Universidad de Navarra

FACULTAD DE FARMACIA

Spent coffee as a new source of bioaccessible and bioactive compounds with antimutagenic and antimicrobial activity

Subproductos de la bebida de café (posos): una nueva fuente de compuestos bioactivos y bioaccesibles con actividad antimutagénica y antimicrobiana

Carmenrosa Monente Ramos

Pamplona, Febrero de 2015

Universidad de Navarra

Spent coffee as a new source of bioaccessible and bioactive compounds with antimutagenic and antimicrobial activity

Subproductos de la bebida de café (posos): una nueva fuente de compuestos bioactivos y bioaccesibles con actividad antimutagénica y antimicrobiana

Memoria presentada por Dña. Carmenrosa Monente Ramos 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.

En Pamplona, Febrero de 2015

Dra. Mª de la Concepción Cid Canda — Dra. Mª Paz de Peña Fariza

acultad de Farmacia
Departamento de Ciencias de la Alimentación y Fisiología

La directora del Departamento, Dra. Diana Ansorena Artieda, CERTIFICA que el presente trabajo de investigación ha sido realizado por la Licenciada Dña. Carmenrosa Monente Ramos, en el Departamento de Ciencias de la Alimentación y Fisiología de la Facultad de Farmacia de la Universidad de Navarra.

Dra. Diana Ansorena Artieda

En Pamplona, a 18 de febrero de 2015

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 (AGL2009-12052)

AGRADECIMIENTOS

A mis tutoras Mª de la Concepción y Mª Paz, por darme la oportunidad de realizar este proyecto, por su dedicación e interés.

A la Universidad de Navarra, la Asociación de Amigos de la Universidad de Navarra, la Comisión de Investigación, la Facultad de Farmacia y al Departamento de Ciencias de la Alimentación y Fisiología por proporcionarme los medios técnicos, humanos y económicos para realizar este proyecto.

A Leire, Anabel, Ángel, y otras personas de la Universidad por su colaboración y ayuda.

To Alan Crozier for welcoming me into his laboratory at the University of Glasgow and for the kind help.

A todos los integrantes de "Broma" los que ya no están, los que continúan y los nuevos. Especialmente a Jimena, Iziar, Lidia, Isabel, Candela, Blanca, Izaskun y Gwen, muchísimas gracias por un inmejorable ambiente de trabajo y todo los buenos momentos dentro y fuera de la Universidad.

A Elena y María por su apoyo constante y paciencia, pero sobre todo por hacer de nuestro piso un Hogar.

A la gente de Venezuela. Mis amigos Carolina, Janni, Grey, Lisbe y Raúl. Mis vecinos Estefanía, Cricri, Madely, Sra. Esperanza, Sr. Luis, Dulce, entre otros, por regalarme tanta alegría y cariño desde la distancia y en cada vuelta a Casa.

A mi familia, especialmente a mis padrinos Mireya y Javier, a mi Tía Mª Angeles, Soto y las pequeñas Angela e Iranzu, por ser un gran apoyo.

A mi Tía Mª Carmen aunque ya no estas con nosotros muchísimas gracias.

Y el mayor de los agradecimientos a mis papas y hermana, por ser el mejor ejemplo de trabajo y constancia.

A mis padres y hermana

ABSTRACT

Spent coffee is the main by-product of the coffee brew preparation and a promising source of bioactive compounds. The aim of this work was to evaluate the main bioactive compounds of spent coffee extracts, their bioaccessibility, as well as their antimutagenic and antimicrobial activity. Previously, the extraction method of antioxidant compounds was improved by applying defatting and lyophilisation. The phenolics and caffeine content of Arabica filter and Robusta espresso spent coffee was measured by HPLC-DAD-MS, and the total content of phenolic compounds (free and bound) was assessed after applying three treatments (alkaline, acid, saline). A total of 36 free chlorogenic acids (CGA) were identified and quantified showing that Arabica and Robusta spent coffee extracts have 329 and 345 µmol of free CGA /g, respectively. Furthermore, bound compounds were estimated as half of the total phenolic content, due to presence of phenolics linked to macromolecules like melanoidins, mainly by non-covalent interactions.

The majority of CGAs of spent coffee extracts remain bioaccessible after an *in vitro* simulated gastroduodenal digestion (89-92%), being the major compounds -caffeoylquinic (CQAs) and feruloylquinic (FQAs) acids- those with a low degradation, whereas lactones were partially degraded. However, CGAs showed very limited absorption and transport (1% initial dose) across the Caco-2 human cell monolayer (intestinal epithelium). Therefore, *in vivo* studies are required to assess bioavailability of spent coffee bioactive compounds.

Spent coffee extracts exhibited strong protection activity against indirect acting mutagen (up to 92%), whereas the protection against a direct acting mutagen was 12-35% (Ames Test), being phenolics with a caffeic acid highly effective.

The growth inhibition of common food-borne pathogen and food spoilage microorganisms by coffee extracts was also studied. Spent coffee extracts showed antimicrobial activity, mainly against Gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes*) and yeast (*Candida albicans*).

In conclusion, all the results support the idea that spent coffee is an accessible, sustainable, and major source of bioactive compounds with potential health benefits in order to be applied as ingredients of functional foods.

RESUMEN

Los subproductos (posos) resultantes de la preparación de la bebida de café son una potencial fuente de compuestos bioactivos. El objetivo de la tesis fue evaluar los principales compuestos bioactivos de los posos del café, su bioaccesibilidad, y su potencial actividad antimutagénica y antimicrobiana. Previamente, se optimizó el método de extracción de antioxidantes aplicando el desgrasado y la liofilización. La cuantificación del contenido de compuestos fenólicos y cafeína de los extractos de posos de café Arábica de filtro y de Robusta expreso, se realizó por HPLC-DAD-MS. Se estimó el contenido total de compuestos fenólicos (libres y unidos) tras aplicar tres tratamientos (alcalino, ácido, salino). Se identificaron y cuantificaron un total de 36 ácidos clorogénicos (CGA) libres, mostrando los extractos de posos Arábica y Robusta una concentración de 329 y 345 µmol/g, respectivamente. Se estimó que la mitad del total de los compuestos fenólicos están unidos a macromoléculas como las melanoidinas, principalmente por enlaces no covalentes.

La mayoría de los CGA de los extractos de posos de café permanecen bioaccesibles tras una digestión gastroduodenal *in vitro* (89-92%). Los mayoritarios –ácidos cafeoilquínicos (CQAs) y feruloilquínicos (FQAs)- son los más estables, mientras que las lactonas se degradan parcialmente. Sin embargo, la absorción y transporte de los CGAs a través una monocapa de células Caco-2 (células intestinales) fue muy limitada (1% dosis inicial). Por tanto, se requerirían estudios *in vivo* para evaluar la biodisponibilidad de los compuestos bioactivos de los extractos de posos del café.

Los extractos de posos de café mostraron una elevada protección frente al mutágeno de acción indirecta (hasta 92%), mientras que frente al mutágeno de acción directa fue del 12-35% (Test de Ames); siendo los fenólicos con ácido cafeico altamente efectivos.

Finalmente, se evaluó la capacidad de los extractos de posos de café para inhibir el crecimiento de microrganismos patógenos y contaminantes comunes en alimentos, mostrando mayor actividad antimicrobiana frente a bacterias Gram-positivas (*Staphylococcus aureus, Listeria monocytogenes)* y levaduras (*Candida albicans*).

En conclusión, todos los resultados muestran que los posos de café son una fuente accesible y sostenible de compuestos bioactivos con potenciales beneficios para la salud, por lo que podrían ser utilizados como ingredientes en alimentos funcionales.

LIST OF ABBREVIATIONS

2-AF	2-Aminofluorene
ABTS•	+2,2'-Azinobis (3-ethylbenzothiazonile-6-sulfonic acid) diammonium salt
CECT	Spanish Collection of Culture Strains
CFQA	Caffeoyferuloylquinic acid
CFU	Colony forming unit
CGA	Chlorogenic acid
CQA	Caffeoylquinic acid
CQL	Caffeoylquinic acid lactones
DAD	Diode-array detector
DHC	Dihydrocaffeic acid
DHF	Dihydroferulic acid
diCQA	Dicaffeoylquinic acid
dm	Dry matter
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH•	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESI	Electrospray interface
FC	Folin-Ciocalteu
FQA	Feruloylquinic acids
FQL	Feruloylquinic acid lactones

GA	Gallic acid
GIT	Gastrointestinal Tract
HMW	High Molecular Weight
ICO	International Coffee Organization
MIC	Minimum Inhibitory Concentration
MRPs	Maillard reaction products
nd	Not detected
NPD	4-Nitro-*O*-phenylenediamine
*p*CoQA	*p*-coumaroylquinic acids
PDA	Photodiode array
ROS	Reactive oxygen species
SD	Standard deviation
TEERS	Transepithelial electrical resistance
tr	Traces
WHO	World Health Organization

CONTENTS

INTRODUCTION **1**

OBJECTIVE/OBJETIVOS **23**

EXPERIMENTAL DESING **27**

RESULTS **29**

OBJETIVE 1 .... **33**

Paper 1 "Influence of extraction process on antioxidant capacity of spent coffee" **35**

Paper 2 "Evaluation of Spent Coffee Obtained from the Most Common Coffeemakers as a Source of Hydrophilic Bioactive Compounds" **43**

OBJECTIVE 2 .... **53**

Paper 3 "Assessment of Total (Free and Bound) Phenolic Compounds in Spent Coffee Extracts" **55**

OBJECTIVE 3 .... **75**

Paper 4 "*In vitro* studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee extracts" **77**

OBJECTIVE 4 .... **99**

Paper 5 "Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms" **101**

GENERAL DISCUSSION **111**

CONCLUSIONS/CONCLUSIONES **131**

DISSEMINATION OF RESULTS **137**

ANNEX **141**

INTRODUCTION

1. Coffee By-products

Coffee is a worldwide food product with a production of 145 million bags in 2013 (International Coffee Organization, 2013). Two major species are cultivated namely, *Coffea Arabica* (75% global production) and *Coffea Canephora*, commonly known as Robusta coffee. After the harvesting, three steps are followed to obtain the final product for consumption, which is the coffee brew. First, the coffee cherries are processed by either one of two methods – a wet or a dry process. Subsequently, a roasting process is applied to the green coffee obtained after the first step. Finally, the roasted coffee is used to produce soluble coffee by the industry or to consume at home or in cafeterias for brewing using different coffeemakers such as filter, espresso, plunger and mocha. Each step generates residues, which might serve as by-products that could have potential applications for the industry (Esquivel and Jiménez, 2012; Murthy and Madhava Naidu, 2012).

1.1 Coffee Pulp and Husk

The coffee pulp is the by-product obtained after the wet processing of coffee and represents 29% dry-weight of the whole berry, whereas coffee husks are the residue generated in the dry processing of coffee berries and represents about 12% on dry-weight basis (Murthy and Madhava Naidu, 2012). These by-products contain carbohydrates, proteins, fibers and minerals (Pandey et al., 2000). Caffeine, tannin and chlorogenic acids (CGA) have also been identified in extracts from these by-products, as well as other minor phenolic compounds (Fan et al., 2000, Andrade et al., 2012). Several potential applications have been proposed such as improved cultivation of mushrooms (Fan et al., 2000; Murthy and Manonmani, 2008), ethanol production (Gouvea et al., 2009), and the production of enzymes, organic acids, flavor and aroma compounds (Pandey et al., 2000; Murthy and Madhava Naidu, 2012).

1.2 Coffee Silverskin

Coffee silverskin is the tegument of the coffee bean and the byproduct obtained after the roasting process. Its composition includes a high content of dietary fiber (50-60%), which is 15% soluble and 85% insoluble (Borrelli et al., 2004; Napolitano

et al., 2007). The amount of melanoidins found in this by-product is around 4.5% (Borrelli et al., 2004). Furthermore, some studies have reported significant caffeine and chlorogenic acids content (caffeoylquinic, feruloylquinic and coumaroylquinic acids) (Narita and Inouye, 2012; Bresciani et al., 2014). All these constituents have shown some functional properties, such as prebiotic effect, or antioxidant capacity (Borrelli et al., 2004; Napolitano et al., 2007; Narita and Inouye, 2012; Bresciani et al., 2014). In fact, some authors have technically tried to add this byproduct as a food ingredient to increase the content of dietary fiber of bread (Pourfarzad et al., 2013), or to enhance the concentrations of caffeine and chlorogenic acids of an antioxidant beverage, which have led to acceptable sensorial properties (Martinez-Saez et al., 2014).

1.3 Spent coffee grounds

The ground roasted coffee is used for consumption. There is an equal split of coffee used to produce soluble coffee, whereas the other 50% is used in the preparation of beverages in cafeterias, restaurants or at domestic level. Both extractions generate large amounts of residues with a potentially great value (Esquivel and Jiménez, 2012; Murthy and Madhava Naidu, 2012). This potential value has been explored resulting in several possible uses that have been proposed and evaluated in these by-products. A general characterization of soluble spent coffee ground showed the presence of a high carbohydrates content (45.3%, w/w), which might be suitable for bioethanol production through a fermentation process (Mussatto et al., 2011a; 2012). Moreover, the lipids extracted from spent coffee grounds could be converted to fatty acids methyl esters for biodiesel manufacture (Al-Hamamre et al., 2012; Kwon et al., 2013; Caetano et al., 2014). This biofuel was found to be stable for more than one month under ambient conditions, due to the presence of phenolic compounds (Kondamudi et al., 2008). Furthermore, spent coffee grounds are suggested as a sustainable and renewable energy resource used for heating purposes (Zuorro and Lavecchia, 2012). Another application could be the potential use as soil fertilizer to increase the quality (bioactive compounds and antioxidant capacity) of vegetables, mainly due to the presence of substantial amounts of minerals (N, K, P, and Mg) and phenolic compounds (Cruz et al., 2012, 2014).

In the last few years, the interest in these by-products has increased the search for a new application as a food ingredient, due to the potential functional properties. Several studies have found high antioxidant capacity in extracts of spent coffee grounds generated from industrial processes (Yen et al., 2005; Ramalakshmi et al., 2009; Mussatto et al., 2011b) or by cafeterias, restaurants and home-brew preparations (Cruz et al., 2012; Bravo et al., 2012; Panusa et al., 2013). Different extraction methodologies have been applied to recover the constituents responsible for that antioxidant activity. Amongst these methodologies there are several solvents namely organic, aqueous or mixture, but the studies, so far, indicated that the water is the most effective in terms of quantities and environmental concerns (Yen et al., 2005; Panusa et al., 2013; Bravo et al., 2013b).

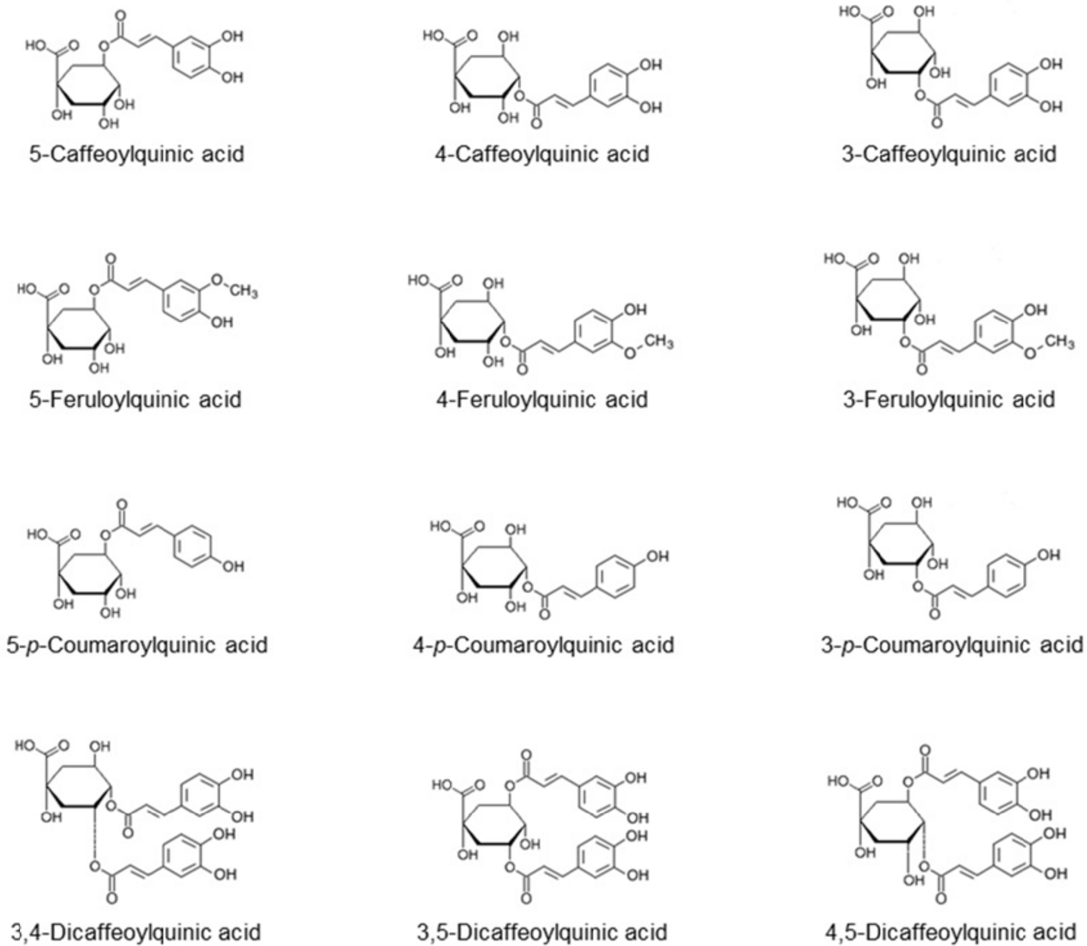
The potential beneficial properties of spent coffee grounds could be associated with the presence of several compounds namely phenolic compounds, caffeine or melanoidins. In a comparative study it was found that the by-products from espresso coffeemaker had higher amounts of soluble natural occurring compounds than the by-products from spent grounds obtained from the soluble coffee industry (Cruz et al., 2012). This differential offers a strong indication of the reuse potential of spent coffee grounds from coffeemakers. Studies of spent coffee from espresso have shown significant amounts of chlorogenic acids (5-caffeoylquinic acid) and caffeine (Cruz et al., 2012; Panusa et al., 2013; Belviso et al., 2014). Moreover, recent data showed that the extraction of coffee chemical compounds is influenced by technological factors, such as water pressure and contact time (Lopez-Galilea et al., 2007; Ludwig et al., 2012). These suggest that the total content of phenolic acids in spent coffee might be related to the brewing method (filter, expresso, moka, plunger coffeemakers). Up to now, limited data have been published about the chlorogenic acids (CGA) detailed profile of coffee brews by-products. The knowledge of the total content of bioactive compounds is crucial for its potential use by the food industry as a functional ingredient.

2. Coffee bioactive compounds

Coffee is a complex mixture of constituents such as phenolic acids, melanoidins and caffeine, which have been linked with beneficial health effects. Particularity, epidemiological studies have shown a positive correlation between the consumption of food with high phenolic compounds content and protection against chronic pathologies, such as cardiovascular diseases, specific cancers, and neurodegenerative diseases (Crozier et al., 2009; del Rio et al., 2013). Chlorogenic acids (CGAs) are the major phenolic compounds of coffee, which are formed between a molecule of quinic acid with one to three molecules of trans-cinnamic acid (Figure 1). The most abundant is 5-caffeoylquinic acids (CQAs), followed by the isomers 4-CQA and 3-CQA. Moreover, feruloylquinic acids (FQAs) are also highly concentrated, mainly in Robusta coffee (Clifford, 1985; Alonso-Salces et al., 2009). On the contrary, *p*-coumaroylquinic acids (*p*CoQAs) have been found in higher amounts in Arabica coffees (Clifford, 1999). Other chlorogenic acids have been identified in coffee, namely caffeoyl-feruloyl-quinic acids (CFQAs), diferuloylquinic acids (diCQAs), di-p-coumaroylquinic acids, dimethoxycinnamoylquinic acids and other minor compounds (Clifford et al., 2006; Alonso-Salces et al., 2009; Jaiswal et al., 2010). Also, cinnamoyl-amino acid conjugates have been characterized in green and roasted coffees (Stark et al., 2006; Clifford and Knight, 2004).

Chlorogenic acids content decreased after coffee roasting, due to a severe degradation process ranged from 60 to 98%, depending of the roasting degree (Clifford, 1985; Moon et al., 2009). Furthermore, changes in the phenolic acid profile have also been reported: (1) isomer migration has been reported to increase the 3 and 4 acyl isomers, (2) hydrolytic reactions separated cinnamic acids and quinic acid molecules and (3) some caffeoylquinic, feruloylquinic and dicaffeoylquinic are transformed into lactones. In this last reaction, a molecule of water from the quinic acid is lost, leading to the formation of an intramolecular ester bond (Clifford, 1999, 2000; Farah et al., 2005, 2006).

Figure 1. Structures of the main CGAs in green coffee beans (Ludwig, 2014a, with the permission of the author)



In the roasting process, the Maillard Reaction also occurs, which is a non-enzymatic browning reaction between reducing sugars and free amino groups. Some of the final products of the reaction are high molecular weight brown compounds named melanoidins (Bekedam et al., 2006). In the coffee brew, melanoidins represent the 25% of the dry matter (Belitz et al., 2009). These compounds have a complex chemical structure, however studies on isolated and purified coffee brew melanoidins have shown strong evidence that polysaccharides, proteins and chlorogenic acids participate in its formation process (Borrelli et al., 2002; Nunes and Coimbra, 2007). Although the mechanism remains unknown, some authors have reported that the thermal oxidative transformations of the hydroxycinnamic

acids may have an important role in melanoidins formation. Also some authors have proposed that certain amount of these phenolic compounds remain linked to the melanoidins structure in coffee (Delgado-Andrade et al., 2005; Nunes and Coimbra, 2010). Moreover, some discrepancies have been arisen about the nature of the chemical bond between phenolics and melanoidins, suggesting covalent and ionic bonds as the most likely interactions.

Several techniques have been applied to break these bonds before the identification and quantitation of phenolic compounds. These have been mainly focused in breaking covalent bonds. For example, alkaline pressure-hydrolysis was one of the first methodologies used to detect compounds attached to the high molecular fraction of coffee extracts (Klöcking et al., 1971). Furthermore, saponification or alkaline hydrolysis is frequently used to release covalently bound phenolic compounds. Previous studies have detected caffeic and ferulic acids after applying this method to coffee brew (Nardini et al., 2002) and high molecular weight fraction in coffee (Bekedam et al., 2008). Recently phenol derivatives and benzoic acid derivatives have been found after alkaline fusion (Nunes and Coimbra, 2007). Acid conditions have also been used on cereals, fruits, vegetables and beverages to release phenolic compounds covalently linked to other structures (Mattila et al., 2006; Alves et al., 2010).

In contrast, non-covalent interactions have been less studied. Barbeau and Kinsella (1983) reported that a high ionic strength medium with NaCl decreased the bindings of chlorogenic acids to a protein fraction. Other recent study showed higher concentrations of phenolic acids after the addition of NaCl, confirming that NaCl breaks the ionic bindings between phenolic compounds and proteins (Xu and Diosady, 2000). Some authors used high ionic strength solutions to break non covalent bonds between melanoidins and low molecular weight compounds, such as phenolic compounds (Borreli et al., 2002; D'Agostina et al., 2004).

Finally, caffeine is the most well know coffee constituent, widely appreciated for the stimulating effects on the central nervous system. Moreover, this natural alkaloid has also been related with some beneficial health properties, such as lower

risk of Diabetes type 2, Parkinson diseases or some types of cancers (van Dam et al., 2006; Costa et al., 2010; Sinha et al., 2012). The negative cardiovascular effects of caffeine seem to be reduced or counteracted by phenolic compounds (Ludwig et al., 2014b). Recent studies reported that coffee is an important source of caffeine in the diet, showing an intake of 49.5 mg/day in United Kingdom, while that amount was doubled in the United Stated (105.4 mg/day) (Fitt et al., 2013; Mitchell et al., 2014). The total content of caffeine in coffee changes among coffee varieties and roasting degree; the Robusta coffees had shown higher levels of caffeine than Arabica coffees (Casal et al., 2000; Ky et al., 2001; Ludwig et al., 2014c).

Free and bound phenolic compounds, caffeine and melanoidins might be present in different concentrations in spent coffee. Thus, a deeper knowledge of the content in these bioactive compounds will provide valuable information for a potential used as food ingredient.

3. Bioaccesibility of phenolic compounds

The bioactive compounds in spent coffee have to be resistant to the conditions of the gastrointestinal tract (GIT) and reach the circulatory system to exert a biological action. The main function of the GIT is the digestion and absorption of food components that are required to maintain good health. Digestion is a series of hydrolytic reactions, where large food molecules are transformed into their monomers. Subsequently, these small molecules are transported through the intestinal wall into the blood system (Fox, 2011). Digestive reactions are caused mainly by the secretion of enzymes, hydrochloric acid, bicarbonate and water. The colonic microbiota also participates actively in the catabolic process (Ludwig et al., 2013) and furthermore, other enzymatic reactions and absorption process occur in the gastric and intestinal cell wall.

The availability of spent coffee compounds may be influenced by the previously described gastrointestinal events. Several parameters could be measured to estimate the amount of bioactive compounds that might reach the blood system. First, the bioaccessibility indicate the amount of compounds available in the

gastrointestinal tract to cross the intestinal barrier (Fernandez-Garcia et al., 2009). Gastrointestinal juices might affect the stability of phenolic compounds, due to the enzymatic activities or pH changes (Friedman and Jürgens, 2000; Rohn et al., 2002). The susceptibility of phenolic acids throughout the digestive tract is a key factor to assess spent coffee significance as a functional ingredient. Furthermore, research has proposed *in vitro* digestion models to simulate gastrointestinal conditions, using commercial digestive enzymes of porcine or bovine origin and bile salts, also regulating the pH, temperature, substrate and incubation time (Hur et al., 2011).

The second relevant parameter is the bioavailability, which asses the absorption, degradation or metabolism of food components in the gastric and intestinal epithelium (Scalbert and Williamson, 2000; Lafay et al., 2006). The bioavailability of phenolic compounds has been measured by *in vitro* and *in vivo* models. *In vitro* studies have shown that gastric and intestinal cells are responsible for the nutrients absorption. Moreover, searches with phenolic compounds standards have repoted that these cells also have catabolic activity, due to the presence of enzymes, namely esterases, methyltranferase sulfatases, glucoronidases (Kern et al., 2003; Farrel et al., 2011). The human epithelial cell lines are commonly used to measure pharmaceutical or phytochemical absorption and transport (Farrel et al., 2011, 2012). In particular Caco-2 cells, these cells are derived from human colon carcinoma, and after around 21 days of cultured they can differentiate into enterocytes showing tight junctions. Furthermore, Caco-2 cells have similar functional properties related to the transport and metabolism of small intestinal enterocyte.

The bioavailability of phenolic compounds has also been investigated with in vivo animal and human feeding studies (Lafay et al., 2006; Stalmach et al., 2010, 2014; Erk et al., 2012). These searches are scared and in some cases with contradictory results. Some research have reported unmetabolized CGAs in plasma after coffee consumption (Monteiro et al., 2007), whereas others have found a wide range of metabolites with low amounts of CQAs and FQAs in plasma (Stalmach et al., 2009, 2010). Furthermore, recent studies have been focused in the study of the impact of CGA dose and their absorption in GIT (Erk et al., 2012; Stalmach et al., 2014).

Measuring both aspects will allow the estimation of the phenolic compounds availability for a potential impact on human health, as well as the amount of components that will reach the colon for further catabolic processes (Ludwig et al., 2013). Previous works on coffee brew have been focused in the bioaccessibility of coffee major components (caffeoylquinic acids or feruloylquinic acids) (Dupas et al., 2006; Tagliazucchi et al., 2012), leaving unknown the fate of other CGAs such as, *p*-coumaroylquinic acids (*p*CoQAs), dicaffeoylquinic acids (diCQAs), and caffeoylquinide acids (CQLs), which may also exert beneficial effects. Up to now, no studies have reported about spent coffee extracts bioaccessibility. A deeper knowledge of the spent coffee bioactive composition and bioaccessibility are required to support their efficacy in health promotion and disease prevention.

4. Spent coffee as a functional ingredient in food

The consumption and development of functional foods have been growing rapidly in recent years, driven by the increased knowledge that diet can improve human health (Fardet and Boirie, 2014). Actually, numerous searches are focused in finding compounds from natural sources with bioactive properties, to develop new functional food products for the prevention of a broad range of diseases. In particular, the ones caused by DNA damage, such as cancer, which are a growing concern for public health institutions and the general population, mainly because of the increasingly mortality rates (8.2 million deaths in 2012) (World Health Organization, 2012). In the case of DNA damage diseases, past research has shown that antioxidant compounds, such as polyphenols, are effective because of their ability to act against reactive oxygen species (ROS) (Farombi and Fakoya, 2005). These molecules induce DNA changes and can lead to cell mutation (Klaunig and Kamendulis, 2004). Coffee has been proposed as a potential functional food due to the presence of caffeine and phenolic compounds (Dorea and Da Costa, 2005). Several studies have positively linked coffee consumption with a decreased risk of oxidative stress-related diseases, such as cancer, cardiovascular diseases, and diabetes, amongst others (Higdon and Frei, 2006; Cano-Marquina et al., 2013).

Furthermore, the spent coffee that it partially retains some of the coffee bioactive compounds, also it might exhibit some of these properties. Moreover, spent coffee could be considered as a novel, suitable and sustainable ingredient to develop value-added foods.

In a recent study, spent coffee was tested for its antioxidant capacity measured by using chemical-based assays and in *in vitro* cell cultures showing the ability to protect against oxidation and DNA damage in human cells (Bravo et al., 2012; 2013a). However, initial studies on coffee found potential mutagenicity of coffee, although excessively heated brewed coffee samples (Kato et al., 1994) or extremely high coffee concentrations (Duarte et al., 1999) were used for these analyses. In addition, non-physiological doses of coffee compounds such as melanoidins or caffeine have been associated with a prooxidant effect (Azam et al., 2003; Caemmerer et al., 2012). On the contrary, a small amount of coffee has a strong protective effect against oxidants (Stadler et al., 1994). Moreover, fruits, vegetables or herbs with antioxidant properties and genoprotective effects have shown antimutagenic effects (Edenharde et al., 2002). Thus, based in these previous knowledge it could be expected that spent coffee exhibit a potential protection against mutagenic agents.

Another consideration that is essential in the development and production of foods, including functional foods, is food safety. This term is used to define all the policies, actions and practices that ensure the quality of food at the different stages of the food chain, from production to consumption. Food safety is aimed at preventing foodborne illnesses, which are caused by various microbiological, chemical or physical contaminants. Microbes are seen as a great challenge due to their impact on health and their ability to proliferate from small quantities to massive proportion (Tent, 1999). The European Food Safety Authority (EFSA) reported high rates of outbreaks per population (1.2 per 100,000) in the EU in 2011 (EFSA, 2013), commonly caused by *Escherichia coli, Salmonella, Bacillus, Shigella* and *Staphylococcus aureus*, amongst others. The severity of foodborne diseases varies from mild symptoms, such as diarrhea or vomiting, to dangerous conditions. In 2011, Salmonella was responsible of the mayor number of outbreaks and *Listeria*

monocytogenes was consider a major risk concern, due to approximately 90% of the cases resulted in hospitalization and with a fatality rate of 10% (EFSA, 2013).

In addition to health consequences, microorganisms may cause food spoilage that can result in considerable economic loss to producers and consumers. The food industry commonly uses preservatives, preferably naturally occurring, to prevent microbial growth. Recent studies have reported microbial growth inhibition after the addition of natural extracts into food model systems. For example Stojkovic et al. (2013) observed that protocatechuic acid inhibited *L. monocytogenes* development in cream cheese. Furthermore, extracts from fruit by-products yield a growth reduction of major foodborne pathogens in fish and cooked ground beef during storage (Ahn et al., 2004; Al-Zoreky, 2009). Coffee has shown antimicrobial activity against a broad range of microorganisms, including foodborne pathogens (Daglia et al., 1994; Almeida et al., 2006; Martínez-Tomé et al., 2011), but to the best of our knowledge, the antimicrobial activity of spent coffee has not yet been evaluated.

References

Ahn, J., Grün, I. U., & Mustapha, A. (2004). Antimicrobial and Antioxidant Activities of Natural Extracts *in Vitro* and in Ground Beef. *Journal of food protection, 67*(1), 148-155.

Al-Hamamre, Z., Foerster, S., Hartmann, F., Kröger, M., & Kaltschmitt, M. (2012). Oil extracted from spent coffee grounds as a renewable source for fatty acid methyl ester manufacturing. *Fuel, 96*(0), 70-76.

Almeida, A. A., Farah, A., Silva, D. A., Nunan, E. A., & Glória, M. B. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry, 54*(23), 8738-8743.

Alonso-Salces, R., Serra, F., Reniero, F., & Héberger, K. (2009). Botanical and Geographical Characterization of Green Coffee (Coffea arabica and Coffea canephora): Chemometric Evaluation of Phenolic and Methylxanthine Contents. *Journal of Agricultural and Food Chemistry, 57*(10), 4224-4235.

Alves, R. C., Almeida, I. M. C., Casal, S., & Oliveira, M. B. P. P. (2010). Method development and validation for isoflavones quantification in coffee. *Food Chemistry, 122*(3), 914-919.

Al-Zoreky, N. S. (2009). Antimicrobial activity of pomegranate (Punica granatum L.) fruit peels. *International journal of food microbiology, 134*(3), 244-248.

Andrade, K. S., Gonçalvez, R. T., Maraschin, M., Ribeiro-do-Valle, R. M., Martínez, J., & Ferreira, S. R. S. (2012). Supercritical fluid extraction from spent coffee grounds and coffee husks: Antioxidant activity and effect of operational variables on extract composition. *Talanta, 88*(0), 544-552.

Azam S., Hadi, N., Khan, N., & Hadi, S. (2003). Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Medical Science Monitor*, 9, 325-330.

Barbeau, W. E., & Kinsella, J. E. (1983). Factors affecting the binding of chlorogenic acid to fraction 1 leaf protein. *Journal of Agricultural and Food Chemistry, 31*(5), 993-998.

Bekedam, E. K., Schols, H. A., van Boekel, M. A. J. S., & Smit, G. (2008). Incorporation of chlorogenic acids in coffee brew melanoidins. *Journal of Agricultural and Food Chemistry, 56*(6), 2055-2063.

Bekedam, E. K., Schols, H. A., van Boekel, M. A. J. S., & Smit, G. (2006). High Molecular Weight Melanoidins from Coffee Brew. *Journal of Agricultural and Food Chemistry, 54*(20), 7658-7666.

Belitz, H., Schieberle, P., & Grosch, W. (2009). Coffee, Tea, Cocoa. In *Food Chemistry,* 4th ed.; Belitz, H., Grosch, W., Schieberle, P., Eds.; Springer-Verlag Berlin: Heidelberg. (pp. 1070).

Belviso, S., Ghirardello, D., Rantsiou, K., Giordano, M., Bertolino, M., Borgogna, D., Cavallero, M. C., Dal Bello, B., Cena, C., Rolle, L., Zeppa, G., & Gerbi, V. (2014). Phytochemical and microbiological stability of spent espresso coffee grounds in capsules. *Food Research International, 61*(0), 93-99.

Borrelli, R., Esposito, F., Napolitano, A., Ritieni, A., & Fogliano, V. (2004). Characterization of a new potential functional ingredient: Coffee silverskin. *Journal of Agricultural and Food Chemistry, 52*(5), 1338-1343.

Borrelli, R., Visconti, A., Mennella, C., Anese, M., & Fogliano, V. (2002). Chemical characterization and antioxidant properties of coffee melanoidins. *Journal of Agricultural and Food Chemistry, 50*(22), 6527-6533.

Bravo, J., Juaniz, I., Monente, C., Caemmerer, B., Kroh, L., De Peña, M. P., & Cid, C. (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds. *Journal of Agricultural and Food Chemistry, 60*(51), 12565-12573.

Bravo, J., Arbillaga, L., de Peña, M. P., & Cid, C. (2013a). Antioxidant and genoprotective effects of spent coffee extracts in human cells. *Food and Chemical Toxicology, 60*(0), 397-403.

Bravo, J., Monente, C., Juániz, I., De Peña, M. P., & Cid, C. (2013b). Influence of extraction process on antioxidant capacity of spent coffee. *Food Research International, 50*(2), 610-616.

Bresciani, L., Calani, L., Bruni, R., Brighenti, F., & Del Rio, D. (2014). Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. *Food Research International, 61*(0), 196-201.

Caemmerer, B., Chodakowski, K., Gienapp, C., Wohak, L., Hartwig, A., & Kroh, L. (2012). Pro-oxidative effects of melanoidin–copper complexes on isolated and cellular DNA. *European food research and technology, 234*(4), 663-670.

Caetano, N., Silva, V., Melo, A., Martins, A., & Mata, T. (2014). Spent coffee grounds for biodiesel production and other applications. *Clean Technologies and Environmental Policy, 16*(7), 1423-1430.

Cano-Marquina, A., Tarín, J. J., & Cano, A. (2013). The impact of coffee on health. *Maturitas, 75*(1), 7-21.

Casal, S., Oliveira, M. B. P. P., Alves, M. R., & Ferreira, M. A. (2000). Discriminate Analysis of Roasted Coffee Varieties for Trigonelline, Nicotinic Acid, and Caffeine Content. *Journal of Agricultural and Food Chemistry, 48*(8), 3420-3424.

Clifford, M. N. (1985). Chemical and Physical Aspects of Green Coffee and Coffee Products. In M. N. Clifford & K. C. Willson, (pp. 305-374). Springer US.

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates ? nature, occurrence and dietary burden. *Journal of the science of food and agriculture, 79*(3), 362-372.

Clifford, M.N. (2000). Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *Journal of the science of food and agriculture, 80*(7), 1033-1043.

Clifford, M. N., & Knight, S. (2004). The cinnamoyl–amino acid conjugates of green robusta coffee beans. *Food Chemistry, 87*(3), 457-463.

Clifford, M. N., Knight, S., Surucu, B., & Kuhnert, N. (2006). Characterization by LC-MSn of Four New Classes of Chlorogenic Acids in Green Coffee Beans: Dimethoxycinnamoylquinic Acids, Diferuloylquinic Acids, Caffeoyl-dimethoxycinnamoylquinic Acids, and Feruloyl-dimethoxycinnamoylquinic Acids. *Journal of Agricultural and Food Chemistry, 54*(6), 1957-1969.

Costa, J., Lunet, N., Santos, C., Santos, J., & Vaz-Carneiro, A. (2010). Caffeine Exposure and the Risk of Parkinson's Disease: A Systematic Review and Meta-Analysis of Observational Studiess. *Journal of Alzheimer's Disease, 20*(0), 221-238.

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural product reports, 26*(8), 1001-1043.

Cruz, R., Cardoso, M. M., Fernandes, L., Oliveira, M., Mendes, E., Baptista, P., Morais, S., & Casal, S. (2012). Espresso Coffee Residues: A Valuable Source of Unextracted Compounds. *Journal of Agricultural and Food Chemistry, 60*(32), 7777-7784.

Cruz, R., Gomes, T., Ferreira, A., Mendes, E., Baptista, P., Cunha, S., Pereira, J. A., Ramalhosa, E., & Casal, S. (2014). Antioxidant activity and bioactive compounds of lettuce improved by espresso coffee residues. *Food Chemistry, 145*(0), 95-101.

Daglia, M., Cuzzoni, M., & Dacarro, C. (1994). Antibacterial activity of coffee. *Journal of Agricultural and Food Chemistry, 42*(10), 2270-2272.

D'Agostina, A., Boschin, G., Bacchini, F., & Arnoldi, A. (2004). Investigations on the High Molecular Weight Foaming Fractions of Espresso Coffee. *Journal of Agricultural and Food Chemistry, 52*(23), 7118-7125.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (Poly)phenolics in Human Health: Structures, Bioavailability, and Evidence of Protective Effects Against Chronic Diseases. *Antioxidants & Redox Signaling, 18*(14), 1818-1892.

Delgado-Andrade, C., Rufían-Henares, J., & Morales, F. (2005). Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *Journal of Agricultural and Food Chemistry, 53*(20), 7832-7836.

Dorea, J., & da Costa, T. (2005). Is coffee a functional food? *The British journal of nutrition, 93*(6), 773-782.

Duarte, M. P., Laires, A., Gaspar, J., Leão, D., Oliveira, J. S., & Rueff, J. (1999). Genotoxicity of instant coffee: possible involvement of phenolic compounds. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 442*(1), 43-51.

Dupas, C., Marsset Baglieri, A., Ordonaud, C., Tomé, D., & Maillard, M. (2006). Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study. *Molecular Nutrition & Food Research, 50*(11), 1053-1060.

Edenharder, R., Sager, J. W., Glatt, H., Muckel, E., & Platt, K. L. (2002). Protection by beverages, fruits, vegetables, herbs, and flavonoids against genotoxicity of 2-acetylaminofluorene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in metabolically competent V79 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 521*(1–2), 57-72.

EFSA, (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control) (2013). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. *EFSA Journal 2013, 11*(4), 3129.

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International, 46*(2), 488-495.

Erk, T., Williamson, G., Renouf, M., Marmet, C., Steiling, H., Dionisi, F., Barron, D., Melcher, R., & Richling, E. (2012). Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. *Molecular Nutrition & Food Research, 56*(10), 1488-1500.

Fan, L., Pandey, A., Mohan, R., & Soccol, C. R. (2000). Use of various coffee industry residues for the cultivation of Pleurotus ostreatus in solid state fermentation. *Acta Biotechnologica, 20*(1), 41-52.

Farah, A., De Paulis, T., Moreira, D. P., Trugo, L. C., & Martin, P. R. (2006). Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees. *Journal of Agricultural and Food Chemistry, 54*(2), 374-381.

Farah, A., De Paulis, T., Trugo, L., & Martin, P. (2005). Effect of roasting on the formation of chlorogenic acid lactones in coffee. *Journal of Agricultural and Food Chemistry, 53*(5), 1505-1513.

Fardet, A., & Boirie, Y. (2014). Associations between food and beverage groups and major diet-related chronic diseases: an exhaustive review of pooled/meta-analyses and systematic reviews. *Nutrition reviews*, 72(12), 741-762.

Farombi, E. F., & Fakoya, A. (2005). Free radical scavenging and antigenotoxic activities of natural phenolic compounds in dried flowers of Hibiscus sabdariffa L. *Molecular nutrition & food research, 49*(12), 1120-1128.

Farrell, T., Poquet, L., Dionisi, F., Barron, D., & Williamson, G. (2011). Characterization of hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC–DAD–MS2: Enhancing chromatographic quantification and application in Caco-2 cell metabolism. *Journal of pharmaceutical and biomedical analysis*, 55(5), 1245-1254.

Farrell, T. L., Poquet, L., Dew, T. P., Barber, S., & Williamson, G. (2012). Predicting Phenolic Acid Absorption in Caco-2 Cells: A Theoretical Permeability Model and Mechanistic Study. *Drug Metabolism and Disposition*, 40(2), 397-406.

Fernández-García, E., Carvajal-Lérida, I., & Pérez-Gálvez, A. (2009). *In vitro* bioaccessibility assessment as a prediction tool of nutritional efficiency. *Nutrition Research, 29*(11), 751-760.

Fitt, E., Pell, D., & Cole, D. (2013). Assessing caffeine intake in the United Kingdom diet. *Food Chemistry, 140*(3), 421-426.

Fox, S. I. (2011). *Human Physiology.* New York; USA: McGraw-Hill (pp. 612).

Friedman, M., & Jürgens, H. S. (2000). Effect of pH on the Stability of Plant Phenolic Compounds. *Journal of Agricultural and Food Chemistry, 48*(6), 2101-2110.

Gouvea, B. M., Torres, C., Franca, A. S., Oliveira, L. S., & Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. *Biotechnology Letters, 31*(9), 1315-1319.

Higdon, J. V., & Frei, B. (2006). Coffee and Health: A Review of Recent Human Research. *Critical reviews in food science and nutrition, 46*(2), 101-123.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry*, 125(1), 1-12.

International Coffee Organization ICO. (2013). Exporting countries: total production http://www.ico.org/prices/po.htm

Jaiswal, R., Sovdat, T., Vivan, F., & Kuhnert, N. (2010). Profiling and Characterization by LC-MS(n) of the Chlorogenic Acids and Hydroxycinnamoylshikimate Esters in Mate (Ilex paraguariensis). *Journal of Agricultural and Food Chemistry, 58*(9), 5471-5484.

Kato, T., Hiramoto, K., & Kikugawa, K. (1994). Possible occurrence of new mutagens with the DNA breaking activity in coffee. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 306*(1), 9-17.

Kern, S. M., Bennett, R. N., Needs, P. W., Mellon, F. A., Kroon, P. A., & Garcia-Conesa, M. (2003). Characterization of Metabolites of Hydroxycinnamates in the *in Vitro* Model of Human Small Intestinal Epithelium Caco-2 Cells. *Journal of Agricultural and Food Chemistry*, 51(27), 7884-7891.

Klaunig, J. E., & Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annual Review of Pharmacology and Toxicology, 44*(1), 239-267.

Klöcking, R., Hofmann, R., & Mücke, D. (1971). Substances of the humic acid type ocurring in extracts of roasted coffee. 3. Detection of phenols in hydrolysates of humic acids from coffee. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, 146(2) 79– 83.

Kondamudi, N., Mohapatra, S. K., & Misra, M. (2008). Spent Coffee Grounds as a Versatile Source of Green Energy. *Journal of Agricultural and Food Chemistry, 56*(24), 11757-11760.

Kwon, E. E., Yi, H., & Jeon, Y. J. (2013). Sequential co-production of biodiesel and bioethanol with spent coffee grounds. *Bioresource technology, 136*(0), 475-480.

Ky, C. L., Louarn, J., Dussert, S., Guyot, B., Hamon, S., & Noirot, M. (2001). Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild Coffea arabica L. and C-canephora P. accessions. *Food Chemistry, 75*(2), 223-230.

Lafay, S., Morand, C., Manach, C., Besson, C., & Scalbert, A. (2006). Absorption and metabolism of caffeic acid and chlorogenic acid in the small intestine of rats. *British Journal of Nutrition, 96*(01), 39-46.

López-Galilea, I., de Peña, M. P., & Cid, C. (2007). Correlation of selected constituents with the total antioxidant capacity of coffee beverages: influence of the brewing procedure. *Journal of Agricultural and Food Chemistry, 55*(15), 6110-6117.

Ludwig, I. A. (2014a).Contribution of Phenolics and Maillard Reaction Products to the Antioxidant Capacity of Coffee Brews. University of Navarra. Navarra

Ludwig, I. A., Clifford, M. N., Lean, M. E. J., Ashihara, H., & Crozier, A. (2014b). Coffee: Biochemistry and potential impact on health. *Food & Function,* 4, 1695-1717.

Ludwig, I. A., Mena, P., Calani, L., Cid, C., Del Rio, D., Lean, M. E. J., & Crozier, A. (2014c). Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? *Food & Function, 5*(8), 1718-1726.

Ludwig, I. A., De Peña, M. P., Cid, C., & Crozier, A. (2013). Catabolism of coffee chlorogenic acids by human colonic microbiota. *BioFactors, 39*(6), 623-632.

Ludwig, I. A., Sanchez, L., Caemmerer, B., Kroh, L. W., De Peña, M. P., & Cid, C. (2012). Extraction of coffee antioxidants: Impact of brewing time and method. *Food Research International, 48*(1), 57-64.

Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. A., Martorell, P., Genovés, S., Ramon, D., & del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based on coffee silverskin. *Food Chemistry, 150*(0), 227-234.

Martínez-Tomé, M., Jiménez-Monreal, A., Garcia-Jiménez, L., Almela, L., García-Diz, L., Mariscal-Arcas, M., & Murcia, M. A. (2011). Assessment of antimicrobial activity of coffee

brewed in three different ways from different origins. *European food research and technology, 233*(3), 497-505.

Mattila, P., Hellstrom, J., & Torronen, R. (2006). Phenolic acids in berries, fruits, and beverages. *Journal of Agricultural and Food Chemistry, 54*(19), 7193-7199.

Mitchell, D. C., Knight, C. A., Hockenberry, J., Teplansky, R., & Hartman, T. J. (2014). Beverage caffeine intakes in the U.S. *Food and Chemical Toxicology, 63*(0), 136-142.

Monteiro, M., Farah, A., Perrone, D., Trugo, L. C., & Donangelo, C. (2007). Chlorogenic Acid Compounds from Coffee Are Differentially Absorbed and Metabolized in Humans. The Journal of nutrition, 137(10), 2196-2201.

Moon, J., Yoo, H. S., & Shibamoto, T. (2009). Role of Roasting Conditions in the Level of Chlorogenic Acid Content in Coffee Beans: Correlation with Coffee Acidity. *Journal of Agricultural and Food Chemistry, 57*(12), 5365-5369.

Murthy, P. S., & Madhava Naidu, M. (2012). Sustainable management of coffee industry by-products and value addition—A review. *Resources, Conservation and Recycling, 66*(0), 45-58.

Murthy, P. S., & Manonmani, H. K. (2008). Bioconversion of coffee industry wastes with white rot fungus *Pleurotus florida*. *Journal of Environmental Sciences,* 2, 145-150

Mussatto, S. I., Ballesteros, L. F., Martins, S., & Teixeira, J. A. (2011b). Extraction of antioxidant phenolic compounds from spent coffee grounds. *Separation and Purification Technology, 83*(0), 173-179.

Mussatto, S. I., Carneiro, L. M., Silva, J. P. A., Roberto, I. C., & Teixeira, J. A. (2011a). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers, 83*(2), 368-374.

Mussatto, S. I., Machado, E. M. S., Carneiro, L. M., & Teixeira, J. A. (2012). Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. *Applied Energy, 92*(0), 763-768.

Napolitano, A., Fogliano, V., Tafuri, A., & Ritieni, A. (2007). Natural Occurrence of Ochratoxin A and Antioxidant Activities of Green and Roasted Coffees and Corresponding By-products. *Journal of Agricultural and Food Chemistry, 55*(25), 10499-10504.

Nardini, M., Cirillo, E., Natella, F., & Scaccini, C. (2002). Absorption of phenolic acids in humans after coffee consumption. *Journal of Agricultural and Food Chemistry, 50*(20), 5735-5741.

Narita, Y., & Inouye, K. (2012). High antioxidant activity of coffee silverskin extracts obtained by the treatment of coffee silverskin with subcritical water. *Food Chemistry, 135*(3), 943-949.

Nunes, F. M., & Coimbra, M. A. (2010). Role of hydroxycinnamates in coffee melanoidin formation. *Phytochemistry Reviews, 9*(1), 171-185.

Nunes, F., & Coimbra, M. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *Journal of Agricultural and Food Chemistry, 55*(10), 3967-3977.

Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R., & Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical engineering journal, 6*(2), 153-162.

Panusa, A., Zuorro, A., Lavecchia, R., Marrosu, G., & Petrucci, R. (2013). Recovery of Natural Antioxidants from Spent Coffee Grounds. *Journal of Agricultural and Food Chemistry, 61*(17), 4162-4168.

Pourfarzad, A., Mahdavian-Mehr, H., & Sedaghat, N. (2013). Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Science and Technology, 50*(2), 599-606.

Ramalakshmi, K., Rao, L. J. M., Takano-Ishikawa, Y., & Goto, M. (2009). Bioactivities of low-grade green coffee and spent coffee in different *in vitro* model systems. *Food Chemistry, 115*(1), 79-85.

Rohn, S., Rawel, H. M., & Kroll, J. (2002). Inhibitory Effects of Plant Phenols on the Activity of Selected Enzymes. *Journal of Agricultural and Food Chemistry, 50*(12), 3566-3571.

Scalbert, A., & Williamson, G. (2000). Dietary Intake and Bioavailability of Polyphenols. *The Journal of nutrition, 130*(8), 2073S-2085S.

Sinha, R., Cross, A. J., Daniel, C. R., Graubard, B. I., Wu, J. W., Hollenbeck, A. R., Gunter, M. J., Park, Y., & Freedman, N. D. (2012). Caffeinated and decaffeinated coffee and tea intakes and risk of colorectal cancer in a large prospective study. *The American Journal of Clinical Nutrition, 96*(2), 374-381.

Stadler, R. H., Turesky, R. J., Müller, O., Markovic, J., & Leong-Morgenthaler, P. (1994). The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 308*(2), 177-190.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., Steiling, H., Williamson G., & Crozier A. (2009). Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition*, 37(8), 1749-1758.

Stalmach, A., Steiling, H., Williamson, G., & Crozier, A. (2010). Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. Archives of Biochemistry and Biophysics, 501(1), 98-105.

Stalmach, A., Williamson, G., & Crozier, A. (2014). Impact of dose on the bioavailability of coffee chlorogenic acids in humans. Food & Function, 5(8), 1727-1737.

Stark, T., Justus, H., & Hofmann, T. (2006). Quantitative Analysis of N-Phenylpropenoyl-l-amino Acids in Roasted Coffee and Cocoa Powder by Means of a Stable Isotope Dilution Assay. *Journal of Agricultural and Food Chemistry, 54*(8), 2859-2867.

Stojković, D. S., Živković, J., Soković, M., Glamočlija, J., Ferreira, I. C. F. R., Janković, T., & Maksimović, Z. (2013). Antibacterial activity of Veronica montana L. extract and of protocatechuic acid incorporated in a food system. *Food and Chemical Toxicology, 55*(0), 209-213.

Tagliazucchi, D., Helal, A., Verzelloni, E., & Conte, A. (2012). The Type and Concentration of Milk Increase the *in Vitro* Bioaccessibility of Coffee Chlorogenic Acids. *Journal of Agricultural and Food Chemistry, 60*(44), 11056-11064.

Tent, H. (1999). Research on food safety in the 21st century. *Food Control, 10*(4–5), 239-241.

van Dam, R. M., Willett, W. C., Manson, J. E., & Hu, F. B. (2006). Coffee, Caffeine, and Risk of Type 2 Diabetes: A prospective cohort study in younger and middle-aged U.S. women. *Diabetes care, 29*(2), 398-403.

World Health Organization (WHO), International Agency for Research on Cancer. (2012). All Cancers (excluding non-melanoma skin cancer) Estimated Incidence, *Mortality and Prevalence Worldwide in 2012*. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

Xu, L., & Diosady, L. L. (2000). Interactions between canola proteins and phenolic compounds in aqueous media. *Food Research International, 33*(9), 725-731.

Yen, W., Wang, B., Chang, L., & Duh, P. (2005). Antioxidant properties of roasted coffee residues. *Journal of Agricultural and Food Chemistry, 53*(7), 2658-2663.

Zuorro, A., & Lavecchia, R. (2012). Spent coffee grounds as a valuable source of phenolic compounds and bioenergy. *Journal of Cleaner Production, 34*(0), 49-56.

OBJECTIVES

OBJETIVOS

Coffee is well known for the presence of large amounts of different components, such as phenolic compounds, caffeine and melanoidins, which have been associated with beneficial health properties. Moreover, the by-product generated during the coffee brew preparation, namely spent coffee, is a promising rich source of bioactive compounds. The large volume of these residues - home, cafeterias or restaurants and industry - indicates the potential use in the industry as a food ingredient. Towards the attainment of this goal, three aspects warrant further study, namely, a detailed analysis of spent coffee components, the effect of human digestion on these compounds and last, to test their potential beneficial health properties.

The aim of this PhD Thesis was to evaluate the composition of spent coffee extracts, as well as the bioaccessibility of their bioactive compounds, and to further investigate potential applications as antimutagenic and antimicrobial agents.

To achieve this aim, the following four objectives were established:

1. To optimize the methodological conditions for the preparation of spent coffee extracts with antioxidant capacity, and to make a selection based on major bioactive compounds content among spent coffee obtained from two coffee varieties (Arabica and Robusta) applying two brewing methods (filter and espresso coffeemakers).
2. To evaluate free and bound phenolic compounds for the assessment of the total phenolics content of spent coffee extract, and to determine the most accurate methodology for this purpose.
3. To assess the bioaccessibility of phenolic compounds of Arabica and Robusta spent coffee extracts using an *in vitro* gastroduodenal digestion, followed by the estimation of the potential absorption of phenolic compounds with a Caco-2 human cell model.
4. To evaluate the antimutagenic and antimicrobial activity of Arabica and Robusta spent coffee extracts for further applications in functional foods.

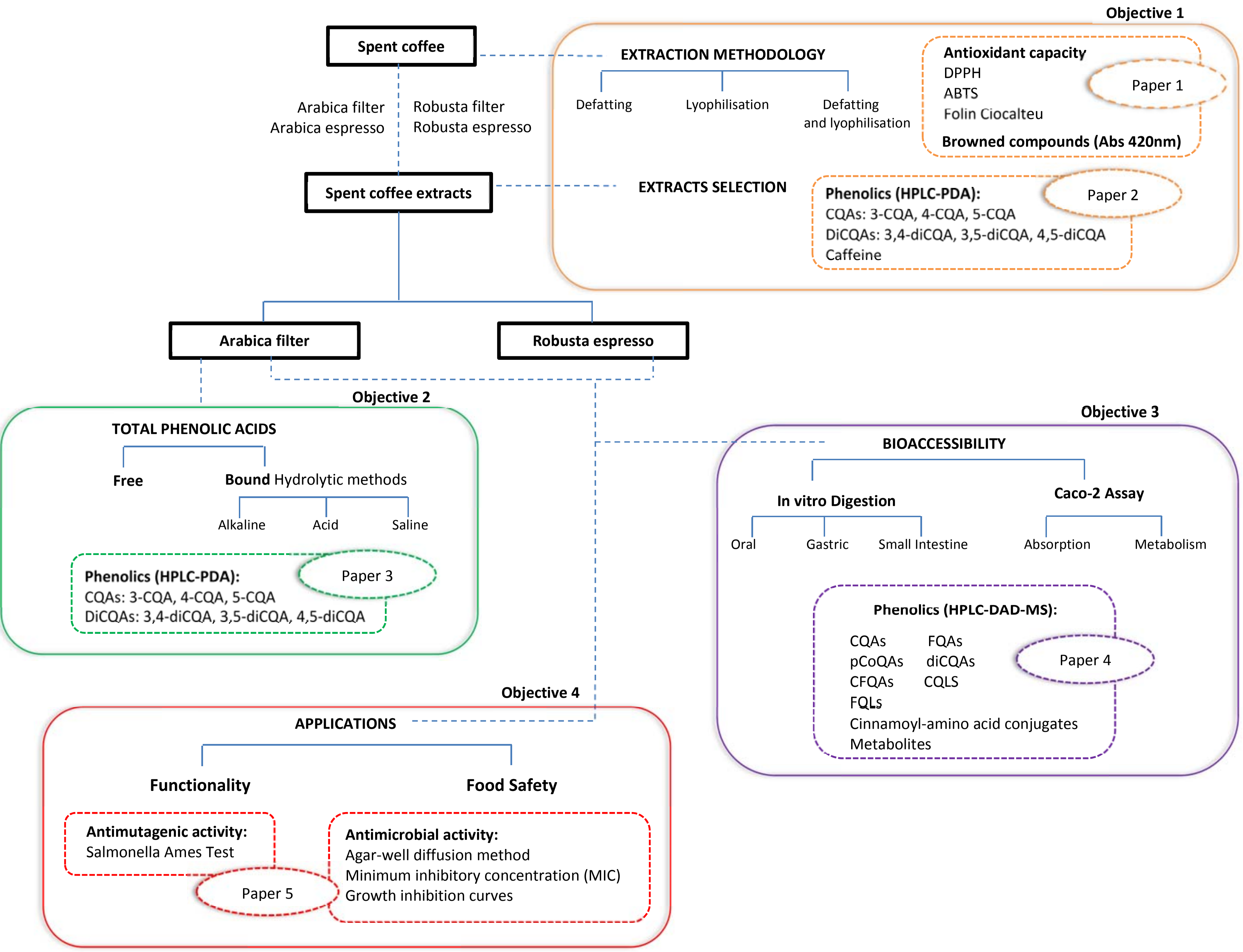
El café es conocido por contener elevadas cantidades de distintos compuestos, como ácidos fenólicos, cafeína y melanoidinas, que presentan propiedades beneficiosas para la salud. De igual modo, los subproductos generados después de la preparación de la bebida de café, denominados posos del café, constituyen una potencial fuente de compuestos bioactivos. La elevada producción de estos residuos, en los hogares, cafeterías o restaurantes e industria, aumenta la probabilidad de su posible uso como ingredientes alimentarios. Sin embargo, esto requiere el estudio previo y en mayor profundidad de la presencia de estos compuestos en dichos subproductos, así como del efecto del proceso de digestión sobre los mismos y comprobar las propiedades beneficiosas para la salud.

El objetivo de esta Tesis Doctoral fue evaluar la composición de los extractos de posos de café, la bioaccesibilidad de sus compuestos bioactivos, y conocer su potencial aplicación como agente antimicrobiano y/o antimutagénico.

Para la consecución de este objetivo se propusieron los siguientes objetivos parciales:

1- Optimizar las condiciones metodológicas para la preparación de extractos de posos de café con capacidad antioxidante, y realizar una selección basada en el contenido de compuestos bioactivos mayoritarios, entre posos de café procedentes de bebidas de café de cafeteras filtro y expreso con dos variedades Arábica y Robusta.
2- Evaluar el contenido de compuestos fenólicos libres y unidos para determinar el contenido total de fenólicos en los extractos de posos del café, y establecer la metodología idónea para dicho propósito.
3- Evaluar la bioaccesibilidad de los compuestos fenólicos de los extractos de posos de café Arábica y Robusta aplicando un modelo de digestión *in vitro*, seguido de la estimación de su potencial absorción en un modelo celular (células humanas Caco-2).
4- Evaluar la actividad antimutagénica y antimicrobiana de los extractos de posos del café Arábica y Robusta para su posterior aplicación en alimentos funcionales.

EXPERIMENTAL DESIGN


Spent coffee
Arabica filter
Arabica espresso
Robusta filter
Robusta espresso
Spent coffee extracts
Arabica filter
Robusta espresso
Objective 1
EXTRACTION METHODOLOGY
Defatting
Lyophilisation
Defatting and lyophilisation
Antioxidant capacity
DPPH
ABTS
Folin Ciocalteu
Browned compounds (Abs 420nm)
Paper 1
EXTRACTS SELECTION
Phenolics (HPLC-PDA):
CQAs: 3-CQA, 4-CQA, 5-CQA
DiCQAs: 3,4-diCQA, 3,5-diCQA, 4,5-diCQA
Caffeine
Paper 2
Objective 2
TOTAL PHENOLIC ACIDS
Free
Bound Hydrolytic methods
Alkaline
Acid
Saline
Phenolics (HPLC-PDA):
CQAs: 3-CQA, 4-CQA, 5-CQA
DiCQAs: 3,4-diCQA, 3,5-diCQA, 4,5-diCQA
Paper 3
Objective 3
BIOACCESSIBILITY
In vitro Digestion
Oral
Gastric
Small Intestine
Caco-2 Assay
Absorption
Metabolism
Phenolics (HPLC-DAD-MS):
CQAs
pCoQAs
CFQAs
FQLs
FQAs
diCQAs
CQLS
Cinnamoyl-amino acid conjugates
Metabolites
Paper 4
Objective 4
APPLICATIONS
Functionality
Food Safety
Antimutagenic activity:
Salmonella Ames Test
Antimicrobial activity:
Agar-well diffusion method
Minimum inhibitory concentration (MIC)
Growth inhibition curves
Paper 5

RESULTS

OBJETIVE 1

To optimize the methodological conditions for the preparation of spent coffee extracts with antioxidant capacity, and to make a selection based on major bioactive compounds content among spent coffee obtained from two coffee varieties (Arabica and Robusta) applying two brewing methods (filter and espresso coffeemakers).

Optimizar las condiciones metodológicas para la preparación de extractos de posos de café con capacidad antioxidante, y realizar una selección basada en el contenido de compuestos bioactivos mayoritarios, entre posos de café procedentes de bebidas de café de cafeteras filtro y expreso con dos variedades Arábica y Robusta.

The results of Objective 1 have been published in the next papers together with other results of the project.

Paper 1:

Influence of extraction process on antioxidant capacity of spent coffee

Jimena Bravo, **Carmen Monente**, Isabel Juániz, M. Paz De Peña and Concepción Cid

Food Research International, 2013, 50, 610-616.

Impact Factor (JCR, 2013): 3.050

Journal Rank in categories:

- Food Science & Technology: 14/123 (Q1)

Paper 2:

Evaluation of Spent Coffee Obtained from the Most Common Coffeemakers as a Source of Hydrophilic Bioactive Compounds

Jimena Bravo, Isabel Juániz, **Carmen Monente**, Bettina Caemmerer, Lothar W. Kroh, M. Paz De Peña and Concepción Cid

Journal Agricultural and Food Chemistry, 2012, 60(51), 12565-12573.

Impact Factor (JCR, 2012): 2.906.

Journal Rank in categories:

- Agriculture, Multidisciplinary: 1/57 (Q1)
- Chemistry, Applied: 12/71 (Q1)
- Food Science & Technology: 15/124 (Q1)

Bravo, J., Monente, C., Juániz, I., De Peña, M. P., & Cid, C. (2013). Influence of extraction process on antioxidant capacity of spent coffee. *Food Research International*, *50*(Stability of phytochemicals during processing), 610-616. doi:10.1016/j.foodres.2011.04.026

Bravo, J., Juániz, I., Monente, C., De Peña, M., Cid, C., Caemmerer, B., & Kroh, L. (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds.*Journal Of Agricultural And Food Chemistry*, *60*(51), 12565-12573. doi:10.1021/jf3040594

OBJETIVE 2

To evaluate free and bound phenolic compounds for the assessment of the total phenolics content of spent coffee extract, and to determine the most accurate methodology for this purpose.

Evaluar el contenido de compuestos fenólicos libres y unidos para determinar el contenido total de fenólicos en los extractos de posos del café, y establecer la metodología idónea para dicho propósito.

Paper 3:

Assessment of total (free and bound) phenolic compounds in spent coffee extracts

Carmen Monente, Iziar A. Ludwig, Angel Irigoyen, M. Paz De Peña and Concepción Cid

Journal Agricultural and Food Chemistry (2nd revision, January 2015)

Impact Factor (JCR, 2013): 3.107

Journal Rank in categories:

- Agriculture, Multidisciplinary: 2/56 (Q1)
- Chemistry, Applied: 11/71 (Q1)
- Food Science & Technology: 13/123 (Q1)

Monente, C., Ludwig, I., De Peña, M., Cid, C., & Irigoyen, A. (2015). Assessment of total (Free and Bound) phenolic compounds in spent coffee extracts. *Journal Of Agricultural And Food Chemistry*, *63*(17), 4327-4334. doi:10.1021/acs.jafc.5b01619

OBJETIVE 3

To assess the bioaccessibility of phenolic compounds of Arabica and Robusta spent coffee extracts using an in vitro gastroduodenal digestion, followed by the estimation of the potential absorption of phenolic compounds with a Caco-2 human cell model.

Evaluar la bioaccesibilidad de los compuestos fenólicos de los extractos de posos de café Arábica y Robusta aplicando un modelo de digestión in vitro, seguido de la estimación de su potencial absorción en un modelo celular (células humanas Caco-2).

Paper 4:

In vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee extracts

Carmen Monente, Iziar A. Ludwig, Maria Paz de Peña, Concepción Cid, Alan Crozier

International Journal of Food Sciences and Nutrition (submitted, February 2015)

Impact Factor (JCR, 2013): 1.202

Journal Rank in categories:

- Food Science & Technology: 63/123 (Q3)
- Nutrition & Dietetics 63/79 (Q4)

TITLE: *In vitro* studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee extracts

AUTHORS: Carmen Monente[a], Iziar A. Ludwig[a], Maria Paz de Peña[a], Concepción Cid[a], Alan Crozier[b]

[a]Department of Nutrition, Food Science and Physiology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain

[b]Department of Nutrition, 3143 Meyer Hall, One Shields Avenue, University of California, Davis, California 95616-5270, USA

*Corresponding author: Alan Crozier

Tel: -1-530-754-0450

E-mail address: alacrozier@ucdavis.ed

ABSTRACT

Spent coffee grounds are the main by-product of coffee beverage production. This waste product could be a valuable source of phenolic compounds, mainly chlorogenic acids (CGAs), The aim of this work was to study the stability of spent coffee CGAs using *in vitro* simulated gastroduodenal digestion and investigate their potential absorption using an *in vitro* Caco-2 model of human small intestinal epithelium. Two spent coffee extracts from filter and espresso coffee brews were submitted to the *in vitro* digestion model. CGAs of nondigested and digested samples were analyzed by HPLC-PDA-MSn. Similar concentrations but different chromatographic phenolic profiles were found during *in vitro* digestion. Lactones were partially degraded and CQA and FQA showed high resistant. Furthermore, the transport and metabolism studies were carried out with Arabica digested sample and standard solutions. Lactones and coumaroylquinic acids showed a higher transport percentage than other phenolic compounds; moreover minimal amounts of the three isomers of CQA and FQA were able to cross the intestinal barrier. The metabolic activity of Caco-2 cells was lower than the expected. In conclusion, spent filter and expresso coffee grounds are rich, unused source of CGAs. The CGAs in spent coffees extracts were largely stable during in vitro gastroduodenal digestion with limited absorption of the intact compounds across the intestinal epithelium.

KEYWORDS: Coffee, by-products, bioaccessibility, Caco-2.

1. Introduction

The consumption and development of functional foods have been growing rapidly in recent years, driven by the increased interest in food that can improve human health. This has resulted in searches for new bioactive ingredients for products with a potential to contribute to the prevention and reduction of risk factor for chronic diseases, or have the capacity to enhance key physiological functions. Flavonoids and related phenolic compounds have proven to be bioactive with a number of beneficial effects on health (Yao et al. 2004, Crozier et al. 2009). One of the main dietary sources of phenolic compounds are the millions cup of coffee consumed every day, due to the presence of substantial amounts of chlorogenic acids (CGAs). The levels do however vary; principally as a consequence of roasting (Moon et al. 2009) and a recent study found the amount per serving in espresso coffees ranging from 24-422 mg (Crozier et al. 2012). Epidemiological studies have linked coffee consumption with a reduced risk of type 2 diabetes (Salazar-Martinez et al. 2004), cardiovascular diseases (Andersen et al. 2006) and some types of cancer notably prostate (Li et al. 2013) and skin (cutaneous melanoma) (Loftfield et al. 2015)

Coffee compounds may be considered as potential functional foods. A sustainable strategy could use the tons of by-products generated after brewing coffee, namely the spent coffee grounds. Currently, limited data are available on the CGA profile of spent coffee grounds. Moreover, the fate of CGAs as they pass along the gastrointestinal tract is another key factor to assess spent coffee significance as a functional ingredient. Previous studies have focused on assessing the absorption and metabolic fate of chlorogenic acids (CGA) from coffee beverage (Stalmach et al. 2009, 2010, 2014; Renouf et al. 2014; Monteiro et al. 2007); studies investigating the gastrointestinal stability, and bioaccessibility of chlorogenic acids from spent coffee grounds are scarce.

The aim of the current study was to assess the CGA profile of the spent coffee grounds remaining after the production of filter and espresso coffees, and to investigate the upper gastrointestinal stability of these compounds using an *in vitro*

model of digestion, and to determine the transport mechanism using Caco-2 cell, an in vitro model of intestinal absorption.

2. Experimental

2.1 Chemicals

Caffeic acid, ferulic acid, sinapic acid, 5-CQA, caffeine, human saliva α-amylase (300-1500 U/mg protein), pepsin (674 U/mg), pancreatin (4 x UPS) and bile salts were purchased from Sigma-Aldrich (Steinheim, Germany). 4,5-DiCQA was obtained from Phytolab (Vestenbergsgreuth, Germany) and dihydrocaffeic acid and dihydroferulic acid from Alfa Aeser (Heysham, UK). Culture flasks, Transwell fitted with polycarbonate membranes were acquired from Corning® Costar® Transwell (Sigma Aldrich, St. Louis, US). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Invitrogen (Paisley, UK). The Caco-2 cell line PD7 clone was kindly provided by Dr. Edith Brot-Laroche (Paris, France). All chemicals and reagents used were of analytical grade from Panreac (Barcelona, Spain), Fisher Scientific (Loughborough, UK), and Sigma Aldrich (Steinheim, Germany).

2.2 Coffee samples

Medium roasted coffee beans from Guatemala (Arabica) and Vietnam (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.

2.3 Spent coffee extract preparation

Arabica filter (24 g coffee/ 400 mL) and Robusta espresso (7g coffee/ 45 mL) coffee brew were prepared to obtain spent coffee. Spent coffee extracts were prepared according to the method described by Bravo et al. (2012). First, dried spent coffee was defatted with petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet

extraction system (Extraction Unit B-811 Standard Büchi, Flawil, Switzerland). Then, spent Arabica coffee was extracted with a volume of 400 mL of water using a filter coffeemaker (model Avantis 70 Inox). Extraction took approximately 6 min at 90 °C. Aqueous spent coffee extracts and coffee brews were freeze-dried and stored at

–18 ºC. Spent coffee extraction yielded 10% (w/w) in terms of final lyophilized powder of spent coffee extract.

2.4 *In vitro* digestion

A three step in vitro digestion was carried out in a bioreactor according to Pastoriza et al. (2011). Briefly, 2 g of each spent extract was dissolved in 200 mL of water and transferred into a vessel placed in a water bath at 37°C. The vessel was magnetically stirred and connected to a pH sensor. Before each step the sample was sparged with N_2 to maintain an reduced O_2 atmosphere. The three steps were carried out in absence of light. First, oral digestion was performed by adding 500 µL of an α-amylase solution (32.5 mg of α-amylase dissolved in 25 ml 1 mM $CaCl_2$, pH 7.0) and incubating for 15 min. The second gastric digestion step was carried out at pH 2.5 with HCl 6N and it was initiated by adding 547 µL of a pepsin solution containing 0.05 g of pepsin/g of lyophilized spent coffee. After a 2 h incubation the gastric digestion was stopped by raising the pH to 6.5 with 0.1 M $NaHCO_3$. Finally for intestinal digestion, 5 mL of a mixture of pancreatin and bile salts (0.1 g of pancreatin and 0.625 g of bile salts in 25 ml of 1 M $NaHCO_3$) was added to the vessel. The pH was adjusted to pH 7.5 with 0.1 M $NaHCO_3$, and samples incubated for 2 h. After each step an aliquot was collected, and the enzymes were inactivated with heat treatment (4 min at 100 ºC), after which the samples were immediately cooled, freeze-dried and stored at –18 ºC prior to analysis. Each experimental condition was tested in triplicate.

For CGA analysis, the freeze dried spent coffee extracts and the digested samples were reconstituted with distilled water, centrifuged at 16.2 g for 5 min and filtered

with 0.45 µm pore size, 13 mm, Milex-HV filters (Millipore, Bedford, MA, USA), and diluted 20-fold with distilled water.

2.5 Transport and metabolism experiments using Caco-2 cells

Caco-2 cells were cultured in DMEM supplemented as previously describe Fanjul et al. (2012). Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin-EDTA and sub-cultured on 25 or 75 cm^2 plastic flasks at a 25×10^4 cells/cm^2 density. Caco-2 cells were seeded on Transwell inserts (12 mm diameter, 1.12 cm^2 growth area) at a density of 6×10^4 cells/cm^2. Culture medium was replaced every 2 days. Cell confluence was confirmed by transepithelial electrical resistance (TEERS) measurements. Transport experiments were performed at 17–21 days post-seeding according to Farrel et al. (2012). Lyophilised, digested Arabica spent coffee was redissolved in DMEM (15 mg/mL, pH 7.4). The culture medium was aspirated and 1 mL test solution was added in the apical side and 2 mL of DMEM pH 7.4 in the basal side. The incubation period was 0.5 and 1 hour at 37ºC. At the end of the incubation period media from the apical and basal sides were collected, the monolayer was washed with DMEM, and cells were scraped in sterile water and sonicated for 30 s. Samples were cooled immediately, freeze-dried and stored at –18 ºC. Each experimental condition was tested in triplicate.

For the metabolism experiment, Caco-2 cells were incubated with (1 mL) standard solutions 5-CQA (0.9 mM), 4,5-DiCQA (0.2 mM), caffeic (2.5 mM), ferulic (0.7 µM), dihydrocaffeic (2.5 µM) and dihydroferulic (0.7 µM) in DMSO (0.1%). The incubation period was 0.5 and 1 hour at 37ºC, then samples were removed and stored as described above. Each experimental condition was tested in triplicate. For CGA analysis culture solution and cells were extracted and deproteinated according Stalamach et al. (2009) with some modifications. Each sample was resuspended in 500 µL of methanol containing 1% formic acid and 20 mmol/L of sodium diethydithiocarbamate and spiked with sinapic acid (20 µL) as an internal standard.

The samples in eppendorf tubes were mixed using a flat shaker at a speed of 400 rpm for 5 min at room temperature, before being centrifuged for 10 min at 4ºC. The supernatant was collected and the pellet was re-extracted as describe above. The two supernatants were combined and reduced to dryness in vacuo and resuspended in HPLC mobile phase.

4.6 Qualitative and quantitative analysis of CGAs and hydroxycinnamates by HPLC-PDA-MS^n

CGAs were analyzed using a Surveyor HPLC with a photodiode array (PDA) detector scanning from 200 to 600 nm, an autosampler cooled at 6 ºC, and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (ESI) (Thermo Fisher Scientific, San Jose, CA). The injection volume was 100 µL of sample. HPLC was performed at 40 ºC with a Synergi 4-mm Polar-RP 250 x 4.6 mm reversed-phase column (Phenomenex, Macclesfield, UK). The mobile phase consisted of 0.1 % aqueous formic acid (solvent A) and 100% methanol (solvent B). Isocratic conditions of 5% solvent B were maintained for 15 min, followed by a gradient from 5 to 10% B over 20 min and maintained for 5 min, then the % B was increased to 40% over 60 min and maintained 20 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 and consecutive reaction monitoring. Calibration curves of standard were used to quantify 5-CQA, hydroxycinnamic acids, dihydroferulic acid (DHF) and dihydrocaffeic acid (DHC). Coefficients of linearity for the calibration curves were typically R^2>0.99. CGAs and hydroxycinnamic acids were quantified by PDA at 325 nm, and 280 nm for DHC and DHF. Quantification of the other CGAs (FQAs, diCQAs, CoQAs, CFQA, CQL, and FQL) 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).

4.7 Statistic

Results are shown as the mean ± standard deviation (SD). Student's t-test was applied for each CGA group to know whether there were differences among spent coffee and the coffee brew. One-way analysis of variance (ANOVA) was applied to determine significant differences ($p \leq 0.05$) between non-digested and digested samples. A T-Tukey test was applied as a test a posteriori with a level of significance of 95%. All statistical analyses were performed using STATA v.12.0.

3. Results

3.1 Characterization of spent coffee extracts

Filter and espresso coffeemakers are commonly used to prepare a cup of coffee, not only at domestic levels but also in workplaces and coffee shops. And Arabica and Robusta coffees are the most consumed varieties around the world. Previous searches have shown that the spent coffees obtained after the preparation of a filter (Arabica coffee) and an expresso (Robusta coffee) coffee brew had the highest antioxidant activity in chemical-based assays and in cell cultures (Bravo et al. 2012; 2013). Thus, these two spent coffee were used to prepare two aqueous extracts which were lyophilised, stored and reconstituted prior to analysis and testing, as well as their respective coffee brews. A total of 36 chlorogenic acids (CGAs) were identified and quantified in both spent coffee extracts and coffee brews with HPLC-PDA-MS^n, including two cinnamoyl-amino acid conjugates (Table 1). Identifications were performed by reference to published CGA fragmentation patterns (Clifford et al. 2003, Clifford and Knight 2004).

Table 1. Identification of chlorogenic acids in spent coffee and coffee brew. HPLC-MS^n rentention time, $[M-H]^-$, 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.

	Rt time	MS^1	MS^2		MS^3	
		Parent ion (m/z)	Base ion m/z	Secondary ions m/z (intensity)	Base ion m/z	Secondary ions m/z (intensity)
O-caffeoylquinic acid	9.6	353	191	179 (90), 135 (35)		
O-caffeoylquinic acid	10.7	353	179	135(25), 191(20)		
3-O-caffeoylquinic acid	12.6	353	191	179(65), 135(15)		
3-O-p-coumaroylquinic acid	20.1	337	163	119(25)		
O-caffeoylquinic acid	22.4	353	179	191(75), 135(25)		
O-caffeoylquinic acid	23.8	353	191	179(10)		
4-O-caffeoylquinic acid	25	353	173	179(95), 191(20), 135(15)		
5-O-caffeoylquinic acid	32.4	353	191	179(5)		
3-O-feruloylquinic acid	33.9	367	193	191(10)		
O-caffeoylquinic acid lactone	38.4	335	179	161(60),135(35)		
4-O-p-coumaroylquinic acid	41.6	337	173	163(10)		
3-O-caffeoylquinic acid lactone	58.2	335	161	135(60),179(10)		
O-caffeoylquinic acid lactone	62.5	335	173	179(25),161(25)		
5-O-p-coumaroylquinic acid	63.2	337	191	179 (5)		
4-O-caffeoylquinic acid lactone	64.6	335	161	135(15),179(15)		
4-O-feruloylquinic acid	67.6	367	173	173(100), 193(30)		
O-feruloylquinic acid	72.4	367	191	173(5)		
5-O-feruloylquinic acid	79	367	191	179(10)		
O-feruloylquinic acid lactone	84,4	349	193	175(30),134(15)		
O-feruloylquinic acid lactone	91.7	349	175	149(50),193(30), 134(15)		
4-O-feruloylquinic acid lactone	93.2	349	175	160(10)		
O-feruloylquinic acid lactone	95.5	349	175	193(85),269(75),173(30),305(70),134(20)		
3,4-di-O-caffeoylquinic acid	98,9	515	353	335(15), 179(15)	179	179(100),173(75),191(60)
1,3 di-O-caffeoylquinic acid	100	515	353	179(25),191(5)	179	179(100),173(25).191(5)
3,5-di-O-caffeoylquinic acid	101.3	515	353	191(15)	191	191(100),179(65),135(15)
1,4-di-O-caffeoylquinic acid	105.6	515	353	335(50), 179(40),191(25)	179	179(100),191(90)
1,5-di-O-caffeoylquinic acid	108.3	515	353	191(50)	191	191(100),179 (35)
4,5-di-O-caffeoylquinic acid	109.8	515	353	173(10),179(10)	179	179(100),173(80),191(25),135(20)
O-caffeoyl-N-tryptophan	111.4	365	229	186(10),135(10)		
3-O-caffeoyl-4-O-feruloylquinic acid	112.1	529	367	173(20),335(10)	173	193(40)
3-O-feruloyl-5-O-caffeoylquinic acid	113	529	367	335(15),173(10)	193	173 (45)
3-O-caffeoyl-5-O-feruloylquinic acid	114.2	529	353	367(40),191(10)	191	179(95)
3-O-feruloyl-4-O-caffeoylquinic acid	115.3	529	353	367(10),335(50)	179	191(20)
p-coumaroyl-N-tryptophan	120.6	349	229	186(15),145(5)		
4-O-feruloyl-5-O-caffeoylquinic acid	121.4	529	367		173	193(50)
4-O-caffeoyl-5-O-feruloylquinic acid	123.8	529	353	367(30)	179	173(90),191(50)

The results indicated that on a weight per weight basis, spent coffee contained higher amounts of CGAs than their respective coffee brew, especially after the production of espresso coffee (Table 2). On average, and a weight/weight basis, there was 6.2±2.5% more CGAs quantified in the Arabica spent coffee compared with the brew, whereas 41±2.4% more CGAs were recovered in Robusta spent coffee compared with the brew, mainly due to the higher extraction of caffeoylquinic acids (CQAs) from the spent coffee (44±2.5%). Spent coffee extracts had a different CGAs profile, depending on the coffee bean variety. Arabica spent coffee contained higher amounts of CQAs, coumaroylquinic acids (*p*CoQAs) and caffeoylquinic acid lactones (CQLs), while Robusta spent coffee was richer in feruloylquinic acids (FQAs), feruloylquinic acid lactones (FQLs), caffeoylferuloylquinic acids (C-FQAs) and cinnamoyl-amino acids.

Table 2. Chlorogenic acids and caffeine content in Arabica and Robusta spent coffees extracts and coffee brews.

Compounds[a]	Arabica		Robusta	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
Caffeoylquinic acids (7)	228 ± 6*	210 ± 2	186 ± 5*	129 ± 3
Feruloylquinic acids (4)	34 ± 1*	28 ± 0	90 ± 3*	48 ± 1
p-Coumaroylquinic acids (3)	9.9 ± 0.3*	9.3 ± 0.3	6.0 ± 0.2*	4.4 ± 0.1
Dicaffeoylquinic acids (6)	3.8 ± 0.0*	2.8 ± 0.0	4.4 ± 0.4*	2.7 ± 0.1
Caffeoylquinic acid lactones (4)	47 ± 3*	53 ± 1	37 ± 2*	43 ± 2
Feruloylquinic acid lactones (4)	4.6 ± 0.6	5.0 ± 0.1	9.0 ± 0.5	9.8 ± 0.1
Caffeoylferuloylquinic acids (6)	0.9 ± 0.0*	0.6 ± 0.0	3.8 ± 0.1*	1.9 ± 0.0
Cinnamoyl-amino acids (2)	0.9 ± 0.0*	0.7 ± 0.0	9.4 ± 0.2*	7.2 ± 0.2
Total chlorogenic acids (36)	329 ± 8*	309 ± 2	345 ± 4*	245 ± 4
Caffeine	406 ± 40	395 ± 35	49 ± 39	671 ± 45

Data expressed in µmol/g of lyophilized spent coffee extract or coffee brew, as mean values ± standard deviation (n = 3). a Number of individual compounds corresponding to the sum for each group of chlorogenic acids. * Significant differences ($p < 0.05$) among spent coffee and its coffee brew (Student's t-test).

3.2 In vitro digestion

The upper gastrointestinal stability of the CGAs, contained in Arabica and Robusta spent coffees, was assessed using a three step *in vitro* model of digestion (Table 3 and Fig. 1). Table 3 summarizes the CGAs content of spent coffee extracts after simulated oral, gastric and intestinal digestion. The results indicated that the total CGA content of spent coffees significantly decreased by 4-14% indicating degradation during the *in vitro* digestion process. However, successive *in vitro* digestions had little impact on CQAs and FQAs degradation, as shown by the stability in the amounts recovered following incubations with digestive juice (Fig. 1). In contrast, the compounds most susceptible to oral, gastric and intestinal digestions were the lactones, CQL and FQL, with losses accounting for 45 to 85% of the initial dose incubated ($p < 0.05$). CGA compounds responded differently to the enzymatic activity and pH changes. Robusta spent coffee showed more degradations of the CQA, FQA, CoQA and diCQA compounds, whereas lactones were more affected in Arabica compared to Robusta spent coffee.

As mentioned above, overall CQAs and FQAs were relatively stable, although after *in vitro* digestion, there were changes due mainly to variations in isomer levels. Firstly, the addition of α-amylase at pH 6.5-7 brought a significant ($p < 0.05$) decrease in the levels of 5-CQA (Fig. 1), and an increase in the 3- and 4-CQA isomers. Following the simulated oral digestion the spent coffees and coffee brews were subjected to in vitro gastric digestion for 2 h. This reduced the concentrations of 3- and 4- isomers, although the final values remained slightly higher than non-digested sample. And finally, the last step was the addition of pancreatin and bile salt (pH 7), which had similar effects to those observed after oral digestion.

Table 3. Chlorogenic acids content of Arabica and Robusta spent coffee extracts after the *in vitro* digestion model.

Compound	Non-digested	Oral digestion	Gastric digestion	Intestinal digestion
Caffeoylquinic acids				
Arabica	228 ± 6[a]	256 ± 8 *(112)*[b]	234 ± 12 *(103)*[a]	244 ± 12 *(107)*[ab]
Robusta	186 ± 5[ab]	194 ± 15 *(104)*[b]	169 ± 6 *(91)*[a]	179 ± 7 *(96)*[ab]
Feruloylquinic acids				
Arabica	34 ± 1[a]	37 ± 1 *(109)*[c]	35 ± 1 *(103)*[ab]	36 ± 1 *(106)*[bc]
Robusta	90 ± 3[a]	99 ± 6 *(110)*[b]	91 ± 4 *(101)*[a]	84 ± 6 *(93)*[a]
p-Coumaroylquinic acids				
Arabica	9.9 ± 0.3[b]	9.0 ± 0.3 *(91)*[a]	9.6 ± 0.6 *(97)*[ab]	9.5 ± 0.3 *(96)*[ab]
Robusta	6.0 ± 0.2[a]	6.4 ± 0.7 *(107)*[a]	5.1 ± 0.3 *(85)*[b]	4.5 ± 0.1 *(75)*[b]
Dicaffeoylquinic acids				
Arabica	3.8 ± 0.0[b]	3.6 ± 0.1 *(95)*[a]	3.5 ± 0.1 *(92)*[a]	3.6 ± 0.0 *(95)*[a]
Robusta	4.4 ± 0.4[b]	3.6 ± 0.5 *(82)*[ab]	3.5 ± 0.3 *(80)*[a]	3.7 ± 0.1 *(84)*[ab]
Caffeoylquinide				
Arabica	47 ± 3[c]	7.7 ± 0.4 *(16)*[a]	17.5 ± 2.1 *(37)*[b]	8.2 ± 0.8 *(17)*[a]
Robusta	37 ± 2[c]	15 ± 3 *(41)*[a]	20 ± 1 *(54)*[b]	17 ± 2 *(46)*[a]
Feruloylquinide				
Arabica	4.6 ± 0.6[b]	1.0 ± 0.1 *(22)*[a]	1.3 ± 0.1 *(28)*[a]	0.9 ± 0.1 *(20)*[a]
Robusta	9.0 ± 0.5[b]	4.6 ± 1.0 *(51)*[a]	5.0 ± 0.8 *(56)*[a]	5.3 ± 0.3 *(59)*[a]
Caffeoylferuloylquinic acids				
Arabica	0.9 ± 0.0[a]	0.9 ± 0.0 *(100)*[a]	0.9 ± 0.0 *(100)*[a]	0.9 ± 0.0 *(100)*[a]
Robusta	3.8 ± 0.1[a]	3.5 ± 0.4 *(92)*[a]	3.3 ± 0.3 *(87)*[a]	3.4 ± 0.1 *(89)*[a]
Cinnamoyl-amino acids				
Arabica	0.9 ± 0.0[a]	1.0 ± 0.1 *(111)*[b]	0.9 ± 0.0 *(100)*[ab]	0.9 ± 0.0 *(100)*[ab]
Robusta	9.4 ± 0.2[a]	8.7 ± 0.8 *(93)*[a]	8.5 ± 0.4 *(90)*[a]	9.0 ± 0.5 *(96)*[a]
Total chlorogenic acids				
Arabica	329 ± 11[b]	317 ± 10 *(96)*[ab]	303 ± 10 *(92)*[a]	304 ± 15 *(92)*[a]
Robusta	345 ± 11[b]	336 ± 28 *(96)*[b]	296 ± 13 *(86)*[a]	306 ± 15 *(89)*[a]

Data expressed in µmol/ g of lyophilized spent coffee extract non-digested or digested. Values are mean ± standard deviation from three independent experiments. Italicized numbers in brackets represent the recovery as a percentage of the non-digested sample. Different superscripts within rows indicate significant differences ($p < 0.05$) between non-digested and digested samples (Analysis of Variance with a pair-wise post-hoc comparison).

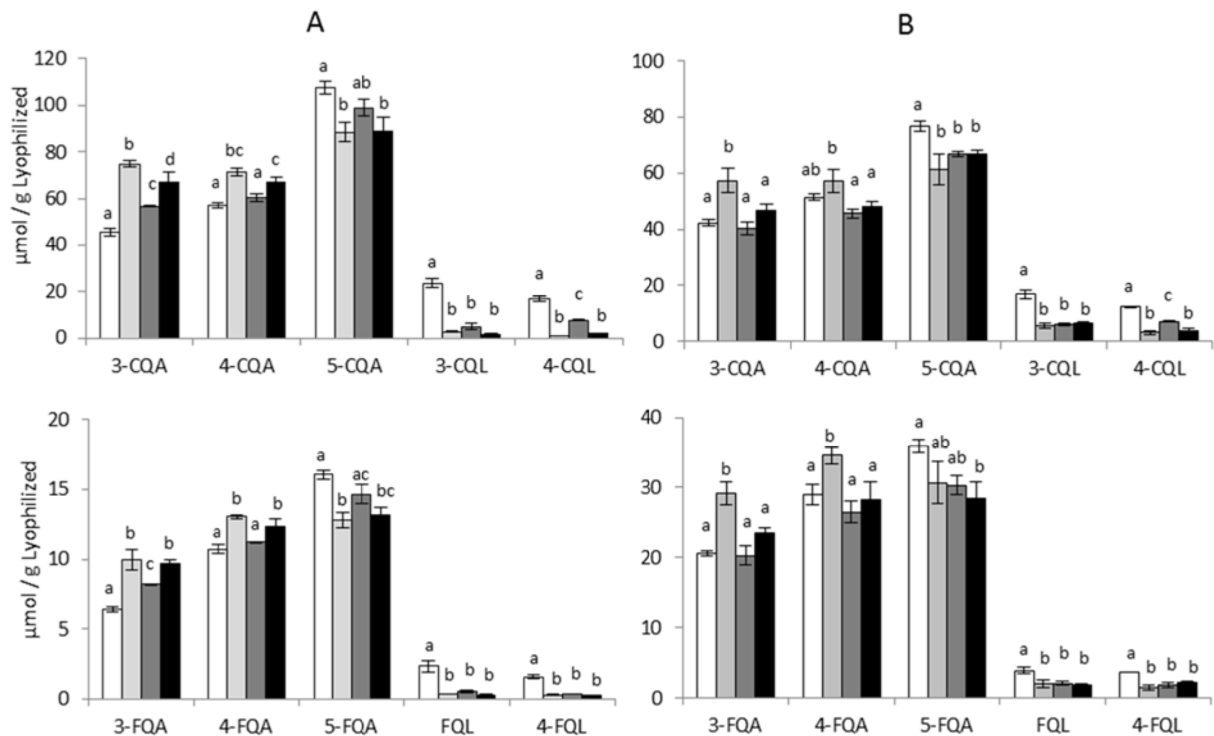


Fig. 1. The impact of *in vitro* digestion model on the stability of the main CGAs isomers in spent coffee extracts. (A) Arabica spent coffee, (B) Robusta spent coffee. (□) Non Digested (■) Oral digestion, (■) Gastric digestion, (■) Intestinal digestion. Data expressed as mean value ± standard deviation. Different letters in each isomer indicate significant differences ($p < 0.05$) between non-digested and digested samples (Analysis of Variance with a pair-wise post-hoc comparison).

3.3 Absorption and metabolism of spent coffee CGAs and hydroxycinnamate standards

The absorption study was performed with the digested Arabica spent coffee extract. The sample was incubated in Transwell cell culture inserts with a Caco-2 cell monolayer, which simulated the intestinal barrier. Table 4 shows the amount of CQAs found in the apical and basal compartments and in the Caco-2 cells as well as the percentage related to the initial amount loaded on the apical side. The results indicate that only 0.55% of the total CGAs were absorbed and transported from the apical to the basolateral side of cell in the first 0.5 h, increased to 1% after 1 h.

quinide lactones and CoQA were recovered in greater amounts on the basolateral side, accounting for 33% and 17% of the amount incubated, respectively. Other CGAs were subject to negligible transport from the apical to the basal cell. DiCQAs showed an absorption of 0.6% and CQAs and FQAs of 0.3% and 0.5%, respectively. There was no difference in the transport of the different CQA and FQA isomers. In contrast to most of the CGAs, there was substantial transport of caffeine with 30% recovered on the basolateral side within 0.5 h and a further 6.5% taking place in the subsequent 0.5 h (Table 4).

Further incubations were carried out to compare the absorption and metabolism of individual coffee components. Caco-2 cells were incubated with 5-CQA, 4,5-diCQA, caffeic acid, ferulic acid, dihydrocaffeic acid and dihydroferulic acid. Table 5 summaries the percentage of the initial dose remaining on the apical side after 0.5 h and 1 h, and that found in the Caco-2 cells and the quantities transported to the basolateral side. Table 5 also provides information on metabolites produced. All the compounds were identified by comparing MS^n fragmentation with patterns of previous works and standard solutions (Stalmach et al. 2009; Farrel et al. 2011b)

Compounds with the highest basolateral recovery were ferulic acid and dihydroferulic acid, accounting for 5.9% and 5.3% of the apical dose, respectively, indicating a highest absorption and basolateral transport compared with caffeic and dihydrocaffeic acids (<0.1% of the apical dose), 5-CQA (0.3%) and 4,5-DICQA (0.5%). There was evidence of substantial metabolism principally in the form of isomerization of 5-CQA and 4,5-DiCQA. There was also low level i) hydrolysis of CQAs and DiCQA releasing caffeic acid, ii) sulphation of caffeic acid and ferulic acid, iii) methylation of caffeic acid yielding ferulic acid and iv) conversion of ferulic acid to dihydroferulic acid (Table 5).

Furthermore, different amounts of CGAs were found among spent coffee extracts. Arabica spent coffee had higher amounts of CQAs, coumaroylquinic acids (CoQAs) and caffeoylquinides (CQLs), while Robusta spent coffee was richer in feruloylquinic acids (FQAs), feruloylquinides (FQLs), caffeoylferuloylquinic acids (C-FQAs) and cinnamoyl-amino acids.

Table 4. Transport of CGAs in spent coffee extracts across the Caco-2 cell monolayer after 0.5 and 1 h of incubation.

Compounds	30 min			1h		
	Apical	Cell	Basolateral	Apical	Cell	Basolateral
Caffeoylquinic acids	1736 ± 31*(99)*	21 ± 2 *(1.2)*	3.6 ± 4 *(0.2)*	1659 ± 45 *(98)*	24.1 ± 3 *(1.4)*	5.4 ± 1.8 *(0.3)*
Feruloylquinic acids	413 ± 10 *(99)*	3.6 ± 0.6 *(0.9)*	1.0 ± 0.0 *(0.3)*	404 ± 39 *(98)*	4.7± 1 *(1.2)*	2.1 ± 0.2 *(0.5)*
Coumaroylquinic acids	34 ± 3 *(90)*	0.4 ± 0.1 *(1.1)*	3.3 ± 0.1 *(8.8)*	33 ± 5 *(82)*	0.5 ± 0.1 *(1.2)*	6.8 ± 0.9 *(17)*
Dicaffeoylquinic acids	27 ± 1 *(97)*	0.8 ± 0.2 *(3.0)*	0.1 ± 0.0 *(0.4)*	23 ± 0 *(96)*	0.9 ± 0.2 *(3.9)*	0.1 ± 0.1 *(0.6)*
Caffeoylquinide	16 ± 7 *(76)*	0.6 ± 0.3 *(3.1)*	4.4 ± 0.2 *(21)*	16 ± 6 *(64)*	0.7 ± 0.0 *(3.0)*	8.2 ± 0.7 *(33)*
Total chlorogenic acids	2225 ± 75 *(98)*	27 ± 5 *(1.2)*	13 ± 1 *(0.6)*	2136 ± 95 *(98)*	31 ± 4 *(1.4)*	23 ± 4 *(1.0)*
Caffeine	3517 ± 387 *(67)*	138 ± 42 *(2.7)*	1563 ± 42 *(30)*	2990 ± 591 *(61)*	139 ± 11 *(2.8)*	1798 ± 67 *(37)*

Data are expressed in nmol as means ± standard deviation from three independent experiments. Italicized numbers in brackets represent the amount of each individual CGA as the percentage of the initial apical dose.

Table 5. Transport and metabolism of CGAs and hydroxycinnamates across the Caco-2 Cell monolayer.

Compounds	% of apical dose					
	0.5 h			1 h		
	Apical	Cell	Basolateral	Apical	Cell	Basolateral
Substrate						
5-*O*-Caffeoylquinic acid	73.1	1.5	0.06	72.0	1.3	0.29
Metabolites						
4-*O*-Caffeoylquinic acid	15.7	0.13	0.001	16.1	0.12	0.29
3-*O*-Caffeoylquinic acid	9.1	0.04	tr	9.4	0.03	0.05
Caffeoylquinic acid-*O*-sulfate	0.05	tr	tr	0.03	tr	0.03
Caffeoylquinic acid-*O*-sulfate	0.23	tr	tr	0.17	tr	0.02
Caffeic Acid	0.04	0.06	0.01	0.04	0.03	0.10
Total	98.2	1.7	0.08	97.7	1.5	0.78
Substrate						
4,5-*O*-Dicaffeoylquinic acid	48.6	1.2	0.11	44.7	0.75	0.47
Metabolites						
3,5-*O*-Dicaffeoylquinic acid	25.7	0.4	0.09	28.5	0.28	0.23
3,4-*O*-Dicaffeoylquinic acid	10.0	0.07	0.03	13.3	0.06	0.06
5-*O*-Caffeoylquinic acid	6.8	0.26	0.15	6.1	0.20	0.20
4-*O*-Caffeoylquinic acid	3.3	tr	Tr	2.6	tr	tr
3-*O*-Caffeoylquinic acid	2.9	tr	Tr	2.1	tr	tr
Caffeic acid	0.27	0.05	0.04	0.23	0.09	0.08
Total	97.6	2.0	0.4	97.5	1.4	1.0
Substrate						
Caffeic acid	94.6	1.7	0.01	92.3	0.01	0.04
Metabolites						
Caffeic acid-3-*O*-sulfate	0.55	0.02	0.03	1.4	0.03	0.03
Ferulic acid	3.0	0.01	0.03	2.6	0.03	0.13
Total	98.2	1.7	0.07	96.3	0.07	0.2
Substrate						
Ferulic acid	95.5	0.9	3.5	92.3	0.6	5.9
Metabolites						
Ferulic acid-4-*O*-sulfate	0.03	0.02	0.04	0.04	0.02	0.07
Dihydroferulic acid	tr	tr	tr	0.79	0.18	0.16
Total	95.5	1.0	3.5	93.1	0.8	6.1
Substrate						
Dihydrocaffeic acid	96.8	3.2	tr	96.8	3.1	0.03
Substrate						
Dihydroferulic acid	96.2	1.7	2.1	93.2	1.5	5.3

Data expressed as a percentage of the apical dose of each standard solution, means from three independent experiments. tr= traces (< LOQ)

4. Discussion

The by-product generated after the brewing process namely, spent coffee, is a rich source of a diversity of CGAs. The aim of the present work was to assess the bioaccessibility of these compounds using Caco-2 cells and determine their stability in the proximal gastrointestinal tract, using a three step in vitro model of digestion to simulate the physiological conditions of the distal gastrointestinal tract.

The stability of the various CGAs in the spent coffee extracts varied depending on the type of compounds, following incubation with simulated digestive juices corresponding to a model of oral, gastric and intestinal digestion. Our study is in line with previous studies reporting that CGAs are stable when incubated with various gastrointestinal fluids (Olthof et al. 2001; Rechner et al. 2001; Farah et al. 2006). Our results showed that the main CGAs in spent coffee, namely CQAs and FQAs were also subjected to isomerisation from the 5-acyl to 3- and 4- acyl loci, as previously reported in ileostomy volunteers (Erk et al. 2014). Moreover, our data showed that the high recoveries of CQA and FQA in the *in vitro* digestion model might be related with the susceptibility and degradation of other components, such as diCQA and lactones, in the spent coffee extracts. These two compounds showed a higher loss of recovery following incubation with gastrointestinal juices, which could have resulted to the release of the hydroxycinnamate moiety of DICQAs or the cleavage of the intramolecular ester bond of lactones. Furthermore, the release of CQAs from other structures, including melanoidins, into which they are incorporated during roasting (Bekedam et al. 2008, Perrone et al. 2012), could also be hypothesised. In general, CGAs in spent coffee extracts were stable in the *in vitro* model of the proximal gastrointestinal tract implying that *in vivo* substantial amounts are likely to reach the colon. This is in keeping with the reported 59-77% recoveries of CQAs in ileal fluid after the ingestion of coffee by ileostomists (Stalmach et al. 2010; Erk et al. 2012).

CQA isomers in spent coffee were transported across the Caco-2 monolayer in only low amounts, corresponding to 0.3% of the apical dose. Similar absorption values have been reported for CQAs from coffee brew and standard solutions using similar

Caco-2 cell models (Konishi and Kobayashi 2004; Dupas et al. 2006). Despite the different functions and morphology between gastric epithelial and Caco-2 cell monolayers, similar absorptive patterns were observed among both cellular lines. The absorption percentage of spent coffee CQAs was 0.3% of the apical dose, in agreement with the findings of Farrell et al. (2012a). In their study, Farrell et al. found that the percentage FQAs and CQLs transported across the gastric cell monolayer was slightly higher than that of CQAs. The only difference among gastric and intestinal cells was the percentage of diCQAs transported, due to the high permeability observed in gastric cells (Farrell et al. 2012a).

Our study demonstrated higher levels of CGAs in spent coffee compared with the corresponding coffee brew, although with similar profiles of the individual compounds. Robusta coffee spent extract contained ca. 40% more CGAs compared with the beverage (only 6% more CGAs in the Arabica spent coffee extract vs brew), which may have been due to the brewing method used (espresso preparation), known for its low extraction efficiency (Cruz et al. 2012).

Previous studies have highlighted the higher content of antioxidant and bioactive compounds in Arabica and Robusta spent coffee extracts compared with their respective coffee brews (Bravo et al. 2012, 2013). Our study also demonstrated the high levels of unextracted CGAs in spent Arabica and Robusta spent coffee compared with the brews, and showed that the aqueous extracts of spent coffee were stable under gastrointestinal conditions and as bioaccessible as the compounds found in coffee beverage.

5. Conclusions

In conclusion, spent filter and expresso coffee grounds are rich, unused source of CGAs. The CGAs in spent coffees extracts were largely stable during in vitro gastroduodenal digestion with limited absorption of the intact compounds across the intestinal epithelium.

Acknowledgments

This research was funded by the Spanish Ministry of Economy and Competitiveness (AGL2009-12052). We thank Ms Pilar Lostao and Ms. Asunción Redín for her kind help. C.M wishes to express her gratitude to the Association of Friends of the University of Navarra for the grant received and to the Unión Tostadora S.A. for providing the coffee.

References

Andersen, L. F., Jacobs, D. R., Carlsen, M. H., Blomhoff, R. (2006). Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. Am J Clin Nutr 83(5): 1039-1046.

Bekedam EK, Schols H, Van Boekel Martinus AJS, Smit G. (2008). Incorporation of chlorogenic acids in coffee brew melanoidins. J Agric Food Chem 56(6): 2055.

Bravo J, Juaniz I, Monente C, Caemmerer B, Kroh L, De Peña MP, Cid C. (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds. J Agric Food Chem 60(51):12565-73.

Bravo J, Arbillaga L, de Peña MP, Cid C. (2013). Antioxidant and genoprotective effects of spent coffee extracts in human cells. Food Chem Toxicol 60: 397-403.

Clifford MN, Knight S. (2004). The cinnamoyl–amino acid conjugates of green robusta coffee beans. Food Chem 87(3): 457-463.

Clifford M, Johnston K, Knight S, Kuhnert N. (2003). Hierarchical scheme for LC-MSn identification of chlorogenic acids. J Agric Food Chem 51(10), 2900-2911.

Crozier A, Jaganath IB, Clifford MN. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 26(8): 1001-1043.

Crozier TWM, Stalmach A, Lean MEJ, Crozier A. (2012). Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. Food Funct 3:30-33.

Cruz R, Cardoso MM, Fernandes L, Oliveira M, Mendes E, Baptista P, Morais S, Casal S. (2012). Espresso coffee residues: a valuable source of unextracted compounds. J Agric Food Chem 60(32): 7777-7784.

Dupas C, Marsset Baglieri A, Ordonaud C, Tomé D, Maillard M. (2006). Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study. Mol Nutr Food Res 50(11): 1053-1060.

Erk T, Williamson G, Renouf M, Marmet C, Steiling H, Dionisi F, Barron D, Melcher R, Richling E. (2012). Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. Mol Nutr Food Res 56(10): 1488-1500.

Erk T, Renouf M, Williamson G, Melcher R, Steiling H, Richling E. (2014). Absorption and isomerization of caffeoylquinic acids from different foods using ileostomist volunteers. Eur J Clin Nutr 53: 159–166.

Fanjul C, Barrenetxe J, Iñigo C, Sakar Y, Ducroc R, Barber A, Lostao MP. (2012). Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2. Acta Psychol 205(1): 82-91.

Farah A, De Paulis T, Trugo L, Martin P. (2005). Effect of roasting on the formation of chlorogenic acid lactones in coffee. J Agric Food Chem 53(5): 1505-1513.

Farah A, Guigon F, Trugo LC. (2006). The effect of human difestive fluids on chlorogenic acids isomers from coffee. In the proceedings of the 21st International Conference on Coffee Science, Montpellier, ASIC: Montpellier 2006, 93-96.

Farrell TL, Dew TP, Poquet L, Hanson P, Williamson G (2011a). Absorption and Metabolism of Chlorogenic Acids in Cultured Gastric Epithelial Monolayers. Drug Metab Dispos 39(12): 2338-2346.

Farrell T, Poquet L, Dionisi F, Barron D, Williamson G. (2011b). Characterization of hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC–DAD–MS2: Enhancing chromatographic quantification and application in Caco-2 cell metabolism. J Pharm Biomed Anal 55(5): 1245-1254.

Farrell TL, Gomez-Juaristi M, Poquet L, Redeuil K., Nagy K., Renouf M, Williamson G. (2012). Absorption of dimethoxycinnamic acid derivatives in vitro and pharmacokinetic profile in human plasma following coffee consumption. Mol Nutr Food Res 56(9): 1413-1423.

Konishi Y, Kobayashi S. (2004). Transepithelial Transport of Chlorogenic Acid, Caffeic Acid, and Their Colonic Metabolites in Intestinal Caco-2 Cell Monolayers. J Agric Food Chem 52(9): 2518-2526.

Li Q, Kakizaki M, Sugawara Y, Tomata Y, Watanabe T, Nishino Y, Tsuji I. (2013). Coffee consumption and the risk of prostate cancer: the Ohsaki Cohort Study. Br J Cancer 108: 2381-2389.

Loftfield E, Freedman ND, Graubard BI, Hollenbeck AR, Shebl FM, Mayne ST, Sinha R. (2015). Coffee drinking and cutaneous melanoma risk in the NIH-AARP Diet and Health Study. J Natl Cancer Inst. 107(2): 1-9

Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C. (2007). Chlorogenic Acid Compounds from Coffee Are Differentially Absorbed and Metabolized in Humans. J Nutr 137(10): 2196-2201.

Moon JK., Yoo HS, Shibamoto T. (2009). Role of roasting conditions in the level of chlorogenic acid content in coffee beans: correlation with coffee acidity. J Agric Food Chem 57(12): 5365-5369.

Olthof MR, Hollman PC, Katan M B. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. J Nutr 131: 66-71.

Pastoriza S, Delgado-Andrade C, Haro A, Rufián-Henares JA. (2011). A physiologic approach to test the global antioxidant response of foods. The GAR method. Food Chem 129(4): 1926-1932.

Perrone D, Donangelo R, Donangelo CM, Farah A. (2010). Modeling Weight Loss and Chlorogenic Acids Content in Coffee during Roasting. J Agric Food Chem 58(23): 12238-12243.

Rechner AR, Spencer JPE, Kunhle G, Hahn U, Rice-Evans CA. (2001). Novel biomarkers of the metabolism of caffeic acid derivatives in vivo. Free Radical Bio Med 30: 1213-1222.

Renouf M, Marmet C, Giuffrida F, Lepage M, Barron D, Beaumont M, Williamson G, Dionisi F. (2014). Dose-response plasma appearance of coffee chlorogenic and phenolic acids in adults. Mol Nutr Food Res 58(2), 301-309.

Salazar-Martinez E, Willett WC, Ascherio A, Manson JE, Leitzmann MF, Stampfer MJ, Hu FB. (2004). Coffee consumption and risk for type 2 diabetes mellitus. Ann Intern Med 140: 1-8.

Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C, Steiling H, Williamson G, Crozier A. (2009). Metabolite Profiling of Hydroxycinnamate Derivatives in Plasma and Urine after the Ingestion of Coffee by Humans: Identification of Biomarkers of Coffee Consumption. Drug Metab Dispos 37: 1749-1758.

Stalmach A, Steiling H, Williamson G, Crozier A. (2010). Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. Arch Biochem Biophys 501 (1): 98-105.

Stalmach A, Williamson G, Crozier A. (2014). Impact of dose on the bioavailability of coffee chlorogenic acids in humans. Food Funct 5(8): 1727 1737

Trugo LC, Macrae R. (1984). A study of the effect of roasting on the chlorogenic acid composition of coffee using HPLC. Food Chem 15(3): 219-227.

Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, Chen SS. (2004). Flavonoids in Food and Their Health Benefits. Plant Foods for Human Nutrition, 59: 113-122.

OBJETIVE 4

To evaluate the antimutagenic and antimicrobial activity of Arabica and Robusta spent coffee extracts for further applications in functional foods.

Evaluar la actividad antimutagénica y antimicrobiana de los extractos de posos del café Arábica y Robusta para su posterior aplicación en alimentos funcionales.

Paper 5:

Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms

Carmen Monente, Jimena Bravo, Ana Isabel Vitas, Leire Arbillaga, M. Paz De Peña and Concepción Cid

Journal of Functional Foods, 2015, I2, 365-374

Impact Factor (JCR, 2013): 4.480

Journal Rank in categories:

- Food Science & Technology: 5/123 (Q1)

Monente, C., Bravo, J., De Peña, M., Cid, C., Vitas, A., & Arbillaga, L. (2015). Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms. *Journal Of Functional Foods*, *12*365-374.
DOI: 10.1016/j.jff.2014.12.006

GENERAL DISCUSSION

Spent coffee is a potential source of compounds with beneficial effects on human health. However, these compounds should be firstly extracted from spent coffee grounds in order to be applied as functional ingredients. Initial analyses carried out in the research group established the most efficient methodological conditions for the extraction of compounds with antioxidant capacity, which are neutral aqueous medium (pH 7.0) using a filter coffeemaker in a proportion of 24 g spent coffee per 400 mL water (Bravo et al., 2013a). Subsequently, two processes, namely defatting and lyophilization, were tested to optimize the extraction of antioxidant compounds. The application of both technological processes was the most efficient methodology. In fact, the extract with the highest antioxidant capacity was obtained when spent coffee grounds were defatted and the extract was lyophilized (Bravo et al., 2013a). Moreover, defatting process showed more influence than lyophilization on radical scavenging activity. Defatting process with organic solvents has been previously used to remove fatty compounds in coffee before extraction of antioxidants or other coffee compounds, such as melanoidins (Rufian-Henares and Morales, 2007; Nunes and Coimbra, 2007; Bekedam et al., 2008a; Ramalakshmi et al., 2009). Thus, the removal of fat could facilitate the extraction of water soluble antioxidants. Defatting process can also prevent fat rancidity and radicals formation during long storage of spent coffee extracts. And lyophilization not only avoids the microorganism growth extending self-life, but also facilitates the handling of spent coffee extracts in powder for further applications, such as an ingredient or an additive in food industry.

This optimized extraction methodology was applied to spent coffee grounds obtained after two of the most common coffee brew preparations (filter and espresso coffeemaker) using two coffee varieties (Arabica and Robusta). All spent coffee extracts showed relevant amounts of total caffeoylquinic acids (CQAs). The distribution of CQAs was identical to that previously reported for coffee brew, where 5-CQA was the most abundant CQAs, followed by 4-CQA and 3-CQA (Clifford, 2000). Our results agree with published data of Cruz et al. (2012), who reported similar amounts of 5-CQA in espresso coffee residues. Furthermore, Arabica spent coffees showed higher concentrations of CQAs than Robusta ones. Higher amounts

of CQAs in Robusta coffees than in Arabica ones have been extensively reported (Farah et al., 2005). However other authors have found higher content in Arabica coffees (Vignoli et al., 2011; Ludwig et al., 2012). This could be because both, the origin of coffee and the higher loss of chlorogenic acids in Robusta coffee during roasting process (Clifford, 1997; Perrone et al., 2012).

Furthermore, other phenolic compounds, such as diCQAs, were found in spent coffee samples. The 3,4-diCQA were the most abundant, followed by 4,5-diCQA and 3,5-diCQA. Some authors observed that diCQAs were extracted rather slowly from coffee in comparison to CQAs (Blumberg et al., 2010; Ludwig et al., 2012). The esterification of an additional caffeic acid molecule in diCQAs increases the number of hydroxyl groups that might be bound to the amide carbonyls of the peptide bond in melanoidins (Bekedam et al., 2008b; Nunes and Coimbra, 2010). Also, other authors corroborated the hypothesis that the chlorogenic acids are incorporated into melanoidins via hydroxycinnamic acids (such as caffeic acid) moieties, mainly through nonester linkages upon coffee roasting (Perrone et al., 2012). Therefore, the release of diCQAs bound to melanoidins is rather difficult compared to monoCQAs.

All spent coffee extracts had concentrations of caffeine ranged from 3.59 mg (from Arabica espresso coffee) to 8.09 mg (from Robusta espresso coffee) per gram of spent coffee. Quite similar amounts of caffeine were reported by Cruz et al. (2012) for espresso coffee residues obtained from Arabica/Robusta blends. These results are in agreement with those of the literature which extensively reports that Robusta coffees are richer in caffeine than Arabica ones (Belitz et al., 2009).

Finally, browned compounds, mainly melanoidins, are originated by Maillard reactions during coffee roasting process. Spent coffee extracts obtained from the filter coffeemaker showed higher browning indices than espresso spent coffees. The absorbance values of spent coffee extracts from filter coffeemaker were similar to those found in filter coffee brews (Bravo et al., 2012). Melanoidins and Maillard reaction products contribute to the antioxidant and other functional properties of coffee by themselves and because of the presence of phenolics in their skeleton (López-Galilea et al., 2006; Rufián-Henares and Morales, 2007).

After this first screening, Arabica coffee residues from filter coffee brew were selected for further experiments because of their highest values of CQAs, diCQAs and melanoidins. Despite the fact that the two Robusta spent coffee extracts had less phenolics content than Arabica ones, Robusta espresso spent coffee extract was also selected for further analyses based on its high antioxidant capacity. This is because Robusta coffees are richer in other CGAs, namely FQAs (Farah et al., 2006). In fact, recent studies have identified the presence of FQAs and other phenolic compounds, such as chlorogenic acids lactones, in spent coffee obtained from espresso coffee capsule or cafeterias of different origins, but their concentration is still unknown (Cruz et al., 2012; Panusa et al., 2013; Belviso et al., 2014). Therefore, a more detailed phenolic profile was determined in the two selected spent coffee samples.

A total of 36 free chlorogenic acids were identified and quantified with HPLC-PAD-MS, including 2 Cinnamoyl-amino acid conjugates. The phenolic acids profiles were different between both spent coffee extracts. Arabica spent coffee extract had higher amounts of CQAs, *p*CoQAs and CQLs, while Robusta spent coffee was richer in FQAs, FQLs, C-FQAs and cinnamoyl-amino acids. Furthermore, the content of other phenolic compounds, such as hydroxycinnamic acids and benzoic acid derivative, was also measured by HPLC-DAD. The results showed low amounts of caffeic acid, ferulic acid, p-coumaric acid, sinapic acid, as well as 4-hydroxybenzoic acid in spent coffee extracts.

Phenolic compounds could be also found in the food matrix attached to other structures such as proteins, polysaccharides, etc. by hydrogen, covalent, ionic bonds and other interactions (Kroll et al., 2003; Pérez-Jiménez and Torres, 2011). As mentioned above, phenolics have been found attached to melanoidins skeleton. These bound compounds might contribute to health related properties, after their release from food matrices by gastrointestinal enzymatic action or further microbiota activity (Andreasen et al., 2001; Manach et al., 2004). The knowledge of the total content of phenolic compounds (free and bound) in spent coffee extracts is important for their potential use as functional ingredients by the food industry. Therefore, after identification and quantitation of free phenolic compounds,

Arabica spent coffee extract, which was the sample with the highest value of melanoidins, was submitted to three hydrolytic methods (alkaline, acid and saline). Then, bound phenolic compounds were identified and quantitated in order to estimate total phenolic compounds.

First, alkaline hydrolysis or saponification is applied to release compounds bound to polymers by covalent interactions (Pérez-Jiménez and Torres, 2011). After hydrolysis, the major CGAs in spent coffee extract completely disappeared while caffeic acid significantly increased, even though not all the expected quantity was found. Some authors have reported that phenolic compounds are susceptible to oxidation at pH 8 and higher, leading to degradation into their corresponding molecules derivatives (Cilliers and Singleton, 1989; Kroll et al., 2003). Alkaline medium can break not only the covalent bonds between melanoidins and phenolic compounds, but also CGAs internal bonds. Our findings suggest that the caffeic acid found in hydrolyzed samples were partially due to the cleavage of the ester linkages in the CGAs with caffeic acid in their structure mentioned above, namely CQAs, CQLs and diCQAs. Numerical data indicate that around 47% (spent coffee extract) and 19% (coffee brew) of the caffeic acid found after the alkaline hydrolysis could come from CGAs or caffeic acids attached to other structures. In the case of coffee brew, this percentage of bound phenolic compounds is in the range of 1 to 29% proposed by Perrone et al. (2012) depending on the roasting degree.

Similarly, the increment of ferulic and coumaric acids concentration could be explained by the release of hydroxycinnamic acids from FQAs and *p*CoQA presents in coffee brew and spent coffee extract. The percentage of the additional ferulic acid found in hydrolyzed samples was similar to the total content of free FQAs and FQLs, which indicate that minor amount of this compound was released from melanoidins and other macromolecules. Even though the concentrations of sinapic and 4-hydroxybenzoic acids were lower than caffeic, ferulic and coumaric acids, an increase was found in comparison to the values in the non-treated samples. There is scarce literature about the presence of sinapic acid in coffee. Up to our knowledge, sinapic acid linked to quinic, caffeoylquinic and feruloylquinic acids has only been reported in Robusta green coffee (Jaiswal et al., 2010). Consequently, part of these

chlorogenic acids could remain attached to melanoidins during roasting process. Finally, the increase of 4-hydroxybenzoic acid agrees with Nunes and Coimbra (2007) who found benzoic acid and derivatives as 4-hydroxybenzoic acid attached to HMW melanoidins fraction of roasted coffee after applying alkaline fusion.

The second hydrolytic treatment was an acid hydrolysis, also to induce the rupture of covalent bonds. In spent coffee, CQAs and diCQAs were completely lost after acid hydrolysis. Also, ferulic, coumaric, sinapic and 4-hydroxybenzoic acids totally disappeared in both spent coffee extracts and coffee brews after acid treatment. A previous study reported that this technique could be used to release and to quantitate phenolic compounds like isoflavones from other coffee components (Alves et al., 2010). However, our results strongly suggest that phenolic acids, such as CGAs and hydroxycinnamic acids are very susceptible to acid hydrolysis. This is in agreement with the study of Mattila et al. (2006), which found that phenolic compounds are affected by extreme pH conditions, but oxidation processes are more likely to occur in acid pH. Consequently, acid hydrolysis is an inadequate technique to release the main bound coffee phenolic compounds (phenolic acids), but it can be applied to evaluate others like isoflavones.

The last methodology to determine bound phenolic compounds in spent coffee extracts was saline treatment. This method has been used to release phenolic compounds ionically bound to proteins or melanoidins. Barbeau and Kinsella (1983) reported that chlorogenic acid carboxyl group is predominately ionized at neutral pH. Therefore, the increase of ionic strength tends to neutralize charge interaction between dissociated carboxylic groups of chlorogenic acid and positively charge side chain groups, like the amide carbonyls of the peptide bonds in proteins and melanoidins, leading to the release of ionically bound phenolics. The extra amounts of CQAs, diCQAs, coumaric, sinapic and 4-hydrobenzoic acids found after the saline treatment means that those compounds are ionically attached to other structures. Thus, it should be highlighted that free and ionically bound CQAs were found in similar amounts in spent coffee extracts. The addition of a caffeic acid molecule in the case of diCQAs increases the hydroxyl groups that can ionically interact with melanoidins explaining the higher amount of bound diCQAs in coffee brews in

comparison to spent coffee extracts. This is in agreement with previous works, which showed that a second extraction of ground coffee to obtain spent coffee extracts using a filter coffeemaker favors the extraction of bound CQAs, mainly diCQAs, probably due to the turbulences which facilitate contact of grounds and water (Bravo et al., 2012; Ludwig et al., 2012).

Some authors did not find phenolic acids increases after applying high ionic strength treatment to coffee brew HMW melanoidins fraction (Nunes and Perrone, 2007; Perrone et al., 2012), whereas others found caffeoylquinic acids ionically bound to the HMW melanoidins core contributing to high antioxidant capacity of coffee (Delgado-Andrade and Morales, 2005). Our data strongly suggest that the extra phenolic compounds found after the ionic treatment have been released from the melanoidins core or other medium and low molecular weight melanoidins or Maillard reaction products. In fact, our results support the theory of Bekedam et al. (2008b) that CGAs are also incorporated into the melanoidins through nonester linkages.

In summary, spent coffee extracts has around 2-fold higher content of total phenolics than those measured directly (without hydrolysis or saline treatment) showing an underestimation of phenolic acids. Phenolic compounds with one or more caffeic acid molecules were approximately 54% linked to macromolecules like melanoidins, mainly by non-covalent interactions (up to 81% of bound phenolic compounds). The rest of the quantitated phenolic acids were mainly attached to other structures by covalent bonds (62-97% of total bound compounds).

Free and bound phenolic compounds in spent coffee should be bioaccessible to cross through the intestinal barrier and reach target cells and organs in order to exert their health benefits. To evaluate the bioaccessibility of these compounds, spent coffee extracts were summited to an *in vitro* simulated gastrointestinal digestion and bioactive compounds were analyzed after oral, gastric, and intestinal digestion. CGAs in the spent coffee extracts responded differently to each step of the *in vitro* digestion. The stability of the CGAs in the spent coffee extracts varied

depending on the type of compounds, but the most abundant ones (CQAs and FQAs) did not significantly change. This is in line with previous studies reporting that CGAs are stable when incubated with various gastrointestinal fluids (Olthof et al., 2001; Rechner et al., 2001; Farah et al., 2006). CQAs and FQAs were also subjected to isomerisation from the 5-acyl to 3- and 4- acyl loci, in agreement with other *in vitro* research (Dupas et al., 2006; Tagliazucchi et al., 2012) as well as in ileostomy volunteers (Erk et al., 2012). Moreover, the data showed that the high recoveries of CQA and FQA in the *in vitro* digestion model might be related with the susceptibility and degradation of other components, such as diCQA and lactones, in the spent coffee extracts. This could be due to the release of the hydroxycinnamate molecule of diCQAs or the cleavage of the intramolecular ester bond of lactones. Furthermore, the release of CQAs from other structures, including melanoidins, into which they are incorporated during roasting (Bekedam et al., 2008, Perrone et al., 2012), could also be hypothesized. In general, CGAs in spent coffee extracts were stable in the *in vitro* model of the proximal gastrointestinal tract implying that *in vivo* substantial amounts are likely to reach the colon. This is in keeping with the reported 59-77% recoveries of CQAs in ileal fluid after the ingestion of coffee by ileostomists (Stalmach et al., 2010; Erk et al., 2012).

Absorption study of the digested Arabica spent coffee extract was performed using a Caco-2 cell model, which simulated the intestinal barrier. CQA isomers in spent coffee were transported across the Caco-2 monolayer in only low amounts. Similar absorption values have also been reported with coffee brew and standard solutions using similar cellular models (Konishi and Kobayashi, 2004; Dupas et al., 2006). Even though, gastric and Caco-2 (intestinal) cells have different functions and morphology, similar absorptive patterns were observed between both cellular lines. The absorption percentage of spent coffee CQAs was in agreement with the findings of Farrel et al. (2011). Furthermore, in this study it was found that the percentage of FQAs and CQLs transported across the gastric cell monolayer was slightly higher than that of CQAs. The only difference between gastric and intestinal cells was the transport percentage of diCQAs, due to the high permeability observed in gastric cells (Farrel et al., 2011).

Coffee brews bioavailability studies have found trace amounts of intact CQAs in plasma after coffee consumption, whereas others have reported Cmax of intact CQAs ranged in 0.16% (Monteiro et al., 2007), or 5-19% (Erk et al., 2012) of the initial ingested dose. Furthermore, FQAs have shown higher transport percentage than CQAs, in agreement with Stalmach et al. (2010, 2014). In reference to CQLs, some authors have reported that these compounds are highly metabolized and absorbed in the small intestine (Erk et al., 2012; Stalmach et al., 2010, 2014), which is partially in agreement with the high permeability of spent coffee lactones in Caco-2 cells. Furthermore, some studies have found substantial amounts of diCQAs in human plasma after coffee consumption (Monteiro et al., 2007; Farah et al., 2008). However, recent feeding studies have not detected intact diCQA in plasma, mainly explained by the high molecular weight of these compounds and the enzymatic reactions in the intestinal barrier (Erk et al., 2012; Stalmach et al., 2010, 2014).

Stalmach et al. (2014) reported that 60-70% of the compounds absorbed in small intestine were metabolites. These compounds are the products of esterases, sulphate, glucuronide and methyltransferases enzymes. In our study, some of these products, namely caffeic acid, ferulic acid, caffeic acid-3'-sulphate or ferulic acid-4'-sulphate, were detected after loading Caco-2 cell with the standard solutions. Consequently, this corroborated the presence of these enzymes in the intestinal cells; but the percentages found were inconsistent with values observed in plasma.

In summary, our study demonstrated that the CGAs in spent Arabica and Robusta spent coffee extracts were stable under gastrointestinal conditions and as bioaccessible as the compounds found in coffee brew.

After characterization of the spent coffee extracts, antimutagenic and antimicrobial activity was evaluated to propose their potential application in functional foods.

Spent coffee extracts showed higher antimutagenic activity than coffee brews against the action of the direct acting mutagen NPD due to the presence of bioactive compounds. First, phenolic compounds might have a positive contribution; in fact, previous findings have reported that these compounds might

play an important role in the antimutagenic activity of fruit, vegetables or herbs (Edenharder et al., 2002). The results with the standard solutions indicated that 5-CQA standard was highly effective in the inhibition of NPD mutagen, mainly due to caffeic acid. Furthermore, the ferulic acid of FQAs also showed an important contribution to the antimutagenic activity of spent coffee extracts. Shushi and Kaur (2008) reported that the methoxy group on the phenyl ring is responsible for the antimutagenic activity of ferulic acid.

Second, caffeine standard solutions also showed high protection against the mutagenic agent. However, 5-CQA and caffeine did not showed any additional or synergistic effects, because similar antimutagenic activity has been found in standard solutions mixtures when comparing with individual standards. The mixtures of similar amounts of both compounds were more efficient. This could partially explain the highest inhibition percentage observed in Robusta spent coffee extract with similar concentrations of naturally-occurring CQAs and caffeine. Nevertheless, coffee samples were less effective than standard solutions, probably due to the presence of many other compounds, which may act as antagonists.

Substances may become mutagenic agents after metabolic process, which it is the case of the aromatic amine 2-aminofluorene (2-AF). S9 mix is rat liver microsomal fraction containing phase I and II metabolic enzymes. Moreover, the metabolic activation involves the cytochrome-based P450 metabolic oxidation system, where the arylamino group of 2-AF is oxidized to the N-hydroxy-derivatives (Wang and Guengerich, 2013). These electrophilic products are highly mutagenic due to their capability to form DNA adducts (Heflich and Neft 1994). The results showed that spent coffee extracts and coffee brews had a stronger response against the mutagenic activity of 2-AF than NPD, yielded almost complete protection against the mutagen action. Similar to the aforementioned results regarding NPD, phenolic compounds and caffeine standard solutions were effective reducing the mutagenicity caused by the mutagen 2-AF. In this case, CQA and caffeic acid showed a higher antimutagenic effect than ferulic acid. Furthermore, the inhibition percentages of the standard mixtures were lower than the ones found for coffee

samples, indicating that other compounds, such as Maillard Reaction Products like melanoidins, might participate actively against indirect acting mutagens.

These data demonstrate that 4.8 mg of both spent coffee extracts was able to reduce the activity of direct and indirect acting mutagens. This quantity of spent coffee extract contains approximately 300 µg of naturally-occurring coffee compounds (CQAs and caffeine). The protection mechanism is not completely understood, but results suggest a possible direct action against free radicals and an indirect mechanism for DNA protection. The antioxidant capacity of phenolic acids, caffeine or melanoidins could be associated with the inhibition of mutagens, through scavenging activity against free radicals (Azam et al., 2005, Rufian-Henares and Morales, 2007). The high level of antioxidant activity of the tested spent coffee extracts (Bravo et al., 2012) and their capability to reduce ROS level and DNA strand breaks induced by H_2O_2 in a human cell line (Bravo et al., 2013b) may support the radical scavenging theory. In addition, the samples might also protect-DNA through other ways. Abraham et al. (1991) found genotoxicity inhibition of several carcinogens by coffee in *in vivo* studies. Indirect acting mutagen could also be blocked by interfering with the enzymatic process or competing for the metabolic paths. Some authors have reported that phenolic acids had selective inhibitory effects on cytochrome P450 (Teel and Huynh, 1998). Furthermore, caffeine is metabolized through cytochrome P450 enzymatic path which might cause a decrease in the revertants number, due to a competitive inhibition (Weisburger et al., 1998).

Thus, spent coffee extract compounds were capable of inhibiting, destroying or avoiding DNA damage caused by direct and indirect acting mutagens, and consequently, might be linked with the prevention of earlier stages of carcinogenesis.

A requirement for a new food ingredient, whether it be functional or not, is that it be safe; this ingredient would become even more valuable if it also contributed to food safety. Spent coffee extracts contain bioactive compounds that would be good candidates as suitable food ingredients for preventing or delaying microbial growth that may cause food borne-diseases or food spoilage. Three assays were used to

evaluate the antimicrobial activity of spent coffee against some common food-borne pathogens (*Salmonella, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli*) and food spoilage microorganisms (*Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger*). First, a screening test was carried out to determine the sensibility of each microorganism to spent coffee and coffee brew samples. Next, the lowest amount of extract capable of inhibiting microorganism growth was estimated. And finally, the antimicrobial effect of spent coffee extracts was measured throughout the time.

In general the results showed that all the tested samples were more active against Gram-positive bacteria (*S. aureus*, *L. monocytogenes*, *B. subtilis*) and the yeast (*Candida albicans)* than against Gram-negative ones (*E. coli*, *S.* Choleraesuis, *Ps. aeruginosa*). *B. subtilis* was the least sensitive Gram-positive bacterium to coffee, coinciding with results reported by Murthy and Manonmani (2009). This higher resistance could be related to the capability to produce endospores, a mechanism linked to the increase of resistance to environmental conditions (Russell, 1991). Furthermore, *S. aureus* showed the lowest Minimum Inhibitory Concentration (MIC), and amounts ranging from 5 to 80 mg/mL of spent coffee were capable of inhibiting the growth of a broad range of microorganisms with concentrations of 10^6 cfu/mL; however, the most resistant bacteria (*E. coli*) required larger quantities. The inhibition curves also showed that *S. aureus* was the most sensitive microorganism to the tested extracts and that small amounts of spent coffee extracts may cause a bacteriostatic effect on microorganisms during long exposures.

The response of each microorganism to coffee samples might be influenced by a number of factors. Firstly, structural differences between Gram-negative and Gram-positive bacteria, specifically in the outer membrane, have been associated with resistance patterns. Secondly, the differences in the antimicrobial activity could also be affected by the variation of the concentrations of the phytochemicals among coffee samples. Our data suggest that Gram-positive bacteria appear to be more susceptible to phenolic acids, coinciding with that which was reported by Del Castillo et al. (2007). Several authors have proposed that the hydroxycinnamic

acids, and specifically the hydroxyl groups on chlorogenic acids, are responsible for the antimicrobial activity, probably due to their capability of disrupting the cell membrane permeability (Lou et al., 2011). In our study, Gram positive bacteria were inhibited by small amounts of spent coffee extracts (CQAs ranged from 0.4 to 4.4 mg/mL), while higher concentrations were needed to inhibit *S*. Choleraesuis, *Ps. aeruginosa, E. coli* and *Candida albicans* (CQAs ranged from 1.6 to 10 mg/mL). Therefore, these results suggest that the antimicrobial activity against more resistant microorganisms was due to other coffee components. Although the data did not show a strong connection between a high content of caffeine and lower inhibition diameters or MICs, some authors have reported that caffeine has antimicrobial activity against Gram-negative bacteria (Almeida et al., 2006, 2012). Melanoidins have been proposed as highly active against resistant microorganisms (Einarsson et al., 1983; Rufián-Henares and Morales, 2008; Stauder et al., 2010). Our results strongly suggest that a high content of melanoidins (Robusta coffee brew) is responsible for the growth inhibition of Gram-negative bacteria. Rufián-Henares and De la Cueva (2009) propose that antimicrobial activity of melanoidins could be mediated by metal chelating mechanisms. Therefore, the complex mixture of compounds found in spent coffee (phenolic compounds, caffeine and melanoidins) is able to act as a suitable antimicrobial agent for extent food shelf-life, mainly against Gram-positive bacteria and yeast.

In summary, the residues obtained from the coffee brew preparation, named spent coffee, are a source of large amounts of bioactive compounds easily extracted after the application of an optimized method. The characterization of the main bioactive compounds in spent coffees extracts showed the presence of caffeine and a wide variety of free CGAs, as well as similar amounts of phenolic compounds linked to macromolecules like melanoidins or other Maillard reaction products, mainly by non-covalent interactions. Phenolic compounds in spent coffee extracts showed stability and bioaccessibility under gastrointestinal conditions. Thus, spent coffee extracts may be able to exert the proved antimutagenic activity and other beneficial health effects. Furthermore, this by-product could be considered as a potential food

ingredient for enhancing functional properties and extending the shelf-life of foods, due to the antimicrobial activity. Although further research is needed to study the stability of compounds in a food matrix and while undergoing industrial processes, the results support the idea that spent coffee is an accessible, sustainable, and major source of bioactive compounds with potential health benefits.

References

Abraham, S. K. (1991). Inhibitory effects of coffee on the genotoxicity of carcinogens in mice. *Mutation Research Letters, 262*(2), 109-114.

Almeida, A. A. P. Naghetini, C C Santos, V R Antonio, A G Farah, A Farah,A.Glória, M.B.A. (2012). Influence of natural coffee compounds, coffee extracts and increased levels of caffeine on the inhibition of Streptococcus mutans. *Food Research International, 49*(1), 459-461.

Almeida, A. A. P., Farah, A., Silva, D. A. M., Nunan, E. A., & Glória, M. B. A. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry, 54*(23), 8738-43.

Alves, R. C., Almeida, I. M. C., Casal, S., & Oliveira, M. B. P. P. (2010). Method development and validation for isoflavones quantification in coffee. *Food Chemistry, 122*(3), 914-919.

Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M. (2001) Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biology & Medicine*, 31, 304-314.

Azam S., Hadi, N., Khan, N., & Hadi, S (2003). Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Medical Science Monitor, 9*, 325-330.

Barbeau, W. E., & Kinsella, J. E. (1983). Factors affecting the binding of chlorogenic acid to fraction 1 leaf protein. *Journal of Agricultural and Food Chemistry, 31*(5), 993-998.

Bekedam, E. K., Roos, E., Schols, H. A., Van Boekel, M. A. J. S., & Smit, G. (2008a). Low Molecular Weight Melanoidins in Coffee Brew. *Journal of Agricultural and Food Chemistry*, 56(11), 4060-4067.

Bekedam, E. K., Schols, H., Van Boekel, M. A. J. S., & Smit, G. (2008b). Incorporation of chlorogenic acids in coffee brew melanoidins. *Journal of Agricultural and Food Chemistry*, 56(6), 2055-2063.

Belitz, H., Grosch, W., & Schieberle, P. (2009). Coffee, Tea, Cocoa. In *Food Chemistry,* 4th ed.; Belitz, H., Grosch, W., Schieberle, P., Eds.; Springer-Verlag Berlin: Heidelberg (pp. 938-969).

Belviso, S., Ghirardello, D., Rantsiou, K., Giordano, M., Bertolino, M., Borgogna, D., Cavallero, M. C., Dal Bello, B., Cena, C., Rolle, L., Zeppa, G., & Gerbi, V. (2014). Phytochemical and microbiological stability of spent espresso coffee grounds in capsules. *Food Research International*, 61(0), 93-99.

Blumberg, S., Frank, O., & Hofmann, T. (2010). Quantitative Studies on the Influence of the Bean Roasting Parameters and Hot Water Percolation on the Concentrations of Bitter Compounds in Coffee Brew *Journal of Agricultural and Food Chemistry,58,* 3720-3728.

Bravo, J., Juaniz, I., Monente, C., Caemmerer, B., Kroh, L., De Peña, M. P., & Cid, C. (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds. *Journal of Agricultural and Food Chemistry, 60*(51), 12565-12573.

Bravo, J., Arbillaga, L., de Peña, M. P., & Cid, C. (2013b). Antioxidant and genoprotective effects of spent coffee extracts in human cells. *Food and Chemical Toxicology, 60*(0), 397-403.

Bravo, J., Monente, C., Juániz, I., De Peña, M. P., & Cid, C. (2013a). Influence of extraction process on antioxidant capacity of spent coffee. *Food Research International, 50*(2), 610-616.

Cilliers, J. J. L., & Singleton, V. L. (1989). Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *Journal of Agricultural and Food Chemistry*, 37(4), 890-896.

Clifford MN (1997) The nature of chlorogenic acids. Are they advantageous compounds in coffee? *In Proceedings of the 17th ASIC colloquium*. Nairobi, pp.79-89.

Clifford, M.N. (2000). Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *Journal of the science of food and agriculture, 80*(7), 1033-1043.

Cruz, R., Cardoso, M. M., Fernandes, L., Oliveira, M., Mendes, E., Baptista, P., Morais, S., & Casal, S. (2012). Espresso Coffee Residues: A Valuable Source of Unextracted Compounds. *Journal of Agricultural and Food Chemistry, 60*(32), 7777-7784.

Del Castillo, M. D., Ferrigno, A., Acampa, I., Borrelli, R. C., Olano, A., Martínez-Rodríguez, A., & Fogliano, V. (2007). In vitro release of angiotensin-converting enzyme inhibitors, peroxyl-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. *Journal of cereal science, 45*(3), 327-334.

Delgado-Andrade, C., & Morales, F. (2005). Unraveling the Contribution of Melanoidins to the Antioxidant Activity of Coffee Brews. *Journal of Agricultural and Food Chemistry, 53*(5), 1403-1407.

Dupas, C., Marsset Baglieri, A., Ordonaud, C., Tomé, D., & Maillard, M. (2006). Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study. *Molecular Nutrition & Food Research, 50*(11), 1053-1060.

Edenharder, R., Sager, J. W., Glatt, H., Muckel, E., & Platt, K. L. (2002). Protection by beverages, fruits, vegetables, herbs, and flavonoids against genotoxicity of 2-

acetylaminofluorene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in metabolically competent V79 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 521*(1–2), 57-72.

Einarsson, H., Snygg, B. G., & Eriksson, C. (1983). Inhibition of bacterial growth by maillard reaction products. *Journal of Agricultural and Food Chemistry, 31*(5), 1043-1047.

Erk, T., Williamson, G., Renouf, M., Marmet, C., Steiling, H., Dionisi, F., Barron, D., Melcher, R., & Richling, E. (2012). Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. *Molecular Nutrition & Food Research, 56*(10), 1488-1500.

Farah, A., De Paulis, T., Trugo, L., & Martin, P. (2005). Effect of roasting on the formation of chlorogenic acid lactones in coffee. *Journal of Agricultural and Food Chemistry, 53*(5), 1505-1513.

Farah, A., Monteiro, M., Donangelo, C. M., & Lafay, S. (2008). Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans. *The Journal of nutrition, 138*(12), 2309-2315.

Farah, A., De Paulis, T., Moreira, D. P., Trugo, L. C., & Martin, P. R. (2006). Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees. *Journal of Agricultural and Food Chemistry*, 54(2), 374-381.

Farrell, T. L., Dew, T. P., Poquet, L., Hanson, P., & Williamson, G. (2011). Absorption and Metabolism of Chlorogenic Acids in Cultured Gastric Epithelial Monolayers. *Drug Metabolism and Disposition, 39*(12), 2338-2346.

Heflich, R. H., & Neft, R. E. (1994). Genetic toxicity of 2-acetylaminofluorene, 2-aminofluorene and some of their metabolites and model metabolites. *Mutation Research/Reviews in Genetic Toxicology, 318*(2), 73-174.

Jaiswal, R., Sovdat, T., Vivan, F., & Kuhnert, N. (2010). Profiling and Characterization by LC-MS(n) of the Chlorogenic Acids and Hydroxycinnamoylshikimate Esters in Mate (Ilex paraguariensis). *Journal of Agricultural and Food Chemistry, 58*(9), 5471-5484.

Konishi, Y., & Kobayashi, S. (2004). Transepithelial Transport of Chlorogenic Acid, Caffeic Acid, and Their Colonic Metabolites in Intestinal Caco-2 Cell Monolayers. *Journal of Agricultural and Food Chemistry, 52*(9), 2518-2526.

Kroll, J., Rawel, H. M., & Rohn, S. (2003). Reactions of Plant Phenolics with Food Proteins and Enzymes under Special Consideration of Covalent Bonds. *Food Science and Technology Research, 9*(3), 205-218.

López-Galilea, I., Andueza, S., Leonardo, I. d., de Peña, M., & Cid, C. (2006). Influence of torrefacto roast on antioxidant and pro-oxidant activity of coffee. *Food Chemistry, 94*(1), 75-80.

Lou, Z., Wang, H., Zhu, S., Ma, C., & Wang, Z. (2011). Antibacterial Activity and Mechanism of Action of Chlorogenic Acid. *Journal of Food Science, 76*(6), M398-M403.

Ludwig, I. A., Sanchez, L., Caemmerer, B., Kroh, L. W., De Peña, M. P., & Cid, C. (2012). Extraction of coffee antioxidants: Impact of brewing time and method. *Food Research International, 48*(1), 57-64.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79, 727-747.

Mattila, P., & Kumpulainen, J. (2002). Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *Journal of Agricultural and Food Chemistry, 50*(13), 3660-3667.

Monteiro, M., Farah, A., Perrone, D., Trugo, L. C., & Donangelo, C. (2007). Chlorogenic Acid Compounds from Coffee Are Differentially Absorbed and Metabolized in Humans. *The Journal of nutrition, 137*(10), 2196-2201.

Murthy, P. S., & Manonmani, H. K. (2009). Physico-chemical, antioxidant and antimicrobial properties of Indian monsooned coffee. *European food research and technology, 229*(4), 645-650.

Nunes, F., & Coimbra, M. (2007). Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. *Journal of Agricultural and Food Chemistry, 55*(10), 3967-3977.

Nunes, F. M., & Coimbra, M. A. (2010). Role of hydroxycinnamates in coffee melanoidin formation. *Phytochemistry Reviews*, 9(1), 171-185.

Olthof M. R., Hollman, P. C., & Katan, M. B. (2001) Chlorogenic acid and caffeic acid are absorbed in humans. *Journal of Nutrition*, 131, 66-71.

Panusa, A., Zuorro, A., Lavecchia, R., Marrosu, G., & Petrucci, R. (2013). Recovery of Natural Antioxidants from Spent Coffee Grounds. *Journal of Agricultural and Food Chemistry*, 61(17), 4162-4168.

Pérez-Jiménez, J., & Torres, J. L. (2011). Analysis of Nonextractable Phenolic Compounds in Foods: The Current State of the Art. *Journal of Agricultural and Food Chemistry, 59*(24), 12713-12724.

Perrone, D., Farah, A., & Donangelo, C. M. (2012). Influence of Coffee Roasting on the Incorporation of Phenolic Compounds into Melanoidins and Their Relationship with Antioxidant Activity of the Brew. *Journal of Agricultural and Food Chemistry*, 60(17), 4265-4275.

Ramalakshmi, K., Rao, L. J. M., Takano-Ishikawa, Y., & Goto, M. (2009). Bioactivities of low-grade green coffee and spent coffee in different in vitro model systems. *Food Chemistry, 115*(1), 79-85.

Rechner, A. R., Spencer, J. P. E., Kunhle, G., Hahn, U., Rice-Evans, C. A. (2001) Novel biomarkers of the metabolism of caffeic acid derivatives in vivo. *Free Radical Biology and Medicine*, 30, 1213-1222.

Rufián-Henares, J. M., & Morales, F. (2008). Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E. coli* and *S. aureus*. *Food Chemistry, 111*(4), 1069-1074.

Rufián-Henares, J. M., & Morales, F. (2007). Functional properties of melanoidins: In vitro antioxidant, antimicrobial and antihypertensive activities. *Food Research International, 40*(8), 995-1002.

Rufián-Henares, J. M., & De la Cueva, S. P. (2009). Antimicrobial activity of coffee melanoidins-a study of their metal-chelating properties. *Journal of Agricultural and Food Chemistry, 57*(2), 432-438.

Russell, A. D. (1991). Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology, 71*(3), 191-201.

Stalmach, A., Steiling, H., Williamson, G., & Crozier, A. (2010). Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Archives of Biochemistry and Biophysics*, 501(1), 98-105.

Stalmach, A., Williamson, G., & Crozier, A. (2014). Impact of dose on the bioavailability of coffee chlorogenic acids in humans. *Food & Function, 5*(8), 1727-1737.

Stauder, M., Papetti, A., Mascherpa, D., Schito, A., Gazzani, G., Pruzzo,C., & Daglia, M. (2010). Antiadhesion and antibiofilm activities of high molecular weight coffee components against Streptococcus mutans. *Journal of Agricultural and Food Chemistry, 58*(22), 11662-1166.

Tagliazucchi, D., Helal, A., Verzelloni, E., & Conte, A. (2012). The Type and Concentration of Milk Increase the in Vitro Bioaccessibility of Coffee Chlorogenic Acids. *Journal of Agricultural and Food Chemistry, 60*(44), 11056-11064.

Teel, R. W., & Huynh, H. (1998). Effect of phytochemicals on cytochrome P450-linked alkoxyresorufin O-dealkylase activity. *Phytotherapy Research, 12*(2), 89-93.

Vignoli, J. A., Bassoli, D. G., & Benassi, M. T. (2011). Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material. *Food Chemistry*, 124(3), 863-868.

Wang, K., & Guengerich, F. P. (2013). Reduction of Aromatic and Heterocyclic Aromatic N-Hydroxylamines by Human Cytochrome P450 2S1. *Chemical research in toxicology, 26*(6), 993-1004.

Weisburger, J. H., Dolan, L., & Pittman, B. (1998). Inhibition of PhIP mutagenicity by caffeine, lycopene, daidzein, and genistein. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 416*(1–2), 125-128.

CONCLUSIONS

CONCLUSIONES

1. The extraction method of spent coffee grounds has been improved by applying two technological processes, namely defatting and lyophilization, before and after an aqueous extraction, respectively. This optimized method allowed us to obtain spent coffee extracts powder with high antioxidant capacity that might be used to enhance health related properties and preservation in foods.

2. Spent coffee extracts have a wide variety of free chlorogenic acids (CGA), quantified in 329 and 345 µmol of free CGA per gram of Arabica filter and Robusta espresso spent coffee extracts, respectively. The caffeoylquinic acids were the most abundant (70% of the free CGAs).

3. The application of both alkaline and saline treatments provides accurate information to estimate total bound and ionically bound phenolic compounds of spent coffee extracts, respectively, whereas acid hydrolysis is an inadequate method to quantify coffee phenolic acids.

4. Bound compounds were estimated as half of the total phenolic content in spent coffee extracts, due to the presence of phenolics linked to macromolecules like melanoidins, mainly by non-covalent interactions. Therefore, the application of hydrolytic methods to evaluate free and bound phenolic compounds is needed to avoid the underestimation of bioactive compounds.

5. The majority of CGA in spent coffee extracts remain bioaccessible after in vitro gastroduodenal digestion (89-92%), being the major compounds -CQAs and FQAs- those with the lowest degradation percentage.

6. The absorption of CGAs is marginal, only 1% of the initial dose, using a Caco-2 human cell model. This demonstrates an underestimation in comparison to in vivo studies of coffee brews. Caco-2 cell monolayer model is suitable for an initial screening to estimate the absorption of phenolic compounds in

the human gastrointestinal epithelium. But, further in vivo models should be used to assess the bioavailability of spent coffee extracts bioactive compounds.

7. Spent coffee extracts exhibit strong protection activity against indirect acting mutagen 2-AF (up to 92%), whereas the protection against direct acting mutagen NPD was 12-35%. Phenolic compounds with caffeic acid molecules were highly effective.

8. Spent coffee extracts have antimicrobial activity, mainly against Gram-positive bacteria (Staphylococcus aureus, Listeria monocytogenes) and yeast (Candida albicans). Amounts ranged from 5 to 80 mg/mL of spent coffee extracts were capable of inhibiting the growth of a broad range of microorganisms.

In conclusion, spent filter and espresso coffee grounds are a rich source of bioaccessible and bioactive compounds, mainly free and bound chlorogenic acids and caffeine, which are easily extracted to obtain a powder with antimutagenic and antimicrobial activity. However, further research is needed (1) to determine the stability of compounds in a food matrix and while undergoing industrial processes, (2) to evaluate the effect of the addition of spent coffee extracts on food sensorial properties, and (3) to assess the bioavailability and functionality of the bioactive compounds using in vivo models. This knowledge is required in order to use spent coffee extracts as a potential ingredient for functional foods.

1. Se optimizó el método de extracción de los posos de café aplicando dos procesos tecnológicos, el desgrasado y el liofilizado, antes y después de la extracción acuosa, respectivamente. Este método optimizado permite la obtención de extractos de posos de café en polvo con alta capacidad antioxidante que podrían ser aplicados para mejorar las propiedades saludables y la conservación de alimentos.

2. Los extractos de posos de café presentan una amplia variedad de ácidos clorogénicos libres (CGA), cuantificados en 329 y 345 µmol de CGA libre por gramo de extractos de posos de café Arábica filtro y Robusta expreso, respectivamente. Los ácidos cafeoilquínicos son los más abundantes (70% de los clorogénicos libres).

3. La aplicación de los tratamientos alcalino y salino aporta información precisa sobre el contenido total de compuestos fenólicos unidos, así como de los unidos de forma iónica, respectivamente; mientras que la hidrólisis ácida no es un método adecuado para la cuantificación de ácidos fenólicos.

4. Se estima que la mitad de los compuestos fenólicos de los extractos de posos de café se encuentran unidos a macromoléculas como las melanoidinas, principalmente por interacciones no covalentes. Por lo tanto, con el fin de evitar subestimar el total de compuestos fenólicos, se requiere la aplicación de métodos hidrolíticos para evaluar tanto los compuestos libres como los unidos.

5. La mayoría de ácidos clorogénicos presentes en los extractos de posos de café permanecen bioaccesibles tras una digestión gastroduodenal in vitro (89-92%), siendo los mayoritarios (CQA y FQA) los que mostraron menor porcentaje de degradación.

6. La absorción de los ácidos clorogénicos fue muy limitada, sólo un 1% de la dosis inicial, con el modelo celular Caco-2. Esto demuestra una subestimación en comparación con estudios in vivo con bebidas de café. El modelo celular Caco-2 ha demostrado ser adecuado para estudios iniciales de absorción de compuestos fenólicos en el epitelio intestinal. Sin embargo, la realización de estudios in vivo podría aportar mayor información sobre la biodisponibilidad de los compuestos bioactivos de los extractos de posos del café.

7. Los extractos de posos de café muestran una elevada protección frente al mutágeno de acción indirecta 2-AF (hasta 92%), mientras que la protección frente al mutágeno de acción directa NPD fue de 12-35%. Los fenólicos con un ácido cafeico en su estructura fueron altamente efectivos.

8. Los extractos de posos de café presentan actividad antimicrobiana especialmente frente a bacterias Gram-positivas (Staphylococcus aureus, Listeria monocytogenes) y levaduras (Candida albicans). Ccantidades entre 5 a 80 mg/mL de extractos de posos de café fueron capaces de inhibir el crecimiento de una amplia variedad de microrganismos.

En conclusión, los subproductos obtenidos de la preparación de la bebida de café filtro y expreso son una importante fuente de compuestos bioaccesibles y bioactivos, en su mayoría ácidos clorogénicos libres y unidos, y cafeína, que pueden ser fácilmente extraíbles para obtener un extracto en polvo con actividad antimutagénica y antimicrobiana. Sin embargo, se requieren estudios adicionales para (1) determinar la estabilidad de los compuestos en los alimentos durante el procesado, (2) valorar el efecto sobre las propiedades sensoriales de los alimentos de la adición de los posos del café, y (3) evaluar su biodisponibilidad y funcionalidad mediante estudios in vivo. Este conocimiento es necesario con la finalidad de utilizar los posos de café como ingredientes en alimentos funcionales.

DISSEMINATION OF RESULTS

Publications

Jimena Bravo, Isabel Juániz, **Carmen Monente**, Bettina Caemmerer, Lothar W. Kroh, M. Paz de Peña, Concepción Cid. (2012). Evaluation of spent coffee obtained from the most common coffeemakers as a source of Hydrophilic bioactive compounds. *Journal of Agricultural and Food Chemistry*, 60(51), 12565-12573.

Jimena Bravo, **Carmen Monente**, Isabel Juániz, M. Paz de Peña, Concepcion Cid. (2013). Influence of extraction process on antioxidant capacity of spent coffee. *Food Research International.* 50, 610-616.

Carmen Monente, Iziar Ludwig, Angel Irigoyen, M. Paz De Peña and Concepción Cid. (2014). Assessment of total (free and bound) phenolic compounds in spent coffee extracts. *Journal of Agricultural and Food Chemistry.* (Under revision).

Carmen Monente, Iziar Ludwig, M. Paz De Peña, Concepción Cid, Alan Crozier. (2014). In vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee extracts. *International Journal of Food Sciences and Nutrition.* (submitted, February 2015).

Carmen Monente, Jimena Bravo, Ana Isabel Vitas, Leire Arbillaga, M. Paz De Peña, Concepción Cid. (2015). Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms. *Journal of Functional Foods,* I2, 365-374.

Conference communications

Jimena Bravo, **Carmen Monente**, Isabel Juániz, M. Paz De Peña, Concepción Cid. (2011). Poster: “Spent coffee: A new source of polyphenols”. In *5th International Conference on Polyphenols and Health*, Sitges. Book of Abstracts 2011; pages 176-177. (see annex)

Jimena Bravo, **Carmen Monente**, Isabel Juániz, M. Paz De Peña, Concepción Cid. (2011). Poster: “Assessment the Antioxidant Capacity of Spent Coffee as a New Source of Antioxidants”. In *First International Congress on CoCoTea*, Novara, Italy.

Book of Abstracts and CD-Proceedings 2011; pages 93 and 196. ISBN: 978-88-903360-0-3. (see annex)

Carmen Monente, Jimena Bravo, M. Paz De Peña, Concepción Cid. (2011). “Influence of grinding on Chemicals composition of coffee brew and spent coffee extracts”. In *Euro Food Chem XVI*, Gdansk, Poland. Polish Journal of Food and Nutrition Sciences 2011; Suppl. 1, Vol. 61, pag. 121. ISSN: 1230-0322. (see annex)

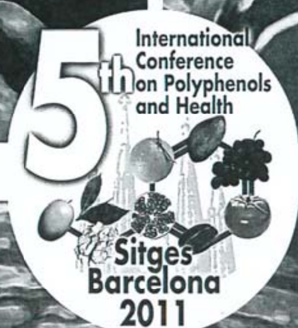
Carmen Monente, Jimena Bravo, Ana Isabel Vitas, Leire Arbillaga, M. Paz De Peña and Concepción Cid. (2014). Oral presentation: Spent coffee grounds as a source of bioactive compounds. *3rd International ISEKI_Food Conference*. Atenas, Grecia. Book of Abstracts 2011; page 44. (see annex)

Carmen Monente, Jimena Bravo, Leire Arbillaga, M. Paz De Peña, Concepcion Cid. (2014). Oral presentation: Antimutagenic activity of coffee brew and spent coffee. *XXI Congreso de la Sociedad Española de Mutagénesis Ambiental*. Navarra, España. Book of Abstracts 2011; page 46. (see annex)

Carmen Monente, Ana Isabel Vitas, M. Paz De Pena, Concepcion Cid. (2014).Poster: Spent coffee: apotential (Poly)phenolic-rich natural food preservative to control microbial growth. *8th World Congress on Polyphenols Applications: ISANH Polyphenols*. Lisboa, Portugal. International Society of Antioxidants in Nutrition & Health; page 204. ISBN: 978-2-35609-073-7. (see annex)

ANNEX

5th International Conference on Polyphenols and Health



Sitges, Barcelona, 17-20 October 2011

FINAL PROGRAMME

www.icph2011barcelona.com

P204

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

Joanna Magielse[1], Teresita Arcoraci[1], Luc Pieters[2], Nina Hermans[1]
Laboratory of Nutrition and Functional Food Science, Department of Pharmaceutical Sciences, University of Antwerp, Antwerp-Belgium[1], Laboratory of Pharmacognosy, Department of Pharmaceutical Sciences, University of Antwerp, Antwerp-Belgium[2]

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.

[1] P. Cos et al. (2002) Free Rad. Res [2] N. Hermans, et al. (2007) J. Phar Pharmacol. [3] N. Hermans, et al. (2005) J. Chrom. B.

P205

UNRAVELLING THE INFLUENCE OF BREWING TIME ON COFFEE CHLOROGENIC ACIDS EXTRACTION

Maria-Paz de Peña, Iziar A. Ludwig, Lidia Sanchez-Ayaso, Concepción Cid
Dept. Nutrition, Food Science Physiology and Toxicology University of Navarra, Pamplona-Spain

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.

Funded by Spanish Ministry of Science (AGL2009-12052)

P206

COMPARISON BETWEEN THE LIPOLYTIC EFFECT OF RESVERATROL AND A MIXTURE OF ANTHOCYANINS IN ISOLATED RAT ADIPOCYTES

Arrate Lasa, Leixuri Aguirre, Jonatan Miranda, Edurne Simón, Itziar Txurruka. María del Puy Portillo
University of the Basque Country, Vitoria-Spain

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.

P207

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

Mariana Monteiro, Adriana Farah
Universidade Federal do Rio de Janeiro, Instituto de Química & Instituto de Nutrição-Brazil

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.

P208

SPENT COFFEE: A NEW SOURCE OF POLYPHENOLS

Maria-Paz de Peña, Jimena Bravo, Carmen Monente, Isabel Juaniz, Concepción Cid
Department Nutrition, Food Science Physiology and Toxicology University of Navarra Pamplona-Spain

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

Hosted by:
The University of Barcelona, UB
UNIVERSITAT DE BARCELONA

different coffeemakers (filter, espresso, plunger and mocha). Spent coffee was dried and defatted. Aqueous extracts were obtained in a filter coffeemaker (24g spent coffee/400mL water) and the extracts were lyophilized. 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.

Results: In all spent coffee extracts relevant amounts of chlorogenic acids were detected, except in mocha ones. Spent coffee from filter coffeemaker showed the highest amount of total caffeoylquinic acids (13.24±0.10 mg/g spent coffee dm), followed by plunger (11.13±0.39 mg/g spent coffee dm) and espresso (11.05±0.13 mg/g spent coffee dm). The content of monoCQAs was higher than diCQAs. Furthermore, diCQAs were 3-4 folds higher in spent coffee extracts than in their respective coffee brews. This evidences that diCQAs were extracted rather slowly from coffee in comparison to monoCQAs, maybe because the presence of two caffeic acid molecules in the chemical structure induces to a higher retention in coffee.

Conclusions: All the coffee residues, with the exception of those obtained with mocha coffee maker, retain relevant amounts of caffeoylquinic acids, being diCQAs in aqueous extracts higher than in coffee brews. For that reason, spent coffee can be proposed as a good source of these polyphenolic compounds in order to obtain potential functional ingredients with applications in new food and pharmaceutical products.

Funded by the Spanish Ministry of Science and Innovation (AGL2009-12052)

P209

CHARACTERIZATION OF POLYPHENOLS IN ORGANIC BLUEBERRY (VACCINIUM SPP.) GROWN IN SOUTHER BRAZIL

Priscilla Reque[1], Marina Pereira[1], Simone Flôres[1], André Jablonski[2], Alessandro Rios[1], Erna Jong[1]

Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul. Porto Alegre. Brazil[1], Escola de Engenharia, Departamento de Engenharia de Minas, Universidade Federal do Rio Grande do Sul. Porto Alegre. Brazil[2].

Blueberry (Vaccinium spp.) is a fruit rich in polyphenols like flavonoids, tannins and phenolic acids. Studies indicate beneficial health properties associated with their high antioxidant activity and free radical-scavenging capacity against the development of chronic diseases, such as diabetes, cancer, cardiovascular and neurodegenerative diseases. Moreover, it also has reports of beneficial actions on the quality of vision. Polyphenols like carotenoids are natural pigments responsible for color of vegetables, fruits and include a large number of compounds, most of those with biological activity. Alpha and beta-carotene have pro-vitamin A activity while lutein and zeaxanthin may play a role in reducing the development and progression of age-related macular degeneration. As there is very little information about the profile of blueberries polyphenols, the aim of the present study was to characterize these compounds in this fruit by HPLC analysis. Rabbiteye organic blueberries were produced in Camaquã (Southern Brazil) and harvested between December 2009 and January 2010. The analyses were carried out in an Agilent high performance liquid chromatography unit, equipped with a degasser, a quaternary solvent pump and a UV/Vis detector. The results show small concentration of zeaxanthin (26,25 µg/100g FW) and beta-carotene (30,64 µg/100g FW), but higher amounts of lutein (158,49 µg/100g FW). It represents 1,53% of the USA recommendation daily intake for beta-carotene and 10,87% for lutein and zeaxanthin or according to the recommendation of Denmark, 1,70% and 26,42% for beta-carotene and lutein, respectively. These results are similar to other two studies with blueberries polyphenols from Bulgaria and Finland but larger than those found by the USDA. Although blueberries are not a great source of carotenoids, these substances, in addition to anthocyanins and other phenolic compounds, are also responsible for the healthy properties of this fruit, especially related to vision.

Key Words: blueberry (Vaccinium spp.), carotenoids, HPLC analysis.

P210

EFFECT OF ENZYMATIC TREATMENT ON POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY IN GRAPE SEED EXTRACT AND GRAPE POMACE

Susana Chamorro[1], Agustin Viveros[2], Inmaculada Alvarez[1], Agustín Brenes[1]

Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN-CSIC, Madrid. Spain[1], Facultad de Veterinaria, Universidad Complutense de Madrid-Spain[2]

In recent years, the interest in the extraction of bioactive compounds from agricultural by-products has promoted research on the use of cell wall hydrolyzing enzymes. The capacity of three fungal enzymatic preparations was used to evaluate the release of low molecular weight of polyphenols and the antioxidant activity of grape pomace and grape seed extract. Cellulase (Laminex, 3174 U/g), pectinase (Pectozyme, 135 U/g) and tannase (10U/g) were added at two concentrations (0.5 and 1%), individually or combined, to grape seed extract and grape pomace. The enzymes have been selected on the basis of the structural composition of grape seeds. HPLC-MS and DPPH radical scavenging activity assays were used to determine phenolic compounds and antioxidant activity. In the case of grape seed extract, tannase treatment increased epicatechin (up to 22%), gallic acid (up to 471%) and procyanidin B2 (42%) content while epigallocatechin gallate, gallocatechin gallate and epicatechin gallate were entirely hydrolysed. In grape pomace, cellulase treatment was not efficient for phenolic release and antioxidant activity improvement. Pectinase, tannase and combination of three enzymes increased the release of epigallocatechin (up to 32, 3 and 29%, respectively), gallic acid (up to 34, 78 and 99%, respectively) and procyanidin B2 (up to 10, 10 and 22%, respectively) while epicatechin gallate was fully hydrolysed. These effects were related with an increase in the antioxidant activity. In conclusion, tannase catalyzed the hydrolysis of the catechin gallates in grape seed by-products releasing mainly gallic acid and increasing the antioxidant activities of the resultant hydrolysates. In grape pomace, the release of phenolic compounds and the antioxidant activity was enhanced with the combination of pectinase and tannase but not with the addition of cellulase.

P211

ORGAN SPECIFIC DIVERSITY IN CONTENT OF POLYPHENOLS AMONG BLACK CURRANT (RIBES NIGRUM L) GERMPLASM CULTIVATED IN SWEDEN

Michael Vagiri[1], Anders Ekholm[1], Eva Johansson[2], Kimmo Rumpunen[1]

Swedish university of agricultural sciences, Department of Plant Breeding and Biotechnology, Balsgard, Kristiansta-Sweden[1], Swedish university of agricultural sciences, Department of Agriculture-Farming Systems, Technology and Product Quality, Alnarp-Sweden[2]

In recent years there has been an increased scientific interest toward the crops belonging to the genus Ribes. Especially black currant has been found an interesting crop, not only due to the taste but also for the health benefits associated with consumption. In addition to the high vitamin C content within the fruit, high levels of other bioactive compounds with potential antioxidant ability have been reported. Recent studies conducted have shown different anatomical parts of the black currant (buds, leaves and fruits) to be good sources of polyphenolic compounds. These compounds have been related to flavor, colour and health benefits, making them desirable extracts for functional foods. The important phenolic compounds that occur in black currants are e.g. anthocyanins, glycosides of myricetin, quercetin, kaempferol, catechins, isorhamnetin and phenolic acids. In numerous in vitro studies conducted, the phenolic extracts of black currant are known to exhibit anti-inflammatory, vasomodulatory and anti-haemostatic activities. Furthermore the black currant anthocyanins have been demonstrated to provide neuroprotection against oxidative stress and in improving vision. The objective of the present study was to biochemically characterizing different organs of the black currant plant for the content of individual polyphenols and estimates the diversity among cultivars. The plant material was cultivated at Balsgård, Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Southern Sweden. Fruits, buds and leaves were hand harvested, freeze-dried, ground and extracted by ethanol. Polyphenols were then separated and analyzed by HPLC-DAD. The composition of flavonols, phenolic acids, catechins and anthocyanidins were investigated. Information about the diversity will be presented in the poster.

P212

IDENTIFICATION OF POLYPHENOLS IN RED WINE BY UHPLC

Isidre Masana[1], Friedrich Mandel[2], Judy Berry[3], William Long[3], John W Henderson Jr.[3]

Agilent Techonologies, Barcelona-Spain[1], Agilent Technologies, Waldbroon-Gremany[2], Agilent TechonologiesWilmington-USA[3]

Generating high peak capacity is necessary for the analysis of complex samples in order to reduce the number of overlapping peaks. Greater peak capacity and resolution can be easily obtained for gradient analysis of complex samples such as wine, by using the higher efficiency of sub-2 micron particles in longer column lengths. This was confirmed by a 43% increase in peak capacity for the analysis of 19 polyphenol standards when the column length of a narrow bore Agilent ZORBAX Rapid Resolution High Definition (RRHD) StableBond SB-C18 column was increased from 100mm to 200mm. An additional 15% improvement in peak capacity was achieved by increasing the column length an extra 100mm to 300mm. The data show that higher quality separations can be achieved using longer column lengths and is demonstrated by the analysis of polyphenols in wine. The Agilent 1290 Infinity LC system was used because the increased column length resulted not only in a significant improvement in resolution, but also system pressures in the 600-1000 bar range.

SPENT COFFEE: A NEW SOURCE OF POLYPHENOLS

Jimena Bravo, Carmen Monente, Isabel Juániz, M. Paz De Peña*,Concepcion Cid
Department of Nutrition, Food Science, Physiology and Toxicology, School of Pharmacy.
University of Navarra, E-31080-Pamplona, Spain. * mpdepena@unav.es

Introduction

Chlorogenic acids (CGA), and mainly caffeoylquinic acids, are the most abundant phenolic compounds in coffee. Despite their contribution to the final acidity, astringency and bitterness of the coffee brew, they are very well known for their antioxidant properties.
Technological factors play an important role in antioxidants extraction during the brewing process (Andueza et al., 2007, López-Galilea et al., 2007, Pérez-Martínez et al., 2010) and, consequently, may have an influence on the presence of remaining antioxidant compounds in spent coffee. Thus, spent coffee that is produced in tons by restaurants and cafeterias, and consumers at domestic levels, could be a good opportunity to have an important source of natural antioxidants, such as chlorogenic acids.

Objective

The aim of the present study was to evaluate the amount of the major chlorogenic acids in spent coffee obtained using the most common coffeemakers (filter, espresso, plunger or french, and mocha).

Results and Discussion

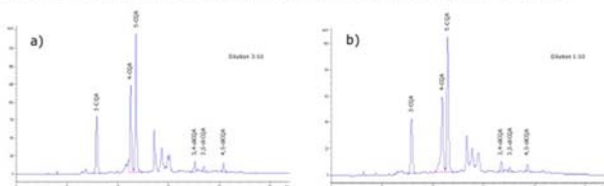
Three monocaffeoylquinic acids (3-CQA, 4-CQA and 5-CQA) and three dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA and 4,5-diCQA) were identified and quantified in spent coffee extracts and their respective coffee brews. The chromatograms of Guatemala filter spent coffee and coffee brew are shown in Figure 1.

Relevant amounts of chlorogenic acids were detected in all spent coffee extracts, except in mocha ones. The most abundant CGA group in spent coffee extracts was the CQA, being 5-CQA the major isomer (2.48-3.59 mg/g spent coffee dm) followed by 4-CQA (1.75-2.51 mg/g spent coffee dm) and 3-CQA (1.1-1.64 mg/g spent coffee dm). A higher extraction of these compounds in the coffee brew led to a lower concentration of monoCQAs in the spent coffee extracts (Figure 2).

In contrast, the amount of diCQAs was 3-4 fold higher in spent coffee extracts than in their respective coffee brews (Figure 3). The range of values for total diCQAs were 0.22-1.94 mg/g coffee dm, and 4.89-5.79 mg/g spent coffee dm in coffee brews and spent coffee extracts, respectively. This evidences that diCQAs were extracted rather slowly from coffee in comparison to monoCQAs, maybe because the presence of two caffeic acid moieties in the chemical structure induces to a higher retention in coffee.

In relation to coffeemaker extraction, spent coffee from filter coffeemaker showed the highest amount of total caffeoylquinic acids (13.24±0.10 mg/g spent coffee dm), followed by plunger (11.13±0.39 mg/g spent coffee dm) and espresso (11.05±0.13 mg/g spent coffee dm). For mocha spent coffee extracts, total caffeoylquinic acids were found below the limit of quantification because mocha coffeemaker extracted the highest amount of total caffeoylquinic acids in the coffee brew.

Figure 1. Chromatogram of a) Guatemala filter spent coffee and b) Guatemala filter coffee brew.



Conclusions

All the coffee residues (spent coffee), with the exception of those obtained with mocha coffeemaker, retain relevant amounts of caffeoylquinic acids. The amount of diCQAs was higher in spent coffee extracts than in coffee brews. For those reasons, spent coffee can be proposed as a good source of these polyphenolic compounds in order to obtain potential functional ingredients with applications in new food and pharmaceutical products.

Acknowledgements

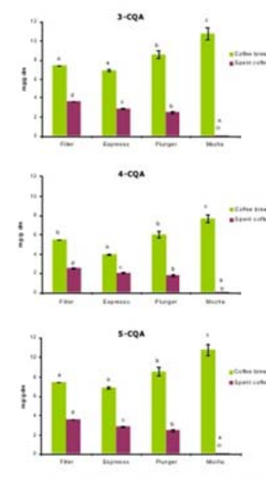
This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Association of Friends of the University of Navarra for the grants given to J.B. and C.M., and the Unión Tostadora for providing the coffee.

Materials and Methods

HPLC-DAD analysis:
- Solid Phase Extraction (Bicchi et al., 1995)
- HPLC method (Farah et al., 2005 with modifications)
- Identification with 5-CQA (Sigma-Aldrich), 3-CQA and 4-CQA (by isomerization of 5-CQA, Trugo and Macrae, 1984), and diCQAs (Phytolab) standards.
- Quantification based on the area of 5-CQA combined with molar extinction coefficients (Trugo and Macrae, 1984)

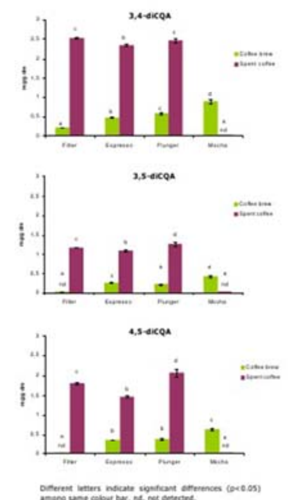
Analysis of variance (ANOVA)

Figure 2. Monocaffeoylquinic acids content of spent coffee in comparison to their respective coffee brews.



Different letters indicate significant differences ($p<0.05$) among same colour bar. tr, traces.

Figure 3. Dicaffeoylquinic acids content of spent coffee in comparison to their respective coffee brews.



Different letters indicate significant differences ($p<0.05$) among same colour bar. nd, not detected.

References

Andueza, S., Vila, M.A., de Peña, M.P., Cid, C. Influence of coffee/water ratio on the final quality of the espresso coffee. J. Sci. Food Agric. **2007**, 87, 586-592.
Bicchi, C. P., Binello, A. E., Pellegrino, G. M., Vanni, A. C. Characterization of Green and Roasted Coffees through the Chlorogenic Acid Fraction by HPLC-UV and Principal Component Analysis. J. Agric. Food Chem. **1995**, 43 (6), 1549-1555.
Bravo, J., Monente, C., Juániz, I., De Peña, M. Paz, Cid, C. Influence of extraction process on antioxidant capacity of spent coffee. Food Res. Int. **2011**, doi:10.1016/j.foodres.2011.04.026.
Farah, A., De Paulis, T., Trugo, L.C., Martin, P.R. Effect of roasting on the formation of chlorogenic acid lactones in coffee. J. Agric. Food Chem. **2005**, 53(5), 1505-1513.
López-Galilea, I., de Peña, M.P., Cid, C. Correlation of selected constituents with the Total antioxidant capacity of coffee beverages: Influence of the brewing procedure. J. Agric. Food Chem. **2007**, 55, 6110-6117.
Pérez-Martínez, M., Cämmerer, B., De Peña, M.P., Cid, C, Kroh, L.W. Influence of brewing methods and acidity regulator on the antioxidant capacity of coffee brews. J. Agric. Food Chem. **2010**, 58(5), 2958-2965.
Trugo, L.C. and Macrae, R. Chlorogenic Acid composition of instant coffees. Analyst **1984**, 109, 263-266.

Book of Abstracts

First International Congress on
Cocoa Coffee and Tea

13th-16th September 2011
Novara, Italy

Edited by F. Travaglia, M. Bordiga, J.D. Coïsson, M. Locatelli, V. Fogliano & M. Arlorio

P 28

ASSESSMENT THE ANTIOXIDANT CAPACITY OF SPENT COFFEE AS A NEW SOURCE OF ANTIOXIDANTS

Bravo J., Monente C., Juaniz I., De Peña M.P., Cid C.

Department of Nutrition, Food Science, Physiology and Toxicology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain.

e-mail: mpdepena@unav.es

Abstract

During last few years, new sources of natural bioactive compounds have been proposed to be added as ingredients for functional foods. Some industrial coffee by-products generated during roasting process or soluble coffee preparation demonstrated to have antioxidant properties (Borrelli et al., 2004, Ramalakshmi et al., 2009). However, the residues generated during coffee brewing procedure, called spent coffee, which are produced in large amounts in cafeterias and restaurants or at domestic levels have not been already studied. Therefore, the aim of the present study was to evaluate the antioxidant capacity of spent coffee to know if this coffee by-product can be considered as a source of antioxidants. Two coffees of different varieties (Arabica and Robusta) were extracted with filter and espresso coffeemakers for obtaining the spent coffee. Spent coffee was dried and defatted and aqueous extracts were obtained in a filter coffeemaker and lyophilized. The antioxidant capacity was evaluated using radical scavenging (ABTS and DPPH), and total phenolic compounds assays in the aqueous spent coffee extracts and the coffee brews (as reference). Guatemala spent coffee obtained from filter coffeemaker showed the highest values in all assays, followed by Vietnam spent coffee obtained from espresso coffeemaker. The antioxidant capacity of Guatemala filter spent coffee was almost the same (88.9-102.3%) than that of the coffee brew. For Vietnam espresso spent coffee, the antioxidant capacity was 59.2- 68.7% than that of the coffee brew. In conclusion, spent coffee obtained after coffee brewing still have antioxidant capacity, consequently this by-product could be consider as a potential source of antioxidant compounds in the food and pharmaceutical industries to increase food nutritional values, products stability and to develop new products.

References

Borrelli, R. et al. Characterization of a New Potential Functional Ingredient: Coffee Silverskin. J. Agric. Food Chem. **2004**, 52, 1338-1343.

Ramalakshmi, K. et al. Bioactivities of low-grade green coffee and spent coffee in different *in vitro* model system. Food Chem. **2009**, 115, 79-85.

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Association of Friends of the University of Navarra for the grants given to J.B. and C.M., and the Unión Tostadora for providing the coffee samples.

ASSESSMENT THE ANTIOXIDANT CAPACITY OF SPENT COFFEE AS A NEW SOURCE OF ANTIOXIDANTS

Bravo J., Monente C., Juaniz I., De Peña M.P.*, Cid C.

Department of Nutrition, Food Science, Physiology and Toxicology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain. * mpdepena@unav.es

Universidad de Navarra

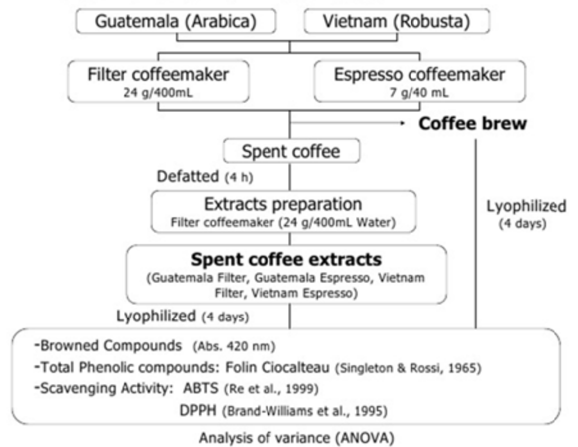
Introduction

During last few years, new sources of natural bioactive compounds have been proposed to be added as ingredients for functional foods. Some industrial coffee by-products generated during roasting process or soluble coffee preparation demonstrated to have antioxidant properties (Borrelli et al., 2004, Ramalakshmi et al., 2009). However, the residues generated during coffee brewing procedure, called spent coffee, which are produced in large amounts in cafeterias and restaurants or at domestic levels have not been already studied.

Objective

The aim of the present study was to evaluate the antioxidant capacity of spent coffee to know if this coffee by-product can be considered as a source of antioxidants.

Materials and Methods



Results and Discussion

Table 1. Browned Compounds, Total Phenolic compounds and Antioxidant Capacity (ABTS, DPPH) of Spent Coffee.

	Filter	Espresso
Browned Compounds		
(Abs 420 nm)		
Guatemala	0.165 ± 0.006	0.102 ± 0.001
Vietnam	0.145 ± 0.005	0.133 ± 0.001
Total Phenolic compounds		
(mg GA / g spent coffee dm)		
Guatemala	24.60 ± 0.18	15.79 ± 0.08
Vietnam	17.54 ± 0.26	19.12 ± 0.14
ABTS		
(μmol Trolox / g spent coffee dm)		
Guatemala	215.12 ± 2.18	131.83 ± 1.49
Vietnam	167.24 ± 5.78	177.63 ± 1.23
DPPH		
(μmol Trolox / g spent coffee dm)		
Guatemala	112.06 ± 2.21	74.57 ± 2.99
Vietnam	74.09 ± 1.00	83.59 ± 2.39

All values are shown as means ± SD (n=3).

Table 2. Browned Compounds, Total Phenolic compounds and Antioxidant Capacity (ABTS, DPPH) of Coffee brews.

	Filter	Espresso
Browned Compounds		
(Abs 420 nm)		
Guatemala (Arabica)	0.155 ± 0.003	0.315 ± 0.008
Vietnam (Robusta)	0.197 ± 0.001	0.418 ± 0.008
Total Phenolic compounds		
(mg GA / g coffee)		
Guatemala (Arabica)	26.70 ± 0.07	23.13 ± 0.23
Vietnam (Robusta)	38.16 ± 1.31	30.55 ± 0.06
ABTS		
(μmol Trolox / g coffee)		
Guatemala (Arabica)	242.08 ± 3.54	206.12 ± 1.68
Vietnam (Robusta)	310.93 ± 2.27	299.86 ± 2.69
DPPH		
(μmol Trolox / g coffee)		
Guatemala (Arabica)	109.49 ± 2.80	87.13 ± 0.40
Vietnam (Robusta)	128.18 ± 5.20	121.72 ± 4.69

All values are shown as means ± SD (n=3).

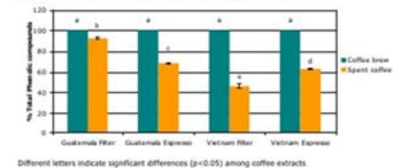
The results (Table 1) indicate that all spent coffee extracts showed antioxidant properties, due to the presence of Browned and Phenolic compounds. Guatemala spent coffee obtained from filter coffeemaker showed the highest values in all assays, followed by Vietnam spent coffees from espresso and filter coffeemakers, and Guatemala spent coffee from espresso coffeemaker, in order.

All Vietnam coffee brews showed higher antioxidant capacity measured by Folin Ciocalteau and scavenging activity assays than Guatemala ones (Table 2). In addition, Vietnam filter coffee brews had higher antioxidant capacity than espresso ones, maybe due to a longer extraction. Thus, in Vietnam spent coffee from espresso coffeemaker remained higher amount of antioxidant compounds.

The antioxidant capacity of Guatemala Filter spent coffee was almost the same (88.9-102.3%) than that of the coffee brew. For Vietnam espresso spent coffee, the antioxidant capacity was 59.2-68.7% than that of the coffee brew.

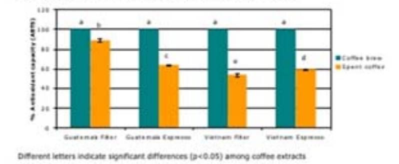
No significant differences ($p>0.05$) were found in DPPH between Guatemala Filter spent coffee and its coffee brew, showing that spent coffee extracts has the same capacity that its coffee brew.

Figure 1. Total Phenolic compounds percentage of Spent coffee in comparison to coffee brew.



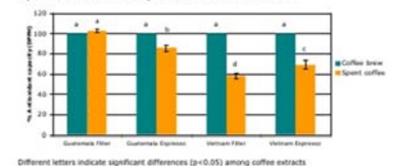
Different letters indicate significant differences ($p<0.05$) among coffee extracts

Figure 2. Antioxidant Capacity (ABTS) percentage of Spent coffee in comparison to coffee brew.



Different letters indicate significant differences ($p<0.05$) among coffee extracts

Figure 3. Antioxidant Capacity (DPPH) percentage of Spent coffee in comparison to coffee brew.



Different letters indicate significant differences ($p<0.05$) among coffee extracts

Conclusion

Spent coffee obtained after coffee brewing still has antioxidant capacity. Consequently this by-product could be consider as a potential source of antioxidant compounds in the food and pharmaceutical industries to increase food nutritional values, products stability and to develop new products.

References

Borrelli, R. et al. Characterization of a New Potential Functional Ingredient: Coffee Silverskin. J. Agric. Food Chem. **2004**, 52, 1338-1343.
Brand-Williams et al. Use of a free radical method to evaluate antioxidant activity. Lebensm.-Wiss. U.-Technol. 1995, 28, 25-30.
Ramalakshmi, K. et al. Bioactivities of low-grade green coffee and spent coffee in different in vitro model system. Food Chem. 2009, 115, 79-85.
Re, R. et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biol med, **1999**, 26(9-10), 1231-1237.
Singleton, V.L. and Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Amer. J. Enol. Viticult.. 1965, 16,144-158.

Acknowledgements

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Association of Friends of the University of Navarra for the grants given to J.B. and C.M., and the Unión Tostadora for providing the coffee samples.

ISSN (1230-0322)
2 0 1 1 , Vol. 61, Suppl. 1

EuroFoodChem XVI
Translating food chemistry into health benefits

Conference Proceedings

Gdansk 6th-8th July, 2011

Polish Journal of Food and Nutrition Sciences
formerly Acta Altimentaria Polonica

Published

by the Division of Food Sciences, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn

P190

Determination of Antioxidant Values of Hawthorn and Cranberry Fruits Growing in Turkey and Its Products Such as Marmelade and Sauces

Ali Batu, Olcay Kaplan

Department of Food Engineering, Faculty of Engineering, Tunceli University, TR6200, Tunceli, Turkey

Key words: antioxidant, phenolic compounds, fruit, sauce, marmalade, health

Phenolic substances which are the most important group of natural antioxidants and very important by any ways such as, aroma formation effects, participation to color formation and change, showing antimicrobial and antioxidative effects, causing enzyme inhibition and purity criteria for some foods. It is indicate that phenolic substances show very useful health effects because of lowering cholesterol, inhibiting oxidation of low density lipoprotein, prevent hypertension and cardiovascular disease, and effects as anticarcinogenic and antimutagenic. For this purpose in recent years these foods are used in the prevention of various diseases so the usage has increased substantially worldwide, and consequently, the higher the antioxidant content of food items for the food market is growing rapidly.

Hawthorn and cranberry are some of the fruits which are rich in terms of phenolic compounds grown in Turkey. Although these fruits are grown so much in Turkey, sauce and marmalades, which have functional properties, producing is not very common from these fruits. From fruit to kitchen process, variation of phenolic compounds and chemical properties of these products have not been studied enough in Turkey. Determination the variation of components which have high significance for health is very important during the producing sauce and marmalade from the fruits which have high antioxidant content. These products which have very high content of antioxidants will be sold at very special markets with high prices. Also new products are presented to consumption as, marmalade at breakfast and sauce will be consumed with cakes and ice creams. In this study, the antioxidant values of hawthorn and cranberry fruits growing in Turkey and their products such as marmelade and sauces will be determined.

Correspondence to: ali_batu@hotmail.com

P191

Influence of Grinding on Chemical Composition of Coffee Brew and Spent Coffee Extracts

Carmen Monente, Jimena Bravo, M. Paz de Peña, Concepción Cid

Department of Nutrition, Food Science, Physiology, and Toxicology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain

Key words: coffee brew, spent coffee, grinding, chlorogenic acids, caffeine

The extraction of coffee brew antioxidant compounds is affected by several technological factors such as grinding, coffee/water ratio, roasting degree and water temperature and pressure. Therefore, could be expected that the composition of the residues generated during brewing procedure, called spent coffee, changes according to the coffee brew. The aim of the present work was to establish if the same time of grinding applies to two different coffee varieties affects the extraction of chlorogenic acids in coffee brews and their corresponding spent coffee. One Arabica coffee from Guatemala and one Robusta coffee from Vietnam with the same roasting degree were ground for 20 s. Particle size distribution of both coffees was measured using a sieve shaker. Filter coffee brews were prepared (24 g coffee/400 mL water), and spent coffees were extracted with water at the same conditions using a filter coffeemaker. Six chlorogenic acids (CGA), three caffeoylquinic acids (3-CQA, 4-CQA, 5-CQA) and three dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA), and caffeine were quantified by HPLC. The results showed that the same grinding time produced an unimodal particle size distribution in Guatemala coffee, whereas for Vietnam coffee was plurimodal. The extraction of the main antioxidant coffee compounds, such as chlorogenic acids (CGA) seems to be favoured by a larger amount of coarse particles, because Guatemala filter coffee brew and spent coffee aqueous extracts exhibited 16.68 mg and 13.24 mg of total CGA per gram of coffee and spent coffee, respectively, whereas Vietnam filter coffee brew and spent coffee aqueous extracts showed 10.13 mg and 6.22 mg of total CGA per gram of coffee and spent coffee, respectively. In contrast, caffeine was in highest concentration in Robusta filter coffee brews and spent coffee aqueous extracts than in Arabica. In conclusion, grinding should be controlled not only by the time because the structure of different coffee beans, and consequently the extraction of antioxidant compounds, can be affected differently with the same grinding time.

Acknowledgements: We thank the Ministry of Science and Innovation of the Spanish Government (AGL2009–12052) and the Asociación de Amigos of the University of Navarra for financial support and for the grants given to C.M and J.B. We also thank the Unión Tostadora for providing the coffee samples.

Correspondence to: mpdepena@unav.es

INFLUENCE OF GRINDING ON CHEMICAL COMPOSITION OF COFFEE BREW AND SPENT COFFEE EXTRACTS

Universidad de Navarra

Carmen Monente, Jimena Bravo, M. Paz De Peña*, Concepcion Cid.

Department of Nutrition, Food Science, Physiology and Toxicology, School of Pharmacy, University of Navarra, E-31080 Pamplona, Spain. *mpdepena@unav.es

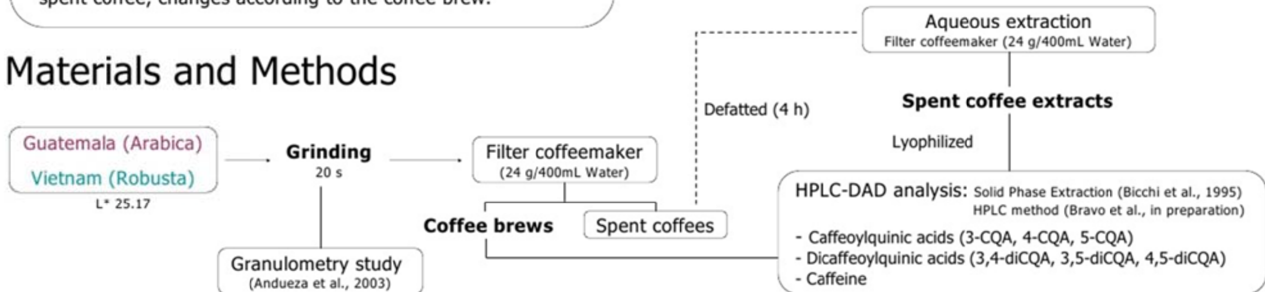
Introduction

The extraction of coffee brew antioxidant compounds is affected by several technological factors such as grinding, coffee/water ratio, roasting degree and water temperature and pressure (Bell et al., 1996, López-Galilea et al., 2006). Therefore, it could be expected that the chemical composition of the residues generated during brewing procedure, called spent coffee, changes according to the coffee brew.

Objective

The aim of the present work was to establish if the same time of grinding applied to two different coffee varieties (Arabica and Robusta) affects the extraction of chlorogenic acids (CGA) in coffee brews and their corresponding spent coffees.

Materials and Methods



Results and Discussion

Figure 1. Percentage of particle size distribution of ground coffees.

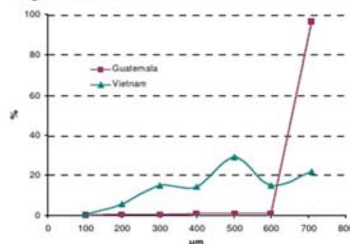


Table 1. Chlorogenic acids and caffeine content in coffee brews and spent coffee extracts.

	Guatemala	Vietnam	LS
3-CQA (mg / g dm)			
Coffee brew	3.54 ± 0.00	2.26 ± 0.13	***
Spent coffee extract	1.64 ± 0.00	0.68 ± 0.03	***
4-CQA (mg / g dm)			
Coffee brew	5.50 ± 0.00	2.93 ± 0.17	***
Spent coffee extract	2.51 ± 0.02	0.97 ± 0.04	***
5-CQA (mg / g dm)			
Coffee brew	7.42 ± 0.01	4.38 ± 0.21	***
Spent coffee extract	3.59 ± 0.02	1.26 ± 0.06	***
3,4-diCQA (mg / g dm)			
Coffee brew	0.22 ± 0.00	0.29 ± 0.01	***
Spent coffee extract	2.53 ± 0.03	1.49 ± 0.07	***
3,5-diCQA (mg / g dm)			
Coffee brew	nd	0.18 ± 0.01	***
Spent coffee extract	1.17 ± 0.01	0.62 ± 0.03	***
4,5-diCQA (mg / g dm)			
Coffee brew	nd	nd	
Spent coffee extract	1.80 ± 0.03	1.20 ± 0.05	***
Caffeine (mg / g dm)			
Coffee brew	10.08± 0.04	22.08 ± 0.06	***
Spent coffee extract	5.20 ± 0.04	7.53 ± 0.35	***

All values are shown as means ± SD (n=3)

The results (Figure 1) showed that the same grinding time produced different patterns in both coffees. In Guatemala coffee, an unimodal particle size distribution was observed with the 96.7% of particles bigger than 710 µm. Whereas for Vietnam coffee was plurimodal, with three maximum peaks at 300 µm, 500 µm and 710 µm.

Table 1 and Figures 2 and 3 show the results of caffeoylquinic acids, which are the most abundant CGA group in coffee, dicaffeoylquinic acids and caffeine. 5-CQA was the major isomer among CQAs in both coffee brews, followed by 4-CQA and 3-CQA. 3,4-diCQA was found in both Guatemala and Vietnam coffee brews, 3,5-diCQA was only found in Vietnam coffee brew whereas 4,5-diCQA was not detected. However, in spent coffee extracts the amount of diCQAs were increased whereas the amount of CQAs were decreased in comparison to coffee brews. This could be due to both the weaker polarity of diCQAs and stronger interactions with melanoidins.

The extraction of the main antioxidant coffee compounds, such as chlorogenic acids (CQAs and diCQAs) seems to be favoured by a larger amount of coarse particles. In fact, Guatemala filter coffee brew and spent coffee aqueous extracts exhibited 16.68 mg and 13.24 mg of total CGA per gram of coffee and spent coffee, respectively. However, Vietnam filter coffee brew and spent coffee aqueous extracts showed 10.13 mg and 6.22 mg of total CGA per gram of coffee and spent coffee, respectively. In contrast, caffeine was in higher concentration in Robusta (Vietnam) filter coffee brews and spent coffee aqueous extracts than in Arabica (Guatemala).

Figure 2. Chromatogram of a) Guatemala coffee brew and b) Guatemala spent coffee extract.

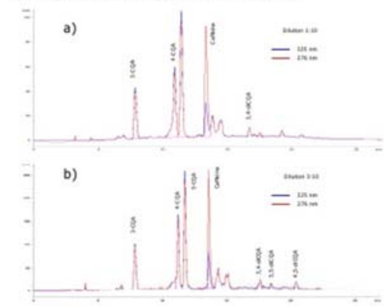
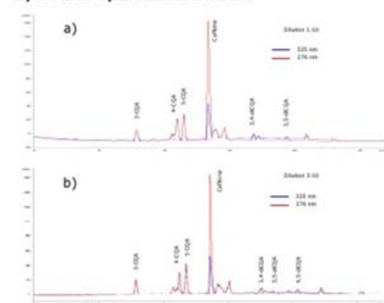


Figure 3. Chromatogram of a) Vietnam coffee brew and b) Vietnam spent coffee extract.



Conclusion

Grinding should be controlled not only by the time, because the structure of different coffee beans (Arabica and Robusta), can be affected differently with the same grinding time giving an unimodal or plurimodal particle size distribution. Consequently the extraction of antioxidant compounds, such as chlorogenic acids, in coffee brews and spent coffee aqueous extracts can also be influenced increasing o reducing their healthy properties.

References

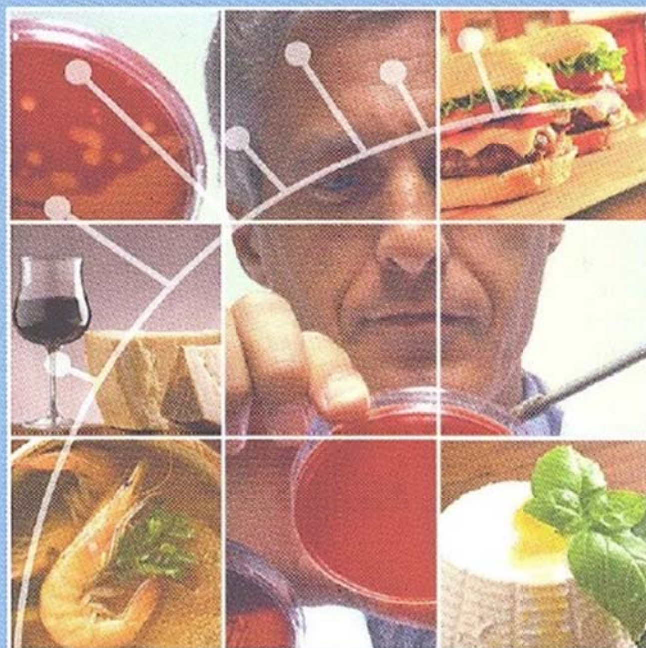
Andueza, S., De Peña, M. P., Cid, C. (2003). Chemical and sensorial characteristics of espresso coffee as affected by grinding and torrefacto roast. *Journal of Agricultural and Food Chemistry*, 51 (24), 7034-7039.
Bell, L. N., Wetzel, C. R., Grand, A. N. (1996). Caffeine Content in Coffee as Influenced by Grinding and Brewing Techniques. *Food Research International*, 29 (8), 785-789.
Bicchi, C. P., Binello, A. E., Pellegrino, G. M., Vanni, A. C. (1995). Characterization of Green and Roasted Coffees through the Chlorogenic Acid Fraction by HPLC-UV and Principal Component Analysis. *Journal of Agricultural and Food Chemistry*, 43 (6), 1549-1555.
López-Galilea, I., Andueza, S., di Leonardo, I., de Peña, M. P., Cid, C. (2006). Influence of torrefacto roast on antioxidant and pro-oxidant activity of coffee. *Food Chemistry*, 94, 75-80.

Acknowledgements

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Association of Friends of the University of Navarra for the grants given to C.M. and J.B., and the Unión Tostadora for providing the coffee samples.

Bridging Training and Research
for Industry and the Wider Community

3rd International ISEKI_Food Conference



"Food Science and Technology Excellence for a Sustainable Bioeconomy"

BOOK *of* ABSTRACTS

21-23 May, 2014
Athens, GREECE

Spent coffee grounds as a source of bioactive compounds

Carmen Monente[1], Jimena Bravo[1], Ana Isabel Vitas[2], Leire Arbillaga[3], M. Paz De Peña[1]* and Concepción Cid[1]

1. Department of Nutrition, Food Science and Physiology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain

2. Department of Microbiology and Parasitology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain

3. Department of Pharmacology and Toxicology, School of Pharmacy, University of Navarra, E-31080-Pamplona, Spain

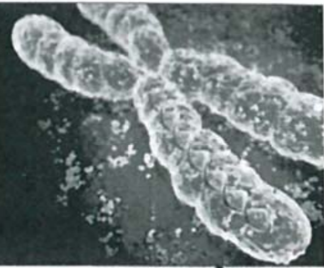
* Corresponding author: mpdepena@unav.es

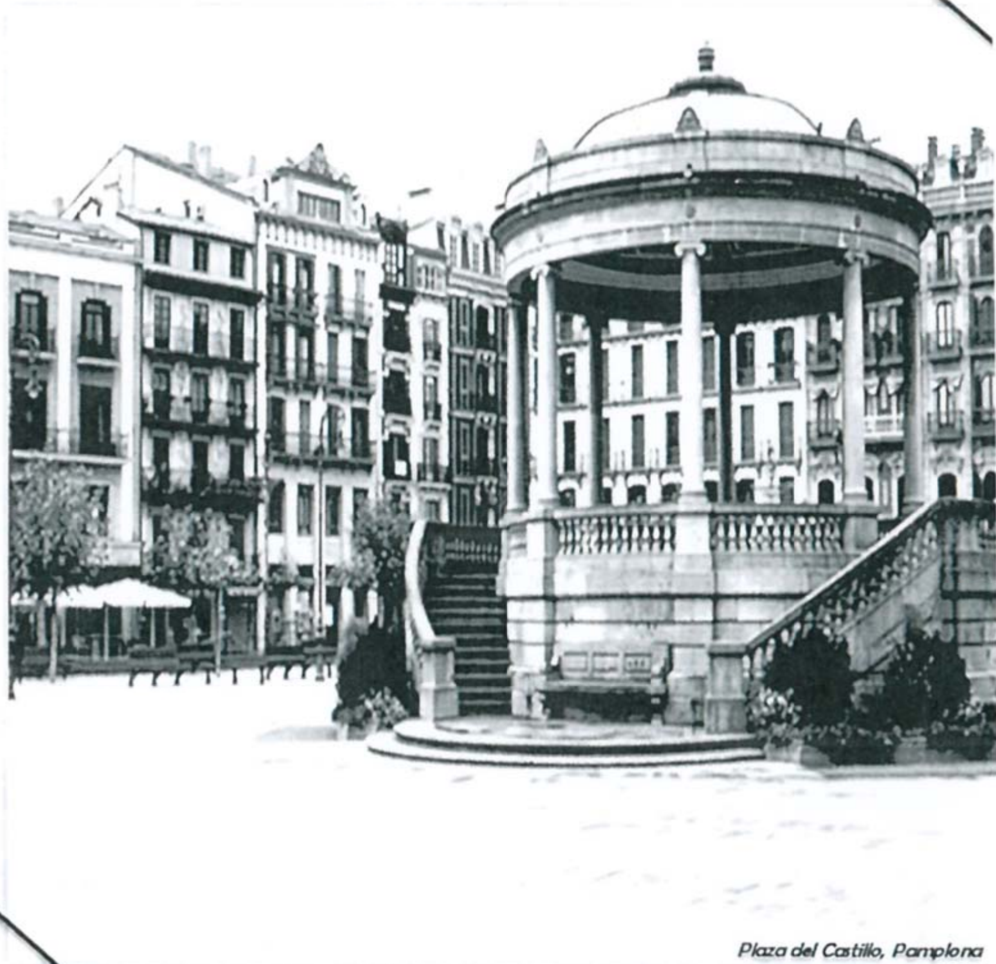
Millions of coffee cups are consumed every day around the world, producing spent coffee grounds in tons in restaurants and cafeterias and also at domestic levels. Coffee is well-known as a rich source of bioactive compounds in human diet that may contribute to the prevention of several diseases. Also that spent coffee grounds could be valuable by-products because of the presence of significant amounts of phenolic (mainly chlorogenic acids) and nonphenolic bioactive compounds. In fact, in a previous work of our research group it was found that spent coffee obtained from the most common coffeemakers (filter and espresso) was an excellent source of hydrophilic bioactive compounds with antioxidant and antigenotoxic properties. Therefore, the aim of this work was to study other functional properties of spent coffee extracts such as antimutagenic and antimicrobial activity in order to propose new and sustainable applications of this coffee by-product.
Spent coffee grounds from filter and espresso coffee were dried and defatted, and then aqueous extracts were obtained using the methodology previously developed. The antimutagenic activity was analysed using the Ames Test. The antimicrobial activity was measured against eight bacterial and two fungal strains (*Staphylococcus aureus, Listeria monocytogenes, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella choleraesuis, Candida albicans* and *Aspergillus niger*). The obtained data prove that extracts of both spent coffee grounds were able to reduce the action of direct and indirect mutagens. Moreover, the antimicrobial assays showed that spent coffees were more active against Gram-positive bacteria than Gram-negative ones. The most susceptible microorganisms were two common food-borne pathogens *S. aureus* and *L. monocytogenes*. In conclusion, spent coffee is an accessible source of natural bioactive compounds. Although further studies are needed to optimize technological extraction conditions, mainly at food industry level, this coffee by-product could be considered as a potential preservative to extent food shelf-life or to be applied to prepare functional foods.

Keywords: antimicrobial, antimutagenic, by-products, coffee

Acknowledgements: This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Association of Friends of the University of Navarra for the grants given to C.M. and J.B. We thank the Unión Tostadora S.A. for providing the coffee.

XXI Congreso de la Sociedad Española de Mutagénesis Ambiental





Plaza del Castillo, Pamplona

Universidad de Navarra

18, 19 y 20 Junio 2014
Facultad de Farmacia
Universidad de Navarra
Pamplona

Antimutagenic activity of coffee brew and spent coffee

Carmen Monente[1], Jimena Bravo[1], Leire Arbillaga[2], M. Paz De Peña[1], Concepción Cid[1]

[1]Department of Nutrition, Food Science and Physiology, School of Pharmacy, University of Navarra, Pamplona, Spain; [2]Department of Pharmacology and Toxicology, School of Pharmacy, University of Navarra, Pamplona, Spain.

Coffee has proven to be an excellent source of bioactive compounds, mainly phenolic acids, such as chlorogenic acids (CGA). These compounds have been extensively associated with a risk decrease in several chronic and degenerative diseases. The by-product generated after brewing processes, named spent coffee, could have similar characteristics and also might contribute with health related properties. In a recent study of our research group, Bravo et al. (2012, 2013) found that spent coffee has antioxidant capacity measured by chemical based assays and in *in vitro* cell cultures, attributed to a high content of phenolic acids, as well as caffeine and Maillard reaction products. The aim of the present study was to evaluate the ability of coffee brew and spent coffee to protect against mutagens responsible of cell mutation. Spent coffee extracts from filter and espresso brewing process, as well as their respective coffee brews, were analyzed using the Salmonella mutagenicity test (Ames Test). Three non-toxic concentrations of the samples were tested with (S9) and without (-S9) metabolic activation. The data prove that both spent coffees extracts and coffee brews were able to reduce the action of direct (NPD) and indirect (2-AF) acting mutagens. The results showed that spent coffee extracts after no metabolic activation (-S9) had higher antimutagenic activity than their respective coffee brews. The protection percentage against direct mutagens (NPD) was up to 35%, showing no dose-dependent pattern. However, spent coffee extracts and coffee brews had a strong protection activity against indirect acting mutagens (2-AF), with a dose-dependent pattern, up to 92% in Arabica spent coffee after metabolic activation (S9). In conclusion, the high antimutagenic activity of spent coffee found in this work suggests that this by-product could be considered as a potential food ingredient to enhance healthy properties of functional foods. However, further experimental and clinical studies would be needed to claim their functional properties.

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Unión Tostadora S.A. for providing the coffee.

Bravo, J., Juaniz, I., Monente, C., Caemmerer, B., Kroh, L., De Peña, M.P., Cid, C., 2012. Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds. J. Agric. Food Chem. 60, 12565-12573.

Bravo, J., Arbillaga, L., De Peña, M.P., Cid, C., 2013. Antioxidant and genoprotective effects of spent coffee extracts in human cells. Food Chem. Toxicol. 60, 397-403.

8th World Congress on

Polyphenols Applications

June 4-6, 2014 – Lisbon, Portugal

Chairmen of the Scientific Committee

Marvin Edeas & Andreas Schieber

Local Organising Committee

Luisa Bivar Roseiro & Fernanda Borges

INTERNATIONAL SOCIETY
OF
ANTIOXIDANTS
IN NUTRITION & HEALTH

ISBN 978-2-35609-073-7

Lisbon Polyphenols 2014 - ISANH© Copyright 2014

SPENT COFFEE: A POTENTIAL (POLY)PHENOLIC-RICH NATURAL FOOD PRESERVATIVE TO CONTROL MICROBIAL GROWTH

CARMEN MONENTE, ANA ISABEL VITAS, M. PAZ DE PENA, CONCEPCION CID.

University of Navarra, Pamplona, Spain.

cmonente@alumni.unav.es

Spent coffee grounds are produced in tons by brewing process in coffee shops, restaurants and at domestic level and could be a good source of phenolic compounds, mainly chlorogenic acids (Bravo et al. 2012, 2013). Coffee has antimicrobial activity against a broad range of microorganisms, including foodborne pathogens, but up to our knowledge, potential antimicrobial activity of spent coffee grounds has not been investigated yet. The aim of the present study was to identify and quantify the main chlorogenic acids of spent coffee extracts and to evaluate their ability to inhibit the growth of some of the most common food-borne pathogen microorganisms.

Two spent coffee extracts (Arabica and Robusta) obtained after filter and espresso brewing process were analyzed by HPLC to measure caffeoylquinic (3-, 4- and 5-CQA) and dicaffeoylquinic acids (3,4-, 3,5- and 4,5-diCQA). Both spent coffee extracts had slightly higher amounts of CQAs and diCQAs than their respective coffee brews. The antimicrobial activity of spent coffee extracts was assessed against six bacterial and one fungal strains (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Choleraesuis, and *Candida albicans*) by using three different tests. The agar well diffusion method (screening test) showed that spent coffee extracts had similar antimicrobial activity than their corresponding coffee brews, being more active against Gram-positive bacteria and yeast (higher inhibition zones diameters). The results indicated that the total amount of CQA and diCQA are highly associated with the inhibition zones diameters of Gram-positive bacteria. Minimum Inhibitory Concentration (MIC) test showed that *S. aureus* was the most sensitive microorganism with both spent coffees extracts (5-10 mg lyophilized/mL), followed by *L. monocytogenes* (20 mg/mL). Finally, the growth inhibition curves of *S. aureus*, *B. subtilis* and *C. albicans* in the presence of low concentrations of both spent coffees extracts (12 mg/mL) was determined. The number of viable cells after 72 hours of exposure was lower than their respective control cultures (2.5 to 3 log CFU/mL in the case of *S. aureus*), suggesting that spent coffee may cause bacteriostatic effect on microorganisms during long exposures. In conclusion, spent coffee could be proposed as an antimicrobial agent that can be used for protection mainly against Gram-positive bacteria and yeast. However, further studies in food matrices are needed to verify the stability during industrial process.

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Unión Tostadora S.A. for providing the coffee.

References:

Bravo, J., Juániz, I., Monente, C., Caemmerer, B., Kroh, L.W., De Peña, M.P., Cid, C. 2012. J. Agric. Food Chem., 60, 12565–12573.

Bravo, J., Monente, C., Juániz, I., De Peña, M. P., Cid, C. 2013. Food Res. Int., 50, 610-616.

SPENT COFFEE: A POTENTIAL (POLY)PHENOLIC-RICH NATURAL FOOD PRESERVATIVE TO CONTROL MICROBIAL GROWTH

Universidad de Navarra

CARMEN MONENTE, ANA ISABEL VITAS, M. PAZ DE PEÑA*, CONCEPCION CID

University of Navarra, Pamplona, Spain *mpdepena@unav.es

Introduction

Spent coffee grounds are produced in tons by brewing process in coffee shops, restaurants and at domestic level and could be a good source of phenolic compounds, mainly chlorogenic acids (Bravo et al. 2012, 2013). Coffee has antimicrobial activity against a broad range of microorganisms, including foodborne pathogens, but up to our knowledge, potential antimicrobial activity of spent coffee grounds has not been investigated yet.

Objective

The aim of the present study was to identify and quantify the main chlorogenic acids of spent coffee extracts and to evaluate their ability to inhibit the growth of some of the most common food-borne pathogen microorganisms.

Material and Methods

1- CHLOROGENIC ACIDS: HPLC-DAD analysis (Bravo et al. 2013)

- Caffeoylquinic acids (3-CQA, 4-CQA, 5-CQA)
- Dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA)

2- ANTIMICROBIAL SUSCEPTIBILITY TEST:

Microbial strains:

Staphylococcus aureus (G+)
Listeria monocytogenes (G+)
Bacillus subtilis (G+)
Escherichia coli (G-)
Pseudomonas aeruginosa (G-)
Salmonella Choleraesuis (G-)
Candida albicans (yeast)

2.1 Agar well diffusion method

10^6 cfu/mL of each microorganism

50 µL of each sample:

Arabica Spent coffee 160mg/mL
Robusta Spent coffee 160mg/mL
Arabica coffee brew 122mg/mL
Robusta coffee brew 260mg/mL

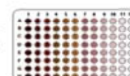
Incubation 37ºC 24h

2.2 Minimum Inhibitory Concentration

200 µL/well Inoculum of each microorganism

Twofold serial dilution of each sample:

Arabica Spent coffee 160- 0.08 mg/mL
Robusta Spent coffee 160-0.08 mg/mL
Arabica coffee brew 122-0.06 mg/mL
Robusta coffee brew 260-0.12mg/mL



Incubation 37ºC 24h (bacterial strains)
30ºC 72h (fungal strain)

2.3 Growth inhibition curve

10^6 cfu/mL of *S. aureus*, *B. subtilis* and *C. albicans*

12mg/mL of **Arabica Spent coffee**
Robusta Spent coffee

Incubation 37ºC 1h, 24h, 48h and 72h

Results and Discussion

Table 1. Characterization of bioactive compounds of spent coffee extracts and coffee brews

Compunds	Concentration (µg/ mg lyophilized)			
	Arabica filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
3-CQA	20.66±0.70	18.10±0.82	18.14±0.48	12.86±0.73
4-CQA	24.75±0.54	21.22±1.09	19.40±2.18	15.43±0.92
5-CQA	36.78±1.01	33.30±0.66	25.74±0.63	20.94±0.84
3,4-diCQA	0.71±0.10	0.50±0.03	0.93±0.02	0.48±0.00
3,5-diCQA	0.54±0.14	0.41±0.10	0.66±0.01	0.41±0.05
4,5-diCQA	0.77±0.12	0.56±0.03	1.21±0.02	0.69±0.09
CQA+diCQA (CGA)	84.22	74.10	66.09	50.80

Values are expressed as means ± standard deviation of three experiments

Table 3. Minimum inhibitory concentration values for coffee extracts and coffee brews against different microorganisms.

Organisms	MIC (mg/mL)			
	Arabica filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
S. aureus	5	7.5	10	8.1
L. monocytogenes	20	30	20	16.3
B. subtilis	40	60	40	32.5
E. coli	80	60	160	32.5
S. choleraesuis	40	30	40	16.3
Ps. aeruginosa	40	60	80	16.3
C. albicans	40	60	40	32.5

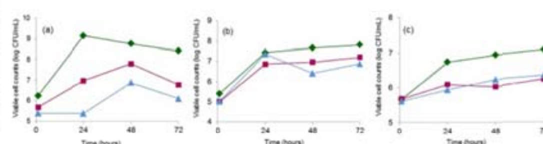
The results (Table 1) indicated that both spent coffee extracts had slightly higher amounts of CQAs and diCQAs than their respective coffee brews. Table 2 summarizes the inhibition diameters obtained with the agar-well diffusion method. The results showed that all tested samples were more active against Gram-positive bacteria and yeast than against Gram-negative bacteria. The total amount of CQA and diCQA are highly associated with the inhibition zones diameters of Gram-positive bacteria. Minimum Inhibitory Concentration (MIC) (Table 3) test showed that *S. aureus* was the most sensitive microorganism with both spent coffees extracts (5-10 mg lyophilized/mL), followed by *L. monocytogenes* (20 mg/mL). Finally, Figure 1 shows the growth inhibition curves of *S. aureus*, *B. subtilis* and *C. albicans* in the presence of low concentrations of both spent coffees extracts (12 mg/mL). The number of viable cells after 72 hours of exposure was lower than their respective control cultures (2.5 to 3 log CFU/mL in the case of *S. aureus*), suggesting that spent coffee may cause bacteriostatic effect on microorganisms during long exposures.

Table 2. Inhibition zones obtained with the agar-well diffusion method (diameter in mm).

Organisms	Inhibition zones (mm)			
	Arabica Filter		Robusta espresso	
	Spent coffee	Coffee brew	Spent coffee	Coffee brew
S. aureus	17.3 ± 0.6[ab]	15.3 ± 0.6[a]	15.3 ± 1.2[a]	19.7 ± 1.2[b]
L. monocytogenes	20.0 ± 3.6[a]	19.0 ± 2.7[a]	18.0 ± 2.0[a]	22.3 ± 3.1[a]
B. subtilis	8.8 ± 0.3[a]	9.7 ± 0.6[a]	ND	11.9 ± 0.2[a]
E. coli	ND	ND	ND	10.3 ± 1.2
S. choleraesuis	ND	ND	ND	10.7 ± 0.6
Ps. aeruginosa	ND	ND	ND	10.3 ± 0.6
C. albicans	15.3 ± 1.2[ab]	13.7 ± 0.6[a]	15.7 ± 1.5[ab]	17.7 ± 0.6[b]

Values are expressed as means ± standard deviation of three experiments. In each row, different superscripts indicate significant differences ($p < 0.05$) among samples.

Figure 1. Growth inhibition curves of spent coffee extracts on (a) *S. aureus*, (b) *B. subtilis*, (c) *C. albicans*. (♦) Control, (■) Arabica spent coffee, (▲) Robusta spent coffee.



Conclusion

Spent coffee could be proposed as an antimicrobial agent that can be used for protection mainly against Gram-positive bacteria and yeast. However, further studies in food matrices are needed to verify the stability during industrial process.

References

Bravo, J., Juániz, I., Monente, C., Caemmerer, B., Kroh, L.W., De Peña, M.P., Cid, C. 2012. J. Agric. Food Chem., 60, 12565–12573.
Bravo, J., Monente, C., Juániz, I., De Peña, M. P., Cid, C. 2013. Food Res. Int., 50, 610-616.
Monente, C., Bravo, J., Vitas, A.I., Arbillaga, L., De Peña, M.P., Cid, C. Food Res. Int. Under Revision

Acknowledgments

This research was funded by the Spanish Ministry of Science and Innovation (AGL2009-12052). We thank the Unión Tostadora S.A. for providing the coffee. We thank the Association of Friends of the University of Navarra for the grant given to C.M.