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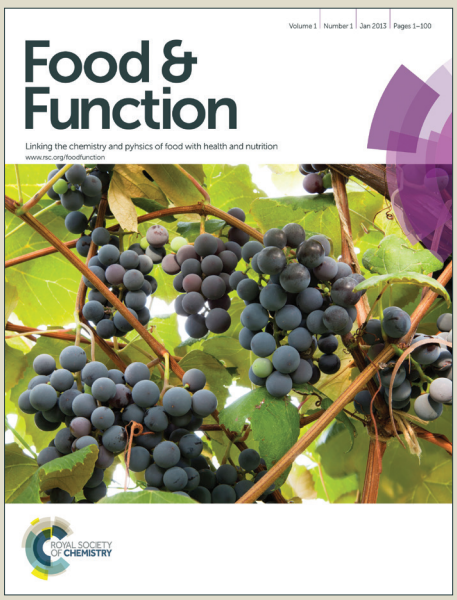

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Impact of dose on the bioavailability of coffee chlorogenic acids in humans

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This article reports and investigation of the effect of dose on the bioavailability of chlorogenic acids in humans based on HPLC-MS^2 and analysis of plasma and urine collected 0-24 h after supplementation

Impact of dose on the bioavailability of coffee chlorogenic acids in humans

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Abstract

Single servings of coffee beverage containing low (412 μmol), medium (635 μmol) and high (795 μmol) amounts of chlorogenic acids were administered to eleven healthy volunteers in a double-blind randomised controlled trial. Analysis of plasma and urine collected for 24-h revealed the presence of 12 metabolites in plasma and 16 metabolites in urine, principally in the form of sulphates, and to a lesser extent glucuronides of caffeic, ferulic, dihydrocaffeic and dihydroferulic acids, as well as intact feruloylquinic and caffeoylquinic acids, and sulphated caffeoylquinic acid lactones. Median values of peak plasma concentrations after increasing doses of chlorogenic acids were 1088, 1526 and 1352 nM. In urine the median amounts of metabolites excreted after 24-h following consumption of the three coffees were 101, 160 and 125 μmol, accounting for 24%, 25% and 16% of the doses ingested. Peak plasma concentration and urinary excretion values showed trends towards a reduced bioavailability of chlorogenic acids associated with the highest dose ingested, when expressed as percentages of intake.

Potential biomarkers of coffee intake were identified as feruloylquinic acids and sulphated caffeoylquinic acid lactones in plasma and urine with positive moderate to strong coefficients of determination for peak plasma concentrations (0.60-0.81) and amounts excreted in urine (0.36-0.73) ($P < 0.05$).

1. Introduction

Coffee is widely consumed throughout the world and the beverage contains high levels of chlorogenic acids with a single shot of espresso from commercial outlets providing between 24 and 422 mg.[1] Although occurring in a diversity of plant-derived foods, coffee is the major dietary source of chlorogenic acids for many people.[2] Chlorogenic acids are a family of non-flavonoid compounds, comprising quinate esters of hydroxycinnamic acids such as caffeic, ferulic, and *p*-coumaric acids. The main chlorogenic acid classes in coffee are caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), *p*-coumaroylquinic acids (*p*CoQAs), dicaffeoylquinic acids (diCQAs), and caffeoylferuloylquinic acids (CFQAs). In addition, caffeoylquinic acid lactones (CQLs) and feruloylquinic acid lactones (FQLs), products of roasting, can also occur in coffee in significant amounts.[3] Depending on the position of the hydroxycinnamic acid on the quinic acid moiety, a wide range of different isomers occur in coffee, with the predominant compounds being 3-, 4- and 5-CQAs.[4]

A limited number of studies have investigated the bioavailability of coffee chlorogenic acids in human subjects. Nardini *et al.*[5] reported an increase of conjugated caffeic acid in human plasma, reaching a maximum concentration 1 h after the ingestion of 200 mL of coffee. Increased amounts of ferulic acid, isoferulic acid, vanillic acid (3-methoxy-4-hydroxybenzoic acid), dihydroferulic acid, hippuric acid and 3-hydroxyhippuric acid were detected in urine samples of five male subjects, after three ingestions of two cups of coffee at 4 h intervals.[6] A wide range of metabolites produced in vivo after coffee intake or chlorogenic acids ingestion has been reported in previous detailed bioavailability studies along with only low nM levels of CQAs and FQAs appearing in plasma and urine

in healthy subjects and volunteers with an ileostomy, and allowed distinguishing between events occurring in the small and large intestine.[7,8] The low nM levels of CQAs and FQAs in plasma is also supported by other studies which have involved enzymic deglucuronidation and desulfation of the plasma samples.[9]

The aim of the current study was to expand the earlier work of Stalmach *et al.*[7,8] and to investigate the impact on the plasma pharmacokinetic profiles and urinary excretion of chlorogenic acid-derived metabolites after the acute ingestion by human volunteers of three instant coffees containing 412, 635 and 795 μmol of chlorogenic acids per 200 mL, equivalent, respectively, to 148, 230 and 292 mg of chlorogenic acids per serving.

2. Results

2.1. Analysis of coffees

The quantities of chlorogenic acids in 200 mL-servings of the three instant coffees (coffee I, II and III) fed to volunteers are summarised in Table 1. Identifications of the chlorogenic acids were based on MS^2 fragmentation patterns of ions at *m/z* 353 (CQAs)[1], *m/z* 367 (FQAs), *m/z* 337 (*p*-CoQAs) and *m/z* 515 (diCQAs), according to Clifford *et al.*[10,11] The identity of 3- and 4-CQAL was ascertained by both the MS^2 profiles of their ions at *m/z* 335 and co-chromatography with standards. The levels of chlorogenic acids in the coffees ranged from 412 ± 9 to 795 ± 14 μmol, and consisted in CQAs (63 - 74 %), FQAs (13 - 18 %), CQALs (5 - 14 %), diCQAs (3 - 8 %) and *p*-CoQAs (1 - 2 %). The 2-fold difference between the highest and the lowest levels was mainly due to

[1] There is much confusion over the names of caffeoylquinic acids with many suppliers, including Sigma, using non-IUPAC nomenclature. They refer to chlorogenic acid as 3-*O*-caffeoylquinic acid when it is 5-*O*-caffeoyquinic acid while their crytochorogenic acid, marketed as 5-*O*-caffeoyquinic acid, is the 3-*O*-isomer

significantly greater amounts of CQAs, FQAs, diCQAs and *p*-CoQAs in coffee III which contained 795 ± 14 μmol of chlorogenic acids. Coffee II with intermediate levels of chlorogenic acids (635 ± 3 μmol) contained significantly more FQAs than the other two beverages (117 μmol compared to 103 and 67 μmol), as well as higher levels of lactones (77 μmol compared to 57 and 40 μmol) per serving (Table 1).

2.2. Identification of chlorogenic acid derivatives and metabolites in plasma and urine samples

The identification and quantification of chlorogenic acid derivatives and metabolites circulating in plasma and excreted in urine was initially carried out on samples collected following the ingestion of coffee I, a commercial instant coffee that contains 412 μmol of chlorogenic acids per 200 mL serving. A total of 21 metabolites, principally sulphates and glucuronides were identified by HPLC-MS^n in the 0-24 h plasma and urine samples. The identifications, summarised in Table 2, were achieved using HPLC-MS^3. The resulting MS^2 and MS^3 daughter ions were then compared to the fragmentation of either the available standards or, in the case of the chlorogenic acids, to their detailed, published MS^n fragmentation patterns.[10,11] The identity of metabolites of chlorogenic acids in plasma and urine samples was also ascertained by comparing their retention time with those of 3'-*O*- and 4'-*O*- sulphate and glucuronide standards. Full details of the basis of the identifications of the metabolites can be found in an earlier publication.[7] There are minor differences in the complex urinary metabolite profiles obtained in the two studies but this involves trace components, such a dihydroisoferulic acid-3′-*O*-glucuronide which was not

detected in quantifiable amounts in the current investigation, rather than major metabolites.

2.2. Chlorogenic acid intake and plasma metabolites

Twelve metabolites were detected in plasma following the ingestion of the three coffees containing different amounts of chlorogenic acids. Peak plasma concentrations (C_{max}), time to reach C_{max} (T_{max}), area-under-the-curve (AUC) and apparent elimination half life[2] ($T_{1/2}$) values were measured following increasing intakes. The pharmacokinetic parameters are summarised in Figure 1 and Table 3.

The metabolic profile of the circulating chlorogenic acid metabolites can be divided into two distinct groups according to their time of appearance in plasma, following coffee intakes (Figure 1). Intact feruloylquinic acids, as well as the sulphated lactones, 5-CQA and sulphated ferulic and caffeic acids reached peak plasma concentrations (C_{max}) between 0.5 and 1.0 h after intake (Table 3). The only exception was ferulic acid-4′-*O*-sulphate, as a second C_{max} peak appeared in plasma, after 4.0 h. Free and sulphated dihydroferulic and dihydrocaffeic acids appeared in plasma at later time points and reached their C_{max} between 4.0 and 6.0 h. Their C_{max} values were also higher, ranging from 33 (26; 41) nM[3] (ferulic acid-4'-*O*-sulphate after ingestion of coffee I) to 518 (462; 771) nM (dihydroferulic acid after ingestion of coffee III). In contrast, C_{max} values of the other compounds ranged from 5.4 (4.5; 5.9) nM (5-FQA after coffee I) to

[2] A true $T_{1/2}$ value can be determined only by intravenous dosing of a metabolite. Assessments based on elimination after oral dosing overestimate the $T_{1/2}$ because the metabolite is still entering the circulatory system when the elimination is being estimated.

[3] Expressed as median and interquartiles (Q1; Q3) values

126 (111; 195) nM (ferulic acid-4′-*O*-sulphate after coffee II). The dihydroferulic and dihydrocaffeic acids also seemed to remain in circulation for a longer period of time, with $T_{1/2}$ times ranging from 0.7 (0.4; 0.8) h (dihydrocaffeic acid after ingestion of coffee I) to 3.9 (3.0; 8.4) h (ferulic acid-4′-*O*-sulphate after coffee III). This large range of $T_{1/2}$ values probably reflects the lack of data points between 8 h and 24 h time points of blood collection. The other compounds appeared to have a more rapid turnover, with $T_{1/2}$ values ranging from 0.2 (0.2; 0.3) h (5-CQA after coffee II) to 1.2 (0.8; 3.4) h (caffeic acid-3′-*O*-sulphate after coffee I).

The total C_{max} of compounds with an early T_{max} of 0.5-1 h (sulphates of caffeic and ferulic acids, sulphates of 3- and 4-caffeoylquinic acid-lactones, 5-CQA and FQAs) with increasing dose were 261, 510 and 244 nM, respectively. The C_{max} of compounds with a later T_{max} of 4-6 h (free and sulphated dihydrocaffeic and dihydroferulic acids and second C_{max} of ferulic acid-4′-*O*-sulphate) following the increasing doses were 808, 1242 and 1164 nM. *AUC* values for dihydrocaffeic acid-3'*O*-sulphate and free dihydroferulic acid and were significantly different between the lowest and highest intakes, with values of 1787 (712; 3210) nmol.h.L^{-1} and 3839 (3002; 7302) nmol.h.L^{-1} for dihydrocaffeic acid-3'*O*-sulphate and 2845 (2255; 3210) nmol.h.L^{-1} and 4549 (3688; 7834) nmol.h.L^{-1} for dihydroferulic acid, respectively. The time to reach C_{max} as well as $T_{1/2}$ for each compound did not differ with the increasing doses. The only CQA that could be quantified in plasma was the main compound in the coffee beverage, namely 5-CQA, with C_{max} of 11 (5.9; 13) nM following the intake of 795 µmol (Table 3).

2.3. Chlorogenic acid intake and urinary excretion of metabolites

A total of 16 metabolites were quantified in the urine samples following increasing doses of the coffee chlorogenic acids. The amounts excreted as well as percentages relative to the levels ingested are summarised in Table 4. The main compounds excreted were dihydrocaffeic acid-3′-*O*-sulphate, with levels excreted ranging from 6.0 % to 8.7 % of the ingested chlorogenic acids. The excretion of other metabolites ranged as follows: feruloylglycine (3.4 – 4.5 %), dihydroferulic acid-4′-*O*-sulphate (2.0 – 2.8 %), ferulic acid-4′-*O*-sulphate (2.0 - 2.4 %), dihydroferulic acid (1.3 - 2.1 %), isoferulic acid-3′-*O*-glucuronide (1.1 - 1.4 %) and caffeic acid-3′- and 4′-*O*-sulphates (0.5 - 1.5 %). The total amounts excreted in the 24-h urine samples increased with increasing intake from 101 (82; 119) to 160 (91; 211) to 125 (109; 241) μmol. The percentages excreted, relative to intake at 24.4 %, 25.2 % and 15.7 % of the dose, were not significantly different, reflecting a large inter-individual variability.

Levels of FQAs excreted for 24-h after coffee intake ranged between 0.61 μmol (5-FQA after intermediate levels coffee) and 9.28 μmol (3-FQA after intermediate levels coffee). When expressed as a percentage of intake, levels of 3-FQA excreted ranged between 4.6 % (high dose) and 6.0 % (low dose), whereas levels of 4-FQA ranged between 2.7 % (intermediate dose) and 5.2 % (low dose) and lowest excretion levels were observed for 5-FQA (1.8 % following intermediate dose and 3.3 % after intake of low dose). Median, ranges and interquartile values are displayed in Figure 2.

2.4. Correlation between doses ingested and levels of metabolites in plasma and urine

Correlation between the doses of chlorogenic acids ingested and levels of parent compounds and metabolites quantified in plasma and urine after intake was assessed using Spearman's rank correlation and results are displayed in Table 5. Compounds and metabolites significantly associated with increasing doses of chlorogenic acids administered were the FQAs and CQAL-S in both plasma and urine, with moderate to strong positive association (correlation coefficients ranging from 0.362 for 5-FQA in urine to 0.807 for 3-FQA in plasma, $p < 0.05$). These metabolites showed an increase with the dose ingested, as shown in Figures 3 and 4. Urinary levels of ferulic acid-4'-*O*-sulphate and isoferulic acid-3'-*O*-glucuronide also significantly increased with increasing intake of chlorogenic acids, with correlation coefficients of 0.409 ($p = 0.018$) and 0.507 ($p = 0.003$).

3. Discusssion

Following the ingestion of coffee there was extensive metabolism of chlorogenic acids. The probable pathways involved in the proximal and distal sections of the gastrointestinal tract, based on feeding studies with volunteers with and without an intact functioning colon, have been elucidated by Stalmach *et al.*[7,8]

Despite being major components in the coffee, and in contrast to the findings of Monteiro *et al.* [12] and Farah *et al.*,[13] in the current study only trace amounts of one of the three CQAs, 5-CQA, appear in the circulatory system. Plasma did however contain low levels of all three FQAs (Figure 1) with absorption occurring, as with 5-CQA, in the small intestine. 5-CQA, which is stable when incubated with gastric juice,[6,14] along with the other CQAs, is probably subjected to the action of esterases in the wall of the small intestine[15,16] releasing caffeic acid which is converted to caffeic acid-3′-*O*-sulphate before

entering the blood stream. It is possible that 3- and 4-CQAL and diCQAs could also be hydrolysed in the small intestine and contribute to the caffeic acid pool. The rapid appearance of ferulic acid-4′-*O*-sulphate in plasma (Fig. 1) could be the result of the FQAs following a parallel pathway being converted to ferulic acid and then ferulic acid-*O*-sulphate, although methylation of caffeic acid via catechol-*O*-methyltransferase (COMT), as well as the conversion of CQAs to FQAs, could also contribute to the ferulic acid pool. Most ferulic acid passes across the small intestine in the unmodified, unconjugated aglycone form,[17] implying that phase II sulphation of the hydroxycinnamate occurs mainly in the liver.

Analysis of ileal fluid collected after acute consumption of the lower dose by ileostomists indicated that in volunteers with a functioning colon ~70 % of the ingested chlorogenic acids will pass from the small to the large intestine,[8] also supported when higher amounts of chlorogenic acids were administered.[18] In vitro faecal fermentations have shown the breakdown of caffeoyl- and feruloylquinic acids, respectively, to caffeic acid and ferulic acid, which, via reduction of the 2,3 double bond on the aliphatic chain, are then converted to their dihydro derivatives by a bacterial reductase prior to phase II sulphation, and to a lesser degree glucuronidation, mediated by mammalian rather than bacterial enzymes.[19] The current study provides evidence of substantial absorption and metabolism in the colon, with C_{max} values mostly well in excess of those obtained with compounds absorbed in the small intestine, being observed 4.0-6.0 h after coffee intake for dihydroferulic acid-4′-*O*-sulphate, dihydrocaffeic acid-3′-*O*-sulphate as well as dihydroferulic acid and dihydrocaffeic acid (Table 3).

The plasma pharmacokinetic data in Table 3 and Figure 1 reveals the appearance in the circulatory system of dihydrocaffeic acid, dihydrocaffeic acid-3′-*O*-sulphate, dihydroferulic acid and dihydroferulic acid-4′-*O*-sulphate 4.0-6.0 h after drinking coffee. This is in agreement with the role of the colonic microflora in the conversion of CQAs to caffeic acid and dihydrocaffeic acid and FQAs to ferulic acid and dihydroferulic acid demonstrated with the *in vitro* based study of Ludwig *et al*.[19] The large intestine is not a site for the *in vivo* sulphation of either hydroxycinnamates,[17] or flavonols.[20] or The sulphation of dihydrocaffeic acid and dihydroferulic acid, therefore, probably occurs in the liver after their absorption into the circulatory systems. The presence of substantial amounts of both free and sulphated dihydroferulic and dihydrocaffeic acids in plasma (Fig. 1) is in keeping with this proposal.

Peak plasma concentrations and urinary excretion could be divided into metabolites absorbed in the small intestine and large intestine, and when expressed as a percent of intake, there was a significant trend towards a reduced absorption in the small intestine associated with the highest dose ingested (Table 6). Similarly, urinary excretion of metabolites absorbed in the small intestine accounted for 5.9% (low dose), 5.4% (medium dose) and 3.9% (high dose), suggesting a reduced bioavailability of coffee chlorogenic acids after consumption of a higher dose. This is in line with a phenomenon noted by Erk *et al.*[18] or in an investigation with ileostomists, demonstrating an increased gastrointestinal transit time associated with the highest dose of chlorogenic acids ingested, albeit at doses that were 2.6 fold (low dose) and 5.7 fold (high dose) greater than those used in the current study. They observed an effect across all three doses, whereas in the current study, we observed a reduced

bioavailability of coffee chlorogenic acids at the higher dose of chlorogenic acids ingested (795 μmol). This was however not reflected on the T_{max} values of the various circulating metabolites identified.

Despite the low plasma and urine concentrations of intact CQAs observed, more of the FQAs were detected in circulation and excreted in urine across all three doses administered. Using an in vitro culture of gastric epithelial monolayers, Farrell *et al.*[21] showed that FQAs are recovered to a lesser extent to the basal side of the cells compared to the CQAs. The presence of catechol-*O*-methyltransferase in gastric[21] and intestinal epithelial cells[22] may explain the presence of FQAs in circulation, as a result of methylation of the absorbed CQAs. In the current study, the urinary excretion levels of 3-FQA were consistently higher compared to those of 4-FQA and 5-FQA, across all three coffees (Fig. 2). This may have resulted from either extensive in vivo isomerisation from 5-FQA to the 4- and 3-acyl, as previously observed in ileostomy volunteers[23] and/or from a preferential absorption with possible involvement of an active transport processed as discussed for the gastric absorption of 4-CQA and 4-FQA over the 5-acyls.[21]

Overall, the excretion of metabolites in urine after ingestion of chlorogenic acids ranged from 15.7 to 25.2 % of intake (Table 4), indicating that the coffee chlorogenic acids are absorbed and excreted to a much greater extent than many other dietary flavonoids and phenolic compounds.[24] In the three coffee feeds total C_{max} values were 1088 (759; 1372), 1526 (919; 2110) and 1352 (1211; 2291) . As noted in other studies with dietary (poly)phenolics this suggests that metabolites and related compounds absorbed into the circulatory

system do not accumulate to any extent as they are treated by the body as xenobiotics and rapidly removed by renal excretion.

Biomarkers of (poly)phenol consumption may be a useful tool to assess dietary intake of certain compounds with potential benefits to human health, thus allowing estimating long-term consumption with better accuracy than dietary questionnaires.[25] The data obtained in the current study with coffee are summarised in Table 5, and show that levels of FQAs and CQAL-*O*-sulphates in plasma and urine significantly and positively correlate with increasing intake of chlorogenic acids. These could serve as biomarkers of coffee intake, as their concentration in body fluids is sensitive to changes in coffee intake, and are also specific to coffee.[26] This is in contrast with the dihydrocaffeic acid and dihydroferulic acid metabolites, which are detected in a wide range of values between individual volunteers, and are also produced from a range of dietary (poly)phenolic compounds.[27,28] The combined presence of these quinic acid ester metabolites in plasma and urine are convenient biomarkers of coffee consumption even after relatively low doses of intake after 'consumer relevant' dosages of chlorogenic acids in the range of 148 – 292 mg per serving, in line with previously reported levels of 24 – 422 mg in espresso coffees purchased in high street outlets in the UK. [1]

4. Experimental

4.1. Chemicals

Ferulic acid, 5-CQA, dihydrocaffeic acid, sinapic acid, and L-ascorbic acid were purchased from Sigma Aldrich Co Ltd (Poole, Dorset, UK). Caffeic acid was obtained from AASC Ltd (Southampton, UK), and dihydroferulic acid from Alfa Aesar (Heysham, Lancashire, UK. EDTA and HPLC grade formic acid were purchased from Fisher Scientific Ltd (Loughborough, Leicestershire, UK), methanol and acetonitrile from Rathburn Chemicals (Walkerburn, Scotland), and acetic acid from WWR International Ltd (Poole, Dorset, UK). 3- and 4-caffeoylquinic acid lactones, 3- and 4-β-*O*-glucuronidated ferulic, isoferulic, caffeic, dihydrocaffeic, dihydroferulic and dihydro(iso)ferulic acids, 3- and 4-*O*-sulphated ferulic, caffeic, dihydrocaffeic and dihydroferulic acids were synthesized as described previously.[29] Feruloylglycine and isoferuloylglycine were prepared by methods outlined in an earlier publication.[8]

4.2. Feeding study design

The feeding study was a double-blinded randomised cross-over trial with a washout period of two weeks between treatment, and involved healthy participants fed a single serving of 200 ml of coffee beverage, containing various doses of chlorogenic acids. The instant coffee powders were contained in colour-coded sachets and neither the researchers involved in the feeding trial and collection of body fluids or the volunteers were made aware of the contents prior to the end of the project.

The criteria for volunteers to participate in the study were to be in good health, non-smoker, and not pregnant. Eleven volunteers participated in the study (eight male and three female) and were aged 19-35 years, with an average

height of 1.70 ± 0.13 m (mean ± standard deviation), an average weight of 70.6 ± 11.5 kg, and an average BMI of 24.3 ± 2.3 kg/m^2.

The study and protocol were reviewed by Glasgow Royal Infirmary NHS Research Ethics Committee and the participating subjects all provided their informed consent prior to the commencement of the study. The volunteers followed a low polyphenol diet for 48 h prior to the beginning of the study. On the night preceding the trial, the subjects stopped consuming food after dinner (8 pm) and came to the trial unit at 8.30 am the next day, in a fasted state. Water was the only liquid allowed *ad libitum*. The 200 ml of coffee beverages were made as described below. The participants were encouraged to drink the beverage within 5 min after it reached a suitable temperature for consumption. All urine excreted over 24 h was collected over the periods: 0-2 h, 2-5 h, 5-8 h and 8-24 h, and blood samples were collected in heparin tubes prior (0 h) and post-ingestion at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 h.

A standard lunch, containing low levels of phenolic and polyphenolic compounds was provided after the 3 h samples were collected. The amount consumed was not limited, water was available all day and the subjects were encouraged to drink. After the 8 h samples were collected, the volunteers remained on the low-phenolic diet until the next morning, prior to collection of the 24-h time point samples. The volumes of urine excreted were measured, and aliquots were stored at –80°C and, likewise, plasma samples were sub-divided into 1 ml aliquots which were stored at –80°C prior to analysis.

4.3. Analysis of coffee, plasma and urine

The instant coffees fed to the volunteers were supplied by the Nestlé Research Centre (Vers-chez-les-Blanc, Lausanne, Switzerland). Beverages were prepared by adding 200 ml of boiled distilled water to 3.4 g of powdered instant coffee and aliquots were stored at –80°C prior to analysis. Samples were defrosted and centrifuged at 16,110 *g* for 3 min at 4°C, and diluted 50-fold with distilled water and 100 µl aliquots analysed in triplicate by HPLC-PDA-MS^n.

Urine samples were defrosted and centrifuged at 16,110 *g* for 3 min at 4°C, prior to the triplicate analysis of 20 µl aliquots by HPLC-PDA-MS^n.

Plasma samples were defrosted and were extracted based on the method of Day *et al.*[30] Plasma (450 µl), acidified with 13.5 µl of 50 % aqueous formic acid, was added drop-wise to a 1125 µL of acetonitrile to which had been added 50 µl of 10 % (m/v) ascorbic acid containing 0.5 µM EDTA, and 1 µg of sinapic acid which was used as an internal standard. Samples were vortexed for 30 sec every 2 min over a 10 min period before centrifuging at 1,500 *g* for 20 min at 4° C. The supernatant was decanted and the pellet re-extracted with 1,125 µl of methanol and after centrifugation the two supernatants were combined and reduced to dryness under a stream of nitrogen at 35°C. The dried samples were then re-suspended in 250 µL of 0.1% aqueous formic acid containing 10% methanol, and centrifuged at 4°C for 20 min at 16,110 *g* in a 0.2 µm Eppendorf filter. Duplicate 100 µl volumes were analyzed by HPLC-PDA-MS^n.

Extraction efficiency experiments were carried out and typical values of 55 ± 1.5 % were achieved for sinapic acid. Extraction efficiencies were also determined for 5-CQA, caffeic acid, ferulic acid, dihydrocaffeic and dihydroferulic

acids, and for 3- and 4-CQA lactones. Ratios of the individual recoveries relative to sinapic acid were computed and used as correction factors in the quantification of metabolites in the plasma samples.

4.4. HPLC-PDA-MS^n

Chlorogenic acids and related compounds were analyzed using a Surveyor HPLC with a PDA detector and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, USA). Separations were performed at 40°C using a SYNERGI 4 µm POLAR-RP 250 x 4.6 mm i.d. reverse phase column (Phenomenex, Macclesfield, UK). Injections were carried out with an autosampler maintained at 4°C. The mobile phase was pumped at a flow rate of 1 ml/min. The column eluate initially passed through the PDA detector and was then split, and 0.3 ml/min directed to the mass spectrometer with ESI operating in full scan negative ionisation mode (100-1000 *m/z*). Analyses of coffee, plasma and urine samples were initially carried out using full scan, data dependent MS^2 scanning from *m/z* 100 to 1000. The tuning of the mass spectrometer was optimised by infusing a standard of ferulic acid, dissolved in the initial HPLC mobile phase, into the source at a flow rate of 0.3 ml/min. Capillary temperature was 300ºC, sheath gas and auxiliary gas were 80 and 60 units/min respectively, and source voltage was 3.0 kV, and collision energy set at 35 %.

Separation of coffee chlorogenic acids was achieved on a 110-min gradient of 5-25 % acetonitrile in 0.1 % aqueous formic acid. Following separation, hydroxycinnamates were detected and quantified with the PDA at 325 nm, and identification was confirmed by mass spectrometry using consecutive reaction monitoring (CRM). Mean data are expressed as µmol ± SE

(n = 3) of 5-CQA equivalents for the CQAs, FQAs, di-CQAs, and *p*-CoQAs, and as µmol ± SE (n = 3) of 3-CQAL and 4-CQAL for the respective lactones quantified.

Plasma and urine were analysed using a 40 min, 5-16 % gradient of acetonitrile in 0.5 % aqueous acetic acid. Following separation, hydroxycinnamates and metabolites were quantified using selective ion monitoring (SIM), based on the calibration curves of the corresponding standard. Sulphated ferulic, isoferulic, caffeic, dihydroferulic, and dihydrocaffeic acids were quantified based on the SIM calibration curves of the respective *O*-sulphated standards. Sulphated 3- and 4-caffeoylquinic acid lactones were quantified based on the SIM calibration curves for 3- and 4-CQAL, respectively. Feruloylglycine was quantified based on the calibration curve of ferulic acid at 325 nm, as the glycine derivative, like ferulic acid, had a λ_{max} at this wavelength. Quantification of FQAs was based on the SIM calibration curve of 5-CQA. Glucuronidated isoferulic acid, dihydroferulic acid, dihydro(iso)ferulic and dihydrocaffeic acids were quantified based on SIM calibration curves of authentic isoferulic-, dihydroferulic- and dihydrocaffeic acid-*O*-glucuronides, respectively. Data are expressed as mean values ± SE (n = 11), in µmol for urine and nM for plasma.

Calibration curves of standards used to quantify chlorogenic acids and derivatives in various samples were drawn from the analysis of 5 µl volumes at concentrations ranging from 0.1 to 100 ng/µl (n = 7) for coffee samples, and 0.2 to 20 ng/µl (n = 5) for plasma and urine samples. Coefficients of linearity for the calibration curves were typically $R^2 > 0.99$. The limits of detection as monitored on PDA (at 325 nm for chlorogenic acids and hydroxycinnamates, and 280 nm for dihydrocaffeic acid and dihydroferrulic acid) and SIM were determined by analysis in triplicate of standards at low concentrations. Typical limits of

detection for PDA were 0.5 ng (CV 4.6-22 %), and 0.1 ng (CV 5.6-33 %) for SIM, except for isoferulic acid where a 5 ng limit of detection (CV 23 %) was obtained with SIM at *m/z* 193, as the acid content of the mobile phase suppresses ionization.[31]

4.5. Pharmacokinetic analysis of chlorogenic acids and their metabolites in plasma

Statistical comparison of the amounts of chlorogenic acids contained in a 200 ml – serving of coffee was achieved using One-way ANOVA. Normality of the data on the pharmacokinetic parameters was assessed using the Shapiro-Wilk test. As the data was not normally distributed, non-parametric statistical tests were used and data are reported as median and interquartiles (Q1; Q3). Statistical analyses testing differences between the three groups for urinary excretion and plasma pharmacokinetic data following increasing doses of chlorogenic acids ingested were performed using Friedman test, followed by pairwise post hoc comparisons. Correlative association between increased doses of chlorogenic acid ingested and levels of plasma and urine metabolites was investigated using Spearman's rank coefficient correlations (IBM®, SPSS® Statistics, Version 21). Statistical significance was set at P-value < 0.05.

5. Conclusions

Coffee is a rich source of chlorogenic acids, principally 5-CQA, 4-CQA and 3-CQA. Analysis of plasma after the acute ingestion of coffees containing increasing amounts of chlorogenic acids of 412, 635 and 795 μmol revealed the presence 13 metabolites and parent compounds. Although FQAs were detected in plasma but only trace amounts of 5-CQA were present. 5-CQA and the FQAs all had plasma

T_{max} times of ~1.0 h indicative of absorption in the small intestine. The main circulatory metabolites absorbed in the small intestine were caffeic acid-3′-*O*-sulphate and ferulic acid-4′-*O*-sulphate. The ferulic acid sulphate had a second T_{max} of 4 h in keeping with colonic absorption. The other plasma metabolites originating from the colon were dihydroferulic acid, dihydrocaffeic acid, dihydroferulic acid-4′-*O*-sulphate and dihydrocaffeic acid-3'-*O*-sulphate. Overall there was a trend, albeit with several exceptions, for increased levels of plasma metabolites with increasing intakes of chlorogenic acids. In contrast, the cumulative 0-24h urinary excretion of 16 metabolites ranged 15.7 to 25.2% of chlorogenic acid intake. The excretion level when expressed as a percentage of intake was not significantly different for the three coffees. However, when divided into metabolites absorbed in the small intestine and large intestine, at the highest dose there is a significant trend towards lower absorption in the small intestine that is not reflected to the same extent with the colonic metabolites. Although not major metabolites, the levels of FQAs and CQAL-*O*-sulphates in plasma and urine did increase significantly and positively correlate with the ingested dose of chlorogenic acids. These compounds are therefore good, specific biomarkers of the extent of coffee consumption, even at low intakes. Further work should be carried out to determine the suitability of these compounds as biomarkers of chlorogenic acids/coffee intake in a wider population of free-living individuals without any dietary restrictions.

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Figure Legends

Fig. 1 Pharmacokinetic profiles of chlorogenic acid derivatives and metabolites in the plasma of human subjects 0-24 h after the ingestion of 200 mL of instant coffee containing 412 (yellow line), 635 (blue line) and 795 (red line) μmol of chlorogenic acids. Data expressed as mean values.

Fig. 2 Box-and-Whisker plots representing median, range and interquartile values of 24-h urinary excretion for 3-FQA, 4-FQA and 5-FQA after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).

Fig. 3 Median and interquartile values of peak plasma concentrations for 3-FQA, 4-FQA, 5-FQA, 3-CQAL sulphate and 4-CQAL sulphate after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).

Fig. 4 Median and interquartile values of peak plasma concentrations for 3-FQA, 4-FQA, 5-FQA, 3-CQAL sulphate and 4-CQAL sulphate after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).

Table 1 Quantification of chlorogenic acids in 200 mL servings of the three different coffee beverages fed to healthy volunteers[a]

Compounds	Coffee I	Coffee II	Coffee III
3-Caffeoylquinic acid	72 ± 1.3 †	101 ± 0.5 *	96 ± 2.6 *
4-Caffeoylquinic acid	78 ± 1.5 †	114 ± 1.3 *	114 ± 3.3 *
5-Caffeoylquinic acid	119 ± 2.1 ‡	186 ± 1.2 †	373 ± 8.3 *
Total Caffeoylquinic acids	**269 ± 4.9** ‡	**401 ± 3.0** †	**583 ± 14** *
3-Feruloylquinic acid	20 ± 1.6 *	30 ± 0.3 †	17 ± 0.3 *
4-Feruloylquinic acid	22 ± 1.8 *	40 ± 0.4 †	21 ± 0.0 *
5-Feruloylquinic acid	25 ± 1.9 ‡	47 ± 0.5 †	65 ± 0.1 *
Total Feruloylquinic acids	**67 ± 5.3** ‡	**117 ± 1.3** †	**103 ± 0.3** *
3-Caffeoylquinic acid lactone	34 ± 2.9 ‡	46 ± 0.5 †	23 ± 0.1 *
4-Caffeoylquinic acid lactone	23 ± 2.0 ‡	32 ± 0.4 †	16 ± 0.1 *
Total Caffeoylquinic acid lactones	**57 ± 4.9** ‡	**77 ± 0.9** †	**40 ± 0.1** *
4-*p*-Coumaroylquinic acid	3.8 ± 0.1 †	4.0 ± 0.1 †	3.2 ± 0.0 *
5-*p*-Coumaroylquinic acid	3.1 ± 0.1 †	3.1 ± 0.0 †	4.8 ± 0.0 *
Total p-Coumaroylquinic acids	**6.8 ± 0.1** †	**7.1 ± 0.1** †	**8.1 ± 0.0** *
3,4-Dicaffeoylquinic acid	5.8 ± 0.6 ‡	13 ± 0.5 †	23 ± 0.1 *
3,5-Dicaffeoylquinic acid	2.8 ± 0.3 ‡	8.1 ± 0.8 †	17 ± 0.1 *
4,5-Dicaffeoylquinic acid	4.0 ± 0.4 ‡	11 ± 0.4 †	21 ± 0.1 *
Total Dicaffeoylquinic acids	**13 ± 1.2** ‡	**32 ± 1.7** †	**61 ± 0.3** *
Total chlorogenic acids	**412 ± 9** ‡	**635 ± 3** †	**795 ± 14** *

[a] Data expressed as mean values ± SE (n = 3) in μmol of 5-CQA equivalents for the CQAs, FQAs, *p*-CoQAs and diCQAs, and 3- and 4-CQAL equivalents for the lactones
Different superscripts within rows indicate statistical difference (One-Way ANOVA, $P < 0.05$)

Table 2 HPLC-MS^n identification of chlorogenic acids and metabolites detected in plasma and urine of human volunteers 0-24 h following the ingestion of 200 mL of coffee [a]

Peak	R_t (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Compounds Identified	Location
1	7.2	433	353	191, 179, 135	3-Caffeoylquinic acid-*O*-sulphate	Urine (traces)
2	10.3	261	181	137, 119	Dihydrocaffeic acid-3-*O*-sulphate	Plasma, Urine
3	11.1	357	181, 175, 137, 113	-	Dihydrocaffeic acid-3-*O*-glucuronide	Urine
4	11.2	433	353	191, 179, 173, 135	4-Caffeoylquinic acid-*O*-sulphate	Urine (traces)
5	11.7	259	179	135	Caffeic acid-4′-*O*-sulphate	Urine (traces in plasma)
6	12.1	275	195	177, 151, 136, 135, 123, 119	Dihydroferulic acid-4′-*O*-sulphate	Plasma, Urine
7	12.8	259	179	135	Caffeic acid-3′-*O*-sulphate	Plasma, Urine
8	13.5	181	137, 119		Dihydrocaffeic acid	Plasma (traces in urine)
9	13.9	371	195, 175, 113	-	Dihydroferulic acid-4′-*O*-glucuronide	Urine (traces in plasma)
10	14.5	273	193	178, 149, 134	Ferulic acid-4′-*O*-sulphate	Plasma, Urine
11	15.4	353	191	-	5-Caffeoylquinic acid	Plasma
12	16.5	367	193	-	3-Feruloylquinic acid	Plasma, Urine
13	16.9	273	193	178	Isoferulic acid-3′-*O*-sulphate	Urine (traces in plasma)
14	17.7	371	195, 175, 113	-	Dihydro(iso)ferulic acid-3′-*O*-glucuronide	Urine
15	19.6	369	193, 175, 113	-	Isoferulic acid-3′-*O*-glucuronide	Urine (traces in plasma)
16	21.9	250	206, 191, 177, 149, 100	-	Feruloylglycine	Urine
17	22.4	415	335	161, 135	3-Caffeoylquinic acid lactone-*O*-sulphate	Plasma, Urine
18	22.9	415	335	161, 135	4-Caffeoylquinic acid lactone-*O*-sulphate	Plasma, Urine
19	24.6	367	173, 193	-	4-Feruloylquinic acid	Plasma, Urine
20	26.4	195	177, 151, 136, 135, 123, 119	-	Dihydroferulic acid	Plasma, Urine
21	27.4	367	191	-	5-Feruloylquinic acid	Plasma, Urine

[a] R_t, 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 3 Pharmacokinetic parameters of chlorogenic acid derivatives and metabolites circulating in plasma of volunteers 0-24 h following the ingestion of coffees containing 412, 635 and 795, µmol of chlorogenic acids [a]

Metabolites	C_{max} (nM)			T_{max} (h)			*AUC* (nmol.h.L^{-1})			$T_{1/2}$ (h)		
	412 µmol	**635 µmol**	**795 µmol**	**412 µmol**	**635 µmol**	**795 µmol**	**412 µmol**	**635 µmol**	**795 µmol**	**412 µmol**	**635 µmol**	**795 µmol**
3-Caffeoylquinic acid lactone-*O*-sulphate	27*† (20; 35)	54† (35; 75)	20* (16; 29)	0.5 (0.5; 0.75)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	26*† (20; 40)	69† (45; 91)	21* (14; 27)	0.5 (0.3; 0.5)	0.5 (0.5; 0.6)	0.3 (0.2; 0.5)
4-Caffeoylquinic acid lactone-*O*-sulphate	21*† (15; 26)	51† (30; 72)	18* (13; 22)	0.5 (0.5; 0.9)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	21* (16; 31)	72† (37; 77)	18* (11; 23)	0.5 (0.4; 0.5)	0.5 (0.5; 0.6)	0.3 (0.1; 0.6)
Caffeic acid-3′-*O*-sulphate	90 (70; 108)	89 (67; 162)	79 (60; 107)	1.0 (0.5; 1.3)	1.0 (0.8; 1.0)	0.5 (0.5; 1.0)	227 (181; 377)	359 (219; 438)	184 (123; 426)	1.2 (0.8; 3.4)	1.2 (0.7; 1.4)	0.9 (0.7; 1.1)
3-Feruloylquinic acid	16*† (13; 17)	43* (29; 46)	13† (10; 16)	0.5 (0.5; 1.0)	1.0 (0.5; 1.0)	0.5 (0.5; 1.3)	25* (23; 33)	78† (53; 102)	24* (20; 25)	0.9 (0.7; 1.1)	1.0 (0.7; 1.0)	1.0 (0.8; 1.3)
4-Feruloylquinic acid	14*† (8.4; 18)	24* (21; 45)	9.2† (7.7; 13)	1.0 (0.5; 1.0)	1.0 (0.8; 1.0)	1.0 (0.5; 1.3)	20* (18; 33)	55† (35; 98)	19* (15; 27)	0.8 (0.6; 0.9)	1.0 (0.8; 1.2)	0.9 (0.8; 1.4)
5-Feruloylquinic acid	5.4* (4.5; 5.9)	12*† (10; 14)	18† (15; 22)	1.0 (0.6; 1.0)	1.0 (0.6; 1.0)	1.0 (1.0; 1.5)	7.9* (6.4; 9.1)	19*† (16; 22)	38† (29; 45)	0.8 (0.6; 1.0)	0.8 (0.5; 1.0)	1.1 (0.8; 1.3)
5-Caffeoylquinic acid	5.9 (5.0; 7.0)	10 (6.6; 13)	11 (5.9; 13)	1.0 (0.9; 1.1)	0.5 (0.5; 1.0)	1.0 (0.5; 1.0)	5.2 (4.2; 12)	7.5 (5.7; 8.9)	14.7 (5.6; 23)	0.3 (0.2; 1.0)	0.2 (0.2; 0.3)	0.6 (0.4; 0.6)
Ferulic acid-4′-*O*-sulphate [b]	65* (61; 90)	126† (111; 195)	68* (47; 94)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	0.5 (0.5; 0.5)	-	-	-	1.2 (1.0; 1.4)	1.1 (0.8; 1.3)	1.1 (0.8; 1.2)
Total (small intestine absorption)	**261 (187;319)**	**510 (336;570)**	**244 (205;297)**	-	-	-	-	-	-	-	-	-
Ferulic acid-4′-*O*-sulphate [b]	33* (26; 41)	54† (44; 73)	35*† (26; 73)	4.0 (4.0; 4.0)	4.0 (3.5; 4.0)	4.0 (4.0; 6.0)	409* (349; 447)	703† (601; 791)	511*† (292; 894)	3.6 (3.3; 4.7)	3.2 (2.6; 7.2)	3.9 (3.0; 8.4)
Dihydrocaffeic acid	49 (34; 54)	56 (39; 101)	72 (56; 102)	6.0 (4.0; 6.0)	6.0 (6.0; 6.0)	6.0 (6.0; 7.5)	118 (92; 209)	481 (333; 604)	631 (230; 989)	0.7 (0.4; 0.8)	3.0 (2.6; 3.5)	2.7 (1.1; 2.9)
Dihydrocaffeic acid-3′-*O*-sulphate	220 (107; 429)	465 (174; 772)	409 (318; 678)	6.0 (4.0; 6.0)	6.0 (6.0; 7.0)	6.0 (6.0; 6.0)	1787* (712; 3210)	3768† (2070; 5062)	3839† (3002; 7302)	2.7 (2.3; 3.9)	2.4 (2.0; 3.9)	2.1 (1.9; 2.2)
Dihydroferulic acid	375 (344; 587)	463 (330; 620)	518 (462; 771)	4.0 (4.0; 6.0)	5.0 (4.0, 7.5)	6.0 (6.0; 6.0)	2845* (2255; 3210)	3792*† (2497; 4797)	4549† (3688; 7834)	2.1 (1.9; 2.2)	2.1 (2.0; 2.2)	2.0 (1.8; 2.0)
Dihydroferulic acid-4′-*O*-sulphate	88 (44; 166)	98 (46; 172)	117 (80; 327)	6.0 (4.0; 6.0)	6.0 (5.0; 8.0)	6.0 (6.0; 6.0)	834 (264; 1132)	1189 (368; 1591)	1043 (780; 3574)	3.3 (2.5; 7.2)	3.5 (2.6; 5.3)	2.9 (2.6; 4.1)
Total (large intestine absorption)	**808** [c] **(470; 1119)**	**1242** [c] **(669; 1594)**	**1164** [c] **(926; 1900)**	-	-	-	-	-	-	-	-	-

[a] Data expressed as median and interquartiles (Q1; Q3) values (n = 11). C_{max}, maximum plasma concentration; T_{max}, time corresponding to C_{max}; *AUC*, area under the curve (0-24 h); $T_{1/2}$, elimination half-life

[b] Double values indicate pharmacokinetic parameters associated with first and second peak plasma concentrations (see Figure 1)

Different superscripts within rows indicate a statistical difference (Friedman's Analysis of Variance with pair-wise post-hoc comparison, $P < 0.05$)

[c] Statistically significant trend between the groups ($P < 0.05$) but no pair-wise significance

Table 4 Quantities of urinary chlorogenic acid derivatives and metabolites excreted by volunteers (0-24 h), following the ingestion of coffees containing 412, 635 and 795, µmol of chlorogenic acids [a]

Metabolites excreted	Dose ingested		
	412 µmol	635 µmol	795 µmol
Small intestine absorption			
Caffeic acid-*O*-sulphates (2)	6.2 (5.5; 7.6) *(1.5 %)* *	5.8 (5.1; 7.2) *(0.9 %)* *†	4.3 (3.3; 5.6) *(0.5 %)* †
Isoferulic acid-3′-*O*-sulphate	0.3 (0.2; 0.4) *(0.1 %)*	0.4 (0.3; 0.7) *(0.1 %)*	0.4 (0.3; 0.5) *(0.1 %)*
Isoferulic acid-3′-*O*-glucuronide	5.0 (3.7; 5.3) *(1.2 %)*	7.1 (5.4; 11.3) *(1.4 %)*	7.7 (5.6; 11.7) *(1.1 %)*
Ferulic acid-4'-*O*-sulphate	9.9 (7.3; 13.7) *(2.4 %)* [c]	14.4 (12.3; 26.7) *(2.3 %)* [c]	16.1 (12.3; 24.8) *(2.0 %)* [c]
3-Caffeoylquinic acid lactone-*O*-sulphate [b]	1.0 (0.9; 1.3) *(3.0 %)*	1.0 (1.0; 1.4) *(2.3 %)*	0.7 (0.6; 0.8) *(3.0 %)*
4-Caffeoylquinic acid lactone-*O*-sulphate [b]	0.8 (0.7; 1.1) *(3.6 %)* *	0.8 (0.7; 0.9) *(2.6 %)* †	0.5 (0.5; 0.6) *(3.3 %)* *†
3-Caffeoylquinic acid-*O*-sulphate [b]	0.0 (0.0; 0.1) *(< 0.1 %)*	0.1 (0.0; 0.1) *(< 0.1 %)*	0.0(0.0; 0.0) *(< 0.1%)*
4-Caffeoylquinic acid-*O*-sulphate [b]	0.1 (0.0; 0.1) *(< 0.1 %)*	0.0 (0.0; 0.1) *(< 0.1 %)*	0.0 (0.0; 0.0) *(< 0.1%)*
3-Feruloylquinic acid [b]	1.2 (0.8; 1.4) *(5.1 %)*	1.6 (1.3; 2.3) *(6.2 %)*	0.7 (0.6; 0.9) *(4.4 %)*
4-Feruloylquinic acid [b]	1.2 (0.9; 1.4) *(5.3 %)* *	1.1 (0.8; 1.7) *(2.7 %)* †	0.7 (0.4; 0.8) *(3.1 %)* †
5-Feruloylquinic acid [b]	0.8 (0.6; 1.0) *(3.3 %)* *	0.9 (0.7; 1.1) *(1.8 %)* †	1.4 (1.1; 1.6) *(2.2 %)* *†
Total excretion after small intestine absorption	**24.4 (23.7; 33.5) *(5.9 %)*** *	**34.4 (30.0; 54.9) *(5.4 %)*** *	**31.2 (26.0; 43.7) *(3.9 %)*** †
Large intestine absorption			
Dihydrocaffeic acid-3′-*O*-sulphate	35.7 (16.3; 43.0) *(8.7 %)*	47.4 (27.8; 71.2) *(7.5 %)*	47.9 (36.6; 89.9) *(6.0 %)*
Dihydroferulic acid	8.7 (4.1; 15.0) *(2.1 %)*	9.2 (7.0; 18.9) *(1.5 %)*	10.6 (6.8; 18.7) *(1.3 %)*
Dihydroferulic acid-4′-*O*-sulphate	11.4 (5.4; 13.9) *(2.8 %)*	12.4 (3.4; 22.5) *(2.0 %)*	17.5 (6.5; 25.8) *(2.2 %)*
Feruloylglycine	18.7 (10.9; 26.4) *(4.5 %)*	26.2 (17.5; 29.9) *(4.1 %)*	27.2 (24.6; 38.9) *(3.4 %)*
Total excretion after large intestine absorption	**76.3 (50.4; 95.5) *(18.5 %)***	**127.7 (60.2; 155.2) *(20.1%)***	**95.6 (83.6; 189.0) *(12.0%)***
Total excretion	**100.7 (82.2; 119.3) *(24.4 %)***	**160.0 (90.7; 211.1) *(25.2 %)***	**125.2 (109.3; 241.0) *(15.7 %)***

[a] Data expressed as median values and interquartiles (Q1; Q3) values (n = 11) in µmol. Italicised figures in brackets represent the amount excreted as a percentage of the dose ingested

[b] Expressed as a percentage of FQAs, CQALs and CQAs contained in coffee beverages

Different superscripts within rows indicate a statistical difference (Friedman's Analysis of Variance with pair-wise post-hoc comparison, $P < 0.05$)

[c] Statistically significant trend between the groups ($P < 0.05$) but no pair-wise significance

Table 5 Spearman's rank coefficient correlation between plasma and urinary metabolites and chlorogenic acid levels after increased doses ingested

Metabolites	Plasma [a]		Urine [b]	
	Rho	p-value	Rho	p-value
3-Caffeoylquinic acid lactone-*O*-sulphate [c]	0.596	0.000	0.643	0.000
4-Caffeoylquinic acid lactone-*O*-sulphate [c]	0.623	0.000	0.577	0.000
Caffeic acid-3′-*O*-sulphate [d]	-0.074	0.682	-0.382	0.028
Ferulic acid-4′-*O*-sulphate [d]	0.047	0.796	0.409	0.018
Isoferulic acid-3′-*O*-sulphate [d]	nd	nd	0.312	0.077
Isoferulic acid-3′-*O*-glucuronide [d]	nd	nd	0.507	0.003
3-Caffeoylquinic acid-*O*-sulphate [c]	nd	nd	0.115	0.525
4-Caffeoylquinic acid-*O*-sulphate [c]	nd	nd	-0.222	0.213
3-Feruloylquinic acid [c]	0.807	0.000	0.733	0.000
4-Feruloylquinic acid [c]	0.670	0.000	0.526	0.002
5-Feruloylquinic acid [c]	0.719	0.000	0.362	0.038
5-Caffeoylquinic acid [c]	0.334	0.150	nd	nd
Dihydrocaffeic acid [d]	0.337	0.069	nd	nd
Dihydrocaffeic acid-3′-*O*-sulphate [d]	0.307	0.087	0.355	0.043
Dihydroferulic acid [d]	0.227	0.227	0.214	0.231
Dihydroferulic acid-4′-*O*-sulphate [d]	0.178	0.331	0.355	0.043
Feruloylglycine [d]	nd	nd	0.335	0.057

[a] Based on C_{max} values in nM
[b] Based on 24-h urinary excretion in µmol
[c] Intake based on amount of individual chlorogenic acid ingested
[d] Intake based on total amount of chlorogenic acid ingested
nd; not detected

Table 6 Peak plasma concentration and urinary excretion of metabolites absorbed in the small intestine and the large intestine, expressed as median values and as percentages of total chlorogenic acid ingested

		Chlorogenic acid doses			p-values
		412 µmol	635 µmol	795 µmol	
Plasma C_{max} (nM)	Small intestine	261.4	510.1	243.7	0.086
	Large intestine	808.4[a]	1241.8[a]	1164.1[a]	0.035
	SI + LI	**1088**[*]	**1526**[†]	**1352**[*†]	**0.009**
Urine excretion (µmol)	Small intestine	24.4[*]	38.4[†]	31.2[*†]	0.012
	Large intestine	76.3	127.7	95.6	0.060
	SI + LI	**100.47**	**160.0**	**125.2**	**0.012**
Urine excretion (%)	Small intestine	5.9[*]	5.4[*]	3.9[†]	0.001
	Large intestine	18.5	20.1	12.0	0.307
	SI + LI	**24.4**	**25.2**	**15.7**	**0.178**

Different superscripts within rows indicate a statistical difference (Friedman's Analysis of Variance with pair-wise post-hoc comparison, $P < 0.05$)
[a] Statistically significant trend between the groups ($P < 0.05$) but no pair-wise significance

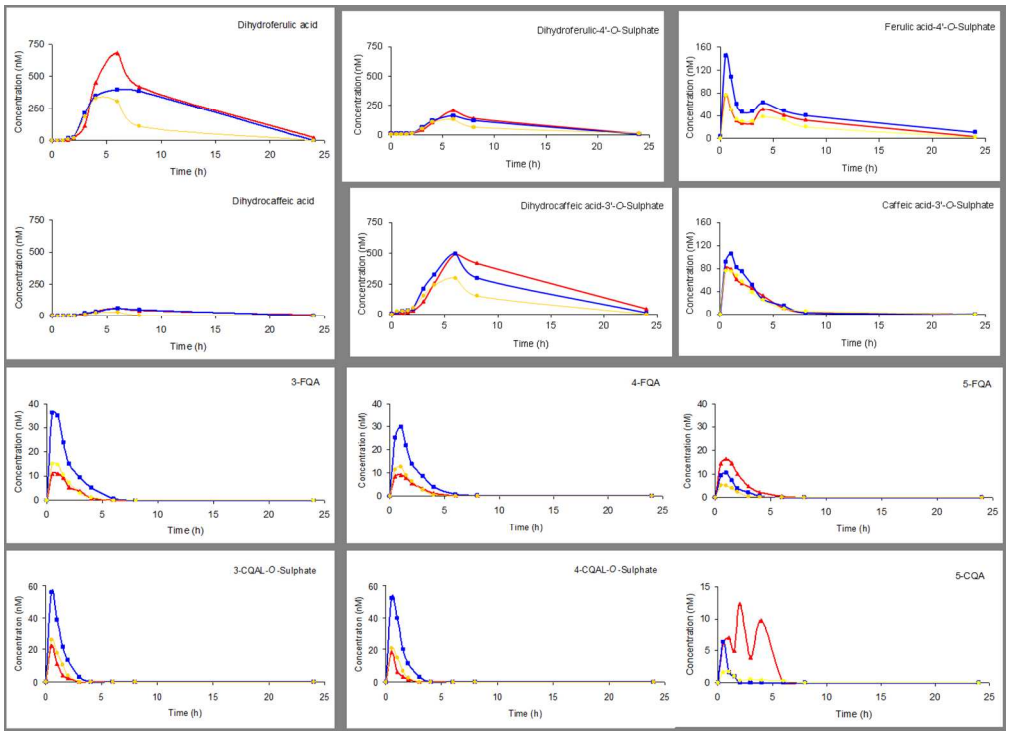

Fig. 1 Pharmacokinetic profiles of chlorogenic acid derivatives and metabolites in the plasma of human subjects 0-24 h after the ingestion of 200 mL of instant coffee containing 412 (yellow line), 635 (blue line) and 795 (red line) μmol of chlorogenic acids. Data expressed as mean values.

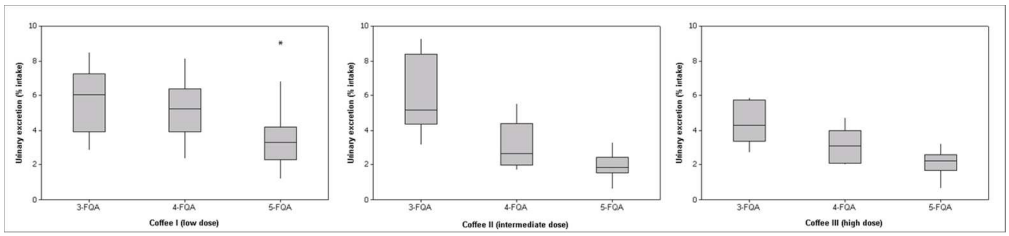

Fig. 2 Box-and-Whisker plots representing median, range and interquartile values of 24-h urinary excretion for 3-FQA, 4-FQA and 5-FQA after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).

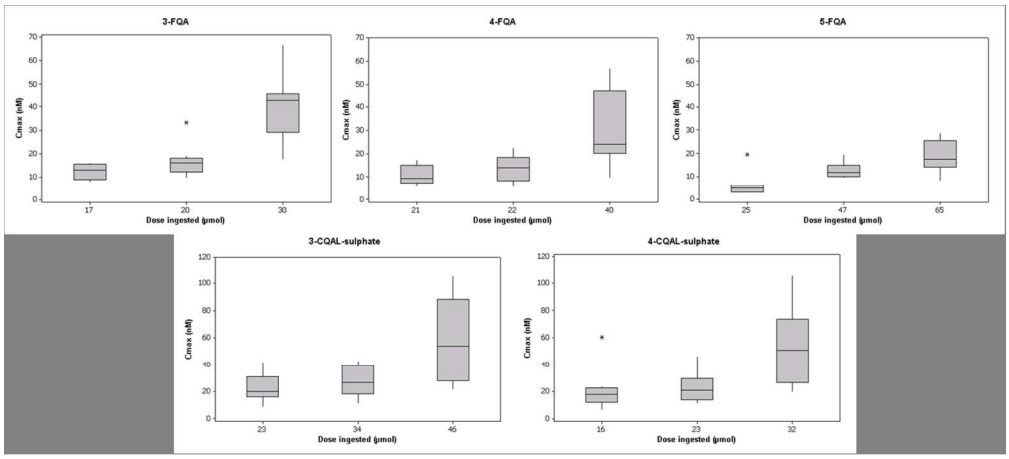

Fig. 3 Median and interquartile values of peak plasma concentrations for 3-FQA, 4-FQA, 5-FQA, 3-CQAL sulphate and 4-CQAL sulphate after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).

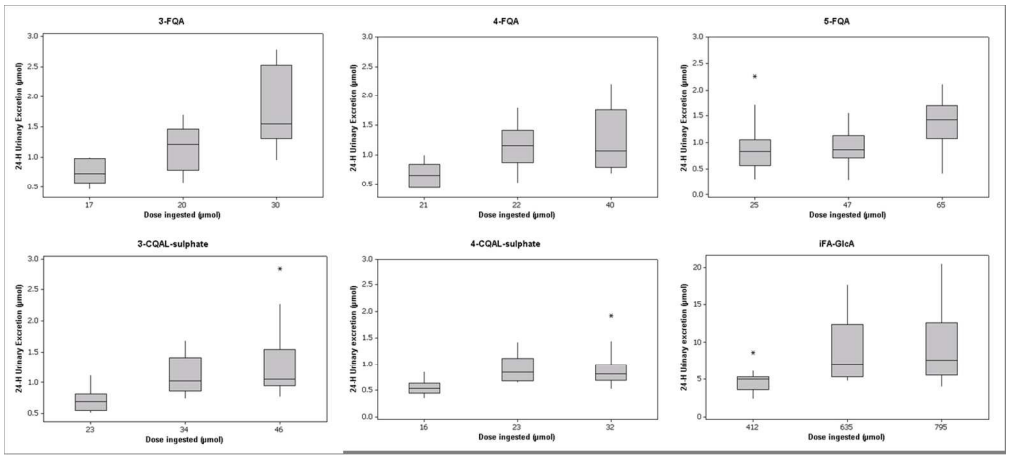

Fig. 4 Median and interquartile values of peak plasma concentrations for 3-FQA, 4-FQA, 5-FQA, 3-CQAL sulphate and 4-CQAL sulphate after intake of increasing doses of the parents compounds by healthy volunteers (n = 11).