

Distribution of procyanidins and their metabolites in rat plasma and tissues in relation to ingestion of procyanidin-enriched or procyanidin-rich cocoa creams

Aida Serra · Alba Macià · Laura Rubió ·
Neus Anglès · Nàdia Ortega · José Ramón Morelló ·
Maria-Paz Romero · Maria-José Motilva

Received: 3 February 2012 / Accepted: 21 June 2012 / Published online: 11 July 2012
© Springer-Verlag 2012

Abstract

Background Procyanidins are extensively metabolized via phase-II and microbial enzymes. However, their distribution in the body is not well characterized.

Aim This study investigates the distribution of procyanidins (monomers and dimers) and their phase-II metabolites in plasma and tissues (thymus, heart, liver, testicle, lung, kidney, spleen and brain).

Methods Wistar rats were fed with 1 g of cocoa cream (CC), 50 mg of procyanidin hazelnut skin extract (PE) and 50 mg PE in 1 g CC (PECC). The rats were killed at 0, 1, 1.5, 2, 3, 4 and 18 h after gavage, and the plasma and tissues were analyzed by UPLC–MS/MS.

Results Epicatechin–glucuronide was the main metabolite in the plasma after the CC intake, with C_{\max} at 423 nM and t_{\max} at 2 h, and methyl catechin–glucuronide (301 nM, 2 h) was the main metabolite in the plasma after the PE intake. As a result of the PECC enrichment, epicatechin–glucuronide (452 nM, 1.5 h) and catechin–glucuronide (297 nM, 2 h) were the main metabolites in the plasma. Methyl catechin–glucuronide was found in the liver after PE (8 nmol/g tissue, 4 h) and PECC (8 nmol/g, 1.5 h). The kidney was found to contain a high concentration of phase-II metabolites of procyanidins and is therefore thought to be the main site of metabolism of the compounds. Methyl

catechin–sulfate (6.4 nmol/g, 4 h) was only quantified in the brain and after PE intake. Catechin metabolites were not found in the spleen or heart. Phenolic acids were detected in all tissues.

Conclusions The formulation of a product enriched or fortified with procyanidins is a way to increase their bioavailability, with clear effects on the plasmatic pharmacokinetics, and a greater accumulation of phenolic metabolites in such tissues as the liver, kidney, lung and brain.

Keywords Food matrix effect · Procyanidins · Plasma · Tissue distribution

Introduction

Food fortification is defined as the addition of one or more micronutrients to a food, with the aim of increasing the intake of these micronutrient(s) to correct or prevent a demonstrated deficiency and/or exert a health benefit. Different methods of food fortification have been described. *Mass fortification* is defined as the fortification of a widely consumed food. When the fortified food is designed for a specific population subgroup, such as complementary foods for young children or rations for displaced populations, the fortified food is called *targeted fortification*. The latter type of fortification is known as *market-driven fortification* and is related to the design of a fortified food based on one generally available on the market, which is fortified voluntarily by the food manufacturers. In industrialized countries, *Market-driven food fortification* has a long history of successful control of deficiencies of vitamins A and D, several B vitamins (thiamine, riboflavin and niacin), iodine and iron [1].

A. Serra · A. Macià · L. Rubió · M.-P. Romero ·
M.-J. Motilva (✉)
Department of Food Technology, XaRTA-UTPV, Escola
Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida,
Avda/Alcalde Rovira Roure 191, 25198 Lleida, Spain
e-mail: motilva@tecal.udl.es

N. Anglès · N. Ortega · J. R. Morelló
R+D+i Department, La Morella Nuts, S.A.U., Camí Ample s/n,
43392 Castellvell del Camp, Spain

Over the last two decades, there has been a great increase in knowledge about the influence of diet on health and welfare. This has led to the creation of new and healthier foods, known as functional foods, designed to reduce the risk of several chronic diseases by modifying their composition slightly [2]. However, there is no universally accepted definition of these foods, and, according to the American Dietetic Association (ADA), functional foods include conventional foods, modified foods (i.e. fortified, enriched or enhanced), medical foods and foods for special dietary use [3]. Unmodified whole foods or conventional foods such as fruit and vegetables could represent the simplest forms of functional foods. An example of their bioactivity is the role played by dark chocolate in reducing cardiovascular disease by improving the endothelial function [4]. Due to the beneficial effects shown to be exerted by functional foods, including the reduction of cholesterol levels, an increase in calcium levels or the antioxidant capacity, prevention of osteoporosis or lowering blood pressure [5–8], they are becoming ever more common in our diet.

Related to functional foods, foods enhanced with bioactive components, such as polyphenols, are attracting growing interest. In this context, while nowadays there is a general consensus about the beneficial effect of dietary flavanols, and their oligomeric derivatives the procyanidins, on the human health, we still lack a comprehensive understanding of their biological properties and a conclusive evidence-based demonstration of a causal relationship between polyphenol intake and a decrease in the likelihood of disease [9].

Another important factor related to the polyphenol supplementation is the relationship between the ingested dose and the metabolism. Large doses are metabolized primarily in the liver, while small doses are metabolized by the intestinal mucosa, as suggested by Shoji et al. [10]. Furthermore, the endogenous action of polyphenols and their metabolites seems to differ, due to a modification of their molecular structure, as occurs with the antioxidant activity, which decreases drastically when the hydroxyl group of the phenolic molecule is modified [11]. As a result of the phase-II metabolism, a wide range of molecules undergo structural modifications, which may affect their binding to proteins and tissue distribution [12]. These modifications may also have potential effects on the biological impact of flavanols. In this context, special attention should be paid to the appropriate doses and the effects of long-term consumption in the development of the next generation of health-promoting cocoa-derived foods with enhanced flavanol contents.

The behavior of a procyanidin supplementation in a cocoa-derived product is evaluated in this present study by comparing the pharmacokinetics, metabolism and tissue

distribution of flavanols and procyanidins (low polymerization grade) after an oral intake of a cocoa cream (CC) and a procyanidin-enriched cocoa cream (PECC) prepared by adding a procyanidin hazelnut skin extract (PE) to the CC. The study also includes the pharmacokinetic study after the ingestion of the PE separately as a means of evaluating the effect of the food matrix.

Materials and methods

Chemical

Internal standard (IS) catechol and the standards of (–)-epicatechin, (+)-catechin, (–)-epigallocatechin, (–)-epigallocatechin-3-*O*-gallate, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, phenylacetic acid and 3-(4-hydroxyphenyl)propionic acid were purchased from Sigma Aldrich (St. Louis, MO, USA), and procyanidin dimer B2 [epicatechin-(4 β -8)-epicatechin], 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and 3-(2,4-dihydroxyphenyl)propionic acid from Fluka Co. (Buchs, Switzerland). The acetonitrile (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade) and glacial acetic acid (99.8 %) were of analytical grade (Scharlab, Barcelona, Spain). Ortho-phosphoric acid 85 % was purchased from MontPlet and 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).

Cocoa cream (CC) and procyanidin extract (PE)

The CC and the procyanidin hazelnut skin extract were kindly supplied by La Morella Nuts S.A.U. (Reus, Spain). The composition of the CC was as follows: 49 % lipids (26.5 % polyunsaturated, 12 % unsaturated and 10.5 % saturated), 38 % carbohydrates (28 % dietary fiber), 9 % protein, 2 % ash and 2 % moisture. The PECC was prepared by adding 50 mg of PE to 1 g of CC. The procyanidin composition of CC, PECC and PE was analyzed by UPLC–MS/MS according to the method described by Ortega et al. [13]. The phenolic composition of the CC, PE and PECC is shown in Table 1.

Treatment of animals and tissue collection

The Animal Ethics Committee of the University of Lleida approved the study (CEEA 03-02/09, 9th November 2009). A total of 57 three-month-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were used in this study. The rats were housed in cages on a 12-h light–12-h

Table 1 Content of procyanidins in the hazelnut skin extract (PE), the cocoa cream (CC) and the enriched cocoa cream (PECC)

Compound	Hazelnut skin extract (PE) (nmol/50 mg extract)	Cocoa cream (CC) (nmol/g cream)	Procyanidin-enriched cocoa cream (PECC) (nmol/g cream)
Catechin	313 ± 28	113 ± 11	434 ± 11
Epicatechin	117 ± 8	506 ± 47	651 ± 17
Epigallocatechin	130 ± 12	Nd	128 ± 15
Epicatechin gallate	Nd	76.0 ± 7.9	29.0 ± 2.5
Epigallocatechin gallate	4.80 ± 0.31	58.0 ± 0.5	32.3 ± 2.9
Dimer	776 ± 76	896 ± 87	1,740 ± 16.5
Trimer	249 ± 13	94.1 ± 7.4	441 ± 37
Tetramer	10.0 ± 1.2	35.2 ± 3.1	85.1 ± 8.0
Total	1,599 ± 137	1,778 ± 162	3,540 ± 322

The results are expressed as nmol/50 mg of extract (ingested dose 50 mg of extract) and as nmol/g of cream (ingested dose 1 g)

Values are mean ± SD ($n = 5$)

Nd not detectable

dark cycle 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 16 h with access to tap water. Subsequently, they were divided into four groups. Group 1 (3 rats): the control group was maintained under fasting conditions without ingestion. Group 2 (18 rats): 1 g of CC was administered, dispersed in 1.5 ml of water. Group 3 (18 rats): 50 mg of PE was administered in 2 ml of water. Group 4 (18 rats): 1 g of PECC was administered in 1.5 ml. The rats were anesthetized with isoflurane (IsoFlo, Veterinaria Esteve, Bologna, Italy) and killed by exsanguinations at 0 h (control group) and at 1, 1.5, 2, 3, 4 and 18 h (3 rats/group/time) after administrating the cream (CC or PECC) or the extract (PE).

The plasma samples were obtained by centrifugation (2,000×g for 30 min at 4 °C) and then stored at −80 °C until the chromatographic analysis of the procyanidins and their metabolites. A range of tissues (thymus, heart, liver, testicle, lung, kidney, spleen and brain) were excised from the rats, stored at −80 °C and freeze-dried for phenolic extraction and chromatographic analysis.

Phenolic extraction of plasma and tissues and chromatographic analysis

The method used to extract procyanidins and their metabolites from plasma and tissues and the chromatographic analysis were based on the methodologies described in our previous papers [14, 15].

Statistical analyses

The data were analyzed by one-way analysis of variance (ANOVA) to assess the significant differences among the CC, PE and PECC groups. All statistical analysis was carried out using Statgraphics Plus 5.1. p values <0.05 were considered statistically significant.

Results

Procyanidin content in extract and creams

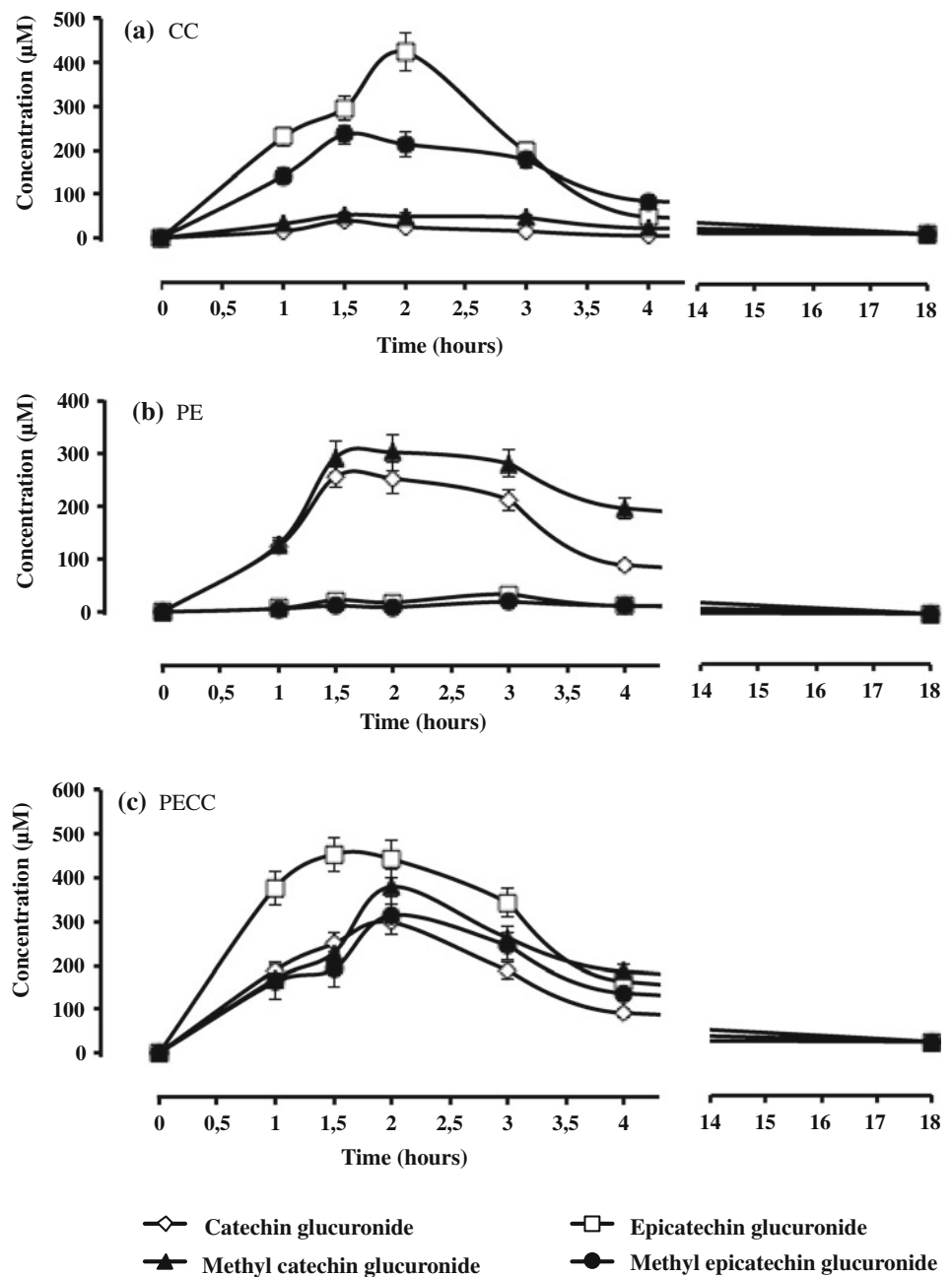
Table 1 shows the phenolic contents of PE, CC and PECC expressed as total nmol of each compound contained in the dose of PE (50 mg) or CC and PECC (1 g) ingested in each treatment. As regards the monomeric forms of procyanidins, catechin was the main monomer in PE (313 nmol/50 mg), and epicatechin was the main monomer found in the CC (506 nmol/g). Concerning the low level of polymerization of proanthocyanidins (dimers–tetramers), dimer was the main compound in PE (776 nmol/50 mg PE) and CC (896 nmol/g); additionally, trimer and tetramer were also determined. As expected, 1 g of PECC contained approximately the amount of procyanidins (flavanols and low molecular weight procyanidins) included in 1 g of CC + 50 mg of PE. This was also observed with the concentrations of dimer, trimer and tetramer.

Procyanidin plasma kinetics

After the CC intake, the main metabolite quantified in plasma was epicatechin–glucuronide (Fig. 1a) reaching the $C_{\max 2h}$ 423 μM, followed by methyl epicatechin–glucuronide. The glucuronide and methyl glucuronide conjugates of catechin were also detected in the plasma samples after an acute intake of the CC at a lower concentration. In contrast, methyl catechin–glucuronide was the main metabolite after the acute intake of the PE with $C_{\max 2h}$ 301 μM, followed by the catechin–glucuronide (Fig. 1b). Dimer was detected with $C_{\max 2h}$ 0.84 μM after the ingestion of the CC, and trimer was also detected with $C_{\max 1h}$ 0.64 μM after the ingestion of the PE (data not shown).

Epicatechin–glucuronide was the main metabolite determined in plasma after the PECC intake, followed at similar concentrations by catechin–glucuronide, methyl catechin–glucuronide and methyl epicatechin–glucuronide (Fig. 1c). Other metabolites were determined at lower concentrations. These included methyl-sulfated conjugates of catechin and epicatechin (Table 2), which were determined in plasma from 1.5 to 3 h after the ingestion of the PECC and reached concentrations of $C_{\max 3h}$ 4.5 and

Fig. 1 Plasma kinetics of procyanidin metabolites detected in rat plasma collected between 0 and 18 h after the ingestion of: **a** cocoa cream (CC), **b** procyanidin-rich hazelnut skin extract (PE) and **c** procyanidin-enriched cocoa cream (PECC). The results are expressed as μM



14 μM , respectively. Dimer and trimer in their free forms were also determined in the plasma from 1 to 2 h with $C_{\text{max } 1\text{h}}$ 1.4 μM and $C_{\text{max } 2\text{h}}$ 0.76 μM , respectively. In all three experiments, the procyanidin metabolites were rapidly cleared from the plasma and were back to the baseline 18 h after ingestion.

To evaluate the effect of the procyanidin enrichment on the procyanidin plasma kinetics, Fig. 2 shows the plasma kinetic (0–18 h) of the total catechin and epicatechin-conjugated metabolites separately, and the plasma kinetic of the total monomeric metabolites (corresponding to the sum of catechin and epicatechin-conjugated metabolites)

for each intake (CC, PE and PECC). In general, catechin, epicatechin and, subsequently, the total of monomeric metabolites, were higher after the ingestion of PECC, highlighting the clear pharmacokinetic curve of total monomeric metabolites that reached a peak of concentration 2 h after the PECC intake. As was expected following the procyanidin concentration in CC, PE and PECC (Table 1), the total quantity of epicatechin metabolites was higher in the plasma from CC and PECC, and the level of catechin metabolites was higher in the plasma from PE and PECC, although epicatechin metabolite level was higher than that of the catechin metabolites after PECC intake.

Table 2 Concentration of minor procyanidin metabolites detected in rat plasma collected between 0 and 18 h after the ingestion of enriched cocoa cream (PECC)

Compound (μM)	Time							
	0 h	1 h	1.5 h	2 h	3 h	4 h	6 h	18 h
Methyl catechin–sulfate	Nd	Nd	2.41 ± 0.13	4.01 ± 0.32	4.50 ± 0.44	Nq	Nd	Nd
Methyl epicatechin–sulfate	Nd	Nd	9.70 ± 0.96	12.2 ± 1.1	14.1 ± 1.0	Nq	Nd	Nd
Dimer	Nd	1.40 ± 0.01	1.21 ± 0.13	0.80 ± 0.76	Nd	Nd	Nd	Nd
Trimer	Nd	0.50 ± 0.03	Nq	0.76 ± 0.03	Nd	Nd	Nd	Nd

The results are expressed as μM

Values are mean \pm SD ($n = 3$)

Nd not detected, Nq not quantified

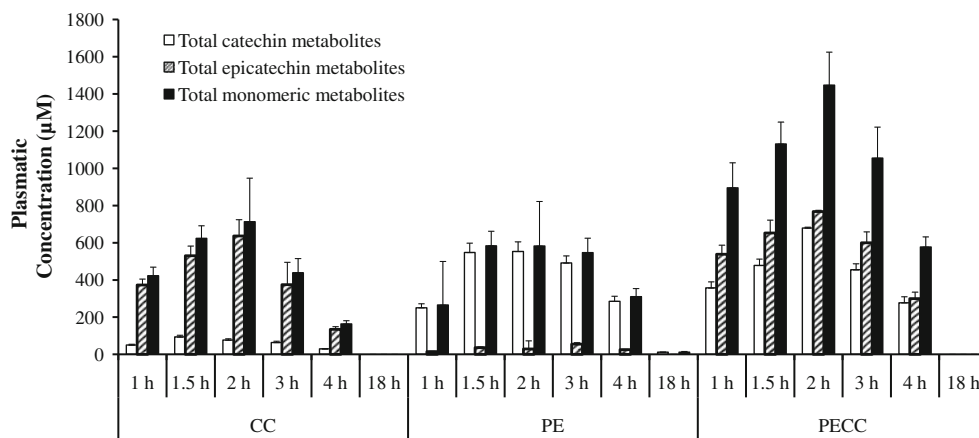


Fig. 2 Total catechin and epicatechin metabolites, and total monomeric metabolites (sum of catechin and epicatechin metabolites) quantified in rat plasma collected between 0 and 18 h after the

ingestion of cocoa cream (CC), procyanidin-rich hazelnut skin extract (PE) and procyanidin-enriched cocoa cream (PECC). The results are expressed as μM

Distribution of procyanidin in tissues

Regarding the tissues that collaborate in the phase-II metabolism, the analysis of the liver showed an increase in the concentration of different compounds compared with the basal conditions (0 h) (Fig. 3). This accumulation corresponded mainly to phenolic acids, independently of the intake (CC, PE or PECC). Only methyl catechin–glucuronide was determined in the liver as a phase-II procyanidin metabolite, and it was only detected after the ingestion of PE and PECC, showing the same C_{max} of 8 nmol/g tissue in both intakes. However, the behavior of the phenolic acids in the liver varied with the product ingested, with a clear peak of *p*-hydroxybenzoic acid 2 h after the PE intake.

Several procyanidin metabolites were determined in the kidney (Fig. 3). There was a clear increase in epicatechin–glucuronide 1 h after the CC intake. Additionally, methyl epicatechin–glucuronide was only determined in the kidney 2 and 3 h after the CC intake, with 5.1 and 2.23 nmol/g, respectively. Methyl catechin–sulfate was also determined

after the ingestion of CC with C_{max} 3h 1.15 nmol/g of tissue (data not shown). Generally, the pharmacokinetic curves obtained after the CC intake showed a slight tendency to form two peaks, the first at 1–2 h, and the second after 3 h. The intake of PE promoted the deposition of catechin–glucuronide and methyl catechin–glucuronide, showing single clear peaks of concentration in the kidney (C_{max} 2h 2.62 and 1.03 nmol/g, respectively). Finally, the combination of PE and CC (PECC) resulted in several procyanidin phase-II metabolites (catechin–glucuronide, epicatechin–glucuronide, methyl catechin–glucuronide and methyl epicatechin–glucuronide) determined in the kidney, all of which showed two peaks of concentration, the first at 1.5 h and the second, 3 h after the PECC intake. The same behavior was detected with the ferulic sulfate acid. Although this phenolic acid was determined in the kidney independently of the ingested product, its concentration increased more after the PECC intake (Fig. 3).

Other peripheral tissues were analyzed to determine the distribution of procyanidin metabolites through the body. The heart was selected to determine procyanidin

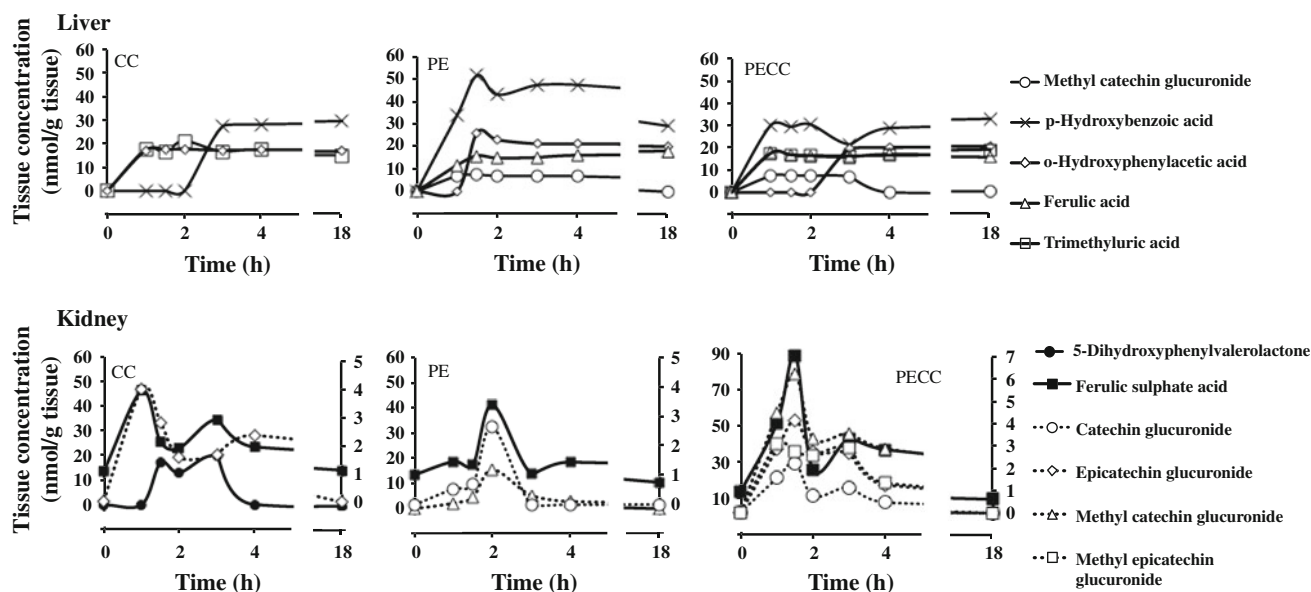


Fig. 3 Increase in the concentration of procyanidin metabolites and phenolic acids in the liver and kidney between 0 and 18 h after the ingestion of cocoa cream (CC), procyanidin-rich hazelnut skin extract

(PE) and procyanidin-enriched cocoa cream (PECC). The results are expressed as nmol/g of tissue. *Discontinuous lines* refer to the right Y axis

disposition in cardiovascular tissue. In that case, no phase-II procyanidin metabolites were found (Fig. 4), but three phenolic acids were determined after the ingestion of CC and PECC. Phenylacetic acid was the main phenolic acid in the heart, with no significant differences between the concentrations detected after the ingestion of CC and the PECC. 5-Dihydroxyphenylvalerolactone reached similar concentrations after the PECC and CC intakes. However, the t_{\max} was different, being delayed until 2 h after the PECC intake compared with 1 h after the CC intake.

By contrast, all the main procyanidin metabolites detected in the plasma were determined in the lung after the ingestion of the PECC, with a higher concentration of epicatechin metabolites in their glucuronide and methyl glucuronide forms (Fig. 4). In contrast, no phase-II metabolites were detected after the ingestion of CC and only methyl catechin–glucuronide was determined in the lung ($C_{\max\ 2h}$ 20 nmol/g of tissue) after the ingestion of the PE.

In the case of the testicles, procyanidin metabolites were found after the PE intake (Fig. 4). Two phase-II procyanidin metabolites were determined in this tissue after the PE and PECC intakes, with a more defined pharmacokinetic curve after the PE intake, drawing a slight peak of concentration 1.5 h after the PE intake. Additionally, three phenolic acids were determined in the tissues after the PE and PECC, including *p*-hydroxybenzoic acid, vanillic acid, 5-dihydroxyphenylvalerolactone.

There was no clear pattern to the accumulation of procyanidin metabolites or phenolic acids in the other studied tissues. Nonetheless, some metabolites were

determined at specific times (0–3 h). Methyl catechin–sulfate was the only phase-II metabolite detected in the brain from the PE in the interval from 1 to 4 h, with an average concentration of 5.5–6.4 nmol/g of tissue. Similarly, 3-hydroxyphenylpropionic acid was determined at all the times, including the basal conditions (26 nmol/g of tissue), and a significant increase in its concentration was observed after the ingestion of the PE and PECC ($C_{PE\ \max\ 1.5h}$ 45 nmol/g of tissue vs. $C_{PECC\ \max\ 1h}$ 33 nmol/g of tissue).

As a primary lymphoid organ, the thymus was analyzed and compared with the basal conditions; increases were only seen in the concentrations of some phenolic acids. These included *p*-hydroxybenzoic acid, which was determined at all the times tested, including the basal conditions (C_{0h} 54 nmol/g) and whose concentration increased from 3 to a $C_{\max\ 18h}$ 102 nmol/g after the ingestion of the PECC. This increase was not observed after the intake of PE and CC. An increase in the concentration of 5-dihydroxyphenylvalerolactone was observed in the thymus 1.5 h after the intake of PECC (24 nmol/g). This concentration remaining constant until 18 h after the PECC intake. Finally, ferulic sulfate acid was determined in the thymus after the intake of CC and PECC; its concentration remaining almost constant (near 17 nmol/g) at all the test times. The spleen was analyzed as a secondary lymphoid organ. Nevertheless, a slight increase was only observed in the concentration of vanillic acid compared with the basal conditions (C_{basal} 17 nmol/g), this being significant after the ingestion of CC and PECC, with $C_{\max\ 4h}$ 27 nmol/g and $C_{\max\ 1h}$ 24 nmol/g, respectively.

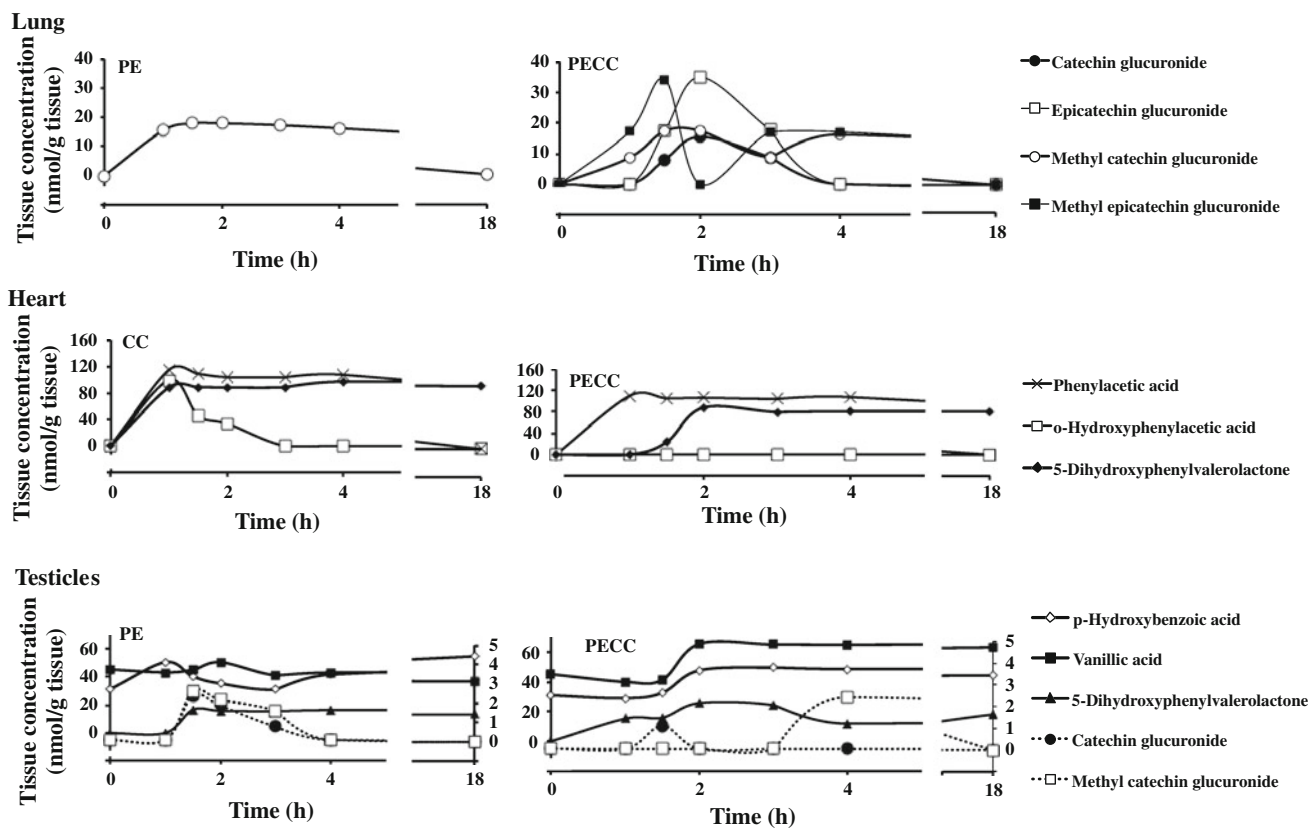


Fig. 4 Increase in the concentration of procyanidin metabolites and phenolic acids (0–18 h) in the lung after the ingestion of PE and PECC; in the heart after the ingestion of CC and PECC; and in the

testicles after the ingestion of PE and PECC. The results are expressed as nmol/g of tissue. *Discontinuous lines* refer to the *right Y axis*

Discussion

The present study aimed to evaluate the differences in the bioavailability, metabolism and tissue distribution of procyanidins comparing a CC and a procyanidin-enriched CC, as examples of acute supplementation of procyanidins, also including the separate ingestion of the procyanidin extract. Clear differences in the procyanidin plasmatic metabolites were detected between intakes. After the ingestion of PECC, the plasmatic metabolites determined resulted from the combination of PE and CC, detecting a dose-dependent metabolism. Additionally, the fact that methyl-sulfated conjugates of procyanidins were only determined in the plasma after the ingestion of PECC reaffirms that procyanidins were metabolized in a dose-dependent way [10]. Some differences in the metabolite bioavailability were detected with the higher procyanidin intake (PECC). With that product, low-grade polymerization procyanidins (dimer and trimer) were found in the plasma for longer post-prandial times and at higher concentrations, similar to those observed by other authors [16–20]. In contrast, free forms of catechin and epicatechin were not detected in the plasma samples, possibly due to

the use of a dietary dose, because when food polyphenols were administered at a pharmacological dose, their free forms could be found in the blood [10, 16, 21].

Figure 5 displays the increase in the total concentration of metabolites (nmol/g), including all the detected phase-II procyanidin metabolites and phenolic acids, for each tissue after a single intake of CC, PE and PECC. A two-peak disposition of phenolic metabolites was detected in practically all the tissues analyzed. The highest accumulation in the liver was observed after the PE intake. On the other hand, the major metabolite accumulation in the kidney was observed after a single dose of CC, followed by the PECC intake. These differences may be due to the vehicle used to deliver the procyanidins, which enhanced their metabolism and elimination. That fact may refine the hypothesis of Shoji et al. [10], who proposed a relation between the sites of the metabolism according to the phenolic concentration administered, adding relevant importance to the food matrix by which the polyphenols are administered. With procyanidin-rich simple food matrices (e.g. PE), liver had more importance in the metabolization process showing a higher metabolite concentration than kidney, while the higher number of

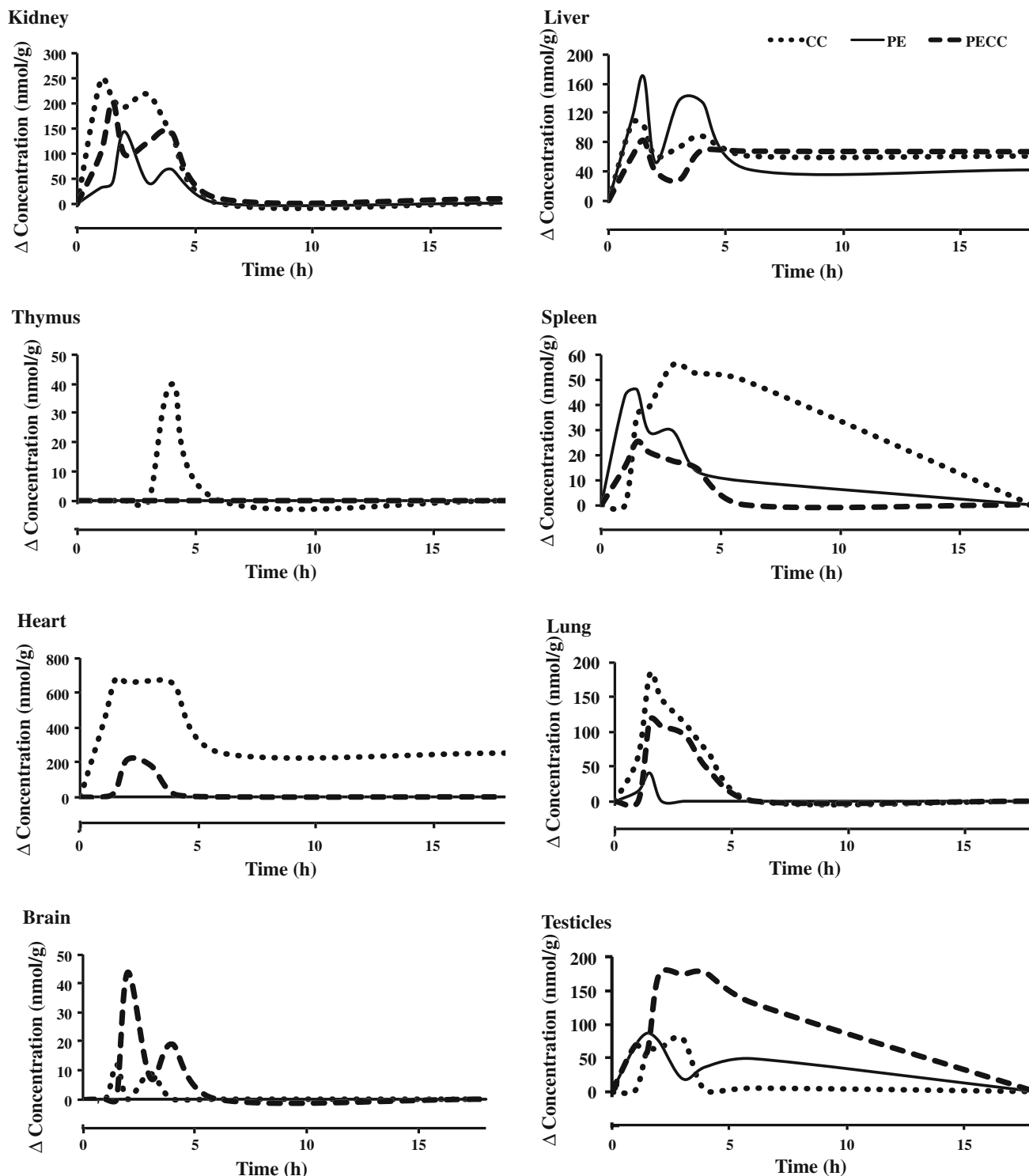


Fig. 5 Kinetic of tissue metabolites expressed as the increase in the concentration (nmol/g) in relation to the basal conditions (0 h) after a single ingestion of cocoa cream (CC), rich procyanidin hazelnut skin extract (PE) and procyanidin-enriched cocoa cream (PECC)

metabolites detected in kidney may be related with the intake of a procyanidin-rich complex food matrix (e.g. CC and PECC). The presence of several procyanidin metabolites in the kidney also reaffirms the urinary path as the main procyanidin excretion pathway [16] and, related to

excretion, glucuronidated and methylated forms of epicatechin and catechin were the main procyanidin conjugates determined in the kidney [19, 22, 23] and the main phase-II metabolites. In contrast, sulpho-conjugates of procyanidins were not found in the kidney.

The procyanidin metabolite detected in the brain and testicles showed two peaks of disposition, with a higher concentration after the PECC intake in both tissues, specifically with methyl catechin–sulfate. However, in a recent study [16], this procyanidin metabolite was not detected after the intake of a high dose of PE (5 g per kg of rat body weight). Thus, a clear food matrix effect was observed in the disposition of procyanidin metabolites in the brain. The intake of a high procyanidin dose that included in a complex food matrix (PECC) could facilitate the disposition of procyanidin metabolites in the brain (Fig. 5). The ability of procyanidin metabolites to cross the blood–brain barrier was first observed by Abd El Mohsen et al. [24] after oral ingestion of epicatechin by rats (100 mg/kg body weight), but the levels found were too low for accurate determination. Similarly, in a recent study by Urpi-Sarda et al. [25], catechin and epicatechin metabolites were found in the brain after 3 weeks of a cocoa diet. Thus, the ability of procyanidin metabolites to cross the blood–brain barrier and target the brain could be affected by the dose and the composition of the food matrix that accompanies the procyanidins in the process of digestion, absorption, metabolism and distribution in the body.

Although no clear kinetics were observed for some individual metabolites in some tissues (Figs. 3, 4), the sum of these showed a clear accumulation of phenolic metabolites (Fig. 5), especially in the range between 1 and 4 h. Based on the results of the study, enriched or rich foods could be proposed as a practical solution to increase the intake of procyanidins with tiny modifications of the diet. This would enhance their described beneficial effects. The presence of phase-II metabolites and fermentation products in such tissues as the brain or heart may be related to the potential health benefits of procyanidins, especially in the context of cardiovascular health [9, 16, 26–28] or a neuroprotective effect [29, 30]. Nonetheless, although the beneficial effect of pure procyanidins may be close to that exerted by a flavanol-rich food [9], the matrix effect should not be forgotten given its ability to modulate the plasmatic bioavailability and disposition in the tissues.

The bioactive action of polyphenol in the body is probably regulated in a dose-dependent way [31–35]. Thus, supplementing phenolic compounds through fortified and enriched food may represent a rich source of polyphenols and increase or expedite the action of these minor dietary compounds in the body. Thus, this study may represent a relevant step toward understanding the importance of the matrix effect and the dose for formulating a phenol-enriched food as a functional food. The results obtained have demonstrated that the formulation of a procyanidin-enriched or fortified product is an option to increase bioavailability. Nevertheless, although a clear effect of the procyanidin metabolites on the plasma pharmacokinetic following the oral administration of a rich

procyanidin extract or an enriched CC was observed, verifying the possible long-term accumulation and bioactive character of these procyanidin metabolites in the tissues would require a long-term study.

Acknowledgments The present study was supported by the CENIT program from the Spanish Minister of Industry and by a consortium of companies led by La Morella Nuts S.A.U. (Reus, Catalonia, Spain) with the following: Shiota Functional Foods, S.L., KRAFT; BTSA, Biotecnologías Aplicadas, S.L., Selección Batallé, S.A., Industrial Técnica Pecuaria S.A., Neuron Bio Pharma, S.A., Grupo Leche Pascual, S.A.U., Innaves, S.A. This study was also supported by the Catalan Government (Interdepartmental Commission for Research and Technological Innovation) through the A. Serra grant. The authors thank Carme Piñol (in charge) from the Animal Facility Service of the University of Lleida for their technical support.

Conflict of interest Authors have declared no conflict of interest.

References

- Allen L, de Bonist B, Dary O, Hurrell R (2006) Guidelines on food fortification with micronutrients. WHO Press, Switzerland
- Kaur S, Das M (2011) Functional foods: an overview. *Food Sci Biotechnol* 20:861–875
- Hasler CM, Brown AC (2009) Position of the American Dietetic Association: functional foods. *J Am Diet Assoc* 109:735–746
- Faridi Z, Njike VY, Dutta S, Ali A, Katz DL (2008) Acute dark chocolate and cocoa ingestion and endothelial function: a randomized controlled crossover trial. *Am J Clin Nutr* 88:58–63
- Gupta AK, Savopoulos CG, Ahuja J, Hatzitolios AI (2011) Role of phytosterols in lipid-lowering: current perspectives. *QJM* 104:301–308
- Franck A (2006) Oligofructose-enriched inulin stimulates calcium absorption and bone mineralisation. *Nutr Bull* 31:341–345
- Bosscher D, Van Loo J, Franck A (2006) Inulin and oligofructose as functional ingredients to improve bone mineralization. *Int Dairy J* 16:1092–1097
- Grassi D, Desideri G, Necozione S, Lippi C, Casale R, Properzi G, Blumberg JB, Ferri C (2008) Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate. *J Nutr* 138:1671–1676
- Schroeter H, Heiss C, Spencer JPE, Keen CL, Lupton JR, Schmitz HH (2010) Recommending flavanols and procyanidins for cardiovascular health: current knowledge and future needs. *Mol Asp Med* 31:546–557
- Shoji T, Masumoto S, Moriichi N, Akiyama H, Kanda T, Ohtake Y, Goda Y (2006) Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J Agric Food Chem* 54:884–892
- Okushio K, Suzuki M, Matsumoto N, Nanjo F, Hara Y (1999) Identification of (–)-epicatechin metabolites and their metabolic fate in the rat. *Drug Metab Dispos* 27:309–316
- Fernández-Murga L, Tarín JJ, García-Perez MA, Cano A (2011) The impact of chocolate on cardiovascular health. *Maturitas* 69:312–321
- Ortega N, Reguant J, Romero MP, Macià A, Motilva MJ (2009) Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an in vitro digestion model. *J Agric Food Chem* 57:5743–5749

14. Serra A, Macià A, Romero MP, Salvadó M, Bustos M, Fernández-Larrea J, Motilva MJ (2009) Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography-tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 877:1169–1176
15. Martí M, Pantaleón A, Rozek A, Soler A, Valls J, Macià A, Romero MP, Motilva MJ (2010) Rapid analysis of procyanidins and anthocyanins in plasma by microelution SPE and ultra-HPLC. *J Sep Sci* 33:2841–2853
16. Serra A, Macià A, Romero MP, Anglès N, Morelló JR, Motilva MJ (2011) Distribution of procyanidins and their metabolites in rat plasma and tissues after an acute intake of hazelnut extract. *Food Funct* 2:562–568
17. Serra A, Macià A, Romero MP, Valls J, Bladé C, Arola L, Motilva MJ (2010) Bioavailability of procyanidin dimers and trimers and matrix food effects in *in vitro* and *in vivo* models. *Br J Nutr* 103:944–952
18. Holt RR, Lazarus SA, Cameron Sullards M, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz HH, Keen CL (2002) Procyanidin dimer B2 [epicatechin-(4 β -8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am J Clin Nutr* 76:798–804
19. Baba S, Osakabe N, Natsume M, Terao J (2002) Absorption and urinary excretion of procyanidin B2 [epicatechin-(4 β -8)-epicatechin] in rats. *Free Radic Biol Med* 33:142–148
20. Zhu QY, Holt RR, Lazarus SA, Ensunsa JL, Hammerstone JF, Schmitz HH, Keen CL (2002) Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *J Agric Food Chem* 50:1700–1705
21. Hackett AM, Shaw IC, Griffiths LA (1982) 3'-O-methyl-(+)-catechin glucuronide and 3'-O-methyl-(+)-catechin sulphate: new urinary metabolites of (+)-catechin in the rat and the marmoset. *Experientia* 38:538–540
22. Natsume M, Osakabe N, Oyama M, Sasaki M, Baba S, Nakamura Y, Osawa T, Terao J (2003) Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat. *Free Radic Biol Med* 34:840–849
23. Tsang C, Auger C, Mullen W, Bornet A, Rouanet J-, Crozier A, Teissedre P (2005) The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br J Nutr* 94:170–181
24. Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P, Rice-Evans CA (2002) Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radic Biol Med* 33:1693–1702
25. Urpi-Sarda M, Ramiro-Puig E, Khan N, Ramos-Romero S, Llorach R, Castell M, Gonzalez-Manzano S, Santos-Buelga C, Andres-Lacueva C (2010) Distribution of epicatechin metabolites in lymphoid tissues and testes of young rats with a cocoa-enriched diet. *Br J Nutr* 103:1393–1397
26. Fraga CG, Litterio MC, Prince PD, Calabró V, Piotrkowski B, Galleano M (2011) Cocoa flavanols: effects on vascular nitric oxide and blood pressure. *J Clin Biochem Nutr* 48:63–67
27. Lee KW, Kang NJ, Oak M-, Hwang MK, Kim JH, Schini-Kerth VB, Lee HJ (2008) Cocoa procyanidins inhibit expression and activation of MMP-2 in vascular smooth muscle cells by direct inhibition of MEK and MT1–MMP activities. *Cardiovasc Res* 79:34–41
28. Cai Q, Li B, Gao H, Zhang J, Wang J, Yu F, Yin M, Zhang Z (2011) Grape seed procyanidin B2 inhibits human aortic smooth muscle cell proliferation and migration induced by advanced glycation end products. *Biosci Biotechnol Biochem* 75:1692–1697
29. Asha Devi S, Sagar Chandrasekar BK, Manjula KR, Ishii N (2011) Grape seed proanthocyanidin lowers brain oxidative stress in adult and middle-aged rats. *Exp Gerontol* 46:958–964
30. Narita K, Hisamoto M, Okuda T, Takeda S (2011) Differential neuroprotective activity of two different grape seed extracts. *PLoS One* 6:e14575
31. Li D, Wang Q, Yuan Z, Zhang L, Xu L, Cui Y, Duan K (2008) Pharmacokinetics and tissue distribution study of orientin in rat by liquid chromatography. *J Pharm Biomed Anal* 47:429–434
32. Ohkita M, Nakajima A, Ueda K, Takaoka M, Kiso Y, Matsumura Y (2005) Preventive effect of flavangenol on ischemia/reperfusion-induced acute renal failure in rats. *Biol Pharm Bull* 28:1655–1657
33. Lin Y, Chen S, Liu C, Nieh S (2011) The chemoadjuvant potential of grape seed procyanidins on p53-related cell death in oral cancer cells. *J Oral Pathol Med* 41:322–331
34. Chung Y, Huang C, Chen C, Chiang H, Chen K, Chen Y, Liu C, Chuang L, Liu M, Hsu C (2011) Grape-seed procyanidins inhibit the *in vitro* growth and invasion of pancreatic carcinoma cells. *Pancreas* 41:447–454
35. Jung M, Triebel S, Anke T, Richling E, Erkel G (2009) Influence of apple polyphenols on inflammatory gene expression. *Mol Nutr Food Res* 53:1263–1280