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Polyphenol metabolism: from in vitro to in vivo approaches

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POLYPHENOL METABOLISM: FROM *IN VITRO* TO *IN VIVO* APPROACHES

Doctoral Thesis

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“Tell me and I’ll forget; show me and I may remember; involve me and I’ll understand.”
Chinese Proverb

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SUMMARY

Phenolic compounds are phytochemicals widely distributed in our diet through the intake of plant-derived products, e.g. fruit and vegetables, cereals, cocoa derivatives, tea, coffee or red wine-. Several beneficial effects from the intake of dietary phenolic compounds have been established. These include the reduction of the risk of cardiovascular and neurodegenerative diseases, prevention of illnesses related to oxidative stress, or the reduction of some cancers. However, less is known about how phenolic compounds act in the body after ingestion and which phenolic metabolites reach the metabolic targets.

This thesis was focus on the evaluation of the bioaccessibility and digestibility of dietary phenolic compounds during the digestion process; and on the study of the absorption, metabolism and tissue distribution of phenolic compounds and their metabolites. The following steps were carried out to reach these objectives, *(i)* the evaluation of the bioaccessibility and digestibility of dietary phenolic compounds by the use of an *in vitro* digestion system; *(ii)* the development of an *in vitro* colonic fermentation model and the evaluation of the colonic fermentation of dietary phenolic compounds; *(iii)* the development of analytical methodologies to quantify polyphenols and their metabolites in biological samples (plasma and tissues) and *(iv)* the study of the absorption, metabolism and tissue distribution of dietary phenolic compounds (mainly procyanidins and olive oil phenolics) and their metabolites *in vivo* using rats as an animal model.

As a result of the experimental work done within this dissertation, the stability during the digestion process of dimer and trimer procyanidins was demonstrated. The colonic metabolic pathways of individual phenolic compounds were successfully established detecting hydroxylated phenolic acids as the main colonic metabolites of flavonoids. Parallel to this, two chromatographic methods combining off-line solid-phase extraction and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) were successfully validated for determining of phenolic compounds and their metabolites in biological samples. These methods were used to detect and quantify polyphenols and their metabolites in the biological samples obtained as a result of the *in vivo* acute and chronic studies, performed using phenolic extract and polyphenol-rich foods as polyphenol sources. From the *in vivo* studies, it was established that phenolic metabolites were widely distributed throughout the body, reaching practically all the organs; procyanidin plasmatic bioavailability and tissue distribution were modulated according to the food matrix composition and the intake of phenols by a naturally phenol-rich or phenol-enriched food could vary the pharmacokinetic parameters of the phenolic metabolites. Additionally, environmental factors, such as the diet, may modulate the hepatic glucuronidation capacity toward flavonoids in the parent and offspring rats, as was demonstrated with in utero exposure to a high fructose and saturated fat diet.

Nonetheless, data from *in vitro* and animal experiments represent a prior step to human clinical studies because they may not be readily extrapolated to humans. Thus, human clinical studies should be the colophon of all nutritional studies related to functional foods.

RESUM

Els compostos fenòlics son fitoquímics àmpliament distribuïts en la nostra dieta degut a la ingesta de productes d'origen vegetal –com són fruites i verdures, cereals, derivats del cacau, té, cafè i vi negre-. Són varis els efectes beneficiosos que han estat relacionats amb la ingesta de compostos fenòlics, com ara, la reducció del risc de malalties cardiovasculars i neurodegeneratives, la prevenció de malalties relacionades amb l'estrès oxidatiu, o la reducció d'alguns tipus de càncers. Però menys n'és sabut sobre com els compostos fenòlics actuen endògenament i sobre quins metabòlits arriben a les dianes metabòliques.

La present tesis s'ha centrat en l'avaluació de la digestibilitat i la bioaccessibilitat dels compostos fenòlics de la dieta, i en l'estudi del metabolisme i la distribució en teixits dels compostos fenòlics i dels seus metabòlits. Per dur a terme els objectius plantejats, *(i)* es va estudiar la digestibilitat i bioaccessibilitat dels compostos fenòlics de la dieta mitjançant l'ús d'un mètode de digestió *in vitro*; *(ii)* es va desenvolupar un mètode de fermentació colònica, per la seva posterior aplicació a l'estudi del metabolisme colònic de compostos fenólics de la dieta; *(iii)* es van desenvolupar metodologies analítiques per la quantificació de compostos fenòlics i els seus metabòlits en mostres biològiques; y finalment, *(iv)* es van realitzar estudis *in vivo*, utilitzant rates Wistar com a model animal, per avaluar l'absorció, el metabolisme i la distribució en teixits dels compostos fenòlics (principalment procianidines i compostos fenòlics de l'oli d'oliva) i els seus metabòlits.

Com a resultats principals de la part experimental destacar que, les procianidines de baix grau de polimerització (dimers i trimers) van mantenir-se estables durant el procés de digestió i que es van caracteritzar les rutes de formació dels productes de fermentació colònica de varis compostos fenòlics, identificant els àcids fenòlics hidroxilats com els principals productes de fermentació dels flavonoids. Paral·lelament, es van desenvolupar i validar dos mètodes cromatogràfics que combinaven l'extracció en fase sòlida amb la cromatografia líquida d'alta resolució acoblada a l'espectrometria de masses en tàndem (UPLC-MS/MS) per determinar compostos fenòlics i els seus metabòlits en mostres biològiques. Aquestes metodologies van ser aplicades en l'anàlisi dels compostos fenòlics i els seus metabòlits en mostres de rata Wistar, obtingudes com a resultat de la realització d'estudis d'ingesta aguda i crònica d'extractes i aliments rics en compostos fenòlics. Els estudis *in vivo* van demostar que la concentració de metabòlits de procianidines en plasma y la seva distribució en teixits es veia modulada per la composició de la matriu alimentaria a través de la qual es vehiculitzaven els compostos fenòlics. A més a més, l'estudi comparatiu dels paràmetres farmacocinètics dels metabòlits va permetre detectar diferències en funció la naturalesa dels compostos fenòlics, essent aquests pròpis d'un aliment de forma natural o bé a través d'un aliment enriquit en compostos fenòlics. Finalment, es va demostrar amb rates com a model animal que una dieta rica en fructosa i greixos saturats aplicada durant el període d'embaràs i lactància podia modular la capacitat de glucuronidació del fetge sobre els flavonoids, en la generació parental i en la generació filla.

No obstant, cal destacar que els resultats obtinguts a partir de models *in vitro* i *in vivo* amb animals d'experimentació, representen el pas previ als estudis clínics, i que els resultats obtinguts poden no ser directament extrapolables a humans. Per la qual cosa, els estudis clínics representen l'objectiu final de tot estudi nutricional relacionat amb el desenvolupament aliments funcionals.

RESUMEN

Los compuestos fenólicos son fitoquímicos ampliamente distribuidos en nuestra dieta debido a la ingesta de productos vegetales –principalmente frutas y verduras, cereales, derivados del cacao, te, café y vino tinto-. Diferentes efectos beneficiosos han sido relacionados con la ingesta de compuestos fenólicos, como por ejemplo la reducción del riesgo de enfermedades cardiovasculares o neurodegenerativas, la prevención de enfermedades relacionadas con el estrés oxidativo, o la reducción de la incidencia de algunos cánceres. Sin embargo, el conocimiento sobre los mecanismos de acción de los compuestos fenòlicos en el organismo y sus dianas metabólicas es limitado.

La presente tesis se ha centrado en la evaluación de la digestibilidad y bioaccesibilidad de los compuestos fenólicos de la dieta, y en el estudio del metabolismo y la distribución en tejidos de los compuestos fenólicos y de sus metabolitos. Para completar los objetivos citados, *(i)* se estudió la digestibilidad y bioaccesibilidad de los compuestos fenólicos de la dieta mediante el uso de un método de digestión *in vitro*; *(ii)* se desarrolló un método *in vitro* de fermentación colónica, para su posterior aplicación al estudio del metabolismo colónico de compuestos fenólicos de la dieta; *(iii)* se desarrollaron metodologías analíticas para la cuantificación de compuestos fenólicos y sus metabolitos en muestras biológicas; y finalmente *(iv)* se realizaron estudios *in vivo*, usando rata Wistar como modelo animal, para evaluar la absorción, el metabolismo y la distribución en tejidos de los compuestos fenólicos (principalmente procianidinas y compuestos fenólicos del aceite de oliva) y sus metabolitos.

Como resultados principales de la parte experimental cabe destacar que, las procianidinas de bajo grado de polimerización (dímeros y trímeros) fueron estables durante el proceso de digestión y que se caracterizaron las rutas de formación de los productos de fermentación colónica de varios compuestos fenólicos, identificando a los ácidos fenólicos hidroxilados como los principales productos de fermentación de los flavonoides. Paralelamente, se desarrollaron y validaron dos métodos cromatográficos, que combinaban la extracción en fase sólida con la cromatografía líquida de alta resolución acoplada a la espectrometría de masas en tándem (UPCL-MS/MS), para la determinación de compuestos fenólicos y sus metabolitos en muestras biológicas. Dichos métodos fueron utilizados para analizar los compuestos fenólicos y sus metabolitos en muestras biológicas, obtenidas como resultado de estudios de ingesta aguda e ingesta crónica de extractos y alimentos ricos en compuestos fenólicos, utilizando ratas Wistar como modelo animal. Los estudios *in vivo* demostraron que la concentración plasmática de metabolitos de procianidinas y su distribución en tejidos fue modulada por la composición de la matriz alimentaria a través de la cual se vehiculizaba a los compuestos fenólicos. Además, el estudio comparativo de los parámetros farmacocinéticos de los metabolitos fenólicos permitió observar diferencias en función de la naturaleza de los compuestos fenólicos, formando parte de un alimento de forma natural o a través de un alimento enriquecido en compuestos fenólicos. Finalmente, se demostró con ratas como modelo animal que una dieta rica en fructosa y grasas saturadas aplicada durante el periodo de embarazo y lactancia podia modular la capacidad de glucuronidación del hígado sobre los flavonoides, en la generación parental y en la generación hija.

No obstante, hay que destacar que los resultados obtenidos a partir de modelos *in vitro* e *in vivo* mediante el uso de animales de experimentación representan el paso previo a los estudios clínicos y que los resultados obtenidos pueden no ser directamente extrapolables a humanos. Por lo que, los estudios clínicos representan el objetivo final de todo estudio nutricional relacionado con el desarrollo de alimentos funcionales.

CONTENT

INTRODUCTION

1. Phenolics: Occurrence, Biochemical Activities and Bioavailability

1.1 Introduction

Phenolics are secondary plant metabolites found in a wide range of plant products, such as fruit and vegetables, cereals, legumes and beverages. Their classification is not fixed and universal accepted, and several versions have been published due to the complexity and the extremely wide diversity of identified polyphenols. After intake, the metabolism of phenolic compounds involves a large number of enzymatic reactions resulting in a high number of metabolites. This is why their bioactivity action is still unclear. Nevertheless, epidemiological studies have suggested that the consumption of phenol-rich products is positively correlated with a decrease in the risk of various cardiovascular and neurodegenerative diseases and even of several cancers. That fact reinforces the interest in the development of functional foods with high content of phenolic compounds.

1.2 Occurrence of phenolics

1.2.1 Origin

Phenolic compounds are synthesized and act as secondary metabolites in plants. The biosynthesis of phenolic compounds in plants requires a complex metabolic route encompassing the flavonoids, shikimate (C_6-C_1 compounds) and phenylpropanoid (C_6-C_3 compounds) pathways (Crozier *et al.* 2006; Fraga 2009; Hoffmann *et al.* 2004; Shimida *et al.* 2003; Tanner *et al.* 2003; Xie and Dixon 2005). In contradistinction with primary metabolites (e.g. phytosterols, acyl lipids, nucleotids, amino acids and organic acids), which have a clear and essential role associated with principal processes of plant metabolism, including photosynthesis, respiration, growth or development, secondary metabolites were ignored for a long time. Nevertheless, secondary metabolites, with a wide variety of molecular structures and irregularly distributed throughout the plant kingdom, have several functions involving defense and survival strategies, such as protecting plants from herbivores and microbial infections, acting as signal molecules in the interaction between plants and their environment, protecting from UV radiation or acting as attractants for pollinators and seed-dispersing animals, among others (Andersen and Markham 2006). Phenolic compounds are sometimes accumulated at high concentration in plant tissues and other structures and are thus abundant micronutrients of our diets (Duthie *et al.* 2003; Manach *et al.* 2004).

1.2.2 Classification

Phenolic compounds are organic chemicals characterized by the presence of at least one aromatic ring with one or more hydroxyl groups attached. The term “polyphenols” should be used to define phenolic compounds with more than

one phenolic ring and devoid of any nitrogen-based functional group in their most basic structural expression (Quideau *et al.* 2011). That definition leaves out all monophenolic structures, such as hydroxytyrosol or gallic acid, which can be either metabolites of polyphenols or biogenetic precursors (Quideau *et al.* 2011) and can share with polyphenols many of their properties and characteristics with polyphenols (Dixon 2004), being thus generally related with the polyphenol research and colloquially included under the polyphenol term. Polyphenols are classified into families according to differences on their carbon skeleton - number and disposition of their carbon atoms- ranging from simple small single aromatic-ring structures to the complex and weighty condensed tannins (Figure 1) (González-Castejón and Rodriguez-Casado 2011; Harbone 1989; Seabra *et al.* 2006).

Following the flavonoids and non-flavonoids classification proposed by Crozier *et al.* 2009, dietary non-flavonoids are characteristics by C_6-C_1 phenolic acids, with gallic acid as the precursor of hydrolysable tannins, hydroxycinammates (C_6-C_3) and their conjugated derivatives, and stilbenes (C_6-C_2-C_6). Nonetheless, this classification omits a sub-class of phenolic compounds named lignans (-(C_6-C_3)$_n$-), a phenolic family of compounds formed by two phenylpropanoid units linked by a hydrogen bridge, these being the monomeric and dimeric forms of hydroxycynammic acid and cynammic alcohol (Chesson *et al.* 1997).

Besides, a number of coumarin-like compounds, known as secoiridoids and exclusive to the *Olea europaea* species, are not included in practically any classification, although the secoiridoid derivatives of oleuropein and ligstroside are the main phenolic compounds of virgin olive oil (Figure 2).

Dietary flavonoids, commonly present in the epidermis of leaves and the skin of fruit are the most numerous and widespread phenolic compounds. Flavonoids

are characterized by a C_{15} phenylchromane core, composed of two aromatic rings linked by a three carbon bridge (C_6-C_3-C_6) (Figure 3) (Crozier *et al.* 2009; Passamonti *et al.* 2009) and are sub-classified into flavonols (e.g. quercetin), flavanols (e.g. catechin, epicatechin), anthocyanins (e.g. cyanidin-3-O-

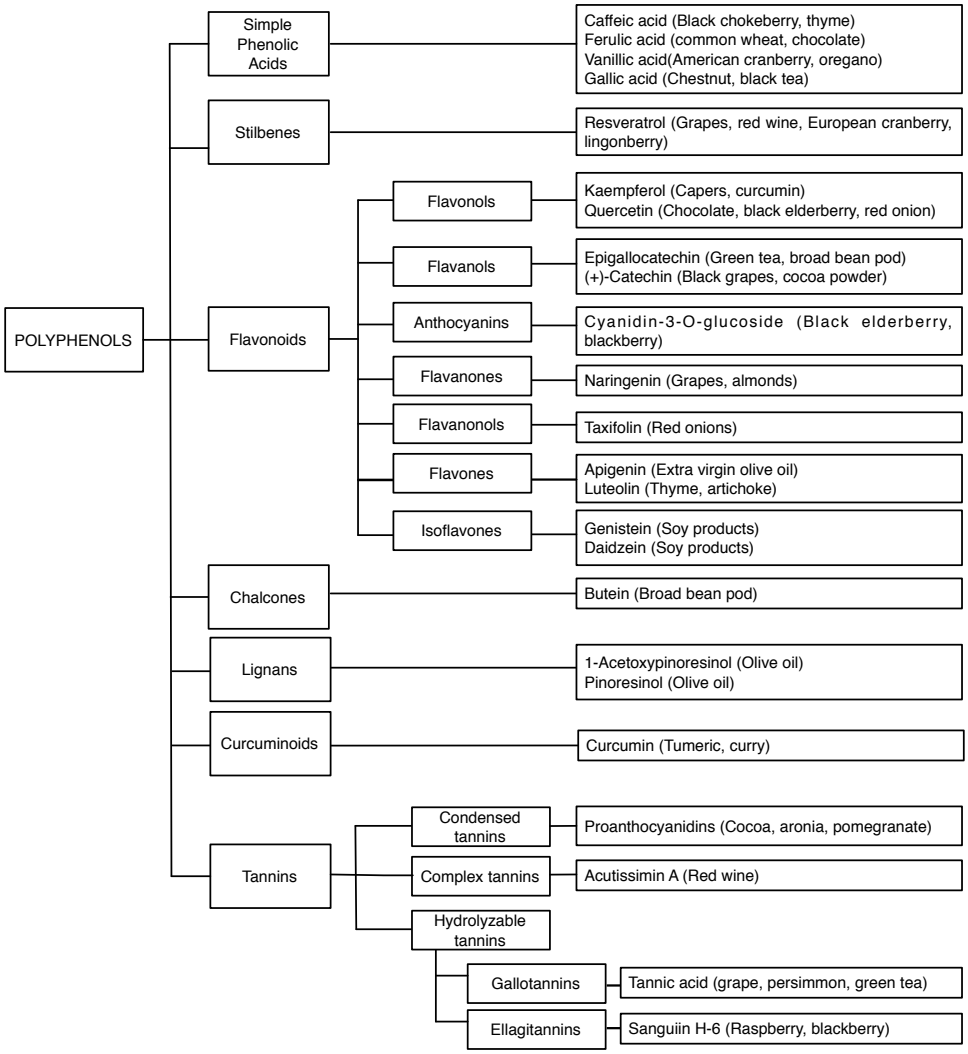

Figure 1. Classification of common dietary polyphenols, with characteristic examples of each phenolic family.

glucoside), flavones (e.g. luteolin and apigenin), flavanones (e.g. naringenin), flavanonols (e.g. taxifolin), and isoflavones (e.g. genistein or daidzein) (Bravo 1998; Hallman and Katan 1997; Harborne and Baxter 1999; Williams *et al.* 2004;) which are sometimes classified into an independent sub-category apart from flavonoids (González-Castejón and Rodriguez-Casado 2011). Moreover, most flavonoids in foods are conjugated to a carbohydrate moiety, representing

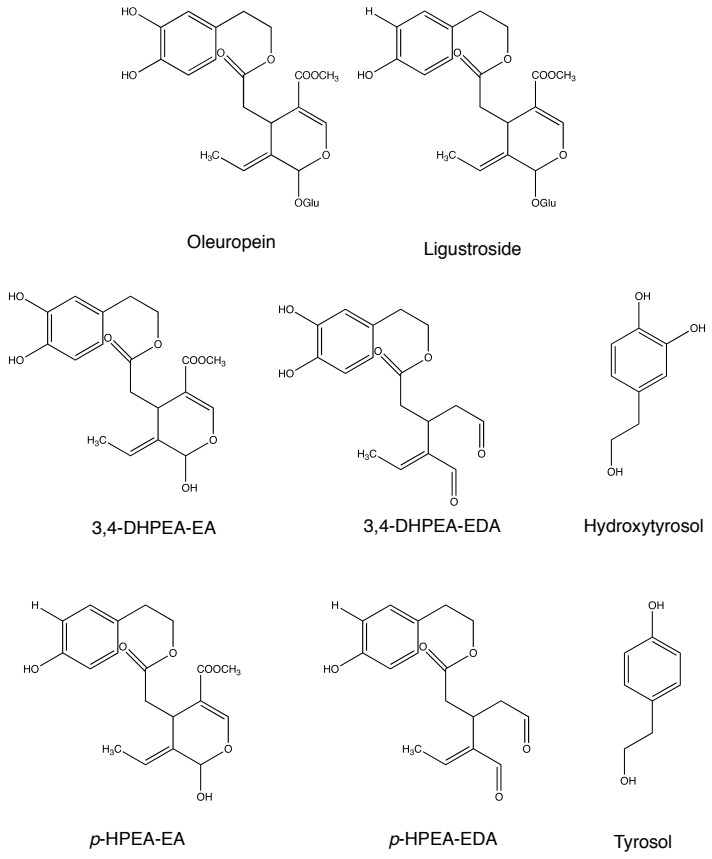

Figure 2. Basic structural skeleton of oleuropein and ligstroside derivatives. 3,4-DHPEA-EA (3,4-dihydroxyphenylethanol-elenolic acid) oleuropein aglycone; 3,4-DHPEA-EDA (3,4-dihydroxyphenylethanol-elenolic acid dialdehyde) dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EA (*p*-hydroxyphenylethanol-elenolic acid) adehydic form of elenolic acid linked to tyrosol; *p*-HPEA-EDA (*p*-hydroxyphenylethanol-elenolic acid dialdehyde) dialdehydic form of elenolic acid linked to tyrosol.

a wide range of combinations depending on the flavonoid, its linkage and the linked mono- and disaccharide (Figure 3) (Passamonti *et al.* 2009) .

The majority of flavonoids present a hydroxylation pattern, usually in 4', 5- and 7-position (Figure 3), or glycosilation pattern that reflects a biological strategy in plant cells to increase their water solubility. The presence of methyl groups or isopentyl units may give a lipophilic character to flavonoid molecules (Crozier *et al.* 2009).

Finally, tannins are defined as either galloyl esters and their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol-, catechin- and triterpenoid cores (gallotannins, ellagitannins and complex tannins,

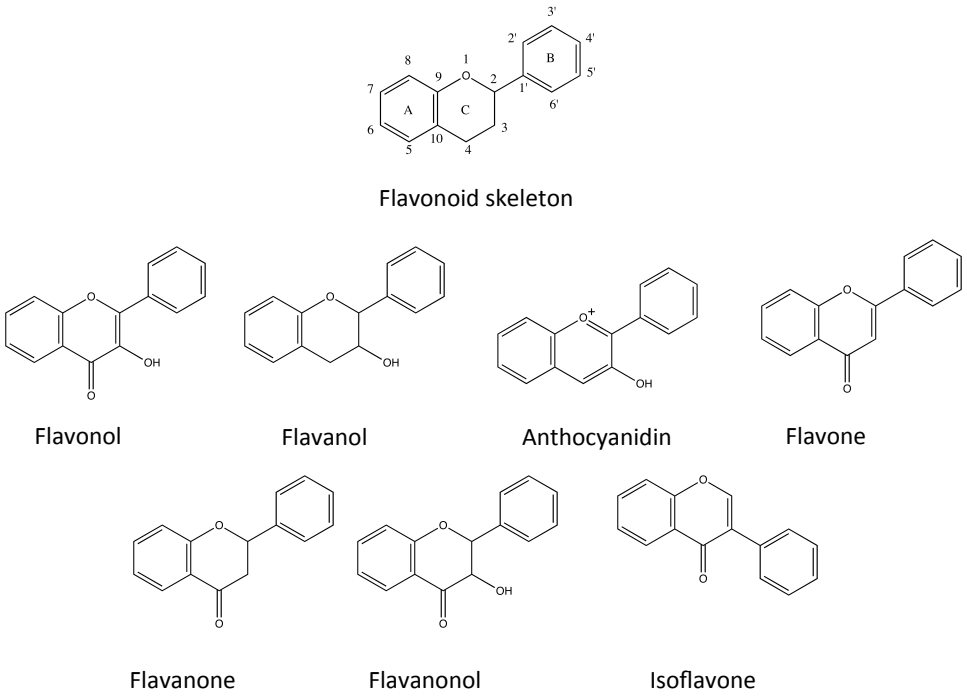

Figure 3. Basic structural skeleton of flavonoids.

respectively), or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (condensed tannins) (Khanbabaee and Van Ree 2001). They can be classified into three groups (Figure 1): condensed tannins, complex tannins and hydrolysable tannins, which include gallotannins and ellagitannins (Jankun *et al.* 1997). With more detail, condensed tannins are polymeric flavonoids consisting of flavanol (catechin) units; gallotannins are hydrolysable tannins with a polyol core (referring to a compound with multiple hydroxyl groups) substituted by 10-12 gallic acid residues; ellagitannins are also hydrolyzable tannins derived from pentagalloylglucose but, unlike gallotannins, they contain additional C-C bonds between adjacent galloyl moieties in the pentagalloylglucose molecule and complex tannins are defined as tannins in which a catechin unit is bound glycosidically to either a gallotannin or an ellagitannin unit (Wilfred and Nicholson 2006).

1.2.3 Polyphenols in foods

Several hundred molecules with polyphenol structure have been identified in edible plants and plant-products, ranging from cereals, fruit, legumes, spices, vegetables to beverages (Ganthavorn and Hughes 1997; Lin *et al.* 1998; Manach *et al.* 2004; Pulido *et al.* 2003). These is generally a direct relation between a characteristic plant, organ or tissue and specific phenolic compounds. Nevertheless, it is impossible to know the nature of all of the polyphenols in our diet precisely (Scalbert and Williamson 2000). Foods normally contains complex and poorly characterized mixtures of polyphenols (Manach *et al.* 2004). Additionally, in some cases the polyphenol composition of a natural product is conditioned by a range of factors, among which are the cultivar, the agronomical and environmental factors, processing and storage (Dabbou *et al.* 2010; Esti *et al.* 1998; Greven *et al.* 2009; Hollman *et al.* 2011;

Manach *et al.* 2004; Servili *et al.* 2003; Servili *et al.* 2004; Servili *et al.* 2007; Visioli *et al.* 2002). For all these reasons, the total intake of polyphenols in the diet is difficult to establish.

Additionally, cultural habits and food preferences lead to unequal intakes between countries. Another important limitation is related to the literature data on the content and composition of food polyphenols, which is incomplete and insufficient to determine dietary intake precisely. This fact is partly related to the use of different analytical methodologies (Hollman *et al.* 2011) and the determination of different fractions of dietary polyphenols (e.g. only extractable polyphenols, or extractable and non-extractable polyphenols) giving rise to very varying results difficult to compare, especially those associated with the quantification of polyphenols with a high degree of polymerization (non-extractable polyphenols) (Saura-Calixto *et al.* 2007). Nonetheless, according to the results obtained by Saura-Calixto and co-workers, who determined the total polyphenol content in vegetable foods and beverages consumed in the Spanish diet (Saura-Calixto and Goñi, 2006) (Table 1) and who determined the intake and potential bioaccessibility of the total polyphenols in the same diet (Saura-Calixto *et al.* 2007) (Table 2), fruits, legumes and nuts were the solid food groups with the highest polyphenol content. Beverages and vegetable oils presented higher polyphenol contents than solid plants and although the lowest polyphenol content was found in cereals, given the high proportion in the diet, they were the principal food source of polyphenols, followed by fruit and beverages.

Simple phenolic acids represent a substantial part of the human diet. Coffee and beans are particularly rich in phenolic acids -with approximately 212 mg of total phenolic acids/100 mL of filtered coffee [(1)] or 31.17 mg of total phenolic acids/100 g of dry beans (mean obtained from 15 varieties of commonly

Table 1. Total polyphenol content of plant foods in the Spanish diet (mg/g original dry sample) (Saura-Calixto *et al.* 2007).

Food group	Extractable polyphenols	Condensed tannins	Hydrolysable polyphenols	Total polyphenols intake range
Cereals	1.07 ± 0.02	n.d.	4.72 ± 0.46	579
Vegetables	2.86 ± 0.13	n.d.	4.56 ± 0.33	742
Legumes	1.54 ± 0.20	7.66 ± 0.40	5.93 ± 0.32	17.43
Fruit	5.38 ± 0.20	12.33 ± 2.90	6.97 ± 0.68	28.38
Nuts	8.94 ± 0.48	2.00 ± 0.10	8.61 ± 0.69	20.15
Beverages	754.6 (mg/100 mL) [a]	-	-	754.6
Oils	31.3 (mg/100 mL) [a]	-	-	31.3

[a] *From Saura-Calixto and Goñi, 2006. n.d.: Not detected.*

Table 2. Total polyphenol intake of plant foods in the Spanish diet (mg/g original dry sample) (Saura-Calixto *et al.* 2007).

Food group	Group intake[a]	Extractable polyphenols	Condensed tannins	Hydrolysable polyphenols	Total polyphenols intake range
Cereals	221.65 ± 4.43	173.75 ± 3.28	n.d.	766.44 ± 74.70	793–1087
Vegetables	280.19 ± 5.61	98.81 ± 4.50	n.d.	157.54 ± 11.41	230–283
Legumes	22.19 ± 0.44	26.08 ± 3.42	129.74 ± 6.79	100.47 ± 5.44	238–275
Fruit	200.60 ± 4.01	134.47 ± 4.98	308.19 ± 72.49	174.24 ± 16.99	470–763
Nuts	5.96 ± 0.12	51.20 ± 2.75	11.45 ± 0.57	49.32 ± 3.95	102–121
Beverages	504.9 (ml)[b] ± 10.10	613.7 ± 13.64 [b]	-	-	580–647
Oils	52.0 (ml) [b] ± 1.04	8.3 ± 1.14 [b]	-	-	5–11
Total		1106.31 ± 16.19	449.38 ± 72.81	1248.01 ± 77.74	2591–3016

[a] *g Edible portion/person/day.*
[b] *From Saura-Calixto and Goñi, 2006. n.d.: Not detected.*

consumed dry bean in the United States (Luthria and Pastor-Corrales 2006))-. Other products, such as apples, blueberries, potatoes and olives, also contain phenolic acids. These acids are commonly conjugated with other natural chemicals such as flavonoids, alcohols, hydroxy fatty acids, sterols and glucosides (González-Castejón and Rodriguez-Casado 2011) and can be divided into two classes, depending on the acid they come from, either derivatives of benzoic acid or derivatives of cinnamic acid (Lafay and Gil-Izquierdo 2008; Manach *et al.* 2004). The derivatives of cinnamic acid are mainly represented by caffeic acid -covering up to 70% of total hydroxycinnamic acids in fruits -e.g. ferulic, sinapic and *p*-coumaric acids-. On the other hand, the concentration of hydroxybenzoic acids in fruit and vegetables is relatively low, excepting the high concentration of hydroxybenzoic acids in red fruits, black radish, onions and potato skin (Lafay and Gil-Izquierdo 2008). However, another important source of phenolic acids comes from the cleavage of the flavonoid skeleton to phenolic acids due to the gut microbiota action, followed by their intestinal absorption.

Although stilbenes only occur naturally in a small number of plant species at low concentrations, contain an extensively studied phenolic compound, mainly due to its consumption in red wine, in which its content is approximately 0.27 ± 0.31 mg/100mL [(1)]. Moreover, resveratrol have been characterized in peanuts (0.04 ± 0.02 mg/100mg fresh weight [(1)]), berries (such as lingonberry (3.00 mg/100mg fresh weight [(1)]) or cranberry (0.27 ± 0.31 mg/100mL[(1)])) and red cabbage, spinach and certain herbs, but at lower concentrations (Bertelli *et al.* 1998; Bhat and Pezzuto 2002; Crozier *et al.* 2009; Vitrac *et al.* 2002).

Flavonoids are commonly consumed with fruit, vegetables, and beverages. Although the occurrence of some classes of flavonoids is restricted to a few

[(1)] The phenolic content has been extracted from the Phenol-Explorer database (Neveu *et al.* 2010)

foodstuffs, e.g. the isoflavones daidzein and genistein in soy, or flavanones in citrus fruits, other flavonoids are extensively distributed in the diet, e.g. quercetin, the main flavonol in our diet, is present in many fruit and vegetables, such as onions (e.g. red onion: 101.26 ± 58.93 mg of quercetin-3,4'-O-diglucoside/100g fresh weight [(1)]) and tea (e.g. black tea: 1.31 ± 0.73 mg of quercetin-3-O-glucoside/100mL[(1)]). Other vegetables that contain flavonols include broccoli and kale. Most red and purple fruits, such as grapes (72 mg total anthocyanins/100g fresh weight [(1)]), cherries (171 mg total anthocyanins/100g fresh weight [(1)]), and blueberries (134 mg total anthocyanins/100g fresh weight [(1)]) have important quantities of anthocyanins. Flavanols, specially catechins, are present in cocoa derivatives (e.g. dark chocolate: 20.50 ± 13.82 mg of catechin/100g fresh weight [(1)]; milk chocolate: 4.64 ± 3.65 mg of catechin/100g fresh weight [(1)]) and other food sources, such as red fruit, including red grapes (5.46 ± 5.74 mg of catechin/100g fresh weight [(1)]), and indirectly in red wine (6.81 ± 6.24 mg of catechin/100g fresh weight [(1)]) (Tsang, 2005). Tea also represents a rich source of flavanols (73 mg of total flavanols/100mL black tea infusion [(1)]) also providing a small quantity of quercetin. Flavones are less common in fruit and vegetables. The most important dietary rich sources of flavones are parsley (apigenin: 302 ± 26.16 mg/100g fresh weight [(2)]; luteolin: 1.24 ± 0.03 mg/100g fresh weight [(2)]) and celery (apigenin: 2.41 mg/100g fresh weight [(2)]). Some cereals, such as millet and wheat, and herbs contain larges quantities of C-glycosides of flavones (González-Castejón and Rodriguez-Casado 2011; Neveu *et al.* 2010). The least known and forgotten sub-class of flavonoids, the flavanonols are found discretely in red wine (Vitrac *et al.* 2002). Dimers, trimers, oligomers and polymers of flavonoids, mainly proanthocyanidins, are present in foods, representing complexes structures, linked through carbon-carbon bonds. These phenols are hydrolysable during digestion resulting in monomeric flavonoids. Moreover, most flavonoids in foods

[(1)] The phenolic content has been extracted from the Phenol-Explorer database (Neveu *et al.* 2010)
[(2)] The phenolic content has been extracted from the USDA database for the flavonoid content (USDA 2012)

are conjugated to a carbohydrate moiety, representing a wide range of combinations depending on the flavonoid, its linkage and the linked mono and disaccharide (Packer *et al.* 1999).

Chalcones are a group of phenolic compounds with a ring-opened structure that are especially abundant in fruit (e.g. citrus fruit, apples, including cider), vegetables (e.g. tomatoes, shallots, bean sprouts, potatoes) and spices (e.g. licorice) (Orlikova *et al.* 2011). Most of the contents of chalcones in citrus fruits and various plants are mediated through the formation of 4, 2', 4', 6'-tetrahydroxychalcone (also known as naringenin chalcone) (Orlikova *et al.* 2011).

Lignans are a polyphenol family of compounds not widely distributed in the plant kingdom. Flax and sesame seed contain high levels of lignans. Lignans are present in cereals and vegetable seed oils, e.g. virgin olive oil (1-acetoxypinoresinol: 0.66 ± 1.08 mg/100g; pinoresinol: 0.42 ± 0.28 mg/100g [(1)]) or sesame seed oil (total lignan content: 1294 mg/100g [(1)]). Besides, vegetables and pulses contain trace levels of lignans. Lignans are metabolized into enterodiol and enterolactone by the colonic microflora, reaching the blood stream and being excreted by the urine (Scalbert and Williamson 2000; Heinonen *et al.* 2001).

Curcuminoids, found in ginger and turmeric, are characteristic because of their coloring and flavoring properties (Omar 1992).

Although some tannins are extremely astringent (Crozier *et al.* 2006), they are abundant in many different edible plants species, highlighting their presence in the fruits, leaves and bark (Wilfred and Nicholson 2006; Han *et al.* 2007). Fruit,

[(1)] The phenolic content has been extracted from the Phenol-Explorer database (Neveu *et al.* 2010)

such as pomegranates, persimmons and berries (e.g. cranberries (Vattem *et al.* 2005), strawberries and red raspberries (Heinonen 2007)); herbs and spices, such as cumin, thyme, vanilla, hops (used to provide bitterness in beer) and cinnamon (Murphy 1999; Peter 2001), and other products such as nuts (Clifford and Scalbert 2000), legumes and chocolate (Han *et al.* 2007), among others, contain tannins. Additionally, smoked foods (smoked fish and meat) may have tannins on their surface due to their presence in the woods used in smoking e.g. mesquite, cherry, oak and others (Maga 1988).

1.3 Biochemical activities

It is well known that polyphenols are food bioactive compounds that display a number of biological activities, such as anti-carginogenic activity (Cossarizza *et al.* 2011), anti-inflammatory effect (Impellizzeri *et al.* 2012; Liu *et al.* 2012; Urpi-Sarda *et al.* 2012), neuroprotective capacity (Asha Devi *et al.* 2011; Bu *et al.* 2007; Mohagheghi *et al.* 2010; Narita *et al.* 2011; Obied *et al.* 2008; Omar 2010; Owen *et al.* 2000; Singh *et al.* 2008; Tuck and Hayball 2002; Vissers *et al.* 2004), antioxidant activity (Impellizzeri *et al.* 2012; Jaganath *et al.* 2009; Obied *et al.* 2008; Owen *et al.* 2000; Spranger *et al.* 2008) or cardioprotective effect (Cai *et al.* 2011; Covas *et al.* 2006; De Rose *et al.* 2001; Fraga *et al.* 2011; Kay *et al.* 2006; Ohkita *et al.* 2011; Omar 2010; Perona *et al.* 2006; Schroeter *et al.* 2010 Urpi-Sarda *et al.* 2012; Vogel *et al.* 2000; Williams *et al.* 2001). But most of the molecular interaction of polyphenols with those cellular targets remains unclear. Nevertheless, some non-specific and specific mechanisms of action have been proposed to explain a few of the biological effects exerted by dietary polyphenols (Fraga *et al.* 2010).

The best-known characteristic of polyphenols is certainly their antioxidant capacity. This antioxidant capacity is due to their ability to donate a hydrogen or transfer an electron and/or to delocalize the unpaired electron within the aromatic structure (Bors *et al.* 1990). Polyphenols, as antioxidant compounds, are able to break free radical chain reactions inhibiting the initiation or propagation of these reactions. On the other hand, in biological systems, redox active metals catalyze free radical-producing reactions. Although in cellular environments some of these metals modulate several physiological pathways, e.g. gene expression related with growth, and development, a disruption of metal homeostasis by an increase in their concentration may lead to uncontrolled metal-mediated formation of deleterious free radicals that could participate in modification of DNA bases, modifying calcium and sulphydryl homeostasis or enhancing lipid peroxidation (Gutteridge 1995; Jomova and Valko 2011; Valko *et al.* 2007). Metal-induced oxidative stress, with metals as catalyst of free radical chain reactions, have been linked to several disease and to a decline of the own antioxidant mechanisms of the body (Jomova and Valko 2011). Sequestration of minerals or trace elements, such as metal ions to prevent the catalyzation actions is another antioxidant strategy (Morel *et al.* 1998; Guo *et al.* 1996; Fraga *et al.* 2010; Fardet *et al.* 2008) exerted by polyphenols. Catechol moieties and combinations of hydroxyl and carbonyl groups are centers of affinity with metal ions (Fraga *et al.* 2010). Nevertheless, it is not clear that the described antioxidant capacity together with the low bioavailability (0.3 to 26%) (Scalbert and Williamson 2000) are sufficient to explain the antioxidant actions of polyphenols *in vivo* (Hollman *et al.* 2011). Their antioxidant mechanisms are probably reinforced by action on the activation/ repression of particular genes via transcription factors or by the activation of the body's own individual antioxidant response, with characteristic enzymes, such as superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx), among others (Lee *et al.* 2003; Na and Surh 2006).

Another non-specific mechanism is the ability of polyphenols to interact with membrane lipids and proteins. That behavior, observed in *in vitro* studies (Hendrich *et al.* 2002; Yoshioka *et al.* 2006; Sirk *et al.* 2009), is due to the presence of both hydrophobic and hydrophilic domains in several polyphenol molecules that allow polyphenols to localize at different levels in the membrane-at the surface absorbed on the polar head of lipids, and/or inserted into the bilayer interacting with the hydrophobic chain of lipids-. The ability to interact with membrane lipids and proteins may affect cell functioning by modifying the plasma membrane structure and physical characteristics, e.g. fluidity or electrical properties, with subsequent changes of several membrane-associated events, such as the activity of membrane-associated enzymes, fluxes of ions and/or metabolites or signal transductions, among others (Fraga *et al.* 2010). Combining both described non-specific mechanisms, polyphenols may act as an antioxidant barrier. Absorbed on the membrane, polyphenol could work as a physical barrier for hydrosoluble radicals and, absorbed in the lipid bilayer, polyphenols may scavenge free radicals and lipid soluble radicals *in vitro* (Verstraeten *et al.* 2003).

Leaving aside the non-specific mechanism, specific interactions between polyphenols and proteins have been described by the assay with cultured human endothelial cells (Lotito and Frei 2006). These interactions take on special relevance since they can be related to positive effects exerted by dietary polyphenols. Polyphenol-enzyme interaction is a clear example. Different polyphenols, especially flavonoids, may inhibit the activity of a wide range of enzymes, mainly ATP-dependent enzymes, through a competition at the ATP-binding site due to the presence of two hydroxyl substitutions in 5,7 positions in the A ring and a 2,3 unsaturation together with a 4-keto group on the C ring (Figure 2) (Lotito and Frei 2006). Nonetheless, not only ATP-dependent enzyme

activity is modified by the presence of dietary polyphenols, NADPH-dependent enzymes (Fraga *et al.* 2010) or enzymes, such as cycloxygenase, phospholipase or lypoxygenase related with Parkinson disease, among others, would be also affected by dietary polyphenols (Mazzio *et al.* 2011).

The interaction of polyphenols with transcription factors or receptor is another examples of polyphenol-protein interaction with positive effects from dietary polyphenols. The first one may modulate *in vitro* the expression of various NF-κB-regulated genes involved in inflammation and carcinogenesis (Park *et al.* 2000; Erlejman *et al.* 2008; Mackenzie *et al.* 2008). On the other hand, the estrogenic effect of polyphenols is related to the second example of polyphenol-protein interaction. Isoflavones are a clear example of polyphenol-receptors able to interact with animal estrogen receptors. A special molecular structure endows them with a large area adequate for interacting with estrogenic receptors (Fraga *et al.* 2010). Other specific mechanisms of action have been described in the literature hindering even more the understanding of the mechanisms by which polyphenols exert positive effects on health. The inhibition of pancreatic lipases preventing hyperlipidemias (Kawaguchi *et al.* 1997) or the decrease of the absorption of oxidized products, such as hydroperoxide and malondialdehyde, present in foods (Gorelik *et al.* 2008) are some examples observed with rats.

1.4 Bioavailability and metabolism

Following the ingestion of dietary polyphenols (Figure 4), the first step of the digestion process takes place in the mouth. During mastication, food is broken up into small portions and also mixed with saliva, which, among other enzymes, has amylases that collaborate in a partially starch degradation. The polyphenol chemical transformation in the oral cavity has not yet been precisely described

due to the limited number of published studies (Vacek *et al.* 2010). Nevertheless, existing evidence describes a partial participation in glycoside hydrolysis by the salivary enzymes (Ice and Wender 1953), microbiota or oral epithelial cells (Walle *et al.* 2005). The glycoside hydrolysis is practically a mandatory step prior to metabolism, because the phenolic compounds are found in plants as glycoside conjugates, often being glucose or rhamnose but they can also be galactose, arabinose, xylose, glucuronic acid or other sugars (Harborne 1994).

In the stomach, food is reduced to smaller particles thus enhancing the release of phenolic compounds from the food matrix (Figure 4) (Scalbert *et al.* 2002). The hydrolysis of the phenolic compounds is continued by the intestinal enzyme systems and the metabolic process of deglycosylation is named phase I metabolism. Phase I metabolism also includes other chemical reactions, such as oxidations, reductions and/or hydrolytic pathways adding a functional group (e.g., OH, SH, or NH_2) to the phenolic molecule (Parkinson 1996). Two possible routes for glycoside conjugate hydrolyzation by which the glycoside conjugate is hydrolyzed and the aglycone appears in the epithelial cells are reported (Crozier *et al.* 2009 ; Vacek *et al.* 2010). The first, called 'LPH/diffusion' is carried out by the action of lactase phloridizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells, due to the LPH exhibiting a specificity for glucoside polyphenols, such as flavonoid-O-β-D-glucosides. The aglycone generated is more lipophilic than the glucoside form and that fact, together with the proximity to the cellular membrane allows it to enter the epithelial cells by passive diffusion (Day *et al.* 2000). The second route is 'transport/CBG'. This route takes place in the epithelial cells, carried out by a cytosolic β-glucosidase (CBG). Nevertheless, transport into the epithelial cells is required, possible involving an active sodium-dependent glucose transporter (Gee *et al.* 2000). Additionally, the glycoside hydrolysis may be carried out enzymatically, in the

food itself, non-enzymatically by gastric juice or by the microflora in the colon (Figure 4) (Scalbert and Williamson 2000; Vacek *et al.* 2010).

Before passing into the blood stream, the aglycones are metabolized suffering a conjugation metabolism (phase II metabolism), resulting in sulphate, glucuronide and/or methylated conjugates by the action of sulphotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UGTs) and catechol-

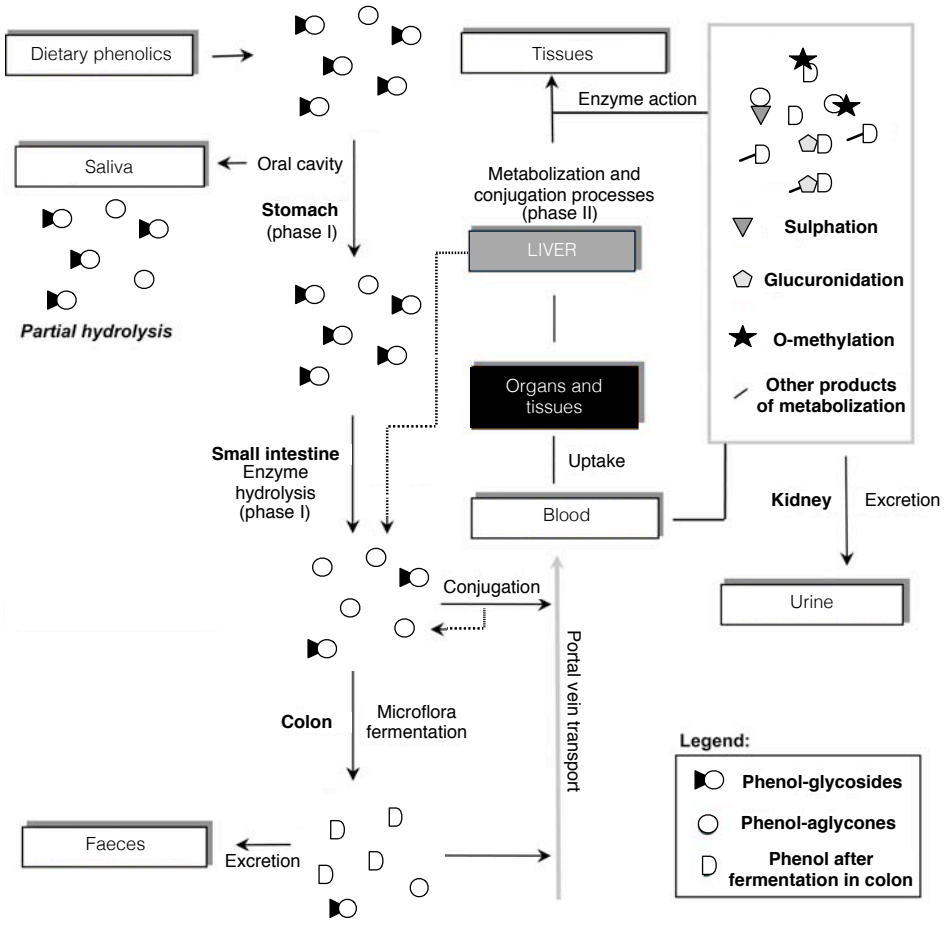

Figure 4. Schematic representacion of digestion, resorption, metabolization, and excretion of plant phenolics in mammals. Modified from Vacek, *et al.* 2010.

O-methyltransferases (COMT), respectively. There is an efflux of metabolized forms back to the small intestine, that involves members of the adenosine triphosphate (ATP-binding) cassette (ABC) family of transporters. Anecdotally, a limited passage of free glycosides across the intestinal brush border has also been reported (Vacek *et al.* 2010).

After their absorption in the digestive tract, phenols may rapidly reach individual tissues, where they may be metabolized. In the liver, the main organ of phase II metabolic transformations, they can be subject to conjugation reactions, undergoing glucuronidation, methylation and/or sulphation as occurs in the brush border of the epithelial cells (Vacek *et al.* 2010). Thus, the metabolites become more hydrophilic and easily excreted through the urine (Cassidy 2003; Wilkening *et al.* 2003). In the phase II metabolism, the functional groups added during the phase I metabolism are modified to O- or N- glucuronides, sulphates, esters, α-carboxyamides and glutathionyl adducts, thus increasing the polarity of the molecules (Parkinson 1996). Simultaneously, there is an enterohepatic recirculation recycling the conjugated forms back to the small intestine through the bile (Crozier *et al.* 2006). Glucuronidation is possibly the major conjugation reaction involved in flavonoid metabolism (Donovan *et al.* 2001; Kuhnle *et al.* 2000; Oliveira *et al.* 2002; Shimoi *et al.* 1998; Spencer *et al.* 1999;). UGT enzymes, the enzymes responsible for the glucuronidation reaction, are found mainly in the liver (Mojarrabi and Mackenzie 1998; Strassburg *et al.* 1998), kidney and intestines. Methylation is conducted by non-specific enzymes found in several tissues, especially the liver and intestine. As occurs with glucuronidation, the liver is the main organ responsible for methylation (Piskula and Terao 1998). Sulphation is described as a common conjugation reaction that take place with low doses of polyphenols and seems to be a highly saturatable pathway as a result of the relative low concentration of sulphoconjugates in the blood and urine (Kay 2006).

Not all the ingested polyphenols are metabolized in the stomach or small bowel and may reach the colon. Additionally, polyphenols that are absorbed, metabolized in the liver (or other tissues) or excreted by bile recirculation or directly by the enterocyte back to the small intestine will reach the colon with a different chemical structure. Luminal phenolic compounds and those still bound to the food matrix enter the colon where the microbial population (obligate anaerobes and facultative anaerobes) degrade the non-digested food matrix and transform components into microbial metabolites (Kleessen *et al.* 2000; Salminen *et al.* 1998;), mainly by deconjugation catalyzed by microbial enzymes (α-rhamnosidase, β-glucosidase and β-glucuronidase) spreading the glycosyl or glucuronosyl moiety from the phenolic backbones, resulting again in aglycone forms (Aura *et al.* 2002; Justesen *et al.* 2000; Rechner *et al.* 2004). Subsequently, a ring-fission (mainly catechol structures of flavonoids) and various enzymatic reactions or cleavages of functional groups (dehydroxylations, demethylations, decarboxylations, hydrolysis, reductions, isomerizations and fissions) carried out by the colonic microflora take place (Selma *et al.* 2009). Colonic metabolites are absorbed in the colon and can again undergo the phase II metabolism, resulting in a conjugated derivatives (Adlercreutz *et al.* 1995; Axelson and Setchell 1981; Lampe 2003).

Pharmacokinetic parameters allow the quantitative study of the evolution of the absorption, distribution, metabolism and elimination over time. When a drug or a bioactive compound, such as polyphenols, is administered, it is rapidly distributed through the blood system. Changes in the plasmatic concentration may indicate their presence in other tissues or excretion. One of the most known pharmacokinetic parameter, and one usually used in pharmacokinetic studies with phenolic compounds, is the Area Under the Curve (AUC). As its name indicates, this is the area obtained when the concentration is plotted against

time (Gunaratna 2001) and it can be used as a measure of exposure. Nonetheless, among other parameters, the dose and the nature of the phenolic compound hinder the comparison between pharmacokinetic studies, as shown in Table 3, which summarizes different AUC values and other pharmacokinetic parameters (T_{max}, plasma concentration, urinary excretion and elimination half-life), extracted from different clinical studies.

Moreover, although the bioavailability, metabolism and bioactivities of phenolic compounds in target tissues have been studied, a large inter-individual variation in the bioavailability has been reported (Bravo 1998; Rice-Evans 2001). Several factors may modify the polyphenol metabolism, including environmental, physiological, epigenetic and genetic factors (Mizuma and Awazu 2004; Williams *et al.* 2004). Some of the enzymes involved in the polyphenol metabolism have genetic polymorphisms and these can also be induced by environmental and physiological factors (Bolling *et al.* 2010; Scalbert and Williamson 2000).

Table 3. Bioavailability studies of phenolic rich foods.

Source	No. of subjects	Dose	T_{max} plasma (h)	Plasma concentration (μmol/L)	AUC (μmol·h/L)	Urinary excretion (% of intake)	Elimination half-life (h)	Ref
Onions	12	100 mg quercetin eq	0.68	7.6	32.1	6.4	10.9	Graefe *et al.* 2001
Orange juice	8	126 mg hesperidin eq	5.4	2.2	10.3	5.3	2.2	Erlund *et al.* 2001
Chocolate	8	82 mg epicatechin eq	2	0.38	1.53	-	1.9	Richelle *et al.* 1999
Soy nuts	10	26.4 mg daidzein	6.0	1.65	18.1	44	7.5	Setchell *et al.* 2003
Coffee	10	96 mg chlorogenic acid	1	0.505 caffeic acid	-	-	-	Nardini *et al. 2002*

Polyphenol bioavailability depends on several factors, including the composition of the food (Manach *et al.* 2004; Serra *et al.* 2010a; Serra *et al.* 2012). Nevertheless, only a few studies have been carried out into the effect of food components on the bioavailability and metabolism of phenolic compounds (Auger *et al.* 2008; Azuma *et al.* 2002; Donovan *et al.* 1999; Hollman *et al.* 2001; Manach *et al.* 2004; Rodríguez-Mateos *et al.* 2012; Serra *et al.* 2010a; Serra *et al.* 2012; Serafini *et al.* 1996; Van het Hof *et al.* 1998). The results published demonstrate that such common macronutrients as carbohydrates could modify the absorption of flavonoids (Rodríguez-Mateos *et al.* 2012), through an effect on the motility of gastric secretions and hepatic blood flow (Schramm *et al.* 2003). Proteins could interact with polyphenols during pancreatic digestion (Laurent *et al.* 2005), and fat could enhance the absorption of phenolic compounds compared with aqueous solutions, modifying the absorption and metabolism of phenolic compounds (Tuck *et al.* 2001; Tulipani *et al.* 2012).

2. Metabolism Approximations

2.1 Introduction

Current food science and nutrition aim to understand the role of nutritional compounds at the molecular level (Herrero *et al.* 2012). This should be the basis for creating functional foods, which must be the result of a long period of research.

The logical stages in the process of developing a food generally starts from an intensive search for functional ingredients, usually guided by a bibliographic search or clear evidence of health benefits related to their ingestion. This preliminary stage is extremely useful for identifying possible functional ingredients, which will be tested *in vitro* to verify their potential functionality, with a preliminary screening to avoid carrying out human clinical trials needlessly. Subsequently, functional ingredients should be tested in animals as a pre-clinical model. Although human clinical trials are possibly the best way to test a functional ingredient, this involves an extremely complex organization of volunteers, generally chosen with a specific profile, and is a huge technical and economical investment. One important aspect of a functional food is the fact that the behavior of the active compounds when included in a food matrix is not the same as when the active compounds are tested as single molecules. The effect exerted by the food matrix during the digestion process may modify

important parameters, such as the stability of the compounds during digestion or their bioavailability.

Thus, prior to carrying out human clinical trial, *in vitro* models (e.g. enzymatic assays, cell cultures, genomic tests or *in vitro* simulation) are very useful techniques for mimicking biological situations and allowing the study of the food matrix effect. *In vitro* models are typically characterized by the fastness and excellent relation cost/effectiveness. The next step is *in vivo* pre-clinical trials using experimental animals, generally rats or mice. Nonetheless, the response obtained by *in vitro* models is not always directly extrapolated to *in vivo* systems. So, and according to the technical guidance for the preparation and presentation of an application for authorization of a health claim draw up by the European Food Safety Authority (EFSA), studies realized in animal or other model systems cannot substitute human data (EFSA 2011).

2.2 Digestion: *In vitro* simulations

The aim of the *in vitro* simulations is to characterize changes that take place in dietary components during the digestion process under physiological conditions, adapting the temperature, the pH, the enzymatic concentrations and, specifically in the colonic fermentation models, the microbiota. In broad terms, there are two types of *in vitro* digestion simulations: the *in vitro* upper intestinal models and the colonic fermentation models. Briefly, the *in vitro* simulation models of the digestion process mimic the physiological conditions and luminal reactions that occur in the mouth, stomach and small intestine, regardless of the metabolism. The *in vitro* upper intestinal models (usually named digestion models) are commonly used to evaluate the digestibility of dietary components and detect changes in the non-digestive fraction. Besides,

the colonic fermentation model includes an inoculum of colonic microbiota and is used to evaluate the colonic metabolism, usually using the non-digestible part obtained after the *in vitro* upper intestinal simulation (Aura 2005).

2.2.1 *In vitro* digestion models

In vitro digestion models are commonly used to evaluate the digestibility, structural changes and release of food components under gastrointestinal conditions representing a cheap and rapid alternative to animal and human studies. *In vitro* digestion models differ from one another mainly in the digestion steps represented in the model (mouth, stomach, small and large intestine), the composition of the biological fluids (e.g. salts, enzymes and buffers) and the fluid flows utilized in each step in the digestion sequence (Hur *et al.* 2011). In general, all the *in vitro* digestion simulations are performed at 37° C and simulating the duration of the digestion times in humans, with slightly modifications, as occurs in the body, according to the nature of the sample being tested. Physiologically large food particles move through the stomach more slowly than smaller ones, and have to be less than 1 mm to cross the pylorus valve.

More than 80 studies related with *in vitro* digestion models have been carried out in the past ten years and these were reviewed by Hur *et al.* 2011. The most appropriate enzymatic composition used in the *in vitro* simulations must be designed taking the composition of the tested samples into account. Related to this, only a few of the reported *in vitro* digestion methods have simulated the mouth stage, which implies an α-amylase treatment of the tested sample (Gawlik-Dziki *et al.* 2009; Kedia *et al.* ; Kiers *et al.* 2000; Kong and Singh 2008; Laurent *et al.* 2007; Ortega *et al.* 2011; Savage and Catherwood 2007;

Versantvoort *et al.* 2005;). Amylase, present in the mouth and stomach, is the enzyme responsible for the conversion of starches into oligosaccharides and monosaccharides. Amylase is usually related to the digestion of starch, lipase to the digestion of fatty product, and pepsin or trypsin to protein digestion. Lipase, present in the stomach and pancreas, transforms the triacylglycerols and diacylglycerols into monoacylglycerols and free fatty acids (Hur *et al.* 2011) and, as occur with the application of amylase in the *in vitro* simulations, the application of lipase is not extensible to all of the reported *in vitro* digestion systems (Versantvoort *et al.* 2005; Kiers *et al.* 2000; Green *et al.* 2007; Bublin *et al.* 2008). Proteases, including stomach protease (pepsin) and small intestine protease (trypsin and chymotrypsin), are responsible for breaking proteins and peptides down into peptides and aminoacids, respectively, and their use is more widespread as they are included in practically all the *in vitro* digestion methods (Savage and Catherwood 2007; Kedia *et al.* 2008 ; Laurent *et al.* 2007; Green *et al.* 2007). Nevertheless, *in vitro* digestion methods that use complex enzyme mixtures are more reproducible than those that use a single enzyme (Fatouros and Mullertz 2008).

Another biological parameter measurable through the use of an *in vitro* upper intestinal models is the bioaccessibility, or the percentage of bioactive compounds that can be absorbed during the digestion process. The bioaccessibility could be approximated *in vitro* by the implementation of a dialysis membrane by which small molecules are separated from the duodenal digestion mixture and considered as the bioaccessible fraction liable to be absorbed (Gil-Izquierdo *et al.* 2002; Ortega *et al.* 2009).

2.2.2 *In vitro* colonic fermentation models

Although gut was long described as an independent organ with immunostimulation properties (Bocci 1992) and the majority of the studies carried out focused on identifying gut microbiota species, scientific studies have demonstrated the relation between various microbial species and some pathologies, such as Crohn's disease, irritable bowel syndrome or obesity (Bischoff 2011; Ley *et al.* 2005; Turnbaugh *et al.* 2006; Malinen *et al.* 2005; Manichanh *et al.* 2006). Moreover, as described previously, the role played by colonic microflora in the metabolism of dietary compounds, e.g. phenolic compounds, is no less important. Non-metabolized polyphenols, polyphenols bonded to the food matrix, and phase II metabolites that reach the colon may suffer the enzymatic action of colonic microflora and prior to their excretion, the new colonic metabolites may exert an specific function in the colon itself (Unno *et al.* 2003), or may be absorbed and returned to the blood stream, reaching phase II metabolism centers of action (Lampe 2003; Axelson and Setchell 1981; Adlercreutz *et al.* 1995).

Several colonic fermentation models have been developed (Figure 5), although the basic principle of the colonic fermentation model is common to all. This is a single or multiple chemostats inoculated with fecal microbiota operating under physiological temperature and pH, in anaerobic conditions (Macfarlane *et al.* 1998; Cinquin *et al.* 2006; Minekus *et al.* 1999; Molly *et al.* 1993). The complexity of *in vitro* colonic fermentation models increases with the number of chemostats operating simultaneously, even mimicking the entire colon, imitating any of the individual sections of the large intestine (e.g. proximal, transverse and distal colon) (Figure 5, R1, R2 and R3).

Briefly, batch culture fermentation models are closed systems containing a pure or mixed bacterial suspensions of faecal material in a selected medium under

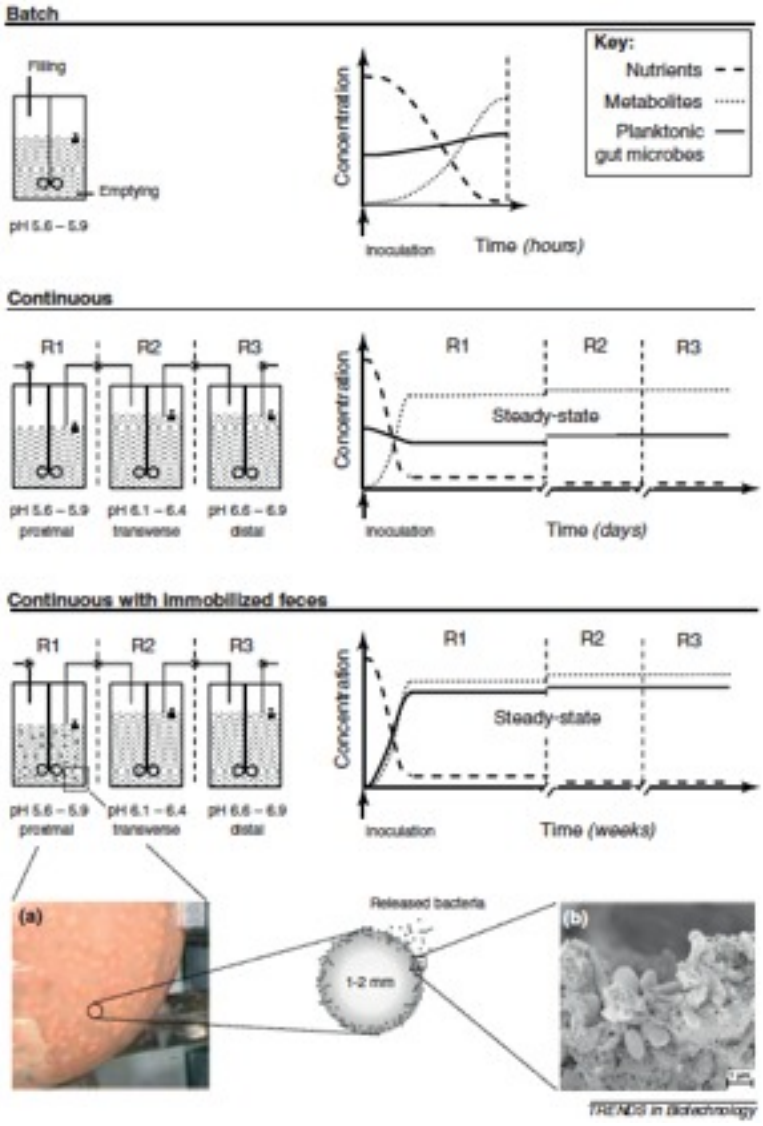

Figure 5. Types and process characteristics of *in vitro* colonic fermentation models simulating proximal (R1), transverse (R2) and distal (R3) colon regions, operated at physiological section-specific constant pH, temperature (37 °C) and under strictly anaerobic conditions (e.g. through continuous CO_2 or N_2 flushing of the headspace). Initial (R1) and steady-state (R1, R2 and R3) fermentation profiles. **(a)** Picture detail of a proximal colon reactor containing polysaccharide beads with immobilized fecal microbiota. **(b)** Electron microscope image of microbes embedded and attached to the surface of an intestinal bead (Payne *et al.* 2012).

anaerobic conditions. They represent the easiest fermentation model and are generally used to perform fermentation studies and substrate digestion assessment (Gumienna *et al.* 2011; Lesmes *et al.* 2008; Pompei *et al.* 2008) with the drawback that only short-term fermentation studies can be performed. More complex fermentation models named continuous fermentation models due to being endowed with a continuous flow mimicking *in vivo* conditions, have been carried out and are extensively used to assess long-term fermentation studies, with the aim of elucidating colon functions (Macfarlane and Macfarlane 2007), metabolic activity (Possemiers *et al.* 2011) or gut bacterial colonizations (Cinquin *et al.* 2004; Le Blay *et al.* 2009), among other applications. Additionally, two types of continuous fermentation models may be differentiated according to their number of stages: single or multistage continuous fermentation models (Payne *et al.* 2012) (Figure 5). Apart from the complexity derived from the number of stages, the technique used for faecal inoculation is a determining factor in their effectiveness in mimicking biological environments. The simplest inoculum is represented by the use of pure cultures or defined mixed cultures (Macfarlane and Macfarlane 2007). Nevertheless, in most *in vitro* fermentation models, a liquid faecal suspension (Lebet *et al.* 1998; Serra *et al.* 2011; Serra *et al.* 2010b; Vernazza *et al.* 2005) has been used as the inoculum, this being a good technique for short-term fermentation simulations (less than 4 weeks) due to liquid fecal inocula generally experiencing a rapid washout of less competitive bacteria the longer the incubation time. To solve problems of inoculum washout, immobilized fecal microbiota have been developed (Cinquin *et al.* 2006; Cinquin *et al.* 2004; Zihler *et al.* 2010), in which fecal beads formed with a polysaccharide matrix are mixed with the growth medium generally in continuous fermentation models. This techniques of fecal inoculation has been identified as another type of continuous fermentation model (Figure 5, a and b).

The common limitation of all of the described fermentation models is the absence of host functionality. This limitation was overcome by developing artificial digestive systems, which represent the most advanced attempt at fermentation models. Artificial digestive systems are able to simulate human digestive functions, including motility, bile secretion, pH and absorption for small intestinal models and peristaltic mixing and water and metabolite absorption for colonic models. The combination of artificial models results in a complex mimicking system of the digestive tract applied generally to advanced pharmaceutical or nutritional studies (Blanquet-Diot *et al.* 2009; Souliman *et al.* 2007; Souliman *et al.* 2006).

The evaluation of the working mechanisms of probiotics and drugs, the metabolism of dietary components and the impact of radioactivity on the gut microbiota are some of the applications of colonic fermentation models usually performed with batch or single-continuous fermentation models (Mennigen and Bruewer 2009; Meunier *et al.* 1995).

2.3 Absorption and metabolism: cell cultures

Cell cultures represent a predictive tool for the intestinal absorption of bioactive compounds (Hur *et al.* 2011). Compared with *in vitro* models, the use of culture cells allows evaluation of the metabolism using human or animal tissue, preserving cell integrity and maintaining the interaction between the cells with good standardization and reproducibility, although it is not always easy to preserve the tissue structure and cellular differentiation in cell cultures (Sambruy *et al.* 2001). The typical polarized organization, the asymmetrical distribution of membrane proteins and lipids and the presence of highly organized structures

of epithelial cells hinder the maintenance of cultured intestinal epithelium. A range of cell models has been developed to preserve the complex functional and morphological organization of the intestinal epithelium *in vitro* (Sambruy *et al.* 2001). Primary cell cultures are cell isolated from the small intestine (Evans *et al.* 1992) or the colon (Fonti *et al.* 1994) maintaining a variable degree of differentiation for three to six days in culture. Nevertheless, this kind of culture cells rapid lose their differentiated characteristics. The second type of culture cells are the cell lines from normal tissues, obtained from primary cultures by isolating homogenous cell lines capable of proliferating and surviving for several *in vitro* passages generally using the small intestine of rats. Normal cell lines transfected with regulatory genes have been reported as the third type of culture cells used to maintain cultured intestinal epithelial cells, and obtained from crypt cell lines. The last type of culture cells, and probably the most widely used of these, are the cell lines of tumoral origin. The use of cell cultures is reinforced by the possibility of using tissue cultures to study the behavior of specific alive cells in a controlled environment (Mather and Roberts 1998).

The caco-2 cell line is an important example of cell lines of tumoral origin used in nutritional modeling for predicting intestinal absorption and metabolism (e.g. transport system -diffusion, endocytosis, transcytosis (Bailey *et al.* 1996)-, conjugations, etc.) at the molecular and cellular level of drugs, nutraceuticals, food additives and dietary constituents, among other compounds, and even for testing possible health benefits (Artursson 1990; Trotter and Storch 1991) or oral absorption (Bailey *et al.* 1996). Caco-2 cell lines were isolated from human colon adenocarcinoma (Fogh *et al.* 1977) during the 70's, and the leading property of these cells is their capacity to differentiate themselves spontaneously in the culture starting at confluency and reaching it in two to three weeks. It allows the formation of a monolayer of highly polarized cells, joined by functional tight junctions, with well-developed and organized microvilli on the apical membrane

(Figure 6). Caco-2 cells normally express hydrolase activities associated with the apical membrane. Some of the intestinal functions are also expressed and, despite their tumoral origin, they exhibit some biochemical characteristics of the normal adult intestine (Harris *et al.* 1992).

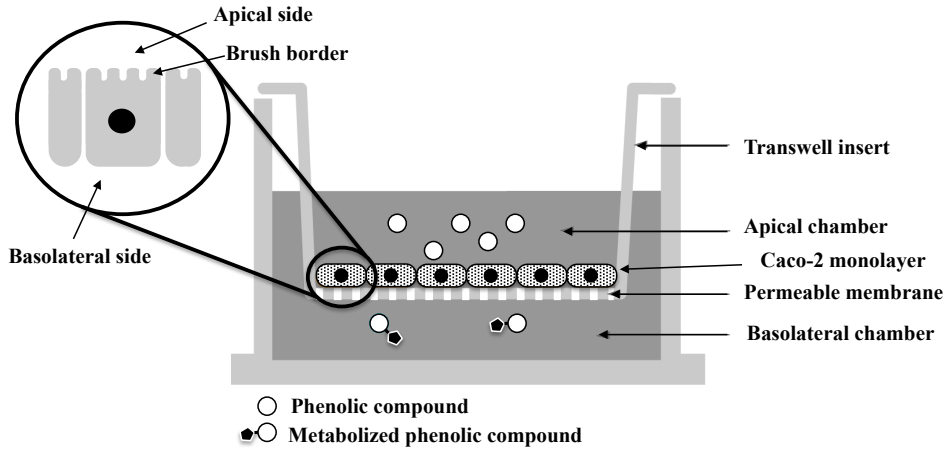

Figure 6: Schematic representation of caco-2 cell monolayer in polarized transwell system.

In the nutritional field, the human hepatoma cell line (HepG2) and the primary hepatocytes are other widely used families of culture cells. Cultured hepatocytes are the most suitable *in vitro* model for evaluating hepatic bio-transformations and are of great relevance in toxicological and pharmaceutical studies (Wilkening *et al.* 2003). Primary hepatocytes do not proliferate and lose their metabolic activity, although this is the best culture cell model for mimicking the hepatic metabolism *in vitro*. Nevertheless, HepG2 is an excellent approximation that provides a reproducible human system diminishing the problems of proliferation and differentiation.

2.4 *In vivo* models

Despite the technologic advances in the *in vitro* models, they cannot fully replace *in vivo* studies. The use of animal models is more widely accepted than *in vitro* models to bridge the gap between *in vitro* studies and the full human organism (Mortensen *et al.* 2008), although data from animal studies are controversially criticized due to the physiological differences between animal and human metabolism (De Kanter *et al.* 1999; Gomes *et al.* 2007). *In vitro* studies and animal experimentation are useful tools for obtaining a valid approximation to human *in vivo* processes (e.g. digestion, absorption, metabolism, tissue distribution, and even health benefits of phenolic compounds). Thus, the data from *in vitro* and *in vivo* studies with animals or other non-human models may be useful as supporting evidences, remembering that human clinical trials cannot be replaced by *in vitro* trials as expressed in the European Food Safety Authority scientific and technical guidelines for preparing and presenting an application for authorization of a health claim (EFSA 2011).

2.4.1 Animal models

An animal model could be defined as a living organism in which some aspect of the physiology can be studied, and in which one or more aspects of the phenomenon resemble the same phenomenon in humans. Animal models are widely applied in nutrition research. Nevertheless, the use of animals as a research model requires an ethical justifications of the pain and suffering caused to the animals compared with the new knowledge obtained from the experimental study. This balance is the well-known concept of cost/benefit (Manciocco *et al.* 2009). Over recent decades, the accepted ethical standards for regulating animal experiments is represented by the principle of the 3Rs

(replacement, reduction and refinement) (Russell and Burch 1959), in which the general principle of the treatment of the animals during experimentation was established with importance given to the intimate relation between humanity and efficiency in animal experimentation. Briefly, replacement means the substitution of animal models with non-animal models (e.g. *in vitro* studies, molecular approaches, mathematical and computer models, etc.) whenever this is possible; reduction refers to the optimization of the number of animals according to the precision obtained in the measurements; several strategies have been developed to reduce the number of animals including choosing the right experimental design and proper statistical analyses (Festing and Altman 2002); and the last R is refinement, which refers to minimizing or avoiding pain, distress and other adverse effects for the animals throughout their life (Buchanan-Smith *et al.* 2005).

Animal studies can be performed in a short period of time, enabling the study from the *in utero* exposure to the animal's death, also including inter-generational experiments. The variability introduced by environmental factors, generally uncontrollable in human studies, is minimized by standardization of laboratory conditions and by the use of 'defined animals' (animals with known genetic and health status) (Öbrink and Rehbinde 2003). The choice of an animal model will depend on its validity with respect to the phenomenon studies (data obtained in the animal model predicts the situation in the non-experimental conditions) and the expression of that phenomenon in the chosen model (Mortensen *et al.* 2008). Ideally, to perform nutrition research anatomy, the biochemistry and physiology of the digestive system should be close to those of humans, and the administered dose should be adjusted by an animal to human compensation (Freireich *et al.* 1966).

In recent years, several animal models have been developed that can be used in practically all areas of research. The use of fish as an animal model has increased for several reasons, some related to the fact that many species are oviparous and their eggs are generally transparent, which allows events in early development to be monitored. The ease of reproduction from a single pair of fish is another important reason for using fish as an animal model (Hau and Van Hoosier 2003). The zebra fish (*Danio rerio*) is an extremely famous fish model specie generally used in large studies of chemical mutagenesis and carcinogenesis, although a recent study has alleged that zebra fish can successfully imitate the current models in elucidating metabolic pathways of model flavonoids (Wei *et al.* 2012). Work with *Drosophila melanogaster* has been carried out over many decades and actually is becoming more important in major advances in animal genetics. The evaluation of polyphenol metabolism is not commonly performed with the fruit fly (*Drosophila melanogaster*) as occurs with the nematode *Caenorhabditis elegans*. Nevertheless, some studies based on the evaluation of the biological effect of phenolic compounds (e.g. flavonoids) have been carried out with these animal models (Sotibrán *et al.* 2011; Luo 2006; Si *et al.* 2011; Wilson *et al.* 2006). Other new animal models could be the African Toad (*Xenopus sp.)* or other fish species, such as *Xiphophorus sp*. and the Japanese medaka, but their use in nutritional studies is limited.

Although the innovative animal models have improved over recent years, the evaluation of the metabolism of phenolic compounds is generally performed by the use of rodents (rats and mice), which represent the overwhelming majority of all the laboratory animals (Hau and Van Hoosier 2003). The digestion and metabolism are similar to the human processes, including absorption, metabolism (phase I and phase II metabolism in liver and intestinal epithelial cells and colonic fermentation) and excretion. Among other reasons, including

their low cost, easy reproduction from a single couple and easy handling, make rodent one of the best models for *in vivo* nutritional research. So, thousand of different nutrition experiments involving dietary polyphenols, as purified molecules (Konishi *et al.* 2006; Okushio *et al.* 1999) and as a component of a food (Donovan *et al.* 2006; Chen *et al.* 1997; Neilson *et al.* 2010; Tsang *et al.* 2005), have been carried out using rat and mice models.

Besides the wild-type animals, there is a wide range of specific animal models useful for mimicking special conditions of interest, generally related to human disease. That specificity can either spontaneously mimic these conditions, such as the Gunn rat for hereditary hyperbilirubinemia, or be induced to simulate those conditions, created by surgical manipulations, chemical manipulation and genetic manipulation (transgenic models). The animal model of obesity induced by the administration *ad libitum* of a cafeteria diet could be a representative example of induced specific animal model (Speakman *et al.* 2008). Although the surgically induced models represent the classical biomedical research models and the chemically induced models represents a useful solution commonly used in many field of research (Chow *et al.* 2007), including nutrition (Speakman *et al.* 2008), transgenic models represent a powerful tool for studying biological functions (Prieto *et al.* 1999). Transgenesis includes the addition of foreign genetic information to animals and specific inhibitions of endogenous gene expression (knockout models) (Houdebine 2007). From the perspective of nutrition and its related areas of research, transgenic animals can be used as *i)* models for studying nutritional stages and metabolic disease and *ii)* sources of modified food products (for example, milk from transgenic animals with high levels of κ-casein could be directly consumed or used to purify κ-casein (Prieto *et al.* 1999)). Nonetheless, the animals used to study nutritional stages and metabolic disease represent the most important group of transgenic animals in

the nutrition research area related to the metabolism of phenolic compounds and their possible effect on metabolic or other human diseases.

Some of the most representative transgenic animal models used in nutritional research are the models for cardiovascular disease based on alterations to renin-angiotensin and other systems (Barret and Mullins 1992), useful for studying the effects of diet on high blood pressure, cardiac hypertrophy and thrombosis; models for studying endocrine disorders, such as diabetes (Bray and Ryan 1996; Stewart 1993); and mice with an alteration in the lipoprotein transport proteins, lipases and receptors used in the study of lipoprotein metabolism and atherosclerosis, which could be one of the most illustrative specific animal models in the nutritional field (Breslow 1993; Prieto *et al.* 1999).

Over the last decade, the introduction of chimeric mice with humanized tissues, specifically the liver (Taleno *et al.* 2004), has been a breakthrough in applied nutrition research. Their capacity to reproduce human-type metabolic responses reduces the species differences between experimental animals and humans, and minimizes the problems derived from the use of primary culture of human tissues (e.g. the inability of cells to proliferate, their quick degradation and the requirement for specific culturing or thechnical conditions) (Li *et al.* 1997).

2.4.2 Human studies

After intense *in vitro* and *in vivo* (animal experimentation) studies and with clear evidence of the planned hypothesis, the last step in all the nutritional research should be a human study, as this is the only manner the real importance of foods or bioactive components can be assess in relation to their health benefits (Lund 2003). Nutritional intervention studies align most closely with

pharmaceutical trials in term of level and type of intervention and ethical considerations, as shown in Table 4.

Human studies could be classified into intervention studies and observational epidemiological studies. In nutrition research, an intervention study is based on applying a nutritional intervention under controlled conditions, and measuring the biological outcome. Subsequently, intervention studies can be divided into

Table 4. A comparison between pharmaceutical trials and nutritional intervention trials. Generally the former is looking to cure illness whilst the latter is ofter aimed at preventing illness or maintenance of health but there is no clear cut boundary particularly in respect to nutraceuticals and functional foods (Lund 2003).

	Pharmaceutical agent	Nutritional Equivalent?
Phase I	Compound has never been given to people before	An isolated food component may not have been given before but probably not equivalent unless given at supra physiological level, e.g. nutraceuticals
	Use of few healthy volunteers	Yes, may use small number for pilot studies or bioavailability studies
	Safety testing - dose response	Dose response may be undertaken but not normally for safety reasons
Phase II	Use a few patients	Could arise in clinical nutrition or when patient is a source of biopsy tissue
	Testing efficacy	Yes, certainly looking for an effect
Phase III	100s-1000s patients	Nutritional studies may use large numbers of people but not normally patients - only potential patients as considering disease prevention
	Efficacy	Yes
	Side effects	Probably No for whole foods but Yes if studying supplements or nutraceuticals
Phase IV	Licensed drug for wider purpose	Might arise with functional foods and nutraceuticals as legislative procedures are developed

double blind/(non-blinded) and randomized/(non-randomized) controlled trials. Commonly, nutritional studies in human may examine the biological effect of a nutrient or food commodities or the effect of a specific type of diet (such as the Mediterranean, Western-style or low-fat dietary patterns). Besides, observational epidemiological studies are based on the ability of the epidemiological instruments to measure the habitual dietary intake and are divided into descriptive (correlational, case-report series, cross sectional) and analytical experimentation (case-control, cohort), and meta-analyses (Mortensen *et al.* 2008).

Although human studies represent the final objective of nutritional research, on occasions the effect of nutrition on health and disease cannot be fully explained through these. Human clinical trials often only provide information about end-points of interest, the intervention studies being much too short. Moreover, the studied phenomenon is generally quantified by the measurement of related chemical biomarkers. Environmental factors, lifestyle, difficult organization, ethical considerations and poor compliance by human subjects hinder the interpretation of the results even more. In addition to all these considerations, the lack of knowledge about how bioactive compounds act at the molecular level and their role in the physiological processes represents the reason why alternative model systems (such as *in vitro* and animal models) are widely used in nutritional research (Mortensen *et al.* 2008).

A multi-disciplinary approach involving epidemiological, cellular or molecular studies and human intervention is necessary for the polyphenol research. The start-point hypothesis is based on epidemiological data relating the intake of a food rich in polyphenols with a specific effect. Subsequently, intervention studies with the food have to be performed to identify changes in the metabolism or health/disease biomarkers. Over recent decades, once the bioactive molecule

had been identified, the next step was to isolate the biactive molecule from the food or chemical synthesis, the potential bioactivity of which was then evaluated *in vitro* (Figure 7, Model A) (Kay 2010). Nonetheless, structural modifications of the phenolic molecules occur during food digestion and metabolism and the absorption, metabolism and tissue distribution can be influenced by the food matrix. Thus, the new tendency is to know the possible modifications that the bioactive compounds can undergo during food processing and establish the post-consumption pharmacokinetics and metabolism of the food that include the bioactive compounds. This way, the physiological concentration of metabolites can be established and the relevant identified structures assayed by *in vitro* or *in vivo* studies to evaluate their bioactivity at physiological levels (Figure 7, Model B) (Kay 2010). Hence the importance of the development of

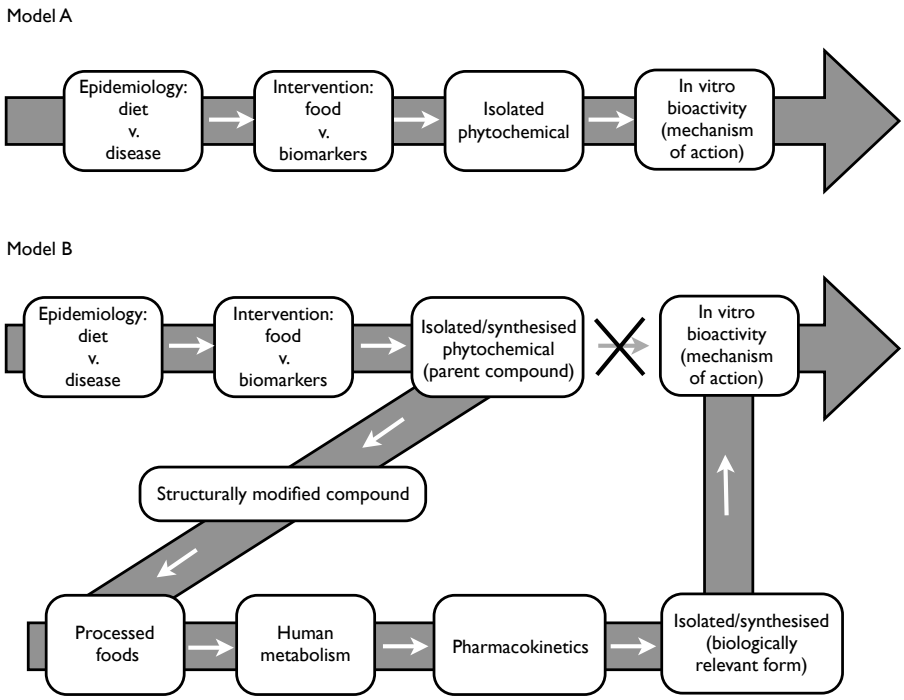

Figure 7: Multi-disciplinary approach of polyphenol research (Kay 2010).

new strategies to investigate the absorption, metabolism and tissue distribution of phenolic compounds and, at the same time, the possible identification of bioactive compounds and their metabolic targets.

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HYPOTHESIS AND OBJECTIVES

The Mediterranean diet is a model of healthy eating promoted around the world due to its association with a lower risk of overall mortality and mortality from chronic metabolic disease, such as cardiovascular disease, cancer, or neurodegenerative disease. Olive oil and nuts are two important Mediterranean vegetable sources, which have been widely studied in relation to their metabolic benefits due to their special lipid profile and polyphenol content, giving rise to documented evidence that has led to the inclusion of these foods in the dietary guidelines of the *American Heart Association*. Furthermore, their consumption have been prompted by the *U.S. Food and Drug Administration* and more recently, the European Union has included olive oil and walnuts in its list of permitted health claims made on foods (Commission Regulation (EU) No. 432/2012), being the only phenolic sources included in that list. According to the Commission Regulation, the permitted claim for olive oil polyphenols is "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"; and the claim permitted for walnuts is "Walnuts contribute to the improvement of the elasticity of blood vessels". Although the walnut is a subject of a health claim only according to their special fatty acid composition, obviating their phenolic profile, there is enough evidence to justify future scientific studies related to the importance of phenolic compounds from nuts (highlighting procyanidins).

In this context, this thesis has focused on developing useful tools for estimating the digestibility, bioavailability, metabolism and distribution of phenolic compounds representing a step prior to human intervention studies. For that purpose, the experiments have focused on the study of flavonoids, as one of the most common groups of phenolic compounds in the human diet, and olive oil phenolic compounds, as a singular phenolic group. The selection of both phenolic families has been reinforced by the Antioxidant Research Laboratory running two projects, the MET-DEV-FUN project, which was mainly focused on

procyanidins as a functional ingredient to prevent cardiovascular disease and the AGL2009-13517-C03-02, which was focused on virgin olive oil phenolic compounds as a functional ingredient to improve the functionality of HDL.

The objectives proposed to reach that target are described below:

I. Study of the digestibility and bioaccessibility of dietary phenolic compounds by the use of an *in vitro* digestion system. Evaluation of the food matrix effect.

II. Development of an *in vitro* colonic fermentation system to mimic the colonic fermentation of dietary phenolic compounds using fresh faecal material. Application of individual phenolic compounds to establish the colonic metabolic pathway previously to the fermentation of a complex phenolic-rich food matrix.

III. Development and validation of sensitive and robust analytical solutions by the use of ultra-performance liquid chromatography coupled to tandem mass spectrometry with electrospray ionization as the ionization technique (UPLC-ESI-MS/MS) to identify and quantify phenolic compounds and their metabolites in biological samples (plasma and tissues).

IV. Evaluation of pharmacokinetic parameters by plasma analysis and tissue distribution of phenols and their metabolites. Analysis of the effect of the food matrix.

    IV. i. Application to procyanidins.

    IV. ii. Application to olive oil phenolic compounds.

V. Study the in utero exposure effect of a high fructose and saturated fat diet on the hepatic glucuronidation capacity toward quercetin.

RESULTS AND DISCUSSION

In vitro models

Digestion and colonic fermentation

Serra et al. British Journal of Nutrition (2010) 103, 944–952
Serra et al. Food Chemistry (2011) 3, 1127-1137
Serra et al. Food Chemistry (2012) 130, 383-393

Bioavailability of procyanidin dimer and trimer and matrix food effects in *in vitro* and *in vivo* models

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BIOAVAILABILITY OF PROCYANIDIN DIMERS AND TRIMERS AND MATRIX FOOD EFFECTS IN *IN VITRO* AND *IN VIVO* MODELS

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Abstract

Among procyanidins (PC), monomers, such as catechin and epicatechin, have been widely studied, whereas dimer and trimer oligomers have received much less attention, despite their abundance in our diet. Recent studies have showed that as dimers and trimers could be important in determining the biological effects of procyanidin-rich food, understanding their bioavailability and metabolism is fundamental. The purpose of the present work is to study the stability of PC under digestion conditions, the metabolism and the bioavailability by using a combination of *in vitro* and *in vivo* models. Simultaneously, the matrix effect of a carbohydrate-rich food on the digestibility and bioavailability of PC is investigated. The results show a high level of stability of PC under gastric and duodenal digestion conditions. However, the pharmacokinetic study revealed limited absorption. Free forms of dimers and trimers have been detected in rat plasma, reaching the maximum concentration 1 h after oral intake of a grape seed extract.

Keywords: Procyanidins; Dimer; Trimer; Digestion; Bioavailability

1 INTRODUCTION

Procyanidins (PC) are found in most plants and in a wide range of foods, such as red wine, cocoa, tea and fruits, and thus they are a part of the human diet. PC belong to the group of flavonoids, and are phenolic compounds mainly formed of (+)-catechin and (−)-epicatechin units with C_4–C_8 and/or C_4–C_6 bonds. PC are

Abbreviations: GSPE, grape seed procyanidin extract; IN, fraction found inside dialysis solution; OUT, fraction found outside dialysis solution; PC, procyanidins.

formed from the condensation of monomeric units, between two and five units for oligomers and over five units for polymers[1].

Grape seed procyanidin extracts (GSPE) have shown several bioactivities. They improve antioxidant cell defences[2] and the plasma lipid profile[3,4], limit adipogenesis[5] and function as insulinomimetic[6] and anti-inflammatory[7] agents. A preliminary study of an *in vitro* model by the authors has also determined that dimeric and trimeric oligomers are the most powerful PC molecules that mimic the complete GSPE [8]. Thus, to explain these health effects of PC and understand the mechanism by which PC act at the cellular level *in vivo*, it is essential to determine PC stability during the digestive process as well as their bioavailability.

Despite it being clear that monomers are absorbed in human subjects and animals, there are controversies about the bioavailability of oligomeric forms. Different studies, following the ingestion of chocolate[9–12], black and green tea[13–16], red wine [17,18] and grape seed extract [1], have shown that during digestion, the oligomers are fragmented into monomeric units of catechin and epicatechin. These are then absorbed, appearing in plasma and urine primarily as glucuronidated, methylated and sulphated metabolites. A study by Rios *et al.*[19] with six healthy subjects who consumed a rich PC cocoa beverage studied the depolymerisation of PC in the stomach and proved that they were remarkably stable in the stomach environment. Another study by Sano *et al.* [20] was the first work to detect procyanidin B1 in human serum after oral intake of a GSPE, the maximum concentration appearing 2 h after intake. As well as stability during digestion, the dose of intake could determine the primary site of phenol metabolism. Large doses are metabolised primarily in the liver, and small doses may be metabolised by the intestinal mucosa, with the liver playing a secondary role to further modify the polyphenol conjugates from the small intestine[21].

The bioavailability of polyphenols has been also reviewed, especially to focus on intestinal absorption and the influence of their chemical structure (for example glycosylation, esterification and polymerisation) and the effect of the food matrix composition[22]. Different studies have been carried out into the interaction between polyphenols and a food matrix such as milk[23, 24], olive oil[25] or sugar[26]. The evidence suggests that variations in polyphenol absorption also occur due to interactions between polyphenols and other food components. For example, some authors suggest that the carbohydrate composition of the meal may influence flavonoid absorption through an effect on the motility of gastric secretions and hepatic blood flow[26]. The polyphenol–protein interaction has also been studied, and shown to be similar to antigen–antibody interactions. The results of these studies suggest that interactions may occur between digestive proteins and monomers and dimers of flavanols from grape seed extract during pancreatic digestion[27].

Nevertheless, one of the most important limitations of the bioavailability studies in human subjects or animal models is related to inter-individual variability. *In*

vitro digestion models are a very useful tool for studying the stability of food components and evaluating the potential effects of the food matrix on their bioavailability. Reviewing the literature, a wide range of *in vitro* digestion methods have been designed to simulate the effect of digestion on food or food components and study its consequences. *In vitro* digestion methods can be classified into two categories[28]: static[29]; dynamic[30, 31] gastrointestinal models. The static models simulate the transit of alimentary bolus through the human digestive tract by sequential exposure of food to simulated mouth, gastric and small intestinal conditions. In contrast, the dynamic gastrointestinal models mimic the gradual transit of ingested compounds through the digestive tract.

Other important limitation related to the study of PC bioavailability concerns the low concentration of their metabolites in biological samples[1]. Therefore, measuring them requires a precise, robust, sensitive and selective analytical technique that allows them to be identified and quantified. Liquid chromatography with MS is a powerful tool for both confirmatory and quantitative analyses in complex matrices due to its high sensitivity and selectivity. Apart from the analytical technique, the use of an adequate extraction procedure to minimise the matrix effects is very important.

As PC dimers and trimers could be important in determining the biological effects of PC-rich food, understanding the bioavailability and metabolism of PC oligomers is fundamental. The objective of the present research is to study the stability of PC under digestion conditions, the metabolism and the bioavailability by using a combination of *in vitro* and *in vivo* models. Simultaneously, the matrix effect of a carbohydrate-rich food on digestibility and bioavailability of PC is investigated.

2 EXPERIMENTAL

2.1 Chemicals and reagent

A GSPE was used as the source of PC. The PC content of the extract was >75 % (monomers 22 %, dimers 20 % and trimers to pentamers 56 %). The composition of commercial cereal-based food, used as carbohydrate-rich food, was as follows: starch (hydrolysed wheat, wheat, barley, maize and oats); malt extract; mineral salts (Ca, Fe and Zn); vitamins (C, niacin, E, thiamine, A, B_6, B_9 and D); flavouring (vanillin).

Internal standard catechol and the standards of (−)-epicatechin and (+)-catechin were purchased from Sigma Aldrich (St Louis, MO, USA); procyanidin B2 from Fluka Co. (Buchs, Switzerland) and trimer was isolated from phenolic extract [32] using the technique described by Serra *et al.*[33]. The cocoa nibs were kindly donated by La Morella Nuts, S.A. (Reus, Spain). Individual stock standard solutions and the internal standard were dissolved in methanol and stored in a dark-glass flask at −18°C. Standard mixtures, at different concentrations, were prepared by appropriate dilution of the stock solutions, and these were stored in dark-glass flasks at 4°C before chromatographic analysis.

Acetonitrile (HPLC grade), methanol (HPLC grade), acetone (HPLC grade) and glacial acetic acid (≥ 99·8 %) were

of analytical grade (Scharlab, Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

2.2 Simulated Gastrointestinal Digestion

A gastrointestinal *in vitro* digestion model was performed based on the methodology described by Ortega *et al.* [34]. The discontinuous model describes a three-step procedure to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (duodenal digestion) (Fig. 1). In order to evaluate the food matrix effect, two different samples were subjected to the digestion model: 300 mg PC extract (GSPE); 300 mg GSPE+600 mg carbohydrate-rich food. After the gastric step, the digestion mixture was placed in tubes and centrifuged for 15 min at 12 000 g at 4°C, yielding the chyme (supernatant or soluble fraction) and the pellet (cloud or non-soluble fraction)[34]. Both fractions, the chyme and pellet, were freeze-dried (BMG Labtech, Offenburg, Germany) and stored for chromatographic analysis of the PC content.

At the end of the dialysed duodenal digestion step, two fractions were collected and analysed separately[34]: the outside dialysis solution (OUT) (Fig. 1), which was considered the dialysable fraction; and the inside dialysis tub content (IN), referring to the non-dialysable fraction. The dialysable fraction (OUT) is considered the fraction that could be available for absorption into the systematic circulation by passive diffusion; while the non-dialysable fraction (IN) is attributed to the digested fraction that would reach the colon fermentation intact. Both fractions were freeze-dried and stored for chromatographic analysis of the PC content.

2.3 Chromatographic analysis of procyanidins in digested fractions

The digested freeze-dried fractions (chyme, pellet, IN and OUT) were dissolved in acetone–Milli-Q water–acetic acid (70:29·5:0·5, v/v/v) at a ratio of 1:5. The tubes were vortexed for 5 min and centrifuged at 12 500 rpm. The supernatant containing the solubilised phenols was filtered through 0·22 μm nylon filters before chromatographic analysis by ultraperformance liquid chromatography–MS/MS using a Waters Acquity ultraperformance liquid chromatography system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters)[34]. The ultraperformance liquid chromatography analyses were performed using an Acquity high strength silica T3 column (100 mm × 2·1 mm inner diameter, 1·8 μm particle size; Waters) with a binary mobile phase. Solvent A was water–acetic acid (0·2 %), and the solvent as acetonitrile. Catechin and epicatechin were quantified using the curves of the respective standards. Epigallocatechin, epicatechin gallate and epigallocatechin gallate were quantified using the epicatechin curve. The PC, dimers and trimers were quantified with the calibration curves of the respective standards. The oligomers from tetramer to heptamer were quantified with the trimer calibration curve. The results of the quantification of the PC in pellet (non-soluble fraction) are expressed as μmol per g of pellet, and the results of the quantification of the PC in the chyme

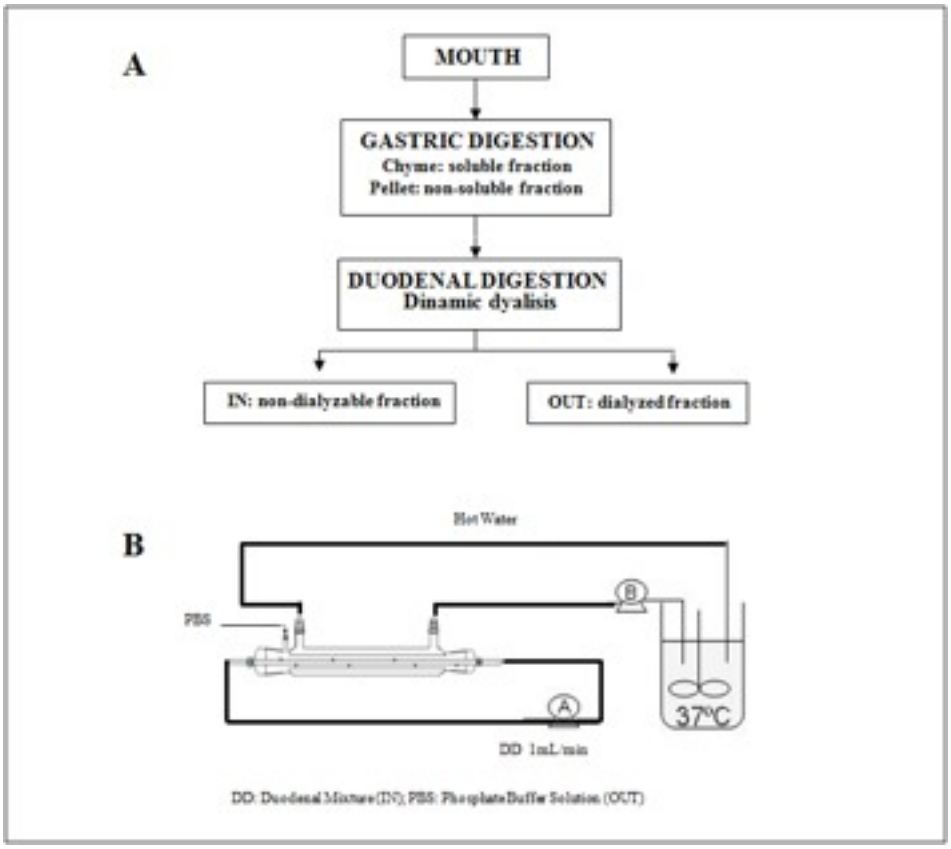

Figure 1. (A) Steps of the proposed *in vitro* digestion model. (B) Schema of the duodenal digestion with dynamic dialysis. DD, duodenal mixture (fraction found inside dialysis solution (IN)); PBS (fractions found outside dialysis solution (OUT)).

(soluble fraction), IN and OUT fractions are expressed in μM.

2.4 Pharmacokinetic study

Rat plasma samples were obtained from 3-month-old male Wistar rats weighing between 370 and 420 g and were purchased from Charles River Laboratories (Barcelona, Spain). The Animal Ethics Committee of the Rovira i Virgili University approved all the procedures. The rats were housed in temperature-controlled rooms (22°C) and subjected to a standard 12 h light–12 h dark cycle (with a light period of 09.00–21.00 hours). All the animals were fed a standard diet of PanLab A04 (Panlab, Barcelona, Spain) and water.

After the animals had been in fasting conditions for between 16.00 and 17.00 hours with only access to tap water, two experiments were carried out: a dose of the PC-rich extract, 1 g GSPE per kg of body weight dissolved in water; a dose of 2 g carbohydrate-rich food+1 g GSPE for

kg of body weight dissolved in water, which was administered to the rats by intragastric gavage. Three animals per experiment were terminally anaesthetised with ketamine–xylazine and euthanised by exsanguinations at 0 h (fasting conditions), 1, 2 and 4 h (postprandial conditions). Blood samples were collected from the abdominal aorta with heparin-moistened syringes. The plasma samples were obtained by centrifugation (2000 g, 30 min at 4°C) and stored at −80°C until the chromatographic analysis of the PC. The identification of the PC metabolites was performed by analyses in MS (full-scan mode) and MS/MS (based on neutral loss scan and product ion scan mode)[33]. First, analyses were carried out in the full-scan mode (from 419 to 1200 m/z) by applying different cone voltages from 20 to 60 V. When low cone voltages were applied, the MS spectrum gave information about the precursor ion or the $[M - H]^-$. In contrast, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their structure. The structural information was also verified by using product ion scan and neutral loss scan in the MS/MS mode.

All the samples were analysed in triplicate by ultraperformance liquid chromatography–MS/MS according to the method in Serra *et al.*[33]. The quantification of the free forms of the PC and their metabolites in the plasma samples was done using the calibration curves of the respective free forms, and the concentrations were expressed as catechin or epicatechin equivalents.

2.5 Statistical analysis

All the data are expressed as the mean of three replicates. In order to simplify the results shown in the tables, we omitted the standard deviation because all these values were lower than 10 %. The data were analysed by a one-way ANOVA test to assess the effect of the carbohydrate-rich food on the *in vitro* digestibility and *in vivo* pharmacokinetics of PC from the GSPE extract. A significant difference is considered at a level of $P < 0{\cdot}05$. All the statistical analysis was carried out using STATGRAPHICS Plus 5.1.

3 RESULTS

3.1 Evaluation of the stability of procyanidins in digestion by the *in vitro* simulated digestion model

The first part of the study was focused on the evaluation of the stability of PC during digestion using an *in vitro* model. The composition of the GSPE used as a PC and proanthocyanidin source in the study is summarised in Table 1. Dimers and trimers were the most abundant compounds with 250·18 and 1568·72 μmol/g extract, respectively. The digestion procedure was applied to GSPE dissolved in water, and the same extract was mixed with cereal-based food in order to evaluate the influence of the food matrix with a high-carbohydrate content on the digestion stability of the PC.

The compounds determined initially in GSPE (Table 1) were analysed in all fractions of *in vitro* digestions to evaluate their digestibility and stability. After the gastric step, the content of PC and proanthocyanidins was measured in the soluble and non-soluble fractions, respectively, called the chyme and pellet

(Fig. 1), and the results were expressed as μmol of concentration in the chyme fraction and as μmol per g of pellet (Table 2). After the gastric step, the amount of PC in the chyme was higher than in the pellet fraction independently of the presence of the carbohydrate-rich food in the digestion mixture. The PC oligomers, dimers to heptamers, showed higher concentrations than the monomers (catechin and epicatechin) in the chime and pellet fractions, the trimer form being the most abundant compound in the gastric fractions, reaching a concentration of 50 279·77 μmol in the chyme fraction after the gastric step. Nevertheless, forms with a high degree of polymerisation (pentamer, hexamer and heptamer) were not detected in pellet fraction.

After the duodenal step, an important loss of PC was observed, and most were retained in the non-dialyzable fraction (IN). Nevertheless, dimers and trimers were quantified in the dialyzable (OUT) fraction (Figure 1), as well as the monomeric structures. As a consequence of the duodenal digestion conditions, decomposition of oligomeric forms was observed in the respective monomeric forms. Additionally to the depolymerization of GSPE, a decrease in the ratio catechin/epicatechin was observed in the digestion mixture. Initially, in the GSPE extract, this ratio was approximately 1·1 and after digestion, a change in the ratio of roughly 0·7 was observed in all fractions.

Finally, the comparative analysis between the concentrations of the different PC in the digestion mixtures revealed that the presence of carbohydrate-rich food, as a source of carbohydrates, showed a significant effect on the digestibility of PC in the *in vitro* digestion model. When PC and proanthocyanidins were submitted to digestion together with carbohydrate-rich food, the amount of catechin and epicatechin present in the digestion mixture after the gastric step was significantly higher ($P < 0·05$) (Table 2). By contrast, the concentration of the other compounds was greater when GSPE was digested without carbohydrate-rich food. Greater differences were observed in the non-soluble fraction (pellet) (Fig. 1), showing a higher concentration of PC ($P < 0·05$) when the extract was digested with carbohydrate-rich food. By contrast, the effect of carbohydrates on the digestibility during duodenal digestion was lower. Thus, the concentration of PC in the dialysed fraction (OUT) was similar independently of the presence of carbohydrates, with the exception of the epigallocatechin gallate that showed a lower concentration ($P < 0·05$) in the presence of carbohydrates. On the other

Table 1. Phenolic composition of grape seed procyanidin extract (GSPE)*.

Compound	Concentration (μmols/g)
Catechin	58
Epicatechin	52
Epigallocatechin	5.5
Epicatechin gallate	89
Epigallocatechina gallate	1.4
Dimer	250
Trimer	1568
Tetramer	8.8
Pentamer	0.73
Hexamer	0.38
Heptamer	0

** The results are expressed as* μmols*/g of GSPE.*

Table 2: Procyanidin and proanthocyanidin contents in the different fractions of the gastric and duodenal steps of the simulated gastrointestinal digestion of grape seed procyanidin extract (GSPE) without and with carbohydrate-rich food.

	GSPE				GSPE+ carbohydrate-rich food			
	Gastric		Duodenal		Gastric		Duodenal	
Compound *	Chyme (μM)	Pellet (μmols/g)	OUT (μM)	IN (μM)	Chyme (μM)	Pellet (μmols/g)	OUT (μM)	IN (μM)
Catechin	1755 [a]	7.4 [a]	95 [a]	451 [a]	1928 [b]	37 [b]	83 [a]	608 [b]
Epicatechin	2221 [a]	9.6 [a]	126 [a]	613 [a]	2499 [b]	42 [b]	114 [a]	823 [b]
Epigallocatechin	258 [a]	0.49 [a]	2.6 [a]	37 [a]	186 [b]	1.41 [b]	2.4 [a]	34.5 [b]
Epicatechin gallate	6305 [a]	29.8 [a]	109 [a]	1772 [a]	5272 [b]	191 [b]	72 [b]	2111 [b]
Epigallocatechin gallate	136 [a]	0.409 [a]	0.80 [a]	28.7 [a]	91 [b]	1.9 [b]	0.65 [a]	25.3 [b]
Dimer	12953 [a]	37.1 [a]	197 [a]	3517 [a]	11485 [b]	124 [b]	168 [a]	4005 [b]
Trimer	50279 [a]	109 [a]	147 [a]	8101 [a]	37505 [b]	356 [b]	111 [a]	8659 [a]
Tetramer	10076 [a]	21.0 [a]	0.25 [a]	1748 [a]	5368 [b]	82 [b]	ND	1673 [b]
Pentamer	16.8 [a]	ND	ND	12.7 [a]	ND	ND	ND	ND
Hexamer	185 [a]	ND	ND	16.9 [a]	49 [b]	ND	ND	6.6 [a]
Heptamer	0.66 [a]	ND	ND	0.73 [a]	2.34 [b]	ND	ND	ND

OUT, fraction found outside dialysis solution; IN fraction found inside dialysis solution; ND, not detected.
[ab]Mean values within a column with unlike superscript letters were significantly different ($p<0.05$) between GSPE + carbohydrate-rich food and GSPE in each digestion step (unpaired Student's t test).

hand, significant differences were observed in the non-dialysable fraction (IN), except the forms with a higher degree of polymerisation (pentamers to heptamers), which showed the same concentration under the two digestion conditions. However, no clear tendency was observed in the results of non-dialysable (IN) fraction in relation to the presence or absence of carbohydrates.

3.2 Pharmacokinetic of procyanidins

Figs. 2 and 3 show the results of the pharmacokinetic study of rat plasma after the ingestion of GSPE without and with carbohydrate-rich food. Under basal conditions (time = 0), PC were not detected in rat plasma. Two hours after the GSPE ingestion, several metabolites, such as catechin glucuronide and epicatechin glucuronide, reached peak plasma concentrations with 6·24 and 9·74 nM, respectively, when GSPE was ingested with carbohydrate-rich food, and 6·32 and 8·71 nM when GSPE is ingested without carbohydrate-rich food. In contrast, the concentration of PC, free forms of dimers and trimers in the plasma reached a maximum 1 h after administration. These free forms appeared in the plasma faster than the glucuronidated forms of the monomers. Dimer concentration was greater after the ingestion of GSPE without the presence of carbohydrate-rich food, reaching a postprandial concentration in plasma of 0·57 nM as against 0·12 nM when the GSPE was ingested with carbohydrate-rich food. Similarly, the presence of the carbohydrate-rich food reduced the maximum concentration of the trimer in the plasma. Non-conjugated forms of

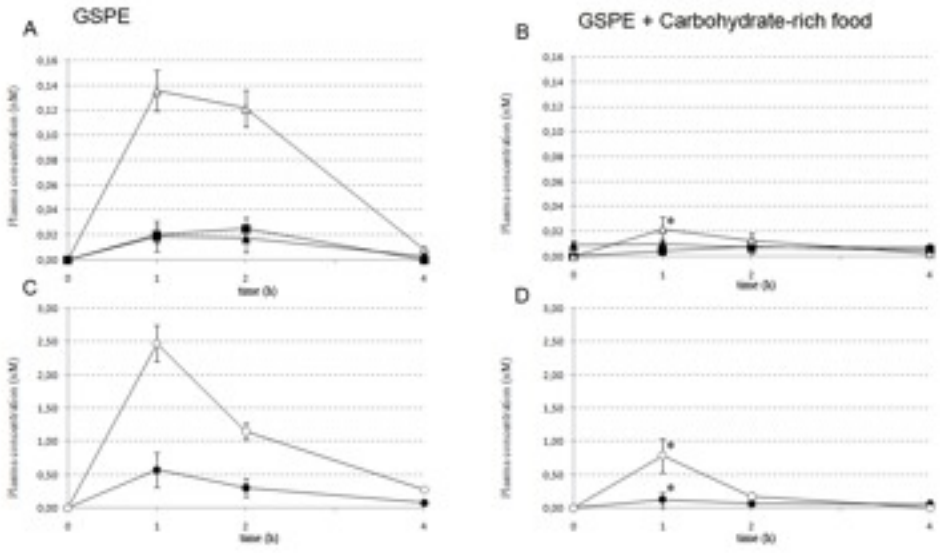

Figure 2. Pharmacokinetic curves of monomeric forms, (■) catechin, (△) epicatechin, (▲) and epicatechin gallate (A,B), and polymeric forms, (●) dimer and (○) trimer (C, D), detected in rat plasma collected between 0h and 4h after the ingestion of GSPE with and without carbohydrate-rich food. The results are expressed as nM. *Means value was significantly different from that without carbohydrate-rich food ($P<0.05$; unpaired *Student's t test*).

monomers (catechin, epicatechin and epicatechin gallate) were present in the plasma at very low concentration, less than 0·03 nM under the two experimental conditions. Glucuronidated forms of catechin and epicatechin were the most abundant, and the second most abundant metabolite was methyl-glucuronidated forms, independently of the presence of the carbohydrate-rich meal.

3.3 Comparison between *in vitro* digestion method and *in vivo* bioavailability

Fig. 4(a) and (b) show the percentage of different PC present in the dialysed fraction (OUT) of the duodenal digestion mixture corresponding to the *in vitro* digestion model (Fig. 1); and Fig. 4(c) and (d) show the percentage of free PC and their metabolites in the rat plasma after the ingestion of the GSPE extract (*in vivo* model). The expression of the quantification of PC as percentages in both the *in vitro* and *in vivo* models aims to normalise the results to facilitate their comparative analysis independently of whether the concentrations of the compounds varied widely in both models. In all the plasma samples, epicatechin was the most abundant compound, glucuronidated forms being 88 and 89 % of the total, when GSPE was digested alone and with carbohydrate-rich food, respectively. This was followed by catechin, which was the second most abundant compound. Nevertheless, the

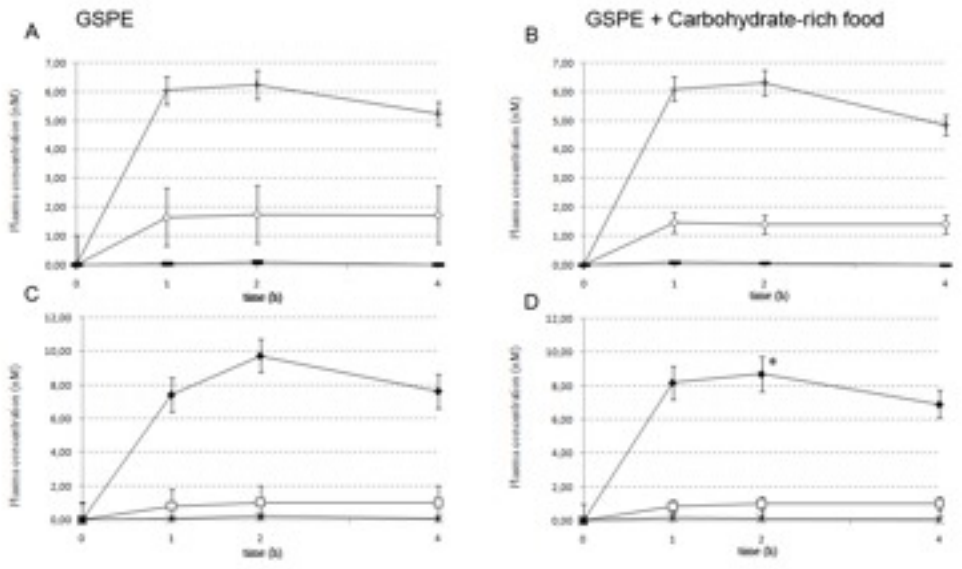

Figure 3. Pharmacokinetic curves of grape seed procyanidin extract (GSPE) metabolites according to the monomer structure, being (—◇—) catechin-methyl-glucuronide, (—+—) catechin glucuronide and (—▬—) catechin-methyl-sulphate (A,B), and (—□—) epicatechin-methyl-glucuronide, (—●—) epicatechin glucuronide and (—✳—) epicatechin-methyl-sulphate (C, D), detected in rat plasma collected between 0h and 4h after the ingestion of GSPE with and without carbohydrate-rich food. The results are expressed as nM. *Means value was significantly different from that without carbohydrate-rich food ($P<0.05$; unpaired *Student's t test*).

dimer structure was the most substantial PC quantified in the dialysed fraction (OUT) after the duodenal step of the *in vitro* digestion model, reaching a percentage of almost 30 % (Fig. 4(a) and (b)); however, this compound was a very low percentage in the plasma samples (1·69 % when the GSPE was administered alone and 0·35 % when it was administered with carbohydrate-rich food) (Fig. 4(c) and (d)). The quantification of the trimer in the plasma samples showed percentages under 1 %, independently of the presence or absence of carbohydrate-rich food in the intake, whereas its percentage in the dialysed fraction (OUT) of the duodenal digestion was higher, close to 20 %. Epicatechin gallate behaved similarly.

4 DISCUSSION

We have shown, using *in vitro* cell lines, that dimer and trimer PC are the most powerful molecules that mimic the *in vivo* effects of GSPE[1]. As there is some controversy about the bioavailability of dimeric and trimeric PC, we used a combination of *in vitro* and *in vivo* models in order to measure the bioavailability of dimer and trimer PC. The effects of the digestion process on their stability and

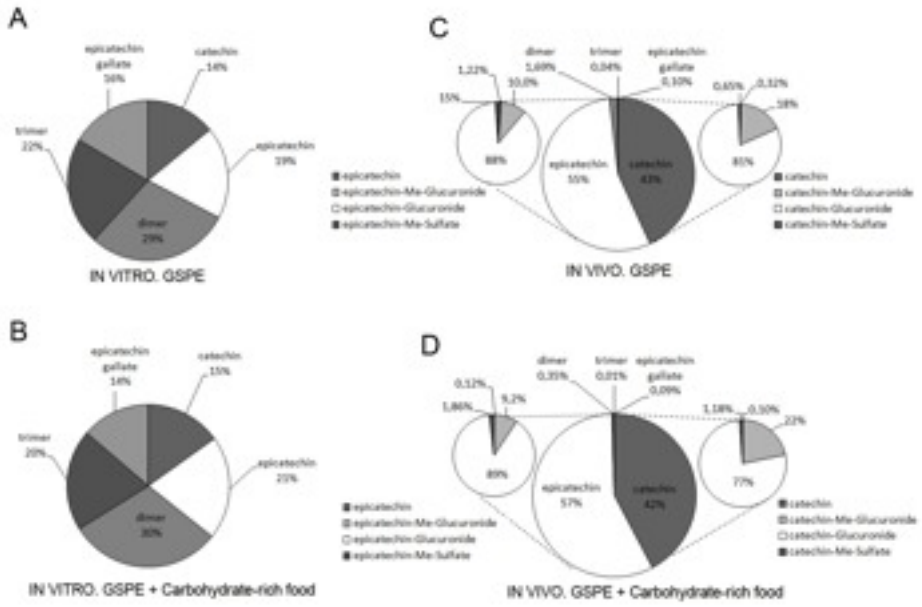

Figure 4. (A and B) Percentage of the catechin, epicatechin, epicatechin gallate, dimer and trimer in the dyalized-fraction (OUT) of the duodenal mixture. (C and D) Percentage of the catechin, epicatechin, and their respective metabolites, epicatechin gallate, dimer and trimer in the postprandial rat plasma.

the food matrix effect on their potential bioaccessibility were also evaluated.

The GSPE used as a source of PC in the present study contained a substantial amount of high molecular weight polymeric PC, dimer and trimer being the most abundant. After the simulated *in vitro* digestion, these PC oligomers were present in high concentration in all digestion fractions, showing an important stability under gastric and duodenal digestion conditions. The most important aspect is related to the increase in their concentration observed in the digestion mixtures after the two steps. This is probably related to the partial hydrolysis of the highly polymerised molecules or condensed tannins in the GSPE. However, the pharmacokinetic study revealed a limited absorption. The limitation in the diffusion across the epithelial cells could explain the nanomolar levels quantified in the postprandial rat plasma after the intake, at micromolar levels, of the oligomeric PC. The pharmacokinetic results shown in the present study agree with previous research that has shown the presence of some dimers (B2 and B5), detected at nanomolar levels, in the plasma of rats given cocoa extracts[35, 36]. By contrast, in other studies, the dimer (B3) and trimer (C2) were not detected in the plasma of rats given purified compounds[37]. With regard to the metabolism, the major metabolites observed in rat plasma in the present study were glucuronidated forms

of catechin and epicatechin, although there were also high levels of methylated-glucuronide forms. Similarly, a previous study by Spencer *et al.*[38] reported that methylated-glucuronide forms were the most bioavailable forms in the small intestine.

When the GSPE extract was submitted to the *in vitro* digestion model, there were high concentrations of the monomers catechin and epicatechin and mainly the dimer and trimer oligomeric flavanols in the gastric fractions (chyme and pellet), showing an important stability under acid conditions. Similar results were observed by Spencer *et al.*[39]. The results of pancreatic digestion showed a lesser decrease in the concentration of PC with a high degree of polymerisation, because a slight decomposition of the oligomeric forms into monomers was seen after the duodenal digestion, being greater when the extract was digested with carbohydrate-rich food. Thus, PC were less stable under alkaline conditions (pH = 6·9) and in the presence of digestive enzymes than under acid conditions in the gastric step. After the evaluation of the stability of the PC under digestion conditions, the next phase was focused on the *in vitro* estimation of the potential bioaccessibility and potential absorption in the systemic circulation in biological systems. For this proposal, a dialysis phase was considered for the duodenal step, estimating the passive diffusion of the digested PC solubilised in the water phase (IN) (Fig. 1). Comparing the total concentration of PC in the duodenal mixture (IN and OUT) after the duodenal digestion with the total concentration in the digestion mixture after the gastric step, a substantial loss was observed. The losses in the PC content of the duodenal digestion mixture could be attributed to the large oligomers being held on the dialysis membrane, reducing the recovery of the PC to be analysed during the washing of the dialysis tub. Additionally, the large oligomers could reduce the membrane's surface. This problem was also reported by Bermúdez-Soto *et al.*[39], who argued that the dialysis membrane as a means of estimating availability for absorption had some limitations. However, some authors have reported that PC are unstable under alkaline conditions[36, 40,41], and this instability also may explain the decrease of PC concentrations in the digestion mixture after pancreatic digestion. According to Rios *et al.*[19], the stability of PC in the stomach and their very limited absorption in the small intestine suggest that they may influence digestion or the physiology of the gut through direct interactions with the gut mucosa and gut lumen solutes.

On the other hand, a decrease in the catechin:epicatechin ratio was observed during digestion in the *in vitro* model in the present study. The increase in epicatechin could be the consequence of the depolymerisation of GSPE, which is mainly composed of epicatechin units, into monomeric forms. However, in the study by Rios *et al.*[19], no epimerisation was observed during the stomach digestion and oligomers to pentamers were stable during stomach transit. The results of the present study corresponding to the *in vivo* model revealed epicatechin and its metabolites as the predominant plasma monomer absorbed (Fig. 2). This fact has been also observed by other authors[42] who

showed in human subjects that after consuming a cocoa beverage containing equal amounts of epicatechin and catechin, the former was identified as the predominant plasma flavanol absorbed.

The pharmacokinetic study of the rat plasma revealed that the glucuronidated forms were the most abundant metabolite. These results agree with those in Vaidyanathan & Walle [43] who observed that rat liver microsomes efficiently glucuronidate epicatechin. Glucuronidation of catechin has been described to be more active in rat livers than in the intestine, in both *in vitro*[44] and *in vivo* models[45]. Traces of glucuronidated and sulphated metabolites from monomers have also been detected in the lumen of gut from rats treated with GSPE[1]. One of the most important factors determining the metabolic fate of polyphenols in human subjects is the quantity ingested[46]. According to Hacket *et al.*[47], when food polyphenols are administered at a pharmacological dose, they are found in free forms in the blood. By contrast, when the dose is decreased to only a few milligrams, the monomers were conjugated and no free forms were detected[18]. Related to this, in the present study, the free forms of catechin and epicatechin were found at very low concentrations in the plasma samples after the ingestion of GSPE. The present results may be coherent with the dose administered to the rats, because the dose used is an intermediate position between the pharmacological dose and typical dose of polyphenols present in rich foods.

The last objective in the present study was to evaluate the effect of carbohydrate-rich foods on the bioavailability of the GSPE. Relatively, little is known about the effect of food on the bioavailability of PC. Food within the gastrointestinal tract is known to markedly alter the oral absorption of many xenobiotic compounds[48]. In the present *in vitro* study, when the extract was ingested with carbohydrate-rich food, an enhanced uptake of the monomeric PC was observed. This result is in agreement with the results in Schramm *et al.*[26], who reviewed that the effect observed with carbohydrate-rich foods (bread, sucrose and grapefruit juice) may have been mediated by a carbohydrate-specific effect on gastrointestinal physiology (e.g. motility and/or secretion) or a carbohydrate-specific enhancement of the activity of a yet unidentified carbohydrate–flavanol transporter.

In conclusion, dimer and trimer PC are absorbable *in vivo*, reaching maximum concentrations in the plasma as soon as 1 h after PC ingestion. Their low concentrations in the plasma are not the consequence of instability during the digestion process as the *in vitro* digestion model shows high levels of dimer and trimer after duodenal digestion. Moreover, absorption of dimer and trimer PC is repressed by the simultaneous presence of carbohydrate-rich food.

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Metabolic pathways of the colonic metabolism of procyanidins (monomers and dimer) and alkaloids

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METABOLIC PATHWAYS OF THE COLONIC METABOLISM OF PROCYANIDINS (MONOMERS AND DIMERS) AND ALKALOIDS

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Abstract
Procyanidins are metabolised by endogenous gastrointestinal microflora into several small molecules that may exert systemic effects. However, the metabolic pathways of procyanidins are still largely uncharacterised. The aim of this study was to evaluate the colonic metabolism of procyanidins (catechin, epicatechin, epicatechin gallate, epigallocatechin gallate and dimer B2) and alkaloids (theobromine and caffeine) by an *in vitro* colonic fermentation model using rat colonic microflora. Simultaneously, a nuts-cocoa cream was incubated and the colonic metabolism of procyanidins and alkaloids was evaluated. Results showed that most of the procyanidins tested were catabolised after 24–48 h of fermentation. Fermentation of the dimer B2 did not produce the same catabolic compounds as epicatechin fermentation and differences were observed between the fermentation of catechin and epicatechin. The alkaloids were not metabolised by the colonic microflora and this fact was verified *in vivo*. The results showed an intense metabolism of procyanidins and poor metabolism of alkaloids.

Keywords: Alkaloids / Colonic microflora fermentation / Digestion / Metabolic pathway / Procyanidins.

1 INTRODUCTION

The concept of fermentation in the digestive tract is defined as the process of deriving energy through the oxidation of organic compounds, such as carbohydrates, not digested in the upper gut (Arasaradnam *et al.*, 2009). The body fermentation process is carrying out by the endogenous gastrointestinal microflora, made up of three domains of life, bacteria, archaea and eukarya (Bäckhed, Ley, Sonnenburg, Peterson, &

Gordon, 2005), including more than 800 different bacterial species (Laparra and Sanz, 2010 and Montalto *et al.*, 2009). Bacterial concentrations in the colon can reach 10^{12} CFU/ml (Colecchia *et al.*, 2009), and mainly comprise obligate anaerobes, such as *Lactobacilli* and *Clostridium*, or bacteroides and bifidobacteria, which are the most important genera of intestinal microbiota in animals and humans (Salminen *et al.*, 1998), and facultative anaerobes such as *Streptococci* and enterobacteria (Salminen *et al.*, 1998). This complex microbial community is able to degrade the non-digested food components, such as non-digestive carbohydrates, and transform these into small molecules called microbial metabolites (Kleessen *et al.*, 2000 and Salminen *et al.*, 1998). Proteins and amino acids can also be effective as growth substrates for colonic microflora (Salminen *et al.*, 1998). With regard to minor food compounds, the dietary intake of polyphenols has been estimated to range between 150 and 1000 mg/day (Rusconi and Conti, 2010, Scalbert *et al.*, 2002 and Scalbert and Williamson, 2000) with some notable differences. For example, the typical Spanish diet contributes a mean daily intake of polyphenols of between 2590 and 3016 mg/day (Saura-Calixto, Serrano, & Goñi, 2007) whilst the mean total intake of polyphenols in Finnish adults is around 860 mg/day (Ovaskainen *et al.*, 2008). Proanthocyanidins are some of the most abundant phenolic compounds in the human diet (Gu *et al.*, 2004) and procyanidins the most common subclass of proanthocyanidins. The intake of this type of minor compounds is linked to the consumption of plant products like cocoa, considered one of the major sources of dietary antioxidants in the American diet after fruits and vegetables, with a mean daily intake of 14.4 g/day (Rusconi & Conti, 2010). Cocoa beans are rich in procyanidins with a concentration in freshly harvested beans of verified genetic origin ranging from 2189 to 4327 mg/100 g of dry defatted samples. Besides procyanidins, alkaloids are another important group of minor compounds present in the cocoa derivates. Theobromine and caffeine are the major alkaloids in cocoa products with 2.4–3.2 g/100 g and 0.3–1 g/100 g of dry defatted sample respectively (Rusconi & Conti, 2010).

Several studies have shown that polyphenols are degraded into phenolic acids, such as phenylvaleric acid, phenylpropionic acid, phenylacetic acid, benzoic acid and hippuric acid. Nevertheless, the type of metabolic products depends on what phenolic compound is metabolised (Das, 1974, Gonthier *et al.*, 2003a, Gonthier *et al.*, 2003b and Rios *et al.*, 2003) and may also depend on the type of colonic flora that performs the catabolism. These microbial metabolites may exert systemic effects (Aura, 2008). Otherwise, these systemic effects may be related to the presence in the lumen intestine of the native forms of polyphenols or their metabolites, including hepatic conjugates and microbial degradation products (Aura, 2008). For example, the flavonoid, quercetin produced by gut microflora enzymes through deglycosylation of quercitrin (3-rhamnosylquercetin) exerts a higher effect on the down-regulation of inflammatory responses (Comalada *et al.*, 2005). Polyphenols and their derived

products can also affect the intestinal ecology by accumulation in the ileal and colorectal lumen of non-digested structures, non-absorbed components and phenol metabolites, like sulphate or glucuronide conjugates, excreted through the bile (Bazzocco, Mattila, Guyot, Renard, & Aura, 2008). In spite of these health benefits, the mechanisms of action, mainly the catabolism of polyphenols in the gut, are still largely uncharacterised (Laparra & Sanz, 2010). The study of the systemic effects of phenol catabolites requires knowing which molecule has the capacity of action and the metabolic pathway through which it is formed. Nevertheless, foods contain a mix of polyphenols, and a complex mixture of fermentation metabolites is obtained when these foods are digested.

Thus, the aim of this research was to study the individual colonic metabolism of procyanidins (catechin, epicatechin, dimer B2, epicatechin gallate (EGC) and epigallocatechin gallate (EGCG)) and alkaloids (theobromine and caffeine) by rat colonic microflora by an *in vitro* model. After the study of the colonic metabolic pathways of individual compounds, the colonic fermentation of procyanidins and alkaloids was evaluated by the fermentation of a nuts-cocoa cream with high content of procyanidins and alkaloids previously digested by an *in vitro* digestion. Finally the colonic metabolism was evaluated *in vivo* by the analyses of rat intestines after a single ingestion of the same nuts-cocoa cream.

2 EXPERIMENTAL

2.1 Chemicals

Internal standard (IS) catechol and the standard of (−)-epicatechin, (+)-catechin, epigallocatechin gallate (EGCG), epicatechin gallate (ECG), theobromine, caffeine, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, phenylacetic acid, 3,4-dihydroxyphenylacetic acid, 2-phenylpropionic acid, 3-(4-hydroxyphenyl)propionic acid and urea were purchased from Sigma Aldrich (St. Louis, MO, USA); procyanidin dimer B2, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)propionic acid from Fluka Co. (Buchs, Switzerland); $NaCO_3$, KCl, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2{\cdot}6H_2O$, Mo_7 $(NH_4)_6O_{24}{\cdot}4H_2O$ were purchased from Panreac Quimica S.A. (Barcelona, Spain); and $CuSO_4{\cdot}5H_2O$, NaCl, $Na_2SO_4{\cdot}10H_2O$ from Scharlau S.L. (Barcelona, Spain).

Individual stock standard solutions and the IS were dissolved in acetone and stored in dark-glass flasks at −18 °C. Standard mixtures were prepared at different concentrations by appropriate dilution of the stock solutions and they were stored in dark-glass flasks at 4 °C before chromatographic analysis. Acetonitrile (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade), hydrochloric acid 37% (reagent grade), ethyl acetate (HPLC-grade) and glacial acetic acid (≥99.8%) were purchased from Scharlab S.L. (Barcelona, Spain). Ortho-phosphoric acid 85% was purchased from MontPlet & Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Nuts-cocoa cream

The nuts-cocoa cream was kindly supplied by La Morella Nuts S.A. (Reus, Spain). The composition was: 49% lipids (26.5% polyunsaturated, 12% unsaturated and 10.5% saturated), 38% carbohydrates (28% dietary fibre), 9% protein, 2% ash and 2% moisture. Total phenol content by Folin–Ciocalteau method: 2.2 mg catechin/g of cream.

2.3 *In vitro* colonic fermentation by rat microflora

The *in vitro* colonic fermentation was based on the methodology described by Appeldoorn, Vincken, Aura, Hollman, and Gruppen (2009) with some modifications. The culture medium was a carbonate–phosphate buffer reduced in an anaerobic chamber for 48 h prior to the fermentation and prepared according to Durand, Dumay, Beaumatin, and Morel (1988). It contained (g/l) $NaHCO_3$ 9.240, $Na_2HPO_4{\cdot}2H_2O$ 3.542, NaCl 0.470, KCl 0.450, $Na_2SO_4{\cdot}10H_2O$ 0.227, $CaCl_2$ (anhydrous) 0.055, $MgCl_2{\cdot}6\ H_2O$ 0.100, urea 0.400 with 10 ml of added trace element solution (trace element solution containing (mg/l) $FeSO_4{\cdot}7H_2O$ 3680, $MnSO_4{\cdot}H_2O$ 1159, $ZnSO_4{\cdot}7H_2O$ 440, $CoCl_2{\cdot}6H_2O$ 120, $CuSO_4{\cdot}5H_2O$ 98, $Mo_7(NH_4)_6O_{24}{\cdot}4H_2O$ 17.4). Fresh faecal material from adult rats (Wistar specie) was stored at 4 °C and then mixed with the culture medium (5 g/l), homogenised in a stomacher for 60 s, filtered and left to rest for 30 min. Next, faecal suspension was distributed in disposable tubs (10 ml/tub/time incubation) and 2 μmols of each compound was added. The mixture was fermented at 37 °C in anaerobic conditions. Samples were taken after 0, 1, 2, 4, 24 and 48 h. All incubations were performed in triplicate.

In parallel, three negative controls were conducted to determine the stability of the compounds during the applied conditions: *(i)* the phenolic compound was incubated for 24 h in buffer solution without microbiota; *(ii)* the phenolic compound was incubated with heat-inactivated microbiota during 24 h and *(iii)* the faecal suspension was incubated without the phenol compound as a negative control for each time point. When the samples were removed, the contents of the fermentation tubs were divided over microtubes (500 μl), and stored at −80 °C until the chromatographic analysis of the phenol metabolites. The contents of one microtube for each time point (fermentation sample) thawed slowly was transferred into a disposable tub, acidified with 60 μl of hydrochloric acid (37%) to inactivate the microbiota and then the phenol metabolites were extracted with ethyl acetate as follows. Five milliliters of ethyl acetate was added, the samples were vortexed for 5 min and centrifuged at 8784g for 10 min at room temperature. Then, the ethyl acetate was recovered and another extraction with 5 ml of ethyl acetate was carried out. The approximately 10 ml of ethyl acetate was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 °C and redissolved with 500 μl of H_2O/ACN/MeOH (86:12:2). Finally, the extract was filtered through a 0.22 μm nylon syringe filter (Teknokroma, Barcelona, Spain) and transferred to the autosampler vial before the chromatographic analysis to determine the procyanidins and alkaloids and their metabolites.

2.4 *In vitro* simulated gastrointestinal digestion

In order to obtain digested samples of nuts-cocoa cream suitable to undergo the colonic fermentation, a gastrointestinal *in vitro* digestion model based on the methodology described by Ortega, Reguant, Romero, Macià, and Motilva (2009) was used. The discontinuous model described a three-step procedure to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (duodenal digestion with dialysis). At the end of the dialyzed duodenal digestion step, the fraction located inside the dialysis tub content (IN fraction), referring to the non-dialyzable fraction, was collected and freeze-dried. Afterwards, it was stored for the analysis of the procyanidins and alkaloids by liquid chromatography and for the experiment as a substrate for the fermentation by rat microflora. For the chromatographic analysis, the digested freeze-dried samples (IN fraction) were dissolved in acetone/Milli-Q water/acetic acid (70/29.5/0.5, v/v/v) at a ratio of 1:5. The tubes were vortexed for 5 min and centrifuged at 12,500 rpm. The supernatant containing the solubilised phenols was filtered through 0.22 μm nylon filters prior to the chromatographic analysis. The experiment was carried out in triplicate.

2.5 Animal and experimental design

Three-month-old male Wistar rats were obtained from the Charles River Laboratories (Barcelona, Spain). The Animal Ethics Committee of the University of Lleida approved all the procedures. The rats were housed in temperature-controlled rooms (22 °C) and subject to a standard 12 h light–12 h dark schedule. All the animals were subjected to a standard diet of PanLab A04 (Panlab, Barcelona, Spain) and water. The animals were then kept in fasting conditions for between 16 and 17 h with access to tap water (total volume of 3 ml). A dose of 1.5 g of nuts-cocoa cream dispersed in water was administered to the rats by intragastric gavage. Finally, the animals were anesthetised with isoflurane (IsoFlo, Veterinaria Esteve, Bologna, Italy) and euthanised by exsanguinations at 24 h. The large intestine was excised, freeze-dried and stored at −80 °C. The experiment was carried out in triplicate. The procedure to extract the procyanidins and alkaloids metabolites from the freeze-dried rat large intestines with ethyl acetate was the same as described above for the *in vitro* fermentation experiment (Section 2.3).

2.6 Chromatographic analysis of procyanidins and alkaloids and their metabolites

The chromatographic analysis of the procyanidins and alkaloids was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford MA, USA) equipped with a binary pump system (Waters, Milford, MA, USA). The chromatographic column was an Acquity HSS T3 (100 mm × 2.1 mm i.d.) with a 1.8 μm particle size (Waters, Milford MA, USA). A binary mobile phase with a gradient elution was used. Eluent A was Milli-Q water:acetic acid (99.8:0.2, v/v) and eluent B was acetonitrile. The gradient was performed as follows: 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; 11.10–12.50 min,

5% B isocratic. The flow rate was 0.4 ml/min and the injection volume was 10 µl.

The tandem mass spectrometry (MS/MS) analyses were carried out on a triple quadrupol detector (TQD) mass spectrometer (Waters, Milford MA, USA) equipped with a Z-spray electrospray interface. The analyses were carried out in the negative ion mode for the polyphenol compounds and the positive ion mode for the alkaloids. The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 l/h and desolvation gas flow rate, 800 l/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. The cone voltages and collision energies were optimised for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50/50, v/v) at a flow rate of 10 µl/min. For each compound, depending on the ionization mode, two characteristic fragments of the deprotonated $[M-H]^-$ or protonated molecular ion $[M-H]^+$ were monitored. The most abundant transition was used for quantification, whilst the second most abundant was used for confirmation purposes. The analytes were quantified by reaction monitored mode (SRM), this being the most sensitive and selective mode. The dwell time established for each transition was 30 ms. Data acquisition was carried out by MassLynx v 4.1 software. In order to determine the different fermentation products generated, analyses in MS (full-scan mode) and MS/MS (based on product ion scan mode) were performed. These techniques (full scan and product ion scan) are excellent tools for verifying structural information about the compounds when standards are not available.

Catechin, epicatechin, dimer B2 [epicatechin-(4β → 8)-epicatechin], ECG, EGCG, caffeine, theobromine, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, phenylacetic acid, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3,4-dihydroxyphenyl)propionic acid were quantified using the calibration curves of the respective standards. Due to the lack of standards, the metabolites 5-(hydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid, 5-(3,4-dihydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 3-(3,4-dihydroxyphenyl)propionic, ferulic and isoferulic acids were quantified using the ferulic acid calibration curve and diarylpropan-2-ol was quantified using the catechin calibration curve.

2.7 Statistical analysis

All the data are expressed as the mean of three replicates plus standard deviation (SD). The data were analyzed using STATGRAPHICS Plus 5.1.

3 RESULTS

3.1 Colonic fermentation of procyanidins

The colonic metabolism of each procyanidin was evaluated by incubation of each individual standard with rat faecal suspension for 48 h. At the same time, the stability of each compound was studied by incubation in buffer medium

without microbiota, with heat-inactivated microbiota with a negative control conducted by incubating active faecal suspension without compounds. In general, a rapid degradation of procyanidins as consequence of the incubation conditions was observed. The highest degradation was observed when each compound was incubated in the absence of microbiota (buffer control), and procyanidins were quantified at low concentration levels at the end of the incubation period (data not shown). The buffer medium without microbiota did not contain metabolites, whereas in the negative control (active faecal suspension without procyanidins) traces of phenylacetic and 4-hydroxyphenylacetic acids were detected (data not shown). As a result of the degradation of the procyanidins, the absolute concentration of each metabolite in the fermentation medium allows a comparative analysis between the different metabolites, but not a real balance in relation to the initial concentration of the compound in the incubation medium.

Differences in the fermentation of the stereoisomers, catechin and epicatechin were observed (Table 1). When epicatechin was fermented, a lower number of fermentation products were obtained, 5-(hydroxyphenyl)-γ-valerolactone being the main fermentation product quantified in the fermentation medium, reaching a concentration of 142 ± 11 pM after 48 h of incubation. However, when catechin was fermented, only 18 ± 1.7 pM of 5-(hydroxyphenyl)-γ-valerolactone was quantified after 48 h of incubation, and phenylacetic acid (24 pM) being the main fermentation product. 5-(Hydroxyphenyl)-γ-valerolactone and 5-(3,4-dihydroxyphenyl)-γ-valerolactone were formed by fermentation of both catechin and epicatechin, reaching the maximum of concentration after 48 h of incubation (Table 1). As has been previously commented, the quantity of metabolites was lower in the epicatechin than in the catechin fermentation medium. Due to this metabolism, a decrease in the concentration of both stereoisomers was observed during incubation from 0 to 48 h. There was a greater decrease in the epicatechin, with complete catabolism at 48 h of incubation, whilst the catechin was not totally catabolised, being quantified at 1.2 ± 0.02 pM in the medium after 48 h of incubation. Different position isomers of hydroxyphenylacetic acid were observed depending on the initial stereoisomer, either catechin or epicatechin. In this way, 3-hydroxyphenylacetic acid was obtained starting from the epicatechin, and 2- and 4-hydroxyphenylacetic acids were obtained starting from the catechin. These hydroxyphenylacetic acids were dehydroxylated to phenylacetic acid, in both the catechin and epicatechin fermentation mediums (Fig. 1). In addition, diarylpropan-2-ol was only detected after 24 h of incubation, reaching concentrations of 21 ± 2.0 and 46 ± 3.7 pM in the catechin and epicatechin fermentation mediums, respectively. Additionally, protocatechuic and *p*-hydroxybenzoic acids were only detected in the catechin fermentation medium. 3-(4-Hydroxyphenyl)propionic acid was only detected in the catechin fermentation medium after 48 h of incubation, whilst 3-(3,4-dihydroxyphenyl) propionic acid was detected in both

Table 1. Metabolism of catechin, epicatechin and dimer B2 by rat colonic microflora.

	Incubation time (hours)					
Compound (pM)	0 h	1 h	2 h	4 h	24 h	48 h
Catechin	30 ± 1.9	27 ± 1.2	28 ± 2.3	24 ± 1.3	3.1 ± 0.03	1.2 ± 0.02
Catechin metabolites						
Phenylacetic acid	1.3 ± 0,09	3.2 ± 0.02	6.0 ± 0.04	5.1 ± 0.4	25 ± 2.4	24 ± 2.3
2-Hydroxyphenylacetic acid	1.0 ± 0,02	0.2 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	n.d.	0.69 ± 0.06
4-Hydroxyphenylacetic acid	n.d.	8.3 ± 0.7	9.24 ± 0.8	9.6 ± 0.7	28 ± 2.1	14 ± 2
Ferulic acid	n.d.	3.0 ± 0.02	3.9 ± 0.03	2.3 ± 0.1	0.28 ± 0.00	0.34 ± 0.01
Isoferulic acid	n.d.	0.30 ± 0.01	0.33 ± 0.01	n.d.	0.35 ± 0.02	0.36 ± 0.03
Protocatechuic acid	0.02 ± 0,00	0.4 ± 0.03	0.47 ± 0.03	0.54 ± 0.03	0.61 ± 0.05	3.9 ± 0.02
p-Hydroxybenzoic acid	n.d.	0.2 ± 0.04	0.14 ± 0.01	0.15 ± 0.01	0.04 ± 0.00	1.1 ± 0.01
3-(4-Hydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	7.5 ± 0.05
3-(2,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.37 ± 0.03
5-(Hydroxyphenyl)-ϫ-valerolactone	n.d.	n.d.	n.d.	n.d.	n.d.	18 ± 1.7
5-(3,4-Dihydroxyphenyl)-ϫ-valerolactone	n.d.	n.d.	n.d.	n.d.	n.d.	1.7 ± 0.01
Diarylpropan-2-ol	n.d.	n.d.	n.d.	n.d.	21 ± 2.1	n.d.
p-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.04
Epicatechin	45 ± 3	31 ± 2	38 ± 4	39 ± 2.5	0.54 ± 0.03	n.d.
Epicatechin metabolites						
Phenylacetic acid	1.5 ± 0.01	5.7 ± 0.4	5.9 ± 0.4	5.7 ± 0.4	26 ± 2.2	58 ± 0.4
3-Hydroxyphenylacetic acid	1.2 ±0.01	3.81 ± 0.02	3.4 ± 0.2	2.9 ± 0.2	1.82 ± 0.01	3.8 ± 0.02
3-(2,4-Dihydroxyphenylpropionic) acid	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00	2.1 ± 0.01
5-(Hydroxyphenyl)-ϫ-valerolactone	n.d.	n.d.	n.d.	n.d.	12 ± 2	142 ± 11
5-(3,4-Dihydroxyphenyl)-ϫ-valerolactone	n.d.	n.d.	n.d.	n.d.	0.16 ± 0.02	0.42 ± 0.03
Diarylpropan-2-ol	n.d.	n.d.	n.d.	0.22 ± 0.01	46 ± 3.7	1.0 ± 0.00
Dimer B2	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	n.d.	n.d.	n.d.
Dimer B2 metabolites						
Epicatechin	0.14 ± 0.00	0.60 ± 0.02	1.07 ± 0.00	0.06 ± 0.00	0	0
Phenylacetic acid	1.3 ± 0.01	3.46 ± 0.01	5.74 ± 0.04	5.0 ± 0.03	30 ± 2.9	29 ± 2.6
4-Hydroxyphenylacetic acid	7.6 ± 0.04	7.57 ± 0.03	12 ± 2	12 ± 2	15 ± 1.6	27 ± 1.8
Protocatechuic acid	0.58 ± 0.04	0.53 ± 0.02	0.43 ± 0.03	0.54 ± 0.04	0.64 ± 0.05	1.2 ± 0.01
p-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.4 ±0.00

Data expressed as mean values ± standard deviation (n = 3). n.d. not detected.

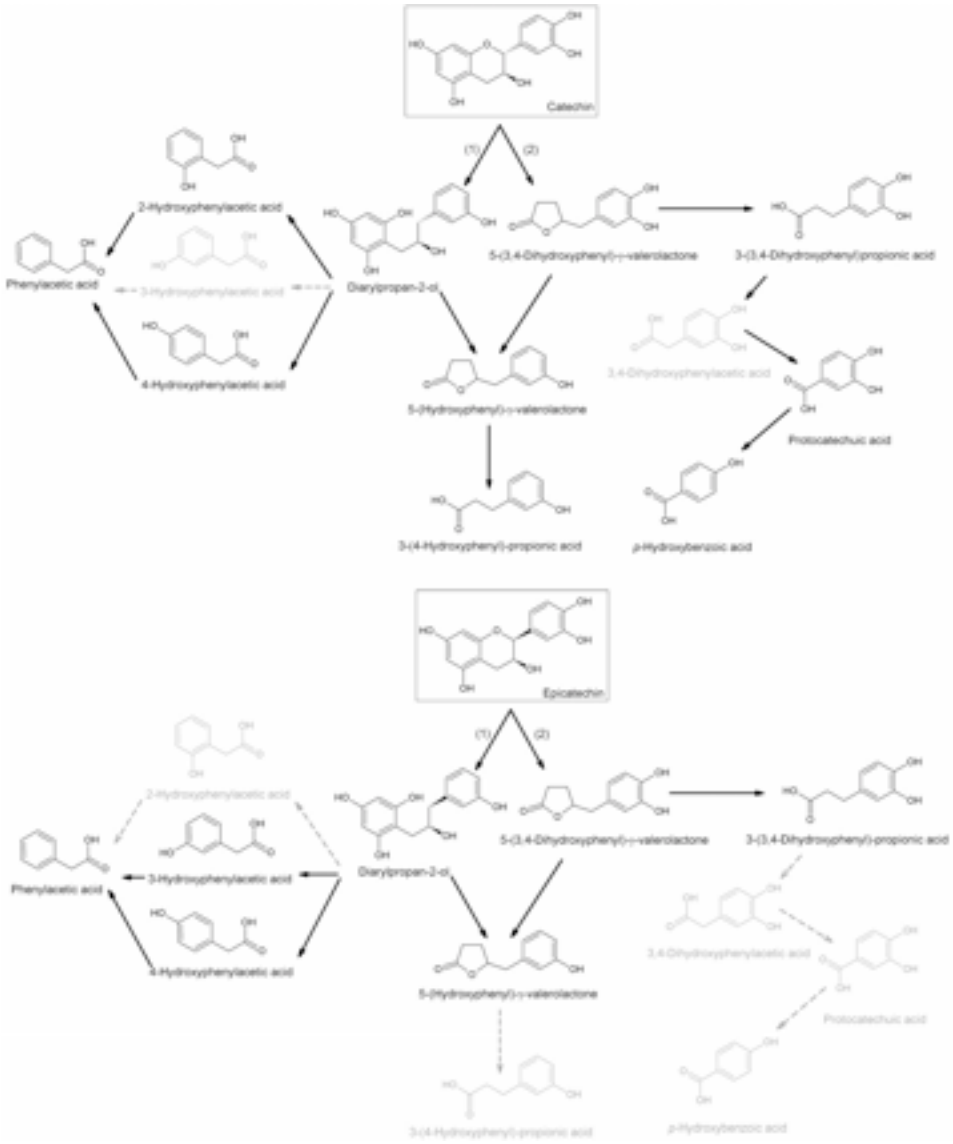

Figure 1. Proposed metabolic pathway of catechin and epicatechin by rat colonic microflora. The numbers (1) and (2) indicate two different metabolic routes. Detected metabolites are in black, not detected metabolites are in grey.

Table 2. Metabolism of epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and alkaloids (theobromine and caffeine) by rat colonic microflora.

	Incubation time (hours)					
Compound (pM)	0 h	1 h	2 h	4 h	24 h	48 h
EGCG	8.5 ± 0.57	5.9 ± 0.45	1.5 ± 0.01	1.4 ± 0.01	0.06 ± 0.00	0.06 ± 0.00
EGCG metabolites						
Epicatechin	0.50 ± 0.03	1.2 ± 0.01	1.7 ± 0.02	0.98 ± 0.06	0.11 ± 0.01	0.08 ± 0.00
Phenylacetic acid	6.7 ± 0.05	8.5 ± 0.84	9.9 ± 0.8	3.5 ± 0.03	17 ± 1.3	35 ± 2.9
2-Hydroxyphenylacetic acid	0.09 ± 0.00	0.09 ± 0.00	0.11 ± 0.02	0.09 ± 0.00	0.09 ± 0.00	0.25 ± 0.01
4-Hydroxyphenylacetic acid	14 ± 0.01	15 ± 0.01	17 ± 1.4	15 ± 1.3	22 ± 2.0	39 ± 3.7
Protocatechuic acid	0.9 ± 0.08	1.1 ± 0.00	1.4 ± 0.01	1.5 ± 0.02	3.3 ± 0.31	11 ± 1.9
p-Hydroxybenzoic acid	0.02 ± 0.00	0.12 ± 0.00	0.19 ± 0.00	0.11 ± 0.01	0.28 ± 0.02	0.84 ± 0.08
3-(3,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.2 ± 0.08
5-(3,4-Dihydroxyphenyl)-ϒ-valerolactone	n.d.	n.d.	n.d.	n.d.	n.d.	0.68 ± 0.06
ECG	3.7 ± 0.02	0.03 ± 0.00	5.1 ± 0.04	9.0 ± 0.67	0.61 ± 0.04	n.d.
ECG metabolites						
Epicatechin	0.5 ± 0.04	n.d.	6.4 ± 0.54	8.2 ± 0.67	n.d.	n.d.
Phenylacetic acid	2.4 ± 0.02	4.4 ± 0.23	4.1 ± 0.35	5.2 ± 0.54	32 ± 3.0	25 ± 2.1
2-Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.52 ± 0.05
4-Hydroxyphenylacetic acid	7.8 ± 0.06	13 ± 0.01	11 ± 1.4	12 ± 1.1	26 ± 1.6	19 ± 0.86
Protocatechuic acid	0.41 ± 0.04	0.5 ± 0.03	0.41 ± 0.03	0.43 ± 0.03	0.78 ± 0.08	3.7 ± 0.31
p-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	0.95 ± 0.08	0.83 ± 0.06
3-(3,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.71 ± 0.03
5-(Hydroxyphenyl)-ϒ-valerolactone	n.d.	n.d.	n.d.	n.d.	n.d.	5.4 ± 0.23
Gallic acid	3.03 ± 0.03	3.5 ± 0.03	7.9 ± 0.78	13 ± 1.3	6.8 ± 0.45	8.9 ± 0.83
Diarylpropan-2-ol	n.d.	n.d.	n.d.	n.d.	1.9 ± 0.07	n.d.
Theobromine	222 ± 19	215 ± 19	186 ± 14	168 ± 13	176 ± 14	190 ± 1.4
Theobromine metabolites						
Phenylacetic acid	9.3 ± 0.07	6.3 ± 0.57	9.3 ± 0.96	7.2 ± 5.3	24 ± 2.4	74 ± 4.8
Protocatechuic acid	2.3 ± 0.01	4.8 ± 0.31	0.6 ± 0.03	0.7 ± 0.04	1.0 ± 0.13	25 ± 1.8
3-(4-Hydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	8.9 ± 0.74
3-(3,4-Dihydroxyphenylpropionic) acid	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.01
Caffeine	0.07 ± 0.00	5.2 ± 0.30	0.44 ± 0.02	n.d.	n.d.	n.d.
Caffeine metabolites						
Phenylacetic acid	0.07 ± 0.00	3.4 ± 0.14	0.36 ± 0.02	n.d.	n.d.	n.d.
4-Hydroxyphenylacetic acid	0.10 ± 0.00	7.3 ± 0.64	0.42 ± 0.04	n.d.	n.d.	n.d.
2-Hydroxyphenylacetic acid	0.08 ± 0.00	4.7 ± 0.43	0.53 ± 0.02	n.d.	n.d.	n.d.
Protocatechuic acid	0.08 ± 0.00	22 ± 1.8	0.58 ± 0.01	n.d.	0.17 ± 0.01	n.d.
3-(4-Hydroxyphenyl)propionic acid	0.18 ± 0.01	44 ± 3.76	1.2 ± 0.03	4.1 ± 0.32	n.d.	n.d.
3-(3,4-Dihydroxyphenylpropionic) acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Data expressed as mean values ± standard deviation (n = 3). n.d. not detected.

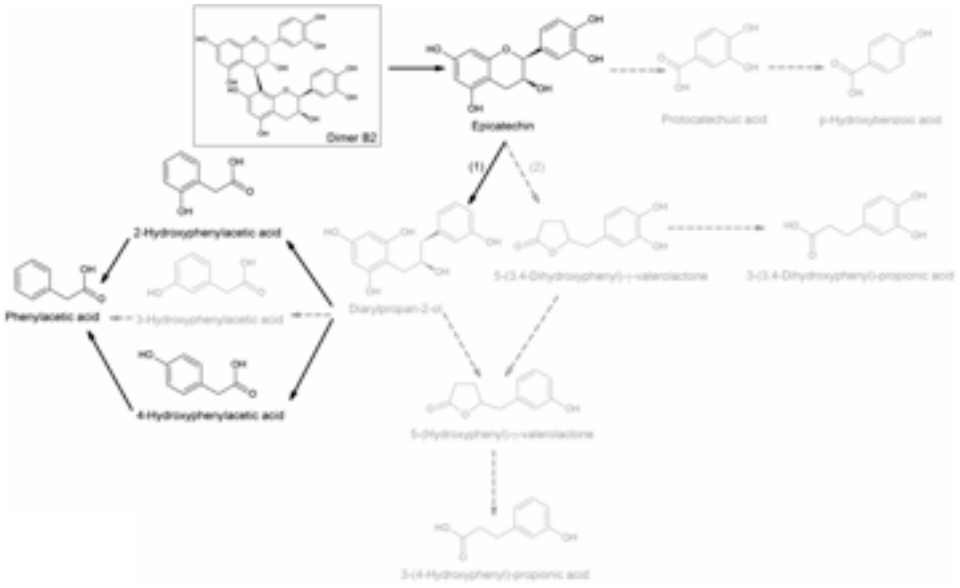

Figure 2. Proposed metabolic pathway of dimer B2 by rat colonic microflora. Detected metabolites are in black, not detected metabolites are in grey.

stereoisomer fermentation mediums, but it appeared earlier when epicatechin was fermented.

When the dimer B2 was fermented with rat colonic microflora, a rapid hydrolysis into epicatechin monomers was observed (Table 1 and Fig. 2). Phenylacetic acid was the major fermentation product, with 30 ± 2.7 pM after 24 h of incubation. The 4-hydroxylated form of phenylacetic acid was detected from the beginning of incubation (0 h), increasing in concentration throughout the incubation period to reach a concentration of 27 ± 1.8 pM at 48 h. Two phenolic acids were also generated by catabolism of the dimer B2, these being *p*-hydroxybenzoic and protocatechuic acids. *p*-Hydroxybenzoic was only detected at 0.4 ± 0.01 pM after 48 h of incubation and the protocatechuic acid was detected in the basal medium (0 h). However, its concentration increased during the fermentation reaching a maximum of 1.2 ± 0.01 pM at the end of the incubation period (48 h). The metabolism of the dimer B2 was nevertheless limited, probably because of its low stability in the fermentation medium, being 0.03 pM the initial concentration of dimer B2 quantified at the beginning of the incubation (0 h).

The metabolism of galloylated epicatechins, ECG and EGCG, resulted in a high number of metabolites (Table 2 and Fig. 3). The *p*-hydroxybenzoic,

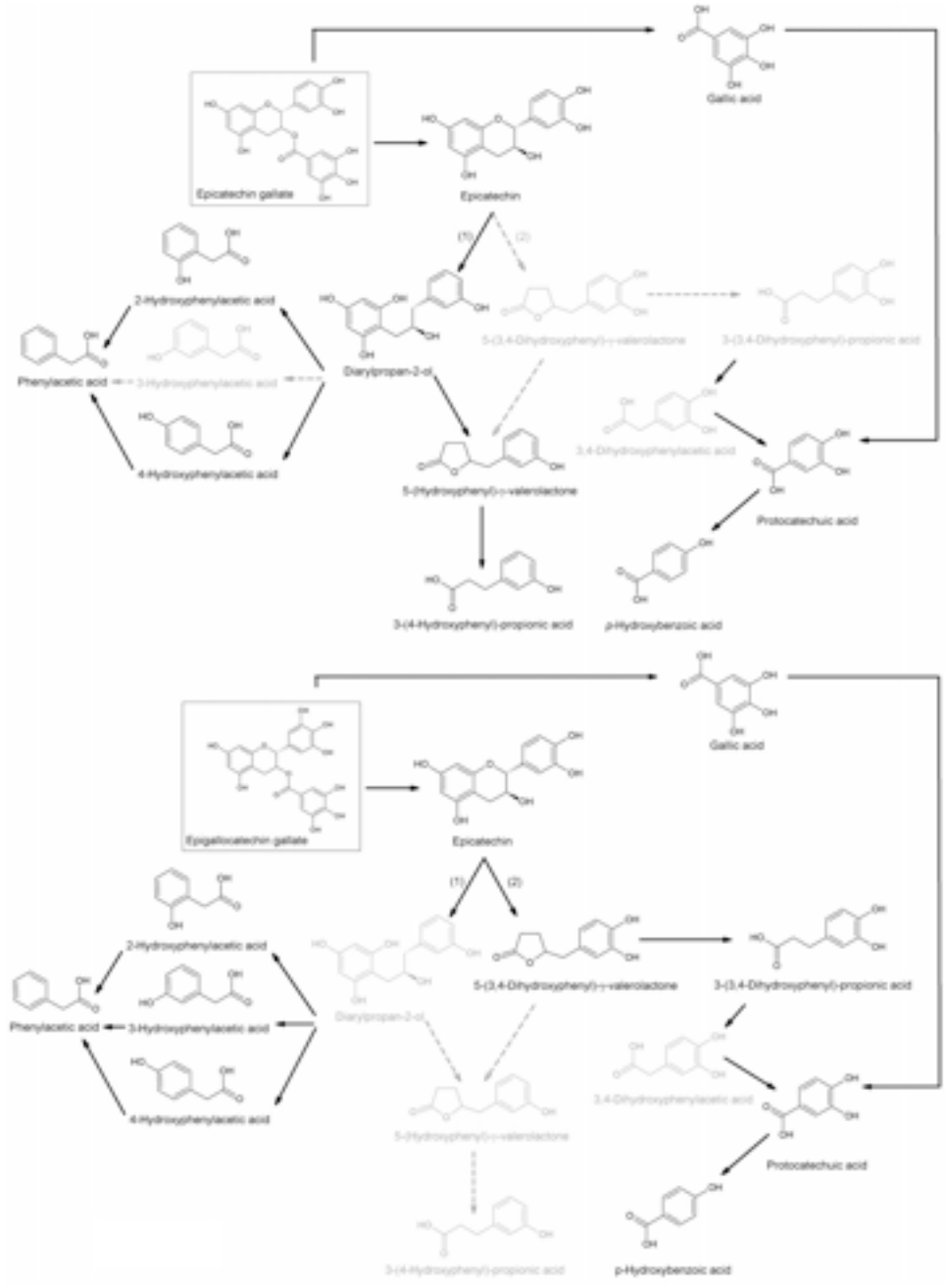

Figure 3. Proposed metabolic pathway of epicatechin gallate and epigallocatechin gallate by rat colonic microflora. Detected metabolites are in black, not detected metabolites are in grey.

protocatechuic, phenylacetic, 2-hydroxyphenylacetic and 4-hydroxyphenylacetic acids were the common metabolites obtained from the metabolism of both galloylated forms. The free form of epicatechin was also detected in the first period of fermentation of both compounds as a result of hydrolysis. Besides those common metabolites, some metabolites were only detected in the ECG fermentation medium. For example, diarylpropan-2-ol was quantified at 1.9 ± 0.07 pM after 24 h of incubation, and gallic acid was quantified at 13 ± 1.1 pM after 4 h of incubation. Additionally, the derivates of valerolactone formed were different depending on the initial gallate form subject to fermentation. 5-(3,4-Dihydroxyphenyl)-γ-valerolactone resulted from the EGCG fermentation, and 5-(hydroxyphenyl)-γ-valerolactone resulted from the ECG fermentation (Table 2). 3-(3,4-Dihydroxyphenyl)propionic was detected in the ECG fermentation medium whilst hydroxyphenylpropionic acid was detected in the EGCG fermentation medium.

3.2 Colonic fermentation of alkaloids

The results in Table 2 show a high stability of theobromine, which was detected throughout the incubation period at a constant concentration of around 200 pM. On the other hand, caffeine showed a major instability in the incubation medium. It was quantified during the first period of incubation (from 0 h to 2 h) at a concentration of 5.2 ± 0.30 pM. With regard to alkaloids metabolism, metabolites with heterocyclic nitrogen, such as theophylline and paraxanthyne, described as alkaloid metabolites in human urine (Schneider, Ma, & Glatt, 2003), were not detected. However low concentrations of a high number of catabolic compounds, such as protocatechuic acid and derivatives of phenylacetic acid and propionic acid, were detected during the first 24 h of incubation and their concentration in the theobromine fermentation medium increased after 24 h of incubation. These types of catabolic compounds (hydroxyphenylacetic and 3-(3,4-dihydroxyphenyl)propionic acids) were also detected in chromatographic analysis of the control mediums (negative control and heat-inactivated microbiota) but the concentrations of these were higher when theobromine was present in the fermentation medium after 24–48 h of incubation (Fig. S1).

3.3 Colonic fermentation of nuts-cocoa cream

After the individual analysis of the colonic metabolism of the procyanidins and alkaloids, the second part of the study was focused on the evaluation of the effect of the food matrix on this metabolism. Prior to the colonic fermentation, the nut-cocoa cream was submitted to a three-step *in vitro* digestion procedure to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (duodenal digestion with dialysis). After the *in vitro* digestion of the nuts-cocoa cream, the greatest decreases in substrate colonic fermentation occurred with caffeine and epicatechin (Table S1). The ECG and EGCG contents increased in the duodenal mixture, probably as consequence of release from the nuts-cocoa cream matrix as previously reported (Ortega *et al.*, 2009).

The non-dialyzable fraction (IN) obtained from the duodenal phase of the *in vitro* digestion of the cream was lyophilised and added to the fermentation medium (100 mg/tube of fermentation) and incubated with the rat faecal slurry for 48 h at 37 °C. Table 3 shows the metabolites detected and quantified in the fermentation medium (*in vitro* colonic fermentation) of the nuts-cocoa cream at 0, 1, 2, 4, 24 and 48 h of incubation, and the concentration (pM of analyte per gramme of large intestine) obtained from rats after 24 h of a single dose of the same cream. Results of the *in vitro* colonic fermentation showed that the procyanidins and alkaloids present in the cream were not detected in the fermentation medium. Nevertheless, a wide range of fermentation products were detected, being phenylacetic acid the main fermentation product with 30 ± 2.0 pM after 48 h of incubation followed by its 4-hydroxylated form with 12 ± 0.99 pM after the same incubation time. The minor fermentation products were 3-(3,4-dihydroxyphenyl)propionic acid, which was only detected after 48 h of incubation with 1.6 ± 0.01 pM, *p*-hydroxybenzoic acid reaching the maximum concentration after 48 h of incubation with 1.0 ± 0.08 pM and gallic acid with 0.95 ± 0.08 pM after 24 h of incubation.

In order to verify the results obtained in the *in vitro* fermentation study of the nuts-cocoa cream, the large intestines including the intestinal contents were obtained from rats after 24 h of a single dose of 1.5 g of the same nuts-cocoa cream. For the analysis and identification of the colonic fermentation products, the large intestine was lyophilised. It was pretreated as the fermentation medium to extract the procyanidin and alkaloid metabolites and analyzed by UPLC–MS/MS. The concentration expressed as nmol of analyte per gramme of sample (large intestine) is shown in Table 4. Only caffeine and theobromine were detected as native forms of alkaloids present initially in the cream, caffeine being the most abundant at 95 ± 4.2 nmol/g of large intestine and theobromine present at a lower concentration of 0.24 ± 0.02 nmol/g of large intestine. Phenylacetic acid was the main metabolite with 18 ± 0.14 nmol/g of large intestine, 3-hydroxyphenylacetic and 4-hydroxyphenylacetic acids were

Table 3: Metabolism of a nuts-cocoa cream as a source of procyanidins and alkaloids by rat colonic microflora.

	Incubation time (h)					
Compound (pM)	0 h	1h	2 h	4 h	24 h	48 h
Phenylacetic acid	0.48 ± 0.03	7.3 ± 0.68	7.2 ± 0.61	8.5 ± 0.65	5.20 ± 0.38	30 ± 2.0
p-Hydroxybenzoic acid	0.09 ± 0.00	0.17 ± 0.01	0.19 ± 0.01	0.22 ± 0.02	0.68 ± 0.04	1.0 ± 0.12
4-Hydroxyphenylacetic acid	2.7 ± 0.16	10 ± 1.72	9.5 ± 0.72	11 ± 0.12	3.18 ± 0.02	12 ± 3.8
Protocatechuic acid	0.29 ± 0.01	3.5 ± 0.02	3.4 ± 0.29	4.8 ± 0.39	5.6 ± 0.32	5.2 ± 0.41
Gallic acid	n.d.	0.09 ± 0.00	n.d.	0.28 ± 0.01	0.95 ± 0.08	0.52 ± 0.04
3-(2,4-Dihydroxyphenylpropionic) acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.6 ± 0.01

Data expressed as mean values±SE (n=3).
n.d. non detected

quantified at 14 ± 1.2 and 10 ± 0.6 nmol/g of large intestine, respectively, and also two valerolactones, 5-(hydroxyphenyl)-γ-valerolactone and 5-(3,4-dihydroxyphenyl)-γ-valerolactone, were found at lower concentrations.

4 DISCUSSION

In general, the procyanidins, except the dimer B2 and the gallates, EGC and EGCG, were catabolised during the final period of fermentation, between 24 and 48 h of incubation. The dimer B2 was hydrolyzed into epicatechin during the first period of incubation (1–4 h), reaching a maximum after 2 h (Table 1) and EGCG and EGC lost the gallate residue to give rise to epicatechin monomer (Fig. 1). The detection of hydroxylated forms and non-conjugated forms of some phenolic acids, like phenyl acetic, phenyl propionic and protocatechuic acids, suggest that the procyanidins may be degraded to low-molecular-weight aromatic compounds in the large intestine due to the action of the colonic microflora. Hydroxylated forms of propionic acids were detected as intermediates in all the procyanidins tested for except the polymerised form of epicatechin (dimer B2), this being the major route for metabolism (Fig. 2). These results agree with previous studies (Déprez *et al.*, 2000 and Meselhy *et al.*, 1997), in which 3,4-dihydroxylated phenolic and 2,4-hydroxylated phenolic acids were also identified after the biotransformation of (−)-epicatechin 3-O-gallate and polymeric proanthocyanidins, respectively.

In our study, procyanidins were metabolised by the rat colonic microflora in the practical absence of carbohydrate energy sources, suggesting that the metabolism of this type of compound may occur even in the absence of more favourable carbon sources (Tzounis *et al.*, 2008). Nevertheless, although the rats used as the source of fresh faecal inoculum followed a standard diet theoretically free of procyanidin and alkaloid specific sources, the detection of phenylacetic and 4-hydroxyphenyl acetic acids in the control medium (without procyanidin) and the initial fermentation medium (0 h) may be explained by the fibre fermentation products present in the rat faecal samples.

In relation to the fermentation of catechin, the presence of diarylpropan-2-ol after 24 h and 5-(3,4-dihydroxyphenyl)-γ-valerolactone after 48 h of incubation may indicate the presence of two parallel but not independent metabolic pathways

Table 4. Concentration of phenolic compounds and metabolites detected in the rat intestine at 18 h after a single dose of 1.5 g of the nuts-cocoa cream. Results are expressed as nmol/g large intestine.

Compound	Concentration (nmol/g)
Theobromine	0.24 ± 0.02
Caffeine	95 ± 4.2
Phenylacetic acid	18 ± 0.14
4-Hydroxyphenylacetic acid	14 ± 1.5
3-Hydroxyphenylacetic acid	10 ± 0.60
5-(Hydroxyphenyl)-ϫ-valerolactone	2.5 ± 1.2
5-(3,4-Dihydroxyphenyl)-ϫ-valerolactone	5.6 ± 2.3

Data expressed as mean values±SE (n=3).

identified as route 1 and route 2 in Fig. 1, in which the metabolic pathway of catechin was represented. Both proposed pathways required the presence of other intermediate compounds not detected in the fermentation medium in our study. However Groenewoud and Hundt (1984) were able to isolate the first intermediate of the metabolic pathway (route 2) (Fig. 1). The determination of diarylpropan-2-ol and dihydroxyphenyl-γ-valerolactone and the order of their appearance may indicate the preferential dehydroxylation in the 4-position (route 1) in relation to the rupture of the 1–2 bond (route 2), according to the results of Groenewoud and Hundt (1984). However, a hydroxy derivate of phenylpropionic acid was isolated in the catechin fermentation medium (Fig. 1). Nevertheless, the presence of hydroxy derivatives of benzoic, phenylacetic and phenylpropionic acids in the catechin fermentation medium was reported in previous studies (Das, 1974 and Harmand and Blanquet, 1978).

As has been proposed for the catechin fermentation, two complementary pathways were also suggested for the stereoisomer epicatechin, identified as route 1 and route 2 (Fig. 1). However, comparing both stereoisomer pathways, epicatechin was less metabolised than catechin, with a lower number of metabolites being obtained, and the formation of protocatechuic, *p*-hydroxybenzoic and 3-(4-hydroxyphenyl) propionic acids not being observed (Lee, Jenner, Low, & Lee, 2006).

The incubation of the stereoisomers (–)-epicatechin or (+)-catechin led to the generation of 5-(3,4-dihydroxyphenyl)-γ-valerolactone, 5-(hydroxyphenyl)-γ-valerolactone and different hydroxylated forms of hydroxyphenylacetic acid, finding 4- and 2-hydroxyphenylacetic acids in the catechin fermentation medium and 3- and 4-hydroxyphenylacetic acids in the epicatechin fermentation medium. When the nuts-cocoa cream was fermented, 4-hydroxyphenylacetic and phenylacetic acids were detected, with the latter being the main fermentation product (Table 3), probably through the fermentation of catechin as a common fermentation product. Similarly, 3-(3,4-dihydroxyphenyl)propionic acid was detected in both fermentation mediums. Nevertheless, 3-(4-hydroxyphenyl) propionic acid was only detected in the catechin fermentation medium. 3-(3,4-Dihydroxyphenyl)propionic acid also was detected in the nuts-cocoa cream fermentation medium as a final fermentation product generated from 5-(3,4-dihiydroxyphenyl)-γ-valerolactone. However, the isomer interconversion between catechin and epicatechin, that Selma *et al.*, 2009 and Tzounis *et al.,* 2008 claim to be necessary for the formation of both metabolites, was not observed in our study, but this type of interconversion may depend on the microorganism responsible for the metabolism (Lee *et al.,* 2006).

The proposed metabolic pathway for the dimer B2 is shown in Fig. 2. In contrast with what was expected, the fermentation of the dimer B2 did not lead to the formation of the same metabolic products of epicatechin and phenylacetic and 4-hydroxyphenylacetic acids were the only common metabolites. The dimer B2 was

hydrolyzed to epicatechin during the first period of incubation (Fig. 2) in contrast to that observed by Appeldoorn *et al.*, 2009 and Aura, 2008. After the first hydrolysis of the dimer B2, several derivatives of benzoic and phenyl acetic acids with different patterns of hydroxylation, and protocatechuic acids were identified, similarly to those observed by other authors (Aura, 2008, Déprez *et al.*, 2000, Gonthier *et al.*, 2003a and Rios *et al.*, 2002), with phenylacetic and 4-hydroxyphenylacetic acids being the main metabolites quantified after 48 h of incubation. By contrast, 2-(3,4-dihydroxyphenyl)acetic acid, the main metabolite described by Appeldoorn *et al.* (2009) was not found in our study. This fact could be explained by differences in the composition of the microflora.

In contrast to the epicatechin fermentation, valerolactones and their precursors were not detected in the fermentation medium of dimer B2, not even in the nuts-cocoa cream fermentation medium. This suggests that the fermentation of the dimer form of epicatechin is slower than the monomer epicatechin and the diarylpropan-2-ol, the major precursor of 5-(hydroxyphenyl)-γ-valerolactone, had not formed after the 48 h of incubation. On the other hand, the non-detection of valerolactones and their precursors could be explained by a limited metabolization of the dimer B2, focused mainly on the upper unit of epicatechin, following the tentative pathway proposed by Winter, Moore, Dowell, and Bokkenheuser (1989). This pathway has previously been shown to be an important mechanism for human microbial degradation of several flavanoids.

In relation to the galloylated forms of epicatechin, ECG and EGCG (Table 1, Fig. 3), the detection of the free epicatechin and gallic acid could confirm the easy cleavage of ester bonds in the galloylated forms (Takagaki & Nanjo, 2010). The predominant faecal degradation product of EGCG and EGC was 4-hydroxyphenylacetic acid indicating a predominant metabolic pathway towards the breakdown of the free epicatechin formed during the first period of incubation. On the other hand, taking into account that the cleavage of the 3-O-gallate group would give rise to the appearance of pyrogallol and its dehydroxylation product pyrocatechol, these metabolites of gallic acid were not detected in our study even after 48 h of incubation, in contrast with what Roowi *et al.* (2010) observed. On the other hand, gallic and 4-hydroxyphenylacetic acids were detected in the nuts-cocoa cream fermentation medium. These facts may confirm the presence of galloylated forms in the cream and the easy cleavage of ester bonds in the galloylated forms (Table 3).

Similarly to the catechin and epicatechin metabolism, 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(hydroxyphenyl)-γ-valerolactone were quantified as the result of the metabolism of EGCG and EGC respectively, but at a lower concentration. However the metabolism of both galloylated forms did not yield the equivalent valerolactone with three hydroxylations detected as major metabolic products by other authors (Roowi *et al.*, 2010). Quantitatively

different results between our experiments and those from other groups can be explained by different composition of the microbiota.

When the *in vitro* digested nuts-cocoa cream (Table 3) was submitted to fermentation, a faster metabolism of procyanidins was observed. So the main metabolites quantified at 48 h of fermentation of individual procyanidins (Table 1 and Table 2) were quantified from the first hours of fermentation. This faster metabolism of the procyanidins could be related to the presence of nutrients, such as sugars (5%) and proteins (9%), from the cream in the fermentation medium.

The metabolism of alkaloids in the fermentation model applied in the present study was low (Table 2) compared with the procyanidins, indicating a reduced use of theobromine and caffeine as a source of carbon by the rat faecal inoculum used. It is not possible to compare our results with preliminary studies by other authors because there are very few works related to the metabolism of alkaloids, probably as a consequence of the poor metabolism of alkaloids by colonic microflora. A study by Madyastha and Sridhar (1998) using a mixed culture consisting of strains belonging to the genera Klebsiella and Rhodococcus revealed oxidation as the major pathway for the degradation of caffeine. This oxidative metabolism of the colonic microflora could explain the rapid degradation of the caffeine in the fermentation medium observed in our study. By contrast, a recent interventional human study allowed the identification and quantification of theobromine and caffeine and their metabolites in such body fluids as urine, plasma and saliva, with paraxanthine and theophylline being found as common metabolites of both alkaloids (Ptolemy, Tzioumis, Thomke, Rifai, & Kellogg, 2010). Nevertheless, these metabolites were not detected in our study, even in the large intestine of rats analyzed after a single ingestion of nuts-cocoa cream with high contents in caffeine and theobromine, the latter being the main alkaloid present in the nuts-cocoa cream (Table 3). In this case, only the alkaloids theobromine and caffeine were detected, theobromine showing a lower concentration than caffeine. In addition, only some metabolites from the procyanidin colonic metabolism were detected, such as 3-hydroxyphenylacetic acid as a characteristic epicatechin fermentation product (Fig. 1) or 4-hydroxyphenylacetic acid, a characteristic catechin fermentation product (Fig. 1). Furthermore, in the negative control tube (control iii, Section, 2.3) containing only the faecal suspension, some phenolic acids, such as phenylacetic, protocatechuic, 3-(4-hydroxyphenyl)propionic, 3-(3,4-dihydroxyphenyl)propionic, 4-hydroxyphenylacetic and 2-hydroxyphenylacetic acids, seem to come to breakage of a diphenylpropane (C_6–C_3–C_6) skeleton. This may suggest that such residual organic matter as fibre added with the faecal content could have fermented resulting in this type of products being detected as metabolites of the colonic fermentation of the different procyanidins tested in the study. However, an increase in the concentration of some of these phenolic acids, such as hydroxyphenylacetic and 3-(3,4-dihydroxyphenyl)propionic acids (Fig. S1)

in the fermentation medium containing caffeine or theobromine during the last period of incubation (24–48 h) confirms the colonic metabolism of alkaloids in our study.

5 CONCLUSIONS

In this investigation, different metabolic pathways for the colonic metabolism of procyanidins and alkaloids were studied and proposed. The metabolism of procyanidins producing a wide range of fermentation products and the null metabolism of alkaloids leads us to wonder if there is an oxidative pathway as the major pathway for the degradation of alkaloids. In the catechin and epicatechin fermentation mediums, the presence of diarylpropan-2-ol and 5-(3,4-dihydroxyphenyl)-γ-valerolactone may indicate the presence of two parallel but not independent metabolic pathways, dehydroxylation or rupture of the 1–2 bond of the C ring. Moreover, the isomer interconversion between catechin and epicatechin was not observed. The metabolism of both galloylated forms, ECG and EGCG, did not yield the equivalent valerolactone with three hydroxylations. In contrast to what was expected, the fermentation of the dimer B2 did not lead to the formation of the same metabolic products of epicatechin, with phenylacetic and 4-hydroxyphenylacetic acids being the only common metabolites. Some of these metabolic pathways were verified by the analysis of the large intestine including the intestinal content from rats after 24 h of a single dose of a nuts-cocoa cream, being phenylacetic acid, 3-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid the main metabolites quantified. Similarly, two valerolactones (5-(hydroxyphenyl)-γ-valerolactone and 5-(3,4-dihydroxyphenyl)-γ-valerolactone were quantified at lower concentration.

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Publication II: Additional Information

Table 1 Additional information. Phenolic composition of the nuts-cocoa cream and the IN fraction obtained by the *in vitro* duodenal digestion of the cream. The results are expressed as nmol compound/g cream.

Compound	Cocoa cream (nmol/g)	IN of cocoa cream(*) (nmol/g)
Alkaloids		
Caffein	7634 ± 121	2680 ± 187
Theobromine	20011 ± 976	10638 ± 981
Procyanidins		
Catechin	1410 ± 210	1296 ± 98
Epicatechin	365 ± 13	127 ± 7
Dimer	77 ± 4	48 ± 3
ECG	13 ± 0.9	95 ± 7
EGCG	3.8 ± 0.25	162 ± 11

(*)The quantification is expressed as nmols of compound in the digestion mixture, corresponding to 1 g of digested nuts-cocoa cream.
Data expressed as mean values ± standard desviation (n=3)

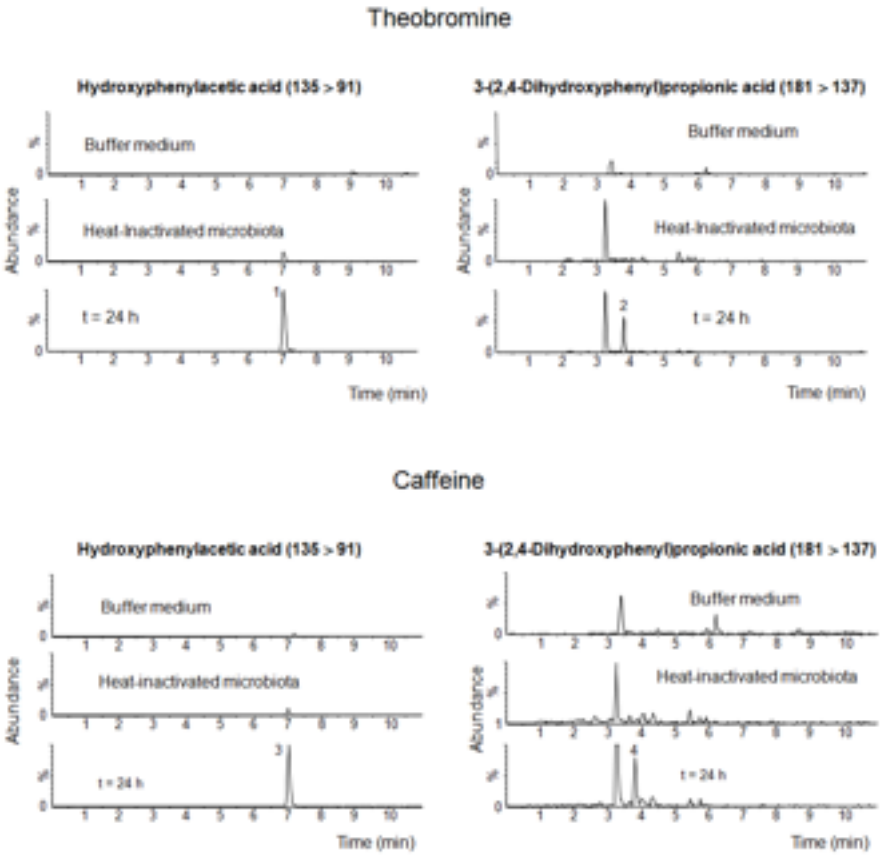

Figure 1 Additional information. Chromatograms of two metabolites, hydroxyphenylacetic acid and 3-(2,4-dihydroxyphenyl)propionic acid present in the negative control, in the heat-innactivated microbiota and in the standard incubation of theobromine and caffeine after 24 h of incubation.

Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids

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METABOLIC PATHWAYS OF THE COLONIC METABOLISM OF FLAVONOIDS (FLAVONOLS, FLAVONES AND FLAVANONES) AND PHENOLIC ACIDS

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Abstract

Flavonoids are metabolized by the gut microflora giving rise to a wide range of phenolic acids that may exert systemic effects in the body. Nevertheless, the colonic metabolism pathways and the function of the metabolites formed are poorly studied. In the present study, the individual colonic metabolism of three subclasses of flavonoids (flavonols, flavones and flavanones) and phenolic acids was evaluated. For this, seven standards of flavonoids (quercetin, quercetin–rhamnoside, quercetin–rutinoside, myricetin, luteolin, naringenin and kaempferol–rutinoside) and two phenolic acids (protocatechuic acid and gallic acid) were submitted to an *in vitro* fermentation model using rat colonic microflora. Simultaneously, a nuts–cocoa cream enriched with these standards of flavonoids was incubated and the colonic metabolism of these compounds was evaluated. The results showed that the greatest number of colonic metabolites came from the fermentation of quercetin and quercetin–rhamnoside, and the maximum concentration of fermentation products was observed after 48 h of fermentation. On the other hand, a smaller number of fermentation products were observed after the colonic fermentation of kaempferol–rutinoside, naringenin, luteolin and myricetin. The phenolic acids were slightly metabolized by the colonic microflora.

Keywords: Colonic microflora fermentation / Digestion / Flavonoids /Metabolic pathway / Phenolic acids.

1 INTRODUCTION

Different epidemiologic studies have shown that there is evidence for the prevention or decrease of cardiovascular diseases related to the intake of flavonoids (Akhlaghi and Bandy, 2009, Geleijnse *et al.*, 2002, Hertog *et al.*, 1993, Mladěnka *et al.*, 2010 and Sesso *et al.*, 2003). Nevertheless the circulation levels reached in plasma after the ingesta of

flavonoids are low (Aura, 2008), which is probably related to their limited absorption (Manach *et al.*, 2005, Rasmussen *et al.*, 2005 and Walle, 2004) or their intense metabolism. Thus, these beneficial effects could be related to the native forms of flavonoids present in vegetables and fresh fruit, or with their metabolites. In general, the absorption of polyphenols in the digestive tract starts in the ileum, where the more complex structures (esters, glycosides, or polymers) that cannot be absorbed in their native form are hydrolysed by intestinal enzymes or the colonic microflora before they can be absorbed (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). During absorption, polyphenols are conjugated in the small intestine and then in the liver. This process mainly includes methylation, sulphation, and glucuronidation (Saude and Sykes, 2007 and Vacek *et al.*, 2010). Subsequently, these new compounds may be excreted as conjugates in bile, pass through the small intestine and reach the colon. On the other hand, the limited absorption of polyphenols in the ileum allows non-absorbed polyphenols to reach the colon whole (Manach *et al.*, 2005, Rasmussen *et al.*, 2005 and Walle, 2004), being transformed by the gut microbiota enzymes (esterase, glucosidase, demethylation, dehydroxylation and decarboxylation enzymatic activities) (Zoetendal, Akkermans, & De Vos, 1998) into a wide range of low-molecular-weight phenolic acids (Jacobs, Gaudier, van Duynhoven, & Vaughan, 2009). Nevertheless, the effect of the new small structures, in particular the health implications of colonic metabolites of flavonoids (Aura, 2008), has been far less studied than the impact of, for example, the metabolites of proteins and carbohydrates (Jacobs *et al.*, 2009).

The metabolic activity of the gut microflora on polyphenols is often responsible for the modulation of the biological activity of these dietary compounds (Setchell *et al.*, 2002 and Xu *et al.*, 1995) and their potential health effects. Several studies have shown that polyphenols are transformed by colonic microflora into phenolic acids, such as phenylvaleric, phenylpropionic, phenylacetic, benzoic and hippuric acids. However, the type of metabolic products depends on what phenolic compound is metabolized (Das, 1974, Gonthier *et al.*, 2003 and Gonthier *et al.*, 2003). Flavonoids are natural products derived from 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone) (flavone). Subcla sses of flavonoids include reduction of the 2(3) carbon–carbon double bond (flavanones), reduction of the keto group (flavanols), and hydroxylation at various positions. Although it is known that the intestinal microflora participate in the metabolism of flavonoids (Griffiths and Barrow, 1972 and Nakagawa *et al.*, 1965), there is a lack of information about the mechanisms involved in this process and the effect of minor differences in their chemical structure.

The aim of this research was to study the individual colonic metabolism of three subclasses of flavonoids, (flavonols, flavones and flavanones) and phenolic acids by rat colonic microflora by an *in vitro* model, with the aim of extending our previous study of the metabolic pathways of the colonic metabolism of procyanidins and alkaloids (Serra *et al.*, 2011). Six flavonoids standards from the three different subclasses were selected: *(i)* Flavonols: quercetin, quercetin–rutinoside, quercetin–rhamnoside, myricetin and kaempferol–rutinoside *(ii)* Flavanones: naringenin, *(iii)* Flavones: luteolin; and two phenolic acids:

protocatechuic acid and gallic acid. After a study of the colonic metabolic pathways of the individual compounds, the colonic fermentation of flavonoids and phenolic acids was evaluated by the fermentation of a nuts-cocoa cream enriched with these standards and those previously digested *in vitro*.

2 EXPERIMENTAL

2.1 Chemicals

The internal standard (IS) catechol and the standard of naringenin, myricetin, gallic acid, protocatechuic acid, phenylacetic acid, *p*-hydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid, 3,4-dihydroxyphenylacetic acid, 2-phenylpropionic acid and urea were purchased from Sigma–Aldrich (St. Louis, MO, USA); quercetin–rhamnoside, *o*-hydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, 3-(2,4-dihydroxyphenyl)propionic acid and kaempferol-rutinoside from Fluka Co. (Buchs, Switzerland); quercetin–rutinoside was acquired from Extrasyntese (Genay, France); $NaCO_3$, KCl, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$ were purchased from Panreac Química S.A. (Barcelona, Spain); and $CuSO_4{\cdot}5H_2O$, NaCl, $Na_2SO_4{\cdot}10H_2O$ from Scharlau S.L. (Barcelona, Spain).

Individual phenol stock standard solutions and the IS were dissolved in acetone and stored in dark-glass flasks at −18 °C. Standard mixtures at different concentrations were prepared by appropriate dilution of the individual phenol stock solutions with methanol, and these solutions were stored in dark-glass flasks at 4 °C before chromatographic analysis. The acetonitrile (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade), hydrochloric acid 37% (reagent grade), ethyl acetate (HPLC-grade) and glacial acetic acid (≥99.8%) were of analytical grade (Scharlab, Barcelona, Spain). Orto-phosphoric acid 85% was purchased from MontPlet & Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Nuts-cocoa cream

The nuts–cocoa cream was kindly supplied by La Morella Nuts S.A. (Reus, Spain). The composition of the cream was determined according to the Spanish Official Methods of analysis (BOE, 1988). The cream moisture content was determined by drying at 130 °C for 2 h, cooled in a desiccator and reweighed. Fat content was determined by continuous extraction with diethyl ether performed on dried samples in a Soxhlet extractor. Carbohydrates were determined by the method of Luff-Schoorl. Dietary fibre was determined by treatment of the dried sample with sulphuric acid and potassium hydroxide solutions, before subsequent weighing of the insoluble residue. Protein was measured by the Kjeldahl method. Ashes were determined by the weighed of the residue obtained by incineration at a temperature of 550 ± 10 °C to complete combustion of organic matter and obtain a constant weight. The composition was: 49% lipids (26.5% polyunsaturated, 12% unsaturated and 10.5% saturated), 38% carbohydrates (28% dietary fibre), 9% protein, 2% ash and 2% moisture. Total phenol content by Folin–Ciocalteau method was 2.2 mg catechin/g of cream. Prior to the *in vitro* digestion and the *in*

vitro fermentation, the cream was spiked with 200 mg/kg of quercetin, quercetin–rhamnoside, quercetin–rutinoside, myricetin, luteolin, naringenin, protocatechuic acid and gallic acid previously dissolved in methanol.

2.3 *In vitro* colonic fermentation by rat microflora

The *in vitro* colonic fermentation was performed following the methodology described in our previous work (Serra *et al.*, 2011), which was based on the methodology described by Appeldoorn, Vincken, Aura, Hollman, and Gruppen (2009), with some modifications. The culture medium was a carbonate–phosphate buffer reduced in an anaerobic chamber for 48 h prior to the fermentation and prepared according to Durand, Dumay, Beaumatin, and Morel (1988). It contained (all in g/l) 9.240 NaHCO3, 3.542 Na2HPO4·2H2O, 0.470 NaCl, 0.450 KCl, 0.227 $Na_2SO_4 \cdot 10H_2O$, 0.055 $CaCl_2$ (anhydrous), 0.100 $MgCl_2 \cdot 6H_2O$, 0.400 urea with 10 ml of added trace element solution (trace element solution containing (mg/l) 3680 $FeSO_4 \cdot 7H_2O$, 1159 $MnSO_4 \cdot H_2O$, 440 $ZnSO_4 \cdot 7H_2O$, 120 $CoCl_2 \cdot 6H_2O$, 98 $CuSO_4 \cdot 5H_2O$, 17.4 $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$). Fresh faecal material from adult rats (Wistar species) was stored at 4 °C and then mixed with the culture medium (5 g of fresh faecal material/l of culture medium), homogenised in a stomacher for 60 s, filtered and left to rest for 30 min. Next, faecal suspension was distributed in disposable tubs (10 ml/tub/time incubation) and 2 μmol of each studied standard compound (quercetin, quercetin–rhamnoside, quercetin–rutinoside, kaempferol–rutinoside, myricetin, luteolin, naringenin, protocatechuic acid and gallic acid) was added. The mixture was fermented at 37 °C under anaerobic conditions by using an anaerobic chamber. Samples were taken after 0, 1, 2, 4, 24 and 48 h. All incubations were performed in triplicate.

At the same time, three negative controls were conducted to determine the stability of the compounds under the applied conditions: *(i)* the phenolic compound was incubated for 24 h in buffer solution without microbiota; *(ii)* the phenolic compound was incubated with heat-inactivated microbiota for 24 h and *(iii)* the faecal suspension was incubated without the phenol compound as a negative control for each time point.

When the samples were removed, the contents of the fermentation tubs were divided between microtubes (500 μl), and stored at −80 °C until the chromatographic analysis of the phenol metabolites. The contents of one microtube for each time point (fermentation sample) thawed slowly, transferred into a disposable tub, and acidified with 60 μl of hydrochloric acid (37%) to inactivate the microbiota. The phenol metabolites were then extracted with ethyl acetate as follows. Five millilitres of ethyl acetate was added, the samples were vortexed for 5 min and centrifuged at 8784g for 10 min at room temperature. The ethyl acetate was then recovered and another extraction with 5 ml of ethyl acetate was carried out. Approximately 10 ml of ethyl acetate was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 °C and redissolved with 500 μl of H_2O/ACN/MeOH (86/12/2, v/v/v). Finally, the extract was filtered through a 0.22 μm nylon syringe filter (Teknokroma, Barcelona, Spain) and transferred to the autosampler vial before the chromatographic analysis to determine the flavonoids and phenolic acids and their metabolites.

2.4 *In vitro* simulated gastrointestinal digestion

In order to obtain digested samples of the enriched nuts–cocoa cream suitable to undergo the colonic fermentation, a gastrointestinal *in vitro* digestion model based on the methodology described by Ortega, Reguant, Romero, Macià, and Motilva (2009) was used. The discontinuous model described a three-step procedure to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (duodenal digestion with dialysis).

At the end of the dialysed duodenal digestion step, the fraction located inside the dialysis tube (IN fraction), referring to the non-dialyzable fraction, was collected and freeze-dried. Afterwards, it was stored for the analysis of flavonoids and phenolic acids by liquid chromatography and for the experiment as a substrate for the fermentation by rat microflora.

For the chromatographic analysis, the digested freeze-dried samples (IN fraction) were dissolved in acetone/Milli-Q water/acetic acid (70/29.5/0.5, v/v/v) at ratio of 1:5. The tubes were vortexed for 5 min and centrifuged at 12.500 rpm. The supernatant containing the solubilised phenols was filtered through 0.22 μm nylon filters prior to the chromatographic analysis. The experiment was carried out in triplicate (Table 1 Additional Information).

2.5 Chromatographic analysis of flavonoids and phenolic acids and their metabolites

The chromatographic analysis of flavonoids and phenolic acids was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford MA, USA), equipped with a binary pump system (Waters, Milford, MA, USA). The chromatographic column was an Acquity HSS T3 (100 mm × 2.1 mm i.d.) with a 1.8-μm particle size (Waters, Milford MA, USA). A binary mobile phase with a gradient elution was used. Eluent A was Milli-Q water:acetic acid (99.8:0.2, v/v) and eluent B was acetonitrile. The gradient was performed as follows: 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; 11.10–12.50 min, 5% B isocratic. The flow rate was 0.4 ml/min and the injection volume was 10 μl.

The tandem mass spectrometry (MS/MS) analyses were carried out on a triple quadrupol detector (TQD) mass spectrometer (Waters, Milford MA, USA) equipped with a Z-spray electrospray interface. The analyses were done in the negative ion mode and the data was acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 l/h and desolvation gas flow rate, 800 l/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimised for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50/50, v/v) at a concentration of 10 mg/l. Two transitions were studied for each compound. The most abundant transition was used for quantification, while the second most abundant was used for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software (Table 2 Additional Information).

Quercetin, quercetin–rutinoside, quercetin–rhamnoside, myricetin, kaempferol–rutinoside, naringenin, luteolin, protocatechuic acid, gallic acid, *p*-hydroxybenzoic acid, phenylacetic acid, *o*-hydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3-(*p*-hydroxyphenyl)propionic acid and 3-(2,4-dihydroxyphenyl)propionic acid were quantified using the calibration curve of the respective standard. Homovanillic acid was quantified using the gallic acid calibration curve.

2.6 Statistical analysis

All the data are expressed as the mean of three replicates plus the standard deviation (SD). The data were analysed using STATGRAPHICS Plus 5.1.

3 RESULTS

3.1 Colonic fermentation of flavonoids

The colonic metabolism of each flavonoid was evaluated by incubation of each individual standard compound with rat faecal suspension for 48 h. Quercetin, quercetin–rutinoside, also called rutin, (glycoside between the quercetin and the disaccharide rutinose) and quercetin–rhamnoside (glycoside formed from the quercetin and the deoxy sugar rhamnose) were incubated to determine if there are differences in the colonic metabolites formed related to the presence of a glycosylated group in the molecule. An important instability of these compounds was observed during the incubation. The results showed that the presence of a saccharide group in the molecule slightly increased its low stability in the incubation medium (Table 1). Quercetin showed the lowest stability with an initial concentration (0 h) of 1.0 ± 0.01 pM compared with the 7.3 ± 0.65 pM and 23 ± 1.9 pM of quercetin–rhamnoside and quercetin–rutinoside, respectively. As a result of the degradation of the some of the flavonoids studied, the absolute concentration of each metabolite in the fermentation medium allows a comparative analysis between the different metabolites, but not a real balance in relation to the initial concentration of the compound in the incubation medium.

Despite the low initial concentration of quercetin present in the fermentation medium (0 h), its fermentation resulted in a wide range of metabolites, including phenylacetic acid, three different mono hydroxylated forms and one dihydroxylated form of phenylacetic acid, protocatechuic acid, 3-(3,4-dihydroxyphenyl)propionic acid, homovanillic acid (2-(4-Hydroxy-3-methoxyphenyl)acetic acid), and *p*-hydroxybenzoic acid. The main fermentation product of quercetin was protocatechuic acid, reaching a concentration of 63 ± 5.9 pM after 24 h of incubation, followed by phenylacetic acid, which reached a concentration of 42 ± 4.0 pM after incubation for 48 h. When quercetin–rhamnoside was fermented, the same fermentation products of quercetin were detected, the main fermentation products being phenylacetic acid (41 ± 0.40 pM) and the *p*-hydroxyphenylacetic acid (34 ± 3.1 pM). However homovanillic acid was not detected. On the other hand, when quercetin–rutinoside was fermented, a lower number of fermentation products were obtained. 3,4-Dihydroxyphenyl acetic acid, the main fermentation product, reached a concentration of 51 ± 4.3 pM after 48 h of incubation. Similarly, when kaempferol–rutinoside was fermented (Table 2) the main metabolite quantified was

Table 1: Metabolism of quercetin, quercetin–rhamnoside and quercetin–rutinoside by rat colonic microflora.

	Incubation time (hours)					
Compound (pM)	0h	1h	2h	4h	24h	48h
Quercetin	1.0 ± 0.01	0.64 ± 0.03	1.1 ± 0.11	3.70 ± 0.31	0.33 ± 0.02	n.d.
Quercetin metabolites						
Phenylacetic acid	2.5 ± 0.21	6.4 ± 0.54	4.0 ± 0.36	6.0 ± 0.56	32 ± 2.6	42 ± 4.0
4-Hydroxyphenylacetic acid	0.30 ± 0.02	11 ± 1.0	11 ± 1.0	11 ± 1.1	35 ± 2.8	14 ± 1.3
3-Hydroxyphenylacetic acid	1.0 ± 0.06	3.0 ± 0.27	2.9 ± 0.28	2.7 ± 0.22	3.1 ± 1.9	23 ± 2.8
2-Hydroxyphenylacetic acid	0.40 ± 0.01	n.d.	n.d.	n.d.	n.d.	1.2 ± 0.12
3,4-Dihydroxyphenylacetic acid	0.02 ± 0.00	n.d.	n.d.	n.d.	4.7 ± 3.1	4.7 ± 0.24
Protocatechuic acid	10 ± 0.9	25 ± 2.1	35 ± 0.27	41 ± 0.40	63 ± 5.9	5.0 ± 0.34
3-(3,4-Dihydroxyphenyl)propionic acid	0.30 ± 0.01	n.d.	n.d.	n.d.	n.d.	0.55 ± 0.04
homovainillic acid	1.0 ± 0.08	n.d.	n.d.	n.d.	n.d.	0.97 ± 0.05
p-Hydroxybenzoic acid	2.0 ± 0.12	n.d.	0.05 ± 0.00	0.05 ± 0.00	0.12 ± 0.01	4.5 ± 0.43
Quercetin-rhamnoside	7.3 ± 0.65	8.1 ± 0.78	7.9 ± 0.76	2.0 ± 0.19	0.56 ± 0.03	0.06 ± 0.00
Quercetin-rhamnoside metabolites						
Quercetin	0.36 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.02 ± 0.00
Phenylacetic acid	4.2 ± 0.34	4.2 ± 0.32	3.1 ± 0.23	4.25 ± 0.41	25 ± 1.8	41 ± 0.40
4-Hydroxyphenylacetic acid	8.9 ± 0.87	9.30 ± 0.93	8.5 ± 0.76	7.34 ± 0.65	27 ± 1.7	34 ± 3.1
3-Hydroxyphenylacetic acid	4.2 ± 0.41	4.0 ± 0.30	3.8 ± 0.30	2.88 ± 0.12	4.12 ± 0.34	13 ± 1.1
2-Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.63 ± 0.04
3,4-Dihydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	6.1 ± 0.54	10 ± 1.0
Protocatechuic acid	0.84 ± 0.07	2.6 ± 0.24	1.6 ± 0.01	0.60 ± 0.02	1.6 ± 0.16	3.83 ± 0.34
3-(3,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	0.09 ± 0.00	0.21 ± 0.02
p-Hydroxybenzoic acid	n.d.	n.d.	0.01 ± 0.00	n.d.	n.d.	0.37 ± 0.01
Quercetin-rutinoside	23 ± 1.9	22 ± 2.1	20 ± 1.7	17.36 ± 1.4	0.35 ± 0.02	0.32 ± 0.02
Quercetin-rutinoside metabolites						
Phenylacetic acid	9.3 ± 0.68	9.3 ± 0.91	9.5 ± 0.87	7.95 ± 0.76	16 ± 1.4	26 ± 2.1
3-Hydroxyphenylacetic acid	5.5 ± 0.43	5.4 ± 0.50	5.9 ± 0.53	4.71 ± 0.32	3.5 ± 0.32	18 ± 1.7
3,4-Dihydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	51 ± 4.3
3-(3,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	3.8 ± 0.3

Data expressed as mean values ± SE (n = 3).
n.d. not detected.

phenylacetic acid, at 15 ± 1.2 pM after 48 h of incubation. However when myricetin (flavonol such as quercetin) was fermented, poor metabolism was observed, with phenylacetic acid being the main metabolite found at 78 ± 6.1 pM after 48 h of incubation.

When the other subclasses of flavonoids tested, namely naringenin (flavanone) and luteolin (flavone), were fermented, differences in the number of fermentation products were obtained (Table 2). The metabolism of naringenin gave a higher number of metabolites with phenylacetic acid, *p*-hydroxyphenylacetic acid and 3-(*p*-hydroxyphenyl)propionic acid being the main fermentation products quantified, respectively, at 40 ± 4.0, 27 ± 2.6 and 45 ± 4.3 pM after 48 h of incubation. By contrast, the metabolism of luteolin was almost null (Table 2). Only 3-(2,4-dihydroxyphenyl)propionic acid was quantified at 0.64 ± 0.05 pM after 48 h of incubation. In relation to this poor metabolism, luteolin was detected in the fermentation medium throughout the incubation period at a concentration between 32 and 5 pM.

3.2 Colonic fermentation of phenolic acids

The results in Table 3 show high stability for gallic and protocatechuic acids, which

Table 2. Metabolism of kaempferol-rutinoside, naringenin, luteolin and miricetin by rat colonic microflora.

	Incubation time (hours)					
Compound (pM)	0h	1h	2h	4h	24h	48h
Kaempferol-rutinoside	9.0 ± 0.67	8.8 ± 0.87	8.9 ± 0.65	7.9 ± 0.32	0.02 ± 0.00	0.008 ± 0.00
Kaempferol-rutinoside metabolites						
Phenylacetic acid	3.4 ± 0.21	4.5 ± 0.35	3.5 ± 0.31	3.6 ± 0.31	1.3 ± 0.12	15 ± 1.2
2-Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.36 ± 0.02
p-Hydroxybenzoic acid	0.007 ± 0.00	0.09 ± 0.00	0.15 ± 0.01	1.0 ± 0.01	4.4 ± 2.0	4.2 ± 0.41
Naringenin	13 ± 0.10	13 ± 0.12	10 ± 0.99	4.7 ± 0.32	6.6 ± 0.64	n.d.
Naringenin metabolites						
Phenylacetic acid	1.4 ± 0.12	2.6 ± 0.24	3.40 ± 0.32	3.6 ± 0.26	4.4 ± 0.41	40 ± 4.0
4-Hydroxyphenylacetic acid	7.8 ± 0.56	11 ± 1.0	10 ± 1.0	11 ± 1.0	12 ± 1.0	27 ± 2.6
2-Hydroxyphenylacetic acid	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.19 ± 0.01
Protocatechuic acid	0.48 ± 0.02	0.53 ± 0.03	0.48 ± 0.02	0.44 ± 0.02	0.49 ± 0.03	1.2 ± 0.12
3-(4-Hydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	4.8 ± 0.47	45 ± 4.3
3-(2,4-Dihydroxyphenyl) propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.01
Luteolin	32 ± 2.9	30 ± 3.0	29 ± 2.1	15 ± 1.4	9.5 ± 0.93	5.0 ± 0.48
Luteolin metabolites						
3-(2,4-Dihydroxyphenyl) propionic acid	n.d.	n.d.	n.d.	n.d.	0.64 ± 0.05	n.d.
Miricetin	n.d.	0.05 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Miricetin metabolites						
Phenylacetic acid	8.1 ± 0.67	7.4 ± 0.72	7.1 ± 0.07	7.1 ± 0.65	60 ± 5.9	78 ± 6.1
3,4-Dihydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	0.69 ± 0.04	2.5 ± 0.06
3-(2,4-Dihydroxyphenyl) propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.4 ± 0.01

Data expressed as mean values±SE (n=3)
n.d. non detected

were detected in the fermentation medium throughout the incubation period (0–48 h). Both phenolic acids were metabolized by colonic microflora. Moreover some metabolites (phenylacetic, *p*-hydroxyphenylacetic, protocatechuic and *p*-hydroxybenzoic acids) were detected at low concentrations at 0 h of incubation. However, an increase in their concentration was observed in all cases throughout the complete incubation period. The main colonic metabolism was observed between 24 and 48 h of incubation. For example, phenylacetic acid and *p*-hydroxyphenylacetic acid were quantified at 15 ± 1.4 and 16 ± 1.2 pM respectively after 48 h of incubation of gallic acid. When the protocatechuic acid was fermented, phenylacetic acid and *p*-hydroxybenzoic acid were the two major fermentation products, with 40 ± 3.6 pM (24 h) and 44 ± 4.3 pM (48 h), respectively (Table 3).

3.3 Colonic fermentation of nuts-cocoa cream

After the individual analysis of the colonic metabolism of the flavonoids, the second part of the study was focused on the evaluation of the effect of the food matrix on this metabolism.

Table 4 shows the metabolites detected and quantified in the fermentation medium (*in vitro* colonic fermentation) of the nuts-cocoa cream after 0, 1, 2, 4, 24 and 48 h of incubation. The results of the *in vitro* colonic fermentation showed that the flavonoids added to the cream were not detected in the fermentation medium. Nevertheless, an increase in some

Table 3. Metabolism of gallic acid and protocatechuic acid by rat colonic microflora.

	Incubation time (hours)					
Compound (pM)	0h	1h	2h	4h	24h	48h
Gallic acid	43 ± 4.0	64 ± 6.1	67 ± 6.0	58 ± 0.54	32 ± 3.1	28 ± 2.3
Gallic acid metabolites						
Phenylacetic acid	1.5 ± 0.01	4.2 ± 3.2	4.3 ± 0.38	3.8 ± 0.23	19 ± 1.3	15 ± 1.4
4-Hydroxyphenylacetic acid	4.7 ± 0.43	10 ± 1.0	10 ± 0.98	10 ± 1.0	11 ± 0.95	16 ± 1.2
Protocatechuic acid	0.53 ± 0.04	0.72 ± 0.05	0.75 ± 0.07	0.66 ± 0.06	n.d.	6.8 ± 0.63
3-(4-Hydroxyphenyl) propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	5,8 ± 0.43
Protocatechuic acid	296 ± 21	284 ± 24	288 ± 17	265 ± 21	273 ± 25	254 ± 21
Protocatechuic acid metabolites						
Phenylacetic acid	4.2 ± 0.02	3.8 ± 0.31	5.0 ± 0.04	5.5 ± 0.43	40 ± 3.6	39 ± 3.9
2-Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0,70 ± 0.06
p-Hydroxybenzoic acid	0.38 ± 0.01	0.39 ± 0.02	0.75 ± 0.03	1.0 ± 0.01	0.47 ± 0.02	44 ± 4.3
3-(2,4-Dihydroxyphenyl) propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.6 ± 1.2

Data expressed as mean values±SE (n=3)
n.d. non detected

Table 4. Metabolism by rat colonic microflora of a 500ppm enriched nuts-cocoa cream with flavonoids (quercetin, quercetin-rhamnoside, quercetin-rutinoside, myricetin, rutin and luteolin) and phenolic acids (protocatechuic acid and gallic acid).

	Incubation time (hours)					
	0h	1h	2h	4h	24h	48h
Enriched nuts-cocoa cream						
Phenylacetic acid	0.48 ± 0.02	7.3 ± 0.54	7.2 ± 0.56	8.5 ± 0.79	5.2 ± 0.45	30 ± 2.7
4-Hydroxyphenylacetic acid	2.7 ± 0.18	10 ± 0.98	9.5 ± 0.94	11 ± 1.0	3.2 ± 0.31	12 ± 0.93
Protocatechuic acid	0.29 ± 0.02	3.5 ± 0.23	3.4 ± 0.32	4.8 ± 0.37	5.6 ± 0.56	5.2 ± 0.52
3-(2,4-Dihydroxyphenyl)propionic acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.6 ± 0.14
p-Hydroxybenzoic acid	0.09 ± 0.00	0.17 ± 0.01	0.19 ± 0.01	0.22 ± 0.020	0.68 ± 0.06	1.0 ± 0.13
Gallic acid	n.d.	0.09 ± 0.00	n.d.	0.28 ± 0.02	0.95 ± 0.07	0.52 ± 0.48

Data expressed as mean values±SE (n=3)
n.d. non detected

fermentation products was observed throughout the fermentation period (from 0 to 48 h), being phenylacetic acid the main fermentation product with 30 ± 2.7 pM after 48 h of fermentation, followed by its *p*-hydroxylated form at 12 ± 0.93 pM after the same incubation time. The minor fermentation products were 3-(3,4-dihydroxyphenyl)propionic acid, which was only detected after 48 h of incubation at 1.6 ± 0.14 pM, *p*-hydroxybenzoic acid reaching the maximum concentration after 48 h of incubation with 1.0 ± 0.13 pM and gallic acid with 0.95 ± 0.07 pM after 24 h of incubation.

4 DISCUSSION

In general, all the flavonoids studied were catabolised during the final period of

fermentation, between 24 and 48 h of incubation. In general, all the flavonoids studied, except quercetin and myricetin, were highly stable under the colonic conditions. The detection of hydroxylated forms and non-conjugated forms of some phenolic acids, such as phenyl acetic, phenyl propionic and protocatechuic acids, suggest that the flavonoids may be degraded to low-molecular-weight aromatic compounds in the large intestine due to the action of the colonic microflora. Hydroxylated forms of propionic acids were detected as intermediates in all the flavonoids tested.

In relation to the fermentation of quercetin, different metabolic pathways were proposed (Fig. 1A). Quercetin could be metabolized to 3-(3,4-dihydroxyphenyl)propionic acid via ring fission of the C-ring in various ways, and this could be subsequently degraded into 3,4-dihydroxyphenylacetic acid. This fermentation product was also produced by taxifolin as reported by Braune *et al.*, 2005 and Jaganath *et al.*, 2009 and Schneider, Schwiertz, Collins, and Blaut (1999). However, taxifolin was not detected in our study. After this step, 3,4-dihydroxyphenylacetic acid could be degraded into protocatechuic acid (the main metabolite quantified in the fermentation medium), to degrade subsequently into hydroxybenzoic acid. On the other hand, 3,4-dihydroxyphenylacetic acid may also be dehydroxylated to *m*- or *p*-hydroxyphenylacetic acid and phenylacetic acid. Besides, homovanillic acid was only quantified in the fermentation medium of quercetin, with it being the exclusive colonic fermentation metabolite of quercetin in our experiment. This has been previously reported in the literature (Jaganath *et al.*, 2009 and Justesen and Arrigoni, 2001), and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) has been determined in human urine as a biomarker for microbial and/or hepatic metabolism of quercetin (Gross *et al.*, 1996). However, the main metabolite formed from the colonic metabolism of quercetin in our study was protocatechuic acid and only small amounts of *p*-hydroxybenzoic acid and 3-(2,4-dihydroxyphenyl)propionic acid were quantified. These three fermentation products were also detected after the colonic fermentation of the flavonoid-enriched nuts–cocoa cream, where phenylacetic acid was the main fermentation product.

The fermentation of glycosylated forms of quercetin (quercetin–rhamnoside and quercetin–rutinoside) led to a lesser number of fermentation products compared with the fermentation of the free form of quercetin (Table 1). Quercetin was only quantified in the fermentation medium as a direct product of deglycosylation after the fermentation of quercetin–rhamnoside (Fig. 1B). Quercetin was not detected in the fermentation medium of quercetin–rutinoside, however, although the deglycosylation could have occurred as a first step of metabolism (Fig. 2A). The non-presence of quercetin was probably because quercetin–rutinoside was deglycosylated to quercetin and quercetin was immediately used as a substrate by the colonic microflora and the liberated sugar moiety could be used as a fermentation cosubstrate, thus accelerating the fermentation process (Justesen & Arrigoni, 2001). A similar situation was observed when enriched nuts–cocoa cream was fermented. Only the final fermentation products were detected, probably because of the presence of sugar and other matrix components that may act as a fermentation substrate, accelerating the

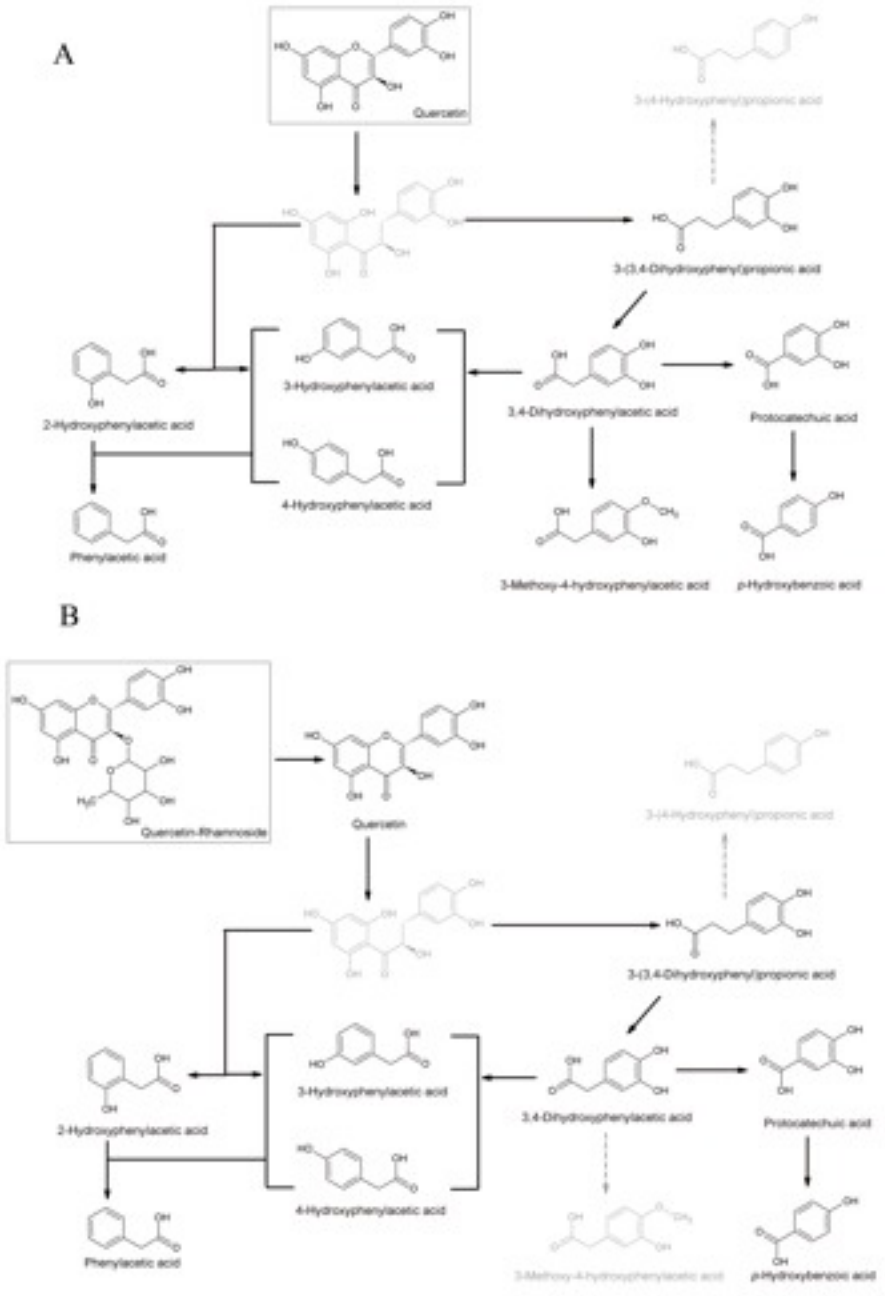

Fig. 1. Proposed metabolic pathway of quercetin (A) and quercetin–rhamnoside (B) by rat colonic microflora. Detected metabolites are in black, non-detected metabolites are in grey.

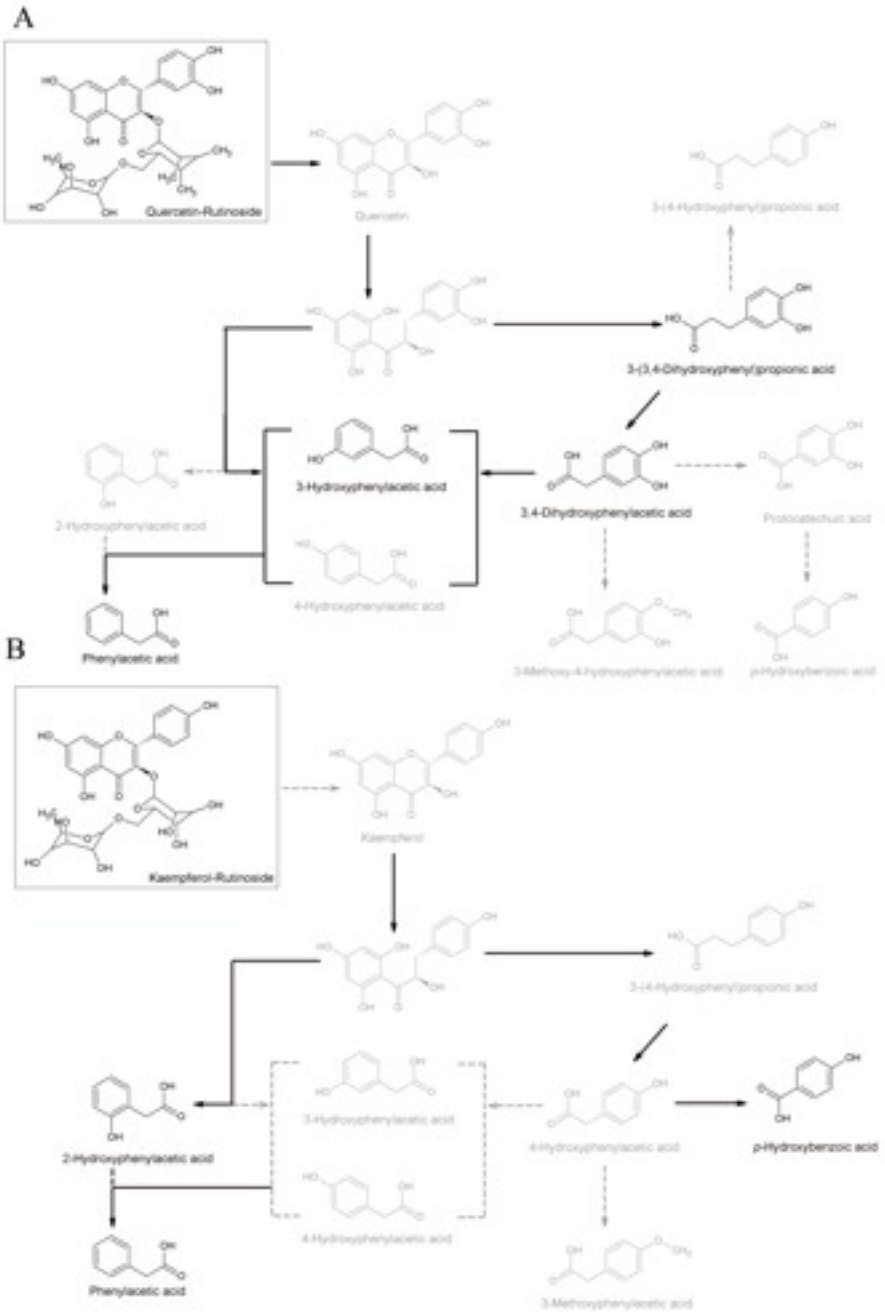

Fig. 2. Proposed metabolic pathway of quercetin–rutinoside (A) and kaempferol–rutinoside (B) by rat colonic microflora. Detected metabolites are in black, non-detected metabolites are in grey.

fermentation of flavonoids and phenolic acids. Quercetin–rutinoside deglycosylation was reported by Aura *et al.* (2002) and Crozier, Jaganath, and Clifford (2009) using human faecal bacteria. However, the deglycosylation pattern of quercetin–rutinoside was dependent upon the interindividual composition of the bacterial microflora. The proposed metabolic pathways of both glycoside forms, quercetin–rutinoside and rhamnoside, are shown in Fig. 1B and Fig. 2A, respectively. These were similar to that proposed for quercetin (Fig. 1A). Nevertheless, the corresponding tautomeric chalcone was not detected in the fermentation medium of the glycosylated quercetin structures. In general, the hydroxyphenylacetic acids have been characterised as specific metabolites of the colonic degradation of quercetin and quercetin glycosides (Aura *et al.*, 2002, Baba *et al.*, 1983, Gross *et al.*, 1996, Rechner *et al.*, 2002, Sawai *et al.*, 1987 and Schneider *et al.*, 1999) and have been also detected as a urine metabolite after their ingestion in flavonol-rich foods or plant extracts (Baba *et al.*, 1983, Booth *et al.*, 1956 and Sawai *et al.*, 1987).

In the present study, three hydroxylated forms of phenylacetic acid, *o*-, *m*- and *p*-, were detected in the quercetin–rhamnoside fermentation medium (Fig. 1B). On the other hand, only *m*-hydroxyphenylacetic acid was found in the quercetin–rutinoside medium (Fig. 2A). 3,4-dihydroxyphenylacetic acid and phenylacetic acid were common metabolites of quercetin–rhamnoside and quercetin–rutinoside, identified by other authors as one of the colonic degradation products of quercetin or quercetin glycosides in colonic fermentation models or human intervention studies (Aura *et al.*, 2002, Aura, 2008, Baba *et al.*, 1983, Gross *et al.*, 1996, Sawai *et al.*, 1987, Schneider and Blaut, 2000, Winter *et al.*, 1989 and Winter *et al.*, 199). Besides, protocatechuic acid and *p*-hydroxybenzoic acid were quantified as metabolites of quercetin–rhamnoside in our study. Human intervention studies have reported 3-methoxy-4-hydroxyphenylacetic (homovanillic acid) acid as a product of phase II metabolism (Jaganath *et al.*, 2009), probably produced in the liver by methylation of 3,4-dihydroxyphenylacetic acid after its transport from the colon to the portal vein. In our study, the methoxy-4-hydroxyphenylacetic acid was not detected after the fermentation of quercetin–rutinoside and quercetin–rhamnoside as the colonic fermentation model does not include a phase II metabolism.

The proposed metabolic pathway of kaempferol–rutinoside is shown in Fig. 2B, being similar to the proposed metabolic pathway of the quercetin–rutinoside. The metabolism of kaempferol–rutinoside showed an initial cleavage of the glycosydic bond, and the free form of kaempferol was not detected in the fermentation medium. The colonic fermentation showed an intense metabolism with phenylacetic acid as the main metabolite. A study by Schneider and Blaut (2000) only detected 2-(4-hydroxyphenyl)acetic acid as the fermentation product of kaempferol.

The proposed metabolic pathway of naringenin is shown in Fig. 3A. An intense metabolism of this compound was observed, thus giving rise to a wide range of metabolic products. The complete degradation of naringenin by colonic microflora may be related to its structure, with hydroxyl groups at the 5-, 7-, and 4′- position, and this structure is appropriate for optimal flavonoid

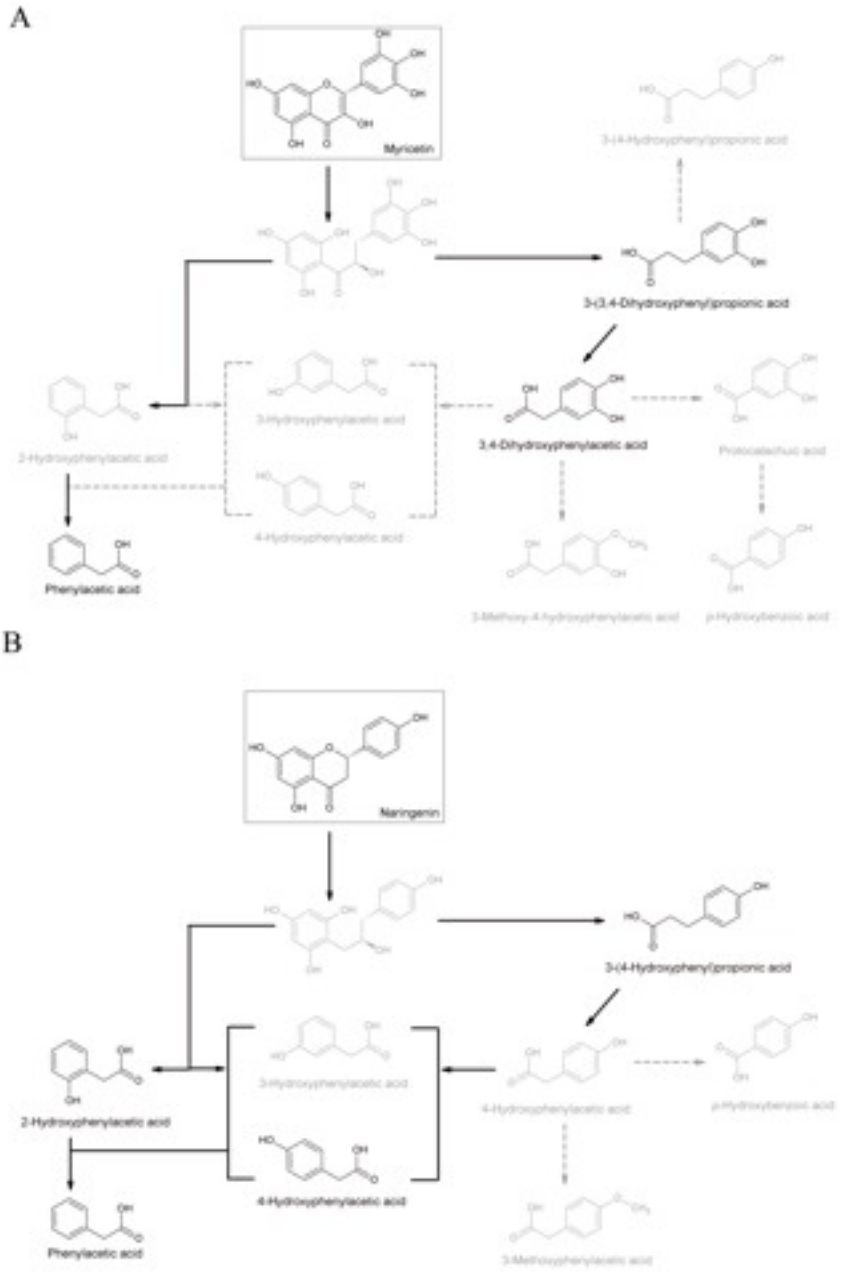

Fig. 3. Proposed metabolic pathway of naringenin (A) and myricetin (B) by rat colonic microflora. Detected metabolites are in black, non-detected metabolites are in grey.

degradation (Simons, Renouf, Hendrich, & Murphy, 2005). The ring cleavage product, 3-(4-hydroxyphenyl)propionic acid, was observed, consistent with the findings by other authors (Miyake *et al.*, 1997 and Yu *et al.*, 1997). However, Rechner *et al.* (2004) proposed phloroglucinol as a complementary metabolite of 3-(4-hydroxyphenyl) propionic acid after fermentation of naringenin, luteolin and kaempferol. However, this metabolite was not detected in our study. Fig. 3B shows the metabolic pathways of myricetin. This is a flavonol with three hydroxylated groups in the B ring, which released three fermentation metabolites, 2-(3,5-dihydroxyphenyl)acetic acid, its dehydroxylated form, phenylacetic acid, and 3-(3,5-dihydroxyphenyl)propionic acid. However, the trihydroxylated form of phenylacetic acid, 2-(3,5-dihydroxyphenyl)acetic acid, was not detected in the myricetin fermentation medium, in contrast to the results reported by Selma, Espín, and Tomás-Barberán (2009). Therefore, as it can be seen in Fig. 3B, myricetin showed the typical structure of degradation, with the formation of the corresponding chalcone with three hydroxylations as a precursor of the detected phenolic acids as final products of fermentation.

Unlike myricetin, luteolin showed poor colonic fermentation. Only 3-(3,4-dihydroxyphenyl)propionic acid was detected as a fermentation product at low concentration, 0.64 pM after 24 h of incubation (Table 2). This low colonic metabolism of luteolin was previously reported by Schneider and Blaut (2000).

In relation to the phenolic acids, gallic acid and protocatechuic acid, a low colonic fermentation was observed, with phenylacetic acid the main metabolite in both cases. In fact, the two phenolic acids were quantified in the fermentation medium during the complete incubation. These monophenol structures are metabolic products of the polyhydroxy polyphenol compounds, like flavonoids, which could explain their poor metabolism. In fact, only phenolic acids were detected in the fermentation medium of the enriched nuts-cocoa cream. The analysis of the metabolites in the fermentation medium showed a similar metabolic pathway for both phenolic acids, which were firstly dehydroxylated resulting in hydroxybenzoic and 3,4-dihydroxybenzoic acids from protocatechuic and gallic acids, respectively.

5 CONCLUSIONS

In this study, different metabolic pathways for the colonic metabolism of different subclasses of flavonoids and phenolic acids were studied and proposed. The metabolism of quercetin and quercetin–rhamnoside produced a wide range of fermentation products. Homovanillic acid was detected as a quercetin fermentation product, and was the exclusive quercetin fermentation metabolite. Only in the quercetin–rhamnoside fermentation was quercetin accumulated as a product of deglycosylation after incubation. On the other hand, a lesser number of fermentation products were observed during colonic fermentation of kaempferol–rutinoside, naringenin, luteolin and myricetin. Regardless of the flavonoid subclasses (flavanols, flavones and flavanones, and their glycosylated forms), phenylacetic acid and its hydroxylated forms were the main fermentation products. Phenolic acids were slightly metabolized by colonic

microflora. The intense metabolism of flavonoids and the accumulation of phenolic acids, mainly phenylacetic acid and *p*-hydroxyphenylacetic acid, were confirmed by the fermentation of a nuts–cocoa cream enriched with the same compounds studied individually.

6 ACKNOWLEDGEMENTS

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Publication III: Additional Information

Table 1 Additional information. Concentration of flavonoids and phenolic acids present in the IN fraction obtained by the *in vitro* duodenal digestion of an enriched nuts-cocoa cream. The results are

Compound	IN of the enriched nuts-cocoa cream (*) (nmol/g)
Quercetin	3.2 ± 0.12
Quercetin-rhamnoside	51 ± 4.8
Quercetin-rutinoside	5.1 ±3.1
Luteolin	2.9 ± 0.29
Myricetin	37 ± 2.6
Naringenin	2.7 ± 0.17
Protocatechuic acid	45 ± 3.8
Gallic acid	109 ± 10

(*)The quantification is expressed as nmols of compound in the digestion mixture, corresponding to 1 g of digested nuts-cocoa cream.
Data expressed as mean values ± standard desviation (n=3)

Method validation

Plasma and tissue samples

Serra et al. Journal of Chromatography B (2009) 877, 1169-1176
Serra et al. Journal of Chromatography B (2011) 879, 1519-1528

Determination of procyanidins and their metabolites in plasma samples by ultra-performance líquid chromatography – tandem mass spectrometry

Journal of Chromatography B (2009) 877, 1169–1176

DETERMINATION OF PROCYANIDINS AND THEIR METABOLITES IN PLASMA SAMPLES BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

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Abstract
An off-line solid-phase extraction (SPE) and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for determining procyanidins, catechin, epicatechin, dimer, and trimer in plasma samples. In the validation procedure of the analytical method, linearity, precision, accuracy, detection limits (LODs), quantification limits (LOQs), and the matrix effect were studied. Recoveries of the procyanidins were higher than 84%, except for the trimer, which was 65%. The LODs and LOQs were lower than 0.003 and 0.01 μM, respectively, for all the procyanidins studied, except for the trimers, which were 0.8 and 0.98 μM, respectively. This methodology was then applied for the analysis of rat plasma obtained 2 h after ingestion of grape seed phenolic extract. Monomers (catechin and epicatechin), dimer and trimer in their native form were detected and quantified in plasma samples, and their concentration ranged from 0.85 to 8.55 μM. Moreover, several metabolites, such as catechin and epicatechin glucuronide, catechin and epicatechin methyl glucuronide, and catechin and epicatechin methyl-sulphate were identified. These conjugated forms were quantified, in reference to the respective unconjugated form, showing concentrations between 0.06 and 23.90 μM.

Keywords: UPLC-MS/MS; Plasma; Procyanidins.

1 INTRODUCTION

Flavanols are found in a wide range of food sources as both monomers and oligomeric procyanidins. Flavanol monomers are (–)-epicatechin and (+)-catechin, and procyanidins are oligomers of epicatechin and catechin. The main food sources of flavanols include cocoa,

red wine, green tea, red grapes, berries and apples [1]. Over the past decade, different studies have reported on the health benefits, mainly cardio-protective effects, of flavanols. These findings have been attributed in part to their antioxidant properties. Thus, research into the bioavailability, metabolism and pharmacokinetics of ingested flavanols in the diets of volunteers is of great importance as it may in turn reflect the antioxidant status of the subjects studied.

Biomarkers are biological molecules found in blood, body tissues or physiological fluids. These biological molecules are useful tools in nutritional epidemiology to determine food or nutrient exposure because they combine the measurement of bioavailability and metabolism. The main advantage of biomarkers is that they are an important alternative to the more traditional dietary assessment tools, such as diet histories, food frequency questionnaire or diet diaries [2] with significant variability related to food processing and storage conditions, and dietetic estimations.

Several studies in humans have demonstrated the relationship between the phenols present in biological fluids, such as blood or urine, and dietary intake [3] and [4]. Dietary polyphenols undergo metabolic conversion in the liver, including methylation of a hydroxyl group and reduction of a carbonyl group, as well as conjugation leading to glucurono and sulphoconjugates. In order to use these specific metabolites as biomarkers, it is essential that the analytical technique for determining them in biological samples to be sensitive, selective, reliable and robust.

Different analytical methodologies have been reported for the analysis of procyanidins in biological samples, such as plasma [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [16], [17], [18], [19] and [20], urine [9], [11], [13], [18] and [19], and body tissues [13], [15] and [19]. These methods include high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [15], [16] and [18], tandem MS [8], [13] and [14], fluorimetric [6] and [17], diode array detection (DAD) [11], and electrochemical detector (ECD) [7], [8], [9] and [12], gas chromatography (GC) coupled to MS [5] and [19], although this technique often requires derivatization, and capillary electrophoresis (CE) coupled to DAD [20].

Recent advances in analytical techniques have improved their effectiveness and could expand their potential for analyzing and identifying biomarkers. The use of MS and tandem MS (MS/MS) as a detector is a useful tool for determining analytes at low concentrations and for the positive identification of compounds in complex samples, such as biological ones.

The aim of this study is to develop and validate a rapid, sensible and reliable method for the determination of monomers, catechin and epicatechin, dimers and trimers in plasma samples by off-line SPE-UPLC-MS/MS. After validation, the method was used to identify and quantify procyanidins and their metabolites in rat plasma obtained 2 h after administration, by intragastric gavage, of a dose corresponding to 1 g of grape seed procyanidin extract (GSPE) per kilogram of body weight.

2 EXPERIMENTAL

2.1 Chemicals and Reagents

Standards of (−)-epicatechin, (+)-catechin and the internal standard (IS)

catechol were purchased from Sigma–Aldrich (St. Louis, MO, USA); and procyanidin B_2 from Fluka Co. (Buchs, Switzerland). Individual stock standard solutions of 2000 mg/l, except to the dimer which was 500 mg/l, were dissolved in methanol and stored in a dark-glass flask at −18 °C. A stock standard mixed solution of catechin, epicatechin and dimer was prepared weekly at a concentration of 100 mg/l in methanol. The working standard solutions were prepared daily by diluting the stock standard solutions with the solution of acetone:water:acetic acid (70:29.5:0.5, v:v:v).

The GSPE, whose commercial name is Vitaflavan® (Les Dérives Résiniques et Terpéniques, Dax, France) was obtained from *vitis vinifera* grape seed from southwest France. The procyanidin content of the extract was >75% (monomers 22%, dimers 20% and trimers to pentamers 56%). The cocoa nib samples corresponded to the Forastero variety from Ghana (West Africa). Cocoa nibs were obtained from roasted cocoa beans separated from their husks and broken into small pieces.

Acetonitrile (HPLC-grade), methanol (HPLC-grade), acetone (HPLC-grade) and glacial acetic acid (≥99.8%) were of analytical grade (Scharlab, Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Isolation of the trimer as standard

The trimer was isolated by using semi-preparative HPLC in normal phase from a phenolic extract obtained from cocoa nibs [21]. The cocoa nib phenolic extract was dissolved in acetone:water:acetic acid (70:29.5:0.5, v:v:v) at a concentration of 100 mg/ml before the chromatographic fractionation.

The semi-preparative HPLC system includes a Waters 1525EF binary HPLC pump, a Waters Flexinject, an Inertsil ODS-3 column (5 mm, 25 cm × 10 mm i.d., GL Sciences Inc.) equipped with a Spherisorb S5 ODS-2 (5 mm, 10 cm × 10 mm i.d., Technokroma, Barcelona, Spain) precolumn, a Waters 2487 λ absorbance detector (280 nm) and a Waters Fraction Collector II. The semi-preparative HPLC system was operated using Brezze software. 50 μl of phenolic extract of cocoa nibs was injected manually into the injector module (1 ml sample loop). The mobile phase was dichloromethane: methanol: water:acetic acid (84:14:2:2, v:v:v:v) as the eluent A, and methanol:water:acetic acid (96:2:2, v:v:v) as the eluent B. The elution started with 0% of eluent B, then was linearly increased to 17.6% of eluent B in 30 min, further increased to 45% of B in 15 min, and an additional increase to 50% B in 5 min. Then, it was kept isocratic for 10 min and returned to the initial conditions for 10 min. The reequilibration time was 10 min. The flow rate was 1 ml/min.

The fractions corresponded to trimer were collected manually by observing the detector output on the recorder and according to their retention time [22] and [23]. After that, the organic solvent was removed by rotary evaporation (Buchi, Labortechnick AG, Switzerland) under a partial vacuum at 30 °C. Finally, the water extract was freeze-dried in Lyobeta 15 lyophilizer (ImaTelstar, Spain) and then the fractions were stored in an N_2 atmosphere at −40 °C. An aliquot of the lyophilized fraction was exactly weighted and dissolved in acetone:water:acetic acid

(70:29.5:0.5, v:v:v) at final concentration of 5.8 μM. Then, this solution was spiked to the plasma matrix in order to study the quality parameters of the developed method.

2.3 Plasma samples

Rat plasma samples were obtained from male Wistar rats (three-month-old). These rats weighed between 370 and 420 g and were purchased from Charles River Laboratories (Barcelona, Spain). The Animal Ethics Committee of the Rovira i Virgili University approved all the procedures. The rats were maintained in air-conditioned quarters at 22 °C under 12 h dark/12 h light cycles (light from 9 a.m. to 9 p.m.). All the animals were fed a standard diet of PanLab A04 (Panlab, Barcelona, Spain) and water.

Procyanidin-rich extract (Vitaflavan®) was administered to the rats by intragastric gavage, after the animals had been in fasting conditions between 16 and 17 h with only access to tap water. The procyanidin extract was administered to the rats at a dose of 1 g GSPE per kilogram of body weight. 2 h after the treatment the rats were anaesthetized with ketamine–xylazine and blood samples were collected from abdominal aorta. The plasma samples were obtained by centrifugation (2000 × g, 30 min at 4 °C) and stored at −40 °C until chromatographic analysis.

2.4 Plasma phenols extraction

Prior to the chromatographic analysis, the rat plasma samples were pretreated by off-line SPE. OASIS HLB (60 mg, Waters Corp., Milford, MA) cartridges were used. These were conditioned sequentially with 5 ml of methanol and 5 ml of Milli-Q water/acetic acid (99.8:0.2, v:v). 20 μl of phosphoric acid 85% and 50 μl of catechol (IS) at a concentration of 20 mg/l were added to 1 ml of plasma sample, and then this solution was loaded into the cartridge. The loaded cartridges were washed with 3 ml of Milli-Q water and 5 ml of Milli-Q water/acetic acid (99.8:0.2, v:v). Then, the retained procyanidins were eluted with 4 ml of solution acetone:water:acetic acid (70:29.5:0.5, v:v:v). The elution solvent was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 °C (PIERCE Model 18780, IL, USA) and reconstituted with 100 μl of elution solution. Finally, the extract was filtered through 0.22 μm nylon syringe filter (Teknokroma, Barcelona, Spain) and transferred to the autosampler vial before the chromatographic analysis. The injection volume was 2.5 μl.

In order to obtain a plasma matrix free from procyanidins, pool plasma from basal conditions was dephenolized. To dephenolization, the plasma was loaded through the cartridge (OASIS HLB 200 mg, Waters Corp., Milford, MA) and the elution of the loaded plasma was collected. This sample matrix was used as the blank plasma to validate the procedure.

2.5 UPLC-ESI-MS/MS

The UPLC analysis of procyanindin extracts was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters, Milford, MA, USA). The separations were achieved using an Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) from Waters (Milford, MA, USA). The analytes were separated with a mobile phase consisting of water:acetic acid (99.8:0.2, v/v) (eluent A) and acetonitrile (eluent B) at a flow rate of 0.4 ml/min. The gradient elution was reported in our previous work [21] and the total analysis

time was 12.5 min. Briefly, it 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; 11.10–12.50 min, 5%B isocratic.

The tandem MS analyses were carried out on a TQD mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. The analyses were done in negative mode, and the data was acquired in selected reaction monitoring (SRM). The ionization source working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 l/h and desolvation gas flow rate, 800 l/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimized by infusion of a standard solution of 10 mg/l of each standard in a mixture of acetonitrile:water (50:50, v/v) at a flow rate of 10 μl/min. First, full-scan spectra were acquired in order to select the most abundant *m/z* value and the cone voltage was optimized. In all cases, $[M-H]^-$ ions were found to be the most abundant. These ions were selected as the precursor ions, and afterwards, the collision energies were studied to find the most abundant product ions. Therefore, the most sensitive transition was selected for quantification purposes and the second one for confirmation. Table 1 shows the MS/MS transitions for quantification and confirmation as well as cone voltage and collision energy values optimized for each of the standard compounds. For catechol, the product ion 91 *m/z* was the only one formed and hence no second MS/MS transition was available for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out by MassLynx v 4.1 software.

2.6 Validation procedure

The method developed was validated using a serial dilution of the blank plasma spiked with the standard catechin, epicatechin, dimer B_2, and trimer. The parameters considered were: recovery, linearity, calibration curves, precision, accuracy, detection limit (LOD), quantification limit (LOQ), and the study of the matrix effect.

The linearity of the method was evaluated using blank plasma spiked with the analytes. Calibration curves (based on peak abundance) were plotted using $y = a + bx$, where y is the (analyte/IS) peak abundance ratio and x is the (analyte/IS) concentration ratio. Concentrations of the procyanidins were calculated by interpolating their (analyte/IS) peak abundance ratios on the calibration curve. The calibration curves were obtained by analyzing five points at different concentration levels and each

Table 1. Optimized SRM conditions for analyzing catechol (IS), catechin, epicatechin, dimer B_2, and trimer by UPLC-MS/MS in negative mode.

Compound	SRM_1 (quantification)	Cone Voltage (V)	Collision energy (eV)	SRM_2 (confirmation)	Cone Voltage (V)	Collision energy (eV)
Catechol (IS)	109 > 91	40	15	-	-	-
Catechin	289 > 245	45	10	289 > 205	45	15
Epicatechin	289 > 245	45	10	289 > 179	45	15
Dimer B_2	577 > 289	45	20	577 > 425	45	15
Trimer	865 > 577	60	20	865 > 695	60	25

standard solution was injected three times.

The precision of the method, expressed by relative standard deviations (% RSDs) of the concentration and peak abundance, was studied at two concentration levels, 0.2 and 1.4 μM, except for the dimer and trimer, which were 0.07 and 0.6 μM, on three different days (inter-day) and one injection each day. The accuracy of the methodology was evaluated by sample quantification at a concentration level of 2 μM for the trimer, and 0.2 μM for the rest of the procyanidins. The accuracy was calculated from the ratio between the concentrations of the procyanidin found compared to the spiked concentration. This quotient was then multiplied by 100.

The LODs and LOQs were calculated using the signal-to-noise criterion of 3 and 10, respectively. The extraction recoveries of the analytes were determined using blank plasma spiked with the analytes at a concentration of 3 μM, except for the dimer and the trimer, which were 1.5 and 5.8 μM, respectively. To determine extraction recoveries, the responses of the analytes spiked in plasma matrices before and after extraction were compared.

The matrix effect was evaluated by comparing peak abundances obtained from blank plasma samples spiked after sample pre-treatment with those obtained from standard solutions.

3 RESULTS AND DISCUSSION

3.1 Trimer isolation

Trimer procyanidin is not commercially available, and so for this study, the trimer was obtained from an extract rich in procyanidins by semi-preparative HPLC-DAD. Two extracts were tested for this purpose, these being the GSPE extract (Vitaflavan®), and the phenolic cocoa nib extract. Semi-preparative HPLC in the normal phase was used because various authors have reported that this mode separates the procyanidins by their degree of polymerization better than in the reverse mode [22] and [23].

The amount of procyanidins in the GSPE extract was higher than in the cocoa extracts. However, the chromatographic peaks corresponding to different procyanidins in the GSPE extract were broader than the peaks from the cocoa extracts. This could be related to the greater presence of the isomeric forms in the structure of the procyanidins (dimer, trimer, etc.) in the GSPE extract than in the procyanidins from the cocoa extract. The major isomerization level of the procyanidins in the GSPE extract made it difficult to isolate the trimer by semi-preparative HPLC.

Fig. 1 shows the extracted ion chromatograms of the trimer procyanidin isolated from the (A) GSPE extract, and the (B) cocoa nib phenolic extract by HPLC-MS/MS in the normal phase. As Fig. 1B shows, the trimer procyanidin isolated from the cocoa extract is very simple, and only two isomeric forms were observed. By contrast, the trimer isolated from the GSPE extract shows a great number of isomeric forms (see Fig. 1A). In consequence, the cocoa extract was selected to purify the trimer because it showed a higher peak efficiency than that obtained by the GSPE extract and this made it easy to study the validation procedure.

3.2 SPE procyanidins extraction

Off-line SPE was used as the sample pretreatment technique to clean-up the plasma sample matrix and determine the

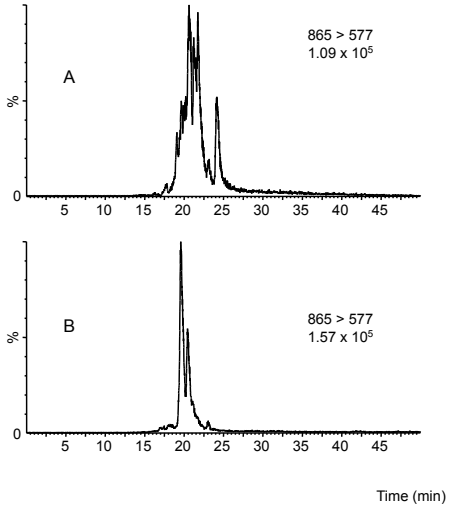

Figure 1. Extracted ion chromatograms of trimer procyanidin isolated by semipreparative HPLC and analyzed by HPLC-MS/MS from (A) GSPE extract, and (B) purified cocoa nib extract. The concentration of each extract was 10mg/ml, and this was dissolved in the extraction solution. See Experimental Section 2.2 for the experimental conditions.

procyanidins at low concentration levels. The main objective of the clean-up step is to remove proteins and endogenous substances present in the biological fluids that could interfere with the chromatographic analysis of the procyanidins. A critical aspect of the clean-up is the optimization of the recovery of the analytes of interest during the elution steps to obtain the maximum recoveries. For this purpose, the commercial standards of the procyanidins (catechin, epicatechin and dimer B_2) and the purified trimer were spiked in blank plasma at different concentrations. Plasma sample acidification was taken into account to disrupt phenol–protein binding and to enhance recovery of the analytes [24].

Different volumes (1–5 ml) of water and acidic water were studied in the clean-up step to remove any possible interference from the plasma matrix without eluting the retained procyanidins. Different volumes were evaluated, and finally we selected 3 ml of water following by 5 ml of acid water (0.2% acetic acid) to clean-up the cartridge, removing the interference substances without elution of the analytes of interest.

Afterwards, the procyanidins elution step was studied. To determine the appropriate composition of the elution solvent, different organic solvents or solutions and different volumes of these were evaluated. The elution solvents studied were acetone:water:acetic acid (70:29.5:0.5, v:v:v), and methanol/acetone at different percentages, from 0% to 100%. The recoveries were higher when acetone:water:acetic acid (70:29.5:0.5, v:v:v) was used as elution solvent. When 1 ml of this solvent was used, almost 100% of the monomers catechin and epicatechin were eluted. However, 4 ml was necessary to elute the procyanidins, dimer and trimer. To increase the pre-concentration factor, evaporation to dryness was carried out in an N_2 stream in an evaporating unit at 30 °C as has been described in Experimental Section 2.4, and reconstituted with 100 μl of elution solution. Then, the extract was re-dissolved in the minimum volume of solvent in order to obtain a high pre-concentration factor. A volume of 100 μl of two different solvents, acetone:water:acetic acid (70:29.5:05, v:v:v) and acetonitrile, were tested to re-dissolve the dried procyanidin plasma extract. When acetonitrile was used, a peak distortion was obtained during the chromatographic analysis. Therefore, acetone:water:acetic acid (70:29.5:05, v:v:v) was selected and the obtained

recoveries were higher than 84% for all the procyanidins, except for the trimer, which was 65% (see Table 2).

As mentioned above, pool plasma from basal conditions was dephenolized in order to obtain a plasma matrix free from procyanidins for the validation procedure. When this plasma sample was pre-treated and pre-concentrated by off-line SPE, no interference peaks were observed. Fig. 2 shows the extracted ion chromatograms of a blank plasma sample spiked with 1.5 μM of catechin and epicatechin, 0.4 μM of dimer B_2, and 3.4 μM of trimer after the SPE extraction.

3.3 Matrix effect

When ESI is used as the ionization technique in MS, one of the main problems is the signal suppression or enhancement of the analyte response due to the other components present in the sample matrix (matrix effect) [25]. To evaluate this matrix effect, the detector responses of the procyanidins spiked in elution solvent (acetone:water:acetic acid (70:29.5:0.5, v:v:v)) were compared to those spiked in plasma at different concentrations. Either a positive or negative effect was observed, lower than 17%, which meant an increase or decrease in the detector response, respectively. Nevertheless, this effect could be considered small. To reduce inaccuracies by matrix, as well as the clean-up step of the plasma sample by off-line SPE, the preparation of the calibration curves with spiked plasma was considered more appropriate.

3.4 Validation of the analytical procedure

Blank plasma spiked with different procyanidin concentrations was analyzed by off-line SPE and UPLC-MS/MS to determine the linearity, calibration curves, reproducibility, accuracy, LODs and LOQs.

Linearity was tested following the procedure developed in the range from 0.02 to 5.8 μM. All the compounds showed R2 higher than 0.998 (Table 2). The precision of the method, calculated as the relative standard deviation (% RSD), in terms of concentration, was calculated at two concentration levels, 0.07 and 0.6 μM for the trimer and dimer, and 0.2 and 1.4 μM for the rest of the procyanidins, and these gave results lower than 8.9% and 4.3%, respectively.

Table 2. Retention time (RT), recovery (%R), linearity, calibration curves, reproducibility, accuracy, LODs and LOQs for the analysis of the studied compounds by UPLC-MS/MS in spiked plasma samples.

Compound	RT (min)	%R	Linearity (μM)	Calibration curve	RSD% (n=3) [a] 1.4 μM	RSD% (n=3) [a] 0.2 μM	Accuracy	LOD (μM)	LOQ (μM)
Catechin	3.68	102	0.02-3	y = 6.913x + 0.252	4.3	8.9	104	0.004	0.013
Epicatechin	4.55	96	0.02-3	y = 5.830x + 0.270	3.9	8.6	99.8	0.004	0.015
Dimer B_2	4.09	84	0.008-0.8	y = 5.524x + 0.030	3.5 [b]	8.5 [c]	98	0.003	0.010
Trimer	4.93	65	0.9-5.8	y = 0.045x – 0.085x	4.2 [b]	8.8 [c]	102	0.800	0.980

a RSD% was calculated as concentration.
b 0.6μM.
c 0.07μM.

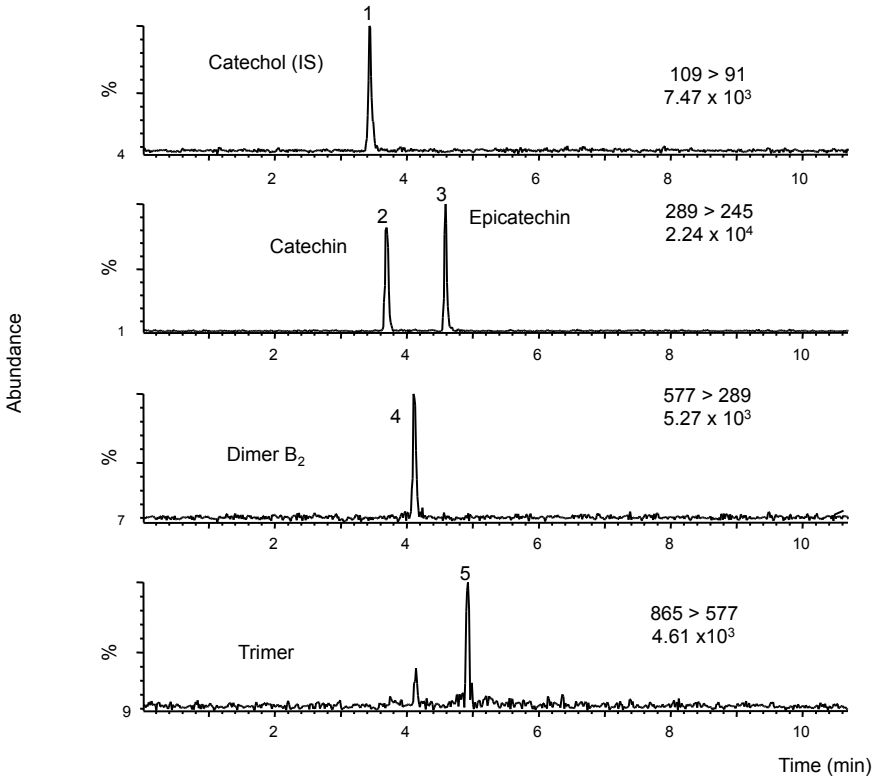

Figure 2. Extracted ion chromatograms from blank plasma sample spiked with catechin, epicatechin, dimmer B_2 and trimer. The peak designation and its concentration was (1) catechol, 9 μM; (2) catechin, 1.5 μM; (3) epicatechin, 1.5 μM; (4) dimer B_2, 0.4 μM; and (5) trimer, 3.4 μM.

The accuracy of the method was between 98% and 104%. The LODs and LOQs were lower than 0.003 and 0.01 μM, respectively, for all the procyanidins studied, except for the trimers, which were 0.8 and 0.98 μM, respectively. These values were similar to and lower than the results reported in the literature [5], [6], [11], [16], [17], [19] and [20]. One of the greatest advantages of the UPLC method developed is the possibility of quantifying the procyanidins studied at low concentrations within 5 min. This analysis time is between 2 and 5-times shorter than the methods reported in the literature for the analysis of these compounds by HPLC [5], [6], [7], [8], [9], [10], [11], [13], [14], [15], [16], [17], [18] and [19].

Additionally, the UPLC system allowed the volume of the sample injected to be between 4 and 40-fold lower than the other methods reported in the literature [14], [15], [16] and [17].

3.5 Application of the method for determining procyanidins in plasma samples

In order to show the applicability of the method developed, rat plasma was analyzed. Procyanidin-rich plasma was obtained from the abdominal aorta of the rats 2 h after administration, via direct stomach intubation, of a dose of GSPE extract corresponding to 1 g/kg of weight. Rat plasma extracted before ingestion of the GSPE extract was used as a control. The UPLC-DAD chromatograms of the procyanidins in the control and procyanidin-rich plasmas are shown in Fig. 3A and B, respectively. Important differences in the chromatographic profile of the two plasma samples were observed. Thus, chromatographic peaks corresponding to monomers (catechin and epicatechin, peaks 2 and 3, respectively), dimers (peak 4) and trimers (peak 5) were only identified in plasma obtained after the ingestion of the GSPE

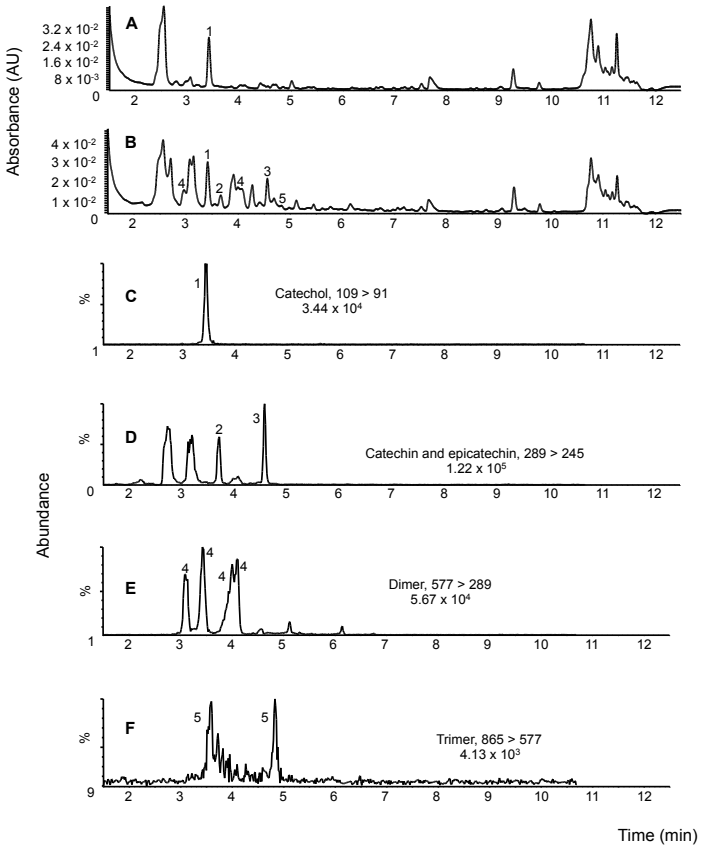

Figure 3. Off-line SPE-UPLC-DAD chromatogram of (A) control rat plasma, and (B) procyanidin rat plasma at 280 nm. Extracted ion chromatograms of (C) catechol (IS), (D) catechin and epicatechin, (E) dimer B_2, and (F) trimer. See the text for the conditions of the experiment.

extract (Fig. 3B). Fig. 3C–F also shows the extracted ion chromatograms of the catechol (IS) and the free procyanidins. The quantification of the free procyanidins (monomers to trimers) showed concentrations ranging from 0.85 to 8.55 μM (Table 3).

The detection and quantification of dimers and trimers in the rat plasma are relevant, because their presence could suggest that these oligomeric forms are absorbed and metabolized in the same way as the monomeric forms. The results obtained agreed with those in the literature, where some authors also detected monomers [10] and [14], dimers [14], [26] and [27], and trimers [14] in plasma samples after intake of procyanidin-rich extracts, such as grape seed extract [27], cocoa [6], [7], [8], [16] and [28], apple [14] and tea [16].

In MS, it is very important to quantify each analyte with the respective calibration curve because the ionization can vary in function of the molecule structure. This was observed in the present study with the quantification of the trimer. When the trimer was quantified using its calibration curve obtained with the purified standard from cocoa phenol extract (see Experimental Section 2.2), the concentration of this oligomer in the plasma was 8.55 μM. In contrast, the quantification of the trimer using the calibration curve of catechin, epicatechin or dimer showed concentrations between 0.05 and 0.06 μM.

During digestion and intestinal absorption, polyphenols are subjected to three main types of conjugation: methylation, sulphation, and glucuronidation [29]. In consequence, apart of the free procyanidins, different conjugated metabolites could be present

Table 3. Procyanidin concentration detected in rat plasma sample by off-line SPE-UPLC-MS/MS.

Compound	Concentration (μM)
Catechin	0.85
Epicatechin	1.28
Dimer	2.40
Trimer	8.55

in rat plasma after the ingestion of GSPE extract. As well as applying the validation method to quantify the free forms of the procyanidins in rat plasma, the identification of the potential generated metabolites was considered. For this purpose, analyses in MS (full-scan mode) and MS/MS (based on neutral loss scan and product ion scan mode) were performed. These techniques (full-scan and product ion scan) are excellent tools for verifying structural information about the compounds when standards are not available.

First, analyses were carried out in the full-scan mode (from 80 to 1200 *m/z*) by applying different cone voltages from 20 to 60 V. When low cone voltages were applied, the MS spectrum gave information about the precursor ion or the $[M–H]^-$. In contrast, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their structure. The structural information was also verified by using product ion scan and neutral loss scan in the MS/MS mode. In product ion scan experiments, the product ions are produced by collision-activated dissociation of the selected precursor ion in the collision cell. Neutral loss scan of 80 and 176 units

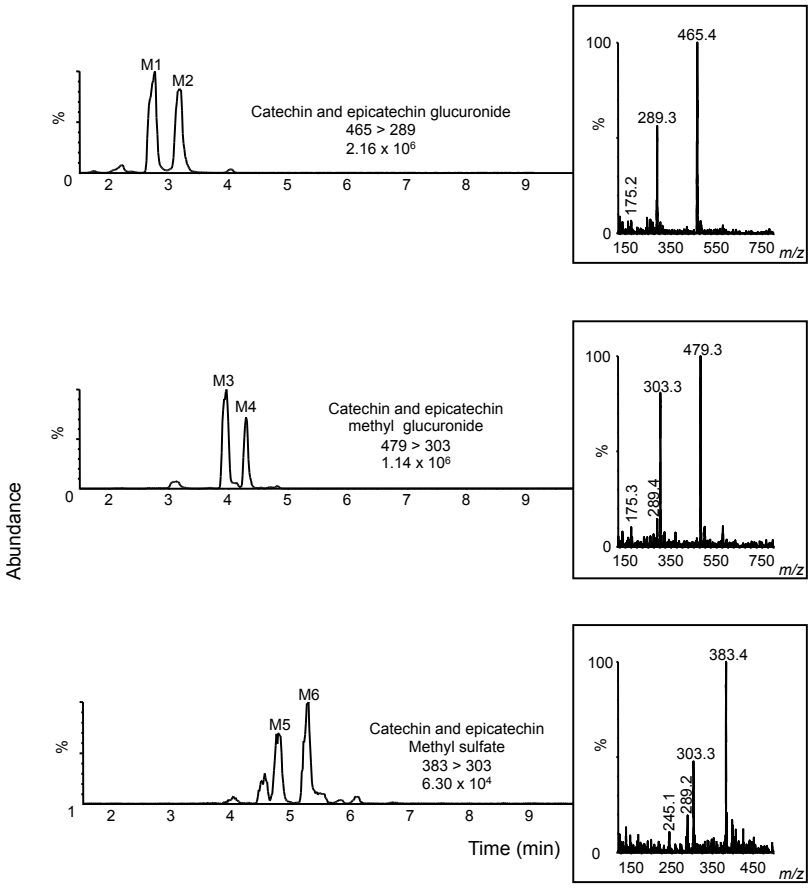

Figure 4. Extracted ion chromatograms and its MS spectra of the generated metabolites: (M1) catechin glucuronide, (M2) epicatechin glucuronide, (M3) catechin methyl-glucuronide, (M4) epicatechin methyl-glucuronide, (M5) catechin methyl-sulphate, and (M6) epicatechin methyl-sulphate. See the text for the conditions of the experiment.

were used to characterize the sulphate and glucuronide forms, respectively.

In the analysis of rat plasma, six procyanidin metabolites could be identified by the fact that their product ions produced ions at m/z 289 that matched that of catechin/epicatechin. The metabolites identified were catechin and epicatechin glucuronide, catechin and epicatechin methyl-glucuronide, and catechin and epicatechin methyl-sulphate, as shown in Table 4. Considering the UPLC elution order of the catechin and epicatechin (Fig. 2) and the elution order of the metabolites (Fig. 4), together with the MS fragmentation pattern, the earlier eluting M1, M3 and M5 peaks have been assigned to catechin metabolites, while the M2, M4 and M6 peaks have been assigned to epicatechin metabolites.

Table 4. Procyanidin metabolites identified in rat plasma sample by off-line SPE-UPLC-MS.

Metabolite[a]	Compound	RT (min)	$[M-H]^-$	MS^2 ions (*m/z*)
M1	Catechin glucuronide	2.59	465	289,175
M2	Epicatechin glucuronide	3.03	465	289,175
M3	Catechin methyl-glucuronide	3.79	479	303,289
M4	Epicatechin methyl-glucuronide	4.16	479	303,289
M5	Catechin methyl-sulphate	4.52	383	303,289,245
M6	Epicatechin methyl-sulphate	5.01	383	303,289,245

[a]*See Fig. 4.*

Two ions gave a precursor ion of *m/z* 479 and product ions of *m/z* 303 and *m/z* 289. These ions could be identified as catechin and epicatechin methyl-glucuronide, because the product ion *m/z* 303 is due to the loss of glucuronide molecule (176 units) and the ion *m/z* 289 to the loss of methyl group (14 units).

The two ions with precursor ion of *m/z* 465 could be identified as catechin and epicatechin glucuronide, respectively, due to the loss of 176 units (*m/z* 289) that could correspond to glucuronide molecule.

Then, the last procyanidin metabolite identified could be catechin and epicatechin methyl-sulphate (precursor ion of 383 *m/z*) whose product ions were *m/z* 303 and *m/z* 289. These could be explained by the loss of a sulphate molecule (80 units) and the methyl group (14 units), respectively. Fig. 4 shows the extracted ion chromatograms of the metabolites generated and their MS spectrum.

The metabolites detected in rat plasma in the present study were in agreement with those of other authors, who detected catechin and epicatechin glucuronide [13] and [30], catechin and epicatechin methyl-glucuronide [13]. However, in others studies, the conjugated forms of procyanidins were not identified in plasma samples like these because previous enzymatic treatments of the sample using β-glucuronidase and sulphatase were applied before the chromatographic analysis [10], [11], [17] and [20]. The recovery and quantification of these metabolites in biological samples are complex because of the lack of standards, those justify the enzymatic treatment of the samples to their tentative quantification in reference to the respective free forms. However the enzymatic treatment could be more an additional confirmation in the identification of the conjugate forms of procyanidins than an accurate quantification. The time and temperature conditions during the enzymatic treatment could result in a loss of the free procyanidins, leading to under-quantification. In this study, the enzymatic treatment was not considered previously to quantification, and the procyanidin metabolites were quantified in catechin

Table 5. Quantification of procyanidin metabolites by off-line SPE-UPLC.MS/MS.

Compound	SRM (quantification)	Cone voltage (V)	E collision (eV)	Concentration (μM)
Catechin glucuronide[a]	465>289	40	20	2.390
Epicatechin glucuronide[b]	465>289	40	20	2.057
Catechin methyl-glucuronide[a]	479>303	40	25	1.375
Epicatechin methyl-glucuronide[b]	479>303	40	25	906
Catechin methyl-sulphate[a]	383>303	40	15	105
Epicatechin methyl-sulphate[b]	383>303	40	15	130

[a] *Quantified with the calibration curve of catechin.*
[b] *Quantified with the calibration curve of epicatechin.*

and epicatechin equivalents by UPLC-MS/MS and two SRM transitions were selected for this. Table 5 shows the quantification of these metabolites in plasma samples using the calibration curves of the respective free forms, and the concentrations are expressed as catechin or epicatechin equivalents. The results confirmed an important metabolism of the procyanidins after the ingestion of the GSPE extract, mainly glucuronoconjugates of catechin and epicatechin.

4 CONCLUSIONS

The present study describes a rapid, simple and sensitive method for determining procyanidins and their metabolites in plasma samples. The developed method, off-line SPE-UPLC-MS/MS, allowed procyanidins to be determined at low μM concentration levels in 5 min, and the metabolites generated after the digestion and intestinal absorption to be identified and tentatively quantified. The detected metabolites were catechin and epicatechin glucuronide, methyl-glucuronide, and methyl-sulphate. The method developed could thus be used successfully for pharmacokinetic and bioavailability studies in humans and can serve as an advantageous alternative to those previously reported due to its speed, sensitivity, selectivity and low sample amount.

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

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RAPID METHODS TO DETERMINE PROCYANIDINS, ANTHOCYANINS AND ALKALOIDS IN RAT TISSUES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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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.

Keywords: Procyanidins / Anthocyanins / Alkaloids / Biological tissues / UPLC-MS/MS

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], [2], [3], [4], [5], [6], [7], [8], [9], [10] and [11], urine [3], [4], [5], [6], [7] and [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] and [14] and anthocyanins [10], [15], [16] and [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 pretreatment 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] and [16] or in strong acids, like trifluoracetic acid (TFA) [10] and [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] and [15] and HPLC–MS [8] and [17] were the chosen analytical technique. LC–MS/MS is a useful tool to 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] and [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 EXPERIMENTAL

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;

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

Compound	ESI	SRM 1 (quantificaction)	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]

the sample was sonicated (S-150D Digital SonifierRCell 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] and [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 mixture 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)

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
Purines						
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

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. 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 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

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 (purines) 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
Purines					
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

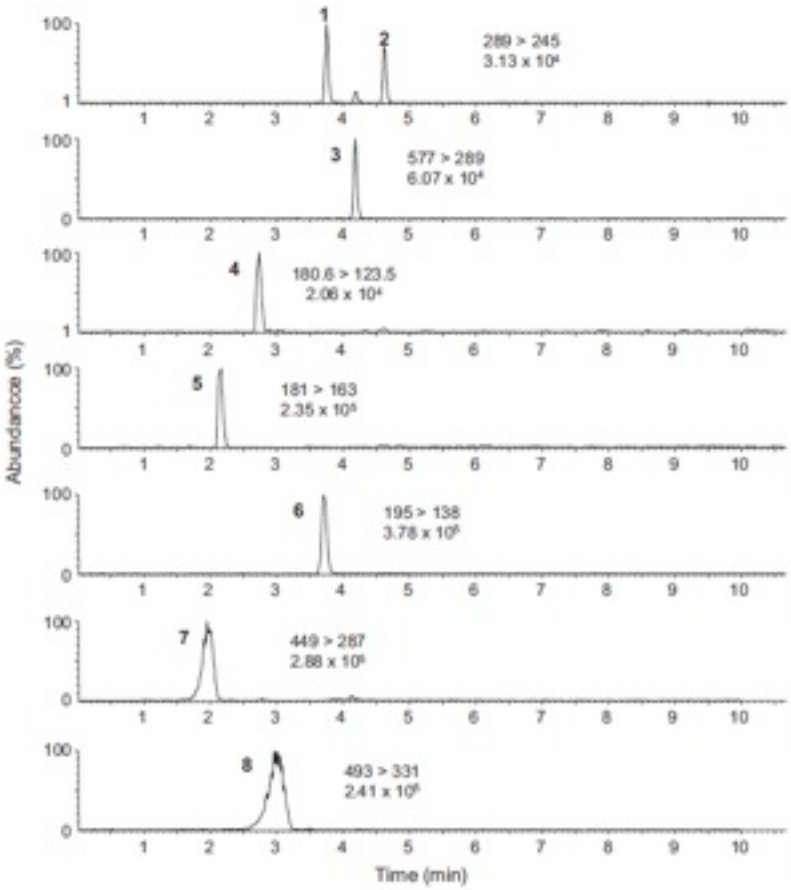

Figure 1. Extracted ion chromatograms from blank liver tissue spiked with the studied compounds. Their designation and their concentrations were (1) catechin, 6 nmols/g fresh tissue; (2) epicatechin, 6 nmols/g fresh tissue; (3) dimer, 3 nmols/g fresh tissue; (4) theophylline, 8.5 nmols/g fresh tissue;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

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

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, heart 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

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] and [14], and anthocyanins [10], [15], [16] and [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] and [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 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

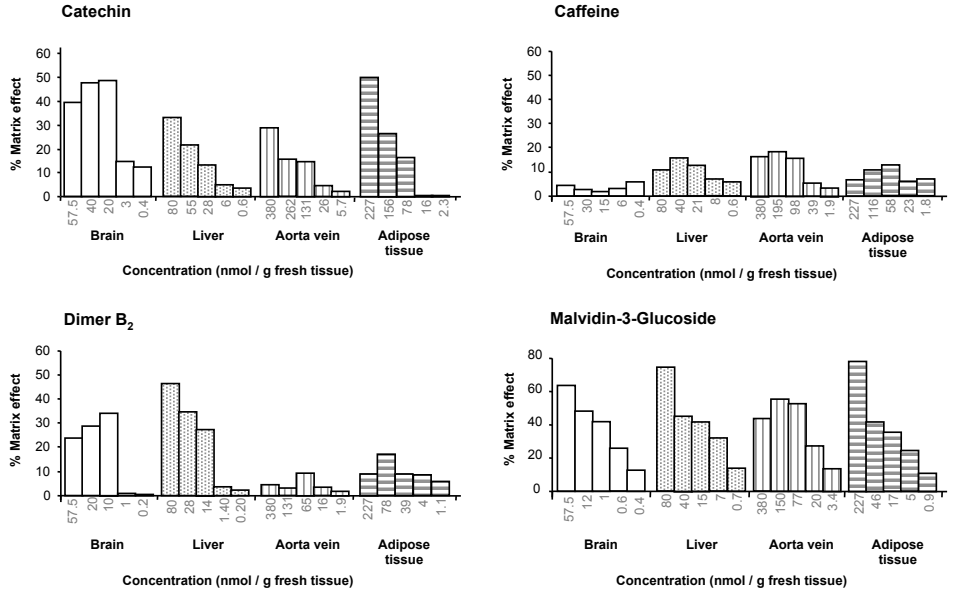

Figure 2. Matrix effect study for the determination of catechin, dimer B_2, caffeine and malvidin-3-glucoside in brain, liver, aorta vein and adipose tissue.

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 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-

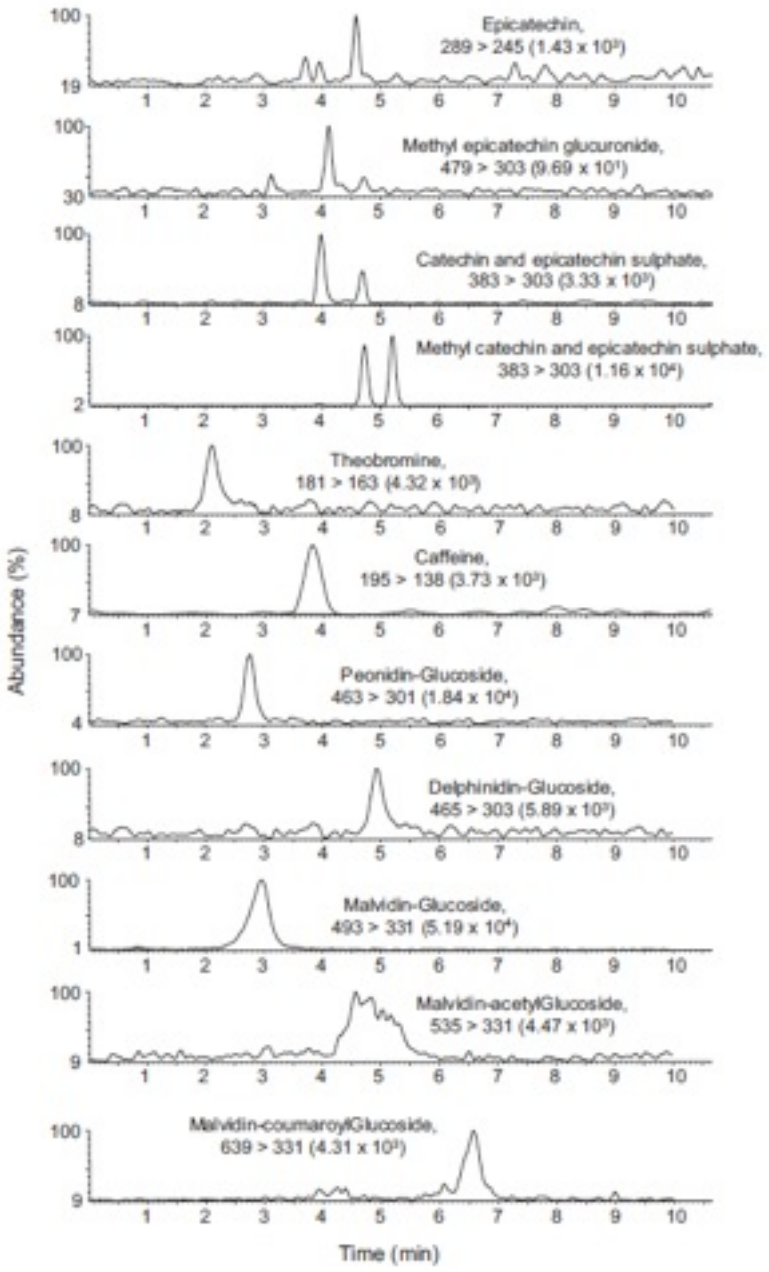

Figure 3. Extract ion chromatograms obtained for the analysis of liver extract after the ingestion of grape seed extract (procyanidins and anthocyanins) or cocoa extract (alkaloids).

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 purines 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.
Purines				
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.

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.

5 ACKNOWLEDGEMENTS

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Publication V: Additional Information

Table 1 Additional Information. Concentration of procyanidins and anthocyanins in grape pomace extract and concentration of alkaloids in cocoa extract.

Grape pomace extract	mg/g extract
Procyanidins	
Catechin	1.8
Epicatechin	4.3
Epigallocatechin	0.26
Epicatechin gallate	0.74
Epigallocatechin gallate	0.73
Dimer	8.91
Trimer	4.9
Anthocyanins	
Cyanidin-glucoside	3.8
Petunidin-glucoside	14.1
Peonidin-gucoside	27.3
Malvidin-glucoside	12.1
Dephinidin-glucoside	8.7

Cocoa extract	
Alkaloids	
Theobromine	43.2
Caffeine	3.3

Table 2 Additional Information. Precision (intra-day and inter-day precision), as % RSD, and accuracy (%) (intra-day and inter-day) for the determination of the studied compounds by off-line LSE and off-line μSPE-UPLC-MS/MS (procyanidins and anthocyanins) and by off-line LSE and off-line SPE-UPLC-MS/MS (alkaloids) in spiked liver, brain, aorta vein and adipose tissue.

LIVER												
Compound	%RSD (n=3), Intra-day			%RSD (n=3), Inter-day			Accuracy (%) (n=3), Intra-day			Accuracy (%) (n=3), Inter-day		
	8.0 nmol/g	2.4 nmol/g	0.6 nmol/g	8.0 nmol/g	2.4 nmol/g	0.6 nmol/g	8.0 nmol/g	2.4 nmol/g	0.6 nmol/g	8.0 nmol/g	2.4 nmol/g	0.6 nmol/g
Procyanidins												
Catechin	4.0	8.3	7.4	4.0	8.7	7.8	101	102	96	100	102	99
Epicatechin	6.2	9.0	8.6	6.5	9.0	9.0	103	104	99	101	103	101
Dimer B_2	1.7	4.1	5.7	2.0	4.5	6.0	105	105	100	104	104	99
Alkaloids												
Theophylline	2.8	3.3	3.9	3.1	3.5	4.1	100	98	96	101	99	97
Theobromine	2.4	4.1	5.0	2.4	4.3	5.0	101	95	105	100	98	104
Caffeine	3.4	3.8	4.2	3.9	4.2	4.5	99	101	100	99	100	102
Anthocyanins												
Cyanidin-3-Glucoside	5.3	5.9	6.4	5.6	6.2	6.7	95	97	99	97	99	99
Malvidin-3-Glucoside	6.3	6.8	7.2	6.7	7.4	7.5	100	103	104	101	105	103

BRAIN												
Compound	%RSD (n=3), Intra-day			%RSD (n=3), Inter-day			Accuracy (%) (n=3), Intra-day			Accuracy (%) (n=3), Inter-day		
	6.0 nmol/g	1.7 nmol/g	0.4 nmol/g	6.0 nmol/g	1.7 nmol/g	0.4 nmol/g	6.0 nmol/g	1.7 nmol/g	0.4 nmol/g	6.0 nmol/g	1.7 nmol/g	0.4 nmol/g
Procyanidins												
Catechin	1.8	2.5	3.3	2.3	2.8	3.5	105	103	99	105	103	101
Epicatechin	3.5	5.2	6.0	3.7	5.5	6.0	99	100	98	100	101	99
Dimer B_2	2.0	6.0	5.8	2.4	6.5	6.1	102	99	100	103	100	101
Alkaloids												
Theophylline	2.0	3.1	3.1	2.4	3.3	3.4	105	99	95	104	100	97
Theobromine	1.6	1.4	6.3	1.8	1.4	6.5	101	95	105	100	97	104
Caffeine	1.1	0.2	0.8	1.3	0.4	1.0	105	98	95	103	97	97
Anthocyanins												
Cyanidin-3-Glucoside	7.7	6.3	6.5	7.9	6.5	6.5	103	101	96	101	100	98
Malvidin-3-Glucoside	5.8	4.3	6.4	6.0	4.5	6.7	96	98	103	98	99	104

Table 2 Additional Information. Continuation.

Compound	AORTA VEIN											
	%RSD (n=3), Intra-day			%RSD (n=3), Inter-day			Accuracy (%) (n=3), Intra-day			Accuracy (%) (n=3), Inter-day		
	38.0 nmol/g	11.5 nmol/g	2.6 nmol/g	38.0 nmol/g	11.5 nmol/g	2.6 nmol/g	38.0 nmol/g	11.5 nmol/g	2.6 nmol/g	38.0 nmol/g	11.5 nmol/g	2.6 nmol/g
Procyanidins												
Catechin	0.5	4.3	7.6	0.5	4.4	7.9	101	102	96	100	103	99
Epicatechin	4.2	2.2	3.0	4.5	2.5	3.3	103	104	99	102	104	99
Dimer B_2	2.6	4.6	5.2	3.0	4.9	5.5	105	105	100	103	103	101
Alkaloids												
Theophylline	2.3	3.6	3.9	2.3	3.6	4.1	99	100	97	901	101	98
Theobromine	2.3	2.7	5.9	2.3	2.7	6.3	98	101	96	99	100	97
Caffeine	2.6	3.5	4.8	2.6	3.5	5.3	97	102	95	97	101	96
Anthocyanins												
Cyanidin-3-Glucoside	3.9	3.2	4.7	4.2	6.4	4.8	95	97	99	97	99	99
Malvidin-3-Glucoside	5.4	0.5	3.6	5.6	0.7	3.7	100	103	104	100	102	103

Compound	ADIPOSE TISSUE											
	%RSD (n=3), Intra-day			%RSD (n=3), Inter-day			Accuracy (%) (n=3), Intra-day			Accuracy (%) (n=3), Inter-day		
	23 nmol/g	6.8 nmol/g	1.6 nmol/g	23 nmol/g	6.8 nmol/g	1.6 nmol/g	23 nmol/g	6.8 nmol/g	1.6 nmol/g	23 nmol/g	6.8 nmol/g	1.6 nmol/g
Procyanidins												
Catechin	4.0	8.3	7.4	4.0	8.5	7.7	103	102	98	102	102	98
Epicatechin	6.2	9.0	8.6	6.4	9.0	8.8	97	99	101	98	100	100
Dimer B_2	1.7	4.1	5.7	1.9	4.3	6.2	95	97	96	96	98	97
Alkaloids												
Theophylline	2.8	3.3	3.9	3.1	3.7	4.2	100	98	96	101	99	97
Theobromine	2.4	4.1	5.0	2.6	4.2	5.3	101	95	105	102	98	104
Caffeine	3.4	3.8	4.2	3.6	4.1	4.5	99	101	100	100	101	100
Anthocyanins												
Cyanidin-3-Glucoside	5.3	5.9	6.4	5.5	6.3	6.6	105	103	98	104	103	99
Malvidin-3-Glucoside	6.3	6.8	7.2	6.5	7.0	7.7	100	98	99	101	99	98

Pharmacokinetic profile

Procyanidins and olive oil phenolic compounds

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Serra et al. European Journal of Nutrition (2012) In press
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PAPER

DISTRIBUTION OF PROCYANIDINS AND THEIR METABOLITES IN RAT PLASMA AND TISSUES AFTER AN ACUTE INTAKE OF HAZELNUT EXTRACT

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Abstract

Procyanidins are present in a wide range of dietary foods and their metabolism is well known. Nevertheless, the biological target and their distribution are topics lacking information. The purpose of the present work was to study the metabolism and distribution of procyanidins and their metabolites in rat plasma and different tissues, such as liver, brain, lung, kidney, intestine, testicle, spleen, heart and thymus, after 2 h of an acute intake of hazelnut extract rich in procyanidins (5 g kg^{-1} of rat body weight). The interest of an acute intake of procyanidins instead of repeated low doses from daily ingestion of is to achieve a concentration of metabolites in the tissues that allows their detection and quantification. The results showed that catechin and epicatechin-glucuronide, methyl catechin and epicatechin-glucuronide and methyl catechin and epicatechin-sulphate were detected in plasma samples at the μmol level. On the other hand, catechin-glucuronide, methyl catechin-glucuronide and methyl catechin-sulphate were identified in some tissues, such as thymus, intestine, lung, kidney, spleen and testicle at the nmol level. Procyanidins with a low grade of polymerization (dimers and trimers) were detected in plasma samples and the intestine. Additionally, a wide range of simple aromatic acids from fermentation by the colonic microflora was detected in all tissues studied.

Keywords: Acute intake / bioavailability / distribution / plasma / procyanidins / tissues

1 INTRODUCTION

It is well known that polyphenols present in food are highly metabolized before their absorption. In recent years, attention has focused on the digestion and gastrointestinal metabolism of procyanidins.[1–6] Prior to absorption, procyanidins are hydrolyzed by digestive enzymes or colonic microflora[7] and during the absorption step, procyanidins are conjugated in the small intestine, resulting in a wide range of conjugated metabolites, from the combination of sulphatation, glucuronidation and methylation .[8–10] Additionally, the colonic microflora also participates in the last step of the procyanidin metabolism, generating new small molecules by hydrolysis, mainly simple aromatic acids. Secondly, procyanidins are metabolized by the liver where they can be modified into a variety of metabolites, mainly glucuronide conjugates.

Throughout digestion, hydrolysis and metabolism change the molecular structure of procyanidins, leading to a large number of different molecules. These structural modifications may exert a negative influence on their biological activities, as occurs with the antioxidant activity, which decreases drastically when the hydroxyl group is modified.[6] Different studies have shown the bioavailability of procyanidins by studying the concentration of their metabolites in plasma and urine.[4,11–13] Nevertheless, determination of the bioavailability of polyphenol metabolites in tissues may be much more important than knowledge of their plasma concentrations.[7]

There is a lack of knowledge about the specific target organs where the metabolites derived from ingested procyanidins accumulate. The existing studies related to the distribution of procyanidins in tissues focus on evaluating the behavior of a single molecule, such as epicatechin, by detecting this compound and its metabolites in some rat tissues.[6,14] However, it is well known that foods contain a complex mixture of phenolic compounds[6,14] making the study of metabolism, distribution and accumulation of procyanidins in the body more difficult.

These are the reasons why we report in this paper on a comprehensive study of the absorption, metabolism and distribution in plasma and body tissues (thymus, heart, brain, spleen, testicle, intestine, kidney, lung and liver) of (+)-catechin and (−)-epicatechin and procyanidins with a low degree of polymerization (dimers and trimers) following the oral intake of a high dose of hazelnut extract in rats (5 g kg^{-1} of rat body weight). To observe and understand the future potential benefits of polyphenols, taking into account their short life in plasma, the studies should be carried out during the postprandial state, immediately after ingestion.[15,16] So, the aim of an acute intake of procyanidins instead of repeated low doses from daily ingestion of them is to achieve a concentration of procyanidin metabolites in the tissues that allow their detection and quantification. This fact may be very useful in future repeated low dose experiments, facilitating the understanding of future results.

2 MATERIALS AND METHODS

2.1 Reagents

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-(4β-8)-epicatechin], 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and 3-(2,4-dihydroxyphenyl)propionic acid from Fluka Co. (Buchs, 125 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& Esteban S. A. (Barcelona, Spain). Formic acid and L (+)-ascorbic acid (reagent grade) were all provided by ScharlauChemie (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Hazelnut procyanidin extract

A hazelnut extract used as a source of procyanidins was kindly supplied by La Morella Nuts S.A. (Reus, Spain). The extract was produced from hazelnut skins by solid/liquid extraction using a mixture of water and acetone based on the previous work by Ortega *et al.*[17]. 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 procyanidin composition of hazelnut skin extract was analysed according to the method in Ortega *et al.*[18].

2.3 Treatment of animals and plasma and tissues collection

Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12h light–12h dark schedule at controlled temperature (22 °C). They were subjected to a standard diet of a commercial chow, PanLab A04 (Panlab, Barcelona, Spain), and water *at libitum*. The animals were then kept in fasting conditions for between 16 and 17 h with only access to tap water. Subsequently, a single acute dose of 5 g of hazelnut extract/kg of body weight dispersed in water was administered to the rats (n = 10) by intragastric gavage. Two hours later, the animals were anaesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and euthanized by exsanguinations. Blood samples were collected from the abdominal aorta with heparin-moistened syringes. The plasma samples were obtained by centrifugation (2000g, 30 min at 4 °C) and stored at −80 °C until the chromatographic analysis of procyanidin metabolites. Additionally, a control group of rats (n = 10) were maintained in fasting conditions with no intake of the extract and were similarly euthanized. The thymus, heart, liver, intestine, testicle, lung, kidney, spleen and brain of rats were excised, stored at −80 °C and freeze-dried for procyanidin extraction and chromatographic analysis. The study was approved by The Animal Ethics Committee of the University of Lleida (CEEA 03-02/09, 9th November 2009). All experiments with rats were performed in

compliance with the relevant laws and University of Lleida guidelines.

2.4 Extraction of procyanidins from plasma and tissues

The method used to extract procyanidins and their metabolites from plasma and tissues was based on the methodologies described in our previous papers.[12,19] In order to clean-up the biological matrix and preconcentrate the phenolic compounds, the plasma samples were pretreated by microelution solid-phase extraction (μSPE), and the rat tissue samples were pretreated by a combination of a liquid-solid extraction (LSE) and μSPE. Briefly, the extraction was realized with 60 mg of freeze-dried tissue in which 50 μl of ascorbic acid 1%, 50 μl of catechol 20 mg l^{-1} (dissolved in phosphoric acid 4%) as an internal standard and 100 μl of phosphoric acid 4% were added. The sample was extracted four times with 400 μl of water/methanol/phosphoric acid 4% (94/4/1, v/v/v). In each extraction, 400 μl of extraction solution was added. The sample was sonicated during 30 s maintaining it in a freeze water bath to avoid heating and it was then centrifuged for 15 min, at 14000 rpm at 20 °C. The supernatants were collected, and then the extracts were treated with μSPE before the chromatographic analysis of the procyanidins and their metabolites.

OASIS HLB μElution Plates 30 μm (Waters, Milford, MA, USA) were used. Briefly, these 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 tissue 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. Then, the retained molecules (procyanidins and their metabolites) were eluted with 2 × 50 μl of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solution was directly injected into the chromatographic system, and the sample volume was 2.5 μl.

2.5 Analysis of procyanidins and their metabolites by UPLC-ESI-MS/MS

Procyanidins were analysed by Acquity Ultra-Performance™ liquid chromatography from Waters (Milford MA, USA) and tandem MS, as reported in our previous studies.[12,20] Briefly, the column was Acquity high strength silica (HSS) T3 (100 mm × 2.1 mm i.d., 1.8 μm particle size) with 100% silica particles, from Waters (Milford MA, USA). The mobile phase was 0.2% acetic acid as eluent A and acetonitrile as eluent B. The flow-rate was 0.4 ml min^{-1} and the analysis time 12.5 min.

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 electrospray ionization (ESI). The procyanidins and their metabolites were analyzed in negative ion mode and the data was acquired through selected reaction monitoring (SRM). Two SRM transitions were studied for each analyte, the most sensitive transition being selected for quantification and a second one for confirmation purposes (Additional Information). The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software.

(+)-Catechin, (–)-epicatechin, dimer B2 [epicatechin-(4β-8)-epicatechin], gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, phenylacetic acid, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(2,4-dihydroxyphenyl)propionic acid were quantified using the calibration curves of the respective standards. Due to the lack of standards for some metabolites, 5-(hydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid, 5-(3,4-dihydroxyphenyl)- γ-valerolactone was quantified using the calibration curve of 3-(2,4-dihydroxyphenyl)propionic acid, and isoferulic acid was quantified using the calibration curve of ferulic acid.

2.6 Statistical analysis

The data on the procyanidin metabolite concentration are expressed as mean values ± standard error (n = 10). The data were analyzed by *Student t* test to assess the significant differences between the control group and the treated group two hours after the acute intake of the hazelnut procyanidin extract. All statistical analysis was carried out using STATGRAPHICS Plus 5.1.

3 RESULTS

The composition of the hazelnut extract used as a source of procyanidins in this study is summarized in Table 1. Procyanidins with low grade of polymerization (dimers) were the most abundant with 15 ± 1.3 μmol g^{-1} of extract, followed by catechin with 6.3 ± 0.54 μmol g^{-1} of extract. Procyanidin trimers and tetramers were present in the extract with 4.9 ± 0.32 μmol and 0.20 ± 0.01 μmol g^{-1} of extract, respectively. Two hours after the acute intake of the hazelnut extract, several metabolites were detected in the rat plasma samples (Table 2). An intense metabolism (methylation, sulphation and glucuronidation) of the monomers, catechin and epicatechin, was observed. The glucuronidated forms of catechin and epicatechin were quantified at 1763 ± 132 μmol l^{-1} and 154 ± 12 μmol l^{-1}, respectively. The methyl-glucuronidated and methyl-sulphate forms of both monomers were also quantified, methyl-catechin-glucuronide being the main metabolite (1103 ± 98 μmol l^{-1}). However, procyanidin dimers and trimers were detected as unconjugated forms with 20 ± 1.3 μmol l^{-1} and 1748 ± 145 μmol l^{-1}, respectively. None of these procyanidin metabolites were detected in plasma samples from the rat control group (data not shown).

Table 1. Procyanidin composition of hazelnut skin extract[a)]

Compound	Concentration (μmol/g)
(+)-Catechin	6.3 ± 0.54
(–)-Epicatechin	2.4 ± 0.13
(–)Epigallocatechin	2.6 ± 0.21
(–)-Epigallocatechin-3-O-gallate	0.09 ± 0.00
Procyanidin dimers[b)]	15 ± 1.3
Procyanidin trimers[b)]	4.9 ± 0.32
Procyanidin tetramers[b)]	0.20 ± 0.01

a)Data expressed as mean values±standard error (n=5).
b) Quantified as dimer B2.

Table 2. Plasma concentration of metabolites after an acute intake of 5 g/kg of body weight of hazelnut skin extract[a)]

Compound	Concentration (μmol/l)
Catechin-glucuronide	1763 ± 132
Epicatechin-glucuronide	154 ± 12
Methyl-Catechin-glucuronide	1103 ± 98
Methyl-Epicatechin-glucuronide	59 ± 3.4
Methyl-Catechin-sulphate	18 ± 1.5
Methyl-Epicatechin-sulphate	21 ± 2.0
Procyanidin dimers	20 ± 1.3
Procyanidin trimers	1748 ± 145

a)Data expressed as mean values±standard error (n=10).

In relation to the distribution and accumulation of procyanidin metabolites in the rat tissues, a wide range of metabolites resulting from small intestine or liver metabolism (conjugated derivatives) or from colonic microflora fermentation (simple aromatic acids) were investigated by HPLC-MS /MS (Table 2, ESI). Differences in the concentration of metabolites between samples from the control group and the rats after the acute intake of hazelnut extract were analyzed with the *Student t* test to assess the significant differences. Table 3 lists the metabolites that showed statistically significant differences ($p < 0.01$ and $p < 0.05$) in their concentration between tissues from the control group and the group treated with the procyanidin extract. The analysis of the intestines showed high concentrations of conjugated derivatives of catechin, such as catechin-glucuronide and methyl-catechin-glucuronide, the latter being the main metabolite quantified (218 ± 20 nmol g^{-1} tissue). Besides, the free form of procyanidin dimers and trimers were detected exclusively in this organ. Related to simple aromatic acids, protocatechuic acid, protocatechuicsulphate acid and gallic acid were only detected in the intestines after the acute intake of the extract. In contrast to the intestines, only protocatechuic acid was detected in the livers after the acute intake of hazelnut extract (15 ± 1.3 nmols g^{-1} tissue).

Methyl-catechin-glucuronide was only quantified (2.7 ± 0.13 nmol g^{-1} tissue) in the thymus from the treated rats. A wide range of simple aromatic acids were quantified in this tissue (Table 2, ESI), but only the concentration of vanillic acid showed significant differences ($p < 0.05$) between the control and treated groups with 76 ± 37 nmol g^{-1} tissue after the acute intake of the hazelnut extract. As in the thymus, methyl-catechin-glucuronide was only detected in the spleens after an acute intake of extract (1.5 ± 0.13 nmol g^{-1} tissue), and the concentration of vanillic acid in the spleens increased significantly ($p < 0.05$) after the intake of the extract.

Two catechin-conjugated metabolites were detected in the testicles after the acute intake of the hazelnut extract, these being catechin-glucuronide with 2.2 ± 0.32 nmol g^{-1} tissue and methyl-catechin-glucuronide with 2.3 ± 0.15 nmol g^{-1} tissue. Additionally, two hydroxylated forms of phenylacetic acid, *p*- and *o*-hydroxyphenylacetic acids were only quantified after the intake of the hazelnut extract with a similar concentration.

Surprisingly, the analysis of the lungs from the treated group of rats revealed

the presence of high concentrations of the free form of epicatechin with 59 ± 5.1 nmol g^{-1} tissue (Table 3). Besides the two conjugate forms of catechin quantified in other tissues, catechin-glucuronide with 19 ± 1.9 nmol g^{-1} tissue and methyl-catechin-glucuronide with 23 ± 2.5 nmol g^{-1} tissue. Additionally, protocatechuic-sulphate acid was only detected after the acute intake of hazelnut extract.

Finally, as an essential pathway of excretion, the kidneys were analyzed and a high number of metabolites were detected. Methyl-catechin-sulphate and catechin-glucuronide were only detected after the acute intake of the extract, with 1.8 ± 0.12 nmol g^{-1} tissue and 5.1 ± 0.45 nmol g^{-1} tissue, respectively. Additionally, methyl-catechin-glucuronide was also detected in the control group, but its concentration was significant lower ($p < 0.05$) than in the kidneys from the treated rats (Table 3). Besides, the concentration of different simple aromatic acids increased significantly ($p < 0.05$ and $p < 0.01$) after the acute intake of hazelnut extract. Methyl gallate was only detected in the kidneys. This metabolite was also detected in the control group, but its level increased significantly ($p < 0.05$) after the acute intake of the extract.

4 DISCUSSION

The main objective of this work was to evaluate the metabolism and distribution of procyanidin metabolites in rat bodies, including their distribution in plasma and tissues. As far as we know, the present study shows for the first time the distribution of procyanidin metabolites in a wide range of rat tissues after an acute intake of a complex mixture of procyanidins contained in a food matrix, this being a hazelnut skin extract. The extract used was rich in procyanidin dimers, and the main monomer present in the extract was catechin (Table 1).

After the ingestion of the hazelnut skin extract, the monomers catechin and epicatechin were absorbed, appearing at high concentrations in the plasma as conjugated forms (Table 2). The main metabolites detected in the plasma in our study were glucuronidated and methyl-glucuronidated conjugates and this agrees with the studies by El Mohsen *et al.*[21] and Harada *et al.*[22] The presence of the methylated forms of the glucuronide and sulphate conjugates of catechin and epicatechin could be explained by the ingestion of a large amount of catechin contained in the hazelnut extract.[6] This high absorption and conjugation of procyanidins in glucuronidated, sulphated and methylated forms observed in the present study is in agreement with previous studies, which also analyzed biological fluids after the ingestion of such rich sources of procyanidins as chocolate,[23–25] tea[26] or grape seed extract.[1,27] Nevertheless, not only were the conjugated forms of catechin and epicatechin able to reach the bloodstream; the low grade of polymerization of procyanidins, such as dimers and trimers, were detected in plasma two hours after the extract intake, similar to that observed by other authors.[1,28–30] However, the free forms of catechin and epicatechin were not detected.

The absorption of procyanidins initially takes place during transfer through the small intestine and subsequently, by the liver,[21] resulting in a wide range of

metabolites. These metabolites may reach other organs through the bloodstream. Fig. 1 shows the distribution and accumulation of procyanidin metabolites in the rat tissues observed in the present study, resulting from metabolism in the small intestine or liver (conjugated derivatives) or from the fermentation of colonic microflora (simple aromatic acids). In order to assess the major metabolites accumulated in different tissues as a result of the ingestion of a procyanidin-rich extract, the difference between the amount quantified in tissues obtained from the treated group and the quantities found in the tissues from the control group for each compound was calculated. The characteristic procyanidin metabolism conducted by the intestine and liver may explain the presence of catechin-glucuronide, methyl-catechin-glucuronide and methyl-catechin-sulphate in some organs, like the thymus, lung, kidney, spleen or testicles, two hours after the acute intake of the hazelnut extract.

The free forms of catechin and epicatechin were not detected in either the plasma or tissues, except in the lungs where the free form of epicatechin was quantified at an even higher level than the conjugated forms of catechin (Fig. 1). On the contrary, the free forms of procyanidin dimers and trimers were only quantified in the plasma but not in the tissues. So, this may confirm the absorption of low grade of polymerization procyanidins but not the disposition in tissues, probably because molecular weight made the interaction of dimers and trimers with tissue proteins difficult, as occurs with similar molecules, such as tannins. This interaction is fundamental to allow the fixation between the bioactive compounds in the tissues or to exert their biological activities.[31] Procyanidin dimers and trimers were only detected in the intestine, probably as a result of the hydrolysis of the most highly polymerized procyanidins in the hazelnut extract that cannot be absorbed in their native form, or as a result of an incomplete hydrolysis into the monomeric forms, catechin and epicatechin, that occurs during digestion and the first 1–4 h of colonic fermentation.[32] However, the accumulation of these in specific target organs has not been demonstrated at least two hours after the ingestion of the extract. Differences in the nature of the tissue metabolites and blood metabolites may be related to the specific uptake or elimination of some of the tissue metabolites or the intracellular metabolism.[7]

In relation to the balance between the stereoisomers catechin and epicatechin, the main metabolites quantified in plasma were the conjugated forms of catechin. However, conjugated forms of epicatechin were also quantified, but at lower concentrations. Despite their presence in plasma, epicatechin conjugate forms were not detected in any tissue except the lungs, where the free form of epicatechin was found. This major accumulation of the conjugated forms of catechin in the tissues compared with epicatechin conjugates may be related to the higher levels of catechin in the hazelnut extract. Another possible explanation could be related to the influence of the stereochemical configuration of flavanols in the level and metabolism of flavanols in humans, recently reported by Ottaviani *et al.*[33] The results of this study showed a major oral

absorbability of epicatechin after the oral intake of a low-flavanol cocoa-based dairy-containing drink matrix, enriched with catechin and epicatechin isolated from cocoa powder preparations. In contrast, our results demonstrate a greater absorbability of catechin, probably as a consequence of the higher concentration of its conjugated metabolites measured in the plasma, and the exclusive accumulation of these metabolites in some tissues.

The analysis of the metabolism and tissue distribution of procyanidins showed important differences in the nature and accumulation of metabolites two hours after the intake of the hazelnut extract (Table 3 and Fig. 1). The concentrations of the catechin metabolites resulting from metabolism in the small intestine or liver (conjugated derivatives) ranged from 1.5 to 23 nmol aglycone equivalents g^{-1} tissue, and the concentration of the potential metabolites formed from colonic microflora fermentation (simple aromatic acids) ranged from 2.6 to 110 nmol g^{-1} tissue (Fig. 1). Additionally, flavonoids are also rapidly excreted in the bile and urine.[34,35] Both excretion pathways were observed in the results obtained. An example of recirculation in bile, corresponding to the phase II biotransformation in the liver, could be the presence of catechin-glucuronide and methyl catechin-glucuronide in the intestine (Fig. 1); and the quantification of some catechin metabolites (methyl catechin-sulphate, catechin-glucuronide and methyl catechin-glucuronide) in the kidney may indicate the excretion of procyanidin metabolites through the urine.

The nature of the intake and the time of tissue sampling may be of great importance, depending on the kinetics of the accumulation and elimination of procyanidins in the tissues. In this study, it was chosen to sample the extract two hours after ingestion so that this time corresponded with the maximum concentration of procyanidin metabolites in the plasma observed in previous studies.[19,36] With regard to the nature of the ingestion, a single and acute intake of procyanidins was done in the present work to carry out a pharmacokinetic study. However, long treatments with procyanidins may provide different kinds and numbers of metabolites in the tissues, as occurred in the study performed by Urpi-Sarda, *et al.*,[14] in which catechin and epicatechin metabolites were found in the brain after three weeks of 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 duration of the treatment with procyanidins. Additionally, the presence of catechin metabolites in the tissues two hours after the ingestion of hazelnut extract, together with the results of Urpi-Sarda, *et al.*,[14] may indicate that, with an adequate combination of time and doses, procyanidin metabolites could accumulate in tissues.

As regards the colonic metabolism, a variety of simple aromatic acids were detected in the intestine and tissues, probably as products of the colonic fermentation of procyanidins. Some of these simple aromatic acids have been quantified in a previous study after the colonic fermentation of a cocoa cream by *in vitro* and *in vivo* models.[32] For example,

Table 3. Quantities of metabolites in different tissues from rats control and from rats after an acute intake of nuts skin extract[a)]

Tissue	Metabolite (nmol /g tissue)	Control	Acute intake
Intestine	**Catechin-glucuronide**	**n.d.** [a]	**42 ± 3.2** [c]
	Methyl-catechin-glucuronide	**n.d.** [a]	**218 ± 20** [c]
	Dimer	**n.d.** [a]	**27 ± 1.9** [c]
	Trimer	**n.d.** [a]	**7 ± 0.4** [c]
	Protocatechuic acid	n.d. [a]	32 ± 1.8 [c]
	Protocatechuic-sulphate acid	n.d. [a]	18 ± 1.7 [c]
	Gallic acid	n.d. [a]	24 ± 1.6 [c]
Liver	Protocatechuic acid	n.d. [a]	15 ± 1.3 [b]
Thymus	**Methyl-catechin-glucuronide**	**n.d.** [a]	**2.7 ± 0.13** [c]
	Vanillic acid	67 ± 6.0 [a]	76 ± 3.7 [b]
Spleen	**Methyl-catechin-glucuronide**	**n.d.** [a]	**1.5 ± 0.13** [b]
	Vanillic acid	17 ± 0.9 [a]	20 ± 1.8 [b]
Testicle	**Catechin-glucuronide**	**n.d.** [a]	**2.2 ± 0.32** [c]
	Methyl-catechin-glucuronide	**n.d.** [a]	**2.3 ± 0.15** [c]
	p-Hydroxyphenylacetic acid	n.d. [a]	19 ± 1.1 [c]
	o-Hydroxyphenylacetic acid	n.d. [a]	19 ± 1.2 [c]
Lung	**Epicatechin**	**n.d.** [a]	**59 ± 5.1** [c]
	Catechin-glucuronide	**n.d.** [a]	**19 ± 1.9** [c]
	Methyl-catechin-glucuronide	**n.d.** [a]	**23 ± 2.5** [c]
	p-Hydroxybenzoic acid	46 ± 2.9 [a]	65 ± 7.0 [b]
	Protocatechuic-sulphate acid	n.d.[a]	18 ± 1.0 [c]
Heart	Vanillic acid	165 ± 15.5 [a]	203 ± 15 [b]
	Protocatechuic acid	n.d. [a]	110 ± 5.5 [c]
	3-Hydroxyphenylvaleric acid	85 ± 8.1 [a]	98 ± 7.6 [c]
	5-Dihydroxyphenyl-γ-valerolactone	n.d. [a]	91 ± 9.0 [c]
	Trimethyluric acid	92 ± 9.2 [a]	124 ± 11 [b]
Brain	Vanillic acid	21 ± 2.1 [a]	18 ± 1.6 [b]
	p-Hydroxyphenylpropionic acid	n.d. [a]	15 ± 1.1 [b]
	m-Hydroxyphenylpropionic acid	21 ± 1.9 [a]	18 ± 1.0 [b]
Kidney	**Methyl-catechin-sulphate**	**n.d.** [a]	**1.8 ± 0.12** [c]
	Catechin-glucuronide	**n.d.** [a]	**5.1 ± 0.45** [c]
	Methyl-catechin-glucuronide	**4.0 ± 0.23** [a]	**17 ± 1.4** [c]
	p-Hydroxybenzoic acid	31 ± 3.0 [a]	36 ± 2.3 [b]
	p-Hydroxyphenylacetic acid	20 ± 1.9 [a]	29 ± 2.1 [c]
	m-Hydroxyphenylpropionic acid	17 ± 1.1 [a]	24 ± 2.6 [b]
	Protocatechuic acid	18 ± 1.8 [a]	39 ± 4.0 [c]
	Protocatechuic-sulphate acid	12 ± 1.1 [a]	22 ± 6.8 [c]
	Methyl gallate	13 ± 1.1 [a]	16 ± 1.0 [c]

Letters in bold represent the phase II metabolites of procyanidins.
a) Data expressed as mean values±standard error (n=10) b) Mean values within a column with unlike superscript letter were significantly different. Signification level (p<0.01) between control tissues and tissues obtained after an acute intake of nuts skin extract. c) Mean values within a column with unlike superscript letter were significantly different. Signification level (p<0.05) between control tissues and tissues obtained after an acute intake of nuts skin extract.

Figure 1. Increase of concentration of phenolic acids and procyanidin metabolites quantified in different tissues. The increase is expressed as nmols, obtained by difference between the amoun quantified in the tissues obtained after an acute intake of the nuts skin extract (treated group) and the amount quantified in the control tissues.

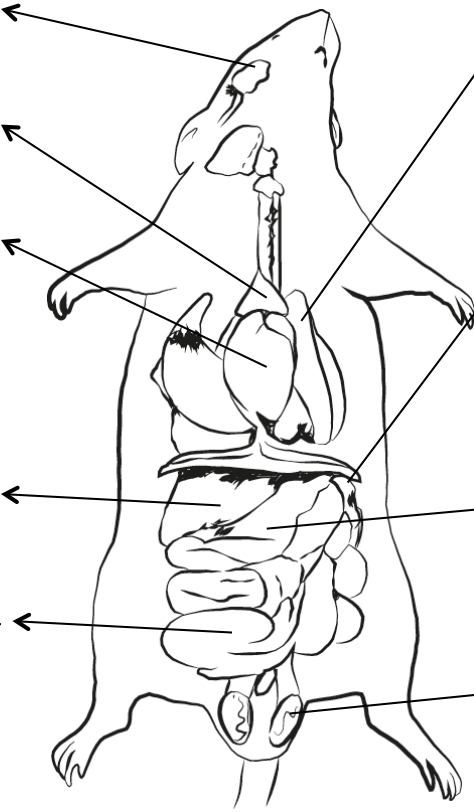
Compound	Brain
p-Hydroxyphenylacetic acid	15

Compound	Thymus
Methyl-Catechin-glucuronide	**2.7**
Vanillic acid	9

Compound	Heart
p-Hydroxyphenylacetic acid	2.6
Protocatechuic acid	110
3-Hydroxyphenylpropionic acid	25
Vanillic acid	38
3-Hydroxyphenylvaleric acid	13
5-Dihydroxyphenyl-γ-valerolactone	91
Trimethyluric acid	31

Compound	Liver
Protocatechuic acid	15

Compound	Intestine
Catechin glucuronide	**42**
Methyl-Catechin-glucuronide	**218**
Procyanidin dimers	**27**
Procyanidin trimers	**6.8**
Protocatechuic acid	32
Protocatechuic-sulphate acid	18
Gallic acid	24



Compound	Lung
Epicatechin	**59**
Catechin-glucuronide	**19**
Methyl-Catechin-glucuronide	**23**
p-Hydroxybenzoic acid	18
Protocatechuic sulphate acid	18

Compound	Kidney
Methyl-Catechin-sulphate	**1.8**
Catechin-glucuronide	**5.1**
Methyl-Catechin-glucuronide	**13**
p-Hydroxyphenylacetic acid	8.9
p-Hydroxybenzoic acid	5
3-Hydroxyphenylpropionic acid	7
Protocatechuic acid	20
Protocatechuic-sulphate acid	9.7
Methyl gallate	3.2

Compound	Spleen
Methyl-Catechin-glucuronide	**1.5**
Protocatechuic acid	17
Vanillic acid	2.3

Compound	Testicle
Catechin-glucuronide	**2.2**
Methyl-Catechin-glucuronide	**2.3**
p-Hydroxyphenylacetic acid	19
o-Hydroxyphenylacetic acid	19

protocatechuic acid, found in all tissues except the testicles, or hydroxyphenylacetic acid, could be intermediate fermentation products of phenylacetic and 3-(4-hydroxyphenyl)-propionic acids.[32] *p*-Hydroxybenzoic acid quantified in the lungs and kidneys has been described as the final fermentation product of catechin.[32]

The presence of protocatechuic sulphate acid in the lungs may indicate enzymatic metabolism after the colonic fermentation, possibly due to a trans-membrane intestine metabolism. Protocatechuic acid has been described as a fermentation product of catechin and dimer B2,[32,35] from the decarboxylation of the 3,4-dihydroxyphenylpropionic acid and its subsequent dehydroxylation to *p*-hydroxybenzoic acid, this being a common compound quantified in tissues in this study. Additionally, some compounds, such as 3-hydroxypenylpropionic acid and *p*-hydroxybenzoic acid, usually found in urine[34,35] were detected in the kidneys. In fact, 3-hydroxyphenylpropionic acid has been described as the main urinary metabolite after an ingestion of dimer B3.[35]

Only protocatechuic acid was detected in the liver, despite its role in the procyanidin metabolism. A previous study by Urpi-Sarda, *et al.*[14] showed the accumulation of some procyanidin metabolites in the liver was probably related to a continuous intake of procyanidins over weeks. On the other hand, the presence of a wide range of simple aromatic acids in heart tissue with significant differences in their concentration compared with the control group , may be related to the potential health benefits of procyanidins, especially in the context of cardiovascular health.[37]

To sum up, after an acute intake of a procyanidin-rich extract, the procyanidins were absorbed, metabolized and distributed around the body. As a consequence, some conjugated derivatives and simple aromatic acids, such as procyanidin metabolites, were detected in the plasma and tissues. The main accumulation of the conjugated metabolites (mainly glucuronide conjugates) of procyanidins was observed in the lung. This disposition may indicate a temporary accumulation of procyanidin metabolites in tissues, probably related to the dose and duration of the treatment. The main accumulation of simple aromatic acids, probably resulting from the hydrolytic metabolism of procyanidins, was observed in the heart. Based on the results of this study, it would be important to consider the possible role of these simple aromatic acids accumulated in the tissues as a result of the intake of procyanidins. So, studying the distribution of procyanidin metabolites in these tissues should be the starting point for knowing the metabolic target and the first step towards understanding how procyanidin acts at a cellular level.

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Publication VI: Additional Information

Table Additional Information. Optimized SRM conditions for analyzing the studied and the generated fermentation compounds.

Compound	SRM_1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM_2 (confirmation)	Cone voltage (V)	Collision energy (eV)
$[M-H]^-$						
Phenylacetic acid	135 > 91	20	5	-	-	-
p-hydroxybenzoic acid	137 > 93	20	15	-	-	-
4-Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-
3-Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-
2-Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-
Protocatechuic acid	153 > 109	45	15	-	-	-
3-(4-Hydroxyphenyl)propionic acid	165 > 121	20	10	165 > 149	20	15
3,4-Dihydroxyphenylacetic acid	167 > 123	20	10	167 > 95	20	15
Gallic acid	169 > 125	35	10	169 > 97	35	15
3-(2,4-Dihydroxyphenylpropionic) acid	181 > 137	20	10	181 > 93	20	15
Homovanillic acid	181 > 137	20	15	181 > 122	20	15
Catechin	289 > 245	45	15	289 > 205	45	15
Epicatechin	289 > 245	45	15	289 > 179	45	15
Dimer B2	577 > 289	45	20	577 > 425	45	15

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

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DISTRIBUTION OF PROCYANIDINS AND THEIR METABOLITES IN RAT PLASMA AND TISSUES IN RELATION TO INGESTION OF RICH OR ENRICHED PROCYANIDIN COCOA CREAMS

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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 number of phase-II metabolites of procyanidins and is therefore thought to be the main site of metabolism of the compounds. Methyl catechin-sulphate (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

1 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].

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.

2 MATERIALS AND METHODS

2.1 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-(4β-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).

2.2 Cocoa cream and procyanidin extract

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.

2.3 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 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.

2.4 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].

2.5 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.

3 RESULTS

3.1 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.

3.2 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

Table 1. Procyanidins content in the hazelnut skin extract (PE), the cocoa cream (CC) and the enriched cocoa cream (PECC). 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).

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	n.d.	128 ± 15
Epicatechin gallate	n.d.	76.0 ± 7.9	29.0 ± 2.5
Epigallocatehin gallate	4.80 ± 0.31	58.0 ± 0.5	32.3 ± 2.9
Dimer	776 ± 76	896 ± 87	1740 ± 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	1599 ± 137	1778 ± 162	3540 ± 322

n.d. not detectable
Values are means ± SD (n = 5)

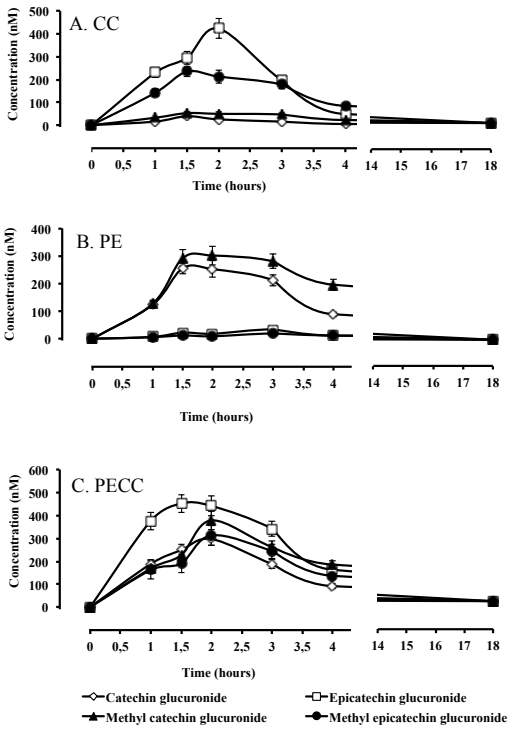

Figure 1. Plasma kinetics of procyanidin metabolites detected in rat plasma collected between 0 and 18 h after the ingestion of: A. cocoa cream (CC), B. procyanidin rich hazelnut skin extract (PE) and C. procyanidin enriched cocoa cream (PECC). The results are expressed as nM.

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-sulphated 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 14 μM, respectively. Dimer and trimer in their free forms were also determined in the plasma from 1 to 2 h with $C_{max\ 1h}$ 1.4 μM and $C_{max\ 2h}$ 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

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). The results are expressed as nM.

Compound (nM)	Time							
	0 h	1 h	1.5 h	2 h	3 h	4 h	6 h	18 h
Methyl catechin sulphate	n.d.	n.d.	2.41 ± 0.13	4.01 ± 0.32	4.50 ± 0.44	n.q.	n.d.	n.d.
Methyl epicatechin sulphate	n.d.	n.d.	9.70 ± 0.96	12.2 ± 1.1	14.1 ± 1.0	n.q.	n.d.	n.d.
Dimer	n.d.	1.40 ± 0.01	1.21 ± 0.13	0.80 ± 0.76	n.d.	n.d.	n.d.	n.d.
Trimer	n.d.	0.50 ± 0.03	n.q.	0.76 ± 0.03	n.d.	n.d.	n.d.	n.d.

n.d. not detected
n.q. not quantified
Values are means ± SD (n = 3)

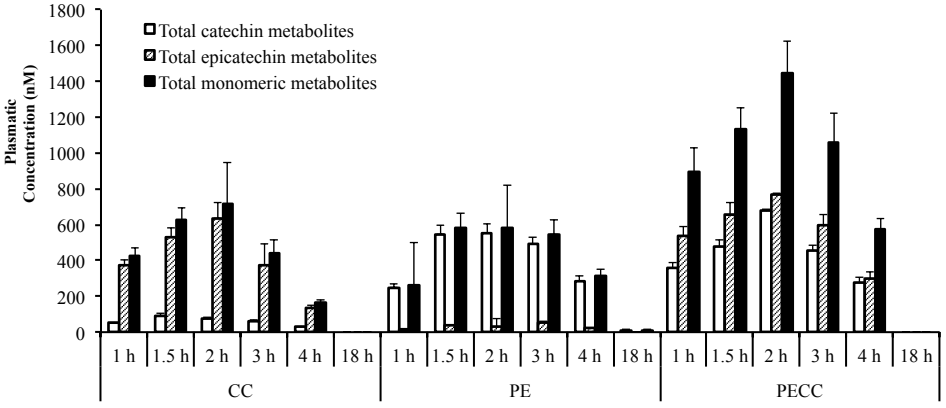

Figure 2. Total catechin and epicatechin metabolites, and total monomeric metabolites (sum of catechin and epicatechin metabolites) quantified in rat plasma collected between 0 and 18 h after the ingestion of cocoa cream (CC), procyanidin rich hazelnut skin extract (PE) and procyanidin enriched cocoa cream (PECC). The results are expressed as nM.

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.

3.3 Distribution of procyanidins 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–sulphate 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,

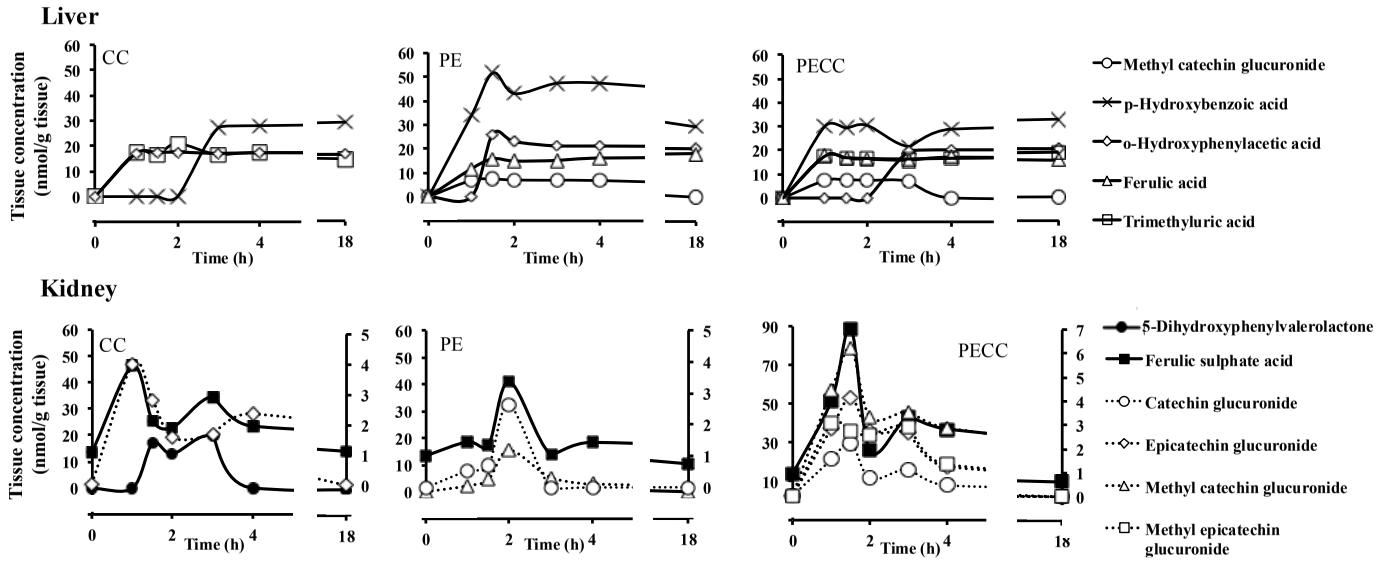

Figure 3. Increase in the concentration of procyanidin metabolites and phenolic acids in the liver and kidney between 0 and 18 h after the ingestion of cocoa cream (CC), procyanidin rich hazelnut skin extract (PE) and procyanidin enriched cocoa cream (PECC). The results are expressed as nmol/g of tissue. Discontinuous lines refers to the right Y-axis.

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 sulphate 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 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–sulphate 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*-

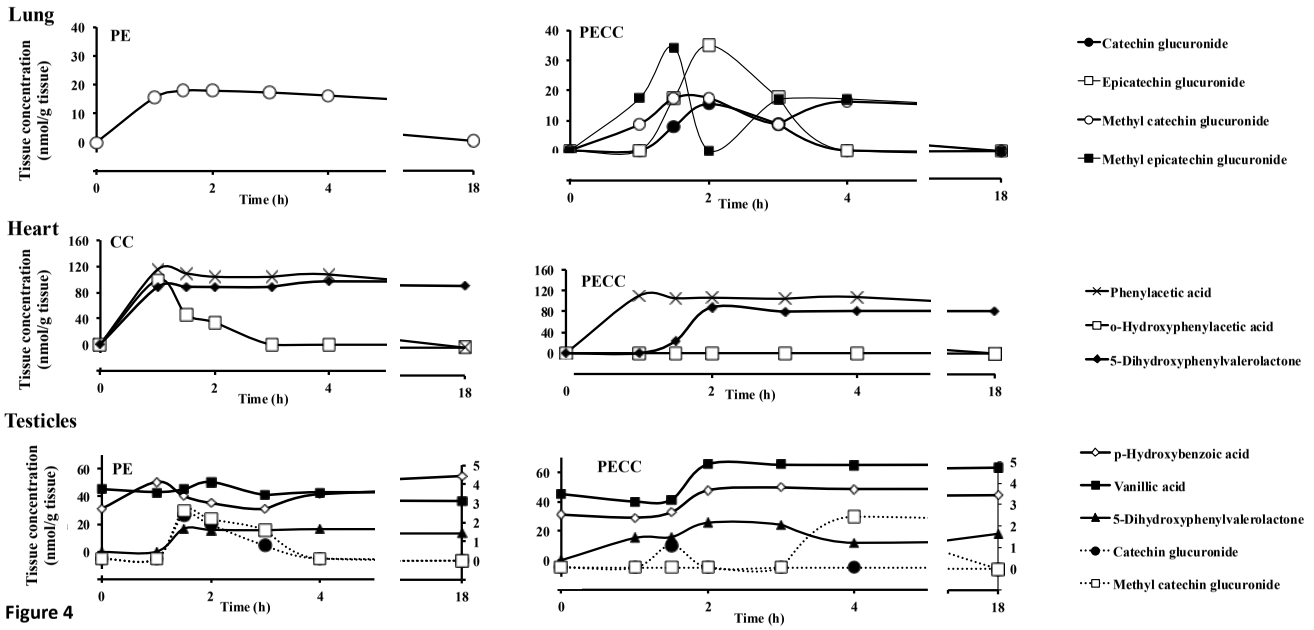

Figure 4

Figure 4. Increase in the concentration of procyanidin metabolites and phenolic acids (0 to 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 refers to the right Y-axis.

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 sulphate 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.

4 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-sulphated 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 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–sulphate. 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 flavonol-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

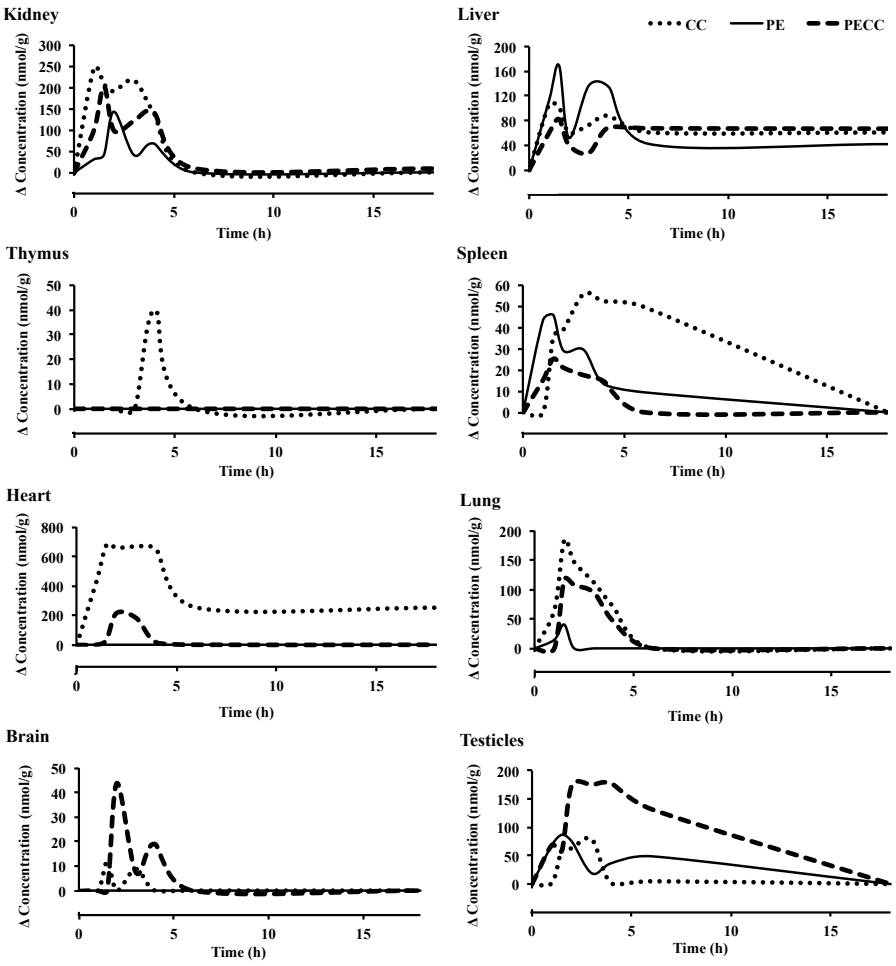

Figure 5. Kinetic of tissue metabolites expressed as the increase in the concentration (nM) in relation to the basal conditions (0 h) after a single ingestion of cocoa cream (CC), rich procyanidin hazelnut skin extract (PE) and procyanidin enriched cocoa cream (PECC).

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.

5 ACKNOWLEDGEMENTS

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Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake

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DISTRIBUTION OF OLIVE OIL PHENOLIC COMPOUNDS IN RAT TISSUES AFTER ADMINISTRATION OF A PHENOLIC EXTRACT FROM OLIVE CAKE

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Abstract

Scope The distribution and accumulation of olive oil phenolic compounds in the body are topics lacked of information. The aim of this study was to evaluate the bioavailability, metabolism and distribution of phenolic compounds from olive cake.

Methods and results The metabolism and distribution of phenolic compounds were examined by UPLC-MS/MS after an acute intake of a phenolic extract from olive cake, analyzing plasma and tissues (heart, brain, liver, kidney, spleen, testicle and thymus) 1, 2 and 4 h after ingestion using Wistar rats as the in vivo model. The results showed a wide distribution of phenolic compounds and their metabolites in the tissues, with a main detoxification route through the kidneys. Highlighting the quantification of the free forms of some phenolic compounds, such as oleuropein derivative in plasma (C_{max} 4 h: 24 μmol/L) and brain ($C_{max\ 2\ h}$: 2.8 nmol/g), luteolin in kidney ($C_{max\ 1\ h}$: 0.04 nmol/g), testicle ($C_{max\ 2\ h}$: 0.07 nmol/g) and heart ($C_{max\ 1\ h}$: 0.47 nmol/g); and hydroxytyrosol in plasma ($C_{max\ 2\ h}$: 5.2 μmol/L), kidney ($C_{max\ 4\ h}$: 3.8 nmol/g) and testicle ($C_{max\ 2\ h}$: 2.7 nmol/g).

Conclusion After a single ingestion of olive oil phenolic compounds, these were absorbed, metabolized and distributed through the blood stream to practically all parts of the body, even across the blood-brain barrier.

Keywords: Distribution / olive oil / phenolic compounds / tissues.

Abbreviations: PEOC, phenolic extract from olive cake; μSPE, microelution solid-phase extraction.

1 INTRODUCTION

One of the most characteristic elements of the Mediterranean diet is undoubtedly olive oil. It is well known that through its well-balanced composition virgin olive oil may have significant positives effects in the body [1-3]. However, it is not only its high content in monounsaturated fatty acids that is important, but also the minor compounds it contains, such as phenolics. The phenolic fraction of virgin olive oil is formed by a wide range of groups, including phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives (the dialdehydic form of elenolic acid linked to hydroxytyrosol or 3,4-DHPEA-EDA, and the dialdehydic form of elenolic acid linked to tyrosol or *p*-HPEA-EDA), phenolic acids (vanillic and *p*-coumaric acids), lignans (pinoresinol and acetoxypinoresinol) and flavonoids (luteolin and apigenin) [1-7].

The antioxidant capacity of olive oil has been widely studied and it is known that after ingestion, the phenolic compounds of olive oil may display local antioxidant capacity in the gastrointestinal tract as affirmed by Owen *et al.* [8]. Moreover, after absorption and metabolism of olive oil, the antioxidant actives may display their activity at cellular level [3]. On the other hand, not all the beneficial effects attributed to the intake of olive oil or its phenolic compounds, such as oleuropein or hydroxytyrosol, are related to its antioxidant capacity; other beneficial properties, such as a neuroprotective effect [3,9], cardioprotective effect [3,10-15], anti-inflamatory effect [16-18] or anti-hypertensive effect [19,20] are being studied.

Some clinical trials have provided evidence that phenolic compounds are absorbed in a dose-dependent way [21,22] and around 98% are metabolized. This leads to a wide range of metabolites that are found in biological fluids, mainly glucuronide and sulphate conjugates [1,23]. Nevertheless, the phenolic compounds of olive oil and their metabolites are present in biological fluids at very low concentrations and some authors are cautious about attributed a direct relation between ingestion and the beneficial effects of this minor fraction of virgin olive oil [1,4].

The key to the relation between the ingestion of virgin olive oil and its beneficial effect is probably not to be found in such widely analyzed biological fluids as plasma and urine, but must be sought in those tissues in which the phenolic compounds and their metabolites and other minor olive oil compounds contribute positively to the normal cell metabolic process. However, the distribution and accumulation of phenolic compounds of olive oil are still not well understood, because only one method has been developed to determine the phenolic compounds of olive oil in various rat tissues (liver, kidney, heart, muscle, testicles, fat and brain) spiked with standards of 3,4-dihydroxyphenylglycol, hydroxytyrosol and tyrosol[24].

To provide a new perspective for understanding the mechanism through olive oil acts in the body, the present work aims to describe the distribution of olive oil phenolic compounds and their metabolites in plasma and rat tissues

after the ingestion of a phenolic extract from olive cake (PEOC).

2 MATERIALS AND METHODS

2.1 Chemical and reagents

The standards of apigenin, luteolin, hydroxytyrosol, tyrosol, and *p*-coumaric acid were purchased from Extrasynthese (Genay, France); Caffeic and homovanillic acids were purchased from Fluka Co. (Buchs, 125 Switzerland). (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol from Sigma Aldrich (Germany). The secoiridoid derivatives 3,4-DHPEA-EDA and *p*-HPEA-EDA, and the lignan acetoxypinoresinol are not available commercially and were isolated from virgin olive by semi-preparative HPLC as described in Suarez *et al.* 2008[25]. A stock solution of each standard compound was dissolved in methanol, and all the solutions were stored in a dark flask at 4° C. Catechol was used as an internal standard (IS) prepared in phosphoric acid 4%.

Acetonitrile (ACN) (HPLC-grade), methanol (HPLC-grade), glaciar acetic acid (≥99.8%), formic acid and L(+)-ascorbic acid (reagent grade) were all provided by ScharlauChemie (Barcelona, Spain). Pure hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Orto-phosphoric acid 85% was purchased from Mont Plet& Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 PEOC

Olive cake is the main byproduct of the olive oil extraction process. PEOC was administrated to the rats in order to study the distribution of the metabolites distributed in the different body tissues. The composition of PEOC and how it was obtained is shown in our previous report [26]. Briefly, an accelerated solvent extractor ASE100 (Dionex, Sunnyvale, CA) was used to extract the phenolic compounds from the olive cake using solvents (ethanol/water, 80/20, v/v) at high temperature (80° C) and pressure. The resulting PEOC was rotary evaporated until all the ethanol had been eliminated. Then, it was freeze-dried and stored at -80° C in N_2 atmosphere. The phenolic composition was analyzed according to the method in Suarez *et al.*[26].

2.3 Treatment of rats and plasma and tissues collection

The Animal Ethics Committee of the University of Lleida approved the study (CEEA 03-02/09, 9th November 2009). Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12h light-12h 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 17h with access to tap water and after this time, a single dose of 3g of PEOC/kg of body weight dispersed in water was administered by intragastric gavage (Figure 1). The rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and euthanized by exsanguinations 1 (n=4), 2 (n=4) and 4 (n=4) h after the ingestion of the PEOC. Additionally, a control group of rats (n=4) was maintained under fasting conditions without PEOC ingestion and then similarly euthanized.

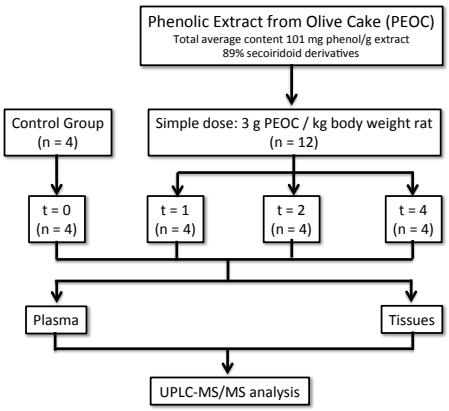

Figure 1. Schema of the treatment of rats and plasma and tissues collection.

Blood samples were collected from the abdominal aorta with heparin-moistened syringes. The plasma samples were obtained by centrifuging (2000 g for 30 min at 4° C) and stored at -80° C until the chromatographic analysis. Different tissues (liver, kidney, testicle, brain, spleen, heart and thymus) were excised from the rats, stored at -80° C and freeze-dried for phenolic extraction and chromatographic analysis.

2.4 Phenolic extraction of plasma and tissues

In order to clean-up the biological matrix and preconcentrate the phenolic compounds, the plasma samples were pretreated by microelution solid-phase extraction (μSPE) [27], and the tissues were sequentially pretreated by a combination of liquid-solid extraction (LSE) and μSPE. To develop the tissue extraction method, a pool of each tissue obtained from rats from the control group was spiked with the phenolic standards (hydroxytyrosol, tyrosol, *p*-coumaric acid, caffeic acid, homovanillic acid, pinoresinol, apigenin and luteolin) dissolved in phosphoric acid 4 %. For the analysis of different tissues by LSE, 60 mg of freeze-dried tissue or 30 mg of freeze-dried heart were weighted, and then 50 μL of ascorbic acid 1% and 100 μl of phosphoric acid 4% were added. The sample was treated 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 SonifierR Cell Disruptor, Branson, Ultrasonidos S.A.E., Barcelona, Spain) for 30 s maintaining the sample in a freeze water bath to avoid heating and then centrifuged for 15 min at 9000 rpm at 20 °C. The supernatants were collected, and then an aliquot of the extract was treated by μSPE to clean up the sample before the chromatographic analysis. The off-line μSPE was based on the methodology described in a previous report where olive oil phenolics were analyzed in plasma samples [27]. Briefly, OASIS HLB μElution Plates 30 μm (Waters, Milford, MA, USA) were used and two methodologies were applied, one to determine hydroxytyrosol, and the second one to determine the other studied phenolic compounds. For the analysis of hydroxytyrosol, the micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of Milli-Q at pH 2 with hydrochloric acid. 200 μL of phosphoric acid 4% was added to 200 μL of tissue extract, and then this mixture was loaded onto the plate. The loaded plates were washed with 75 μL of Milli-Q water and 75 μL of Milli-Q water at pH 2. The retained hydroxytyrosol was then eluted with 2 x 25 μL of acetonitrile/Milli-Q water solution (50/50, v/v). On the

other hand, for the analysis of the rest of the studied phenolic compounds, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of Milli-Q at pH 2 with hydrochloric acid. 350 µL of phosphoric acid 4% was added to 350 µL of tissue 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 Milli-Q water at pH 2. The retained phenolic compounds were then eluted with 2 x 50 µL of methanol. The eluted solutions (phenolic extracts) were directly injected into the UPLC-MS/MS, and the injection volume was 2.5 µL.

2.5 Quantitative analysis of plasma and tissues metabolites

Phenolic compounds were analyzed by Acquity Ultra-PerformanceTM liquid chromatography and tandem MS from Waters (Milford MA, USA), as reported in our previous studies [27-30]. Briefly, the column was an Acquity UPLCTM BEH C18 (100 mm x 2.1 mm i.d., 1.7 µm particle size), also from Waters (Milford MA, USA). The mobile phase was 0.2% acetic acid as eluent A and acetonitrile as eluent B, and the flow-rate was 0.4 mL/min. The elution started at 5% of eluent B for 2.1 min, then was linearly increased to 40% of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. Then, back to initial conditions in 0.1 min, and the reequilibration time was 2.9 min.

The UPLC system was coupled to a PDA detector AcQuity UPLCTM and a triple quadrupole detector (TQDTM) mass spectrometer (Waters, Milford, MA, USA). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters and the transition acquired for each compound (one for quantification and a second one for confirmation purposes) as reported in our previous study [27].

2.6 Statistical analysis

The data on polyphenol metabolite levels are presented as mean values± standard error (n=4). In order to simplify the results, the standard error was omitted because all values were lower than 10%. The data were analysed by a multifactor ANOVA test to determine significant differences between post-ingesta times (1, 2 and 4 h) and to compare these with the control group. A significant difference was considered at a level of $p \leq 0.05$. All the statistical analysis was carried out using STATGRAPHICS Plus 5.1.

3 RESULTS

The composition of PEOC used in this study is shown in Table 1. The extract contained the most representative phenolic compounds of virgin olive oil, including phenyl alcohols, phenolic acids, secoiridoid derivatives, lignans and flavonoids. Hydroxytyrosol showed a high concentration with 3.45 mg/g PEOC, and the main phenolic compound quantified in PEOC was 3,4-DHPEA-EDA with 60.2 mg/g PEOC.

In relation to the bioavailability (Table 2), the main phenolic family detected in the plasma at different times after the PEOC ingestion were the phenolic alcohols. Hydroxytyrosol-sulphate was the main plasma metabolite with approximately 55% of the total phenolic alcohols

quantified in all tested times (1, 2 and 4 h), followed by tyrosol-sulphate. Of the other conjugated metabolites, hydroxytyrosol-glucuronide and tyrosol-glucuronide were detected in all the plasma samples, hydroxytyrosol-glucuronide being the most abundant. Moreover, the free form of hydroxytyrosol was detected at concentrations between 4.3 and 5.2 μmol/L after the ingestion of PEOC. In relation to the secoiridoids, a metabolite identified as oleuropein derivate was detected and quantified in the plasma samples reaching maximum concentration (24 μmol/L) 4 h after ingestion. The second main group of phenolic metabolites quantified in plasma was the phenolic acids, homovanillic-sulphate acid being the main phenolic acid quantified.Vanillic-sulphate, elenolic, hydroxybenzoic and hydroxyphenylacetic acids were also quantified but at lower concentrations. Nevertheless, hydroxybenzoic and hydroxyphenyl acetic acids were detected in the plasma from the control group at trace level (data not shown). Luteolin in its free form was detected at trace levels in all the samples, with no significant differences between the times after the ingestion of PEOC and the control group (data not shown). However, its glucuronide conjugate was quantified in the plasma samples at low levels (less than 1 μmol/L) only after the ingestion of the PEOC (Table 2). Analyzing the importance in percentage of the phenolic families (phenyl alcohols, phenolic acids and secoiridoid derivatives) quantified in the phenolic extract (PEOC) and in rat plasma 1h, 2h and 4h post-ingestion of 3 g of PEOC/kg of rat body weight (Figure 2), the high percentage of total phenyl alcohols (mainly metabolites of hydroxytyrosol and tyrosol) in the plasma was noteworthy in relation to their low concentration in the extract. The increase of hydroxytyrosol metabolites in plasma after the PEOC ingestion may be related to the high content of secoiridoids in the

Table 1. Phenolic composition of the olive extract.

Compound	mg/g extract
Tyrosol	0.08
Hydroxytyrosol	3.45
Total phenyl alcohols	3.51
Vanillin	0.02
p-Coumaric acid	0.05
Vanillic acid	0.03
Caffeic acid	0.04
Total phenolic acids	0.10
Elenolic acid	25.9
3,4-DHPEA-AC	0.34
p-HPEA-EA	0.11
p-HPEA-EDA	0.19
3,4-DHPEA-EDA	60.2
3,4-DHPEA-EA	1.43
Methyl 3,4-DHPEA-EA	0.12
Ligstroside derivative	0.30
Oleuropein derivative	0.08
Total secoiridoid derivatives	89
Pinoresinol	0.07
Acetoxypinoresinol	0.14
Total lignans	0.21
Apigenin	0.40
Luteolin	1.82
Apigenin-7-O-glucoside	0.21
Luteolin-7-O-glucoside	3.07
Rutin	1.71
Total flavonoids	7.2
Verbascoside	0.61
Total average content	101

3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; p-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; p-HPEA-EA, aldehydic form of elenolic acid linked to tyrosol, 3,4-DHPEA-AC, 4 (acetoxyethyl)-1,2-dihydroxy- benzene; 3,4-DHPEA-EA, oleuropein aglycone; methyl 3,4-DHPEA-EA, methylated form of oleuropein aglycone.

Table 2. Plasma concentration of phenol metabolites following the acute intake of 3 g/kg of body weight of olive extract*. Results are expressed as µmol phenolic metabolite/L plasma.

	Time (hours) after extract intake**		
Metabolite (µmol/L)	1 h	2 h	4 h
Hydroxytyrosol	4.3±0.4[b]	5.2±0.5[a]	5.1±0.6[ab]
Hydroxytyrosol-sulphate	51±4[b]	89±7[a]	82±8[a]
Hydroxytyrosol-glucuronide	1.5±0.2[b]	1.8±1.4[a]	1.8±1.6[a]
Tyrosol-glucuronide	1.0±0.2[a]	1.2±0.2[a]	0.44±0.07[b]
Tyrosol-sulphate	36±3.1[b]	53±6.0[a]	50±4.3[a]
Total Phenolic Alcohols	*94±7.9[b]*	*150±15[a]*	*139±14[a]*
Vanillin-sulphate	1.4±0.1[b]	2.5±0.2[a]	1.1±0.2[b]
Vanillic-sulphate acid	2.9±0.1[b]	4.5±0.4[a]	4.8±0.5[a]
Homovanillic-sulphate acid	16±1.3[b]	36±3.8[a]	13±1.5[b]
Hydroxybenzoic acid	1.2±0.1[c]	2.3±0.2[a]	1.6±0.2[b]
Hydroxyphenylacetic acid	1.5±0.1[ab]	1.6±0.1[a]	1.2±0.2[b]
Elenolic acid	1.1±0.3[a]	1.1±0.2[a]	0.89±0.09[b]
Total phenolic acids	*24±3.9[b]*	*48±6.3[a]*	*23±4.2[b]*
Oleuropein derivate	13±1.2[b]	21±2.3[a]	24±2.3[a]
Total secoiridoid derivatives	13±1.2[b]	21±2.3[a]	24±2.3[a]
Luteolin-glucuronide	0.68±0.07[a]	0.58±0.03[ab]	0.54±0.2[b]
Total flavonoids	*0.68±0.07[a]*	*0.58±0.03[ab]*	*0.54±0.2[b]*
Total phenolic metabolites	*131±13[b]*	*198±23[a]*	*186±20[a]*

[a]*Data expressed as mean values±standard error (n=4).*
[b]*Different letters within the same row indicate a significant difference (p<0.05).*

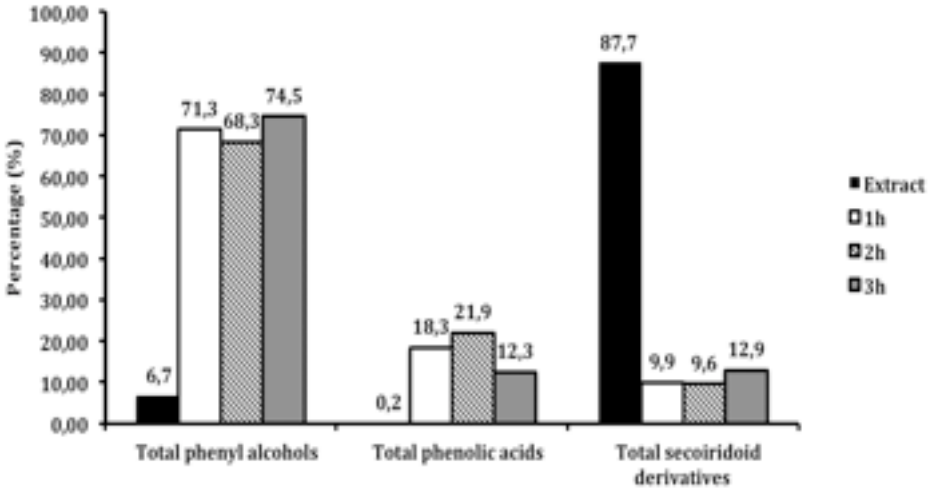

Figure 2. Importance in percentage of phenolic families (phenyl alcohols, phenolic acids and secoiridoid derivatives) quantified in the phenolic extract (PEOC) and in rat plasma at 1h, 2h and 4h post-ingestion of 3 g of PEOC/kg of rat body weight.

extract acting as a source of hydroxytyrosol.

After the absorption of phenols from the PEOC, a wide range of phenolic compounds was quantified in practically all the tissues analyzed, showing a wide distribution though the body. Nevertheless, oral bioavailability was low, with values lower than 5%. For example, the main phenolic compound of PEOC, hydroxytyrosol, showed a low oral bioavailability with 2,35% (1h), 3,75% (2h) and 2,00% (4h) including hydroxytyrosol and its derivates and taking into account all analyzed tissues. The liver and kidney were the organs with the greatest presence of phenolic metabolites, followed by the testes (Table 3). The heart, brain, spleen and thymus (Table 4) showed a lower number of metabolites with phenolic acids being the main metabolites quantified. Oleuropein derivate was present in all the tissues analysed 1 h after the PEOC ingestion, except in the thymus. In general, the maximum tissue concentration of this compound (C_{max}) was reached in all the tissues at 2 h (t_{max}) (Table 3). However, the maximum concentration was observed in the kidney, 71 nmol/g tissue 4 h after the PEOC ingestion. Phenyl alcohols were the main metabolites quantified, with hydroxytyrosol-sulphate as the main metabolite detected in all the analyzed tissues. Hydroxytyrosol-sulphate was also present in the kidneys from the control rats (0h), but at very a low concentration (16 nmol/g tissue). Nevertheless, its concentration increased rapidly, reaching values of 3163 nmol/g tissue 1 h (t_{max}) after the PEOC ingestion, and then decreased slowly until 1979 nmol/g tissue 4 h after ingestion. The testes also presented a high concentration of hydroxytyrosol-sulphate, but the accumulation started later in that tissue, with 1005 nmol/g tissue 2 h (t_{max}) after the PEOC ingestion. Its concentration in the liver was not as high as in the testicle and kidney (C_{max}237 nmol/g tissue, t_{max}2 h). On the other hand, hydroxytyrosol-sulphate was detected in the heart, thymus, brain and spleen at lower concentrations, under 17 nmol/g tissue (Table 4). Hydroxytyrosol-glucuronide was another quantified conjugated form of hydroxytyrosol (kidney: C_{max} 15 nmol/g tissue, t_{max} 1h; liver: C_{max} 2.1 nmol/g tissue, t_{max} 2h; testicle: C_{max} 3.18 nmol/g tissue, t_{max} 2h) and also the free form of hydroxytyrosol was detected in the kidney (C_{max} 3.8 nmol/g tissue, t_{max} 4h) and testicle (C_{max} 2.68 nmol/g tissue, t_{max} 2h).

Tyrosol-sulphate was quantified in all the tissues except in brain, and as with the hydroxytyrosol conjugate, the maximum concentration was detected in the kidney (C_{max} 684 nmol/g tissue, t_{max} 1h) but no significant differences were observed between 1, 2 and 4 h and a lower concentration (24 nmol/g tissue) was quantified in the kidney from rats of control group (0h). The testes showed the C_{max} (410 nmol/g tissue) 2 h after the ingestion of the extract. Lower concentrations of tyrosol-sulphate were detected in the heart, thymus and spleen (less than 23 nmol/g tissue). Besides, the glucuronidated form of tyrosol was present in the kidney and testes. The free or, in some cases, conjugated form (glucuronidated or sulphated) of some characteristic phenolic acids of virgin olive oil, such as caffeic acid or vanillic acid were present in tissues. Specifically,

Table 3. Quantities of phenolic metabolites in liver, kidney and testicle from control rats (0h) and rats after an acute intake of olive oil extract (1, 2 and 4 h)*. Results are expressed as nmol/g tissue.

	Liver [b]				Kidney [b]				Testicle [b]			
Metabolite (nmol/g tissue)	0h	1h	2h	4h	0h	1h	2h	4h	0h	1h	2h	4h
Hydroxytyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	1.9 [b]	1.3 [c]	3.8 [a]	n.d.	n.d.	2.68 [a]	1.40 [b]
Hydroxytyrosol-sulphate	n.d.	122 [c]	237 [a]	184 [b]	16 [a]	3163 [a]	3100 [a]	1979 [b]	n.d.	n.q.	1005 [b]	201 [c]
Hydroxytyrosol-glucuronide	n.d.	1.7 [a]	2.1 [a]	1.0 [b]	n.d.	15 [b]	12 [b]	2.6 [c]	n.d.	n.d.	3.18 [a]	1.70 [b]
Tyrosol-sulphate	1.3 [b]	68 [a]	73 [a]	70 [a]	24 [b]	684 [a]	599 [a]	605 [a]	n.d.	100 [b]	410 [a]	129 [b]
Tyrosol-glucuronide	n.d.	n.d.	n.d.	n.d.	n.d.	1.9 [a]	2.5 [a]	n.q.	n.d.	n.q.	6.9	n.q.
Oleuropein derivate	n.d.	5.3 [a]	20 [b]	14 [b]	n.d.	52 [b]	60.8 [b]	71 [b]	n.d.	3.88 [b]	16.63 [a]	7.73 [b]
Vanillin-sulphate	n.d.	0.4 [b]	1.0 [a]	0.4 [b]	0.15 [d]	2.7 [b]	3.0 [a]	1.4 [c]	n.d.	0.46 [b]	2.0 [a]	0.54 [b]
Cumaric-sulphate acid	n.d.	0.5 [a]	0.6 [a]	0.8 [a]	2.2 [d]	22 [a]	12 [c]	18 [b]	n.d.	0.36 [b]	1.5 [a]	0.43 [b]
Cumaric-glucuronide acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.19 [a]	0.3 [a]	n.q.	n.d.	n.d.	n.d.	n.d.
4-Hydroxy-3-metoxyphenylacetaldehid	n.d.	12 [b]	16 [a]	11 [b]	93 [b]	191 [a]	99 [b]	74 [c]	39 [a]	27.39 [a]	86.20 [b]	n.d.
Vanillic acid	0.74 [b]	2.7 [a]	2.7 [a]	2.4 [a]	2.8 [a]	3.6 [b]	3.3 [a,b]	3.8 [b]	n.d.	n.d.	n.d.	0.06
Vanillic-sulphate acid	n.d.	2.4 [b]	4.5 [a]	3.2 [b]	1.3 [a]	38 [c]	34 [b,c]	32 [b]	n.d.	0.41 [b]	4.56 [a]	0.52 [b]
Cafeic acid	n.d.	0.28 [b]	0.14 [a,b]	0.10 [a]	n.d.	1.5 [b]	2.1 [a]	1.2 [b]	n.d.	n.d.	n.d.	n.d.
Cafeic-sulphate acid	n.d.	n.d.	n.d.	n.d.	n.d.	2.1 [a]	1.2 [b]	1.1 [b]	n.d.	n.d.	n.d.	n.d.
Homovanillic acid	n.d.	0.11 [a]	0.19 [b]	n.d.	n.d.	2.5 [a]	2.6 [a]	1.3 [b]	n.d.	n.q.	6.37 [a]	1.68 [b]
Homovanillic-sulphate acid	n.d.	2.2 [b]	7.4 [a]	3.2 [b]	4.3 [a]	25 [c]	24 [c]	12 [b]	3 [a]	6.86 [b]	24.96 [c]	8.54 [b]
Ferulic-sulphate acid	n.d.	n.d.	n.d.	n.d.	6.3 [a]	4.4 [c]	3.5 [d]	5.5 [b]	n.d.	0.12 [a]	0.72 [b]	0.21 [a]
Hydroxyphenylacetic acid	0.01 [a]	0.5 [b]	0.63 [b]	0.52 [b]	1.5 [b]	6.9 [a]	6.7 [a]	2.2 [b]	n.d.	n.d.	n.d.	n.d.
Enterolactone	1.44 [a]	6.4 [a]	5.2 [b]	4.0 [b]	n.d.	2.7 [a]	2.3 [a]	0.67 [b]	n.d.	n.d.	n.d.	n.d.
Enterolactone-sulphate	2.0 [a]	5.2 [b]	5.6 [b]	4.2 [b]	n.d.	5.8 [a]	5.0 [a]	10 [a]	n.d.	n.d.	n.d.	n.d.
Enterolactone-glucuronide	0.01 [a]	0.4 [b,c]	0.5 [c]	0.3 [b]	1.9 [a]	3.3 [b,c]	5.4 [d]	3.6 [c]	n.d.	n.d.	n.d.	n.d.
Luteolin	n.d.	n.d.	n.d.	n.d.	n.q.	0.04	n.q.	n.q.	n.d.	n.q.	0.07 [a]	0.02 [a]

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

[a] *Data expressed as mean values (n=4). Standard error was omitted because all values were lower than 10%.*

[b] *For each tissue, different letters within the same row indicate a significant difference ($p<0.05$).*

Table 4. Quantities of metabolites in brain, spleen, heart and thymus from control rats (0h) and rats after an acute intake of olive oil extract (1, 2 and 4 h)*. Results are expressed as nmol/g tissue.

	Brain [b]				Spleen [b]			
Metabolite (nmol/g tissue)	0h	1h	2h	4h	0h	1h	2h	4h
Hydroxytyrosol sulphate	n.d.	28.4[b]	50[a]	27[b]	n.d.	0.09 [a]	12 [a]	34 [a]
Tyrosol sulphate	n.d.	6.81[b]	10.14[a]	10.49[a]	n.d.	13 [b]	21 [c]	23 [c]
Oleuropein derivate	n.d.	0.44 [c]	2.8[a]	1.3[b]	n.d.	3.1 [b]	4.8 [a]	3.3[a,b]
Vanillin sulphate	n.d.	0.02 [a]	0.02 [a]	0.03 [a]	n.d.	n.d.	n.d.	n.d.
Vanillic acid	0.86 [a]	1.8 [b]	1.7 [b]	2.0 [b]	1.1 [a]	1.6 [a,b]	1.9 [b]	1.1 [a]
Vanillic sulphate acid	n.d.	0.38 [b]	0.73 [c]	0.29 [b]	n.d.	2.8 [b]	5.6 [c]	2.6 [b]
Cafeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.16 [a]	0.25 [a]	3.4 [b]
Homovanillic sulphate acid	0.77 [a]	2.0 [b]	1.8 [b]	2.1 [b]	n.d.	n.d.	0.12 [a]	n.q.
Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.37 [b]	0.45 [c]	n.q.
	Heart [b]				Thymus [b]			
Metabolite (nmol/g tissue)	0h	1h	2h	4h	0h	1h	2h	4h
Hydroxytyrosol sulphate	n.d.	157 [b]	263 [a]	128 [b]	n.d.	n.d.	n.d.	n.d.
Tyrosol sulphate	n.d.	13 [b]	17 [a]	6.9 [c]	n.d.	n.d	4.80 [b]	9.99 [a]
Oleuropein derivate	n.d.	2.6 [c]	8.4 [a]	4.9 [b]	n.d.	n.d.	n.d.	n.d.
Vanillin sulphate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	2.95
Cumaric sulphate acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.08 [b]	0.36 [a]	0.06 [b]
Vanillic sulphate acid	n.d.	0.13 [a]	0.74 [b]	0.35 [c]	n.d.	1.31 [a]	1.80 [a]	n.q.
Homovanillic sulphate acid	n.d.	1.4	n.q.	n.q.	n.d.	n.d.	n.d.	n.d.
Hydroxyphenylacetic acid	n.q.	0.14 [b]	0.25 [a]	n.q.	n.q.	n.q.	n.q.	1.80
Luteolin	n.d.	0.47[a]	0.16[b]	0.27[b]	n.d.	n.d.	n.d.	n.d.

n.d.: not detected; n.q.: not quantified.
[a] *Data expressed as mean values (n=4). Standard error was omitted because all values were lower than 10%.*
[b] *For each tissue, different letters within the same row indicate a significant difference ($p<0.05$).*

vanillic-sulphate acid was quantified in all the tissues. However, 4-hydroxy-3-methoxyphenylacetaldehid was the most abundant phenolic acid present in the kidney (C_{max} 191, t_{max} 1h), liver (C_{max} 16, t_{max} 2h) and testes (C_{max} 86, t_{max} 2h) but this compound was also detected in the kidney from the control rats (0h) at 93 nmol/g tissue. Among the flavonoids, only luteolin was quantified in some tissues (kidney, testicle and heart) at low concentrations, less than 0.47 nmol/g tissue, and no lignans were quantified in their native form in the tissues. In addition, enterolactone and its glucuronide and sulphate conjugated metabolites were quantified in the liver and kidney (Table 3).

4 DISCUSSION

Because of the short life of polyphenols in plasma, to observe its potential benefit the studies should be carried out during the postprandial state, immediately after its intake [28,29]. Thus, the acute intake of olive oil phenols may be useful in a future repeated low dose experiments obtaining a concentration of phenolic metabolites in the tissues that allow their detection and quantification.

After ingestion, the secoiridoid derivatives may be partially modified in the acidic

environment of the stomach, suffering a non-enzimatic metabolism that considerably decreases their concentration and increases moderately the total phenyl alcohols and the total phenolic acids [30,31]. A rapid absorption was observed in the current study, with phenolic compounds being detected in the plasma 1h after ingestion of PEOC, although the t_{max} was at 2h for most of the quantified compounds. Similarly, different studies have reported the C_{max} of the phenolic compounds in the plasma between 1
and 2h after ingestion of virgin olive oil [22,32].

As expected, most of the metabolites that were identified in the plasma appeared in their conjugated form, mainly sulphated followed by glucuronidated forms [22], indicating that rat hepatocytes were capable of sulphating these phenols [33-35]. Additionally, the large amount of sulpho-conjugated may reflect a high-sulphotransferase activity of platelets [36]. On the contrary, Miro-Casas *et al.* [23] reported that 98% of hydroxytyrosol in human plasma and urine was mainly glucurono-conjugated. D'Angelo *et al.*[35] proposed a metabolic pathway for hydroxytyrosol that could be similarly applied to tyrosol, in which the action of cathecol-O-methyltransferases promoted the appearance of homovanillic acid and its conjugated forms. Related with this metabolism, hydroxytyrosol, hydroxytyrosol-sulphate, homovanillic acid, homovanillic-sulphate acid, and the mono-hydroxylated form of phenylacetic acid were quantified in rat plasma samples at all the tested times. As previously reported for human plasma [22], this suggests that all the intermediate forms were rapidly subjected to the UDP-glucuronosyltransferase and sulphotransferase enzymatic activities resulting in conjugated forms.

Instead of this first-step metabolism in the gut and liver, the free form of hydroxytyrosol was detected in the plasma (Table 2), in contrast to the results of previous studies in which only the conjugated forms of hydroxytyrosol were quantified in human plasma after the ingestion of virgin olive oil [1,23,31]. Similarly, a phenolic structure, identified as oleuropein derivate, was the only secoiridoid derivate detected as an unconjugated form in the plasma. Nevertheless, this could be explained because the parental form of the secoiridoids are not the major bioavailable form *in vivo* [37], which explains the absence of other secoiridoids in the plasma after the ingestion of a secoiridoid rich extract like PEOC. Probably, the first step of the intestine/hepatic metabolism could be related to the ingested dose. High doses could saturate the conjugation metabolism of the olive phenolic compounds and this may allow the detection of free forms in the plasma in the present study, these probably being absorbed by passive diffusion. Of special interest is the presence of these free aglycones circulating in the blood, probably with biological effects different from those of conjugated metabolites.

Little data are available in the literature on the distribution and accumulation of olive phenolic compounds in tissues. Our results showed that the phenolic metabolites were uniformly distributed in

all the tested tissues with a high concentration and number of metabolites in the liver and kidney, showing a preferential renal uptake of the metabolites, as observed by d'Angelo *et al.*[35] using intravenously injected [^{14}C] hydroxytyrosol. The presence of phenolic metabolites in the liver (Table 3) could be explained by the functionality of this organ in the metabolism of phenolic compounds. Linked to this functionality, the presence of free and conjugated (sulphated and glucuronidated) forms of the phenolic acids and phenolic alcohols suggest the importance of the liver in the conjugation metabolism and suggest an active metabolism during the experimental time. This metabolic detoxification process, common to many xenobiotics, facilitates the urinary elimination of phenolics [38]. In relation to this, the high concentration of sulphateconjugated forms of hydroxytyrosol and tyrosol quantified in the kidney could indicate the main excretion path for olive phenolic compounds [39]. By contrast, glucuronidated forms have been described mainly as metabolites in human urine [21,39,40].

A wide range of metabolites were detected in testes (Table 3). Despite the extensive presence of metabolites in this tissue, there are no studies about the function of olive phenolic compounds in testes. Nevertheless, procyanidins were widely distributed in lymphoid tissues, as Urpi-Sarda *et al.*[41] reported in their study, and possibly the lymphoid nature could be related to the accumulation of phenolic metabolites. Moreover, Wang *et al.* [42] reported a protective effect of phenolic compounds from Ginkgo biloba on an injury produced by an inducing stress agent in testicles.

It is of interest the detection in heart of conjugated forms of hydroxytyrosol, mainly hydroxytyrosol-sulphate (Table 4), and the free forms of oleuropein derivate and luteolin. These phenolic metabolites could exert a direct protective effect related to oxidative process in the arterial wall. In this sense some studies [43-47] have postulated that LDL oxidation does not take place in the circulation, and occur in the arterial wall because serum lipoprotein lipids are well protected in circulation from oxidation by the robust antioxidant defenses.

The increase of some phenolic metabolites in the brain as a consequence of the ingestion of the phenolic extract is of special interest too. The presence of phenolic compounds from PEOC in the brain could exert a protective effect against stress of different nature, such as oxidative stress in dopaminergic neurons [48], or nitrosative stress [49]. Additionally, the interest of the results of this study could be reinforced by the absence of free homovanillic and 3,4-dihydroxyphenylacetic acids, these being the main oxidized metabolites deriving from dopamine metabolism in the central nervous system [35].

The spleen is an important cross-point for antigenic information transported by the blood and immune system, and a major antibody producing organ [50]. The close relationship between the spleen and blood could explain the presence of some metabolites in this tissue, although the functionality of these metabolites in the spleen is not clear due to a lack of

information. However, other phenolic compounds, such as protocatechuic acid, showed a strong ability to attenuate the ageing alterations of the antioxidative defense systems in the spleen and liver [51]. So, given their antioxidant nature, olive phenolic compounds could also affect it.

The quantification of metabolites of the phenolic compounds in the plasma and multiple tissues indicated that after an acute ingestion of olive phenolic compounds, they were absorbed, metabolized and distributed though the blood stream to practically all the parts of the body, even across the blood-brain barrier. Sulphate conjugates of phenyl alcohols (mainly hydroxytyrosol and tyrosol) were the main metabolites quantified in the plasma and tissues and free forms of some phenolic compounds, such as oleuropein derivate in the plasma and brain, luteolin in the kidney, testicle, brain and heart, or hydroxytyrosol in the plasma, kidney and testicle were quantified. That work may suppose a breakthrough in the research about how olive oil acts positively in the prevention and improvement of some diseases, such as cardiovascular disease; or how olive oil exert positive effects in specific parts of the body, such as neuroprotective effect. Nevertheless, further experiments are required to investigate the effect of olive phenolic compounds on the functions of such organs as the spleen, liver, brain or heart.

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Rich or enriched foods? An example of olive oil phenolic compounds

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RICH OR ENRICHED FOODS? AN EXAMPLE OF OLIVE OIL PHENOLIC COMPOUNDS

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Abstract

The daily consumption of olive oil, as a fatty product, is limited and phenol enrichment of virgin olive oil could be a good option to increase phenolic intake without increasing calorie intake. Nevertheless, there is evidence to suggest that added phenolic compounds do not behave the same as own phenolic compounds. The aim of the study was to investigate the differences on the absorption, metabolism and tissue distribution of olive oil phenol of a phenol-enriched olive oil (EVOO) comparing with a naturally phenol-rich virgin olive oil (HVOO) at same level of phenolic concentration. Additionally, a virgin olive oil with naturally low phenolic content (LVOO) was included in the experiment. For this purpose, rat plasma and tissues (liver, kidney, brain, spleen, and heart) were obtained after an acute intake of 5 g of oil per kilogram of rat weight and the phenolic metabolites were analyzed. The behavior exerted by the phenolic compounds and the oily matrix seemed to be different when the phenolic compounds were not in the oil itself. Significant differences were found in the total plasma phenolic metabolites with a higher concentration after the EVOO intake (18 ± 1.8 μmol/l of plasma), followed by the HVOO (16 ± 1.6 μmol/l of plasma). Nevertheless, the behavior of phenolic compounds in the analyzed tissues seemed to not follow the same pattern of disposition in each tissue according to the phenolic nature.

Keywords: Olive oil / phenolic enrichment / phenolic metabolism / plasma phenolic metabolites / tissue distribution.

1 INTRODUCTION

Over recent decades, important epidemiological studies have established the protective effect of the Mediterranean Diet (MD) against the risk of breast, prostate, and

colorectal cancer and cardiovascular disease, which are among the most prevalent disabling and fatal diseases (Pauwels, 2011). These interesting data provide further support to the pioneering Seven Countries Study conducted by Keys *et al.* (Karon & Parker, 1988) in which a relatively low incidence of cardiovascular disease and cancer in the Mediterranean cohorts were found, and the basis of the MD concept was proposed. However, it is difficult to clarify this concept precisely because there is "no single ideal Mediterranean diet" (Noha & Trustwell, 2001). An excellent review by Bond, *et al.* (Bond, 2008) examines the defining characteristics and health benefits associated with the MD and lists its key components. Drawing on this review of the literature, the general characteristics of the ideal MD can be defined follows: a primarily plant-based, whole-food diet with an abundance of healthful plant-derived bioactive compounds (such as polyphenols, phytosterols, vitamins, minerals, and fiber) and with a low intake of atherogenic saturated fat, dietary cholesterol, and trans fat (via infrequent consumption of such animal protein sources as red meat and meat products and processed foods).

The Mediterranean people have used olive oil for culinary purposes for centuries. The health benefits of virgin olive oil (VOO) have been attributed to two main constituents: the high oleic acid (monounsaturated fatty acid) content and the presence of phytochemicals, such as phenolic compounds, squalene and alpha-tocopherol (Ortega, 2006).

VOO contains several groups of phenolic compounds that are also common in other vegetable products. On the contrary, a number of coumarin-like compounds, known as secoiridoids, are exclusive to the O. europaea species. It is accepted that the secoiridoid derivatives of oleuropein and ligstroside are often the main phenolic compounds in fresh VOO. Due to its natural origin, the phenolic content of VOO varies considerably with agronomic factors (such as olive cultivar, harvesting time, growing area, water availability and the health status of the fruit, among others) and the conditions under which the oil was extracted from the olive fruit (El Riachy, Priego-Capote, León, Rallo & Luque de Castro, 2011). In fact, the secoiridoid derivatives of hydroxytyrosol are the major phenolic compounds encountered in the 'Arbequina', 'Cornicabra', 'Picolimón' and 'Picual' cultivars, with values ranging from 105 to 1113, expressed as mg of caffeic acid/kg of VOO (El Riachy, Priego-Capote, León, Rallo & Luque de Castro, 2011).

In relation to the presence of these powerful antioxidants, the health benefits of consumption of VOO, compared with refined olive oil, of reducing the risk of heart attacks and attenuating the risk factors for cardiovascular diseases, have been scientifically documented, and cardioprotection via inhibition of LDL oxidation has been proposed (Visioli & Bernardini, 2011). Recently, the European Food Safety Authority (EFSA) released a report about the benefits of ingesting 5mg/day of phenolic compounds from olive oil (hydroxytyrosol, tyrosol, and their secoiridoids) to prevent oxidation of LDL (EFSA Panel on Dietetic Products, 2011). Considering the Food and Drug

Administration recommended daily intake limit of 23 g of olive oil, and taking into account that the low phenolic concentration detected in commercial VOOs, the consumption of 5 mg of hydroxytyrosol and its derivatives per day could be extremely difficult or even impossible, so new alternative strategies need to be considered. The first way would be selecting existing olive cultivars or the developing new ones with higher contents of phenolic compounds, leading to VOO with high phenolic content. Another interesting way is to develop high-phenol olive oils by adding phenolic extracts obtained from different olive byproducts. There have been a notable number of patents published over the last decade regarding the synthesis, extraction and application of hydroxytyrosol (Visioli & Bernardini, 2011). This indicates a growing interest in exploiting hydroxytyrosol as a potential supplement or preservative to be employed in the nutraceutical, cosmeceutical, and food industries. Therefore, extensive knowledge of the bioavailability of VOO polyphenols is necessary if their health effects are to clearly elucidated and pharmacologically enhanced.

In general, the hydrophilic phenolic compounds of VOO are absorbed in a dose-dependent manner in animals and humans, and are excreted in the urine, mainly as hydroxytyrosol glucuronide conjugates (Visioli, Grande, Bogani & Galli, 2006). The absorption of hydroxytyrosol differs with the vehicle in which it is delivered, being higher when the hydroxytyrosol is administered in an oily vehicle (90%) than when it is administered in an aqueous solution (75%) (Tuck, Freeman, Hayball, Stretch & Stupans, 2001). Although researchers have emphasized the study of phenolic metabolites in biological fluids (plasma and urine), these metabolites may accumulate in certain target tissues with hydrolytic enzymatic activity (e.g., sulphatase) that can reconvert conjugates back to active aglycones (Liu & Hu, 2007). In a previous study, we observed that the hydroxytyrosol and secoiridoid derivatives were absorbed, metabolized and distributed through the blood stream to practically all parts of the body, even across the blood-brain barrier after a single ingestion of an olive phenolic extract. So, knowledge of the absorption, metabolism and distribution of key bioactive metabolites of hydroxytyrosol in the plasma and tissues and the modulation caused by nature and concentration of phenolic compounds and the food matrix may be useful for enhancing the bioavailability of hydroxytyrosol and secoiridoids.

The interest of this study is based on the duality of the existence of VOO with naturally high phenolic content, which depends on a range of agronomic and technological factors, and the possibility of preparing fortified olive oils with a controlled and standard phenolic concentration. The aim of the study was to investigate the differences in the absorption, metabolism and tissue distribution of olive oil phenol from a phenol-enriched olive oil comparing with a naturally phenol-rich VOO at the same level of phenolic concentration. Additionally, a VOO oil with naturally low phenolic content was included in the experiment. For this proposal, rat plasma and tissues (liver, kidney, brain, spleen,

and heart) were obtained after an acute intake of 5 g of oil per kilogram of rat weight and the phenolic metabolites were analyzed by UPLC-MS/MS.

2 MATERIALS AND METHODS

2.1 Chemical and reagents

The standards of hydroxytyrosol, tyrosol, *p*-coumaric acid, apigenin and luteolin were purchased from Extrasynthese (Genay, France); caffeic and homovanillic acids were purchased from Fluka Co. (Buchs, 125 Switzerland). (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol from Sigma Aldrich (Germany). The secoiridoid derivatives 3,4-DHPEA-EDA and *p*-HPEA-EDA, and the lignan acetoxypinoresinol were not available commercially and were isolated from virgin olive by semi-preparative HPLC, as described in Artajo *et al.* (Artajo, Romero, Morelló & Motilva, 2006). A stock solution of each standard compound was dissolved in methanol, and all the solutions were stored in dark flasks at 4° C. Catechol prepared in phosphoric acid 4% was used as an internal standard (IS).

Acetonitrile (HPLC-grade), methanol (HPLC-grade), glaciar acetic acid (≥99.8%), formic acid and L(+)-ascorbic acid (reagent grade) were all supplied by Scharlau Chemie (Barcelona, Spain). Pure hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Orto-phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Olive oils

Three VOOs with different total phenolic contents were used in the experiment to determine the effect of the phenol-enrichment of VOO oil compared with a naturally phenol-rich VOO. VOO with high phenolic content (average 550 mg phenols/kg oil) (HVOO) was from the Verdal cultivar and obtained from a commercial olive oil mill (Molí Els Torms, Lleida, Catalonia, Spain). The phenol-enriched virgin olive oil (EVOO) was prepared by adding an extract rich in the main olive oil phenolic compounds to a VOO from the Negral cultivar with low phenolic content (average 290 mg phenols/kg of oil) (LVOO) following the procedure described in our previous study (Suárez *et al.*, 2011). The amount of phenolic extract added to the LVOO used as matrix of enrichment was adjusted to achieve a phenolic content similar to the phenol-rich oil (HVOO). The phenolic composition of the tree olive oils was analyzed by UPLC-MS/MS following the method in Suarez *et al.* (Suárez, Macià, Romero & Motilva, 2008).

2.3 Treatment of the rats and plasma and tissue collection

The Animal Ethics Committee of the University of Lleida approved the study (CEEA 03-02/09, 9th November 2009). Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12h light-12h dark schedule at controlled temperature (22° C). They were given a commercial feed, PanLab A04 (Panlab, Barcelona, Spain), and water *ad libitum*. The rats (n=12) were later kept under fasting conditions for between 16 and 17h with access to tap water. Subsequently, the rats were

divided into four groups. Groups 1, 2 and 3 (n =3) were fed with 1 g of each VOO (LVOO, HVOO and EVOO respectively)/ kg of body weight by intragastric gavage. One hour after the oil ingestion, the rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and euthanized by exsanguinations. Additionally, a control group of rats (n=3) was maintained under fasting conditions without ingestion and then similarly euthanized.

Blood samples were collected from the abdominal aorta with heparin-moistened syringes. The plasma samples were obtained by centrifuging (2000 g for 30 min at 4º C) and stored at -80º C until the chromatographic analysis of the phenolic metabolites. Different tissues (liver, kidney, brain, spleen, heart) were excised from the rats, stored at -80º C and freeze-dried for phenolic extraction and chromatographic analysis.

2.4 Phenolic extraction of plasma and tissues and chromatographic analysis

In order to clean-up the biological matrix and preconcentrate the phenolic compounds, the plasma samples were pretreated by microelution solid-phase extraction (µSPE) (Serra, Rubió, Borrás, Macià, Romero & Motilva, 2012), and the tissues were sequentially pretreated by a combination of liquid-solid extraction (LSE) and µSPE. 60 mg of freeze-dried tissue were weighed, and then 50 µl of ascorbic acid 1% and 100 µl of phosphoric acid 4% were added. The samples were treated 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 samples were sonicated (S-150D Digital SonifierR Cell Disruptor, Branson, Ultrasonidos S.A.E., Barcelona, Spain) for 30 s while maintained in a freezing water bath to avoid heating and then centrifuged for 15 min at 9000 rpm at 20º C. The supernatants were collected, and then an aliquot of the extracts was treated by µSPE (OASIS HLB µElution Plates 30 µm) (Waters, Milford, MA, USA) to clean up the samples before the chromatographic analysis (Serra, Macià, Romero, Piñol & Motilva). For the analysis of hydroxytyrosol, the micro-cartridges were conditioned sequentially with 250 µl of methanol and 250 µl of Milli-Q at pH 2 with hydrochloric acid. 200 µl of phosphoric acid 4% was added to 200 µl of tissue extract, and then this mixture was loaded onto the plate. The loaded plates were washed with 75 µl of Milli-Q water and 75 µl of Milli-Q water at pH 2. The retained hydroxytyrosol was then eluted with 2 x 25 µl of acetonitrile/Milli-Q water solution (50/50, v/v). On the other hand, to analyze the rest of the phenolic compounds studied the micro-cartridges were conditioned sequentially with 250 µl of methanol and 250 µl of Milli-Q at pH 2 with hydrochloric acid. 350 µl of phosphoric acid 4% was added to 350 µl of tissue 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 Milli-Q water at pH 2. The phenolic compounds retained were then eluted with 2 x 50 µl of methanol. The eluted solutions (phenolic extracts) were directly injected into the UPLC-MS/MS, and the sample volume was 2.5 µl.

The phenolic compounds were analyzed by Acquity Ultra-PerformanceTM liquid chromatography and tandem MS from Waters (Milford MA, USA), as reported in

our previous studies (Suárez *et al.*, 2009). The detection and quantification of the phenolic compounds and their metabolites in plasma and tissues were based on their ion fragmentation in the MS/MS mode using SRM. Due to the lack of standards for these metabolites, they were tentatively quantified by using the calibration curves corresponding to their phenolic precursors. This way, hydroxytyrosol and tyrosol metabolites were quantified using the calibration curves of hydroxytyrosol and tyrosol, respectively; homovanillic acid, vanillic acid and vanillin metabolites were quantified using the calibration curve of homovanillic acid; *p*-coumaric, *p*-hydroxybenzoic and ferulic acid metabolites were quantified by means of the *p*-coumaric acid calibration curve. Apigenin metabolites were quantified with the apigenin calibration curve. Enterodiol and enterolactone were quantified with the pinoresinol calibration curve.

2.5 Statistical analysis

The data on polyphenol metabolite levels are presented as mean values ± standard error (n=9). The data were analyzed by a multifactor ANOVA test to determine significant differences between olive oils (LVOO, HVOO and EVOO) and to compare these with the control group. A significant difference was considered to be at a level of $p \leq 0.05$. All the statistical analyses were carried out using STATGRAPHICS Plus 5.1.

3 RESULTS

3.1. Quantitative analysis of the phenolic compounds in the olive oils

Three VOOs were used to study the bioavailability and tissue distribution of olive oil phenols. The phenolic contents of the HVOO (538 ± 59 mg phenols/kg oil) and EVOO (555 ± 50 mg phenols/kg oil) were similar (Table 1), but naturally acquired or through enrichment by adding a phenolic extract respectively. The LVOO (293 ± 31 mg phenols/kg oil), used as matrix enrichment to prepare the EVOO, was included in the study to determine the dose-effect on the bioavailability and tissue distribution of phenolic compounds. The three VOOs had similar proportions of secoiridoid derivatives (average 90-97 %), the main phenolic fraction, followed by phenolic acids (average 1.3-6.8 %), phenolic alcohols, lignans and flavonoids as minor fractions.

3.2 Quantitative analysis of plasma metabolites

The main phenolic metabolites quantified in the plasma 1 h after the oil intakes (LVOO, HVOO and EVOO) are shown in Figure 1. Significant differences were determined in the total plasma phenolic metabolites with higher concentrations after the EVOO intake (18 ± 1.8 µmol/l of plasma), followed by the HVOO (16 ± 1.6 µmol/l of plasma). There were significant differences in the concentrations of individual metabolites, such as sulphate conjugated forms of hydroxytyrosol, homovanillic alcohol and tyrosol, these being higher after the intake of EVOO. No significant differences in these compounds were observed between the HVOO and LVOO. The concentration of tyrosol sulphate was higher in plasma from the LVOO and HVOO, and the concentration of hydroxyphenyl propionic acid sulphate was significantly higher after the intake of the HVOO, with no significant differences between the LVOO

Table 1. Phenolic composition of virgen olive oils with different phenolic content: low phenolic-content virgin olive oil (LVOO), high phenolic-content virgin olive oil (HVOO) and phenol-enriched virgin olive oil (EVOO). Total phenolic content in each family of phenolic compounds is expressed as mg/kg of oil (percentage in relation to the total phenolic content).

Compound (mg(kg oil)	Low phenolic-content virgin olive oil (LVOO)	High phenolic-content virgin olive oil (HVOO)	Phenol-enriched virgin olive oil (EVOO)
Hydroxytyrosol	0.18±0.02	0.18±0.01	5.7 ± 0.56
Tyrosol	0.39±0.03	0.26± 0.03	0.93± 0.10
Total phenolic alcohols	*0.57 ± 0.06 (0.19%)*	*0.44 ± 0.42 (0.08%)*	*6.6 ± 0.65 (1.18%)*
Homovanillic acid	0.06±0.00	0.05±0.00	0.07±0.00
Vanillin	0.09±0.00	0.12±0.02	0.51±0.06
p-Hydroxybenzoic acid	0.02±0.00	0.03± 0.01	0.19± 0.02
Elenolic acid	20 ± 1.4	7.1 ± 0.71	28 ± 2.9
Total phenolic acids	*20 ± 2.1 (6.8%)*	*7.3 ± 0.71 (1.3%)*	*29 ± 2.7 (5.2%)*
3,4-DHPEA-AC	n.d.	7.5 ± 0.04	17 ± 1.6
3,4-DHPEA-EDA	4.4 ± 0.48	94 ± 9.6	202 ± 22
3,4-DHPEA-EA	63 ± 6.2	100 ± 11	85 ± 8.3
Methyl 3,4-DHPEA-EA	1.5 ± 0.12	2.0 ± 0.26	1.1 ± 0.14
Oleuropein derivative	0.22±0.02	0.25 ±0.03	0.45 ± 0.43
p-HPEA-EDA	9.3 ± 0.91	15 ± 1.9	7.5 ± 0.76
p-HPEA-EA	188 ± 15	306 ± 31	180 ± 11
Ligstroside derivative	0.17±0.02	1.3 ± 0.13	12 ± 1.0
Total secoiridoid derivatives	*266 ± 24 (90%)*	*526 ± 55 (97%)*	*505 ± 47 (90%)*
Pinoresinol	0.37±0.04	0.07±0.01	0.25±0.02
Acetoxypinoresinol	2.1 ± 0.20	0.09±0.01	2.3 ± 0.22
Total lignans	2.5 ± 0.23 (0.85%)	0.16 ± 0.02 (0.02%)	2.5 ± 0.31 (0.45%)
Apigenin	1.4 ± 0.12	1.1 ±0.1	1.6 ± 0.18
Luteolin	1.3 ±0.13	2.5 ± 0.28	10 ± 0.11
Total flavonoids	*2.7 ± 0.31 (0.92%)*	*3.6 ± 0.34 (0.66%)*	*12 ± 1.3 (2.16%)*
Total phenolic content	*293 ± 31*	*538± 59*	*555± 50*

n.d. not detected
3,4-DHPEA-AC: 4-(acetoxyethyl)-1,2-dihidroxybenzene; 3,4-DHPEA-EDA: dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA: oleuropein aglycone; p-HPEA-EDA: dialdehydic form of elenolic acid linked to tyrosol; p-HPEA-EA: ligstroside aglycone.

and EVOO. There were no significant differences between oils in the plasma concentration of such other phenolic metabolites quantified in the plasma, as the glucuronide conjugates of hydroxytyrosol, ferulic acid and coumaric acid, and the sulphate conjugates of hydroxytyrosol acetate, apigenin and coumaric acid. None of these phenolic metabolites were detected in rat plasma from the control group (without olive oil intake). No free forms were quantified in the plasma after any olive oil intake and

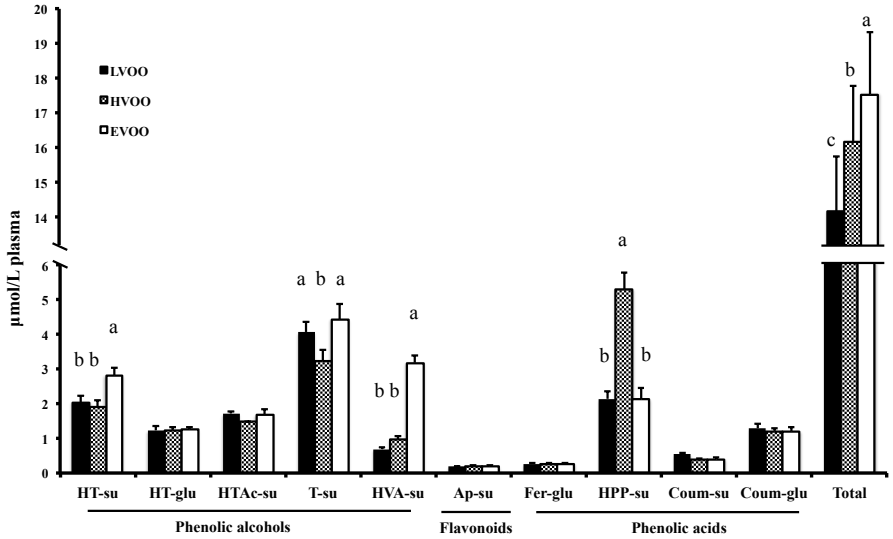

Figure 1. Plasmatic metabolites detected 1h after an acute intake of virgin olive oil with different phenolic content. Low phenolic-content virgin olive oil (LVOO), high phenolic-content virgin olive oil (HVOO), and phenol-enriched phenolic-content virgin olive oil (EVOO). [ab] Means significant differences between oils ($p \leq 0.05$). Nomenclature used, HT-su: Hydroxytyrosol sulphate; HT-glu: Hydroxytyrosol glucuronide; HTAc-su: Hydroxytyrosol acetate sulphate; T-su: Tyrosol sulphate; HVA-su: Homovanillic alcohol sulphate; Ap-su: Apigenin sulphate; Fer-glu: Ferulic glucuronide acid; HPP-su: Hydroxyphenylpropionic sulphate acid; Coum-su: Coumaric sulphate acid; Coum-glu: Coumaric glucuronide acid.

no phenolic compounds were quantified at basal conditions (data not shown).

3.3 Quantitative analysis of metabolites in tissues

Once absorbed, the phenolic metabolites spread throughout the body and were found in all the tissues analyzed (Tables 2 and 3). No metabolites were quantified in the control group (before oil intake) with exception of vanillic acid, which was quantified in the brain (0.86 ± 0.07 nmol/g of tissue) and liver (0.74 ± 0.06 nmol/g of tissue). The metabolic profile after the intake of the three different oils was similar in liver and kidney, independently of the olive oil and the phenolic doses (Table 2). Nevertheless, significant differences were observed in the concentration of some phenolic metabolites in both tissues depending on the phenolic dose. There were significant differences in the concentration of hydroxytyrosol sulphate, tyrosol sulphate, 4-hydroxy-3-methoxyphenyl acetaldehyde and vanillic acid sulphate in the liver, and higher concentrations of these were found after the intake of the EVOO. Between the LVOO and HVOO, only pinoresinol glucuronide and 4-hydroxy-3-methoxyphenyl acetaldehyde showed significant differences. In general, the concentration of phenolic metabolites in the liver was higher after the intake of the EVOO than with the HVOO although the ingested dose of

Table 2. Concentration of phenolic metabolites in spleen and kidney tissues of the control rats (before acute intake of oil) and rats 1 h after the acute intake of 1 g of low phenolic-content VOO (LVOO), high phenolic-content VOO (HVOO) and phenol-enriched VOO (EVOO).

Tissue	Compound (nmols/g)	Control	Low phenolic-content virgin olive oil (LVOO)	High phenolic-content virgin olive oil (HVOO)	Phenol-Enriched virgin olive oil (EVOO)
Liver	Hydroxytyrosol sulphate	n.d.	1.6 ± 0.1^{b}	1.6± 0.1^{b}	3.9 ± 0.2^{a}
	Tyrosol sulphate	n.d.	16 ± 1^{b}	11 ± 1^{b}	20 ± 2.3^{a}
	Vanillin sulphate	n.d.	0.07 ± 0.00^{a}	0.04 ± 0.00^{a}	0.05 ± 0.00^{a}
	Vanillic sulphate acid	n.d.	0.27 ± 0.34^{b}	0.03 ± 0.00^{c}	0.36 ± 0.12^{a}
	Homovanillic sulphate acid	n.d.	0.14 ± 0.17^{a}	0.28 ± 0.3^{a}	0.20 ± 0.16^{a}
	Pinoresinol glucuronide	n.d.	0.79 ± 0.05^{a}	2.4 ± 0.2^{b}	0.90 ± 0.12^{a}
	Vanillic acid	0.74 ± 0.06^{a}	2.5 ± 0.1^{b}	2.2 ± 0.2^{b}	2.7 ± 0.3^{b}
	4-Hydroxy-3-methoxyphenyl acetaldehyde	n.d.	6.9 ± 0.88^{b}	39 ± 4.2^{a}	42 ± 3.6^{a}
Kidney	Hydroxytyrosol sulphate	n.d.	9.9 ± 1.2^{b}	10 ± 1.1^{b}	20 ± 0.20^{c}
	Tyrosol sulphate	n.d.	39 ± 0.4^{b}	31 ± 2.8^{b}	32 ± 2.9^{b}
	Coumaric sulphate acid	n.d.	5.4 ± 1.8^{d}	1.6 ± 0.9^{b}	3.5 ± 0.32^{c}
	Vanillic sulphate acid	n.d.	0.69 ± 0.52^{b}	1.0 ± 0.12^{b}	1.9 ± 0.22^{c}
	Homovanillic sulphate acid	n.d.	5.5 ± 0.50^{c}	4.0 ± 0.42^{b}	5.1 ± 0.42^{c}
	Ferulic sulphate acid	n.d.	3.0 ± 0.30^{d}	2.0 ± 0.20^{c}	1.4 ± 0.12^{b}
	Enterolactone sulphate	n.d.	2.8 ± 0.12^{b,c}	3.3 ± 0.35^{c}	2.2 ± 0.19^{b}
	Enterolactone glucuronide	n.d.	1.6 ± 0.21^{a}	2.5 ± 0.31^{b}	1.9 ± 0.18^{a}
	Hydroxyphenyl acetic acid	1.5 ± 0.14^{a}	1.7 ± 0.13^{b}	1.2 ± 0.23^{a}	1.8 ± 0.15^{b}
	4-Hydroxy-3-metoxyphenyl acetaldehyde	n.d.	26 ± 2.1^{c}	85 ± 8.0^{a}	17± 1.9^{b}

n.d. no detected
Data expressed as mean values±standard error (n=9)
Different letters within the same row indicate a significant difference ($p<0.05$).

phenols was similar. Unlike what was observed in the liver, the concentration of some phenolic metabolites in the kidney was higher after the intake of HVOO, especially enterolactone sulphate and glucuronide conjugates, which were only found in this tissue. As occurred in the liver, higher concentrations of hydroxytyrosol sulphate and vanillic acid sulphate were quantified after the intake of the EVOO.

As regard to the other analyzed tissues (Table 3), two metabolites were quantified in the heart only after the EVOO intake, these being hydroxytyrosol sulphate and 4-hydroxy-3-methoxyphenyl acetaldehyde. The spleen was the tissue with more differences in phenolic distribution between the virgin olive oil intakes. After the LVOO and HVOO, only hydroxytyrosol sulphate was quantified (0.43 ± 0.03 nmol/g of tissue and 2.42 ± 0.21 nmol/g of tissue, respectively), but this metabolite was not found after the EVOO intake. Nevertheless, several phenolic metabolites were quantified in the spleen after the EVOO intake, including tyrosol sulphate, this being the main metabolite (4.49 ± 0.6 nmol/g of

Table 3. Concentration of phenolic metabolites in heart, spleen and brain tissues of the control rats (before acute intake of oil) and rats 1 h after the acute intake of 1 g of low phenolic-content VOO (LVOO), high phenolic-content VOO (HVOO) and phenol-enriched VOO (EVOO).

Tissue	Compound (nmols/g)	Control	Low phenolic-content virgin olive oil (LVOO)	High phenolic-content virgin olive oil (HVOO)	Phenol-Enriched virgin olive oil (EVOO)
Heart	Hydroxytyrosol sulphate	n.d.	n.d.	n.d.	4.9 ± 0.3
	4-Hydroxy-3-metoxyphenyl acetaldehyde	n.d.	n.d.	n.d.	19 ± 2.1
Spleen	Hydroxytyrosol sulphate	n.d.	0.43 ± 0.03 [a]	2.42 ± 0.21 [b]	n.d.
	Tyrosol sulphate	n.d.	n.d.	n.d.	4.49 ± 0.6
	Vanillic sulphate acid	n.d.	n.d.	n.d.	0.48 ± 0.39
	Cafeic acid	n.d.	n.d.	n.d.	0.29 ± 0.22 [a]
	Hydroxyphenyl acetic acid	n.d.	n.d.	n.d.	0.14 ± 0.10
	4-Hydroxy-3-methoxyphenyl acetaldehyde	n.d.	0.48± 0.03 [a]	n.d.	0.53± 0.04 [b]
Brain	Hydroxytyrosol sulphate	n.d.	0.77± 0.01[a]	1.1± 0.13 [a,b]	1.7 ± 0.01[b]
	Tyrosol sulphate	n.d.	n.d.	0.09 ± 0.00	n.d.
	Vanillin sulphate	n.d.	n.d.	0.01 ± 0.00	n.d.
	Homovanillic sulphate acid	n.d.	1.2 ± 0.09 [b]	3.0 ± 0.3 [c]	0.79 ± 0.06[a,b]
	Vanillic sulphate acid	n.d.	n.d.	0.07 ± 0.08 [a]	0.06± 0.003 [a]
	Vanillic acid	0.86 ± 0.07 [a]	1.3 ± 0.11[b]	2.2 ± 0.03 [c]	1.4 ± 0.13 [b]
	Ferulic sulphate acid	n.d.	n.d.	0.01 ± 0.00	n.d.
	4-Hydroxy-3-methoxyphenyl acetaldehyde	n.d.	1.0 ± 0.01[a]	0.47 ± 0.03 [a]	n.d.

n.d. no detected
Data expressed as mean values±standard error (n=9)
Different letters within the same row indicate a significant difference ($p<0.05$).

tissue), vanillic sulphate acid, hydroxyphenylacetic acid and cafeic acid. Finally, note the large number of metabolites deposited in the brain after the intake of the HLOO. Nonetheless, the concentration of these phenolic metabolites in the brain was lower than the amount quantified in the other analysed tissues, except the spleen. Hydroxytyrosol sulphate was quantified in the brain after the intake of all the virgin olive oils tested, with higher concentrations according to the phenolic content ingested. Additionally, a small quantity of tyrosol sulphate was quantified after the HVOO intake. It is notable that the intake of this oil with naturally high phenolic content (HVOO) resulted in higher levels of phenolic metabolites in the brain, including higher concentrations of homovanillic sulphate acid, vanillic acid, ferulic sulphate acid, vanillic sulphate and tyrosol sulphate.

4 DISCUSSION

The absorption of phenolic compounds differs according to the vehicle in which they are carried. In general, due to the

digestion process, the phenolic compounds of the VOO are absorbed worse in aqueous solutions than in oily matrices (Tuck & Hayball, 2002, Tulipani *et al.*, 2012). That fact gives the VOO a special character as a vehicle for increasing the daily phenolic intake and they could be the basis for developing the hypothesis for an enriched VOO. Additionally, VOO may have a positively effect on the bioavailability of the phenolic compounds in cooked (Fielding, Rowley, Cooper & O'Dea, 2005) and raw (Ninfali, Mea, Giorgini, Rocchi & Bacchiocca, 2005) phenol-rich foods. Moreover, the differences between the bioavailability and metabolism of the oil's own or added phenolic compounds in an oily matrix were not well determined in the bibliography. As our results suggested, the behavior exerted by the phenolic compounds and the oily matrix seemed to be different when the phenolic compounds were not from the oil itself. Although the number of plasma phenolic metabolites was the same in all the tested olive oils (LVOO, HVOO and EVOO), significant differences were observed in the plasmatic concentration, mainly among the sulphated conjugates. At the same phenolic concentration, the phenolic compounds added to the olive oil increased the plasmatic bioavailability. The results of the latter study showed that the plasmatic concentration of catechin, epicatechin and subsequently, the total monomeric metabolites, increased with a procyanidin enrichment of a cocoa cream reaching the maximum of concentration 2 h after the intake. The results of the present study, related to the plasmatic concentration of the olive oil phenolic metabolites, are summarized in Figure 2. To analyze the ratio between sub-families, the results are expressed as percentage of the total phenolic acids and total phenolic alcohols, with both families sub-dividing into the glucuronidated and sulphated forms, quantified in the plasma 1 h after the oil ingestion. After the intake of the LVOO, the proportions of phenolic acids and phenolic alcohols were similar (50 % and 48 % respectively), while this proportion varied when the intake of phenols increased, reaching 64% and 57% of the total phenolic alcohols, for HVOO and EVOO respectively. Regarding the types of conjugation, sulphation was clearly the main reaction type with approximately the 85% of the total conjugates, regardless of the oil ingested. The proportion of the sulphate/ glucuronide conjugates of the phenolic acids only varied after the intake of the HVOO, with the percentage of glucuronidated metabolites increasing to 32 % and the sulpho-conjugates decreasing to 68%.

The phenolic metabolites were uniformly distributed in all the tissues studied, with some differences in the number and amount of the metabolites detected according to the quantity and nature of the phenolic compounds. With the aim of evaluating the effective accumulation of phenolic metabolites, Figure 3 shows the total concentration of the metabolites found in each tissue after the intake of each three oils. The liver and kidney played a crucial role in the phenolic metabolism and excretion, and differences in the accumulation of phenolic metabolites were observed depending on the nature of the phenolic compounds, whether these were the oil's own or added. While a higher concentration of the phenolic metabolites

was detected in liver after the intake of the EVOO (added phenolic compounds), the intake of the same amount of phenols through an olive oil with a high phenolic content (HVOO - with its own phenolic compounds) resulted in a higher concentration of metabolites in the kidney. Statistically, the kidney was the tissue with the highest accumulation of phenolic metabolites, although the peak of the excretion of hydroxytyrosol and tyrosol was described in the first 6 h after

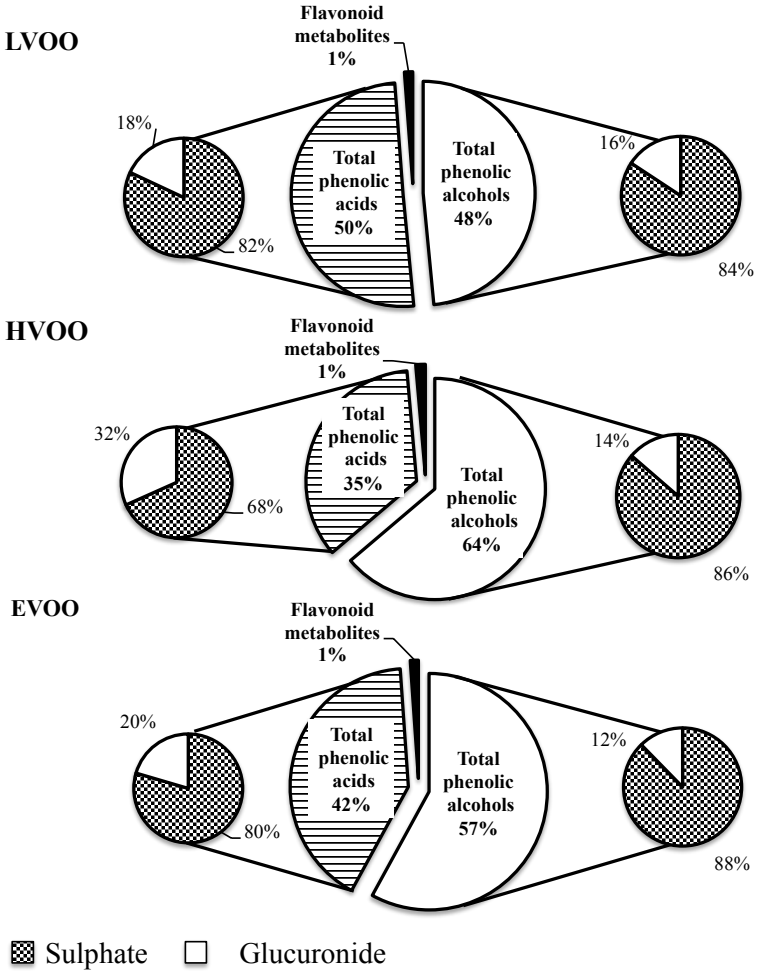

Figure 2. Proportion of each family of metabolites and the conjugation pattern, expressed as percentages, detected in the plasma samples 1h after an acute intake of virgin olive oil with different phenolic content. Low phenolic-content virgin olive oil (LVOO), high phenolic-content virgin olive oil (HVOO), and phenol-enriched phenolic-content virgin olive oil (EVOO).

the administration of the virgin olive oil (D'Angelo *et al.*, 2001). The concentration of metabolites was approximately 10 times higher in the kidney than in other peripheral tissues (D'Angelo *et al.*, 2001). The intense sulphatation and glucuronidation (phase II metabolism) could be related to a metabolic detoxification process, common to many xenobiotics, facilitating urinary excretion (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). This might explain the high concentration of phenolic metabolites found in the kidney in the present study, independently of the oil ingested.

As regards the peripheral tissues, the brain showed a similar distribution pattern to the kidney and the spleen showed a similar distribution pattern to the liver (Figure 3). Although the quantification of hydroxytyrosol and some of its metabolites in the brain could be a combination of the exogenous and endogenous metabolites, due to

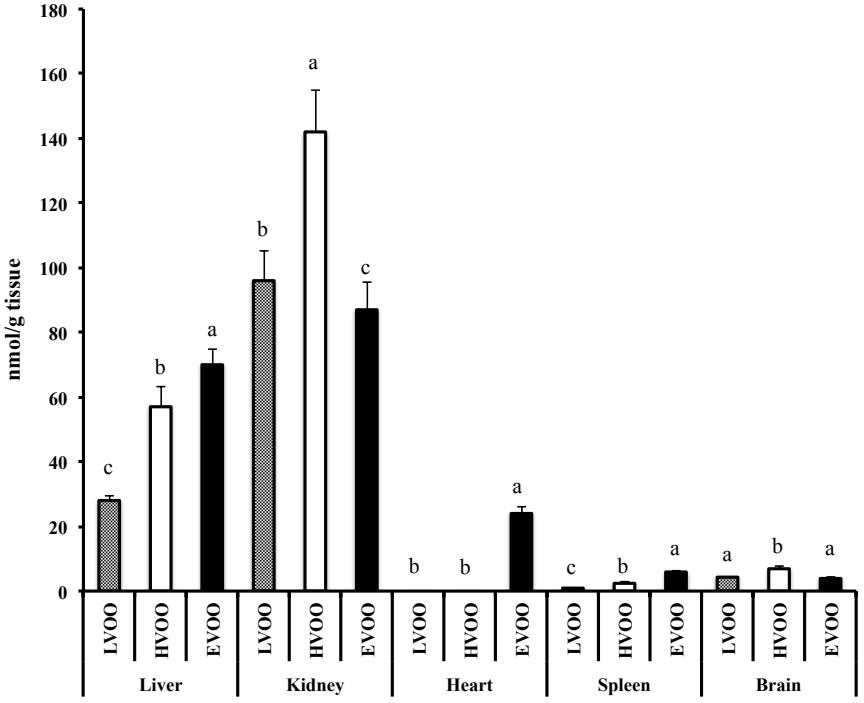

Figure 3. Total phenolic content expressed as nmol/g of tissue detected 1h after an acute intake of virgin olive oils with different phenolic content. Low phenolic-content virgin olive oil (LVOO), high phenolic-content virgin olive oil (HVOO), and enriched phenolic-content virgin olive oil (EVOO). Total phenolic alcohols included: Hydroxytyrosol sulphate, hydroxytyrosol glucuronide, hydroxytyrosol acetate sulphate, tyrosol sulphate and homovanillic alcohol sulphate and Total phenolic acids included: Ferulic glucuronide acid, hydroxyphenylpropionic sulphate acid, coumaric sulphate acid and coumaric glucuronide acid. [ab] Means significant differences between intakes ($p \leq 0.05$).

hydroxytyrosol may be generated endogenously from dihydroxyphenylacetic acid through dihydroxyphenylacetic reductase of the brain (Xu & Sim, 1995) and from the dopamine metabolism (Caruso, Visioli, Patelli, Galli & Galli, 2001, De La Torre, 2008, Edwards & Rizk, 1981, Goldstein, Eisenhofer & Kopin, 2003); several olive oil phenolic compounds, mainly sulphated forms, were able to cross the blood-brain barrier. The fact that these metabolites were not detected in the control group (basal conditions) confirms that the phenolic metabolites quantified in the brain could be from the intake of olive oil, with the exception of vanillic acid. However, the concentration of this compound increased after intake of the three oils, especially after the intake of the HVOO. The presence of more metabolites in the brain after the HVOO intake than after the EVOO intake could be related to the natural microemulsion of virgin olive oils during the mechanical extraction process of the oil. Briefly, virgin olive oil is produced industrially by pressing the olives mechanically. The malaxation step aims to destroy the oil bodies (inner structure of the olives acting as tiny reservoirs) and free the oil. This leads to the formation of an emulsion of olive oil with the internal water from the olives, naturally stabilized in the hydrophobic matrix by proteins and phospholipids. The olive oil producers almost always accelerate the breakdown of the emulsion by centrifuging and filtering it, in order to offer the consumers a clear olive oil. This procedure is a only macroscopic phase separation of the hydrophobic triglycerides from the aqueous phase and the therein solubilized hydrophilic substances (mainly phenolic compounds), because an important amount of these constituents remain in the olive oil (Xenakis, Papadimitriou & Sotiroudis, 2010). Thus, possible effects of the microstructure, where the hydrophilic minor components are dispersed in the VOO, could offer a favorable environment for crossing the blood brain barrier better than the microstructures formed during the preparation of phenol-enriched olive oil by the addition of phenolic extract previously solubilized in water.

The close relationship between the spleen and the blood (Timens & Leemans, 1992) could explain the presence of phenolic metabolites. However, their presence in the spleen did not follow a dose dependent pattern, and the amount detected with a dietary dose was similar to the amount detected with a pharmacological dose of 3g of olive cake phenolic extract/kg weight administered to the rats as an aqueous solution in a previous study (Serra, Rubió, Borrás, Macià, Romero & Motilva, 2012). On the other hand, in tissues such as the brain or the heart there was a clear reduction in the number and quantity of phenolic metabolites after a dietary dose compared with the study cited above (Serra, Rubió, Borrás, Macià, Romero & Motilva, 2012).

5 CONCLUSIONS

Depending on the metabolic target, the phenolic dose should be varied to improve the functionality. If the metabolic target is the brain, the VOO phenolic content should be lower than if the metabolic target is the cardiovascular system, where an enriched VOO could be more appropriate. The matrix

characterization is an important point to take into account when developing new functional phenolic-enriched or -fortified food due to its possible interference with the bioavailability and metabolism of the phenolic compounds. The use of rich-phenolic or enriched-phenolic products could vary the metabolite pharmacokinetic profile, due to differences according to the nature of the phenolic compounds, either the oil's own or added to the matrix. Phenolic-enriched VOO could be the best option to increase the plasma phenolic content. On the other hand, if there is a metabolic target, a prior dose-tissue disposition study could be performed.

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Flavanol metabolites accumulate in visceral adipose depots after chronic intake of grape seed proanthocyanidin extract in rats

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FLAVANOL METABOLITES ACCUMULATE IN VISCERAL ADIPOSE DEPOTS AFTER CHRONIC INTAKE OF GRAPE SEED PROANTHOCYANIDIN EXTRACT IN RATS

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Abstract
A considerable number of epidemiological investigations and intervention studies support an association between the intake of flavanol- and proanthocyanidin-containing foods and a decreased risk of metabolic diseases. Nonetheless, less is know about which metabolites reach to specific cells and the capacity of tissues to accumulate flavanols and/or their metabolites. The main objective of this study was to determine in rat the plasmatic bioavailability and disposition and accumulation in liver, muscle, brown adipose tissue and white adipose tissues (mesenteric and perirenal) after a chronic consumption of three doses of grape seed phenolic extract (5, 25 and 50 mg /kg rat body weight) for 21 days in order to determine if there is a dose-response. Glucuronidated conjugates followed by methyl glucuronidated conjugates were the main flavanol metabolites quantified in plasma, also detecting dimer in its free form. Each of the studied organs has a particular behavior of accumulation and response to the assayed grape seed extract doses, with a clear dose response in brown adipose tissue, in which flavanols could play an important role in the reduction or prevention of obesity modulating the functionality of that tissue.

Keywords: Flavanols / chronic intake / tissue accumulation / adipose tissue.

1 INTRODUCTION

Flavan-3-ol and proanthocyanidins are the most abundant flavonoid subgroups in the human diet (Zamora-Ros *et al.*, 2010). These compounds are mainly found in apples, legumes, grapes, nuts,

red wine, tea and cocoa (Wang, Chung, Song & Chun, 2011). A considerable number of epidemiological research and intervention studies support an association between the intake of foods containing flavanol and proanthocyanidin and a decreased risk of diseases, in particular cardiovascular diseases (McCullough, Peterson, Patel, Jacques, Shah & Dwyer, 2012, Schroeter, Heiss, Spencer, Keen, Lupton & Schmitz, 2010) and cancer (Cutler *et al.*, 2008). Moreover, the consumption of flavanol- and proanthocyanidin-rich foods improves dyslipidemia (Bladé, Arola & Salvadó, 2010; Mellor, Sathyapalan, Kilpatrick, Beckett & Atkin, 2010), insulin sensitivity (Hooper *et al.*, 2012) and obesity (Basu *et al.*, 2010) in humans.

Grape seed proanthocyanidin extract (GSPE) improves the atherosclerotic risk index and reduces postprandial triglyceridemia, in both healthy (Del Bas *et al.*, 2005; Quesada *et al.*, 2011) and dyslipidemic rats (Quesada *et al.*, 2009). Moreover, GSPE improves glycemia in rats with altered glucose homeostasis (Pinent, Blay, Bladé, Salvadó, Arola & Ardévol, 2004; Pinent, Cedó, Montagut, Blay & Ardévol, 2012). In addition, GSPE modulates the mitochondrial function, increasing their oxidative capacity in muscle (Pajuelo *et al.*, 2011), white adipose tissue (WAT) (Pajuelo *et al.*, 2011) and brown adipose tissue (BAT) (Pajuelo *et al.*, 2012) in rats.

Over recent years, great effort has been made to determine flavanol and procyanidins with a low grade of polymerization bioavailability, confirming dimer and trimer absorption (Baba, Osakabe, Natsume & Terao, 2002; Holt *et al.*, 2002; Serra *et al.*, 2010; Zhu *et al.*, 2002). Furthermore, the plasma kinetics of parental molecules and metabolites have been defined (Natsume *et al.*, 2003; Okushio, Suzuki, Matsumoto, Nanjo & Hara, 1999). However, less is known about which metabolites reach specific cells and the capacity of organs to accumulate flavanols and/or their metabolites. Previous experiments have demonstrated a tissue distribution of flavanol metabolites throughout practically all the body, even crossing the blood-brain barrier, after an acute intake of a rich-flavanol extract (Serra, Macià, Romero, Anglès, Morelló & Motilva, 2011) and a similar profile of tissue disposition was detected after an acute intake of a dietary dose of flavanols and proanthocyanidin with a low grade of polymerization, using cocoa cream as a source of polyphenols (Serra *et al.*, 2012). High levels of flavanol metabolites and phenolic acids were detected in tissues, such as the heart, lung and liver. However, the information derived from an acute intake experiment is not sufficient to evaluate correctly the tissue accumulation of flavanols and proanthocyanidins with a low grade of polymerization and/or their metabolites in tissues.

To gain insight into the molecular mechanisms used by flavonoids to modify the lipid, glucose and energy metabolism, it is essential to determine which flavanol and/or metabolite reaches and accumulates in the liver and adipose tissues. As mentioned above, the concentrations of flavonoid metabolites in the organs have been measured with acute and high doses of flavonols (Serra, Macià, Romero, Anglès, Morelló &

Motilva, 2011; Serra *et al.*, 2012). Thus, the aim of this study was to determine flavanol metabolites in rat liver, muscle and adipose tissues after a chronic consumption of lower doses of GSPE than previously studied, this being a situation closer to those of the real human consumption of proanthocyanidins. The experiment was done with three doses of GSPE (5, 25 or 50 mg /kg rat body weight) for 21 days in order to determine if there is a dose-response metabolite accumulation.

2 MATERIAL AND METHODS

Chemicals and Reagent

The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained monomeric (21.3 %), dimeric (17.4 %), trimeric (16.3 %), tetrameric (13.3 %) and oligomeric (5–13 units, 31.7 %) proanthocyanidins.

Internal standard (IS) catechol, and the standards of (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, (−)-epigallocatechin-3-O-gallate were purchased from Sigma Aldrich (St. Louis, MO, USA) and proanthocyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] from Fluka Co. (Buchs, 125 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& 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).

Treatment of animals and plasma and tissue collection

Twenty female Wistar rats weighing 150-175 g were purchased from Charles River (Barcelona, Spain). The animals were housed in animal quarters at 22° C with a 12-h light/dark cycle (light from 08:00 to 20:00) and were fed ad libitum with a standard chow diet (Panlab, Barcelona, Spain) and tap water. After 1 week of adaptation, the rats were trained to lick condensed milk (1 ml), which was used as a vehicle for administering GSPE, for an additional week. After this period, the animals were randomly divided into four groups (n = 5). Each group was treated with 5, 25 or 50 mg of GSPE /kg body weight/day dispersed in condensed milk. GSPE was administered every day at 09.00 hours for 21 days. On day 21, five hours after the GSPE treatment, the rats were anaesthetized with ketamine/xylazine and killed by exsanguination from the abdominal aorta using syringes, with heparin as the anticoagulant. Plasma was obtained by centrifugation and stored at -80° C until analysis. Liver, muscle, brown adipose tissue and mesenteric and perirenal white adipose tissue were excised and frozen immediately in liquid nitrogen and stored at -80° C until the analysis of the phenolic metabolites. All experimental procedures were performed according to the current national and institutional guidelines for animal care and in place at Universitat Rovira i Virgili. The Animal Ethics Committee of the Universitat Rovira i Virgili approved all the procedures.

Extraction of flavanols and low grade of polymerization proanthocyanidins from plasma and tissues

The method used to extract flavanols and

proanthocyanidins with a low grade of polymerization and their metabolites from the plasma and tissues was based on the methodologies described in our previous papers (Martí *et al.*, 2010; Serra, Macià, Romero, Piñol & Motilva, 2011). In order to clean-up the biological matrix and preconcentrate the phenolic compounds, the plasma samples were pretreated by microelution solid-phase extraction (μSPE), and the rat tissue samples were pretreated by a combination of a liquid-solid extraction (LSE) and μSPE. Briefly, the extraction was done with 60 mg of freeze-dried tissue to which 50 μL of ascorbic acid 1%, 50 μL of catechol 20 mg/L (dissolved in phosphoric acid 4%) as an internal standard and 100 μL of phosphoric acid 4% were added. The sample was extracted four times with 400 μL of water/methanol/phosphoric acid 4% (94/4/1, v/v/v). 400 μL of extraction solution was added to each extraction. The sample was sonicated during 30 s maintaining it in a freeze water bath to avoid heating, and it was then centrifuged for 15 min, at 14,000 rpm at 20º C. The supernatants were collected, and then the extracts were treated with μSPE before the chromatographic analysis of the flavanols and proanthocyanidins with a low grade of polymerization and their metabolites. OASIS HLB μElution Plates 30 μm (Waters, Milford, MA, USA) were used. Briefly, these 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 tissue extract or plasma, 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. Then, the retained molecules (flavanols and low grade of polymerization proanthocyanidins and their metabolites) were eluted with 2×50 μL of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solution was directly injected into the chromatographic system, and the sample volume was 2.5 μL.

Analysis of flavanols and pranthocyanidins with a low grade of polymerization and their metabolites by UPLC-ESI-MS/MS

The flavanols and proanthocyanidins with a low grade of polymerization were analysed by Acquity Ultra-Performance-liquid chromatography from Waters (Milford MA, USA) and tandem MS, as reported in our previous studies (Martí *et al.*, 2010, Serra *et al.*, 2009). Briefly, the column was Acquity high strength silica (HSS) T3 (100 mm × 2.1 mm i.d., 1.8 μm particle size) with 100% silica particles, from Waters (Milford MA, USA). The mobile phase was 0.2% acetic acid as eluent A, and acetonitrile as eluent B. The flow-rate was 0.4 ml min^{-1} and the analysis time, 12.5 min. 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 electrospray ionization (ESI). The flavanols and proanthocyanidins with a low grade of polymerization and their metabolites were analyzed in the negative ion mode and the data was acquired through selected reaction monitoring (SRM).

Two SRM transitions were studied for each analyte, the most sensitive transition being selected for quantification and a

second one for confirmation purposes (Additional Information). The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software. (+)-Catechin, (−)-epicatechin and dimer B2 [epicatechin-(4β-8)- epicatechin], were quantified using the calibration curves of the respective standards. The flavanol metabolites were identified tentatively using the calibration curve of the respective monomeric flavanol.

Statistical analysis

The data were analyzed by ANOVA to assess the significant differences between the administered chronic doses for each metabolite in each tissue and between tissues. All statistical analysis was carried out using JMP 8.

3 RESULTS

After a regular 21-day consumption of GSPE assaying different doses (5, 25 and 50 mg/kg of body weight), several flavanol metabolites were identified in the plasma (Fig. 1). Glucuronidated conjugates followed by methyl glucuronidated conjugates were the main flavanol metabolites quantified in the plasma and methyl catechin sulphate was quantified at lower concentrations. In contrast, methyl epicatechin sulphate was only quantified at trace level (0.02

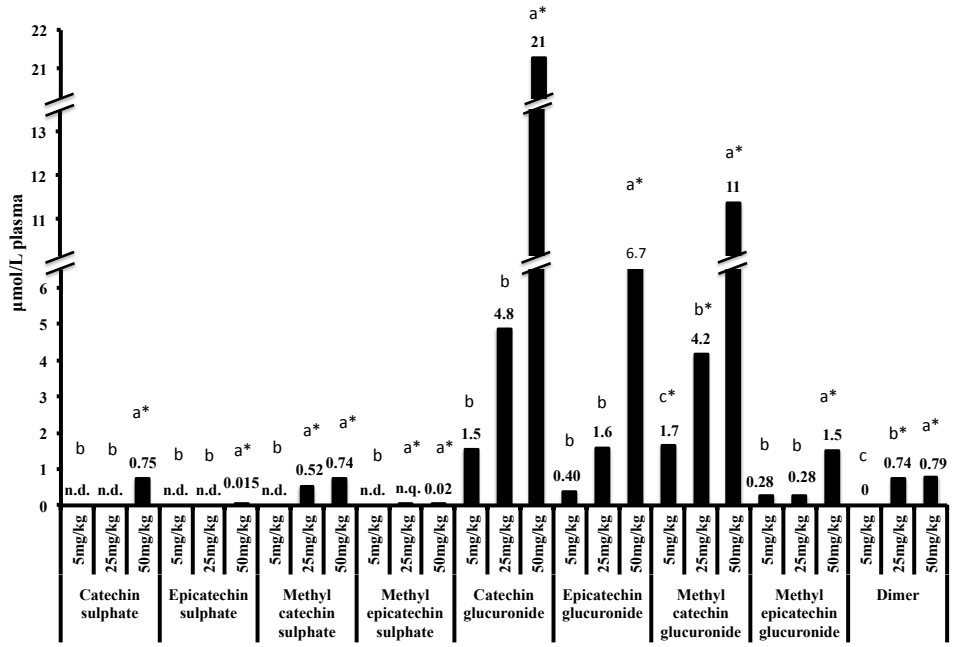

Figure 1. Flavanol metabolite and procyanidin with a low grade of polymerization concentrations quantified in plasma after a 21-day chronic intake of GSPE at different doses (5, 25 and 50 mg/kg body weight). The results are expressed as µM. . [ab] Different letters within the same metabolite concentration indicate a significant difference between doses ($p < 0.05$). *Indicates a significant difference between the tested dose and the control.

µmol/L plasma) after the higher tested chronic dose (50 mg/kg). Additionally, dimer was also quantified in the plasma samples. Significant differences ($p<0.05$) were observed on the flavanol plasmatic concentrations between doses. For the plasmatic glucuronidated forms, significant differences were only detected with the highest tested dose ($C_{Cat\_50\ mg/kg}$ 21 µmol/L plasma and $C_{Epi\_50\ mg/kg}$ 11 µmol/L, for catechin and epicatechin, respectivelly), although glucuronidated forms of catechin and epicatechin were quantified in the plasma 21 days after all the tested chronic intakes. Similar behavior, with significant differences at 50 mgkg, was observed for methyl epicatechin glucuronide. On the other hand, dimer was quantified at similar concentrations (approximately 0.75 µmol/L plasma) after the 25 and 50 mg/kg chronic doses. However, dimer was not detected after the 5 mg/kg dose. Methyl catechin sulphate was quantified at lower concentration in the plasma than the glucuronide and methyl glucuronide conjugates after the 25 and 50 mg/kg chronic doses, and catechin sulphate was only quantified with the 50 mg/kg dose.

Related to the accumulation of flavanol metabolites in the tissues, as Fig. 2 displays, the liver and muscle showed a direct relation between the accumulation and the administered doses of the GSPE extract for all the quantified flavanol metabolites. No flavanol metabolites were quantified in the liver after the lower chronic dose (5 mg/kg). Besides, high concentration levels were detected with 25 mg/kg and 50 mg/kg. The methyl glucuronide conjugates were the most abundant flavanol metabolites quantified in the liver. The glucuronidated conjugates were the most abundant metabolites detected in the muscle, followed by the methyl glucuronidated conjugates. As regards the adipose tissues (Fig. 3), a significant dose-dependent accumulation was observed in the brown adipose tissue for the total flavanol metabolites. Significant differences were detected for all the quantified metabolites 21 days after the 50 mg/kg dose. However, no significant differences were detected in the brown adipose tissue between 5 mg/kg and 25 mg/kg. Glucuronidated and methyl glucuronidated conjugates of catechin and epicatechin were determined as phase II metabolites in the brown adipose tissue. On the other hand, in the mesenteric and perirenal adipose tissue, no dose dependent accumulation was observed. Also glucuronidated and methyl glucuronidated conjugates of catechin and epicatechin were quantified in both adipose tissues. Nonetheless, with the 5-mg/kg dose, practically no metabolites were quantified and significant differences for epicatechin and catechin glucuronide were only detected in the mesenteric adipose tissue ($p<0.05$) between 25 mg/kg and 50 mg/kg were detected. Note that dimer was not detected in any of the studied tissues.

Table 1 allows the detection of significant differences between tissues for each metabolite differentiating between ingested doses of GSPE extract. For catechin glucuronide, with the dose 5 mg/kg, the muscle was the tissue with the greatest accumulation. In contrast, the tissue distribution was more homogeneous at higher doses, with no significant differences between the

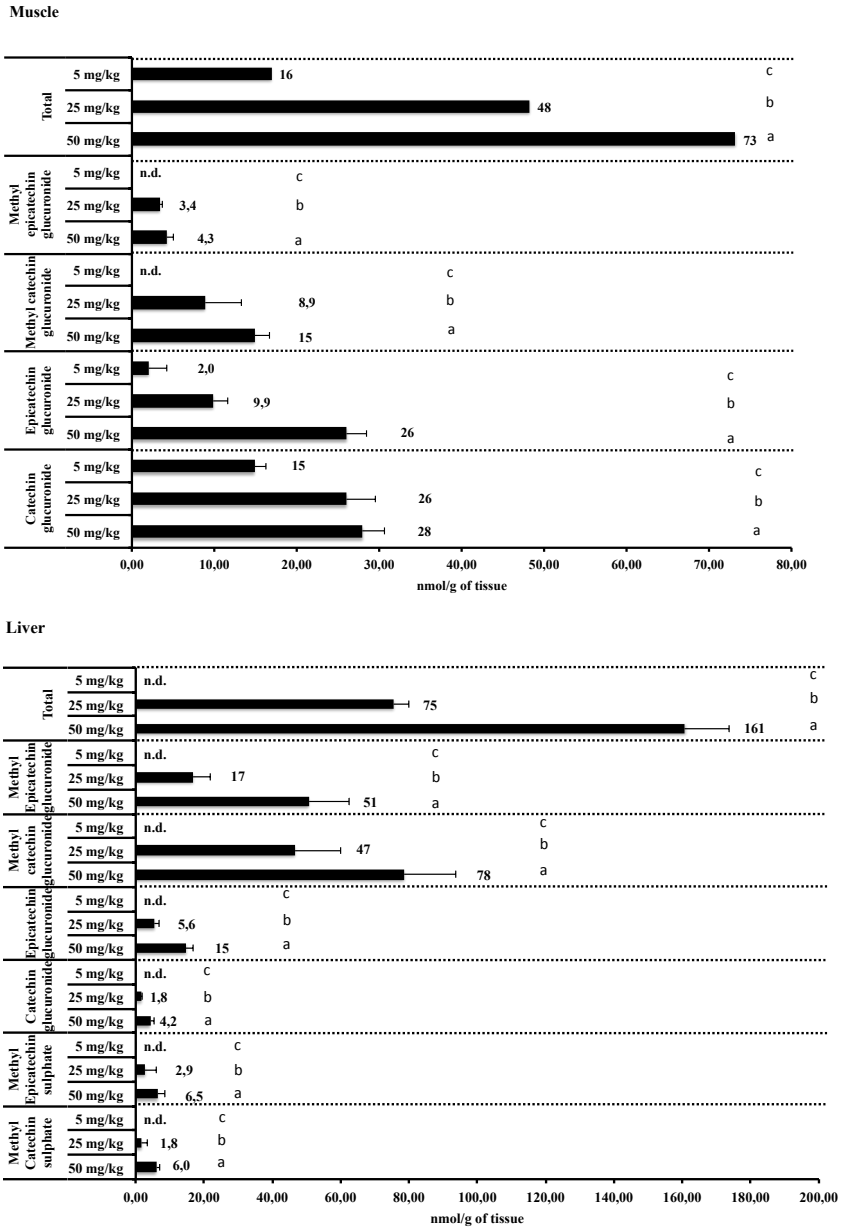

Figure 2. Flavanol metabolite concentrations quantified in muscle and liver after a 21-day chronic intake of GSPE at different doses (5, 25 and 50 mg/kg body weight). The results are expressed as nmol/g of tissue. [ab] Different letters within the same metabolite concentration indicate a significant difference between doses ($p < 0.05$).

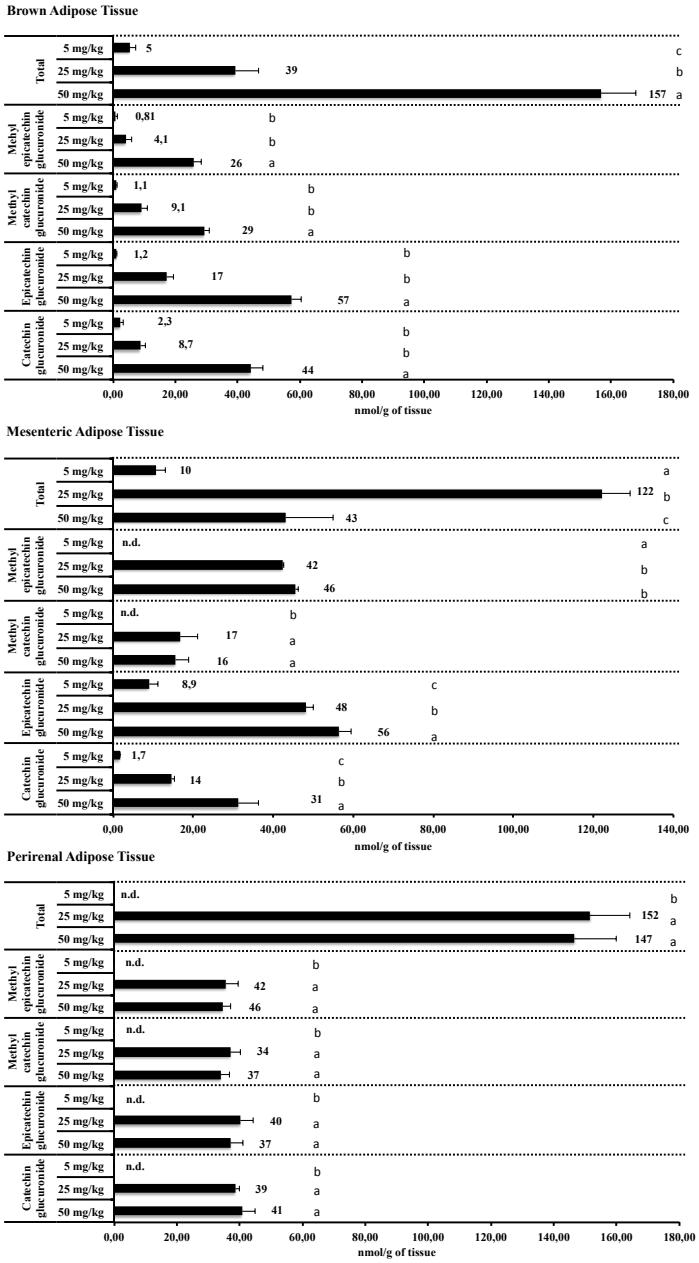

Figure 3. Flavanol metabolite concentrations quantified in white adipose tissues (mesenteric and perirenal) and brown adipose tissue after a 21-day chronic intake of GSPE at different doses (5, 25 and 50 mg/kg body weight). The results are expressed as nmol/g of tissue. [ab] Different letters within the same metabolite concentration indicate a significant difference between doses ($p < 0.05$).

Table 1. Flavanol metabolite concentration in liver, muscle, Brown adipose tissue and White adipose tissues (mesenteric and perirenal) after a 21-chronic intake of GSPE at different doses (5, 25 and 50 mg/kg body weight). Results are expressed as nmol/g of tissues. [ab] Different letters within the same column indicate a significant difference between tissues. Significantion level (p) is included for each metabolite and dose.

	Ingested Dose		
Tissue	50mg/kg	25mg/kg	5mg/kg
	Catechin glucuronide		
Liver	4,2 ± 1,1 [b]	1,8 ± 0,07 [c]	n.d. [b]
Muscle	27 ± 2,6 [a]	26 ± 3,5 [ab]	15 ± 1,4 [a]
Brown Adipose Tissue	44 ± 3,9 [a]	8,7 ± 1,6 [cb]	2,3 ± 0,98 [b]
Mesenteric Adipose Tissue	31 ± 4,9 [a]	15 ± 0,75 [cb]	1,7 ± 0,00 [b]
Perirenal Adipose Tissue	41 ± 4,2 [a]	39 ± 1,3 [a]	n.d. [b]
Signification Level (p)	0,0000	0,0000	0,0000
	Methyl catechin glucuronide		
Liver	78 ± 15,2 [a]	47 ± 13 [a]	n.d. [b]
Muscle	15 ± 1,2 [b]	8,9 ± 4,4 [b]	n.d. [b]
Brown Adipose Tissue	29 ± 1,7 [b]	9,1 ± 1,9 [b]	1,1 ± 0,22 [a]
Mesenteric Adipose Tissue	16 ± 3,2 [b]	17 ± 4,3 [ab]	n.d. [b]
Perirenal Adipose Tissue	34 ± 2,7 [b]	37 ± 3,2 [ab]	n.d. [b]
Signification Level (p)	0,0000	0,0048	0,0000
	Methyl catechin sulphate		
Liver	6,0 ± 0,98 [a]	1,8 ± 1,6	n.d.
Muscle	n.d. [b]	n.d.	n.d.
Brown Adipose Tissue	n.d. [b]	n.d.	n.d.
Mesenteric Adipose Tissue	n.d. [b]	n.d.	n.d.
Perirenal Adipose Tissue	n.d. [b]	n.d.	n.d.
Signification Level (p)	0,0000	0,0507	-
	Epicatechin glucuronide		
Liver	15 ± 2,1 [b]	5,6 ± 1,3 [c]	n.d. [c]
Muscle	26 ± 2,4 [b]	9,9 ± 1,7 [bc]	2,0 ± 0,1 [b]
Brown Adipose Tissue	57 ± 3,1 [a]	17 ± 2,2 [b]	1,2 ± 0,01 [bc]
Mesenteric Adipose Tissue	56 ± 2,9 [a]	48 ± 1,7 [a]	8,9 ± 2,3 [a]
Perirenal Adipose Tissue	37 ± 3,8 [ab]	40 ± 4,2 [a]	n.d. [c]
Signification Level (p)	0,0000	0,0000	0,0000
	Methyl epicatechin glucuronide		
Liver	51 ± 11 [a]	17 ± 4,9 [b]	n.d. [b]
Muscle	4,3 ± 0,7 [c]	3,4 ± 0,3 [c]	n.d. [b]
Brown Adipose Tissue	26 ± 2,4 [b]	4,1 ± 1,8 [c]	0,81 ± 0,7 [a]
Mesenteric Adipose Tissue	46 ± 0,69 [a]	42 ± 0,34 [a]	n.d. [b]
Perirenal Adipose Tissue	35 ± 2,7 [ab]	36 ± 3,9 [a]	n.d. [b]
Signification Level (p)	0,0000	0,0000	0,0001
	Methyl epicatechin sulphate		
Liver	6,5 ± 1,2 [a]	2,9 ± 0,15	n.d.
Muscle	n.d. [b]	n.d.	n.d.
Brown Adipose Tissue	n.d. [b]	n.d.	n.d.
Mesenteric Adipose Tissue	n.d. [b]	n.d.	n.d.
Perirenal Adipose Tissue	n.d. [b]	n.d.	n.d.
Signification Level (p)	0,0000	0,0735	-

muscle, brown adipose tissue, mesenteric adipose tissue and perirenal adipose tissue. The opposite situation was observed for methyl catechin glucuronide. Initially, at a low dose (5 mg/lg), it was mainly accumulated in the brown adipose tissue. Nevertheless, with increasing doses, the accumulation was concentrated in the liver. Regarding the methyl sulphated conjugates, practically no tissue accumulation was detected. A low accumulation of metabolites after the treatment with doses of GSPE of 25 and 50 mg/kg was only observed in the liver. The epicatechin glucuronidated conjugate was accumulated mainly in the mesenteric adipose tissue during the 5-mg/kg dose. With 25 mg/kg and 50 mg/kg the accumulation was concentrated in the three adipose tissues (brown, perirenal and mesenteric) with no significant difference between them. Methyl epicatechin glucuronide, at the 5-mg/kg dose was only accumulated in the mesenteric adipose tissue. Nonetheless, at higher doses of the GSPE extract (25 mg/kg and 50 mg/kg), the mesenteric and perirenal adipose tissues showed the highest accumulations, followed by the liver.

4 DISCUSSION

The liver, muscle and adipose tissues are crucial in the homeostasis of triglycerides and glucose. Moreover, obesity depends on fat accumulation in white adipose tissue and of energy wasted in brown adipose tissue (Zamora-Ros *et al.*, 2010). Therefore, in order to understand the role of flavanols improving hypertriglyceridemia (Quesada *et al.*, 2011; Quesada *et al.*, 2009; Ruzaidi, Amin, Nawalyah, Hamid & Faizul, 2005; Ruzaidi, Abbe, Amin, Nawalyah & Muhajir, 2008), hyperglucemia (Caimi, Carollo & Lo Presti, 2003; Ceriello *et al.*, 2001; Grassi *et al.*, 2008; Landrault *et al.*, 2003; Maritim, Dene, Sanders & Watkins III, 2003) and obesity (Basu *et al.*, 2010), it is essential to known if the flavanols reach these organs and which metabolites could be responsible for their effects. The present study shows the plasmatic bioavailability and tissue accumulation of the flavanol metabolites in the white adipose tissues (mesenteric and perirenal), brown adipose tissue, muscle and liver after a regular 21-day intake of GSPE. The results obtained show that each of the organs studied has a specific pattern of metabolite accumulation and response to the assayed GSPE doses.

The presence of several catechin and epicatechin metabolites in the plasma reinforces the fact that procyanidins are intensely metabolized by the small intestine and liver. Plasmatic catechin conjugates were more abundant than epicatechin conjugates, a fact directly relatable to the proportion of catechin and epicatechin in the GSPE (6.3 ± 0.54 μmol/g of GSPE and 2.4 ± 0.13 μmol/g of GSPE for catechin and epicatechin respectively, data shown in a previous work (Serra *et al.*, 2010)). Glucuronidated conjugates, followed by methyl glucuronidated conjugates, were the main flavanol metabolites detected in the plasma and this agrees with the existent literature (Abd El Mohsen *et al.*, 2002; Harada *et al.*, 1999; Ottaviani, Momma, Kuhnle, Keen & Schroeter, 2012; Serra, Macià, Romero, Anglès, Morelló & Motilva, 2011; Tsang *et al.*, 2005).

Previous studies have shown a high number of phase II metabolites in the liver after a chronic intake of catechin (Urpi-Sarda *et al.*, 2010). By contrast, after an acute intake of a proanthocyanidin-rich extract, these phenolic metabolites were not detected in the liver (Serra, Macià, Romero, Anglès, Morelló & Motilva, 2011). However, the results of the present study show that the sulphate conjugates of catechin and epicatechin were only deposited in the liver, showing a dose response accumulation and indicating a sulphation capacity of rat hepatocytes. Nonetheless, these sulphated conjugates were not detected in the liver after the treatment with 5 mg GSPE/kg body weight. Moreover, their presence in the plasma at low concentrations could reflect a possible sulphotransferase activity of the platelets, as was observed by Anderson *et al.* (Anderson, Garcia, Liebentritt & Kay, 1991) incubating hydroxytyrosol labeled, a phenyl alcohol typical of virgin olive oil, in whole blood.

Glucuronide conjugates were detected in all the organs studied, but their distribution and concentration may depend on methylation. Non-methylated glucuronide derivatives were mainly accumulated in the brown adipose tissue and in the two white adipose depots studied, the mesenteric and perirenal. On the other hand, methyl catechin glucuronide was accumulated mainly in the liver, whereas methyl epicatechin glucuronide was accumulated mainly in the brown and white adipose tissues. It is important to highlight that the accumulation of glucuronide derivatives in the white adipose depots was not dose responsive. In both the white adipose tissues studied (perirenal and mesenteric), the levels of glucuronide derivatives were similar, at 25 and 50 mg of GSPE/kg body weight. Moreover, the concentration of some of these metabolites was even higher than in the other tissues studied. All this together suggested that visceral white adipose tissue could be a store for flavanol metabolites in the body. Nonetheless, further studies are needed to understand if flavanols accumulate in adipocytes or other cell types in the adipose tissue.

Brown adipose tissue could represent a very important target for flavanol metabolites, due to, for example, their improvement of the mitochondrial function related to the energy homeostasis of the brown adipose tissue (Pajuelo *et al.*, 2011). This tissue is the only tissue with detectable levels of all the glucuronidated derivatives at the lowest GSPE dose (5 mg /kg body weight). Moreover, the brown adipose tissue showed a dose response in the concentration of all the conjugate-metabolites of catechin and epicatechin. Moreover, some of these metabolites reached high concentrations, probably related to the high irrigation of this type of adipose tissue by blood vessels (Ravussin & Galgani, 2011). These results, together with the possible enhancement of the thermogenic capacity and the improvement of the mitochondrial function exerted by a chronic supplementation of proanthocyanidins in brown adipose tissue (Pajuelo *et al.*, 2011; Pajuelo *et al.*, 2012), suggest that proanthocyanidins may play an important role in reducing or preventing obesity by modulating the functionality of the brown adipose tissue at low doses.

The dose-dependent disposition of the phase II metabolites of the procyanidins detected in the muscle may also be related to an improvement in the mitochondrial function in skeletal muscle detected in a previous experiments (Pajuelo *et al.*, 2012), suggesting an improvement in the activity of enzymes involved in oxidation and metabolism of pyruvate, and a shift in priority to the glycosidic metabolism rather than lipid metabolism.

5 CONCLUSIONS

In this investigation, after a chronic intake of flavanol-rich extract at different doses, the plasmatic bioavailability and the distribution and accumulation in the adipose tissues, muscle and liver of flavanols and their metabolites were studied. Each of the studied organs has a specific behavior of accumulation and response to the assayed GSPE doses, with a clear dose response in the brown adipose tissue, in which the flavanols could play an important role in reducing or preventing obesity by modulating the functionality of that tissue. The results of this experiment could be useful for future in-vitro research with adipose cell cultures giving information about the physiological concentrations reached in specific adipose tissues after a chronic intake of flavanols.

6 ACKNOWLEDGMENTS

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Fetal programming on hepatic glucuronidation

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THE FETAL PROGRAMMING OF DIETARY FRUCTOSE AND SATURATED FAT ON HEPATIC QUERCETIN GLUCURONIDATION IN RATS

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Abstract

Objective: Phase II biotransformation of flavonoids generates bioactive metabolites *in vivo*. However, data on the effect of environmental and physiological factors and fetal programming on phase II pathways toward flavonoids are limited. We examined the effect of parental exposure to a diet high in saturated fats and fructose diet 1 mo before conception through lactation on *in vitro* hepatic UDP-glucuronosyltransferase (UGT) activity toward quercetin in both parent (F0) and offspring (F1) rats and comparing between sexes. *Methods:* Parents were fed a diet containing 9.9% coconut fat, 0.5% cholesterol, 30% fructose, and 30% glucose (SFF) or a control (C) diet containing 11% corn oil and 60% glucose. After weaning, offspring were fed the C diet for an additional 12 wk. Glucuronidation rate of microsomal UGT was determined with 30 μM quercetin and 12.5 μg protein in a total volume of 100 μL after a 15-min incubation at 37°C. Three quercetin glucuronides (7-OH, 3'-OH, and 4'-OH) were quantified. *Results:* In the F0 females, the SFF diet decreased by 29 and 19% the production rate of 3'- and 4'-OH quercetin glucuronides, respectively, as compared to the C diet ($P \leq 0.05$). Production rate of 7-OH quercetin glucuronide in the female F1 rats born to C dams was 59% larger than in their male counterparts ($P < 0.05$) but no difference was observed in the offspring of SFF dams. *Conclusion:* High dietary fructose and saturated fat decreased UGT capacity toward quercetin in female rats and in utero exposure to the diet decreased the glucuronidation capacity of their pups.

Keywords: Quercetin / rat / UDP-glucuronosyltransferase / fetal programming / fructose / saturated fats

1 INTRODUCTION

Flavonoids are ubiquitous in plant foods and products derived from them, e.g., fruits, vegetables, tea, and red wine, and in many observational studies their intake is inversely associated with the risk of chronic diseases, e.g., certain cancers, cardiovascular disease, and neurodegenerative disorders [1], [2], [3], and [4]. While bioavailability, metabolism, and bioactions of flavonoids have been partly characterized [5] and [6], these results reveal a marked inter-individual variation [7], likely due to a combination of environmental, physiological, epigenetic, and genetic factors. As the majority of consumed flavonoids are extensively transformed by phase II metabolic pathways, e.g., glucuronidation, sulphation, and methylation, that facilitate their elimination [8], the variation in the capacity of phase II enzymes toward flavonoids may be partially responsible for this inter-individual variation in bioavailability.

The capacity of phase II metabolism toward flavonoids is subject to the influence of environmental and physiological factors and polymorphisms. We have previously demonstrated that age and sex affect hepatic and intestinal UDP-glucuronyl-transferase (UGT) activity toward flavonoids [9] and [10]. Phytochemicals, including carotenoids, polyphenols (including flavonoids), indoles, and allyl sulphides have also been shown modulate the activities of phase II enzymes [11]. While diabetes and obesity as well as dietary protein and energy have been reported to modulate liver drug-metabolizing phase I enzyme composition [12] and [13], relatively little information is available regarding the impact of nutrition status, lifestyle, pathological conditions on phase II metabolism [14]. Thus, a greater characterization is warranted of the factors that modulate the capacity of phase II metabolism toward flavonoids to help us understand their mechanisms of action in promoting health.

A growing body of evidence indicates that fetal and neonatal exposure to poor nutrition and environment can modulate metabolic homeostasis and predispose individuals to an increased risk of diseases later in life through epigenetic mechanisms [15]. To our knowledge, there have been no investigations on the impact of nutrient exposure in utero or during early postnatal life on the capacity of phase II metabolism. However, Guillemette *et al.* [16] suggested that epigenetic modifications such as DNA methylation and histone acetylation might regulate UGT gene expression.

The typical Western diet, high in saturated fats and sugars, appears to modify fetal programming and lead to a predisposition for the risk of obesity and metabolic abnormalities when the offspring reach adulthood [17]. Since the ontogenetic development of hepatic UGT occurs in early life [18], we investigated in rats the fetal programming effect of a diet high in saturated fats and fructose on hepatic UGT activity toward quercetin, the most abundant dietary flavonol. We also examined the impact of this diet on hepatic UGT activity in breeding pairs of rats. In both generations the comparison between sexes on the hepatic UGT activity toward quercetin was investigated.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Thermo Fisher Scientific, Rockford, IL). Quercetin dihydrate, UDP-glucuronic acid, alamethicin, and all other chemical and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 Animals and Diets

The detailed information about the diet can be found in our previous report [19]. Briefly, two diets, control (C) and saturated fat+fructose (SFF), were prepared using amino acid defined ingredients and manufactured by Dyets Inc. (Bethlehem, PA, USA). The main difference between two diets were 60% glucose in the C vs. 30% glucose and 30% fructose in the SFF and 11% corn oil in the C vs. 9.9% coconut fat and 0.5% cholesterol in the SFF.

Proven male and female Sprague Dawley breeders (8 M, 8 F) were obtained from Charles River Laboratories (Wilmington, MA). After arrival, female and male rats were assigned randomly to one of the two diets (Fig. 1). All rats were single housed with a 12:12 h light:dark cycle and fed *ad libitum* for 4 wk. Subsequently, each female was mated with a male in the same dietary group. During the pregnancy and lactation, dams remained on their designated diet. Litter size was adjusted 3-d postpartum to 8–10 to ensure that each pup received comparable nutrition. At weaning (d 21), 5 male and female offspring from each group were fed with the C diet for an additional 12 wk. At weaning, parental rats (F0 generation) were killed with terminal exsanguinations under isoflurane (Aerrane™) anesthesia. At the age of 15 wk, all offspring (F1 generation) were

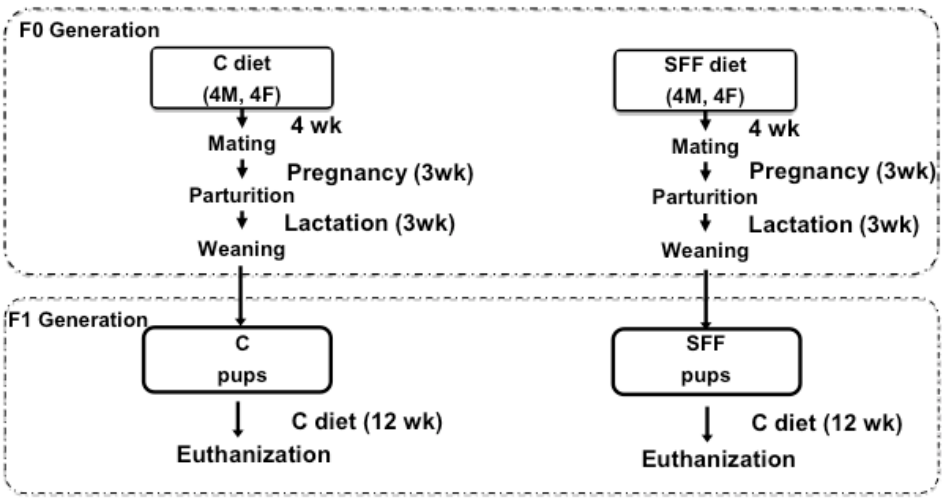


Figure 1. Study design. Rats in the F0 generation were killed at weaning and in the F1 generation killed at age of 15 wk. Abbreviation for diets: C, control; F + SFA, fructose and saturated fats.

killed by the same protocol. Livers were harvested and stored at -80°C until collection of hepatic microsomes. The protocol was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

2.3 Preparation of liver microsomes

Hepatic microsomal fractions were prepared according to Chen *et al.* [9]. Briefly, whole liver was pulverized in liquid nitrogen and stored at -80°C until homogenization in ice-cold sucrose homogenization buffer using SDT-1810 Tekman Tissumizer (Cincinnati, OH) at 85% power for 2 min. Microsomes were collected after centrifugation at 1000 x g for 15 min at room temperature, 10,000 x g for 20 min at 4°C, and then at 100,000 x g for 60 min at 4°C. The resulting microsomal fraction was suspended in 400 µL of potassium phosphate buffer (0.1 mol/L, pH 7.5, containing 20% glycerol). Microsomal protein content was determined with a Pierce BCA kit (Thermo Fisher Scientific, Boston, MA) and adjusted to 5 mg/mL with phosphate buffer (0.1 mol/L, pH 7.5). The microsomal suspension was aliquoted and stored at -80°C until use.

2.4 Glucuronidation Assay

The glucuronidation assay was performed according to Bolling *et al.* [9]. Briefly, microsomal glucuronidation of quercetin (Q) was initiated with an incubation of microsomal protein and alamethicin at 37°C for 15 min in microcentrifuge tubes containing 30 µmol/L quercetin (final concentration). The quercetin concentration was selected based on our previous study [9] because the concentration was near the K_m value of hepatic microsomal UGT in rats. A cofactor solution of UDP-glucuronic acid was added to initiate the reaction in a final assay volume of 100 µL. After incubation for selected duration ranging from 0 to 30 min at 37°C, the reaction was terminated with 100 µL ice-cold methanol containing 33 µmol/L daidzein as an internal standard. The supernatant was dried under nitrogen gas at room temperature and then reconstituted in 50% methanol for HPLC analysis. Experiments to determine the linearity of the glucuronidation reaction were performed with a pooled microsomal protein sample to establish the assayed condition of microsomal protein concentration (5-25 µg/100 µL) and enzymatic reaction time (5-30 min).

2.5 Determination of quercetin glucuronides

Quercetin glucuronides generated from microsomal glucuronidation were determined using HPLC, according to the method of Bolling *et al.* [9] and Boersma *et al.* [20]. Briefly, quercetin and its glucuronide metabolites were monitored at 370 nm after elution from a Phenomenex Synergi 10 µm Hydro-RP80 250x4.6 mm column (Torrance, CA). Three principal quercetin glucuronide metabolites were quantified, with the following retention times: 11.5 min for 7-O-quercetin glucuronide (7-OH), 14.3 min for 4′-O-quercetin glucuronide (4'-OH) and 14.8 min for 3′-O-quercetin glucuronide (3'-OH). After normalization with the internal standard, daidzein, glucuronide metabolites were quantified using a standard curve of quercetin established in the absence of UDPGA.

2.6 Statistical analysis

Results are expressed as mean ± SE. Natural logarithmic transformation of data was applied prior to statistical analysis, to normalize unequal variance. The effects of generation, diet, and sex on the Q production rates catalyzed by UGT were assessed using a 3-way ANOVA with interactions between 3 independent variables. Since there were significant sex and diet interactions, the effects of sex and diet and their interaction in F0 and all groups combined were assessed using a two-way ANOVA. When P values for the interaction between sex and diet were ≤0.05, post-hoc analysis was performed using Tukey's honestly significant difference test. The difference in production rate of Q metabolites between sexes in the F1 rats was tested using a *Student's t-test*, as well as between F0 and F1. Pearson's correlation test was performed to assess the association between production rates of Q metabolites. A P ≤0.05 was considered significant. All statistical analyses were performed using JMP IN (SAS Inc., Cary, NC).

3 RESULTS

The enzymatic linearity of microsomal UGT activity toward 30 μmol/L quercetin was established in triplicate using a pooled sample with protein concentrations ranging from 5 to 25 μg in a 100-μL assay volume and incubation time from 5 to 30 min (Fig. 2). The r2 value for both regression lines was >0.94. A decrease in the quercetin concentration was observed along with an increase in microsomal protein concentration or incubation time, as well as increases in concentrations of 3 quercetin metabolites. The assay condition of 12.5 μg protein/100 μL and 15 min that fell in the middle of the linear range of the regression curve was selected to test the effects of generation, diet, and sex on microsomal UGT activity toward 30 μmol/L quercetin.

Three quercetin glucuronide metabolites (7-, 3'-, and 4'-OH) were quantified. Hepatic UGT favored production of 7-OH, followed by 3'-OH. The effect of generation on UGT activity in production of 3'-OH and 4'-OH-quercetin glucuronides was independent of sex and diet (P ≤0.0001) (Table 1 and Fig. 3). There were significant interactions between sex and diet on 7-OH, 3'-OH, and total quercetin glucuronide metabolite (TQG) so 2-way ANOVA tests were performed to assess the relationship between sex and diet on the production rate of quercetin UGT metabolites. Figures 4, 5, and 6 show the results of the effect of sex and diet on the production rates of quercetin UGT metabolites in the all rats of both groups, F0 and F1 rats, respectively. In the combined generations, in which the global effect of a SFF diet in the context of an utero exposure and an inter-generation experiment was evaluated, the significant effect of diet on the production rate was dependent on sex and metabolite (Fig. 4). The P value of the sex-diet interaction for 7-OH and TQG produced in the 2-way ANOVA tests was 0.0012 and 0.0022, respectively. For 3'-OH and 4'-OH glucuronides, the interaction of diet and sex was not statistically significant. The production rate of 7-OH and TQG in C females was 63 and 33% larger than the C males, respectively (P ≤0.05) while no difference was found in the SFF rats. The diets did not affect production rate of quercetin glucuronides in males. The SFF

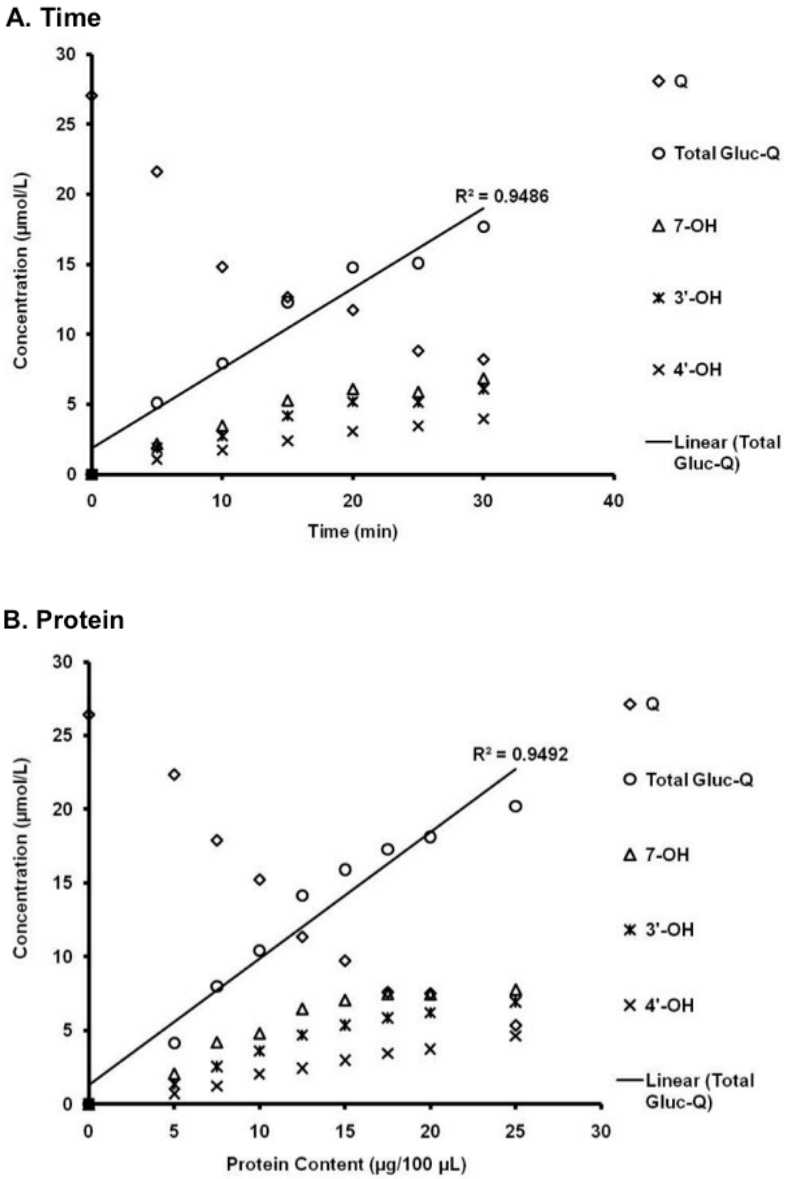


Figure 2. The linearity of UGT activity toward 30 µmol/L quercetin during incubation with microsomal protein concentration at 12.5 µg/100 µL (A) and microsomal protein content when incubation time was set at 15 min (B). The R^2 values are calculated from the regression lines of quercetin metabolites. The assay condition of 15 min incubation and 12.5 µg/100 µL microsomal protein was utilized in all experiments.

Table 1. P-value summary of 3-way ANOVA tests.

Source	7-OH	3'-OH	4'OH	TQG
Generation	0.753	≤0.0001	≤0.0001	0.030
Sex	0.240	0.834	0.801	0.313
Diet	0.055	0.083	0.210	0.053
Generation*sex	0.724	0.091	0.072	0.271
Generation*diet	0.630	0.884	0.651	0.860
Sex*diet	0.001	0.015	0.038	0.001
Generation*sex*diet	0.460	0.032	0.014	0.053

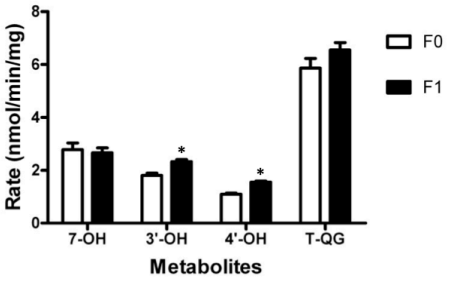


Figure 3. The UGT activity toward quercetin is generation dependent independently to the diet. The production rate for 3'- and 4'-OH-quercetin glucuronide was larger in F1 rats than in F0 rats. *Means of the same metabolite differ, P ≤0.0001, tested by a *Student's t-test.*

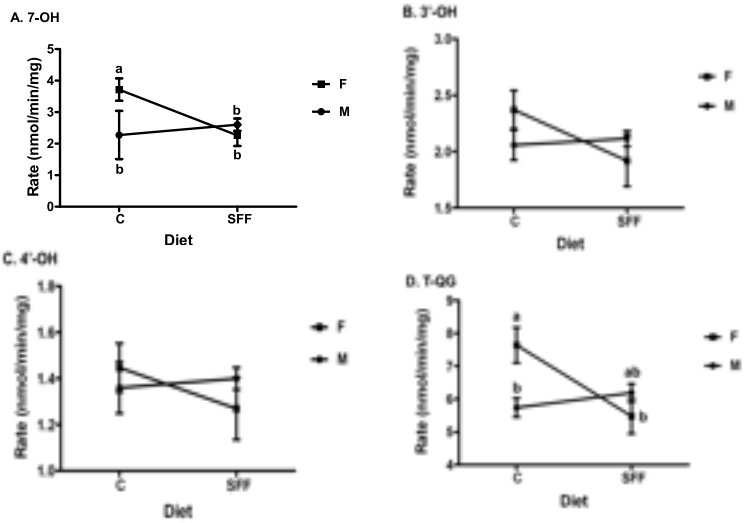


Figure 4. The dietary effect on hepatic UGT activity toward quercetin in all rats of both generations is sex and metabolite dependent, (A) 7-OH quercetin glucuronide (7-OH), (B) 3'-OH quercetin glucuronides (3'-OH), (C) 4'-OH quercetin glucuronides (4'-OH), (D) total quercetin metabolites (T-QG). [ab]Means with different letters in each panel differ, tested by a two-way ANOVA followed by Tukey Kramer HSD Multi-Comparison, P ≤0.05.

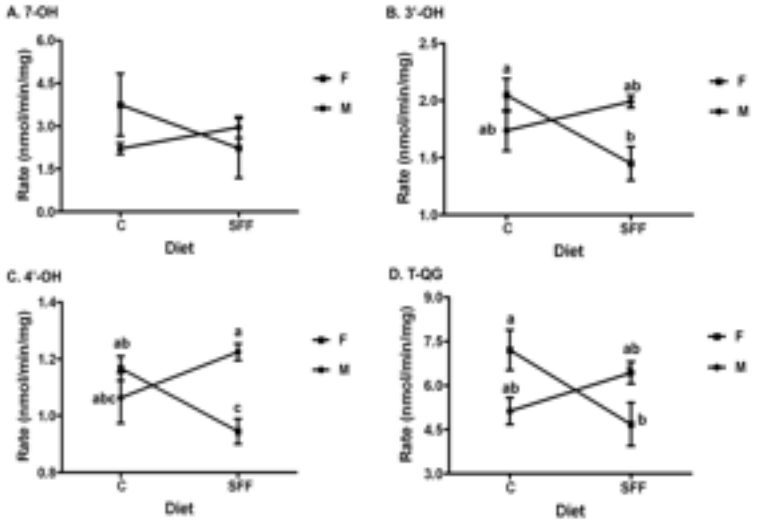
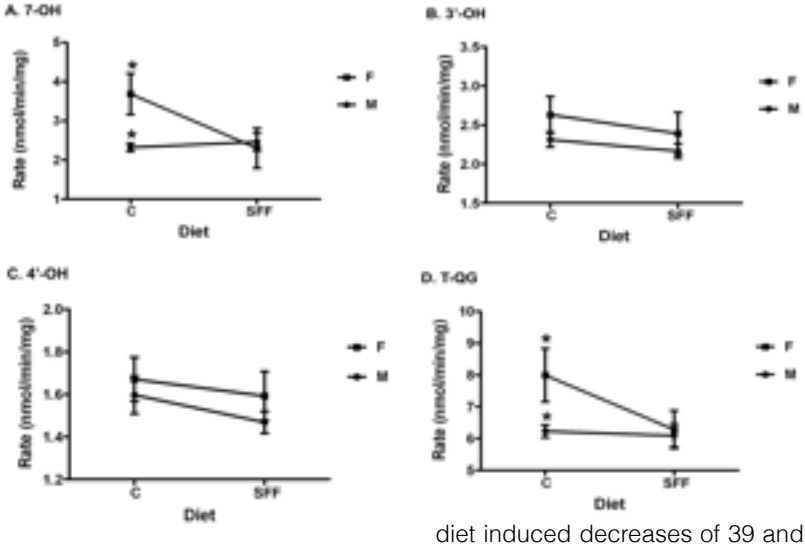


Figure 5. The dietary effect on hepatic UGT activity toward quercetin in F0 rats is sex and metabolite dependent, (A) 7-OH quercetin glucuronide (7-OH), (B) 3'-OH quercetin glucuronides (3'-OH), (C) 4'-OH quercetin glucuronides (4'-OH), (D) total quercetin metabolites (T-QG). [ab]Means with different letters in each panel differ, tested by a two-way ANOVA followed by Tukey Kramer HSD Multi-Comparison, $P \leq 0.05$.



diet induced decreases of 39 and 28% in

Figure 6. The effect of diets on hepatic UGT activity toward quercetin in the F1 rats is sex and metabolite dependent, (A) 7-OH quercetin glucuronide (7-OH), (B) 3'-OH quercetin glucuronides (3'-OH), (C) 4'-OH quercetin glucuronides (4'-OH), (D) total quercetin metabolites (T-QG). *Means with the mark in each panel differ, tested by a *Student's t-test*, $P \leq 0.05$.

the production rate of 7-OH and TQG in females compared to the C diet (P ≤0.05).

The magnitude of the diet and sex interaction in production rate of quercetin glucuronides were more marked in the F0 than in the F1 rats (Fig. 5 and 6). The effect of the SFF diet on hepatic UGT activity was dependent on sex and quercetin metabolite (Fig. 5) with P values (2-way ANOVA) for 7-OH, 3'-OH, 4'-OH, and TQG of 0.0194, 0.0095, 0.0051, and 0.0063, respectively. The production rate of quercetin metabolites was not different between male and female rats fed C diet. The SFF diet led to a 22% lower production rate of 4'-OH in females than in males, but not of the other metabolites. In female rats, SFF diet resulted in a 29, 19, and 35% decrease in the production rate of 3' -OH, 4'-OH, and TQG as compared to C diet (P ≤0.05).

In the F1 rats, the sex and parental diet interaction was significant for 7-OH and TQG (P=0.0398 and 0.016, respectively from 2-way ANOVA) (Fig. 6). Neither parental diets nor sex affected production rate of 3'- and 4'OH quercetin glucuronides. As statistical significant was not found with Tukey's multi-comparison test, a *Student's t-test* was performed to evaluate whether means within each dietary group were different. The production rate of 7-OH quercetin glucuronide and TQG in the female rats born to C dams was 59 and 28% larger than in their male counterparts (P=0.035 and ≤0.05, respectively). No such difference was observed in the offspring born to SFF dams.

A series of Pearson's correlation tests were performed to provide some insight into the impact of diet on UGT isoenzymes. The magnitude of correlation between production rates of quercetin metabolites: in all rats, TQG and 7-OH (r = 0.91, P ≤0.0001), TQG and 3'-OH (r = 0.83, P ≤0.0001), TQG and 4'-OH (r = 0.62, P ≤0.0001), 7-OH and 3'OH (r = 0.53, P = 0.0005), 3'-OH and 4'-OH (r = 0.89, P ≤0.0001); in the F0 rats, TQG and 7-OH (r = 0.96, P ≤0.0001), TQG and 3'-OH (r = 0.89, P ≤0.0001), TQG and 4'-OH (r = 0.68, P = 0.004), 7-OH and 3'OH (r = 0.73, P = 0.0011), 3'-OH and 4'-OH (r = 0.89, P ≤0.0001); and in the F1 rats, TQG and 7-OH (r = 0.95, P ≤0.0001), TQG and 3'-OH (r = 0.86, P ≤0.0001), TQG and 4'-OH (r = 0.77, P = 0.004), 7-OH and 3'-OH (r = 0.67, P = 0.0004), 3'-OH and 4'-OH (r = 0.86, P ≤0.0001), 7-OH and 4'-OH (r = 0.56, P = 0.0046).

4 DISCUSSION

Dietary polyphenols are subject to extensive phase II biotransformation, which make them more hydrophilic and readily excreted [5]. Most absorbed polyphenols are found in circulation and tissues predominately in conjugated forms - glucuronized, sulphated, methylated or as a combination of these forms, though some may be present in aglycone and/or their parent plant forms. Therefore, the variation in the capacity of phase II metabolism in humans may partly account for the markedly varied pharmacokinetics and bioefficacy of these nutrients [7]. Court [21] indicated that age, sex, enzyme inducers, and genetic polymorphisms have all been implicated as souces of variability in UGT capacity. We previously observed that the *in vitro* production rate of flavonoid metabolites catalyzed by hepatic microsomal UGT of F344 rats was

dependent on age, UGT isoenzymes, and flavonoid structure [9]. We have also observed that advanced age modulates *in vitro* production rate of quercetin glucuronides catalyzed by microsomal UGT present in the small intestine of F344 rats, with the degree and direction of changes dependent upon the intestinal segment examined [10]. The effects of drug, nutrient, or dietary pattern on phase II metabolism have been recognized [11] and [12]; e.g., Navarro *et al.* [22] noted that consumption of cruciferous vegetables was associated with a decrease in excretion of salicylic acid glucuronide. In addition, Iwuchukwu *et al.* [23] reported that resveratrol, curcumin, and chrysin induced UGT1A1 mRNA expression in Caco-2 cells. Our study provides another line of evidence that high dietary fructose and saturated fat may modulate UGT activity toward quercetin.

Quercetin is biotransformed extensively via glucuronidation, sulphation, and methylation. We previously characterized 8 quercetin phase II metabolites in liver of F344 rats fed a 0.45% quercetin (w/w) diet for 6 wk, and noted that 92% of quercetin detected in the liver were metabolites with 67.6% being conjugated with a glucuronide [24]. Consistent with the results of Boersma *et al.* [20], our *in vitro* microsomal UGT reaction generated 3 quercetin metabolites, with 7-OH being dominant. However, the production of quercetin glucuronides by UGT could vary as the capacity of phase II enzymes is subject to modulation of environmental and chemopreventive agents as well as and nutritional status in the context of the totality of genetic background [25]. Osabe *et al.* [14] reported that mRNA and protein of hepatic UGT1A1 and 1A6 increased in male rats fed a diet containing 10% lard and 60% surcose, but not in females, ascribing the increases to dietary modulation of constitutive androstane receptor and peroxisome proliferator-activated receptor-α. In contrast to the report of Osabet *et al.* [14], our study showed that a diet containing 9.9% coconut fat, 0.5% cholesterol, 30% fructose, and 30% glucose decreased *in vitro* hepatic UGT activity toward quercetin in the F0 female rats as compared to the control diet containing 11% corn oil and 60% glucose. Interestingly, we did not find this change in the F0 male rats. It is also worth noting that impact of diet appears dependent on the UGT isoenzyme as decreases were found only in the production rate of of 3'-OH and 4'-OH quercetin glucuronides. Based on the high correlation coefficients between these two metabolites shown, it is possible they were produced by overlapping UGT isoenzymes. The difference between these two studies may be due to the rodent species (Wistar vs. F344), methodology (protein and gene expression vs. enzyme activity), and diet (sucrose and lard [long-chain saturated fats] vs. fructose, glucose, and coconut oil [medium-chain saturated fats]). Further, since quercetin glucuronides were generated through the collective effort of all microsomal UGT isoenzymes, we were not able to identify a change in individual UGT isoenzymes. Boersma *et al*. [20] found that using human recombinant UGT, several UGT isoenzymes metabolized quercetin with different conversion rates, with UGT1A9, 1A1, 1A3, 1A8, and 2B7 being the most efficient.

Sexual dimorphism is apparent in rodent models of the biotransformation of endobiotics and xenobiotics through phase II pathway but equivocal in humans [21] and [26]. The divergence in UGT activity and protein and gene expression between sexes is substrate, species, tissue, and UGT isoenzyme dependent [27, [28], and [29]. For example, hepatic Ugt1a5, 1a8, 2b1, and 2b2 mRNA level in female rats are about 35, 130, 50, and 60% larger, respectively, than those in male rats [30]. Mazur *et al.* [31] noted that hepatic microsomal glucuronidation capacity toward bisphenol appeared to be larger in females than males, and Buckley and Klaassen [27] and Osabe *et al.* [14] found that female rodents expressed larger mRNA and protein of certain UGT isoenzymes. However, Dai *et al.* [32] found that overall *in vivo* acetaminophen glucuronidation was lower in female than in male mice. Regarding glucuronidation capacity in humans, Court [21] did not find a sex-dependent effect on *in vitro* transformation of drugs using 55 human livers. In this study, we found that there was a gender dependent effect on *in vitro* hepatic quercetin glucuronidation in adult F1 rats born to the control dams, as well as all rats of both generations in the control group. It is worth noting that such an effect of sex was only significant in the production of 7-OH quercetin glucuronide and total quercetin glucuronides, but not of 3'-OH and 4'-OH metabolites. As regioselectivity of UGT isoenzymes dictates what biotransformed products are produced [33], our results may be a consequence of the differential influence of sex on individual UGT isoenzymes. Thus, our results suggest the bioavailability and bioefficacy of quercetin or other polyphenols may be sex-dependent. Thus, further *in vivo* studies examining the pharmacokinetics of quercetin and its functional endpoints are warranted.

During the last decade, the potential impact of fetal programming on molecular and biochemical phenotypes and subsequent risk for chronic disease has become widely appreciated. However, little is known of the effect of fetal programming on developmental plasticity of detoxification mechanisms in later life. In an ontogeny study of hepatic UGT of inbred Gunn and Wistar rats, Bustamante *et al.* [34] detected the activity of UGT1A1 on day 22 of gestation found it reached its highest level at adult life. Thus, it is plausible that capacity of detoxification mechanisms such as UGT in adult life could be predetermined by dietary factors in early life. Using a guina pigs, Smith *et al.* [35] found that intermittent in utero exposure to morphine enhanced gene expression of hepatic UGT 2A3 by ~150% in 7-d-old female offspring compared to untreated controls. We show here that exposure to a high fat and fructose diet during gestation and lactation diminishes UGT activity toward quercetin in female adult offspring. This fetal programming effect elicited by the diet might be limited to certain UGT isoenzymes as suggested by the unchanged production rate of 3'- and 4'-OH quercetin glucuronides. As UGT activity is dependent on tissue type, isoenzyme, species, sexual hormones and substrate, our results help inform a new direction to our understanding of the underlying mechanisms for the marked inter-individual variation in polyphenol pharmacokinetics.

There are a few limitations in our study. First, only one condition was employed to assess hepatic microsomal UGT activity toward quercetin (30 μmol/L quercetin, 12.5 μg/100 μL microsomal protein, and 15 min incubation). The protein amount and incubation time were selected based on our linearity experiments. Quercetin concentration was selected based on our previous rat study [9] that the concentration was close to Km value of hepatic UGT. Thus, it is possible that our results are generalizable to other experimental conditions. Second, our results might not be readily applicable to *in vivo* pharmacokinetics and bioefficacy of quercetin and other flavonoids because of the complexity of quercetin biotransformation with its concurrent reactions of glucuronidation, sulphation, and methylation in the small intestine, liver, and kidney [24].

5 CONCLUSIONS

The biotransformation of polyphenols via phase II enzymes may contribute to the marked inter-individual variation in their pharmacokinetics. Our observation that a diet high in saturated fat and fructose diminishes *in vitro* UGT activity toward quercetin in female rats suggests a potential change in bioefficacy of quercetin because catechol moeity in the B ring plays an important role in its antioxidant capacity [36]. Fetal programming by specific dietary factors in utero can decrease the capacity of selected hepatic UGT isoenzymes toward quercetin in female adult.

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CONCLUDING REMARKS

The concluding remarks section intends to give an overview vision of the results obtained in each proposed objective of the thesis while comparing them with the recent literature. The study of the metabolism of phenolic compounds by *in vitro* and *in vivo* studies was focused on flavonoids (mainly procyanidins) and phenolic compounds of virgin olive oil. Nonetheless, several global conclusions have been obtained from the full set of experiments and these are summarized in that section, taking into account that the general basis of the phenolic metabolism are common for all the phenolic families.

In general, dietary phenolic compounds undergo several transformations during their passage through the body. Many studies have demonstrated their bioactivity by *in vitro* (Corona *et al.* 2009; Jaganath *et al.* 2009; Schaffer *et al.* 2007) and *in vivo* studies (Schaffer *et al.* 2007; Terra *et al.* 2011) but less is known about how phenolic compounds behave in the organism. Understanding the absorption, distribution and metabolism of phenolic compounds is crucial for the proper comprehension of their *in vivo* significance and bioactivities (Spencer *et al.* 2000). For this, several studies of polyphenol bioavailability were performed using a pure single molecule chemically synthesized or isolated from food (El Mohsen *et al.* 2006; Konishi *et al.* 2006; Matsumoto *et al.* 2004; Miro-Casas *et al.* 2003; Okushio *et al.* 1999; Urpi-Sarda *et al.* 2010). Nevertheless, the bioavailability of phenolic compounds from whole foods may be extremely different and they are present in the diet as complex mixtures formed mainly by food macro-components (carbohydrates, proteins and lipids) (Saura-Calixto *et al.* 2007). Thus, the use of whole food as polyphenol sources could be probably the best option for phenolic metabolism studies.

1.1 Digestion and colonic fermentation

Polyphenol stability during the digestion process, the food matrix effect and the release of phenolic compounds from the food matrix are important parameters directly related to polyphenol absorption. In relation to the first objective proposed, *in vitro* models represent a convincing tools for obtaining adequate results regarding to the polyphenol metabolism that take place in the digestion tract lumen. Polyphenol have to be accessible and reach the target tissue to exert their biological activity (Saura-Calixto *et al.* 2007). Although phenolic compounds was repeatedly claimed to have low stability under gastric conditions for various years (Aura 2008; Felgines *et al.* 2003), several *in vitro* studies have contradicted this (Serra *et al.* 2010; Soler *et al.* 2010; Spencer *et al.* 2000). Despite not all the ingested phenolic compounds surviving the intense digestion conditions, as they are more resistant under gastric conditions than under intestinal conditions (Serra *et al.* 2010; Soler *et al.* 2010), approximately 48% of dietary polyphenols are bioaccessible in the small intestine, while that value falls to 6% in the large intestine (Saura-Calixto *et al.* 2007). The extreme conditions in the stomach and intestine can hydrolyze oligomeric forms into more simple structures, as occurred in our experiment in which grape seed procyanidin extract (GSPE) was submitted to *in vitro* digestion, and polymeric procyanidins gave rise to low-grade polymerization structures or monomeric units (Ortega *et al.* 2011; Serra *et al.* 2010). That intense hydrolysis is necessary, prior to the *in vivo* absorption of complex proanthocyanidins, for them to be degraded into low polymerization or monomeric forms during digestion (Rios *et al.* 2002). Similarly, during the digestion process, secoiridoid derivatives of olive oil polyphenols result in a significant increase in total amounts of phenyl alcohols and phenolic acids in the digestion mixture (Corona *et al.* 2006; Serra *et al.* 2012d; Soler *et al.* 2010; Visioli *et al.* 2003; Vissers *et al.* 2004).

Phenolic compounds appear to be linked to the food matrix and they are released from it, due to the action of the digestive enzymes, becoming bioaccessible (Aura 2008), with a direct relation between polyphenol release from the food matrix and absorption (Scholz and Williamson 2007). The absorption of phenolic compounds differs according to the vehicle they are carried in. In general, due to the digestion process, the phenolic compounds are better absorbed when they are included in oily matrices than in aqueous solutions (Tuck and Hayball 2002; Tulipani *et al.* 2012). This fact could be related to the protective effect of fat forming micellar structures within the digested matrix (Ortega *et al.* 2009), and/or with the presence of surface-active agents, e.g. bile salts, which may form micellar and vesicular structures within the digest providing protection to the digested phenols (Carey and Cohen 1995).

Little is known about the effect of the food matrix on polyphenol bioavailability and metabolism. Comparing the few results available, not all the food matrices act in the same way on the polyphenol bioaccessibility and research into the specific interaction between polyphenols and macro and micronutrients in the digestive tract will be critical for understanding how food formulations could be a tool for improve circulating and tissue levels of minor dietary compounds. As examples of macro nutrient modulation of polyphenol digestion processes, carbohydrates may enhance the uptake of the monomeric procyanidins, as was observed in our *in vitro* digestion study (Serra *et al.* 2010), possibly by a specific effect on the gastrointestinal physiology (e.g. motility and/or secretion) or a carbohydrate-specific enhancement of the activity of a yet unidentified carboydrate-flavonol transporter (Serra *et al.* 2010; Schramm *et al.* 2003). Fatty matrices may facilitate the absorption of phenolic compounds, as was demonstrated with virgin olive oil secoiridoids, comparing in rats a oral dosage of a hydroxytyrosol oily solution with an intravenous hydroxytyrosol aqueous

solution (Tuck and Hayball 2002); or as was demonstrated with procyanidins comparing the bioavailability of procyanidins of a procyanidin enriched cocoa cream with the non-enriched cocoa cream, in which the procyanidin metabolite plasmatic concentration and the tissue disposition was modulated according to the fatty food matrix (Serra *et al.* 2012b). Additionally, according to Tulipani *et al.* 2012, lipid matrices may enhance the re-absorption by enterohepatic recirculation affecting the plasma concentration of phenolic compounds, demonstrated with tomato phenolic compounds using an olive oil enriched tomato sauce. On the other hand, the polyphenol bioavailability may be impaired by the presence of dietary proteins, such as casein and albumine, which are able to bind phenolic compounds, e.g. chlorogenic acids, by covalent and non-covalent interactions. So, the common action of mixing phenolic-rich sources, such as coffee or cocoa, with milk could be negative for the polyphenol bioavailability (Arts *et al.* 2001; Duarte and Farah 2011; Dupas *et al.* 2006; Manach *et al.* 2004). Additionally, polyphenol-rich foods are consumed as formulated products with other minor nutrients and secondary ingredients or additives, such as sweeteners or antioxidants (e.g. ascorbic acid), which may influence the bioaccessibility and intestinal absorption of polyphenols (Ferruzzi 2010). Thus, future research goals should have to be addressed towards thorough research into the effect of formulation and matrix on the bioavailability and metabolism of phenolic compounds.

Despite the intense release of phenolic compounds from the food matrix, approximately 10% of the phenolic compounds ingested are always inaccessible, not leaving the food matrix throughout the digestion process (Saura-Calixto *et al.* 2007). The majority of phenolic compounds linked to the non-digestible fraction are associated with the insoluble indigestible fraction not passing through the intestinal barrier (Saura-Calixto *et al.* 2007) but reaching the colon, in which they may act, e.g. as antioxidant or prebiotic (Bialonska *et al.*

2010; Scalbert 1991; Silva *et al.* 1997). Polyphenol phase II metabolites (sulphated, glucuronidated and methylated conjugates) in the digestive tract lumen due to enterohepatic recirculation can also reach the colon and are subject to colonic metabolism. Due to the wide range of existing dietary phenolic compounds, the complexity of the phase II metabolites and the microbiota diversity, several new phenolic metabolites may be generated in the colon, and these could enter the blood stream and reach the target tissues after being reabsorbed in the colon.

Generalizing, phenolic acids are the main colonic metabolites of phenolic compounds, generated by the ring-fission and cleavages of functional groups (Fogliano *et al.* 2011; Serra *et al.* 2011; Serra *et al.* 2012c). Nevertheless, little is known about the polyphenol catabolism as an individual phytochemical, and this is the main reason why the second objective was proposed. Using *in vitro* colonic fermentation methods with fresh fecal material, the symbiotic microbiota is maintained and individual phenolic compounds may be fermented to draft the catabolism pathway. Knowing the individual pathways of each phenolic compound could be an advantage for understanding the catabolism of complex polyphenol-rich foods (Serra *et al.* 2011; Serra *et al.* 2012c). Our results of colonic fermentation of individual phenolic compounds suggested that most of the colonic metabolites are common to several phenolic compounds (Figure 8), and that could be the main reason why matches between fermentations of polyphenol-rich foods have been detected in the colonic metabolites (Dall'Asta *et al.* 2012).

During colonic fermentation the first described catabolic reaction is a previous hydrolysis of polymeric and conjugated forms of polyphenols (Figure 8) (Roowi *et al.* 2010; Serra *et al.* 2011; Serra *et al.* 2012c; Takagaki and Nanjo 2010), whereby low-grade polymerization flavonoids (e.g. procyanidin dimer),

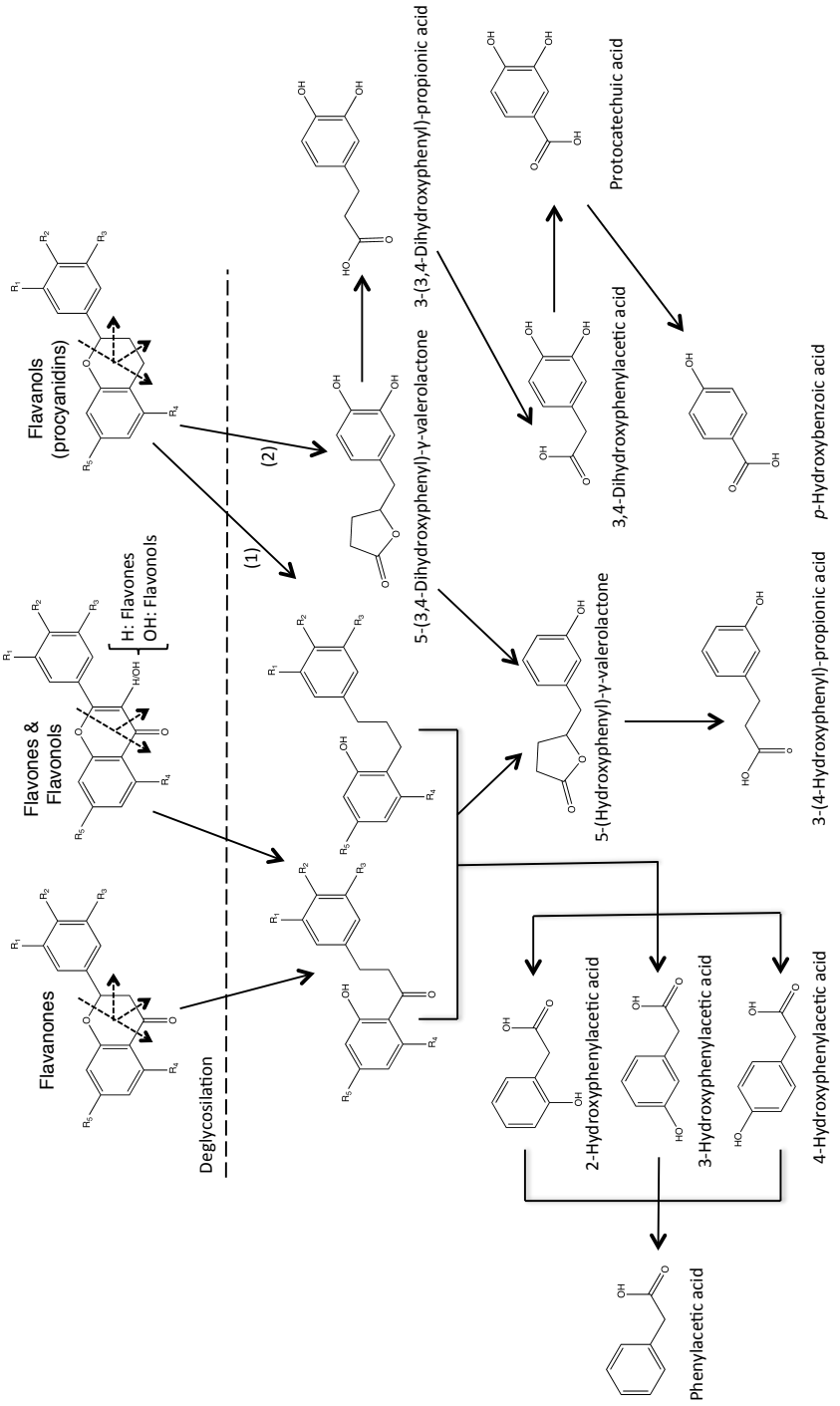


Figure 8. General proposed metabolic pathway of some flavonoids.

glycosilated and galloylated forms (e.g. quercetin-rhamnoside and quercetin-rutinoside or epicatechin gallate and epigallocatechin gallate, respectively) were hydrolyzed *in vitro* into their monomeric forms (Serra *et al.* 2011; Serra *et al.* 2012c). Subsequently, hydroxylated forms and non-conjugated forms of some phenolic acids (phenyl acetic, phenyl propionic and protocatechuic acids) were quantified in the fermentation mediums, suggesting that flavonoids were degraded into low-molecular-weight aromatic compounds through the action of the colonic microflora (Serra *et al.* 2011; Serra *et al.* 2012c). The hydroxylated phenolic acids are the first colonic metabolites generated after hydrolysis by the break down of carbon linkages, which are dehydroxylated progressively throughout the fermentation period giving rise to the aglycone form of phenolic acids, such as *p*-hydroxybenzoic acid or phenylacetic acid (this being the principal colonic metabolite of flavonoids) (Aura *et al.* 2002; Aura 2008; Baba *et al.* 1983; Gross *et al.* 1996; Sawai *et al.* 1987; Schneider and Blaut 2000; Serra *et al.* 2012c; Serra *et al.* 2011; Winter *et al.* 1989; Winter *et al.* 1991). The dehydroxylation pattern may be slightly different for each flavonoid skeleton structure, e.g. procyanidins showed two different fermentation pathways. The first proposed route (Figure 8, pathway$^{(1)}$) may indicate preferential dehydroxylation in the 4-position (Serra *et al.* 2011) and the second proposed pathway (Figure 8, pathway$^{(2)}$) was based on the rupture of the 1-2 bond (Serra *et al.* 2011; Groenewoud and Hundt 1984). On the other hand, flavanols, flavones and flavanones did not show two different patterns of colonic fermentation. Nonetheless, the colonic fermentation products were practically the same as procyanidins (Figure 8). However, not all the fermentation products are common to all the flavonoids, e.g. homovanillic acid was detected in our fermentation study exclusively as a quercetin fermentation product (Serra *et al.* 2012c) and according to that, homovanillic acid was detected as a catabolic metabolite of black tea, which contains some quercetin derivatives, such as quercetin rutinoside (Dall'Asta *et al.* 2012).

In summary, the *in vitro* digestion models are excellent tools for mimicking the structural modifications that take place during the digestion process, evaluating the release of phenolic compounds from the food matrix and evaluating the hydrolysis that gives rise to the smaller phenolic structures. Additionally, it is well known that during the colonic fermentation process, phenolic compounds undergo a complex and intense metabolism through the action of the colonic microflora and the application of an *in vitro* fermentation model using fresh faecal material, as occurs with the assays with culture cells to mimic phase II metabolism, allows the fermentation of individual phenolic compounds and identifies every breakage minutely.

1.2 Analytical methodologies for biological samples

The presence of phenolic compounds in foods at low concentration levels and the low described bioavailability, together with the food complexity, represents a challenge for quantifying phenolic metabolites in biological samples, such as blood and tissues. Thus, for the correct interpretation of nutritional findings the analysis of polyphenols and their metabolites from biological samples requires the use of a sensible and reliable analytical technique (Panteghini 2006; Peters *et al.* 2007). The sensitivity is an extremely important quality parameter of the analytical method because phenolic compounds are present at low concentration levels (nM or low μM range), and normally represent only a very small percentage of the amount of phenols consumed found in the biological samples. On the other hand, the quality and the relevance of the results are related to the accuracy of the measurements (Panteghini 2006), and unreliable results could represent underestimations of effects, false interpretations, and unwarranted conclusions (Peters *et al.* 2007). On the other hand, a careful analytical method development is almost important as its validation. Therefore,

only a validated methodology can be objectively judged as an adequate methodology (Peters *et al.* 2007).

The most common method for analyzing plant phenols and their metabolites in biological samples is high-performance liquid chromatography (HPLC) with reverse-phase columns in combination with diverse detectors, such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) (Vacek *et al.* 2010). Related to this, we have developed two validated chromatographic methodologies using the ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), with electrospray ionization (ESI) as the ionization technique, prior to the quantification of phenolic metabolites in the plasma (procyanidins and their metabolites) (Serra *et al.* 2009) and tissues (procyanidins, anthocyanins and their metabolites) (Serra *et al.* 2011b). In both methods, different quality parameters of the method were studied, such as recovery, linearity, calibration curves, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD). In order to avoid inaccuracies from the sample matrix, the method was validated by spiking the analytes in the biological sample. Due to the use of the ESI, the matrix effect, defined as the signal suppression or signal enhancement of the analyte response by comparing it with when the analyte was prepared in an organic solvent, was part of the validation process because the matrix effect is one of the main problems linked with the presence of other components in the sample that could affect the signal and consequently the analyte response (Annesley 2003; Matuszewski *et al.* 2003; Maurer 2005; Shah *et al.* 2000; Shishikura *et al.* 2006). With the sample matrix, always a sample pre-treatment technique previous to the chromatographic analysis should always be applied to clean the sample up and determine the phenolic compounds at low concentration levels and eliminating interfering components present in the sample matrix (Serra *et al.* 2009; Serra *et al.* 2011b). The chosen sample pre-treatment technique for both developed

methodologies was solid phase extraction. The use of a solid phase extraction (SPE) using OASIS Hydrophilic-Lipophilic-Balanced (HLB) (60 mg, Waters Corp., Milford, MA) cartridges (Serra *et al.* 2009) was initially planned for plasma samples. Nonetheless, that type of cartridge required a considerable amount of the sample, loading 1 mL of plasma, and a long time to evaporate the eluted fraction with nitrogen stream to pre-concentrate the analytes. Thus, in order to mitigate these disadvantages, the use of a micro-solid phase extraction (μSPE) method, developed by Martí, *et al.* 2010 was proposed for subsequent experiments. Microplates using an OASIS HLB μElution Plate 30 μm was proposed as the device format instead of cartridges, loading 350 μL of sample (Serra *et al.* 2011a; Serra *et al.* 2011b; Serra *et al.* 2012b). Independently of the use of SPE or μSPE, the clean-up and elution steps of the solid phase extractions must be optimized for practically each phenolic family to obtain the maximum recoveries. For procyanidins, the clean-up of loaded cartridges was established with Milli-Q water and Milli-Q water/acetic acid (99.8:0.2,v:v). The applied volumes for SPE were 3 mL and 5 mL, respectively (Serra *et al.* 2009). On the other hand, the volumes were considerable reduced when biological samples were pre-treated by μSPE, in which 200μL of Milli-Q water and 200μL of Milli-Q water/acetic acid (99.8:0.2,v:v) were applied (Martí *et al.* 2010). The same happened with the elution step. Initially, 4 mL of solution acetone:water:acetic acid (70:29.5:0.5, v:v:v) was used with SPE (Serra *et al.* 2009); which was reduced to 2 × 50 μL of the same solution using μSPE (Martí *et al.* 2010). μSPE was also used to analyze the procyanidin metabolites in the tissue samples (Serra *et al.* 2011b). The recoveries obtained in both developed methodologies were over 65% for procyanidins, and the reduction of the required sample weight was considerable, bearing in mind the small amount of the available biological samples.

In general, both validated methodologies showed low LOD and LOQ. Nonetheless, the quality parameters obtained should not be compared with other literature because the validation process of the analytical methods was not performed in the previous research works, in which procyanidins (García-Ramírez *et al.* 2006; Urpi-Sarda *et al.* 2010; Prasain *et al.* 2009; Tsang *et al.* 2005;) and anthocyanins (El Mohsen *et al.* 2006; Miyazawa *et al.* 1999; Talavera *et al.* 2005; Vanzo *et al.* 2008) were detected in tissue samples. Comparing both validation procedures, the analysis of polyphenols and their metabolites in plasma was less influenced by the interaction between the analytes and the matrix than the analysis of polyphenols and their metabolites in the tissue samples, similar to what was reviewed by Vacek *et al.* 2010.

Phenolic compounds are retained by the cartridge sorbent when they are in their neutral form. Due to differences in the acid dissociation constant (pka), between procyanidins and the olive oil phenolic compounds, their behavior during the extraction step is different. In order to retain procyanidins (pka ~ 9), these were load to the cartridge with 0.2% of acetic acid, because in these conditions procyanidins were in their neutral form. Besides, olive oil phenolic compounds were loaded to the cartridge with water at pH 2 because their pka value is lower (pka ~ 3-5). Thus, a different methodology is required to analyze olive oil phenolic compounds. Moreover, procyanidins are better eluted with acetone. On the other hand, methanol is better for phenolic compounds of olive oil. For that reason, a methodology previously developed and validated by a co-worker (Suárez *et al.* 2009) was used in the experimental studies related to olive oil phenolics. Olive oil phenolic metabolites were identified and quantified in biological samples by μSPE and UPLC-ESI-MS/MS and the pre-treatment step was optimized to maximize the recoveries. Thus, two different methods were used, one was specifically for isolating hydroxytyrosol metabolites and the other one was applied to the other phenolic compounds under study. Unlike the other

phenolic compounds, hydroxytyrosol was eluted with acetonitrile. The recovery values described by Suárez, *et al.* (Suárez *et al.* 2009) for that methodology were over 67%.

1.3 Absorption, metabolism and distribution

Besides the use of very sensitive and robust methodologies and taking into account the relatively low sensitive of the full-scan mode of the ESI-MS/MS detector, the targeted metabolomics requires detailed knowledge of the potential analytes that could be detected after the ingestion of phenolic compounds. Due to the lack of commercial standards of phenolic compound metabolites, and the complexity and high cost of their chemical synthesis, carrying out an acute intake study with experimental animals (rats in our study) allows valuable biological samples with high concentration of phenolic metabolites to be obtained. These biological samples (plasma and tissues, in our case) could be useful for identifying and tentatively quantifying even the trace level metabolites generated after the intake of phenolic compounds, developing complete methodologies. That, together with the lack of information about some aspects of the metabolism of phenolic compounds, such as the tissue distribution or the *in vivo* food matrix effect on the polyphenol metabolism, represents one of the proposed objectives. Related to this, phenolic compounds are rapidly absorbed and metabolized, their metabolites rapidly appearing in plasma and showing a maximum concentration (C_{max}) at between 1.5-3h, when polyphenols are fed as a component of a complex food (Serra *et al.* 2010; Serra *et al.* 2012b; Serra *et al.* 2012e), or in less than 1h when polyphenols are fed as a pure chemical compound (Baba *et al.* 2002; Piskula and Terao 1998). However, the low stability of pure phenolic compounds under gastric conditions

is a drawback for oral administration. Thus, intravenous administration is an effective alternative to avoid gastric degradation.

In general, phenolic compounds are absorbed as their aglycones after a prior hydrolysis of the glycosides (Walle 2004) and metabolized reaching the blood stream as conjugated forms. Nonetheless, our studies confirmed the presence of free forms in the plasma when the polyphenols were administered at a pharmacological dose (Hackett *et al.* 1983; Serra *et al.* 2010; Serra *et al.* 2011a; Serra *et al.* 2012d). A clear example was quantifying epicatechin and catechin in a free form in the plasma after an acute intake of grape seed procyanidin extract (GSPE) at a pharmacological dose (1 g of GSPE/kg of rat body weight) (Serra *et al.* 2010) or the presence of unconjugated hydroxytyrosol in the plasma after an acute intake of a olive cake phenolic extract (PEOC) (3 g of PEOC/kg of rat body weight) (Serra *et al.* 2012d). High doses could saturate the conjugation metabolism of the phenolic compounds, as we observed with olive phenolic compounds and procyanidins, allowing the detection of their free forms in rat plasma samples (Serra *et al.* 2010). Another important fact possibly related with the saturation of the conjugation metabolism is the presence of low grade polymerization procyanidins (dimer and trimer) in plasma samples after an acute intake at a pharmacological dose (Baba *et al.* 2002; Holt *et al.* 2002; Serra *et al.* 2010; Zhu *et al.* 2003; Zhu *et al.* 2002) or even after a chronic intake (Serra *et al.* 2012). There are also indications that differences on the primary metabolism site could be related to the ingested phenolic dose (Shoji *et al.* 2006). Large doses are metabolized primarily in the liver, while small doses are metabolized by the intestinal mucosa, shifting the sulphation and glucuronidation of phenolic compounds. So, the quantity of polyphenol ingested is probably one of the most important factors determining their metabolic fate (Koster *et al.*1981).

With *Wistar* rats as the animal model used in our studies, the main conjugated phase II metabolites of the procyanidins detected in plasma were the glucuronidated forms (Serra *et al.* 2010; Serra *et al.* 2011a; Serra *et al.* 2012b), similarly to what other authors observed (Abd El Mohsen *et al.* 2002; Harada *et al.* 1999; Vaidyanathan and Walle 2002) followed by the methyl glucuronidated conjugates. Although glucuronidation has been described in the liver and intestine, rat liver microsomes have shown an extremely efficient glucuronidation capacity in front of flavonoids (Vaidyanathan and Walle 2002). Nonetheless, after an acute intake of a PEOC rich in secoiridoid derivatives, the sulphated forms were the most abundant metabolites detected in the plasma (Serra *et al.* 2012d); indicating that rat hepatocytes were also capable of sulphated these phenols (D'Angelo *et al.* 2001; Tuck *et al.* 2001; Tuck and Hayball 2002) and reflecting a possible high-sulphotransferase activity of the platelets (Anderson *et al.* 1991). Apart from the differences on the metabolism according to the structural variations of each phenolic compound, it has been demonstrated that there is a wide divergence in the bioavailability and metabolism of phenolic compounds (Manach *et al.* 2005). The existent inter-individual variability, which is generally small in animal models but could represent a problem for the interpretation of results in human clinical studies, could be explained by modulations exerted by the environment, physiological, polymorphisms, epigenetic and genetic factors, among others factors, on the capacity of phase II enzymes toward phenolic compounds (Bolling, 2009; Serra *et al.* 2012a). On the other hand, other parameters related to the experimental design, such as the food matrix or the dose administered, may affect the pharmacokinetic parameters globally. Even significant differences in the metabolism of phenolic compounds have been detected depending on the nature of these (Serra *et al.* 2012e). The duality of the naturally existence of foods with high phenolic contents, such as virgin olive oil, in which these contents may depend on a range of agronomical and technological factors, and the possibility of preparing

fortified foods, with a controlled and standardized phenolic composition, requires knowledge about the differences between the origin of the phenolic compounds of the foods, either inherent or added, on their bioavailability and metabolism. Nowadays, there is a growing trend towards developing polyphenols enriched or fortified foods as possible functional foods (Gaudette and Pickering 2011; Rubió *et al.* 2012; Suárez *et al.* 2010; Trigueros *et al.* 2011). Nonetheless, the use of phenolic-rich or phenolic-enriched products could vary the pharmacokinetic profile of the phenolic metabolites, e.g. the behavior exerted by the phenolic compounds and an oily matrix seemed to be different when the phenolic compounds were not from the olive oil itself, with significant differences in the plasmatic concentration of the olive oil phenolic metabolites. Although the detected phenolic metabolites were similar, when the phenolic compounds were administered with a phenol-enriched olive oil, an increase was detected in the plasmatic concentration of phenolic metabolites comparing with the intake of natural rich olive oil (Serra *et al.* 2012e).

Besides, a clear relation between plasmatic metabolites and the health benefits exerted by phenolic compounds has yet to be established, so studying the distribution of the polyphenols in the tissues could be the starting point for knowing the metabolic target and the first step toward understanding how polyphenols acts at a cellular level. The distribution and accumulation of phenolic compounds in tissues is a recent research topic with little available information (El Mohsen *et al.* 2006; Serra *et al.* 2012e; Serra *et al.* 2012d; Serra *et al.* 2012b; Serra *et al.* 2011b; Serra *et al.* 2011a; Talavéra *et al.* 2005; Urpi-Sarda *et al.* 2010). The use of pure chemical phenolic compounds in some of the polyphenol tissue distribution studies, generally administered intravenously (D'Angelo *et al.* 2001) or intragastrically (El Mohsen *et al.* 2006; Zafra-Gómez *et al.* 2010), hinders the extrapolation when the phenolic compounds are administered as a component of a food. Moreover, doing human clinical studies

to evaluate the phenolic tissue distribution is controversial and sometimes impossible. However, experiments such as the one reported by Henning *et al.* 2006, in which the presence of tea polyphenol in prostate tissue was evaluated in humans after a chronic intake of tea over 5 days, have been reported. All of these facts lead to concluding that animal models are the best solution for studying the tissue distribution.

Related with this, according to the existent results about tissue distribution, our results suggest that dietary phenolic metabolites present in plasma are widely distributed throughout the body by the blood stream, reaching practically all the organs, even crossing the blood brain barrier (Figure 9 and 10) (Serra *et al.* 2011a; Serra *et al.* 2012b; Serra *et al.* 2012d; Serra *et al.* 2012e) and, as was demonstrated by other authors, they may reach fetal organs after maternal exposure (Chu *et al.* 2007). A relation between tissue disposition and blood irrigation could be a coherent hypothesis for explaining the presence of the polyphenols in practically all parts of the body at different concentrations. However, that fact cannot explain the significant differences detected between tissues in our studies in the profile of phenolic metabolites (Serra *et al.* 2011a; Serra *et al.* 2012b; Serra *et al.* 2012d; Serra *et al.* 2012e) The polyphenol-tissue protein interaction seems fundamental for allowing the fixation between the bioactive compounds in the tissue and exerting their biological activities (Frazier *et al.* 2010). So, together with the blood flow, it could contribute to the significant differences observed between tissues in the polyphenol profile. The higher concentrations of phenolic metabolites detected in the liver and kidney could be explained by their role in the drug detoxification metabolism (D'Angelo *et al.* 2001; Manach *et al.* 2004; Serra *et al.* 2011a; Serra *et al.* 2012b; Serra *et al.* 2012d; Serra *et al.* 2012e) with the urinary being the main excretion path for the phenolic compounds (Serra *et al.* 2012d; Caruso *et al.* 2001). The presence of glucuronidated and methyl glucuronidated flavonoids in the intestinal content

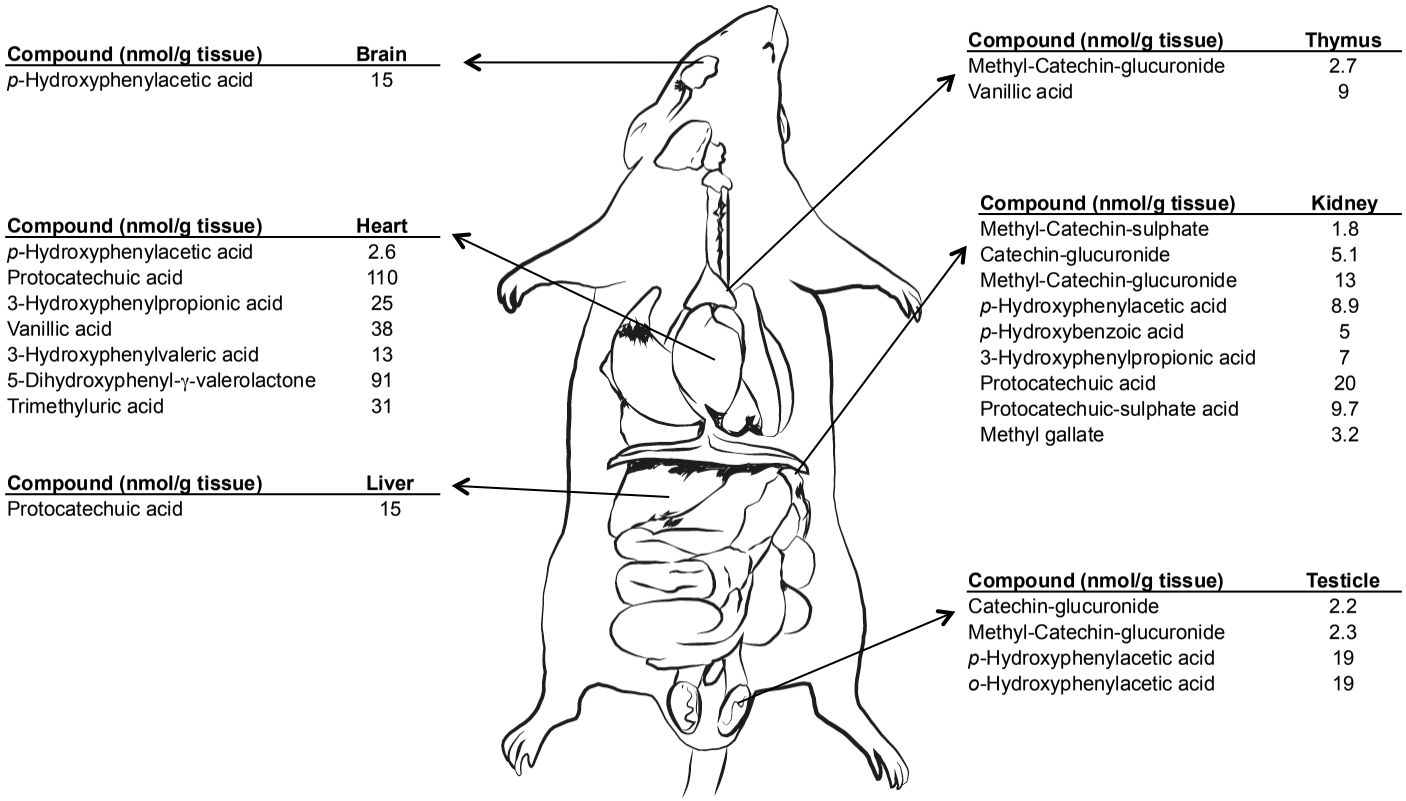
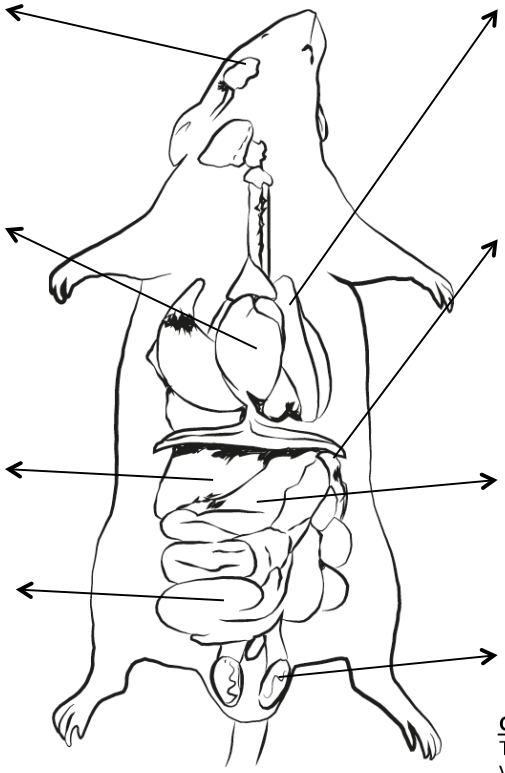


Figure 9. Procyanidin metabolite concentrations detected in some tissues after an acute intake of 5 g of hazelnut skin extract/kg of rat body weight (Serra *et al.* 2011a).

Compound (nmol/g tissue)	Brain
Hydroxytyrosol sulphate	50
Tyrosol sulphate	10.14
Oleuropein derivate	2.8
Vanillin sulphate	0.02
Vanillic acid	1.7
Vanillic sulphate acid	0.73
Homovanillic sulphate acid	1.83

Compound (nmol/g tissue)	Heart
Hydroxytyrosol sulphate	263
Tyrosol sulphate	17
Oleuropein derivative	8.4
Vanillic sulphate acid	0.74
Hydroxyphenylacetic acid	0.25
Luteolin	0.16

Compound (nmol/g tissue)	Liver
Hydroxytyrosol sulphate	237
Hydroxytyrosol glucuronide	2.1
Tyrosol sulphate	73
Oleuropein derivate	20
Vanillin sulphate	1.0
Coumaric sulphate acid	0.6
4-Hydroxy-3-methoxyphenylacetaldehid	16
Vanillic acid	2.7
Vanillic sulphate acid	4.5
Caffeic acid	0.14
Homovanillic acid	0.19
Homovanillic sulphate acid	7.4
Hydroxyphenylacetic acid	0.63
Enterolactone	5.2
Enterolactone sulphate	5.6
Enterolactone glucuronide	0.5



Compound (nmol/g tissue)	Thymus
Tyrosol sulphate	4.80
Coumaric sulphate	0.36
Vanillic sulphate acid	1.80

Compound (nmol/g tissue)	Kidney
Hydroxytyrosol	1.3
Hydroxytyrosol sulphate	3100
Hydroxytyrosol glucuronide	12
Tyrosol sulphate	599
Tyrosol glucuronide	2.5
Oleuropein derivate	60.8
Vanillin sulphate	3.0
Coumaric sulphate acid	12
Coumaric glucuronide acid	0.3
4-Hydroxy-3-metoxyphenylacetaldehid	99
Vanillic acid	3.3
Vanillic sulphate acid	34
Caffeic acid	2.1
Caffeic sulphate acid	1.2
Homovanillic acid	2.6
Homovanillic sulphate acid	24
Ferulic sulphate acid	3.5
Hydroxyphenylacetic acid	6.7
Enterolactone	2.3
Enterolactone sulphate	5.0
Enterolactone glucuronide	5.4

Compound (nmol/g tissue)	Testicle
Tyrosol sulphate	1.3
Vanillic acid	0.74
Hydroxyphenylacetic acid	0.01
Enterolactone	1.44
Enterolactone sulphate	2.0
Enterolactone glucuronide	0.01

Figure 10. Olive oil polyphenol metabolite concentrations detected in some tissues after an acute intake of 3 g of PEOC/kg of rat body weight (Serra *et al.* 2012c)

after the ingestion of 5 g of hazelnut extract/kg of body weight (Serra *et al.* 2011b) could verify the recirculation through the bile of phase II biotransformation metabolites from the liver. On the other hand, the presence of free and conjugated (sulphated and glucuronidated) forms of the phenolic acids in practically all the tissues analyzed, especially the liver, verifies the colonic absorption and metabolization of catabolic metabolites and suggests the importance of the liver in the conjugation metabolism (Serra *et al.* 2011a; Serra *et al.* 2012b; Serra *et al.* 2012d; Serra *et al.* 2012e).

The metabolism of phenolic compounds is generally a common process for all the phenolic compounds, with an intense hydrolysis during the digestion step, followed by a conjugation in the brush border membrane of the small intestine through which phenolic compounds cross into the bloodstream, reaching all parts of the body. Nonetheless, the results of the *in vivo* experiments with flavonoids and olive oil phenolic compounds showed differences in the metabolism (Figures 9 and 10), with a clear predominance of glucuronide conjugates from procyanidins and sulphated conjugates from olive oil phenolic compounds. Moreover, the concentrations of phenolic metabolites detected in tissues were completely different for procyanidins and olive oil phenolic compounds, with a lower dose of olive oil phenolic compounds (3 g of PEOC/kg of body weight) than procyanidins (5 g of hazelnut skin extract/kg of body weight), the phenolic concentration detected in the tissues was higher with PEOC and a wider range of metabolites was detected after the PEOC intake. So, differences appear in the tissue distribution between families of phenolic compounds and each phenolic family has its own pattern of metabolism.

Despite the widespread presence of phenolic metabolites in tissues, their presence in some specific tissues, such as the testicles or thymus, has yet to be related to any functionality. Nonetheless, several health benefits in specific areas

of the body, such as protection against cardiovascular disease or neuroprotection, have been related to the intake of polyphenol and the specific information about which phenolic metabolite is deposited in each tissue could be extremely useful for identifying the bioactive compounds with specific *in vivo* bioactivity and the mechanisms by which phenolic compounds act at the cellular level. Moreover, that information could be helpful for developing new polyphenol-enriched foods with a specific functionality in a specific organ in the body, because according to the nature of the phenolic compounds, whether added or not, the tissue distribution could vary modifying the metabolic target tissue (Serra *et al.* 2012e).

The results of the experiments included in that thesis and carried out using Wistar rats (Serra et al. 2012b; Serra et al. 2012d; Serra et al. 2012e; Serra et al. 2011a; Serra et al. 2010) have confirmed that the acute intake experiments are useful tools for obtaining information about the phenolic absorption, metabolism and tissue distribution quickly. Furthermore, acute intake experiments allow cost reductions compared with long-term experiments. However, the presence of polyphenol metabolites in tissues hours after the ingestion of polyphenol rich foods (Serra et al. 2012b; Serra et al. 2011a), together with the existent results of short chronic polyphenol intakes (Urpi-Sarda et al. 2010), may indicate that polyphenol metabolites could accumulate in tissues with an adequate combination of time and doses. As was demonstrated in our chronic procyanidin intake experiment (Serra et al. 2012), after a 21-day intake of grape seed procyanidin extract (GSPE) with different doses assayed (5 mg, 25 mg and 50 mg GSPE/kg of rat body weight), each of the organs studied (muscle, liver, white adipose tissues (mesenteric and perirenal) and brown adipose tissue) has its own specific accumulation of, and response to, the assayed GSPE doses. For example, the levels of glucuronide derivatives in both the white adipose tissues studied (perirenal and mesenteric) were similar, at 25 and 50 mg GSPE/

kg body weight. In contrast, the brown adipose tissue showed a dose response in the concentration of all the conjugate-metabolites of catechin and epicatechin quantified. This is of great importance due to the relation between flavanol metabolites and improvement of the mitochondrial function related to the energy homeostasis of the brown adipose tissue (Pajuelo et al. 2011).

Nowadays, researchers all over the world are investigating the mechanisms through which phenolic compounds exert their bioactivity in the metabolic target. Future research studies should clarify *i)* if the bioactivity of phenolic compounds is caused by the action exerted by phenolic metabolites in the tissues, with a possible deconjugation pathway that leads to more bioactive aglycone polyphenols, or, *ii)* if phenolic compounds acts by inducing or modifying the gene expression of specific responses in the body. Other interesting studies more related to the metabolism of phenolic compounds could be the *in vivo* study of the absorption, metabolism, tissue distribution and excretion of dietary phenolic compounds by the use of labeled phenolic compounds to draft the origin of each metabolite precisely.

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CONCLUSIONS

CONCLUSIONS

The starting hypothesis postulated for this thesis was focused on the development of useful tools for estimating the digestibility, bioavailability, metabolism and distribution of phenolic compounds and their metabolites, as a preliminary step towards human clinical studies. Related to this and answering the defined targets, the main achievements of the research project are summarized below:

Targets 1 and 2: *In vitro* digestion and colonic fermentation models.

1. The application of an *in vitro* digestion model to the study of the digestibility and potential bioaccessibility of procyanidins demonstrated that proanthocyanidins with a low level of polymerization (dimers and trimers) are stable under the conditions in the different stages of the digestion process, demonstrating their potential *in vivo* bioavailability.

2. By applying a simple *in vitro* colonic fermentation model, the metabolic pathways of some individual components of the procyanidin and flavonoid phenolic groups were established.

3. The application of that *in vitro* model to a complex food matrix, specifically a cocoa cream with high carbohydrate and lipid contents, allowed the matrix effect on the metabolism of phenolic compounds (procyanidins and flavonoids) spiked in that matrix to be evaluated.

Target 3. Development and validation of methodologies for the analysis of phenolic compounds in biological samples.

4. The development and validation of the chromatographic methodologies

combining the solid phase micro-extraction (μSPE) and ultra-performance liquid chromatography (UPLC-MS/MS) enabled the identification and quantification at nanomolar and micromolar level of the metabolites of the procyanidins and phenolic compounds from virgin olive oil in biological samples (plasma and tissues).

Target 4. Metabolism and distribution of procyanidins and phenolic compounds of virgin olive oil.

5. The use of Wistar rats as a pre-clinical model proved useful for the study of the absorption, metabolism and distribution of the phenolic metabolites in tissues, analyzed after an acute intake of phenolic-rich extracts (procyanidins or olive cake phenolic extracts) or phenolic-rich foods (cocoa cream and virgin olive oil).

6. The analysis of rat plasma obtained 2 hours after the intake of a procyanidin-rich extract (5 g/kg of body weight) revealed an intense phase II metabolism (glucuronidation, sulphation and methylation) of catechin and epicatechin. The main phenolic metabolites detected in the plasma were the glucuronidated conjugates of catechin and epicatechin, followed by the methyl sulphated and methyl glucuronidated forms of catechin and epicatechin. There was a notable presence of the free forms of dimers and trimers in the plasma.

7. In relation to the accumulation and distribution of procyanidin metabolites in tissues, a wide range of phase II metabolites (conjugated forms) and/or colonic fermentation metabolites (phenolic acids) were detected. The glucuronidated conjugates of catechin and methyl catechin were detected in practically all the tissues tested. The lung was the tissue with the

greatest accumulation of phenolic metabolites, with the presence of free epicatechin. The highest levels of phenolic compounds were found in the heart, possibly as a result of a prior hydrolysis or colonic fermentation of procyanidins. The presence of phenolic acids in tissues could be interesting given their possible bioactivity in metabolite targets after an intake of procyanidins.

8. The analysis of rat plasma obtained 2 hours after ingestion of an extract rich in hydroxytyrosol derivatives and oleuropein (3 g / kg body weight) has revealed an intense sulfation and, to a lesser extent, glucuronidation, mainly of phenolic alcohols and phenolic acids, with hydroxytyrosol sulfate and tyrosol sulfate being the most abundant metabolites derived from the phase II metabolism in the plasma.

9. Detection of different metabolites of hydroxytyrosol and tyrosol (mainly as sulfate and, to a lesser extent, the glucuronidated form) in various tissues has confirmed its distribution through the bloodstream, even crossing the blood brain barrier. The kidney and liver were the organs in which the highest concentrations of tyrosol and hydroxytyrosol metabolites were detected after ingestion of an extract rich in phenolic compounds of olive oil, showing an intense detoxification metabolism. Several phenolic acids and products of lignan metabolism, such as enterolactone, were also detected in different organs, mainly in their sulfated forms.

10. Parameters related to the experimental design, such as the composition of the food matrix in which phenolic compounds are vehiculized and the ingested phenolic dose, modified the pharmacokinetic parameters of the phenolic metabolites in the plasma. The dose allows the modulation of the absorption, metabolism and tissue distribution of phenolic metabolites.

High doses may even saturate the enzymatic conjugation system of the phenolic compounds allowing the presence of free forms in the plasma. The composition of the food matrix, through which phenolic compounds are vehiculized, specifically the presence of fat and carbohydrates, directly affects the bioavailability of procyanidins with their distribution in the tissues favoring the absorption of the monomeric forms. However, the presence of fat in the food matrix impeded the detection of the dimer and trimer forms in the plasma.

11. The concentration of phenolic metabolites in the plasma and tissues may be modulated by the fact that phenols are either naturally present in food or added. The comparative study of the intake of olive oil (5g/kg) enriched with phenolic compounds and the intake of the same dose in an olive oil with a high phenolic content showed high differences in concentrations in the plasma of some metabolites, such as the sulfated forms of hydroxytyrosol, tyrosol and homovanillic alcohol, whose levels were higher in the plasma samples obtained after the ingestion of enriched olive oil. However, the effect of the intake of olive oil rich in phenolic compounds or enriched with these on the distribution of the phenolic metabolites in the tissues depends on the organ studied. After the intake of the enriched olive oil, higher concentrations of the metabolites of hydroxytyrosol and tyrosol were observed in the liver, heart and spleen. By contrast, after ingestion of a virgin olive oil with high phenolic content, a greater concentration of these metabolites was observed in the brain and kidney. This may represent a strategy for developing foods enriched with phenolic compounds aimed at a specific metabolic target.

12. Glucuronidated conjugates, followed by methyl glucuronidated conjugates of catechin and epicatechin, were the main procyanidin

metabolites detected in the plasma after a chronic intake of a procyanidin-rich extract at different doses. Glucuronide conjugates were detected in all the organs. Each of the studied organs (muscle, liver, white adipose tissue (mesenteric and perirenal) and brown adipose tissues) has a specific behavior of accumulation and response to the assayed GSPE doses, with a clear dose response in the brown adipose tissue, where the flavanols could play an important role in reducing or preventing obesity by modulating the functionality of that tissue. The results of this experiment could be useful for future in-vitro research with adipose cell cultures giving information about the physiological concentrations reached in specific adipose tissues after a chronic intake of flavanols.

Target 5: Study of the glucuronidation capacity of the liver.

13. The capacity for the hepatic glucuronidation (UGT) of quercetin is modulated by a diet rich in fructose and saturated fats during pregnancy and lactation in female rats of the parental generation and females in the following generation of rats. Since the ontogeny development of hepatic UGT occurs in early life, a modulation of liver enzymes could lead to an increased possibility metabolic diseases in adult life.

CONCLUSIONS

La hipòtesi de partida de la present Tesis Doctoral es va centrar en la necessitat de desenvolupar eines útils per estimar la digestibilitat, la biodisponibilitat, el metabolisme i la distribució de compostos fenòlics i els seus metabòlits, com a fase prèvia als estudis clínics. En relació amb dita hipòtesi, i responent als objectius plantejats, les fites aconseguides durant el desenvolupament d'aquesta Tesis Doctoral es detallen a continuació i s'estructuren en base als objectius plantejats inicialment.

Objectius 1 i 2. Models *in vitro* de digestió i fermentació colònica.

1. L'aplicació d'un model de digestió *in vitro* per a l'estudi de la digestibilitat i de la bioaccessibilitat de les procianidines va demostrar que les molècules de baix grau de polimerització (dimers i trimers) són estables en les condicions de les diferents etapes de la digestió, demostrant així la seva potencial bioaccessibilitat *in vivo*. Paral·lelament, el procès de digestió facilita la hidròlisi de les proantocianidines de baix pes molecular donant lloc a la formació dels monòmers, catequina i epicatequina.

2. A partir de l'aplicació d'un model senzill de simulació de l'etapa de fermentació colònica *in vitro* s'han pogut establir les rutes metabòliques de diferents compostos fenòlics, procianidines i flavonoids, d'una manera individualitzada.

3. L'aplicació d'aquest model de fermentació colònica *in vitro* a una matriu alimentària complexa, com és una crema de cacau amb un elevat contingut en carbohidrats i lípids, ha permès avaluar l'efecte de la matriu alimentària sobre el metabolisme fenòlic de les procianidines i d'alguns flavonoids.

Objectiu 3. Desenvolupament i validació de mètodes analítics per la determinació de compostos fenòlics i els seus metabòlits en mostres biològiques.

4. El desenvolupament i validació de mètodes analítics que combinen la micro-extracció en fase sòlida (μSPE) i la cromatografia líquida d'alta resolució acoplada a l'espectrometria de masses en tàndem (UPLC-MS/MS) ha permès l'identificació i la quantificació de metabòlits de procianidines i de fenols de l'oli d'oliva en mostres biològiques (plasma i teixits) a baixos nivells de concentració, en el rang de nanomolar (nM) i micromolar (μM).

Objectiu 4. Metabolisme i distribució de les procianidines i dels fenols de l'oli d'oliva.

5. L'ús de rates Wistar com a model experimental pre-clínic ha mostrat una alta efectivitat en els estudis d'absorció, metabolisme i distribució de metabòlits de compostos fenòlics en diferents teixits. Aquests estudis s'han realitzat després d'una ingesta aguda d'un extracte ric en compostos fenòlics (extracte ric en procianidines o en fenols d'oli d'oliva), i també d'un aliment enriquit amb compostos fenòlics (crema de cacao o oli d'oliva).

6. L'anàlisi de plasma de rata obtingut després de 2 hores de l'ingesta d'un extracte ric en procianidines (5 g d'extracte/kg pes de la rata) ha permès observar un intens metabolisme de fase II (glucuronidació, sulfatació i metilació) dels monòmers catequina i epicatequina. Els principals metabòlits detectats en plasma han estat les formes glucurònides de catequina i epicatequina, i les formes metil-sulfat i metil-glucurònid de

catequina i epicatequina. És destacable també la presència de dimer i trimer en la seva forma lliure.

7. S'han detectat en teixits un elevat nombre de metabòlits de fase II de procianidines (formes conjugades) i/o de metabòlits de la fermentació colònica (àcids fenòlics simples) com a resultat de la distribució i accumulació d'aquests metabòlits en teixits. Les formes glucuronidades de catequina i metil-catequina han estat els metabòlits més comuns detectats en la majoria dels teixits analitzats. La principal acumulació de la forma glucuronidada de catequina s'ha detectat en el pulmó. En aquest órgan cal destacar també l'acumulació d'epicatequina en la seva forma lliure. La principal acumulació d'àcids fenòlics simples, probablement resultant de l'hidròlisi o de la fermentació colònica de procianidines, s'ha observat en el cor. En base als resultats d'aquest estudi, es podria valorar l'interés de la funció dels àcids fenòlics simples a nivell de teixits diana com a metabòlits resultats de l'ingesta de procianidines.

8. L'anàlisi del plasma de rata obtingut 2 hores després de l'ingesta d'un extracte ric en compostos fenòlics derivats de l'hidroxitirosol i de l'oleuropeïna (3 g d'extracte/kg pes de la rata) ha permès observar un intens metabolisme de sulfatació, i en menor proporció glucuronidació, principalment dels alcohols fenòlics i dels àcids fenòlics. L'hidroxitirosol sulfat i el tirosol sulfat han estat els metabòlits, derivats del metabolisme en fase II, més abundants en plasma.

9. La detecció de diferents metabòlits d'hidroxitirosol i tirosol (principalment en la seva forma sulfatada i en menor proporció en la forma glucuronidada) en diversos teixits ha permès confirmar la seva distribució a través del torrent sanguini, inclús creuant la barrera hematoencefàlica.

Ronyó i fetge han estat els teixits en els que s'ha detectat una major concentració de metabòlits de tirosol i hidroxitirosol després de l'ingesta d'un extracte ric en compostos fenòlics de l'oli d'oliva. Aquest fet indica un intens metabolisme de detoxificació. Diferents àcids fenòlics i productes del metabolisme dels lignans, com la enterolactona, s'han detectat i identificat també en diferents teixits, principalment en la seva forma sulfatada.

10. Paràmetres relacionats amb el disseny experimental, com la composició de la matriu alimentària en la que es vehiculitzen els compostos fenòlics i la dosis fenòlica ingerida, han modificat els paràmetres farmacocinètics dels metabòlits fenòlics en plasma. La dosis també permet modular l'absorció, el metabolisme i la distribució dels metabòlits fenòlics en teixits. Altes dosis podrien inclús saturar el sistema enzimàtic de conjugació dels compostos fenòlics permetent la presència de les formes lliures en plasma. La composició de la matriu alimentària a través de la qual els compostos fenòlics són vehiculitzats, concretament la presència de greix i carbohidrats, afecta directament la biodisponibilitat de les procianidines i la seva distribució en teixits; aquest fet afavoreix l'absorció de formes monomèriques. No obstant, la presència de greix en la matriu alimentària no ha permès la detecció en plasma de les formes dimer i trimer.

11. La concentració de metabòlits fenòlics en plasma i en teixits podria estar modificada en funció de si els fenols estan en la seva forma natural en l'aliment (formen part de l'aliment) o si s'han addicionat mitjançant un enriquiment. L'estudi comparatiu de l'ingesta d'oli d'oliva (5 g d'oli/kg pes de la rata) enriquit en compostos fenòlics en relació amb l'ingesta de la mateixa dosis d'un oli verge d'alt contingut en compostos fenòlics ha

mostrat diferències en la concentració plasmàtica d'alguns metabòlits, (les formes sulfatades de l'hidroxitirosol, de l'alcohol homovainillic i del tirosol) sent superiors en les mostres de plasma obtingudes després d'una ingesta de l'oli enriquit. Cal destacar també que l'efecte de l'ingesta d'un oli ric o d'un oli enriquit en compostos fenòlics sobre la disposició dels metabòlits fenòlics en teixits depèn del teixit estudiat. Després d'una ingesta d'un oli d'oliva enriquit amb compostos fenòlics s'ha detectat una major concentració de metabòlits d'hidroxitirosol i tirosol en el fetge, en el cor i en el melsa. En canvi, després d'una ingesta d'un oli d'oliva verge d'alt contingut fenòlic la major concentració de metabòlits fenòlics va ser en el cervell i en el ronyó. Aquest fet pot representar una estratègia pel desenvolupament d'aliments enriquits en compostos fenòlics dirigits a una diana metabòlica concreta.

12. Després d'una ingesta crònica d'un extracte ric en procianidines administrat en rates a diferents dosis (5 mg/kg de pes, 25 mg/kg de pes i 50 mg/kg de pes), els conjugats glucurònid de catequina i epicatequina, seguits dels conjugats metil-glucurònid d'aquests monòmers, van ser els metabòlits de procianidines més abundants en plasma. Addicionalment, els conjugats glucurònid van ser detectats en tots els òrgans analitzats, múscul, fetge, teixit adipós blanc (mesenteric i perirenal) i teixit adipós marró. No obstant, cadascun d'aquests òrgans va mostrar un comportament diferent en front a l'acumulació de metabòlits respecte a la dosis d'extracte administrada. El teixit adipós marró va mostrar una clara dosis-resposta. En aquest teixit els metabòlits dels flavanols podrien tenir un paper important en la reducció o prevenció de l'obesitat a través de la modulació de la funcionalitat d'aquest teixit. Els resultats d'aquest experiment d'ingesta crònica ofereixen informació sobre les concentracions fisiològiques en teixit adipós dels metabòlits de

procianidines; aquesta informació podria ser útils en futures investigacions *in vitro* amb cèl·lules de teixit adipós.

Objectiu 5: Estudi de la capacitat de glucuronidació a nivell hepàtic.

13. La capacitat de glucuronidació hepàtica (UGT) sobre la quercetina és veu afectada per l'exposició, durant l'embaràs i la lactància, a una dieta rica en fructosa i greixos saturats, en rates femella de la generació parental i en rates femella de la generació filla. Donat que el desenvolupament ontogènic de la UGT hepàtica té lloc durant el primer període de la vida, una modulació dels enzims hepàtics podria generar una certa predisposició en el desenvolupament de malalties metabòliques en la vida adulta.

CONCLUSIONES

La hipótesis de partida de la presente Tesis Doctoral se centró en la necesidad del desarrollo de herramientas útiles para estimar la digestibilidad, biodisponibilidad, metabolismo y distribución de compuestos fenólicos y de sus metabolitos, como fase previa a los estudios clínicos. En relación con dicha hipótesis, y respondiendo a los objetivos planteados, los hitos conseguidos durante el desarrollo de la Tesis Doctoral se detallan a continuación y se estructuran en base a los objetivos de la tesis.

Objetivos 1 y 2. Modelos *in vitro* de digestión y fermentación colónica.

1. La aplicación de un modelo de digestión *in vitro* para el estudio de la digestibilidad y potencial bioaccesibilidad de procianidinas demostró que las moléculas de bajo grado de polimerización (dimeros y trimeros) son estables a las condiciones de las diferentes etapas del proceso de digestión, lo que demuestra su potencial bioaccesibilidad *in vivo*. Paralelamente, el proceso de digestión facilita la hidrólisis de las proantocianidinas de bajo peso molecular dando lugar a la formación de los monómeros, catequina y epicatequina.

2. A partir de la aplicación de un modelo sencillo de simulación de la etapa de fermentación colónica *in vitro* se han podido establecer las rutas metabólicas de forma individual de diferentes componentes de los grupos fenólicos procianidinas y flavonoides.

3. La aplicación de este modelo de fermentación colónica *in vitro* a una matriz alimentaria compleja, concretamente crema de cacao con un elevado contenido en hidratos de carbono y lípidos, ha permitido evaluar el efecto de la matriz alimentaria sobre el metabolismo fenólico de

procianidinas y otros flavonoides.

Objetivo 3. Desarrollo y validación de métodos de análisis de fenoles y sus metabolitos en muestras biológicas.

4. El desarrollo y validación de los métodos analíticos que combinan la micro-extracción en fase sólida (μSPE) y la cromatografía líquida de alta resolución acoplada a la espectrometría de masas en tándem (UPLC-MS/MS) ha permitido la identificación y cuantificación de metabolitos de procianidinas y de fenoles de aceite de oliva en muestras biológicas (plasma y tejidos) a bajos niveles de concentración, en el rango de nanomolar (nM) y micromolar (μM).

Objetivo 4. Metabolismo y distribución de procianidinas y fenoles de oliva.

5. El uso de ratas Wistar como modelo experimental pre-clínico se ha mostrado útil para los estudios de absorción, metabolismo y distribución en tejidos de metabolitos fenólicos, analizados tras una ingesta aguda de extracto rico en compuestos fenólicos (extracto rico en procianidinas o en fenoles del aceite de oliva), o de alimentos enriquecidos con extractos fenólicos (crema de cacao o aceite de oliva).

6. El análisis de plasma de rata obtenido a las 2 horas después de la ingesta de un extracto rico en procianidinas (5 g de extracto/kg peso de la rata) ha permitido observar un intenso metabolismo de fase II (glucuronidación, sulfatación y metilación) de catequina y epicatequina. Los principales metabolitos detectados en plasma han sido las formas glucuronidadas de catequina y epicatequina, y las formas metil-sulfato y metil-glucuronide de catequina y epicatequina. Es destacable la presencia de las formas

dimero y trimero en su forma libre.

7. En relación con la acumulación y distribución en tejidos de los metabolitos de procianidinas, se han detectado un elevado número de compuestos resultantes del metabolismo de fase II (formas conjugadas) y/o de la fermentación colónica (acidos fenólicos simples). Las formas glucuronidadas de catequina y metil-catequina han sido los metabolitos más comunes detectados en la mayor parte de los órganos estudiados. La principal acumulación de la forma glucuronidada de catequina se ha detectado en el pulmón, siendo destacable la acumulación en este mismo órgano de epicatequina en su forma libre. La principal acumulación de ácidos fenólicos simples, probablemente resultantes de la hidrólisis o de la fermentación colónica de procianidinas, se ha observado en el corazón. En base a los resultados de este estudio, se podría valorar el interés de la función de los ácidos fenólicos simples a nivel de tejidos diana, como metabolitos resultantes de la ingesta de procianidinas.

8. El análisis del plasma de rata obtenido a las 2 horas después de una ingesta de un extracto rico en compuestos fenólicos derivados del hidroxitirosol y de la oleuropeina (3 g de extracto/kg peso de la rata) ha permitido observar un intenso metabolismo de sulfatación, y en menor medida glucuronidación, principalmente de los alcoholes fenólicos y de los ácidos fenólicos; siendo el hidroxitirosol sulfato y el tyrosol sulfato los metabolitos derivados del metabolismo en fase II más abundantes en plasma.

9. La detección de diferentes metabolitos de hidroxitirosol y tirosol (principalmente su forma sulfatada y en menor medida la forma glucuronidada) en diferentes órganos ha permitido confirmar su

distribución a través del torrente sanguíneo, incluso atravesando la barrera hematoencefálica. Riñón e hígado han sido los órganos en los que se ha detectado una mayor concentración de metabolitos de tirosol e hidroxitirosol después de la ingesta de un extracto rico en compuestos fenólicos del aceite de oliva, mostrando un intenso metabolismo de detoxificación. Varios ácidos fenólicos y productos del metabolismo de los lignanos, como la enterolactona, han sido detectados también en diferentes órganos, principalmente en su forma sulfatada.

10. Parámetros relacionados con el diseño experimental, como son la composición de la matriz alimentaria en la que se vehiculizan los compuestos fenólicos, así como la dosis fenólica ingerida, han modificado los parámetros farmacocinéticos en plasma de los metabolitos fenólicos. La dosis permite modular la absorción, el metabolismo y la distribución en tejidos de los metabolitos fenólicos; altas dosis podrían incluso saturar el sistema enzimático de conjugación de los compuestos fenólicos permitiendo la presencia de formas libres en plasma. La composición de la matriz alimentaria, a través de la cual son vehiculizados los compuestos fenólicos, concretamente la presencia de grasa e hidratos de carbono, afecta directamente a la biodisponibilidad de las procianidinas y a su distribución en tejidos favoreciendo la absorción de formas monoméricas. Sin embargo, la presencia de grasa en la matriz alimentaria ha impedido la detección en plasma de las formas dimero y trímero.

11. La concentración de metabolitos fenólicos en plasma y órganos podría estar modulada por el hecho que, para una misma concentración fenólica, los fenoles estén de forma natural en el alimento o bien adicionados mediante un enriquecimiento. El estudio comparativo de la ingesta de aceite de oliva (5 g de aceite/kg de peso de rata) enriquecido

en compuestos fenólicos en relación con la ingesta de la misma dosis de un aceite de oliva virgen de alto contenido fenólico ha mostrado diferencias en la concentración plasmática de algunos metabolitos, como las formas sulfatadas de hidroxitirosol, de alcohol homovaníllico y de tirosol, siendo superior en las muestras de plasma obtenidas tras la ingesta del aceite de oliva enriquecido. Sin embargo, el efecto de la ingesta de un aceite de oliva rico o enriquecido en compuestos fenólicos sobre la disposición de los metabolitos fenólicos en tejidos depende del órgano estudiado. Después de la ingesta de aceite de oliva enriquecido se ha observado una mayor concentración de metabolitos de hidroxitirosol y tirosol en el hígado, corazón y bazo. Por el contrario, después de la ingesta de un aceite de oliva virgen de alto contenido fenólico se ha observado una mayor concentración de estos metabolitos en cerebro y riñón. Este hecho puede representar una estrategia para el desarrollo de alimentos enriquecidos en compuestos fenólicos dirigidos a una diana metabólica específica.

12. La formas glucuronidadas de catequina y epicatequina fueron los metabolitos mayoritarios cuantificados en plasma de rata obtenido tras una ingesta crónica durante 21 días de un extracto rico en procianidinas, administrado a diferentes dosis (5 mg/kg, 25 mg/kg y 50 mg/kg peso). Cabe destacar la presencia en plasma de dimero en su forma libre. En relación con los tejidos y órganos analizados (músculo, hígado, tejido adiposo blanco (mesentérico y perirenal) y tejido adiposo marrón) las formas glucuronidadas de catequina y epicatequina fueron los metabolitos más comunes. No obstante, la respuesta de cada órgano, en relación con la concentración de metabolitos depositada, fue distinta según la dosis de extracto administrada. El tejido adiposo marrón, que juega un papel importante en la reducción o prevención de la obesidad,

mostró una clara dosis-respuesta. Esta respuesta podría permitir la modulación de la funcionalidad de este tejido a través de los metabolitos de las procianidinas. Los resultados de este estudio ofrecen información sobre las concentraciones fisiológicas de metabolitos de procianidinas que se pueden alcanzar en tejido adiposo después de una ingesta continuada de extracto rico en procianidinas, información útil para futuras investigaciones sobre mecanismos relacionados con la regulación de la obesidad en modelos de líneas celulares de tejido adiposo.

Objetivo 5: Estudio de la capacidad de glucuronidación a nivel hepático.

13. La capacidad de glucuronidación hepàtica (UGT) sobre la quercetina es modulada por la exposición, durante el período de embarazo y lactancia, a una dieta rica en fructosa y grasas saturadas en ratas hembra de la generación parental y en ratas hembra de la generación hija. Dado que el desarrollo ontogénico de la UGT hepática ocurre en la vida temprana, una modulación de los enzimas hepáticos podría generar una cierta predisposición al desarrollo de enfermedades metabólicas en la vida adulta.

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