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First identification of dimethoxycinnamic acids in human plasma after coffee intake by liquid chromatography–mass spectrometry

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ABSTRACT

There is a substantial amount of published literature on the bioavailability of various coffee components including the most abundant metabolites, caffeic and ferulic acids. Surprisingly, to date, the appearance of dimethoxycinnamic acid derivatives in humans has not been reported despite the fact that methylated form of catechol-type polyphenols could help maintain, modify or even improve their biological activities. This study reports an LC–MS method for the detection of dimethoxycinnamic acid in human plasma after treatment with an esterase. Liquid chromatography, including the combination of methanol and acetonitrile as organic eluent, was optimized to resolve all interferences and enable reliable detection and identification of 3,4-dimethoxycinnamic and 3,4-dimethoxy-dihydrocinnamic acids. In addition to the good mass accuracy achieved (better than 5 ppm), tandem mass spectrometric and co-chromatography experiments further confirmed the identity of the compounds. The optimized method was applied to analyze samples obtained immediately, 1 and 10 h after coffee ingestion. The results show that in particular 3,4-dimethoxycinnamic acid appears in high abundance (∼380 nM at 60 min) in plasma upon coffee intake, indicating that it is important to consider these derivatives in future bioavailability and bioefficacy studies.

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1. Introduction

Coffee is rich in polyphenols such as chlorogenic acids, and thus it is frequently studied in relation to various health benefits. Its consumption has been associated with decreased risk of endometrial cancer, especially among women with excessive body weight [1] and data from Inioue et al. also suggests that coffee consumption may reduce the risk of liver cancer [2].

Coffee's most abundant polyphenolic constituents are chlorogenic acids (quinic acid esters of hydroxycinnamates) accounting for 6–10% of coffee on a dry-weight basis [3]. Characterization of the composition of coffee beans by reversed phase liquid chromatography ultraviolet absorbance detection and electrospray ionization tandem mass spectrometry has been reported [4–6]. Occurrence of dimethoxycinnamic acid in coffee has been reported by Alonso-Salces et al. [4] and Andrade et al. [7], and the compound was shown to discriminate between different coffee varieties [7]. The relative amount of free dimethoxycinnamic acid was estimated to be less than 0.7 g/kg. Various conjugates of dimethoxycinnamic acids with quinic acid and coumaric acid were also confirmed by tandem mass spectrometry [5,6], but again in minor (∼0.2%) amounts on a dry mass basis.

Bioavailability of the major green coffee constituents in humans has been studied by Farah et al. [8], including quantification of chlorogenic and cinnamic acids in plasma and urine after ingestion of green coffee extract capsules. Metabolic pathways of hydroxycinnamates and ferulates were proposed by Stalmach et al. [9], including single methylation of the phenol group of the caffeic acid by catechol-O-methyltransferase (COMT). Note that in the case of phenolic acids, the term "methylation" can refer to methyl-esterification of the carboxyl group and methylation of the phenol group. In this study it refers to the methylation of the phenol group, unless stated otherwise. In general, methylation reduces the *in vitro* antioxidant function of polyphenols [10,11], however, its impact on biological activities of polyphenols remains largely unknown. There is emerging evidence that methylation could help maintain or even improve the biological activities of polyphenols while increasing their metabolic stability due to blocking the sites of glucuronidation and sulfation and thereby reducing their excretion rate [12–15]. Interestingly, while mono-methylation of caffeic acid by COMT has been reported in hepatic cells [16,17], Caco-2 cells [18,19] and in humans [9,20], the methylation of both hydroxyl groups to yield dimethoxy derivatives has not been observed. The latter has been

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explained by the *ortho*-diphenolic structure selectivity of COMT [17].

Conventionally, identification and quantification of chlorogenic acid metabolites is performed after protein precipitation induced by the addition of methanol. Then enzymatic treatment with β-glucuronidase and sulfatase enzymes is carried out. The resulting free phenolic acids are then extracted by ethyl acetate [21,22]. Liquid chromatographic separation of coffee phenolics is most commonly achieved on octadecylsilica stationary phases under reversed-phase conditions using highly acidic eluents such as 1 mM trifluoroacetic acid [18,21] or 4% formic acid [17]. Detection and subsequent quantification of phenolics is most often performed using UV–VIS absorbance [17,18]. Gas chromatography–mass spectrometry analysis including silylation has been also deployed to identify polyphenol metabolites including ferulic and dihydroferulic acid [23]. Atmospheric pressure ionization mass spectrometry is increasingly applied to identify metabolites of hydroxycinnamates using mainly electrospray ionization and various mass analyzers [9,17,18]. While semi-quantitative approaches based on caffeic- and ferulic acid equivalents have also been demonstrated [22], the lack of authentic standards hinders accurate calibration and absolute quantification of these metabolites.

In the present paper, we report the *in vivo* occurrence of double methylated caffeic acid (3,4-dimethoxycinnamic acid) and its dihydro-derivative (3,4-dimethoxy-dihydrocinnamic acid). Liquid chromatography combined with high resolution mass spectrometry has been optimized to detect these new metabolites with special emphasis on optimization of the organic modifier to eliminate false positives and use of tandem mass spectrometry for structural elucidation.

2. Experimental

2.1. Chemicals

HPLC grade water, methanol and acetonitrile were obtained from Chemie Brunschwig AG, Basel, Switzerland. Acetic acid (100%) was from Merck KGaA, Darmstadt, Germany. Phenolic acid standards (caffeic acid, ferulic acid, isoferulic acid, dihydrocaffeic acid, dihydroferulic acid) were purchased from Extrasynthese, Genay Cedex, France. 3-(4-Hydroxyphenyl)-propionic acid was obtained from Fluka, Buchs, Switzerland. 3,4-Dimethoxycinnamic acid, 3,4-dimethoxy-dihydrocinnamic acid and methylferulate were synthesized in-house using trimethylsilyldiazomethane obtained from Sigma–Aldrich, Buchs, Switzerland. Silylation for gas chromatographic analysis was carried out using N,O-bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane, 99:1 (v/v) obtained from Sigma, Buchs, Switzerland. The enzyme mixture used for liberating quinic-, glucuronic- and sulfate conjugates was 1000 units of β-glucuronidase (Sigma, Buchs, Switzerland), 60 units of sulfatase (Sigma, Buchs, Switzerland) and 0.1 unit of chlorogenate esterase (Kikkoman, Japan) in 400 μL of 50 mM sodium phosphate buffer (pH 7.0). The enzyme mixture used for treating the soluble coffee was 0.1 unit of chlorogenate esterase (Kikkoman, Japan) in 400 μL of 50 mM sodium phosphate buffer (pH 7.0).

2.2. Synthesis of 3,4-dimethoxycinnamic acids

3,4-Dimethoxycinnamic and 3,4-dimethoxy-dihydrocinnamic acids were prepared from ferulic and dihydroferulic acids, respectively. Ferulic acid (566 mg) was solubilised in a 250 mL flask using 50 mL methanol:diethylether 1:1. Then 10 mL 2 M trimethylsilyldiazomethane in diethylether was added to start the reaction. The flask was flushed with nitrogen, tightly closed and kept at 4 °C for 16 h. Then solvents and trimethylsilyldiazomethane were removed by evaporation under vacuum. The purity of synthesis for methyl 3,4-dimethoxy-cinnamate was estimated 99% by gas-chromatography–mass spectrometry. Obtained mass-spectrum of methyl 3,4-dimethoxy-cinnamate is shown in Fig. 1A. The methyl 3,4-dimethoxy-cinnamate was then saponified by dissolving the dry residue in 100 mL 0.2 M sodium hydroxide and heating at 70 °C under partial vacuum (699 mbar) for 3 h. The reaction was stopped by adding 100 mL 2 N hydrochloric acid. 3,4-dimethoxycinnamic acid was extracted using ethyl acetate/diethylether (1:1, v/v, 300 mL). The organic phase was washed 3 times with water (200 mL) and after drying over anhydrous sodium sulfate, the solvent was removed under vacuum. The purity of the residue obtained after saponification was assessed by GC–MS analysis after silylation. Briefly, 2 mg of the dry residue was dissolved in 0.4 mL pyridine and 0.1 mL silylation agent mix (see Section 2). The derivatization reaction was conducted at 70 °C for 40 min. The GC–MS analysis indicated an approximate purity of 98%. The mass-spectrum of the prepared di-trimethylsilyl ester derivative of 3,4-dimethoxycinnamic acid is provided in Fig. 1B. The total yield of the reaction based on weight recovery of the product and GC–MS analysis was estimated to about 80%.

Similarly, 3,4-dimethoxy-dihydrocinnamic acid was prepared from dihydroferulic acid using the same protocol. The analysis of the product obtained after saponification showed 3,4-dimethoxy-dihydrocinnamic acid (83%) as the main product of the reaction. Obtained mass spectra of methyl- and di-trimethylsilyl ester derivatives of 3,4-dimethoxy-dihydrocinnamic acid are shown in Fig. 1C and D.

2.3. Gas-chromatography–mass-spectrometry (GC–MS)

GC–MS analyses were performed to assess the yield of synthesis of 3,4-dimethoxycinnamic and 3,4-dimethoxy-dihydrocinnamic acids using a 6890N Agilent GC (Agilent Technologies, Palo Alto, CA) connected to an Agilent 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA). The separation was achieved using an HP-5 MS capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm, J&W Scientific, Palo Alto, CA). Splitless injection (1 mL/min for 1 min) was performed at 250 °C and the transfer line was heated at 280 °C. Oven temperature programming was the following: 70 °C isothermal for 1 min then increased to 190 °C at 20 °C/min and kept for 1 min, then heat to 260 °C at 10 °C/min and keep for 1 min. Finally heat to 350 °C at 30 °C/min and keep for 5 min. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Conventional electron ionization at 70 eV was used to generate ions, which were detected by scanning *m*/*z* 50–350 range at automatically selected scan speed.

2.4. Standard solutions

A stock solution of commercially available standards was prepared at 10 μg/mL in water:acetonitrile 7:3 containing 1 % acetic acid.

2.5. Study design

The original protocol was a controlled 4-treatment crossover study, which was approved by the ethical committee of clinical research of the University of Lausanne, Switzerland (Protocol reference 136/07). Three of the treatments were considered for other objectives and will not be discussed in the present paper. Twelve healthy subjects were recruited, from which ten subjects completed the study. Inclusion criteria included age 18–50 years, healthy and non-smokers, body mass index range of 18–25. One week prior to the first treatment, body mass index was measured. Twenty four hours prior to treatment until the end of the sampling

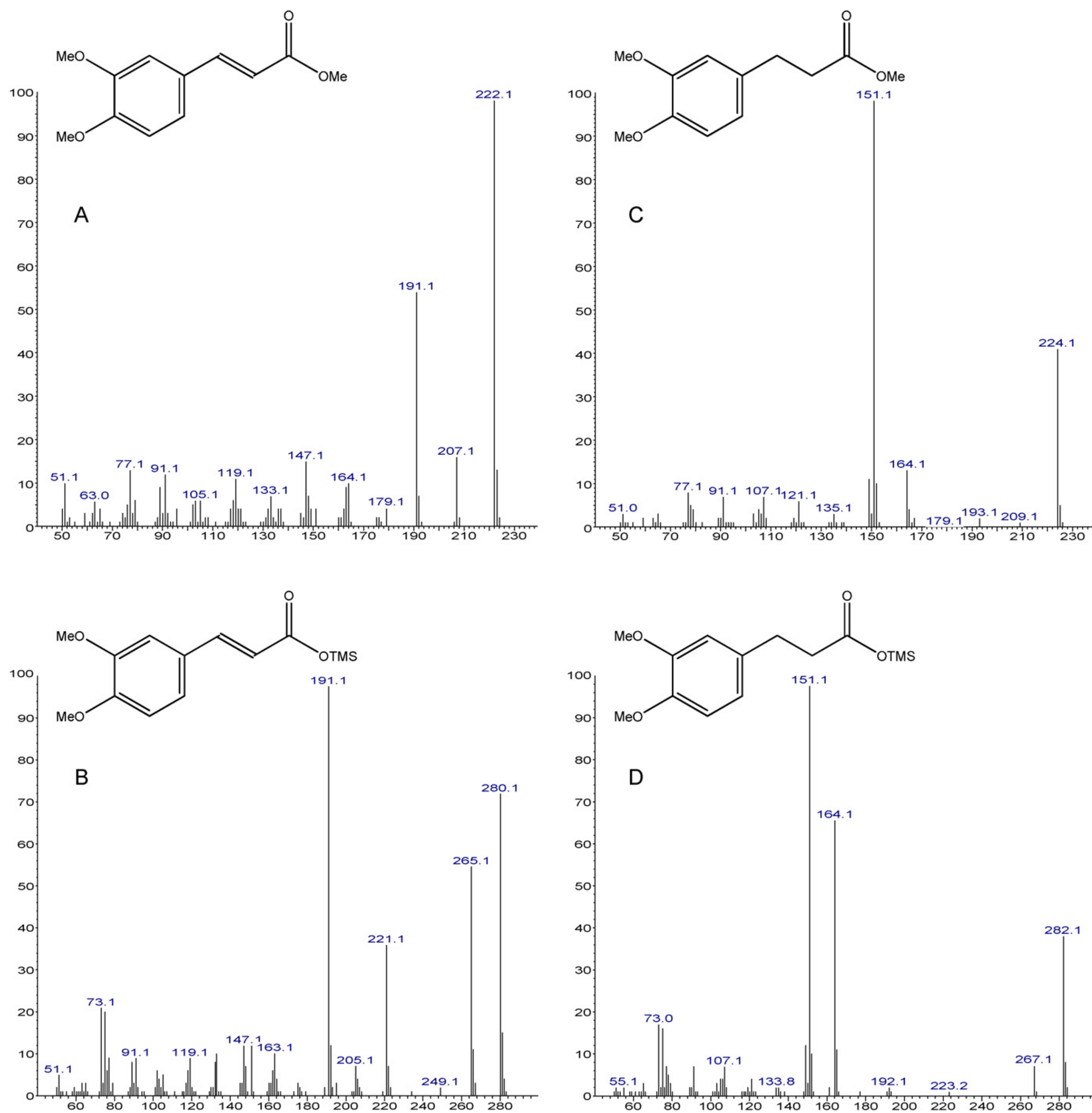

Fig. 1. Electron ionization mass-spectra of 3,4-dimethoxycinnamic acid methyl ester (A); di-trimethylsilylether derivative of 3,4-dimethoxycinnamic acid (B); 3,4-dimethoxy-dihydrocinnamic acid methyl ester (C) and di-trimethylsilylether derivative of 3,4-dimethoxy-dihydrocinnamic acid (D).

period, the ingestion of coffee, tea, cola, alcohol, whole grain cereal (white bread allowed) or any medication was not allowed. Baseline blood was sampled, and then subjects received 400 mL of 1% (w/v) commercially available soluble coffee. Blood was collected at 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*.

2.6. Sample preparation

Extraction of phenolic acids from plasma has been described elsewhere in detail [22]. In summary, 380 μL of plasma and 20 μL of 3-(4-hydroxyphenyl)-propionic acid (internal standard for other assays, 500 nM in water/acetonitrile (95:5, v:v) containing 0.1% formic acid) were precipitated with 3 volumes of ethanol. The ethanol supernatant was poured into a 2 mL Eppendorf tube. The protein precipitation procedure was repeated twice by adding 1 volume of ethanol and the pooled ethanol was dried under nitrogen flow at room temperature for ∼2 h. The dry residues were resuspended in 400 μL of enzyme mix described above. The samples were incubated for 60 min at 37 °C. At the end of the incubation, 42 μL of 1 N HCl and 240 mg NaCl were added. Liquid–liquid extraction with ethyl acetate (800 μL) was done four times and the pooled organic phases were dried under nitrogen. Directly before analysis,

Table 1
LC gradient parameters for the separation of phenolic acids.

Time [min]	Solvent A [%]	Solvent B [%]	Flow rate [μL/min]	Curve value
0	99	1	300	1
2	99	1	300	6
9.5	65	35	300	6
11.5	1	99	300	6
14.5	1	99	300	6
15	99	1	300	6
19	99	1	300	1

the samples were dissolved in 200 μL water:acetonitrile 7:3 containing 1% acetic acid and 10 μL was injected into the LC–MS/MS system.

Enzymatic treatment of soluble coffee was done by dissolving 10 mg coffee powder in 1 mL chlorogenate esterase solution. Incubation and extraction was performed as described above for plasma samples. Directly before analysis, the samples were dissolved in water:acetonitrile 7:3 containing 1% acetic acid to obtain a solution of 0.2 g/mL. 10 μL was injected into the LC–MS/MS system.

2.7. Liquid chromatography

LC separation of phenolic acids was achieved on an Acquity HSS T3 C18 column (1.8 μm, 150 × 2.1 mm, Waters: 186003534) at room temperature using a Waters Acquity UPLC system. Mobile phase A was water:acetic acid (99:1), mobile phase B was acetonitrile:methanol (4:1). The gradient is summarized in Table 1.

2.8. Mass spectrometry

Mass spectrometry was performed on a Waters Synapt High Definition Mass Spectrometer in negative Electrospray (ESI) mode at a resolution of 10,000 full width at half maximum (FWHM) in V mode. A detailed description of the instrument can be found elsewhere [24]. Electrospray capillary voltage was 2.5 kV, source temperature was 150 °C, vaporizer temperature was 400 °C. Desolvation gas (nitrogen) flow was 800 L/h, trapping gas (argon) was 1.5 mL/min, while cone and source gas flows (nitrogen) were switched off. The instrument was operated in enhanced duty cycle (EDC) mode with an EDC mass of 207. Scan time was 1 s in an m/z range of 10–4000, cone voltage was 20 V. Centroid data was acquired with automatic accurate mass assignment using a lock mass of 209.0044 $[C_5H_8O_6Na_2-H]^-$. Calibration was performed every day by introducing sodium formate (2-propanol:water:formic acid:0.1 M NaOH 810:135:1:10) into the ion source via the Lock-spray assembly at a flow rate of 30 μL/min. The software MassLynx 4.1 (SCN version 566/662) was used to operate the mass spectrometer and the liquid chromatograph.

2.9. Quantification

Calibration curves were constructed by analyzing samples spiked with known amounts (50, 150, 300, 600, 1200, and 2400 nM) of analytes. The obtained peak areas were plotted in function of analyte enrichment. Quantity of dimethoxycinnamic acids was assessed by comparing their peak area to that of an internal standard, in our case ferulic acid. The concentration of ferulic acid was known in all samples, since it was determined based on calibration curves (see Table 2). The responses of ferulic acid and dimethoxycinnamic acids were determined and compared in spiked plasma samples. The measured responses of 3,4-dimethoxycinnamic and 3,4-dimethoxy-dihydrocinnamic acids were 0.97 and 2.27 relative to that of ferulic acid. Based on these, the quantitation of dimethoxycinnamic acids in plasma samples was possible by comparing their peak areas to the peak area and concentration of ferulic acid.

3. Results and discussion

3.1. The role of the organic modifier in the detection of dimethoxycinnamic acids

Both high resolution mass spectrometry coupled with liquid chromatography and gas chromatography–mass spectrometry are common approaches for discovering new or unexpected metabolites. In our case, LC–MS and MS/MS were chosen to (1) obtain accurate mass information to associate the observed peaks with elemental composition; (2) perform tandem mass spectrometry to unambiguously assign product ions to precursor ions for structural elucidation; (3) avoid unexpected artifact formation caused by chemical derivatization. In cases where no or limited preliminary information is available about the analytes, application of multiple reaction monitoring (MRM, or selected reaction monitoring, SRM) is not possible. Consequently, the selectivity of such approaches is based on the chromatographic separation and the resolution of the mass spectrometer (single stage mode). The primary readout is the retention time and the accurate mass, the latter relying heavily on complete mass resolution of the analyte peaks from interferences. In the present work, optimization of the mass spectrometric conditions was carried out by analyzing the standard solutions at various sample cone voltage values (5 V increments) to achieve maximal sensitivity for the molecular ions $[M-H]^-$. The liquid chromatography gradient was optimized using water enriched by 1% acetic acid and acetonitrile as eluents to achieve separation of all compounds. Acetonitrile was selected as the organic component instead of methanol due to its lower viscosity. This latter point was particularly important, since the applied long column in combination with the small particle size resulted in pressures above 900 bars. Acetic acid was selected to achieve low pH, since it has been reported to yield better sensitivity compared to formic acid [25]. This method has been applied to human plasma samples to profile their phenolic acid content. Most phenolic acids yielded the expected peaks with excellent mass accuracy values (between 1 and 4 ppm mass errors). Caffeic acid, however, exhibited an unusual, truncated peak shape (Fig. 2B). The lack of signals between retention time 7.0–7.1 min implies that, in this region, the centroided accurate mass values are shifted outside the plotted m/z window. Indeed, when a wider window (m/z 0.5) is selected, the signals reappear in the form of a major peak; see Fig. 2B dashed line.

Table 2
Calibration slopes, linearity and detection limits obtained in human plasma matrix. Note that for dihydroferulic acid no detection limits could determined, since the blank plasma already contained this compound in substantial amounts. LLOD: lower limit of detection; LLOQ: lower limit of quantification.

Analyte	Slope	Linearity coefficient [R^2]	LLOD [nM]	LLOQ [nM]	Precision (%)
Caffeic acid	0.0867	0.9986	50	150	16
Dihydrocaffeic acid	0.0723	0.9985	100	300	25
Ferulic acid	0.1097	0.9996	50	150	20
Isoferulic acid	0.1242	0.9990	50	150	18
Dihydroferulic acid	0.2866	0.9992	–	–	–

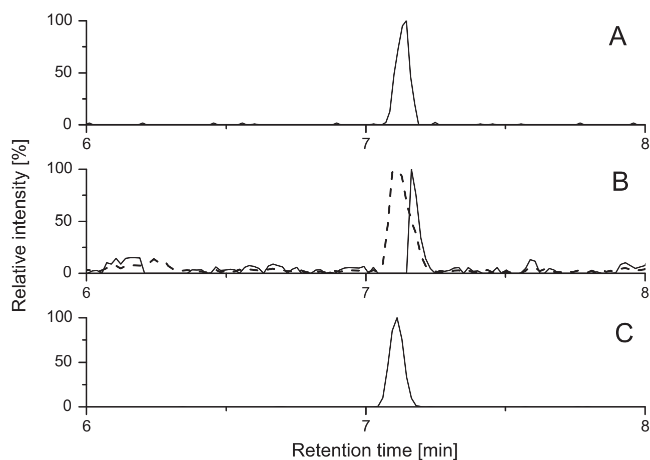

Fig. 2. Ion chromatograms of caffeic- and hippuric acid. Trace A depicts the *m*/*z* 179.0350 channel of caffeic acid standard (10 μg/mL). Trace B illustrates the interference between caffeic and hippuric acid by showing the *m*/*z* 179.0350 channel obtained from a spiked plasma sample. The continuous line depicts a truncated peak corresponding to a *m*/*z* 0.01 wide channel, while the dashed line represents the peak if extracted using *m*/*z* 0.05 width. Panel B thus embraces both the signals of caffeic acid (molecular ion) and hippuric acid (first isotope). Trace C depicts the *m*/*z* 178.0510 channel of hippuric acid in a plasma sample.

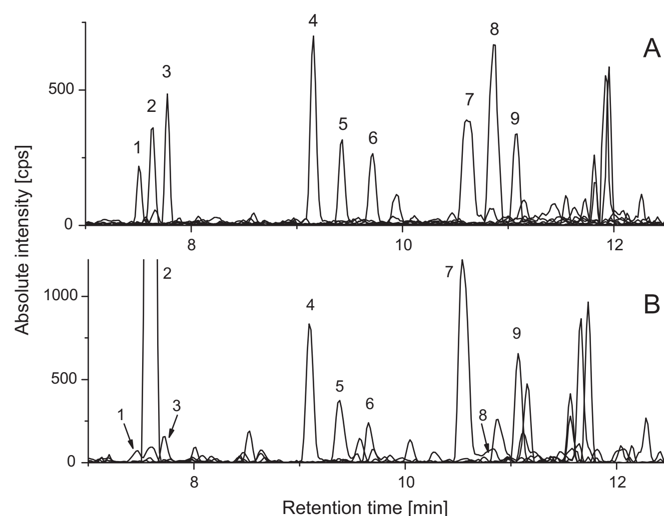

Fig. 3. RP-LC–MS chromatograms obtained from spiked (400 nM for each compound, A) human plasma sample and non-spiked plasma sample drawn at time 60 min in the intervention (B). The traces were obtained by extracting the following ion currents in an *m*/*z* 0.01 wide channel: 1 – dihydrocaffeic acid (*m*/*z* 181.0506); 2 – hippuric acid (*m*/*z* 178.0510); 3 – caffeic acid (*m*/*z* 179.0350); 4 – dihydroferulic acid (*m*/*z* 195.0663); 5 – ferulic acid (*m*/*z* 193.0506); 6 – isoferulic acid (*m*/*z* 193.0506); 7 – unknown interference with *m*/*z* 209.0796; 8 – 3,4-dimethoxy-dihydrocinnamic acid (*m*/*z* 209.0819); 9 – 3,4-dimethoxycinnamic acid (*m*/*z* 207.0663).

Scrutinizing the mass spectra reveals that the origin of this phenomenon is an abundant compound with *m*/*z* 178.0510 at 7.15 min (assigned as hippuric acid, an abundant component of both human plasma and urine). Independent mass measurement of the isotope peak of hippuric acid (*m*/*z* 179.0543) from the signals of caffeic acid (*m*/*z* 179.0350) would require a mass resolution of >15,000 FWHM. The high abundance of hippuric acid compared to caffeic acid further increases this number. Since the available resolution on the used instrument is ~10,000, this has two major consequences: (1) accurate mass values of caffeic acid are erroneous (~50 ppm); and (2) quantification of caffeic acid is not feasible since one half of the peak is masked. To resolve these issues, methanol was added to eluent B (methanol:acetonitrile 1:2) in order to alter the chromatographic selectivity. Using this solvent composition, complete resolution of caffeic acid and hippuric acid was achieved. The modified method has been applied to human plasma samples before and after coffee consumption. Proper integration of all peaks was possible and peaks of potential dimethoxycinnamic acids were detected at 11.02 (*m*/*z* 209.0794) and 11.25 min (*m*/*z* 207.0654). The peak at 11.25 min was tentatively assigned as 3,4-dimethoxy caffeic acid (mass error −4.3 ppm). The appearance of its spectrum was not affected by which region of the chromatographic peak was averaged (front or rear), suggesting that the peak represents one compound. The other peak at 11.02 min was assigned as 3,4-dimethoxy-dihydrocaffeic acid (mass error −12.1 ppm). The mass spectrum of this compound, however, did depend on which region of the chromatographic peak was averaged. This observation and the large mass error suggested that the peak represents two closely eluting compounds with similar molecular weights and unresolved MS signals. In order to separate these two compounds, the composition of solvent B was further optimized. Applying a ratio of methanol:acetonitrile 1:4, complete separation of the two compounds with the molecular ion of 209 was achieved (see peaks 8 and 9 in Fig. 3A). The same solvent composition and gradient also enabled resolution of all other phenolic acids investigated (Fig. 3A), therefore it could be applied for the samples obtained from the human study described in the Experimental section. Ion chromatograms obtained from a plasma sample drawn at time 60 min in the intervention are shown in Fig. 3B, depicting dihydroferulic acid (4), ferulic acid (5), isoferulic acid (6) and 3,4-dimethoxycinnamic acid (9) as the main peaks.

3.2. Confirmation of dimethoxycinnamic acids by co-chromatography and tandem mass spectrometry

In addition to the good mass accuracy (better than 5 ppm) obtained on the hypothetical dimethoxy derivatives, co-chromatography and tandem mass spectrometric experiments were carried out. The identical retention times and similar product ion spectra obtained (Fig. 4A and B) from standard solutions and plasma samples confirmed the identity of 3,4-dimethoxycinnamic acid. Possible ion structures corresponding to the major fragments are also postulated in Fig. 4. To provide more evidence that the methyl group is present as the methoxy and not the methyl-ester, the methyl ester of ferulic acid (isomer of 3,4-dimethoxycinnamic acid) was synthesized and analyzed by LC–MS. As expected, the methyl ester eluted later (11.60 min) than the methoxy-form according to its reduced acidity, thus complete chromatographic separation of the two isomers was achieved.

The low abundance of 3,4-dimethoxy-dihydrocinnamic acid did not allow obtaining its product ion spectrum from plasma samples. This compound was thus identified based on its accurate mass and retention time only.

3.3. Detection of dimethoxycinnamic acids in human plasma and soluble coffee

The optimized method was used to analyze plasma samples from ten healthy individuals after coffee consumption at three different time points: 0 min (baseline), 60 min and 600 min. The results show that dimethoxycinnamic acid and dimethoxy-dihydrocinnamic acid derivatives appear in human plasma after ingestion of coffee (Fig. 5). 3,4-Dimethoxycinnamic acid was present at the highest concentration at 60 min, at the same time as ferulic acid. 3,4-Dimethoxy dihydrocaffeic acid was present at the highest concentration at 600 min, at the same time as dihydroferulic acid. On the other hand, since the samples were treated with esterase, it is not possible to distinguish what proportion of these derivatives was in the free aglycone form or still esterified to quinic acid (as in the original coffee).

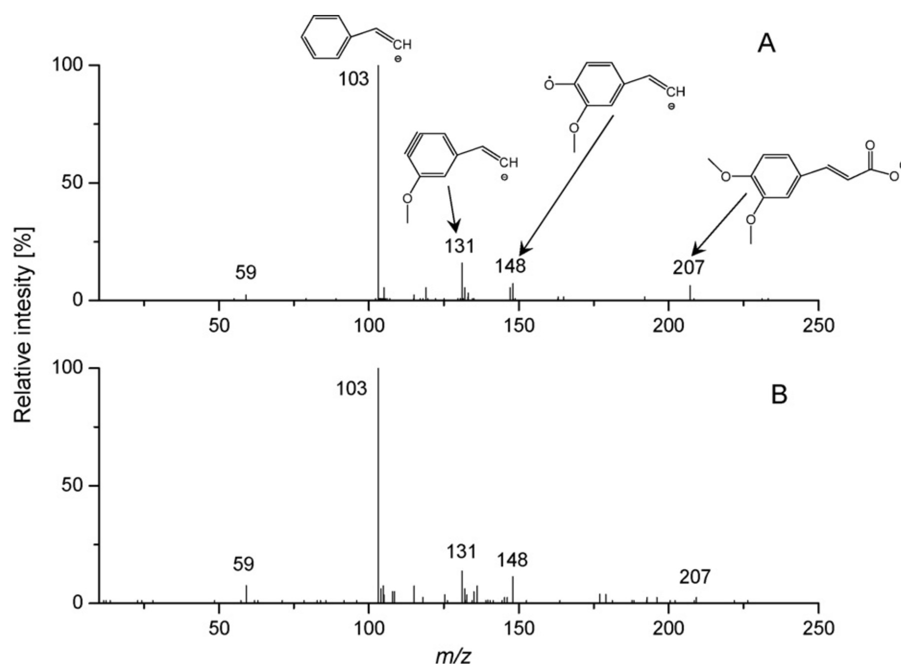

Fig. 4. Collision-induced dissociation product ion spectra of 3,4-dimethoxycinnamic acid. Panel A depicts the fragmentation pattern obtained from a standard solution (retention time 11.07 min), while panel B shows the spectrum of the postulated 3,4-dimethoxycinnamic acid obtained from human plasma (retention time 11.09 min).

There are several possible scenarios explaining the source of these compounds. One possibility is that ferulic (or caffeic) acid is methylated *in vivo* on the second hydroxyl group. This latter phenomenon has never been reported before, and the COMT enzyme is known to methylate only one hydroxyl group of catechols [9].

The other possibility could be that the dimethoxycinnamic acid derivatives are present already in the coffee itself and they are absorbed preferentially compared to ferulic/caffeic acid. In order to explore this latter possibility, the soluble coffee used for the intervention was enzymatically cleaved using an esterase to free phenolics from quinic acid esters and subsequently analyzed by LC–MS. The peak area of 3,4-dimethoxycinnamic acid was found to be approximately 3% of the peak area of ferulic acid. Based on the similar LC–MS response (see above), this translates into 3% relative amount of 3,4-dimethoxycinnamic acid compared to ferulic acid derivatives. This result is in accordance with the literature, since dimethoxycinnamic acid has indeed been reported in coffee beans, although in very low amounts [4–7]. The relative abun-

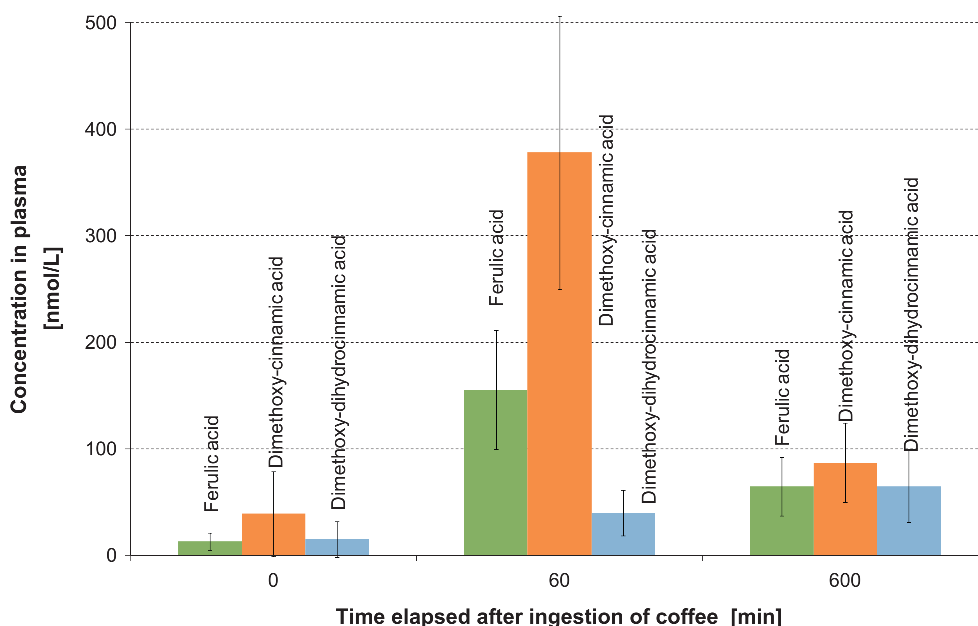

Fig. 5. Concentrations of ferulic acid, 3,4-dimethoxy- and 3,4-dimethoxydihydrocinnamic acid in human plasma after coffee intake. Three time points were analyzed in duplicate: T0, T60 and T600: immediately, 60 and 600 min after coffee consumption, respectively. Student's paired t-test ($n = 10$) results between T0 and T60 samples were 1.30E−07; 1.24E−07 and 0.005179327 for ferulic acid, 3,4-dimethoxycinnamic acid and 3,4-dimethoxy-dihydrocinnamic acids, respectively. Student's paired t-test ($n = 10$) results between T0 and T600 samples were 1.0026E−05; 0.005916314 and 0.000304994 for ferulic acid, 3,4-dimethoxycinnamic acid and 3,4-dimethoxy-dihydrocinnamic acids, respectively.

dance of 3,4-dimethoxycinnamic acid compared to ferulic acid is thus very different in the coffee extract and in plasma (3% and 240%). This observation could have various explanations, e.g. (1) the quinic acid esterases liberate more ferulic acid than dimethylcinnamic acid; (2) during metabolism both hydroxyl groups on the aromatic ring undergo methylation, hereby converting ferulic acid into dimethoxycinnamic (so far never observed in humans); (3) dimethoxycinnamic acids could be preferentially absorbed in the small intestine.

Further experiments will be necessary to address these possibilities and determine the reason why dimethoxycinnamic acids appear at this concentration in plasma. The presented results however indicate that it is important to consider these derivatives in future bioavailability and bioefficacy studies. To estimate quantitative performance of the method used in this work, calibration slope, linearity and detection limits were determined for the available compounds using the time-of-flight mass spectrometer, see Table 2. Precision was calculated at the respective LLOQ levels by spiking blank (T0) plasma samples from seven individuals. This way, the given values in Table 2 represent not only the uncertainties of the method, but also the different matrix effects originating from seven individuals. Method transfer to triple quadrupole instruments and validation will be needed to routinely quantify these compounds in human studies.

4. Conclusions and outlook

An LC–MS approach has been developed to detect potential dimethoxy-phenolic acids in human plasma. A combination of methanol:acetonitrile 1:4 as organic phase was found to enable simultaneous quantification of all phenolics investigated. The method was applied to confirm the appearance of dimethoxycinnamic acids in human plasma upon coffee intake. The high abundance of 3,4-dimethoxycinnamic acid emphasizes the need to consider these compounds in future coffee bioavailability and metabolic studies.

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