JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development and Validation of Methods for the Extraction of Phenolic Acids from Plasma, Urine, and Liver and Analysis by UPLC-MS

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ABSTRACT: This study developed and validated a method for the extraction and determination of 11 phenolic acids in rat plasma, urine, and liver by ultraperformance liquid chromatography−mass spectrometry (UPLC-MS). A system suitability test (instrumental linearity, area, and retention time precision) was performed and recovery, intraday and between-day precisions, detection limits (LOD), and quantification limits (LOQ) were determined for all compounds in each biological matrix. Recoveries varied between 88 and 117% in plasma, between 87 and 102% in urine, and between 38 and 100% in liver. Precision was higher than 13.7% intraday and 14.0% interday in all matrices, at three concentration levels. To demonstrate the applicability, the method was used to estimate the concentrations of phenolic acids in samples from animals that received 5-caffeoylquinic acid (5-CQA) by gavage. The excellent validation results and the applicability of the method to real samples confirmed the suitability for studies on absorption, bioavailability, and pharmacokinetics of phenolic acids derived from foods rich in phenolic compounds.

KEYWORDS: *phenolic acids, solid phase extraction, plasma, urine, tissue, UPLC-MS*

INTRODUCTION

Phenolic compounds are potentially bioactive substances that occur naturally in plants and derived foods, with broad scientific evidence supporting their beneficial role on human health and prevention of degenerative diseases, summarized in reviews.[1,2] Approximately 8000 substances belong to the category of "plant phenolics", all of which share at least one aromatic ring bearing one or more hydroxyl groups, comprising different compounds: simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolyzable and condensed tannins, lignans, and lignins.[2,3]

Phenolic acids are hydroxylated derivatives of benzoic or cinnamic acids.[4] The most abundant phenolic acids in foods are the hydroxycinnamates, such as caffeic, ferulic, and *p*-coumaric, which occur mainly as ester conjugates with quinic acid, collectively referred to as chlorogenic acids.[2] These compounds are broadly present in fruits and vegetables and are the main phenolic compounds in coffee and yerba maté, contributing significantly to daily dietary intake of phenolics.[5,6]

Although beneficial properties of plant phenolics are often attributed to antioxidant activities, emerging findings suggest a variety of potential mechanisms of action in vivo, beyond antioxidant functions.[2,7] However, the compounds present in food may not reach physiological targets in their native forms, but probably as metabolites.[8,9] It is actually known that phenolic compounds undergo several modifications due to the action of phase II enzymes and gut microbiota metabolism.

In fact, following ingestion, a small part of chlorogenic acids may be absorbed intact or after cleavage of ester bonds by membrane[10] or microbial esterases,[11] appearing in plasma and urine mainly as phase II conjugates of chlorogenic acids and hydroxycinnamates (methyl, glucuronide, and sulfate derivatives).[12−14] Metabolization of chlorogenic acids and flavonoids by colonic microbiota leads to the production of other phenolic acids, such as hydroxyphenylpropionic, dihydrocaffeic, hippuric, and hydroxybenzoic acids,[6,15] which are more hydrophilic and may be present in body fluids and tissues in larger quantities than the parent compounds.[2]

Despite the potential role of chlorogenic and other phenolic acids in human health, due to their broad presence in foods and/or potentially in the body as metabolites of other phenolic compounds, their bioavailability has not received as much attention as that of flavonoids.[6,16] For the determination of phenolic compounds and their metabolites in biological matrices, it is necessary to ensure an efficient and reproducible extraction, coupled to separation techniques sensitive enough to detect the low concentrations found in biological fluids and tissues.[17]

A limited number of studies have reported methods for the extraction and analysis of chlorogenic acids or other phenolic acids from biological matrices. Two studies that evaluated the bioavailability of chlorogenic acids from coffee have described methodologies for liquid−liquid extraction of phenolic acids from plasma,[12,18] and urine,[12] with analysis performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The first study[18] presents a validated methodology for only three hydroxycinammic acids (caffeic, ferulic, and

Received: January 9, 2013
Revised: May 27, 2013
Accepted: May 28, 2013
Published: May 28, 2013

isoferulic) and two phenolic acids formed by the gut microbiota (dihydrocaffeic and dihydroferulic acids), because full enzymatic hydrolysis was applied (esterase, β-glucuronidase, and sulfatase). The second study[12] evaluated 21 compounds, including intact chlorogenic acids, but as validation was not a main goal, the respective parameters were not fully evaluated or reported.

Recently, methods coupling solid phase extraction (SPE) or liquid−liquid extraction with LC-MS techniques have been described for analyses of phenolic acids formed by metabolization of cocoa polyphenols in urine,[19] plasma phenolic acids, and polyphenols from thyme,[20] urinary phenolic acids formed by metabolization of polyphenols from cranberry,[21] and urine phenolic metabolites from tomato.[17] However, only the last two include chlorogenic acids among the evaluated compounds. Besides, to our knowledge, there are no published studies with validated methods for extraction of chlorogenic acids and other phenolic acids from tissues.

The present work describes a new method for the extraction and analysis of phenolic acids from plasma, urine, and tissues (liver). The method comprises extraction from biological matrices using off-line solid phase cartridges and ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) as the analytical and separation technique. Validation was performed using 11 phenolic acids, which represent native forms broadly found in foods such as chlorogenic acids (5-caffeoylquinic acid, 5-CQA), intermediate metabolites (hydroxycinnamates caffeic, ferulic, isoferulic, and *p*-coumaric acids), and also compounds that may be formed after metabolization of chlorogenic acids and polyphenols by the gut microbiota (dihydrocaffeic, hydroxybenzoic, *m*-coumaric, vanillic, hippuric, and hydroxyphenylpropionic acids). After validation, the method was applied to samples from rats that received 5-CQA orally by gavage.

MATERIALS AND METHODS

Materials. The phenolic acids 5-CQA, caffeic, ferulic, isoferulic, hippuric, *p*-coumaric, *m*-coumaric, vanillic, 3-hydroxybenzoic, dihydrocaffeic, and 2-hydroxyphenylpropionic acids, enzymes β-glucuronidase (type IX from *Escherichia coli*) and sulfatase (type VIII from abalone entrails), MOPS, Na_2EDTA, sodium dithionite, and ascorbic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Solid phase extraction cartridges (Oasis HLB, 1 cm^3 30 mg) were provided by Waters (Milford, MA, USA). Formic acid (98%) and phosphoric acid (85%) were obtained from Merck (Darmstadt, Germany) and sodium acetate and organic solvents (acetonitrile and methanol) of HPLC grade from Carlo Erba (Rodano, Italy). Deionized water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA, USA).

Standard Preparations. Stock solutions (1 mg/mL) were prepared separately in methanol for each analyte. They were further diluted with methanol to obtain a work solution with all of the compounds at 100 μg/mL, which was divided in small aliquots and kept at −70 °C. Dilutions were prepared in water (recovery experiments and calibration curves) or mobile phase after the methanol had been dried under nitrogen and the solution had been resuspended in the same amount of deionized water.

Experimental Animals. Twelve male Wistar rats (6 weeks old) were housed in groups of three per cage in an atmosphere of 55 ± 10% relative humidity at 25 ± 2 °C, with a 12 h light/dark cycle (lights on at 7:00 a.m.) with free access to water and a commercial feed for laboratory rats (Nuvilab CR1, Nuvital, Curitiba, PR, Brazil) for 3 weeks. At 9 weeks of age, animals (∼260 g body weight) were kept under fasting conditions overnight with access to water and were divided in two groups (6 rats each). One group received a single dose of 5-CQA (240 mg/kg of body weight) diluted in water by gavage, whereas the second group (control) received the corresponding amount of water. Rats were placed in metabolic cages for urine collection for 2 h, and after this period, they were anesthetized with IP ketamine and xylazine mixture, blood was collected by cardiac puncture, and the liver was excised and perfused with saline solution.

Blood was centrifuged for 10 min at 3000*g* (4 °C), and 40 μL of sodium acetate buffer (250 mM pH 5.0, 20 mg/mL ascorbic acid, 1 mg/mL Na_2EDTA)[18,22] was added to each 1 mL of plasma and urine. All samples were immediately frozen in liquid nitrogen and kept at −80 °C prior to analyses.

All animal procedures were in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College for Animal Experimentation according to the American Psychological Association Guidelines for Ethical Conduct in the Care and Use of Animals. The study protocols were approved by the Ethical Committee of the Tropical Medicine Institute, University of São Paulo (Protocol 037/2009).

Sample Preparation and Solid Phase Extraction. *Plasma and Urine.* Aliquots of 200 μL of control plasma or urine, previously treated with antioxidant solution (20 mg/mL ascorbic acid and 1 mg/mL Na_2EDTA in sodium acetate buffer, 250 mM, pH 5.0), were defrosted on ice in the dark, spiked with the standard solution (or corresponding amount of water), and incubated with β-glucuronidase (500 U for plasma and 1000 U for urine) and sulfatase (2.5 U for plasma and 5 U for urine). Enzymes were prepared previously in sodium acetate buffer (250 mM, pH 5.0), aliquoted, and kept at −70 °C. For urine samples, 20 μL of MOPS buffer (625 mM, pH 6.8) was added to adjust the pH. Samples were briefly vortexed, purged with nitrogen, and incubated for 1 h at 37 °C, with shaking. At the end of the incubation period, 400 μL of 4% phosphoric acid and 200 μL of water were added to each tube.

Liver. Liver samples were freeze-dried for 12 h before extraction. Individual tissue weights were recorded before and after the freeze-drying process, so the results obtained using the methodology can be expressed per gram of fresh tissue.

Freeze-dried control tissue samples (30 mg) were spiked with the standard solution (or corresponding amount of water) and homogenized in 300 μL of methanol, 0.2% formic acid, and 300 μL of 0.3 M sodium dithionite/0.1% (w/v) Na_2EDTA in a 2 mL tube on ice.[22] The homogenate was centrifuged at 5000*g* for 10 min (4 °C). The supernatant was collected on a glass tube containing 8 μL of aqueous ascorbic acid (10 mg/mL) and kept on ice. Extraction was repeated with 200 μL of each solution, and the sample was vortexed for 1 min and centrifuged at 10000*g* for 10 min (4 °C). The combined supernatants were partially vacuum evaporated for 40 min at 35 °C on a CentriVap concentrator (Labconco, Kansas City, MO, USA). The tube was washed with 0.3 mL of 625 mM MOPS buffer (pH 6.8), and samples transferred to a new 2 mL tube. After purging with nitrogen, samples were incubated at 37 °C for 30 min with β-glucuronidase (1000 U) and sulfatase (5 U) with shaking. Reaction was stopped by adding 300 μL of 4% phosphoric acid.

Solid Phase Extraction. Solid phase cartridges (Oasis HLB) were conditioned sequentially with 1 mL of methanol and 0.2% formic acid in water and loaded with the previously prepared plasma, urine, or liver samples. After two washings of the cartridge with 1 mL of 0.2% formic acid, phenolic acids were eluted with 3 × 0.5 mL of methanol acidified with 0.2% formic acid into a tube containing 8 μL of ascorbic acid solution (10 mg/mL). The eluate was vacuum evaporated at 33 °C (∼1 h), until dryness. The residues were reconstituted with 200 μL of mobile phase (94% water/6% acetonitrile with 0.1% formic acid) and filtered through 0.22 μm PVDF membrane syringe filters (Millipore, Billerica, MA, USA) directly on a 96-well plate before injection into UPLC-MS.

Liquid Chromatography−Mass Spectrometry Analyses. Analyses were carried out on an Agilent 1200 SL binary pump LC system (Agilent Technologies, Santa Clara, CA, USA), coupled to an Agilent single-quadrupole mass spectrometer (6150B) equipped with electrospray ionization (ESI). Chromatographic runs were also monitored with a diode array detector (DAD, Agilent Technologies),

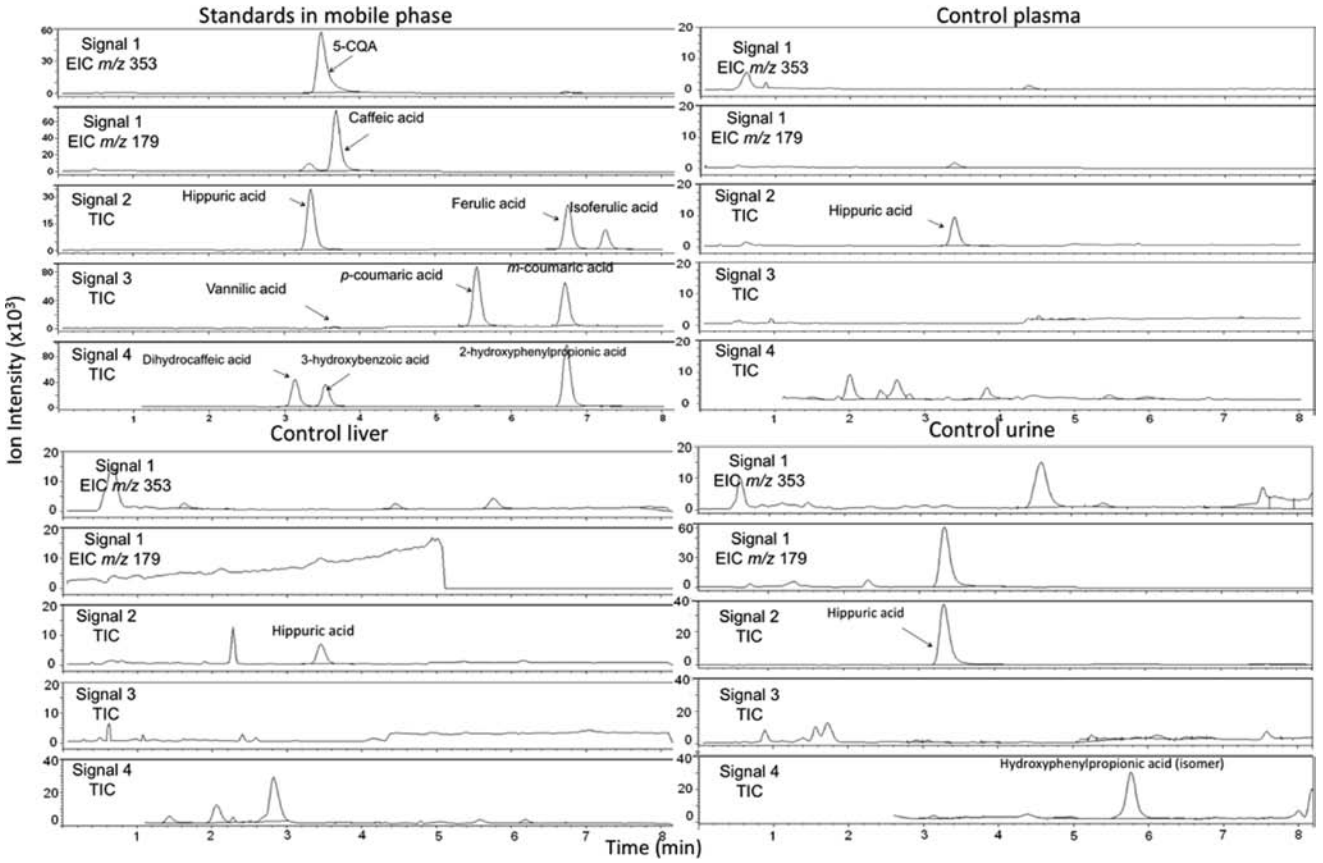

Figure 1. Representative UPLC-MS chromatograms of phenolic acid standards in mobile phase (1 μg/mL) and control plasma, urine, and liver.

and UV spectra were used, when possible, to confirm the identity of phenolic acids.

Separation was achieved on a Zorbax SB-C18 column (50 × 2.1 mm, 1.8 μm) with a Zorbax SB-C8 guard column (12.5 × 2.1 mm, 5 μm) (Agilent Technologies), operating at 45 °C. Mobile phases were constituted with water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid. The best separation was achieved with the following gradient program: 0−0.5 min, 6% B; 0.5−8.5 min, 20% B; 8.5−9.5 min, 90% B; 9−12 min, held at 90%, 12−12.5 min, back to 6% B; and a 10 min post run re-equilibration at 6% B. Mass spectrometric data were collected between 0.5 and 8.5 min. The flow rate was 330 μL/min and the injection volume, 5 μL.

MS analyses were performed in the negative mode, with nitrogen as nebulizer (35 psi) and drying gas (9 L/min) at 350 °C with a capillary voltage set at −4 kV. First, a solution with all of the standards in mobile phase was infused in scan mode, so the deprotonated molecule and the most intense product ion of each compound were recorded and selected for single ion monitoring (SIM), the mode used for method optimization and quantitative analysis of samples. The cone voltage (collision-induced dissociation, CID) for all compounds was 100 V. Chromatographic data were recorded and integrated using LCD/MSD ChemStation software.

System Suitability Test (SST). The SST[23] was performed under the optimized chromatographic conditions for the separation of 11 phenolic acids. A standard solution with all of the analytes in mobile phase was injected 10 times in two different days, to verify the instrumental precision (intra- and interday), determined as relative standard deviation (%RSD) of retention time and peak areas. Linearity, expressed as the determination coefficient (R^2), was checked from 0.25 to 20.0 ng (50−4000 ng/mL) and calculated by plotting the peak area of each concentration against the respective known concentration.

Method Validation. The validation was performed according to the International Conference on Harmonization Guidance for the Validation of Analytic Methods.[23] It was based on the following criteria: limit of detection (LOD), limit of quantification (LOQ), precision (intra- and interday variability), recovery, and linearity.

LOD and LOQ were determined for each biological matrix (plasma, urine, and liver) using the standard of the response method, with the formulas

$$\mathrm{LOD} = (3.3\sigma)/S$$

$$\mathrm{LOQ} = (10\sigma)/S$$

where σ is the standard deviation of the blank (control samples) and S the slope of the calibration curve. Standard deviation of the blank was calculated by analyzing the background (noise) of three pooled samples from the group, as close as possible to the expected retention time for each analyte, using ChemStation software. External calibration curves were built with spiked pooled control samples (for plasma and liver) or standard solutions (for urine), with different concentration levels.

Extraction recoveries (%R) were determined by comparing the absolute response of the analytes spiked in control samples before and after the extraction procedure, using nine determinations (three concentrations/three replicates each) for all of the compounds.

Method precision (intraday and interday precision) was determined as the relative standard deviation (%RSD) with three replicates and three concentrations, on two different days by the same analyst. Analytes were added to samples before the extraction procedure.

Method Applicability. The method was applied to plasma, urine, and liver samples from animals that received 5-CQA by gavage (240 mg/kg body weight). Samples were prepared and analyzed exactly as described above.

Phenolic acid concentrations were calculated with external calibration curves (minimum of five points), built by spiking control plasma and liver with standard solutions or with standards diluted in mobile phase for quantification of phenolic acids from urine, as some

Table 1. LC-MS Parameters and Instrumental Precision (Intra- and Interday) for Phenolic Acids with the Developed Chromatographic Method

				RT precision (%RSD)			area precision (%RSD)		
				intraday (n = 10)		interday	intraday (n = 10)		interday
phenolic acid	RT (min)	$[M - H]^-$ (m/z)	product ion (m/z)	batch 1	batch 2		batch 1	batch 2	
5-CQA[a]	3.5	353	191	0.37	0.67	0.57	0.90	1.13	1.86
caffeic	3.8	179	135	0.22	0.42	0.37	1.07	1.24	3.38
ferulic	6.8	193	178	0.12	0.11	0.14	0.55	0.86	1.42
isoferulic	7.3	193	178	0.12	0.10	0.13	0.83	1.74	2.13
m-coumaric	6.8	163	119	0.10	0.10	0.14	0.82	0.82	1.87
p-coumaric	5.6	163	119	0.11	0.16	0.18	0.54	1.25	3.18
dihydrocaffeic	3.3	181	137	0.28	0.64	0.49	2.39	1.80	2.62
hippuric	3.4	178	134	0.22	0.46	0.37	0.40	0.96	2.86
3-hydroxybenzoic	3.7	137	93	0.18	0.40	0.34	1.12	0.46	3.24
2-hydroxyphenylpropionic	6.9	165	121	0.09	0.11	0.13	1.37	1.26	3.22
vanillic	3.8	167	152	0.22	0.44	0.36	4.24	2.93	3.69

[a]5-CQA, 5-caffeoylquinic acid.

Table 2. Extraction Recovery of Phenolic Acids in Spiked Plasma, Urine, and Liver Submitted to Solid Phase Extraction and Analyzed by UPLC-MS

	recovery %[a] (mean ± SD)								
	plasma			urine			liver		
phenolic acid	250 ng/mL	500 ng/mL	1 μg/mL	250 ng/mL	1 μg/mL	15 μg/mL	250 ng/mL	500 ng/mL	1 μg/mL
5-CQA[b]	100 ± 4.7	88 ± 2.0	98 ± 0.8	91 ± 1.0	97 ± 0.6	99 ± 1.0	38 ± 3.0	41 ± 1.5	49 ± 2.4
caffeic	105 ± 3.2	114 ± 9.1	108 ± 2.6	94 ± 3.6	98 ± 0.6	99 ± 1.2	72 ± 4.2	64 ± 5.0	69 ± 1.9
ferulic	95 ± 4.8	102 ± 1.5	96 ± 0.2	89 ± 5.5	96 ± 2.5	99 ± 1.1	93 ± 0.6	69 ± 1.6	75 ± 1.1
isoferulic	100 ± 5.4	101 ± 3.2	96 ± 1.2	102 ± 8.8	99 ± 0.8	99 ± 1.7	87 ± 5.0	68 ± 5.3	77 ± 1.4
m-coumaric	100 ± 3.2	106 ± 1.7	102 ± 1.0	93 ± 2.9	96 ± 3.9	99 ± 0.7	85 ± 0.8	61 ± 3.0	75 ± 2.3
p-coumaric	101 ± 3.6	104 ± 1.9	97 ± 0.5	96 ± 1.5	101 ± 0.7	99 ± 2.1	81 ± 1.6	60 ± 3.1	76 ± 3.7
dihydrocaffeic	106 ± 2.6	110 ± 0.5	102 ± 1.0	91 ± 0.3	98 ± 1.4	100 ± 1.4	84 ± 2.8	70 ± 4.4	68 ± 1.3
hippuric	110 ± 1.4	117 ± 3.4	109 ± 0.8	98 ± 1.0	100 ± 1.6	102 ± 1.0	100 ± 1.8	74 ± 4.2	84 ± 0.6
3-hydroxybenzoic	108 ± 1.5	114 ± 3.4	94 ± 0.7	95 ± 1.7	94 ± 3.1	100 ± 1.4	90 ± 2.0	74 ± 9.1	78 ± 0.9
2-hydroxyphenylpropionic	107 ± 2.3	107 ± 2.6	103 ± 1.4	87 ± 3.9	96 ± 1.0	96 ± 1.0	82 ± 2.9	60 ± 2.2	74 ± 2.4
vanillic		90 ± 3.6	91 ± 4.0		91 ± 8.0	102 ± 1.8		82 ± 7.7	88 ± 5.0

[a]n = 3 replicates in each level. [b]5-CQA, 5-caffeoylquinic acid.

Table 3. Limits of Detection (LOD, ng/mL) and Quantification (LOQ, ng/mL) for Analysis of Phenolic Acids by UPLC-MS in Plasma, Liver, and Urine Samples

	plasma		liver		urine	
phenolic acid	LOD	LOQ	LOD	LOQ	LOD	LOQ
5-CQA[a]	16	52	10	30	14	48
caffeic	12	38	6	20	16	50
ferulic	16	52	10	30	30	96
isoferulic	20	60	14	46	66	202
m-coumaric	10	30	10	30	18	54
p-coumaric	30	94	8	26	16	50
dihydrocaffeic	24	72	6	20	12	40
hippuric	64	200	8	28	22	68
3-hydroxybenzoic	16	46	8	26	32	102
2-hydroxyphenylpropionic	14	40	12	32	22	66
vanillic	436	1320	390	1176	472	1430

[a]5-CQA, 5-caffeoylquinic acid.

of the studied compounds were present in control urine in relatively high concentrations.

Two compounds with retention times of 5.9 and 6.4 min, UV spectra >95% similar to 5-CQA's spectra, and m/z 367 and 193 were present in plasma, urine, and liver samples. On the basis of these data and by comparison with recent literature,[12] compounds were tentatively identified as feruloylquinic acid isomers. Another compound with retention time of 5.9 min was tentatively identified as an isomer of hydroxyphenylpropionic acid (m/z 165 and 121, UV spectra >99% similar to the available standard 2-hydroxyphenylpropionic acid). The concentrations of these compounds were estimated using the calibration curves of 5-CQA and 2-hydroxyphenylpropionic acid, respectively.

dx.doi.org/10.1021/jf401534d | J. Agric. Food Chem. 2013, 61, 6113−6121

Table 4. Intraday and Interday Area Precisions of Phenolic Acids from Spiked Plasma, Liver, and Urine at Three Levels[a]

Plasma									
	precision (%RSD)								
	intraday (batch 1)			intraday (batch 2)			interday		
phenolic acid	250 ng/mL	500 ng/mL	1 μg/mL	250 ng/mL	500 ng/mL	1 μg/mL	250 ng/mL	500 ng/mL	1 μg/mL
5-CQA[b]	1.5	9.0	4.6	2.1	3.4	5.2	11.2	9.5	6.5
caffeic	1.3	3.8	3.4	4.9	8.9	2.9	11.0	6.5	4.5
ferulic	3.0	7.8	3.2	2.5	0.5	0.7	10.2	5.3	2.1
isoferulic	1.5	8.1	5.7	3.9	1.3	2.3	12.1	6.7	3.9
m-coumaric	1.3	8.6	3.5	3.9	1.5	0.4	8.1	5.6	2.2
p-coumaric	1.9	5.6	0.8	4.2	1.1	1.4	7.5	4.3	1.4
dihydrocaffeic	1.1	5.5	3.6	3.2	3.9	3.9	7.8	10.3	5.7
hippuric	0.8	11.2	4.5	3.4	2.0	7.2	10.0	8.9	5.6
3-hydroxybenzoic	1.7	10.2	12.1	5.7	1.1	3.8	5.7	7.5	11.7
2-hydroxyphenylpropionic	2.8	6.5	2.6	2.5	0.4	2.2	9.9	4.4	2.4
vanillic		9.5	6.1		0.6	0.7		8.4	3.9
Liver									
	precision (%RSD)								
	intraday (batch 1)			intraday (batch 2)			interday		
phenolic acid	250 ng/mL	500 ng/mL	1 μg/mL	250 ng/mL	500 ng/mL	1 μg/mL	250 ng/mL	500 ng/mL	1 μg/mL
5-CQA	7.9	12.9	4.9	10.0	3.7	5.0	8.1	13.5	4.8
caffeic	5.8	10.3	2.7	10.8	6.8	8.7	9.6	9.1	10.1
ferulic	0.6	13.3	1.4	10.3	2.3	2.7	7.7	14.0	5.1
isoferulic	5.8	9.9	1.8	11.8	7.8	0.9	9.7	10.5	6.9
m-coumaric	1.0	9.5	3.1	10.9	5.0	2.5	9.5	10.9	8.1
p-coumaric	1.9	9.2	4.9	7.8	5.3	7.4	7.9	9.0	8.7
dihydrocaffeic	3.4	12.2	1.9	10.5	6.4	4.6	8.2	9.7	7.8
hippuric	1.8	13.7	0.7	2.0	5.7	5.5	2.9	12.6	7.5
3-hydroxybenzoic	2.2	11.9	1.1	5.7	12.3	5.8	5.6	11.5	6.8
2-hydroxyphenylpropionic	3.6	9.5	3.2	7.6	3.6	5.1	7.1	12.5	7.6
vanillic		7.1	5.7		9.2	6.3		7.4	7.2
Urine									
	precision (%RSD)								
	intraday (batch 1)			intraday (batch 2)			interday		
phenolic acid	250 ng/mL	1 μg/mL	15 μg/mL	250 ng/mL	1 μg/mL	15 μg/mL	250 ng/mL	1 μg/mL	15 μg/mL
5-CQA	1.1	0.6	1.8	1.7	2.0	1.2	4.0	3.4	1.5
caffeic	3.8	0.6	1.3	1.3	2.6	2.5	3.1	2.5	1.8
ferulic	6.1	2.7	1.2	3.9	3.9	3.1	6.9	3.9	2.2
isoferulic	8.6	0.8	2.1	4.9	2.4	2.0	9.9	3.1	2.0
m-coumaric	3.1	4.1	0.7	0.2	1.0	2.2	2.2	5.1	1.5
p-coumaric	1.5	0.7	2.4	4.0	2.0	0.6	3.2	2.1	1.6
dihydrocaffeic	0.3	1.5	1.6	2.2	1.8	4.4	2.7	1.5	3.0
hippuric	1.0	1.6	1.0	0.3	0.5	2.6	4.2	2.2	1.8
3-hydroxybenzoic	1.8	3.1	4.1	1.4	2.0	6.3	2.7	2.3	4.7
2-hydroxyphenylpropionic	4.4	1.0	1.0	3.8	2.4	1.1	6.3	1.8	1.1
vanillic		8.0	4.5		12.9	9.2		12.0	6.5

[a]Samples were submitted to solid phase extraction and analyzed by UPLC-MS. [b]5-CQA, 5-caffeoylquinic acid.

RESULTS AND DISCUSSION

UPLC-MS Analyses and SST. Phenolic acids mass spectra were determined by injection of each compound separately in negative ion mode. ESI conditions (fragmentor voltage, drying gas flow, and nebulizer pressure) were optimized with a mixture of all studied compounds to improve sensitivity and reduce baseline noise (see Liquid Chromatography–Mass Spectrometry Analyses).

An optimized chromatographic run of 8.5 min yielded symmetric peaks for all phenolic acids. The cleaning step after each run (90% solvent B for 3 min) was crucial for the adequate performance of repeated sample injections and to improve column lifetime.

Chromatographic peaks were separated in four main total ion count (TIC) signals (SIM mode chromatograms) with the deprotonated molecule $[M - H]^-$ and the most abundant product ion selected for each compound. Extracted ion chromatograms (EIC) of the deprotonated molecule were used to achieve additional separation for adequate quantification of 5-CQA and caffeic, *p*-coumaric, *m*-coumaric, 3-hydroxybenzoic, and dihydrocaffeic acids. One of the main advantages of MS detectors is that compounds can be separated according to the different m/z values, even if they coelute.

Table 5. Concentration (Mean ± SEM) of Phenolic Acids in Plasma, Liver, and Urine of Rats 2 h after Administration of 5-Caffeoylquinic Acid (5-CQA) by Gavage (240 mg/kg Body Weight)

phenolic acid	plasma (ng/mL)	liver (ng/g fresh tissue)	urine (μg/mL)
5-CQA	1165.6 ± 28.6	360.3 ± 18.9	12.3 ± 3.8
feruloylquinic acid (isomer 1)[a]	486.3 ± 52.6	123.3 ± 21.6	48.0 ± 12.4
feruloylquinic acid (isomer 2)[a]	414.2 ± 47.8	388.4 ± 57.1	25.3 ± 9.1
caffeic	217.8 ± 38.3	37.8 ± 3.9	2.8 ± 0.9
ferulic	NQ[b]	NQ	0.89 ± 0.14
isoferulic	ND[c]	ND	0.36 ± 0.03
m-coumaric	ND	ND	ND
p-coumaric	ND	ND	ND
dihydrocaffeic	NQ	NQ	0.09 ± 0.03
hippuric	NQ	65.2 ± 5.5	40.9 ± 3.5
3-hydroxybenzoic	NQ	ND	ND
2-hydroxyphenylpropionic	ND	ND	ND
hydroxyphenylpropionic (isomer)[d]	NQ	NQ	0.39 ± 0.06
vanillic	ND	ND	ND

[a]Concentration estimated as 5-CQA equivalents. [b]NQ, not quantified. [c]ND, not detected. [d]Concentration estimated as 2-hydroxyphenylpropionic acid equivalents. ,

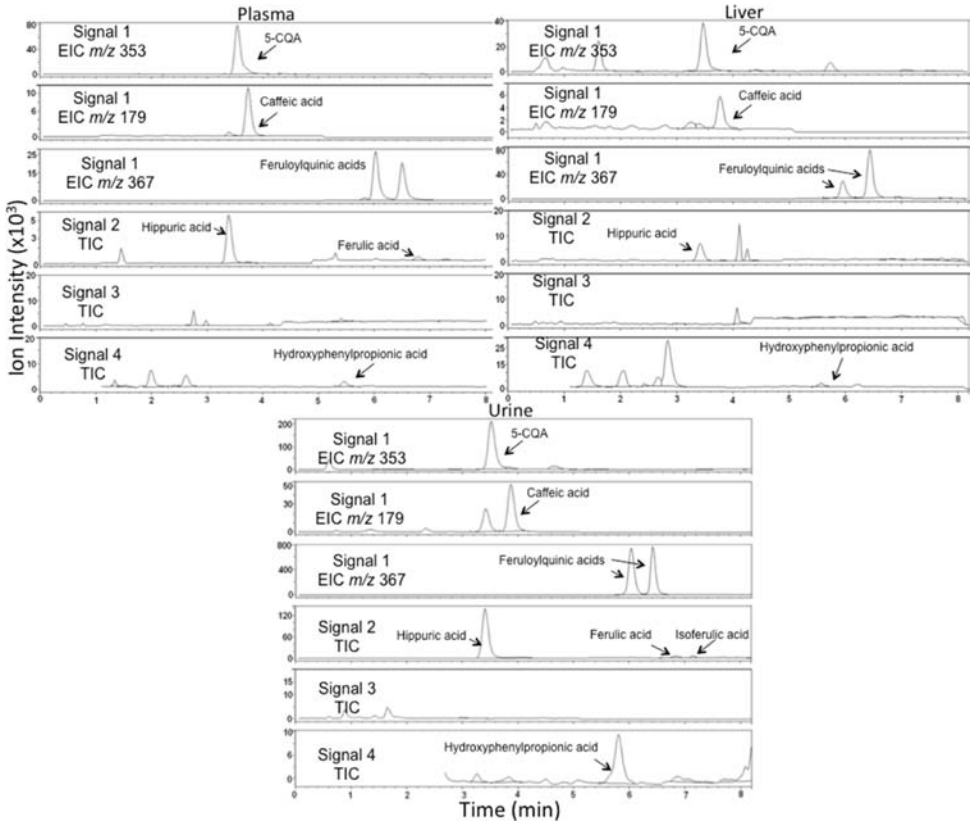

Figure 2. Representative UPLC-MS chromatograms of phenolic acids from Wistar rat plasma, urine, and liver 120 min after administration of 5-caffeoylquinic acid (5-CQA) by gavage (240 mg/kg body weight).

Phenolic acids and matrix interferents with the same *m*/*z* were sufficiently separated chromatographically. Figure 1 presents the chromatograms of standards in mobile phase and chromatograms of control plasma, liver, and urine. Hippuric acid was found in all three control matrices, and an isomer of 2-hydroxyphenylpropionic acid was also found in urine.

The SST showed satisfactory precision for peak areas and retention times, intra- and interday (Table 1).

Linearity of detector response was checked by linear regression. The coefficient of determination (R^2) was >0.99 from 0.25 to 20.0 ng (50−4000 ng/mL) for 5-CQA and caffeic, hippuric, *p*-coumaric, isoferulic, 2-hydroxyphenylpropionic, dihydrocaffeic, and 3-hydroxybenzoic acids. For ferulic and *m*-coumaric acids, the linear range was from 0.25 to 10.0 ng (50−2000 ng/mL) and for vanillic acid, from 1.0 to 20.0 ng (200−4000 ng/mL).

Sample Preparation and Solid Phase Extraction. Plasma and urine samples were treated with ascorbic acid/Na_2EDTA antioxidant solution before freezing. Liver was freeze-dried to eliminate water, thus concentrating the sample and improving the contact between solvent and tissue.[24] Sodium dithionite and Na_2EDTA were used as antioxidants

during the homogenization instead of ascorbic acid, because the latter can act as a pro-oxidant in the presence of catalytic ions such Fe^{2+} and Cu^{2+}, as tested by Chu et al.[22]

Samples were hydrolyzed with β-glucuronidase and sulfatase enzymes prior to extraction, because phenolic acids may be modified by phase II metabolism in vivo and appear in the body mainly conjugated with β-glucuronide acid and/or sulfate groups.[12−14] As these modified compounds are not all commercially available to date, the hydrolysis is necessary for quantification of the substances as their nonconjugated equivalents.

Enzyme activities in the presence of phosphate or MOPS buffers were evaluated according to standard Sigma-Aldrich protocols.[25,26] Phosphate buffer was shown to inhibit β-glucuronidase activity (data not shown), in accordance with previous observations.[27] MOPS buffer did not decrease enzymatic activity and was used to adjust the pH of urine and liver samples to the optimal range for enzymatic hydrolysis (pH 6.5−7.0). It was not necessary to adjust plasma pH, as it was already in the optimal range for β-glucuronidase. Although sulfatase has an optimal pH of 5.0, this enzyme showed good activity at pH 6.5−7.0, whereas β-glucuronidase was more sensitive to pH changes.

Enzymatic hydrolysis conditions were tested by evaluating different incubation times (30 min and 1 and 2 h) and enzyme amounts (500, 1000, and 2000 units of β-glucuronidase and 2.5, 5, and 10 U of sulfatase). Conditions were assessed by monitoring the increase on chromatographic peak areas of 5-CQA and caffeic and ferulic acids in pooled plasma, urine, and liver samples from the animals that received 240 mg/kg body weight of 5-CQA by gavage. When larger amounts of enzymes and prolonged incubation time did not increase peak areas of the cited compounds, the smaller amount of enzymes and the incubation time that resulted in similar chromatographic peak areas were chosen.

For plasma samples, 500 U of β-glucuronidase and 2.5 U of sulfatase were sufficient to hydrolyze the samples after 1 h of incubation, as no further increase in peak areas was observed with higher enzyme amounts or prolonged incubation time. It was necessary to double the enzyme amounts for treatment of urine samples. With regard to the liver, recovery was dramatically reduced by the incubation step, although antioxidants were used and samples were purged with nitrogen. The tests confirmed that the reduced recovery was a result of the incubation time and not the enzymes used. Therefore, the incubation period was reduced to 30 min and the amounts of enzymes were the same as applied to urine (1000 U of β-glucuronidase and 5 U of sulfatase), to ensure the release of all conjugates.

Following enzymatic hydrolysis, samples were submitted to SPE for extraction of phenolic acids. Initially, two liquid−liquid extraction methods were tested with plasma samples: protein precipitation with methanol[28] and liquid partition using ethyl acetate,[18,29] both followed by vacuum evaporation and resuspension in mobile phase. Although recovery values were satisfactory for most analytes (>90%), results for 5-CQA were low with the ethyl acetate method (40% recovery), and samples remained with a lot of impurities with methanol precipitation, which compromised the sub 2-μm column lifetime and the quality of chromatographic data. Therefore, SPE was the method chosen for further optimization.

Most extractions of phenolic compounds are carried out under acidic conditions because these compounds are generally more stable in low pH and kept neutral, being readily extracted into organic solvents, whereas dissociated forms may remain in the aqueous phase.[1,30] Although HCl has been used in many methods for the extraction of phenolic acids from biological fluids,[28,31] aqueous HCl has been reported to destroy hydroxycinnamic acids.[3] Formic acid was chosen on the basis of the literature[12,18,19] and for being a weak acid compatible with ESI/MS interface.

The SPE procedure was based on the generic Oasis HLB protocol from Waters, which includes dilution of samples with 4% H_3PO_4. This acid solution breaks the bonds between phenolic compounds and proteins and also modifies the pH, stopping β-glucuronidase and sulfatase activities. First, cartridges were conditioned and equilibrated, and samples were applied. Different washing procedures were tested to remove most of interference from the biological matrices without eluting the retained phenolic acids: one or two washings with 0.2% formic acid in water; one washing with 0.2% formic acid in water followed by 0.2% formic acid + 5% methanol in water. Best recoveries were obtained with two consecutives washings (1 mL each) with 0.2% formic acid in water. Finally, samples were eluted with 0.2% formic acid in methanol into tubes containing ascorbic acid, to avoid oxidation during vacuum evaporation.

Method Validation. Standard mixture solutions of phenolic acids were spiked in control plasma, urine, and liver samples at known concentrations. After extraction, samples were analyzed by UPLC-MS, and recovery, intra- and interday precision, and limits of determination and quantification were evaluated according to ICH guidelines.[23]

The extraction recoveries obtained at three contamination levels are shown in Table 2. Results are among the highest in the literature for extraction of phenolic acids from plasma and urine, especially for hydroxycinnamates (caffeic, ferulic, isoferlic, *p*-coumaric, and *m*-coumaric acids) and 5-CQA.[13,18,19,21,32−36]

Tissues are more complex matrices than plasma and urine, due to cellular structures with high contents of proteins, collagen, and fat, depending on the tissue.[24] The presence of metal ions and microsomal enzymes in liver may also lead to oxidation of phenolic acids.[22] As a result, recovery values for liver were lower than those obtained for plasma and urine. These results are in accordance with other authors who reported extraction recovery of different phenolic compounds from tissues.[22,24,35] In a recent publication,[24] the extraction recoveries of catechin and epicatechin from rat tissues were increased when four liquid extractions were carried out instead of two, before cleanup with SPE. Therefore, performing more extractions may also be useful to increase the recoveries of phenolic acids from tissues.

Table 3 presents the LOD and LOQ obtained for plasma, urine, and liver. Values varied among different compounds, but followed a similar trend in the different matrices. Vanillic acid limits were the highest among the studied compounds, which is probably a result of poor ionization on the electrospray interface, under the conditions tested.

The LOD and LOQ calculated in the present study are below or within the concentration ranges applied in other studies that quantified phenolic compounds in human plasma and urine after coffee consumption (from 200 ng/mL to 20 μg/mL)[12] and in urine of rats fed cranberry (50−2000 ng/mL).[21] Furthermore, concentrations of 5-CQA, its isomers (3- and 4-CQA), and caffeic acid in plasma of humans after coffee

consumption are higher than the LOQs reported here.[13] This indicates that the method described in the present paper is suitable for bioavailability studies of phenolic compounds in animals and humans. Furthermore, the sensitivity of chromatographic analyses depends not only on the conditions applied but mainly on the equipment used. Thus, different values for LOD and LOQ may be obtained with this method when using different LC-MS equipment.

The intraday and interday precisions for each biological matrix are summarized in Table 4 and show the good precision of the proposed method, with RSD values lower than 15% for all of the compounds. These results are similar to those obtained with other methods described for the analysis of phenolic acids by LC-MS techniques in different biological matrices, such as feces,[37] plasma, and urine.[17,34]

Application of the Developed Method. The method described above was applied to samples obtained from rats that received a single dose of 5-CQA by gavage (240 mg/kg of body weight). Samples of each animal (n = 6) were analyzed in triplicate. The absolute amount of 5-CQA ingested was 72 ± 8 mg (mean ± SD). For comparison, a cup of coffee (200 mL) contains approximately 70−350 mg of chlorogenic acids.[12,38]

Results of phenolic acid quantification in plasma, liver, and urine of rats 2 h after receiving 5-CQA are shown in Table 5 and Figure 2. Some of the studied compounds were present in control plasma (hippuric acid), liver (hippuric acid), and urine (hippuric and hydroxyphenylpropionic acids), as shown in Figure 1. Concentrations of these compounds found in control samples were set as "zero" for plasma and liver, as the calibration curves were built by spiking control samples with standard solutions. In control urine, the concentration of hippuric acid was 21.1 ± 1.9 μg/mL and that of hydroxyphenylpropionic acid, 0.38 ± 0.12 μg/mL (mean ± SEM).

5-CQA, feruloylquinic acids (isomers 1 and 2), and caffeic acid were found in all samples. Feruloylquinic acids (FQAs) are methylated forms of 5-CQA. Their presence was reported in human plasma and urine after consumption of chlorogenic acids from coffee, which also contains FQAs.[12] Caffeic acid is a product from the cleavage of 5-CQA by esterase enzymes. The presence of this compound in the biological fluids and liver 2 h after ingestion of 5-CQA indicates that the cleavage of 5-CQA occurred as a result of gastrointestinal esterases, [10] rather than esterase from microbial origin. Ferulic and isoferulic acids are methylated derivatives of caffeic acid and were present in quantifiable amounts only in urine.

With regard to the metabolites originated by activity of colonic microbiota, hippuric acid was quantified in urine and liver, but not in plasma, because its concentration was between the LOD and LOQ. Dihydrocaffeic acid and a hydroxyphenylpropionic isomer were quantified in urine and detected in plasma and urine, whereas 3-hydroxybenzoic, vanillic, and *m*-coumaric acids were not detected in any of the samples. The fact that the animals were euthanized 2 h after the gavage explains the low concentrations or absence of these microbial metabolites in the samples.

Previous studies showed that higher concentrations of compounds formed by colonic microbiota are found in human plasma and/or urine after 8 h of coffee ingestion.[12,39,40] In the present work, besides the microbial metabolites, the aim was to test the application of the developed method on the quantification of 5-CQA and other metabolites formed earlier in the body, so a smaller time interval after the gavage (2 h) was chosen for collection of biological samples.

In conclusion, the assays described feature efficient sample preparation procedures by SPE with good recovery rates and a relatively short chromatographic run time for the separation and quantification of 11 phenolic acids, which can be formed in the body after consumption of foods rich in chlorogenic acids or other phenolic compounds. The application to samples from animals that received 5-CQA by gavage showed that the method described can be used for absorption, pharmacokinetic, and bioavailability studies of phenolic acids in rodents and also in humans.

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Funding

We thank FAPESP for financial support (Grants 2009/14853-0 and 2008/10308-4) and a Ph.D. fellowship to D.M.d.O. (Process 2009/01275-8).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

5-CQA, 5-caffeoylquinic acid; ESI, electrospray ionization; EIC, extracted ion chromatogram; HCl, hydrochloric acid; IP, intraperitoneal; LOD, limit of detection; LOQ, limit of quantification; Na_2EDTA, disodium ethylenediaminetetraacetate dehydrate; MOPS, 4-morpholinepropanesulfonic acid; H_3PO_4, orthophosphoric acid; R^2, determination coefficient; %RSD, relative standard deviation; SD, standard deviation; SEM, standard error of the mean; SIM, single ion monitoring; SPE, solid phase extraction; SST, system suitability test; TIC, total ion count; UPLC-MS, ultraperformance liquid chromatography coupled to mass spectrometry

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