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Quantitative analysis of plasma caffeic and ferulic acid equivalents by liquid chromatography tandem mass spectrometry

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ABSTRACT

A validated method was developed for the simultaneous determination of the hydroxycinnamates caffeic acid (CA), dihydrocaffeic acid (DHCA), ferulic acid (FA), dihydroferulic acid (DHFA), and isoferulic acid (IFA) in human plasma as metabolites derived from coffee consumption. The method includes a protein precipitation step prior to enzymatic hydrolysis of the conjugated metabolites (sulfate, glucuronide, and/or ester) back to their aglycone forms. After liquid–liquid extraction, the reconstituted extract was analysed by high-performance liquid chromatography coupled to negative electrospray ionisation tandem mass spectrometry. Calibration curves were constructed from spiked human plasma samples in the range of 0–4800 nM for each of the targeted analytes. Two internal standards, 3-(4-hydroxyphenyl)-propionic acid (500 nM) and 1,3-dicaffeoylquinic acid (200 nM), were spiked at the beginning of the sample preparation and before analysis, respectively. Good performance data were obtained with limits of detection and quantification of the five hydroxycinnamates ranging between 1–15 nM and 3–50 nM, respectively. Within and between-days precisions were respectively calculated between 8–18% and 8–30% (at 50 nM added initially), between 6–9% and 6–12% (at 200 nM), and between 5–9% and 5–9% (at 500 nM). Precision calculated from different analysts ranged from 18% to 44% (at 50 nM), from 8% to 16% (at 200 nM), and from 4% to 8% (at 500 nM). Using this method, we determined plasma levels in humans and measured the efficiency of deconjugation using our enzymatic cocktail.

1. Introduction

Coffee contains phenolic compounds called hydroxycinnamates, consisting principally of chlorogenic acids (CGAs), a family of trans-cinnamic acids esterified with quinic acid. The CGAs found in green and roasted coffee beans consist mainly of the three isomers each of caffeoylquinic acids (3-, 4- and 5-CQAs), feruloylquinic acids (3-, 4- and 5-FQAs) and dicaffeoylquinic acids (3,4-, 4,5- and 3,5-diCQAs).

Human epidemiological studies have indicated potential health benefits associated with coffee consumption on chronic human diseases [1,2]. Antioxidants in coffee and in particular chlorogenic acids might be partially responsible for these effects. Natella et al. observed a significant 5.5% increase ($P < 0.05$) in plasma antioxidant activity in humans following a single intake of brewed coffee, further suggesting that coffee possesses antioxidant properties [3]. Other investigators have reported that chlorogenic acid (5-CQA) has anticarcinogenic effects in animal models [4,5] and inhibits LDL cholesterol oxidation *ex vivo* [6]. In addition, some of the hydroxycinnamates found in fruits, vegetables and grains but also in coffee, such as caffeic, ferulic, ρ-coumaric, have been shown to protect against the oxidation of human LDL particles *in vitro* [7–10], which is a key step in the formation of atherosclerotic plaques [12]. Using an LDL oxidation assay, Richelle et al. showed that phenolic compounds in coffee have antioxidant activity which varied depending on the coffee bean source and the degree of roasting [11]. The potential health benefits related to the consumption of antioxidant phenolic compounds in coffee may, therefore, have implications on public health. Oxidative stress is involved in the pathology of many diseases and coffee consumption has been associated with reduced risk of human diseases including Parkinson and Alzheimer's diseases, liver cirrhosis, diabetes type 2 and certain cancers (e.g. liver, colon) [1,2]. However, further human studies with coffee are required now in order to better understand the absorption, bioavailability and the production of metabolites with antioxidants activity *in vivo*.

In addition to the antioxidant and anticarcinogenic properties of hydroxycinnamate conjugates (caffeoylquinic, feruloylquinic, ρ-coumaroylquinic and dicaffeoylquinic acids) [13] and free hydroxycinnamates (i.e. caffeic, ferulic and ρ-coumaric acids) [14],

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coffee also contains alkaloids (i.e. caffeine and smaller amounts of its precursor, theobromine) [15], cafestol and kahweol [16], some Maillard reaction products (melanoidins) [17–19], and quinolactones (with the 3- and 4-isomers being the most abundant) [20,21]. These compounds have also been shown to possess various *in vitro* or *ex vivo* biological properties.

A few studies have investigated the metabolic fate of free hydroxycinnamates and related CGA esters in animal model systems. The issue of the bioavailability of chlorogenic acids is controversial mainly due to analytical limitations. Azuma et al. reported that after ingestion of 5-CQA by rats, metabolites in plasma were found in the form of glucuronide and/or sulfate conjugates of CA and FA [22]. Almost all ingested 5-CQA was recovered intact in the small intestine, indicating poor absorption. Similarly, previous studies established that 5-CQA was poorly absorbed in the human small intestine, but hydrolyzed by colonic microflora to CA and quinic acid [23], and subsequently was hydrolyzed to hippuric acid [24]. After oral ingestion of foods containing CGAs, only CA and FA along with their conjugated metabolites (glucuronides and sulfates) have been detected [25,26]. However, other authors have shown that chlorogenic acids (including diCQAs) were absorbed intact and were therefore bioavailable in humans [24,27,28]. Dupas et al. also showed a very limited but significant *in vitro* and *in vivo* absorption of CQA by Caco2 cells and rats, respectively, suggesting that absorption of intact chlorogenic acids may occur [29–31]. Moreover, the levels reached may be below limits of detection [29–31]. In human studies CQA was also detected in urine at low levels, further suggesting that minor part of it may be absorbed and excreted without structural modifications [25,28,32].

Several analytical techniques have been reported for the qualitative and quantitative measurements of CA, FA and their conjugates in humans or animals from plasma or urine samples after ingestion of CGAs. Liquid chromatography coupled to electrochemical or ultraviolet detectors have been described [26,33–35]. Alternatively, gas chromatography coupled to mass spectrometry detection has been also reported [24].

Ideally, the best approach will be to quantitatively measure all types (aglycone, sulfate, glucuronide, methyl or even double conjugations) of parents and metabolites present in plasma. This method could easily ends-up by monitoring more than 50 compounds but there are no currently proper standards in any lab for most of them. Moreover, trying to identify so many compounds simultaneously may result that a significant number of metabolites will fall under their LLOQ.

The present work describes a novel method to quantify CA, DHCA, FA, DHFA and IFA, from human plasma after a full enzymatic (glucuronidase, sulfatase, and esterase) cleavage to free glucuronide, sulfate and ester conjugated metabolites. These five compounds were analysed by LC-ESI–MS/MS using 3-(4-hydroxyphenyl)-propionic acid and 1,3-dicaffeoylquinic acid as internal standards.

2. Materials and method

2.1. Chemicals and reagents

Ethanol and 3-(4-hydroxyphenyl)-propionic acid (IS1) and dihydroferulic acid (DHFA) were supplied by Fluka (Buchs, Switzerland). CA, FA, IFA and DHCA were supplied by Extrasynthese (Lyon, France). Ferulic acid-4′-*O*-sulfate (FA-4′-SO_4), isoferulic acid-3′-*O*-sulfate (IFA-3′-SO_4), caffeic acid-4′-*O*-sulfate (CA-4′-SO_4), dihydroferulic acid-4′-*O*-sulfate (DHFA-4′-SO_4), dihydrocaffeic acid-4′-*O*-sulfate (DHCA-4′-SO_4), 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), 5-feruloylquinic acid (5-FQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), dihydroferulic acid-4′-*O*-glucuronide (DHFA-4′-gluc), ferulic acid-4′-*O*-glucuronide (FA-4′-gluc), isoferulic acid-3′-*O*-glucuronide (IFA-3′-gluc), dihydrocaffeic acid-4′-*O*-glucuronide (DHCA-4′-gluc), dihydrocaffeic acid-3′-*O*-glucuronide (DHCA-3′-gluc), caffeic acid-4′-*O*-glucuronide (CA-4′-gluc), caffeic acid-3′-*O*-glucuronide (CA-3′-gluc) were chemically synthesized from our NRC collection of reference compounds (under submission in a peer-reviewed journal). Methanol, sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), formic acid, hydrogen chloride, sodium chloride, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Acetonitrile ultra gradient HPLC grade was provided from JT Baker (P.H. Stehelin & Cie AG, Basel, Switzerland). 1,3-Dicaffeoylquinic acid (1,3-diCQA, IS2) was supplied by Chromadex (LGC Promochem, Teddington, UK). Sulfatase (from abalone entrails Type VIII, 20–40 units/mg) and β-glucuronidase (from *Escherichia coli* Type IX-A, 1,000,000–5,000,000 units/g) were provided from Sigma (Buchs, Switzerland). Chlorogenate esterase (*Aspergillus japonicus*, 15 units/g) was from Kikkoman Biochemicals (Tokyo, Japan). Deionised and distilled water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA, USA).

2.2. Standard solutions

Stock standard solutions were prepared individually by dissolving each analyte in methanol at a concentration of 100 μM. Further successive dilutions were made to prepare the spiking experiments and to optimise the MS/MS parameters.

2.3. Plasma sample

The clinical study was approved by ethical commission (Nestlé Ethical committee 07.15 MET). After 24 h of a wash-out period with diet free from any coffee, tea, cola, alcohol, or wholegrain cereals, the human volunteers arrived in fasted state at the metabolic unit. Human blood samples were collected in EDTA tubes at the following time points: 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8 and 24 h. At $t = 0$, the volunteer received an equivalent of 4 g instant coffee (containing 900 μM total chlorogenic acids) dissolved in 400 mL hot water and consumed a controlled diet (low in phenolic acid-containing foods) the entire day. Blood was centrifuged for 10 min at $3500 \times g$ (+4 °C). An aliquot of 1 mL of the supernatant was transferred into a 3 mL cryotube (several aliquots of 1 mL were made for each plasma sample). A 40 μL NaH_2PO_4 (0.4 M) buffer containing 20% ascorbic acid and 0.1% EDTA (pH 3.6) was added to each cryotube to prevent oxidation of the phenolic compounds. Samples were then sealed and frozen at −80 °C prior to analysis.

2.4. Sample preparation

An aliquot of 380 μL plasma sample was introduced in a 2 mL Eppendorf tube, in which 20 μL of IS1, solubilised in water/acetonitrile (95:5, v:v) containing 0.1% formic acid (to give a final concentration of 500 nM), was previously added. After adding three volumes of ethanol (1.2 mL), the vial was vortexed for 5 min at 775 g (IKA MS2 minishaker) and centrifuged for an additional 5 min at $17,500 \times g$ at 4 °C. The ethanol supernatant fraction was poured into a clean 2 mL Eppendorf tube. The protein precipitation procedure was repeated twice by adding one volume (400 μL) of ethanol. The pooled ethanol phases were dried under a nitrogen stream at room temperature (~2 h), then reconstituted with freshly prepared 400 μL of 50 mM sodium phosphate buffer (pH 7.0) containing an enzymatic cocktail of 1000 units of β-glucuronidase, 60 units of sulfatase and 0.1 unit of chlorogenate esterase. The sample was briefly vortexed (775 g) and incubated for 60 min min at 37 °C in a thermomixer (10 g, Eppendorf Thermomixer Compact).

At the end of the incubation, 42 μL of 1N HCl and 240 mg NaCl were added and briefly vortexed (775 g). A liquid–liquid extraction was realised four times by adding 800 μL of ethyl acetate, followed by a 5 min vortex at 230 g, centrifugation for 5 min at 3000 × *g* and collection of the ethyl acetate upper phases. The latter organic phases were dried under a nitrogen stream at room temperature. The dried extract was reconstituted with 400 μL of methanol/water/acetonitrile (20/76/4, v:v:v) containing 0.08% formic acid and vortexed for 5 min (230 g). After centrifugation at 17,500 × *g* for 5 min, the plasma extract was filtered on a 0.45 μm nylon membrane filter (VWR International AG, Dietikon, CH). An aliquot of 5 μL IS2 (to give a final concentration of 200 nM) was added to 95 μL filtered plasma extract in a LC vial and placed in the autosampler for LC-ESI–MS/MS analysis.

2.5. LC-ESI–MS/MS

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

2.6. Method

2.6.1. Calibration

Calibration curves were performed using three different human plasma samples fortified with our targeted analytes. Blood samples were taken and processed following the description mentioned in the above paragraph. The matrix-matched calibration curve was built by spiking each analyte (before sample preparation) at concentration levels of 0, 25, 50, 100, 200, 400, 800, 1600, 4800 nM (constant 500 nM for IS1 and of 200 nM for IS2 which was spiked before LC-ESI–MS/MS analysis). To evaluate the quantification results, the values obtained from these matrix-matched calibration curves were compared by plotting the analyte area against the concentration and the analyte area ratio (analyte/IS1) against the concentration.

2.6.2. Confirmation criteria

An analyte was considered as positively identified when the following criteria were met: (i) the ratio of the chromatographic retention time of the analyte to that of the same analyte in the spiked plasma sample were within ±2.5% tolerance; (ii) the presence of a signal at each of the two SRMs for the analyte (the use of two SRMs per compound counts for four identification points as defined by the EU Commission Decision 2002/657/EC and ensure the good selectivity of the method); and (iii) the peak area ratio of SRM2 against SRM1 was within the tolerance values fixed by the EU directives [36].

2.6.3. Extraction recovery

The recovery experiments were carried out at three concentrations (50, 200 and 500 nM) with six independent replicates. A human plasma sample was spiked at the very beginning of the sample preparation and the area obtained for each analyte was compared to the mean area ($n = 6$) calculated from the same human plasma extract, spiked at identical concentrations, but just prior to LC-ESI–MS/MS analysis.

2.6.4. Repeatability (r) and intermediate reproducibility (iR)

Accuracy and precision (within- and between-day) of free CA, FA, IFA, DHCA and DHFA were calculated from the analysis of six human plasma replicates fortified with all five analytes at each of the three specified fortification levels (50, 200 and 500 nM; 500 nM (IS1) and 200 nM (IS2)) and performed by the same operator on four separate occasions. Within-laboratory precision was obtained by following the same protocol but analyses were performed by two other operators in triplicate experiments on three separate occasions following the FDA guidance [37].

2.6.5. Detection (LOD) and quantification (LLOQ) limits

These two limits were calculated from the calibration curve data obtained from the three human plasma samples (see above paragraph) injected three times, and analysed in three distinct days over a week-period to average potential instrumental drifts ($n = 9$). The signal-to-noise ratio (S/N) at the expected retention times for each analyte was calculated (using Analyst software) from the non-spiked samples (to evaluate the presence of endogenous compounds) and at the lowest 25 and 50 nM fortification levels. LOD and LLOQ were then calculated from the median values of these set of data to meet S/N of respectively three and ten.

3. Results and discussion

3.1. Mass spectrometry

MS source parameters were evaluated for CA, FA, IFA, DHCA and DHFA, in positive and negative ionisation full scan mode. As these analytes contain an acidic group (Fig. 1), negative ionisation gave a better overall MS response for all the targeted compounds as deprotonated species. Collision induced dissociation (CID) experiments were carried out under various collision energies and two major SRMs were kept to monitor the chosen five analytes and to enable an unambiguous confirmation of the analytes. The most intense one (SRM1) was used for quantification while the second (SRM2) was for analyte confirmation based on an appropriate area ratio fitting within the expected percentage tolerances defined by the EU guidelines concerning the performance of analytical methods and interpretation of results [36]. Table 1 summarises the key MS/MS parameters used to monitor our five targeted analytes along with our two internal standards. Fang et al. reported the fragmentation

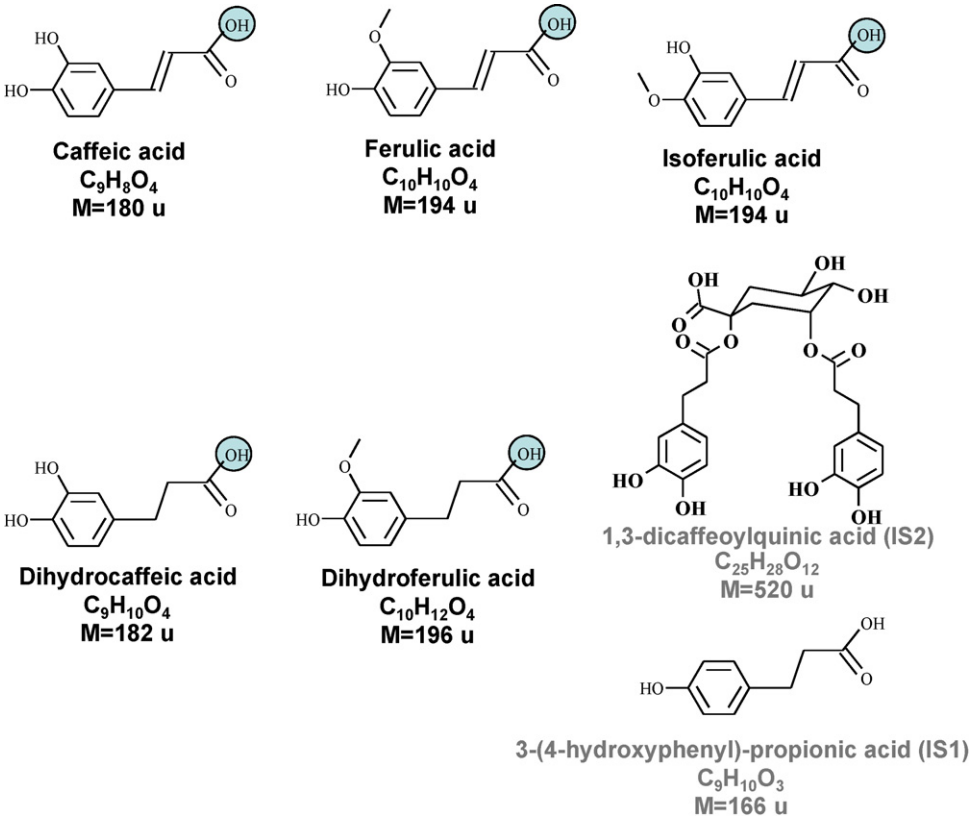

Fig. 1. Chemical structure of the aglycone chlorogenic acids.

patterns of CQAs, FQAs, diCQAs, CA and FA using LC-ESI–MS/MS in negative ionisation mode [38] and Clifford et al. completed this postulate by introducing a hierarchical key for identification of mono- and diacylchlorogenic acid compounds using an ion trap instrument [39]. Basically, a decarboxylation (loss of CO_2, 44 u) occurs directly from the deprotonated species of CA, DHCA and 3-(4-hydroxyphenyl)-propionic acid to give respectively *m/z* 135, *m/z* 137 and *m/z* 121 (corresponding to M-CO_2-H^-). Similar loss was also observed for FA, IFA and DHFA but after a demethylation (FA and IFA: *m/z* 178, DHFA: *m/z* 180) to give a radical anion species at respectively *m/z* 134, and *m/z* 136 (corresponding to [M-CH_3-CO_2-H]$^{\bullet -}$). In addition, *m/z* 89 was observed as major ion for CA and described by Matsui et al. to [M-CO_2-H_2O-CO-H]$^-$ [40]. 1,3-DiCQA loses either the quinic acid moiety to give a caffeic acid ion at *m/z* 179 or the 1,3-dicaffeoyl moieties to give a quinic acid ion at *m/z* 191.

3.2. Liquid chromatography

Adequate chromatographic separation is absolutely necessary to differentiate the isomers FA and IFA. Indeed, these two molecules fragment similarly and the two SRM transitions monitored (193 → 134 and 193 → 178) are identical. The only difference is in the MS/MS response where FA revealed a higher signal compared to IFA. Moreover, the SRM 193 → 134 was chosen to quantify FA whereas this transition was used to confirm the presence of IFA (less intense as compared to *m/z* 193 → 178). Several HPLC columns and solvents were evaluated to ensure an adequate chromatographic separation. A Zorbax SB-C18 column showed a good retention of all analytes and was kept for further optimisation. Ion pairing agent such as nonylfluoropentanoic acid was assessed to better separate the two isomers but the MS/MS response was highly suppressed even at a concentration of 1 mM. Formic and acetic acids were also evaluated at different percentages, and the latter gave more satisfactory results (analyte retention and MS/MS response) at 1% (v:v). This result is in agreement with Cremin et al., but we used acetic acid only in solvent A to provide both enough separation on the LC column and MS/MS response in negative ionisation mode [25]. By using ternary solvents, the gradient was rapidly optimised to meet a good compromise between enough separation and limited run time (19 min including re-equilibration time) for the analysis. Fig. 2 depicts the typical MS/MS traces observed

Table 1
SRM monitored for the chlorogenic acids with their area ratio (± allowed tolerances in absolute and percentage values).

Analyte	RT (min)	DP (V)	Quantification (SRM1)	CE (eV)	Confirmation (SRM2)	CE (eV)	Area ratio	LOD (nM)	LLOQ (nM)
Caffeic acid (CA)	7.3	−27	179 → 135	−22	179 → 89	−40	0.03 ± 0.02 (50%)	3	9
Dihydrocaffeic acid (DHCA)	6.5	−22	181 → 137	−17	181 → 59	−25	0.97 ± 0.19 (20%)	15	50
Ferulic acid (FA)	10.1	−24	193 → 134	−22	193 → 178	−18	0.65 ± 0.13 (20%)	1	3
Dihydroferulic acid (DHFA)	9.5	−28	195 → 136	−20	195 → 121	−28	0.39 ± 0.01 (25%)	4	12
Isoferulic acid (IFA)	10.7	−24	193 → 178	−18	193 → 134	−20	0.87 ± 0.17 (20%)	3	11
3-(4-Hydroxyphenyl)-propionic acid (IS1)[a]	8.4	−25	165 → 121	−17	165 → 59	−21	0.57 ± 0.11 (20%)		
1,3-diCQA (IS2)[a]	8.9	−51	515 → 191	−42	515 → 179	−39	0.72 ± 0.14 (20%)		

[a] IS1 was spiked (500 nM) at the beginning of the sample preparation, whereas IS2 (200 nM) was spiked prior to LC-ESI–MS/MS analysis.

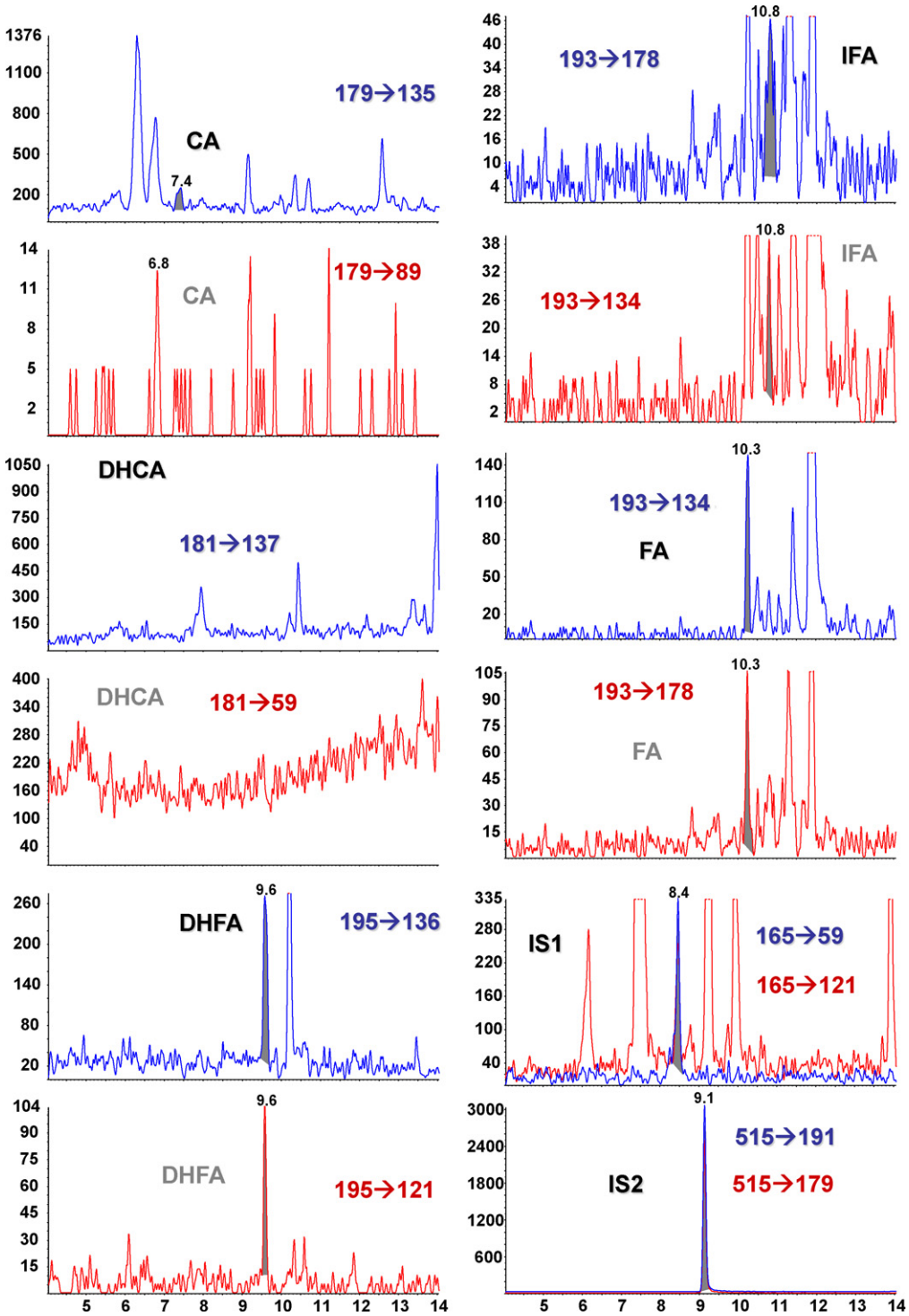

Fig. 2. LC-ESI–MS/MS chromatograms of a typical human plasma sample submitted to a 24-h dietary polyphenols restriction. IS1 (3-(4-hydroxyphenyl)-propionic acid) was added at the beginning of the sample preparation at a fortification level of 500 nM.

for a human plasma sample, where the volunteer followed a diet low in phenolic compounds 24 h before blood sampling. Such a chromatogram can be considered as a typical baseline level. Alternatively, Fig. 3 depicts the SRM traces of the five analytes (plus two internal standards) when the same human plasma sample was spiked with our targeted analytes at a concentration level of 50 nM. The elution order of our analytes was as follows: DHCA (6.5 min), CA (7.4 min), IS1 (8.4 min), IS2 (9.1 min), DHFA (9.6 min), FA (10.3 min), and IFA (10.8 min). As seen in Fig. 2, almost no signal was present for CA, IFA and DHCA, whereas a small amount of FA and DHFA was present (endogenous amount was estimated at 4 and 10 nM, respectively). After spiking the same plasma sample with 50 nM of each analyte (500 nM for IS1 and 200 nM for IS2), a suitable response was observed for all targeted analytes (Fig. 3), with a lower S/N ratio for DHCA.

3.3. Extraction of plasma sample

Part of the goal of this work was to develop an extraction method which released conjugated metabolites (sulfate, glucuronide and

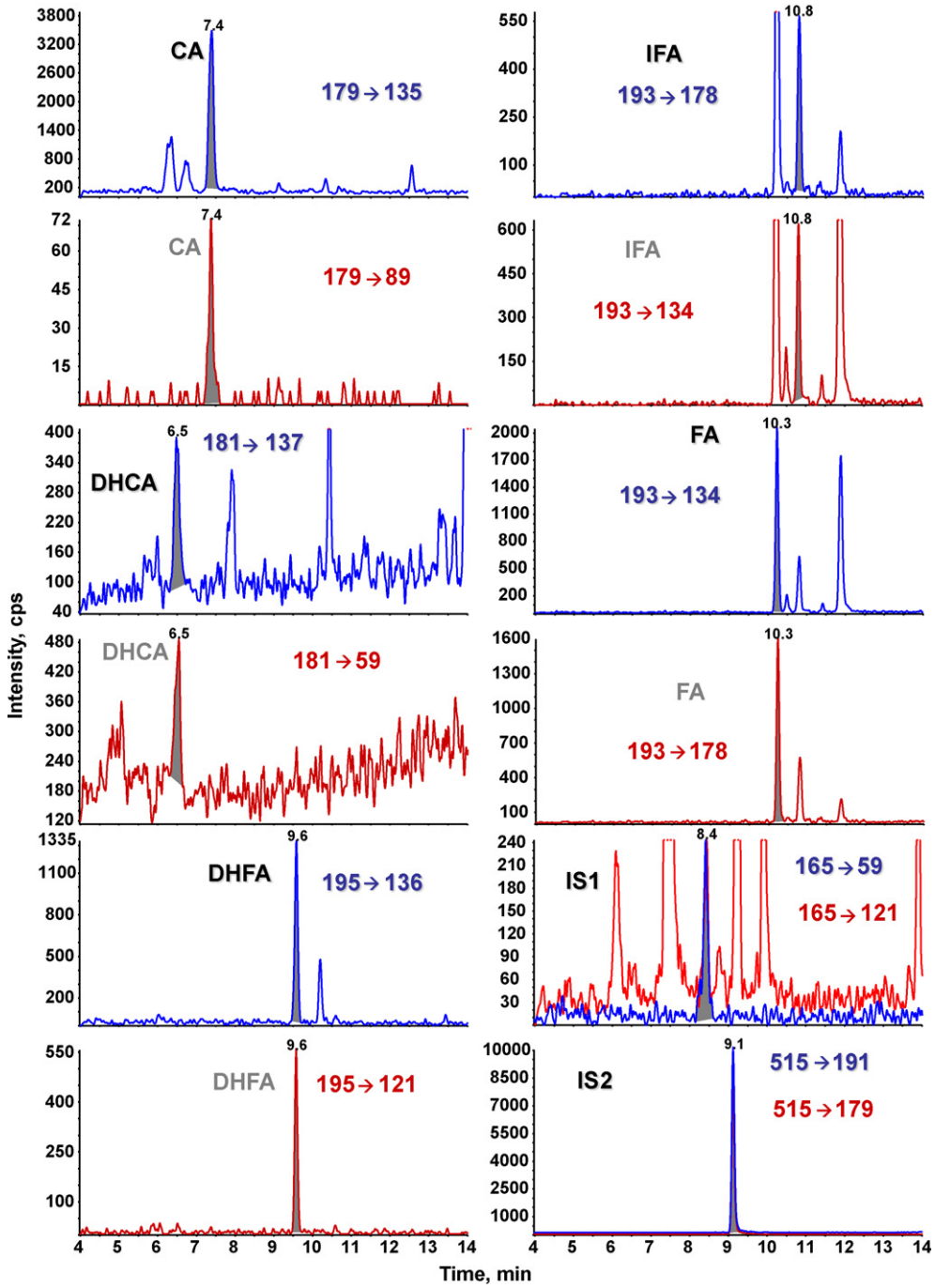

Fig. 3. LC-ESI–MS/MS chromatograms of a typical human plasma sample spiked at a concentration level of 50 nM for each analyte. In both cases, IS1 (3-(4-hydroxyphenyl)-propionic acid) was added at the beginning of the sample preparation (500 nM) and IS2 (1,3-diCQA) was added before LC-ESI–MS/MS (200 nM).

ester) using a novel enzymatic cocktail, and to measure the resulting DHCA, FA, IFA, CA and DHFA equivalents. Therefore, after the ethanol protein precipitation step, the hydrolysis conditions of β-glucuronidase, sulfatase and esterase were tested by evaluating different buffers (sodium acetate, sodium phosphate at 50 mM each), time/temperature parameters (30, 45, 60, 90, 120 min) and modulating various enzyme amounts (500 and 1000 units of β-glucuronidase, 10 and 60 units of sulfatase, 0.025 and 0.1 unit of chlorogenate esterase). The conditions were assessed by monitoring the respective areas of the aglycone peaks obtained from several plasma samples (no conjugated metabolites were available at this time of the work). In addition to these enzymatic digestions, a liquid–liquid extraction was also added as a clean-up step prior to LC-ESI–MS/MS analysis, as previously reported by Nardini et al. [33].

3.4. Method validation

3.4.1. Calibration curve

As no isotopically labelled internal standards were commercially available, a matrix-matched calibration curve in plasma was realised by adding the analytes at the right beginning of the sample preparation using the zero-time point (after a wash-out period of low phenolic acid-containing food). Several analogue molecules were evaluated for such purpose. Thus, 3-(4-hydroxyphenyl)-propionic acid was used as IS1 and spiked at the beginning of the

Table 2
Performance data of the LC-ESI–MS/MS method to measure chlorogenic acids in human plasma from spiking experiments of various aglycone metabolites.

Spiking level of 50 nM					
	CA	DHCA	FA	DHFA	IFA
Repeatability conditions					
Accuracy[a]	105 ± 4%	98 ± 16%	101 ± 10%	101 ± 4%	99 ± 4%
Within-day	8%	18%	16%	16%	8%
Between-day	8%	30%	18%	16%	8%
Intermediate reproducibility conditions					
Accuracy[a]	107 ± 13%	109 ± 20%	106 ± 13%	102 ± 8%	102 ± 12%
Within-lab.	30%	44%	28%	18%	28%
Spiking level of 200 nM					
	CA	DHCA	FA	DHFA	IFA
Repeatability conditions					
Accuracy[a]	100 ± 4%	101 ± 6%	102 ± 5%	101 ± 4%	100 ± 3%
Within-day	8%	9%	9%	7%	6%
Between-day	8%	12%	9%	7%	6%
Intermediate reproducibility conditions					
Accuracy[a]	100 ± 4%	101 ± 5%	102 ± 6%	99 ± 3%	100 ± 4%
Within-lab.	10%	12%	16%	8%	9%
Spiking level of 500 nM					
	CA	DHCA	FA	DHFA	IFA
Repeatability conditions					
Accuracy[a]	100 ± 4%	100 ± 3%	100 ± 5%	100 ± 4%	100 ± 3%
Within-day	7%	6%	9%	7%	5%
Between-day	7%	6%	9%	7%	5%
Intermediate reproducibility conditions					
Accuracy[a]	100 ± 3%	100 ± 3%	100 ± 3%	101 ± 2%	100 ± 2%
Within-lab.	8%	8%	8%	5%	4%

CA: caffeic acid; DHCA: dihydrocaffeic acid; FA: ferulic acid; DHFA: dihydroferulic acid; IFA: isoferulic acid.
[a] Accuracy values were calculated at a 95% confidence level.

sample preparation, whereas 1,3-dicaffeoyl quinic acid (IS2) was spiked before LC-ESI–MS/MS analysis. The latter IS was used to assess the potential drift of the LC-MS instrument over time and to ensure good data quality (both retention time and ion response). As example, the coefficient of variation obtained from the area (MS/MS response) of IS2 over 46 runs was calculated at 6%. As the chemical structure of IS1 differs slightly from the five CGAs to quantify, two calibration curves were built for each analyte either by plotting the analyte area against the concentration or the analyte area ratio (analyte *versus* IS1) against concentration. Indeed, the choice of an appropriate IS requires several controls (when isotopically labelled compounds are not available), as they may lead to erroneous quantitative results. From our experiments, the MS/MS response of IS1 was lower than IS2 (roughly 20-fold), although the spiking level was higher (2.5-fold). However, the coefficient of variation of the IS1 area was calculated at 10% ($n = 46$). The correlation coefficients obtained by plotting the analyte area and area ratio were compared to justify the appropriate IS. Therefore, 3-(4-hydroxyphenyl)-propionic acid gave satisfactory results as good linearity was observed for all analytes over the concentration range chosen and all correlation coefficients were improved when plotting the area ratio against the concentration.

3.4.2. Limit of detection (LOD) and lower limit of quantification (LLOQ)

These limits were calculated from three human subjects following a polyphenol-free diet prior to taking the blood sample. Very weak or almost no signals were observed in the non-spiked plasma samples, demonstrating that the one-day restriction without phenolic compounds minimised the presence of endogenous compounds. LOD and LLOQ were calculated from the non-spiked and spiked plasma samples at concentrations of 25 and 50 nM ($n = 9$ for each fortification level). The signal-to-noise (S/N) ratio of each analyte was calculated with the Analyst software at both 25 and 50 nM spiking levels. LOD and LLOQ values were then deduced to provide S/N ratio of 3 and 10, respectively (Table 1). The higher values obtained for DHCA may suggest that relatively higher ion suppression occur during the retention time of this analyte and lower recovery data compared to the other CGAs.

3.4.3. Repeatability and intermediate reproducibility

This set of experiments, described in Section 2, was performed for the five free aglycones. Table 2 summarises the performance data obtained on the repeatability (r) and iR for the free species. Good accuracy was obtained with r and iR ranging between 98–105% and 99–109%, and CVs below 20%. Within and between-days precisions were respectively calculated between 8–18% and 8–30% (at 50 nM added initially), between 6–9% and 6–12% (at 200 nM), and between 5–9% and 5–9% (at 500 nM). Precision calculated from different analysts ranged from 18% to 44% (at 50 nM), from 8% to 16% (at 200 nM), and from 4% to 8% (at 500 nM). The repeatability values ranged from 5.0 to 10.7 nM, and from 11.2 to 29.1 nM for the intermediate reproducibility values (lowest concentration level).

3.4.4. Recovery experiments

This set of experiments was realised by spiking a human plasma sample at three concentration levels (50, 200 and 500 nM) of each aglycone at the beginning of the sample preparation and prior to LC-ESI–MS/MS analysis. The recovery values were calculated from the area ratio of the former MS/MS trace (spiking experiments before sample preparation) *versus* the latter one (spiking before analysis) multiplied by 100. In such case, matrix effects are omitted reflecting only the true recovery of the sample preparation. The mean value

Table 3
Recovery values (expressed as median) after adding chlorogenic acids into human plasma measured by LC-ESI–MS/MS ($n = 6$ independent replicates for each spiking level).

	Spiking concentration		
	50 nM	200 nM	500 nM
CA	110 ± 12%	67 ± 5%	79 ± 10%
DHCA	56 ± 8%	44 ± 10%	102 ± 10%
FA	99 ± 14%	106 ± 7%	118 ± 16%
DHFA	107 ± 19%	96 ± 6%	114 ± 11%
IFA	67 ± 6%	56 ± 5%	63 ± 5%

and coefficient of variation obtained for each analyte is reported in Table 3.

Thus, the recovery values for CA, FA, and DHFA ranged between 99% and 110%, at the lowest fortification level, with CVs below 19%. DHCA and IFA gave lower recovery values of 56% and 67% respectively (50 nM spiking level), with CVs below 8%. Consistent data were also obtained when increasing the spiking level (except for DHCA at 500 nM).

3.5. Kinetic of CGAs in human plasma sample

To demonstrate the validity of our analytical method, one human subject was submitted to a one-day polyphenol-free diet before consuming a controlled diet and a single dose of coffee. Plasma samples were collected over 24 h. From the first time point (0 min), a matrix-match calibration curve was built on 8-points, in a concentration range of 25–4800 nM for each analyte (500 nM of IS1 and 200 nM of IS2). Two distinct replicates were carried out for each time point and analysed by LC-ESI–MS/MS. Fig. 4 depicts the concentrations observed for CA, DHCA, FA, IFA and

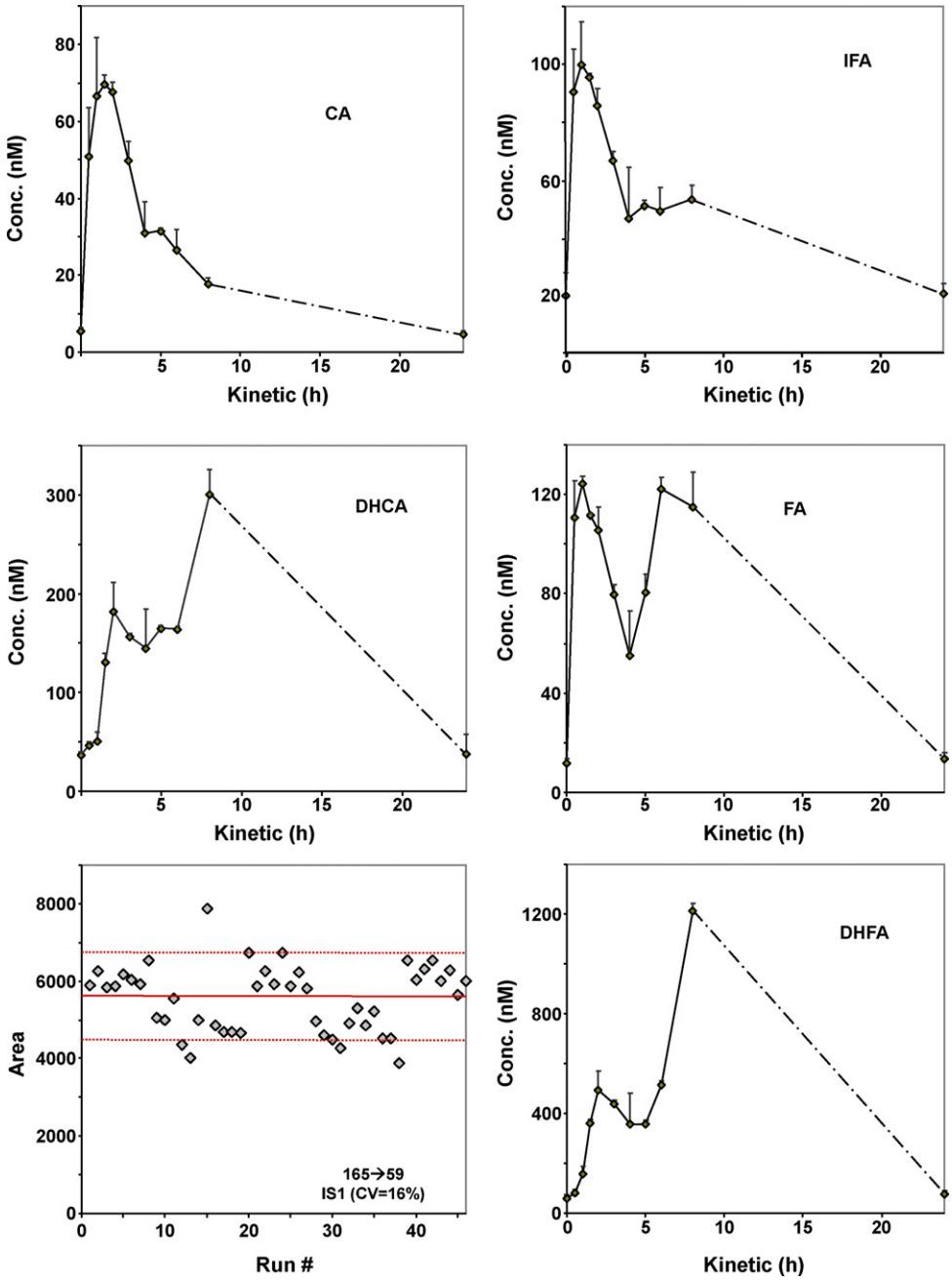

Fig. 4. Absorption of CGAs in plasma from a human volunteer over a 24 h period.

Table 4
Assessment of the enzymatic digestion conditions measured as recovery values (expressed as median) of the conjugated glucuronide, sulfate, and ester species. These data were obtained by dividing the peak areas (LC-ESI–MS/MS) of the conjugated compound added in human plasma at the beginning of the sample preparation against those obtained when spiking similar amount of the aglycone prior to the analysis ($n = 6$ independent replicates for each spiking level).

		Spiking level		
		50 nM	200 nM	500 nM
Glucuronidase				
Conjugated metabolite				
CA-3′-gluc, CA-4′-gluc[a]	CA	81 ± 5%	93 ± 8%	97 ± 7%
DHCA-3′-gluc, DHCA-4′-gluc[a]	DHCA	86 ± 5%	84 ± 8%	119 ± 8%
FA-4′-gluc	FA	93 ± 8%	107 ± 17%	104 ± 10%
DHFA-4′-gluc	DHFA	117 ± 6%	106 ± 3%	114 ± 4%
IFA-3′-gluc	IFA	75 ± 2%	90 ± 5%	89 ± 4%
Sulfatase				
Conjugated metabolite				
CA-4′-SO_4	CA	24 ± 2%	11 ± 1%	10 ± 2%
DHCA-4′-SO_4	DHCA	50 ± 19%	29 ± 3%	14 ± 2%
FA-4′-SO_4	FA	80 ± 10%	49 ± 6%	31 ± 9%
DHFA-4′-SO_4	DHFA	62 ± 11%	37 ± 10%	18 ± 4%
IFA-3′-SO_4	IFA	67 ± 9%	45 ± 6%	27 ± 5%
Esterase				
Conjugated metabolite				
3-CQA	CA	70 ± 22%	57 ± 1%	31 ± 5%
4-CQA	CA	40 ± 12%	53 ± 15%	49 ± 7%
5-CQA	CA	123 ± 7%	95 ± 19%	94 ± 10%
3,5-diCQA[b]	CA	62 ± 1%	45 ± 1%	33 ± 6%
5-FQA	FA	89 ± 1%	95 ± 17%	61 ± 2%

[a] For these experiments, the fortification level was 25, 100 and 250 nM each.
[b] 3,5-diCQA was spiked at a concentration of 25, 100 and 250 nM, respectively (to give ultimately 50, 200 and 500 nM of CA).

DHFA over time. The concentration *versus* time profiles were biphasic for all these compounds, with a rapid distribution phase and a slower terminal elimination phase lasting up to 24 h after coffee intake. This preliminary study indicated that more time points are required between 8 and 24 h to provide a full pharmacokinetic study. Wittemer et al. have reported similar biphasic profiles for CA, FA and IFA [26]. In our study, two maxima concentrations (C_{max}) were obtained for CA at 69.6 ± 2.4 nM (1.5 h) and at 31.6 ± 0.5 nM (5 h), for FA at 124.1 ± 3.2 nM (1 h) and at 114.8 ± 14.4 nM (6 h), for IFA at 99.5 ± 14.4 nM (1 h) and at 52.9 ± 4.6 nM (8 h). The biphasic behaviour of CA, FA and IFA suggest that some absorption takes place in the upper gut, with a cleavage of the caffeoylquinic acid ester bond. After entering the systemic circulation, CA conjugates are most likely methylated during the first liver passage as maximum concentrations of both FA and IFA were obtained after 1 h.

DHCA and DHFA revealed respectively 4- and 10-fold higher peak plasma maxima concentrations with 301.1 ± 24.3 nM and 1214.5 ± 26.9 nM. These maximal concentrations were observed after 8 h coffee intake; suggesting that beside the upper gut, the colon is another absorption site of caffeoylquinic acids resulting in different metabolites [23,26,41]. A full bioavailability and pharmacokinetic study is on-going to confirm these preliminary findings.

3.6. Assessment of the enzymatic hydrolysis

The method allowed us to evaluate the efficiency of the enzymatic digestion step, which is commonly used for this type of research. Such assessment is usually omitted due to lack of commercially available conjugated species. Here, several conjugated standards were chemically synthesized (Section 2) and added to plasma samples to ensure appropriate conditions for enzymatic hydrolysis. Thus, similar experiments as reported above for the aglycones were carried out at 50, 200, and 500 nM ($n = 6$ for each concentration level, cf. Section 2).

As a first set of experiments, CA-3′-gluc, CA-4′-gluc, DHCA-3′-gluc, DHCA-4′-gluc, FA-4′-gluc, DHFA-4′-gluc, and IFA-3′-gluc were added to a plasma sample at the beginning of the sample preparation. Recovery values after incubation with glucuronidase were calculated by dividing the peak areas obtained for the released aglycones *versus* those obtained when adding similar amounts of the aglycones prior to LC-ESI–MS/MS analysis (data were not normalised against the analogue IS used to provide more realistic values). Similarly, the hydrolytic efficiency of sulfatase was checked under identical conditions by adding CA-4′-SO_4, DHCA-4′-SO_4, FA-4′-SO_4, DHFA-4′-SO_4, and IFA-3′-SO_4. Finally, ester metabolites (3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, and 5-FQA) were used to assessed the performance of the esterase activity.

Table 4 summarises the recovery data obtained under these conditions. Regarding the glucuronidase efficiency, the recovery values ranged between 75% and 117%, with CVs below 8% (at the 50 nM spiking level). It is worth noting that similar data were obtained when increasing the spiking levels, with consistent CV values.

The efficiency of both esterase and sulfatase were not as high as those observed for the aglycones and glucuronide conjugates. Indeed, the recovery values obtained for the sulfate conjugates ranged from 24% to 80% at the lowest concentration levels (50 nM) with CVs up to 19% (for DHCA). This relatively high CV may be partly explained by the fortification level at the LLOQ value for such analyte (LLOQ: 50 nM for DHCA) and/or link to instrument fluctuations. These values decreased when increasing the concentration levels for all the tested compounds, while showing lower CV values (below 9% at a spiking level of 500 nM).

The recovery data for the ester compounds observed at a fortification level of 50 nM ranged between 40% and 123% (with CVs calculated up to 22% for 3-CQA). It is worth mentioning that the isomer position of caffeoylquinic acid showed a different hydrolysis rate with the esterase (5-CQA: 123% > 3-CQA: 70% > 4-CQA: 40%). Apart from 4-CQA and 5-CQA, the recovery values decreased when increasing the spiking level. This findings suggest that additional amounts of enzyme would be required and/or with modification/optimisation of the reaction conditions (buffer, concentration, pH). Compared to a single step hydrolysis conditions (pH 7) used in this work, a sequential enzymatic digestion (pH 7 followed by adjusting the pH at 5) would be a good alternative option to meet the optimal pH of esterase and β-glucuronidase first, before the sulfatase one. Very limited information is currently available to

assess hydrolytic efficiency of enzymes on conjugated metabolites. There are some examples where sulfate esters are resistant to sulfatase hydrolysis [42], while on the other hand certain phosphate esters can be substrates of sulfatase [43]. For most studies, the corresponding conjugated compounds are not available to ensure optimisation of appropriate enzymatic conditions. Overall, it is worth mentioning that even with relatively poor recovery values, the coefficients of variation were low (good repeatability).

4. Conclusion

This work describes a method for quantifying CA, DHCA, IFA, FA and DHFA from plasma samples using LC-ESI–MS/MS. Good performance data was obtained for the measurement of these analytes with LOD and LOQ values ranging from 1 to 15 nM and 3 to 50 nM, respectively. DHCA was less extractable from the plasma matrix and resulted in an overall 5-fold less sensitivity compared to most of the other analytes. This method was applied to plasma from human volunteers, submitted to a polyphenol-free diet 24 h prior to blood sampling, and the kinetics of absorption of CGAs measured after coffee intake. Adequate sensitivity was obtained on these preliminary measurements, which revealed a first biphasic absorption profile for all monitored analytes, followed by an increased absorption of DHCA and DHFA at a latter time. These data will serve to support further bioavailability and pharmacokinetic studies on chlorogenic acids in coffee.

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