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

Carmen Monente[1], Iziar A. Ludwig[1], Angelique Stalmach[2], Maria Paz de Peña[1], Concepción Cid[1], Alan Crozier[3]

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[1]Department of Nutrition, Food Science and Physiology, School of Pharmacy, University of Navarra Pamplona, Spain, [2]School of Medicine, University of Glasgow, Glasgow, UK, [3]Department of Nutrition, University of California, Davis, California, USA

Correspondence: Alan Crozier, Department of Nutrition, 3143 Meyer Hall, One Shields Avenue, University of California, Davis, California 95616-5270, USA

Tel: -1-530-754-0450

E-mail address: alan.crozier44@gmail.com

Abstract

Spent coffee grounds are a potential commercial source of substantial amounts of chlorogenic acids (CGAs). The aim of this study was to evaluate the stability of spent coffee CGAs using in vitro simulated gastroduodenal digestion and to investigate their potential absorption using an in vitro Caco-2 model of human small intestinal epithelium. During in vitro digestion, lactones were partially degraded while caffeoylquinic and feruloylquinic acids were much more stable. Transport and metabolism studies showed that 1% of the total CGAs were absorbed and transported from the apical to the basolateral side of a Caco-2 cell monolayer after 1 h. Lactones and coumaroylquinic acids showed the rate of highest absorption. Caco-2 cells possessed low metabolic activity. In conclusion, spent coffee extracts contain large amounts of CGAs, which remained bioaccessible across the intestinal barrier, albeit to a relatively low degree.

Keywords

Spent coffee grounds, chlorogenic acids, *in vitro* gastrointestinal stability, in vitro bioaccessibility

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 the risk factor for chronic diseases, or which 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; Del Rio et al. 2013; Rodrigues-Mateos et al. 2014). 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). However, the levels vary principally as a consequence of roasting (Moon et al., 2009) and one study found the CQAs per serving in espresso coffees ranging from 24-422 mg (Crozier et al., 2012). As reviewed by Ludwig et al. (2014a), 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, most notably of the prostate (Li et al., 2013) and skin (cutaneous melanoma) (Loftfield et al., 2015).

In excess of a million tons of spent coffee grounds, remaining after the commercial production of coffee beverage, are produced each year and at one time they were used to feed racehorses until the authorities realised that the grounds contained caffeine, a well-known stimulant. Starbucks are reported to be working to convert coffee grounds into laundry detergents and bioplastics, while Nestlé incinerate the grounds and use it as a heat source to cook their food products (Kennedy, 2013). On a smaller scale, coffee grounds are used for compost and seemingly are much appreciated by earthworms and acid-loving plants such as blueberries

(Martin & Gershuny, 1992). There are also anecdotal reports of the use of grounds as a slug and snail repellent, probably at least partially because of the presence of caffeine which acts as a neurotoxin (Hollingsworth et al., 2002).

The data of Ludwig et al. (2014b) suggests that spend coffee grounds may contain sizable amounts of residual CGAs which when isolated could offer potential beneficial effects on health. However, limited data are currently available on the CGA profile of spent coffee grounds and earlier studies on the absorption and metabolic fate of CGAs have followed the ingestion of coffee beverage rather than isolated CGAs (Monteiro et al., 2007; Stalmach et al., 2009, 2010, 2014; Renouf et al., 2014). The aim of the current study was therefore i) to assess the CGA profile of the spent coffee grounds remaining after the production of filter and espresso coffees, ii) to investigate the upper gastrointestinal stability of these compounds using an in vitro model of digestion, and iii) to determine the transport of spent coffee CGAs using Caco-2 cell, an in vitro model of intestinal absorption.

Materials and methods

Chemicals

Caffeic acid, ferulic acid, sinapic acid, 5-*O*-caffeoylquinic 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-Dicaffeoylquinic acid (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).

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.

Spent coffee extract preparation

Arabica filter (24 g coffee/ 400 ml) and Robusta espresso (7g coffee/ 45 ml) coffee brews 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 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. Both spent coffee extracts yielded a recovery of 8 mg lyophilized per ml of extract. Coffee brews recovery were for Arabica filter 11 mg lyophilized/ ml and for Robusta expresso 25 mg lyophilized /ml.

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 a 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.

126 **Transport and metabolism experiments using Caco-2 cells**

127 Caco-2 cells were cultured in DMEM supplemented as previously describe Fanjul et al. (2012).
128 Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin-EDTA and
129 sub-cultured on 25 or 75 cm^2 plastic flasks at a 25×10^4 cells/cm^2 density. Caco-2 cells were
130 seeded on Transwell inserts (12 mm diameter, 1.12 cm^2 growth area) at a density of
131 6×10^4 cells/cm^2. Culture medium was replaced every 2 days. Cell confluence was confirmed by
132 transepithelial electrical resistance (TEERS) measurements. Transport experiments were
133 performed at 17–21 days post-seeding according to Farrell et al. (2012). Lyophilised, digested
134 Arabica spent coffee was redissolved in DMEM (15 mg/ml, pH 7.4). The culture medium was
135 aspirated and 1 ml test solution was added in the apical side and 2 ml of DMEM pH 7.4 in the
136 basal side. The incubation period was 0.5 and 1 hour at 37ºC. At the end of the incubation period
137 media from the apical and basal sides were collected, the monolayer was washed with DMEM,
138 and cells were scraped into sterile water and sonicated for 30 s. Samples were cooled
139 immediately, freeze-dried and stored at –18 ºC. Each experimental condition was tested in
140 triplicate.

141 For the metabolism experiment, Caco-2 cells were incubated with 1 ml of standard
142 solutions of either 5-CQA (0.9 μM), 4,5-DiCQA (0.2 μM), caffeic acid (2.5 μM), ferulic acid
143 (0.7 μM), dihydrocaffeic acid (2.5 μM) or dihydroferulic acid (0.7 μM) in DMSO (0.1%). The
144 incubation period was 0.5 and 1 h at 37ºC, then samples were removed and stored as described
145 above. Each experimental condition was tested in triplicate. For CGA analysis culture solution
146 and cells were extracted and deproteinated according Stalmach et al. (2009) with some
147 modifications. Each sample was resuspended in 500 μl of methanol containing 1% formic acid
148 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. Each experimental condition was tested in triplicate.

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, namely feruloylquinic acids (FQA), dicaffeoylquinic acids (diCQA), *p*-coumaroylquinic acid (CoQA), caffeoyl-feruolylqunic aicd (CFQA), caffeoylqunic acid-lactone (CQAL) and feruloylqunic acid-lactone (FQAL) was performed using a 5-CQA calibration curve combined with molar extinction coefficients of the respective compound as reported by Trugo and Macrae (1984) and Farah et al. (2005).

Statistics

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.

Results

Characterization of spent coffee extracts

Filter and espresso coffeemakers are commonly used to prepare a cup of coffee, not only in the home but also in workplaces and coffee shops. Arabica and Robusta coffees are the most consumed varieties. Previously it was shown that the spent coffees obtained after the preparation of a filter (Arabica coffee) and an espresso (Robusta coffee) coffee brew had the highest antioxidant activity in chemical-based assays and in cell cultures (Bravo et al. 2012; 2013). Thus,

194 the two spent coffees were each used to prepare an extract which were lyophilised, stored and
195 reconstituted prior to analysis and testing, along with their respective coffee brews. A total of 33
196 CGAs were identified and quantified in both spent coffee extracts and coffee brews with HPLC-
197 PDA-MS^n, including two cinnamoyl-amino acid conjugates (Table 1). Identifications were
198 performed by reference to published CGA fragmentation patterns (Clifford et al., 2003; Clifford
199 & Knight, 2004).

200 The results indicated that on a weight per weight basis, spent coffee contained higher
201 amounts of CGAs than their respective coffee brew, especially after the production of espresso
202 coffee (Table 2). On average, and a weight/weight basis, there was 6.2 ± 2.5% more CGAs
203 quantified in the Arabica spent coffee compared with the brew, whereas 41 ± 2.4% more CGAs
204 were recovered in Robusta spent coffee compared with the brew, mainly due to the higher CQAs
205 from the spent coffee (44 ± 2.5%). Both spent coffee extracts showed similar amounts of total
206 CGAs, but with profiles of different compounds, depending on the coffee bean variety. Arabica
207 spent coffee contained higher amounts of CQAs, CoQAs and CQLs, while Robusta spent coffee
208 was richer in FQAs, FQLs, C-FQAs and cinnamoyl-amino acids (Table 2).

209

210 **In vitro digestion**

211 The proximal gastrointestinal stability of the CGAs contained in Arabica and Robusta spent
212 coffees was assessed using a three step in vitro model (Table 3 and Fig. 1). Table 3 summarizes
213 the CGAs content of spent coffee extracts after simulated oral, gastric and intestinal digestion.
214 The results indicated that the total CGA content of spent coffees decreased significantly by 4-
215 14% indicating degradation during the in vitro digestion process. However, successive in vitro
216 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 equivalent to 45-85% of the initial dose incubated ($p < 0.001$). CGA compounds responded differently to the enzymatic activity and pH changes. Robusta spent coffee showed more degradation 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 (Fig. 1). Firstly, the addition of α-amylase at pH 6.5-7 brought a decrease in the levels of 5-CQA, 17% (Arabica filter) and 20% (Robusta espresso) of the initial value ($p <0.01$), while the 3- and 4-CQA isomers increased significantly. The 3-acyl isomer showed an increase of 36-64% compared with the non-digested sample, while 4-CQA increased 11-25%. Similar results were observed for FQAs.

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-CQA, although the final values remained, respectively, ca. 25 and 5% higher than non-digested sample. The last step was the addition of pancreatin and bile salt (pH 7), which had similar effects to those observed after oral digestion. The final concentration of the 5-acyl isomers were ca. 12% (CQA) and 19% (FQA) lower compared with the non-digested sample in both spent coffee samples. Moreover, the increment average of 3-acyl isomers was 50% of the initial value, and 16% for 4-acyl isomers for Arabica filter spent coffee. In contrast, the increments of 3 and 4-acyl isomers were lower in Robusta espresso spent coffee, showing no significant differences with the non-digested sample.

240 **Absorption and metabolism of spent coffee CGAs and hydroxycinnamate standards**

241 The absorption study was performed with the digested Arabica spent coffee extract. The sample
242 was incubated in Transwell cell culture inserts with a Caco-2 cell monolayer, which simulated
243 the intestinal barrier. Table 4 shows the amount of CQAs found in the apical and basal
244 compartments and in the Caco-2 cells as well as the percentage related to the initial amount
245 loaded on the apical side. The results indicate that only 0.55% of the total CGAs were absorbed
246 and transported from the apical to the basolateral side of cell in the first 0.5 h, and this increased
247 to 1% after 1 h. Quinide lactones and CoQA were recovered in greater amounts on the
248 basolateral side, accounting, respectively, for 33% and 17% of the amount incubated. Other
249 CGAs were subject to negligible transport from the apical to the basal cell. DiCQAs showed an
250 absorption of 0.6% and CQAs and FQAs of 0.3% and 0.5%, respectively. There was no
251 difference in the transport of the different CQA and FQA isomers. In contrast to most of the
252 CGAs, there was substantial transport of caffeine with 30% recovered on the basolateral side
253 within 0.5 h and a further 6.5% taking place in the subsequent 0.5 h (Table 4).

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

261 Compounds with the highest basolateral recovery were ferulic acid and dihydroferulic acid,
262 accounting for 5.9% and 5.3% of the apical dose, respectively, indicating a higher absorption and

basolateral transport than 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).

Discussion

The by-product generated in the manufacture of coffee namely, spent coffee grounds, is a rich source of a diversity of CGAs. The aim of the present study 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 data are in line with previous reports 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). CGA isomer acyl migration was highly pH dependent. Basic pH condition (oral and intestinal digestion) showed an increase in the migration mainly to 3-acyl isomers, and this process was reversed in acid condition (gastric digestion), these are in agreement with Deshpande et al. (2014). Moreover, our data showed that

286 the seemingly high recoveries of CQA and FQA in the in vitro digestion model might be a
287 consequence of the degradation of other components, such as diCQA and lactones in the spent
288 coffee extracts. These two compounds showed a higher losses following incubation with
289 gastrointestinal juices, which could have resulted to the release of the hydroxycinnamate moiety
290 of diCQAs or the cleavage of the intramolecular ester bond of lactones. Previous research have
291 reported positive health effect of lactones (de Paulis et al., 2002, 2004), however these activities
292 might be limited, due to the high losses during the gastrointestinal digestion. Furthermore, the
293 release of CQAs from other structures, including melanoidins, into which they are incorporated
294 during roasting (Bekedam et al., 2008; Perrone et al., 2012), could also be hypothesised. In
295 general, CGAs in spent coffee extracts were stable in the in vitro model of the proximal
296 gastrointestinal tract implying that in vivo substantial amounts are likely to reach the colon. This
297 is in keeping with the reported 59-77% recoveries of CQAs in ileal fluid after the ingestion of
298 coffee by ileostomists (Stalmach et al., 2010; Erk et al., 2012).

299 CQA isomers in spent coffee were transported across the Caco-2 monolayer in only low
300 amounts, corresponding to 0.3% of the apical dose. Similar absorption values have been reported
301 for CQAs from coffee brew and standard solutions using similar Caco-2 cell models (Dupas et
302 al., 2006; Farrell et al., 2012). Despite the different functions and morphology between gastric
303 epithelial and Caco-2 cell monolayers, similar absorptive patterns were observed among both
304 cellular lines. The absorption percentage of spent coffee CQAs was 0.3% of the apical dose, in
305 agreement with the findings of Farrell et al. (2011a). In their study, Farrell et al. found that the
306 percentage FQAs and CQLs transported across the gastric cell monolayer was slightly higher
307 than that of CQAs. The only difference between gastric and intestinal cells was the percentage of
308 diCQAs transported, due to the high permeability observed in gastric cells (Farrell et al., 2011a).

The present study showed the first complete profile of phenolic compounds in spent coffee extracts, moreover cinnamoyl-amino acids had not been previously reported to occur in spent coffee grounds. Furthermore, our study demonstrated higher levels of CGAs in spent coffee compared with the corresponding coffee brew, although with similar profiles of the individual CGAs. 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 espresso brewing method used which is known for its low extraction efficiency (Cruz et al., 2012).

Conclusion

Previous studies have highlighted a 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 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. Spent coffee extracts may therefore be suitable commercial source of CGAs as ingredients in functional foods or supplements.

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Declaration of interests

The authors declare no conflicts of interest.

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436 **Figure Legend**

437 Figure 1. The impact of *in vitro* digestion model on the stability of the main CGAs isomers in
438 spent coffee extracts. (**A**) Arabica spent coffee, (**B**) Robusta spent coffee. (■■) non-digested,
439 (■■) oral digestion; (■■) gastric digestion, (□□) intestinal digestion. Data expressed as mean
440 values ± standard deviation. Different letters in each isomer indicate significant differences ($p < 0.05$)
441 between non-digested and digested samples (Analysis of variance with a pair-wise
442 post-hoc comparison).

443

444

Table 1. Identification of chlorogenic acids in spent coffee and coffee brew*.

Chlorogenic acids	Rt time (min)	MS^1 Parent ion *(m/z)*	MS^2 Base ion *m/z*	MS^2 Secondary ions *m/z (intensity)*	MS^3 Base ion *m/z*	MS^3 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	25.0	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)
3,5-Di-*O*-caffeoylquinic acid	101.3	515	353	191(15)	191	191(100),179(65),135(15)
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)

* HPLC-MS^n retention 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.

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 (3)	3.6 ± 0.0*	2.7 ± 0.0	4.2 ± 0.4*	2.5 ± 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 (33)	*329 ± 8**	*309 ± 2*	*345 ± 4**	*245 ± 4*
Caffeine	406 ± 40	395 ± 35	649 ± 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).

462 Table 3. Chlorogenic acids content of Arabica and Robusta spent coffee extracts after the *in vitro*
463 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	3.6 ± 0.0^{b}	3.5 ± 0.1 *(95)*^{a}	3.4 ± 0.1 *(92)*^{a}	3.4 ± 0.0 *(95)*^{a}
Robusta	4.2 ± 0.4^{b}	3.4 ± 0.5 *(82)*^{ab}	3.3 ± 0.3 *(80)*^{a}	3.5 ± 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}	316 ± 10 *(96)*^{ab}	303 ± 10 *(92)*^{a}	304 ± 15 *(92)*^{a}
Robusta	345 ± 11^{b}	335 ± 28 *(96)*^{b}	296 ± 13 *(86)*^{a}	306 ± 15 *(89)*^{a}

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

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)*
p-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.

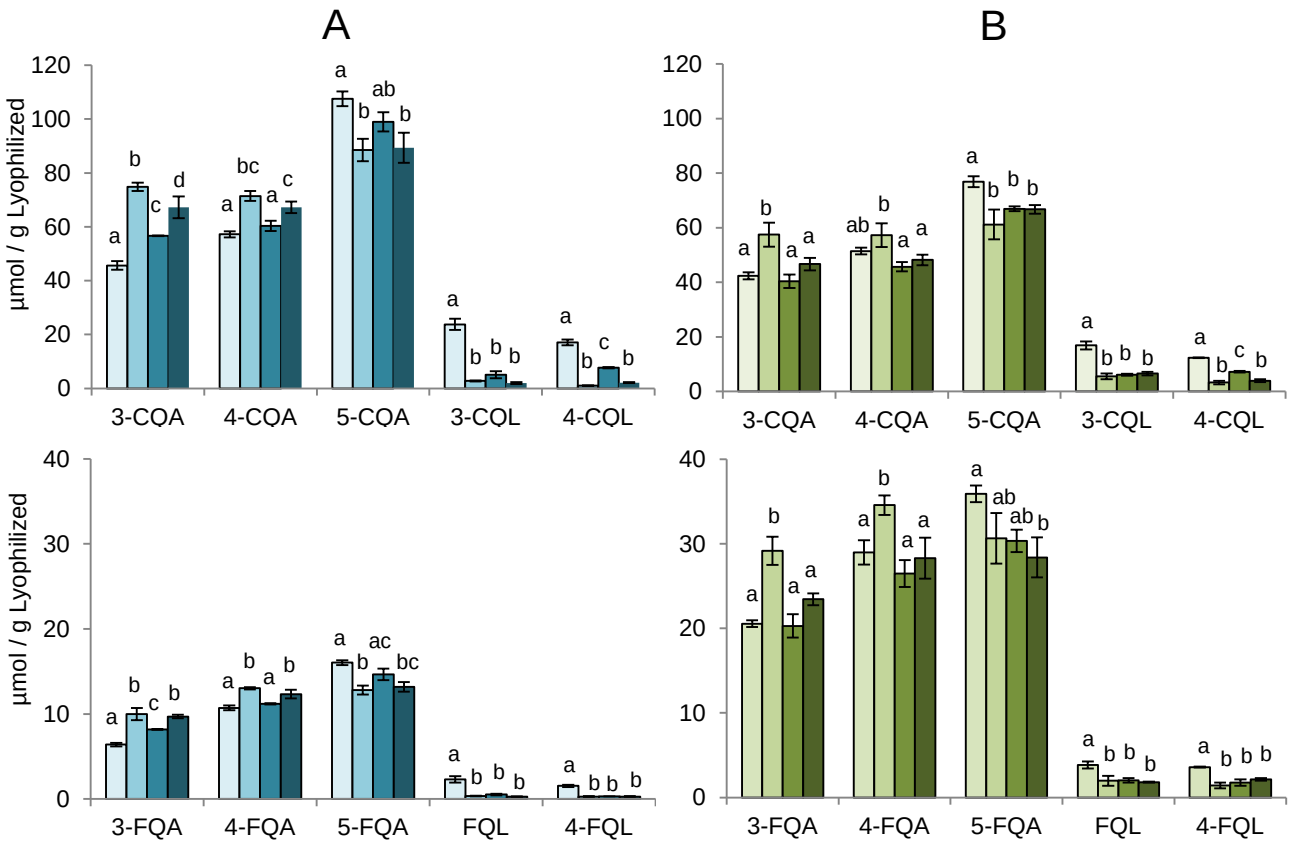
476 Table 5. Transport and metabolism of CGAs and hydroxycinnamates across the Caco-2 Cell
477 monolayer[#].

478

Compounds	% of apical dose					
	0.5 h			1 h		
	Apical	Cell	Basolateral	Apical	Cell	Basolateral
Substrate						
5-*O*-Caffeoylquinic acid	73.1	1.5	0.1	72.0	1.3	0.3
Metabolites						
4-*O*-Caffeoylquinic acid	15.7	0.1	–	16.1	0.1	0.3
3-*O*-Caffeoylquinic acid	9.1	–	tr	9.4	–	0.1
Caffeoylquinic acid-*O*-sulfate	0.1	tr	tr	–	tr	–
Caffeoylquinic acid-*O*-sulfate	0.2	tr	tr	0.2	tr	–
Caffeic Acid	–	0.1	0.0	0.0	–	0.1
Total	98.2	1.7	0.1	97.7	1.5	0.8
Substrate						
4,5-*O*-Dicaffeoylquinic acid	48.6	1.2	0.1	44.7	0.8	0.5
Metabolites						
3,5-*O*-Dicaffeoylquinic acid	25.7	0.4	0.1	28.5	0.3	0.2
3,4-*O*-Dicaffeoylquinic acid	10.0	0.1	–	13.3	0.1	0.1
5-*O*-Caffeoylquinic acid	6.8	0.3	0.2	6.1	0.2	0.2
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	0.3	0.1	–	0.2	0.1	0.1
Total	97.6	2.0	0.4	97.5	1.4	1.0
Substrate						
Caffeic acid	94.6	1.7	–	92.3	–	–
Metabolites						
Caffeic acid-3-*O*-sulfate	0.6	–	–	1.4	–	–
Ferulic acid	3.0	–	–	2.6	–	0.1
Total	98.2	1.7	0.1	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	–	–	–	–	–	0.1
Dihydroferulic acid	tr	tr	tr	0.8	0.2	0.2
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	0.0
Substrate						
Dihydroferulic acid	96.2	1.7	2.1	93.2	1.5	5.3

479
480 [#]Data expressed as a percentage of the apical dose of each standard solution, means from three independent
481 experiments; tr = traces (< the limit of quantification); – not detected
482

483


A
B
µmol / g Lyophilized
120
100
80
60
40
20
0
3-CQA
4-CQA
5-CQA
3-CQL
4-CQL
40
30
20
10
0
3-FQA
4-FQA
5-FQA
FQL
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