RESEARCH ARTICLE

Measurement of caffeic and ferulic acid equivalents in plasma after coffee consumption: Small intestine and colon are key sites for coffee metabolism

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Previous studies on coffee examined absorption of phenolic acids (PA) in the small intestine, but not the contribution of the colon to absorption. Nine healthy volunteers ingested instant soluble coffee (~335 mg total chlorogenic acids (CGAs)) in water. Blood samples were taken over 12 h, and at 24 h to assess return to baseline. Many previous studies, which used glucuronidase and sulfatase, measured only PA and did not rigorously assess CGAs. To improve this, plasma samples were analyzed after full hydrolysis by chlorogenate esterase, glucuronidase and sulfatase to release aglycone equivalents of PA followed by liquid–liquid extraction and ESI-LC-ESI-MS/MS detection. Ferulic, caffeic and isoferulic acid equivalents appeared rapidly in plasma, peaking at 1–2 h. Dihydrocaffeic and dihydroferulic acids appeared in plasma 6–8 h after ingestion (T_{max} = 8–12 h). Substantial variability in maximum plasma concentration and T_{max} was also observed between individuals. This study confirms that the small intestine is a significant site for absorption of PA, but shows for the first time that the colon/microflora play the major role in absorption and metabolism of CGAs and PA from coffee.

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1 Introduction

Coffee contains high levels of phenolic antioxidants consisting principally of chlorogenic acids (CGAs). They are a family of esters formed between a *trans*-cinnamic acid, *e.g.* caffeic acid (CA) or ferulic acid (FA), and quinic acid, which has axial hydroxyls on carbon 1 and 3 and equatorial hydroxyls on carbon 4 and 5. The main CGA in coffee is 5-caffeoylquinic acid (5-CQA) [1], although other CQAs, feruloylquinic (FQAs) and di-di-CQAs acids were present in significant quantities. The total content of CGA in a "classic" cup of coffee (200 mL) varies between 70 and 350 mg [2]. Therefore, if coffee is consumed throughout the day, it may provide up to two-thirds of total daily dietary antioxidants [3]. While phenolic metabolites of CGA have been studied for potential bioefficacy, controversy remains as to how efficient those compounds actually are in reducing the risk of chronic disease [4, 5]. In addition, there is a lack of data on the metabolism and bioavailability of the parent compounds. Human plasma kinetics have been assessed only up to 8 h after coffee ingestion [6–8] and missed the potential role of the colon and microflora. Studies involving ileostomy patients showed that 33% of CGAs are absorbed, thus leaving a potential 66% to reach the colon for further metabolism [9].

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Abbreviations: CA, caffeic acid; **CGA,** chlorogenic acid; **CQA,** caffeoylquinic acid; C_{max}, maximum plasma concentration; **DHCA,** dihydrocaffeic acid; **DHFA,** dihydroferulic acid; **FA,** ferulic acid; **FQA,** feruloylquinic acid; **iFA,** isoferulic acid; **PA,** phenolic acids; **SRM,** selected reaction monitoring; T_{max}, time needed to reach maximum plasma concentration

Some metabolism studies were also reported using animal models. Using a gastric infusion model, Lafay *et al.* [10] showed that from a 35 μmol dose of 5-CQA, 16% were absorbed intact in the stomach. Analysis of plasma from the gastric vein and aorta of these rats revealed a concentration of 5-CQA of 3.3 and 1.6 μmol/L, respectively. In a further experiment intestinal absorption was assessed by small intestinal perfusions with 50 μmol 5-CQA [11]. A net transfer of 9.2% into the enterocytes was observed, of which 8% entered the circulation, whereas the remaining 1.2% was effluxed back into the lumen. Interestingly and as reported by others previously [12], the analysis of mesenteric plasma showed only phenolic acids (PA) (mainly FA and traces of CA) but no intact 5-CQA [11]. Results from these two animal models, as well as other animal [12, 13] and human studies [14] suggest that nutritional doses of intact 5-CQA are absorbed in the stomach, and compounds not absorbed in the stomach will pass to the small then large intestine where they are cleaved prior to absorption. Gonthier *et al.* [13] investigated the urinary excretion of metabolites after ingestion for 8 days of either 250 μmol/day 5-CQA or 250 μmol/day CA in rats. In the group fed 5-CQA, 0.86% of the ingested dose was recovered in the urine as intact 5-CQA. CA, FA and isoferulic acid (iFA) were also identified in urine, in even lower amounts (0.5%). By comparison, in the rats fed CA, about 13% of the ingested dose was recovered as CA, FA and iFA in the urine. Quantifiable amounts of FA and iFA in urine after feeding CA support the idea that CA can be methylated *in vivo* into FA or iFA. Hence, animal evidence suggest that CGA are potentially absorbed and metabolized into PA after coffee ingestion, but the human evidence of PA metabolism and pharmacokinetics are still limited.

This study describes a novel method involving a full enzymatic cleavage (glucuronidase, sulfatase and esterase) into constituent phenolic equivalents to measure accurately with all relevant corresponding standards the appearance of PA in plasma after consumption of CGA rich coffee. PA detected here are CA, FA, dihydroferulic acid (DHFA), dihydrocaffeic acid (DHCA), and iFA.

2 Materials and methods

2.1 Subjects

Nine healthy subjects (four males, five females) were recruited for this study. Subjects were 34 ± 7 years of age, weighted 70 ± 10 kg and measured 170 ± 8 cm. Volunteers were informed of all the details of the study before giving their informed consent. The study was approved by the ethical committee of clinical research of the University of Lausanne (Protocol reference 136/07). Inclusion criteria: 18–45 years, healthy as determined by the medical questionnaire, average coffee consumption of 1–5 cups *per* day, BMI 18–25, non-smoker and given informed consent. Exclusion criteria: intestinal or metabolic diseases/disorders such as diabetic, renal, hepatic, hypertension, pancreatic or ulcer, food allergy, major gastrointestinal surgery, difficulty to swallow, regular consumption of medication, high alcohol consumption (more than four drinks/day), have given blood within the last 3 wk or currently participating or having participated in another clinical trial during the last 3 wk prior to the beginning of this study.

2.2 Study design

One week prior to the first treatment, BMI was measured. Twenty four hours prior to receiving the treatment until the end of the present sampling period, the ingestion of coffee, tea, cola, alcohol, whole grain cereal (white bread allowed) or any medication was prohibited. Only water was allowed during the night and in the morning before the treatment. On the day of treatment, the subjects arrived fasting early in the morning at the metabolic unit with the filled pre-intervention diet recording sheets of the previous 2 day. They received a form to report any deviations from the study plan or non-beneficial effects of the treatment. Baseline blood was sampled, and then subjects received the treatment (4 g soluble instant coffee dissolved in 400 mL water). Blood was collected with EDTA 5 mL blood collection tubes at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 11 and 12 h after treatment. Polyphenol-free lunch and dinner were provided at the metabolic unit. The subjects were encouraged to drink as much as they wanted, and water was available *ad libitum*.

Twenty four hours after receiving the treatment, a final blood sample was taken.

CGA content was determined as a percentage dry basis *via* HPLC analysis (Table 1).

2.3 Plasma extraction

An aliquot of plasma (380 μL) was introduced into a 2 mL Eppendorf tube, in which 20 μL of 3-(4-hydroxyphenyl)-propionic acid (internal standard, 200 nM, from Fluka, Buchs, Switzerland) was previously added. Three volumes (1.2 mL) of ethanol was added, vortexed for 5 min at

Table 1. CGA content in dry soluble coffee

CGA	% Dry basis
3-CQA	1.02
4-CQA	1.38
5-CQA	4.00
4,5-di-CQA	0.54
3,4-di-CQA	0.50
4-FQA	0.18
5-FQA	0.75
Total	8.80

2700 rpm (Vortex-Genie®2, Scientific Industries, New York, USA) and centrifuged for 5 min at 17 500 × *g* at 4°C. The ethanol supernatant was poured into a clean 2 mL Eppendorf tube. The protein precipitation procedure was repeated twice by adding one volume (400 μL) of ethanol. The pooled ethanol phases were dried under nitrogen flow at room temperature for ~2 h. The dry residue was resuspended in 400 μL of 50 mM sodium phosphate buffer (pH 7.0) freshly prepared containing an enzymatic cocktail of 1000 units of β-glucuronidase (Sigma, Buchs, Switzerland), 60 units of sulfatase (Sigma) and 0.1 unit of chlorogenate esterase (Kikkoman, Japan). The sample was briefly vortexed (2700 rpm) and incubated for 60 min at 37°C in a thermomixer (300 rpm, Eppendorf Thermomixer Compact, Schönenbuchs, Switzerland). At the end of the incubation, 42 μL of 1 N HCl and 240 mg NaCl were added and briefly vortexed (2700 rpm). A liquid–liquid extraction was carried out four times by adding 800 μL of ethyl acetate, vortexing for 5 min at 1600 rpm, centrifuging at 3000 × *g* for 5 min and collecting the ethyl acetate upper phases. The pooled organic phases were dried under nitrogen at room temperature for ~30 min. The residue was then dissolved in 400 μL of methanol/water/ACN 20:76:4 v/v/v) containing 0.08% formic acid vortexed for 5 min (1600 rpm). After centrifugation at 17 500 × *g* for 5 min, the plasma extract was filtered on a 0.45 μm nylon membrane filter.

To properly quantify the compounds of interest, baseline plasma samples were used to create a standard curve (50–2400 nM) by spiking 20 μL of buffer solution containing known concentration of CA, FA DHCA (from Extrasynthese, Lyon, France), iFA, DHFA (synthesized in house) and internal standard. Samples were then extracted together with the unknown plasma samples collected throughout the 12 h kinetics.

2.4 LC-ESI-MS/MS analyses and quantification of coffee phenolics in plasma

Analysis of extracted plasma samples was performed on an Agilent 1100 quaternary pump LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 3200 Q TRAP mass spectrometer instrument equipped with a TurboIonSpray® ionisation source (Applied Biosystems, Foster City, CA, USA). HPLC analyses were run on a Zorbax SB-C18 RP column (2.1 × 50 mm, 1.8 μm) (Agilent Technologies, Basel, Switzerland). The mobile phases were constituted with solvent A: water containing 1% acetic acid, solvent B: methanol and solvent C: ACN. The gradient program was: 0 min 100% A, 0–10 min 60% A (30% B and 10% C), 10–11 min 10% A (60% B and 30% C), 11–13 min held at 10% A (60% B and 30% C), 13–14 min back to 100% A, 14–19 min re-equilibration at 100% A; running at a flow rate of 0.3 mL/min. The injection volume was 25 μL and the LC column was thermostated at 40°C. The LC flow was directed into the MS detector between 4 and 14 min using a VICI diverter (Valco Instrument, Houston, TX, USA). MS tuning was performed in negative ESI by infusing (Pump-11, Harvard Apparatus, Holliston, MA, USA) individual solutions of each analyte (5 μg/mL in methanol) mixed with a HPLC flow made of solvents A, B and C 50:40:10 v/v/v; 0.3 mL/min) using a Tee-connector. Nitrogen was used for the nebuliser (GS1 and GS2) and curtain (CUR) gases at pressures of 70, 20 and 10 psi, respectively. The interface heater was activated and the block source temperature was maintained at 600°C with a capillary voltage set at −4 kV. Nitrogen was also used as collision gas at a medium pressure selection. MS/MS detection was achieved using the selected reaction monitoring (SRM) acquisition mode. The two most intense fragment ions of each compound were selected using a constant dwell times of 50 ms, resulting in a total scan time of 0.7 s (including a 5 ms pause time between each SRM). Quantitative analysis was performed using the most intense SRM signal (SRM1) whereas the second SRM transition (SRM2) was used for analyte confirmation based on appropriate area ratio calculated from standard solutions. Data processing was performed using Analyst 1.4.2 software (Applied Biosystems MDS/SCIEX).

Recovery experiments for extraction efficiency of phenolic equivalents of CA and FA were conducted. After spiking at 50 nM, three replicates were reproduced three times by the same operator. Recovery is reported as a percentage of the concentration spiked into blank plasma samples, and for CA and FA was 106 ± 8% and 99 ± 14%, respectively.

2.5 Statistical analysis

All statistical analyses are done with SAS software (version 9.1). The rejection level in statistical tests is equal to 5%, and standard deviation is shown.

The AUC is the area under the available plasma curve of coffee PA against time after treatment (hours). It is calculated by the trapezoidal rule as follows:

$$\mathrm{AUC} = \frac{1}{2}\sum_{i=1}^{n-1}(t_{i+1} - t_i)(H_{i+1} + H_i)$$

where n is the number of blood samples, t_i is the time of the i-th blood sample (hours) and H_i is the i-th available coffee PA value. If H_i is inferior to the LOQ as determined by ESI-MS/MS (signal-to-noise ratio = 10), H_i is set to LOQ.

3 Results

Total CGA content in the instant coffee (4 g in 400 mL water) used for this study corresponded to 335 mg (900 μmol) of total CGA ingested as a single dose (Table 1). After consumption, the plasma concentration over time curves suggested two groups of metabolites obtained after

full enzymatic cleavage. Both groups exhibited biphasic behaviour with a first peak at ~1 h after ingestion and a second 8–12 h after ingestion. The first group (CA, FA and iFA equivalents) show a maximum plasma concentration (C_{max}) within the first hour after ingestion (Fig. 1). The second group (DHCA and DHFA) show the maximum concentration in plasma at 8–12 h after ingestion (Fig. 2). The biphasic phenomenon observed was the strongest for FA equivalents and much less pronounced for the other metabolites.

Calculation of AUC was done up to 12 h without further extrapolation (Fig. 2). The 24 h time point was used to assess return of plasma concentration back to baseline. For DHCA and DHFA, there was considerable inter-individual variation: Some subjects showed levels close to baseline by 12 h (Fig. 3A), whereas in some other subjects, time needed to reach maximum plasma concentration (T_{max}) was not even reached by 12 h (Fig. 3B). Therefore, mean C_{max} and T_{max} could not be properly calculated for those colonic metabolites.

Substantial interindividual variation was observed for C_{max} and T_{max} (Table 2) of the first group (''early appearing'') of metabolites. For CA, FA or iFA equivalents, we observed a C_{max} value between ~50 and ~150 nM with a T_{max} between 30 and 240 min after ingestion.

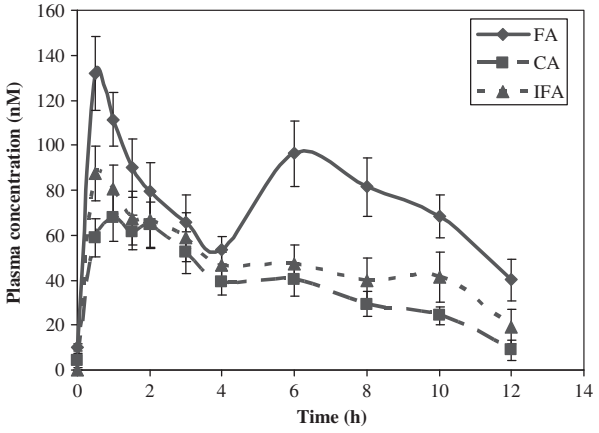

Figure 1. Mean plasma concentration of intestinal metabolites CA, FA and iFA equivalents in nine healthy volunteers after ingestion of instant coffee.

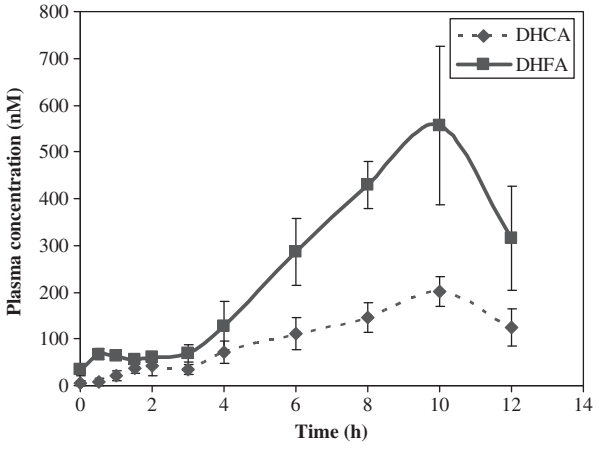

Figure 2. Mean plasma concentration of proposed colonic metabolites DHCA and DHFA in nine healthy volunteers after ingestion of instant coffee.

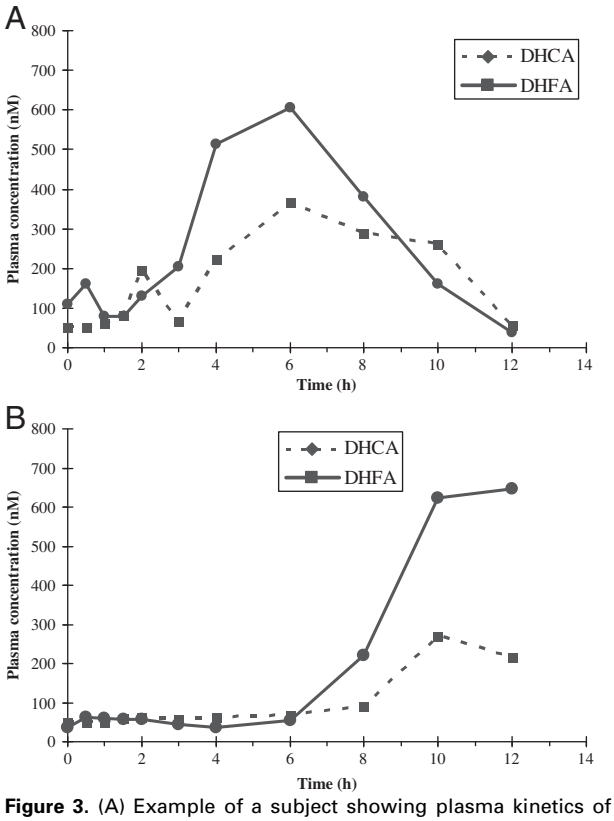

Figure 3. (A) Example of a subject showing plasma kinetics of colonic metabolites reaching levels close to baseline 12 h after ingesting coffee. (B) Example of a subject showing plasma kinetics of colonic metabolites not reaching C_{max} 12 h after ingesting coffee.

Table 2. C_{max} and T_{max} for ''early-appearing'' intestinal metabolites expressed as phenolic acid equivalents

	Mean	SD	Min	Max
C_{max} CA (nM)	81.1	29.7	38	129
C_{max} FA (nM)	139.4	43.8	91	205
C_{max} iFA (nM)	97.6	32.4	62	163
T_{max} CA (min)	100.0	100.6	30	360
T_{max} FA (min)	143.9	47.9	30	360
T_{max} iFA (min)	109.2	36.4	30	360

4 Discussion

Coffee contains a complex profile of different isomers of CQAs, FQAs and diCQAs which upon absorption and metabolism will increase in number, especially since early work indicated that cleavage of CGA into CA and quinic acids is an important metabolic pathway [15–17]. Our goal in this study was to include CGA in the total estimation of PA equivalents and not to estimate how much was in the intact CGA form. Currently, there are no proper standards of some CGAs and of any CGAs conjugate so that the data obtained would have been semi-quantitative only. Our strategy was to quantify CA and FA equivalents from all sources in plasma after coffee consumption. This provides us with general but accurate information on levels of CA and FA moieties appearing in plasma as we have all the standards of the relevant phenolics we wanted to measure to produce precise quantification. From the CQA composition in our coffee beverage (Table 1), this should, following absorption and metabolism, lead to only five major primary fragments, *i.e.* CA, FA, iFA, DHCA and DHFA.

Originally, we assessed three options as to which method could be used in this study (Fig. 4). Method 1, used here, is the simplest and most accurate to perform as all parents or metabolites appearing in plasma are cleaved to release CA, FA and iFA moieties. In addition, we could also accurately detect colonic metabolites such as DHFA or DHCA with their corresponding standards.

Method 2 does not involve the use of esterase, but the list of compounds to be identified in plasma goes from 5 (CA, FA, iFA, DHFA, and DHCA) to at least 14 (3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4 di-CQA, 4,5 di-CQA, 3,5 di-CQA, CA, FA, iFA, DHFA, and DHCA) (plus possibly other conjugates no one has yet identified), which is a method more difficult to put in place and to validate. In fact, some papers reported this method and ignored plasma appearance of CGA [6]. In addition, some standards are not commercially available (3-FQA for example) and must be quantified based on another isomer (5-FQA for example). Hence, this method is only semi-quantitative and the data produced should be interpreted with caution. However, we believe that using this simplified method, although we lose information on specific metabolites in the process, we will still end up producing much stronger and reliable data and represent a contribution from all metabolites as PA ''equivalents''.

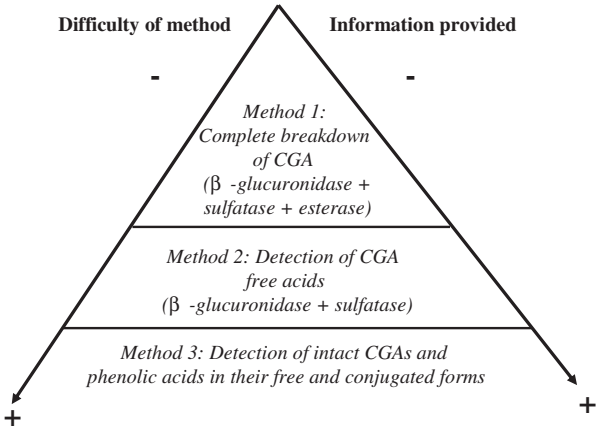

Figure 4. Possible analytical strategies to detect chlorogenic and PA in plasma.

Method 3 is the theoretically ideal detection and quantification method, but is not possible at the moment. Indeed, we are far from knowing which metabolites (aglycone, sulfate, glucuronides, methyl or even double conjugation) of parents or metabolites are present in plasma. We could easily reach more than 50 compounds to be detected with this method and there are no proper standards in any lab for most of them. We believe that with our current knowledge, this method would not produce reliable data and, to our knowledge, no one will be capable to measure all these compounds in the near future. The complexity of CGAs and PAs metabolism does not allow us at this stage to fully comprehend within the same study and by using one single validated method all potential metabolites appearing in plasma after coffee ingestion. In addition, by trying to identify so many compounds, it is likely that a significant number of metabolites will fall under their LOQ and will not be considered. By using a simpler method as presented here (method 1 with glucuronidase, sulfatase and esterase), we consider all metabolites as CA or FA equivalents and get a more precise idea of how much of those equivalents are present in plasma over time as all of the compounds considered are above our precisely defined LOQs. This method could also be used to assess in a simple, efficient and time-saving manner inter- or intra-individual differences in clinical studies.

Before this study, only three other reports have investigated absorption of CGAs after coffee ingestion in plasma of healthy volunteers. They reported data over 2 h [6], 4 h [7] and 8 h [8] after ingestion of coffee, which underlined the importance of the small intestine in the metabolism and absorption of CGA and PA in humans. However, detection of what appear to be major colonic metabolites (appearing 8–10 h after ingestion) has not been previously investigated for coffee, since none of these reports looked for the dihydro-forms of the PA nor at longer time points. Other published studies demonstrated a rapid absorption of intact CGA [7, 18] although some studies could not detect any CQAs in plasma even after deconjugation with glucuronidase and sulfatase enzymes [6, 19–21]. Monteiro *et al.* [7] showed the presence of parent compounds (CQA, FQA and di-CQAs isoforms) up to 4 h after ingestion at a significant concentration in plasma after consumption of coffee at a very high dose (3395 μmol *versus* 900 μmol total CGA in this study). In addition, Nardini *et al.* [6] measured the appearance of CA 1 and 2 h after ingestion. No other PA and CGA have been measured at time points later than 2 h. Recently, Farah *et al.* [8] have investigated and quantified CQAs,

di-CQAs, and phenolics in plasma for up to 8 h after ingestion of 451 μmol total CGA. However, there are several differences in study design, CGA delivery (extract or soluble coffee) and analytical tools between those human studies and the present one so comparison of bioavailability data should be done with caution.

With the chosen analytical strategy explained F in Figure 4, we readily detected CA, FA, iFA, DHCA and DHFA equivalents following ingestion of 350 mg of total CGAs (900 μmol) corresponding to two cups of coffee. Frequent blood sampling over 12 h also allowed us to detect what appeared to be major colonic metabolites (8–10 h after ingestion). Previous studies reported the presence of CA in plasma after coffee ingestion, and the experimental approach adopted in this study has allowed us to identify additionally the presence of FA and iFA moities. These follow similar kinetics to CA equivalents (maximum plasma appearance 1–2 h after ingestion) and so are more likely to be absorbed by the small intestine.

Another key discovery in this study was the detection of colonic metabolites, namely DHFA and DHCA, in significant concentrations ($C_{max} > 1\,\mu M$ in some subjects for DHFA). Presence in plasma of those colonic metabolites from coffee has not been previously reported in the literature. Surprisingly, we did not obtain return to baseline of those dihydro-metabolites for most healthy subjects even 12 h after ingestion, since T_{max} was still not reached for some subjects or was not back to baseline for others (Fig. 3). To our knowledge, only two other reports showed the presence of DHCA and DHFA in plasma after CGA consumption, but from artichoke leaf extract [18, 19]. T_{max} of those compounds was earlier, 6 h after ingestion. In this study, T_{max} for DHFA and DHCA was estimated to be ~10–12 h after ingestion, much later than reported by Wittemer *et al.* [19]. Due to the lack of complete DHCA and DHFA kinetics for most subjects that did not reach their T_{max} by 12 h, estimating a mean C_{max} and T_{max} was impossible for this study. Differences in CGAs profile between artichoke extract and coffee might also be the cause of the observed differences in plasma T_{max}. Although artichoke contains significant amounts of 5-CQA, it also contains 1-CQA, 1,5-di-CQA and 3,5-di-CQA that are not present in coffee [22]. The ingestion of CGA together with food (breakfast given with beverage in this study), or without [19], might also explain those differences. Thus, the matrix and form of delivery of the CGA modulates pharmacokinetics parameters (especially T_{max}). Colonic metabolites were also detected in urine after coffee consumption but were either not investigated or found in plasma [8].

This study on healthy volunteers showed that the small intestine is a significant site for absorption of phenolic or CGA as expressed by kinetics of corresponding PA equivalents. Importantly it also shows for the first time that the colon and the microflora play the major role in the absorption and metabolism of CGA from coffee, leading to relatively high levels of late-appearing (>8 h) dihydro-phenolic acids in plasma.

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The authors have declared no conflict of interest.

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