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Rapid Liquid Chromatography Tandem Mass Spectrometry Assay To Quantify Plasma (−)-Epicatechin Metabolites after Ingestion of a Standard Portion of Cocoa Beverage in Humans

ELENA ROURA,† CRISTINA ANDRÉS-LACUEVA,† OLGA JÁUREGUI,‡ EVA BADIA,§ RAMON ESTRUCH,§ MARIA IZQUIERDO-PULIDO,† AND ROSA M. LAMUELA-RAVENTÓS*,†

Department of Nutrition & Food Science-CeRTA, University of Barcelona, Spain, Scientific and Technical services, University of Barcelona, Spain, and Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer, University of Barcelona, Barcelona, Spain

A rapid liquid chromatography electrospray ionization tandem mass spectrometry with negative ion detection method was developed and validated to determine cocoa flavonoid metabolites in human plasma and urine after the intake of a standard portion of a cocoa beverage. A chromatographic run time of only 9 min provided clear separation of all metabolites and internal standards. Samples were analyzed in a product-ion scan of *m*/*z* 289, 369, and 465 to identify the metabolites and in multiple reaction monitoring acquisition mode to quantify (−)-epicatechin ((−)-Ec) (289/ 245), (−)-epicatechin−glucuronide ((−)-EcG) (465/289), and (−)-epicatechin−sulfate ((−)-EcS) (369/289). One (−)-Ec-G and three (−)-Ec-S were identified and confirmed in urine as the major metabolites, and one (−)-Ec-G was the only metabolite present in plasma volunteers ($n = 5$) at a mean concentration of 625.7 ± 198.3 nmol/L at 2 h after consumption of a cocoa beverage containing 54.4 mg of (−)-Ec.

KEYWORDS: Cocoa flavonoids; (−)-epicatechin; metabolites; human plasma; quantification; confirmation; LC/ESI-MS/MS

INTRODUCTION

Phenolic compounds are a complex and important group of compounds naturally found in the plant kingdom (*1*). More than 8000 polyphenolic structures are currently known, the common feature of which is an aromatic ring bearing at least one hydroxyl substituent. Polyphenols can be divided into different classes based upon their chemical structure: phenolic acids, flavonoids, stilbenes, and lignans, with flavonoids being the largest class of polyphenols and the main one in the cocoa beans (*2*).

Recent reports indicate that the main flavonoids, flavan-3-ols ((−)-epicatechin ((−)-Ec) and (+)-catechin)) and their oligomeric derivatives (procyanidins) found in cocoa beans, and its derived products popular worldwide such as cocoa powder, chocolate, and cocoa beverages, have a variety of beneficial actions, including antioxidant protection as a defense against reactive oxygen species and prevention of LDL cholesterol oxidation, modulation of vascular homeostasis, and inhibition of platelet aggregation (*3*, *4*). A study by Schramm et al. provides evidence that some of the effects of chocolate on platelet activity may be secondary to beneficial changes in eicosanoid metabolism (a decrease in leukotriene and an increase in prostacyclin) as a consequence of the capacity of cocoa flavonoids to inhibit mammalian 15-lipoxygenase at low concentrations (*5*).

The study of the bioavailability of these flavonoids and the concentration of its metabolites in human plasma is necessary to understand its effects on health. It is important to know which metabolites are present, since they are the compounds that reach the target cell or tissues and their activity could be different from that observed by the polyphenols present in food. Since flavanols are metabolized in the enterocytes and the liver, their metabolites are the major forms found in human fluids. However the activity of these metabolites differed. Thus, Spencer et al. (*6*) showed that 3′-*O*-methyl-Ec prevents the activation of caspase-3 induced by hydrogen peroxide in cortical neurones and dermal fibroblasts; however, glucuronidated (−)-Ec possesses no such activity. In contrast, Harada et al. (*7*) showed that Ec-5-*O*-glucuronide and 3′-*O*-methyl-(−)-Ec-5-*O*-glucuronide have superoxide anion radical scavenging activity.

On the other hand, usually the (−)-Ec concentration in rat and human plasma samples is determined after a pretreatment with a solution of glucuronidase and sulfatase. Thus, methylated and nonmethylated plasma conjugates are measured as free forms (*8*−*11*), and the amounts of each (−)-Ec metabolite in plasma and urine are calculated as the amount of (−)-Ec detected after enzymatic treatment minus the amount of (−)-Ec detected without enzymatic treatment. The (−)-Ec levels

* Corresponding author. Tel: +34-93.403.48.43. Fax: +34-93.403.59.31. E-mail address: lamuela@ub.edu.
† Department of Nutrition & Food Science-CeRTA.
‡ Scientific and Technical Services.
§ Department of Internal Medicine.

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found in the literature following cocoa ingestion after sample treatment with glucuronidase and sulfatase enzymes were about 257 ± 66 nM after the ingestion of 80 g of procyanidin-rich semisweet chocolate, containing 557 mg of procyanidins (*10*), 427 ± 249 nM after consumption of 300 mL of cocoa containing 897 mg of flavanols and procyanidins (*12*), and 5.92 ± 0.60 μM after ingestion of 0.375 g cocoa/kg weight as a beverage (*11*).

Baba et al. (*13*) also analyzed the metabolites of (−)-Ec in plasma and urine after ingestion of cocoa products by liquid chromatography mass spectrometry (LC-MS) after glucuronidase and/or sulfatase treatment describing in plasma the presence of glucuronide, sulfate, and sulfoglucuronide forms from nonmethylated (−)-Ec and sulfate and sulfoglucuronide forms from methylated (−)-Ec. Other literature works have reported the analysis of (−)-Ec metabolites but after the ingestion a high dose of (−)-Ec as a solution.

The aim of this study was to develop a rapid and reproducible method for the qualitative and quantitative analysis of (−)-Ec metabolites in human fluid samples by solid-phase extraction (SPE) and LC-MS/mass spectroscopy (MS) after cocoa consumption. Therefore we have developed a fast and accurate SPE-LC-MS/MS method that allows the determination of (−)-Ec metabolites in plasma and urine without enzymatic sample treatment. The low limit of quantification of the proposed method allows the analysis of (−)-Ec and their metabolites after ingestion of a moderate dose of (−)-Ec present in food matrix, such as a cocoa beverage. The method will allow high sample studies in future (−)-Ec availability and epidemiology studies.

MATERIALS AND METHODS

Reagents and Standards. Reagents were obtained from the following sources: methanol and acetonitrile (high-performance LC (HPLC) grade) from Scharlau (Barcelona, Spain), *o*-phosphoric acid from Panreac (Barcelona, Spain), and formic acid from Sigma (Steinheim, Germany). Standards were obtained as follows: (−)-Ec from Sigma (St. Louis, MO) and taxifolin from Extrasynthese (Genay, France). Their purity was >95%. Blank human plasma was purchased from Sigma-Aldrich Co. (Steinheim, Germany). All the chemicals used were of analytical or chromatographic grade. The water was purified in a Milli-Q water purification system (Millipore, Molsheim, France).

Cocoa Flavonoids Determination. The determination and quantification of (−)-Ec and other phenolic compounds in a cocoa powder used in this study were performed as described by Andrés-Lacueva et al. (*17*), with minor modifications. In brief, 0.5 g of cocoa powder was mixed with 5 mL of deionized water (at 100 °C) and 20 mL of methanol. The extract was shaken in a vortex for 1 min and subsequently centrifuged for 15 min at 2000*g* and 4 °C. The supernatant was then concentrated to remove methanol and filtered through a 4-mm poly(tetrafluoroethylene) (PTFE) 0.45 μm filter. The extract (100 μL) was injected in a Hewlett-Packard HP1050 gradient liquid chromatograph with a DAD 1050M with a Chemstation HP Rev. Asterix 05.02, monitoring the phenolic compounds at 280 nm. The column used was a Nucleosil 120 C_{18} (250 × 4 mm^2, 5 μm), and the solvents were acidulated water (0.1% HCOOH) and acetonitrile (0.1% HCOOH).

Biological Samples Collection and Storage. Subjects and clinical study design: 5 nonsmoking healthy volunteers (2 women and 3 men aged from 18 to 49 years) with no history of heart disease or homeostatic disorders were included in a randomized, crossover trial. Participants were instructed to abstain from vitamin supplements, drugs, alcoholic beverages, and any polyphenol containing foods for at least 24 h before and during the test day. Subjects fasted at least 8 h before test food consumption. All volunteers consumed a cocoa beverage containing 40 g of cocoa powder (Nutrexpa, Spain) in 250 mL of whole milk (Ato, Spain) or 250 mL of whole milk in a random order. Biological samples were obtained before and 2 h after consumption of the test foods (cocoa beverage or milk control). Venous blood (20 mL) was collected in 5-mL vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (Becton Dickinson, Franklin Lakes, NJ). The plasma was obtained after blood centrifugation at 13000*g* during 15 min and stored in Eppendorf tubs at −80 °C until analysis.

Immediately after the blood draw, the subjects consumed the test foods (cocoa beverage or milk control). Additional blood samples were taken 2 h later and processed as indicated. All subjects were given a light meal of bread and cheese 4 h after the first blood collection. Urine samples were collected in sterilized bottles of 1.5 mL before cocoa beverage intake and all urine for 6 h after ingestion. The urine before storage at −80°, was acidified with HCl in order to preserve the phenolic compounds.

The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 1996, the protocol was approved by the Institutional Review Board of the Hospital Clinic, Barcelona, and all volunteers gave written informed consent before their inclusion in the trial.

Instrumentation. Samples were extracted by an SPE-Vacuum Visiprep Manifold from Supelco (Barcelona, Spain). LC analyses were performed using a Perkin-Elmer series 200 (Norwalk, CT) with a quaternary pump. An API 3000 triple-quadrupole mass spectrometer (Perkin-Emer Sciex, Concord, ON, Canada) equipped with a Turbo ionspray source in negative-ion mode was used to obtain the MS and MS/MS data. A Luna C18 column (50 × 2 mm i.d., 5 μm) (Phenomenex, Torrance, CA) was used.

Sample Preparation. Plasma samples were treated after collection and storage as follows. Plasma (1.5 mL) was mixed with 555 μL of antioxidant solution containing 0.2 g/mL ascorbic acid, 1 mg/mL EDTA, and with 30 μL of *o*-phosphoric acid to break the protein−phenol bond. After vortex mixing for 2 min, the mixture underwent a SPE with a Waters Oasis HLB 3 cm^3 (60 mg) cartridge (Milford, MA).

The SPE procedure consisted of the application of the acidulated plasma to an activated Waters Oasis HLB extraction cartridge. Cleanup of the cartridge was done with 1.5 M formic acid (2 mL) and with water−methanol (95:5) (2 mL). (−)-Ec and its metabolites were eluted with 2 mL of methanol with 0.1% formic acid. The eluted fraction was evaporated under nitrogen, and the residue was reconstituted with mobile phase up to 150 μL and filtered through a 4-mm, 0.45-μm PTFE filter (Waters) into an amber vial insert for LC-MS/MS analysis. The urine samples were treated in the same way.

The preparation of standards and the processing of the samples were performed in a darkened room with a red safety light to avoid the oxidation of the analytes during the process.

LC-MS/MS Conditions. For the analysis of (−)-Ec and its metabolites, a triple-quadrupole mass spectrometer was used. The elution gradient was carried out with binary solvent system consistent in water−0.1% formic acid (solvent A) and acetonitrile−0.1% formic acid (solvent B) at a constant flow rate of 800 μL min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent B was applied (*t*(min), %B): (0, 9), (4, 20), (9, 100). Taking into account that the sample is dissolved in the LC initial mobile phase, injection volume was increased up to 40 μL, thus observing a peak width increase going from 15 to 40 μL, so a decision was made to inject 15 μL.

MS/MS conditions were optimized for (−)-Ec as described by Sánchez-Rabaneda et al. (*18*). Because of the absence of commercially available metabolites of (−)-Ec, the same MS/MS conditions were used in this work. The turbo ionspray source settings were: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, −200 V; entrance potential, −10 V; drying gas (N_2) heated to 300 °C and introduced at a flow rate of 6000 cm^3 min^{-1}. The declustering potential was −50 and the collision energy−20. In all experiments, the Q1 and Q3 quadrupoles both operated at unit resolution.

RESULTS

Identification and Confirmation of (−)-Ec Metabolites in Human Plasma and Urine. To identify and quantify the (−)-Ec metabolites in human plasma and urine, several extraction procedures and analytical methods were used. First of all,

Table 1. Molecular Weight and *m*/*z* of the Main Metabolites of (–)-Ec Described in the Literature and Investigated in This Study and the Corresponding Traces in MRM

metabolites	Mw	trace
(–)-epicatechin ($C_{15}H_{14}O_6$)	290	*m*/*z*/ 289 → *m*/*z* 245
O-methyl epicatechin	304	M/*z* 303 → *m*/*z* 289
(–)-epicatechin-*O*-*β*-D-glucuronide	466	*m*/*z* 465 → *m*/*z* 289
O-methyl epicatechin glucuronide	480	*m*/*z* 479 → *m*/*z* 289
sulfated epicatechin	370	*m*/*z* 369 → *m*/*z* 289
sulfated *O*-methyl epicatechin	384	M/*z* 383 → *m*/*z* 289
sulfated epicatechin-*O*-*β*-D-glucuronide	546	*m*/*z* 545 → *m*/*z* 289
sulfated *O*-methyl epicatechin glucoronide	560	*m*/*z* 559 → *m*/*z* 289

the phenolic fraction of the plasma samples was isolated by precipitating the proteins with different organic solvents, methanol or acetonitrile acidulated with HCl or TFA acids, then the supernatant was evaporated and filtered as described by Morand et al. (*19*). However, the results were not those expected. The extraction with methanol−HCl gave (−)-Ec recoveries lower than 50%, while with acetonitrile−TFA, a good recovery (>80%) was obtained. However the extraction with acetonitrile−TFA gave a low ratio of signal-to-noise in the MS/MS analysis, thus decreasing the metabolite's responses. Therefore, we tested and developed a SPE method to improve the signal-to-noise ratio for (−)-Ec metabolites. The SPE with Oasis HLB cartridges served as a simple and rapid method to remove sugars, proteins, and other substances in samples so as to extract and concentrate the (−)-Ec metabolites from their matrix for further analyses.

Because of the absence of available (−)-Ec metabolite standards, a urine sample from 6 h after the cocoa beverage intake was investigated using LC-MS/MS in MRM to check the traces of all (−)-Ec metabolites described in the literature (*13*−*16*, *19*). The objective was to identify the metabolites and to establish their retention time (t_r). The molecular weight and *m*/*z* of the investigated (−)-Ec metabolites are summarized in **Table 1**. These structures were identified in plasma and urine by Natsume et al. (*14*), who analyzed these compounds by LC-MS and NMR with a chromatographic run time of 30 min. **Figure 1** shows the chromatogram obtained from the MRM experiment where five different (−)-Ec metabolites appear; the M1 shows *m*/*z* 369/289 corresponding to three epicatechin−sulfates (EcS) M1a (t_r = 5 min), M1b (t_r = 6.3 min), and M1c (t_r = 7.8 min). M2 shows *m*/*z* 465/289 (t_r = 2.3 min), corresponding to a epicatechin−glucuronide (EcG). M1 (a, b, and c) and M2 were confirmed in a second experiment, a product-ion scan (PIS) mode. M3, M4, and M5 show *m*/*z* of 479/289, 545/289, and 559/289, corresponding to a methyl-glucuronide, sulfo-glucuronide, and methyl-sulfo-glucuronide of (−)-Ec, respectively.

Preliminary examination of the chromatograms in the MRM acquisition mode revealed the possible presence of five epicatechin metabolites in urine, but this presence had to be confirmed with experiments in a PIS. In this experiment, the fragment ions which arise from a precursor ion were determined, thus allowing the confirmation of some of the metabolites identified in the MRM scan mode. To confirm the structures of these compounds, different subfractions were studied in PIS mode of *m*/*z* 369, 465, 479, 545, and 559 (corresponding to sulfates, glucuronide, methyl-glucuronide, sulfo-glucuronide, and methyl-sulfo-glucuronide of (−)-Ec respectively).

Figure 2 shows the PIS of (−)-EcS, where three sulfates were confirmed showing different relative intensities in mass spectra. This was due to the presence of the sulfate group in different

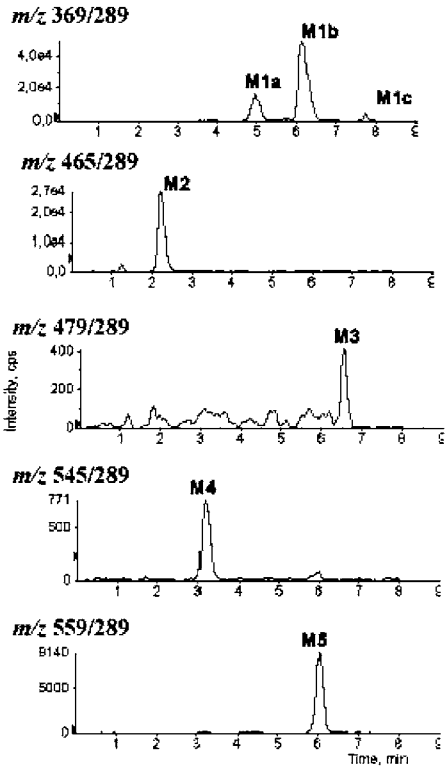

Figure 1. Traces for the (–)-Ec metabolites of urine samples in MRM mode. Peaks: (M1), (–)-EcS; (M2), (–)-EcG; (M3), methyl (–)-EcG; (M4), sulfate (–)-EcG; (M5), sulfate-methyl (–)-EcG.

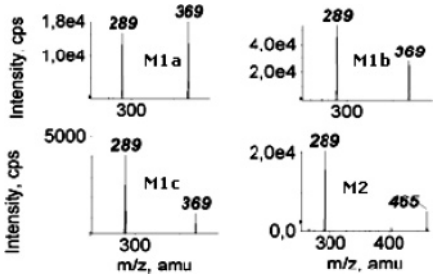

Figure 2. Product-ion spectrum of M1 and M2 (–)-Ec metabolites detected in the urine samples. M1a, b, and c correspond and confirm three (–)-Ec sulfates where the peak at 289 *m*/*z* derives from the loss of a sulfate group in a different position. M2 confirms the presence the (–)-EcG; the peak at 289 *m*/*z* derives from the loss of a glucuronic acid.

positions that could not be assigned due to the lack of reference standards. Also, the PIS of (−)-EcG has confirmed one glucuronide.

PIS spectra for *m*/*z* 369 produced an ion at *m*/*z* 289 due to the loss of 80 units due to the loss of a sulfate unit, and product-ion scan for *m*/*z* 465 produced an ion at *m*/*z* 289 due to the loss of 176 units and the loss of a glucuronide unit. **Figure 2** shows the mass spectra generated by the product ion scan of M1 and M2 where M1 showed $[M - H]^-$ at *m*/*z* 369 and a minor ion at *m*/*z* 289, indicating that a, b, and c contained a sulfate group attached to (−)-Ec. M2, showed $[MH]^-$ at *m*/*z* 465 and a minor ion at *m*/*z* 289, indicating that it contains a glucuronide group attached to (−)-Ec. In **Figure 3**, the MRM chromatogram of a plasma sample and the mass spectra generated by the PIS where the only peak detected and confirming is the (−)-EcG, at the retention time of 2.3 min as in urine samples, is shown. The position of glucuronidation and sulfatation was not concisely

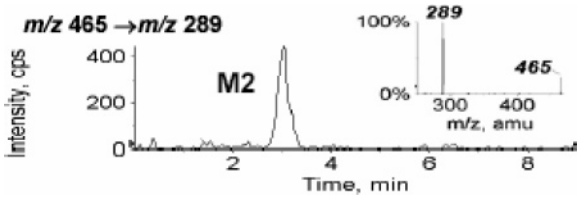

Figure 3. Trace for (–)-EcG of plasma sample in MRM 2 h after consumption of a cocoa beverage and its product ion spectrum, confirming that the peak at 289 *m/z* derives from the loss of a glucuronic acid.

identified by LC-MS/MS analysis. However, Natsume et al. (*14*) stated that one of the main metabolites of (−)-Ec in human plasma and urine was (−)-epicatechin-3′-*O*-glucuronide, so it may be supposed that the glucuronide detected in our study is also glucuronidated in the 3′ position.

Performance Characteristics. *Linearity.* Standard addition (−)-Ec curves were prepared from the pure standard in a blank matrix containing taxifolin as an internal standard. The peak areas were plotted against the corresponding concentration to obtain the calibration curve. The method was linear over the working range between 15 and 300 μg/L.The least-squares regression calibration curve for (−)-Ec standard was (mean (SD)): slope, 0.000825 (9.09×10^{-5}); intercept, 0.05 (0.01); r^2 0.999 (0.0009). The residuals analysis for this range of concentration was (mean (SD)): 100.6% (±6.7) (*21*).

Sensitivity. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by repeated injections of diluted solutions of epicatechin in plasma blanks. The LOD was estimated as the concentration of (−)-Ec that generated a peak with an area at least 3 times higher than the baseline noise. LOQ was calculated at a signal-to-noise ratio of 10 for (−)-Ec compounds. The LOD and LOQ for (−)-Ec standard in plasma samples was 4 μg/L (cyclic voltammetry (CV) = 7.5%, $n = 9$) and 13.3 μg/L (CV = 6.6%, $n = 9$), respectively.

Precision and Accuracy. To assess within run imprecision, we analyzed a sufficient number of samples at normal ($n = 10$) concentration from (−)-Ec standard solution in plasma blank (50 μg/L) and from a real plasma sample ($n = 10$) where we analyzed the precision on retention time and concentration with the (−)-EcG metabolite. The bioanalytical precision and accuracy were assessed through replicate analysis of samples containing known amounts of (−)-Ec and taxifolin prepared in blank human plasma by SPE; we analyzed three (−)-Ec standard solutions at low (18 μg/L) ($n = 3$), normal (40 μg/L) ($n = 3$), and above normal concentration (300 μg/L) ($n = 3$) in three replicates each over 7 days. **Table 2** shows the precision and accuracy of this method. Satisfactory results were obtained giving precision and accuracy that was acceptable according to the criteria (*22*, *23*).

Recovery. To assess the recovery of the proposed method, we studied the (−)-Ec recovery in two different matrixes: in water to assess the SPE-LC-MS/MS method and in blank human plasma to observe the matrix influence, both at a concentration range from 10 to 100 μg/L with taxifolin as internal standard. The recovery in water was 93.5% (CV = 3.14%), while in the matrix it was 71% (CV = 5.3%) possibly due to the complex matrix that could interfere in the (−)-Ec metabolite's ionization or due to the possible interaction of phenols with plasma components such as protein which can occur during the extraction.

Quantification of (−)-Ec Metabolites in Plasma. The concentrations of identified (−)-Ec metabolites were measured using standard addition curves of (−)-Ec with taxifolin as internal standard done in plasma blank matrix.

In our study, a plasma (−)-Ec concentration was found to be 625.7 ± 198.3 nmol/L in five healthy subjects 2 h after consumption of a cocoa drink that provided 54.4 mg (−)-Ec (11.05 $nM_{plasma\ EC}/mg_{ingested\ EC}$), while after the consumption of only milk by the same subjects this value was below the detection limit. Similar results were observed by Baba et al. (*13*), who reported nonmethylated plasma (−)-Ec concentration of 3.46 μmol/L in healthy subjects 2 h after they had consumed a cocoa drink that provided 220 mg of epicatechin (15.72 $nM_{plasma\ EC}/mg_{ingested\ EC}$). Our results obtained with the proposed method were comparable to those obtained by other authors (*10*, *12*, *13*), which quantify the (−)-Ec metabolites as (−)-Ec but after their enzymatic hydrolysis, with glucuronidases and sulfatases. However, the direct determination of the extract allowed us to consider all the metabolite profiles of the (−)-Ec.

Nevertheless to corroborate that the quantification using (−)-Ec was acceptable, we performed an enzymatic hydrolysis: for this an enzymatic method described by Piskula and Terao (*24*) was used to demonstrate that the combined quantities of (−)-Ec metabolites measured with the proposed method are in agreement with the measured quantity of (−)-Ec released after enzymatic treatment. Before the enzymatic hydrolysis the mean concentration of (−)-EcG expressed as (−)-Ec ($n = 10$) was 80.30 ± 7.92 μg/L while after the enzymatic hydrolysis the (−)-EcG peak completely disappears and the (−)-Ec was the only peak in a concentration of 70.86 ± 9.25 μg/L. After statistics analysis (Student's t test), we can assess that the quantification of (−)-EcG as (−)-Ec without enzymatic hydrolysis is an acceptable way to do it because both means were not significant differences ($P < 0.05$).

Table 2. Within- and Between-Run Precision and Accuracy of the Assay

Within-Run Precision of the Assay

	R_t (min)			concn (μg/L)		
compound (concn)	mean (min)	SD	CV (%)	mean (μg/L)	SD	CV (%)
(–)-Ec (normal) ($n = 10$)	2.83	0.0 4	1.4	37.62	2.8	7.4
(–)-EcG (normal) ($n = 10$)	3.18	0.1 6	5.0	148.7	7.1	4.8

Between-Day Precision of the Assay

	R_t (min)			concn (μg/L)			
compound (concn)	mean (min)	SD	CV (%)	mean (μg/L)	SD	CV (%)	accuracy (%)
(–)-Ec (low, 18 μg/L) ($n = 3$)	2.68	0.2 5	9.5	15.45	0.2	1.5	86
(–)-Ec (normal, 40 μg/L) ($n = 3$)	2.81	0.0 2	0.7	43.78	2.3	5.2	109
(–)-Ec (above normal, 300 μg/L) ($n = 3$)	2.78	0.0 2	0.6	296.9	28. 8	9.7	99

DISCUSSION

Our findings show that the main (−)-Ec metabolites in urine after cocoa consumption were glucuronide and sulfate conjugates of nonmethylated forms, while only the glucuronidate form is found in plasma. Similar results were observed by Harada et al. (*7*) where the (−)-Ec 5-*O*-*β*-glucuronide was the only metabolite found in the plasma without any enzymatic treatment using rats after the ingestion of (−)-Ec solution. In urine, Lee et al. *(25)* also observed glucuronide and sulfate results when tea (rich (−)-Ec source) was administered to humans.

Contrary to our results, Natsume et al. (*14*) describes only the presence of the glucuronide form in urine, while Baba et al. (*13*) detected sulfated and sulfoglucuronide forms as the major metabolites in urine. These contradictory results could be explained by the differences in the analytical method, or a concomitant enzyme action when sulfatase and glucuronidase were used, or by the (−)-Ec dose.

The most important advantages of the method presented over the reported methods for (−)-Ec metabolites determination are its accuracy, speed, and sensitivity, and the ability of the method to quantify the (−)-Ec metabolites in human plasma directly without enzymatic pretreatment.

In conclusion, a SPE-LC/ESI-MS/MS method has been developed that allows an accurate, precise, and rapid determination of cocoa (−)-Ec metabolites in human fluids after intake of a regular quantity of (−)-Ec present in a food matrix such as a cocoa beverage in a chromatographic run time of only 9 min.

This method will make it possible to screen urine and plasma samples for the determination of metabolites in kinetic and future bioavailability studies. In urine, the major (−)-Ec metabolites found were the sulfate conjugates, but glucuronide conjugate was also detected. However, one glucuronide conjugate of epicatechin was the only metabolite present in plasma. To our knowledge, this is the first time that epicatechin metabolites have been quantified in human plasma without any enzymatic hydrolysis after the consumption of a real portion of a cocoa product.

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