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Identification of novel circulating coffee metabolites in human plasma by liquid chromatography–mass spectrometry

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ABSTRACT

This study reports a liquid chromatography–mass spectrometry method for the detection of polyphenol-derived metabolites in human plasma without enzymatic treatment after coffee consumption. Separation of available standards was achieved by reversed-phase ultra performance liquid chromatography and detection was performed by high resolution mass spectrometry in negative electrospray ionization mode. This analytical method was then applied for the identification and relative quantification of circulating coffee metabolites. A total of 34 coffee metabolites (mainly reduced, sulfated and methylated forms of caffeic acid, coumaric acid, caffeoylquinic acid and caffeoylquinic acid lactone) were identified based on mass accuracy (<4 ppm for most metabolites), specific fragmentation pattern and co-chromatography (when standard available). Among them, 19 circulating coffee metabolites were identified for the first time in human plasma such as feruloylquinic acid lactone, sulfated and glucuronidated forms of feruloylquinic acid lactone and sulfated forms of coumaric acid. Phenolic acid derivatives such as dihydroferulic acid, dihydroferulic acid 4′-*O*-sulfate, caffeic acid 3′-*O*-sulfate, dimethoxycinnamic acid, dihydrocaffeic acid and coumaric acid *O*-sulfate appeared to be the main metabolites circulating in human plasma after coffee consumption. The described method is a sensitive and reliable approach for the identification of coffee metabolites in biological fluids. In future, this analytical method will give more confidence in compound identification to provide a more comprehensive assessment of coffee polyphenol bioavailability studies in humans.

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1. Introduction

Coffee is one of the most widely consumed beverages throughout the world and contains bioactive compounds [1] with antioxidant properties, which provide several potential health benefits. The major coffee polyphenolic components are chlorogenic acids (quinic acid esters of hydroxycinnamates) accounting for 6–10% of coffee on a dry-weight basis [2] as well as di-caffeoylquinic acids (diCQA) and caffeoylquinic lactone (CQAL) [3].

Coffee has often been studied in relation to health benefits and its consumption may reduce the risk of diabetes [4], cardiovascular diseases [5], and certain cancers [6,7]. However, human bioavailability and metabolism of coffee phenolics are not fully understood [8]. Chlorogenic acids can be cleaved prior to absorption [9,10], resulting in the appearance of phenolic acids such as caffeic acid derivatives. Up to now, identification and quantification of chlorogenic acid metabolites in human plasma have been performed after protein precipitation induced by the addition of methanol followed by enzymatic treatment with β-glucuronidase,

Abbreviations: ACN, acetonitrile; CA, caffeic acid; CE, collision energy; CID, collision induced dissociation; C_{max}, maximum plasma concentration; COMT, catechol-*O*-methyltransferase; CGA, chlorogenic acid; CoA, coumaric acid; CQA, caffeoylquinic acid; CQAL, caffeoylquinic acid lactone; diCQA, di-caffeoylquinic acid; diFQA, di-feruloylquinic acid; DHCA, dihydrocaffeic acid; DHDMCA, dihydro-dimethoxycinnamic acid; DHFA, dihydroferulic acid; DHiFA, dihydro-isoferulic acid; DMCA, dimethoxycinnamic acid; DMCQA, dimethoxycinnamoylquinic acid; EC, epicatechin; EDC, enhanced duty cycle; EDTA, ethylenediaminetetraacetic acid; EGC, epigallocatechin; EIC, extracted ion chromatogram; ESI, electrospray; EtOH, ethanol; FA, ferulic acid; FQA, feruloylquinic acid; (i)FQAL, (iso)feruloylquinic acid lactone; HDMS, high definition mass spectrometry; HRMS, high-resolution mass spectrometry; iFA, isoferulic acid; LC, liquid chromatography; LLOQ, lower limit of quantification; MeOH, methanol; MS, mass spectrometry; PA, peak area; RMS, root mean square; RS, relative sensitivity; R_t, retention time; ST, sulfuryl-*O*-transferase; T_{max}, time needed to reach maximum plasma concentration; UPLC, ultra-performance liquid chromatography.

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sulfatase and sometimes an esterase. The resulting free phenolic acids, also called aglycones, were then extracted with ethyl acetate [11,12] and separated using reversed-phase liquid chromatography with highly acidic eluents such as 1 mM trifluoroacetic acid [11,13] or 4% formic acid [14]. Detection and subsequent quantification of phenolics is most often performed using UV–VIS absorbance [13,14]. Bioavailability of the major green coffee constituents in humans has been studied by Farah et al. [15], including quantification of chlorogenic and cinnamic acids in plasma and urine after ingestion of green coffee extract capsules. Although semi-quantitative approaches based on caffeic- and ferulic acid equivalents have also been demonstrated [12], the lack of authentic standards hinders accurate calibration and absolute quantification of these metabolites. Indeed, to evaluate the amount of conjugated forms of phenolic acids, Azuma et al. [16] and Nardini et al. [17] compared the concentration of aglycone forms (e.g. caffeic acid (CA) and ferulic acid (FA)) in plasma with and without enzymatic treatment. Stalmach et al. [18] studied the bioavailability of coffee chlorogenic acids in volunteers by identifying conjugated forms of caffeic and ferulic acids in human plasma and urine. Stalmach et al. [18] proposed metabolic pathways of chlorogenic acids following the ingestion of instant coffee in human. This involved catechol-*O*-methyltransferase (COMT) for methylation, the sulfuryl-*O*-transferase (ST) for sulfation, the UDP-glucuronosyl transferase for glucuronidation, esterase for hydrolysis of the ester bond and reductases for conversion to dihydroforms.

Expanding our knowledge towards the metabolic fate of dietary antioxidants such as chlorogenic acids present in coffee is the key to understanding their actual health benefits. In the present paper, we report 19 novel circulating coffee metabolites in human blood as well as the occurrence of already reported conjugates. For this purpose, a methodology based on ultra performance liquid chromatography (UPLC) combined with high resolution mass spectrometry (HRMS) is proposed to detect, identify and quantify these metabolites.

2. Experimental

2.1. Chemicals

LC grade water, acetonitrile (ACN) and methanol (MeOH) were obtained from Chemie Brunschwig AG (Basel, Switzerland). LC–MS grade acetic acid was obtained from Fluka/Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Ethanol (EtOH, analytical grade) was purchased from Merck (Darmstadt, Germany). Dihydrocaffeic acid (DHCA), isoferulic acid (iFA), 3-caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA), para-coumaric acid (*p*-CoA) were purchased from Extrasynthese (Genay Cedex, France). Phenolic acids: CA, FA and dimethoxycinnamic acid (DMCA) were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Dihydroferulic acid (DHFA) was purchased from alfa-chemcat (Ward Hill, USA).

Stable isotope labelled $d^{13}C_2$-caffeic acid was purchased from Orphachem S.A. (Clermont-Ferrand Cedex, France). All other available standards (mainly phenolic and chlorogenic metabolites) were synthesized as described previously [19].

2.2. Standard solutions

Stock solutions (named A–F) were prepared in methanol or water:acetonitrile (1:1) as given in Table 1. These were combined and diluted to obtain the working solution for instrument parameter optimization (10 μg/mL per each analyte) and the solution to optimize the chromatography (1 μg/mL per each analyte).

Internal standard $d^{13}C_2$-caffeic acid was dissolved in pure methanol to obtain 1 mg/mL of working solution.

2.3. Subject characteristics

Nine healthy subjects (four male, five female) were recruited for this study. Subjects were 34 ± 7 years of age, weighed 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 were 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 were 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 weeks or currently participating or having participated in another clinical trial during the last 3 weeks prior to the beginning of this study.

2.4. Study design

The original protocol was a 4-treatment crossover controlled study. Three of the treatments were considered for other objectives and will not be discussed in the present paper. One week prior to the first treatment, BMI was measured. Twenty four hours prior to treatment until the end of the sampling period, the ingestion of coffee, tea, cola, alcohol, whole grain cereal (white bread allowed) or any medication was not allowed. Only water could be drunk during the night and in the morning before the treatment. Subjects arrived fasted early in the morning at the metabolic unit. Baseline blood was sampled, and then subjects received 400 mL of instant coffee. Blood was collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 11, 12 and 24 h after drinking the beverage. A standard lunch and dinner were provided at the metabolic unit. Water was available *ad libitum*. Twenty four hours after receiving the treatment, a final blood sample was taken to assess return to baseline. From blood samples, EDTA plasma was collected and stored at −80 °C.

2.5. Sample preparation

Plasma samples of one subject (14 timepoints) were thawed, vortexed for homogenization and 400 μL was added dropwise to 1000 μL of acetonitrile (ACN) (containing 2 mg/mL of ascorbic acid and 100 ng/mL of $d^{13}C_2$-caffeic acid as internal standard). The mixture was vortexed for 10 s, left for 5 min at room temperature and vortexed again for 10 s to allow protein precipitation. The samples were centrifuged at $2500 \times g$ (3290 rpm) for 10 min at 4 °C in a Sigma 3–16 K centrifuge. The supernatant was transferred into amber vials and evaporated under nitrogen flow. Before analysis, samples were reconstituted in 100 μL of solvent A (water, 1% acetic acid (v/v)) and 10 μL was injected into the UPLC–MS system.

2.6. UPLC

LC separation of phenolic acid and chlorogenic metabolites was achieved on a reversed-phase column HSS T3, 150 mm × 1.0 mm, 1.8 μm at room temperature using a Waters Acquity UPLC system. Mobile phase A was water containing 1% acetic acid, mobile phase B was methanol/acetonitrile (1:3, v/v). Final mobile phase gradient is summarized in Table 2.

Table 1
List of available compounds.

Analyte number	Analyte name	Molecular weight	Conc. stock solution [mg/mL]	Solution name
1	CA	180.16	1.00	A
2	DHCA	182.17	1.00	A
3	CA 3′-*O*-glucuronide	356.28	1.00	A
4	CA 4′-*O*-glucuronide	356.28	1.00	A
5	DHCA 3′-*O*-glucuronide	358.30	1.00	A
6	DHCA 4′-*O*-glucuronide	358.30	4.00	A
7	CA 3′-*O*-sulfate	260.22	1.00	A
8	CA 4′-*O*-sulfate	260.22	1.00	A
9	DHCA 3′-*O*-sulfate	262.24	1.00	A
10	DHCA 4′-*O*-sulfate	262.24	1.00	A
11	*p*-CoA	164.16	2.00	A
12	FA	194.18	1.00	B
13	iFA	194.18	1.00	B
14	DHFA	196.20	1.00	B
15	DHiFA	196.20	2.00	B
16	FA 4′-*O*-glucuronide	370.31	1.00	B
17	iFA 3′-*O*-glucuronide	370.31	1.00	B
18	DHFA 4′-*O*-glucuronide	372.32	1.00	B
19	DHiFA 3′-*O*-glucuronide	372.32	1.00	B
20	FA 4′-*O*-sulfate	274.25	1.00	B
21	iFA 3′-*O*-sulfate	274.25	1.00	B
22	DHFA 4′-*O*-sulfate	276.26	1.00	B
23	DHiFA 4′-*O*-sulfate	276.26	1.00	B
24	DMCA	208.21	2.00	B
25	DHDMCA	210.23	4.00	B
26	3-CQA	354.31	1.00	C
27	4-CQA	354.31	1.00	C
28	5-CQA	354.31	1.00	C
29	5-CQA 3′-*O*-sulfate	434.37	1.00	E
30	5-CQA 4′-*O*-sulfate	434.37	2.00	E
31	3-CQA 1,5 lactone	336.29	1.00	C
32	4-CQA 1,5 lactone	336.29	1.00	C
33	3-DMCQA	382.36	1.00	C
34	4-DMCQA	382.36	1.00	C
35	3-FQA	368.34	2.00	D
36	4-FQA	368.34	1.00	D
37	5-FQA	368.34	1.00	D
38	5-FQA 4′-*O*-glucuronide	544.46	1.00	E
39	5-FQA 4′-*O*-sulfate	448.40	1.00	E
40	3-FQA 1,5 lactone	350.32	1.00	D
41	4-FQA 1,5 lactone	350.32	1.00	D
42	3,4-diCQA	516.45	1.00	D
43	3,5-diCQA	516.45	1.00	D
44	4,5-diCQA	516.45	1.00	D
45	3-Caffeoyl 4-FQA lactone	512.46	0.85	F
46	3-DMC 4-FQA 1,5 lactone	540.52	1.00	D
47	3-Feruloyl 4-DMCQA 1,5 lactone	540.52	1.00	E
48	3-Caffeoyl 4-DMCQA 1,5 lactone	526.49	1.00	E
49	3-DMC 4-CQA lactone	526.49	1.00	F
50	3,4-diFQA lactone	526.49	1.00	F
51	d$^{13}C_2$-caffeic acid	183.15	1.00	A

2.7. Mass spectrometry

Mass spectrometry was performed on a Waters Synapt High Definition Mass Spectrometer (HDMS) in negative electrospray ionization (ESI^-) operating mode at a resolution of 10,000 full width at half maximum in V mode. A detailed description of the instrument can be found elsewhere [20]. Electrospray capillary voltage was set at 1.8 kV, source temperature at 120 °C, vaporizer temperature at 450 °C and cone voltage was 25 V. Desolvation gas (nitrogen) flow was set at 1000 L/h, trapping gas (argon) at 2.0 mL/min, while cone and source gas flows (nitrogen) were switched off.

Table 2
Applied UPLC gradient parameters for the elution of the phenolic and chlorogenic acids.

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate [μL/min]	Curve value
0	95	5	120	1
2	95	5	120	6
10	88	12	120	6
15	85	15	120	6
20	75	25	120	6
35	55	45	120	6
36	5	95	120	6
40	5	95	120	6
41	95	5	120	6
45	95	5	120	6

Calibration was performed every day by introducing sodium formate (composition of 2-propanol:water:formic acid: 0.1 M NaOH; 810:135:1:10 (v/v/v/v)) into the ion source via the Lock-spray assembly at a flow rate of 50 μL/min. The software MassLynx 4.1 (SCN version 639) was used to operate the mass spectrometer and the UPLC system.

For mass accuracy measurements, the full scan acquisition mode was selected. Trap and transfer collision energies (CEs) were set at 6.0 V and 4.0 V, respectively. Scan time was 1 s in an m/z range of 100–750. Centroid data was acquired with automatic accurate mass assignment using a lock mass of 262.9761 $[C_5H_7O_8Na_3-H]^-$,

corresponding to a sodium formate cluster from the calibration solution infused online.

For structural elucidation, the product ion scan mode was selected to obtain fragmentation pattern of each metabolite. In this case, the instrument was operated in enhanced duty cycle (EDC) mode with an EDC mass of 249 for phenolic acid derivatives and 427 for chlorogenic acid derivatives. Under these conditions, trap and transfer CEs were set up based on the optimized parameters obtained when infusing the available standards. Scan time was 1 s in an m/z range of 10–4000.

The selected resolution (for maximized sensitivity) enabled a calibration which delivered mass accuracy of better than 1 ppm RMS mass error for further measurements of the calibrant solution.

2.8. Mass accuracy measurements

Processed methods with defined parameters such as expected retention time (when standards available), chemical formulas of potential metabolites were created using the MetaboLynx software. Acquired data (in full scan acquisition mode) were processed with these methods. As a result, a list of potential metabolites was created where several informative data such as name of potential metabolite, mass error and retention time could be retrieved.

2.9. Relative quantification

After compound identification, all coffee metabolites were quantified using their accurate mass channel selected in the MetaboLynx method (mass window = 0.050 amu) based on the calibration curves of available standards taken as reference. All caffeic acid derivatives were quantified based on the CA calibration curve and their relative sensitivity factors towards CA. All CoA derivatives were quantified based on the *p*-CoA calibration curve and relative sensitivity factors of CA *O*-sulfates towards CA. All feruloylquinic acids (FQAs) were quantified based on the 3-FQA calibration curve and their relative sensitivity factors towards 3-FQA. All caffeoylquinic acid lactone (CQAL) derivatives were quantified based on 3-CQAL calibration curve and the relative sensitivity factors of 3-feruloylquinic acid lactone (FQAL) towards 3-CQAL. Due to the lack of commercially available standards, the concentration of CQAL sulfate was calculated using the 3-CQAL calibration curve and the concentration of FQAL sulfate and FQAL *O*-glucuronide were obtained using the 3-CQAL calibration curve and applying the 3-FQAL relative sensitivity factor.

Calibration curves of CA, *p*-CoA, 3-FQA and 3-CQAL were established applying the method of standard addition using blank human plasma, containing no analyte of interest. Each calibration point was then processed as described in Section 2.5 and analyzed in duplicate. Outliers (>15% of deviation and >20% of deviation for the Lower Limit Of Quantification (LLOQ)) were excluded from the calibration. Peak integrations were performed using the software QuanLynx and peak area (PA) ratios (peak area of analyte/peak area of internal standard).

3. Results and discussion

3.1. MS parameter optimization

The mass spectrometric behavior of all standards was studied using negative-ion ESI. Optimization of the mass spectrometric conditions was carried out by infusing the standard solutions individually and manually increasing the sample cone voltage (5 V increments) to achieve maximized sensitivity for the molecular ions $[M-H]^-$, while minimizing in-source collision induced dissociation (CID). Chromatography dependent mass spectrometric parameters such as desolvation gas flow rate, desolvation gas temperature, and cone gas flow rate were optimized stepwise, manually, by infusing standard mix solutions.

3.2. LC parameter optimization

The solvent mixture methanol/acetonitrile 1:4 (v/v) reported previously for analysis of methoxy-cinnamic acids [21] was further optimized to enable separation of the glucuronide, sulfate and quinic acid conjugates. Acids were tested to achieve low pH and acetic acid (1%) was selected, since it yielded better sensitivity compared to formic acid (data not shown), which is also consistent with the literature [22]. Ultimately, a gradient (Table 2) using acidified water (1% acetic acid) and methanol:acetonitrile 1:3 (v/v) was found to be the best performing system for the separation of the above mentioned analytes. Due to the number of metabolites, it was not possible to separate them all: compounds 14 and 20; 46 and 49 and 41 and 47 coeluted. A typical chromatogram for a mixture of 50 analytes is shown in Fig. 1 with the identities of the peaks given in Table 1.

This method was then applied to human plasma samples to profile their phenolic and chlorogenic acid content. Most phenolic acids yielded chromatograms with excellent accurate mass accuracy values (between 1 and 8 ppm mass errors, depending on the peak intensity of the analyte).

3.3. Extraction optimization

Several solvent combinations were tested for optimal extraction of all available analytes including acetonitrile and ethanol with different acidification levels in single and double protein precipitation. In the presence of MeOH, 5-CQA 4′-*O*-sulfate isomerized into 4-CQA 4′-*O*-sulfate and 3-CQA 4′-*O*-sulfate and 5-FQA 4′-*O*-sulfate isomerized into 4-FQA 4′-*O*-sulfate and 3-FQA 4′-*O*-sulfate (data not shown). This solvent was not pursued. To recover the maximum number and amount of compounds, acetonitrile was selected as the best compromise of the choice for organic solvents to remove the proteins. The presence of acid (formic or acetic acid) did not improve the extraction of analytes.

3.4. Identification of phenolic acid derivatives in human plasma

Recent investigations revealed the presence of sulfated and glucuronidated forms of phenolic acids after coffee consumption [18]. To better demonstrate the bioavailability of coffee phenolics, it is crucial to go further in the understanding of the metabolic fate of these compounds.

The strategy applied in the present work on the identification of phenolic acid metabolites was to determine the accurate mass of molecular ions using the MetaboLynx software, obtain their fragmentation pattern and perform co-chromatography with available reference standards. Besides the 12 previously reported phenolic acid derivatives [18,21], 10 additional metabolites, mainly reduced and/or sulfated and/or methylated forms of CA and CoA, could be detected and identified for the first time in plasma samples (without enzymatic treatment) of volunteers after ingestion of a-400 mL cup of coffee. Among these 22 metabolites, 5 could only be identified based on their accurate mass and co-chromatography data. The other 17 metabolites exhibit a significant MS signal to be able to perform CID fragmentation.

Metabolite M12 was chosen as an example for the identification and elucidation of the molecular structure. M12 eluted at R_t = 11.5 min. For each human plasma sample, a list of metabolites was created with their calculated accurate mass. For each metabolite, the related mass error (expressed in ppm) towards its theoretical mass was obtained by deduction of the theoretical mass from its calculated accurate mass. These results are summarized

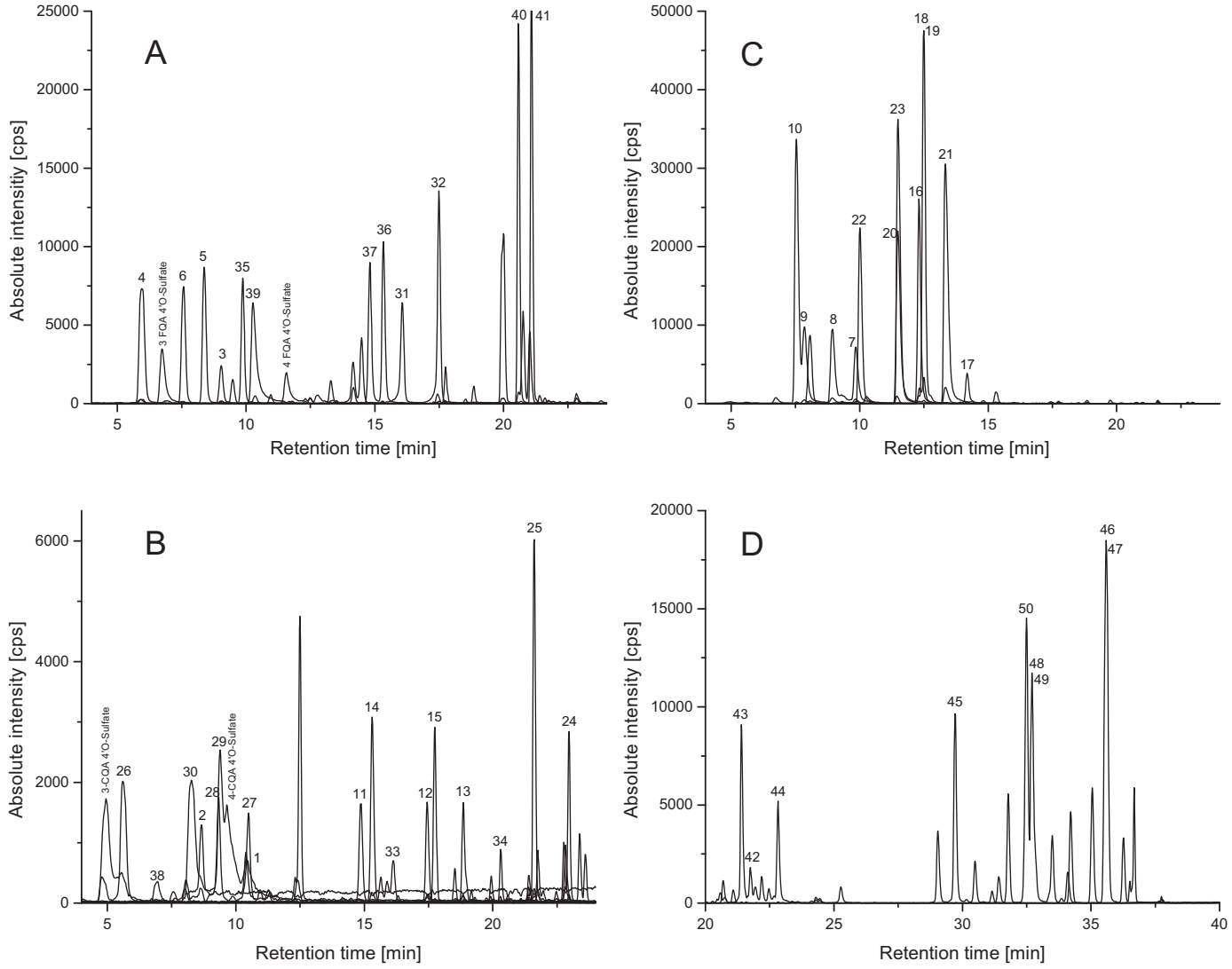


Fig. 1. Reference UPLC-accurate mass extracted ion chromatogram (EIC) for the separation of phenolic and chlorogenic acids. The ion chromatograms are presented in four separate windows (A–D) in order to facilitate visualization. Peak identity is given by numbers, which correspond to the analytes listed in Table 1. *m*/*z* window was set up at 0.1 Da.

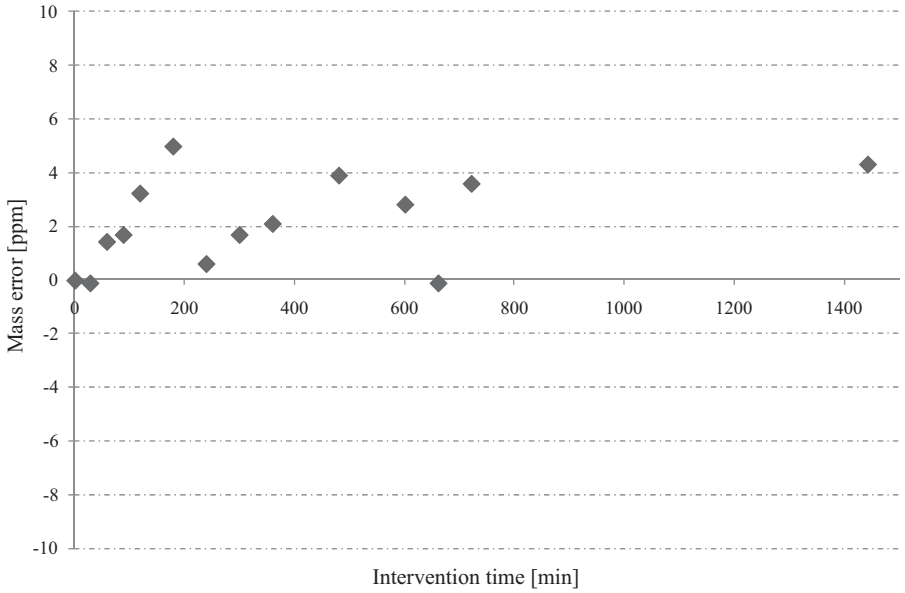


Fig. 2. Mass error of metabolite M12 for each timepoint of one human plasma time curve.

Table 3
UPLC–HRMS and UPLC–MS^2 of discovered metabolites in human plasma after coffee consumption. Listed fragment in bold is the major fragment in CID spectrum with information on the corresponding loss in brackets.

Metabolite ID	Retention time [min]	Observed ion accurate mass [*m*/*z*]	Averaged mass error [ppm]	Selected $[M-H]^-$ for fragmentation [*m*/*z*]	Main product ions [*m*/*z*]	Proposed metabolite identity	Remark
M1	17.4	193.0495	−5.7	193	–	FA	Traces
M2	18.7	193.0492	−7.3	193	–	iFA	Traces
M3	8.7	181.0510	2.2	181	**137 (CO_2 loss)**; 135; 121; 119	DHCA	
M4	15.2	195.0656	−3.2	195	**136 (CO_2 + Me loss)**; 135; 121; 119	DHFA	
M5	17.8	195.0664	0.5	195	151; 149; **136 (CO_2 + Me loss)**; 135; 134; 121; 119	DHiFA	
M6	22.9	207.0667	1.9	207	163; 148; 133; 131; **103 (CO_2 + double methoxy loss)**	DMCA	
M7	21.7	209.0827	3.8	209	–	DHDMCA	Traces
M8	8.9	258.9931	5.0	259	179; **135 (SO_3 + CO_2 loss)**; 134	CA 4′-*O*-sulfate	
M9	9.8	258.9934	6.1	259	179; **135 (SO_3 + CO_2 loss)**; 134	CA 3′-*O*-sulfate	
M10	7.5	261.0080	2.1	261	**181 (SO_3 loss)**; 137; 121; 119	DHCA 4′-*O*-sulfate	
M11	7.9	261.0083	3.4	261	**181 (SO_3 loss)**; 137; 135; 121; 109	DHCA 3′-*O*-sulfate	
M12	11.5	273.0081	2.3	273	**193 (SO_3 loss)**; 178; 149; 134	FA 4′-*O*-sulfate	
M13	13.4	273.0092	6.4	273	**193 (SO_3 loss)**; 178; 134	iFA 3′-*O*-sulfate	
M14	9.9	275.0241	3.6	275	**195 (SO_3 loss)**; 151; 136	DHFA 4′-*O*-sulfate	
M15	11.5	275.0219	−4.4	275	**195 (SO_3 loss)**; 193; 151; 149; 136; 135; 134; 119	DHiFA 3′-*O*-sulfate	
M16	8.5	357.0859	8.7	357	–	DHCA 3′-*O*-glucuronide	Traces
M17	12.4	369.0837	2.6	369	**193 (glucuronide loss)**; 178; 175; 134; 113	FA 4′-*O*-glucuronide	
M18	14.3	369.0837	2.8	369	–	iFA 3′-*O*-glucuronide	Traces
M19	12.6	371.0996	3.3	371	309; **195 (glucuronide loss)**; 175; 113	DH(i)FA *O*-glucuronide	
M20	7.5	242.9973	1.8	417	**163 (SO_3 loss)**; 119	*m*-CoA *O*-sulfate	
M21	8.6	242.9973	1.8	417	**163 (SO_3 loss)**; 119	*o*-CoA *O*-sulfate	
M22	9.8	245.0129	1.6	419	**165 (SO_3 loss)**; 121	*p*-DHCoAO-sulfate	
M23	9.8	367.1040	1.4	367	**193 (quinic acid loss)**; 191; 134; 117	3-FQA	
M24	14.8	367.1037	0.8	367	**191 (FA loss)**; 173; 85	5-FQA	
M25	15.3	367.1035	0.2	367	193; **173 (FA loss)***; 155; 134; 93	4-FQA	
M26	20.6	349.0918	−3.0	349	193; **175 (quinide loss)**	3-FQAL	
M27	21.1	349.0941	3.5	349	**175 (quinide loss)**; 160; 132	4-FQAL	
M28	16.0	415.0347	1.7	415	**335 (SO_3 loss)**; 161	CQAL *O*-sulfate 1	
M29	17.3	415.0349	2.1	415	**335 (SO_3 loss)**	CQAL *O*-sulfate 2	
M30	18.3	415.0340	−0.3	415	**335 (SO_3 loss)**; 161	CQAL *O*-sulfate 3	
M31	17.1	429.0516	4.5	429	**349 (SO_3 loss)**; 175	(i)FQAL *O*-sulfate 1	
M32	18.1	429.0507	2.2	429	**349 (SO_3 loss)**; 175	(i)FQAL *O*-sulfate 2	
M33	12.5	525.1261	2.1	525	**349 (SO_3 loss)**; 193; 175	(i)FQAL *O*-glucuronide 1	
M34	17.7	525.1223	−5.2	525	**349 (SO_3 loss)**	(i)FQAL *O*-glucuronide 2	

in Fig. 2. All obtained mass errors for one dedicated metabolite were then averaged so that we reported one averaged mass accuracy and one mass error per discovered metabolite, which is summarized in Table 3. M12 yielded a $[M-H]^-$ ion with averaged accurate mass at *m*/*z* = 273.0081. The CID product ion spectrum of the ion exhibited fragment ions at *m*/*z* = 193, *m*/*z* = 178, *m*/*z* = 149 and *m*/*z* = 134 as illustrated in Fig. 3. These fragment ions can originate from a consecutive loss of 80 amu corresponding to a sulfate group and/or loss of 44 amu corresponding to a carbon dioxide and/or loss of 15 amu corresponding to a methyl radical. Considering that fragment ions *m*/*z* = 193, *m*/*z* = 178, *m*/*z* = 149 and *m*/*z* = 134 are characteristic of FA, but not iFA, and that FA 4′-*O*-sulfate and iFA 3′-*O*-sulfate can be chromatographically separated, the identity of M12 could be unambiguously confirmed. Based on mass accuracy, co-chromatography with reference standard synthesized as described previously [19] and same product ion spectrum from reference standard, M12 was assigned to be FA 4′-*O*-sulfate with an averaged mass error = 2.3 ppm. The identical strategy (accurate mass of molecular ions combined with fragmentation pattern and co-chromatography data from reference standards) was applied for the identification of the other 21 phenolic acid metabolites. A detailed description on the identification of all metabolites can be found in the Supplementary files.

3.5. Identification of chlorogenic acid derivatives in human plasma

Recent investigations revealed the presence of few chlorogenic derivatives after coffee consumption [18].

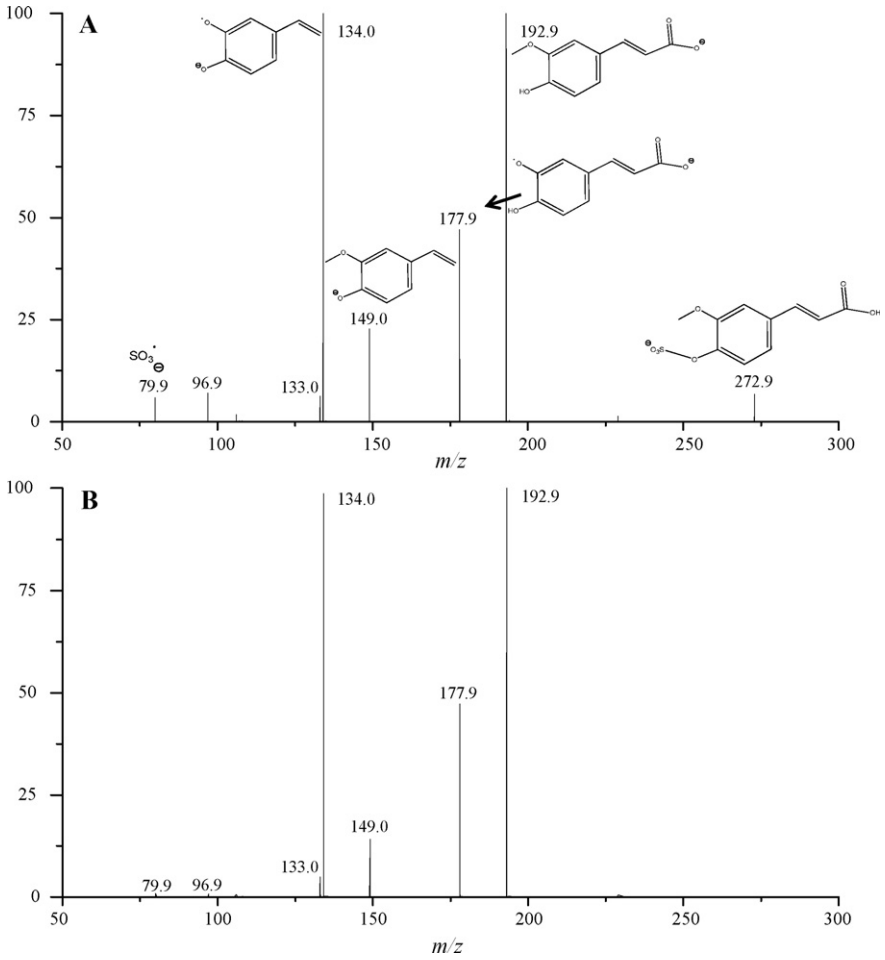


Fig. 3. CID product ion spectra of FA 4′-*O*-sulfate standard (A) and of metabolite M12 in human plasma (B).

12 chlorogenic acid derivatives mainly sulfated and/or methylated forms of CQAL, could be detected and identified. Among them 9 metabolites were identified for the first time in plasma samples (without enzymatic treatment) of volunteers after ingestion of a-400 mL cup of coffee.

M23, M24 and M25 were chosen as an example for the identification and elucidation of the molecular structure. M23, M24 and M25 eluted at R_t = 9.8 min, R_t = 14.8 min and R_t = 15.3 min, respectively. For each timepoint from the plasma time curve (if the metabolite was present), an accurate mass was calculated with the related mass error expressed in ppm. For each human plasma sample, a list of metabolites was created with their calculated accurate mass. For each metabolite, the related mass error (expressed in ppm) towards its theoretical mass was obtained by deduction the theoretical mass from its calculated accurate mass. These results are summarized in Fig. 2. All obtained mass errors for one dedicated metabolite were then averaged so that we reported one averaged mass accuracy and one mass error per discovered metabolite, which is summarized in Table 3. These three metabolites exhibited an averaged accurate mass between m/z = 367.1035 and m/z = 367.1040. M23 MS/MS fragmentation was dominated by the elimination of the quinic acid group leading to product ion at m/z = 193 (ferulic acid) whereas M24 MS/MS fragmentation was dominated by the elimination of the ferulic acid moiety leading to product ion m/z = 191 (quinic acid ion) which was also reported by Clifford et al. [23]. M25 yielded a major product ion at m/z = 173, characteristic of a conjugation on position 4 of quinic acid [23,24] and additional product ions at m/z = 193 (quinic acid loss), m/z = 155, m/z = 134 and m/z = 93.

These data matched with the one generated from the 3-FQA, 4-FQA and 5-FQA reference standards. Based on their specific fragmentation patterns, it was possible to discriminate and identify these three metabolites. Therefore, M23 was identified as 3-FQA (averaged mass error = 1.4 ppm), M24 as 5-FQA (averaged mass error = 0.8 ppm) and M25 as 4-FQA (averaged mass error = 0.2 ppm). The identical strategy (accurate mass of molecular ions combined with fragmentation pattern and co-chromatography data from reference standards) was applied for the identification of the 9 novel chlorogenic acid metabolites. A detailed description on the identification of all metabolites can be found as Supplementary information.

3.6. Relative quantification of identified coffee metabolites

Up to now, quantification of metabolites not commercially available was performed by using the aglycone form of the metabolite (e.g. CA calibration curve to quantify CA 4′-*O*-sulfate). Indeed, to quantify epicatechin (EC) metabolites in human plasma after cocoa consumption, Roura et al. [24] used (−)-EC calibration curves. To quantify EC and epigallocatechin (EGC) metabolites in human plasma after green tea ingestion, Del Rio et al. [25,26] used EC and EGC calibration curves, respectively. Performing the quantification of metabolites this way implies that the mass spectrometric (MS) responses of these compounds are identical.

Experiments were performed by analyzing blank human plasma spiked with all available standards at different concentrations (that underwent the sample preparation process) to assess their actual MS response. Relative sensitivity (MS response factor) of all

Table 4
Relative sensitivity factors of metabolites.

Analyte	Relative sensitivity	Remark
CA	1.00000	Reference
DHCA	1.67882	
CA 4′-*O*-sulfate	0.13720	
CA 3′-*O*-sulfate	0.38740	
DHCA 4′-*O*-sulfate	0.11989	
DHCA 3′-*O*-sulfate	0.09387	
DHCA 3′-*O*-glucuronide	0.20473	
FA	3.91610	
iFA	1.23990	
DHFA	0.61412	
DHiFA	1.71749	
DMCA	0.83841	
DHDMCA	0.41264	
FA 4′-*O*-sulfate	0.08881	
iFA 3′-*O*-sulfate	0.09968	
DHFA 4′-*O*-sulfate	0.10204	
DHiFA 3′-*O*-sulfate	0.10394	
FA 4′-*O*-glucuronide	0.25589	
iFA 3′-*O*-glucuronide	2.29134	
DHFA 4′-*O*-glucuronide	0.09522	
p-CoA	1.01613	
3-FQA	1.00000	Reference
4-FQA	1.21013	
5-FQA	1.35697	
3-CQAL	1.00000	Reference
3-FQAL	0.71163	
4-FQAL	0.37687	

metabolites was obtained by calculating: PA (reference analyte)/PA (metabolite), where the reference analyte was either CA, *p*-CoA, 3-FQA or 3-CQAL. All relative sensitivities (RS) for available standards are reported in Table 4 and metabolites listed below their reference analyte (e.g. all phenolic compounds had CA as reference standard). These results clearly indicate how misleading it would be to postulate that the MS response of, for example, CA 4′-*O*-sulfate would be the same as its aglycone caffeic acid. Indeed, in that case, the sulfated conjugate is ionized much more easily compared to its aglycone. Due to the presence of the sulfate group, this could lead to an over-estimation of the plasma concentration of the sulfated conjugate by a factor of 1/RS = 7.3 if the quantification was based on the aglycone calibration curve only. In other examples, an under-estimation of the metabolite concentration might occur. It was also noticeable that the presence of an additional hydroxyl group (comparison *p*-CoA and CA) did not affect the ionization process. Hence, the average of the relative sensitivity factors of CA 4′-*O*-sulfate and CA 3′-*O*-sulfate; and DHCA 4′-*O*-sulfate and DHCA 3′-*O*-sulfate was used to estimate the concentration of novel coumaric sulfate and dihydrocoumaric sulfate conjugates, respectively.

For quantification purposes, CA, *p*-CoA, 3-FQA or 3-CQAL were the selected reference standards for building calibration curves. The coefficients of linearity were $R^2 > 0.9970$ for CA, *p*-CoA and 3-FQA; and $R^2 > 0.9889$ for 3-CQAL. Lower limit of quantification (LLOQ) was at 37.5 nM for CA and *p*-CoA and 5.0 nM for 3-FQA and 3-CQAL. Averaged accuracy for these 4 reference standards varied between 100.0% and 101.1%. The relative quantitative data were obtained by applying the selected reference standard calibration curve and the metabolite relative sensitivity factor towards its reference standard.

3.7. Application to bioavailability

After ingestion of coffee beverage, chlorogenic acids undergo extensive metabolism in the human body [17]. The common approach applied for the quantification of coffee metabolites so far has been to quantify the aglycone forms of the metabolites by treating the plasma samples with an enzymatic cocktail containing β-glucuronidase, sulfatase and sometimes chlorogenic acid esterase as described by Renouf et al. [8], Guy et al. [12] and Monteiro et al. [27]. Knowing the metabolic fate of the conjugates in plasma will help understand their bioavailability and metabolism. The present paper reports the presence of more than 30 different circulating forms of chlorogenic acids metabolites upon coffee intake, among them 19 being identified for the first time. The plasma appearance of the 34 above mentioned coffee metabolites is shown in Fig. 4.

As demonstrated, the metabolized forms of coffee components in human plasma are mainly reduced and/or sulfated and/or methylated forms of phenolic acids and CQAL. Similar results were observed by Stalmach et al. [18]. Additionally in the present work, FQAL, sulfated and glucuronidated forms of FQAL and sulfated forms of CoA have been newly identified in human plasma (without enzymatic treatment) after coffee consumption. DMCA and DHDMCA [21] are also present, DMCA reaching its highest concentration at 30 min after coffee intake. The absence in human plasma of complex chlorogenic acids, such as diCQAs, did not match the findings of Farah et al. [15] but is in agreement with the findings of Stalmach et al. [18]. Conjugated CQA or FQA were not detected, which was also in agreement with the reported literature [18]. The absence of CQAs in human plasma is in agreement with the reported literature [16,17], confirming that they may be cleaved prior to absorption [9,10]. The presence of lactone derivatives in human plasma and their kinetic profile implies that they are markers of coffee intake. Indeed, significant amounts of CQAL were quantified in roasted coffee [15,28,29]. The presence of CGA lactone derivatives and sulfated/glucuronidated CGAL may suggest that this chemical group may facilitate its absorption. The first identification of 9 lactone derivatives in our study supports this hypothesis. Generally speaking, sulfation is the major conjugation reaction occurring in the human body as at least half of the metabolites of coffee phenolics present in human plasma are sulfated, which is also supported by in vitro studies [30].

All reported metabolites appear in two distinct patterns (Fig. 4). The first group of metabolites appeared in plasma early after coffee ingestion (T_{max} between 0.5 h and 2 h), suggesting a rapid absorption and metabolism (glucuronidation or sulfation by the intestine/liver [30]). Absorption in the upper part of the small intestine or the stomach could explain why metabolites appear so quickly in plasma. The second pattern of metabolites (reduction) appears much later (T_{max} between 8 and 12 h). This has also been shown by Stalmach et al. [18], Renouf et al. [8] and Guy et al. [12]. Stalmach et al. [18] reported T_{max} of reduced forms of metabolites to be at around 5 h with volunteers ingesting 200-mL serving of instant coffee while our findings demonstrate a T_{max} of reduced forms of metabolites to be at 10–12 h with volunteers ingesting 400-mL serving of instant coffee. The late plasma appearance of reduced forms of the phenolic acids emphasizes the important contribution of the colonic microflora in the metabolism of chlorogenic acids. DHFA appeared as a major coffee metabolite detected in human plasma reaching a C_{max} higher than 800 nM followed by CA 3′-*O*-sulfate, DMCA and DHFA 4′-*O*-sulfate with a C_{max} around 300 nM. The high plasma abundance of DHFA deserves further investigation for bioefficacy testing. In addition, compounds absorbed in the small intestine and excreted in the bile also contribute to that second wave of coffee polyphenols as they are most likely metabolized and re-absorbed in the colon (enterohepatic circulation, so that one compound can appear more than once in plasma under different potentially bioactive forms). These findings, taken altogether, show the great complexity of polyphenol absorption and metabolism. Their chemical structure is shown in Fig. 5.

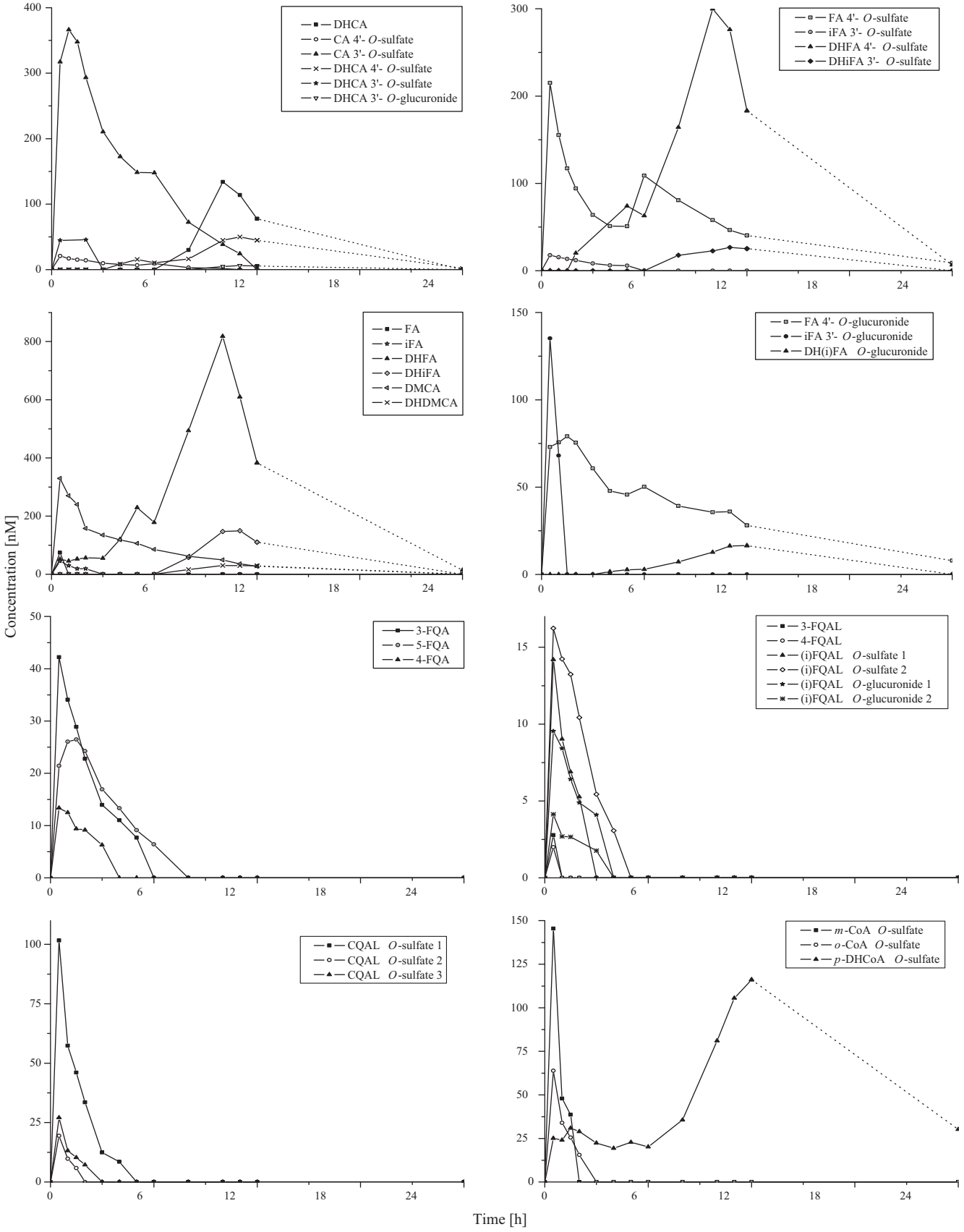
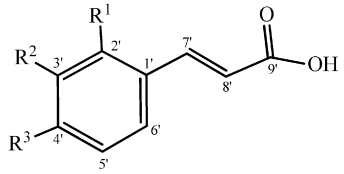
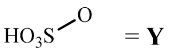
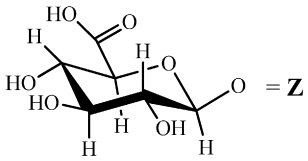


Fig. 4. Plasma kinetic profile of coffee metabolites in human plasma upon coffee intake.

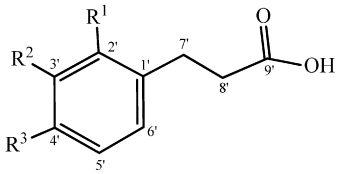




= **Y**

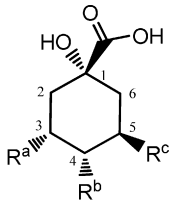


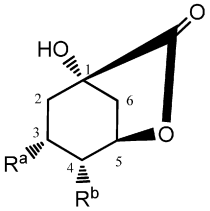
= **Z**



M20: R^1 = Y, R^2=R^3=H: *o*-CoA *O*-sulfate
M21: R^1=R^3=H, R^2 =Y: *m*-CoA *O*-sulfate
M1: R^1=H, R^2=OCH3, R^3=OH: FA
M2: R^1=H, R^2=OH, R^3=OCH_3: iFA
M6: 1:R^1=H, R^2=R^3=OCH_3: DMCA
M9: R^1=H, R^2=Y, R^3=OH: CA 3'-*O*-sulfate
M8: R^1=H, R^2=OH, R^3=Y: CA 4'-*O*-sulfate
M12: R^1=H, R^2=OCH_3, R^3=Y: FA 4'-*O*-sulfate
M13: R^1=H, R^2=Y, R^3=OCH_3: iFA 3'-*O*-sulfate
M17: R^1=H, R^2=OCH_3, R^3=Z: FA 4'-*O*-glucuronide
M18: R^1=H, R^2=Z, R^3=OCH_3: iFA 3'-*O*-glucuronide

M3: R^1=H, R^2=R^3=OH, : DHCA
M4: R^1=H, R^2=OCH_3, R^3=OH: DHFA
M5: R^1=H, R^2=OH, R^3=OCH_3: DHiFA
M7: R^1=H, R^2=R^3=OCH_3: DHDMCA
M11: R^1=H, R^2=Y, R^3=OH: DHCA 3'-*O*-sulfate
M10: R^1=H, R^2=OH, R^3=Y: DHCA 4'-*O*-sulfate
M14: R^1=H, R^2=OCH_3, R^3=Y: FA 4'-*O*-sulfate
M15: R^1=H, R^2=Y, R^3=OCH_3: iFA 3'-*O*-sulfate
M16: R^1=H, R^2=Z, R^3=OH: DHCA 3'-*O*-glucuronide
M19: R^1=H, R^2=Z, R^3=OCH_3: DHFA 4'-*O*-glucuronide or
R^1=H, R^2=OCH_3, R^3=Z: DHiFA 3'-*O*-glucuronide
M22: R^1=R^2=H, R^3 = Y: *p*-DHCoA *O*-sulfate





M23: R^a=M1, R^b=R^c=OH: 3-FQA
M24: R^a=R^b=OH, R^c=M1: 5-FQA
M25: R^a=R^c=OH, R^b=M1: 4-FQA

M26: R^a=M1, R^b=OH: 3-FQAL
M27: R^a=OH, R^b=M1: 4-FQAL
M28: R^a=M9, R^b=OH: CQAL *O*-sulfate 1
M29: R^a=M8, R^b=OH: CQAL *O*-sulfate 2
M30: R^a=OH, R^b=M8: CQAL *O*-sulfate 3
M31: R^a=M12 or M13, R^b=OH: (i)FQAL *O*-sulfate 1 or
R^a=OH, R^b=M12 or M13
M32: R^a=OH, R^b=M12 or M13: (i)FQAL *O*-sulfate 2
R^a=OH, R^b=M12 or M13
M33: R^a=M17 or M18, R^b=OH: (i)FQAL *O*-glucuronide 1
R^a=OH, R^b=M17 or M18
M34: R^a=OH, R^b=M17 or M18: (i)FQAL *O*-glucuronide 2
R^a=OH, R^b=M17 or M18

Fig. 5. Chemical structures of coffee metabolites detected in human plasma as a consequence of coffee intake. The numbers correspond to Table 1 (lactone derivatives are only postulated forms).

4. Conclusions and outlook

As a conclusion, a versatile analytical method based on liquid chromatography–high resolution mass spectrometry enabling the accurate identification and the relative quantification of novel circulating coffee metabolites in human plasma was successfully developed. A total of 22 phenolic acid derivatives and 12 chlorogenic acid derivatives appeared in human plasma as a result of coffee consumption. Among them, 19 novel circulating coffee metabolites were identified for the first time in human plasma (without enzymatic treatment) such as feruloylquinic acid lactone, sulfated and glucuronidated forms of feruloylquinic acid lactone and sulfated forms of coumaric acid. The variety of metabolites also highlights the complexity of the human metabolic pathways that chlorogenic acids undergo. In this sense, the non-negligible abundance of (dihydro)coumaric acid *O*-sulfates highlights the need to consider these metabolites in future coffee bioavailability studies.

The approach described here provides an analytical basis for capturing a global picture of the metabolism of coffee components to help better understand their bioavailability in humans. This work also highlights the importance of having reliable reference standards to accurately quantify metabolites to avoid misleading results, which can occur when quantifying new metabolites using their aglycone equivalent calibration curve and will give more confidence in the absolute quantification of all these metabolites in further intervention studies.

Authors' statement of contributions

K.R., K.N., M.R., G.W. designed research; K.R. conducted research; C.S.M. provided essential reagents or materials; K.R., M.R. analyzed data; K.R., P.G., G.W., K.N., M.R. wrote the paper; K.R., M.R. had primary responsibility for final content: K.N., S.R. helped with the interpretation of data, discussion and consulting.

All authors read and approved the final manuscript.

Conflict of interest

All authors are employees of Nestec. SA, which fully funded the study presented in this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.050.

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