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Amarante da Encarnação, J, Farrell, TL, Ryder, A et al. (2 more authors) (2015) In vitro enzymic hydrolysis of chlorogenic acids in coffee. Molecular Nutrition and Food Research, 59 (2). pp. 231-239. ISSN 1613-4125

https://doi.org/10.1002/mnfr.201400498

In vitro enzymic hydrolysis of chlorogenic acids in coffee

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Key words: instant coffee, phenolic acids, pancreatic digestion, human plasma, Caco-2 cells

Abbreviations: **CA**, caffeic acid, **3-CQA**, 3-O-caffeoylquinic acid, **4-CQA**, 4-O-caffeoylquinic acid, **5-CQA**, 5-O-caffeoylquinic acid, **DHCA**, dihydrocaffeic acid, **DHFA**, dihydroferulic acid, **3,4-DMCIN**, 3,4-dimethoxycinnamic acid, **DMEM**: Dulbecco's modified Eagle's medium, **FA**, ferulic acid, **FBS**, fetal bovine serum, **GC**, instant coffee made from 65 % roasted and 35 % unroasted green coffee beans, **GoC**, golden roast caffeinated instant coffee, **GoD**, golden roast decaffeinated instant coffee, **HBSS,** Hank's balanced salts solution, **HCAs**, hydroxycinnamic acids, **iFA**, isoferulic acid.

Abstract

Scope: Coffee is rich in quinic acid esters of phenolic acids (chlorogenic acids) but also contains some free phenolic acids. A proportion of phenolic acids appear in the blood rapidly after coffee consumption due to absorption in the small intestine. We investigated in vitro whether this appearance could potentially be derived from free phenolic acids in instant coffee or from hydrolysis of chlorogenic acids by pancreatic or brush border enzymes.

Methods/results: We quantified 6 free phenolic acids in instant coffees using HPLC-DAD-ESI-QQQ. The highest was caffeic acid, but all were present at low levels compared to the chlorogenic acids. Roasting and decaffeination significantly reduced free phenolic acid content. We estimated, using pharmacokinetic modelling with previously published data, that the contribution of these compounds to small intestinal absorption is minimal. Hydrolysis of certain chlorogenic acids was observed with human differentiated Caco-2 cell monolayers and with porcine pancreatin, which showed maximal rates on 3- and 5-O-caffeoylquinic acids respectively.

Conclusions: The amounts of certain free phenolic acids in coffee could only minimally account for small intestinal absorption based on modelling. The hydrolysis of caffeoyl quinic, but not feruloylquinic acids, by enterocyte and pancreatic esterases is potentially a contributing mechanism to small intestinal absorption.

1 Introduction

Coffee is a rich source of chlorogenic acids, a diverse family of esters formed between quinic acid and certain phenolic acids, mainly hydroxycinnamic acids such as caffeic, ferulic and p-coumaric acids [1]. A limited number of studies have identified small amounts of free phenolic acids in green coffee beans and commercially available instant coffee [2, 3]. Variation in chlorogenic acid content can result from different bean varieties and from different climates [4]. While extensive changes to chemical composition and final chlorogenic acid content of the coffee bean can result from decaffeination and roasting [1, 5], very little is known about the impact of coffee processing on generating free phenolic acids. Investigations using cell models of the intestinal epithelium indicate that phenolic acids are rapidly transported into the blood [6] and human intervention studies investigating the appearance of phenolic acids in human plasma have indicated that free phenolic acids and especially dimethoxycinnamic acid are highly bioavailable after coffee consumption [7].

A very limited absorption of intact chlorogenic acids occurs after consumption of coffee, and the levels of compounds such as caffeoyl quinic or feruloyl quinic acids in plasma are at very low nM levels [8]. During digestion, chlorogenic acids are hydrolysed to quinic acid and the constituent phenolic acid. The phenolic acid appears in plasma in various forms, but mostly as sulphate conjugates [8]. Most absorption occurs after 4-6 hours, owing to hydrolysis of chlorogenic acids by gut microbiota in the colon [9]. However, a significant amount of the phenolic acids appear at earlier time points in the blood (1-3 hours), especially for metabolites such as ferulic acid sulphate and dimethoxycinnamic acid [7] , but it is not known if these are derived from free phenolic acids already present in the coffee, or from hydrolysis of chlorogenic acids by enzymes present in the small intestine. The latter could include pancreatic secretions and brush border enzymes. A previous study on human tissues indicated no hydrolysis upon incubation of chlorogenic acid (mainly 5-O-caffeoylquinic acid) with extracts of human intestinal tissue, liver or plasma. On the other hand, chlorogenic acid was efficiently converted into caffeic acid when incubated with faecal extracts [9].

There is also some evidence for a small amount of gastric absorption of intact chlorogenic acids in animal and cell models [3, 10].

Phenolic acids may be the principal bioactive compounds responsible for the observed beneficial health effects associated with regular coffee consumption [11]. There is growing evidence that chlorogenic acid metabolites may contribute to improved intestinal health by increasing mucosal membrane integrity [12]. Additionally, studies with animal models of type 2 diabetes indicated favourable antiglycemic effects and modulation of oxidative enzymes following consumption of caffeic acid [13] and ferulic acid [14]. It is now widely appreciated that chronic low grade inflammation plays a key role in development of degenerative diseases [15], and the reported bioactivity of phenolic acids may in part contribute to their control or prevention.

The aim of this study was to verify the presence of free phenolic acids in a variety of instant coffees and estimate the impact of roasting and decaffeination on the phenolic acid content. Pharmacokinetic modelling was then used to assess whether free phenolic acids, present in coffee, can account for the levels of respective metabolites observed in human plasma after coffee consumption. Pancreatic secretions and preparations of Caco-2 cells monolayers were used to investigate the hydrolysis of individual caffeoylquinic acids and chlorogenic acids, as present in coffee.

2 Materials and Methods

2.1 Chemicals and materials

All chemicals used in this investigation were purchased from Sigma-Aldrich (Berkshire, UK) unless otherwise stated. Acetonitrile (HPLC-MS grade) was purchased from Fisher Scientific Ltd (Leicestershire, UK). Ferulic acid (≥ 99 %) and sinapic acid (≥ 99 %, HPLC) were purchased from Fluka Analytical; dihydroferulic acid (97 %), dihydrocaffeic acid (98+ %) and methyl ferulate were obtained from Alfa Aesar (Lancashire, UK); isoferulic acid was from Extrasynthèse (Genay France) and DMSO from Riedel-de Haën. The human colon adenocarcinoma cell line, Caco-2 (HTB-37), was obtained from American Type Culture Collection at passage 18 (LGC Promochem, Middlesex, UK). Culture flasks, Transwell plates fitted with polycarbonate semi-permeable inserts of pore size 0.4 μm and area 4.67 cm^2 were obtained from Corning Life Sciences (Appleton Woods, Birmingham, UK). All water refers to deionized water, Millipore UK Ltd (Hertfordshire, UK).

The instant coffees used were obtained from a local supermarket: A coffee made from 35 % unroasted and 65 % roasted green coffee beans (GC), a golden roast with caffeine (GoC), a decaffeinated golden roast (GoD), a medium-dark roast with caffeine (MC), and a decaffeinated medium-dark roast (MD).

2.2 Sample preparation

Standards: For analysis of phenolic acids in coffee solutions, calibration curves with concentrations ranging from 0.05 to 100 μM for caffeic acid, dihydrocaffeic acid, ferulic acid, dihydroferulic acid, isoferulic acid and 3,4-dimethoxycinnamic acid were prepared in premixed 5 % acetonitrile and 94.9 % water acidified with 0.1 % formic acid (solvent A). Sinapic acid was used as internal standard at a final concentration of 100 μM.

For analysis of free caffeic and ferulic acids after pancreatin treatment of coffee, caffeoylquinic acids (3-, 4-, 5-CQA) and feruloylquinic acids (3-, 4-, 5-FQA) calibration curves were prepared by dissolving a 10 mM stock solution of free phenolic acid in 20 mM potassium phosphate buffer (pH

7.4). Final concentrations ranged from 1 to 34 µM and from 1 to 27 µM for caffeic and ferulic acid, respectively, for the analysis of phenolic acids after pancreatic digestion of coffee. Final concentrations ranged from 0.02 to 34 µM and from 0.01 to 27 µM for caffeic and ferulic acid, respectively, for the analysis of phenolics acids after pancreatic digestion of caffeoyl- and feruloylquinic acids, respectively.

For analysis of metabolites in cell culture solutions, calibration curves with final concentration ranging from 0.1 to 2400 µM for caffeic acid, 5-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, ferulic acid, methyl caffeate and methyl ferulate were prepared by dissolving a high concentration of test compound in dimethylsulfoxide and diluting with solvent A. The mixture was added to HBSS to create a matrix-matched calibration curve based on peak area at 320 nm. All samples were normalized to the internal standard, p-coumaric acid, which was maintained at 50 µM. Ferulic acid was used to determine diode array analytical performance based on triplicate injections on the same day: the limit of quantification was 0.1 µM, accuracy and precision at this concentration were calculated at < ± 15 % and < ± 5 % R.E. and R.S.D respectively.

Preparation of coffee solutions: Triplicates of spiked and non-spiked coffee samples were prepared from two different batches of commercially available instant coffees. Phenolic extraction was done by adding boiling deionized water to the coffee granules to a final 50 mg/mL concentration. This first extracted stock was then diluted to 5 mg/mL in solvent A. Spiked samples were supplemented with free phenolic acids to a final concentration of 100 µM and sinapic acid was used as internal standard at a final concentration of 100 µM. For the pancreatin digestion of coffee, a 20 mg/mL GC coffee solution was prepared in potassium phosphate buffer (pH 7.4).

Preparation of pancreatin solution: A 100 mg/mL porcine pancreatin (4 x USP; Sigma-Aldrich (Berkshire, UK)) suspension was prepared in potassium phosphate monobasic buffer (pH 7.4), briefly vortexed and centrifuged for 5 min at 20,000 g and 4 °C. A control solution was prepared by boiling

an aliquot of the supernatant. A further aliquot was used to quantify the protein content using the Bradford assay. Both active and inactive pancreatin solutions were kept on ice during the experiment. Pancreatic digestion was carried out for 60 min at 37 ºC. An aliquot (100 µL) was collected at 0, 10, 20, 40 and 60 min after incubation start, and 10 % HCl (10 µL) was added to each collected sample to stop the reaction. The inactivated sample was centrifuged for 10 min at 20,000 g at 4 ºC, and 5 µL of the supernatant was injected in duplicate on an HPLC-DAD-ESI-QQQ for quantitation.

Preparation of Caco-2 cells: Caco-2 cells were cultured as previously published [16] with the following modifications: On or after 22 d, metabolism studies (n = 3 per test compound) were initiated by replacement of DMEM culture medium with transport buffer (HBSS modified with 1.8 mM calcium chloride, pH 7.4) in the apical (2 mL) and basal (2 mL) compartments. Plates were incubated at 37 °C in a 5 % CO_2-humidified atmosphere for 15 min to allow equilibration of tight junction integrity. Apical and basal solutions were carefully aspirated and 2 mL of a 1 mM phenolic acid ester test solution was added to the apical compartment, while all basal solutions were transport buffer only. The test solution consisted of test compound (methyl ferulate, methyl caffeate, 5-O-caffeoylquinic acid or 3-O-caffeoylquinic acid) dissolved in a small amount of DMSO, which was diluted 500 times with transport buffer (0.2 % DMSO). Trans-epithelial electrical resistance (TEER) was recorded using a Millicell ERS volt-ohm meter fitted with a chopstick probe (Millipore Ltd, Watford, UK). To assess the stability of the test compounds during the experiment, 2 mL of the test solution was added to a Transwell plate, in the absence of cells, as a reference sample. Both the Transwell plate and reference sample were incubated at 37 °C in a 5 % CO_2-humidified atmosphere for 2 h after which TEER measurements were repeated. 1 mL of the apical, basal and reference sample solutions were then collected, acetic acid was added to obtain a final concentration of 10 mM and samples stored at -80 °C until analysis. Immediately before HPLC-DAD analysis, samples were thawed and centrifuged (17,000 × g, 5 min, RT) to remove particulate matter. 95 µL were removed to an amber vial, spiked with 5 µL of internal standard (p-coumaric acid, 50 µM final concentration) and placed in the HPLC autosampler for analysis.

Caco-2 cell metabolism: Cellular hydrolysis was calculated by subtracting the amount of metabolite degraded in the reference sample after incubation (2 h), from the amount of metabolite (ferulic acid or caffeic acid) detected in the apical and basal compartments after incubation (2 h) with the Caco-2 monolayers. Values are expressed as mean ± SD (n = 3). For TEER measurements, the mean final unit area resistance values for the control and test conditions were 1037 ± 104 Ωcm^2 and 960 ± 112 Ωcm^2 respectively which is indicative of a monolayer with established tight junctions and a well-developed apical-brush border [17].

2.3 Sample analysis

Analysis of the coffee solutions and metabolites resulting from in vitro pancreatic hydrolysis was performed on an HPLC-DAD-ESI-QQQ. For the cell culture metabolites, this was done on a Rapid Resolution HPLC-DAD (1200 series Agilent Technologies, Berkshire, UK). Dihydrocaffeic (DHCA) and dihydroferulic acids (DHFA) were analysed at 280 nm, and caffeic (CA), ferulic (FA), isoferulic (iFA) and 3,4-dimethoxycinnamic acids (3,4-DMCIN) at 325 nm. Upon optimization of the standards, the mass spectrometric variables shown in Table 1 were used.

5 µL of either coffee solution, cell culture solution or standard was injected. Chromatographic separation was achieved on an Eclipse plus C18 column (30 °C, 2.1 mm x 100 mm, 1.8 µm pore size; Agilent Technologies) using a 61-min gradient of solvent A (premixed 94.9 % water in 5 % acetonitrile modified with 0.1 % formic acid) and B (premixed 5 % water in 94.9 % acetonitrile modified with 0.1 % formic acid), based on a previously established gradient [3]. Identification of metabolites in cell culture solutions was confirmed by comparison of the UV spectra and retention time with commercial standards. Caffeic acid and ferulic acid from the pancreatic digestion were also quantified using an Eclipse plus C18 column (30 °C, 2.1 mm x 100 mm, 1.8 µm pore size; Agilent Technologies), however, chromatographic separation of the 5 µL injected sample was achieved in a 30-min gradient with a flow rate of 0.3 mL/min. The gradient increased from 0 % to 8 % B within the first 14.0 min and was kept at 19 % B from 14.1 to 20.0 min. The column was washed with 100 % B

from 20.1 to 24.0 min prior to equilibration with 0 % B from 24.1 to 30.0 min. Chromatograms were recorded at 280 and 320 nm and DAD spectra were stored for quality control purposes. The flow was evaporated with an electrospray unit operated in negative mode at 350 °C. The nebulizer gas was nitrogen set to a flow rate of 13 L/min and a pressure of 60 psi.

2.4 Recoveries

Recovery rates of phenolic acids extracted from coffee were estimated from the comparison of triplicate samples of non-spiked 5 mg/mL coffee solutions with 100 µM-spiked 5 mg/mL coffee solutions.

2.5 Pharmacokinetic calculations

To estimate the possible contribution of unmodified free phenolic acids to the early plasma pool, pharmacokinetic curves of 4 g of GC were computed assuming the following variables: 100 % bioavailability (F), a volume of distribution measured for another polyphenol, (-)-epigallocatechin gallate, equivalent to that of 200 mg in healthy human [18] (V_β/F 2009 +/- 1514), an administration dose of free phenolic acid as quantified in 4 g GC (FA: 310.2 µg, iFA: 7.2 µg, DHFA: 17.2 µg, CA: 1116 µg, DHCA: 173 µg, 3,4-DMCIN: 149 µg), a maximal concentration (C_{max}) and half-life ($t_{1/2}$) taken from existing pharmacokinetic curves [19], an elimination constant (ke) calculated from $t_{1/2}$ (ke = ln(2)/ $t_{1/2}$) and a ka calculated using the following formula: $k2 * t_{max} - ln(ka) - ke * t_{max} + ln(ke) = 0$.

2.6 Statistical analyses

The statistical software R was used for analyses of data. Each free phenolic acid was compared between coffees, using the Welch two-sample t-test. Only values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Free phenolic acid content of coffee

The conditions established for analysis of free phenolic acids by HPLC-DAD-ESI-QQQ are shown in Table 1 and Figure 1. The limit of quantification per injection was 7.5, 45, 5.6 and 4.8 pg for CA, DHCA, FA and DHFA respectively. Using this method, the content of 6 free phenolic acids was determined in 5 different instant coffees (Table 2). All compounds were above the limit of quantification. Amongst the 5 coffees analysed, the coffee originating from a mixture of roasted and unroasted beans (GC) had the highest content of CA and FA ($p < 0.001$ and $p < 0.05$ respectively). On the other hand, the medium dark roast caffeinated coffee (MC) contained the highest content of DHCA, DHFA and iFA, and the golden roast caffeinated coffee (GoC) contained the highest level of 3,4-DMCIN. Finally, the two decaffeinated coffees tested had, when compared to their corresponding roast caffeinated coffee, lower levels of all free phenolic acids. These observations suggest that not only roasting, but also decaffeination, may affect the content of free phenolic acids. The levels of free phenolic acids are > 2 orders of magnitude lower than that of chlorogenic acids typically present in the GC coffee [20], see Table 2.

3.2 Estimation of the contribution of free phenolic acids to the early appearing blood phenolic acid conjugates

Phenolic acid metabolites were reported in plasma in a previous human study where subjects consumed a cup of GC coffee beverage at different concentrations [19] . To ascertain the contribution of phenolic acids present in coffee to the early appearance of phenolic acids metabolites in plasma, the contents in the GC coffee from the current investigation were compared to computed pharmacokinetic curves (Figure 2) and to the maximum plasma values previously reported obtained after consumption of a cup of 4 g GC coffee [19]. Assumptions made are shown in the Material and Methods section. However, our values are the maximum estimates and real values are likely to be less. Even in the unlikely event that our assumptions are underestimated by 10-fold, the amount of free phenolic acid in

coffee is still not enough to account for the early appearing blood peaks by at least an order of magnitude, and more for some compounds. As an example, the free ferulic acid fraction in coffee would give rise to a peak of 0.5 nM in blood assuming 100% absorption, compared to an obtained value of 200 nM after drinking a cup of coffee, i.e. ~400-fold higher. From this calculation, we estimate that most of the early appearing phenolic acids would be due to hydrolysis of the constituent chlorogenic acids, which must occur either in the stomach or in the small intestine. We therefore tested some possible sources of esterase in the small intestine for ability to hydrolyse the various chlorogenic acids present in coffee.

3.3 Pancreatic activity on chlorogenic acids

Since the amount of free phenolic acids in coffee could not account for the early peaks in plasma, we then tested sites in the small intestine which might facilitate hydrolysis of chlorogenic acids. Intestinal and liver tissue extracts did not hydrolyse 5-O-caffeoylquinic acid [9]. We therefore tested the pancreatic secretion which is secreted into the small intestine. Incubation of the coffee solution with porcine pancreatin at 8.5 mg protein/mL (Figure 3) led to hydrolysis of chlorogenic acids present in the coffee to give free caffeic acid, with a rate of 203 pmol/(mg protein*min). We then tested pure chlorogenic acid (50 μM each) as pancreatin substrates. Linear rates over 60 min were obtained, equivalent to 1.6, 2.4 and 5.4 pmol/(mg protein*min) for 3- CQA, 4- CQA and 5-CQA, respectively. No significant increase in ferulic acid was observed after pancreatin treatment of coffee, and also no ferulic acid was detected after incubation of pancreatin with 50 μM 3-FQA, 4-FQA and 5-FQA. These data suggest that CQAs and more specifically 5-CQA is a better substrate for pancreatin, a complex mixture of various lipases, peptidases, amylases and non-specific carboxylesterases. No digestion was observed when the coffee solution and each of the chlorogenic acids were incubated with an inactivated pancreatin preparation.

3.4 Esterase activity

In the cell culture medium, the phenolic acid esters exhibited good stability (Table 3). In total, 19.8 % of the methyl ferulate was hydrolyzed, at a 0.25 mol/h rate, following incubation with Caco-2 monolayers, releasing ferulic acid which was detected in both the apical and basal compartments in similar amounts. In comparison, 11.4 % methyl caffeate was metabolized at a 0.10 mol/h rate, but the caffeic acid was distributed mainly in the basal compartment (i.e. 9.5 %). The hydrolysis of the caffeoylquinic acid isomers was distinctly lower than observed for the methyl ester analogue, methyl caffeate. Interestingly, 3-O-caffeoylquinic acid was hydrolysed ~10-fold more rapidly than 5-O-caffeoylquinic acid; 0.18 % (1.7 mmol/h) and 0.02 % (0.23 mmol/h) respectively.

4 Discussion

Amongst a variety of commercially available instant coffees, the caffeinated instant coffee made from 35 % green and 65 % roasted beans water extract (GC) had the highest content of free CA and FA. The medium dark roast caffeinated coffee (MC) had the highest content of free DHFA, iFA and DHCA and the golden roast caffeinated coffee (GoC) had the highest content of 3,4-DMCIN. Additionally, when comparing caffeinated and decaffeinated coffees of a same roast, the decaffeinated coffees contained less of all free phenolic acids tested. These data suggest that not only chlorogenic acids [1], but also some free HCAs decrease with roasting intensity and decaffeination.

As expected, when compared to the amount of intact chlorogenic acids measured previously [20], the amounts of free phenolic acids present in GC are substantially lower (Table 2). However, free CA and FA are readily absorbed through the intestinal wall without further modifications [21] and less abundant compounds could have the potential to contribute significantly to the pool of metabolites measured in human fluids. Our pharmacokinetic simulation suggests that the free phenolic acids are not sufficient to make a significant contribution to the plasma phenolic acids, even if 100% absorption is assumed. The in vitro esterase experiments using pancreatic secretions and small intestinal cells suggest, however, that the hydrolysis of chlorogenic acids into free phenolic acids are major contributing mechanisms to the early appearance of free phenolic acids in plasma. Results from chlorogenic acid treatment by pancreatic enzymes indicated a rate of hydrolysis of 5-CQA up to 5-fold higher when compared to 3-CQA and 4-CQA. The pancreatic secretion used in these experiments is a complex mixture of lipases, trypsin, peptidases, proteases, RNAses, DNAses, amylases and unspecific carboxylesterases, and so it is not possible at this stage to attribute the hydrolysis to a single enzyme. The hydrolysis of methyl esters in Caco-2 cells has been previously reported [22] and was used in this study to verify the presence of esterase activity in our Caco-2 cell model. Less than 7% of chlorogenic acid (isomer composition not specified) was lost when incubated with human gastric fluid, duodenal fluid and ileostomy effluent [23]. The expression of carboxylesterase(s), which

286 is believed to be localized on the endoplasmic reticulum [24] has been previously characterized in
287 both the Caco-2 cell model and human intestinal tissue [25].
288
289 Intestine, liver and tissue extracts were unable to hydrolyse 5-O-caffeoylquinic acid [9]. Our results
290 are consistent with this since we found a very low rate of hydrolysis of 5-O-caffeoylquinic acid by the
291 intestinal model, differentiated Caco-2 cells. However, 3-O-caffeoylquinic acid was hydrolysed at a
292 much higher rate (~10-fold). After hydrolysis, the product caffeic acid, and its methylated derivative
293 ferulic acid, are transported differently, probably due to the differing lipophilicity of the methyl esters
294 and caffeoylquinic acids, Log D ~ 2 and – 3.5 respectively (reference: MarvinSketch, 2012; version
295 5.3.1, ChemAxon). The hydrophilic nature of the caffeoylquinic acids and paracellular permeation
296 [26] may explain the lower rates of hydrolysis compared to the methyl esters. Steric hindrance in the
297 active site of human carboxylesterase-2, the predominant isoform in the small intestine [24], has been
298 reported to occur with substrates containing a bulky acyl-moiety (representing the phenolic acid part
299 of the quinic acid ester) which interfere with the formation of the enzyme-acyl intermediate during the
300 first stage of hydrolysis. Steric interference may in part explain the differential hydrolysis of
301 caffeoylquinic acids, and the lack of hydrolysis of the feruloylquinic acids. Interestingly, these
302 findings may support data from human bioavailability studies in vivo which showed that only 5-O-
303 caffeoylquinic acid could be detected in plasma after coffee consumption [8], perhaps suggesting that
304 the susceptibility of the 3-acyl isomer to hydrolysis and relative resistance of the 5-acyl may
305 contribute to the differential amounts of chlorogenic acids observed in vivo. An additional site of
306 absorption is the stomach; cultured gastric epithelial cells have the capacity to hydrolyse chlorogenic
307 acids into phenolic acids, with the esterase action favouring hydrolysis of caffeic and
308 dimethoxycinnamic acid-containing substrates [3]. Several publications have also shown that intact
309 chlorogenic acids can pass across the rat stomach and Caco-2 cell monolayers, but only minor
310 hydrolysis into phenolic acids occurred in the rat stomach [10, 27].
311

In conclusion, this study contributes significant knowledge to our understanding of the metabolic fate of chlorogenic acids (Figure 4) in the small intestine and demonstrates that free phenolic acids present in coffee are not present in sufficient quantity to explain the early appearance of phenolic acids metabolites following consumption of a coffee drink. Further investigation of possible sources of free phenolic acids indicated that pancreatin efficiently hydrolysed 5-CQA and the later, more than 3-CQA could therefore be an important contributor to the early peak of caffeic acid.

We would like to thank Nestlé Research Center, Lausanne, Switzerland for partially funding the Ph.D. studentships of JAE and NUK.

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393
394

Figure legends

Figure 1: Typical diode array detector and total ion count chromatograms of the coffee sample containing 35 % of green coffee beans. Absorbance profile at 280 nm (A), Total ion counts from mass spectrometry (B, C), Individual transitions for indicated mass-to-charge ratio (m/z).

Figure 2: Calculated pharmacokinetic curves for phenolic acids in plasma based on free phenolic acid content in coffee. These pharmacokinetic curves were computed assuming a 100 % bioavailability of the compound and an administrated dose of free phenolic acids equivalent to that found in 4 g of GC. From published data, the small intestinal C_{max} values (i.e. < 3 hours) after consumption of this amount of coffee were approximately: DHCA, 50 nM; DHFA, 60 nM; 3,4-DMCIN, 500 nM; FA, 210 nM; CA, 180 nM; iFA, 80 nM.

Figure 3: Treatment of instant coffee by porcine pancreatin as estimated by formation of caffeic and ferulic acids.
Caffeic and ferulic acids estimated by LC-MS during the incubation of instant coffee (20 mg/ml CG) with porcine pancreatin (8.5 mg protein/mL).
Data is presented as mean ± SD, n=4.

Figure 4: Sites of hydrolysis of chlorogenic acids derived from this and previous studies.

Tables

Table 1 Optimization variables for phenolic acids of interest

	RT (min)	λ (nm)	$[M-H]^-$ (m/z)	Fragmentor (eV)	MS^2 (m/z)	CE1 (eV)	CE2 (eV)
DHCA	12.45	280	181	90	137, 59	8	15
CA	15.77	325	179	90	135, 89	12	28
DHFA	33.60	280	196	90	136, 121	12	25
FA	36.31	325	193	90	178, 134	8	12
SA	38.33	325	223	100	208, 164	5	10
iFA	38.60	325	193	80	178, 134	5	12
3,4-DMCIN	45.95	325	207	80	103, 163	8	10

Values obtained from optimization of the different phenolic acids and used for analysis on HPLC-DAD-ESI-QQQ. RT: retention time (min), λ: wavelength of maximal absorbance (nm), $[M-H]^-$: mass-to-charge ratio (m/z) of negatively charged molecular ion; Fragmentor voltage (eV); MS2: mass-to-charge ratio of product ion produced by fragmentation of $[M-H]^-$ or $[M-H]^+$;CE1 and CE2: collision energy for first or second transition.

Table 2 Free phenolic acid content in 5 instant coffees

µg phenolic acid/g coffee	DHCA	CA	DHFA	FA	iFA	3,4-DMCIN
GC	43 ± 1	279 ± 16	4.3 ± 0.2	77 ± 5	1.8 ± 0.1	37 ± 0
GoC	56 ± 1	107 ± 3	4.1 ± 0.2	23 ± 1	2.7 ± 0.4	48 ± 1
GoD	44 ± 2	72 ± 4	3.6 ± 0.2	22 ± 2	1.7 ± 0.6	19 ± 0
MC	71 ± 1	87 ± 2	6.7 ± 0.2	29 ± 4	3.2 ± 0.3	32 ± 2
MD	46 ± 1	75 ± 4	4.6 ± 0.2	22 ± 1	1.7 ± 0.0	15 ± 0

Free phenolic acid content in 5 instant coffees: GC, 35 % unroasted and 65 % roasted coffee caffeinated; GoC, golden roast caffeinated; GoD, golden roast decaffeinated; MC, medium roast caffeinated; MD, medium roast decaffeinated. Values are given in µg phenolic acid per gram of dry weight of instant coffee. Standard deviations are for the two batches of coffee analysed, with three biological replicates, each with two technical replicates. The content of chlorogenic acids, previously reported [20], is: 3-CQA: 42,000 µg/g coffee; 4-CQA, 49,600; 5-CQA, 162,400; 4,5-diCQA, 23,200; 3,5-diCQA,16,000; 3,4-diCQA, 22,800; 4-FQA, 8,800; 5-FQA, 31,600.

437

438 **Table 3 Summary of hydrolysis of phenolic esters by Caco-2 cell monolayers**

Phenolic acid ester	Caco-2 study			
	Mean apical dose (μM)	Hydrolysis (%)		Mass balance (%)
		Apical	Basal	
methyl ferulate	1285	11.1 ± 0.4	8.7 ± 0.4	88 ± 2
methyl caffeate	894	1.9 ± 0.1	9.5 ± 0.3	97 ± 1
5-O-caffeoylquinic acid	1134	n.d.	0.02 ± 0.001	98 ± 1
3-O-caffeoylquinic acid	940	0.18 ± 0.01	n.d.	88 ± 3

439

Caco-2 monolayers (n = 3) were incubated (2 h) with approximately 1 mM of phenolic acid ester and the release metabolite (caffeic acid or ferulic acid) was quantified by HPLC-DAD. n.d.: not detected. Values are the mean of 3 cultures ± SD, experiments performed using passages 48 to 50.

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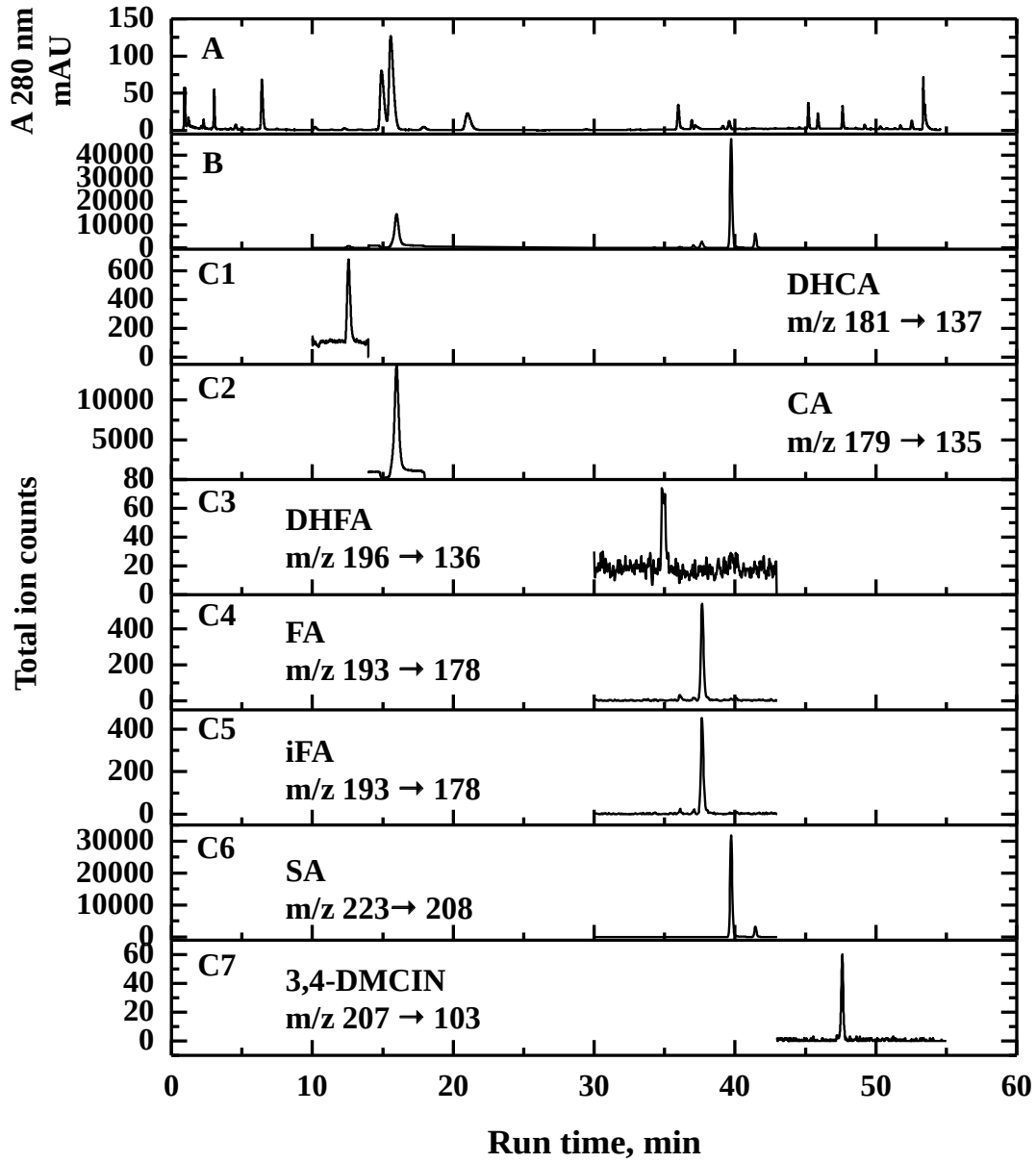
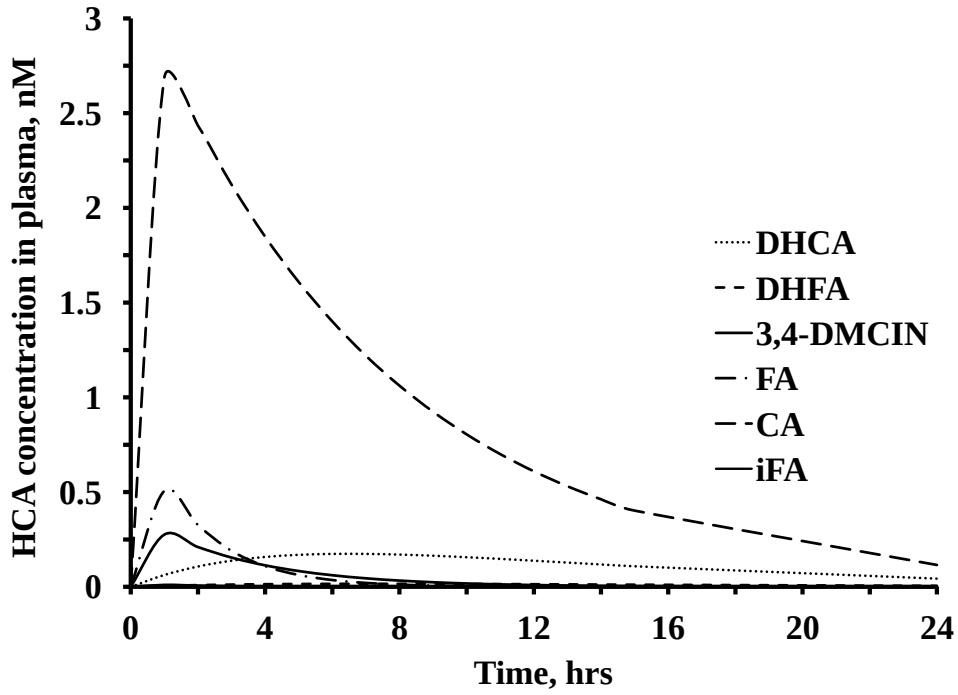

A 280 nm
mAU
A
B
C1
DHCA
m/z 181 → 137
C2
CA
m/z 179 → 135
Total ion counts
C3
DHFA
m/z 196 → 136
C4
FA
m/z 193 → 178
C5
iFA
m/z 193 → 178
C6
SA
m/z 223→ 208
C7
3,4-DMCIN
m/z 207 → 103
Run time, min

442

443 Fig 1



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445 Fig 2

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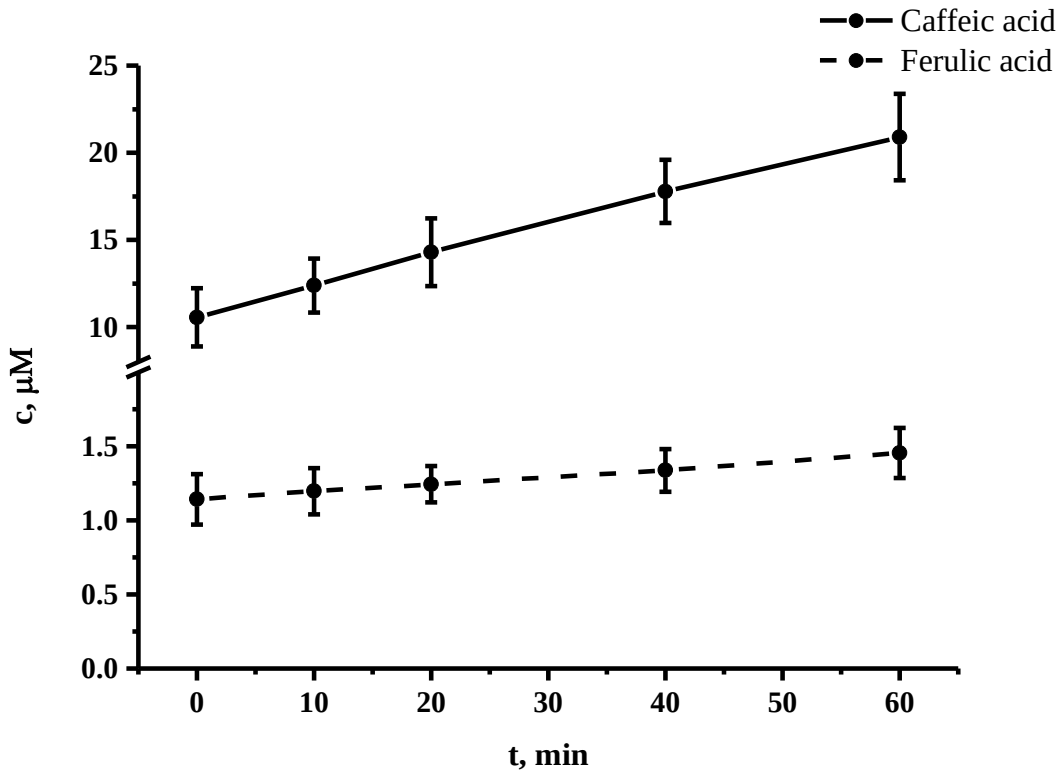

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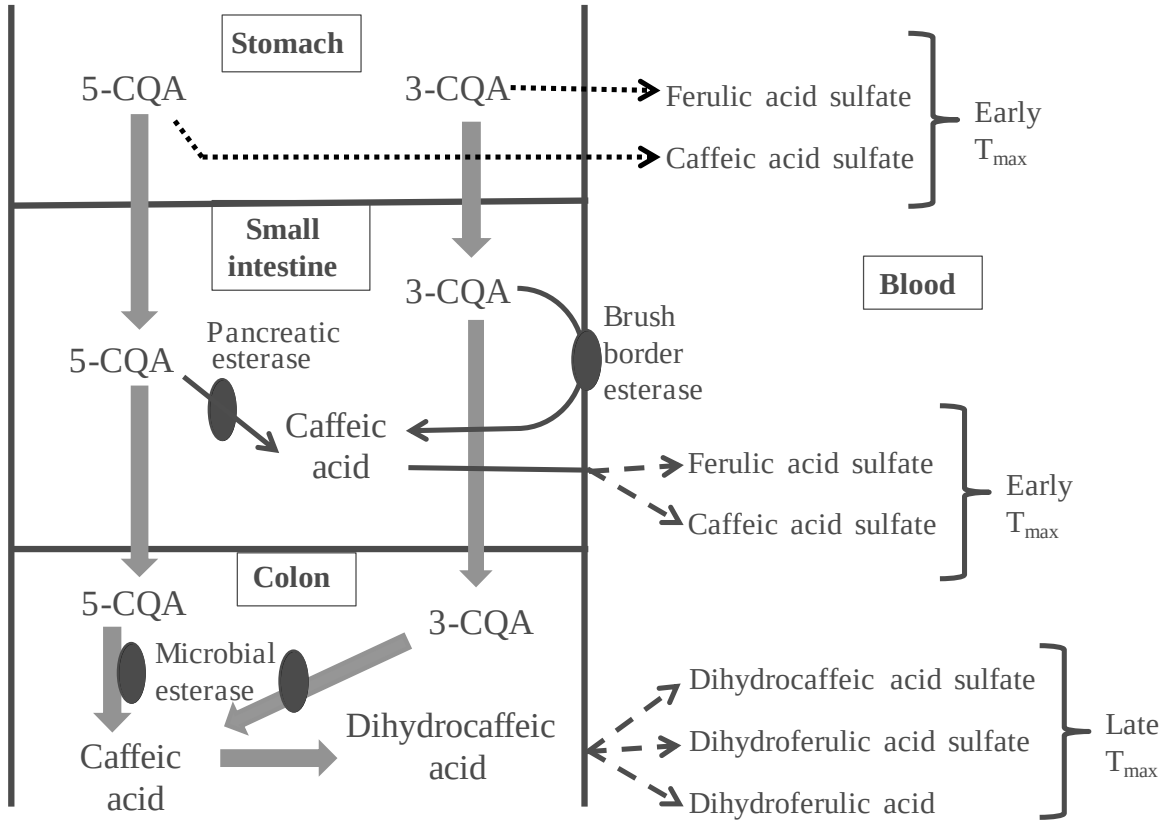
449 Fig3



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452 Fig 4

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