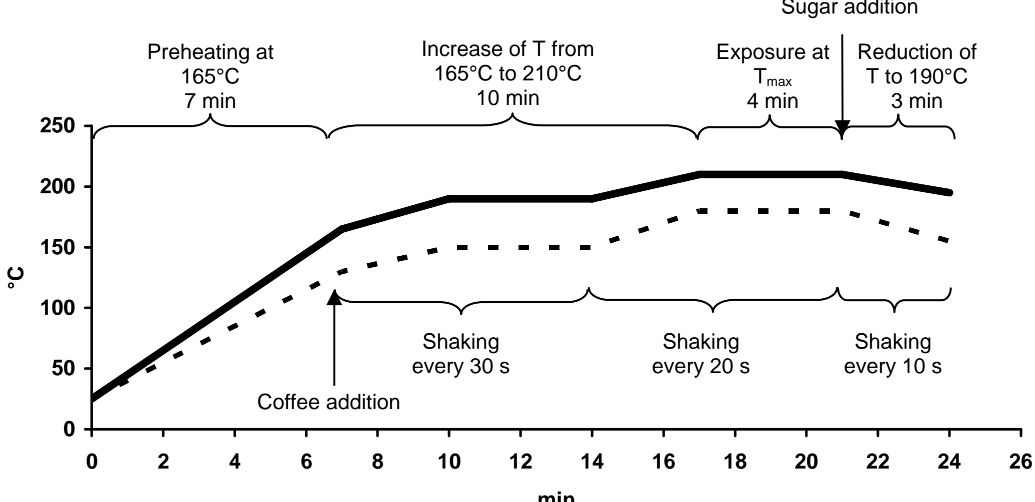

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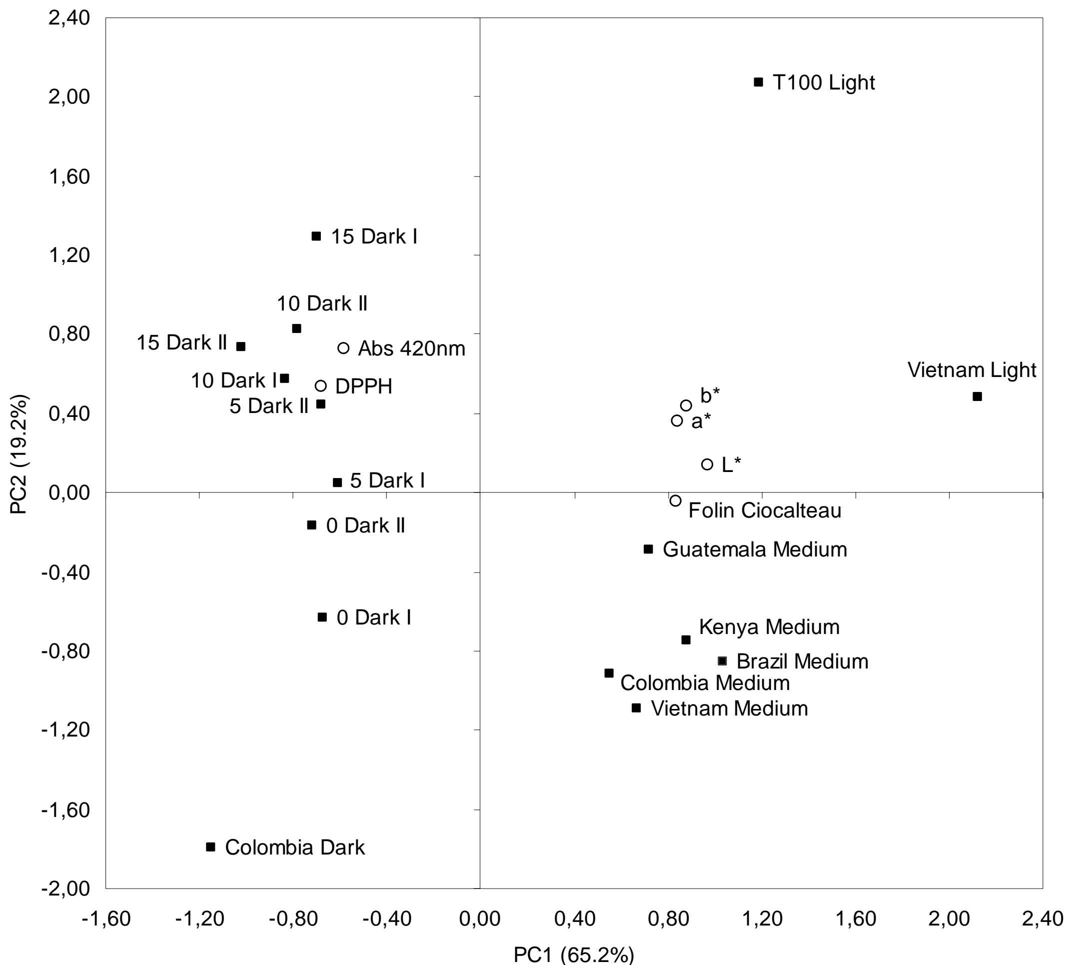

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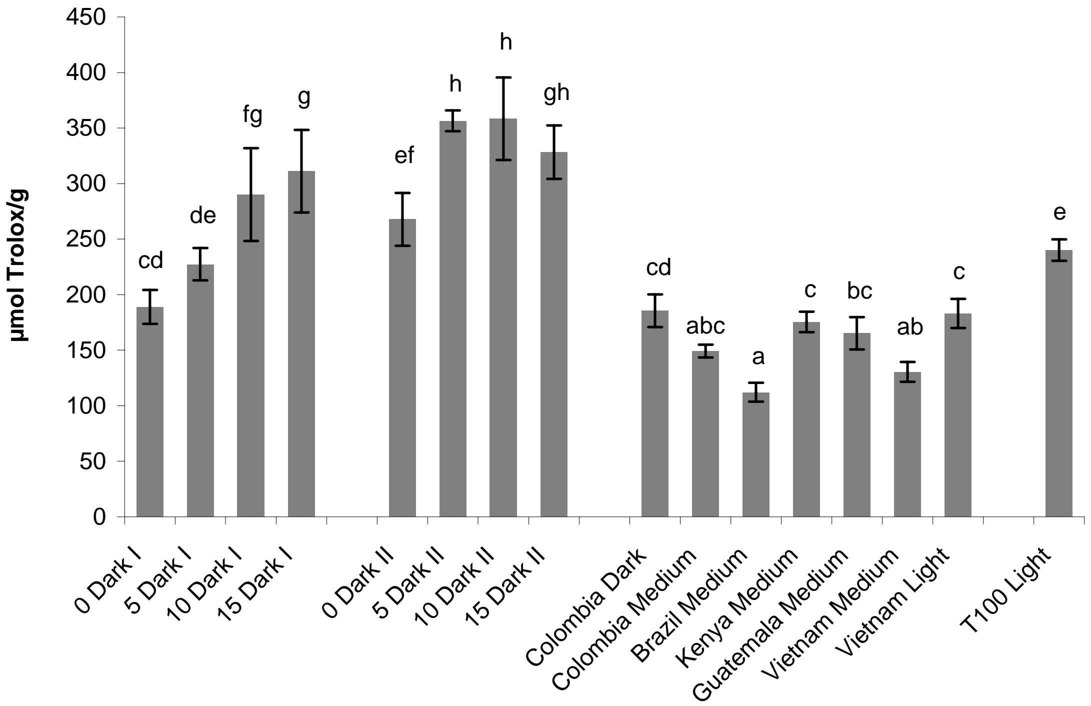

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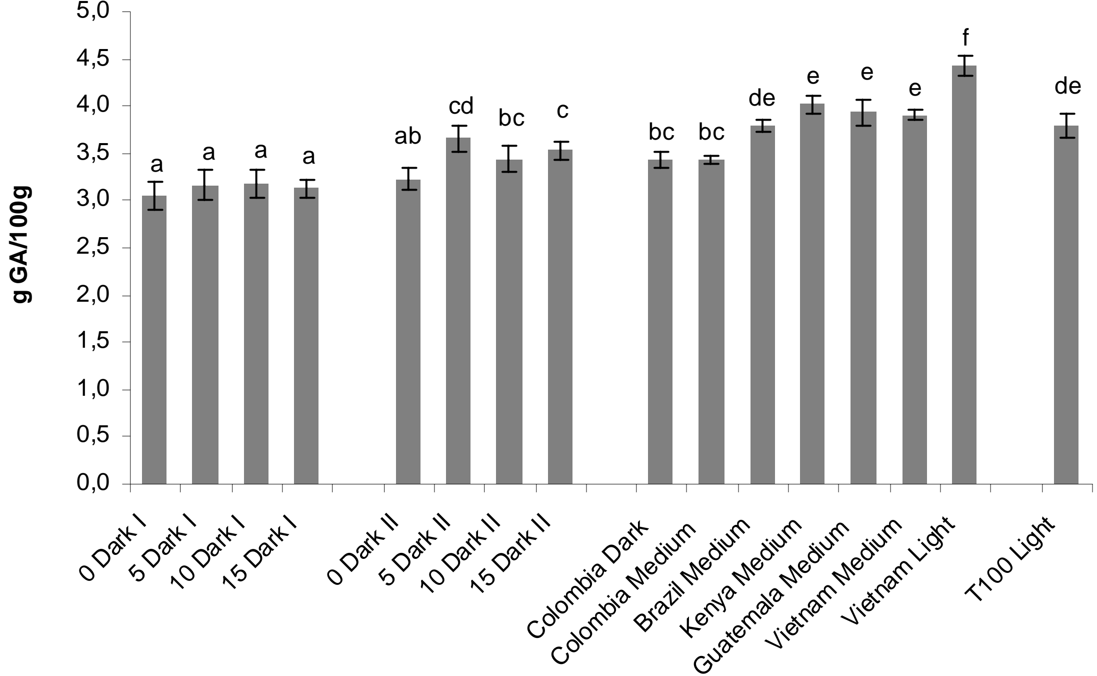

Objective 2

Study of the extractability of the main coffee antioxidant compounds during the most common brewing process (espresso and filter coffeemakers).

Estudio de la extracción de los principales compuestos antioxidantes durante la preparación de la bebida de café mediante los sistemas de extracción más habitualmente utilizados (cafeteras expreso y de filtro).

Paper 2:

Extraction of coffee antioxidants: impact of brewing time and method

Iziar A. Ludwig, Lidia Sanchez, Bettina Caemmerer, Lothar W. Kroh, M. Paz De Peña, Concepción Cid

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ABSTRACT

The aim of this work was to study the extraction behavior of the main coffee antioxidants (caffeoylquinic acids, melanoidins and caffeine) and the antioxidant capacity, during brewing time in the most widely consumed coffee brew methods (filter and espresso) in coffee. Antioxidant capacity by colorimetric assays (Folin-Ciocalteau, ABTS and DPPH) and electron spin resonance spectroscopy techniques (Fremy's salt and TEMPO) were analyzed. In espresso coffee, more than 70% of the antioxidants (except dicaffeoylquinic acids, diCQA) of a coffee brew were extracted during the first 8 s. In filter coffee, a U-shape antioxidants extraction profile was observed, starting later (after 75s) in Vietnam coffee than in Guatemala one, probably due to different wettability. Other technological parameters, such as turbulences and a longer contact time between water and ground coffee in filter coffeemaker, increased extraction efficiency, mainly in less polar antioxidant compounds as diCQA. In conclusion, these technological factors should be considered to optimize coffee antioxidants extraction that can be used as ingredients for functional foods.

KEYWORDS: Antioxidants, brewing time, coffee, Maillard reaction products, phenolics.

1. INTRODUCTION

Several chronic diseases, such as cancer, cardiovascular, inflammatory, and neurogenerative pathologies are associated with oxidative stress (Aruoma, 1999; Beal, 1995; Dorea & da Costa, 2005). Beside fruits and vegetables, plant beverages such as coffee brew have been proposed as an important source of antioxidants in human diet (Pulido, Hernandez Garcia, & Saura Calixto, 2003; Svilaas, Sakhi, Andersen, Svilaas, Strom, & Jacobs, 2004). The antioxidant capacity of coffee brew is attributed to both antioxidants originally present in coffee beans, like phenolic compounds, and roasting-induced antioxidants, like melanoidins and other Maillard Reaction Products (MRP) (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Crozier, Jaganath, & Clifford, 2009; del Castillo, Ames, & Gordon, 2002).

The most abundant phenolic compounds of coffee are chlorogenic acids (CGA). CGA are known for their contribution to the final acidity, astringency, and bitterness of the coffee brew, but also for their potent antioxidant properties (Moreira, Monteiro, Ribeiro-Alves, Donangelo, & Trugo, 2005; Natella, Nardini, Giannetti, Dattilo, & Scaccini, 2002; Trugo & Macrae, 1984; Variyar, Ahmad, Bhat, Niyas, & Sharma, 2003). During roasting, CGA are partially degraded and at least partly incorporated in coffee melanoidins through non-covalent or covalent bounds (Bekedam, Schols, van Boekel, & Smit, 2008; Nunes & Coimbra, 2010). Melanoidins are generally defined as the browned-colored, high-molecular-weight, nitrogenous end products of the Maillard reaction. They are formed during roasting process of coffee. Beside its contribution to flavor and color, one of the important functional properties of melanoidins is its antioxidant activity (Caemmerer & Kroh, 2006; C. Delgado-Andrade & Morales, 2005; López-Galilea, Andueza, Leonardo, de Peña, & Cid, 2006; Rufián-Henares & Morales, 2007). Although there is still a discussion about their bioavailability, it is clear that at

least they may act as prebiotic or even antimicrobial depending on their nature and concentration (Borrelli & Fogliano, 2005; Rufián-Henares & de la Cueva, 2009). Also caffeine or its metabolites in humans have been proposed as antioxidant compounds against lipid peroxidation induced by reactive oxygen species (Devasagayam, Kamat, Mohan, & Kesavan, 1996; Lee, 2000). However, although caffeine has been extensively studied from the pharmacological point of view, less attention has been paid to its potential antioxidant activity that may be overshadowed by phenolic compounds and MRP.

Brewing process is essential for the antioxidant composition and health properties of a coffee brew, because the contact of water with roasted coffee grounds is the crucial step for extraction of coffee compounds. Other factors, such as origin or variety of coffee beans, blending, roasting degree and grinding also play a key role in coffee composition. Among the several brewing techniques, filter coffee (drip filter) is the most widely used coffee brew obtained by infusion method, whereas espresso coffee is the most appreciated coffee brew produced by pressure method. In drip filtration methods, water at 92-96 ºC flows through a hardly compressed ground coffee bed and the extract drips from the brewing chamber into the pot. Turbulence in the brewing chamber prevents water from becoming saturated (Lingle, 1996). In pressure methods, water at approximately 9 bars and 88-92ºC is forced to go through coffee grounds compacted in a small brewing chamber (coffee cake). Also rapid brewing time and fine particle size are necessary (Lingle, 1996). Many chemical species identified in roasted coffee, including antioxidants, exhibit different extraction rates that may also be influenced by the choice of brewing technique and conditions (Peters, 1991; Petracco, 2001; Petracco, 2005).

Even though the brewing time is given by the coffee brewing technique, the knowledge of extraction behavior of the main coffee antioxidants during this time might induce to know the technological factors with major impact on antioxidants extraction. Thus, it could be possible to obtain not only coffee brews with higher antioxidant capacity, but also coffee extracts with health properties that can be used as ingredients in functional foods. For these reasons, the aim of this work was to study the extraction behavior of the main coffee antioxidants and the antioxidant capacity, during brewing time in the most widely consumed coffee brew procedures (filter and espresso).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents. The methanol (spectrophotometric and HPLC grade) and Folin-Ciocalteau reagent were from Panreac (Barcelona, Spain). ABTS (2,2'-Azino-bi(3-ethylbenzo-thiazonile-6-sulfonic acid) diammonium salt), potassium persulfate, $DPPH^{\cdot}$ (2,2-Diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, Fremy's salt (potassium nitrosodisulfonate) and TEMPO (2,2,6,6-tetramethyl-1-piperidin-1-oxyl) were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid was from Fluka (Madrid, Spain). Pure reference standards of 5-caffeoylquinic acid (5-CQA) and caffeine were obtained from Sigma-Aldrich (Steinheim, Germany) and pure reference standards of 3,4-, 3,5- and 4,5-dicaffeoylquinic acids were purchased from Phytolab (Vestenbergsgreuth, Germany). A mixture of 3-CQA, 4-CQA, and 5-CQA was prepared from 5-CQA using the isomerization method of Trugo and Macrae (1984), also described in Farah et al. (2005).

2.2. Coffee brew samples. Roasted coffee from Guatemala (*Coffea arabica*, 3.03 % water content, L* = 25.40 ± 0.69, roasted at 219 ºC for 905 s) and Vietnam (*Coffea*

canephora var. robusta, 1.59 % water content, L* = 24.92 ± 0.01, roasted at 228 ºC for 859 s) was provided by a local factory. The L* value was analyzed by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant and CIE 1931 standard observer. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in an l cm Petri plate, and the L* value was measured in triplicate on the CIELab scale.

Roasted coffee beans were ground to a powder in a Moulinex coffee grinder (model Super Junior "s", Paris, France) for 20 s immediately before sample preparation. Filter Coffee Brew was prepared from 36 g of ground roasted coffee for a volume of 600 mL, using a filter coffee machine (model Avantis 70 Aroma plus, Ufesa, Spain). Extraction took approx. 6 min at 90 ºC. Five fractions for filter coffee were collected sequentially every 75 s. Espresso Coffee Brew was prepared from 7 g of ground roasted coffee for a volume of 45 mL using an espresso coffee machine (model Saeco Aroma, Italy). Three fractions for espresso coffee were collected sequentially every 8 s. Coffee brews and fractions were lyophilized using a CRYODOS Telstar (Terrassa, Spain) and stored at -18ºC until sample analysis.

2.3. pH. The pH measurements of coffee brews and fractions were performed with a Crison Basic 20pH-meter.

2.4. Browned compounds (Abs 420 nm). Fifty microliters of coffee brew or fraction were diluted up to 2 mL with deionized water. Browned compounds were quantified by measuring the absorbance of the sample at 420 nm after exactly 1 min, in a 3 mL capacity cuvette (1 cm length) with a Lambda 25 UV-VIS spectrophotometer (Perkin-Elmer Instruments, Madrid, Spain) connected to a thermostatically controlled chamber (25 ºC) and equipped with UV Win-Lab software (Perkin Elmer).

2.5. Folin-Ciocalteau (FC) assay. The Folin-Ciocalteau reducing capacity of coffee or fractions was performed according to the Singleton´s method (Singleton & Rossi, 1965). For every coffee sample, 1:10 dilutions with demineralized water were prepared, and 500 μL of Folin-Ciocalteau reagent were added to 100 μL of the coffee sample solution. After 2 min delay, 1.5 mL of a 7.5% sodium carbonate solution was added. Next, the sample was incubated in darkness at room temperature for 90 min. The absorbance of the sample was measured at 765 nm in a Lambda 25 UV-VIS spectrophotometer (Perkin Elmer Instruments, Madrid, Spain). Gallic acid (GA) was used as reference, and the results were expressed as milligrams of GA per mililiter of coffee brew or fraction.

2.6. Antioxidant capacity by ABTS assay. The antioxidant capacity measured with ABTS was carried out according to the method described by Re et al. (1999) with some modifications. The radicals $ABTS^{\cdot+}$ were generated by the addition of 2.45 mM potassium persulfate to an 7 mM ABTS solution prepared in phosphate-buffered saline (PBS, pH 7.4) and allowing the mixture to stand in darkness at room temperature for at least 12 h before use. The $ABTS^{\cdot+}$ stock solution was adjusted with PBS to an absorbance of 0.7 (±0.02) at 734 nm in a 1 cm cuvette at 25 ºC (Lambda 25 UV, VIS spectrophotometer, Perkin Elmer Instruments, Madrid, Spain). An aliquot of 50 μL of coffee sample diluted with demineralized water (5:1000 to 15:1000) was added to 2 mL of $ABTS^{\cdot+}$ reagent and the absorbance was monitored for 18 min at 25 ºC. Calibration was performed with Trolox solution (a water-soluble vitamin E analogue) and total antioxidant capacity was expressed as micromoles (μmol) of Trolox per mililiter of coffee brew or fraction.

2.7. Antioxidant capacity by DPPH assay. The antioxidant capacity was measured using the DPPH decolorization assay (Brand-Williams, Cuvelier, & Berset, 1995). A

$6.1x10^{-5}$ M DPPH· methanol solution was prepared immediately before use. The DPPH· solution was adjusted with methanol to an absorbance of 0.7 (±0.02) at 515 nm in a 1 cm cuvette at 25 ºC (Lambda 25 UV, VIS spectrophotometer, Perkin Elmer Instruments, Madrid, Spain). Fifty microliters of appropriate diluted coffee sample (1:100 to 3:100) was added to DPPH· solution (1.95 mL). After mixing, the absorbance was monitored at 515 nm for 18 min at 25 ºC. Calibration was performed with Trolox solution and the total antioxidant capacity was expressed as micromoles (µmol) of Trolox per mililiter of coffee brew or fraction.

2.8. Antioxidant capacity by Electro Spin Resonance (ESR) spectroscopy. The ESR spectroscopy measurements were performed with Fremy´s salt and TEMPO as stabilized radicals with the same procedure described by Roesch et al. (2003) and modified by Caemmerer & Kroh (2006). For the investigation with Fremy's salt, 100 µL of every coffee sample diluted 250-fold with demineralized water was allowed to react with an equal volume of an aqueous 1 mM Fremy's salt solution prepared in 50 mM phosphate buffer (pH 7.4). ESR spectra were recorded every 40 s for 30 min. For the investigation with TEMPO, aliquots of 300 µL of coffee sample were allowed to react with 100 µL of 1 mM TEMPO solution. ESR spectra were obtained after 120 min, by which time the reaction was complete. Microwave power was set at 10 dB. Modulation amplitude, center field, and sweep width were set at 1.5, 3397, and 71 G, respectively. Both Fremy's salt and TEMPO antioxidant activity were calculated as Trolox equivalents and expressed as micromoles (µmol) of Trolox per mililiter of coffee brew or fraction.

2.9. Chlorogenic acids (CGA) and caffeine. Extract preparation and cleanup were carried out according to Bicchi et al. (1995). The compounds were analyzed by HPLC following the method described by Farah et al. (2005), with some modifications. HPLC

analysis was achieved with an analytical HPLC unit model 1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump and an automated sample injector. A reversed-phase Hypersil-ODS (5 μm particle size, 250 x 4.6 mm) column was used at 25 ºC. The sample injection volume was 100 μL. The chromatographic separation was performed using a gradient of methanol (solvent A) and Milli-Q water acidulated with phosphoric acid (pH 3.0, solvent B) at a constant flow of 0.8 mL/min starting with 20% solvent A. Then solvent A was increased to 50% within 15 min to be maintained at 50% for 9 min and, finally, to return to initial conditions (20% solvent A) in 3 min. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 325 nm for CGA and 276 nm for Caffeine. Identification of CGA and caffeine was performed by comparing the retention time and the photodiode array spectra with those of their reference compounds. Quantification of 5-caffeoilquinic (5-CQA) and caffeine was made by comparing the peak areas with those of the standards. Quantification of the other chlorogenic acids (CGA) was performed using the area of 5-CQA standard combined with molar extinction coefficients of the respective CGA as reported by Trugo and Macrae (1984) and Farah et al. (2005).

2.10. Statistical analysis. Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. Student's t-test was applied for each antioxidant capacity assay to know whether there were differences between both coffees in each coffee brew. One-way analysis of variance (ANOVA) was applied for each parameter to compare antioxidants extraction among fractions in each coffee brew sample. A T-Tukey test was applied as a test a posteriori with a level of significance of 95%. All statistical analyses were performed using the SPSS v.15.0 software package.

3. RESULTS AND DISCUSSION

3.1. Coffee fractions Volumes

The volumes of the coffee brews and fractions obtained by espresso and filter coffeemakers are shown in Table 1**.** The volumes of the three espresso coffee fractions were quite similar, ranging from 14 to 17 mL. In contrast, the volumes of the filter coffee fractions increased from F1 (76-80 mL) up to F3 (160-186 mL) and then decreased to F5 (26-54 mL), showing an inverted U-shape profile.

To extract coffee compounds during the brewing process, the dry coffee grounds must first absorb water. Once the water has completely surrounded a coffee particle, both inside and out, the coffee extractable material begins to move out of the bean's cellular structure and into the surrounding water. Because espresso coffeemaker applies constant pressure that forces water through the coffee grounds with a constant flow, the coffee fraction volumes were similar among each other. However, in filter coffee no mechanical forces are applied, and the brew volume dripping out from the extraction chamber depends on the water amount, and consequently on the water pressure in the extraction chamber of the coffeemaker according to Darcy's law (Petracco, 2005). Furthermore, at the beginning of the filter extraction process, part of the water is absorbed by coffee grounds. In an espresso coffeemaker, water is forced to go through the coffee cake, but, in a filter coffeemaker, during wettability, 1 g of coffee will absorb 2 mL of water as a general rule (Lingle, 1996). This fact explains the low volume obtained for F1 (0-75 s). With time, water fills the extraction chamber increasing the pressure and favoring that water passes through the coffee bed, which leads to higher volumes in the middle fractions. At the end of the brewing procedure, pressure decreases when the water reservoir depletes, giving the lowest volume in the last fraction (F5).

3.2. Antioxidant capacity of coffee fractions

The antioxidant capacity of the coffee brews and fractions obtained by espresso and filter coffeemakers was measured by means of three colorimetric assays (Folin-Ciocalteau, ABTS and DPPH) and two electron spin resonance (ESR) spectroscopy techniques (Fremy´s salt and TEMPO) and the results are shown in Figure 1 to 5.

The Folin-Ciocalteau assay is based on an electron-transfer reaction. Although this is the most popular method to evaluate the total phenolic compounds, the Folin-Ciocalteau reagent can be reduced by many electron-donors, not only phenolic compounds (Huang, Ou, & Prior, 2005). Two different stable radicals ($ABTS\cdot^{+}$ and DPPH·) were chosen to assess the radical scavenging activity in coffee fractions. These radicals react energetically with hydrogen-donors, such as phenolic compounds, being DPPH· likely more selective in the reaction with H-donors than $ABTS\cdot^{+}$ (Huang et al., 2005). In these three colorimetric assays, Vietnam coffee brews showed significantly ($p<0.01$) higher antioxidant capacity than Guatemala ones. The results were similar to those reported by other authors in espresso and filter coffee brews (Pérez-Martinez, Caemmerer, De Peña, Cid, & Kroh, 2010; Sánchez González, Jiménez Escrig, & Saura Calixto, 2005).

Espresso coffee fractions from both coffees showed a remarkable decrease in antioxidant capacity with brewing time. More than 70% of the overall antioxidant capacity of an espresso coffee brew was found in F1 (0-8 s), whereas F3 accounted for less than 12 %. These results demonstrate that the compounds responsible for the antioxidant activity of an espresso coffee brew are mainly extracted at the beginning of the brewing process and, afterwards, are diluted. Similar results were found by Alves et al (2010) for DPPH antioxidant activity in espresso coffees with different brew lengths (“short” to “long”). These authors also observed that the antiradical or reducing activity of espresso coffee brew is not only dependent on total phenolic amounts measured by Folin-Ciocalteau assay. This may be due to the fact that the Folin-Ciocalteau assay not

only evaluates phenolic compounds, but also because it is well known that roasting-induced antioxidants like Maillard reaction products (MRP), contribute to the overall antioxidant capacity of coffee (Delgado-Andrade, Rufián-Henares, & Morales, 2005; Pérez-Martinez et al., 2010).

To go deeper into the influence of brewing time on antioxidant capacity due to phenolics or MRP, Electron spin resonance (ESR) spectroscopy was applied using Fremy's salt and TEMPO radicals. Mainly phenolic compounds can be detected when Fremy's salt is used as the stabilized radical, whereas TEMPO is mainly scavenged by Maillard reaction products (MRP), such as melanoidins (Caemmerer & Kroh, 2006). The results obtained with ESR spectroscopy (Figure 4 and 5) showed that Fremy´s salt scavenging capacity was almost four times higher than TEMPO. Similar results were reported by other authors who proposed that the phenolic antioxidants evaluated by Fremy´s salt dominate the overall antioxidant capacity of coffee brews, whereas the contribution of roasting-induced antioxidants is rather limited (Bekedam, Schols, Cämmerer, Kroh, van Boekel, & Smit, 2008; Pérez-Martinez et al., 2010).

The ESR antioxidant capacity of espresso coffee fractions showed that F1 (0-8 s) accounted for 75-81 % and for 86-89 % of the Fremy´s salt and TEMPO scavenging capacity of an espresso coffee brew, respectively. Although antioxidant capacity due to phenolics and measured by Fremy's salt assay was the highest in the first fraction, 20-25 % of the scavenging capacity was still found in F2 and F3. This could be due to a slower extraction of those phenolics retained in the inner coffee particles and those bound to melanoidins that need more time and water pressure to be released. The highest percentages observed for TEMPO scavenging capacity in F1 indicate that MRP antioxidants were mainly extracted during the first 8 seconds, whereas the last fraction (16-24 s) only accounted for 1-2 %. These results agree with the significantly highest

values of Browned compounds (Abs 420 nm) showed in the first fraction (Table 2) that clearly decreased in the next ones (F2 and F3).

Filter coffee fractions showed different antioxidant capacity extraction behaviors, being also different in the two coffee samples in comparison to espresso coffee. In Guatemala filter coffee, all antioxidant capacity assays showed a U-shape profile with the highest concentration in F1 (0-75 s) and F5 (300-375 s) and the lowest in F3 (150-225 s). However, in Vietnam coffee the U-shape antioxidant capacity extraction started after 75 s, showing F1 the lowest values. This could be due to a higher water absorption in Vietnam coffee that leads to a longer wetting stage. The wettability depends on the particle shape and size that may be different depending on factors like grinding that is also influenced by coffee origin or variety and roasting degree (Lingle, 1996). In this work, taking into account that roasting degree and grinding conditions were controlled to be the same, different wettability may be due to the different brittleness of the coffee beans. The increase of antioxidant capacity in the last fractions (F4 and F5) of filter coffee brews could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee. In fact, because the last fraction (F5) had the lowest volumes (26 mL and 54 mL for Guatemala and Vietnam coffees, respectively), their contribution to the antioxidant capacity of the overall coffee brew was rather limited (~9 % and ~14 %, respectively).

The results of the antioxidant capacity due to phenolics and MRP, measured by ESR spectroscopy in filter coffee fractions using Fremy's salt and TEMPO as stabilized radicals (Figures 4 and 5), also corroborate that the antioxidants extraction seems to be delayed in Vietnam filter coffee. This was more pronounced in TEMPO antioxidant capacity that mainly evaluates the scavenging activity of melanoidins which are polymeric compounds with more difficult to be released without water pressure. In fact,

the Absorbance at 420 nm of Vietnam filter F1 fraction was significantly the lowest as shown in Table 3. Moreover, taking into account the brew volume, only ~3 % of TEMPO antioxidant capacity of the overall Vietnam filter coffee brew was extracted during the first 75 seconds (F1), whereas ~37 % was found in F2 (75-150 s). So that, the contribution of the first two fractions of Vietnam filter coffee to the overall TEMPO antioxidant capacity was similar to the ~40 % found in Guatemala filter coffee F1.

3.3. Antioxidant compounds extraction

The antioxidant capacity of coffee brew is attributed to both, natural antioxidants, like phenolic compounds, and roasting-induced antioxidants, like melanoidins and other MRPs. To know the influence of brewing time on the main antioxidant compounds, browned compounds (Abs 420 nm), caffeine and caffeoylquinic acids in coffee brews fractions were quantified and the results are shown in Table 2 and 3. Browned compounds, as previously discussed, were mainly extracted in those coffee fractions with high TEMPO antioxidant capacity showing a high correlation ($r=0.969$, $p<0.001$). Also caffeine has been proposed as an antioxidant compound against lipid peroxidation induced by reactive oxygen species (Lee, 2000). Caffeine was in significantly higher concentration in Vietnam espresso and filter coffee brews and fractions. It is very well known that Robusta coffees are richer in caffeine than Arabica ones (Belitz, Grosch, & Schieberle, 2009). Thus, caffeine might partially explain the higher antioxidant capacity of Vietnam coffee brews that could not be attributed to the main chlorogenic acids that were found in lower amounts in these coffee brews, as will be discussed later. Traditionally, the higher antioxidant capacity of Robusta coffee brews has been attributed to higher total phenolic compounds (usually measured by Folin Ciocalteau technique), and then to chlorogenic acids because 5-CQA is the most abundant phenolic in coffee. However, other authors (López-Galilea, de Peña, & Cid, 2007; Vignoli,

Bassoli, & Benassi, 2011) also observed higher antioxidant capacity but lower 5-CQA amounts in brews prepared with Robusta coffee or torrefacto blends. These authors reported high correlations between antioxidant capacity of coffee brews and caffeine, suggesting that caffeine might be a good contributor to the antioxidant capacity or reducing power of coffee brews. In the present work, also high correlations have been found between antioxidant capacity assays and caffeine (r values ranging from 0.906 for Fremy's salt assay to 0.968 for DPPH).

Chlorogenic acids (CGA) are water soluble esters formed between *trans*-cinnamic acids, such as caffeic acid, and quinic acid. They may be subdivided according to the nature, number and position of the cinnamic substituents (Clifford, 1999). Caffeoylquinic acid (CQA) is the most abundant chlorogenic acid class accounting for 76-84% of the total CGA in green coffee (Perrone, Farah, Donangelo, de Paulis, & Martin, 2008). Although during roasting CGA are lost up to 95%, CQA still are the predominant CGA in roasted coffee (Trugo & Macrae, 1984). Monocaffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA) were identified and quantified by HPLC-DAD in each fraction and coffee brew, and the results are shown in Tables 4 and 5. 5-CQA was the major compound among CQAs in all samples, followed by 4-CQA and 3-CQA. The diCQAs were in lower concentration than CQAs. The abundance of 3,4-diCQA and 4,5-diCQA was similar in every coffee fractions or brews, whereas 3,5-diCQA was the least abundant isomer. These results are in agreement with those reported by other authors in roasted coffee (Perrone et al., 2008) and in coffee brew (Alves et al., 2010). Higher amounts of CQA in Robusta coffees than in Arabica ones have been extensively reported (Farah et al., 2005). However, in this study less amounts of CQA were found in Vietnam coffee than in Guatemala ones. Also Vignoli et al. (2011) observed higher amount of 5-CQA

in Arabica soluble coffee. This could be due to several factors, such as the origin of coffee and the higher loss of chlorogenic acids in Robusta coffee during roasting process (Clifford, 1997; Perrone, Donangelo, Donangelo, & Farah, 2010).

Fractions obtained from espresso coffeemaker showed in both coffees a steep decrease with extraction time in all three CQA isomers (3-, 4-, and 5-CQA). F1 (0-8s) accounted for about 70 %, F2 (8-16 s) for 17 % and F3 (16-24 s) for less than 14 % of the total CQA amounts found in an espresso coffee brew. The CQA extraction behavior was similar to that of the antioxidant capacity measured by colorimetric assays and Fremy´s salt, showing high correlations (r values ranging from 0.727 for 5-CQA and DPPH to 0.903 for 4-CQA and Fremy's salt, $p<0.001$), maybe because monocaffeoylquinic acids are the most abundant phenolic compounds in coffee. In contrast, diCQAs were extracted more slowly, accounting F1 for ~50 %, F2 for ~30 % and F3 still for ~20 %, showing correlations coefficients lower than 0.700 (except for 3,4-diCQA with r values ranging from 0.906 for Fremy's salt to 0.968 for DPPH). The esterification of an additional caffeic acid moiety in diCQA increases the number of hydroxyl groups and might favor the retention of these compounds by interaction with melanoidins or other polymeric compounds (Bekedam, Schols, van Boekel et al., 2008; Kroll, Rawel, & Rohn, 2003), reducing the release of diCQA. In fact, the hydrogen bonding between hydroxyl groups of the phenolic compounds and the amide carbonyls of the peptide bond were found to be a common non-covalent interaction between phenolics and melanoidins (Nunes & Coimbra, 2010). Also the weaker polarity of the diCQA compared to the CQA might explain the slower release of these compounds during extraction with water (Kroll et al., 2003). Blumberg et al. (2010) studied the influence of hot water percolation on the concentration of monocaffeoylquinic acids and

chlorogenic acid lactones and reported that dicaffeoylquinic lactones were extracted rather slowly in comparison to monocaffeoylquinic ones.

Caffeoylquinic acids extraction behavior was different in filter coffee, as can be seen in Table 5. Different extraction profiles were also found for the two coffee samples. In Guatemala filter coffee, CQAs and diCQAs extraction showed a U-shape profile with the highest concentration in F1 (0-75 s) and F5 (300-375 s) and the lowest in F3 (150-225 s), similar to that observed for antioxidant capacity according to the correlations showed before. However, in Vietnam filter coffee the U-shape extraction of caffeoylquinic acids started after 75 s, and F1 exhibited the significantly lowest caffeoylquinic acids concentration. The delay in caffeoylquinic acids extraction might be attributed to the longer wetting stage observed in Vietnam coffee, as described above. On the other hand, the increased extraction of caffeoylquinic acids in the last stage of the brewing process, mainly observed in F5 in both coffee samples, could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee. This might facilitate the hydrolysis of caffeoylquinic acids bound to melanoidins inducing their release during advanced stages of filter coffee brewing (Lingle, 1996). However, when the lowest volumes of these fractions are taking into account, it could be observed that caffeoylquinic acids only accounted for ~8 % and ~11 % of the total in Guatemala and Vietnam filter coffee brews, respectively. Unlike in espresso coffee, similar extraction percentages among CQAs and diCQAs in each coffee fraction along the filter brewing process were observed. Moreover, when the concentration of antioxidants is calculated per gram of coffee taking into account the different fractions volumes, higher extraction of these phenolic compounds per gram of coffee was obtained in filter coffee brews than in espresso ones, in agreement with Pérez-Martinez et al. (2010). This may be due to the technological differences between

espresso and filter coffeemaker. Although the high water pressure applied in espresso coffeemaker favors the extraction process, the short contact time between water and coffee grounds, the high coffee/water ratio and the limited space in coffee cake does not allow equilibrium to be reached (Petracco, 2005). In contrast, longer time and turbulences in the extraction chamber of the filter coffeemaker allow the water in immediate contact with the coffee to extract additional compounds when it has not become so saturated with dissolved material. Thus, both technological factors might favor the extraction of both CQAs and diCQAs, free and bound with melanoidins. In fact, turbulences are considered, after time and temperature, the third most important factor in filter coffee brewing (Lingle, 1996). Less turbulences during sequential coffee percolation could also be the reason why Blumberg et al. (2010) found that monocaffeoylquinic acids and monocaffeoyl and dicaffeoyl quinides extraction behaviors were more similar to those of our espresso coffee fractions than filter ones, i.e. higher extraction in the first fractions and slower release of dicaffeoyl quinides.

In conclusion, brewing time plays a key role in antioxidants extraction of coffee. To optimize their extraction in order to obtain antioxidants that can be used as ingredients for functional foods, several technological factors should be taken into account. Thus, higher water pressure increases antioxidants extraction speed like in the first fraction of espresso coffee. Nevertheless, parameters like turbulence and longer contact time, typically of a filter coffeemaker, should be considered in order to increase extraction efficiency, mainly in less polar antioxidant compounds as diCQA. Moreover, extraction conditions should also be adjusted for each coffee because cellular structure of coffee beans may also influence. Further research in the influence of technological parameters on chemical composition of coffee brew fractions, as well as their sensory properties, should be needed before to industrial development.

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Table 1. Volumes of coffee brews and fractions obtained by espresso and filter coffeemakers.

	Espresso		Filter	
	$t_{extraction}$ (s)	V (mL)	$t_{extraction}$ (s)	V (mL)
Guatemala				
Coffee brew	24	47	375	532
F1	0-8	16	0-75	80
F2	8-16	14	75-150	146
F3	16-24	17	150-225	186
F4	-	-	225-300	94
F5	-	-	300-375	26
Vietnam				
Coffee brew	24	46	375	520
F1	0-8	17	0-75	74
F2	8-16	14	75-150	120
F3	16-24	15	150-225	160
F4	-	-	225-300	112
F5	-	-	300-375	54

Table 2. pH, browned compounds and caffeine in espresso coffee brews and fractions. All values are shown as mean ± standard deviation (n=3). Different letters indicate significant differences ($p<0.05$) among different coffee fractions in each coffee.

	pH	Browned compounds [Abs_{420}]	Caffeine [mg/100mL]
Guatemala			
Coffee brew	4.01 ± 0.01	0.391 ± 0.013	141.4 ± 2.4
F1	4.83 ± 0.01 a	0.903 ± 0.008 c	296.8 ± 1.6 c
F2	4.90 ± 0.01 b	0.253 ± 0.005 b	82.66 ± 0.7 b
F3	5.09 ± 0.01 c	0.128 ± 0.004 a	39.6 ± 0.4 a
Vietnam			
Coffee brew	5.76 ± 0.00	0.458 ± 0.011	253.3 ± 2.0
F1	5.57 ± 0.01 a	1.172 ± 0.008 c	575.4 ± 3.9 c
F2	6.08 ± 0.01 b	0.297 ± 0.004 b	159.2 ± 0.1 b
F3	6.38 ± 0.00 c	0.133 ± 0.007 a	74.7 ± 0.2 a

Table 3. pH, browned compounds and caffeine in filter coffee brews and fractions. All values are shown as mean ± standard deviation (n=3). Different letters indicate significant differences ($p<0.05$) among different coffee fractions in each coffee.

	pH	Browned compounds [Abs_{420}]	Caffeine [mg/100mL]
Guatemala			
Coffee brew	5.29 ± 0.01	0.200 ± 0.003	57.1 ± 0.1
F1	5.12 ± 0.01 a	0.275 ± 0.002 c	106.8 ± 0.1 e
F2	5.25 ± 0.01 b	0.200 ± 0.002 b	57.1 ± 0.2 c
F3	5.39 ± 0.01 c	0.160 ± 0.005 a	35.7 ± 0.0 a
F4	5.37 ± 0.02 c	0.171 ± 0.005 a	48.6 ± 0.4 b
F5	5.12 ± 0.00 a	0.266 ± 0.007 c	89.0 ± 0.6 d
Vietnam			
Coffee brew	6.07 ± 0.01	0.205 ± 0.001	115.3 ± 0.4
F1	6.14 ± 0.01 c	0.132 ± 0.005 a	65.9 ± 0.6 a
F2	5.93 ± 0.01 a	0.298 ± 0.010 c	158.1 ± 0.3 e
F3	6.06 ± 0.01 b	0.210 ± 0.012 b	112.9 ± 0.2 c
F4	6.19 ± 0.01 d	0.193 ± 0.007 b	104.4 ± 0.8 b
F5	6.08 ± 0.01 b	0.273 ± 0.015 c	117.6 ± 0.9 d

Table 4. Chlorogenic acids in espresso coffee brews and fractions. All values are shown as mean ± standard deviation (n=3). Different letters indicate significant differences ($p<0.05$) among different coffee fractions in each coffee.

	3-CQA [mg/100mL]	4-CQA [mg/100mL]	5-CQA [mg/100mL]	3,4-diCQA [mg/100mL]	3,5-diCQA [mg/100mL]	4,5-diCQA [mg/100mL]
Guatemala						
Coffee brew	43.2 ± 0.1	55.6 ± 0.7	96.7 ± 1.8	5.1 ± 0.1	2.8 ± 0.2	5.0 ± 0.1
F1	91.3 ± 1.3 c	114.6 ± 0.6 c	201.1 ± 1.6 c	9.8 ± 0.2 c	4.2 ± 0.1 c	9.6 ± 0.4 c
F2	26.4 ± 0.4 b	33.8 ± 0.1 b	56.3 ± 0.6 b	6.5 ± 0.4 b	3.1 ± 0.1 b	5.7 ± 0.1 b
F3	15.0 ± 0.0 a	22.4 ± 0.1 a	29.8 ± 0.1 a	3.2 ± 0.0 a	1.8 ± 0.0 a	2.9 ± 0.0 a
Vietnam						
Coffee brew	25.8 ± 1.2	35.0 ± 0.2	52.9 ± 2.0	4.1 ± 0.0	2.0 ± 0.0	3.8 ± 0.1
F1	49.3 ± 0.6 c	70.4 ± 0.5 c	108.0 ± 2.9 c	7.8 ± 0.2 c	2.7 ± 0.2 c	5.4 ± 0.1 c
F2	16.0 ± 0.1 b	20.5 ± 0.1 b	30.8 ± 0.2 b	4.6 ± 0.0 b	1.7 ± 0.1 b	3.8 ± 0.1 b
F3	9.4 ± 0.3 a	13.0 ± 0.2 a	16.0 ± 0.4 a	2.1 ± 0.0 a	0.8 ± 0.0 a	1.7 ± 0.1 a

Table 5. Chlorogenic acids in filter coffee brews and fractions. All values are shown as mean ± standard deviation (n=3). Different letters indicate significant differences ($p<0.05$) among different coffee fractions in each coffee.

	3-CQA [mg/100mL]	4-CQA [mg/100mL]	5-CQA [mg/100mL]	3,4-diCQA [mg/100mL]	3,5-diCQA [mg/100mL]	4,5-diCQA [mg/100mL]
Guatemala						
Coffee brew	17.0 ± 0.1	25.3 ± 0.0	38.7 ± 0.1	3.8 ± 0.0	2.0 ± 0.0	3.2 ± 0.0
F1	31.0 ± 0.3 e	40.9 ± 0.1 d	70.1 ± 0.3 e	6.1 ± 0.3 d	2.9 ± 0.1 c	6.0 ± 0.0 d
F2	16.8 ± 0.4 c	26.2 ± 0.2 c	38.2 ± 0.7 c	3.8 ± 0.1 b	2.0 ± 0.1 b	3.9 ± 0.1 b
F3	11.0 ± 0.1 a	16.8 ± 0.0 a	24.8 ± 0.2 a	2.7 ± 0.1 a	1.4 ± 0.0 a	2.7 ± 0.0 a
F4	14.7 ± 0.2 b	23.0 ± 0.2 b	34.1 ± 0.4 b	3.8 ± 0.2 b	2.1 ± 0.1 b	3.7 ± 0.0 b
F5	24.5 ± 0.8 d	42.0 ± 0.9 d	61.3 ± 1.0 d	4.4 ± 0.0 c	3.0 ± 0.1 c	4.3 ± 0.1 c
Vietnam						
Coffee brew	15.0 ± 0.1	19.4 ± 0.0	21.8 ± 0.2	3.1 ± 0.0	0.7 ± 0.0	1.2 ± 0.0
F1	10.6 ± 0.2 a	13.1 ± 0.1 a	14.3 ± 0.2 a	2.0 ± 0.0 a	0.3 ± 0.0 a	0.7 ± 0.0 a
F2	18.5 ± 0.2 d	24.4 ± 0.0 e	28.3 ± 0.4 d	4.2 ± 0.1 e	0.9 ± 0.0 d	1.6 ± 0.0 d
F3	14.6 ± 0.1 bc	19.1 ± 0.0 c	21.2 ± 0.2 b	2.9 ± 0.0 c	0.7 ± 0.0 b	1.2 ± 0.0 b
F4	14.5 ± 0.3 b	18.3 ± 0.0 b	20.4 ± 0.5 b	2.8 ± 0.0 b	0.7 ± 0.0 b	1.2 ± 0.0 b
F5	15.2 ± 0.4 c	20.4 ± 0.1 d	22.6 ± 0.6 c	3.6 ± 0.0 d	0.8 ± 0.0 c	1.5 ± 0.0 c

FIGURE CAPTIONS

Figure 1. Antioxidant capacity (Folin-Ciocalteau method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences (p<0.01) between coffee brews. Different letters indicate significant differences (p<0.05) among coffee fractions in each coffee.

Figure 2. Antioxidant capacity (ABTS method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences (p<0.01) between coffee brews. Different letters indicate significant differences (p<0.05) among coffee fractions in each coffee.

Figure 3. Antioxidant capacity (DPPH method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences (p<0.01) between coffee brews. Different letters indicate significant differences (p<0.05) among coffee fractions in each coffee.

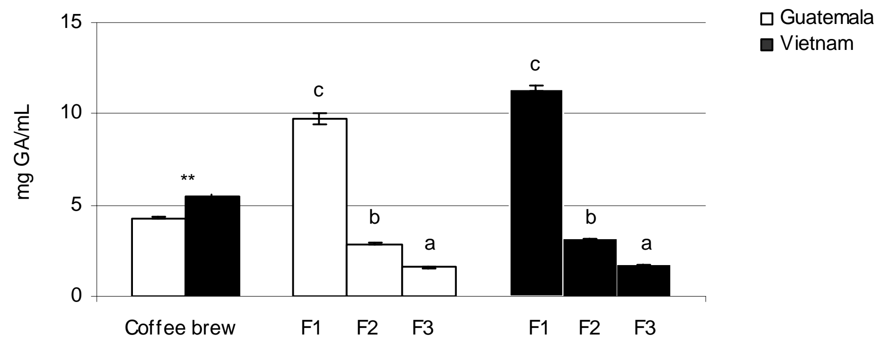
Figure 4. Antioxidant capacity (Fremy's Salt method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences (p<0.01) and ns nonsignificant differences (p>0.05) between coffee brews. Different letters indicate significant differences (p<0.05) among coffee fractions in each coffee.

Figure 5. Antioxidant capacity (TEMPO method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences (p<0.01) and ns

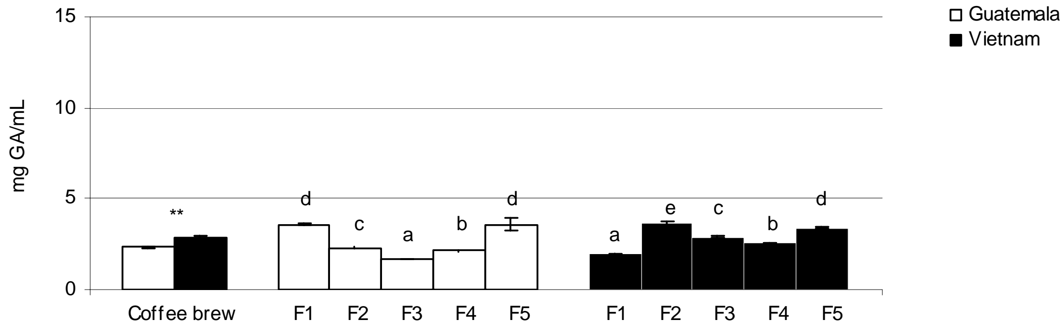
nonsignificant differences ($p>0.05$) between coffee brews. Different letters indicate significant differences ($p<0.05$) among coffee fractions in each coffee.

Figure 1. Antioxidant capacity (Folin-Ciocalteau method) of coffee brews and fractions obtained by espresso (**a**) and filter coffeemaker (**b**). All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences ($p<0.01$) between coffee brews. Different letters indicate significant differences ($p<0.05$) among coffee fractions in each coffee.

a) Espresso coffeemaker



b) Filter coffeemaker



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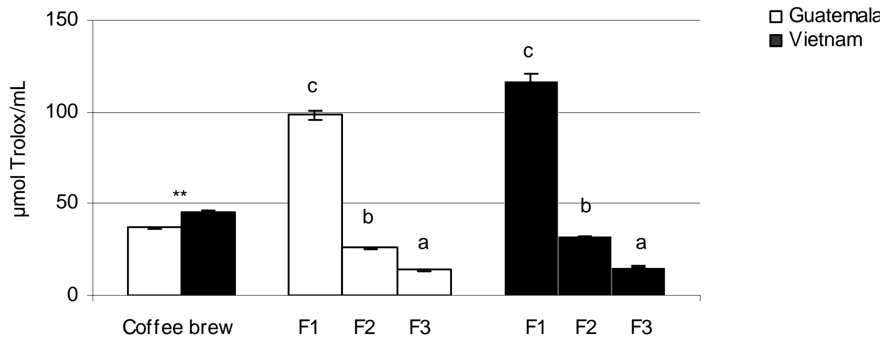
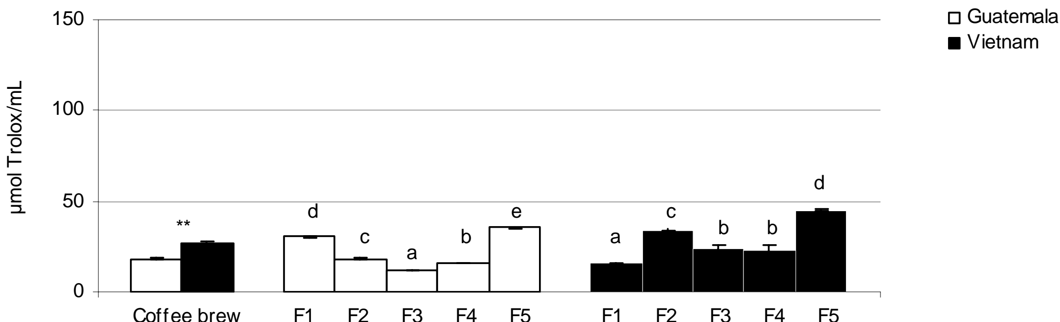


b) Filter coffeemaker



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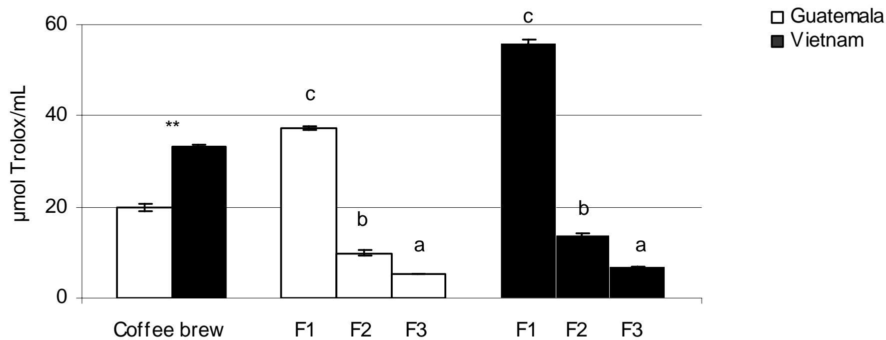
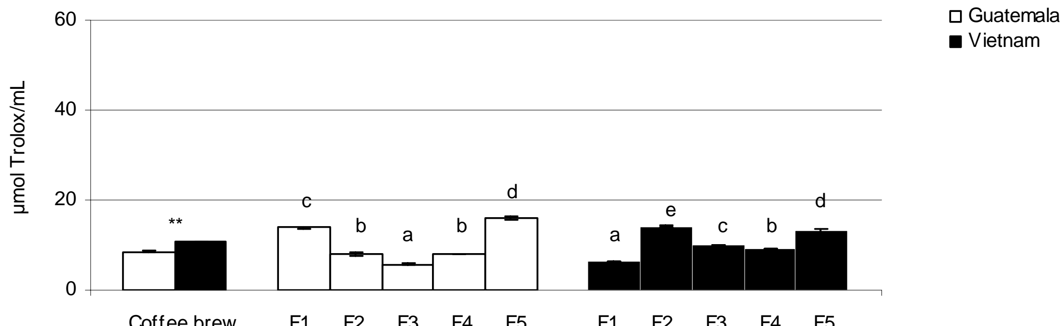


b) Filter coffeemaker



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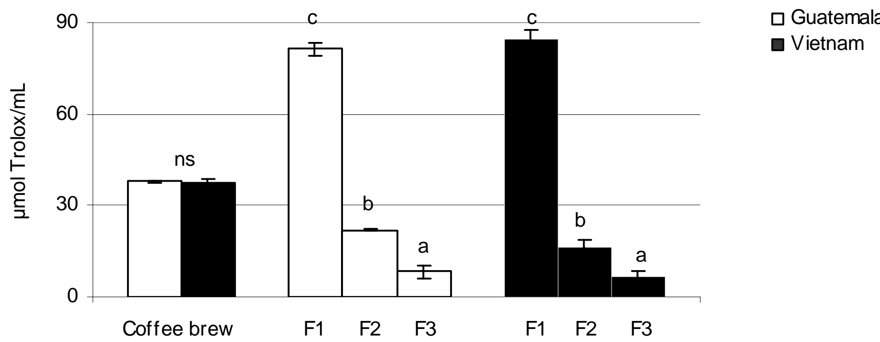

b) Filter coffeemaker

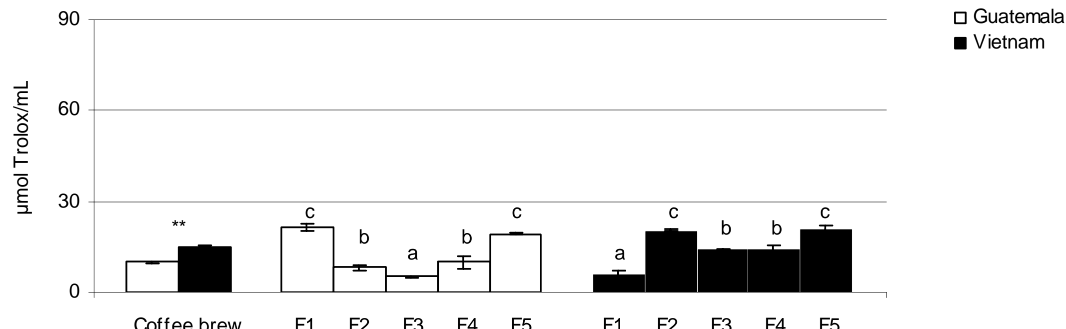

Figure 5. Antioxidant capacity (TEMPO method) of coffee brews and fractions obtained by espresso **(a)** and filter coffeemaker **(b)**. All values are shown as mean ± standard deviation (n=3). ** indicates highly significant differences ($p<0.01$) and ns nonsignificant differences ($p>0.05$) between coffee brews. Different letters indicate significant differences ($p<0.05$) among coffee fractions in each coffee.

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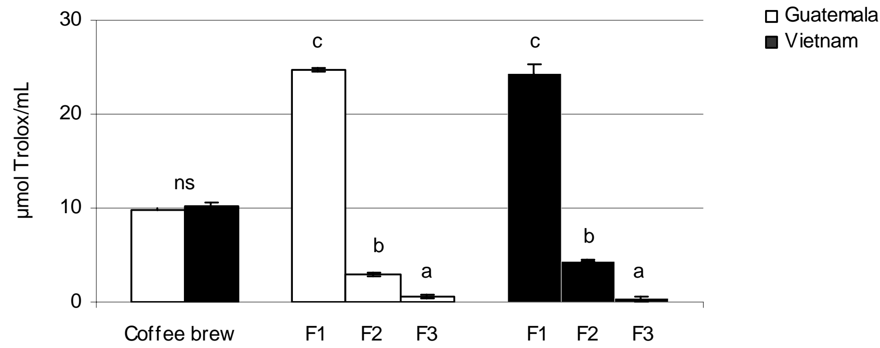
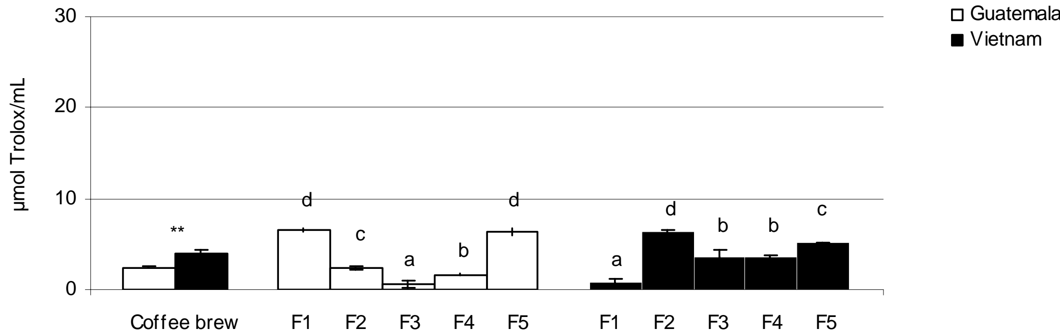


b) Filter coffeemaker



Objective 3

Evaluation of the contribution of volatile Maillard reaction products generated during roasting, to the antioxidant capacity of coffee.

Evaluación de la contribución de los compuestos volátiles, resultantes de la reacción de Maillard y generados durante el tueste, sobre la capacidad antioxidante de café.

Paper 3:

Modelling the contribution of volatile compounds to the antioxidant capacity of coffee

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- Chemistry, applied: 12/71 (Q1)
- Food Science and Technology: 11/128 (Q1)

TITLE: Modelling the contribution of volatile compounds to the antioxidant capacity of coffee

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ABSTRACT

Several authors have proposed some volatile compounds present in coffee as potent antioxidants (Fuster et al., 2000, Yanagimoto et al., 2002). However, the contribution of the volatile compounds to the antioxidant capacity of coffee is still unclear and controversial mainly because of their low concentrations in coffee brews (Lopez-Galilea et al., 2007). The aim of this study was to evaluate the antioxidant capacity of those volatile compounds reported as antioxidants and present in coffee brews. Seven furans, 3 pyrroles, and 2 thiophenes identified in filter coffee brews (by SH-GC-MS) were selected to prepare model systems at different concentrations including those found in coffee. All model systems were analysed using $ABTS^+$ as free stable radical to asses the antioxidant capacity. Results were compared with the antioxidant capacity of coffee brew. The model system containing all the three chemical groups (pyrroles, furans and thiophenes) was the most active in scavenging $ABTS^+$, followed by pyrroles and furans. Thiophenes were ineffective as radical scavengers at all concentrations including 100-fold. However, in comparison with the antioxidant capacity exhibited by the coffee brew sample, the contribution of the volatile compounds assayed was insignificant. In addition, results showed that the heterocyclic compounds analysed exhibited non-linear concentration-dependent antioxidant capacity.

1 INTRODUCTION

Among other volatiles, heterocyclic compounds formed during the roasting process have been extensively studied from the viewpoint of flavour chemistry (Flament, 2001). However, recent research has proposed some heterocyclic compounds formed during heat treatment as being potentially antioxidant. Severini et al. (2000) observed that certain volatile compounds produced in the roasting process of almonds displayed an antioxidant effect. Furthermore, typical volatile compounds formed in Maillard reaction model systems have been reported to inhibit oxidation of lipids (Elizalde et al., 1992; Osada & Shibamoto, 2006). Besides their contribution to flavour, some typical volatile heterocyclic compounds found in coffee have been investigated for their antioxidant properties. Fuster et al. (2000) and Yanagimoto et al. (2002) analyzed one by one the inhibitory effect of isolated volatile compounds on hexanal oxidation and reported considerable antioxidant capacity for some pyrrols, furans and thiophenes. Most of these studies have been carried out at concentration levels higher than those present in coffee. Later Yanagimoto et al. (2004) analyzed the antioxidant activity of chromatographic fractions obtained from a dichloromethane extract of coffee brew. This study suggested that some volatile compounds were responsible for the antioxidant activity exhibited by dichloromethane fractions. More recently Lopez-Galilea et al. (2008) studied the correlation between total antioxidant capacity of coffee and some selected constituents, including heterocyclic volatile compounds, and found that these latter were negatively correlated with radical quenching activity. However, in this latter study, those coffees that exhibited higher antioxidant activity showed lower amounts of volatile compounds due to botanic variety and roasting process.

For the reasons outlined above, the aim of this study was to assess the actual contribution of heterocyclic volatile compounds to the total antioxidant activity of coffee. Therefore antioxidant activity of model systems of potentially antioxidant heterocyclic compounds was analyzed at different concentration levels including those found in coffee. Furthermore, possible antagonistic/synergic effects between compound classes were studied.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents. ABTS (2,2'-Azino-bi(3-ethylbenzo-thiazonile-6-sulfonic acid) diammonium salt), potassium persulfate, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, and pure reference standards for furan, 2-methylfuran, 2,5-dimethylfurane, 2-methyl-tetrahydrofuran-3-one, furfural, 5-methylfurfural, 2-furfurylacetate, 1-methylpyrrole, pyrrole, 2-formyl-1-methyl-pyrrole, thiophene and 2-methylthiophene were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2 Coffee samples. Roasted coffee from Colombia (*Coffea arabica*, named Arabica, L* = 24.82±0.81, roasted at 219 ℃ for 905 s) and Vietna m (*Coffea canephora* var. robusta, named Robusta, L* = 24.92±0.01, roasted at 228 ℃ for 859 s) were provided by a local roasting company. The L* value was analyzed by means of a tristimulus colourimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant and CIE 1931 standard observer. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in a 1 cm Petri plate and the L* value was measured in triplicate on the CIELab scale. Coffee beans were ground for 20 s using a grinder (model Moulinex super junior "s", París, France).

Filter Coffee Brew was prepared from 36 g of ground roasted coffee for a volume of 600 mL, using a filter coffee machine (model Avantis 70 Aroma plus, Ufesa, Spain). Extraction took approx. 6 min at 90 ℃.

2.3 Volatile Compounds Analysis. Volatile compounds were analysed according to the method described by Maeztu et al. (2001) using static headspace-gas chromatography-mass spectrometry (SH-GC-MS). Six mL of coffee or volatile compound standard solution was introduced into a 20 mL vial, which was immediately sealed with a silicone rubber Teflon cap. Each vial was equilibrated at 40 ℃ for 15 min in t he headspace sampler (model 7694E, Agilent Technologies, Palo Alto, CA). Each vial was pressurized with carrier gas for 12 s, and 1 mL of the headspace sample was injected into an HP-Wax glass capillary column (60 m × 0.25 mm × 0.5 μm film thickness) in an HP 7890 gas chromatograph (Agilent Technologies). The injector temperature was 60 ℃, and the carrier gas was heli um (1 mL/min linear speed). The oven temperature was maintained at 40 ℃ for 3 min and t hen raised at 2.5 ℃/min to 205 ℃ and maintained for 10 min. Mass spectrometry analysis was performed with a mass selective detector (model 5975C, Agilent Technologies) operating in the electron impact ionization mode

(70 eV), with a scan range of 30-160 amu. Ion source temperature was set at 230 ℃. Each sample was analyzed in triplicate.

Identification and Quantification of Volatile Compounds. The volatile compounds were identified by comparing their mass spectra with those of pure reference compounds and also by comparing their Kovats indices with those of standard compounds. The Kovats indices were calculated according to the method of Tranchant (1982). Peak areas were measured by calculation of each volatile total area based on integration of a single ion. The quantification ion of each volatile compound is given in **Table 1.** Volatile compounds selected for model systems were additionally quantified by calibration curves to determine their concentrations in coffee. Aqueous solutions of each standard volatile compound were analysed at different known concentrations and chromatographic areas obtained using selective ion monitoring (SIM) were plotted against concentration. Equations for each compound obtained by linear regression included the areas found in the coffee samples in all cases. Coefficients of linearity for the calibration curves were typically R^2>0.99.

2.4 Volatiles model systems. Twelve volatile heterocyclic compounds (7 furans, 3 pyrroles, and 2 thiophenes) identified in roasted coffee by SH-GC-MS were selected to prepare 4 model systems: Furans (Fu), Pyrroles (Py), Thiophenes (Th) and Furans-Pyrroles-Thiophenes (Fu-Py-Th). Pure reference standards were dissolved in deionized water at concentration equivalent to those found in coffee (**Table 2**). Additionally, 10- and 100-fold concentrated model systems were prepared to analyze dose dependent antioxidant activity.

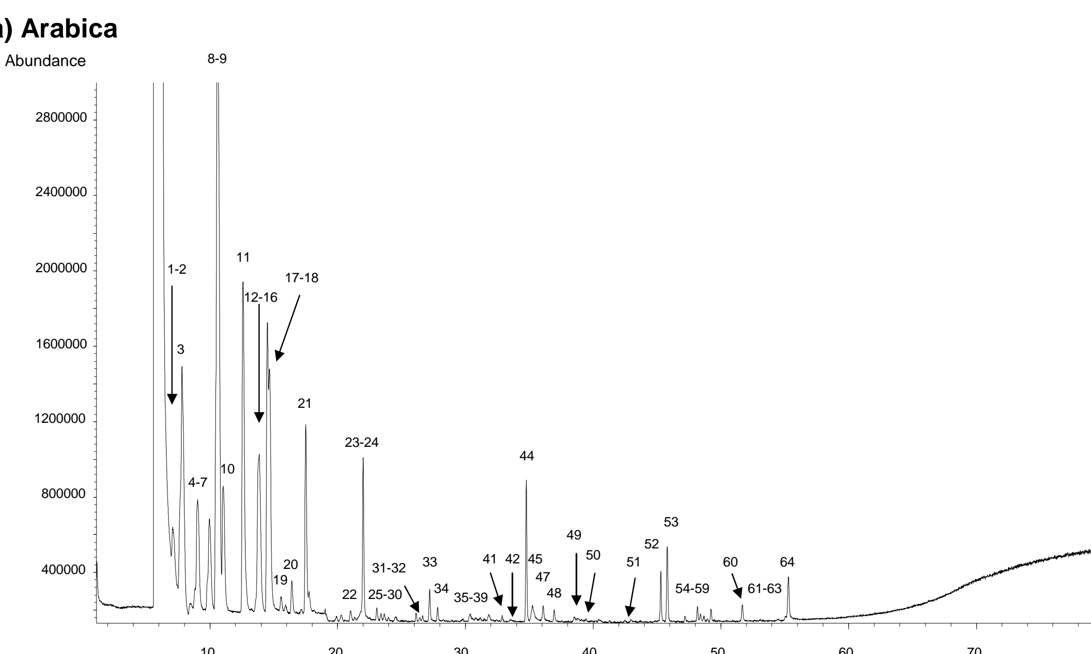
2.5 Antioxidant capacity by ABTS assay. The antioxidant capacity measured with ABTS was carried out according to the method described by Re et al. (1999) with some modifications. The $ABTS\cdot^{+}$ radicals were generated by the addition of 2.45 mM potassium persulfate to an 7 mM ABTS solution prepared in phosphate-buffered saline (PBS, pH 7.4) and allowing the mixture to stand in darkness at room temperature for at least 12 h before use. The ABTS·+ stock solution was adjusted with PBS to an absorbance of 0.7 (±0.02) at 734 nm in a 1 cm cuvette at 25 ℃ (Lambda 25 UV, VIS spectrophotometer, Perkin Elmer Instruments, Madrid, Spain). An aliquot of 50 µL of coffee sample diluted with demineralized water (15:1000) or 50 µL of each model system was added to 2 mL of ABTS·+ reagent and the absorbance was monitored for 18 min at 25 ℃. Calibration was performed with Trolox soluti on (a water-soluble vitamin E analogue) and total antioxidant capacity was expressed as milimoles (mmol) of Trolox per liter of coffee brew or model system.

2.5 Statistical analysis. Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. All statistical analyses were performed using the SPSS v.15.0 software package.

3 RESULTS AND DISCUSSION

Figure 1 shows the chromatographic profiles of the volatile compounds of roasted Arabica and Robusta coffee. **Table 1** shows the chromatographic areas of the identified compounds in both samples. A total of sixty-two and sixty-four volatile compounds were identified and quantified for Arabica and Robusta coffee, respectively. They comprised 4 sulfur compounds, 8 aldehydes, 6 esters, 15 furans, 8 ketones, 5 alcohols, 2 thiophenes, 6 pyrroles, 2 pyridines, 4 pyrazines, 2 thiazoles, 1 lactone, 2 phenolic compounds, 1 alkene, and 1 ether.

(a) Arabica



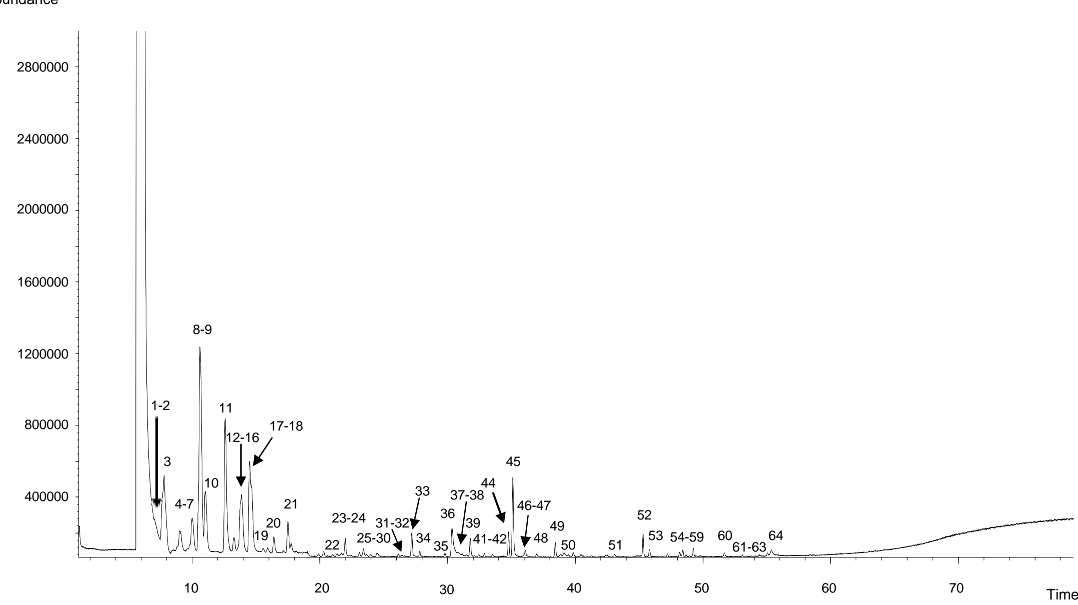


Figure 1. SH-GC-MS chromatogram of Arabica **(a)** and Robusta **(b)** coffee. For peak identification see **Table 1**.

Arabica coffee showed a significantly higher total area of volatiles than Robusta ($2.1x10^6$ vs $0.7x10^6$), mainly because the most abundant volatile chemical classes (aldehydes, furans, ketones and esters) were significantly higher in Arabica samples. Aldehydes and esters are responsible for fruity and malty coffee flavour notes, whereas diketones contribute to the buttery aroma, and furans are considered to be responsible for the typical roasted coffee aroma (Semmelroch & Grosch, 1995; Maeztu et al., 2001; Flament, 2001). Similar results were reported by other authors (Sanz et al., 2002; Lopez Galilea et al., 2008a) when they compare the volatile compounds of Arabica coffee with those found in Arabica-Robusta coffee blends roasted by conventional or torrefacto techniques. In contrast, chromatographic areas of pyrazines and pyridines, and in less proportion thiazoles, were higher in Robusta coffee. Pyrazines are responsible for roasty, earthy, musty and woody flavour notes characteristic of Robusta coffee (Blank et al., 1991; Semmelroch & Grosch, 1995; Lopez Galilea et al., 2006) and pyridines contribute to smoky aroma (Flament, 2001). Also, low molecular weight phenolic compounds, and mainly 2-methoxyphenol (guaiacol) that is a key odorant (Semmelroch & Grosch, 1995; Sanz et al., 2002) responsible of phenolic and burnt aroma (Lopez Galilea et al., 2006), were only detected in Robusta coffee samples at low levels but not in Arabica. Similar results were found by other authors in conventional roasted Arabica and Robusta coffee (Semmelroch & Grosch, 1995; Maeztu et al., 2001; Lopez Galilea et al., 2008b) and coffee brews (Maeztu et al., 2001).

Table 1. Areas ($x10^{-3}$) of volatile compounds identified in the headspace of Arabica and Robusta coffee.

Peak nr. [b]	*QI* [c]	*ID* [d]	*KI* [e]	*Compounds*	**Arabica**	**Robusta**
Sulfur compounds						
2	47	B	633	methanethiol	10639 ± 283	2171 ± 204
4	62	A	673	dimethyl sulfide	21413 ± 645	4288 ± 506
26	94	A	1079	dimethyl disulfide	3677 ± 402	4126 ± 432
51	114	A	1432	2-furanmethanethiol (furfurylthiol)	70818 ± 5636	48049 ± 4299
				Total Sulfur compounds	**106547**	**58634**
Aldehydes						
3	43	A	649	acetaldehyde	86612 ± 9108	18058 ± 1704
6	58	A	710	propanal	23479 ± 2675	4132 ± 477
8	41	A	754	2-methylpropanal	205848 ± 6800	41530 ± 5784
12	72	B	841	butanal	5007 ± 223	2133 ± 160
17	39	A	883	2-methylbutanal	120962 ± 6328	33608 ± 3009
18	44	A	888	3-methylbutanal	120636 ± 6882	83086 ± 1081
27	56	A	1086	hexanal	3683 ± 241	1526 ± 305
30	84	A	1103	2-methyl-2-butenal	2283 ± 151	1709 ± 155
				Total Aldehydes	**568510**	**185782**
Esters						
5	60	B	682	formic acid, methyl ester	244615 ± 31288	41513 ± 5307
10	43	B	791	acetic acid, methyl ester	22410 ± 1352	9909 ± 1081
13	43	B	854	acetic acid, ethyl ester	traces	638 ± 315
16	57	B	876	propanoic acid, methyl ester	1592 ± 154	884 ± 91
52	43	B	1484	1-hydroxy-2-propanone acetate	13511 ± 1617	6455 ± 826
58	57	B	1554	1-hydroxy-2-butanone acetate	819 ± 22	366 ± 54
				Total Esters	**282947**	**59765**
Furans						
7	68	A	724	furan	37036 ± 3267	28830 ± 5724
11	82	A	832	2-methylfuran	216408 ± 7745	93177 ± 5589
14	82	A	862	3-methylfuran	8897 ± 807	3219 ± 225
20	96	A	934	2,5-dimethylfuran	16239 ± 635	14123 ± 976
25	94	B	1077	2-vinylfuran	3179 ± 102	1021 ± 159
34	108	B	1162	2-vinyl-5-methylfuran	4656 ± 271	1449 ± 238
44	43	A	1284	2-methyltetrahydrofuran-3-one	52497 ± 2324	9506 ± 991
53	96	A	1491	2-furancarboxaldehyde (furfural)	23557 ± 205	8778 ± 326
54	81	A	1517	2-furfuryl methyl sulfide	148 ± 30	225 ± 105
55	81	B	1520	2-furfuryl formate	645 ± 134	234 ± 70
56	95	A	1537	2-acetylfuran	3726 ± 415	1541 ± 262
59	81	A	1560	2-furfuryl acetate	2895 ± 291	1633 ± 201
60	110	A	1606	5-methylfurfural	1189 ± 62	438 ± 29
61	91	B	1631	2-furfurylfuran	391 ± 71	335 ± 82
64	98	A	1687	furfuryl alcohol	11148 ± 997	2964 ± 251
				Total Furans	**382611**	**167473**
Ketones						
9	58	A	763	2-propanone	297481 ± 24952	107777 ± 27101
15	43	A	869	2-butanone	60667 ± 1544	22195 ± 2774
21	43	A	965	2,3-butanedione	96526 ± 4428	14796 ± 1501
23	57	B	1055	3-hexanone	97686 ± 4562	10822 ± 1047
24	43	A	1060	2,3-pentanedione	66523 ± 3292	6705 ± 1552
31	69	A	1138	3-penten-2-one	1829 ± 139	709 ± 83
32	57	B	1144	3,4-hexanedione	3188 ± 252	643 ± 56
48	43	B	1323	1-hydroxy-2-propanone	230 ± 58	62 ± 10
				Total Ketones	**624130**	**163709**
Alcohols						
19	45	B	917	ethanol	15764 ± 1482	3636 ± 186
28	43	A	1105	2-methyl-1-propanol	259 ± 134	516 ± 69
37	56	B	1221	3-methylbutan-1-ol	1452 ± 223	586 ± 185
42	41	B	1265	3-methyl-3-buten-1-ol	657 ± 37	858 ± 119

47	71	B	1338	3-methyl-2-buten-1-ol	621 ± 117	714 ± 109
				Total Alcohols	**18753**	**6310**
Thiophenes						
22	84	A	1023	thiophene	2324 ± 91	2144 ± 154
29	97	A	1099	2-methylthiophene	1671 ± 29	1599 ± 135
				Total Thiophenes	**3995**	**3743**
Pyrroles						
33	81	A	1151	1-methylpyrrole	13884 ± 602	9870 ± 675
35	80	B	1195	1-ethyl-1*H*-pyrrole	1072 ± 132	1354 ± 245
38	94	B	1226	2,5-dimethylpyrrole	561 ± 59	541 ± 82
57	67	A	1543	1*H*-pyrrole	2719 ± 187	2686 ± 302
62	109	B	1662	2-formyl-1-methylpyrrole	1459 ± 139	539 ± 82
65	81	B	1833	*N*-furfurylpyrrole	482 ± 26	331 ± 17
				Total Pyrroles	**20177**	**15321**
Pyridines						
36	79	A	1205	pyridine	3492 ± 123	25078 ± 75
40	93	B	1240	2-methylpyridine	traces	nd
				Total Pyridines	**3492**	**25078**
Pyrazines						
39	80	A	1232	pyrazine	4902 ± 372	8616 ± 318
45	94	A	1289	2-methylpyrazine	4249 ± 561	20163 ± 1804
49	108	A	1348	2,5-dimethylpyrazine	1500 ± 126	3413 ± 141
50	108	B	1373	2,3-dimethylpyrazine	693 ± 81	1514 ± 400
				Total Pyrazines	**11344**	**33706**
Thiazoles						
43	85	B	1271	1,3-thiazole	442 ± 26	148 ± 24
46	99	B	1303	4-methylthiazole	nd	391 ± 108
				Total Thiazoles	**442**	**539**
Lactones						
63	42	B	1674	γ-butyrolactone	1589 ± 231	1015 ± 126
				Total Lactones	**1589**	**1015**
Phenolic compounds						
-	94	A	1075	Phenol	nd	traces
66	109	B	-	2-methoxyphenol (guaiacol)	nd	62 ± 57
				Total phenolic compounds	**-**	**62**
Others						
1	67	A	624	1,3-pentadiene	62095 ± 1585	5672 ± 543
41	81	A	1252	2-furfuryl methyl ether	2207 ± 157	1247 ± 115
				Total others	**64302**	**6919**

[a] All values are shown as means ± standard deviations. nd, not detected.[b] Peak number corresponding to chromatograms in **Figure 1**.[c] Ion used for the compound quantification. [d] The reliability of the identification proposal is indicated by the following: A, mass spectrum, KI, and retention time according to standards; B, tentative identification by comparing mass spectrum with Wiley mass spectral database and retention indices with literature data [e] Kovats index calculated for the HP-Wax capillary column.

Several studies suggest that some heterocyclic compounds developed during coffee roasting such as furans, pyrroles and thiophenes, exhibit antioxidant properties (Fuster et al., 2000; Yanagimoto et al., 2002; Yanagimoto et al., 2004). However, Lopez-Galilea et al. (2008b) found significant negative correlations of these compounds with antioxidant capacity suggesting a prooxidant capacity. Despite the different approach in these studies, the results might not be considered as contradictory if it is taken into account that some antioxidant compounds may act as prooxidant at different doses. Most of the work which studies the antioxidant capacity of volatiles has been carried out using standard compounds at higher concentration than those present in coffee. Thus, these results cannot be directly transferred to the knowledge of the antioxidant capacity of coffee. For those reasons, the concentration of heterocyclic volatile

compounds in coffee was firstly measured to further assess their actual contribution to the total antioxidant capacity of coffee at the concentration usually found in coffee.

Seven furans (furan, 2-methylfuran, 2,5-dimethylfuran, 2-methyl-tetrahydrofuran-3-one, furfural, 5-methylfurfural and 2-furfurylacetate), three pyrrols (1-methylpyrrole, pyrrole, and 2-formyl-1-methyl-pyrrole), and two thiophenes (thiophene and 2-methylthiophene) were initially chosen because they were previously proposed by other authors as potential antioxidants, but also because their chromatographic areas were the highest in analyzed coffee samples in most cases. The concentration of these volatile compounds, were quantified in both Arabica and Robusta coffees based on the calibration curves of the corresponding standard (**Table 2**). Except in thiophenes with the same concentrations, Arabica coffee exhibited higher concentration in all analyzed compounds, showing considerably higher amounts of 2-methyl-tetrahydrofuran-3one (more than 5-fold) and 5-methylfurfural (almost 3-fold) than in Robusta coffee. The volatiles with the highest chromatographic areas, 2-methylfuran and 1-methylpyrrole, were not the most abundant in coffee because the relationship between the chromatographic area and the concentration is not the same for every volatile compounds. For that reason, the ranking order among volatiles in each chemical family group was also different.

Table 2. Concentration of antioxidant volatile compounds in Arabica and Robusta coffee. All values are shown as mean ± standard deviation (n=3).

	Coffee (µg/mL)	
Compounds	Arabica	Robusta
Furans		
furan	0.23 ± 0.02	0.19 ± 0.04
2-methylfuran	1.25 ± 0.06	0.56 ± 0.04
2,5-dimethylfurane	0.24 ± 0.01	0.22 ± 0.01
2-methyl-tetrahydrofuran-3-one	95.72 ± 5.93	18.09 ± 2.53
furfural	24.23 ± 0.28	10.04 ± 0.44
5-methylfurfural	7.05 ± 0.53	2.50 ± 0.25
2-furfurylacetate	2.93 ± 0.32	1.95 ± 0.22
Pyrroles		
1-methylpyrrole	0.44± 0.03	0.30 ± 0.03
pyrrole	1.17 ± 0.10	1.15 ± 0.16
2-formyl-1-methyl-pyrrole	1.37 ± 0.17	0.56 ± 0.10
Thiophenes		
thiophene	0.10 ± 0.00	0.10 ± 0.00
2-methylthiophene	0.02 ± 0.00	0.02 ± 0.00
Aldehydes		
acetaldehyde	95.66 ± 10.03	20.48 ± 1.90
propanal	92.64 ± 10.56	18.52 ± 2.13
2-methylpropanal	16.91 ± 0.51	4.03 ± 0.56
2-methylbutanal	3.50 ± 0.18	0.82 ± 0.07
3-methylbutanal	4.82 ± 0.17	3.22 ± 0.04
Ketones		
2,3-butanedione	62.48 ± 2.88	8.88 ± 0.92
2,3-pentanedione	32.02 ± 1.60	3.85 ± 0.85

Model systems with the selected furans (Fu), pyrroles (Py), thiophenes (Th), and one with all of them (Fu-Py-Th) were prepared based on the mean concentrations of each compound found in Arabica and Robusta coffee (**Table 3)**. The antioxidant capacity of each model system was

assessed using the $ABTS^+$ radical quenching assay and results are shown in **Figure 2**. For each model system, three different concentration levels including that found in coffee (coffee, 10-fold, 100-fold) were analyzed to evaluate the dose dependent antioxidant activity.

Table 3. Concentrations of volatile compounds present in each model systems at concentractions equivalent to coffee.

Compounds	**Model system** (µg/mL)					
	Fu	**Py**	**Th**	**Fu-Py-Th**	**Ald**	**Ke**
Furans (Fu)						
Furan	0.20			0.20		
2-methylfuran	0.90			0.90		
2,5-dimethylfuran	0.20			0.20		
2-methyl-tetrahydrofuran-3-one	55.00			55.00		
Furfural	17.00			17.00		
5-methylfurfural	5.00			5.00		
2-furfurylacetate	2.50			2.50		
Pyrroles (Py)						
1-methylpyrrole		0.35		0.35		
Pyrrole		1.00		1.00		
2-formyl-1-methyl-pyrrole		1.00		1.00		
Thiophenes (Th)						
Thiophene			0.10	0.10		
2-methylthiophene			0.02	0.02		
Aldehydes (Ald)						
Acetaldehyde					58.00	
Propanal					55.50	
2-methylpropanal					10.50	
2-methylbutanal					2.15	
3-methylbutanal					4.00	
Ketones (Ke)						
2,3-butanedione						35.00
2,3-pentanedione						18.00

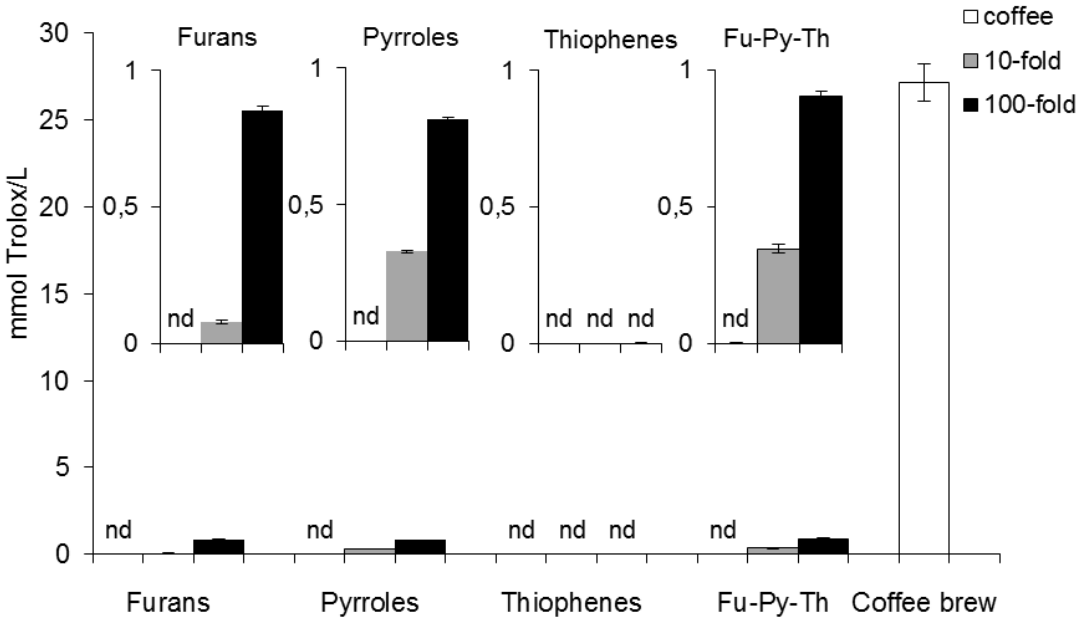


Figure 2. Antioxidant activity (ABTS) of model systems and filter coffee brew.

Furans are cyclic ethers present in heated and roasted foods. A great variety of furans are originated during roasting process in coffee as Maillard-reaction products, but they are also the result of thermal oxidation of lipids and thermal degradation of thiamine, nucleotides, and terpenes (Flament, 2001). In furan model system (Fu), any appreciable antioxidant activity at coffee furan concentration was observed. However, for the 10-fold and 100-fold concentrated furan model systems, $ABTS^+$ quenching activities equivalent to 0.08 and 0.85 mmol Trolox per liter were found, showing a linear dose dependent increase in antioxidant capacity. To ascertain the contribution of each single furan to the overall Fu-model system antioxidant activity, volatile compounds were tested individually. Because the furan model system showed no antioxidant activity at concentration levels actually present in coffee, 10-fold concentrated solutions of each furan were used to measure the $ABTS^+$ antioxidant activity. Results are shown in **Figure 3**. From the 7 analyzed furans, only 2-methyl-tetrahydrofuran-3-one exhibits antioxidant activity. This was no significant different to that of the Fu-model system at the same concentration level (10-fold), showing that the antioxidant activity of the main coffee furans might be mainly attributed to this volatile compound, maybe because 2-methyl-tetrahydrofuran-3-one was by far the most abundant furan in coffee. Although five of the furans analyzed (namely furan, 2-methylfuran, furfural, 5-methylfurfural and 2-furfurylacetate) have been reported as potent antioxidants (Fuster et al., 2000; Yanagimoto et al., 2002; Yanagimoto et al., 2004), results obtained in this study show that even at concentrations 10-fold higher than actually present in coffee, only 2-methyl-tetrahydrofuran-3-one exhibited a very limited radical scavenging activity.

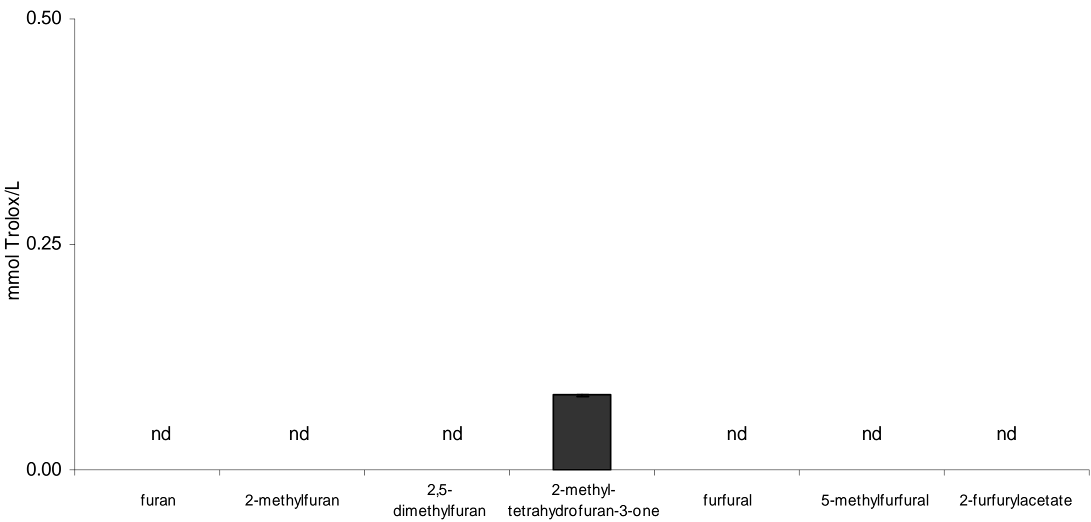


Figure 3. Antioxidant activity (ABTS) of furans.

Pyrroles are formed during roasting process. Pyrrole and 1-methyl-pyrrole are formed in the pyrolysis of proline and threonine alone or combined with glucose or sucrose, and in the pyrolysis of trigonelline (Flament, 2001). 2-formyl-1-methylpyrrole is formed from 1-methylpyrrole and also when D-xylose reacted thermally with various amines or amino acids (glycine, alanine, beta-alanine, leucine). In Pyrrole model system (Py), no appreciable antioxidant activity at concentration levels equivalent to coffee was found. The 10-fold and 100-fold concentrated pyrrole systems exhibited antioxidant activity equal to 0.32 and 0.81 mmol Trolox per liter, respectively, showing a non-linear dose dependent antioxidant activity increase. In comparison with furans, pyrroles showed a 4 times higher radical quenching activity at 10-fold concentrations, but similar antioxidant activity at 100-fold concentrations. These results suggest a higher effectiveness of pyrroles at lower concentrations, as proposed by other authors (Fuster et al., 2000) but still undetectable at coffee concentration. To assess the contribution of each pyrrole, the three compounds were analyzed separately at 10-foldconcentration by the ABTS assay. Results shown in **Figure 4** reveal that the antioxidant activity measured for the Py model system might be totally attributed to pyrrole, whereas 1-methylpyrrole and 2-formyl-1-methylpyrrol seem to be ineffective in quenching $ABTS^{+}$ radicals at the tested concentrations. Also, Yanagimoto et al (2002) observed that pyrrole inhibited hexanal oxidation higher than 1-methylpyrrole, but the inhibition were quite low for both volatile compounds (<10% at 10 µg/mL for pyrrole and <3% at 5-20 for µg/mL for 1-methylpyrrole). However, 2-formyl-1-methylpyrrole seems to be more effective as a lipophilic antioxidant inhibiting hexanal oxidation in dichloromethane solutions (Yanagimoto et al., 2002) than as a hydrophilic antioxidant quenching ABTS radicals in aqueous solutions similar to coffee brews.

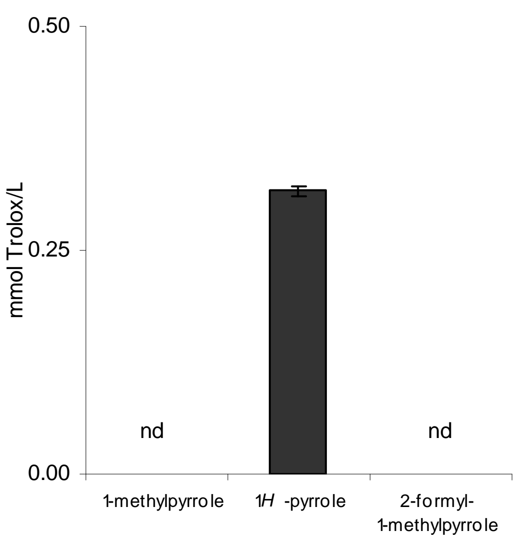


Figure 4. Antioxidant activity (ABTS) of pyrrols.

Thiophenes present in roasted coffee can be formed by pyrolysis of sulfur amino acids as methionine or cysteine and cystine alone, or by browning reactions in the presence of sugars (Flament, 2001). Although some authors (Fuster et al., 2000; Yanagimoto et al., 2002) reported that both thiophenes found in coffee exhibit antioxidant activity, the results obtained in this study did not show radical quenching activity at any analyzed concentration level (coffee, 10-fold and 100-fold). This could probably be due to the very low amounts of thiophenes used in this study to evaluate the $ABTS^+$ antioxidant activity even at the highest concentration (100-fold, with 10 µg/mL for tiophene and 2 µg/mL for 1-methylthiophene). Actually, these compounds are present in coffee in very low amounts and therefore, although their antioxidant capacity was demonstrated at high concentrations (more than 50 µg/mL), tiophenes barely contribute to the antioxidant capacity of coffee.

When the antioxidant activity of a model system containing all the 12 selected heterocyclic compounds (furans, pyrroles and thiophenes) was analyzed, no radical quenching activity was detected at concentration levels similar to coffee brew, whereas $ABTS^+$ quenching activity equal to 0.35 and 0.90 mmol Trolox per liter were observed for 10-fold and 100-fold concentrated samples, respectively. Thus, the radical quenching activity of the Fu-Py-Th model system was slightly higher than the maximum value showed for each concentration (pyrroles at 10-fold and furans at 10-fold), but not the sum of the model systems of furans and pyrroles. These results suggest antagonistic effects among furans, pyrroles and thiophenes. Moreover, when the $ABTS^+$ quenching activity of model systems was compared to the overall antioxidant activity of a filter coffee brew, the results clearly showed the almost insignificant contribution of these heterocyclic volatile compounds to the antioxidant activity of coffee, even at the 100-fold concentrated Fu-Py-Th model system, which exhibited the highest $ABTS^+$ quenching activity, accounting only for 3.3% of the overall activity of a filter coffee brew (**Figure 2**).

The most abundant volatile compounds in coffee are aldehydes and ketones and some of them are also Maillard reaction products. For those reasons, we decided to test the antioxidant

activity of two new model systems, one with aldehydes (Ald) and another with ketones (Ke). In terms of chromatographic areas, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal (Strecker degradation products of valine, isoleucine, and leucine), were the most abundant aldehydes followed by acetaldehyde and propanal that are formed by pyrolysis of alanine and serine, and/or sugar. However, acetaldehyde and propanal were in significantly higher concentrations than Strecker aldehydes in both coffee brews. Also, two diones (2,3-butanedione and 2,3-pentanedione) were selected for quantification and further evaluation of their antioxidant activity in a model system. All aldehydes and ketones were in significantly higher amounts in Arabica coffee than in Robusta one, in agreement with other studies (Grosch, 1996). As in the previous model systems, mean concentrations of the selected aldehydes and ketones found in Arabica and Robusta coffee were used to prepare the Aldehydes and the Ketones model systems (**Table 3**). The antioxidant capacity of each model system at three different concentration levels (coffee, 10-fold and 100-fold) was assessed using the $ABTS^{+}$ radical quenching assay. **Figure 5** shows that, even at the highest concentration, the antioxidant capacity of the aldehydes and ketones were negligible in comparison to that of the coffee brew and also to those of the heterocyclic volatiles model systems.

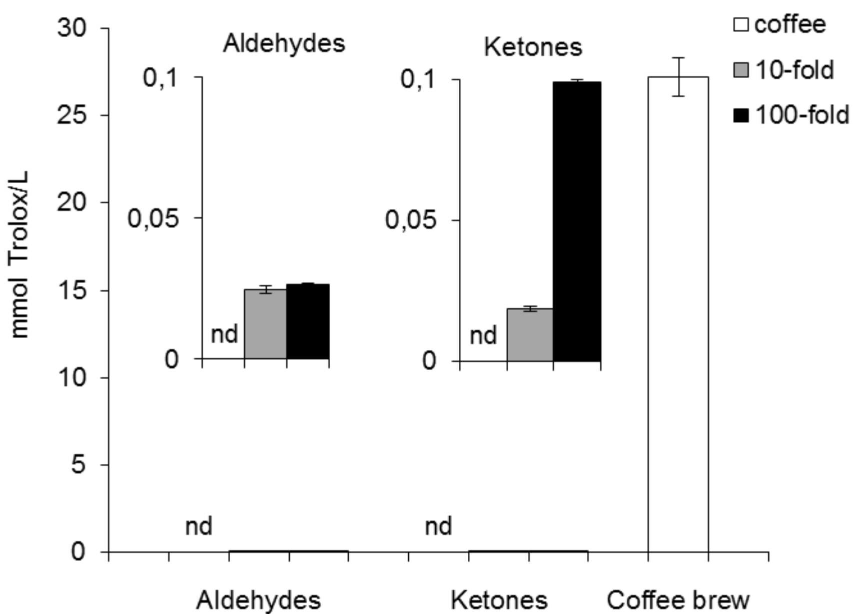


Figure 5. Antioxidant activity (ABTS) of aldehydes and ketones model systems.

In summary, volatile compounds present in coffee contribute very little to the antioxidant capacity of coffee in comparison to other coffee antioxidants, such as caffeolylquinic acid and melanoidins. The results of the present study also indicate that, although some volatile compounds may act as antioxidant in high doses, it is necessary to evaluate their capacity at the actual concentrations in food samples.

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Objective 4

Evaluation of the contribution of phenolic and non-phenolic compounds to the antioxidant capacity of coffee brews (espresso and filter).

Evaluación de la contribución de los compuestos fenólicos y no-fenólicos a la capacidad antioxidante de la bebida de café (expreso y de filtro).

1 INTRODUCTION

In the last years, due to an increasing interest in the health implications of foods, the potential benefits of coffee consumption on health have been extensively studied. Epidemiological evidence has shown that moderate coffee consumption is associated with a reduction in the relative risk of development of several diseases, such as coronary heart disease, type-2 diabetes, liver and colon cancer (Van Dam & Hu, 2005; Bonita et al., 2007; Arab, 2010; Galeone et al., 2010). These benefits might be ascribed to the brews' antioxidant activity. The contribution of different coffee components to the antioxidant activity of the brew is also a topic of great interest in the literature. Chlorogenic acids (CGA), a group of non-flavonoid (poly)phenolic compounds, comprising esters of quinic acid with hydroxycinnamic acids (caffeic, *p*-coumaric, and ferulic acid), are considered to be major contributors to the antioxidant activity of coffee brew (Svilaas et al., 2004; Vignoli et al., 2011). Moreover, coffee has been proposed as the principal source of chlorogenic acids (Clifford, 2000; Pulido et al., 2003) in the human diet. The amounts of chlorogenic acids present in coffee brew depend on several factors, such as coffee variety, roasting degree, and brewing procedure (Clifford, 2000; Farah et al., 2005; Ludwig et al., 2012). Thus, the aim of this study was to study the influence of the coffee species (Robusta and Arabica) and the coffee making procedure (espresso and filter coffeemaker) on the CGA composition of coffee brews and to evaluate the contribution of these compounds to the antioxidant capacity. Furthermore, extraction behaviour of CGAs during the brewing process was studied for espresso and filter coffeemaker.

MATERIALS AND METHODS

2.1 Chemicals. Pure reference standard of 5-Caffeoylquinic acid, di-ammonium salt of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), and formic acid were purchased from Sigma-Aldrich (Poole, Dorset, UK). HPLC-grade methanol was obtained from Fisher Scientific (Loughborough UK).

2.2 Coffee. Medium roasted Vietnamese coffee beans (*Coffea canephora* var. Robusta) and Guatemalan coffee beans (*Coffea Arabica)* were provided by Unión Tostadora S.A (Logroño, Spain). The beans were ground to a powder in a Moulinex coffee grinder for 20 seconds immediately before sample preparation. Espresso coffee, 45 mL, was prepared from 7 g of ground roasted coffee (Robusta or Arabica) using an espresso coffee machine (model Saeco Aroma, Italy). In addition, three fractions for espresso coffee were collected sequentially every 8 s. Filter coffee, 600mL, was prepared from 36 g of ground roasted coffee, using a filter coffee machine (model Avantis 70 Aroma plus, Ufesa, Spain). Extraction took approx. 6 min at 90 ℃. Five fractions for filter coffee were collected sequentially every 75 s. Coffee brews and fractions were freeze-dried using a CRYODOS Telstar (Terrassa, Spain) and stored at -18℃ until sample analysis.

2.3 Qualitative and quantitative analysis of chlorogenic acids by HPLC-PDA-MS^n. For HPLC analysis of coffee brews and fractions, the freeze-dried sample was reconstituted with distilled water, centrifuged at 16.2 g for 5 min and diluted 20-fold with distilled water. Chlorogenic acids were analysed using a Surveyor HPLC with a PDA detector scanning from 200-600 nm, an autosampler cooled at 6 ℃ and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific, San José, USA). Injection volume was 100 µL. Chromatographic separation was performed at 40 ℃ using a Sinergy 4 µm Polar-RP 250 x 4,6 mm reversed phase column (Phenomenex, Macclesfield, UK) and a mobile phase consisting of two solvents, 1 % aqueous formic acid (solvent A) and 100 % methanol (solvent B). Isocratic conditions of 95 % solvent A and 5 % solvent B were maintained for 30 min, followed by a linear gradient from 5 to 20 % solvent B over 40 min and a linear gradient from 20 to 60 % solvent B over 50 min. The flow rate was 1 mL/min. After passing the PDA flow cell, the eluate was split and 0.3 mL/min was directed to the mass spectrometer with ESI operating in negative ionization mode. Analysis was initially carried out in full-scan, data-depending scanning from *m/z* 100 to 600 and identification was confirmed by single reaction monitoring (SRM) and consecutive reaction monitoring (CRM). CQAs were quantified by PDA at 325 nm, by reference to a 20-1000 ng 5-CQA calibration curve (R^2=0.9999). Quantification of the other chlorogenic acids (FQAs, diCQAs, CFQA, CQAL, FQAL) was performed using the area of 5-CQA standard combined with molar extinction coefficients of the respective compound as reported by Trugo and Macrae (1984) and Farah et al. (2005). Results were expressed in µmol per 100 mL.

2.4 On-line HPLC $ABTS^+$ decolourization assay. The antioxidant capacity of individual chlorogenic acids was analysed with an on-line HPLC system according to the method described by Stalmach et al. (2006). The $ABTS\cdot^+$ radical stock solution was prepared by adding 3.5 mM potassium persulfate to a 2 mM $ABTS^+$ solution prepared in distilled water. This mixture was incubated in darkness at room temperature for at least 12 h before use. $ABTS^+$ reagent was prepared by diluting the stock solution 8-fold with phosphate buffer (pH 8). Freeze-dried coffee brew samples were reconstituted with distilled water, centrifuged at 16.2 g for 5 min and diluted 20-fold with distilled water. Chlorogenic acids were analysed using a Surveyor HPLC with a PDA detector. Injection volume was 100 µL and separation and quantification were carried out as described in the previous section. After passing the PDA flow cell, the eluate was directed to a "T"-piece where the $ABTS^+$ reagent (0.25 mM) was added at a flow rate of 0.5 mL per minute delivered by a liquid chromatography pump (Shimadzu LC-10 AD VP). After mixing by passing through a 1.5 m x 0.44 mm loop, the absorbance at 720 nm was measured using an UV/VIS detector (Spectra System UV 2000, Finnigan MART). The overall antioxidant capacity of each coffee brew and fraction was also determined using the method described above after removing the column from the HPLC Surveyor system. The antioxidant capacity of the individual chlorogenic acids and of the coffee brews and fractions was quantified against Trolox calibration curves. The results were expressed as concentration of Trolox equivalents in µmol per 100 mL. Trolox equivalent antioxidant capacity (TEAC) values for CGA subgroups were expressed as

the ratio between the amount of each CGA subgroup (µmol) and the corresponding antioxidant capacity (µmol Trolox equivalents).

2.5 Statistical analysis. Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. All statistical analyses were performed using the SPSS v.15.0 software package.

3 RESULTS AND DISCUSSION

3.1 Qualitative and quantitative analysis of coffee chlorogenic acids. Retention times and EI-mass spectra of the CGAs detected in this study are presented in **Table 1**. Identification was based on the MS fragmentation patterns reported by Clifford et al. (2003, 2005).

Table 1. HPLC-MS^3 identification of chlorogenic acids. $[M-H]^-$, negatively charged molecular ion; MS^2, daughter ions produced from $[M-H]^-$ fragmentation; MS^3, daughter ions produced from fragmentation of MS^2 base ion. For peak number, see Figures 1 and 2.

Peak nr	Compound	Rt min	*[M-H]⁻* *m/z*	*MS^2* *m/z (intensity)*	*MS^3* *m/z (intensity)*
1	CQA	11.5	353	191 (100) 179 (90), 135 (30	
2	CQA	12.0	353	179 (100) 135 (20), 191 (5)	
3	3-CQA	14.3	353	191 (100) 179 (60), 135 (10)	
4	3-*p*CoQA	22.6	337	163 (100) 119 (10)	
5	CQA	25.6	353	179 (100) 191 (50), 135 (25)	
6	CQA	27.7	353	191 (100) 179 (5)	
7	4-CQA	28.7	353	173 (100) 179 (80, 191 (20)	
8	5-CQA	38.9	353	191 (100) 179 (5)	
9	3-FQA	40.7	367	193 (100) 191 (5)	
10	CQL	44.5	335	179 (100) 161 (70) 135 (30)	
11	4-*p*CoQA	46.2	337	173 (100) 163 (15) 191 (10)	
12	3-CQL	55.6	335	161 (100) 135 (60)	
13	CQL	57.8	335	173 (100) 179 (50) 161 (40) 135 (25)	
14	4-CQL	58.9	335	161 (100) 135 (15)	
15	4-FQA	61.4	367	173 (100) 193 (25)	
16	1,3-diCQA	64.8	515	353 (100) 335 (25) 179 (20) 191 (5)	191 (100) 179 (60)
17	FQA	64.4	367	191 (100)	
18	5-FQA	70.9	367	191 (100) 173 (5)	
19	FQL	79.7	349	175 (100) 149 (40), 193 (25)	
20	4-FQL	80.7	349	175 (100) 160 (10),193 (25)	
21	3,4-diCQA	84.9	515	353 (100) 335 (20), 173 (20)	173 (100) 179 (90), 191 (50)
22	3,5-diCQA	86.9	515	353 (100)	191 (100) 179 (70), 173 (5)
23	1,5-diCQA	91.7	515	353 (100) 191 (15)	191 (100)
24	4,5-diCQA	93.2	515	353 (100)	173 (100) 179 (70), 191 (20)
25	3-F-5-CQA	96.1	529	367 (100)	193 (100) 173 (30)
26	3-F-4-CQA	94.1	529	353 (100) 367 (80) 335 (30)	173 (100) 179 (80) 191 (20)
27	3-C-4-FQA	94.8	529	367 (100) 335 (15), 173 (15)	173 (100) 193 (20)
28	3-C-5-FQA	96.5	529	353 (100) 367 (40), 191 (20)	191 (100) 179 (50)
29	4-F-5-CQA	97.8	529	367 (100)	173 (100) 193 (60)
30	4-C-5-FQA	98.7	529	353 (100) 367 (20)	173 (100) 179 (80), 191 (30)

A total of 22 CGAs were identified in the coffee samples: three caffeoylquinic acid (CQA) isomers, three feruloylquinic acid (FQA) isomers, two p-coumaroylquinic acid (pCoQA) isomers, five dicaffeoylquinic acid (diCQA) isomers, six caffeoylferuloylquinic acid (CFQA) isomers, two caffeoylquinic acid lactones (CQL) and one feruloylquinic acid lacton (FQL). Four additional components were partially identified as CQAs, one as FQA, two as CQLs, and one as a FQL

but it was not possible to determine the respective position of the caffeic acid and ferulic acid moiety.

Representative HPLC-325 nm chromatograms of Robusta and Arabica coffee are illustrated in **Figures 1** and **2**. The amounts of the individual compounds found in espresso and filter coffee brew are presented in **Table 2** and **3**, respectively. The main CGAs were the CQAs, followed by smaller amounts of FQAs, CQLs, diCQA, FQLs, CFQAs and *p*CoQAs. When comparing the chlorogenic acids contents of Arabica and Robusta in both extraction systems (espresso and filter), it can be said that, although the overall contents of CGAs were not greatly different for both species, the CGAs composition differed considerably. Arabica coffee had approx. 30 % higher amounts 3-, 4-, and 5-caffeoylquinic acids, whereas Robusta coffee showed 2-fold higher levels of FQAs and FQLs and almost 5-fold higher amounts of CFQAs. These findings are in accordance with other studies on coffee chlorogenic acids that report higher amounts of CGAs with a ferulic acid moiety in Robusta coffee varieties (Clifford, 1997; 2000).

As regards the coffee making procedure, filter and espresso, higher amounts of CGAs were found in espresso coffee, most likely due to the higher coffee to water ratio and the pressure applied during extraction that leads to a more concentrated coffee brew.

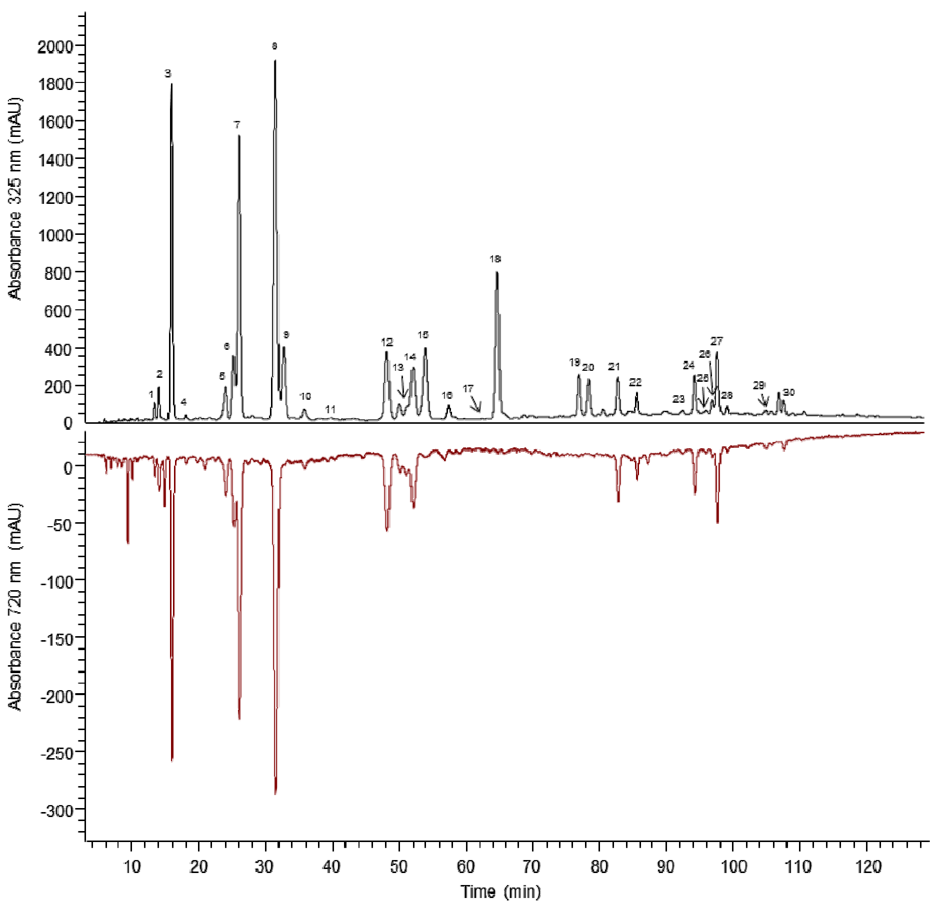

Figure 1. On-line HPLC ABTS^{+} analysis of Robusta coffee. For peak identification see **Table 1**.

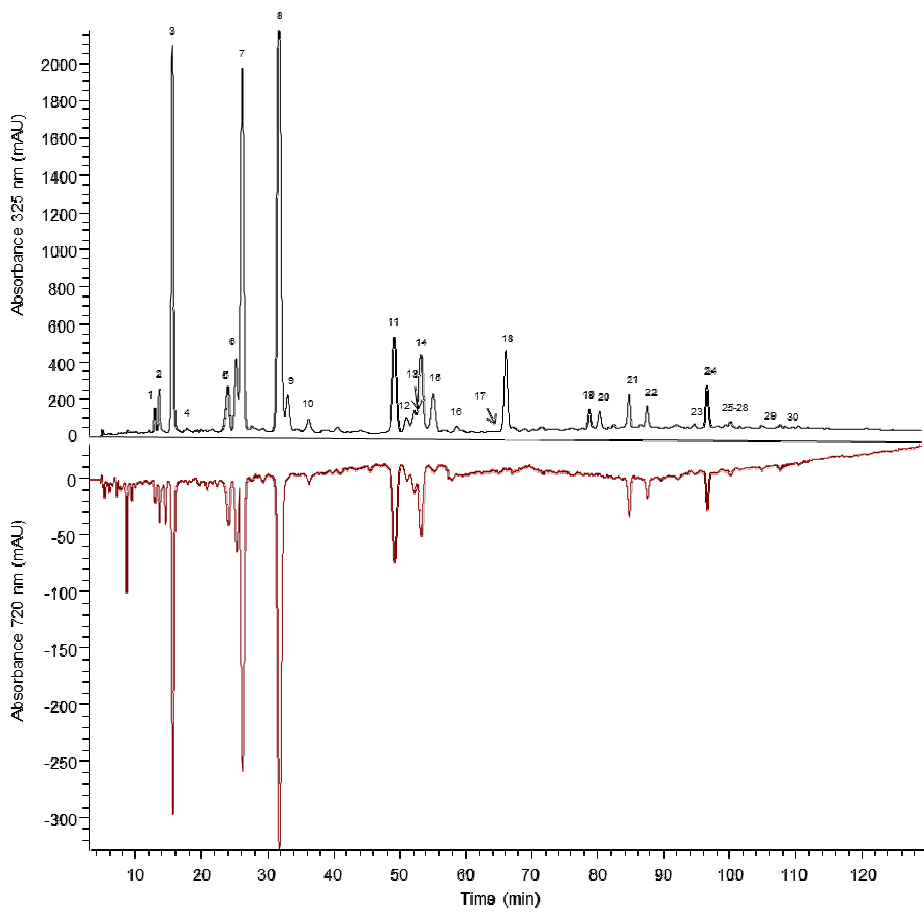

Figure 2. On-line HPLC ABTS+ analysis of Arabica coffee. For peak identification see **Table 1**.

Table 2. Concentration and antioxidant capacity of individual chlorogenic acids in Robusta and Arabica **espresso coffee.** [a]

Compound (Peak nr) [b]	Robusta Espresso Coffee			Arabica Espresso Coffee		
	Concentration [µmol/100mL]	Trolox equivalents [µmol/100mL]	% of total	Concentration [µmol/100mL]	Trolox equivalents [µmol/100mL]	% of total
CQA (1)	2.6 ± 0.0	1.4 ± 0.1	0.7	3.0 ± 0.2	1.6 ± 0.1	0.6
CQA (2)	5.3 ± 0.3	2.8 ± 0.2	1.3	5.9 ± 0.2	3.0 ± 0.1	1.1
3-*O*-CQA (3)	57.0 ± 1.5	34.5 ± 3.6	16.4	74.9 ± 0.8	46.5 ± 4.3	18.0
CQA (5)	9.3 ± 0.7	5.6 ± 0.5	2.7	10.6 ± 0.1	6.8 ± 0.6	2.6
1-*O*-CQA (6)	15.0 ± 0.4	8.2 ± 0.1	3.9	14.3 ± 0.1	8.2 ±0.8	3.2
4-*O*-CQA (7)	67.9 ± 1.3	36.4 ± 1.7	17.3	89.7 ± 1.0	46.8 ±3.6	18.1
5-*O*-CQA (8)	104.4 ± 3.6	68.1 ± 6.7	32.4	153.4 ± 0.5	103.0± 10.0	38.8
Total CQA	*261.4*	*157.0*	*74.6*	*351.8*	*213.1*	*82.4*
3-*O*-FQA (9)	19.4 ± 0.5	n.d.	-	8.8 ± 0.1	n.d.	-
4-*O*-FQA (15)	21.8 ± 0.6	n.d.	-	10.9 ± 0.1	n.d.	-
1-*O*-FQA (17)	3.4 ± 0.0	n.d.	-	1.6 ± 0.0	n.d.	-
5-*O*-FQA (18)	34.7 ± 1.0	n.d.	-	18.8 ± 0.1	n.d.	-
Total FQA	*79.2*	*n.d.*	-	*40.1*	*n.d.*	-
3-*O*-*p*CoQA (4)	2.8 ± 0.0	1.0 ± 0.0	0.5	n.q.	-	-
4-*O*-*p*CoQA (11)	2.0 ± 0.0	0.6 ± 0.0	0.3	1.2 ± 0.1	0.4 ± 0.0	0.2
Total pCoQA	*4.8*	*1.6*	*0.8*	*1.2*	*0.4*	*0.2*
1,3-*O*-diCQA (16)	0.4 ±0.0	n.q.	-	n.q.	n.q.	-
3,4-*O*-diCQA (21)	4.7 ± 0.0	5.6 ± 0.0	2.7	4.1 ± 0.2	4.5 ± 0.4	1.8
3,5-*O*-diCQA (22)	2.7 ± 0.1	2.5 ± 0.02	1.2	2.4 ± 0.1	2.2 ± 0.2	0.8
1,5-*O*-diCQA (23)	0.7 ± 0.0	0.7 ± 0.0	0.3	0.5 ± 0.0	0.5 ± 0.0	0.2
4,5-*O*-diCQA (24)	4.9 ± 0.0	5.7 ± 0.3	2.7	5.1 ± 0.1	4.8 ± 0.3	1.9
Total diCQA	*13.4*	*14.5*	*6.9*	*12.1*	*12.1*	*4.7*
3-F,5-CQA (25)	0.9 ± 0.0	0.4 ± 0.0	0.2	0.4 ± 0.0	0.2 ± 0.0	0.1
3-F,4-CQA (26)	0.5 ± 0.0	0.3 ± 0.0	0.1	0.4 ± 0.0	0.2 ± 0.0	0.1
3-C,4-FQA (27)	7.4 ± 0.0	4.2 ± 0.3	2.0	0.8 ± 0.0	0.4 ± 0.0	0.2
3-C,5-FQA (28)	1.7 ± 0.0	0.9 ± 0.1	0.4	0.5 ± 0.0	0.3 ± 0.0	0.1
4-F,5-CQA (29)	0.6 ± 0.1	0.3 ± 0.0	0.2	0.3 ± 0.0	n.q.	-
4-C,5-FQA (30)	1.6 ± 0.0	0.9 ± 0.1	0.4	0.3 ± 0.0	n.q.	-
Total CFQA	*12.7*	*7.0*	*3.3*	*2.7*	*1.2*	*0.5*
Total CGA	**371.5**	**180.2**	**85.7**	**407.9**	**226.7**	**87.7**
O-CQL (10)	3.7 ± 0.1	1.9 ± 0.1	0.9	3.8 ± 0.0	1.9 ± 0.2	0.7
3-*O*-CQL (12)	27.6 ± 1.5	14.7 ± 2.0	7.0	27.9 ± 0.1	15.0 ± 0.9	5.8
O-CQL (13)	5.7 ± 0.2	2.6 ± 0.2	1.3	6.3 ± 01	2.9 ± 0.6	1.1
4-O-CQL (14)	22.5 ± 0.2	11.0 ± 0.1	5.2	24.0 ± 0.0	11.9 ± 0.2	4.6
Total CQL	*59.5*	*30.2*	*14.3*	*62.0*	*31.8*	*12.3*
O-FQL (19)	9.4 ± 0.0	n.d.	-	4.6 ± 0.1	n.d.	-
4-*O*-FQL (20)	8.6 ± 0.1	n.d.	-	4.0 ± 0.1	n.d.	-
Total FQL	*18.0*	*n.d.*	-	*8.6*	*n.d.*	-
Total CGL	**77.6**	**30.2**	**14.3**	**70.6**	**31.8**	**12.3**
Total	**449.1**	**210.3**	**100**	**478.5**	**258.5**	**100**

[a] All values are shown as means ± standard deviations. nd, not detected. n.q. not quantified. [b] Peak numbers correspond to chromatograms in **Figures 1** and **2**.

Table 3. Concentration and antioxidant capacity of individual chlorogenic acids in Robusta and Arabica **filter coffee**.

Compound (Peak nr)	Robusta Filter Coffee			Arabica Filter Coffee		
	Concentration [µmol/100mL]	Trolox equivalents [µmol/100mL]	% of total	Concentration [µmol/100mL]	Trolox equivalents [µmol/100mL]	% of total
CQA (1)	1.7 ± 0.0	0.9 ± 0.0	0.6	1.4 ± 0.2	0.7 ± 0.1	0.5
CQA (2)	3.7 ± 0.3	1.9 ± 0.0	1.2	3.2 ± 0.3	1.7 ± 0.2	1.1
3-*O*-CQA (3)	44.1 ± 1.3	26.1 ± 1.5	16.9	45.6 ± 0.3	26.8 ± 1.3	17.6
CQA (5)	7.0 ± 0.6	4.5 ± 0.3	2.9	6.0 ± 0.4	3.7 ± 0.3	2.4
1-*O*-CQA (6)	10.1 ± 0.0	5.9 ± 0.1	3.8	7.8 ± 0.5	3.8 ± 0.3	3.0
4-*O*-CQA (7)	51.5 ± 1.6	26.8 ± 1.5	17.3	53.7 ± 1.1	27.2 ± 2.2	17.9
5-*O*-CQA (8)	77.5 ± 3.0	50.7 ± 3.4	32.8	95.4 ± 2.5	62.7 ± 2.7	41.3
Total CQA	*195.7*	*116.7*	*75.4*	*213.0*	*127.4*	*83.9*
3-*O*-FQA (9)	15.2 ± 0.1	n.d.	-	5.3 ± 0.5	n.d.	-
4-*O*-FQA (15)	18.6 ± 0.3	n.d.	-	6.5 ± 0.6	n.d.	-
1-*O*-FQA (17)	1.5 ± 0.1	n.d.	-	0.9 ± 0.1	n.d.	-
5-*O*-FQA (18)	30.6 ± 1.4	n.d.	-	11.4 ± 0.4	n.d.	-
Total FQA	*65.8*	*n.d.*	-	*24.2*	*n.d.*	-
3-*O*-*p*CoQA (4)	0.3 ± 0.0	n.q.	-	0.3	n.q.	-
4-*O*-*p*CoQA (11)	0.8 ± 0.0	0.2 ± 0.0	±	0.7 ± 0.0	0.2 ± 0.0	0.2
Total pCoQA	*1.1*	*0.2*	*0.2*	*1.0*	*0.2*	*0.2*
1,3-*O*-diCQA (16)	1.7 ± 0.0	1.5 ± 0.1	1.0	0.3 ± 0.0	n.q.	-
3,4-*O*-diCQA (21)	3.6 ± 0.1	4.3 ± 0.3	2.8	2.2 ± 0.3	2.6 ± 0.1	1.7
3,5-*O*-diCQA (22)	1.9 ± 0.0	1.7 ± 0.1	1.1	1.3 ± 0.1	1.3 ± 0.1	0.8
1,5-*O*-diCQA (23)	0.5 ± 0.0	0.5 ± 0.1	0.3	0.3 ± 0.0	n.q.	-
4,5-*O*-diCQA (24)	3.3 ± 0.1	3.8 ± 0.3	2.4	2.8 ± 0.4	2.8 ± 0.0	1.8
Total diCQA	*11.0*	*11.8*	*7.6*	*6.9*	*6.6*	*4.4*
3-F,5-CQA (25)	0.9 ± 0.0	0.6 ± 0.0	0.4	0.3 ± 0.0	n.q.	-
3-F,4-CQA (26)	1.9 ± 0.0	1.1 ± 0.0	0.7	0.3 ± 0.0	n.q.	-
3-C,4-FQA (27)	8.2 ± 0.0	4.8 ± 0.0	3.1	0.7 ± 0.0	0.4 ± 0.0	0.2
3-C,5-FQA (28)	0.9 ± 0.0	0.5 ± 0.0	0.3	0.4 ± 0.0	0.2 ± 0.0	0.1
4-F,5-CQA (29)	0.7 ± 0.0	0.4 ± 0.0	0.2	0.3 ± 0.0	n.q.	-
4-C,5-FQA (30)	1.9 ± 0.0	1.0 ± 0.1	0.7	0.3 ± 0.0	n.q.	-
Total CFQA	*14.4*	*8.3*	*5.4*	*2.3*	*0.6*	*0.2*
TotalCGA	**288.0**	**136.8**	**88.4**	**247.3**	**134.8**	**88.8**
O-CQL (10)	2.5 ± 0.0	1.2 ± 0.0	0.8	2.1 ± 0.2	1.0 ± 0.1	0.7
3-*O*-CQL (12)	16.6 ± 0.5	8.9 ± 0.1	5.8	16.1 ± 0.4	8.5 ± 0.5	5.6
O-CQL (13)	2.8 ± 0.3	1.5 ± 0.1	0.9	3.1 ± 0.2	1.4 ± 0.1	0.9
4-O-CQL (14)	13.2 ± 0.1	6.3 ± 0.7	4.1	13.6 ± 0.6	6.0 ± 0.7	4.0
Total CQL	*35.1*	*17.9*	*11.6*	*34.9*	*16.9*	*11.2*
O-FQL (19)	7.3 ± 0.7	n.d.	-	2.4 ± 0.1	n.d.	-
4-*O*-FQL (20)	6.4 ± 0.6	n.d.	-	2.1 ± 0.0	n.d.	-
Total FQL	*13.7*	*n.d.*	-	*4.5*	*n.d.*	-
Total CGL	**48.8**	**17.9**	**11.6**	**39.4**	**16.9**	**11.2**
Total	**336.8**	**154.8**	**100**	**286.8**	**151.8**	**100**

[a] All values are shown as means ± standard deviations. nd, not detected. n.q. not quantified.[b] Peak numbers correspond to chromatograms in **Figures 1** and **2**.

3.2 HPLC-derived and overall antioxidant capacity of coffee brews. In this study on-line ABTS chromatographic analysis was used to assess the antioxidant capacity of each single CGA present in the coffee samples. After separation of coffee chlorogenic acids on the HPLC column, the eluate was mixed with stabilized $ABTS^+$ radical solution, which has a deep blue colour. Any quenching of the radical resulted in a loss of colour, which was detected by monitoring the absorbance at 720 nm giving a negative peak on the HPLC traces as illustrated in **Figures 1** and **2**. The antioxidant capacities of individual compounds were added together to give the total HPLC-derived antioxidant capacity, that was used to determine the contribution of each single compound to the total antioxidant capacity of CGAs. As can be seen in **Tables 2** and **3**, caffeoylquinic acids showed the highest contribution to the total antioxidant capacity (75-84 %), followed by CQLs (11-14%), diCQA (4-8 %), CFQA (0-5 %), and *p*CoQAs (0-1 %). None of the FQAs and FQLs exhibited $ABTS^+$ scavenging capacity. Similar findings were reported by Rice-Evans et al. (1997) and Stalmach et al. (2006) indicating that methylation of the 3'-hydroxyl group has an adverse effect on antioxidant activity. To further investigate the influence of chemical structure on antioxidant capacity, Trolox equivalent antioxidant capacity (TEAC) values were calculated for the different chlorogenic acid subgroups. Results are presented in **Table 4**.

Table 4. TEAC values for CGA subgroups as influenced by the hydroxycinnamic moiety.

Compound	TEAC [a]	Structure [b]
CQAs	0.5-0.6	
FQAs	-	
*p*CoQAs	0.3	
diCQAs	0.9-1.2	
CFQAs	0.5-0.6	
CQL	0.4-0.5	
FQL	-	

[a] Ratio between the amount of each CGA subgroup (µmol) and the corresponding antioxidant capacity (µmol Trolox equivalents). [b] Structures shown correspond to a representative compound of each chlorogenic acid subgroup.

The antioxidant capacity exhibited by the different chlorogenic acid subgroups depends on the number of hydroxyl groups in the molecule (Nardini et al., 1995; Rice-Evans et al., 1996). This was also observed in the current study. Dicaffeoylquinic acids exhibited the highest antioxidant capacity because of the presence of two caffeic acid moieties, each one with two hydroxyl groups. CQAs, with only one caffeic acid group exhibited half the antioxidant capacity of diCQAs. The same values were observed for CFQAs with one caffeic and one ferulic acid moiety, most likely due to the absence of antioxidant capacity of ferulic acid groups as discussed above. CQLs, typical products of coffee roasting (Farah et al., 2005) showed slightly lower TEAC values than their precursors, the CQA. This indicates that lactonization seems to have a reducing effect on the $ABTS^+$ scavenging capacity of the compound. Chlorogenic acids with *p*-coumaroylquinic acid moieties exhibited the lowest TEAC values. The presence of only one hydroxyl group in the hydroxycinnamic acid moiety of these chlorogenic acids resulted in a reduction of the antioxidant capacity by 50% compared to CQAs. These results corroborate that the antioxidant capacity of chlorogenic acids depends primarily on the number of hydroxyl groups but is also influenced by other structural changes like methylation or lactonization.

Regarding the total antioxidant capacities of CGAs in the two coffee varieties analysed in this study (**Table 2** and **3)**, the higher values obtained for Arabica coffee could be explained by differences in the chlorogenic acid composition. As described above, Arabica coffee showed higher levels of CQAs which possess high TEAC values, whereas Robusta coffee was rich in FQAs and FQL, which do not exhibit any $ABTS^+$ scavenging activity at all.

To assess the contribution of the CGAs to the overall antioxidant capacity of coffee, samples were analysed using the on-line ABTS method described above but after removing the column from the HPLC system. Data obtained allowed us to determine the antioxidant capacity of all coffee components including those that did not elute from the chromatographic column, such as high molecular weight Maillard reaction products. Results are shown in **Table 5**.

The comparison of the overall antioxidant capacity with the HPLC-derived antioxidant capacity revealed that only 7-16 % of the antioxidant capacity of coffee brews corresponds to the chlorogenic acids (**Table 4**). These values were lower for espresso coffee brews (7-11 %) than those for filter coffee brews (12-16 %). Moreover, Robusta coffee exhibited lower values than Arabica coffee when the same extraction procedure (espresso or filter coffeemaker) was used. The results obtained in the current study indicate that the remaining 84-93% of the overall antioxidant capacity must be ascribed to other compounds different to chlorogenic acids that did not elute from the HPLC column. These compounds were extracted in higher amounts by the espresso coffeemaker and were present in higher amounts in Robusta coffee. As mentioned previously, those compounds that did not elute from the HPLC column might correspond to the high molecular weight Maillard reaction products (MRPs) resulting from the coffee roasting process. MRPS exhibit antioxidant capacity and depending on the roasting degree may contribution to higher degree to the overall antioxidant capacity of coffee than chlorogenic acids (Smrke et al., 2013).

Table 5. Overall antioxidant capacity of coffee in Robusta and Arabica espresso and filter coffee brews.

	Trolox equivalents [µmol/100mL] (% of total)	
	Robusta Coffee	Arabica Coffee
Espresso		
Overall antioxidant capacity	2856.6 ± 200.2 (100%)	2378.1 ± 194.4 (100%)
HPLC-derived antioxidant capacity	210.3 ± 16.4 (7%)	258.5 ± 22.6 (11%)
Difference	2646.3 (93%)	2119.6 (89%)
Filter		
Overall antioxidant capacity	1256.0 ± 52.0 (100%)	972.9 ± 35.9 (100%)
HPLC-derived antioxidant capacity	154.8 ± 8.6 (12%)	151.8 ± 8.7 (16%)
Difference	2763.33 (88%)	821.1 (84%)

3.3 Chlorogenic acids extraction. HPLC-PDA-MS^n was used to assess the extraction behaviour of the chlorogenic acids identified in the coffee samples. Three fractions for espresso coffee (F1-F3) and 5 fractions for filter coffee (F1-5) were collected sequentially during the brewing process. The volumes of the coffee brews and fractions obtained by espresso and filter coffeemakers are shown in **Table 6**. The volumes of the three espresso coffee fractions were quite similar, ranging from 14 to 19 mL. In contrast, the volumes of the filter coffee fractions increased from F1 (87-88 mL) up to F3 (167-170 mL) and then decreased to F5 (27-34 mL), showing an inverted U-shape profile. The technological factors responsible for these differences were discussed in a previous work (Ludwig et al., 2012).

Table 6. Volumes of coffee brews and fractions obtained by espresso and filter coffeemakers.

	Espresso		**Filter**	
	$t_{extraction}$ (s)	V (mL)	$t_{extraction}$ (s)	V (mL)
Arabica				
Coffee brew	24	51	375	519
F1	0-8	17	0-75	87
F2	8-16	15	75-150	158
F3	16-24	19	150-225	170
F4	-	-	225-300	77
F5	-	-	300-375	27
Robusta				
Coffee brew	24	46	375	520
F1	0-8	16	0-75	88
F2	8-16	14	75-150	148
F3	16-24	16	150-225	167
F4	-	-	225-300	77
F5	-	-	300-375	35

The amounts of each chlorogenic acid found in the fractions of espresso and filter coffee brews are shown in **Figure 3** and **4**. The extraction profile for espresso coffees revealed that approx. 70 % of the chlorogenic acids present in the coffee brew were extracted during the first 8 seconds (F1) of the extraction process. During the next 8 seconds (F2) approx. 20% were extracted, whereas the remaining 10% were extracted during the final stage of the brewing process (last 8 seconds, F3).The extraction profiles of the individual compounds were very similar, except for diCQAs and CFQAs which showed a slower extraction accounting F1 for ~62 %, F2 for ~24 % and F3 for ~13 %. This could be because these compounds are those with the highest molecular weight.

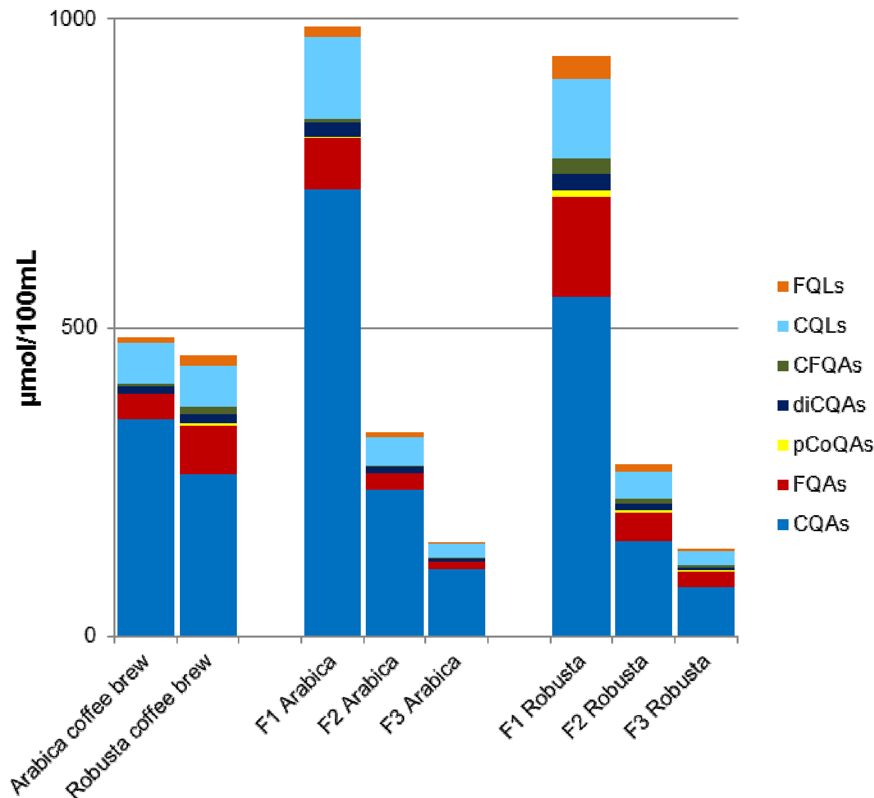

Figure 3. Chlorogenic acid composition of coffee brews and fractions obtained by espresso coffeemaker.

In contrast to espresso coffee, extraction profiles of filter coffee showed lower amounts of chlorogenic acids per 100mL in F1 (first 75 seconds) than in F2 (75-150s). The lowest chlorogenic acid concentration was obtained for F3 (150-225s),but CGAs concentration showed a steady increase (F4 and F5) towards the end of the extraction process. However, when the volumes of the different fractions were taken into account, data revealed that only about 18 % of the chlorogenic acids present in a coffee brew were extracted during the first stage of the brewing process (F1), whereas approx. 60% of the total CGAs were extracted during the next 150 seconds (F2 and F3), and only 20% of these compounds were extracted during the final 150 seconds (F4 and F5).

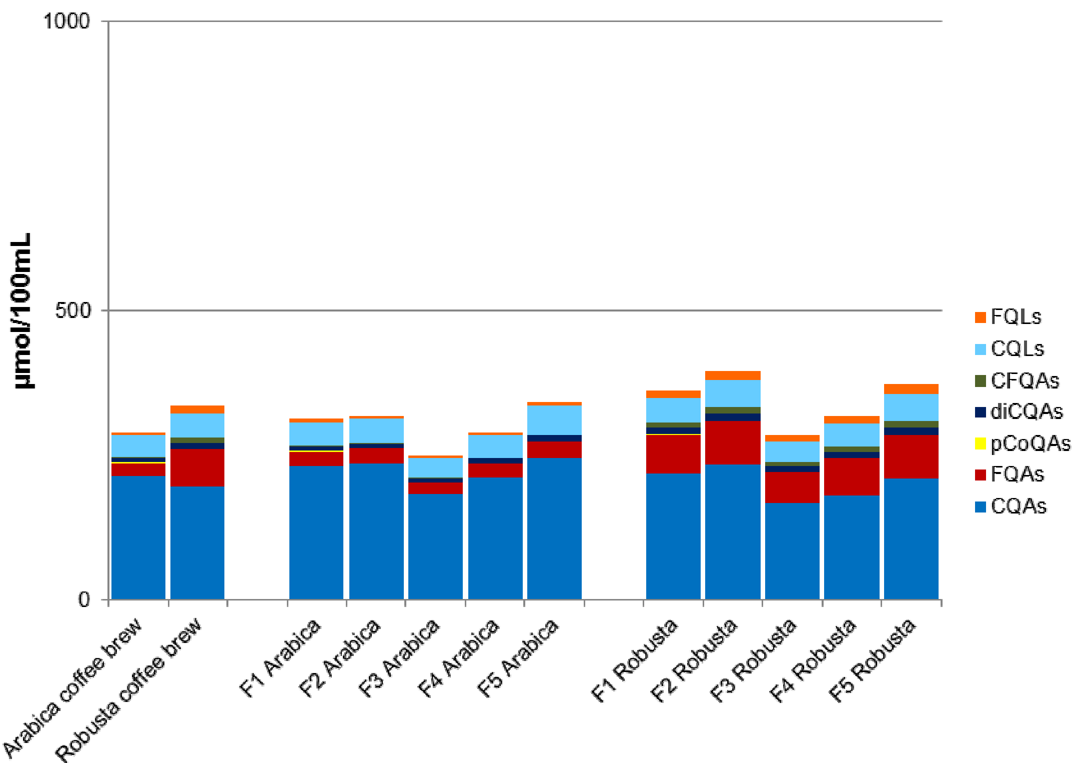

Figure 4. Chlorogenic acid composition of coffee brews and fractions obtained by filter coffeemaker.

In summary, although total CGA amounts were not greatly different for both species, Robusta and Arabica, the latter one showed higher levels of CQAs, whereas Robusta coffee was rich in FQAs and FQL. It was also confirmed that an increase in hydroxyl group promotes the free radical scavenging activities of chlorogenic acids, whereas methylation of the hydroxyl group at the position 3 of the hydroxycinnamic acid and lactonization of the quinic acid moiety resulted in its decrease. Radical scavenging capacity of chlorogenic acids, however, accounted for only 7-16 % of the overall capacity of coffee brews. Thus, further studies about compounds responsible for the antioxidant capacity of coffee brews are needed.

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Objective 5

Study of the fate of coffee phenolics at colon level as influenced by the metabolism of colonic microbiota.

Estudio de la influencia del metabolismo de la microflora intestinal humana sobre los compuestos fenólicos a nivel del colon.

Paper 3:

Catabolism of coffee chlorogenic acids by human colonic microbiota

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TITLE: Catabolism of coffee chlorogenic acids by human colonic microbiota

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ABSTRACT

Several studies have indicated potential health benefits associated with coffee consumption. These benefits might be ascribed in part to the chlorogenic acids (CGAs), the main (poly)phenols in coffee. The impact of these dietary (poly)phenols on health depend on their bioavailability. As they pass along the gastrointestinal tract CGAs are metabolised extensively and it is their metabolites rather than the parent compounds which predominate in the circulatory system. This paper reports on a study in which after incubation of espresso coffee with human faecal samples, HPLC-MS and GC-MS were used to monitor CGA breakdown and identify and quantify the catabolites produced by the colonic microflora. The CGAs were rapidly degraded by the colonic microflora and over the 6 h incubation period, 11 catabolites were identified and quantified. The appearance of the initial degradation products, caffeic and ferulic acid, was transient, with maximum quantities at 1 h. Dihydrocaffeic acid, dihydroferulic acid, and 3-(3′-hydroxyphenyl)propionic acid were the major end products, comprising 75-83 % of the total catabolites, whereas the remaining 17-25% consisted of six minor catabolites. The rate and extent of the degradation showed a clear influence of the composition of the gut microbiota of individual volunteers. Pathways involved in colonic catabolism of CGAs are proposed and comparison with studies on the bioavailability of coffee CGAs ingested by humans helped distinguish between colonic catabolites and phase II metabolites of CGAs.

1 INTRODUCTION

Dietary (poly)phenolic compounds are receiving much attention because of the health benefits ascribed to their antioxidant, anti-inflammatory, cardioprotective, anticancer, chemopreventive, and neuroprotective properties (Del Rio et al., 2013). Among dietary components with a high (poly)phenol content, coffee, one of the most consumed beverages, is an extremely rich source of chlorogenic acids (CGAs) (Clifford, 2000; Clifford et al., 2005; Crozier et al., 2012). CGAs are a family of non-flavonoid compounds, comprising quinate esters of hydroxycinnamic acids such as caffeic, ferulic, and *p*-coumaric acids. The main classes of CGAs in coffee are caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), *p*-coumaroylquinic acids (*p*CoQAs), dicaffeoylquinic acids (diCQAs), and caffeoylferuloylquinic acids (CFQAs). In addition, caffeoylquinic acid lactones (CQLs) and feruloylquinic acid lactones (FQLs), products of roasting, can also occur in coffee in significant amounts. Depending on the position of the hydroxycinnamic acid on the quinic acid moiety, a wide range of different isomers occur in coffee, with the predominant compounds being 3-, 4- and 5-CQAs (Clifford, 2000; Clifford et al., 2005; Crozier et al., 2012; Farah et al., 2005).

Several studies have indicated potential health benefits associated with coffee consumption (Bøhn et al., 2012; Higdon & Frei, 2006; Nkondjock, 2009; Williamson & Stalmach, 2012). For example, epidemiological evidence has shown significant association between habitual coffee consumption and a lower risk of type-2 diabetes (Van Dam & Hu, 2005), a preventive effect in hepatocellular and endometrial cancer (Arab, 2010), and a reduced risk of colourectal cancer (Galeone et al., 2010). These benefits might be ascribed in part to the CQAs which possess

antioxidant activity (Stalmach et al., 2006). *In vitro* CQAs scavenge radicals (Foley et al., 1999; Rice-Evans et al., 1996), increase the resistance of LDL to lipid peroxidation (Abu Amsha et al., 1996; Nardini et al., 1995) and inhibit DNA damage (Kasai et al., 2000; Shibata et al., 1999), and *in vivo* have anticarcinogenic effects in animal models (Huang et al., 1988; Tanaka et al., 1993). However, it should be noted that the health effects of CQAs *in vivo* are not necessarily a consequence of their antioxidant activity. As they pass along the gastrointestinal tract CQAs are metabolised extensively and it is their metabolites rather than the parent compounds which predominate in the circulatory system, albeit it in sub-µmol concentrations, after ingestion of coffee (Stalmach et al., 2009; Stalmach et al., 2010).

In a study using an *in vitro* model with rat small intestine, very little absorption of 5-CQA was observed (Spencer et al., 1999), pointing towards the large intestine as the most likely site for CQA absorption and metabolism. The important involvement of the colon is also supported by feeding studies involving humans with an ileostomy. Levels of ingested CGAs recovered in ileal fluid indicate that about one-third is absorbed in the stomach and/or small intestine (Olthof et al., 2001; Stalmach et al., 2010). In subjects with an intact functioning colon the remaining two-thirds will reach the large intestine, where the gut microbiota will mediate breakdown to a wide range of low-molecular catabolites. Because the fate of (poly)phenols is very much dependent upon any structural changes that occur during the passage through the gastrointestinal tract (Selma et al., 2009; Williamson & Clifford, 2010), the identification and quantification of microbial catabolites produced in the large intestine are of importance in the context of overall bioavailability and potential health benefits of dietary (poly)phenolics.

In order to further elucidate colonic catabolism of CGAs this paper reports on a study in which after incubation of espresso coffee with human faecal samples, HPLC-MS and GC-MS were used to monitor CGA breakdown and identify and quantify the catabolites produced. The *in vitro* investigation supplements earlier bioavailability studies in which the fate of CGAs was monitored after the ingestion of coffee by healthy subjects and volunteers with an ileostomy (Stalmach et al., 2009; Stalmach et al., 2010).

2 METHODS

2.1 Chemicals. 3-(4'-Hydroxyphenyl)propionic acid, 3-(phenyl)propionic acid, 3',4'-dihydroxyphenylacetic acid, 3'-hydroxyphenylacetic acid, 4'-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, catechol, sinapic acid, 2',4',5'-trimethoxycinnamic acid and 5-CQA were purchased from Sigma-Aldrich (Poole, Dorset, UK). 3,4-Dihydroxybenzoic acid, ferulic acid, and caffeic acid were obtained from AASC Ltd (Southampton, UK) and 3-(3',4'-dihydroxyphenyl)propionic acid and benzoic acid were supplied by Extrasynthese (Gebay, France). 3-(3'-Methoxy-4'-hydroxyphenyl)propionic acid was supplied by Alfa Aeser (Heysham, UK), and 3-(3'-hydroxyphenyl)propionic acid by Fluorochem (Derby, UK). All chemicals and reagents used in the preparation of buffers and macromineral, micromineral, and reducing solutions were of analytical grade from Acros Organics (Geel, Belgium), AnalaR (Pool, UK), Fisher Scientific (Loughborough, UK), and Sigma Aldrich (Steinheim, Germany). HPLC-grade

methanol and acetonitrile were obtained from Fisher Scientific (Loughborough UK). Pyridine 99.8 % and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (synthesis grade) were purchased from Sigma Aldrich (Steinheim, Germany).

2.2 Coffee. Medium roasted Vietnamese coffee beans (*Coffea canephora* var. Robusta) were provided by Unión Tostadora S.A (Logroño, Spain). The beans were ground to a powder in a Moulinex coffee grinder for 20 seconds immediately before sample preparation. Espresso coffee, 45 mL, was prepared from 7 g of ground roasted coffee using an espresso coffee machine (Model Saeco Aroma, Italy). The coffee brew was freeze-dried and stored at –18 °C.

2.3 Faecal sample preparation. The project was approved by Ethics Committee of the University of Glasgow College of Medical, Veterinary and Life Sciences (Ref: 2011023). Faecal samples were obtained from three volunteers (V1-3), two females and one male, aged 27-45, who were non-smokers, and had not consumed antibiotics for at least 3 months before the study. The volunteers followed a low (poly)phenol diet for 48 h before faecal sample collection. The samples were collected by the donors in plastic tubes containing an AnaeroGen sachet (Oxoid Ltd., Cambridge, UK) to maintain anaerobic conditions during transport, and were processed within 30 min of passage. For each volunteer 24 g freshly voided sample was homogenised with 75 mL phosphate buffer (pH 7) to obtain a 32% faecal slurry. The slurries were filtered through muslin to remove particulate material.

2.4 Fermentation medium. Fermentation medium prepared according to Roowi et al. (2010) was adjusted to pH 7 with HCl, sterilised by being boiled during 5 min and purged with oxygen-free nitrogen (OFN). Reducing solution (containing 312 mg cysteine hydrochloride, 312 mg $Na_2S \cdot 9H_2O$, 2 mL 1M NaOH, and distilled water made up to 50 mL) was added at 0.5 mL per 10 mL of medium after which the medium was purged again with OFN until anaerobic conditions as indicated by a colour change from pale indigo to pink.

2.5 *In vitro* incubations. Five mL of the faecal slurry was added to 44 mL of the pre-reduced medium in 100-mL fermentation bottles. Each batch culture consisted of 49 mL of the above described inoculated medium and one of the following substrates:

(a) 0.5 g freeze-dried espresso coffee plus 0.5 g glucose in 1 mL distilled water.

(b) 1 mL distilled water for the controls.

After the addition of the substrate the bottles were purged with OFN and sealed airtight. The bottles were incubated in a shaking water bath (60 strokes/min) at 37 °C for 6 h, aiming to simulate the condition in the colonic lumen (Edwards et al., 1996). Aliquots (6 mL) of faecal suspensions were taken after 0 h, 1 h, 2 h, 3 h, 4 h, and 6 h and stored immediately at –80 °C.

2.6 Qualitative and quantitative analysis of CQAs by HPLC-PDA-MS^n. For CGA analysis of the coffee brew, the freeze-dried sample was reconstituted with distilled water, centrifuged at 16.2 *g* for 5 min and diluted 20-fold with distilled water. Extraction of CGAs from faecal incubates was adapted from Stalmach et al. (2010) with some modifications. For each sample 250 µL defrosted faecal suspension was spiked with 250 µg sinapic acid, which was used as an

internal standard. The supernatants obtained after methanol extraction were reduced to dryness in vacuo and resuspended in 2.25 mL 1 % formic acid and 250 µL methanol. CGAs were analysed using a Surveyor HPLC with a PDA detector scanning from 200-600 nm, an autosampler cooled at 6 ℃ and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (ESI) (Thermo Fisher Scientific, San José, USA). The injection volume was 100 µL for both coffee samples and faecal extracts. Chromatographic separation was performed at 40 ℃ using a Synergi 4 µm Polar-RP 250 x 4.6 mm reversed phase column (Phenomenex, Macclesfield, UK) and a mobile phase consisting of 1 % aqueous formic acid (solvent A) and 100 % methanol (solvent B). Isocratic conditions of 5 % solvent B were maintained for 30 min, followed by a linear gradient from 5 to 20 % B over 40 min and a linear gradient from 20 to 60 % B over 50 min. The flow rate was 1 mL/min. After passing the PDA flow cell, the eluate was split and 0.3 mL/min was directed to the mass spectrometer with the ESI operating in negative ionization mode. Analysis was initially carried out in full-scan, data-depending scanning from *m/z* 100 to 600 and identification was confirmed by single reaction monitoring (SRM) and consecutive reaction monitoring (CRM). CGAs were quantified by PDA at 325 nm, by reference to a 20-1000 ng 5-CQA calibration curve (R^2=0.9999). Quantification of the other CGAs (FQAs, diCQAs, CFQA, CQAL, FQAL) was performed using a 5-CQA calibration curve combined with molar extinction coefficients of the respective compound as reported by Trugo and Macrae (1984) and Farah et al. (2005).

2.7 Qualitative and quantitative analysis of catabolites by GC-MS. Extraction of phenolic acids from faecal incubates was carried out according to method described by Rowi et al. (2010) with some modifications. For each sample 500 µL defrosted faecal suspension was spiked with 60 µg 2′,4′,5′-trimethoxycinnamic acid, as an internal standard, and acidified with 3 mL of 0.2 M HCl. After loading the samples onto preconditioned styrene divinylbenzene SPE cartridges (Strata SDB-L 200 mg, Phenomenex, Macclesfied, U.K.), and washing with 3 mL 0.1 M HCl, the cartridges were dried under a stream of nitrogen. Cartridges were then eluted with 3 mL of dry ethyl acetate. Extracts were reduced to dryness in amber glass vials under nitrogen flow after which 300 µL derivatisation reagent (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide:pyridine, 1:4, v/v) was added. The vials were flushed with nitrogen before sealing. Derivatisation was achieved by heating the vials at 80 ℃ for 20 min. Identification and quantification of catabolites was carried out with a gas chromatograph (Agilent 6890 Series GC-System Plus) equipped with an autosampler (Agilent 7683 Series) and a split/splitless injector and interfaced to a mass spectrometer (Agilent 5973 Network Mass selective Detector). Chromatographic separation was performed using a ZB-5MS Zebron 30 m x 0.25 mm i.d. x 0.25 µm capillary column (Phenomenex, Cheshire, U.K.) with helium as carrier gas at a flow rate of 1.2 mL/min. Samples (1 µL) were injected in split mode with a 25:1 ratio and chromatographic conditions were as follows: The inlet temperature was maintained at 220 ℃. The oven was programmed from 4 ℃ to 160 ℃ at 20 ° C/min, to 200 ℃ at 1.5 ℃/min, to 250 ℃ at 10 ℃/min, to 300 ℃ at 40 ℃/min and held for 5 min at 300 ℃. Phenolic compounds were identified by NIST05 library screening and comparison with retention times and mass spectra of

authentic standards. Quantification was based on 5-500 ng calibration curves constructed for a set of phenolic and aromatic acid standards that were derivatized, as described above, prior to analysis (R^2≥0.97).

2.10 Statistical analysis. Each sample was analysed in triplicate. Results are shown as mean values ± standard deviations. Student's *t* test was applied to ascertain any statistical differences between two groups of means. One-way analysis of variance (ANOVA) was used to look for inter-individual differences between the volunteers. All statistical analyses were performed using the SPSS v.15.0 software package.

3. RESULTS

3.1 CGA content of espresso coffee. Identifications were based on the MS fragmentation patterns reported by Clifford et al. (2003, 2005). A total of 15 CGAs were identified and quantified in the espresso coffee along with four additional components that were partially identified as CQAs and one as a FQL, but it was not possible to determine the respective positions of the caffeic acid and ferulic acid moiety. The amounts of the individual compounds are shown in **Table 1**.

Table 1. Contents of individual chlorogenic acids found in espresso coffee. Data expressed as mean value, standard deviation <7% of the mean in all instances (n = 3)

Compound (HPLC Peak no.)	Concentration [µmol/0.5g]
5-*O*-Caffeoylquinic acid (7)	19.5
4-*O*-Caffeoylquinic acid (6)	12.7
3-*O*-Caffeoylquinic acid (3)	10.6
Caffeoylquinic acid (1)	2.8
Caffeoylquinic acid (2)	0.5
Caffeoylquinic acid (4)	1.0
Caffeoylquinic acid (5)	1.7
Total Caffeoylquinic acids	**48.8**
5-*O*-Feruloylquinic acid (12)	6.5
4-*O*-Feruloylquinic acid (11)	4.1
3-*O*-Feruloylquinic acid (8)	3.6
Total Feruloylquinic acids	**14.2**
3,4-*O*-Dicaffeoylquinic acid (15)	0.9
3,5-*O*-Dicaffeoylquinic acid (16)	0.5
4,5 *O*-Dicaffeoylquinic acid (17)	0.9
Total Dicaffeoylquinic acid	**2.3**
3-*O*-Caffeoyl-4-*O*-feruloylquinic acid (18)	1.4
3 *O*-Caffeoyl-5-*O*-feruloylquinic acid (19)	0.3
4-*O*-Caffeoyl-5-*O*-feruloylquinic acid (20)	0.3
Total Caffeoylferuloylquinic acids	**2.0**
3-*O*-Caffeoylquinide (9)	5.1
4-*O*-Caffeoylquinide (10)	4.2
Total Caffeoylquinides	**9.3**
Feruloylquinide (13)	1.8
4-*O*-Feruloylquinide (14)	1.6
Total Feruloylquinides	**3.4**
Total Chlorogenic acids	***80.0***

The overall CGA content of the espresso coffee was 80.0 ± 2.4 µmol per 0.5 g of freeze dried coffee. The main group of CGAs, the CQAs, represent 61 % of the total CGA content, followed by the FQAs (18 %), the CQLs (12 %), the FQL (4 %), the diCQAs (3 %), and the CFQAs (2 %). 5-CQA was the main compound and accounted for 24 % of the total CGA content. HPLC retention times and EI-mass spectra of the CGAs detected in this study are presented in **Table 1** of the supplementary information.

3.2 CGA degradation during faecal fermentation. Five hundred mg of freeze-dried espresso coffee, containing 80 µmol of CGAs was incubated in the presence of human faecal microbiota from three volunteers for up to 6 h and the samples were analysed by HPLC-PDA-MS^n. Representative HPLC-325 nm chromatograms of a fermentation experiment are illustrated in **Figure 1** and time course profiles the degradation of CGAs are present in **Figure 2**. The CGAs present in coffee were completely degraded after 3–4 h of fermentation, except for volunteer 2, where ~60 % of the initial amount of FQAs and FQL, and residual amounts of the other CGAs were still present after 6 h.

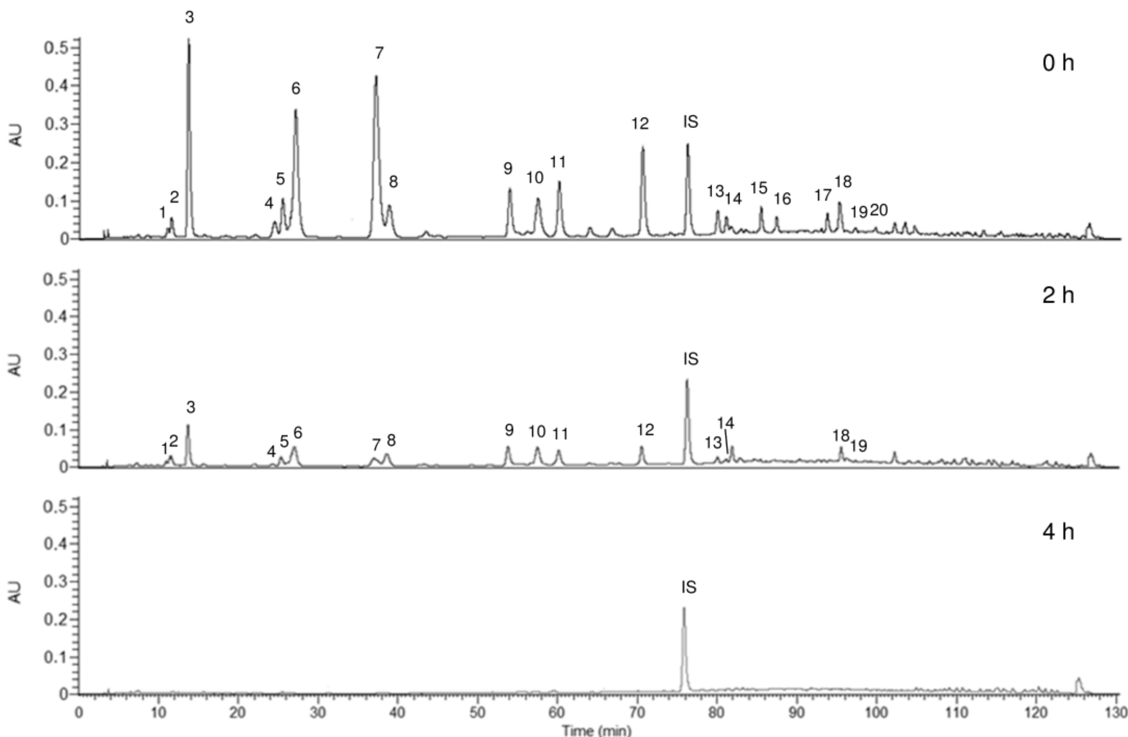

Figure 1. Reversed phase HPLC analysis with detection at 325 nm. Chromatograms of faecal incubates from volunteer 3 after incubation with 500 mg freeze-dried coffee for 0, 2, and 4 h. IS, internal standard (sinapic acid). For identification of peaks and peak numbers, see **Table 1**.

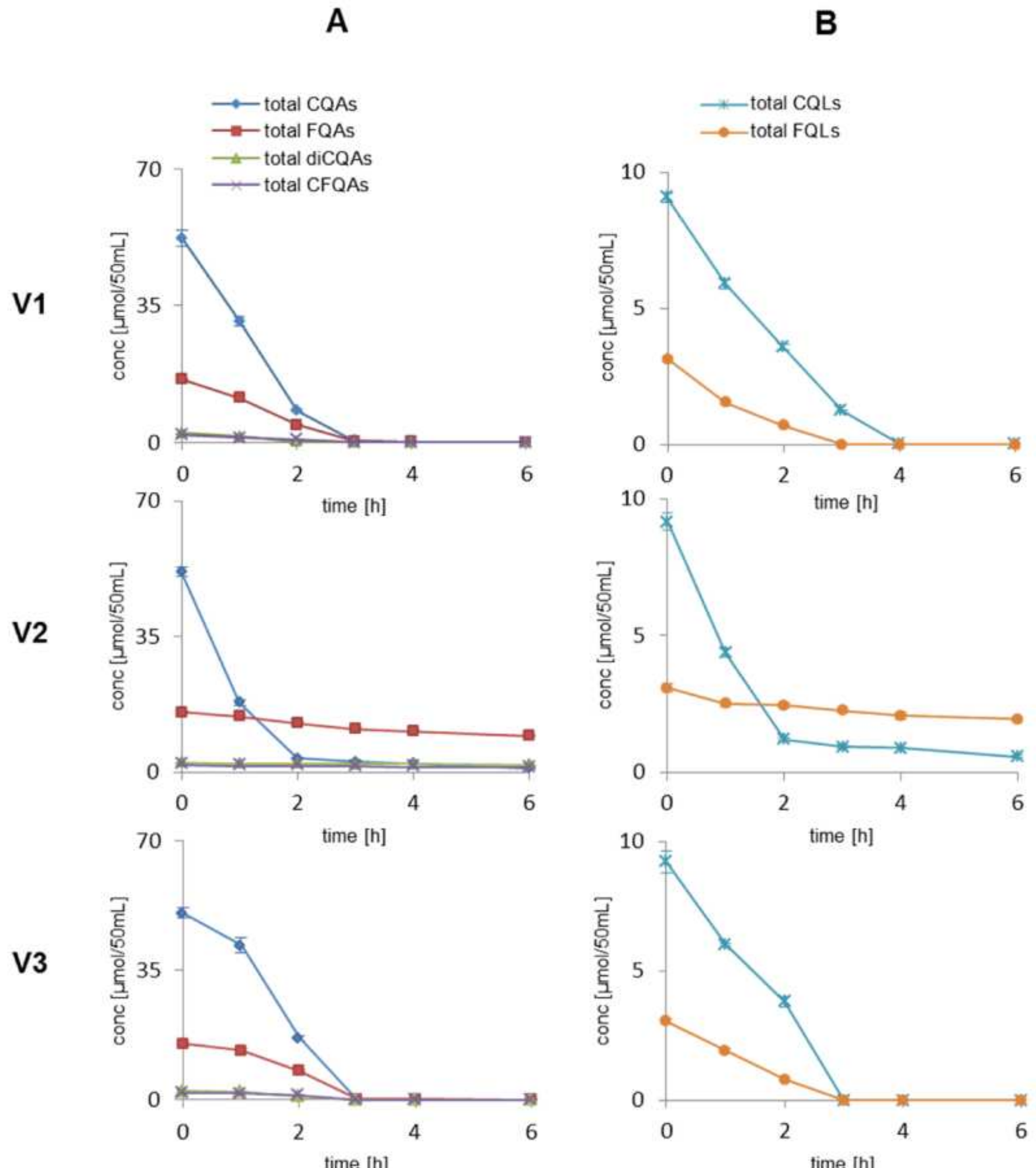

Figure 2. CGA degradation profiles of faecal fermentation samples from three volunteers over 6 h of incubation.

3.3 Identification and quantification of CGA catabolites. GC-MS analysis of the faecal incubates resulted in the identification and quantification of 11 catabolites (see **Table 2** of supplementary information). Catabolite profiles for the three faecal donors are presented in **Figures 3** and **4**. The results shown are corrected for endogenous levels of phenolic acids such as 3-(phenyl)propionic acid, phenylacetic acid, and benzoic acid which were detected in very low levels in the control fermentations of the faecal samples.

The initial degradation products of CGAs were caffeic acid and ferulic acid (**Figure 3A**), resulting from cleavage of the quinic acid moiety. Both reached a maximum after 1 h (0.8-9.8 µmol and 0.3-2.0 µmol, respectively) and declined thereafter. Much smaller amounts of these compounds accumulated in samples from volunteer 2 than volunteers 1 and 3.

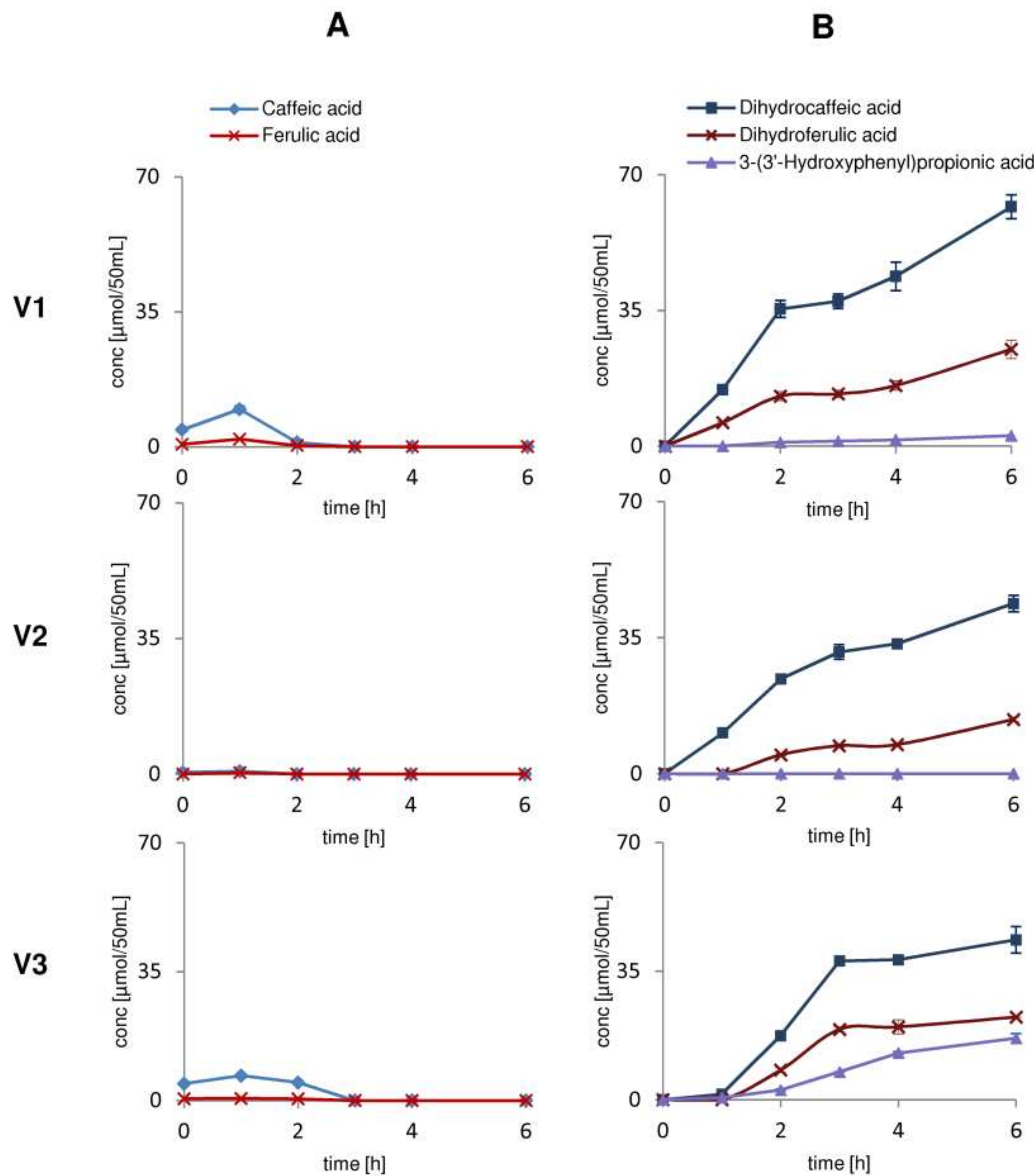

Figure 3. Profiles of main catabolites produced during faecal fermentation from three volunteers over 6 h of incubation.

The next catabolic event was the appearance of dihydrocaffeic acid [aka 3-(3',4'-dihydroxyphenyl)propionic acid] and dihydroferulic acid [aka 3-(3'-methoxy-4'-hydroxyphenyl)propionic acid] after 1 h (**Figure 3B**). The levels of both these compounds increased steady throughout the faecal fermentation. The quantities present at 6 h were 43.8-61.7 µmol for dihydrocaffeic acid, corresponding to 64-91 % of the initial amount of caffeic acid moieties present in CGAs, and 13.9-25.0 µmol for dihydroferulic acid, corresponding to 68-119 % of the initial amount of ferulic acid derived CGAs.

After 2 h, 3-(3′-hydroxyphenyl)propionic acid, resulting from 4′-dehydroxylation of dihydrocaffeic acid, was detected in incubates from two of the three volunteers (**Figure 3B**). Thereafter the

levels increased during the fermentation and reach a maximum of 2.6-16.7 µmol at the 6 h time point, accounting for 4-25 % of the initial amount of CGA-derived caffeic acid.

A further six phenolic compounds were found in the *in vitro* incubates in low and variable amounts, suggesting that they were the products of minor catabolic pathways. These were 3-(4'-hydroxyphenyl)propionic acid, 3-(phenyl)propionic acid, phenylacetic acid, benzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), and 1,2-dihydroxybenzene (catechol) (**Figures 4A** and **B**). In addition, trace quantities of 3'-hydroxyphenylacetic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were also detected.

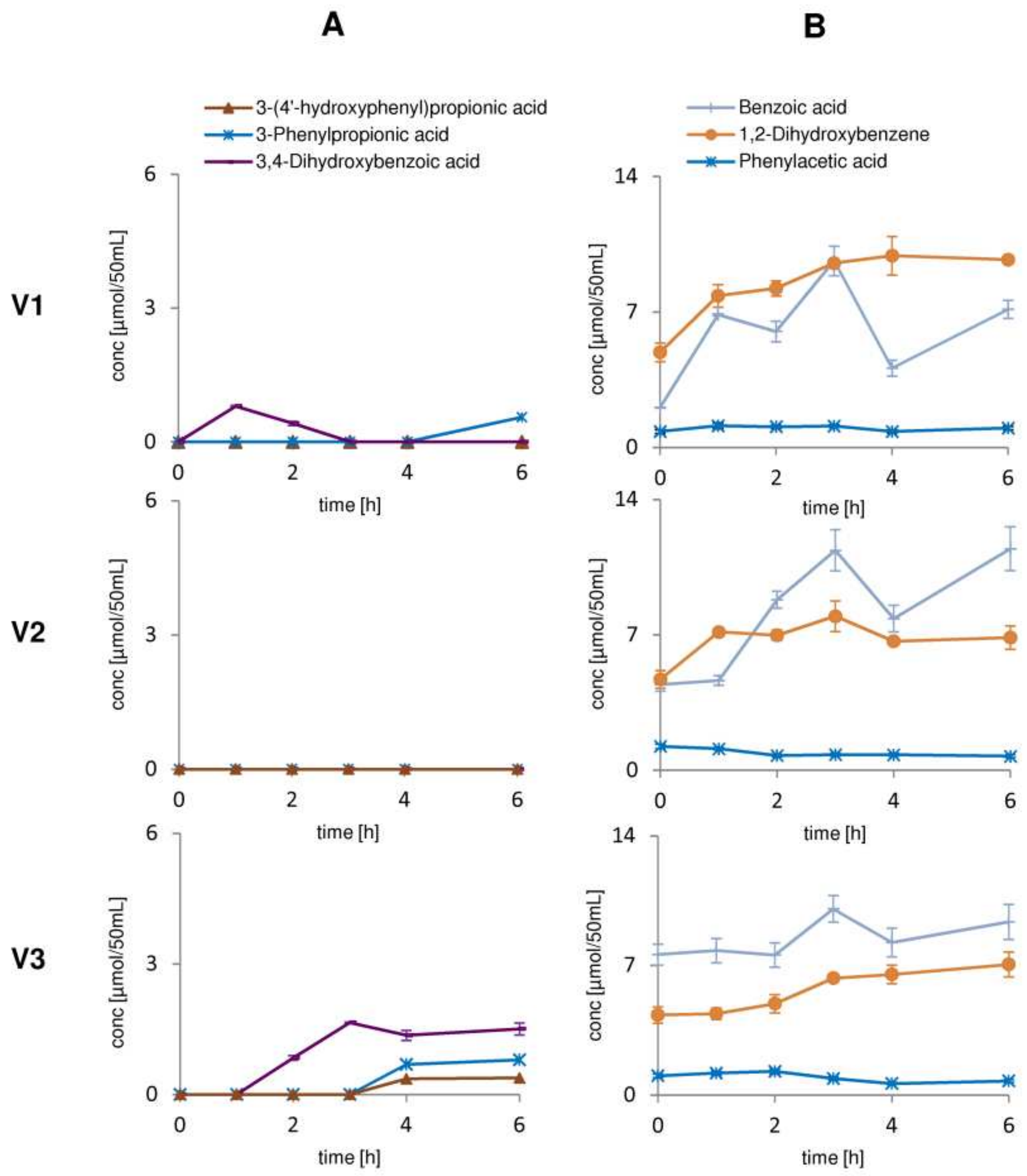

Figure 4. Profiles of minor catabolites produced during faecal fermentation from three volunteers over 6 h of incubation.

The overall quantities of catabolites present at the end of the 6 h fermentation were 107.8 ± 6.3 µmol, 76.7 ± 4.8 µmol, and 102.3 ± 6.8 µmol for volunteers 1-3, respectively. Dihydrocaffeic acid, dihydroferulic acid, and 3-(3′-hydroxyphenyl)propionic acid, the major end products, comprising 75-83 % of the total catabolites, whereas the remaining 17-25% correspond to the six minor catabolites described above.

4. DISCUSSION

For the *in vitro* fermentation, 500 mg freeze-dried espresso coffee was incubated 0 h, 1 h, 2 h, 3 h, 4 h, and 6 h, under anaerobic conditions, with fresh faecal samples from three healthy donors in the presence of glucose. Analysis of aliquots of incubates by HPLC-PDA-MS^n and GC-MS revealed that that CGAs are a good substrate for the enzymes of the human gut microbiota as they were almost completely degraded after 3-4 h of faecal fermentation (**Figure 2**).

Potential catabolic steps occurring in the faecal incubates are illustrated in **Figure 5**. The breakdown of the principal CGAs in the coffee, CQAs and FQAs is probably the result of the action of bacterial esterases, in as much as the first catabolic event was the appearance of caffeic acid and ferulic acid resulting from cleavage of quinic acid from the hydroxycinnamate moiety. Several colonic bacteria, including *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri*, express cinnamoyl esterase (Couteau et al., 2001), which would catalyse such a cleavage. The appearance of low amounts of caffeic acid and ferulic acid was transient, with maximum quantities occurring at 1 h (**Figure 2**). This would appear to be a consequence of their further metabolism, via reduction of the side chain double bond by reductase activity, which led to the appearance of dihydrocaffeic acid and dihydroferulic acid. The two dihydro derivatives were the major end products of the faecal fermentation of the coffee CGAs.

As well as dihydrocaffeic acid and dihydroferulic acid, 3-(3'-hydroxyphenyl)propionic acid was detected after 1-2 h of fermentation. Formation of the phenylpropionic acid was concomitant with reduced concentrations of dihydrocaffeic acid, as a result of 4'-dehydroxylation. 3-(3'-Hydroxyphenyl)propionic acid was previously reported to be the main catabolite found after incubation of 1 µmol 5-CQA with human faecal microbiota (Gonthier et al., 2006). In the present study with a more substantial amount of CGA substrate much higher variable amounts of 3-(3'-hydroxyphenyl)propionic acid were found depending on the donor as illustrated in **Figure 3B**.

Further degradation of 5-CQA to form 3-(phenyl)propionic acid, phenylacetic acid, benzoic acid and their respective hydroxy derivatives, has been reported previously (Selma et al., 2009). However this study, and that of Gonthier et al. (2006), focused on the colonic catabolism of pure reference compounds and did not take into account possible matrix effects occurring in incubations with the complex mixture of dietary CGAs present in coffee that are likely to reach the colon *in vivo* (Stalmach et al., 2010). In the current investigation six catabolites, resulting from demethoxylation, dehydroxylation and decarboxylation were found in low and variable amounts (**Figure 3**). Removal of the 3′-methoxy group of dihydroferulic acid would lead to the

formation of 3-(4'-hydroxyphenyl)propionic acid, levels of which increased after 4-6 h fermentation (**Figure 4**). Dehydroxylation of 3-(4'-hydroxyphenyl)propionic acid and/or its 3'-hydroxy isomer would result in the small increases in 3-(phenyl)propionic acid observed after 3-4 h of fermentation. This compound has been proposed as a biomarker of colonic degradation of 5-CQA (Rechner et al., 2004). Shortening of the three carbon side chain of 3-(phenyl)propionic acid) would lead to the formation of phenylacetic acid. However, this would appear not to be a major catabolic step since low levels of phenylacetic acid were detected at the 0 h time point of fermentation and the amounts did not increase substantially during incubation possibly as a consequence of decarboxylation resulting in its conversion to benzoic acid as illustrated in **Figure 5**.

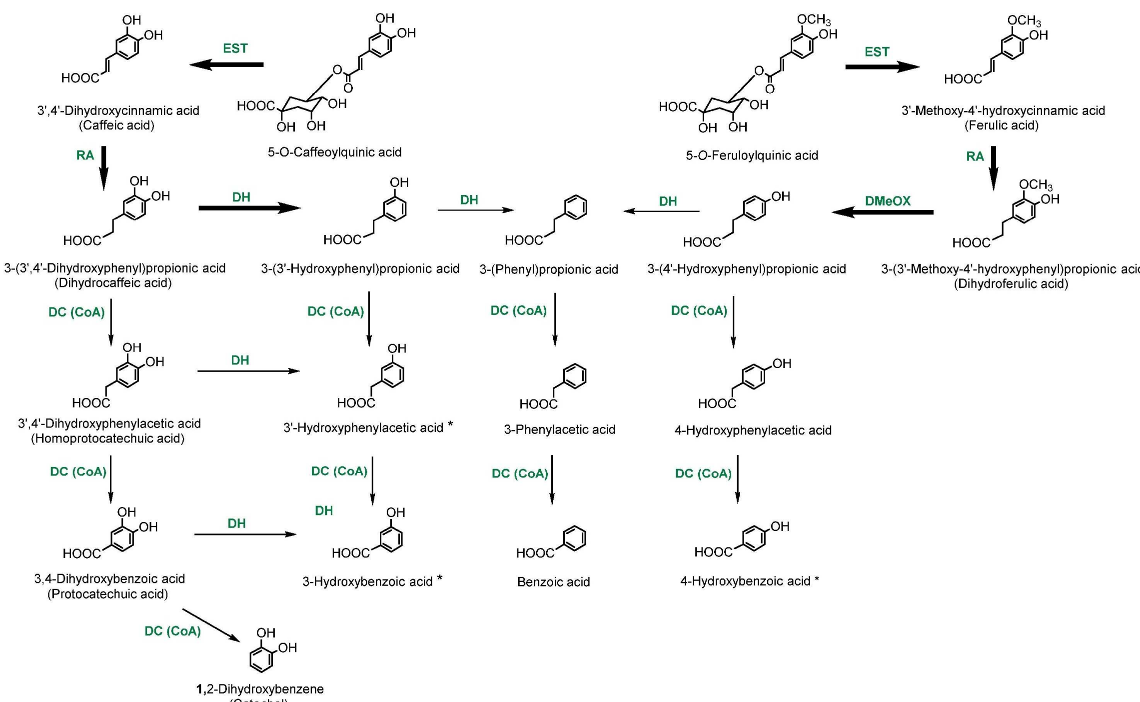

Figure 5. Proposed catabolic pathways for CGA microbial degradation in the colon after ingestion of espresso coffee. Asterisk next to a compound name indicates trace amounts. Bold arrows indicate major pathways. EST, esterase; RA, reductase; DH, dehydrogenase; DMeOX, demethoxyesterase; DC (CoA), decarboxylation (co-enzyme A mediated).

3,4-Dihydroxybenzoic acid (protocatechuic acid) was found in faecal samples from two volunteers and in trace amounts from the third (**Figure 4**). This compound could be formed via α-oxidation steps from dihydrocaffeic acid with 3′,4′-dihydroxyphenylacetic acid (homoprotocatechuic acid) acting as an intermediate. The dihydroxyphenylacetic acid was, however, not detected in any of the faecal samples, which, arguably, is indicative of its rapid rate of conversion to 3,4-dihydroxybenzoic acid. 4′-Dehydroxylation would then convert 3,4-dihydroxybenzoic acid to 3-hydroxybenzoic acid while, alternatively, decarboxylation would result in the production of 1,2-dihydroxybenzene (catechol) as illustrated in **Figure 5**.

Benzoic acid and 1,2-dihydroxybenzene occur in coffee as a result of the roasting process (Nunes & Coimbra, 2010; Rahn & König, 1978) which would explain why they were already present in the samples taken at 0 h. Nevertheless the levels of both compounds varied over the 6 h faecal fermentation period indicating that their presence might at least in part be the result of bacterial catabolism of CGAs. Benzoic acid, however, can also have been formed from quinic acid by the gut microbiota (Williamson & Clifford, 2010). Three further phenolic acids were detected in the faecal incubates in trace amounts, namely 3′-hydroxyphenylacetic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid that might result from 3-(3'-hydroxyphenyl)propionic acid and 3-(4'-hydroxyphenyl)propionic acid being subject to α-oxidation. None of these phenolic acids were present in the control or in the coffee samples indicating they are CGA degradation products.

The data obtained with the faecal fermentations suggest that bacteria present in the human gut are capable of further demethoxylation, dehydroxylation and decarboxylation of dihydrocaffeic acid, dihydroferulic acid and 3-(3'-hydroxyphenyl)propionic acid. However, the corresponding catabolic routes can be considered as minor pathways of coffee CGA degradation, since the amounts found for the resulting compounds were very low. From the catabolites detected during the faecal fermentation we propose pathways with major and minor routes of colonic CGA degradation after coffee ingestion (**Figure 5**).

Total amounts of catabolites found in V1 and V3 after 6 h of incubation, 107.8 ± 6.3 µmol and 102.3 ± 6.8 µmol, respectively, were significantly higher ($p<0.05$) than 80.0 ± 2.4 µmol CGA content of the coffee used for the faecal fermentation. Several studies have confirmed the incorporation of CGAs and other phenolic compounds into coffee melanoidins, the high molecular brown end products of the Maillard reaction formed during coffee roasting (Bekedam et al., 2008; Nunes & Coimbra, 2010). A partial release of these compounds during the faecal fermentation could have increased the amounts of phenolic acids and might explain the differences found between amounts of CGAs incubated and the amounts of catabolites found at the end of the fermentation.

Inter-individual differences in the catabolism of phenolic compounds by gut microbiota have been extensively reported (González-Barrio et al., 2011; Gross et al., 2010; Jaganath et al., 2009; Selma et al., 2009) and were also found in the present study. The observed differences can be attributed to the well-known different bacterial populations present in the individual faecal microbial communities, harbouring specific enzymatic capacities (Zoetendal et al., 2008). The rate and extent of the degradation of the coffee CGAs showed a clear and statistically significant ($p<0.05$) influence of the composition of the gut microbiota. The inter-individual differences were particularly noticeable for volunteer 2 (**Figure 2**). While degradation profiles obtained for V1 and V3 differed only in the breakdown rate, V2 showed an incomplete degradation of FQAs and FQL, with recovery rates up to 60 % after 6 h of faecal fermentation. Other minor CGAs, such as diCQAs, CFQAs, were also degraded to a lesser extent in samples from V2. Breakdown rates for CQAs and CQLs, however, were similar for all volunteers, and as these

representatives made up 72 % of the total CGAs, CGA degradation by gut microbiota was 80-100 % for all volunteers. Differences between the donors were also observed in the catabolite profiles (**Figures 3** and **4**). Samples from volunteer 2 showed significantly lower levels of caffeic acid and ferulic acid. Nevertheless dihydrocaffeic acid and dihydroferulic acids were the main end catabolites of faecal fermentation for volunteer 2. In contrast to volunteers 1 and 3, 3-(3′-hydroxyphenyl)propionic acid and its 4′-hydroxy isomer were not found in fermentations with faecal material from volunteer 2. The inter-individual differences indicate that the composition of the gut microflora and thus the presence of certain bacterial species might have an influence on the formation of catabolites, especially those produced by minor pathways.

The results obtained in this *in vitro* study are in accordance with those on the bioavailability of CGAs in humans in which the main colon-derived metabolites in plasma and/or urine after ingestion of coffee were dihydrocaffeic acid, dihydroferulic acid and their sulfated and glucuronide metabolites (Stalmach et al., 2009). There was an absence of glucuronide and sulfate conjugates in faecal fermentations demonstrating that they are not of bacterial origin but instead are probably phase II hepatic products and/or are formed in epithelial cells during passage through the colon en route to the circulatory system. The same probably also applies i) to co-enzyme A-mediated metabolism of ferulic acid to feruloylglycine and ii) to the catechol-*O*-methyltransferase catalysed reactions that *in vivo* result in metabolism of caffeic acid to isoferulic acid (Stalmach et al., 2009; Stalmach et al., 2010), conversions that did not occur in the faecal incubations.

In conclusion, all coffee CGAs were rapidly degraded by the colonic microflora. A total of 11 catabolites were identified during the 0–6 h of faecal fermentation showing that the CGAs were extensively catabolized by the microorganisms present in the human faecal samples. The rate and extent of the degradation showed a clear influence of the composition of the gut microbiota of individual volunteers. However, the amounts found indicate major degradation pathways leading to the accumulation of dihydrocaffeic acid and dihydroferulic acid. It is known that these catabolites are better absorbed that their CQAs and FQAs, and *in vivo* studies have confirmed free and sulfated dihydrocaffeic and dihydroferulic acids as main metabolites in plasma and urine after ingestion of coffee. Thus, bioavailability of CGAs depends largely on microbial conversions in the colon. Nevertheless, dihydrocaffeic, dihydroferulic acids, other minor catabolites and their parent CGAs that are present in upper regions of the large intestine, where they might act as antioxidants and prebiotics explaining at least partly some of the health benefits at colon level ascribed to coffee consumption. It is important that investigations of the potential protective colonic effects of coffee consumption (Galeone et al., 2010) with *ex vivo* cell systems should make use of this mixture of ingested CGAs and their colonic catabolites at doses that prevail in the proximal gastrointestinal tract.

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SUPPLEMENTARY INFORMATION

Catabolism of coffee chlorogenic acids by human colonic microbiota

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Table 1. HPLC-MS^n identification of chlorogenic acids. $[M-H]^-$, negatively charged molecular ion; MS^2, daughter ions produced from $[M-H]^-$ fragmentation; MS^3, daughter ions produced from fragmentation of MS^2 base ion. For peak number, see Figure 1.

			MS^1	MS^2		MS^3	
			Parent ion	Base ion	Secondary ions	Base ion	Secondary ions
Peak No.	Compound	R_t min	*[M-H]⁻* *(m/z)*	*m/z (intensity)*	*m/z (intensity)*	*m/z (intensity)*	*m/z (intensity)*
1	CQA	11.5	353	191 (100)	179 (90), 135 (30)		
2	CQA	12.0	353	179 (100)	135 (20), 191 (5)		
3	3-CQA	14.3	353	191 (100)	179 (60), 135 (10)		
4	CQA	25.6	353	179 (100)	191 (50), 135 (25)		
5	CQA	27.7	353	191 (100)	179 (5)		
6	4-CQA	28.7	353	173 (100)	179 (80, 191 (20)		
7	5-CQA	38.9	353	191 (100)	179 (5)		
8	3-FQA	40.7	367	193 (100)	191 (5)		
9	3-CQL	55.6	335	161 (100)	135 (60)		
10	4-CQL	58.9	335	161 (100)	135 (15)		
11	4-FQA	61.4	367	173 (100)	193 (25)		
12	5-FQA	70.9	367	191 (100)	173 (5)		
13	FQL	79.7	349	175 (100)	149 (50), 193 (30)		
14	4-FQL	80.7	349	175 (100)	160 (10),193 (25)		
15	3,4-diCQA	84.9	515	353 (100)	335 (20), 173 (20)	173 (100)	179 (90), 191 (50)
16	3,5-diCQA	86.9	515	353 (100)		191 (100)	179 (70), 173 (5)
17	4,5-diCQA	93.2	515	353 (100)		173 (100)	179 (70), 191 (20)
18	3-C-4-FQA	94.8	529	367 (100)	335 (15), 173 (15)	173 (100)	193 (20)
19	3-C-5-FQA	96.5	529	353 (100)	367 (40), 191 (20)	191 (100)	179 (50)
20	4-C-5-FQA	98.7	529	353 (100)	367 (20)	173 (100)	179 (80), 191 (30)

Table 2. GC-MS identification of colonic catabolites. Main ion, main fragment produced from the trimethylsilyl derivative of the corresponding phenolic or aromatic compound; Secondary ions, typical fragment produced from the trimethylsilyl derivative of the corresponding phenolic or aromatic compound.

Compound	**R_t (min)**	**Main ion *(m/z)***	**Secondary ions *(m/z)***
Benzoic acid	6.2	179	105, 135, 77, 194
Phenylacetic acid	6.6	73	164, 193, 91
1,2-Hydroxybenzene	6.7	73	254, 239, 149, 79
3-(Phenyl)propionic	7.8	104	73, 207, 91, 222
3-Hydroxybenzoic acid	9.7	267	193, 223,282, 73
3'-Hydroxyphenylacetic acid	10.5	73	164, 147, 281, 296
4-Hydroxybenzoic acid	10.9	267	223, 193, 73, 282
3-(3'-Hydroxyphenyl)propionic acid	13.3	205	192, 310, 177, 73
3-(4'-Hydroxyphenyl)propionic acid	14.2	179	192, 310, 73
3,4-Dihydroxybenzoic acid	16.0	193	370, 73
Dihydroferulic acid	18.6	340	209, 192, 73, 179
Dihydrocaffeic acid	20.6	179	398, 267, 73, 280
Ferulic acid	27.1	338	308, 323, 249, 73
Caffeic acid	29.4	396	219, 73, 381, 307

GENERAL DISCUSSION

Influence of torrefacto roasting process on coffee antioxidants

The effect of sugar addition during the roasting process of coffee (torrefacto) on browned compounds (caramelization and Maillard reaction products) and antioxidant capacity was evaluated in Colombian coffees roasted in the laboratory with increasing amounts of sugar (0%, 5%, 10%,15%) using the following colourimetric methods: CIELab parameters and absorbance at 420nm for colour formation and DPPH· radical quenching assay and the Folin-Ciocalteau (FC) assay for antioxidant capacity. Results obtained were compared with one commercial torrefacto coffee and seven commercial conventional roasted coffees of different origins (Colombia, Brazil, Kenya, Guatemala, and Vietnam) and different roasting degrees (Dark, Medium and Light).

Brown colour development is one of the most visual changes in heat-treated foods, such as coffee, cereal, cookies, etc. during processing. In the present work, the colour of ground roasted coffees was measured by means of the CIELab parameters (L* or lightness, and a* and b* as the chromaticity parameters) and the Absorbance at 420nm (browned compounds). As expected, low L* values were observed for all dark roasted coffee samples (torrefacto and conventional roasted) whereas higher values were obtained for the medium and light roasted samples (conventional). Although a slight tendency to increase L* value with the addition of sugar was observed, this increase was not significant and, in fact, no significant correlation ($p>0.05$) between L* value and the amount of added sugar has been found. Similar results were found for the chromaticity parameters a* (+red) and b*(+yellow), which were significantly higher in the light roasted coffees than in the medium and dark roasted coffees. Again no significant correlations have been found between any of the chromaticity parameters (a* or b*) and the amount of added sugar during roasting. Thus, the CIELab parameters seem to be related to the roasting degree but independent of the type of roasting process (conventional or torrefacto), maybe because torrefacto roasting process only induces the formation of an external caramel coating and hardly affects the interior of the coffee beans.

The absorbance at 420nm has been commonly used to characterize melanoidins, which are mainly originated by Maillard reactions during the coffee roasting process and other heat-treated foods (Morales, 2005; Nunes & Coimbra, 2007). However, it has been reported that melanoidins accounted for only 65 % of the colour potency of the high molecular weight fraction obtained from light roasted coffee, and for only 39 % from dark roasted coffee (Nunes & Coimbra, 2007). Many other brown-coloured products appear to be sugar (retro)aldolization/ dehydration and carbohydrate condensation products, which may or may not be attached to proteins or other amino nitrogen structures in a similar way to the Maillard reactions (Rizzi, 1997; Hofmann, 1999). So that, hypothetically, the addition of sugar to coffee during the torrefacto roasting process might induce a higher formation of brown-coloured Maillard reactions and caramelization products that, if soluble in water, can be measured by the absorbance at 420nm. Those coffees with sugar added during roasting (torrefacto) showed significantly ($p<0.05$) higher absorbance at 420nm than those roasted conventionally with the same roasting degree. In fact,

highly significant ($p<0.001$) and excellent correlation (0.876) between the absorbance at 420nm and the amount of sugar added during the roasting process has been found showing that this parameter might be proposed as a marker for the torrefacto roasting process. The highest absorbances at 420nm in torrefacto coffees also explain that caramelization products in torrefacto roasted coffee are mainly water soluble.

The antioxidant capacity of the torrefacto roasted coffees in comparison with conventional roasted coffees was evaluated by two colourimetric assays, the DPPH· radical quenching assay and the Folin-Ciocalteau (FC) assay. Focusing on the influence of sugar addition during the roasting process, correlation results show a clear and significant (0.701, $p<0.001$) tendency to increase the DPPH antioxidant capacity with the amount of sugar added during roasting process. In fact, significant correlation between DPPH and absorbance at 420nm, proposed as a good marker for the torrefacto roasting process, was found (0.721, $p<0.001$). These findings are in agreement with those obtained in commercial torrefacto roasted coffee blends (López-Galilea et al., 2006b; López-Galilea et al., 2007). Thus, the higher DPPH quenching activity found in torrefacto coffees can be attributed mainly to the formation of Maillard reactions and caramelization antioxidant products (Manzocco et al., 2000). Commercial conventional roasted coffees showed lower DPPH antioxidant capacity results than lab-roasted torrefacto roasted coffees. This might be explained by a longer storage (from roasting to purchase) under less controlled conditions (room temperature) in commercial samples, because during storage the antioxidant capacity decreases due to the presence of residual oxygen, and other radicals or pro-oxidant compounds formed during the roasting process (Manzocco et al., 2002). Moreover, roasting degree seems to influence conventional roasted coffees in different ways, depending on the origin or variety because DPPH increased with a higher roasting degree (Dark vs Medium) for Colombia coffee (Arabica), but decreased for Vietnam one (Robusta) (Medium vs Light). This could be due to the fact that although Robusta coffee has higher amounts of phenolic compounds than Arabica ones, roasting induces a higher loss of these antioxidant compounds in Robusta coffees (Clifford, 1997; Perrone et al., 2010). In fact, only moderate correlation between DPPH and L* values (-0.483, $p<0.001$) has been found. In regard to the provenance of coffee, conventional roasted coffees of different origins and the same roasting degree (Medium) can be ranked according to the DPPH quenching activity in increasing order as follows: Brazil < Vietnam < Colombia < Guatemala < Kenya.

The Folin Ciocalteau method is traditionally used to measure phenolic compounds, but several authors have reported that this method also evaluates other reducing nonphenolic compounds, such as melanoidins, proteins and thiols, and thus should be seen as a measure of total antioxidant capacity rather than phenolic content (Caemmerer & Kroh, 2006; Everette et al., 2010; Perez-Martinez et al., 2010). A decrease in the antioxidant capacity measured by the Folin-Ciocalteau technique in conjunction with the increase of roasting degree can be observed. A highly significant ($p<0.001$) and good correlation (0.785) with L* values has been found. Similar patterns were reported by other authors (Bekedam et al., 2008a; Sacchetti et al., 2009) in conventional roasted coffees, mainly due to a higher degradation of chlorogenic acids. Loss

of phenolic compounds during roasting is very well known and losses of 8-10% for every 1% loss of dry matter (Clifford, 1997; Clifford, 1999; Clifford, 2000) up to 95% of the chlorogenic acid content in green coffee with drastic roasting conditions were reported (Trugo & Macrae, 1984). Higher FC reducing capacities were found in those coffees roasted with sugar (torrefacto) in comparison with their respective conventional roasted ones (same origin, same roasting degree). For that reason, the influence of torrefacto roasting on phenolic compounds was studied more deeply in those coffees roasted with increasing amounts of sugar addition. 5-caffeoilquinic, caffeic and ferulic acids, and 4-vinylguaiacol were quantified in these latter samples by HPLC analyses and results were similar to those reported for commercial Colombian coffees by Lopez Galilea et al. (2008b). Little differences among coffees samples in the four phenolic compounds (3.48-6.34 mg 5-CQA, 4.03-5.16 μg caffeic acid, 37.22-49.15μg ferulic acid, and 6.60-7.87 μg 4-vinylguaiacol per g of coffee) were observed. These differences, most of them statistically non-significant ($p<0.05$), seem to be due to normal variations during the roasting process, but not to the addition of sugar during the torrefacto roasting process. The addition of sugar during the torrefacto roasting process did not reduce the degradation of the main phenolic compounds caused by heat treatment as was suggested by the Folin-Ciocalteau technique if it is used as a measurement of the total phenolic compounds. This discrepancy in the results might be explained by the presence of other reducing nonphenolic compounds formed during the torrefacto roasting process that react with the Folin Ciocalteau reagent, but not by a protective effect of torrefacto roasting against the degradation of phenolic compounds. This agrees with the results of Lopez Galilea et al. (2008b) who do not find any correlations between phenolic compounds and torrefacto roast in commercial coffee blends. Thus, it could be said that torrefacto roasting process hardly affects the final content of phenolic compounds that seems to be more influenced by other factors such as roasting degree, the variety of coffee, etc. (Farah et al., 2006).

To evaluate at a glance the influence of the roasting type (conventional or torrefacto) and the roasting degree (Dark, Medium and Light) on the antioxidant activity and colour of coffee samples, Principal Component Analysis (PCA) has been applied to the results obtained from the present study. **Figure 1** shows a bidimensional representation of all the variables and coffee samples according to the two selected Principal Components (PC). PC1 (65.2% of the total variance) was mainly characterized by the CIELab colour parameters (L*, a* and b*) and the Folin-Ciocalteau reducing capacity. It could be observed that PC1 distributed all the coffee samples according to the roasting degree, the dark samples being on the left half of the graphic, but independently of the origin, variety and type of roasting process (conventional or torrefacto). PC2 (19.2% of the total variance) was mainly and positively characterized by the absorbance at 420nm. Moreover, DPPH quenching activity contributed partially to the PC2. Thus, those coffees roasted with sugar addition (torrefacto) were mapped in the top half-part of the graphic. Roasting degree also exerts influence on brown compounds formation because dark roasted coffees showed significantly ($p<0.05$) higher absorbances at 420nm than conventional medium and light roasted coffees. However, this influence was much lower than that induced by

torrefacto roast because (1) there were no significant differences between medium and light conventional coffees in agreement with other authors (Del Castillo et al., 2002), and (2) PC2 cannot discriminate between different roasting degree coffees. In conclusion, the absorbance at 420nm might be proposed as a good marker for the torrefacto roasting process, whereas the roasting degree might be better characterized by L* values.

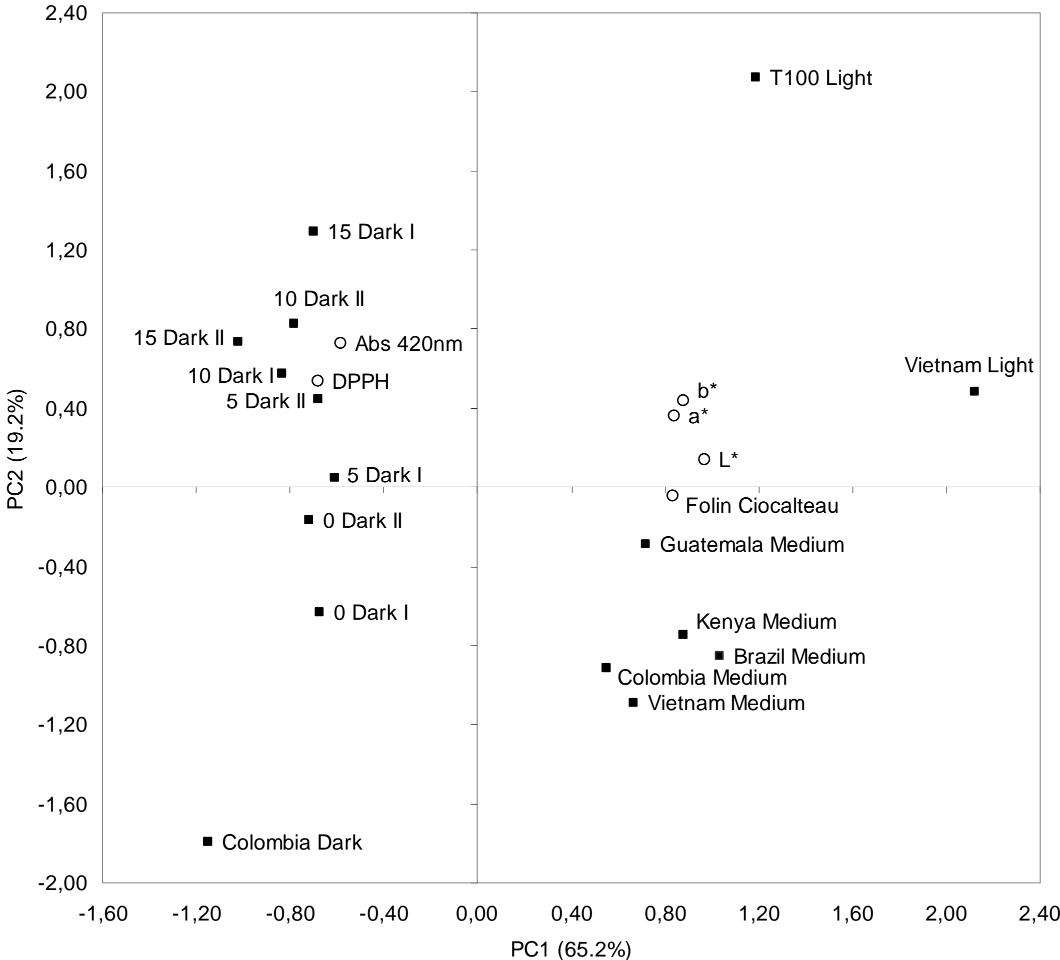

Figure 1. Principal Component Analysis of ground roasted coffee. 0, 5, 10 and 15 is the amount of sugar added (g per 100 g of coffee) during roasting in lab-roasted coffees. Dark, Medium and Light are the roasting degrees. I and II are the batch number in lab-roasted coffees. The origin of coffee is indicated with the name of the country. T100 is commercial 100% Torrefacto roasted coffee.

In summary, although the addition of sugar during roasting (torrefacto) increases the radical quenching capacity (DPPH), other factors, such as coffee variety and origin, and roasting degree, have been shown as factors with higher influence on the presence of antioxidant compounds and, consequently, overall antioxidant activity. For those reasons, for the evaluation of the contribution of coffee compounds to the antioxidant capacity in next experiments, two conventional roasted coffees (one Arabica and one Robusta) with the same roasting degree (medium) were chosen.

Extractability of coffee antioxidants

Brewing process is essential for the antioxidant composition and health properties of a coffee brew, because the contact of water with roasted coffee grounds is the crucial step for extraction of coffee compounds. Previous works showed that some chemical species identified in roasted coffee, including antioxidants, exhibit different extraction rates that may also be influenced by the choice of brewing technique (Petracco, 2001, 2005; Lopez-Galilea et al., 2007) and technological conditions (water pressure and temperature, and coffee/water ratio) (Andueza et al., 2002, 2003, 2007). However, in these studies, among coffee phenolics, only 5-caffeoylquinic acid has been quantified. Although this is the most abundant phenolic in coffee, many other chlorogenic acids may contribute to the antioxidant capacity. The knowledge of extraction behaviour of the main coffee antioxidants during brewing time might lead to identify the technological factors with major impact on antioxidant extraction. Thus, it could be possible to obtain not only coffee brews with higher antioxidant capacity, but also coffee extracts with health properties that can be used as ingredients in functional foods. For these reasons, before to evaluate the contribution of Maillard reaction products and phenolic compounds to the antioxidant capacity of coffee brews, it is of a great interest to study the extraction behaviour of the main coffee antioxidants and the antioxidant capacity, during brewing time in the most widely consumed coffee brew procedures (espresso and filter coffeemakers).

One Arabica coffee (origin Guatemala that was one of the coffees with the highest antioxidant capacity, Ludwig et al., 2013) and one Robusta coffee (origin Vietnam) with the same roasting degree (medium) were selected for the preparation of filter and espresso coffee brews. To study the extractability of coffee antioxidants, three fractions for espresso coffee (F1-F3) and 5 fractions for filter coffee (F1-F5) were collected sequentially every 8 s and every 75 s, respectively, during the brewing process.

The volumes of the three espresso coffee fractions were quite similar, ranging from 14 to 17 mL because espresso coffeemaker applies constant pressure that forces water through the coffee grounds with a constant flow. In contrast, the volumes of the filter coffee fractions increased from F1 (76-80 mL) up to F3 (160-186 mL) and then decreased to F5 (26-54 mL), showing an inverted U-shape profile. In filter coffee no mechanical forces are applied, and the brew volume dripping out from the extraction chamber depends on the water amount, and consequently on the water pressure in the extraction chamber of the coffeemaker according to Darcy's law (Petracco, 2005). This fact explains the low volume obtained for F1 (0-75 s). With time, water fills the extraction chamber increasing the pressure and favouring that water passes through the coffee bed, which leads to higher volumes in the middle fractions. At the end of the brewing procedure, pressure decreases when the water reservoir depletes, giving the lowest volume in the last fraction (F5).

The antioxidant capacity of the coffee brews and fractions obtained by espresso and filter coffeemakers was measured by means of three colourimetric assays (Folin-Ciocalteau, ABTS

and DPPH) and two electron spin resonance (ESR) spectroscopy techniques (Fremy´s salt and TEMPO).

The Folin-Ciocalteau assay, based on an electron-transfer reaction, is the most popular method to evaluate the total phenolic compounds. However, the Folin-Ciocalteau reagent can be reduced by many electron-donors, not only phenolic compounds (Huang et al., 2005). $ABTS\cdot^{+}$ and DPPH· were chosen to assess the radical scavenging activity in coffee fractions. These radicals react energetically with hydrogen-donors, such as phenolic compounds, being DPPH· likely more selective in the reaction with H-donors than $ABTS\cdot^{+}$ (Huang et al., 2005). In these three colourimetric assays, Robusta coffee brews showed significantly ($p<0.01$) higher antioxidant capacity than Arabica ones. The results were similar to those reported by other authors in espresso and filter coffee brews (Sanchez-Gonzalez et al., 2005; Perez-Martinez et al., 2010).

Espresso coffee fractions from both coffee varieties showed a remarkable decrease in antioxidant capacity with brewing time in all three colourimetric assays. More than 70% of the overall antioxidant capacity of an espresso coffee brew was found in F1 (0-8 s), whereas F3 accounted for less than 12 %. These results demonstrate that the compounds responsible for the antioxidant activity of an espresso coffee brew are mainly extracted at the beginning of the brewing process and, afterwards, are diluted. Similar results were found by Alves et al. (2010) for DPPH antioxidant activity in espresso coffees with different brew lengths (“short” to “long”).

To go deeper into the influence of brewing time on antioxidant capacity due to phenolics or Maillard reaction products (MRP), Electron spin resonance (ESR) spectroscopy was applied using Fremy’s salt and TEMPO radicals. Mainly phenolic compounds can be detected when Fremy’s salt is used as the stabilized radical, whereas TEMPO is mainly scavenged by Maillard reaction products (MRP), such as melanoidins (Caemmerer & Kroh, 2006). The results obtained with ESR spectroscopy showed that Fremy´s salt scavenging capacity was almost four times higher than TEMPO. Similar results were reported by other authors who proposed that the phenolic antioxidants evaluated by Fremy´s salt dominate the overall antioxidant capacity of coffee brews, whereas the contribution of roasting-induced antioxidants is rather limited (Bekedam et al., 2008b; Perez-Martinez et al., 2010).

The ESR antioxidant capacity of espresso coffee fractions showed that F1 (0-8 s) accounted for 75-81 % and for 86-89 % of the Fremy´s salt and TEMPO scavenging capacity of an espresso coffee brew, respectively. Whereas antioxidant capacity due to phenolics and measured by Fremy’s salt assay showed a similar profile to the results obtained with the colourimetric assays, the high percentages observed for TEMPO scavenging capacity in F1 indicate that MRP antioxidants were extracted mainly during the first 8 seconds and more quickly than phenolics. In fact the last fraction (16-24 s) only accounted for 1-2 % of the TEMPO scavenging capacity.

Filter coffee fractions showed different antioxidant capacity extraction behaviours in comparison to espresso coffee, being also different in the two coffee samples, Arabica and Robusta. In Arabica filter coffee, all antioxidant capacity assays showed a U-shape profile with the highest

concentration in F1 (0-75 s) and F5 (300-375 s) and the lowest in F3 (150-225 s). However, in Robusta coffee the U-shape antioxidant capacity extraction started after 75 s, showing F1 the lowest values. This could be due to a higher water absorption in Robusta coffee at the beginning of the brewing process that leads to a longer wetting stage. The wettability depends on the particle shape and size that may be different depending on factors like grinding that is also influenced by coffee origin or variety and roasting degree (Lingle, 1996). In this work, taking into account that roasting degree and grinding conditions were maintained the same, different wettability may be due to different brittleness of Arabica and Robusta coffee beans. The increase of antioxidant capacity in the last fractions (F4 and F5) of filter coffee brews could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee. Nevertheless, it should be stressed that the last fraction (F5) had the lowest volume (26 mL and 54 mL for Arabica and Robusta coffees, respectively) and thus, its contribution to the antioxidant capacity of the overall coffee brew was rather limited (~9 % and ~14 %, respectively). The results of the antioxidant capacity due to phenolics and MRP, measured by ESR spectroscopy in filter coffee fractions using Fremy's salt and TEMPO, also corroborate that the antioxidants extraction seems to be delayed in Vietnam filter coffee. This was more pronounced in TEMPO antioxidant capacity that mainly evaluates the scavenging activity of melanoidins which are polymeric compounds with more difficult to be released without water pressure.

The antioxidant capacity of coffee brew is attributed to both, natural antioxidants, like phenolic compounds, and roasting-induced antioxidants, like melanoidins and other MRPs. To know the influence of brewing time on the main antioxidant compounds, browned compounds (Abs 420 nm), caffeine and caffeoylquinic acids were quantified in the coffee samples. Extraction behaviour of browned compounds was in all samples similar to TEMPO scavenging capacity measured by ESR spectroscopy. In fact, high correlation ($r=0.969$, $p<0.001$) between absorbance at 420 nm and TEMPO antioxidant capacity was found. These results support the findings described above about extraction behaviour of MRP.

Among coffee components, caffeine has been proposed as an antioxidant compound against lipid peroxidation induced by reactive oxygen species (Lee, 2000). Caffeine was found in significantly higher concentration in Robusta espresso and filter coffee brews and fractions. It is very well known that Robusta coffees are richer in caffeine than Arabica ones (Belitz et al., 2009). Thus, caffeine might partially explain the higher antioxidant capacity of Robusta coffee brews that could not be attributed to the caffeoylquinic acids that were found in lower amounts in these coffee brews, as will be discussed later. In fact, high correlations have been found between antioxidant capacity assays and caffeine (r values ranging from 0.906 for Fremy's salt assay to 0.968 for DPPH). Other authors also reported similar findings, suggesting that caffeine might be a good contributor to the antioxidant capacity or reducing power of coffee brews (López-Galilea et al., 2007; Vignoli et al., 2011). Nevertheless a more in-depth analysis of the compositional differences between Arabica and Robusta coffees is necessary to confirm these results.

Caffeoylquinic acids (CQAs) are the most abundant chlorogenic acid class accounting for 76-84% of the total CGA in green coffee (Perrone et al., 2008). Although during roasting CGAs are lost up to 95%, CQA still are the predominant CGA in roasted coffee (Trugo & Macrae, 1984). Monocaffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA) were identified and quantified by HPLC-PDA in each fraction and coffee brew. 5-CQA was the major compound among CQAs in all samples, followed by 4-CQA and 3-CQA. The diCQAs were present in much lower concentration than CQAs. These results are in agreement with those reported by other authors in roasted coffee (Perrone et al., 2008) and in coffee brew (Alves et al., 2010). Higher amounts of chlorogenic acids in Robusta coffees than in Arabica ones have been extensively reported (Farah et al., 2005). However, in this study lower amounts of CQA were found in Robusta coffee than in Arabica ones. Also Vignoli et al. (2011) observed higher amount of 5-CQA in Arabica soluble coffee. This could be due to several factors, such as the origin of coffee and the higher loss of chlorogenic acids in Robusta coffee during roasting process (Clifford, 1997; Perrone et al., 2010).

Fractions obtained from espresso coffeemaker showed in both coffee varieties a steep decrease with extraction time in all three monocaffeoylquini acid isomers (3-, 4-, and 5-CQA). F1 (0-8s) accounted for about 70 %, F2 (8-16 s) for 17 % and F3 (16-24 s) for less than 14 % of the total CQA amounts found in an espresso coffee brew. The CQA extraction behaviour was similar to that of the antioxidant capacity measured by colourimetric assays and Fremy´s salt, showing high correlations (r values ranging from 0.727 to 0.903, $p<0.001$), most likely because monocaffeoylquinic acids are the most abundant phenolic compounds in coffee. In contrast, diCQAs were extracted more slowly, accounting F1 for ~50 %, F2 for ~30 % and F3 still for ~20 %, showing correlations coefficients lower than 0.700 The esterification of an additional caffeic acid moiety in diCQA increases the number of hydroxyl groups and might favor the retention of these compounds by interaction with melanoidins or other polymeric compounds (Kroll et al., 2003; Bekedam et al., 2008c), reducing the release of diCQA. In fact, the hydrogen bonding between hydroxyl groups of the phenolic compounds and the amide carbonyls of the peptide bond was found to be a common non-covalent link between phenolics and melanoidins (Nunes & Coimbra, 2010). Also the weaker polarity of the diCQA compared to the CQA might explain the slower release of these compounds during extraction with water (Kroll et al., 2003). Blumberg et al. (2010) studied the influence of hot water percolation on the concentration of monocaffeoylquinic acids and chlorogenic acid lactones and reported that dicaffeoylquinic lactones were extracted rather slowly in comparison to monocaffeoylquinic ones.

Caffeoylquinic acids extraction behaviour was different in filter coffee. Different extraction profiles were also found for the two coffee samples. In Arabica filter coffee, CQAs and diCQAs extraction showed a U-shape profile with the highest concentration in F1 (0-75 s) and F5 (300-375 s) and the lowest in F3 (150-225 s), similar to that observed for antioxidant capacity according to the correlations showed before. However, in Robusta filter coffee the U-shape extraction of caffeoylquinic acids started after 75 s, and F1 exhibited the significantly lowest caffeoylquinic acids concentration. The delay in caffeoylquinic acids extraction might be

attributed to the longer wetting stage observed in Robusta coffee, as described above. On the other hand, the increased extraction of caffeoylquinic acids in the last stage of the brewing process, could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee. This might facilitate the hydrolysis of caffeoylquinic acids bound to melanoidins increasing their release during advanced stages of filter coffee brewing (Lingle, 1996). However, when the low volumes of these fractions are taken into account, it could be observed that caffeoylquinic acids from F5 only accounted for ~8 % and ~11 % of the total contents in Arabica and Robusta filter coffee brews, respectively.

Unlike in espresso coffee, similar extraction percentages among CQAs and diCQAs in each coffee fraction along the filter brewing process were observed. Moreover, when the concentration of antioxidants is calculated per gram of coffee taking into account the different fraction volumes, higher extraction of these phenolic compounds per gram of coffee was obtained in filter coffee brews than in espresso ones, in agreement with Pérez-Martinez et al. (2010). This may be due to the technological differences between espresso and filter coffeemaker. Although the high water pressure applied in espresso coffeemaker favors the extraction process, the short contact time between water and coffee grounds, the high coffee/water ratio and the limited space in the coffee cake does not allow equilibrium to be reached (Petracco, 2005). In contrast, longer contact time and turbulences in the extraction chamber of the filter coffeemaker allow the water in immediate contact with the coffee to extract additional compounds when it has not become so saturated with dissolved material. Thus, both technological factors might favor the extraction of CQAs and diCQAs, free and bound with melanoidins. In fact, turbulences are considered, after time and temperature, the third most important factor in filter coffee brewing (Lingle, 1996).

In summary, brewing time plays a key role in antioxidants extraction of coffee. To optimize their extraction in order to obtain antioxidants that can be used as ingredients for functional foods, several technological factors should be taken into account. Thus, higher water pressure increases antioxidants extraction speed as observed in espresso coffee. Nevertheless, parameters like turbulence and longer contact time, typically of a filter coffeemaker, should be considered in order to increase extraction efficiency, mainly of less polar antioxidant compounds as diCQA. Further research in the influence of technological parameters on chemical composition of coffee brew fractions, as well as their sensory properties, should be needed before industrial development.

Contribution of volatile Maillard reaction products to the antioxidant capacity of coffee

Roasting process induces the formation of a great variety of volatile compounds, most of them Maillard reaction products, which contribute to aroma that is one of the most valuable properties of roasted coffee. In addition, some studies have proposed several heterocyclic compounds formed during heat treatment as being potentially antioxidant (Fuster et al., 2000; Yanagimoto et al., 2002). For this work, one Arabica coffee (origin Colombia that is one of the most aromatic coffee) and one Robusta coffee (origin Vietnam) with the same roasting degree (medium) were

selected to study the profile of their volatile compounds and later to quantify those who are proposed as potent antioxidants and to evaluate their contribution to the overall antioxidant capacity of coffee.

A total of sixty-two and sixty-four volatile compounds were identified and quantified for Arabica and Robusta coffee, respectively. They comprised 4 sulfur compounds, 8 aldehydes, 6 esters, 15 furans, 8 ketones, 5 alcohols, 2 thiophenes, 6 pyrroles, 2 pyridines, 4 pyrazines, 2 thiazoles, 1 lactone, 2 phenolic compounds, 1 alkene, and 1 ether.

Arabica coffee showed a significantly higher total area of volatiles than Robusta (2.1×10^6 vs 0.7×10^6), mainly because the most abundant volatile chemical classes (aldehydes, furans, ketones and esters) were significantly higher in Arabica samples. Aldehydes and esters are responsible for fruity and malty coffee flavour notes, whereas diketones contribute to the buttery aroma, and furans are considered to be responsible for the typical roasted coffee aroma (Semmelroch & Grosch, 1995; Maeztu et al., 2001; Flament, 2001). Similar results were reported by other authors (Sanz et al., 2002; Lopez Galilea et al., 2008a) when they compared the volatile compounds of Arabica coffee with those found in Arabica-Robusta coffee blends roasted by conventional or torrefacto techniques. In contrast, chromatographic areas of pyrazines and pyridines, and in less proportion thiazoles, were higher in Robusta coffee. Pyrazines are responsible for roasty, earthy, musty and woody flavour notes characteristic of Robusta coffee (Blank et al., 1991; Semmelroch & Grosch, 1995; Lopez Galilea et al., 2006a) and pyridines contribute to smoky aroma (Flament, 2001). Also, low molecular weight phenolic compounds, and mainly 2-methoxyphenol (guaiacol) that is a key odorant (Semmelroch & Grosch, 1995; Sanz et al., 2002) responsible for phenolic and burnt aroma (Lopez Galilea et al., 2006a), were only detected in Robusta coffee samples at low levels but not in Arabica. Similar results were found by other authors in conventional roasted Arabica and Robusta coffee (Semmelroch & Grosch, 1995; Maeztu et al., 2001; Lopez Galilea et al., 2008b) and coffee brews (Maeztu et al., 2001).

Several studies suggest that some heterocyclic compounds developed during coffee roasting such as furans, pyrroles and thiophenes, exhibit antioxidant properties (Fuster et al., 2000; Yanagimoto et al., 2002; Yanagimoto et al., 2004). However, Lopez Galilea et al. (2008b) found significant negative correlations of these compounds with antioxidant capacity suggesting a prooxidant capacity. Despite the different approach in these studies, the results might not be considered as contradictory if it is taken into account that some antioxidant compounds may act as prooxidant at different doses. Most of the work which study the antioxidant capacity of volatiles have been carried out using standard compounds at higher concentration than those present in coffee. Thus, these results cannot be directly transferred to the knowledge of the antioxidant capacity of coffee. For those reasons, the concentration of heterocyclic volatile compounds in coffee was firstly measured to further assess their actual contribution to the total antioxidant capacity of coffee at the concentration usually found in coffee.

Seven furans (furan, 2-methylfuran, 2,5-dimethylfuran, 2-methyl-tetrahydrofuran-3-one, furfural, 5-methylfurfural and 2-furfurylacetate), three pyrrols (1-methylpyrrole, pyrrole, and 2-formyl-1-methyl-pyrrole), and two thiophenes (thiophene and 2-methylthiophene) were initially chosen because they were previously proposed by other authors as potential antioxidants, but also because their chromatographic areas were the highest in analyzed coffee samples in most cases. The concentration of these volatile compounds, were quantified in both Arabica and Robusta coffees based on the calibration curves of the corresponding standard. Except in thiophenes with the same concentrations, Arabica coffee exhibited higher concentration in all analyzed compounds, showing considerably higher amounts of 2-methyl-tetrahydrofuran-3one (more than 5-fold) and 5-methylfurfural (almost 3-fold) than in Robusta coffee. The volatiles with the highest chromatographic areas, 2-methylfuran and 1-methylpyrrole, were not the most abundant in coffee because the relationship between the chromatographic area and the concentration is not the same for every volatile compounds. For that reason, the ranking order among volatiles in each chemical family group was also different.

Model systems with the selected furans (Fu), pyrroles (Py), thiophenes (Th), and one with all of them (Fu-Py-Th) were prepared based on the mean concentrations of each compound found in Arabica and Robusta coffee. The antioxidant capacity of each model system was assessed using the $ABTS^+$ radical quenching assay. For each model system, three different concentration levels including that found in coffee (coffee, 10-fold, 100-fold) were analyzed to evaluate the dose dependent antioxidant activity.

Furans are cyclic ethers present in heated and roasted foods. A great variety of furans are originated during roasting process in coffee as Maillard-reaction products, but they are also the result of thermal oxidation of lipids and thermal degradation of thiamine, nucleotides, and terpenes (Flament, 2001). In furan model system (Fu), any appreciable antioxidant activity at coffee furan concentration was observed. However, for the 10-fold and 100-fold concentrated furan model systems, $ABTS^+$ quenching activities equivalent to 0.08 and 0.85 mmol Trolox per liter were found, showing a linear dose dependent increase in antioxidant capacity. To ascertain the contribution of each single furan to the overall Fu-model system antioxidant activity, volatile compounds were tested individually. Because the furan model system showed no antioxidant activity at concentration levels actually present in coffee, 10-fold concentrated solutions of each furan were used to measure the $ABTS^+$ antioxidant activity. From the 7 analyzed furans, only 2-methyl-tetrahydrofuran-3-one exhibits antioxidant activity. This was no significant different to that of the Fu-model system at the same concentration level (10-fold), showing that the antioxidant activity of the main coffee furans might be mainly attributed to this volatile compound, maybe because 2-methyl-tetrahydrofuran-3-one was by far the most abundant furan in coffee. Although five of the furans analyzed (namely furan, 2-methylfuran, furfural, 5-methylfurfural and 2-furfurylacetate) have been reported as potent antioxidants (Fuster et al., 2000; Yanagimoto et al., 2002; Yanagimoto et al., 2004), results obtained in this study show that even at concentrations 10-fold higher than actually present in coffee, only 2-methyl-tetrahydrofuran-3-one exhibited a very limited radical scavenging activity.

Pyrroles are also formed during roasting process. Pyrrole and 1-methyl-pyrrole are formed in the pyrolysis of proline and threonine alone or combined with glucose or sucrose, and in the pyrolysis of trigonelline (Flament, 2001). 2-formyl-1-methylpyrrole is formed from 1-methylpyrrole and also when D-xylose reacted thermally with various amines or amino acids (glycine, alanine, beta-alanine, leucine). In Pyrrole model system (Py), no appreciable antioxidant activity at concentration levels equivalent to coffee was found. The 10-fold and 100-fold concentrated pyrrole systems exhibited antioxidant activity equal to 0.32 and 0.81 mmol Trolox per liter, respectively, showing a non-linear dose dependent antioxidant activity increase. In comparison with furans, pyrroles showed a 4 times higher radical quenching activity at 10-fold concentrations, but similar antioxidant activity at 100-fold concentrations. These results suggest a higher effectiveness of pyrroles at lower concentrations, as proposed by other authors (Fuster et al., 2000) but still undetectable at coffee concentration. To assess the contribution of each pyrrole, the three compounds were analyzed separately at 10-fold concentration by the ABTS assay. Results reveal that the antioxidant activity measured for the Py model system might be totally attributed to pyrrole, whereas 1-methylpyrrole and 2-formyl-1-methylpyrrol seem to be ineffective in quenching $ABTS^+$ radicals at the tested concentrations. Also, Yanagimoto et al. (2002) observed that pyrrole inhibited hexanal oxidation higher than 1-methylpyrrole, but the inhibition were quite low for both volatile compounds (<10% at 10 µg/mL for pyrrole and <3% at 5-20 µg/mL for 1-methylpyrrole). However, 2-formyl-1-methylpyrrole seems to be more effective as a lipophilic antioxidant inhibiting hexanal oxidation in dichloromethane solutions (Yanagimoto et al., 2002) than as a hydrophilic antioxidant quenching $ABTS^+$ radicals in aqueous solutions similar to coffee brews.

Thiophenes present in roasted coffee can be formed by pyrolysis of sulfur amino acids as methionine or cysteine and cystine alone, or by browning reactions in the presence of sugars (Flament, 2001). Although some authors (Fuster et al., 2000; Yanagimoto et al., 2002) reported that both thiophenes found in coffee (thiophene and 2-methylthiophene) exhibit antioxidant activity, the results obtained in this study did not show radical quenching activity at any analyzed concentration level (coffee, 10-fold and 100-fold). This could probably be due to the very low amounts of thiophenes used in this study to evaluate the $ABTS^+$ antioxidant activity even at the highest concentration (100-fold, with 10 µg/mL for tiophene and 2 µg/mL for 1-methylthiophene). Actually, these compounds are present in coffee in very low amounts and therefore, although their antioxidant capacity was demonstrated at high concentrations (more than 50 µg/mL), tiophenes barely contribute to the antioxidant capacity of coffee.

When the antioxidant activity of a model system containing all the 12 selected heterocyclic compounds (furans, pyrroles and thiophenes) was analyzed, no radical quenching activity was detected at concentration levels similar to coffee brew, whereas $ABTS^+$ quenching activity equal to 0.35 and 0.90 mmol Trolox per liter were observed for 10-fold and 100-fold concentrated samples, respectively. Thus, the radical quenching activity of the Fu-Py-Th model system was slightly higher than the maximum value showed for each concentration (pyrroles at 10-fold and furans at 10-fold), but not the sum of the model systems of furans and pyrroles. These results

suggest antagonistic effects among furans, pyrroles and thiophenes. Moreover, when the $ABTS^{+}$ quenching activity of model systems was compared to the overall antioxidant activity of a filter coffee brew, the results clearly showed the almost insignificant contribution of these heterocyclic volatile compounds to the antioxidant activity of coffee, even at the 100-fold concentrated Fu-Py-Th model system, which exhibited the highest $ABTS^{+}$ quenching activity, accounting only for 3.3% of the overall activity of a filter coffee brew.

The most abundant volatile compounds in coffee are aldehydes and ketones and some of them are also Maillard reaction products. For those reasons, we decided to test the antioxidant activity of two new model systems, one with aldehydes (Ald) and another with ketones (Ke). In terms of chromatographic areas, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal (Strecker degradation products of valine, isoleucine, and leucine), were the most abundant aldehydes followed by acetaldehyde and propanal that are formed by pyrolysis of alanine and serine, and/or sugar. However, acetaldehyde and propanal were in significantly higher concentrations than Strecker aldehydes in both coffees. Also, two diones (2,3-butanedione and 2,3-pentanedione) were selected for quantification and further evaluation of their antioxidant activity in a model system. All aldehydes and ketones were in significantly higher amounts in Arabica coffee than in Robusta one, in agreement with other studies (Grosch, 1996). As in the previous model systems, mean concentrations of the selected aldehydes and ketones found in Arabica and Robusta coffee were used to prepare the aldehydes and the ketones model systems The antioxidant capacity of each model system at three different concentration levels (coffee, 10-fold and 100-fold) was assessed using the $ABTS^{+}$ radical quenching assay. The results shown that even at the highest concentration, the antioxidant capacity of the aldehydes and ketones were negligible in comparison to that of the coffee brew and also to those of the heterocyclic volatiles model systems.

In summary, volatile compounds present in coffee contribute very little to the antioxidant capacity of coffee in comparison to other coffee chemical compounds, such as phenolics and melanoidins. The results of the present study also indicate that, although some volatile compounds may act as antioxidant in high doses, it is necessary to evaluate their capacity at the actual concentrations in food samples. For these reasons, next experiments were mainly focused on non-volatile compounds (phenolics and non-phenolics) present at the most common coffee brews (espresso and filter) prepared with Arabica and Robusta roasted coffee.

Contribution of phenolic and non-phenolic compounds to the antioxidant capacity of coffee

Despite the fact that technological factors influence the extractability of the main antioxidant compounds in coffee, it should be taken into account that, similar to the volatiles, each antioxidant present in coffee may contribute in different percentage to the overall antioxidant capacity of coffee brews. In addition, as it was suggested before, it is necessary to evaluate their antioxidant capacity at the actual concentrations in food samples. Thus, on-line ABTS chromatographic analysis was used to evaluate the contribution of non-volatile compounds to

the antioxidant capacity of coffee brews (espresso and filter). This methodology allows us to know both the antioxidant capacity of each single chlorogenic acid and the overall antioxidant capacity of the coffee brews. Moreover, HPLC-PDA-MS^n was used to identify and quantify many other chlorogenic acids beside 3-, 4- and 5-caffeoylquinic and 3,4-, 3,5- and 4,5-dicaffeoylquinic acids in espresso and filter coffee brews (Arabica from Guatemala and Robusta from Vietnam) and their fractions.

A total of 22 CGAs were identified in the coffee brew samples: three caffeoylquinic acid (CQA) isomers, three feruloylquinic acid (FQA) isomers, two p-coumaroylquinic acid (pCoQA) isomers, five dicaffeoylquinic acid (diCQA) isomers, six caffeoylferuloylquinic acid (CFQA) isomers, two caffeoylquinic acid lactones (CQL) and one feruloylquinic acid lactone (FQL). Four additional components were partially identified as CQAs, one as FQA, two as CQLs, and one as a FQL but it was not possible to determine the respective position of the caffeic acid and ferulic acid moiety. Identification was based on the MS fragmentation patterns reported by (Clifford et al. (2003; 2005). **Figure 2** shows a representative HPLC-325 nm chromatogram of Robusta coffee. The most abundant CGAs were the CQAs, followed by smaller amounts of FQAs, CQLs, diCQA, FQLs, CFQAs and *p*CoQAs.

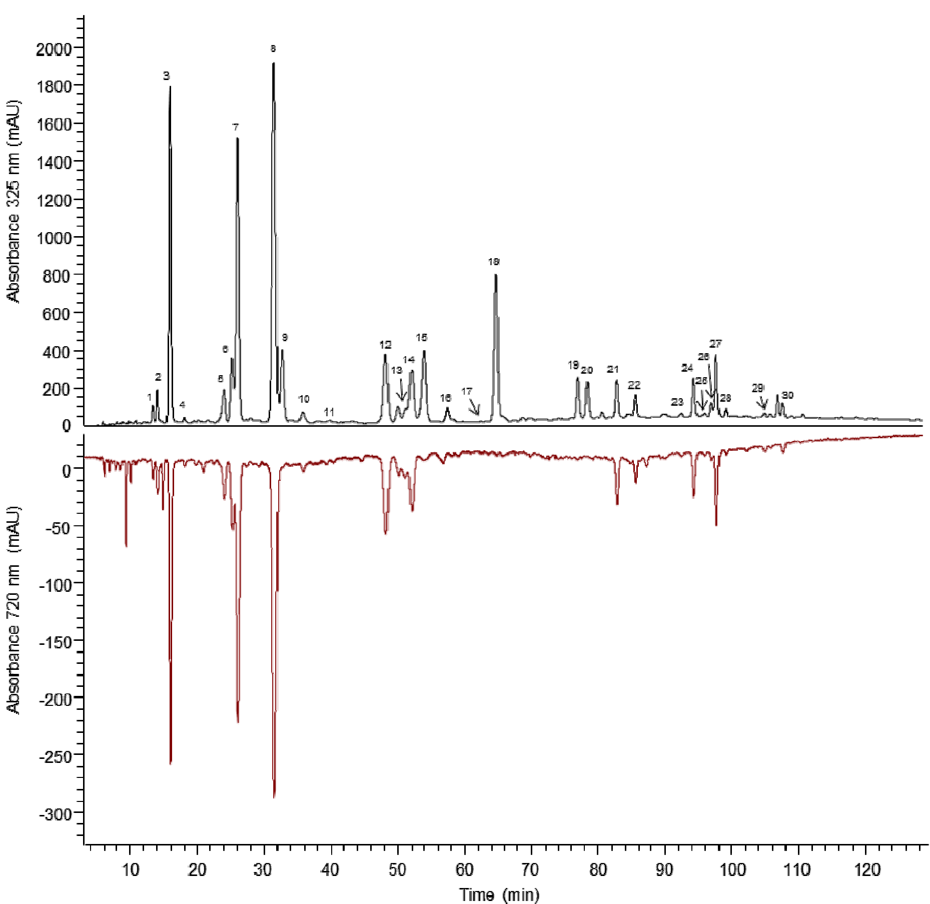

Figure 2. On-line HPLC ABTS$^+$ traces of Robusta coffee. 1=CQA, 2=CQA, 3=3-CQA, 4=3-*p*CoQA, 5=CQA, 6=1-CQA, 7=4-CQA, 8=5-CQA, 9=3-FQA, 10=CQAL, 11=4-*p*CoQA, 12=3-CQAL, 13=CQAL, 14=4-CQAL, 15=4-FQA, 16=1,3-diCQA, 16= FQA, 18=5-FQA, 19=FQAL, 20=4-FQAL, 21=3,4-diCQA, 22=3,5-diCQA, 23=1,5-diCQA, 24=4,5-diCQA, 25=3F5CQA, 26=3F4CQA, 273C4FQA, 28=3C5FQA, 29=4F5CQA, 30=4C5FQA.

Although the overall contents of CGAs were not greatly different for both species, the CGAs composition differed considerably. Arabica coffee had approx. 30 % higher amounts 3-, 4-, and 5-caffeoylquinic acids, whereas Robusta coffee showed 2-fold higher levels of FQAs and FQLs and almost 5-fold higher amounts of CFQAs. These findings are in accordance with other studies on coffee chlorogenic acids that report higher amounts of CGAs with a ferulic acid moiety in Robusta coffee varieties (Clifford, 1997; Clifford, 2000). As regards the coffee making procedure, filter and espresso, higher amounts of CGAs were found in espresso coffee, most likely due to the higher coffee to water ratio and the pressure applied during extraction that leads to a more concentrated coffee brew.

On-line ABTS chromatographic analysis was used to assess the antioxidant capacity of each single CGA present in the coffee samples. After separation of coffee chlorogenic acids on the HPLC column, the eluate was mixed with stabilized $ABTS^+$ radical solution, which has a deep blue colour. Any quenching of the radical resulted in a loss of colour, which was detected by monitoring the absorbance at 720 nm giving a negative peak on the HPLC traces as illustrated. The antioxidant capacities of individual compounds were added together to give the total HPLC-derived antioxidant capacity, that was used to determine the contribution of each single compound to the total antioxidant capacity of CGAs. Caffeoylquinic acids showed the highest contribution to the total antioxidant capacity (75-84 %), followed by CGLs (11-14%), diCQAs (4-8 %), CFQAs (0-5 %), and *p*CoQAs (0-1 %). None of the FQAs and FQLs exhibited $ABTS^+$ scavenging capacity. Similar findings were reported by Rice-Evans et al. (1997) and Stalmach et al. (2006) indicating that methylation at the 3'-hydroxyl group has an adverse effect on antioxidant activity.

To further investigate the influence of chemical structure on antioxidant capacity, Trolox equivalent antioxidant capacity (TEAC) values were calculated for the different chlorogenic acid subgroups. The antioxidant capacity exhibited by the different chlorogenic acid subgroups depends on the number of hydroxyl groups in the molecule (Nardini et al., 1995; Rice-Evans et al., 1997). This was also observed in the current study. Dicaffeoylquinic acids exhibited the highest antioxidant capacity because of the presence of two caffeic acid moieties, each one with two hydroxyl groups. CQAs, with only one caffeic acid group exhibited half the antioxidant capacity of diCQAs. The same values were observed for CFQAs with one caffeic and one ferulic acid moiety, most likely due to the absence of antioxidant capacity of ferulic acid groups as discussed above. CQLs, typical products of coffee roasting (Farah et al., 2005) showed slightly lower TEAC values than their precursors, the CQAs. This indicates that the lactonization seems to have a reducing effect on the $ABTS^+$ scavenging capacity of the compound. Chlorogenic acids with *p*-coumaroylquinic acid moieties exhibited the lowest TEAC values. The presence of only one hydroxyl group in the hydroxycinnamic acid moiety of these chlorogenic acids resulted in a reduction of the antioxidant capacity by 50% compared to CQAs. These results corroborate that the antioxidant capacity of chlorogenic acids depends primarily on the number of hydroxyl groups but is also influenced by other structural changes like methylation or lactonization.

Regarding to the total antioxidant capacities of CGAs of the two coffee varieties analysed in this study, the higher values obtained for Arabica coffee could be explained by differences in the chlorogenic acid composition. As described above, Arabica coffee showed higher levels of CQAs which possess high TEAC values, whereas Robusta coffee was rich in FQAs and FQLs, which do not exhibit any $ABTS^+$ scavenging activity at all.

To assess the contribution of the CGAs to the overall antioxidant capacity of coffee, samples were analysed using the on-line ABTS method described above but after removing the column from the HPLC system. Data obtained allowed to determine the antioxidant capacity of all coffee components including those that did not elute from the chromatographic column, such as high molecular weight Maillard reaction products. The comparison of the overall antioxidant capacity with the HPLC-derived antioxidant capacity revealed that only 7-16 % of the antioxidant capacity of coffee brews corresponds to the chlorogenic acids. These values were lower for espresso coffee brews (7-11 %) than those for filter coffee brews (12-16 %). Moreover, Robusta coffee exhibited lower values than Arabica coffee when the same extraction procedure (espresso or filter coffeemaker) was used. The results obtained in the current study indicate that the remaining 84-93% of the overall antioxidant capacity must be ascribed to other compounds different to chlorogenic acids that did not elute from the HPLC column. These compounds were extracted in higher amounts by the espresso coffeemaker and were present in higher amounts in Robusta coffee. As mentioned previously, those compounds that did not elute from the HPLC column might correspond to the high molecular weight Maillard reaction products (MRPs) resulting from the coffee roasting process. Other authors suggested that MRPs may contribute to higher degree to the overall antioxidant capacity of coffee than chlorogenic acids in dark roasted coffee (Smrke et al., 2013). These findings seem to contradict the previously discussed ESR spectroscopy results which showed that phenolic antioxidant capacity evaluated by Fremy´s salt was almost four times higher than roasting-induced antioxidant capacity measured with TEMPO. The apparent inconsistency might be explained by the fact that the on-line ABTS assay measures the antioxidant capacity of the free CGAs present in the coffee samples but does not take into account CGAs and other coffee phenolics bound to melanoidins, whereas Fremy´s salt assay evaluates the antioxidant capacity of free and bound coffee phenolics (Bekedam et al., 2008b). In addition, synergistic effects between CGAs and other coffee antioxidants might increase the antioxidant capacity exhibited by individual CGAs as measured by the on-line ABTS technique.

To go deeper into the knowledge of the extractability of the phenolics in the coffee brews, HPLC-PDA-MS^n was used to identify and quantify all the chlorogenic acids found in coffee brews in the three fractions for espresso coffee (F1-F3) and 5 fractions for filter coffee (F1-5) collected sequentially during the brewing process.

Extraction profile for espresso coffees revealed that approx. 70 % of the chlorogenic acids present in the coffee brew were extracted during the first 8 seconds (F1) of the extraction process. During the next 8 seconds (F2) approx. 20% were extracted, whereas the remaining

10% were extracted during the final stage of the brewing process (last 8 seconds, F3). The extraction profiles of the individual compounds were very similar, except for diCQAs and CFQAs which showed a slower extraction accounting F1 for ~62 %, F2 for ~24 % and F3 for ~13 %, This could be because these compounds are those with the highest molecular weight.

In contrast to espresso coffee, extraction profiles of filter coffee showed lower amounts of chlorogenic acids per 100mL in F1 (first 75 seconds) than in F2 (75-150s). The lowest chlorogenic acid concentration was obtained for F3 (150-225s), but CGAs concentration showed a steady increase (F4 and F5) towards the end of the extraction process. However, when the volumes of the different fractions were taken into account, data revealed that only about 18 % of the chlorogenic acids present in a coffee brew were extracted during the first stage of the brewing process (F1), whereas approx. 60% of the total CGAs were extracted during the next 150 seconds (F2 and F3), and only 20% of these compounds were extracted during the final 150 seconds (F4 and F5). The different extraction profiles found in espresso and filter coffee brews are consistent with the reults previously described on CQAs extractabilty. The technological factors responsible for these differences were already discussed.

In summary, although total CGA amounts were not greatly different for both species, Robusta and Arabica, the latter one showed higher levels of CQAs, whereas Robusta coffee was rich in FQAs and FQLs. It was also confirmed that an increase in hydroxyl group promotes free radical scavenging activities of chlorogenic acids, whereas methylation of the hydroxyl group at the position 3 of the hydroxycinnamic acid and lactonization of the quinic acid moiety resulted in its decrease. Radical scavenging capacity of chlorogenic acids, however, accounted for only 7-16 % of the overall capacity of coffee brews suggesting that high molecular weight Maillard reaction products might highly contribute to the antioxidant capacity of coffee brews. Thus, further studies about responsible compounds on antioxidant capacity of coffee brews are needed.

Catabolism of coffee phenolics by human colonic microbiota

The health benefits ascribed to the chlorogenic acids (CGAs), the main (poly)phenols in coffee depend on their bioavailability. Because the fate of (poly)phenols is very much dependent upon any structural changes that occur during the passage through the gastrointestinal tract (Selma et al., 2009; Williamson & Clifford, 2010), the identification and quantification of microbial catabolites produced in the large intestine are of importance in the context of overall bioavailability and potential health benefits of dietary (poly)phenolics. Thus, espresso coffee was incubated with human faecal samples, and HPLC-MS and GC-MS were used to monitor CGA breakdown and identify and quantify the catabolites produced by the colonic microflora.

A total of 15 CGAs were identified and quantified in the espresso coffee along with four additional components that were partially identified as CQAs and one as a FQL but it was not possible to determine the respective position of the caffeic acid and ferulic acid moiety. The overall CGA content of the espresso coffee was 80.0 ± 2.4 μmol per 0.5 g of freeze dried coffee.

The main group of CGAs, the CQAs, represent 61 % of the total CGA content, followed by the FQAs (18 %), the CQLs (12 %), the FQL (4 %), the diCQAs (3 %), and the CFQAs (2 %). 5-CQA was the main compound and accounted for 24 % of the total CGA content.

For the in vitro fermentation, 500 mg freeze-dried espresso coffee was incubated 0 h, 1 h, 2 h, 3 h, 4 h, and 6 h, under anaerobic conditions, with fresh faecal samples from three healthy donors in the presence of glucose. Analysis of aliquots of incubates by HPLC-PDA-MS^n and GC-MS revealed that that CGAs are a good substrate for the enzymes of the human gut microbiota as they were almost completely degraded after 3-4 h of faecal fermentation.

Potential catabolic steps occurring in the faecal incubates are illustrated in **Figure 3**. The breakdown of the principal CGAs in the coffee, CQAs and FQAs is probably the result of the action of bacterial esterases, in as much as the first catabolic event was the appearance of caffeic acid and ferulic acid resulting from cleavage of quinic acid from the hydroxycinnamate moiety. Several colonic bacteria, including *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri*, express cinnamoyl esterase (Couteau et al., 2001), which would catalyse such a cleavage. The appearance of low amounts of caffeic acid and ferulic acid was transient, with maximum quantities occurring at 1 h This would appear to be a consequence of their further metabolism, via reduction of the side chain double bond by bacterial reductase activity, which led to the appearance of dihydrocaffeic acid and dihydroferulic acid. The two dihydro derivatives were the major end products of the faecal fermentation of the coffee CGAs.

As well as dihydrocaffeic acid and dihydroferulic acid, 3-(3'-hydroxyphenyl)propionic acid was detected after 1-2 h of fermentation. Formation of the phenylpropionic acid was concomitant with reduced concentrations of dihydrocaffeic acid, as a result of 4'-dehydroxylation. 3-(3'-hydroxyphenyl)propionic acid was previously reported to be the main catabolite found after incubation of 1 µmole 5-CQA with human faecal microbiota (Gonthier et al., 2006). In the present study with a more substantial amount of CGA substrate much higher but variable amounts of 3-(3'-hydroxyphenyl) propionic acid were found depending on the donor.

Further degradation of 5-CQA to form 3-(phenyl)propionic acid, phenylacetic acid, benzoic acid and their respective hydroxy derivatives, has been reported previously (Selma et al., 2009). However this study, and that of Gonthier et al. (2006), focused on the colonic catabolism of pure reference compounds and did not take into account possible matrix effects occurring in incubations with complex mixture of dietary CGAs present in coffee. In the current investigation six catabolites resulting from demethoxylation, dehydroxylation and decarboxylation were found in low and variable amounts. Removal of the 3′-methoxy group of dihydroferulic acid would lead to the formation of 3-(4'-hydroxyphenyl)propionic acid, levels of which increased after 4-6 h fermentation. Dehydroxylation of 3-(4'-hydroxyphenyl)propionic acid and/or its 3'-hydroxy isomer would result in the small increases in 3-(phenyl)propionic acid observed after 3-4 hours of fermentation. This compound was proposed as a biomarker of colonic degradation of 5-CQA by Rechner et al. (2004). Shortening of the three carbon side chain of 3-phenylpropionic acid would lead to the formation of phenylacetic acid. However, this would appear not to be a major

catabolic step since low levels of phenylacetic acid were detected at the initial time point of fermentation (0 h) and the amounts did not increase substantially during incubation possibly as a consequence of decarboxylation resulting in its conversion to benzoic acid as illustrated in **Figure 3**.

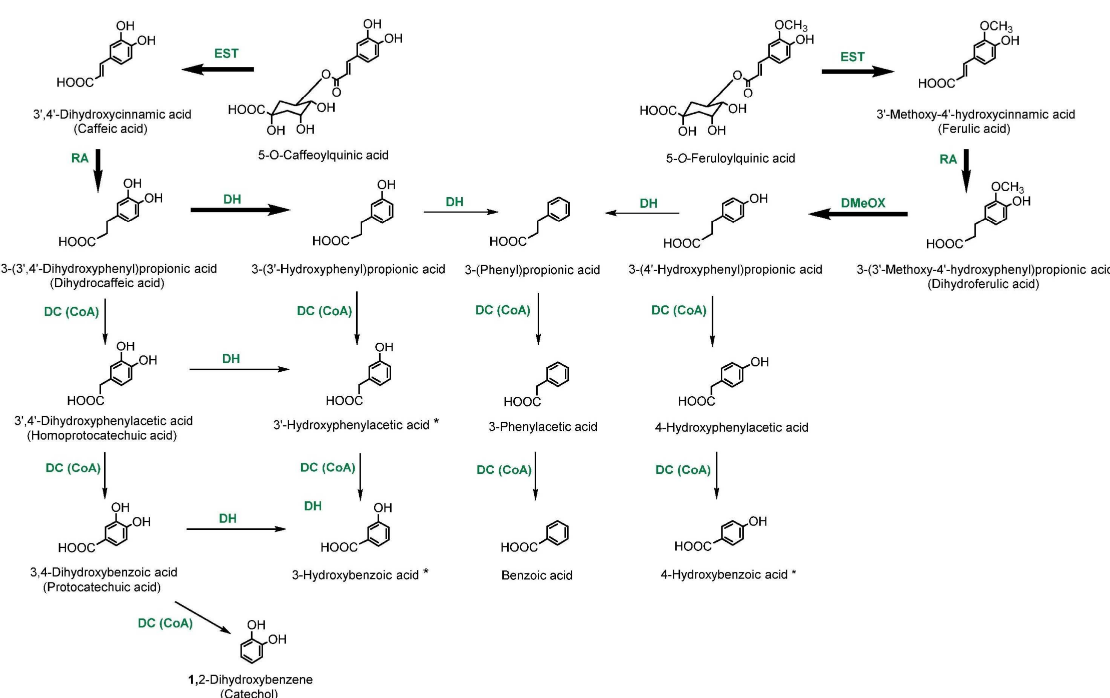

Figure 3. Proposed catabolic pathways for CGA microbial degradation in the colon after ingestion of espresso coffee. Asterisk next to a compound name indicates trace amounts. Bold arrows indicate major pathways. EST, esterase; RA, reductase; DH, dehydrogenase; DMeOX, demethoxyesterase; DC (CoA), decarboxylation (co-enzyme A mediated).

3,4-Dihydroxybenzoic acid (protocatechuic acid) was found in faecal samples from two volunteers and in trace amounts from the third. This compound could be formed via α-oxidation steps from dihydrocaffeic acid with 3′,4′-dihydroxyphenylacetic acid (homoprotocatechuic acid) acting as an intermediate. The dihydroxyphenylacetic acid was, however, not detected in any of the faecal samples, which, arguably, is indicative of its rapid rate of conversion to 3,4-dihydroxybenzoic acid. 4′-Dehydroxylation would then convert 3,4-dihydroxybenzoic acid to 3-hydroxybenzoic acid while, alternatively, decarboxylation would result in the production of 1,2-dihydroxyphenol (catechol) as illustrated in **Figure 3**.

Benzoic acid and 2-dihydroxyphenol occur in coffee as a result of the roasting process (Rahn & König, 1978; Nunes & Coimbra, 2010) which would explain why they were already present in the samples taken at 0 h. Nevertheless the levels of both compounds varied over the 6 hour faecal fermentation period indicating that the amounts found might be partially the result of bacterial catabolism of CQAs. Benzoic acid, however, can also have been formed from quinic acid by the gut microbiota (Williamson & Clifford, 2010). Three further phenolic acids were detected in the faecal incubates in trace amounts, namely 3′-hydroxyphenylacetic acid, 3-

hydroxybenzoic acid and 4-hydroxybenzoic acid that might result from 3-(3'-hydroxyphenyl)propionic acid and 3-(4'-hydroxyphenyl)propionic acid being subject to α-oxidation. None of these phenolic acids were present in the control or in the coffee samples indicating they are CGA degradation products.

The data obtained with the faecal fermentations suggest that bacteria present in the human gut are capable of further demethyoxylation, dehydroxylation and decarboxylation of dihydrocaffeic acid, dihydroferulic acid and 3-(3'-hydroxyphenyl)propionic acid. However, the corresponding catabolic routes can be considered as minor pathways of coffee CGA degradation, since the amounts found for the resulting compounds were very low. From the catabolites detected during the faecal fermentation and the amounts found we propose a pathway with major and minor routes of colonic CGA degradation after coffee ingestion (**Figure 3**).

Total amounts of catabolites found in V1 and V3 after 6 h of incubation were significantly higher ($p<0.05$) than the CGA content of the coffee used for the faecal fermentation. Several studies have confirmed the incorporation of chlorogenic acids and other phenolic compounds into coffee melanoidins, the high molecular brown end products of the Maillard reaction formed during coffee roasting (Bekedam et al., 2008c; Nunes & Coimbra, 2010). A partial release of these compounds during the faecal fermentation could have increased the amounts of phenolic acids and might explain the differences found between amounts of CGAs incubated and the amounts of catabolites found at the end of the fermentation.

Interindividual differences in the catabolism of phenolic compounds by gut microbiota have been extensively reported (Selma et al., 2009; Jaganath et al., 2009; Gross et al., 2010; González-Barrio et al., 2011) and were also found in the present study. The observed differences can be attributed to the well-known different bacterial populations present in the individual faecal microbial communities, harbouring specific enzymatic capacities (Zoetendal et al., 2008). The rate and extend of the degradation of the coffee CGAs showed a clear and statistically significant ($p<0.05$) influence of the composition of the gut microbiota. The inter-individual differences were particularly noticeable for volunteer 2. While degradation profiles obtained for V1 and V3 differed only in the breakdown rate, V2 showed an incomplete degradation of FQAs and FQL, with recovery rates up to 60 % after 6 h of faecal fermentation. Other minor CGAs, such as diCQAs, CFQAs, were also degraded to a lesser extent in samples from V2. Breakdown rates for CQAs and CQLs, however, were similar for all volunteers, and as these representatives made up 72 % of the total chlorogenic acid amount, CGA degradation by gut microbiota was for all volunteers between 80-100 %. Differences between the donors were also observed in the catabolite profiles. Samples from volunteer 2 showed significantly lower levels of caffeic acid and ferulic acid. Nevertheless dihydrocaffeic acid and dihydroferulic acids were the main end catabolites of faecal fermentation also for volunteer 2. Contrary to volunteers 1 and 3, 3-(3′-hydroxyphenyl)propionic acid and its 4′-hydroxy isomer were not found in fermentations with faecal material from volunteer 2. The inter-individual differences indicate that the composition of the gut microflora and thus the presence of certain bacterial species might

have a great influence on the formation of catabolites, especially those resulting from minor pathways.

The results obtained in this in vitro study are in accordance with those on the bioavailability of CGAs in humans in which the main colon-derived metabolites in plasma and/or urine after ingestion of coffee were dihydrocaffeic acid, dihydroferulic acid and their sulfated and glucuronide metabolites (Stalmach et al., 2009). There was an absence of glucuronide and sulfate conjugates in faecal fermentations demonstrating that they are not of bacterial origin but instead are probably phase II hepatic products and/or are formed in epithelial cells during passage through the colon en route to the circulatory system. The same probably also applies i) to co-enzyme A mediated metabolism of ferulic acid to feruloylglycine and ii) to the catechol-*O*-methyltransferase catalysed reactions that *in vivo* result in the metabolism of caffeic acid to isoferulic acid (Stalmach et al. 2009, 2010), conversions that did not occur in the faecal incubations.

In conclusion, all coffee CGAs were rapidly degraded by the colonic microflora. A total of 11 catabolites were identified during the 0–6 h of faecal fermentation showing that the CGAs were extensively catabolized by the microorganisms present in the human faecal samples. The rate and extent of the degradation showed a clear influence of the composition of the gut microbiota of individual volunteers. However, the amounts found indicate major degradation pathways leading to the accumulation of dihydrocaffeic acid and dihydroferulic acid. It is known that these catabolites are better absorbed that their CQAs and FQAs and *in vivo* studies have confirmed free and sulfated dihydrocaffeic and dihydroferulic acids as main metabolites in plasma and urine after ingestion of coffee. Thus, bioavailability of CGAs depends highly on microbial conversions in the colon. Nevertheless, dihydrocaffeic, dihydroferulic acids, other minor catabolites and their parent CGAs that are present in upper regions of the large intestine where they might act as antioxidants and prebiotics explaining at least partly some of the health benefits at colon level ascribed to coffee consumption. It is important that investigations of the potential protective colonic effects of coffee consumption with ex vivo cell systems should make use of this mixture of ingested CGAs and their colonic catabolites at doses that prevail in the proximal gastrointestinal tract.

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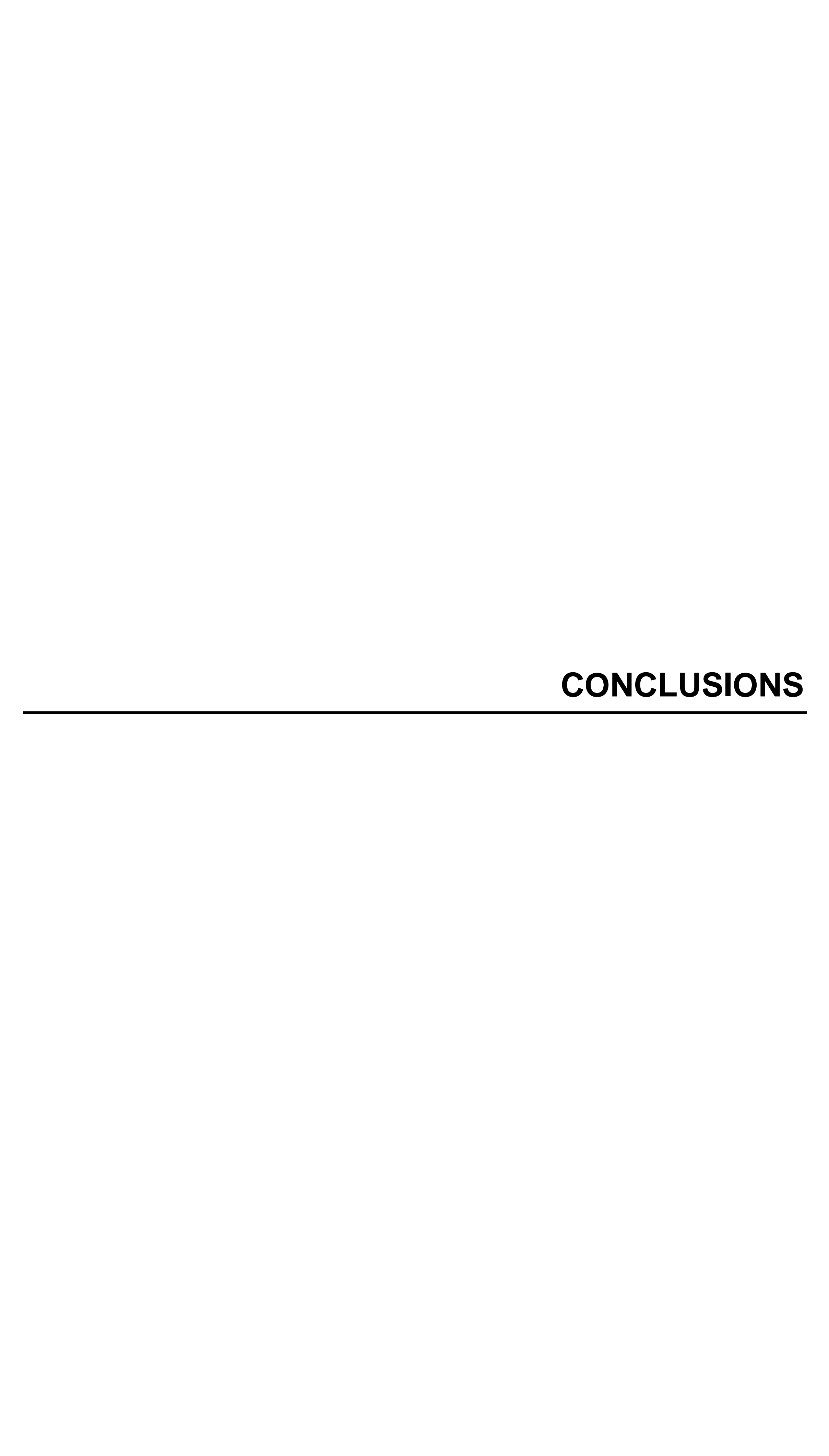


CONCLUSIONS

Objective 1

1. The addition of sugar during roasting (torrefacto) is a technological process with a lower influence on the presence of antioxidant compounds and, consequently, overall antioxidant capacity than roasting degree, coffee variety (Arabica and Robusta) and origin. In fact, although torrefacto increases the radical quenching capacity (DPPH) in coffee, it hardly affects the final content of antioxidant compounds like phenolics.
2. The absorbance at 420 nm is a good marker for the torrefacto roasting process, whereas the roasting degree might be better characterized by L* values.

Objective 2

3. Brewing time plays a key role in antioxidants extractability of coffee. Moreover, higher water pressure increases phenolic and non-phenolic antioxidants extraction speed like in espresso coffee, but parameters like turbulence and longer contact time, typically of a filter coffeemaker, should be considered in order to increase extraction efficiency, mainly in less polar antioxidant compounds like dicaffeoylquinic acids.

Objective 3

4. Volatile Maillard reaction products present in coffee contribute very little (<3.3 %) to the antioxidant capacity in comparison to other coffee antioxidants, such as phenolics and melanoidins, even at 100-fold coffee concentration.
5. Although some coffee volatile compounds like heterocyclic ones (furans, pyrroles and thiophenes) may act as antioxidants in high doses, it is necessary to evaluate their antioxidant capacity at the actual concentrations in foods.

Objective 4

6. The identification and quantification of 22 chlorogenic acids by HPLC-PDA-MS^n shows that the total chlorogenic acids content in coffee brews is not greatly different for both species, but Arabica coffee shows higher levels of caffeoylquinic acids, whereas Robusta coffee is rich in feruloylquinic acids and their lactones.
7. The antioxidant capacity of coffee chlorogenic acids can be mainly attributed to caffeoylquinic acids (75-84 %), followed by their lactones (11-14%), dicaffeoylquinic acids (4-8 %), caffeoyl-feruloyl-quinic acids (0-5 %), and *p*-coumaroylquinic acids (0-1 %). Moreover, none of the feruloylquinic acids and their lactones exhibit $ABTS^+$ scavenging capacity. The TEAC values for the chlorogenic acids subgroups confirm that an increase in hydroxyl groups promotes free radical scavenging activities of chlorogenic acids, whereas methylation of the hydroxyl group at the position 3 of the hydroxycinnamic acid and lactonization of the quinic acid moiety results in its decrease.
8. The $ABTS^+$ scavenging capacity of chlorogenic acids accounts for only 7-16 % of the overall capacity of coffee brews suggesting that high molecular weight Maillard reaction products, as well as phenolics bound to melanoidins might contribute to a higher degree to the antioxidant capacity of coffee brews.

Objective 5

9. Faecal fermentation results show that all coffee clorogenic acids are rapidly and extensively catabolized by the colonic microflora, leading to the formation of up to 11 catabolites.
10. An incomplete degradation of feruloylquinic acids and their lactones (40 % after 6 h) and significantly lower levels of caffeic and ferulic acids in faecal fermentation samples for volunteer 2 indicate that the inter-individual differences in composition of the gut microflora might have an influence on the rate and extent of the chlorogenic acids degradation.
11. The amounts of the different colonic catabolites found indicate major degradation pathways leading to the accumulation of dihydrocaffeic acid and dihydroferulic acid that are better absorbed than their parent compounds. Thus, bioavailability of chlorogenic acids depends greatly on microbial conversions in the colon.
12. Dihydrocaffeic, dihydroferulic acids, other minor catabolites and their parent chlorogenic acids that are present in the large intestine where they might act as antioxidants and prebiotics explaining at least partly some of the health benefits at colon level ascribed to coffee consumption.

Objetivo 1

1. El tueste torrefacto influye en menor proporción que el grado de tueste, y la variedad y origen del café en el contenido de compuestos antioxodantes y, por consiguiente, en la capacidad antioxidante del café. De hecho, aunque el tueste torrefacto aumenta la capacidad de captación de radicales libres (DPPH), apenas afecta al contenido de compuestos antioxidantes como los fenólicos.
2. La absorbancia a 420 nm es un buen marcador del tueste torrefacto, sin embargo el grado de tueste se caracteriza mejor por el valor L*.

Objetivo 2

3. El tiempo de elución en la preparación de una bebida de café tiene un papel clave en la extracción de antioxidantes. Además, una mayor presión del agua, propia de la cafetera expreso, aumenta la velocidad de extracción de compuestos fenólicos y no fenólicos. En cambio, otros factores tecnológicos como las turbulencias y un mayor tiempo de contacto entre el agua y el café, típicos de una cafetera filtro, favorecen la eficacia de la extracción de los compuestos antioxidantes, principalmente los de menor polaridad, como los diCQA.

Objetivo 3

4. Los compuestos volátiles resultantes de la reacción de Maillard presentes en el café contribuyen mínimamente (<3,3 %) a la capacidad antioxidante global del café en comparación con otros compuestos antioxidantes, como los fenólicos y las melanoidinas, incluso a concentraciones 100 veces superiores a las encontradas en el café.
5. Aunque algunos compuestos volátiles heterocíclicos (furanos, pirroles y tiofenos) pueden actuar como antioxidantes a altas concentraciones, es necesario evaluar su capacidad antioxidante a las concentraciones realmente presentes en los alimentos.

Objetivo 4

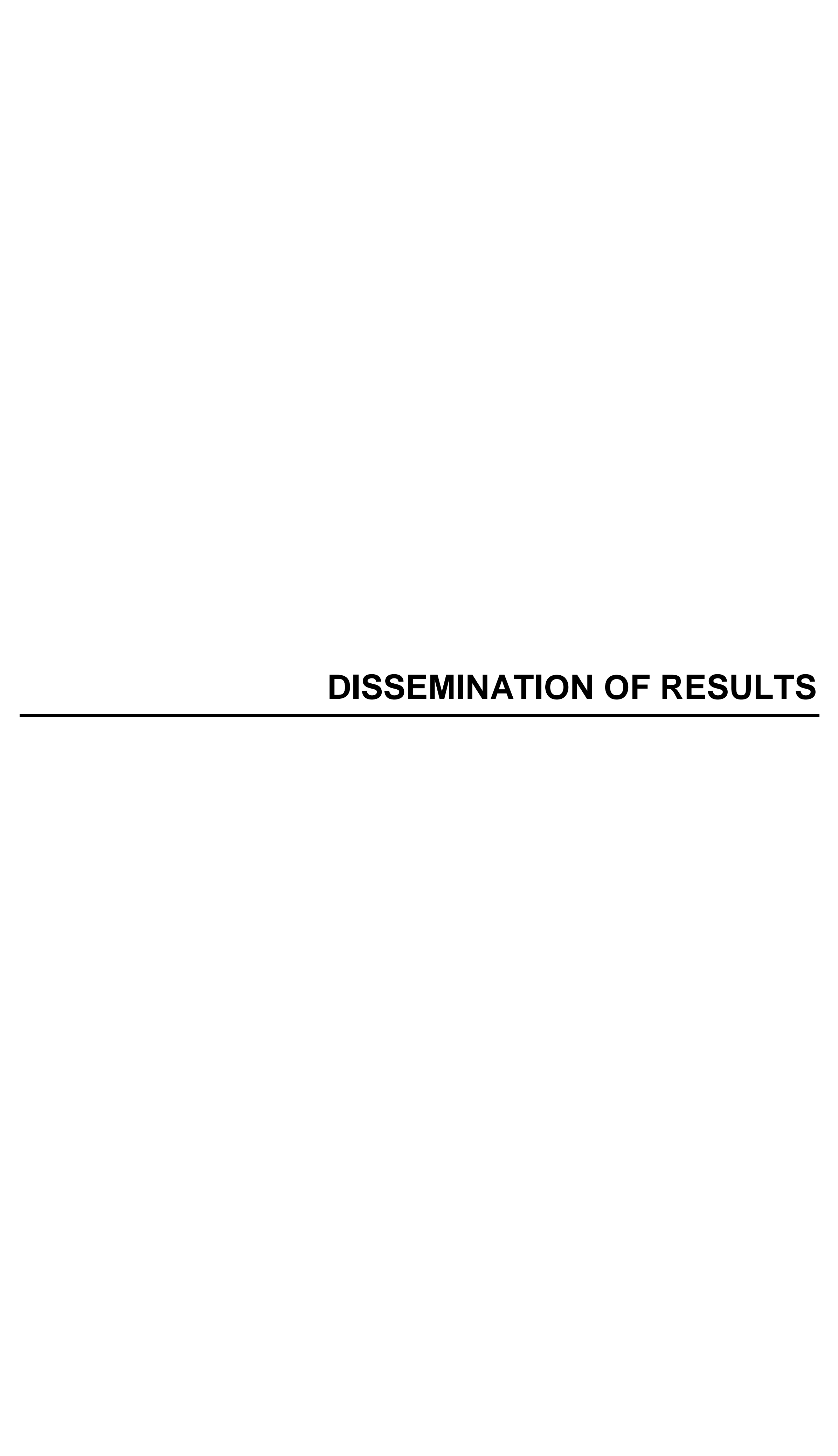
6. La identificación y cuantificación de 22 ácidos clorogénicos por HPLC-PDA-MS^n lleva a concluir que el contenido total de ácidos clorogénicos no es muy diferente entre las dos especies de café, aunque el café Arábica presenta mayores niveles de ácidos cafeoilquínicos, mientras que la variedad Robusta es más rica en ácidos feruloilquínicos y sus correspondientes lactonas.
7. La capacidad antioxidante de los ácidos clorogénicos presentes en el café se debe mayoritariamente a los ácidos cafeoilquínicos (75-84%), seguidos por sus correspondientes lactonas (11-14%), los ácidos dicafeoilquínicos (4-8%), los cafeoilferuloilquínicos (0-5%), y los *p*-cumaroilquínicos (0-1%). Además, ninguno de los ácidos feruloilquínicos y sus correspondientes lactonas presentan capacidad reductora del radical $ABTS^+$. Los valores de la capacidad antioxidante equivalente al Trolox (TEAC) de los diferentes subgrupos de ácidos clorogénicos confirman que un

incremento en el número de grupos hidroxilo en la estructura de los ácidos clorogénicos aumenta su capacidad reductora del radical $ABTS^+$, mientras que la metilación del grupo hidroxilo en la posición 3 del ácido hidroxicinámico y la lactonización del ácido quínico disminuyen dicha capacidad.

8. La capacidad reductora del radical $ABTS^+$ de los ácidos clorogénicos supone solo un 7-16 % de la capacidad antioxidante de las bebidas de café, lo cual sugiere que los compuestos de alto peso molecular resultantes de la reacción de Maillard así como compuestos fenólicos unidos a melanoidinas podrían contribuir en una alta proporción a la capacidad antioxidante de las bebidas de café.

Objetivo 5

9. Los resultados obtenidos durante la fermentación fecal del café muestran que la microflora del colon metaboliza rápida y ampliamente los ácidos clorogénicos presentes en el café dando lugar a la formación de 11 catabolitos.
10. La degradación incompleta de los ácidos feruloilquínicos y sus lactonas (40% después de 6 h) y los niveles significativamente más bajos de los ácidos cafeico y ferúlico en las muestras procedentes de uno de los voluntarios indican que las diferencias inter-individuales en la composición de la microflora intestinal puede tener influencia en la velocidad y extensión de la degradación de los ácidos clorogénicos.
11. Las principales rutas de degradación de los ácidos clorogénicos muestran una acumulación de los ácidos dihidrocafeico y dihidroferúlico que se absorben mejor que los ácidos clorogénicos originales. Por lo tanto la biodisponibilidad de los ácidos clorogénicos depende en gran medida del metabolismo de la microbiota del colon.
12. Los ácidos dihidrocafeico y dihidroferúlico, otros catabolitos minoritarios y los ácidos clorogénicos no catabolizados que están presentes en el intestino grueso podrían actuar como antioxidantes y prebióticos explicando, al menos en parte, algunos de los efectos beneficios para la salud a nivel del colon atribuidos al consumo de café.



DISSEMINATION OF RESULTS

Publications

Iziar A. Ludwig, Jimena Bravo, M. Paz De Peña, Concepción Cid. (2013). Effect of sugar addition (torrefacto) during roasting process on antioxidant capacity and phenolics of coffee. *LWT – Food Science and Technology*, 51, 553-559.

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Iziar A. Ludwig, Lidia Sánchez, M. Paz De Peña, Concepción Cid. Modelling the contribution of volatile compounds to the antioxidant capacity of coffee. *Journal of Agricultural and Food Chemistry* (submitted).

Iziar A. Ludwig, , M. Paz De Peña, Concepción Cid, Alan Crozier. Assesing the contribution of phenolic and non-phenolic compounds to the antioxidant capacity of coffee brews (in preparation).

Conference communications

Iziar A. Ludwig, Ana I. Mudarra, Concepción Cid, and M. Paz De Peña. (2007). Oral communication: “Increasing antioxidant capacity of coffee by addition of sugar during roasting process”. In *Thermally Processed Foods: Possible Health Implications (COST-927),* Ebersberg, Germany. Book of Abstracts; page 9. (see annex).

Jimena Bravo, **Iziar A. Ludwig**, Isabel Juániz, M. Paz De Peña y Concepción Cid. (2010). Poster: “Influencia del origen en la capacidad antioxidante del café”. In *II Congreso de la Federación Española de Sociedades de Nutrición, Alimentación y Dietética (FESNAD*), Barcelona, Spain. Nutrición Hospitalaria 2010; Supl. 1, Vol. 25, pag. 100. ISSN: 0212-1611. (see annex)

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Iziar Ludwig, M. Paz De Peña, and Concepción Cid. (2011). Oral communication: “Unravelling the contribution of volatile compounds to the antioxidant capacity of coffee- A study on model systems”. In *First International Congress on CoCoTea*, Novara, Italy. Book of Abstracts and CD-Proceedings; pages 24 and 31. ISBN: 978-88-903360-0-3. (see annex)

Iziar Ludwig, Lidia Sánchez Ayaso, M. Paz De Peña, and Concepción Cid. (2011). Poster: “Unravelling the influence of brewing time on coffee chlorogenic acids extraction”. In *5th International Conference on Polyphenols and Health*, Sitges, Spain. Libro de Abstracts; page 176. (see annex)

Iziar Ludwig, Maizatul H. Omar, M. Paz De Peña, and Concepción Cid, Alan Crozier. (2012). Oral comunication: “Coffee phenolics degradation and formation of bioactive metabolites by human colonic microflora”. *Chemical Reactions in Foods VII*, Prague, Czech Republic. Book of Abstracts; page 59. ISBN: 978-80-7080-836-8. (see annex)

Annex

COST-927

Thermally processed foods: possible health implications

September 5th - 6th, 2007

Ebersberg – Germany

INCREASING ANTIOXIDANT CAPACITY OF COFFEE BY ADDITION OF SUGAR DURING ROASTING PROCESS

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Torrefacto is a roasting technique in which sugar is added to coffee at the end of the process. Initially, the aim of the sugar addition was to mask the negative sensory characteristics of low quality robusta coffees. However, the higher intensity of brown colour and body of coffee brews of torrefacto blends lead some segments of population to their consumption in several countries of Latin America and Southern Europe, like Spain and Portugal. The addition of sugar during roasting process (no more than 15 % by weight according to Spanish legislation RD 1372/1997) may increase the antioxidant capacity of coffee because sugar is one of the main precursors in the Maillard reaction. This hypothesis seemed to be confirmed in previous experiments of our group (Lopez-Galilea et al, 2006) in commercial coffees where coffee blends with high percentages of torrefacto roast had stronger antioxidant capacity than conventional roasted coffee. However, other factors, such as coffee variety and roasting degree, could also influence.
The aim of this work was to study the influence on the antioxidant capacity (DPPH and potential redox), browned compounds (A420nm) and polyphenols of the addition of increased sucrose amounts (0%, 5%, 10% and 15%) to Arabica Colombian coffee during roasting. Roasting process was standardized following the same temperature and time conditions, and the degree of roasting was controlled by weight loss (17-18%) and colour luminosity (L* 19-21).

DPPH and browned compounds were proportionally increased with sugar addition. In fact, an excellent and significant correlation between sucrose amount and browned compounds has been found. In torrefacto coffee, browned compounds could be Maillard reaction products, such as melanoidins, but mainly sugar caramelization products, and these compounds seems to be the most relevant in the antioxidant capacity of coffee measured by DPPH. In contrast, total polyphenols, 5-caffeoyl quinic, caffeic and ferulic acids and potential redox apparently were not affected by the addition of sugar. As potential redox evaluates the oxidation/reduction efficiency of all antioxidants, including the slow ones, and pro-oxidants in coffee, further researches should be needed to know the overall antioxidant capacity of torrefacto coffee.

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Lopez-Galilea, I., Andueza, S., di Leonardo, I., de Peña, M.P. and Cid, C. (2006). Influence of torrefacto roast on antioxidant and pro-oxidant activity of coffee. *Food Chem.,* 94, 75-80.

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II Congreso Fesnad, Barcelona 2010, “Hacia una alimentación responsable” 3 a 5 de marzo de 2010

http://www.fesnad.org/congresos/II_congreso_Fesnad/congreso.asp

INFLUENCIA DEL ORIGEN EN LA CAPACIDAD ANTIOXIDANTE DEL CAFÉ

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INTRODUCCIÓN

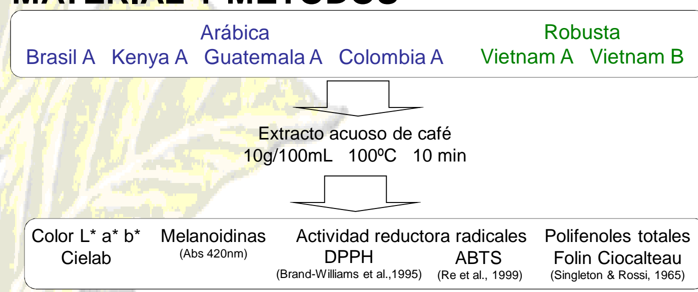
El café se ha propuesto como la primera fuente de antioxidantes entre las bebidas de la dieta española (Pulido et al., 2003) pudiendo tener un papel significativo en la prevención de enfermedades relacionadas con el estrés oxidativo como la diabetes, arteriosclerosis, enfermedades neurodegenerativas, cáncer y obesidad, con alta incidencia en los países más desarrollados.

Estudios previos de nuestro grupo de investigación han mostrado la influencia del tueste torrefacto y la preparación de la bebida de café en la capacidad antioxidante del café (López-Galilea et al., 2006 y 2007).

OBJETIVO

El objetivo de este trabajo consistió en el análisis de la influencia del origen y grado de tueste del café sobre su capacidad antioxidante.

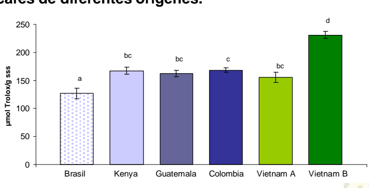
MATERIAL Y MÉTODOS



Análisis de Varianza (ANOVA)
Análisis Componentes Principales (PCA)

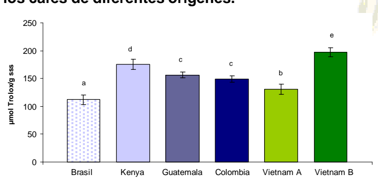
RESULTADOS Y DISCUSIÓN

Figura 1. Actividad reductora del radical ABTS de los cafés de diferentes orígenes.



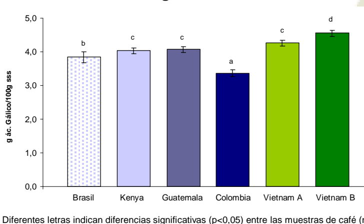
Diferentes letras indican diferencias significativas ($p<0,05$) entre las muestras de café (n=6).

Figura 2. Actividad reductora del radical DPPH de los cafés de diferentes orígenes.



Diferentes letras indican diferencias significativas ($p<0,05$) entre las muestras de café (n=6).

Figura 3. Contenido de los polifenoles totales de cafés de diferentes orígenes.



Diferentes letras indican diferencias significativas ($p<0,05$) entre las muestras de café (n=6).

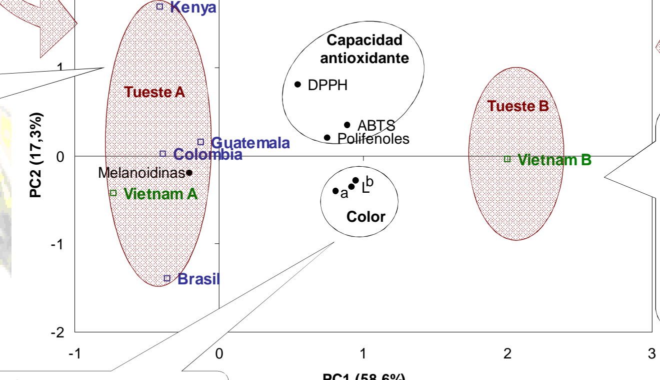
Tabla 1. Caracterización de los cafés de diferentes orígenes.

	Arábica				Robusta	
	Brasil A	Kenya A	Guatemala A	Colombia A	Vietnam A	Vietnam B
Melanoidinas (Abs. 420 nm)	$0.369±0.011^{c}$	$0.342±0.010^{a}$	$0.414±0.010^{d}$	$0.348±0.008^{a}$	$0.367±0.008^{bc}$	$0.354±0.006^{ab}$
L*	$25.70±0.15^{b}$	$25.11±0.01^{ab}$	$24.92±0.01^{a}$	$25.37±0.01^{ab}$	$25.41±0.69^{ab}$	$31.13±0.43^{c}$
a*	$12.88±0.09^{c}$	$12.40±0.02^{bc}$	$12.69±0.02^{ab}$	$12.75±0.02^{bc}$	$11.73±0.19^{a}$	$13.31±0.31^{c}$
b*	$16.51±0.08^{b}$	$15.58±0.02^{ab}$	$15.73±0.03^{a}$	$15.38±0.01^{a}$	$15.61±0.41^{a}$	$21.40±0.33^{c}$

Resultados expresados como media ± desviación estándar (n=6)
En cada fila, diferentes letras indican diferencias significativas ($p<0,05$) entre las muestras.

Valores altos de L* (Luminosidad) indican un grado de tueste inferior. El café Vietnam B muestra un grado de tueste significativamente inferior ($p<0,05$) a los cafés tueste A.

Figura 4. Análisis de Componentes Principales de cafés de diferentes orígenes.



Los cafés con similar grado de tueste (Tueste A: L*~25,21) aunque de diferentes orígenes presentaron capacidad antioxidante semejante.

Los cafés con diferente grado de tueste (Vietnam A L* 25,21, Vietnam B L* 31,13) presentan diferencias muy significativas ($p<0,05$) en la capacidad antioxidante (ABTS, DPPH, polifenoles totales).

Los cafés analizados se distinguieron significativamente por el grado de tueste (Tueste A- Tueste B).

CONCLUSIÓN

El Análisis de Componentes Principales permitió concluir que el tueste del café tiene mayor influencia sobre la capacidad antioxidante que el origen del mismo.

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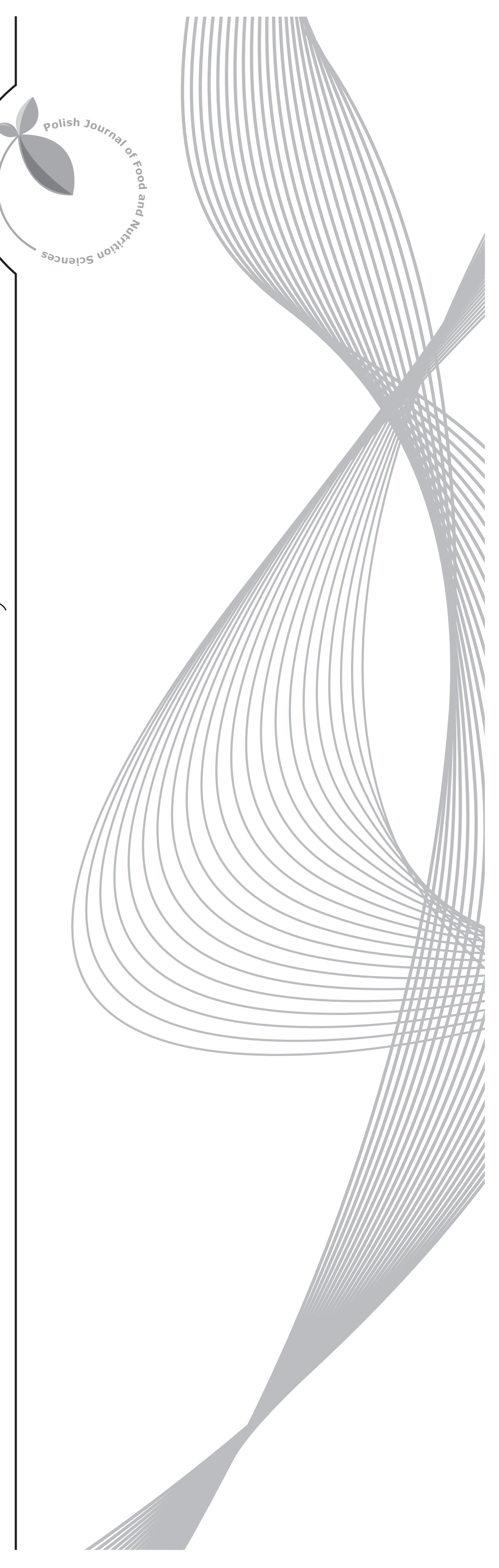

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mulated with F-100% flour, respectively. Similarly, total EAA content was found 9% and 6% higher in rye bread and ginger cake formulated with F-100% flour, respectively. In addition, rye bread formulated with whole rye flour exhibited larger content of total EAA (16%) than WB. Regarding protein quality indexes, F100% rye bread showed greater CS values compared to either F92% rye bread and WB, however, PER values were similar among wheat and rye breads. In the case of ginger cakes, CS and PER values were found slightly lower compared to WB. Hence, whole rye flour (100% extraction rate) could be use as an approach to improve the nutritional quality of traditional rye-based products.

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P199

Effects of Microwave and Hot-Air Popper Methods on Some Characteristics of Popcorn

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Key words: popcorn, microwave, hot-air popper, hexanal, textural properties

Popcorn is one of the most popular snack foods in the world. In this study three types of commercial popcorns seed packages including light butter flavour (14% fat), butter flavour (32% fat) and great buttery taste (44% fat) popped with two different methods; microwave oven and hot-air popper. The effects of popping methods and the types of popcorn seed products on popping volume (cm^3/g), popped kernel size (kernel number/10 g), moisture content, color values (L, a and b), hexanal content as indicator of oil oxidation and some textural characteristics (crispness and firmness) have been determined. Popcorn seed samples popped with hot-air popper showed higher popping volume and popped kernel size. Higher oil level increased the popped kernel size, but decreased the popping volume ($p<0.001$). The moisture contents of the samples in both methods varied between 0.89 and 3.03%. Samples popped with hot-air popper had higher moisture contents and higher L and a values ($p<0.05$). In both methods, oil levels did not significantly alter the moisture content and color values ($p>0.05$). Higher oil contents leaded to higher hexanal concentrations. Popcorn samples popped with microwave method had lower hexanal level ($p<0.001$) than their counterparts produced with hot-air popper. The oil level and popping method significantly ($p<0.05$) affected the textural properties of popcorn. Increasing the oil level and microwave application also increased the crispness and the firmness values. Overall quality of popcorns popped with hot-air method look preferable, but lower hexanal content of popcorns prepared with microwave method should be considered.

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P200

Influence of Temperature and Equilibration Time on the Quantification of Aroma Impact Compounds in Coffee Brews

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Key words: coffee, aroma, static headspace, coffee brews, aroma impact compounds

One of the most contributory factors for the high acceptability of coffee is its aroma, which involves more than 800 volatile compounds. The aim of this study was to optimize the methodology of the main aroma impact compounds extraction in coffee brews with the highest efficiency at lowest time using static headspace-gas chromatography-mass spectrometry (HS-GC-MS). Equilibration time and temperature were the factors studied to choose the optimal conditions for analyzing aroma compounds in coffee brews by a static headspace sampling extraction method.

Five temperatures of equilibration (50, 55, 60, 65 and 70°C) were studied. Seventy one volatile compounds were identified in Arabica filter coffee brews and the main aroma impact compounds in coffee brews (methanethiol, acetaldehyde, propanal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2,3-butanedione, 2,3-pentanedione, 3,5-dimethyl-3-ethylpyrazine and guaiacol) were quantified. Increased amounts of aroma impact compounds with temperature increase were found, except in methanethiol, 3,5-dimethyl-3-ethylpyrazine and guaiacol that decreased at 70°C due to thermal degradation. Consequently, 65°C was selected as extraction temperature. Then, the extraction of the aroma impact compounds was studied at five different equilibration times: 15, 25, 35, 45 and 55 min at 65°C. The maximum concentration in the majority of the quantified aroma impact compounds was obtained at an equilibration time of 25 min. In conclusion, the optimal conditions in static headspace for quantification of aroma impact compounds in coffee brews were 25 min at 65°C.

Acknowledgements: This research was funded by the Spanish Ministry of Science and Innovation (AGL2009–12052). We thank the Government of Navarra (Dpt. Industry and Dpt. Edu-

cation) for the grants given to L.S.A. and I.A.L, and the Unión Tostadora for providing the coffee samples.

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P201

How Does Affect Thermal Processing to Functional Properties of Bean Flours?

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Key words: dehydration, bean flours, functional properties

Research has emphasized the utilization of beans in the form of meal and flour for using as functional ingredients in food products. Dehydration is a technology classified as a high temperature process to produce a variety of foods and ingredients and offers numerous advantages including prolonged preservation time, high productivity and quality of resulting products. The objective of this study was to determine changes in functional properties of bean flours as affected by soaking, cooking and dehydration, with a view to providing useful information towards effective utilization of these legumes in various food applications. The raw legume flours exhibited low oil holding capacities, 1.10 mL/g and did not show any change by thermal processing, instead water holding capacities reached 3.45 mL/ g sample after dehydration. Emulsifying activity and foam capacity were higher in raw legumes than thermal processed samples. Nevertheless, swelling capacity showed great increases in legume flours after dehydration process. Thus, the study of the effect of dehydration on functional properties provides useful information for bean flours. These legume flours could be used as functional ingredients in food systems and incorporated into products such as bakery products, seasonings, and sausages among other but sensory and texture analyses of the products would be necessary.

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Effect of Frying Process on Antioxidant Capacity of Vegetable Oils, Meat and Potatoes

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Key words: antioxidant capacity, food, total phenolic content, frying

Vegetable oils, meats and French fries hold an important place in human diet. Vegetable oils are consumed as salad oils, cooking oils, and frying oils. Fried foods are very popular worldwide due to their delicious sensory characteristics. Despite common opinion, the frying process have almost the same or even lower effect on nutrient losses compared to other cooking methods [1,2]. In the frying process, vegetables, meat, potatoes or seafood, are brought in direct contact with hot oil. The food surface becomes golden yellow to dark brown and develops a pleasant fried food flavor. Frying temperature and frying time varies depends on products fried.

The antioxidant activity of studied food products and oils before and after the frying process was determined by a ferric reducing antioxidant power (FRAP) method. Moreover, the total content of phenolic compounds by the Folin-Ciocalteau method was analyzed. Meat products (poultry and pork) and French fries were fried in rapeseed oil, palm oil and olive oil with garlic. The antioxidant activity of meat and French fries before frying ranged between 9.0–15.3 μmol TE (Trolox)/100 g and 273.8– 276.7 μmol TE/100 g, whereas after frying values increased for meet samples from 31.1 μmol TE/100 g to 69.7 μmol TE/100 g, and decreased for French fries 75.4 μmol TE/100 g – 121.1 μmol TE/100 g. However, the antioxidant activity of oils varied from 113.3 μmol TE/100 g to 231.0 μmol TE/100g. The total phenolic compounds in all oils decreased in the range 3.3– 80.2% after frying. Principal component analysis (PCA) was applied for analysis of frying process impact on the antioxidant capacity and TPC of studied food samples.

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INFLUENCE OF TEMPERATURE AND EQUILIBRATION TIME ON THE QUANTIFICATION OF AROMA IMPACT COMPOUNDS IN COFFEE BREWS

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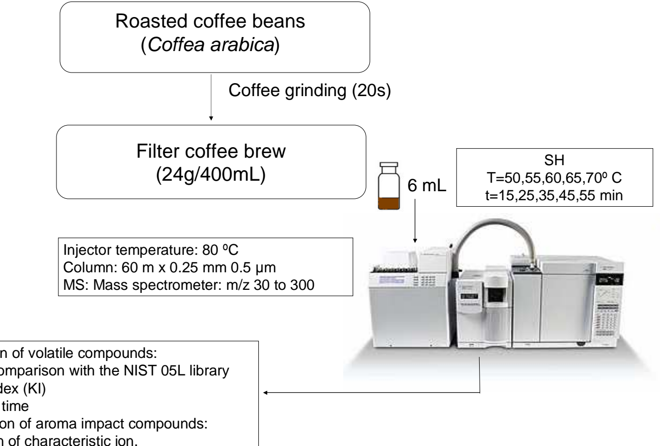
INTRODUCTION

One of the most contributory factors for the high acceptability of coffee is its aroma, which involves more than 800 volatile compounds. The coffee aroma is characterized by the presence of a wide range of volatiles belonging to several classes of compounds, present in variable concentrations. Although SPME is one of the most common methods to extract volatiles of headspace (López-Galilea et al., 2006), static headspace (SH) allows the extraction and injection automatically and directly to GC-MS.

OBJECTIVE

The aim of this study was to optimize the methodology of the main aroma impact compounds extraction in coffee brews (Sanz et al., 2001) with the highest efficiency at shortest time using static headspace-gas chromatography-mass spectrometry (SH-GC-MS). Equilibration time and temperature were the factors studied to choose the optimal conditions for analyzing aroma compounds in coffee brews by a static headspace sampling extraction method.

MATERIALS AND METHODS



RESULTS AND DISCUSSION

Figure 1. Chromatograms obtained at 50 and 65 ºC at 20 min.

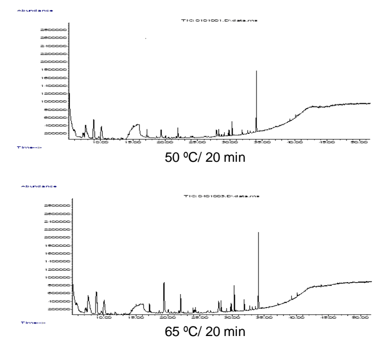


Figure 2. Effect of equilibration temperature on aroma impact compounds in Coffee Brews.

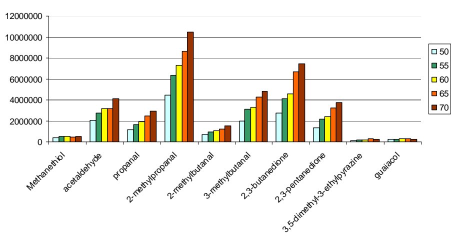
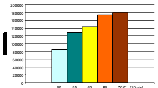


Figure 3. Effect of equilibration temperature on total volatiles area (x10^{-3}) in Coffee Brews.



Seventy one volatile compounds were identified in Arabica filter coffee brews. Three are sulfur compounds, 7 aldehydes, 3 esters, 16 furans, 8 ketones, 4 alcohols, 2 thiophenes, 6 pyrroles, 3 pyridines, 13 pyrazines, 2 thiazoles, 1 lactone, 1 phenolic, 1 acid and 1 alkadiene. The main aroma impact compounds in coffee brews (methanethiol, acetaldehyde, propanal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2,3-butanedione, 2,3-pentanedione, 3,5-dimethyl-3-ethylpyrazine and guaiacol) were quantified.

Five equilibration temperatures (50, 55, 60, 65 and 70ºC) in headspace were studied (Figures 1 to 3). Increased amounts of aroma impact compounds with temperature were found, except in methanethiol, 3,5-dimethyl-3-ethylpyrazine and guaiacol that decreased at 70ºC maybe due to thermal degradation. Moreover, the total volatiles areas also increased with equilibration temperature up to 65ºC. It was observed that there were not significantly differences between 65ºC and 70ºC. Consequently, 65ºC was selected as extraction temperature because higher temperatures might favour thermal degradation and artefacts formation.

To optimize static headspace equilibration time in aroma impact compounds extraction, five different equilibration times (15, 25, 35, 45 and 55 min) at 65ºC were studied (Figures 4 to 6). The maximum concentration in methanethiol, acetaldehyde, propanal, 2,3-butanedione and 2,3-pentanedione was obtained at 25 min of equilibration time. Moreover, the minimum time at which the maximum total volatiles area was obtained, was 25 min. So that, the optimal conditions in static headspace to evaluate the main aroma impact compounds in coffee brews are 65ºC at 25 min.

Figure 4. Chromatograms obtained at 25 and 55 min at 65 ºC.

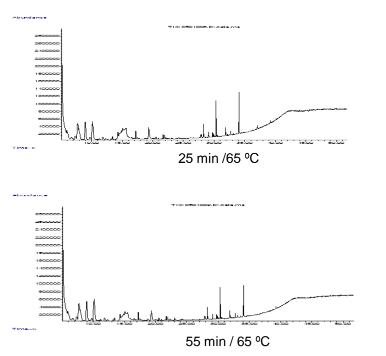


Figure 5. Effect of equilibration time on aroma impact compounds in Coffee Brews.

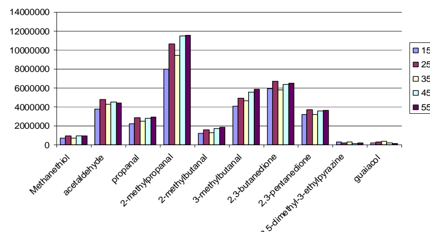
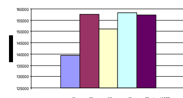


Figure 6. Effect of equilibration time on total volatiles area (x10^{-3}) in Coffee Brews.



CONCLUSIONS

In conclusion, the optimal conditions in static headspace for quantification of aroma impact compounds in coffee brews are 25 min at 65 ºC.

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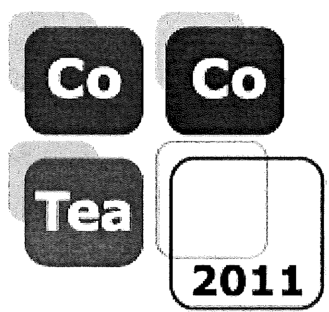
CD-PROCEEDINGS



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P 29

INFLUENCE OF THE EXTRACTION TIME ON THE ANTIOXIDANT ACTIVITY OF COFFEE BREWS

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Introduction

Espresso and filter coffee are the most common brewing methods to prepare a cup of coffee. In previous works, it could be obsewed that espresso coffee brew is the richest in terms of antioxidant intake, whereas filter coffee is the most efficient in antioxidants extraction per gram of coffee (Pérez-Martínez et al., 2010). Several technological factors, such as water pressure and contact time, might influence the extraction of antioxidants (Andueza et al., 2002, López-Galilea et al., 2007). The aim of this work was to study the influence of extraction time on antioxidant capacity of espresso and filter coffee by means of three colorimetric assays (Folin-Ciocalteau, ABTS and DPPH) and two electron spin resonance (ESR) spectroscopy techniques (Fremy's salt and TEMPO). This research is of great interest in order to optimize the antioxidants extraction to obtain antioxidant-rich coffee brews.

Materials and Methods

Three fractions for espresso coffee (14g/80 mL, Aroma Saeco model) collected sequentially every 8 seconds, and five fractions for filter coffee (24g/400 mL, UFESA Avantis 70 inox model) collected sequentially every 75 seconds, were obtained. The coffee used in this study was a 100% Coffea Arabica from Guatemala. To measure the antioxidant capacity, Folin-Ciocalteau colorimetric assay according to Singleton & Rossi method (1965); ABTS scavenging activity technique described by Re et al. (1999); and DPPH scavenging activity assay according to Brand-Williams et al. (1995), and two electron spin resonance (ESR) spectroscopy techniques (Fremy's salt and TEMPO) as described by Roesch et al. (2003) were applied. Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. One-way analysis of variance (ANOVA) with a T-Tukey· a *posteriori* test was applied for each parameter. All statistical analyses were performed using the SPSS v.15.0.

Results and Discussion

Table 1. Time of extraction and volumes obtained in Filter and Espresso coffee brew fractions.

	Espresso coffee brew		Filter coffee brew	
	$t_{extraction}$(sec)	V (mL)	$t_{extraction}$(sec)	V (mL)
F1	8	16	75	80
F2	8	14	75	146
F3	8	17	75	186
F4	-	-	75	94
F5	-	-	75	26

The antioxidant capacity of Guatemala coffee brews and fractions obtained by espresso and filter coffeemakers was measured by means of three colorimetric assays (Folin-Ciocalteau, ABTS and DPPH) and two electron spin resonance (ESR) spectroscopy techniques (Fremy's salt and TEMPO) and the results are shown in Figure 1 to 3. In all assays, espresso coffee fractions showed a remarkable decrease in antioxidant capacity with extraction time. More than 70% of the overall antioxidant capacity of an espresso coffee brew was found in F1 (first 8 seconds), whereas F3 accounted for less than 12 %. These results demonstrate that the compounds responsible for the antioxidant activity of an Espresso coffee brew are mainly extracted at the beginning of the brewing process and, afterwards, are diluted.

In contrast, in Filter coffee brew, antioxidants extraction showed a U-shape profile with the highest antioxidant activity in fractions one (0-75 s) and five (300-375 s), whereas in the middle stages of extraction little or lesser active antioxidant compounds were extracted. The increase of antioxidant capacity in the last two fractions (F4 and F5) of filter coffee brew could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee.

Figure 1. Folin Ciocalteau antioxidant capacity of Guatemala coffee fractions obtained by espresso (a) and filter coffeemaker (b).

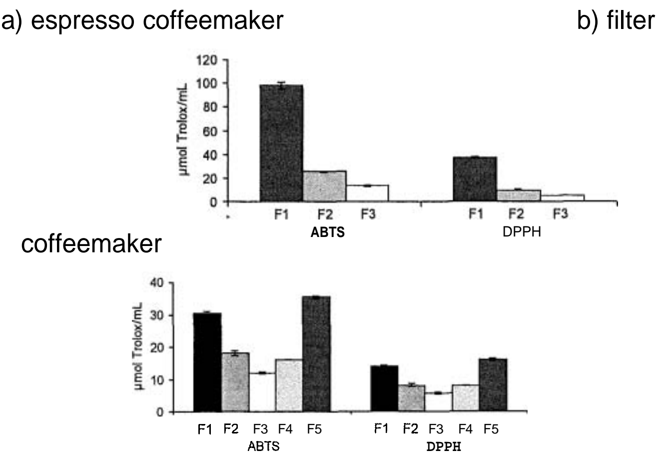

Figure 2. ABTS and DPPH antioxidant capacity of Guatemala coffee fractions obtained by espresso (a) and filter coffeemaker (b).

To go deeper into the influence of brewing time on antioxidant capacity due to phenolics or MRP, Electron spin resonance (ESR) spectroscopy was applied using Fremy's salt and TEMPO radicals.

Mainly phenolic compounds can be detected when Fremy's salt is used as stabilized radical, whereas TEMPO is mainly scavenged by Maillard reaction products (MRP), such as melanoidins (Perez-Martinez 2010). The results obtained with ESR spectroscopy (Figure 3) showed that Fremy's salt scavenging capacity was almost four times higher than TEMPO. Similar results were reported by other authors (Perez-Martinez et al. 2010; Bekedam et al., 2008) who proposed that the phenolic antioxidants evaluated by Fremy's salt dominate the overall antioxidant capacity of coffee brews, whereas the contribution of roasting-induced antioxidants is rather limited.
The ESR antioxidant capacity obtained for Espresso coffee fractions showed that F1 (0-75s) accounted for 75-81 % and for 86-89 % of the Fremy's salt and TEMPO scavenging capacity of an Espresso coffee brew, respectively. The highest value observed for TEMPO scavenging capacity indicates that MRP antioxidants were mainly extracted during the first 8 seconds, whereas the last fraction (16-24s) only accounted for 1-2 %. Although antioxidant capacity due to phenolics, measured by Fremy's salt assay, was also the highest in the first fraction, 20-25 % of the scavenging capacity was still found in F2 and F3. This could be due to a slower extraction of those phenolics retained in the inner coffee particles and those bound to melanoidins that need more time and water pressure to be released.

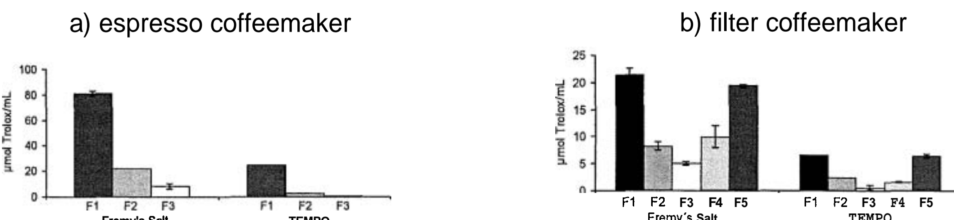

Figure 3. Fremy's Salt and TEMPO antioxidant capacity of Guatemala coffee fractions obtained by espresso (a) and filter coffeemaker (b).

Conclusions

In conclusion, extraction time plays a key role in antioxidants extraction of coffee, maybe due to water pressure. Consequently, in order to optimize the extraction of coffee antioxidants, water pressure should be increased, reducing extraction time like in the first fraction of espresso coffee. This might be a good way to obtain antioxidants that can be used as ingredients for functional foods. However, further research in the chemical composition of coffee brew fractions, as well as sensory properties, should be needed before to industrial development.

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UNRAVELLING THE CONTRIBUTION OF VOLATILE COMPOUNDS TO THE ANTIOXIDANT CAPACITY OF COFFEE: A STUDY ON MODEL SYSTEMS

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Abstract

Several authors have proposed some volatile compounds present in coffee as potent antioxidants (Fuster et al., 2000, Yanagimoto et al., 2002). However, the contribution of the volatile compounds to the antioxidant capacity of coffee is still unclear and controversial mainly because of their low concentrations in coffee brews (Lopez-Galilea et al., 2007). The aim of this study was to evaluate the antioxidant capacity of those volatile compounds reported as antioxidants and present in coffee brews. Seven furans, 3 pyrroles, and 2 thiophenes identified in filter coffee brews (by HS-GC-MS) were selected to prepare model systems at different concentrations including those found in coffee. All model systems were analysed using ABTS as free stable radical to assess the antioxidant capacity. Results were compared with the antioxidant capacity of a filter coffee sample. The model system containing all the three chemical groups (pyrroles, furans and thiophenes) was the most active in scavenging ABTS, followed by pyrroles and furans. Thiophenes were ineffective as radical scavengers at all concentrations including 100-fold. However, in comparison with the antioxidant capacity exhibited by the filter coffee sample, the contribution of the volatile compounds assayed was insignificant (0.13%). In addition, results showed that the heterocyclic compounds analysed exhibited non-linear concentration-dependent antioxidant capacity. In conclusion, volatile compounds contribute very little to the antioxidant capacity of coffee in comparison to other coffee antioxidants such as caffeoylquinic acids and melanoidins.

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EVALUATION OF THE IN VITRO AND IN VIVO ANTIOXIDATIVE ACTIVITIES OF CYNARA SCOLYMUS LEAF EXTRACT

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Cynara scolymus is known for its hepatoprotective effects and positive effects on gastrointestinal disorders. It also has antioxidative effects. In this study, in vitro and in vivo antioxidative properties of a standardised extract of C. scolymus are determined. With respect to its in vitro antioxidant activity screening, different assays are used to establish an antioxidative profile. [1] The radical scavenging capacity is examined using the DPPH-assay. The reducing power is analysed in the FRAP-assay. The biomimetic in vitro lipid peroxidation assay is used as a third assay to evaluate antioxidative potential. The in vitro antioxidative properties of the C. scolymus extract and its main constituents, chlorogenic acid, cynarin and luteolin-7-O-glucoside are determined. Quercetin and rutin are used as reference compounds. For the in vivo antioxidative evaluation of C. scolymus extract, the streptozotocin induced diabetic rat model is used [2]. Three weeks after induction of diabetes, oxidative stress is developed and supplementation with C. scolymus leaf extract is started. The test group receives the C. scolymus extract (500 mg/kg) by oral gavage daily during four weeks. A positive (diabetes, Vitamin E (50 mg/kg)), a negative (diabetes, no treatment) and a healthy (no diabetes, no treatment) control group are also included. Blood samples are taken at the beginning and end of the gavage period. In order to evaluate oxidative stress status, different biomarkers are analysed by HPLC in the plasma samples: malondialdehyde (in vivo lipid peroxidation), alpha- and gamma-tocopherol, retinol and coenzyme Q9 (fat-soluble antioxidants) [3]. In addition, glutathione is determined by HPLC in erythrocytes. By evaluating these markers of oxidative stress, the in vivo antioxidative activity of C. Scolymus leaf extract is determined.

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UNRAVELLING THE INFLUENCE OF BREWING TIME ON COFFEE CHLOROGENIC ACIDS EXTRACTION

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Introduction: Chlorogenic acids, the major phenolics of coffee, are the main antioxidants in coffee brews. The aim of this work was to study the influence of brewing time on the concentration of caffeoylquinic acids in coffee brews in order to optimize their extraction.
Materials and methods: Three fractions (FE1-FE3) for Espresso coffee (7g coffee/40mL) collected sequentially every 8 seconds, and five fractions (FF1-FF5) for Filter coffee (24g coffee/400mL) collected sequentially every 75 seconds, were obtained from Guatemala (Arabica) and Vietnam (Robusta) coffees. Monocaffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA) were identified and quantified by HPLC-DAD in each fraction and coffee brew.
Results: Espresso coffee fractions in both coffees showed a steep decrease with brewing time in CQA isomers. Approximately 70% of the totals CQAs of an Espresso coffee brew were found in FE1 (0-8s). In comparison, diCQAs were extracted slower, accounting FE1 for ~50%, FE2 for ~30% and FE3 still for ~20%. Filter coffee fractions revealed different CQAs and diCQAs extraction patterns, being also different in Guatemala and Vietnam coffees. In Guatemala Filter coffee, CQAs and diCQAs extraction showed a U-shape profile with the highest concentration in FF1 (0-75s) and FF5 (300-375s) and the lowest in FF3 (150-225s). However, in Vietnam coffee the U-shape caffeoylquinic acids extraction started after 75 s, maybe because Vietnam coffee has a longer wetting stage. The increase of caffeoylquinic acids in the last two fractions (FF4 and FF5) of Filter coffee brews could be due to the water pressure decrease that induces a lower flow and a longer contact time between water and ground coffee.
Conclusions: Caffeoylquinic acids are mainly extracted during first 8 seconds in espresso coffee, while in Filter coffee a longer extraction time is necessary to obtain a polyphenol-rich coffee brew.
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COMPARISON BETWEEN THE LIPOLYTIC EFFECT OF RESVERATROL AND A MIXTURE OF ANTHOCYANINS IN ISOLATED RAT ADIPOCYTES

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Introduction: Resveratrol, a polyphenol present in grapes and its derivative drinks, is known to reduce adipose tissue weight in some animal models by decreasing lipogenesis and increasing fat oxidation. Although several studies have attributed to resveratrol a lipolytic activity, this effect has not been widely analyzed. Anthocyanins, flavonoids present mainly in berries, have also been described as functional ingredients due to their beneficial properties as antioxidants or vessel dilatators. They have also been described as hypolipidemic molecules but no data exits in the literature according their lipolytic effect.
Objective: To compare the lipolytic effect of resveratrol and anthocyanins in rat white adipose tissue.
Methods: Eight male Wistar rats (body weight 250g) were sacrificed after a 12 hour-fasting. Epididymal and subcutaneous adipose tissues were dissected and digested by collagenase. Lipolysis was measured in presence of resveratrol and Medox®, a mixture of 17 different natural anthocyanins, at a dose of 10-5 M. Lipolysis was quantified under basal and isoproterenol (ISO, 10-5M)-stimulated conditions. Glycerol release was measured by spectrofotometry and total lipids by gravimetry using Dole method. Results were expressed as percentage of basal lipolysis.
Results: Resveratrol increased glycerol release under basal conditions in both epididymal and subcutaneous adipose tissues (242% and 227% of control basal lipolysis), but Medox® only showed the lipolytic effect in the epididymal tissue (132%). The ISO-stimulated lipolysis was enhanced after the addition of resveratrol only in the subcutaneous adipose tissue (233%).
Conclusion: Resveratrol seems to be a more potent lipolytic molecule than anthocyanins in both adipose tissue locations.

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CHLOROGENIC ACIDS CONTENTS IN BRAZILIAN COFFEA ARABICA CULTIVARS FROM VARIOUS CONSECUTIVE CROPS

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In the last few years chlorogenic acids (CGA) have been widely studied due to their potential bioactive properties in humans. Because of coffee's high CGA contents, this beverage is known as the major antioxidants source in the Western human diet and this led to a search for cultivars that may be at the same time of good sensorial quality and rich in CGA. C. arabica is the best quality coffee species and represents about 75% of the world market, being Brazil the main producer and exporter. In this work, we compared the contents of CGA in the most economically important cultivars in Brazil Mundo Novo, Bourbon, Red Catuai and Yellow Catuai-cultivated in one farm in Minas Gerais, Brazil, and harvested in four or five consecutive years, depending on the cultivar. CGA content was determined by LC-MS. Eight CGA 3-; 4- and 5-caffeoylquinic acids (CQA); 4- and 5-feruloylquinic acids (FQA) and 3,4-; 3,5- and 4,5-dicaffeoylquinic acids (diCQA) were evaluated and identified in all green coffee samples. CGA average contents in green coffee seeds from all cultivars and all crops ranged from 6.1±0.7 to 6.6±1.1 g100g-1 (dry weight). The distribution of CGA classes was similar in all evaluated cultivars and crops, being CQA the major CGA class identified in all samples, followed by diCQA and FQA, respectively. CGA content did not vary significantly among cultivars when all crops were considered, but when selecting only one crop, different cultivars presented higher contents in different years. This may be explained by the fact that these are secondary metabolites that have protective role against pathogens and abiotic stresses and their contents may vary significant according to the plant needs. The present results raise the importance of considering at least three crops when selecting coffee cultivars with higher phenolic compounds contents.

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SPENT COFFEE: A NEW SOURCE OF POLYPHENOLS

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Introduction: Chlorogenic acids are the main phenolic compounds in coffee. Some coffee brewing techniques extracted less chlorogenic acids than others. Thus, the coffee residues, named spent coffee, still may have relevant amounts of these antioxidant compounds. Therefore, the aim of the present study was to evaluate the content of the major chlorogenic acids in spent coffee obtained from different coffeemakers.
Materials and methods: An Arabica coffee (Guatemala) was extracted with four

UNRAVELLING THE INFLUENCE OF BREWING TIME ON COFFEE CHLOROGENIC ACIDS EXTRACTION

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INTRODUCTION

Several chronic diseases, such as cancer, cardiovascular and neurogenerative pathologies are associated with oxidative stress. Plant beverages such as coffee brew have been proposed as an important source of antioxidants in human diet (Pulido et al., 2003). The contact of water with roasted coffee grounds during brewing is the crucial step for the extraction of coffee bioactive compounds. The knowledge of the extraction behavior of the main coffee antioxidants during brewing time might induce to know the technological factors with major impact on antioxidants extraction. Thus, it could be possible to obtain not only coffee brews with higher antioxidant capacity, but also coffee extracts with health properties that can be used as ingredients in functional foods.

The aim of this work was to study the extraction behavior of the main coffee antioxidants, chlorogenic acids (CGA), during brewing time in the most widely consumed coffee brew procedures (espresso and filter).

RESULTS AND DISCUSSION

Three fractions (F1-F3) for espresso coffee collected sequentially every 8 seconds, and five fractions (F1-F5) for filter coffee collected sequentially every 75 seconds, were obtained from Guatemala (Arabica) and Vietnam (Robusta) coffees. Monocaffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA) were identified and quantified by HPLC-DAD in each fraction and coffee brew.

Figure 1 shows the chromatograms of Arabica (Guatemala) and Robusta (Vietnam) filter coffee brews. Surprisingly, less amounts of caffeoylquinic acids were found in Robusta (Vietnam) coffee than in Arabica (Guatemala) ones. This could be due to both, the influence of the origin and the higher loss of chlorogenic acids in Robusta coffee during roasting process (Clifford, 1997, Perrone et al., 2010).

Figure 2. 5-CQA, 4-CQA and 3-CQA of Arabica and Robusta coffee brews and fractions obtained by espresso (**a**) and filter (b) coffeemakers.

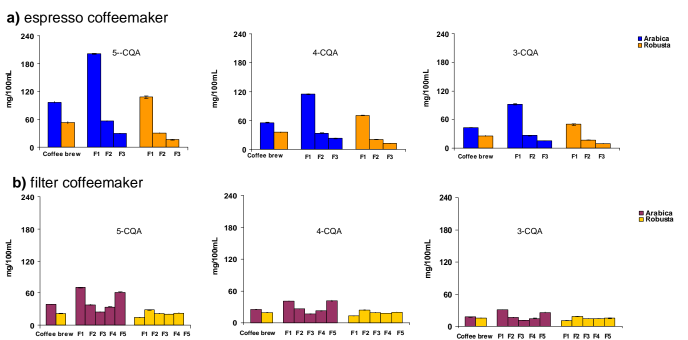
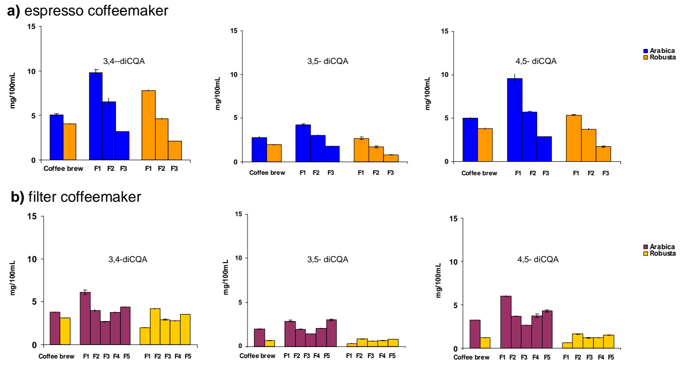


Figure 3. 3,4-diCQA; 3,5-diCQA and 4,5-diCQA of Arabica and Robusta coffee brews and fractions obtained by espresso (**a**) and filter (b) coffeemakers.



CONCLUSIONS

Brewing time plays a key role in the extraction of the main antioxidants (chlorogenic acids) of coffee. To optimize their extraction, some technological factors should be taken into account:

- High water pressure increases caffeoylquinic acids speed like in the first fraction (0-8s) in espresso coffee.
- Turbulences and longer contact time, typically of a filter coffeemaker, are needed to obtain a polyphenol-rich coffee brew and to increase extraction efficiency, mainly in diCQAs.
- Extraction conditions should be adjusted for each coffee origin because cellular structure of coffee beans may also influence.

ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Government of Navarra (Dpt. Education and Dpt. Industry) for the grants given to I.A.L., and L.S.A. and the Unión Tostadora for providing the coffee samples.

MATERIALS AND METHODS

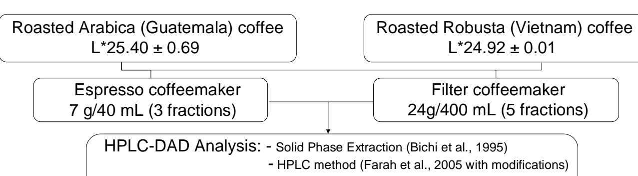
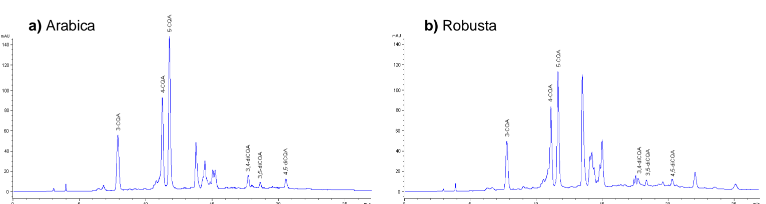


Table 1. Extraction times and volumes of coffee brews and fractions.

	Espresso coffee brew			**Filter coffee brew**		
		Arabica	Robusta		Arabica	Robusta
	$t_{extraction}$ (s)	V (mL)	V (mL)	$t_{extraction}$ (s)	V (mL)	V (mL)
coffee brew	24	47	46	375	532	520
F1	0-8	16	17	0-75	80	74
F2	8-16	14	14	75-150	146	120
F3	16-24	17	15	150-225	186	160
F4	-	-		225-300	94	112
F5	-	-		300-375	26	54

Figure 1. Chromatogram of Arabica (**a**) and Robusta (**b**) Filter coffee brews.



5-CQA was the most abundant chlorogenic acid in all samples, followed by 4-CQA and 3-CQA (**Figure 2**). The abundance of 3,4-diCQA and 4,5-diCQA was quite similar in every coffee fractions or brews, whereas 3,5-diCQA was the least abundant isomer (**Figure 3**).

Espresso coffee fractions in both coffees showed a steep decrease with brewing time in CQA isomers. Approximately 70% of the total CQAs of an espresso coffee brew were found in F1 (0-8s). In comparison, diCQAs were extracted more slowly, accounting F1 for ~50%, F2 for ~30% and F3 still for ~20%. The higher number of hydroxyl groups in diCQAs might favour higher retention by interaction with melanoidins or other polymeric compounds that slow down their release during extraction with water when the extraction time is short.

Filter coffee fractions revealed different CQAs and diCQAs extraction patterns, being also different in Arabica (Guatemala) and Robusta (Vietnam) coffees. In Arabica filter coffee, CQAs and diCQAs extraction showed a U-shape profile with the highest concentration in F1 (0-75s) and F5 (300-375s) and the lowest in F3 (150-225s). However, in Robusta filter coffee the U-shape extraction of caffeoylquinic acids started after 75 s, and F1 exhibited the significantly lowest caffeoylquinic acids concentration, due to a longer wetting stage that also explains the low volume obtained for F1 (**Table 1**) . On the other hand, the increased extraction of caffeoylquinic acids in the last two fractions (F4 and F5) of the filter coffee brews could be due to the longer contact time between water and ground coffee which facilitates the hydrolysis of caffeoylquinic acids bound to melanoidins.

Higher efficiency of phenolic compounds extraction per gram of coffee was obtained in filter coffee brews than in espresso ones **(Table 2)**, in agreement with Perez-Martinez et al. (2010). This may be due to the longer time and turbulences that allow the water in immediate contact with the coffee to extract additional compounds, like CQA and diCQAs, free and bound with melanoidins.

Table 2. Efficiency of phenolic compounds extraction (mg/g coffee) obtained by espresso and filter coffeemakers.

	Espresso (mg/g coffee)		Filter (mg/g coffee)	
	Arabica	Robusta	Arabica	Robusta
ΣCQA	12.79	7.49	13.49	9.38
ΣdiCQA	0.99	0.68	1.59	0.84
Total	13.78	8.17	15.08	10.22

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COFFEE PHENOLICS DEGRADATION AND FORMATION OF BIOACTIVE METABOLITES BY HUMAN COLONIC MICROFLORA

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Coffee is one of the most consumed beverages and a rich source of chlorogenic acids, a group of phenolics comprising hydroxycinnamates such as caffeic acid and ferulic acid, linked to quinic acid. However, only ca. 30% of the chlorogenic acids are absorbed in the small intestine, whereas the remaining ca.70% reaches the colon, where they can be fermented by the gut microbiota to a wide range of low-molecular metabolites. These metabolites might play an important role in the biological activity ascribed to phenolics, such as antioxidant, anticancerogenic and antiinflammatory activity at colon level. The aim of this work was to study the effect of the colonic microflora on the breakdown of coffee chlorogenic acids and the formation of metabolites using an in vitro fermentation model. The colonic metabolism of single compounds like caffeoylquinic and caffeic acids has been investigated, but up to our knowledge, this is the first time that coffee brew is used. Previous to the in vitro fermentation, the phenolic compounds present in espresso coffee brew were identified and quantified by HPLC-PDA-MS^3. 0.5 g freeze-dried espresso coffee was incubated with fresh faecal samples from three healthy donors in the presence and absence of glucose. Samples were analyzed by HPLC-PDA-MS^2 at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h and 24 h. A total of 12 chlorogenic acids and 4 chlorogenic acid lactones were found in the espresso coffee brew and at the initial point of faecal fermentation (0 h). Additionally, traces of caffeic and ferulic acids were also found. Generally, the addition of glucose enhanced the degradation of coffee phenolics, although there were significant differences among the individuals. After 3-6h only traces of phenolic compounds initially present in the fermentation medium were found, except for volunteer 2 that showed an inhibited degradation of dicaffeoylquinic acids, 4-caffeoylquinic acid lactone, feruloylquinic acids and their lactones that still remained in significant amounts after 6h. This may be attributed to the well-known different bacterial populations present in the individual faecal microbial communities, harboring specific enzymatic capacities. Caffeic acid was the first metabolite detected during the fermentation but it was degraded after 4–6 h. The major phenolic end-product identified was 3-(3,4-dihydrophenyl)propionic acid (dihydrocaffeic acid), which was still present in significant amounts after 24 h. Also, 3,4-dihydroxybenzoic acid (protocatechuic acid) was detected at low levels during fermentation, and traces of 3-(3-hydroxyphenyl)propionic acid were found at 24 h. In conclusion, coffee chlorogenic acids were rapidly degraded by the colonic microflora. However, low amounts of them, and their degradation products or metabolites, remained for several hours during fermentation. Therefore, the presence of these bioactive compounds might explain some of the health benefits of coffee brews, mainly those in colon, including prebiotic effects.

Keywords: Coffee, chlorogenic acids, gut microflora, colonic metabolism, bioactive compounds

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