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ORIGINAL PAPER

Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats

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Abstract Proanthocyanidins, flavonoids exhibiting cardiovascular protection, constitute a major fraction of the flavonoid ingested in the human diet. Although they are poorly absorbed, they are metabolized by the intestinal microbiota into various phenolic acids. An analytical method, based on an optimized 96-well plate solid-phase extraction (SPE) procedure and liquid chromatography tandem mass spectrometry (SPE-LC-MS/MS) for the analysis of 19 phenolic microbial metabolites and monomeric and dimeric flavanols in urine samples, was developed and validated. Human urine samples were obtained before and after ingestion of an acute consumption of 40 g of soluble cocoa powder and rat urines before and after the prolonged administration (2 weeks) of different diets composed of natural cocoa powder. The mean recovery of analytes using the new SPE-LC-MS/MS method ranged from 87% to 109%. Accuracy ranged from 87.5% to 113.8%, and precision met acceptance criteria (<15% relative standard deviation). Procyanidin B2 has been detected and quantified for the first time in human and rat urine after cocoa consumption. Changes in human and rat urinary levels of microbial phenolic acids and flavanols were in the range of 0.001–59.43 nmol/mg creatinine and of 0.004–181.56 nmol/mg creatinine, respectively. Major advantages of the method developed include reduction of laboratory work in the sample preparation step by the use of 96-well SPE plates and the sensitive measurement of a large number of metabolites in a very short run time, which makes it ideal for use in epidemiological studies.

Keywords Cocoa · Epicatechin · Procyanidin · Microbial metabolites · LC-MS/MS

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Introduction

Polyphenols are among the most abundant antioxidant compounds in our diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases and cancer [1]. These antioxidants are widely distributed in plants and are present in fruits, cocoa, legumes, and in fruit-derived products such as juices, wine, beer, and cider [1]. Health effects derived from polyphenol consumption depend on their bioavailability (absorption, distribution, metabolism, and elimination), a factor which is also influenced by their chemical structure [2]. Among the

flavonoids, isoflavones, which are limited to soya and derived products, present the best bioavailability. In contrast, oligomers and polymers of flavan-3-ol (proanthocyanidins), which are very abundant in our diet, are poorly absorbed [2]. These polyphenols reach the colon and are metabolized by the intestinal microbiota into various phenolic acids, mainly including phenylpropionic, phenylacetic, and benzoic acid derivatives [3]. Recently, it has been reported that some of these metabolites may also present biological properties including antioxidant, antiplatelet aggregation [4], and antiproliferative activities [5].

Accurate estimation of polyphenol intake or exposure is of high importance in order to determine the bioavailability of these compounds and to be able to calculate the polyphenol doses that could be related to certain health effects in epidemiological studies. Although normally based on food composition tables, which are often incomplete, polyphenol intake can also be estimated by measuring biomarkers of exposure in plasma or urine, such as phenolic microbial metabolites [6, 7]. This is particularly important in the case of proanthocyanidins for which intake doses are very difficult to estimate due to their structural complexity and lack of adequate analytical methods. These facts, together with recent findings demonstrating that some microbial metabolites derived from proanthocyanidin consumption possess certain biological properties, prompt the need for analytical methods that allow a rapid and sensitive measuring of these compounds in biological samples generated in large epidemiological studies.

Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) has been demonstrated to be highly suitable for the analysis of phenolic metabolites [10–12]. However, sample preparation is a very important step to reduce matrix effects and increase sensitivity when quantifying phenolic metabolites by LC-MS/MS [12]. Liquid–liquid extraction (LLE) is the most common technique used for the extraction of phenolic microbial metabolites in urine samples [13–15]. However, LLE involves multistep sample extraction and cleanup procedures that are time-consuming and that use large amounts of solvent, which can result in analyte loss and large matrix interferences under LC-MS/MS. To improve these procedures, in the present work we have introduced the use of solid-phase extraction (SPE) in 96-well plates for the extraction of phenolic microbial metabolites in order to increase sample throughout and minimize matrix effects [12]. In addition, a very short run time and highly sensitive LC-MS/MS method has been developed for the simultaneous determination of epicatechin, procyanidins, and phenolic microbial metabolites in urine samples. The analytical method has been validated and applied to both human and rat urines collected after ingestion of nutritional doses of different cocoa products, which is known to be a very rich source of proanthocyanidins.

Experimental

Standards and reagents

The following standards (% purity) were used. Phenylacetic acid (≥98%); 3-hydroxyphenylacetic acid (≥97%); 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid; 98%); 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid; 99%); 4-hydroxyphenylpropionic acid (phloretic acid; ≥98%); 3,4-dihydroxyphenylpropionic acid (hydrocaffeic acid) (≥98%); *p*-coumaric acid (≥98%); caffeic acid (≥95%); ferulic acid (≥98%); protocatechuic acid (>97%); 4-hydroxybenzoic acid (≥98%); 3-hydroxybenzoic acid (≥98%); hippuric acid (98%); enterodiol (95%); enterolactone (95%); ethyl gallate (≥96%); (−)-epicatechin (≥98%), procyanidin B2 (≥90%), creatinine and β-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxyhippuric acid (>99%) was purchased from PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). Vanillic acid, 4-*O*-methylgallic acid, and *m*-coumaric acid were purchased from Extrasynthèse (Genay, France). HPLC-grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Human subjects and study design

Twenty-one nonsmoking healthy volunteers (nine women and 12 men) between 18 and 50 years old with a body mass index of (mean±SD) 21.6±2.1 were recruited [16]. None reported any history of heart disease, homeostatic disorders, or other medical disease nor received any medication or vitamin supplement. All gave written informed consent before their inclusion in the trial, and the Institutional Review Board of the Hospital Clínic of Barcelona (Spain) approved the study protocol.

Participants were instructed to abstain from polyphenol-rich foods for at least 48 h before and during the intervention day. After overnight fasting, they were provided with a single dose of 40 g of cocoa powder with 250 mL water. After 4 h of the cocoa intake, a light meal consisting of bread and cheese was provided. Urine samples were collected before and after 24 h of the cocoa consumption and stored at −80 °C until analysis.

The soluble cocoa powder used in the study contained 57% of carbohydrates (sucrose, 46%; starch, 1%; complex carbohydrates, 10%), 16% of fiber, 5.4% of fat, 14.1% of protein, 3.97% of moisture, 1.3% of theobromine, 0.13% of caffeine, and 2% of ash. The phenolic composition (mean±SD) of the cocoa powder was determined according to the methodology of Andres-Lacueva et al. [17] and Roura et al.

[18]: 23.1% of monomers with 0.71±0.09 mg/g of (−)-epicatechin and 0.21±0.01 mg/g of (+)-catechin, 13.4% of dimers, among which are 0.64±0.06 mg/g of procyanidin B_2, 63.6% of 3–8mers [16,19], and flavonols including 33.87 μg/g isoquercitrin, 5.74 μg/g quercetin, 4.33 μg/g quercetin-3-glucuronide, and 36.32 μg/g quercetin-3-arabinoside. The total polyphenolic content was 11.51±0.95 mg catechin/g cocoa powder.

Animal study design

Dams with 15 day-old Wistar rat litters were obtained from Harlan (Barcelona, Spain). Rats were housed in cages of 10 pups per lactating mother in controlled conditions of temperature and humidity in a 12:12 light/dark cycle.

At day 21, pups were weaned and randomly distributed in three different groups (n=7 for each group) receiving the following diet: (1) 4.8 g natural cocoa powder/kg/day by oral gavage with free access to control chow and water (this dose corresponded to a chow containing ~4% (*w*/*w*) natural cocoa powder), (2) chow containing 10% (*w*/*w*) natural cocoa and free access to water, and (3) control diet group [20]. After 2 weeks of dietary treatment, rats were maintained in metabolic cages for 24 h to collect urine output. Urine samples were frozen at −80 °C for further analysis.

The chow (AIN-93G formulation) used in this study had the following composition: 200 g/kg of casein, 3 g/kg of L-cystine, 397.5 g/kg of corn starch, 132 g/kg of maltodextrin, 100 g/kg of sucrose, 70 g/kg of soybean oil, 50 g/kg of cellulose, 35 g/kg of mineral mix (TD94046), 10 g/kg of vitamin mix (TD94047), 2.5 g/kg of choline bitartrate, and 0.014 g/kg of *tert*-butylhydroquinone, equivalent to an intake of 3,700 kcal/kg diet. The natural cocoa powder contained 22% of protein, 16% of carbohydrate, 11% of lipid, 32% of fiber, 4% of moisture, 12.3% of ash, 2.5% of theobromine, and 0.1% of caffeine. The 10% cocoa diet was prepared from the AIN-93G formulation control removing 72.8 g/kg (16 g/kg of corn starch, 11 g/kg of soybean oil, 25.5 g/kg of cellulose, and 22 g/kg of casein) and adding natural cocoa [20].

The phenolic composition (mean±SD) of the natural cocoa powder (Forastero variety) and the chow containing 10% (*w*/*w*) natural cocoa was: 2.2±0.1 and 0.34±0.01 mg/g of (−)-epicatechin, respectively; 0.74±0.05 and 0.10±0.004 mg/g of (+)-catechin, respectively; 1.68±0.11 and 0.23±0.01 mg/g of procyanidin B2, respectively; 0.05±0.001 and 0.02±0.00 mg/g of isoquercetin, respectively; 0.03±0.00 and 0.01±0.00 mg/g of quercetin, respectively; the total polyphenolic content was 21.85±1.08 and 0.90±0.54 mg catechin/g cocoa, respectively [20].

The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 3131).

Enzymatic hydrolysis

One milliliter of urine sample was spiked with 100 μL of 10.1 nmol/mL ethyl gallate as internal standard (IS). Ethyl gallate was selected as the IS due to its absence in human body fluids, its selectivity, recovery (93%), and precision (relative standard deviation (RSD)=8%). Samples were then hydrolyzed as previously described, although some modifications were applied [8]. Briefly, samples were acidified with 50 μL of 0.58 mol/L acetic acid to pH 4.9 and incubated with β-glucuronidase/sulfatase at 37 °C for 45 min. Straight afterwards, samples were acidified to pH 2 with 6 mol/L HCl.

Preparation of standard solutions

The different phenolic standards (n=19), and (−)-epicatechin and procyanidin B2 standards, were dissolved in 80% methanol to prepare 800 mg/L stock solutions. A standard pool solution (16 mg/L of each analyte) was prepared in Milli-Q ultrapure water from the stock solution and stored at 4 °C. For calibration purposes, a wide range of standards (0.5–500 μg/L) at six different concentration levels were prepared from the pool in synthetic urine containing ten solutes with the pH adjusted to 6.5 [21].

Solid-phase extraction

SPE was performed using both Oasis® MCX (mixed-mode-cation-exchange/reversed-phase sorbent) and MAX (mixed-mode-anion-exchange/reversed-phase sorbent) 96-well plates (Waters, Mildford, Massachusetts), a vacuum manifold, and a vacuum source. Firstly, optimization of the extraction conditions was carried out taking into account the fabricant recommendations (Waters Oasis®). Extraction recovery test was then performed in both cartridges in order to select the most suitable cartridges for the extraction of phenolic metabolites from urine samples.

The MCX 96-well plate was conditioned with 1 mL of methanol followed by 1 mL of 2% formic acid. One milliliter of the analyte standard solution in synthetic urine or hydrolyzed urine sample was then loaded onto the plate and washed with 1 mL of 2% formic acid. The plates were thoroughly dried by vacuum (1.69×10^4 to 5.07×10^4 Pa). Analytes were then eluted with methanol (1.5 mL) by gravity and finally by vacuum (max. 1.69×10^4 Pa).

The MAX 96-well plate was conditioned with 1 mL of methanol followed by 1 mL of water. One milliliter of the analyte standard solution in synthetic urine or hydrolyzed urine sample was then loaded onto the plate and washed with 1 mL of 5% ammonium hydroxide. The plates were

thoroughly dried by vacuum (1.69×10^4 to 5.07×10^4 Pa). Analytes were then eluted with methanol (1.5 mL) and with 1 mL of 2% formic acid in methanol by gravity and finally by vacuum (max. 1.69×10^4 Pa).

Eluates from both cartridges were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 μL of initial mobile phase.

LC-MS/MS

LC analyses were performed using a Perkin Elmer series 200 (Norwark CT) equipped with a quaternary pump, a refrigerated autosampler (set at 4 °C), and a column oven (set at 35 °C). An Applied Biosystems API 3000 Triple Quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray ionizing in negative mode was used. Separation was performed in a Phenomenex Luna C_{18} analytical column (50×2.0 mm i.d., 5 μm; Torrance, CA) applying a linear gradient consisting of two mobile phases, A (water/acetonitrile/formic acid, 94.9:5:0.1, *v*/*v*/*v*) and B (acetonitrile/formic acid, 99.9:0.1, *v*/*v*) at a flow rate of 400 μL/min, as follows: 0–1 min, 4–40% B; 1–3 min, 40–100% B; 3–5 min, 100% B; 6–10 min, 4% B. Then, the column was washed and re-equilibrated for 6 min. The injected volume was 15 μL. MS/MS parameters for phenolic acid compounds were optimized by infusion and flow injection analysis (FIA). Direct infusion experiments were performed at a constant flow rate of 5 μL/min to optimize the following MS parameters: capillary voltage, focusing potential, entrance potential, declustering potential, and collision energy. Nebulizer, curtain, collision, and auxiliary (nitrogen) gas flow rates, as well as the auxiliary gas temperature were optimized using FIA experiments.

For quantification purposes, data were collected under the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound. A dwell time of 100 ms was used for each injection. Due to its high concentration in urine, hippuric acid was analyzed at detector wavelength of 240 nm and identified by comparing with the retention time of the authentic standard.

Human and rat urine creatinine concentrations were measured by a colorimetric assay using picric acid [18].

Statistical analysis

SPSS Statistical Analysis System, version 14.0 (SPSS) was used to perform the statistical analysis. Because the data were nonparametric (Kolmogorov test) and presented nonhomogeneous variances (Levene test), the Wilcoxon test for related samples (human urines) and the Mann–Whitney test for unrelated samples (rat urines) were used to compare changes in outcome variables in response to the intervention period or type of intervention. Significant increases between 0 and 24 h for human urine samples and between the control group and the cocoa groups for rat urine samples were expressed as means±standard error of the mean (SEM). Statistical tests were two-tailed, and the significance level was 0.05.

Results and discussion

Quality parameters of the method

After optimizing the LC-MS/MS conditions for each analyte, the quality of the method was evaluated according to the criteria of the Food and Drug Administration for method validation [22]: selectivity, linearity, sensitivity, recovery, precision, and accuracy.

LC-MS/MS optimization The optimized MS/MS parameters for the different analytes after performing infusion and FIA analyses were as follows: capillary voltage, −3,700 V; focusing potential, −200 V; entrance potential, −10 V; declustering potential, −50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6,000 cm^3/min. The collision energy (V) obtained for each phenolic compound was: 3,4-dihydroxyphenylpropionic acid (−16); 4-hydroxyphenylpropionic acid (−16); *m*- and *p*-coumaric acids (−30); caffeic acid (−21); ferulic acid (−25); 3,4-dihydroxyphenylacetic acid (−12); 3-methoxy-4-hydroxyphenylacetic acid (−10); 3-hydroxyphenylacetic acid (−12); phenylacetic acid (−12); protocatechuic acid (−20); vanillic acid (−20); 3- and 4-hydroxybenzoic acids (−16); 4-*O*-methylgallic acid (−26); 4-hydroxyhippuric acid (−20); enterodiol (−30); enterolactone (−26); (−)-epicatechin (−25), and procyanidin B2 (−25). The MRM chromatograms of the different analytes in synthetic urine under the optimized MS/MS conditions are shown in Fig. 1.

Selectivity Under the chromatographic and MS/MS conditions used for the assay, metabolites and standards were well resolved, and no interferences from matrix components at the mass transition and retention time of the analytes were observed in synthetic urine.

Detection and quantification limits The limit of detection (LOD) was calculated as the concentration of analyte in synthetic urine that produced a signal-to-noise ratio equal to 3. The limit of quantification (LOQ) was considered as the lowest standard concentration in the calibration curve prepared with synthetic urine showing high reproducibility (precision of 20% and accuracy of 80–120%) [22]. The

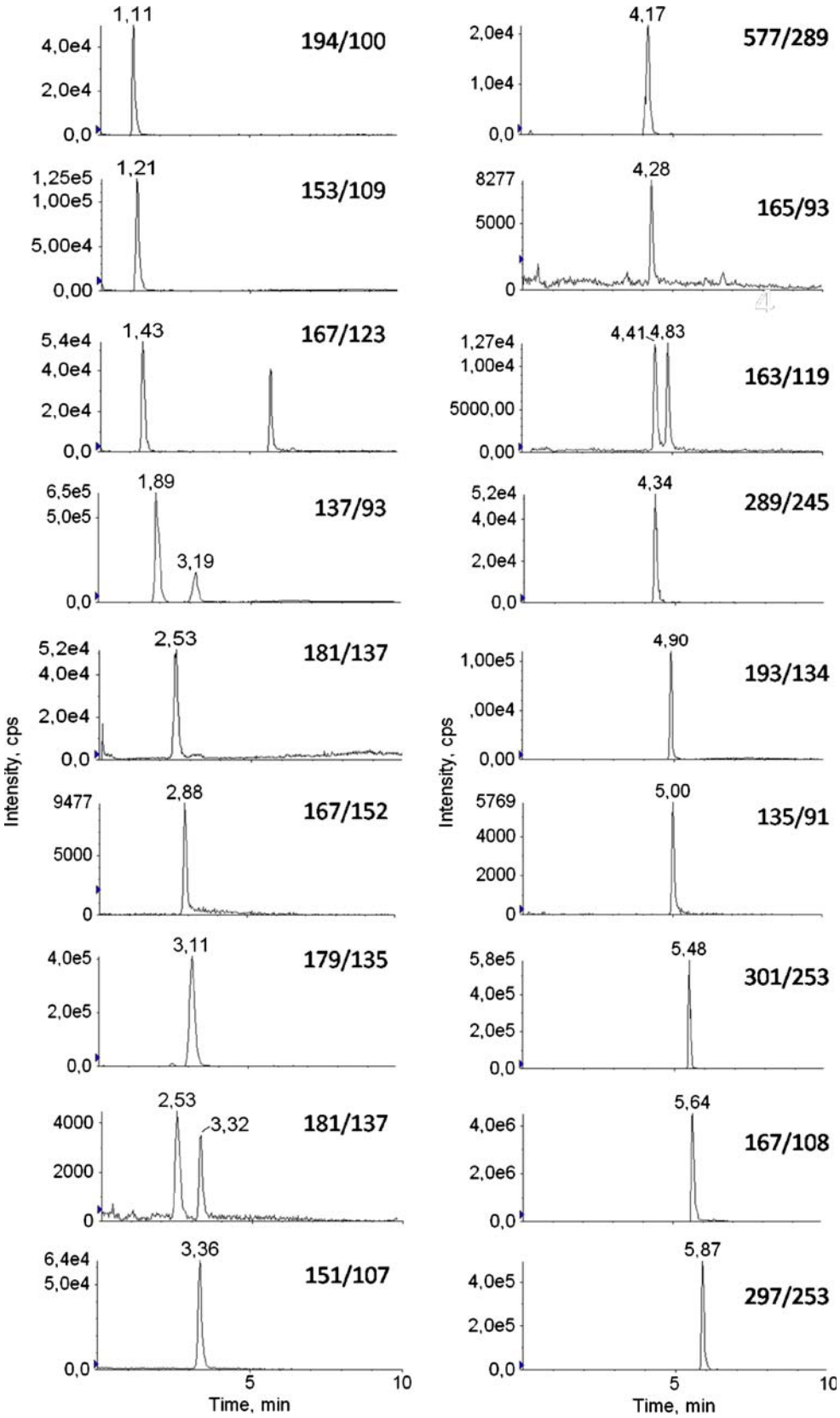

Fig. 1 MRM chromatograms of the standards in synthetic urine. MRM transitions are referred to analytes presented in Table 1

LODs (LOQs) varied between 0.030 μg/L (0.50 μg/L) for enterodiol and 44.4 μg/L (50 μg/L) for phenylacetic acid, indicating a wide range of sensitivity between the different analytes (Table 1). The sensitivity of this method represents a significant improvement (6–100 times better) for most of the analystes when compared to other LLE-LC-MS/MS published methods [13, 14]. This will prove useful in nutritional studies when low levels of metabolites must be measured.

Extraction recovery, linearity, and residuals SPE was compared between MCX and MAX 96-well plates in their optimum conditions. Similar recoveries were obtained with both cartridges when standards were compared. However, in the case of urine or hydrolyzed urine samples, the recovery was about 20% higher with MCX than with MAX cartridges, indicating that the former support resulted in a larger retention of interfering matrix components that could lead to signal suppression of analytes of interest under LC-MS/MS.

The total extraction recovery was defined as the detector response obtained from an amount of analyte added to and extracted from a standard in synthetic urine compared to the detector response obtained for the true concentration of that standard solution. The total recovery values, expressed as the mean percentage value (% ±SD) are shown in Table 1. A low extraction recovery was obtained for procyanidin B2, but since it met the acceptance criteria in terms of accuracy and precision, quantification was carried out with the corresponding calibration curve. However, in order to confirm the presence of procyanidin B2 in the human and rat urine samples (see "*Procyanidin in urine*" section in "Analysis of flavanols and phenolic acids in human and rat urines" below), further experiments were performed, and the extraction recovery was improved up to 80%±9.5 using

Table 1 Retention time, recovery, calibration curves, residual analysis, and limits of detection, and quantification of the SPE-LC-MS/MS method

Analyte	MRM	Retention time ± %RSD	Recovery (% ±SD)	Calibration curve (R^2)[a]	Residuals % mean (SD)	LOD (μg/L)	LOQ (μg/L)
C_6C_3							
3,4-Dihydroxyphenylpropionic acid	181/137	2.53±0.9	94±9.0	y=0.31x+0.02; 0.991	95±2.8	1.67	5.0
4-Hydroxyphenylpropionic acid	165/93	4.28±5.2	104±7.0	y=5.69e−4x+1.1e−4; 0.999	103±6.8	16.50	100.0
m-Coumaric acid	163/119	4.87±3.5	91±4.5	y=1.06x+0.005; 0.953	97±5.6	0.26	0.5
p-Coumaric acid	163/119	4.39±3.9	94±1.8	y=0.42x+0.002; 0.986	98±12.8	0.52	1.0
Caffeic acid	179/135	3.11±5.8	102±7.0	y=2.02x+0.03; 0.999	102±5.4	0.05	0.50
Ferulic acid	193/134	4.90±1.3	92±4.8	y=0.06x+0.002; 0.997	98±9.8	0.49	1.0
C_6C_2							
3,4-Dihydroxyphenylacetic acid	167/123	1.43±2.7	109±4.0	y=0.28x+0.003; 0.998	100±10.2	0.71	1.0
3-Methoxy-4-hydroxyphenylacetic acid	181/137	3.22±8.1	93±8.0	y=0.02x+0.003; 0.999	97±7.9	37.11	50.0
3-Hydroxyphenylacetic	151/107	3.36±3.7	91±4.9	y=0.07x+0.001; 0.996	102±7.0	2.75	5.0
Phenylacetic acid	135/91	5.00±3.8	95±6.0	y=0.02x+0.0001; 0.995	106±8.3	44.40	50.0
C_6C_1							
Protocatechuic acid	153/109	1.21±2.5	96±14.7	y=0.93x+0.01; 0.988	100±13.1	0.63	1.0
Vanillic acid	167/152	2.88±11.2	95±10.0	y=7.00e−3x+0.004; 0.999	95±7.5	5.12	10.0
4-Hydroxybenzoic acid	137/93	1.86±6.2	97±4.7	y=0.32x+0.004; 0.993	98±10.4	1.68	5.0
3-Hydroxybenzoic acid	137/93	3.19±3.3	91±4.9	y=0.13x+0.004; 0.998	103±10.0	9.09	10.0
4-Hydroxyhippuric acid	194/100	1.11±3.5	86±3.9	y=0.42x+0.01; 0.993	99±11.1	0.18	0.5
Hippuric acid	–	2.17±2.2	94±5.5	y=3.67e−3x+1.73; 0.999	98±0.1	4.84	100.0
4-*O*-Methylgallic acid	167/108	5.64±0.4	89±4.0	y=3.06x+0.005; 0.996	98±12.4	0.03	0.5
Enterolignans							
Enterodiol	301/253	5.48±0.5	97±9.5	y=1.60x+0.002; 0.993	98±8.9	0.03	0.50
Enterolactone	297/253	5.87±0.4	89±3.3	y=1.60x+0.004; 0.988	100±7.6	0.06	0.50
Flavanols							
(−)-Epicatechin	289/245	4.34±2.0	87±3.0	y=0.405x+2.69e−4; 0.996	96±10.3	0.49	1.0
Procyanidin B2	577/289	4.17±7.8	10±8.5	y=3.88e−3x−2.33e−4; 0.996	100.0±8.5	4.68	100.0

[a] Calibration curves were performed from the LOQ to 500 or 1,000 μg/L, depending on the analyte response

methanol containing 0.006% of ammonia (adjusted to pH 6) as elution solvent.

Calibration curves were performed over a wide range of concentrations (0.5–500 μg/L). Depending on the analyte response, the concentration range used varied from the LOQ to 500 μg/L, or from the LOQ to 1,000 μg/L. The six-point calibrator concentrations showed a linear and reproducible curve for standards. Weighted ($1/x^2$) least-square regression analysis was applied to obtain the equation regression lines, correlation coefficients, and residual analysis [mean range (SD)] as shown in Table 1.

Precision and accuracy Accuracy and precision (repeatability) were determined using three different concentration levels for each analyte in synthetic urine in the range of expected concentrations (low, medium, and high, in function of the LOD of each particular analyte) and performing five different determinations per concentration level over a 5-day period. The precision of the method, expressed by the relative standard deviation (%RSD), met acceptance criteria since % RSD was lower than 15% at each tested concentration level (Table 2) [22]. The accuracy of the method, expressed as [(mean observed concentration)/(added concentration)×100], was acceptable at each concentration level: 90.3–113.8% for the low concentration level, 89.5–112.1% for the medium concentration level, and 87.5–108.7% for the high concentration level (Table 2) [22].

The precision of the retention time of the different analytes was also evaluated (Table 1 and Fig. 1). In general, the % RSD was lower than 10% for most of the analytes. The high % RSD for vanillic acid is consistent with a lower precision in the retention of this compound.

Analysis of flavanols and phenolic acids in human and rat urines

The validated method was applied to determine the concentration of phenolic acid microbial metabolites, as well as (−)-epicatechin and dimeric procyanidin metabolites in urine samples collected after the consumption of nutritional doses of cocoa in humans and rats. The identification and quantification of flavanols and phenolic acid metabolites in urines was done by comparing their MRM transition, MS spectra, and retention time with that of authentic standards.

Phenolic acid metabolites in urine Table 3 shows the changes in concentration and the percentage of increase of the different phenolic acid metabolites identified in human and rat urine samples after cocoa consumption.

In humans, with the exception of phenylacetic acid, an increase in the concentration of all the metabolites was registered after consumption of soluble cocoa powder. In particular, caffeic acid, ferulic acid, 3-hydroxyphenylacetic acid, vanillic acid, 3-hydroxybenzoic acid, 4-hydroxyhippuric acid, hippuric acid, (−)-epicatechin, and procyanidin B2 showed a statistically significant ($p<0.05$) increase after 24 h of cocoa consumption in human volunteers (Table 3). Large inter-individual differences were observed in this study as has been also observed in other phenolic bioavailability studies [23]. These results are in accordance with those reported in the study carried out by Rios et al. [15] in which volunteers ingested a double dose of cocoa. However, in contrast to our findings, these authors did not find significant changes in the urinary levels of 3-hydroxyphenylacetic, 3-hydroxybenzoic, 4-hydroxyhippuric, and hippuric acid after 24 h of cocoa consumption. In another human feeding trial, in which six different polyphenol-rich beverages were compared [14], a significant increase ($p<0.05$) in (−)-epicatechin was found after 24 h of cocoa beverage intake, which is also in agreement with the present results.

In rats, a significant increase in the concentration of the following compounds was observed after the gavaging of 4.8 g of natural cocoa powder per kilograms per day during 2 weeks when compared to the control diet (Table 3): 3,4-dihydroxyphenylpropionic acid, *m*-coumaric acid, 3-hydroxyphenylacetic acid, protocatechuic acid, vanillic acid, and (−)-epicatechin. In addition to the above compounds, after consumption of the diet containing 10% (*w*/*w*) natural cocoa, a significant increase was also recorded for caffeic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, enterolactone, and procyanidin B2 (Table 3). Finally, significant differences were also observed between the two different cocoa treatment groups for *m*-coumaric acid, caffeic acid, 3-hydroxyphenylacetic acid, protocatechuic acid, vanillic acid, 3-hydroxybenzoic acid, enterolactone, (−)-epicatechin, and procyanidin B2 in favor of the 10% *w*/*w* natural cocoa diet (Table 3). In general, these results indicate that major differences were registered for phenolic acids containing hydroxyl groups at both C3 and C4 position or at C3 position. These results are consistent with those reported by Gonthier et al., who identified C3-hydroxylated phenolic acids as the major microbial metabolites of flavanols after administration of a catechin diet to rats [9].

It is important to highlight that this is the first study related to the microbial metabolism of cocoa polyphenols in rats. Differences in the percent increase in metabolite concentration between the human and rat urines as well as the difference in predominant phenolic acids observed between the two species could be due to the differences in the ingested dose of cocoa and to the different microbiota present in the intestine of each species, human and rodent. The ingested dose of cocoa in humans was ~10-fold less

Table 2 Precision and accuracy data obtained from the SPE-LC-MS/MS method in different days at three different concentration levels

Analyte	Low concentration (μg/L)				Medium concentration (μg/L)				High concentration (μg/L)			
	Added	Mean	Accuracy (%)	Precision (%RSD)	Added	Mean	Accuracy (%)	Precision (%RSD)	Added	Mean	Accuracy (%)	Precision (%RSD)
C_6C_3												
3,4-Dihydroxyphenylpropionic acid	5.0	4.8	96.0	14.4	50.0	56.1	112.1	10.9	500.0	448.4	89.7	11.9
4-Hydroxyphenylpropionic acid	100.0	103.9	103.9	15.0	250.0	238.1	95.2	10.7	500.0	543.3	108.7	13.0
m-Coumaric acid	0.5	0.5	100.4	3.6	10.0	9.4	94.0	5.5	100.0	102.9	103.0	6.5
p-Coumaric acid	1.0	1.0	96.8	8.5	50.0	55.9	111.8	14.0	500.0	463.4	89.4	14.5
Caffeic acid	0.5	0.5	95.8	7.5	50.0	53.3	106.6	13.2	500.0	478.2	95.6	4.4
Ferulic acid	1.0	1.1	113.8	14.7	50.0	48.1	96.2	15.0	500.0	475.1	95.0	4.7
C_6C_2												
3,4-Dihydroxyphenylacetic acid	1.0	1.0	95.9	2.2	10.0	9.65	95.5	8.3	250.0	242.6	97.0	11.4
3-Methoxy-4-hydroxyphenylacetic acid	50.0	47.3	94.6	10.5	100.0	90.7	90.7	10.7	500.0	478.0	96.0	11.2
3-Hydroxyphenylacetic	5.0	4.9	99.5	12.8	100.0	105.5	105.4	8.8	500.0	442.4	88.5	4.5
Phenylacetic acid	50.0	56.2	112.4	4.1	250.0	278.9	111.6	2.9	500.0	499.0	99.8	4.8
C_6C_1												
Protocatechuic acid	1.0	0.9	90.3	8.8	10.0	10.8	108.0	14.9	250.0	245.2	98.1	9.4
Vanillic acid	10.0	9.6	96.4	5.3	50.0	47.3	94.6	13.0	250.0	234.3	93.7	9.5
4-Hydroxybenzoic acid	5.0	5.0	100.0	3.6	50.0	55.3	110.4	14.3	250.0	237.1	94.8	10.0
3-Hydroxybenzoic acid	10.0	9.4	94.0	8.4	100.0	102.4	102.4	6.2	500.0	457.4	91.5	12.4
4-Hydroxyhippuric acid	0.5	0.5	98.0	9.9	10.0	11.2	112.1	6.6	250.0	213.6	85.4	7.2
Hippuric acid	100.0	91.3	91.3	11.7	500.0	501.5	100.3	0.1	1,000.0	995.0	99.5	6.9
4-*O*-Methylgallic acid	0.5	0.5	98.7	4.8	10.0	9.0	89.8	0.1	250.0	232.8	93.1	13.8
Enterolignans												
Enterodiol	0.5	0.5	104.1	3.0	50.0	44.7	89.5	3.5	500.0	437.5	87.5	3.4
Enterolactone	0.5	0.5	97.9	5.3	10.0	9.9	99.2	3.4	100.0	97.9	97.9	4.0
Flavanols												
(−)-Epicatechin	1.0	0.9	93.5	12.6	100.0	92.5	92.6	10.3	500.0	498.4	99.7	6.9
Procyanidin B2	100.0	102.4	998.5	3.0	250.0	226.5	90.6	13.3	500.0	535.3	107.1	8.5

Table 3 Changes in concentration levels and percentage of increase of microbial-derived phenolic metabolites and flavanols in human and rat urine samples after cocoa consumption

Analyte	Human urine, 0–24h		Rat urine, control—cocoa (4.8g/kg/day)		Rat urine, control—cocoa diet (10% *w/w* cocoa chow)	
	Δ±SEM (nmol/mg creatinine)	Increase (%)	Δ±SEM (nmol/mg creatinine)	Increase (%)	Δ±SEM (nmol/mg creatinine)	Increase (%)
C_6C_3						
3,4-Dihydroxyphenylpropionic acid	0.79±2.4	11	0.37±0.2*	106	0.93±0.2*	267
4-Hydroxyphenylpropionic acid	<LOQ		n.d.		n.d.	
m-Coumaric acid	0.014±0.007	37	14.64±2.1*	7,381	46.13±4.6**	23,250
p-Coumaric acid	0.057±0.03	467	0.17±0.7	4	1.73±0.8	40
Caffeic acid	0.39±0.13*	183	0.04±0.3	5	1.26±0.4**	160
Ferulic acid	10.52±2.3*	619	3.48±2.5	38	−1.94±2.0	−21
C_6C_2						
3,4-Dihydroxyphenylacetic acid	−0.02±0.2	6	0.94±0.6	118	0.40±0.3	50
3-Methoxy-4-hydroxyphenylacetic acid	1.54±1.4	23	5.92±4.6	52	11.27±2.7*	99
3-Hydroxyphenylacetic	6.53±4.0*	193	67.67±23.6*	998	181.56±18.6**	2,677
Phenylacetic acid	−35.84±19.8*	−36	36.70±27.4	31	20.52±46.2	17
C_6C_1						
Protocatechuic acid	0.51±1.4	8	5.66±1.8*	352	18.84±2.5**	1,173
Vanillic acid	1.14±0.6*	265	15.78±6.6*	183	50.57±7.5**	586
4-Hydroxybenzoic acid	0.55±1.2	15	20.08±13.8	27	10.28±14.0	14
3-Hydroxybenzoic acid	0.45±0.2*	413	0.73±0.3	141	7.20±1.1**	1,397
4-Hydroxyhippuric acid	1.27±0.7*	15	−4.97±8.1	−15	−0.79±11.0	−2
Hippuric acid	59.43±20.1*	73	62.45±56.4	42	75.34±71.7	50
4-*O*-Methylgallic acid	n.d.		n.d.		n.d.	
Enterolignans						
Enterodiol	0.001±0.002	27	0.004±0.004	0	0.004±0.005	154
Enterolactone	0.11±0.2	39	0.26±0.3	22	2.05±0.8**	178
Flavanols						
(−)-Epicatechin	0.53±0.35*	167	5.49±2.0*	7,160	36.62±3.4**	47,739
Procyanidin B2	0.20±0.2*	354	0.49±0.3	88	3.10±0.5**	556

LOQ limit of quantification; *n.d.* not detected

*$P<0.05$, significant difference with respect to the control diet group; **$P<0.05$, significant difference with respect to both control and 4.8 g cocoa/kg/day groups

than the 4.8 g natural cocoa powder/kg/day diet and 20-fold less than the 10% *w/w* natural cocoa diet in rats.

Besides the above mentioned metabolites, the phenyl-valerolactones, 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone (DHPV), and 5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (MHPV) were also identified in human and rat urine samples after cocoa consumption based on their mass spectra. These flavanol-derived microbial metabolites have been previously identified after tea [24] and wine powder consumption [8] but are identified herein for the first time in humans and rat urine after cocoa consumption. In order to identify these metabolites, hydrolyzed urine samples were injected in the product ion scan mode of deprotonated molecules in negative mode ($[M-H]^-$). The MS spectra and the fragmentation pattern generated for DHPV (*m/z* 207) and MHPV (*m/z* 221) are shown in Fig. 2. The fragment at *m/z* 163, previously described [24], as well as the generation of other characteristic fragment ions, support the identification of these compounds.

Procyanidin in urine Procyanidin B2 has been identified and quantified in human and rat urine after cocoa consumption. MRM chromatograms of procyanidin B2 (*m/z* 577/289) in human and rat urine are shown in Fig. 3. It is important to highlight that other peaks at the same transition (MRM 577/289) were also observed. For example, peak at retention time at 6 min in human urine could be another procyanidin that could not be confirmed due to the lack of standard. The rat urine chromatogram showed two major unidentified peaks that did not correspond to

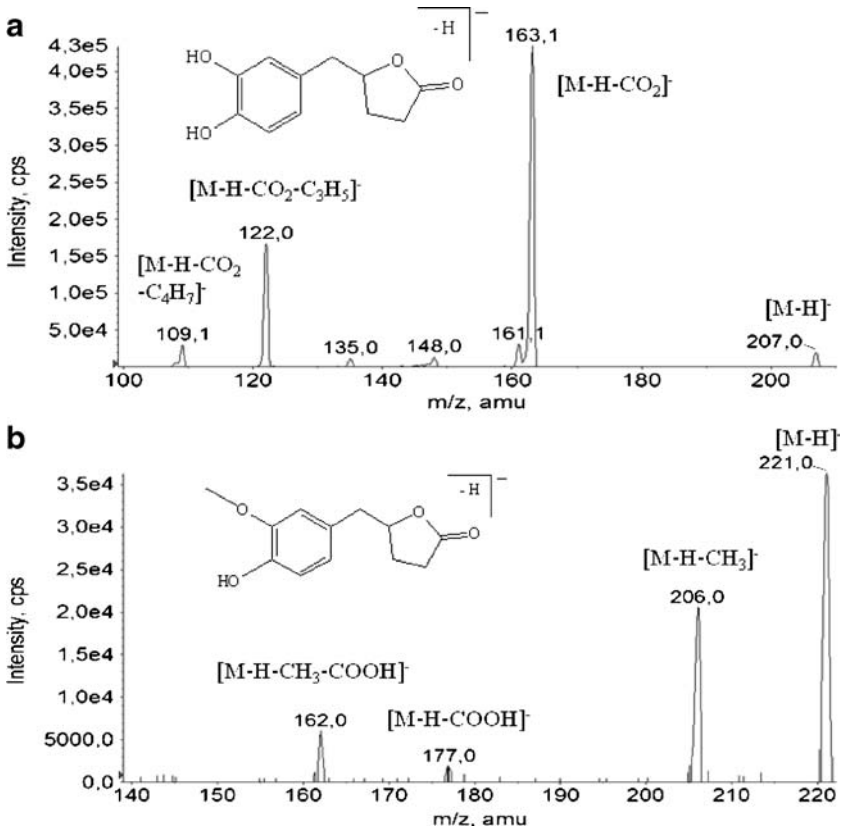

Fig. 2 Negative ion MS/MS spectra and proposed fragmentation pattern for 5-(3′4′-dihydroxyphenyl)-γ-valerolactone (**a**) and 5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (**b**) in human urine after cocoa consumption

procyanidin B1 or fragments from trimeric compounds (procyanidin C1) due to closer retention times to procyanidin B2 [25]. Changes in concentration levels in procyanidin B2 recorded in humans and rats after cocoa consumption are shown in Table 3.

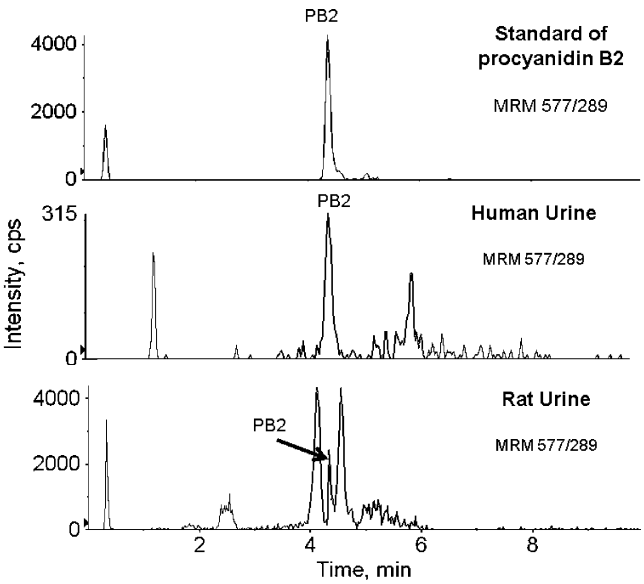

Fig. 3 MRM chromatogram of procyanidin B2 in synthetic urine, in human urine after acute intake of 40 g soluble cocoa powder, and in rat urine after prolonged administration of a 10% (*w*/*w*) natural cocoa diet

Dimeric procyanidins have been detected in both human and rat biological fluids, although in humans, the evidence is very scarce. Procyanidin B2 has been detected in human plasma presenting a maximum peak concentration at 2 h after acute consumption of a flavanol-rich cocoa, whereas procyanidin B1 has been detected in human serum 2 h after consumption of the pure compound [26,27]. In contrast, in the case of rats, there is a large number of scientific evidence. Procyanidin B2 was quantified in plasma and urine after oral administration of procyanidin B2 showing maximum concentration in plasma at 0.5 h and an excretion of 83.0 nmol in 18 h [28]. Dimeric and trimeric procyanidins had also been detected in rat plasma after the consumption of apple procyanidins showing a maximum peak at 2 h [29]. Donovan et al., in 2002 [30], determined conjugated forms of catechin and epicatechin in both plasma and urine of rats fed with catechin or procyanidin dimer B3 or with a grape seed extract containing catechin, epicatechin, and a mixture of procyanidins, but they did not detect procyanidins or conjugates in the plasma or urine of rats. Tsang et al. [31] detected phase II catechin metabolites in plasma, liver, kidneys, and urine of rats fed with a grape seed extract containing catechin, epicatechin, and dimers, trimers, tetramers, and polymeric procyanidins, and furthermore,

they also found low amounts of the procyanidin dimers B1, B2, B3, and B4, trimer C2 and an unknown trimer in urine [31]. Recently, Prasain et al. detected monomeric catechins, their methylated metabolites, and proanthocyanidins up to trimers in rat blood samples after the administration of grape seed extract [32].

Conclusions

The SPE-LC-MS/MS method developed in the present work allows the simultaneous determination of 19 microbial phenolic metabolites, as well as monomeric and dimeric flavanols in human and rat urine samples collected after cocoa consumption. This method offers significant improvement in sensitivity and recovery allowing its application to studies with large numbers of samples. Although the method was applied to the determination of flavanol-derived microbial metabolites, it is also applicable for other phenolic metabolites including enterolactone and enterodiol, which are produced by the microbial metabolism of lignans, and for 4-*O*-methylgallic acid, associated with the metabolism galloylated flavanols and described as a biomarker of wine or tea consumption [6, 33]. Procyanidin B2 has also been detected for the first time in human and rat urine after cocoa consumption. Furthermore, at a qualitative level, two poorly described metabolites (DHPV and MHPV) derived from the microbial metabolism of (epi) catechin and/or procyanidin have also been confirmed. Major advantages over previous reported methods also include the reduction of laboratory work in the sample-preparation step by the use of 96-well SPE plates and the sensitive measurement of a large number of metabolites in a very short run time, which makes it ideal for use in epidemiological studies. More studies about the effects of food consumption on the urine metabolome are required. Some of the microbial metabolites determined by the method developed in the present work could be accurate biomarkers of proanthocyanidin consumption, phenolic compounds that represent a major fraction of the flavonoid intake in the human diet.

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References

1. Aron PM, Kennedy JA (2008) Mol Nutr Food Res 52:79–104
2. Manach C, Williamson G, Morand C, Scalbert A, Remesy C (2005) Am J Clin Nutr 81:230–242
3. Deprez S, Brezillon C, Rabot S, Philippe C, Mila I, Lapierre C, Scalbert A (2000) J Nutr 130:2733–2738
4. Rechner AR, Kroner C (2005) Thromb Res 116:327–334
5. Gao K, Xu A, Krul C, Venema K, Liu Y, Niu Y, Lu J, Bensoussan L, Seeram NP, Heber D, Henning SM (2006) J Nutr 136:52–57
6. Mennen LI, Sapinho D, Ito H, Bertrais S, Galan P, Hercberg S, Scalbert A (2006) Br J Nutr 96:191–198
7. Spencer JP, Abd El Mohsen MM, Minihane AM, Mathers JC (2008) Br J Nutr 99:12–22
8. Gonthier MP, Cheynier V, Donovan JL, Manach C, Morand C, Mila I, Lapierre C, Remesy C, Scalbert A (2003) J Nutr 133:461–467
9. Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C, Scalbert A (2003) Free Radic Biol Med 35:837–844
10. Barnes S, Prasain JK, Wang CC, Moore DR (2006) Life Sci 78:2054–2059
11. Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos R, Cherubini A, Jauregui O, de la Torre R, Covas MI, Estruch R, Jaeger W, Andres-Lacueva C (2007) Clin Chem 53:292–299
12. Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas MI, Andres-Lacueva C (2005) Anal Chem 77:3149–3155
13. Gonthier MP, Rios LY, Verny M, Remesy C, Scalbert A (2003) J Chromatogr B Analyt Technol Biomed Life Sci 789:247–255
14. Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Remesy C, Scalbert A (2005) Br J Nutr 94:500–509
15. Rios LY, Gonthier MP, Remesy C, Mila I, Lapierre C, Lazarus SA, Williamson G, Scalbert A (2003) Am J Clin Nutr 77:912–918
16. Roura E, Almajano MP, Bilbao ML, Andres-Lacueva C, Estruch R, Lamuela-Raventos RM (2007) Free Radic Res 41:943–949
17. Andres-Lacueva C, Lamuela-Raventos RM (2000) LC-GC Eur.902–905
18. Roura E, Andres-Lacueva C, Estruch R, Lamuela-Raventos RM (2006) Clin Chem 52:749–752
19. U.S.Department of Agriculture. USDA Database for the Proanthocyanidin Content of Selected Foods. http://www.nal.usda.gov/fnic/foodcomp.2004
20. Ramiro-Puig E, Urpi-Sarda M, Perez-Cano FJ, Franch A, Castellote C, Andres-Lacueva C, Izquierdo-Pulido M, Castell M (2007) J Agric Food Chem 55:6431–6438
21. Miro-Casas E, Farre AM, Covas MI, Rodriguez JO, Menoyo CE, Lamuela Raventos RM, de la Torre R (2001) Anal Biochem 294:63–72
22. U.S.Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry. Bioanalytical Method Validation*; May 2001
23. Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, Lambert G, Mohr S, Yang CS (2002) Cancer Epidemiol. Biomarkers Prev 11:1025–1032
24. Li C, Lee MJ, Sheng SQ, Meng XF, Prabhu S, Winnik B, Huang BM, Chung JY, Yan SQ, Ho CT, Yang CS (2000) Chem Res Toxicol 13:177–184
25. Andres-Lacueva C, Monagas M, Khan N, Izquierdo-Pulido M, Urpi-Sarda M, Permanyer J, Lamuela-Raventos RM (2008) J Agric Food Chem 56:3111–3117
26. Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz HH, Keen CL (2002) Am J Clin Nutr 76:798–804
27. Sano A, Yamakoshi J, Tokutake S, Tobe K, Kubota Y, Kikuchi M (2003) Biosci Biotechnol Biochem 67:1140–1143
28. Baba S, Osakabe N, Natsume M, Terao J (2002) Free Radical Biology Medicine 33:142–148

29. Shoji T, Masumoto S, Moriichi N, Akiyama H, Kanda T, Ohtake Y, Goda Y (2006) J Agric Food Chem 54:884–892
30. Donovan JL, Manach C, Rios L, Morand C, Scalbert A, Remesy C (2002) Br J Nutr 87:299–306
31. Tsang C, Auger C, Mullen W, Bornet A, Rouanet JM, Crozier A, Teissedre P (2005) Br J Nutr 94:170–181
32. Prasain JK, Peng N, Dai Y, Moore R, Arabshahi A, Wilson L, Barnes S, Michael WJ, Kim H, Watts RL (2009) Phytomedicine 16:233–243
33. Waffo-Teguo P, Hawthorne ME, Cuendet M, Merillon JM, Kinghorn AD, Pezzuto JM, Mehta RG (2001) Nutr. Cancer 40:173–179