Journal of Chromatography B, 879 (2011) 1519–1528

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid methods to determine procyanidins, anthocyanins, theobromine and caffeine in rat tissues by liquid chromatography-tandem mass spectrometry

Aida Serra [a], Alba Macià [a], Maria-Paz Romero [a], Carme Piñol [b], Maria-José Motilva [a,*]

[a] Department of Food Technology, XaRTA-UTPV, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Avda/Alcalde Rovira Roure 191, 25198 Lleida, Spain
[b] Animal Facilities, Campus Ciències de la Salut, Facultat de Medicina, Universitat de Lleida, C/Montserrat Roig 2, 25008 Lleida, Spain

ARTICLE INFO

Article history:
Received 5 January 2011
Accepted 21 March 2011
Available online 29 March 2011

Keywords:
Anthocyanins
Biological tissues
Procyanidins
Theobromine
Caffeine
UPLC–MS/MS

ABSTRACT

Rapid, selective and sensitive methods were developed and validated to determine procyanidins, anthocyanins and alkaloids in different biological tissues, such as liver, brain, the aorta vein and adipose tissue. For this purpose, standards of procyanidins (catechin, epicatechin, and dimer B_2), anthocyanins (cyanidin-3-glucoside and malvidin-3-glucoside) and alkaloids (theobromine, caffeine and theophylline) were used. The methods included the extraction of homogenized tissues by off-line liquid–solid extraction, and then solid-phase extraction to analyze alkaloids, or microelution solid-phase extraction plate for the analysis of procyanidins and anthocyanins. The eluted extracts were then analyzed by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, using a triple quadrupole as the analyzer. The optimum extraction solution was water/methanol/phosphoric acid 4% (94/4.5/1.5, v/v/v). The extraction recoveries were higher than 81% for all the studied compounds in all the tissues, except the anthocyanins, which were between 50 and 65% in the liver and brain. In order to show the applicability of the developed methods, different rat tissues were analyzed to determine the procyanidins, anthocyanins and alkaloids and their generated metabolites. The rats had previously consumed 1 g of a grape pomace extract (to analyze procyanidins and anthocyanins) or a cocoa extract (to analyze alkaloids) per kilogram of body weight. Different tissues were extracted 4 h after administration of the respective extracts. The analysis of the metabolites revealed a hepatic metabolism of procyanidins. The liver was the tissue which produced a greater accumulation of these metabolites.

1. Introduction

Over recent years, research into the bioavailability of minor food compounds such as polyphenols (procyanidins and anthocyanins) or alkaloids, has focused on the detection of metabolites in such biological fluids as plasma [1–11], urine [3–7,12], milk [13], or saliva [5]. However, pharmacokinetics tend to go further, investigating the behavior in all the tissues of the body. Until now, there have been few studies in the literature that have measured procyanidins [2,6,8,14] and anthocyanins [10,15–17] in biological tissues, such as the liver and brain. On the other hand, in the literature there are no studies about the determination of alkaloids (theobromine and caffeine) in these samples.

Due to their complexity, an intense pre-treatment of the samples is necessary to analyze biological tissues, to break down the collagen structure and free the analytes. Generally, the most common sample extraction method for measuring phenols includes a homogenization step in saline solution [6,8,16] or in strong acids, like trifluoracetic acid (TFA) [10,17], followed by an organic solvent extraction. However, the use of electrospray ionization (ESI), as the ionization technique in mass spectrometry (MS), is not compatible with the use of saline solutions for phenol extraction from tissues. Thus, it is necessary to eliminate salts, prior to chromatographic analysis or develop new extraction methods without the use of saline solutions.

The pretreatment methodology has to allow working with small sample quantities because the availability of this kind of samples is limited. Recently, microelution SPE (μSPE) was used to determine procyanidins and anthocyanins in plasma samples satisfactorily [9] loading the micro-cartridge with such small sample quantities as 350 μl.

As well as the sample pretreatment method, the analytical separation technique is also very important. It is essential that this analytical technique is sensitive, selective and reliable in order to determine the target compounds at low concentration levels in complex matrices, such as biological tissues. In the few studies reported in the literature into measuring phenolic metabolites in these samples, high-performance liquid chromatography (HPLC) coupled to tandem MS (MS/MS) [2,6,14,15] and HPLC–MS [8,17] were the chosen analytical technique. LC–MS/MS is a useful tool to

* Corresponding author. Tel.: +34 973 702817; fax: +34 973 702596.
E-mail address: motilva@tecal.udl.es (M.-J. Motilva).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.03.042

determine and identify the phenolic metabolites at low concentration levels in these biological complex matrixes (tissue samples).

Additionally, the speed of an analytical methodology (sample pretreatment and analysis) is very important. The use of μSPE reduces the analysis time because the evaporation and reconstitution steps are avoided [9,18]. Additionally, the use of columns with low particle size, such as 1.7 μm in UHPLC, instead of 5 μm in HPLC, also reduces the analysis time by a factor of to five [19].

In order to extend and improve the developed methodologies, in terms of speed, sensitivity and precision, and due to the lack of validated analytical methods reported in the literature for the determination of procyanidins, anthocyanins and alkaloids in biological tissues, this study was conceived to develop and validate rapid, sensitive, selective and reliable methods for the measuring these compounds in different biological tissues, such as liver, brain, aorta vein and adipose tissue. In addition, to our knowledge, this is the first study where the alkaloids (theobromine and caffeine) are determined in tissue samples. The sample pretreatment method was first studied and optimized in order to extract and preconcentrate the studied compounds and clean up the sample matrix. The analytical separation technique was ultra-performance LC (UPLC)–ESI–MS/MS, with quadrupole as the analyzer. Then, the developed methods were applied to identify and quantify the studied compounds and their metabolites in different rat tissue samples obtained 4 h after the administration of a dose corresponding to 1 g of grape pomace extract (to study the procyanidins and anthocyanidins) or 1 g of cocoa extract (to study the alkaloids) per kilogram of body weight.

2. Experiment

2.1. Chemical and reagents

The standards of (−)-epicatechin, (+)-catechin, theobromine, theophylline, and caffeine were purchased from Sigma Aldrich (St. Louis, MO, USA); procyanidin dimer B_2 from Fluka Co. (Buchs, 125 Switzerland). The anthocyanins, cyanidin-3-glucoside and malvidin-3-glucoside, were purchased from Extrasynthese (Genay, France). Acetonitrile (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade) and glacial acetic acid (≥99.8%) were of analytical grade (Scharlab, Barcelona, Spain). Pure hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Ortho-phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Formic acid and L (+)-ascorbic acid (reagent grade) were all provided by Scharlau Chemie (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Extracts

A grape pomace extract rich in procyanidins and anthocyanidins and a cocoa extract rich in alkaloids were used to show the applicability of the developed methods. The extracts were obtained as follows.

Cocoa extract: An accelerated solvent extractor ASE100 (Dionex) was used to extract the alkaloid compounds from cocoa powder. This extractor allows faster extractions using solvents at high temperatures and pressures [20]. 10 g of cocoa powder, which was previously cleaned with cold water to remove sugars, was mixed with 2.5 g of diatomaceous earth and was extracted with acetone/water/acetic acid (70/29.5/0.5, v/v/v) at 40 °C using a 100-ml extraction cell. The flush volume was set at 50% and four static cycles of 5 min were carried out. After that, the sample was purged with nitrogen (≥99.99% purity, Air Liquide, Madrid, Spain). The resulting extract was rotary evaporated until all of the acetone and acetic acid were eliminated, and was then freeze-dried and stored at −18 °C in N_2 atmosphere. The main alkaloids quantified in the extract were theobromine and caffeine (Table 1 and Figure Additional Information).

Grape pomace extract: An accelerated solvent extractor ASE100 (Dionex) was also used to extract the phenolic compounds (procyanidins and anthocyanins) from grape pomace. 15 g of grape pomace mixed with 5 g of diatomaceous earth was extracted twice with acetone/water (25/75, v/v) at 80 °C using a 100-ml extraction cell. The flush volume was set at 50% and three static cycles of 5 min were carried out. The sample was then purged with nitrogen (≥99.99% purity, Air Liquide, Madrid, Spain). The resulting extract was rotary evaporated until all of the acetone was eliminated, and then freeze-dried and stored at −18 °C in N_2 atmosphere. The main procyanidins quantified in the extract were catechin, epicatechin, dimer and trimer, and the main anthocyanins were cyanidin-glucoside, petunidin-glucoside, delphinidin-glucoside, peonidin-3-glucoside and malvidin-3-glucoside (Table 1 and Figure Additional Information).

2.3. Treatment of rats and tissue collection

Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12 h light-12 h dark schedule at controlled temperature (22 °C). They were given a commercial feed, PanLab A04 (Panlab, Barcelona, Spain), and water *ad libitum*. The rats were later kept under fasting conditions for between 16 and 17 h with access to tap water. Two different experiments were carried out.

Experiment 1 (five rats): a single dose of 1 g of grape pomace extract/kg of body weight dispersed in water, as a source of procyanidins and anthocyanins, was administered by intragastric gavage.

Experiment 2 (five rats): a single dose of 1 g of cocoa extract/kg of body weight dispersed in water, as a source of alkaloids, was also administered by intragastric gavage.

Additionally, a control group (five rats) rats was maintained under fasting conditions without extract ingestion and then similarly euthanized. The animals were anesthetized with isoflurane (IsoFlo, Veterinaria Esteve, Bologna, Italy) and euthanized by exsanguinations at 18 h. The liver, brain, aorta vein and adipose tissue were excised from the rats. The tissues were stored at −80 °C and freeze-dried for procyanidin, anthocyanin and alkaloid extraction and chromatographic analysis. The study was approved by the Animal Ethics Committee of the University of Lleida.

2.4. Pre-treatment method of tissue sample

As the sample pretreatment, off-line liquid–solid extraction (LSE)-SPE and off-line LSE-μSPE was used to analyze the phenolic compounds (procyanidins and anthocyanins) and alkaloids respectively. LSE was chosen because a liquid organic solvent was used to extract the analytes in a solid matrix tissue. The biological matrices were cleaned-up and the studied compounds were preconcentrated. To optimize the extraction step, a pool of liver tissue from the control group was spiked with standards of procyanidins (catechin, epicatechin and dimer B_2), anthocyanins (cyanidin-3-glucoside and malvidin-3-glucoside) and alkaloids (theophylline, caffeine and theobromine) dissolved in phosphoric acid 4%.

The LSE was optimized to increase its suitability. The extraction procedure was as follows; 50 μl of ascorbic acid 1% and 100 μl of phosphoric acid 4% were added to 60 mg of freeze-dried tissue. The liver sample was extracted four times with 400 μl of water/methanol/phosphoric acid 4% (94/4.5/1.5, v/v/v). In each extraction, 400 μl of extraction solution was added; the sample was sonicated (S-150D Digital Sonifier^{R}Cell Disruptor, Branson,

Ultrasonidos S.A.E., Barcelona, Spain) during 30 s, maintaining the sample in a freeze water bath to avoid heating and then centrifuged for 15 min at 17,150 × *g* at 20 °C. The supernatants were collected, and then the extracts were cleaned-up by using μSPE to determine procyanidins and anthocyanins and SPE to determine alkaloids.

The off-line μSPE was based on the methodology described in a previous study [9] using OASIS HLB μElution Plates 30 μm (Waters, Milford, MA, USA). Briefly, the micro-cartridges were conditioned sequentially with 250 μl of methanol and 250 μl of 0.2% acetic acid. 350 μl of phosphoric acid 4% was added to 350 μl of liver extract, and then this mixture was loaded onto the plate. The loaded plates were washed with 200 μl of Milli-Q water and 200 μl of 0.2% acetic acid. The retained procyanidins and anthocyanins were then eluted with 2 x 50 μl of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solution was directly injected in the UPLC–MS/MS, and the sample volume was 2.5 μl.

On the other hand, the recovery of the alkaloids from the liver extract was by off-line SPE using OASIS HLB cartridges (60 mg, Waters Corp., Milford, USA). These were conditioned sequentially with 1 ml of methanol and 1 ml of Milli-Q water. 1 ml of liver extract was loaded onto the cartridge. The loaded cartridges were washed with 1 ml Milli-Q water. The retained alkaloids were then eluted with 1 ml of methanol. This solution was directly injected into the UPLC–MS/MS, and the sample volume was also 2.5 μl.

2.5. UPLC–ESI–MS/MS

Procyanidin, anthocyanin and alkaloid tissue extracts were analyzed by Acquity Ultra-Performance™ liquid chromatography and tandem MS from Waters (Milford MA, USA), as reported in our previous studies [1,9,21]. Briefly, the column was an Acquity high-strength silica (HSS) T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) with 100% silica particles, also from Waters (Milford MA, USA). Two different mobile phases were used to analyze these compounds [9]. The procyanidins and alkaloids were separated with a mobile phase that consisted of 0.2% acetic acid (eluent A) and acetonitrile (eluent B), and the anthocyanins with 10% acetic acid (eluent A) and acetonitrile (eluent B). The flow-rate was 0.4 ml/min. The elution gradient was 0–10 min, 5–35% B; 10–10.10 min, 35–80% B; 10.10–11 min, 80% B isocratic; 11–11.10 min, 80–5% B; and 11.10–12.50 min, 5% B isocratic.

Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford MA, USA) equipped with a Z-spray electrospray interface. The ionization technique was ESI. The procyanidins were analyzed in the negative ion mode, and the anthocyanins and alkaloids in the positive ion mode, and the data was acquired through selected reaction monitoring (SRM). Two SRM transitions were studied, selecting the most sensitive transition for quantification and a second one for confirmation purposes. The SRM transitions and the individual cone voltage and collision energy for each analyte are shown in Table 1. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software.

2.6. Analytical characteristics of the method

After the development of the extraction methods, liver, brain, aorta and adipose tissue (obtained from the control rats) were spiked with the standards of procyanidins, anthocyanins and alkaloids at different concentrations before the sample pre-treatment. Then the instrumental quality parameters: linearity, extraction recovery, calibration curves, intra-day and inter-day precision, intra-day and inter-day accuracy, detection limit (LOD), quantification limit (LOQ), and the study of the matrix effect were evaluated.

The linearity of the method was evaluated using control tissue samples spiked with the standards. Calibration curves (based on peak area abundance) were plotted using $y = a + bx$, where y is the analyte peak abundance and x is the analyte concentration. Concentrations of the procyanidins, anthocyanins and alkaloids were calculated by interpolating the analyte peak abundance on their calibration curves. These curves were obtained by analyzing five points at different concentration levels and each standard solution was injected three times. The studied standard compounds in the tissue samples were quantified by using their calibration curves. On the other hand, due to the lack of standards, the metabolites of catechin and epicatechin were tentatively quantified by using the calibration curve of catechin and epicatechin, respectively. Other anthocyanins detected in tissues after the ingested of grape pomace extract were tentatively quantified by using the calibration curve of malvidin-3-glucoside.

Extraction recoveries (%*R*) were determined by comparing the absolute response of the analytes spiked in control tissues before and after the sample pre-treatment. The precision of the methods (intra-day and inter-day precision) were determined as the relative standard deviation (% RSD) of the concentration ($n = 3$). The matrix effect was evaluated by comparing peak abundances of the analytes spiked in the tissue matrices after the sample pre-treatment, LSE-μSPE (procyanidins and anthocyanins) and LSE-SPE (alkaloids), with the peak abundances of the analytes spiked in the elution solvent, (acetone/Milli-Q water/acetic acid, 70/29.5/0.5) and methanol, respectively. The intra-day and inter-day accuracy ($n = 3$) were calculated as the ratio between the mean measured concentration and the nominal concentration multiplied by 100. The extraction recovery, precisions of the method and accuracy were studied at three concentration levels ($n = 3$), 8.0, 2.4 and 0.6 nmol/g tissue for the liver, 6.0, 1.7 and 0.4 nmol/g tissue for the brain, 38.0, 11.5 and 2.6 nmol/g tissue for the aorta vein, and 23.0, 6.8 and 1.6 nmol/g tissue for the adipose tissue. The LODs and LOQs were calculated using the signal-to-noise criterion of 3 and 10, respectively.

3. Results and discussion

3.1. Pre-treatment of tissue sample

Initial experiments for measuring procyanidins, anthocyanins and alkaloids and their metabolites were based on the conditions previously reported for the analysis of procyanidins and anthocyanins in plasma samples [9]. In that study, these compounds and their metabolites were extracted by μSPE with good extraction recoveries. Tissues are more complex matrices than biological fluids, such as plasma, saliva and urine, because the former are complex cellular structures with high contents of proteins, collagen and fat, depending on the tissue. For this reason, as well as μSPE, another sample pre-treatment technique, such as LSE, was combined to extract the soluble phenolic compounds and alkaloids by diffusion from the solid tissue matrix to a liquid extraction solvent.

Prior to the LSE extraction, the tissues were lyophilized to eliminate water and thus improve the contact between the solvent extraction and the matrix tissue. 50 μl of ascorbic acid 1% was added to the tissue sample to avoid phenol oxidation during extraction, and 100 μl of phosphoric acid 4% was added to denaturalize proteins. Then, some parameters were studied in order to optimize the extraction. These were sample weight, extraction solvent, number of extractions, and disruption treatment (sonicator/vortex).

Firstly, the sample pre-treatment studies were evaluated using the liver as the tissue sample. The weight of freeze-dried tissue subjected to extraction was the first parameter to optimize. Different weights were tested, from 60 to 100 mg. The use of weights higher than 60 mg was discarded because the supernatant could not be completely separated from the solid residue when the mix-

Table 1
Optimized SRM conditions for the analyses of the studied compounds and their metabolites by UPLC–MS/MS.

Compound	ESI	SRM 1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM 2 (confirmation)	Cone voltage (V)	Collision energy (eV)	Reference
Standards								
Catechin	Negative	289 > 245	45	15	289 > 205	45	10	[1,2]
Epicatechin	Negative	289 > 245	45	15	289 > 179	45	10	[1,2]
Dimer B_2	Negative	577 > 289	45	20	289 > 425	45	15	[1,2]
Theophylline	Positive	180.6 > 123.5	45	15	180.6 > 96	40	25	
Theobromine	Positive	181 > 163	45	15	181 > 140	45	10	[2]
Caffeine	Positive	195 > 138	45	15	195 > 110	45	20	[2]
Cyanidin-3-Glucoside	Positive	449 > 287	40	20	–	–	–	[3]
Malvidin-3-Glucoside	Positive	493 > 331	40	25	493 > 315	40	20	[3]
Metabolites								
Catechin glucuronide	Negative	465 > 289	40	20	289 > 245	45	15	[1,2]
Epicatechin glucuronide	Negative	465 > 289	40	20	289 > 245	45	15	[1,2]
Methyl catechin-glucuronide	Negative	479 > 303	40	25	289 > 245	45	15	[1,2]
Methyl epicatechin-glucuronide	Negative	479 > 303	40	25	289 > 245	45	15	[1,2]
Catechin sulphate	Negative	369 > 289	40	20	289 > 245	45	15	
Epicatechin sulphate	Negative	369 > 289	40	20	289 > 245	45	15	
Methyl catechin-sulphate	Negative	383 > 303	40	15	289 > 245	45	15	[1,2]
Methyl epicatechin-sulphate	Negative	383 > 303	40	15	289 > 245	45	15	[1,2]
Peonidin-3-Glucoside	Positive	463 > 301	40	25	–	–	–	[3]
Delfinidin-3-Glucoside	Positive	465 > 303	40	20	–	–	–	[3]
Petunidin-3-Glucoside	Positive	479 > 317	40	20	–	–	–	[3]
Cyanidin-3-AcetylGlu	Positive	491 > 287	40	20	–	–	–	[3]
Peonidin-3-AcetylGlu	Positive	505 > 301	40	25	–	–	–	[3]
Delfinidin-3-AcetylGlu	Positive	507 > 303	40	25	–	–	–	[3]
Petunidin-3-AcetylGlu	Positive	521 > 317	40	30	–	–	–	[3]
Malvidin-3-AcetylGlu	Positive	535 > 331	40	25	535 > 315	40	20	[3]
Cyanidin-3-CoumaroylGlu	Positive	595 > 287	40	30	–	–	–	[3]
Peonidin-3-CoumaroylGlu	Positive	609 > 301	40	25	–	–	–	[3]
Delfinidin-3-CoumaroylGlu	Positive	611 > 303	40	35	–	–	–	[3]
Petunidin-3-CoumaroylGlu	Positive	625 > 317	40	25	–	–	–	[3]
Malvidin-3-CoumaroylGlu	Positive	639 > 331	40	25	639 > 315	40	20	[3]

ture was centrifuged. When the extraction was done with 60 mg of freeze-dried tissue, full separation of the solid residue and solvent extraction was obtained. 60 mg of sample was accordingly chosen.

Afterwards, following the work by Urpi-Sarda et al. [14], who used 1.5 M f formic acid with 5% methanol, six different extraction solvents were tested to determine the more appropriate composition to obtain the maximum extraction of the different analytes: 1.5 M formic acid (solvent 1), 1.5 M formic acid with 5% of methanol (solvent 2) [14] and 1.5 M formic acid with 5% of acetone (solvent 3). Water (solvent 4) was tested as a control solvent extraction. Hydrochloric acid (solvent 5) was also tested to evaluate the influence of the pH of the medium on the extraction efficiency of compounds, in line with Zafra-Gómez et al. [22]. These authors reported that the extraction efficiency increases for acid pH values because the dissociated forms may remain in the aqueous phase. Finally, water/methanol/H_3PO_4 4% (94/4.5/1.5, v/v/v) (solvent 6) was chosen as a mix between the control tested and the method proposed by Urpi-Sarda et al. [14]. To optimize the extraction solvent, freeze-dried liver tissue was spiked with the studied analytes, and the extraction recoveries (%*R*) are shown in Table 2. The recovery studies used 60 mg of sample, sonicator and two extractions. No differences were observed between the procyanidin and anthocyanin recoveries obtained when 1.5 M formic acid (solvent 1), 1.5 M formic acid with 5% of methanol (solvent 2) and 1.5 M formic acid with 5% of acetone (solvent 3) were tested. However, 1.5 M formic acid with 5% of acetone (solvent 3) was discarded because the separation of the supernatant was more difficult than with the other extraction solvents. Nevertheless, Garcia-Viguera et al. [23] reported that acetone is more efficient than acidified methanol for extracting anthocyanins from red fruit. On the other hand, when water (solvent 4) was used as the extraction solvent, high anthocyanin recoveries were obtained, 98% for cyanidin-3-glucoside and 100% for malvidin-3-glucoside. In contrast, the extraction recoveries of procyanidins were lower than 50%. In order to improve this low percentage of procyanidin recovery, different proportions of methanol, water and diluted phosphoric acid were tested [24]. Water/methanol/H_3PO_4 4% (94/4.5/1.5, v/v/v) (solvent 6) was shown to be a more appropriate extraction solvent for procyanidins, reaching recoveries of 77%, 80% and 65% for catechin, epicatechin and dimer B_2, respectively. In contrast to the results obtained by Zafra-Gómez et al. [22], in the present study, no compounds were recovered when hydrochloric acid (solvent 5) was tested as the extraction solvent.

Once the optimal extraction solvent had been selected, the number of extractions, from 1 to 5, was studied. No differences were observed between 4 and 5 extractions. Thus, the number of extractions selected was 4. As an example, the extraction recoveries for catechin and epicatechin improved from 77% to 100% and from 80% to 100%, respectively, when 4 extractions were carried out instead of 2.

Finally, a sonicator and vortex were compared to disrupt the tissue. Only the sonicator was able to break the tissue and free the analytes from the matrix. This extraction system was previously applied by Talavéra et al. [15]. Sonication is faster and more efficient than such traditional methods as maceration/stirring, because the surface area in contact between the solid and liquid phases is much greater due to particle disruption taking place. On the other hand, other authors, such as Vanzo et al. [16], carried out the extraction satisfactorily using vortex.

For the analyses of alkaloids, the LSE-μSPE recoveries were lower than 5% in all the extraction solvents tested. In order to improve the retention of these analytes in the sorbent and increase the extraction recovery, these compounds were analyzed by off-line SPE, instead of μSPE. The LSE conditions were those used for the analysis of procyanidins and anthocyanins. Firstly, the off-line SPE method was based on the recommended generic Oasis® HLB SPE method from Waters in which the cartridges were conditioned by adding sequentially 1 ml of methanol and 1 ml of Milli-Q water.

Table 2
Extraction recovery (%*R*) for the determination of the studied compounds by off-line LSE and μSPE-UPLC–MS/MS in spiked liver (mean ± SE).

	Solvent extraction					
	1.5 M formic acid	1.5 M formic acid + 5% methanol	1.5 M formic acid + 5% acetone	Water	0.1 M HCl	Water/methanol/H_3PO_4 4% (94/4.5/1.5, v/v/v)
Procyanidins						
Catechin	62 ± 2	56 ± 2	50 ± 3	49 ± 3	0	77 ± 2
Epicatechin	51 ± 2	51 ± 2	47 ± 3	43 ± 3	0	80 ± 3
Dimer B_2	0	0	0	0	0	65 ± 4
Alkaloids						
Theophylline	0	0	0	0	0	2 ± 0
Caffeine	0	0	0	0	0	4 ± 1
Theobromine	0	0	0	0	0	5 ± 1
Anthocyanins						
Cyanidin-3-Glucoside	100 ± 2	98 ± 3	89 ± 4	98 ± 3	0	65 ± 4
Malvidin-3-Glucoside	71 ± 2	69 ± 2	66 ± 3	100 ± 4	0	60 ± 2

For experimental conditions see in Section 2.4: 60 mg tissue sample, sonicator, two extractions.

Extractions were done by loading 1 ml of extract that had previously been mixed with 20 μl of phosphoric acid 85% to break the bonds between the proteins and alkaloids. The loaded cartridges were washed with 1 ml of Milli-Q water. Then, in order to elute the studied compounds, and based on the literature, different elution solvents, such as chloroform [12], ethyl acetate/2-propanol (93/7, v/v) [12], water and chloroform/2-propanol (80/20, v/v) [12], were tested. However the best extraction recoveries were obtained when 1 ml of methanol was used as the elution solvent, and the extraction recoveries were 52%, 61% and 72% for theophylline, caffeine and theobromine, respectively. Fig. 1 shows the extraction ion chromatograms obtained from the analysis of liver tissue spiked with the studied compounds under optimum extraction conditions.

Once the method had been developed and optimized to extract the studied compounds (LSE-μSPE for procyanidins and anthocyanins and LSE-SPE for alkaloids) using liver as the tissue, the extraction recoveries were also determined for the analysis of brain, aorta vein and adipose tissue (Table 3). The results showed that

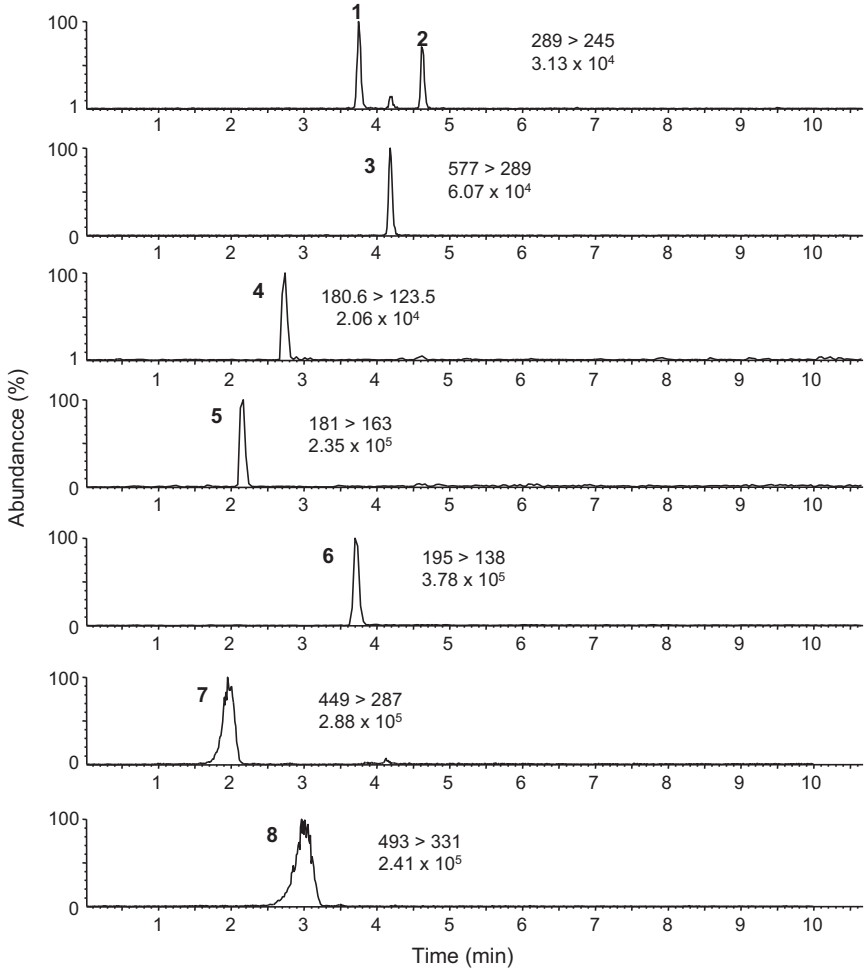

Fig. 1. Extracted ion chromatograms from blank liver tissue spiked with the studied compounds. Their designation and concentrations were (1) catechin, 6 nmol/g tissue; (2) epicatechin, 6 nmol/g tissue; (3) dimer, 3 nmol/g tissue; (4) theophylline, 8.5 nmol/g tissue; (5) theobromine, 14.5 nmol/g tissue; (6) caffeine, 7 nmol/g tissue; (7) cyanidin-3-glucoside, 3 nmol/g tissue; (8) malvidin-3-glucoside, 3 nmol/g tissue.

Table 3
Extraction recovery (%*R*) for the determination of the studied compounds by off-line LSE and μSPE-UPLC–MS/MS (procyanidins and anthocyanidins) or SPE-UPLC–MS/MS (alkaloids) in spiked liver, brain, aorta vein and adipose tissue (mean ± SE).

	Sample pre-treatment	Tissue samples			
		Liver	Brain	Aorta vein	Adipose tissue
Procyanidins					
Catechin	LLE-μSPE	100 ± 3	100 ± 3	100 ± 2	100 ± 2
Epicatechin	LLE-μSPE	100 ± 3	96 ± 2	100 ± 2	100 ± 2
Dimer B_2	LLE-μSPE	85 ± 2	94 ± 4	100 ± 3	100 ± 3
Anthocyanins					
Cyanidin-3-Glucoside	LLE-μSPE	60 ± 2	65 ± 2	84 ± 2	90 ± 2
Malvidin-3-Glucoside	LLE-μSPE	50 ± 1	60 ± 2	92 ± 3	100 ± 3
Alkaloids					
Theophylline	LLE-SPE	75 ± 2	57 ± 1	100 ± 2	63 ± 1
Theobromine	LLE-SPE	78 ± 2	92 ± 2	100 ± 2	79 ± 2
Caffeine	LLE-SPE	82 ± 3	66 ± 2	62 ± 1	74 ± 2

For experimental conditions see in Section 2.4: 60 mg tissue sample, sonicator, four extractions, extraction solvent; water/methanol/H_3PO_4 4% (94/4.5/1.5, v/v/v).

almost 100% of the monomers catechin and epicatechin were recovered in all tissues. The dimer B_2 showed recoveries from 85% in the liver to 100% in the adipose tissue. The recoveries of cyanidin-3-glucoside and malvidin-3-glucoside ranged from 50% to 65% in the liver and the brain tissue, respectively, and these were higher than 84% in the aorta vein and adipose tissue.

Finally, in relation to the extraction recoveries of alkaloids, it was noted that 100% of theophylline was recovered from the aorta vein. In the other tissues studied, this extraction recovery was lower, and the lowest extraction recovery was in the liver (57%). Similarly, theobromine and caffeine generally showed higher extraction recoveries than theophylline. The lowest extraction recovery values were observed with caffeine from the brain and the aorta vein, which were 66% and 62%, respectively.

3.2. Quality parameters

The quality parameters of the developed methods for the measuring the studied compounds in the four tissues are shown in Table 4 (linearity, LOD and LOQ). The linearity was evaluated following the procedure developed in the range from 0.2 to 80 nmol/g liver, from 0.2 to 57.5 nmol/g brain, from 0.4 to 380 nmol/g aorta vein, and from 0.1 to 227 nmol/g adipose tissue. The calibration curves were plotted as the peak areas according to analyte concentration. The functions were linear, with mean correlation coefficients >0.99.

The intra-day precision (within-day precision, $n = 3$) of the methods, obtained for each analyte during the same day that the tissues were spiked with the analytes, was expressed as the relative standard deviation (% RSD) of the concentration, and was calculated at three different concentration levels according to the tissue matrix (Table 2 Additional Information). These values were lower than 9.0% in the liver, 7.7% in the brain, 7.6% in the aorta vein and 9.0% in the adipose tissue. The lowest intra-day precision of procyanidins (catechin, epicatechin and dimer B_2) was obtained in the brain, with values between 1.8% and 6.0%, and the aorta vein, with values between 0.5% and 7.6%. On the other hand, the intra-day precision values obtained in the adipose tissue were the highest, with values between 6.0% and 10.1%. The intra-day precision of alkaloids obtained in liver, vein aorta and adipose tissue was similar, with values between 2.8% and 5% for the liver, 2.3% and 5.9% for the aorta vein and 2.7% and 5.7% for the adipose tissue. In contrast, the intra-day precision values obtained in the brain covered a wide range, with values between 0.2% and 5.9%. The inter-day precision (different-day precision) was similar or slightly higher than the intra-day precision.

The accuracy of the intra-day and inter-day methods developed ($n = 3$) was also calculated at three different concentration levels, according to the tissue matrix studied, and the values were between 95% and 103%. No differences were shown between intra-day and inter-day accuracy. The results for precision and accuracy appear to indicate that the methodology for extracting the compounds from the studied tissues is highly reproducible and robust.

The LODs (concentration for signal/noise = 3) were lower than 0.9 nmol/g fresh liver, 1.1 nmol/g fresh brain, 13.3 nmol/g fresh aorta vein and 5.7 nmol/g fresh adipose tissue. The LOQs (concentration for signal/noise = 10) were lower than 3.3 nmol/g fresh liver, 4.6 nmol/g fresh brain, 35.8 nmol/g fresh aorta vein and 19.0 nmol/g fresh adipose tissue. However, the highest LOQ and LOD values in all the studied tissues corresponded to theobromine. The other LOQs and LODs were practically ten times lower.

The quality parameters obtained in the methods validation were not compared with the results obtained by other authors because the research works in which procyanidins [2,6,8,14], and anthocyanins [10,15–17] were detected in tissue samples, the validation procedure was not studied.

3.3. Matrix effect

The signal response of the analytes obtained from standard solutions prepared with organic solvent, acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v) or methanol, and matrix samples may differ significantly [25,26]. This fact, known as the matrix effect, occurs when the molecules originating from the sample matrix coelute with the analytes of interest and can compete for ionization capacity and interfere with the ionization process of the analytes in LC–ESI–MS/MS. This coextracted sample matrix can suppress or, less frequently, enhance the analyte signal response, according to whether the signal response of the analytes in the standard solutions is lower or higher than the response of the analytes in the sample matrix, respectively.

In order to study the influences on the MS signal responses by coeluting substances originated from complex biological tissues (brain, liver, aorta vein and adipose tissue), the matrix effect (%) was studied at different concentration levels. This was evaluated by comparing the peak abundances obtained from a pool of control samples spiked after sample pre-treatment with those obtained from standard solutions (acetone/Milli-Q water/acetic acid (70/29.5/0.5, v/v/v) for procyanidins and anthocyanins, or methanol for alkaloids) at different concentrations.

Although the studied compounds could be present in biological tissues at low concentration levels, the matrix effect was studied in all the linearity range in order to compare this effect at different concentrations. Fig. 2 shows the absolute matrix effect (%) for catechin, dimer B_2, caffeine and malvidin-3-glucoside, as the representative studied compounds, in the four tissues studied (the

Table 4
Retention time (RT), linearity (nmol/g tissue), LOD and LOQ to determine the studied compounds by off-line LLE and off-line μSPE-UPLC–MS/MS in spiked liver, brain, aorta vein and adipose tissue.

Tissue/Compound	RT (min)	Linearity (nmol/g fresh tissue)	LOD (nmol/g fresh tissue)	LOQ (nmol/g fresh tissue)
Liver				
Catechin	3.98	1.0–80	0.4	1.0
Epicatechin	4.55	1.0–80	0.6	1.0
Dimer B_2	4.09	0.2–80	0.08	0.2
Cyanidin-3-Glucoside	1.90	1.6–80	0.6	1.6
Malvidin-3-Glucoside	2.96	1.5–80	0.5	1.5
Theophylline	2.72	1.0–80	0.4	1.0
Theobromine	2.01	3.3–80	0.9	3.3
Caffeine	3.61	1.5–80	0.5	1.5
Brain				
Catechin	3.98	0.9–57.5	0.3	0.9
Epicatechin	4.55	0.9–57.5	0.4	0.9
Dimer B_2	4.09	0.2–57.5	0.06	0.2
Cyanidin-3-Glucoside	1.90	1.3–57.5	0.5	1.3
Malvidin-3-Glucoside	2.96	1.1–57.5	0.3	1.1
Theophylline	2.72	0.9–57.5	0.3	0.9
Theobromine	2.01	4.6–57.5	1.1	4.6
Caffeine	3.61	0.9–57.5	0.3	0.9
Aorta vein				
Catechin	3.98	11.4–380	3.4	11.4
Epicatechin	4.55	9.2–380	3.0	9.2
Dimer B_2	4.09	0.4–380	0.07	0.4
Cyanidin-3-Glucoside	1.90	6.84–380	1.9	6.8
Malvidin-3-Glucoside	2.96	4.5–380	1.5	4.5
Theophylline	2.72	3.8–380	1.1	3.8
Theobromine	2.01	35.8–380	13.3	35.8
Caffeine	3.61	3.0–380	1.1	3.0
Adipose tissue				
Catechin	3.98	6.1–227	1.8	6.1
Epicatechin	4.55	4.5–227	1.6	4.5
Dimer B_2	4.09	0.1–227	0.03	0.1
Cyanidin-3-Glucoside	1.90	2.5–227	0.7	2.5
Malvidin-3-Glucoside	2.96	1.0–227	0.2	1.0
Theophylline	2.72	3.6–227	1.1	3.6
Theobromine	2.01	19.0–227	5.7	19.0
Caffeine	3.61	3.4–227	1.1	3.4

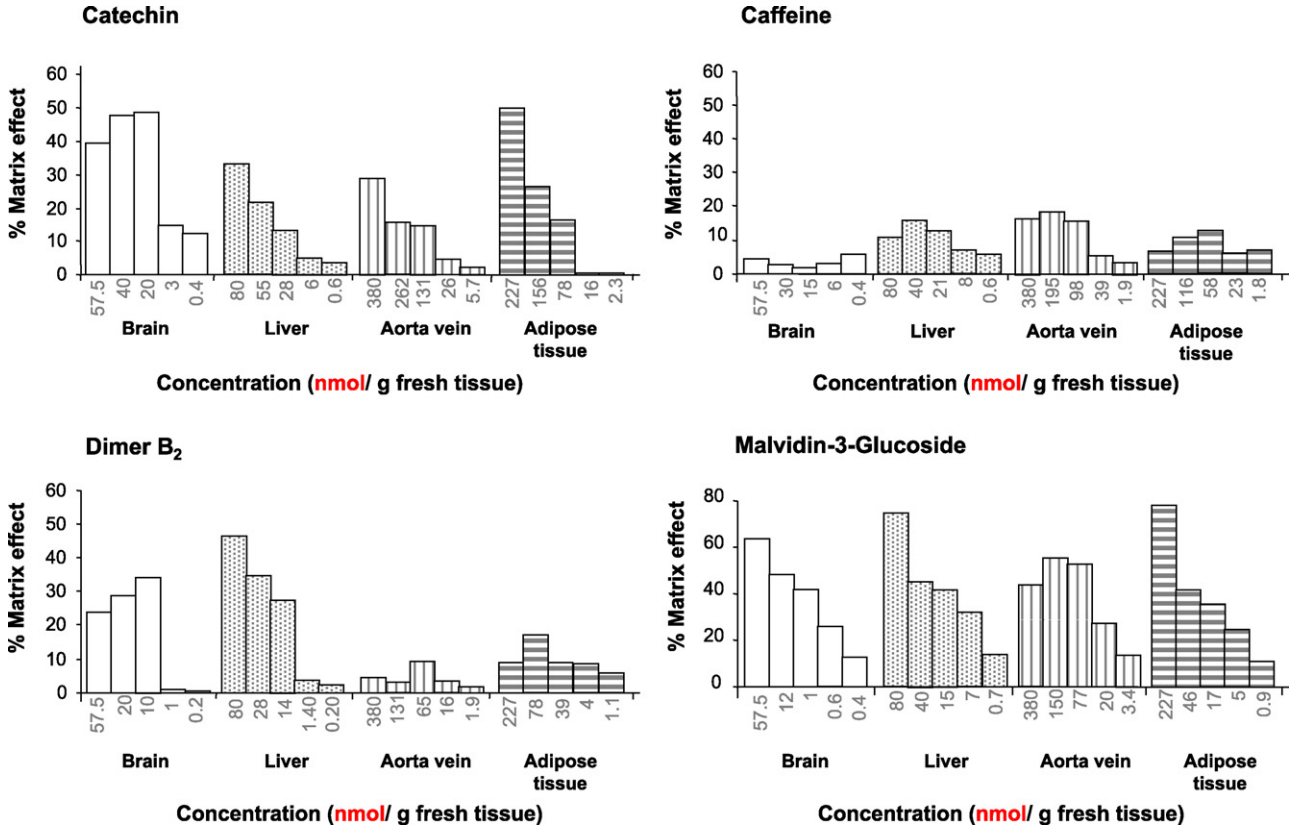

Fig. 2. Matrix effect study for the determination of catechin, dimer B_2, caffeine and malvidin-3-glucoside in brain, liver, aorta vein and adipose tissue.

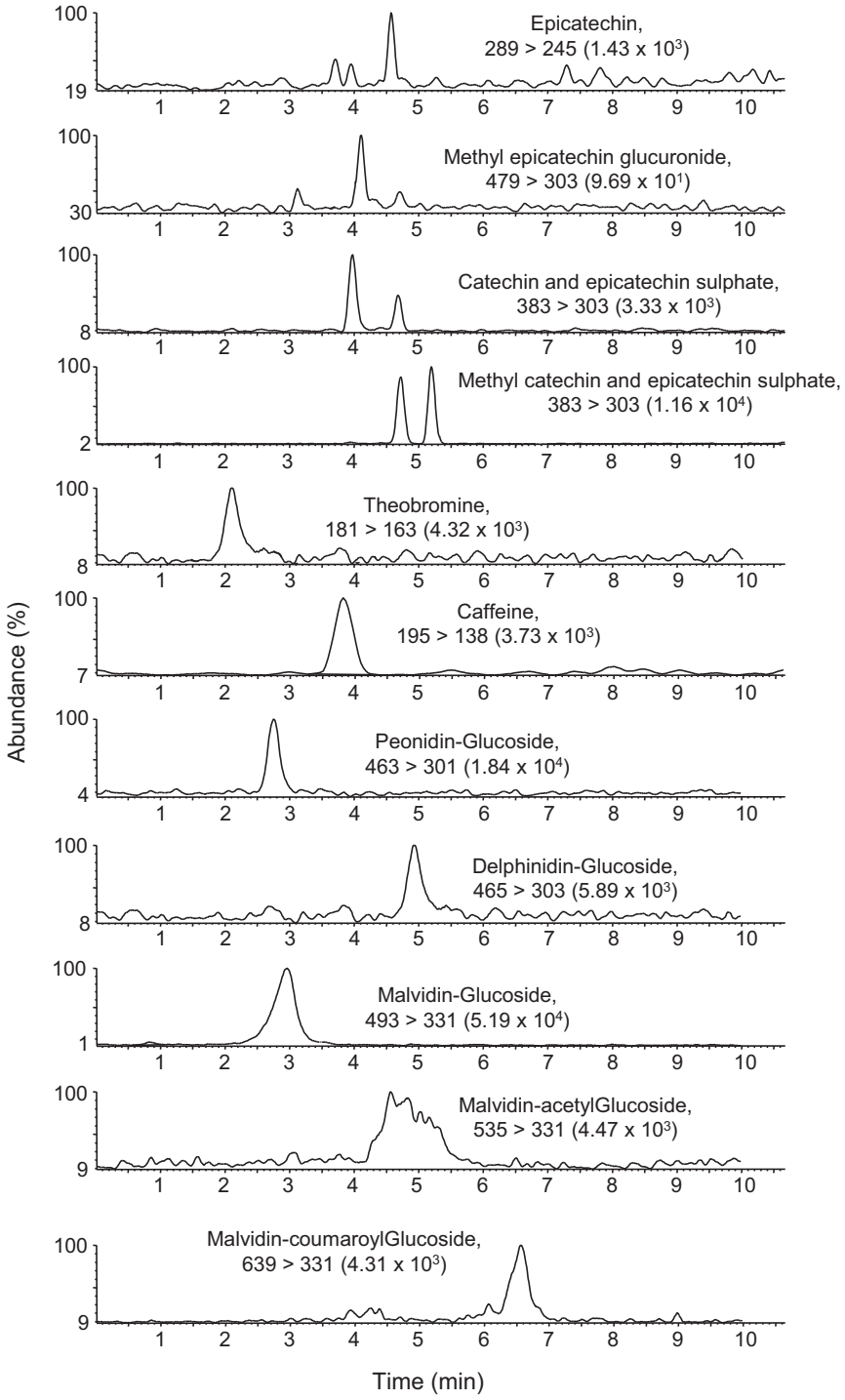

Fig. 3. Extract ion chromatograms obtained for the analysis of the liver extract after the ingestion of grape seed extract (procyanidins and anthocyanins) or cocoa extract (alkaloids).

brain, liver, aorta vein and adipose tissue) at the different concentrations of the linearity range. The matrix effect (%), expressed as the signal suppression (%), was calculated for each compound as the percentage decrease in signal intensity in the biological tissue matrix versus the elution solvent [26]. Although the Fig. 2 shows the absolute matrix effect (%), both positive and negative effects were observed, which meant the signal of the analyte in the spiked matrix was respectively higher or lower than the signal in the standard solution. The matrix effect was reduced as the analyte concentration decreased. At high concentration levels, the signal abundances of the analytes in the standard solutions were higher than the signal abundances of the same analytes at the same concentration extracted from the spiked tissue. This means that the coeluting matrix substances reduced the ion intensity of the studied compounds and caused signal suppression. On the other hand, at low concentration levels (for example lower than 3, 6, 26 and 16 nmol/g tissue for the determination of catechin in brain, liver, aorta vein and adipose tissue, respectively) the matrix effect was small, being less than 10% for catechin, dimer B_2 and caffeine, and 17% for malvidin-3-glucoside in all the tissues. In summary, the alkaloid

Table 5
Concentration (nmol analyte/g tissue) of procyanidins and anthocyanins and their metabolites at 4 h after the administration of a 1 g of grape pomace extract/kg rat weight (Experiment 1), and concentration of alkaloids and their metabolites at 4 h after the administration of a 1 g of cocoa extract/kg rat weight (Experiment 2) (mean ± SE).

	Liver	Brain	Aorta vein	Adipose tissue
Procyanidins				
Catechin	n.d.	n.d.	n.d.	n.d.
Epicatechin	13.6 ± 0.10	n.d.	n.d.	n.d.
Catechin glucuronide	n.d.	2.12 ± 0.14	n.d.	n.d.
Epicatechin glucuronide	n.d.	5.48 ± 0.36	n.d.	n.d.
Methyl catechin-glucuronide	n.d.	1.87 ± 0.00	n.d.	n.d.
Methyl epicatechin-glucuronide	13.5 ± 0.07	1.60 ± 0.08	n.d.	n.d.
Catechin sulphate	16.1 ± 0.34	n.d.	n.d.	n.d.
Epicatechin sulphate	14.0 ± 0.07	n.d.	n.d.	n.d.
Methyl catechin-sulphate	32.8 ± 0.78	0.45 ± 0.09	n.d.	n.d.
Methyl epicatechin-sulphate	30.3 ± 0.54	0.51 ± 0.19	n.d.	n.d.
Dimer B_2	n.d.	1.16 ± 0.11	1.05 ± 0.10	0.17 ± 0.01
Anthocyanins				
Malvidin-glucoside	3.55 ± 0.07	n.d.	n.d.	n.q.
Peonidin-glucoside	2.40 ± 0.01	n.d.	n.d.	n.q.
Delfinidin-glucoside	2.53 ± 0.01	n.d.	n.d.	n.d.
Malvidin-acetylglucoside	2.51 ± 0.01	n.d.	n.d.	n.d.
Malvidin-coumarylglucoside	2.41 ± 0.01	n.d.	n.d.	n.d.
Alkaloids				
Theophylline	n.d.	n.d.	n.d.	n.d.
Theobromine	3.82 ± 0.10	25.6 ± 1.42	289 ± 6.00	n.q.
Caffeine	5.24 ± 0.18	2.36 ± 0.08	27.2 ± 1.14	n.q.

n.d.: not detected. n.q.: not quantified.

caffeine showed the lowest matrix effect and the anthocyanin malvidin-3-glucoside the highest matrix effect in the four tissues. The matrix effect (%) of epicatechin was similar to catechin; theobromine and theophylline to caffeine; and cyanidin-3-glucoside to malvidin-3-glucoside.

Due to the complexity of the biological sample matrix, the sample preparation and the use of an exhaustive sample extraction step is essential to maintain high sensitivity and signal reproducibility to qualitatively and quantitatively determine metabolites at very low concentrations.

3.4. Application of the developed methods

The first method described above was applied to determine the procyanidins and anthocyanins and their metabolites in the liver, brain, aorta vein and adipose tissue obtained from rats 4 h after a single administration of a grape pomace extract (Experiment 1 of Section 2.3). Similarly, the second method was applied to determine alkaloids and their metabolites in the same tissue samples obtained from rats 4 h after a single administration of a cocoa extract (Experiment 2 of Section 2.3). Fig. 3 shows, as an example, the extract ion chromatograms of the generated metabolites of procyanidins and anthocyanins, obtained by UPLC–MS/MS, for the analysis of liver extract after the ingestion of grape pomace extract.

Table 5 shows the results of the quantification of procyanidins, anthocyanidins and alkaloids in the different tissues analyzed. The analysis of procyanidins showed intense metabolism in the liver and brain. The free form of epicatechin was only quantified in the liver (13.6 nmol/g tissue), and the free form of dimer B_2 in the brain, aorta vein and adipose tissue (1.16, 1.05 and 0.17 nmol/g tissue, respectively). Among the anthocyanins, malvidin-glucoside was quantified in the liver at a concentration of 3.55 nmol/g tissue but not in the adipose tissue, because its concentration was between its LOQ and LOD. In the other tissues, namely the brain and aorta vein, the anthocyanins were not detected. Theobromine and caffeine, the free forms of the alkaloids, were detected in all the tissues analyzed, with concentrations ranging between 2.82 and 289 nmol/g tissue and 5.24 and 27.2 nmol/g tissue, respectively, except in the adipose tissue where they were not quantified (their concentrations were between their LOQ and LOD). The amount of theobromine and caffeine in the aorta vein was higher than in the other tissue analyzed (289 and 27.2 nmol/g tissue, respectively). Despite the high concentration of alkaloids quantified in the different tissues, theophylline was not detected.

After determining the studied phenolic compounds and alkaloids (included in the study of analytical characteristics of the methods) in the tissues, the developed methods were also applied to identify and quantify metabolites resulting from the hepatic metabolism. The metabolites generated from the standard compounds were identified by using full-scan mode, by the MS mode, and by neutral-loss scan and product ion scan, by the tandem MS mode. These techniques are excellent tools for verifying the chemical structure when standards are not available. The catechin and epicatechin metabolites showed the same MS fragmentation pattern but different retention times. In order to identify these metabolites, the same UPLC elution order of catechin and epicatechin standards was considered. Therefore, the catechin metabolites eluted earlier than the epicatechin metabolites. A wide range of metabolites of catechin and epicatechin were identified and quantified, including catechin and epicatechin glucuronide, methyl catechin and epicatechin glucuronide, catechin and epicatechin sulphate, and methyl catechin and epicatechin sulphate. These metabolites were quantified in the liver and brain, and the highest concentration was found in the liver tissue. Therefore, as can be seen in Table 5, the main procyanidin metabolites found in the liver was methyl catechin-sulphate with 32.8 nmol/g tissue, followed by methyl epicatechin-sulphate with 30.3 nmol/g tissue. The glucuronide forms were not found in the liver, with the exception of methyl epicatechin-glucuronide, but they were quantified in the brain at much lower concentrations. No procyanidin metabolites were detected in the aorta vein and adipose tissue.

Apart from malvidin-glucoside, other anthocyanins, such as peonidin-glucoside, delphinidin-glucoside, malvidin-acetylglucoside and malvidin-coumaroyl-glucoside were also determined in the liver, these being present in the grape pomace extract administered to the rats (Table 1 Additional Information). The concentrations of these compounds were between 2.41 and 3.55 nmol/g tissue. Delphinidin-glucoside was also detected in the liver, as reported by Vanzo et al. [16]. No anthocyanins were detected in the brain and adipose tissue. Peonidin-glucoside was identified in the adipose tissue, and its concentrations were between its LOQ and its LOD.

4. Concluding remarks

In the present study, rapid, selective and sensitive methods were developed to determine procyanidins, anthocyanins and alkaloids in biological tissues. The use of the off-line LSE and off-line μSPE or off-line SPE sample preparation with UPLC–MS/MS allows the rapid determination of these compounds and their metabolites at low concentration levels in different tissue samples. The matrix effect was small, lower than 10% for procyanidins and alkaloids and lower than 17% for anthocyanins, and the calibration curves were prepared with spiked tissue samples to reduce inaccuracies by sample matrix (coeluting matrix substances). The application of the developed methods to analyze different tissues (liver, brain, aorta vein and adipose tissue) allowed the identification and quantification of procyanidins, anthocyanidins and alkaloids and their metabolites at different concentration levels. The analysis of the metabolites revealed a hepatic metabolism of procyanidins, the liver being the tissue which produced the greatest accumulation of these metabolites.

Acknowledgements

This work was supported by the Spanish Ministry of Education and Science financing the project AGL2009-13517-C13-02 and the present work was also supported by the Catalan Government (Interdepartmental Commission for Research and Technological Innovation) through the A. Serra grant.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.042.

References

[1] A. Serra, A. Macià, M.P. Romero, M.D. Salvadó, M. Bustos, J. Fernández-Larrea, M.J. Motilva, J. Chromatogr. B 877 (2009) 1169.
[2] J.K. Prasain, N. Peng, Y. Dai, R. Moore, A. Arabshahi, L. Wilson, S. Barnes, J. Michael Wyss, H. Kim, R.L. Watts, Phytomedicine 16 (2009) 233.
[3] J. Wang, H. Luo, P. Wang, L. Tang, J. Yu, T. Huang, S. Cox, W. Gao, Food Chem. Toxicol. 46 (2008) 232.
[4] B. Bartolomé, M. Monagas, I. Garrido, C. Gómez-Cordovés, P.J. Martín-Álvarez, R. Lebrón-Aguilar, M. Urpí-Sardà, R. Llorach, C. Andrés-Lacueva, Arch. Biochem. Biophys. 501 (2010) 124.
[5] A.S. Ptolemy, E. Tzioumis, A. Thomke, S. Rifai, M. Kellogg, J. Chromatogr. B 878 (2010) 409.
[6] C. Tsang, C. Auger, W. Mullen, A. Bornet, J. Rouanet, A. Crozier, P. Teissedre, Br. J. Nutr. 94 (2005) 170.
[7] M.J. Arnaud, Handb. Exp. Pharmacol. 200 (2011) 33.
[8] B. García-Ramírez, J. Fernández-Larrea, M.J. Salvadó, A. Ardèvol, L. Arola, C. Bladé, J. Agric. Food Chem. 54 (2006) 2543.
[9] M.P. Martí, A. Pantaleón, A. Rozek, A. Soler, J. Valls, A. Macià, M.P. Romero, M.J. Motilva, J. Sep. Sci. 33 (2010) 2841.
[10] T. Miyazawa, K. Nakagawa, M. Kudo, K. Muraishi, K. Someya, J. Agric. Food Chem. 47 (1999) 1083.
[11] M. Murkovic, H. Toplak, U. Adam, W. Pfannhauser, J. Food Compos. Anal. 13 (2000) 291.
[12] M. Zydroń, J. Baranowski, I. Baranowska, J. Sep. Sci. 27 (2004) 1166.
[13] A. Aresta, F. Palmisano, C.G. Zambonin, Food Chem. 93 (2005) 177.
[14] M. Urpi-Sarda, E. Ramiro-Puig, N. Khan, S. Ramos-Romero, R. Llorach, M. Castell, S. Gonzalez-Manzano, C. Santos-Buelga, C. Andres-Lacueva, Br. J. Nutr. (2010) 1.
[15] S. Talavéra, C. Felgines, O. Texier, C. Besson, A. Gil-Izquierdo, J. Lamaison, C. Rémésy, J. Agric. Food Chem. 53 (2005) 3902.
[16] A. Vanzo, M. Terdoslavich, A. Brandoni, A.M. Torres, U. Vrhovsek, S. Passamonti, Mol. Nutr. Food Res. 52 (2008) 1106.
[17] M.A. El Mohsen, J. Marks, G. Kuhnle, K. Moore, E. Debnam, S.K. Srai, C. Rice-Evans, J.P.E. Spencer, Br. J. Nutr. 95 (2006) 51.
[18] M. Suárez, M.P. Romero, A. Macià, R.M. Valls, S. Fernández, R. Solà, M.J. Motilva, J. Chromatogr. B 877 (2009) 4097.
[19] D. Guillarme, J. Schappler, S. Rudaz, J. Veuthey, Trends Anal. Chem. 29 (2010) 15.
[20] M. Suárez, M.P. Romero, T. Ramo, A. Macià, M.J. Motilva, J. Agric. Food Chem. 57 (2009) 1463.
[21] N. Ortega, M.P. Romero, A. Macià, J. Reguant, N. Anglès, J.R. Morelló, M.J. Motilva, J. Food Compos. Anal. 23 (2010) 298.
[22] A. Zafra-Gómez, B. Luzón-Toro, I. Jiménez-Diaz, O. Ballesteros, A. Navalón, J. Pharm. Biomed. Anal. 53 (2010) 103.
[23] C. Garcia-Viguera, P. Zafrilla, F.A. Tomás-Barberán, Phytochem. Anal. 9 (1998) 274.
[24] C. Santos-Buelga, G. Williamson, Methods in Polyphenol Analysis, The Royal Society of Chemistry, Cambridge, UK, 2003.
[25] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. B 877 (2009) 2198.
[26] M.J. Gómez, M. Petrović, A.R. Fernández-Alba, D. Barceló, J. Chromatogr. A 1114 (2006) 224.