Bioavailability of hydroxycinnamates in an instant green/roasted coffee blend in humans. Identification of novel colonic metabolites.

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ABSTRACT

Roasting greatly reduces the phenolic content in green coffee beans. Considering the beneficial effects of coffee polyphenols, blends containing green coffee beans are being consumed as a healthier alternative to roasted coffee. This study was aimed at assessing the absorption and metabolism of hydroxycinnamates in an instant green/roasted (35/65) coffee blend in healthy humans. Twelve fasting men and women consumed a cup of coffee containing 269.5 mg (760.6 μmoles) of chlorogenic acids. Blood and urine samples were taken before and after coffee consumption at different times and analyzed by LC–MS-QToF. Up to 25 and 42 metabolites were identified in plasma and urine, respectively, mainly in the form of sulfate and methyl derivatives, and to a lower extent as glucuronides. Un-metabolized hydroxycinnamate esters (caffeoyl-, feruloyl-, and coumaroylquinic acids), hydroxycinnamic acids (caffeic, ferulic and coumaric acids) and their phase II metabolites, in addition to phase II derivatives of lactones, represented a minor group of metabolites (16.3% of the metabolites excreted in urine) with kinetics compatible with small intestine absorption. Dihydrohydroxycinnamic acids and their phase II derivatives, in addition to feruloylglycine, showed delayed kinetics due to their colonic origin and represented the most abundant group of metabolites (75.7% of total urinary metabolites). Dihydrohydroxycinnamate esters (dihydroferuloyl-, dihydrocaffeoyl- and dihydrocoumaroylquinic acids) have been identified for the first time in both plasma and urine, with microbial origin (excreted 8-12 h after coffee intake) amounting to 8% of total urinary metabolites. In conclusion, coffee polyphenols are partially bioavailable and extensively metabolized, mainly by the colonic microbiota.

KEYWORDS: hydroxycinnamate derivatives, instant green/roasted coffee blend, bioavailability, human, plasma pharmacokinetics, LC-MS-QToF, colonic bacteria.

1. Introduction

Coffee is the most widely consumed beverage with over 2 billion cups daily consumed in industrialized countries due to its unique aroma and stimulatory effects[1]. This makes coffee a key beverage at breakfast, mid morning or after-meals. Considering the high intake of this beverage, coffee may have a relevant impact on health of widespread populations.

Although coffee consumption is frequently restricted in patients suffering from hypertension, arrhythmia or other kind of cardiovascular condition, recent findings point to an inverse association between coffee consumption and certain chronic disorders such as cardiovascular diseases and related pathologies[2,3]. Recently, Bravo *et al*[4] showed that balancing the divergent results of observational and intervention studies, the detrimental effects of moderate coffee consumption on cardiovascular diseases risk are not supported. In fact, there is a protective effect associated to moderate consumption (around 3-4 cups per day) of filtered coffee, reducing the risk of coronary and cerebrovascular heart diseases and heart failure, with no negative impact on blood pressure.

A cup of coffee (ranging from 25 mL in expresso coffee to 180 mL in American type filter coffee, otherwise 100 mL is taken as a reference) is a wide and interesting source of bioactive compounds that, synergistically or not, produce health benefits after acute or regular consumption. Depending on the type of bean, the content in hydroxycinnamic acids varies between 15-325 mg[5], and methylxanthines from 60-85 mg, mainly caffeine and minor amounts of theobromine and theophylline[5-7]. Coffee is also an important source of the diterpenoids cafestol and kahweol (from 0.1 to 10 mg for filtered and unfiltered coffee, respectively[8]); trigonelline (40-110 mg[9]), soluble dietary fiber (0.14-0.75 g[10]), and melanoidins, which are formed during green coffee roasting (0.25-0.81 g/100 mL in brewed dark coffee[11]).

Polyphenols are considered the healthiest constituents of coffee, with estimated intakes up to 1-2 g/day following regular coffee intake[5]. Polyphenols contained in coffee are hydroxycinnamate esters, collectively known as hydroxycinnamic acids or more commonly chlorogenic acids, considering the abundance of the latter. Hydroxycinnamates derived from the binding of quinic acid with one or two hydroxycinnamic acid moieties (caffeic, ferulic and/or *p*-coumaric acid) lead to different isomers of caffeoylquinic, feruloylquinic and *p*-coumaroylquinic acids, respectively, along with dicaffeoylquinic and caffeoylferuloylquinic acids as the main phenolic compounds identified in green coffee beans. In addition, glycosides and amides derivatives of hydroxycinnamic acids complete the complex phenolic profile of green coffee beans[12,13]. However, part of the hydroxycinnamic acids are lost during roasting, with reductions of over 50% of the initial phenolic content[14,15]. Hydroxycinnamic acid

derivatives undergo isomerization and transformation into lactones during roasting, with different bioactivity potential than the precursor hydroxycinnamic acids in the green coffee bean[15]. Therefore, the benefits associated with the consumption of coffee depend on the type of coffee consumed, either green, roasted or green/roasted coffee blends.

The biological activity of phenolic compounds depends on their bioavailability and metabolic fate, as well as on their digestive accessibility, which is determined by the release from the food matrix and efficiency in trans-epithelial passage. Recently Del Rio et al[16] reviewed the rate and extent of absorption of coffee polyphenols in humans, as well as the metabolic pathways involved, showing that hydroxycinnamic acids are extensively metabolized and partially absorbed in healthy subjects. These polyphenols are partially absorbed in the upper gastrointestinal tract, being hydrolyzed by intestinal esterases into free caffeic, ferulic and coumaric acids and conjugated by phase II enzymes into methylated, sulfated and/or glucuronidated metabolites. Maximum plasma concentrations of these metabolites appear 1-3 h after coffee intake in nM concentrations[17,18]. Polyphenols not absorbed in the small intestine reach the colon where they are metabolized by the intestinal microbiota mainly to reduced forms of hydroxycinnamic acids, which are absorbed through the colonic epithelium and conjugated by phase II enzymes. The colonic metabolites appear in plasma 5-9 h after coffee consumption at higher concentrations in the μM range, although some in the nM range[17,18].

Since roasting greatly reduces the content of hydroxycinnamic acids in coffee beans, consuming a green/roasted coffee blend is a healthier option to roasted coffee. In fact, a soluble coffee product containing 35% of green coffee and 65% of roasted beans, which presented the distinct aroma and taste of roasted coffee, has shown interesting health benefits. In our research group, healthy and hypercholesterolemic subjects who regularly consumed 3 cups of this coffee blend (providing 496 mg/day of hydroxycinnamic derivatives) distributed along the day, showed positive effects on blood pressure, blood glucose and triglyceride levels, also improving the lipid profile in hypercolesterolemic subjects[19,20]. In order to further understand the results derived from the intervention studies, it is important to study the bioavailability and metabolism of polyphenols in coffee and this may also enable the identification of the biological mechanisms associated to the beneficial health effects. In addition, the results here presented could be extrapolated to the biotransformation of polyphenols contained in other green/roasted coffee blends, or green coffee consumed as an infusion or as a nutraceutical, which consumption is increasing. Up to date, most of the studies on the bioavailability of coffee polyphenol have been carried out with roasted coffee[17-18;21-24]. Therefore, the aim of the present work was to evaluate the bioavailability of hydroxycinnamates after consuming a realistic amount of a soluble green/roasted (35/65)

coffee blend in healthy humans. In addition, an important effort has been made to identify new metabolites derived from the microbiota, showing the importance of gut bacteria on polyphenol absorption and metabolism.

2. Materials and Methods

2.1. Chemical reagents and materials

A commercial brand of soluble green/roasted coffee blend (Nescafe Green Blend©) was purchased in a local supermarket in Madrid (Spain). All solvents and reagents were of analytical grade unless otherwise stated. Ascorbic acid, 5-caffeoylquinic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, 4-hydroxy-3-metoxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylbenzoic acid and 4-hydroxyphenylbenzoic acid were from Sigma-Aldrich (Madrid, Spain). 3,5-dicaffeoylquinic acid was purchased from PhytoLab (Vestenbergsgreuth, Germany). Methanol, formic acid, and acetonitrile (HPLC grade) were acquired from Panreac (Madrid, Spain).

2.2. Characterization and quantification of the phenolic content of the soluble green/roasted coffee blend by HPLC-MS and HPLC-DAD

Soluble coffee was prepared according to manufacturer's instructions. 3.5 g of green/roasted coffee blend was dissolved in 240 mL of boiling water. After shaking for 5 min, the sample was made up to 250 mL and filtered through filter paper. Then, an aliquot of the coffee beverage was filtered through a PVDF 0.45 μm filter prior to analysis. Phenolic compounds in green/roasted coffee blend infusion were analysed using an Agilent 1200 series liquid chromatographic system equipped with an autosampler, quaternary pump, diode array detector (DAD) and simple quadrupole (sQ) mass spectrometer (Agilent Technologies, Waldrom, Germany). Samples (20 μL) were injected into a Superspher RP18 column (4.6 mm × 250 mm i.d., 4 μm; Agilent Technologies) preceded by an ODS RP18 guard column kept in a thermostatic oven at 30°C. Elution was performed at a flow rate of 1 mL/min using a binary system consisting of 1% formic acid in deionized water (solvent A) and 1% formic acid in acetonitrile (solvent B). The solvent gradient changed from 10% to 20% solvent B over 5 min, 20% to 25% solvent B over 30 min, 25% to 35% solvent B over 10 min, then maintained isocratically for 5 min, and returning to the initial conditions over 10 min. Chromatograms were recorded at 320 nm. The mass spectrometer was fitted to an atmospheric pressure electrospray ionization (ESI)

source, which operated in negative ion mode. Capillary voltage was set to 3500 V, with nebulizing gas flow rate of 12 h/L, drying gas temperature of 350°C and nebulizer pressure of 45 psig. Mass spectrometry data were acquired in scan mode (mass range m/z 100–1000). Data acquisition and analysis were carried out in an Agilent ChemStation.

An Agilent 1200 series liquid chromatographic system (Agilent Technologies) equipped with a quaternary pump, column oven, autosampler and DAD was used to quantify the identified polyphenols in green/roasted coffee blend by high-performance mass spectrometry (HPLC–MS). The chromatographic conditions (column, guard column, binary gradient, injection volume, etc.) were as described above. For quantitative analysis the external standard method was used. Due to the lack of standards for certain phase II metabolites, they were tentatively quantified by using the calibration curves corresponding to their phenolic precursors. Thus, 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acids were used to calculate the mono- and di-acylcinnamate esters content, respectively. Samples were prepared and analyzed in triplicate and the results were expressed as the mean value.

2.3.Subjects and study design

The study protocol was conducted in accordance with the ethical recommendations of the Declaration of Helsinki and approved by the Ethics Committee of Hospital Universitario Puerta del Hierro in Majadahonda (Madrid, Spain). Volunteers recruitment was carried out through placing advertisements at the Institute of Food Science, Technology and Nutrition (ICTAN).

The study was carried out in twelve healthy subjects (7 men and 5 women); men's average age and body mass index were 27.86 ± 3.48 y and 23.42 ± 2.52 kg/m^2, respectively, and women's 28.88 ± 3.56 y and 22.43 ± 3.33 kg/m^2, respectively. They were non-smoker, non-vegetarian, non-pregnant women, who were not taking any medication or nutritional supplements, not suffering from any chronic pathology or gastrointestinal disorder. The sample size was estimated attending to similar previous bioavailability studies[17,21,25]. The volunteers gave their informed consent prior to participation.

The study was carried out at the Human Nutrition Unit of ICTAN. After an overnight fast, volunteers consumed 3.5 g of instant soluble green/roasted coffee blend in 250 mL of hot water. A polyphenol-free breakfast, lunch and afternoon snack were provided 2 h, 6 h and 10 h after consumption of coffee by the volunteers, who remained at the Human Nutrition Unit throughout the duration of the study. Water and isotonic beverages were available *ad libitum*. On the three days previous to the intervention, participants were also instructed not to consume coffee, others infusions (yerba mate, chamomile, etc.), tea, beer, red wine or their

199 derived products, whole grain cereals (white bread was allowed), as well as fruits, fruit juices
200 and vegetables, except banana, watermelon, cantaloupes and potatoes. In addition,
201 consumption of legumes, virgin olive oil, vinegar and dried fruits was also restricted.
202 Volunteers were asked to complete a 24 h food intake recall the day before each intervention
203 in order to control any possible food restriction incompliance.

204 Prior to coffee intake, a nurse inserted a cannula in the cubital vein of the non-prevailing
205 arm of the volunteers and blood samples were collected into EDTA-coated tubes at baseline
206 (t=0) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 9, 10, and 12 h after consuming a cup of coffee. Plasma was
207 separated by centrifugation (10 minutes, 3000 rpm, 4°C) and stored at -80°C until further
208 analysis. Urine samples were collected in 24 h urine collection containers, which contained 5
209 mg of ascorbic acid, at different time intervals (t=-2-0, 0-2, 2–5, 5–8, 8–12, 12–24h), and were
210 aliquoted and frozen at -20°C until analysis.

211

212 ***2.4. Extraction of phenolic metabolites from biological samples***

213 A liquid-liquid extraction and protein precipitation with acetonitrile and methanol was used
214 to isolate metabolites from plasma following the procedure described by Day et al[26] with some
215 modifications. A 400 μL defrosted plasma sample was acidified with 12 μL of 50% (v/v)
216 aqueous formic acid. After vortexing the aqueous mixture, it was added drop wise to 1000 μL
217 of cold acetonitrile, containing 50 μL of 10% (m/v) ascorbic acid, and vortexed three times for
218 20 s before centrifuging at 10000 rpm for 10 min at 4°C. The supernatant was separated, and
219 the pellet was re-extracted with 1000 μL of methanol. After centrifugation (10000 rpm, 10
220 min), the two supernatants were combined and reduced to dryness under a stream of nitrogen
221 at 30°C. The dried samples were resuspended in 100 μL of aqueous formic acid (0.1%)
222 containing 10% acetonitrile acidified with 0.1% formic acid and centrifuged at 4°C for 20 min at
223 14000 rpm. The final supernatant was collected, filtered (0.45 μm pore-size, cellulose-acetate
224 membrane filters, Albet) and 20 μL were analyzed by LC-MS-QToF. Recoveries of the standards
225 chlorogenic, caffeic and ferulic acids ranged from 95 to 99%.

226 Urine samples were diluted with an equivalent volume of Milli-Q water (50%) and
227 centrifuged at 14000 rpm (10 min, 4°C). Supernatants were filtered (0.45 μm pore-size
228 cellulose-acetate membrane filters) and a 5 μL aliquot was directly injected into the LC-MS-
229 QToF equipment.

230

231 ***2.5.Metabolite identification by HPLC-ESI-QToF analysis***

Analysis were performed on an Agilent 1200 series LC system coupled to an Agilent 6530A Accurate-Mass Quadrupole Time-Of-Flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). Compounds were separated on a reverse-phase Ascentis Express C18 (15 cm x 3 mm, 2.7 μm) column (Sigma-Aldrich Química, Madrid) preceded by a Supelco 55215-U guard column at 30°C. The test samples, either 30 μL of plasma or 5 μL of urine, were injected and separated using a mobile phase consisting of Milli-Q water (phase A) and acetonitrile (phase B), both containing 0.1% formic acid, at a flow rate of 0.3 mL/min. The mobile phase was initially programmed with 90% of solvent A and 10% of B. The elution program increased to 30% of solvent B in 10 min, 40% solvent B in 5 min and 50% solvent B in 5 min. Then, the initial conditions (10% solvent B) were recovered in 2 min and maintained for 8 min. The Q-ToF acquisition conditions were as follows: drying gas flow (nitrogen, purity > 99.9%) and temperature were 10 L/min and 325°C, respectively; sheath gas flow and temperature were 6 L/min and 250°C, respectively; nebulizer pressure was 25 psi; cap voltage was 3500 V and nozzle voltage was 500 V. Mass range selected was from 100 up to 970 *m/z* in negative mode and fragmentor voltage of 150 V. Data were processed in a Mass Hunter Workstation Software.

Due to the lack of standards for some phase II metabolites, they were tentatively quantified using the calibration curves of their corresponding phenolic precursors. Thus, 5-caffeoylquinic acid was used to quantify monohydroxycinnamoyl derivatives; caffeic, ferulic, dihydrocaffeic, dihydroferulic and dihydrocoumaric acids were used to quantify their respective free hydroxycinnamic acids and phase II metabolites. The rest of microbial metabolites identified, derivatives of hydroxyphenylacetic and hydroxyphenylbenzoic acids, were quantified using their respective commercially available standards. Urine concentration of excreted metabolites was normalized by the volume excreted in each studied interval. A linear response was obtained for all the standard curves (from 1 to 1000 nM), as checked by linear regression analysis. Calibration curves were freshly prepared in a pool of both plasma and urine due to matrix effects. Limits of detection and quantification in plasma ranged from 1 to 3 nM and from 2 to 9 nM, respectively, while limits of detection and quantification in urine ranged from 3 to 20 nM and from 30 to 80 nM, respectively. The inter- and intra-day precision of the assay (as the coefficient of variation, ranging from 2.1 to 10%) were considered acceptable and allowed the quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecules). The recovery ranged between 95% and 105% in plasma and between 93% and 98% in urine samples.

2.5. Nutrikinetic analysis

Statistical analyses were carried out using the program SPSS (version 19.0, SPSS, Inc., IBM Company). To determine the absorption and elimination of hydroxycinnamic metabolites after consumption of the soluble green/roasted coffee blend, metabolie nutrikinetics were studied using the pharmacokinetic functions of Microsoft Excel. Maximum concentration (C_{max}), area under curve (AUC) and time to reach maximum concentration (T_{max}) were calculated. Data are given as mean ± standard deviation.

3. Results

3.1. Phenolic content of instant green/roasted coffee blend

The phenolic constituents present in the instant green/roasted coffee blend were analyzed by diode-array detection. A representative HPLC-DAD chromatogram of the phenolic fraction of soluble green/roasted coffee blend at 320 nm has been included in the supplementing information (Supplementary Table 1). The 250 mL serving of coffee was prepared from 3.5 g of the green/roasted coffee blend providing 760.6 μmoles (269.5 mg) of hydroxycinnamic acids (Table 1). The most abundant phenolic compounds in this coffee were monoacylquinic derivatives (83.5% of the total polyphenols in coffee), constituted mainly by caffeoylquinic acids (66.8%), feruloylquinic acids (13.5%), coumaroylquinic acids (2.2%), and dimethoxycinnamoylquinic acids (1.0%). The next most abundant group was diacylquinic acids (12.3%), headed by dicaffeoylquinic acids (10.9%), and followed by minor amounts of caffeoylquinic lactones (2.2% of total polyphenols), caffeoyl-*N*-tryptophan (1.7% of total polyphenols) and caffeoylshikimic acids (0.3% of total polyphenols).

3.2. LC-QToF identification of hydroxycinnamates and metabolites in plasma and urine

Table 2 shows the retention time (RT), molecular formula, accurate mass of the quasimolecular ion $[M-H]^-$ after negative ionization, MS^2 fragments and location (U: urine or P: plasma) of the main compounds identified in plasma and urine samples by LC-QToF. The characterization of the identified compounds was supported by commercial standards and/or previously published results.

Un-metabolized compounds originally present in the coffee beverage were detected in both plasma and urine samples. 3-, 4- and 5-caffeoylquinic acids were identified in urine and only 5-caffeoylquinic acid in plasma. Likewise, 3-, 4- and 5-feruloylquinic acids were detected in urine and 4- and 5-feruloylquinic acids were also found in plasma. In addition, coumaroylquinic acid was detected in urine (Table 2).

Caffeic and dimethoxycinnamic acids in urine and *trans*- and *iso*ferulic acids in plasma were characterized, which were formed from their respective monohydroxycinnamoylquinic acid. Phase II derivatives of these free hydroxycinnamic acids (caffeic acid and iso- and *trans*ferulic acids) were also detected in biological fluids. In particular, caffeic acid 3-sulfate, ferulic acid 3-glucuronide and ferulic acid 3-sulfate were detected in urine while ferulic acid 4-glucuronide and ferulic acid 4-sulfate were observed in both plasma and urine samples. No free coumaric acid was detected in contrast to their phase II derivatives. Specifically, a sulfated derivative of coumaric acid was detected in plasma and two isomers of coumaric acid glucuronide were detected in different samples; the isomer with shorter retention time appeared in both plasma and urine while that with longer retention time only in urine.

After green/roasted coffee intake, an important group of metabolites formed were reduced hydroxycinnamate esters, which were extensively transformed into phase II derivatives. Dihydrocaffeic, dihydro*trans*ferulic and dihydrodimethoxycinnamic acids were identified in both plasma and urine, whereas dihydrocoumaric acid was only found in urine and dihydro*iso*ferulic acid was only observed in plasma. In addition, phase II derivatives of these reduced forms were also detected. Dihydrocaffeic acid 3-glucuronide, dihydrocaffeic acid 4-sulfate and dihydrocaffeic acid 3-sulfate were detected in urine, and dihydrocaffeic acid 4-sulfate in both urine and plasma. Additionally, two isomers of each glucuronidated and sulfated derivatives of dihydroferulic acid were detected in plasma and urine samples; in contrast, an isomer of glucuronidated and sulfated dihydrocoumaric acid were detected only in urine.

A new group of reduced forms resulting from the microbial metabolism of hydroxycinnamates, namely dihydrohydroxycinnamoylquinic acids, has been identified for the first time. Thus, 3-, 4- and 5-dihydrocaffeoylquinic acids were identified in urine with quasimolecular ion at *m/z* 355.1035 and fragment ion at *m/z* 181 corresponding to dihydrocaffeic acid. Likewise, 3-, 4- and 5-dihydroferuloylquinic acids were identified based on their $[M-H]^-$ at *m/z* 369.1191 and fragment ion at *m/z* 195 corresponding to dihydroferulic acid. Three isomers of dihydroferuloylquinic acid were observed in urine whereas the 3- and 5-isomers were detected in plasma. Lastly, two chromatographic peaks at 14.8 and 15.3 min showed a quasimolecular ion at *m/z* 339.1085 and fragment ion at *m/z* 165, which were compatible with dihydrocoumaroylquinic acid, detected in both plasma and urine samples.

Feruloylglycine has been previously described after coffee intake[17]. However, in the present study two chromatographic peaks at 8.8 and 9.5 min showed a chemical structure compatible with this compound ($[M-H]^-$ at *m/z* 250.0721 and fragment ions at *m/z* 191 and 134), being tentatively assigned as feruloylglycine and *iso*feruloylglycine, respectively, according to the

order of elution of their precursors (ferulic and *iso*ferulic acids). While feruloylglycine was detected in both plasma and urine, *iso*feruloylglycine was only detected in urine.

Phase II derivatives of lactones were also characterized. Although hydroxycinnamoyl lactones were detected in biological samples, up to three sulfated derivatives of caffeoylquinic lactone and one glucuronidated derivative of feruloylquinic lactone were observed in urine and plasma, respectively, in the present study.

Lastly, derivatives of hydroxyphenylpropionic, hydroxyphenylacetic, hydroxybenzoic and hydroxyhippuric acids were detected in plasma and urine samples.

3.3. Quantification of plasma metabolites and nutrikinetic parameters

Out of the 48 metabolites identified after coffee consumption, 25 were detected in plasma although only 20 showed levels above limit of quantification. The kinetics of plasma appearance and clearance of these metabolites up to 12 h post-intake are represented in Figure 1. Nutrikinetic parameters are summarized in Table 3.

Un-metabolized hydroxycinnamate esters such as caffeoyl- and feruloylquinic acids were detected in plasma. While 5-caffeoylquinic acid was at trace levels (Table 3), the concentrations of 4- and 5-feruloylquinic acids showed a rapid increase between 1 and 2 h after coffee consumption and complete clearance at 5 h (Figure 1A). Hydroxycinnamic acids such as *iso*ferulic and dimethoxycinnamic acids (Figure 1B) along with trace levels of ferulic acid were detected in plasma, together with phase II derivatives of hydroxycinnamic acids such as ferulic acid 4-glucuronide, ferulic acid 4-sulfate, coumaric acid glucuronide (Figure 1C), and coumaric acid sulfate at trace levels (Table 3). Hydroxycinnamic acids and their phase II derivatives showed similar kinetic profiles with a rapid increase in concentration between 1 and 2 h after coffee consumption and slow clearance, maintaining or even showing a second maxima between 4 and 5 h in the particular cases of dimethoxycinnamic acid (Figure 1B) and ferulic acid 4-glucuronide, or even at 6-7 h in the case of ferulic acid 4-sulfate (Figure 1C), with subsequent clearance at 10-12 h post-intake. Additionally, trace amounts of feruloylquinic lactone glucuronide was detected in plasma. All these compounds were present in low concentrations in plasma, showing C_{max} ranging from traces to 24 nM (Table 3). The time to reach maximum concentration (T_{max}) ranged between 0.5 and 1.2 h, which pointed to absorption taking place in the small intestine.

Reduced forms of hydroxycinnamic acids, dihydrocaffeic, dihydroferulic, dihydro*iso*ferulic acid (Figure 1D) and dihydrodimethoxycinnamic acid, along with their phase II derivatives, dihydrocaffeic acid 4-sulfate, dihydroferulic acid 4-glucuronide, dihydro*iso*ferulic acid 3-glucuronide, dihydroferulic acid 4-sulfate and dihydro*iso*ferulic acid 3-sulfate (Figure 1E),

formed the main group of metabolites detected in plasma (Table 3). The plasmatic profile of these metabolites showed maxima concentrations between 5.4 and 7.0 h (T_{max}) post-intake and minor second maxima between 8 and 9 h in the particular case of dihydro*iso*ferulic acid, dihydrocaffeic acid 4-sulfate, dihydroferulic acid 4-sulfate and dihydro*iso*ferulic 3-sulfate, compatible with a biphasic kinetic. Dihydroferulic acid and dihydrocaffeic acid 4-sulfate were the predominant metabolites, showing C_{max} values of 300 and 260 nM, respectively, followed by sulfated and glucuronidated derivatives of ferulic acid and dihydro*iso*ferulic acid with C_{max} ranging from 90 to 50 nM. Dihydrocaffeic acid, phase II derivatives of *iso*ferulic acid (sulfated and glucuronidated derivatives) and dihydrodimethoxycinnamic acids were less abundant, with C_{max} values ranging from 30 nM to trace levels.

Feruloylglycine, as a ferulic acid derivative, was detected with a plasmatic profile similar to that of the reduced forms of hydroxycinnamic acids (Figure 1D), as its concentration rapidly increased with T_{max} at 7 h decreasing afterwards, although not recovering baseline levels 12 h after coffee intake (Figure 1D). Their presence in plasma was relatively low (C_{max} 20 nM, Table 3).

Furthermore, for the first time reduced forms of hydroxycinnamate esters (3- and 5-dihydroferuloylquinic acids and two isomers of dihydrocoumaroylquinic acid) have been identified in plasma. These compounds showed the most delayed kinetic of all the mentioned metabolites, with maxima concentration about 9 h (T_{max} 8.4-9.4 h) and partial clearance 12 h post-intake (Figure 1F). Among these microbial metabolites, 5-dihydroferuloylquinic acid was the most abundant (C_{max} 40 nM), followed by 3-dihydroferuloylquinic acid and the two isomers of dihydrocoumaroylquinic acids with C_{max} values ranging from 9 to 11 nM (Table 3).

Lastly, metabolites related with hydroxyphenylpropionic, hydroxyphenylacetic and hydroxybenzoic acids were also detected in plasma, although no differences were observed in their concentrations along the 12 h blood sampling period (data not shown).

3.4. Quantification of urinary metabolites

Table 4 shows the metabolites quantified in 24 h urine samples, where 42 metabolites of the 48 metabolites identified after coffee consumption were identified. Additionally, eleven microbial metabolites derivatives of hydroxyphenylpropionic, hydroxyphenylacetic, and hydrophenylbenzoic acids along with hydroxylhippuric acid and phloroglucinol were also detected in urine.

Monoacylquinic acids (3-, 4- and 5-caffeoylquinic acids, 3-, 4- and 5-feruloylquinic acids, and coumaroylquinic acid) were excreted unmetabolized in small amounts in the urine, accounting for 3.3% of the total metabolites quantified. Among these acids, feruloylquinic acids were the

406 most abundant compounds, accounting for 84.9% of the total un-metabolized compounds,
407 followed by caffeoylquinic acids (8.8%) and coumaroylquinic acid (6.3%).
408 Phase II derivatives of free hydroxycinnamic acids (caffeic, *trans*ferulic, *iso*ferulic and
409 coumaric acids) along with caffeic acid accounted for 12.0% of the total metabolites excreted
410 in urine. Ferulic acid 4-sulfate was the most abundant hydroxycinnamic acid (8 μmoles/24h),
411 followed by caffeic acid 3-sulfate, ferulic acid 4-glucuronide and *iso*ferulic acid 3-sulfate with
412 urinary excretions as high as 2.6, 2.3 and 1.7 μmoles, respectively (Table 4). The concentration
413 of *iso*ferulic acid 3-glucuronide and two isomers of coumaric acid glucuronide ranged from
414 0.11 to 0.24 μmoles in 24h. Additionally, three isomers of caffeoylquinic lactone sulfate were
415 excreted in urine, recovering up to 1290 nmoles in 24 h urine, equivalent to 1% of the
416 metabolites excreted (Table 4).
417 All the aforementioned metabolites, hydroxycinnamates, hydroxycinnamic acids and their
418 phase II derivatives along with sulfated lactone derivatives, were preferentially excreted
419 between 2 and 5 h after coffee ingestion and amounted to 16.3% of the total urinary
420 metabolites excreted in 24 h. It is worth noting that glucuronidated and sulfated ferulic acids
421 were also largely excreted between 8 and 12h, in agreement with the biphasic profile
422 observed in plasma (Figure 1C).
423 However, reduced forms of hydroxycinnamic acids and their phase II derivatives were the
424 most important group of metabolites quantified in urine after coffee ingestion, which were
425 largely excreted between 5 and 12 h post-intake, representing 64.4% of the total urinary
426 metabolites. Excretion of dihydrocaffeic acid and its phase II derivatives added up to 35
427 μmoles in 24h, being dihydrocaffeic acid 3-sulfate the most abundant urinary metabolite (31
428 μmoles). Similarly, excretion of dihydroferulic acid and its phase II derivatives amounted up to
429 37 μmoles in 24 h, in line with the higher excretion of unmetabolized feruloylquinic acids
430 compared to caffeoylquinic acids previously described (Table 4). Among dihydroferulic acid
431 metabolites, the 4-sulfate and 4-glucuronide derivatives were the main urinary metabolites (13
432 and 8 μmoles in 24h, respectively). In accordance with the lower presence of coumaroylquinic
433 acids in the coffee beverage, excretion of dihydrocoumaric acid and its sulfated and
434 glucuronidated metabolites amounted to 9 μmoles in 24 h, with an important concentration of
435 dihydrocoumaric acid sulfate (8 μmoles in 24 h).
436 Interestingly, reduced forms of hydroxycinnamates were also characterized in urine for the
437 first time; 3-, 4- and 5-dihydrocaffeoylquinic acids, 3-, 4- and 5-dihydroferoylquinic acids, and
438 two isomers of dihydrocoumaroylquinic acid. These novel metabolites were quantified in
439 noticeabe amounts, accounting for 8% of the total urinary metabolites, and were specially

excreted between 8 and 12 h after coffee intake, in agreement with their delayed appearance in plasma (Figure 1F).

Feruloylglycine and *iso*feruloylglycine were also extensively excreted in the 5-8 h period. Their contribution to the total amount of identified metabolites was relevant (11.2%), mainly constituted by feruloylglycine (14 μmoles in 24h), thus actually being the second most abundant urinary metabolite after dihydrocaffeic acid-3 sulfate (Table 4).

Finally, the majority of microbial metabolites such as derivatives of hydroxyphenylpropionic, hydroxyphenylacetic and hydrophenylbenzoic acids, along with hydroxyhippuric acid and phloroglucinol, were present in basal urine before coffee intake and their levels increased minimally after coffee consumption (0-24 h), peaking between 8 and 12 h compared to baseline values (Table 4). These compounds were not included in the calculation of the recovery of polyphenols after coffee consumption since they are not exclusively derived from the biotransformation of hydroxycinnamic acids.

Taken together, the total amount of hydroxycinnamate metabolites excreted in urine 24 h after the intake of a single serving of the green/roasted coffee blend added to 127 μmoles, which represents only 16.7% of the 760.6 μmoles of polyphenols consumed.

3. Discussion

Although absorption and metabolism of hydroxycinammate esters in coffee have been previously reported[17-18;21-24], the novelty of the present work consisted in evaluating the bioavailability of the phenolics in a green/roasted coffee blend (35:65, v/v) using a realistic dose of coffee, namely 3.5 g of the instant soluble green/roasted coffee blend added to 250 mL of boiling water, following manufacturer's instructions. This yielded a beverage containing 269.5 mg (760.6 μmoles) of hydroxycinnamic acids. A higher dose of coffee would have altered the kinetics of appearance, clearance and biotransformation pathways, not reflecting the response to nutritional doses. Furthermore, special attention was given to searching for novel microbial metabolites, considering that the identification of these metabolites could contribute to shed light on the biotransformation pathway of hydroxycinnamate esters in coffee and, therefore, to understand the bioactivity associated to coffee consumption.

Results showed that hydroxycinnamate esters present in the green/roasted blend were partially absorbed and extensively metabolized, so that most of the metabolites were produced by the gut microbiota. Reduced forms of hydroxycinnamic acids (dihydrohydroxycinnamic acids), mainly as phase II conjugated metabolites formed after

absorption in the colon, were the predominant metabolites in plasma and urine, underlying the importance of the microbiota in the metabolism of hydroxycinnamic compounds.

Whereas no free dicaffeoylquinic and caffeoylferuloylquinic acids were detected in the collected biological samples, small amounts of monoacylquinic acids (caffeoyl-, feruloyl- and *p*-coumaroylquinic acids) were quantified, reaching 3.3% of the metabolites excreted in urine. The characterized caffeoyl- and feruloylquinic acids in both plasma and urine, and *p*-coumaroylquinic acids in urine would derive from the green/roasted coffee blend after their direct absorption without being metabolized in the small intestine, although part of the excreted monohydroxycinnamoylquinic acids may also result from hydrolysis by intestinal esterases of diacyl derivatives of hydroxycinnamic acids (dicaffeoylquinic and caffeoylferuloylquinic acids)[27] (Figure 2). These results are in agreement with other recent studies in humans, that failed to observe diacylquinic derivatives and reported low amounts of monoacylquinic acids (C_{max} < 0.1 μM at T_{max} 0.5-2.1) after the consumption of 300 mg of hydroxycinnamates in a green coffee extract[28], 146 mg of hydroxycinnamates in roasted coffee[17], 400 mL (the amount of hydroxycinnamates was not specified) of instant roasted coffee[21], and three doses of roasted coffee providing 176, 352 and 704 mg of hydroxycinnamates[18]. These results contrast with earlier bioavailability studies in roasted coffee where high plasmatic levels of both monoaylquinic acids (3-, 4- and 5-caffeoylquinic acids) and diacylquinic derivatives (3,4-, 3,5- and 4,5-dicaffeoylquinic acids) with C_{max} of 1.0 – 3.14 μM and 0.9 – 1.11 μM, respectively, were reported after the ingestion of 1200 mg of hydroxycinnamates[29], and C_{max} of 0.9 – 5.9 μM and 1.5 – 2.5 μM, respectively, after the ingestion of 170 mg of hydroxycinnamates[30]. However, these authors did not detect diacyl derivatives of hydroxycinnamic acids in urine. Matsui et al.[28] had already observed a dicaffeoylquinic acid isomer in plasma after the ingestion of 300 mg of hydroxycinnamates from a green coffee extract although in trace amount. Additionally, to our knowledge, this is the first study reporting the presence of coumaroylquinic acid in urine.

Most of the compounds identified in plasma and urine were metabolites derived from the hydrolysis of mono- and diacyl derivatives of hydroxycinnamic acids by esterases present in the small intestinal wall[27], yielding free hydroxycinnamic acids (caffeic, ferulic and p-coumaric acids). The hydroxycinnamic acids followed two different pathways; a minor ratio of metabolites was subsequently metabolized by phase II enzymes into sulfated, glucuronidated and methylated derivatives in the intestinal epithelium afterwards entering the bloodstream, whereas most hydroxycinnamic derivatives reached the colon and were substrates for microbial reductases prior to absorption and conjugation into phase II metabolites.

Free hydroxycinnamic acids and their phase II derivatives accounted for 12.0% of total urinary metabolites. It is important to note the biphasic kinetics observed for some phase II derivatives of hydroxycinnamic acids, namely sulfated and glucuronidated derivatives of ferulic acid, showing a second although less intense maximum in plasma and higher urinary excretion between 5 and 12 h. This outcome may extend the *in vivo* bioactivity of polyphenols and may be due to either enterohepatic recirculation[31] or colonic absorption, both pathways would explain the longer permanence of polyphenols in the body, and therefore extended bioactivity. Among this type of metabolites, ferulic acid 4-sulfate and ferulic acid 4-glucuronide were the most abundant. The aforementioned results on chemical nature, kinetics and relative abundance of the detailed metabolites are in line with those reported previously[17,18,21].

Special attention deserves the content of dimethoxycinnamic acid in plasma (C_{max} 8 nM, T_{max} 0.7 h), which contrasts with the high concentration in plasma (C_{max} 380 nM) at 60 min after consuming 400 mL of 1% (w/v) soluble coffee described by Nagy et al.[32] The same research group explained that the result could be due to dimethoxycinnamic acid being preferentially formed by dimethoxycinnamoylquinic acid hydrolysis and absorbed at the intestinal level by passive diffusion[33]. These researchers emphasized the importance of considering the abundant 3,4-dimethoxycinnamic acid metabolite in future coffee bioavailability and metabolic studies. However, other recent bioavailability studies have not reported the presence of these derivatives in biological fluids after coffee consumption[17,21,23], in line with the low amount of dimethoxycinnamic acid determined in the present study.

Lactone derivatives were also absorbed in the small intestine without previous hydrolysis and were transformed into phase II derivatives, representing 1.0% of the total urinary metabolites. Stalmach et al.[17] characterized for the first time the presence of two isomers of caffeoylquinic lactone sulfate in both plasma and urine with similar nutrikinetic profile as here reported. Likewise, Redeuil et al.[21] characterized phase II derivatives of caffeoyl- and feruloylquinic lactone in plasma (urine analysis was not carried out), and Marmet et al.[23] characterized caffeoyl- and feruloylquinic lactones in plasma after oral ingestion of soluble coffee, observing early appearance in both plasma and urine in accordance with the results reported here.

In all, after consumption of the green/roasted coffee blend, the low levels of metabolites of hydroxycinnamic acids, hydroxycinnamate esters and lactone derivatives in plasma and urine, accounting for 16.3% of the urinary metabolites, confirm the low bioavailability in the upper gastrointestinal tract.

It is well known the importance of the colonic microbiota in the metabolism of hydroxycinnamates, already demonstrated by Stalmach et al.[34] in a study carried out in the in a

542 study carried out in ileostomist individuals. In the present study, dihydrocaffeic, dihydroferulic
543 and dihydrocoumaric acids, derived from microbial metabolism, and their phase II metabolites
544 were identified, accounting for 64.4% of the total urinary metabolites (Tables 3 and 4).
545 Another microbial metabolite derived from ferulic acid extensively excreted in urine was
546 feruloylglycine, which was also detected in plasma (C_{max} 22 nM). Additionally, an isomer of this
547 compound, *iso*feruloylglycine, was identified in low amounts only in urine. Both compounds
548 (*iso*- and *trans*feruloylglycine) were formed by CoA enzyme in the colon, which is consistent
549 with the nutrikinetic parameters observed. Feruloylglycine had already been reported in other
550 studies after the consumption of coffee rich in hydroxycinnamic acids, although only in
551 urine[17,22]. Therefore, to our knowledge, this is the first time that feruloylglycine has been
552 detected in plasma and urine, together with *iso*feruloylglycine in urine. Noticeably,
553 feruloylglycine was the second most abundant urinary metabolite (14 μmoles in 24 h) after
554 dihydrocaffeic acid 3-sulfate (31 μmoles in 24 h). Consequently, the presence of dihydrocaffeic
555 acid-3-sulfate and feruloylglycine in urine could be very sensitive biomarkers of green/roasted
556 coffee intake, considering that the excreted amount of both metabolites reached 24.4% and
557 11.0% of the excreted metabolites, respectively. Urinary excretion of *iso*- and
558 *trans*feruloylglycine together accounted for 11.3% of the total metabolites.
559 An important and novel contribution of the present work compared to previous studies on
560 the bioavailability of coffee hydroxycinnamate studies was the identification in plasma and
561 urine of reduced forms of monoacylquinic acids, namely dihydrocaffeoyl-, dihydroferuloyl- and
562 dihydrocoumaroylquinic acids (Tables 3 and 4). Additionally, the presence of low amounts of
563 dihydrocaffeoylquinic acid glucuronide in urine was also identified. Only one *in vitro* study on
564 the microbial biotransformation of chlorogenic acid had reported the formation of
565 dihydrocaffeoylquinic acid.[35] In the present *in vivo* study, relevant amounts of
566 dihydromonoacylquinic acids were detected in plasma (C_{max} 9-42 nM) and urine (10 μmoles
567 excreted in 24 h), amounting to 8% of the total urinary metabolites with the most delayed
568 kinetic out of all the characterized metabolites.
569 Reduced forms of hydroxycinnamic acids and their phase II derivatives, together with
570 reduced forms of hydroxycinnamates and feruloylglycine, accounted for 83.7% of the total
571 excretion, highlighting the relevance of the colon in the metabolism of hydroxycinnamates.
572 In general, ferulic acid derivatives were formed in higher amounts than those derived from
573 caffeic acid due to the double biotransformation pathway followed by hydroxycinnamates:
574 minimally by hydrolysis of their precursors and preferentially by methylation of caffeoylquinic
575 acids via catechol-*O*-methyltransferase (COMT). Additionally, it is worth noting that sulfation

576 was the predominant phase II transformation, followed by methylation and, to a lower extent,
577 glucuronidation, in agreement with Marmet et al.[23] and Sanchez-Bridge et al[24].
578 Finally, derivatives of hydroxyphenylacetic, hydroxybenzoic, and hydroxyhippuric acids
579 were also characterized in both plasma and urine, amounting up to 10 μmoles in 24 h urine
580 (Table 4). However, these metabolites were not considered in the recovery of the ingested
581 green/roasted coffee blend phenols, since they are not exclusively formed during the
582 biotransformation of hydroxycinnamic acids. In all, the total urinary excretion of coffee phenol
583 metabolites presented a recovery of just 16.7% of the phenols ingested, pointing to a low
584 bioavailability of the green/roasted coffee blend polyphenols. Previous studies also showed a
585 limited bioavailability of coffee phenolics, with recoveries up to 29% of the ingested
586 hydroxycinnamtes (146 mg, 412 μmoles[17]). The same research group evaluated the impact of
587 dose of chlorogenic acid in coffee on the bioavailability of this phenol in humans[22], and
588 observed recoveries of 24%, 25% and 16% after the intake of single servings of coffee
589 containing low (412 μmoles ~ 146 mg), medium (635 μmoles ~ 225 mg) and high (795 μmoles
590 ~ 282 mg) contents of the studied phenol, respectively, and plasmatic kinetics coherent with
591 that observed in urine. Considering that in the present study the ingested amount of
592 polyphenols (760.6 μmoles ~ 269.5 mg) was in line with the highest dose reported by Stalmach
593 et al.[22], both studies are in agreement and suggest that hydroxycinnamates absorption
594 saturation may take place with high intake doses. Contrary to these results, Renouf et al.[18] and
595 later Sánchez-Bridge et al.[24] showed a dose-response appearance of metabolites in plasma
596 (urine was not analyzed). The highest doses used in both studies (970 μmoles ~ 344 mg and
597 1485 μmoles ~ 526mg, respectively) were higher than the almost 761 μmoles used in the
598 present study and in Stalmach et al.[22] Moreover, Sánchez-Bridge et al.[24], evaluated the
599 influence of roasting on the extent of conjugation by comparing three types of coffees (high,
600 low and unroasted coffees), observing similar extent of conjugation with roasted or unroasted
601 coffees.
602 A limitation of the present study was the reduced urinary collection time. Most of the
603 microbial metabolites showed relevant amounts in the 12-24 h interval, not returning to basal
604 levels, and therefore it would have been interesting to extend the collection to at least 48 h. It
605 is likely that the amount of urinary metabolites has been underestimated and thus a higher
606 bioavailability of coffee polyphenols cannot be ruled out. Another limitation was the lack of
607 certain metabolite standards, mainly phase II derivatives, forcing to express the results as
608 equivalents of the corresponding precursor compound. Therefore, the results here indicated
609 did not accurately measure the concentrations of the metabolites described in the biological

610 samples. Nevertheless, the results are in line with other studies on the bioavailability of coffee
611 hydroxycinnamates[17,22].

612 In summary, polyphenols contained in a commercial instant green/roasted coffee blend
613 were partially absorbed and extensively metabolized. The predominant metabolites were
614 phase II derivatives of reduced forms of hydroxycinnamic acids, sulfated, methyl derivatives
615 and to a lower extent glucuronidated conjugates, in addition to feruloylglycine. Reduced forms
616 of hydroxycinnamates were identified for the first time in an *in vivo* bioavailability study of
617 phenols in coffee, accounting for 8% of the total urinary metabolites, completing the
618 knowledge on the coffee polyphenol bio-transformations. Attending to their high urinary
619 elimination, dihydrocaffeic acid-3-sulfate and feruloylglycine can be used as biomarkers of
620 intake of hydroxycinnamate-rich foods.

621 In conclusion, the bioavailability of hydroxycinnamates in green/roasted coffee blend is
622 discreet but should not be disregarded considered the high intake of coffee. The phenols in
623 this beverage are extensively metabolized, mainly by the microbiota, and remain for long time
624 in the body of coffee consumers which favors the possible bioactivity of these compounds.

625

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635

636 **Conflict of interest statement**

637 The authors declare no conflicts of interest.

638

639

640 **References**

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Figure legends

Figure 1. Plasma concentrations of the identified metabolites after consuming a cup of instant green/roasted coffee blend containing 269.5 mg of hydroxycinnamic acids. Results represent concentration (μM) as mean ± standard deviation (n=12).

Figure 2. Biotransformation pathways in humans of the main hydroxycinnamate esters contained in the green/roasted green coffee. COMT: catechol-O-methyl transferases; DC: decarboxylases; DH: dehydrogenases; EST: esterases; RED: reductases.

Figure 1.

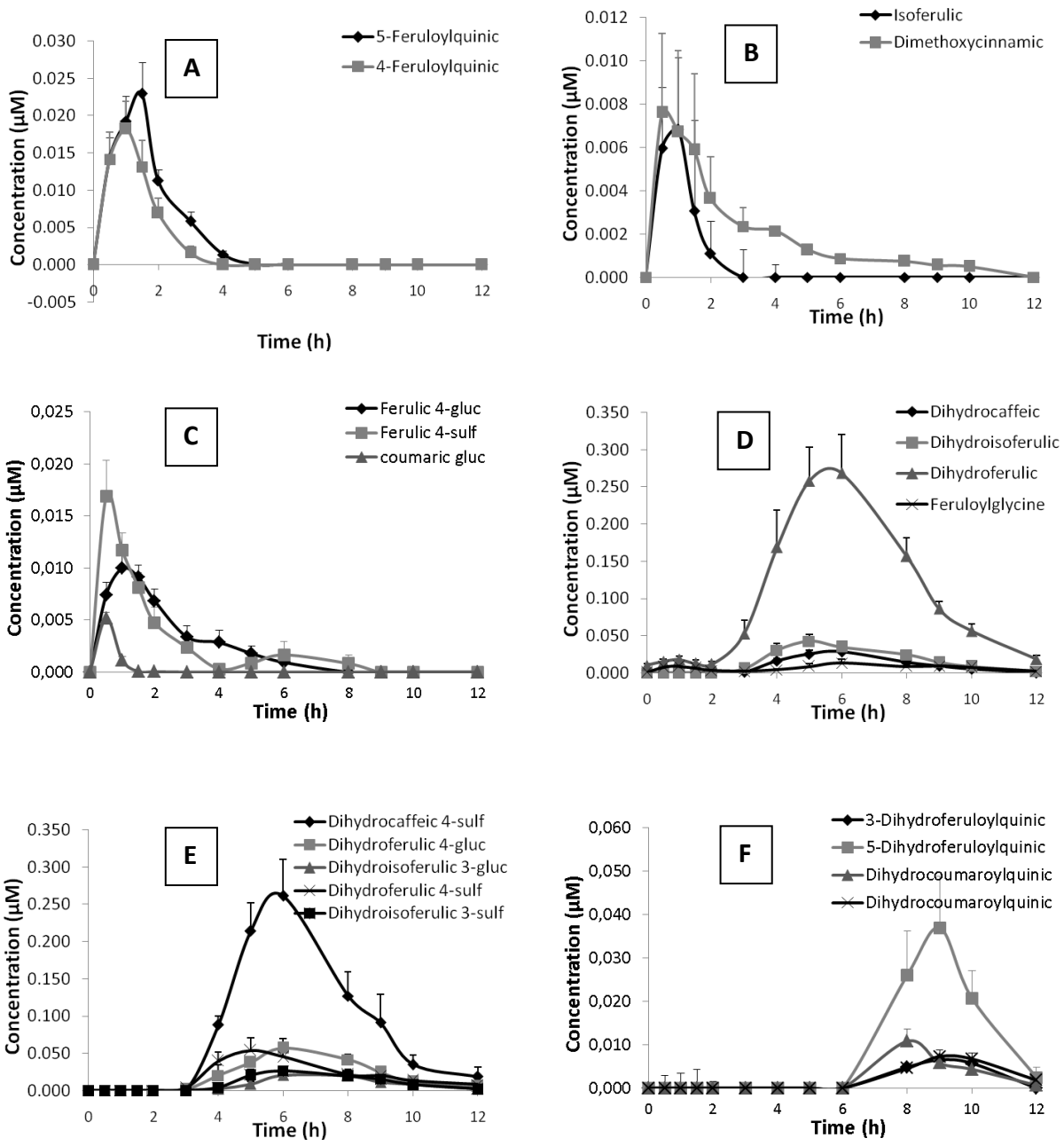
A
5-Feruloylquinic
4-Feruloylquinic
Concentration (µM)
Time (h)
B
Isoferulic
Dimethoxycinnamic
Concentration (µM)
Time (h)
C
Ferulic 4-gluc
Ferulic 4-sulf
coumaric gluc
Concentration (µM)
Time (h)
D
Dihydrocaffeic
Dihydroisoferulic
Dihydroferulic
Feruloylglycine
Concentration (µM)
Time (h)
E
Dihydrocaffeic 4-sulf
Dihydroferulic 4-gluc
Dihydroisoferulic 3-gluc
Dihydroferulic 4-sulf
Dihydroisoferulic 3-sulf
Concentration (µM)
Time (h)
F
3-Dihydroferuloylquinic
5-Dihydroferuloylquinic
Dihydrocoumaroylquinic
Dihydrocoumaroylquinic
Concentration (µM)
Time (h)

Figure 2.

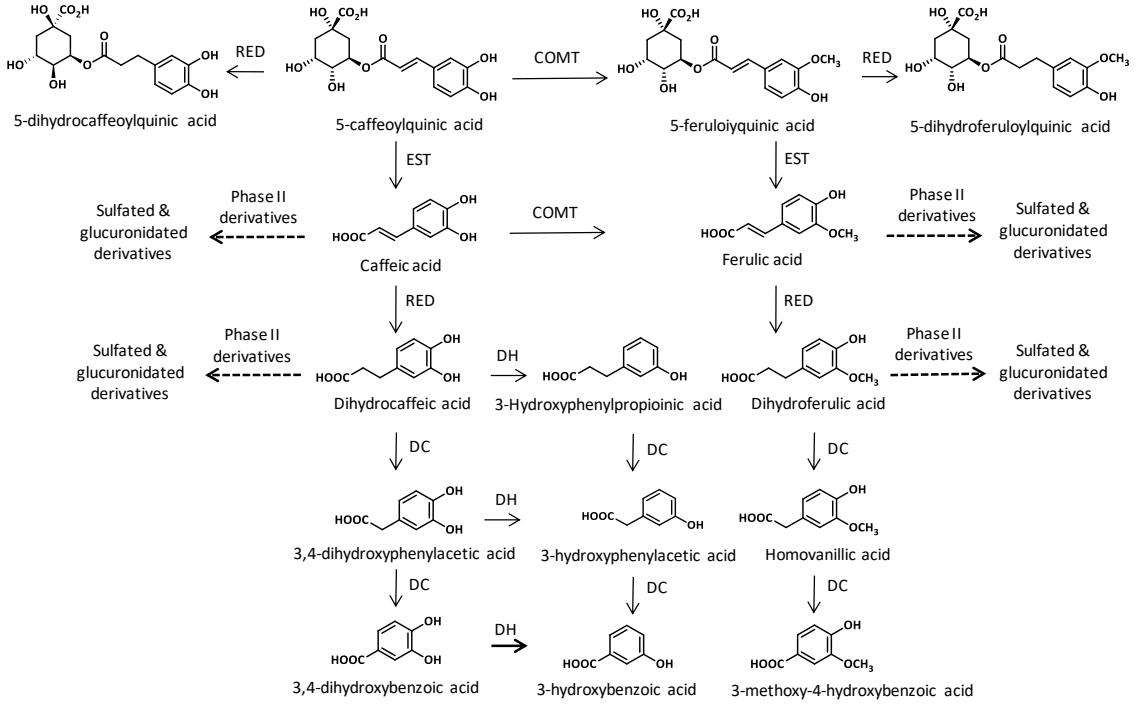
5-dihydrocaffeoylquinic acid
RED
5-caffeoylquinic acid
COMT
5-feruloiyquinic acid
RED
5-dihydroferuloylquinic acid
EST
EST
Sulfated & glucuronidated derivatives
Phase II derivatives
Caffeic acid
COMT
Ferulic acid
Phase II derivatives
Sulfated & glucuronidated derivatives
RED
RED
Sulfated & glucuronidated derivatives
Phase II derivatives
Dihydrocaffeic acid
DH
3-Hydroxyphenylpropioinic acid
Dihydroferulic acid
Phase II derivatives
Sulfated & glucuronidated derivatives
DC
DC
DC
3,4-dihydroxyphenylacetic acid
DH
3-hydroxyphenylacetic acid
Homovanillic acid
DC
DC
DC
3,4-dihydroxybenzoic acid
DH
3-hydroxybenzoic acid
3-methoxy-4-hydroxybenzoic acid

Table 1

Quantities of polyphenols in soluble green/roasted coffee blend determined by HPLC-DAD, expressed in mg per gram of dried material (6.5 % of moisture). Values represent mean ± standard deviation (n =3) and in parenthesis is the percentage of total polyphenols.

Polyphenols	**(mg/g, d.m.)**
Caffeoylquinic acids	51.2 ± 0.7 (66.8%)
Coumaroylquinic acids	1.7 ± 0.1 (2.2%)
Feruloylquinic acids	10.4 ± 0.5 (13.5%)
Dimethoxycinnamoylquinic acids	0.8 ± 0.1 (1.0%)
Dicaffeoylquinic acids	8.3 ± 0.6 (10.9%)
Caffeoylferuloylquinic acids	1.0 ± 0.1 (1.4%)
Caffeoylshikimic acids	0.26 ± 0.02 (0.3%)
Caffeoylquinic lactones	1.70 ± 0.08 (2.2%)
Caffeoyl-*N*-tryptophan (mg/g)	1.3 ± 0.1 (1.7%)
Total Hydroxycinnamic acids	**77 ± 2**

Table 2

LC-QToF identification of hydroxycinnamate derived metabolites in plasma (P) and urine (U) samples of volunteers after the intake of a cup of instant green/roasted coffee blend containing 269.5 mg (760.6 μmol) of hydroxycinnamic acids.

Identified Compound	RT (min)	Molecular formula	$[M-H]^-$	Fragment MS^2	Location
Caffeic acid metabolites					
3-Caffeoylquinic acid	4.0	$C_{16}H_{18}O_9$	353.0878	191	U
5-Caffeoylquinic acid	6.2	$C_{16}H_{18}O_9$	353.0878	191	P, U
4-Caffeoylquinic acid	10.1	$C_{16}H_{18}O_9$	353.0878	191	U
Caffeic acid	7.9	$C_9H_8O_4$	179.0350	135	U
Caffeic acid 3-sulfate	10.7	$C_9H_8O_7S$	258.9918	179; 135	U
Dihydrocaffeic acid	7.0	$C_9H_{10}O_4$	181.0506	137	P, U
Dihydrocaffeic acid 3-glucuronide	6.5	$C_{15}H_{18}O_{10}$	357.0827	181; 137	U
Dihydrocaffeic acid 4-sulfate	7.8	$C_9H_{10}O_7S$	261.0074	181	P, U
Dihydrocaffeic acid 3-sulfate	10.4	$C_9H_{10}O_7S$	261.0074	181	U
3-Dihydrocaffeoylquinic acid	8.5	$C_{16}H_{20}O_9$	355.1035	181	U
5-Dihydrocaffeoylquinic acid	13.1	$C_{16}H_{20}O_9$	355.1035	181	U
4-Dihydrocaffeoylquinic acid	14.8	$C_{16}H_{20}O_9$	355.1035	181	U
Dihydrocaffeoylquinic acid glucuronide	11.3	$C_{22}H_{28}O_{15}$	531.1355	355; 181	U
Ferulic acid metabolites					
3-Feruloylquinic acid	7.1	$C_{17}H_{20}O_9$	367.1035	191	U
5-Feruloylquinic acid	9.6	$C_{17}H_{20}O_9$	367.1035	191	P, U
4-Feruloylquinic acid	10.2	$C_{17}H_{20}O_9$	367.1035	191	P, U
Ferulic acid	11.4	$C_{10}H_{10}O_4$	193.0506	134	P
*iso*Ferulic acid	12.9	$C_{10}H_{10}O_4$	193.0506	134	P
Ferulic acid 4-glucuronide	8.4	$C_{16}H_{18}O_{10}$	369.0827	193; 134	P, U
*iso*Ferulic acid 3-glucuronide	9.2	$C_{16}H_{18}O_{10}$	369.0827	193; 134	U
Ferulic acid 4-sulfate	8.7	$C_{10}H_{10}O_7S$	273.0074	193; 134	P, U
*iso*Ferulic acid 3-sulfate	10.4	$C_{10}H_{10}O_7S$	273.0074	193; 134	U
Dihydroferulic acid	10.8	$C_{10}H_{12}O_4$	195.0663	136	P, U
Dihydro*iso*ferulic acid	11.6	$C_{10}H_{12}O_4$	195.0663	136	P
Dihydroferulic acid 4-glucuronide	6.5	$C_{16}H_{20}O_{10}$	371.0984	195; 136	P, U
Dihydro*iso*ferulic acid 3-glucuronide	8.4	$C_{16}H_{20}O_{10}$	371.0984	195; 136	P, U
Dihydroferulic acid 4-sulfate	8.8	$C_{10}H_{12}O_7S$	275.0231	195; 136	P, U
Dihydro*iso*ferulic acid 3-sulfate	10.4	$C_{10}H_{12}O_7S$	275.0231	195; 136	P, U
3-Dihydroferuloylquinic acid	15.1	$C_{17}H_{22}O_9$	369.1191	195	P, U
5-Dihydroferuloylquinic acid	15.4	$C_{17}H_{22}O_9$	369.1191	195	P, U
4-Dihydroferuloylquinic acid	15.6	$C_{17}H_{22}O_9$	369.1191	195	U
Feruloylglycine	8.8	$C_{12}H_{13}O_5N$	250.0721	191; 134	P, U
*iso*Feruloylglycine	9.5	$C_{12}H_{13}O_5N$	250.0721	191; 134	U
Coumaric acid metabolites					
Coumaroylquinic acid	16.4	$C_{16}H_{18}O_8$	337.0929	163	U
Coumaric acid glucuronide	12.3	$C_{15}H_{16}O_9$	339.0722	163	P, U
Coumaric acid glucuronide	12.9	$C_{15}H_{16}O_9$	339.0722	163	U
Coumaric acid sulfate	18.5	$C_9H_8O_6S$	242.9969	163	P
Dihydrocoumaric acid	8.5	$C_9H_{10}O_3$	165.0557	121	U
Dihydrocoumaric acid glucuronide	6.8	$C_{15}H_{18}O_9$	341.0878	165	U
Dihydrocoumaric acid sulfate	10.6	$C_9H_{10}O_6S$	245.0125	165	U

Dihydrocoumaroylquinic acid	14.8	$C_{16}H_{20}O_8$	339.1085	165	P, U
Dihydrocoumaroylquinic acid	15.3	$C_{16}H_{20}O_8$	339.1085	165	P, U
Dimethoxycinnamic acid metabolites					
Dimethoxycinnamic acid	15.1	$C_{11}H_{12}O_4$	207.0652	103	P
Dihydrodimethoxycinnamic acid	13.8	$C_{11}H_{14}O_4$	209.0819		P, U
Lactone derivatives					
Caffeoylquinic lactone sulfate	11.8	$C_{16}H_{16}O_{11}S$	415.0330	335	U
Caffeoylquinic lactone sulfate	12.3	$C_{16}H_{16}O_{11}S$	415.0330	335	U
Caffeoylquinic lactone sulfate	13.5	$C_{16}H_{16}O_{11}S$	415.0330	335	U
Feruloylquinic lactone glucuronide	12.0	$C_{23}H_{26}O_{14}$	525.1261	349	P
Phenolic acids					
3-Hydroxyphenylpropionic acid	11.1	$C_9H_{10}O_3$	165.0557	121	P, U
3,4-Dihydroxyphenylacetic acid	5.6	$C_8H_8O_4$	167.0350	123	P, U
Methoxy-hydroxyphenylacetic acid	6.5	$C_9H_{10}O_4$	181,0506	137	P, U
Methoxy-hydroxyphenylacetic acid	8.8	$C_9H_{10}O_4$	181,0506	137	P, U
3-Hydroxyphenylacetic acid	7.4	$C_8H_8O_3$	151,0401	107	P, U
3,4-Dihydroxybenzoic acid	3.8	$C_7H_6O_4$	153,0193	109	P, U
3-Methoxy-4-hydroxybenzoic acid	6.3	$C_8H_8O_4$	167.0350	123	P, U
Hydroxybenzoic acid	6.1	$C_7H_6O_3$	137.0244	93	P, U
3-Hydroxyhippuric acid	11.0	$C_9H_9O_4N$	194.0459	100	P, U
4-Hydroxyhippuric acid	14.2	$C_9H_9O_4N$	194.0459	150	P, U
Phloroglucinol	6.5	$C_6H_6O_3$	125.0244	79	P, U

Table 3

Nutrikinetic parameters of metabolites detected in human plasma after consuming a cup of instant green/roasted coffee blend containing 269.5 mg (760.6 μmoles) of hydroxycinnamic acids. Values represent mean ± standard deviation (n=12).

Metabolite	C_{max} (μM)	T_{max} (h) or Range*	AUC (μM/min)
Intestinal Absorption			
5-Caffeoylquinic acid	Traces**	(1.0 – 1.5)*	-
5-Feruloylquinic acid	0.02 ± 0.01	1.2 ± 0.4	0.04 ± 0.02
4-Feruloylquinic acid	0.02 ± 0.01	0.9 ± 0.3	0.03 ± 0.02
Ferulic acid	Traces**	(1.0 – 1.5)*	-
*iso*Ferulic acid	0.008 ± 0.001	0.9 ± 0.2	0.1 ± 0.2
Dimethoxycinnamic acid	0.008 ± 0.002	0.7 ± 0.3	0.02 ± 0.02
Ferulic acid 4-glucuronide	0.011 ± 0.004	1.1 ± 0.5	0.03 ± 0.02
Ferulic acid 4-sulfate	0.02 ± 0.01	0.7 ± 0.2	0.03 ± 0.02
Coumaric acid glucuronide	0.005 ± 0.002	0.5 ± 0.1	0.003 ± 0.001
Coumaric acid sulfate	Traces**	(1.0 – 1.5)*	-
Feruloylquinic lactone glucuronide	Traces**	(1.0 – 1.5)*	-
Microbial metabolites			
Dihydrocaffeic acid	0.03± 0.01	6 ± 1	0.12 ± 0.08
Dihydroferulic acid	0.3 ± 0.1	6 ± 1	1.3 ± 0.6
Dihydro*iso*ferulic acid	0.05 ± 0.03	5 ± 1	0.2 ± 0.1
Dihydrodimethoxycinnamic acid	Traces**	(6.0-8.0)*	0.013 ± 0.004
Dihydrocaffeic acid 4-sulfate	0.26 ± 0.09	6.0 ± 0.1	1.1 ± 0.4
Dihydroferulic acid 4-glucuronide	0.06 ± 0.04	6 ± 1	0.3 ± 0.2
Dihydro*iso*ferulic acid 3-glucuronide	0.02 ± 0.01	7 ± 1	0.10 ± 0.08
Dihydroferulic acid 4-sulfate	0.09 ± 0.05	5.6 ± 0.7	0.4 ± 0.2
Dihydro*iso*ferulic 3-sulfate	0.03 ± 0.02	5.8 ± 0.5	0.1 ± 0.2
Feruloylglycine	0.02 ± 0.01	7 ± 2	0.09 ± 0.06
3-Dihydroferuloylquinic acid	0.009 ± 0.004	9.2 ± 0.8	0.02 ± 0.01
5-Dihydroferuloylquinic acid	0.04 ± 0.02	9.3 ± 0.5	0.11 ± 0.08
Dihydrocoumaroylquinic acid	0.011 ± 0.008	8.4 ± 0.7	0.03 ± 0.02
Dihydrocoumaroylquinic acid	0.009 ± 0.005	9.4 ± 0.7	0.03 ± 0.01

* Range where the metabolite showed the highest value.

** No pharmacokinetic parameters of these metabolites were determined because their content was at trace levels.

Table 4

Amount of metabolites excreted in urine from 0 to 24 h of healthy volunteers, after consuming of a cup of instant green/roasted coffee blend containing 269.5 mg (760.6 μmoles) of hydroxycinnamic acids. Values represent mean ± standard deviation (n=12). N.D.: Not detected. < L.C. Lower than quantification limit.

Metabolites	**-2-0 h**	**0- 2h**	**2-5 h**	**5-8 h**	**8-12 h**	**12-24h**	**TOTAL (μmoles)**
Intestinal absorption							
3-Caffeoylquinic acid	N.D.	0.014 ± 0.003	0.023 ± 0.003	0.007 ± 0.003	**N.D.**	**N.D.**	**0.044 ± 0.009**
5-Caffeoylquinic acid	N.D.	0.056 ± 0.009	0.14 ± 0.05	0.013 ± 0.006	**N.D.**	**N.D.**	**0.21 ± 0.07**
4-Caffeoylquinic acid	N.D.	0.04 ± 0.01	0.032 ± 0.005	0.017 ± 0.009	0.02 ± 0.01	N.D.	**0.11 ± 0.04**
3-Feruloylquinic acid	N.D.	0.24 ± 0.04	0.27 ± 0.06	0.13 ± 0.02	0.09 ± 0.03	0.15 ± 0.02	**0.9 ± 0.2**
5-Feruloylquinic acid	N.D.	0.41 ± 0.07	0.7 ± 0.1	0.21 ± 0.04	0.16 ± 0.02	0.008 ± 0.008	**1.5 ± 0.3**
4-Feruloylquinic acid	N.D.	0.44 ± 0.05	0.56 ± 0.07	0.15 ± 0.03	N.D.	N.D.	**1.2 ± 0.1**
Coumaroylquinic acid	N.D.	N.D.	0.041 ± 0.008	0.057 ± 0.008	0.094 ± 0.004	0.07 ± 0.02	**0.26 ± 0.04**
Caffeic acid	N.D.	0.02 ± 0.01	0.04 ± 0.02	0.10 ± 0.04	0.17 ± 0.01	0.107 ± 0.008	**0.45 ± 0.09**
Caffeic acid 3-sulfate	N.D.	0.4 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	0.30 ± 0.05	N.D.	**2.6 ± 0.5**
Ferulic acid 4-glucuronide	N.D.	0.32 ± 0.06	0.6 ± 0.1	0.7 ± 0.1	0.63 ± 0.04	0.01 ± 0.01	**2.3 ± 0.3**
*iso*Ferulic acid 3-glucuronide	N.D.	0.17 ± 0.03	0.07 ± 0.02	N.D.	N.D.	N.D.	**0.24 ± 0.06**
Ferulic acid 4-sulfate	N.D.	0.9 ± 0.3	2.1 ± 0.5	2.2 ± 0.2	2.3 ± 0.3	0.04 ± 0.04	**8 ± 1**
*iso*Ferulic acid 3-sulfate	N.D.	1.6 ± 0.2	0.05 ± 0.03	N.D.	N.D.	N.D.	**1.7 ± 0.2**
Coumaric acid glucuronide	N.D.	0.11 ± 0.02	0.11 ± 0.03	0.02 ± 0.02	N.D.	N.D.	**0.23 ± 0.06**
Coumaric acid glucuronide	N.D.	0.07 ± 0.02	0.03 ± 0.02	N.D.	N.D.	N.D.	**0.11 ± 0.04**
Caffeoylquinic lactone sulfate	N.D.	0.18 ± 0.07	0.1 ± 0.1	N.D.	N.D.	N.D.	**0.3 ± 0.2**
Caffeoylquinic lactone sulfate	N.D.	0.34 ± 0.06	0.36 ± 0.08	N.D.	N.D.	N.D.	**0.7 ± 0.1**
Caffeoylquinic lactone sulfate	N.D.	0.13 ± 0.02	0.16 ± 0.03	N.D.	N.D.	N.D.	**0.29 ± 0.04**
TOTAL – Intestinal absorption	**N.D.**	**5 ± 1**	**7 ± 2**	**4.3 ± 0.6**	**3.8 ± 0.5**	**0.4 ± 0.1**	**21 ± 4**
Colonic absorption							
Dihydrocaffeic acid	0.06 ± 0.01	0.061 ± 0.009	0.24 ± 0.07	1.2 ± 0.2	0.8 ± 0.2	0.06 ± 0.02	**2.5 ± 0.5**
Dihydroferulic acid	0.06 ± 0.03	0.09 ± 0.03	1.2 ± 0.4	5.5 ± 0.9	2.5 ± 0.7	0.03 ± 0.03	**9 ± 2**
Dihydrocoumaric acid	0.01 ± 0.01	0.04 ± 0.02	0.16 ± 0.04	0.30 ± 0.05	0.23 ± 0.08	0.02 ± 0.02	**0.7 ± 0.2**
Dihydrodimethoxycinnamic acid	N.D.	N.D.	N.D.	N.D.	<L.C.	N.D.	**Traces**
Dihydrocaffeic acid 3- glucuronide	N.D.	N.D.	0.05 ± 0.02	0.23 ± 0.04	0.34 ± 0.03	0.01 ± 0.01	**0.6 ± 0.1**
Dihydrocaffeic acid 4-sulfate	0.36 ± 0.07	0.14 ± 0.04	0.06 ± 0.03	N.D.	N.D.	N.D.	**0.6 ± 0.1**
Dihydrocaffeic 3-sulfate	N.D.	0.15 ± 0.07	1.4 ± 0.4	13 ± 3	15 ± 3	3 ± 1	**31 ± 8**
Dihydroferulic acid 4-glucuronide	0.02 ± 0.01	0.02 ± 0.02	0.5 ± 0.2	3.5 ± 0.8	3.5 ± 0.5	0.22 ± 0.05	**8 ± 2**
Dihydro*iso*ferulic acid 3-glucuronide	N.D.	0.010 ± 0.008	0.4 ± 0.2	1.5 ± 0.5	1.8 ± 0.2	0.16 ± 0.03	**4 ± 1**
Dihydroferulic acid 4-sulfate	N.D.	0.4 ± 0.3	1.3 ± 0.5	6 ± 1	5 ± 1	0.10 ± 0.07	**13 ± 3**
Dihydro*iso*ferulic acid 3-sulfate	N.D.	N.D.	0.11 ± 0.06	1.0 ± 0.2	1.5 ± 0.2	0.20 ± 0.07	**2.8 ± 0.5**

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Continuation							
Metabolites	**-2-0 h**	**0- 2h**	**2-5 h**	**5-8 h**	**8-12 h**	**12-24h**	**TOTAL (μmoles)**
Colonic absorption (continuation)							
Dihydrocoumaric glucuronide	N.D.	0.09 ± 0.02	N.D.	N.D.	N.D.	N.D.	**0.09 ± 0.02**
Dihydrocoumaric sulfate	N.D.	0.05 ± 0.05	0.19 ± 0.04	0.7 ± 0.2	3.1 ± 0.8	4 ± 1	**8 ± 2**
Dihydrocaffeoylquinic acid glucur	N.D.	N.D.	N.D.	0.010 ± 0.005	0.089 ± 0.006	0.003 ± 0.003	**0.10 ± 0.01**
3-Dihydrocaffeoylquinic acid	N.D.	N.D.	0.05 ± 0.02	0.13 ± 0.02	0.31 ± 0.04	0.15 ± 0.03	**0.6 ± 0.1**
5-Dihydrocaffeoylquinic acid	N.D.	N.D.	N.D.	0.008 ± 0.006	0.14 ± 0.01	0.035 ± 0.007	**0.19 ± 0.03**
4-Dihydrocaffeoylquinic acid	N.D.	N.D.	N.D.	0.01 ± 0.01	0.24 ± 0.01	0.04 ± 0.01	**0.29 ± 0.03**
3-Dihydroferuloylquinic acid	N.D.	N.D.	N.D.	0.04 ± 0.04	0.8 ± 0.3	0.02 ± 0.02	**0.9 ± 0.3**
5-Dihydroferuloylquinic acid	N.D.	N.D.	N.D.	0.08 ± 0.05	5 ± 3	0.45 ± 0.08	**6 ± 3**
4-Dihydroferuloylquinic acid	N.D.	N.D.	N.D.	0.03 ± 0.02	0.18 ± 0.04	0.01 ± 0.01	**0.22 ± 0.08**
Dihydrocoumaroylquinic acid	N.D.	N.D.	0.13 ± 0.08	0.13 ± 0.05	0.7 ± 0.3	0.05 ± 0.02	**1.0 ± 0.4**
Dihydrocoumaroylquinic acid	N.D.	N.D.	N.D.	0.06 ± 0.05	0.51 ± 0.07	0.16 ± 0.03	**0.7 ± 0.1**
Feruloylglycine	0.34 ± 0.07	0.54 ± 0.09	1.4 ± 0.2	9 ± 2	2 ± 1	0.63 ± 0.08	**14 ± 4**
*Iso*Feruloylglicine	N.D.	N.D.	0.006 ± 0.005	0.09 ± 0.02	0.111 ± 0.007	N.D.	**0.21 ± 0.04**
TOTAL – colonic absorption	**0.8 ± 0.2**	**1.6 ± 0.6**	**7 ± 2**	**42 ± 10**	**45 ± 12**	**9 ± 3**	**106 ± 28**
TOTAL - INTESTINAL + COLONIC absorp	**0.8 ± 0.2**	**7 ± 2**	**14 ± 4**	**47 ± 10**	**49 ± 12**	**10 ± 3**	**127 ± 32**
Other microbial metabolites							
3-Hydroxyphenylpropionic acid	N.D.	0.024 ± 0.002	0.036 ± 0.004	0.10 ± 0.02	0.26 ± 0.06	0.08 ± 0.01	**0.5 ± 0.1**
3,4-Dihydroxyphenylacetic acid	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.06 ± 0.03	**0.3 ± 0.1**
3-Methoxy-4-hydroxyphenylacetic acid	N.D.	0.03 ± 0.01	0.2 ± 0.1	1.4 ± 0.4	0.9 ± 0.3	N.D.	**2.6 ± 0.8**
4-Methoxy-3-hydroxyphenylacetic acid	0.14 ± 0.04	0.18 ± 0.03	0.16 ± 0.04	0.19 ± 0.02	0.34 ± 0.03	0.16 ± 0.03	**1.2 ± 0.2**
3-Hydroxyphenylacetic acid	0.19 ± 0.04	0.21 ± 0.03	0.23 ± 0.04	0.38 ± 0.08	0.65 ± 0.08	0.35 ± 0.07	**2.0 ± 0.3**
3,4-Dihydroxybenzoic acid	0.06 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	**0.34 ± 0.09**
3-Methoxy-4-hydroxybenzoic acid	0.17 ± 0.03	0.10 ± 0.02	0.11 ± 0.02	0.14 ± 0.02	0.5 ± 0.2	0.34 ± 0.06	**1.3 ± 0.4**
Hydroxybenzoic acid	0.10 ± 0.03	0.06 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.15 ± 0.01	0.29 ± 0.08	**0.8 ± 0.2**
4-Hydroxyhippuric acid	N.D.	N.D.	N.D.	N.D.	0.09 ± 0.01	N.D.	**0.09 ± 0.01**
3-Hydroxyhippuric acid	N.D.	N.D.	N.D.	N.D.	0.08 ± 0.01	N.D.	**0.08 ± 0.01**
Phloroglucinol	0.10 ± 0.01	0.04 ± 0.02	0.06 ± 0.02	0.09 ± 0.02	0.17 ± 0.02	0.22 ± 0.05	**0.69 ± 0.14**
Total other microbial metabolites	**0.8 ± 0.2**	**0.8 ± 0.1**	**1.0 ± 0.3**	**2.5 ± 0.6**	**3.3 ± 0.8**	**1.6 ± 0.6**	**10 ± 2**