Maria Margalef Jornet

External and internal factors affecting the metabolism, bioavailability, tissue distribution, and bioactivity of grape seed flavanols

DOCTORAL THESIS

Supervised by Dr. Anna Arola Arnal

And

Dr. Begoña Muguerza Marquínez

Biochemistry and Biotechnology Department

UNIVERSITAT ROVIRA I VIRGILI

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UNIVERSITAT
ROVIRA I VIRGILI
Departament de Bioquímica i Biotecnologia
c/ Marcel·lí Domingo s/n
Campus Sescelades
43007 – Tarragona
Telèfon: (+34) 977 558 103
Fax: (+34) 977 559 528

FEM CONSTAR que aquest treball titulat **"External and internal factors affecting the metabolism, bioavailability, tissue distribution, and bioactivity of grape seed flavanols"**, que presenta la Maria Margalef Jornet per l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a Menció Europea.

Tarragona, 3 de juliol de 2015

La Directora de la Tesi Doctoral

Dra. Anna Arola Arnal

La codirectora de la Tesi Doctoral

Dra. Begoña Muguerza Marquínez

ALS MEUS PARES

“Serem allò que vulguem ser”

Miquel Martí i Pol

AGRAÏMENTS

Aquesta aventura va començar fa quatre anys. I sembla impossible, però ja s'acaba. Abans de començar-ne una altra:

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TABLE OF CONTENTS

RESUM

Els flavanols són compostos polifenòlics abundants en fruites i vegetals. Aquests compostos de la dieta milloren la salut al afectar processos cel·lulars i fisiològics. A més, el seu consum regular ha estat associat amb una baixa mortalitat i un baix risc de patir malalties cardiovasculars. No obstant, es creu que els efectes beneficiosos dels flavanols són deguts als productes del seu metabolisme. En aquest sentit, els flavanols que s'absorbeixen al nivell de l'intestí prim són reconeguts com a xenobiòtics, de tal manera que són ràpidament conjugats a formes glucuronidades, sulfatades i metilades per enzims de fase II tant a l'intestí prim com al fetge. Aquelles formes de flavanols que arriben al còlon són també metabolitzats per la microbiota a àcids fenòlics de baix pes molecular. Els metabòlits dels flavanols són redirigits a la circulació sistèmica per ser distribuïts al llarg del cos. Està descrit que diverses condicions afecten al metabolisme dels xenobiòtics, i en conseqüència dels flavanols. Per tant, l'objectiu d'aquesta tesi és determinar si el metabolisme dels flavanols , la seva biodisponibilitat i la seva distribució al llarg dels teixits es veu afectada per diferents factors externs i interns, afectant d'aquesta manera a la bioactivitat d'aquests compostos. Per això, es van administrar diferents quantitats de flavanols de llavor de raïm a rates sota diferents condicions experimentals i es van analitzar per HPLC-MS/MS les concentracions de flavanols i els seus metabòlits en plasma i teixits. Els resultats van mostrar que factors externs com la quantitat i la durada del consum dels flavanols, i factors interns com l'edat, el gènere i l'estat de salut de l'hoste afecten al metabolisme, la biodisponibilitat i la distribució en teixits dels flavanols de la dieta, afectant així a les bioactivitats fisiològiques d'aquests compostos. Paradoxalment, la durada del consum de flavanols no contribueix a la seva acumulació en els teixits.

ABBREVIATIONS

BMI: Body mass index

CBG: Cytosolic β-glucosidase

CE: Capillary electrophoresis

COMT: Catechol-*O*-methyltransferase

CVD: Cardiovascular diseases

DAD: Diode array detector

DOCA-salt: Deoxycorticosterone acetate

EGC: Epigallocatechin

EGCG: Epigallocatechin gallate

FLD: Fluorescence detector

FXR: Farnesoid-X-receptor

GC: Gas chromatography

GSPE: Grape seed proanthocyanidin extract

HPLC: High performance liquid chromatography

IHD: Index of hydrogen defficency

LC: Liquid chromatography

LDL: Low-density lipoprotein

LLE: Liquid-liquid extractions

LPH: Lactase phlorizin hydrolase

LSE: Liquid-solid extractions

m/z: mass-charge ratio

MeS: Metabolic Syndrome

miRNA: micro-RNA

MS: Mass spectrometry

MS/MS: Mass spectrometry in tandem mode

NEP: Non-extractable polyphenols

ABBREVIATIONS

NRM: Nuclear magnetic resonance

NSAID: Nonsteroidal anti-inflamatory drugs

PAPS: 3'-phosphoadenosine 5'-phosphosulfate

PAs: Proanthocyanidins

PCs: Procyanidins

Ph: Simple phenols

PhA: Phenolic acids

POL: Propan-2-ol

Q-TOF: Quadrupole hyphenated to a time of flight

Q-TRAP: Quadrupole coupled to an ion trap

QqQ: Triple quadrupole

ROS: Reactive oxygen species

S/N: Signal-to-noise ratio

SAR: Structure-activity relationship

SHR: Spontaneously hypertensive rats

SPE: Solid phase extractions

SULT: Sulfotransferase

TG: Triglycerides

TOF: Time of flight

UDPGA: Uridine-5'-diphospho-α-D-glucuronic acid

UGT: UDP-Glucuronosyltransferase

UHPLC: Ultra high performance liquid chromatography

UV: Ultraviolet

V: Valerolactone

VA: Valeric acid

I. INTRODUCTION

The Seven Countries Study was the first multi-country epidemiological longitudinal study to begin at the end of the 50s. It demonstrated that a diet rich in saturated fats increased cardiovascular disease (CVD) mortality risk [1]*. After that, in the early 80s it was observed that French people, having a high fat diet, did not present the high cholesterol levels to be expected as in all the other countries with similar dietary patterns. This fact was attributed to red wine consumption and was named as The French Paradox* [2]*. It is not known yet whether the cardioprotective effect of red wine is due to alcohol* [3]*, antioxidant compounds, also known as polyphenols, present in this beverage* [4]*, or the combination of both of them* [5]*.*

1. POLYPHENOLS

Plant secondary metabolism is not required for the plant to survive, as it is not essential for a successful growth and development, but produces metabolites that protect against herbivores, microbial infections, and are considered UV protectors, etc. Polyphenols, some of the most important plant secondary metabolites, are not essential compounds for short-term animal well being, but can have long-term benefits on the incidence of cancers and chronic diseases, including CVD, type-II diabetes, and the main modern-Western related diseases [6].

1.1. Classification and structure

The chemistry, biochemistry, of dietary polyphenols have been widely studied [7,8,6], and their classifications have been established by different criteria such as their source of origin, biological function, and chemical structure. The classification of polyphenols by their chemical structure [7,8] places them in to 2 main subgroups: flavonoid forms and non-flavonoid forms [6].

1.2. Flavonoids

Flavonoids are characterized by 2 aromatic rings (A and B) connected by a 3-carbon bridge (C-ring). This main 15-carbon skeleton can be sub-classified in flavones, isoflavones, flavanones, anthocyanins, flavonols, and flavanols (also called flavan-3-ols), depending on the hydroxylation pattern, the distribution of the C_6-C_3-C_6 structures, their index of hydrogen deficiency (IHD), and/or their no-hydroxylated functional groups (Figure 1).

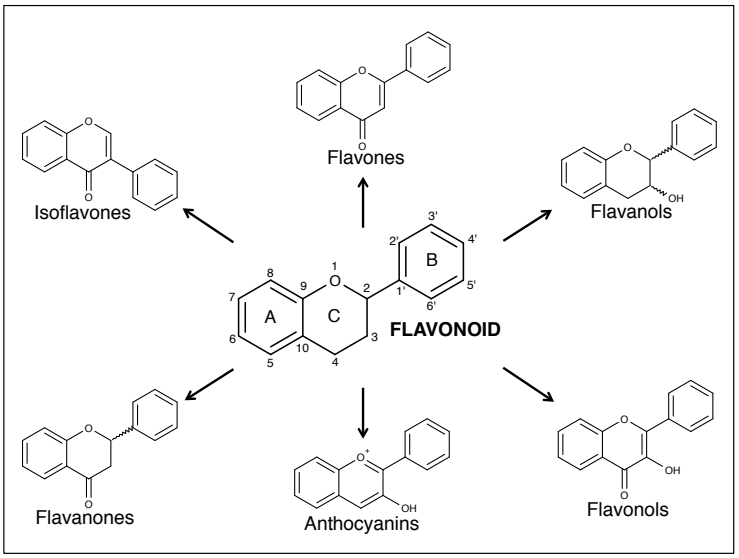

Figure 1. Flavonoids general structure and different flavonoid-type molecules depending on their hydroxylation pattern.

Flavones, with a lack of hydroxylation at C-3, and a double bound between C-2 and C-3, have several possible substitutions including hydroxylation, methylation, *O*- and *C*-glycosylation, and alkylation. Flavones are not widely distributed, found mainly in celery, parsley, and some herbs. Many of them occur as apigenin or luteolin 7-*O*-glycosilates, although their 8- and 6-*O*-glucosides can also be found in the rooibos tea [9]. Isoflavones differ to flavones because their B-ring is attached at the C-3 instead of the C-2 like

flavones and the other flavonoids. They occur mostly in leguminous plants such as soybean, being daidzein and genistein 7-*O*-(malonyl)glucosides [10]. Isoflavones are also classified as phytoestrogens [6] because of their structural similarities to estrogens. Naringenin, and hesperitin, both well-know flavonones, are characterized by the absence of the C-2 – C-3 double bound and the presence of a chiral centre at the C-2 position. This flavonoid-type structure is found mainly in citrus fruits as the *S*- or (-)-enantiomer with the C-ring attached to the B-ring at C-2 in the α-configuration [11,6]. They can occur as hydroxyl, glycosylated, and *O*-methylated derivatives, being hesperitin-7-*O*-rutinoside the most common flavanone glycoside [6]. Anthocyanidins form conjugates with sugars and organic acids producing several anthocianins with different colours ranging from orange to purple, being easily identifiable in fruits and flowers. The most common anthocyanins aglycones are pelargonidin, cyaniding, delphinine, peonidin, petunidin, and malvidin [12]. Flavonols, also regarded as 3-hydroxyflavones [13] can be found in almost all plant kingdom except in fungi and algae, kaempferol, quercetin, isorhamnetin, and miercin being the most common structures. They are mainly found as glycosides at the 5, 7, 3', 4', and 5' positions. Finally, flavanols, also known as flavan-3-ols, the compounds in which this thesis is based on, are the most complex subclass of flavonoids and will be extensively descried in the next section (Section 1.2.1). In brief, flavanols can be found in grapes, cocoa, apples, pomegranate, and red wine and occur in nature in their aglycone forms [14].

The different flavonoid-type structures, food sources, and the most important health effects are summarised in Table 1.

1.2.1. Flavanols

Flavanols, as described above, are the most complex subclass of flavonoids, which use to be found in nature in their aglycone forms or esterified with Gallic acid [14], contrary to other flavonoids which exist in plants basically in glycoside forms (Table 1). Flavanols range from monomers to their oligomeric and polymeric forms. Flavanol monomers, which are also known

as flavan-3-ols, have 2 chiral centres at C-2 and C-3 (Figure 1) lead to 4 different non-planar isomers, although the *R*-structures are the only forms present in nature [9].

Table 1. Main flavonoid families, sources, monomeric components and bioactivity examples

Flavonoid	Bioactivity Examples	Sources	Main Monomeric Components	Ref.
Flavones	Antioxidants Lipid metabolism Cancer	Lemons Olives red pepper parsley celery	Apigenin Rutine Luteolin Luteolin glucosides	15–17
Isoflavones	Antioxidant Breast cancer Fertility	Soya beans Legumes	Genistin Genistein Daidzin	15,18,19
Flavanones	Antioxidant Arterial stiffness Lung Cancer	Citrus fruits Peel of citrus fruits	Naringenine Naringine Taxifolin Hesperidin	6,15,16
Anthocyanins	Antioxidant Inflammation Cardiovascular disease Obesity control Diabetes	Cherries Grapes	Apigenin Cyanidin	20
Flavonols	Antioxidant Coronary heart disease Cancer Inflammation	Onions Broccoli Apples Tea Berries Grapes Red wine	Kaempferol Quercetin	6,15,21
Flavanols	Hypertension Diabetes Lipid homeostasis Inflammation Antioxidant	Green tea Black tea Grapes Grape seeds Red wine	(+)-Catechin (-)-Epicatechin Epigallocatechin gallate	8

In this sense, flavanol monomers (Figure 2) (+)-catechin, and (-)-epicatechin are the most common structures, and (-)-catechin, and (+)-epicatechin have a more limited distribution [22,14]. Flavan-3-ols can also be found as their gallated forms, such as (+)-gallocatechin, (-)-epigallocatechin, and (-)-epigallocatechin gallate (EGCG), depending on the position of the galloylated bond. In contrast, oligomeric and polymeric forms of flavanols, widely known as proanthocyanidins (PAs) or condensed tannins, are the most complex and studied subclass of flavonoids, and the main constituents of the phenolic intake in human diet [8].

Different structures of PAs can be classified owing to [23]: I) degree of polymerization, II) hydroxylation patterns, II) stereochemistry at the 3 chiral centres, and IV) locations and types of the inter-flavanic bond. Flavanol PAs can also be found as procyanidins (PCs) this name being attributed by their hydroxylation standard classification. They are polymeric forms of catechin and epicatechin units (with a specific hydroxylation pattern at [3, 5, 7, 3', 4'] positions, and an additional chiral centre at C-4 in both lower and upper units

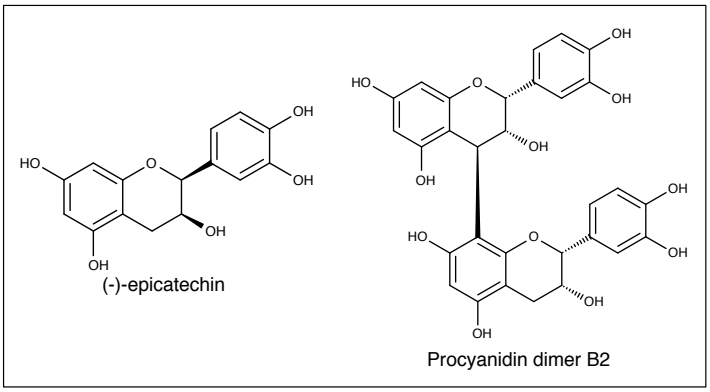

Figure 2. Flavanol monomeric and type B dimeric structures.

[6,8]. From now, all the flavanol polymers will be named as PAs (from the general classification for flavonoid polymeric structures). They can be classified as type A or type B PAs, being the last PAs type formed by oxidative coupling between the upper monomer C-4 and the C-6 or C-8 of the lower unit (Figure 2). The A-type PAs have an additional ether bond between the B-ring of one monomer and the C-ring from the other [6].

Flavanols which represent an important part of the polyphenols in the Mediterranean diet [24], have high concentrations in red wine, cocoa, green tea, fruits, and vegetables in general [25]. Grapes are considered as an important source of dietary flavanols due to their high intake, highlighting skin and seeds being especially suitable sources of these phytochemicals [26]. In fact, data released by the Agriculture, Food, and Environment Spanish Office, grape consumption reached levels of 99 M kg in April 2015 [27].

Epidemiological and cohort prospective studies support the thesis that flavanol-rich diets and high intake may be associated with a reduced

incidence of chronic diseases such as CVD, type-II diabetes, neurodegenerative diseases, and cancer among others [28–30]. However, it is difficult to conclude whether the beneficial effects can be directly attributed to a specific flavanol or flavanol rich foods as human studies have been performed using flavanol-rich foods that contain other nutrients and phytochemicals [31,32]. In addition, there is a possible relationship between the age of the target population and the CVD-factors protection effectiveness of cocoa flavanols [33]. Moreover, flavanol antioxidant activity is the most studied functionality, and the inhibition of the reactive oxygen species (ROS) formation; the ROS scavenging; and the antioxidant defences enhancing, being the best-described mechanisms [34–36]. In contrast, some roles involving cell signalling pathways, and epigenetic factors, such as DNA methylation, histone modification and miRNA regulation pathways have been recently studied as flavanol health promoting mechanisms [37]. In addition, there is also the suggestion that healthy effects of flavanols could be caused by an indirect health benefit provided by the regulation of gut microbiota [38].

The health benefits of specific flavanols are well documented in several diseases and/or pathologies such as hypertension [39–42]. For example, it has been reported that flavanol (-)-epicatechin is able to lower blood pressure, restore endothelial function through the nitric oxide (NO) mediated mechanism [43], whereas administration of EGCG had no effects on subjects with metabolic syndrome (MeS) [44]. These compounds also have beneficial effects on other MeS parameters such as inflammation produced by the diet [45], lipid homeostasis (i.e. triglycerides (TG), cholesterol and low-density lipoprotein (LDL)-cholesterol plasmatic and hepatic levels) [8], and glucose homeostasis [46,47]. For example, it has been reported that a grape seed PA extract (GSPE) reduces TG, apolipoprotein and LDL-cholesterol levels in plasma of healthy rats after an acute administration of the extract [48] and dyslipidemic rats after chronic administration [49]. Furthermore, it has been demonstrated that this GSPE hypolipidemic effect is caused by the activation of nuclear receptor farnesoid-X-receptor (FXR), improving the fatty acid oxidation over TG synthesis [50]. Other molecular mechanisms involved in the

health promoting effects of flavanols are those related to the modulation of mi(cro)RNAs regulators of lipid metabolism [51]. Regarding glucose homeostasis, flavanols act through different mechanisms such as insulin secretion increasing by pancreatic β-cells, lowering hyperglycaemia by the repression of glucose production in hepatocytes and/or reducing insulin resistance, and enhancing glucose uptake in muscle and adipose tissue [52,53]. Specifically, PAs are shown to exhibit, on short time scales, the same affect as insulin in rats [53].

1.3. Non-Flavonoids

The most important source of polyphenols is the flavonoids family, but the non-flavonoid compounds are also dietary significant. This group of compounds can be divided into phenolic acids and non-phenolic acids, Gallic acid [6], and resveratrol [54], respectively (Figure 3). They are examples of the commonest non-flavonoid structures with an important role in the polyphenol research, for their significant presence in foods [6] or their reported health effects [55–58].

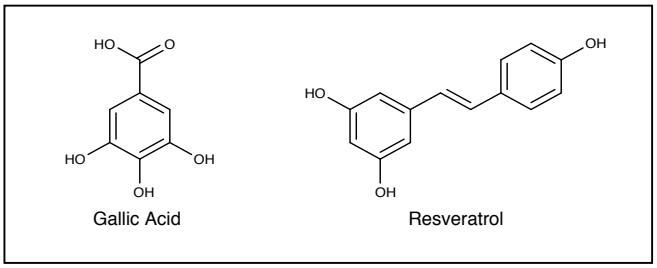

Figure 3. General structures of the main non-flavonoid compounds.

For example, resveratrol, also known as 3,5,4'-trihydroxy-*trans*-stilbene (Figure 3) is a stilbenoid produced by injured plants or after exposure to pathogens [55]. This stilbene is a polyphenol characteristic of red wine [59], although it can be found in several fruits like grapes, raspberries, mulberries, or blueberries [56] and in unusually high concentrations in the Japanese knotweed or Mexican bamboo [6]. The health benefits reported for resveratrol

ranges from cardiopreventive [60,61] to anticarcinogenic [56,62]. Some authors have also reported that resveratrol could have a tentative potential benefit in type-II diabetes [63].

Gallic acid, also known as 3,4,5-trihydroxybenzoic acid (Figure 3), is a C_6-C_1 phenolic acid that can be present in plants as its free form in some fruits [64,65], vegetables and white wine [66], or as a conjugate of flavanols such as epigallocatechin (EGC), epicallocatechin galate (EGCG), or PC dimer B2 gallate [41]. The health benefits attributed to Gallic acid have been reported for *in vitro* [67,68,57] and *in vivo* [69–71] studies, specially as anticarcinogen [72], cardioprotectant [58], lipid homeostasis modulator, and insulin secretor [73].

2. POLYPHENOLS ABSORTION AND METABOLISM

The digestion and fate of polyphenols and their related compounds after ingestion has been a topic of increasing research in recent years (Figure 4). It is widely know that after ingestion they are recognized as xenobiotics undergoing the characteristic detoxification metabolism, starting from the mouth. In this first step, polyphenols cause astringency, and their modification by salivary proteins is significantly restricted [74]. In the stomach, they can resist the acidic conditions and may be transported bound to other ingested plant polysaccharides.

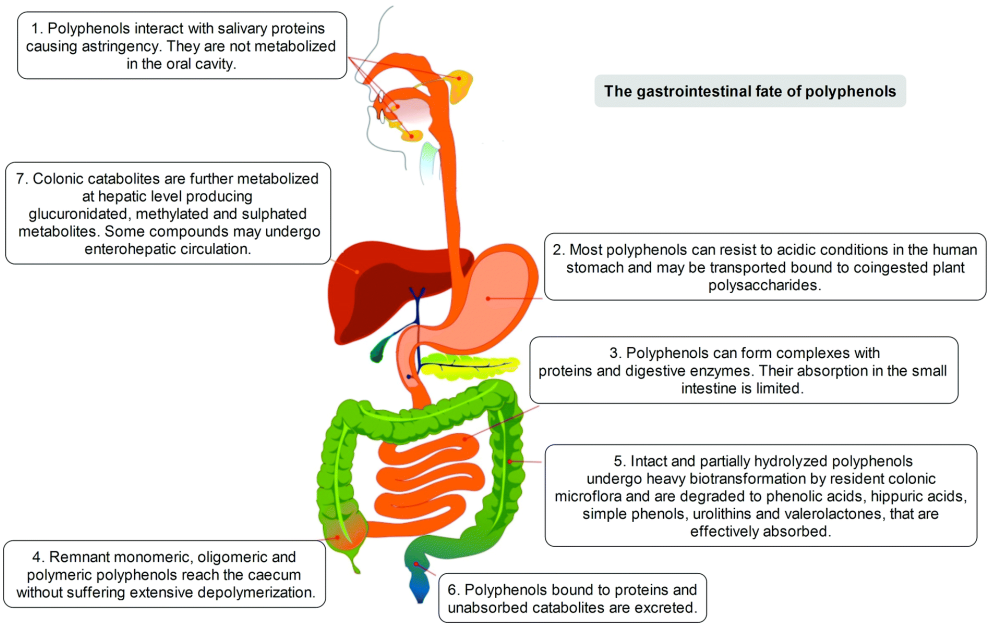

Figure 4. Physiological fate of dietary polyphenols after their ingestion. Extracted from Zanotti et al. 2015 [74].

The first organ strongly involved in their metabolic modification and digestion is the small intestine. Glycosilated polyphenols can become aglycones by the action of different hydrolysing enzymes, such as the intestinal lactase phlorizin hydrolase (LPH), and cytosolic β-glucosidase (CBG) [25]. Polyphenols

of a very low degree of polymerization (i.e. flavanol monomers) can be absorbed in the small intestine by both passive and facilitated diffusion [75]. In addition, it has been reported that polyphenol glycosides enter into the enterocytes by the active sodium-dependent glucose transporters (SGLT-1) [76]. Once absorbed, aglycones can undergo phase-II metabolism *in situ* or in the liver, producing their glucuronidated, sulfated, and/or methylated conjugates [6,77] (Figure 5). It has been estimated that only a small part of the ingested polyphenols are absorbed through intestinal cells (5-10%), independent of glycosilation. The remaining unmodified polyphenols (90-95%), especially those with a high degree of polymerization (higher than 2), and the conjugated metabolites excreted through the enterohepatic circulation, reach the colon where they are subjected to the microbial metabolism. In this manner small phenolic structures are able to cross the enterocytes and reach the liver where they can undergo phase-II metabolism [78,79]. After that, metabolites are transported through the systemic circulation to be distributed to different tissues and finally reach the kidneys to be excreted through the urine. Non-absorbed polyphenols and catabolites, with protein-bound polyphenols are excreted through the faeces [74,77].

2.1. Phase-II metabolism

Phase-II is defined, together with the phase-I metabolism, as the responsible processes for the biotransformation of xenobiotics and hence polyphenols (Figure 5). Phase-I enzymes (mainly cytochromes P450, CYPs) are those involved in the transformation of a parent compound to a more polar metabolite by unmasking/making *de novo* functional groups (i.e. -OH, -NH_2, -SH). They are also involved in biosynthetic processes [80].

Phase-II enzymes are those responsible for making more easily extractable forms of xenobiotics. They have an important role for the inactivation of pharmacologically active substances. The main purpose of phase-II biotransformation is to perform reactions such as glucuronidation, sulfation, methylation, acetylation and glutathione conjugations. Their respective

conjugates are more hydrophilic than the parent compound. The main phase-II enzymes involved in polyphenol detoxification are:

- UDP-glucuronosyltransferase (UGT)
- Sulfotransferase (SULT)
- Catechol-*O*-methyltransferase (COMT)

2.1.1 UGT (E.C. 2.4.1.17)

UGTs are a superfamily of membrane-bond enzymes generally linked to the endoplasmic reticulum having the substrate binding sites exposed to the lumen. They are key enzymes in the detoxification process responsible for glucuronidated metabolites production in all vertebrates (40-70% of all clinical drugs are subjected to UGT-glucuronidation in humans) [81] and some UGTs also have a biosynthetic role [82]. UGTs are not only responsible for the metabolism of xenobiotics (drugs, polyphenols) but also endobiotics (bilirubin, steroid-hormones, bile acids, and fat soluble vitamins) [83,84]. They catalyse the chemical bond between a nucleophilic atom (-O, -N, -S, or -C) with uridine-5'-diphospho-α-D-glucuronic acid (UDPGA), leading to the formation of D-glucuronides [85]. The glucuronic acid is in the α-configuration at the C-1 position when bound to the coenzyme (UDP). Specifically, when the transfer occurs, this configuration is inverted in order to form *O*-linked

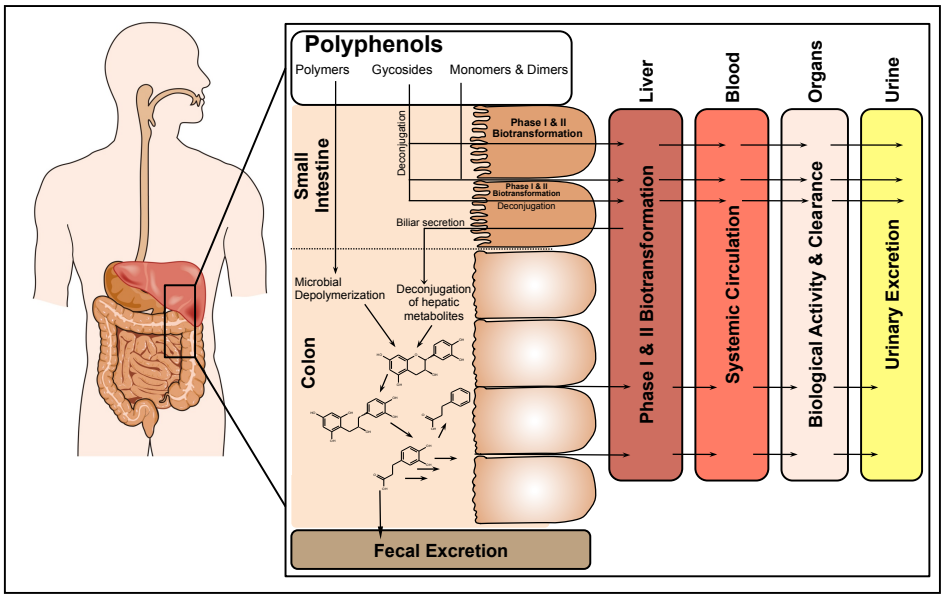

Figure 5. Representation of both phase-II and microbial metabolism of polyphenols.

glucuronides through the conjugation of glucuronic acid with the flavanol hydroxyls at the C-7, C-5, and C-3 positions [86,87]. Analysis of the crystal structure of human UGT predicted that human UGTs catalytic site uses the His35 to deprotonate the lipophilic substrate (such as flavanols), which is bound in the active site by ring-stacking interactions with Phe90. Then, the deprotonated flavanol plays as a nucleophile acceptor facilitating the nucleophilic attack at the of the glucuronic acid C-1 position. The resulting protonated histidine residue is stabilized by Asp151 [88] (Figure 6).

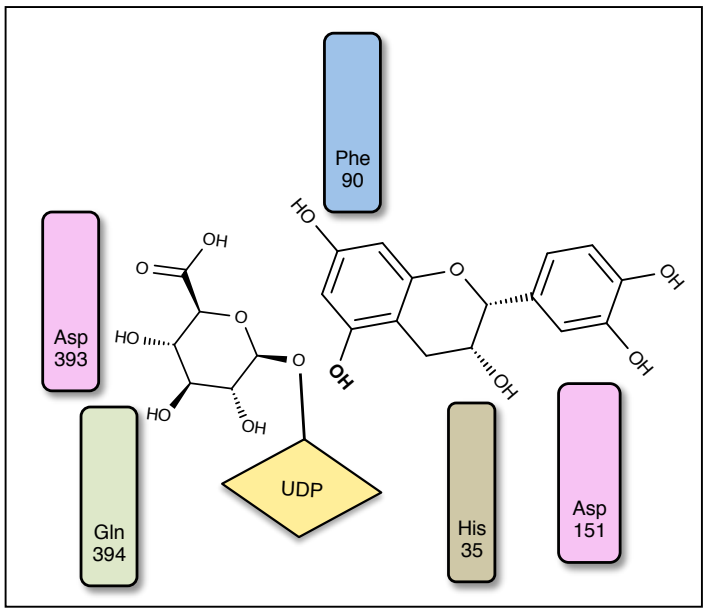

Figure 6. UDP-Glucuronyltransferase (UGT) catalytic site representation. Adapted from Radominska et al. 2010 [88]

Some studies have reported interspecies differences in UGT enzymes between human, monkey, pig, dog and rats [89]. It has been reported that mutations in UGT1A1 are the main cause of Crigler syndrome (hyperbilirubinemia in new-born children, giving them an orange characteristic colour) [90].

Mammalian UGTs are mainly located in the intestine, the liver (first pass metabolism of dietary supplements and drugs), kidneys, brain, pancreas, placenta, and nasal epithelium [86]. In addition, most of them exhibit an overlap

in substrate specificities, being UGT1A9, UGT1A3 [91], and UGT1A10 polymorphisms specific for the flavonoid glucuronidation process [84].

2.1.2 SULT (E.C. 2.8.2.1)

SULTs belong to a superfamily of enzymes that catalyse the conjugation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) with an -O, -N, -S acceptor group. PAPS, synthesized by almost all tissues in mammals, is a universal sulfate donor required for all sulfation reactions. The most important sulfation reaction is done in –O acceptor substrates (e.g. polyphenols). The hydroxyl group is located within the hydrogen bonding distance of the donor sulfonate group of PAPS and of the catalytic residue His108, in addition residues Phe84 and Phe76 form stacking interactions with the ligand. However, the side chain of Phe81, the gate residue at the active site, forms an unfavourable interaction with the ligand [92] (Figure 7).

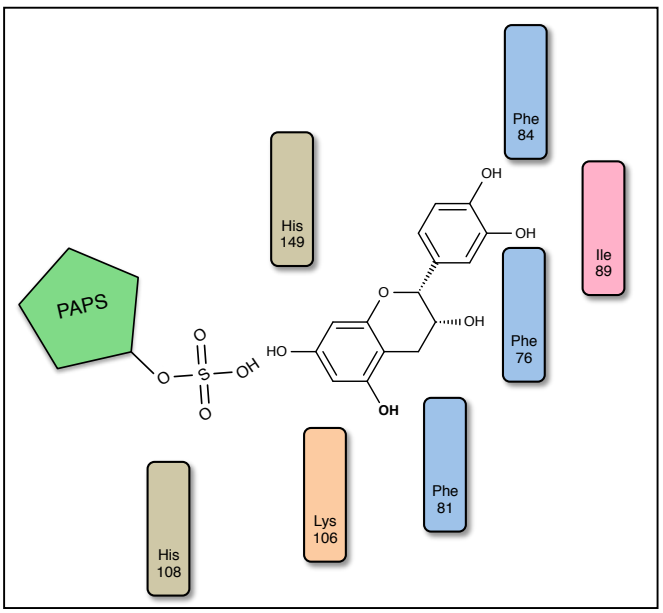

Figure 7. Sulfotransferase (SULT) catalytic site illustration. Adapted from Gamage et al. 2006 [92]

SULT enzymes are wide distributed in tissues such as intestine, jejunum, colon, and liver. In addition, these enzymes can also be found in blood

platelets, and tissues of the brain, adrenal gland, breast, lung, endometrium, placenta, prostate, ovary, testis, and kidney [86].

Two different types of the SULT enzyme have been identified:

- Cytosolic SULT: catalyse the sulfation of xenobiotics and small endobiotic molecules (i.e. steroids, bile acids, and neurotransmitters)
- Membrane-bond SULT: placed in the Golgi apparatus, they are involved in the sulfation of peptides, proteins, lipids, and glycosaminoglycans.

There are different substrate preferences for SULTs, even though there is evidence of substrate overlap at the level of families and subfamilies, SULT1A1, and SULT1A3 being recognized as phenol SULT enzymes [93,94].

Interestingly, several flavonoids such as curcumin, quercetin, and catechins have been described as a specific subfamily SULT inhibitors [95–97].

There is not much information about SULT interspecies differences, that said, some investigations have reported differences between human and rat SULT enzymes [98].

2.1.3 COMT (E.C. 2.1.1.6)

COMT is probably the most studied phase-II enzyme as it is a key enzyme in the degradation of catecholamine neurotransmitters [99], and plays an important role in the modulation of catechol-dependent functions, such as cognition, cardiovascular function, and pain processing [86]. This enzyme introduces a methyl group ($-CH_3$) from S-Adenosyl methionine (SAM) to one of the catechol-structure hydroxyl groups of several substrates including, apart from catecholamines, drugs and xenobiotics. This $-CH_3$ transference, always in the presence of Mg^{2+}, is more common in 3'-hydroxyl positions than in the 4'-hydroxyl positions because it acts through a direct nucleophilic attack, by one of the hydroxyl groups of the catechol substrate, on the methyl carbon of SAM [100]. Lysine residue (Lys144), is present close to one of the substrate hydroxyl groups, acting as a general catalytic base, and responsible of the proton acceptance. Subsequently, the methyl group from

SAM is transferred to the hydroxyl group. Mg^{2+} has an octahedral coordination to Asp141, Asp169, Asn170, both catechol hydroxyls, and a water molecule. Therefore, Mg^{2+} ions are in charge of the orientation of the catechol moiety. In addition, Trp38, Trp143, and Pro174 (the gate keepers) define the selectivity of COMT, keeping the planar catechol ring in the correct position (Figure 8). High activities of COMT have been found in intestine, liver, brain and kindney[101].

As described in SULT enzymes, two different COMT proteins have been reported for 1 gene [86]:

- Soluble COMT: Suspended in the cytoplasmic side of the intracellular membranes.
- Membrane-bound COMT: Located in the cytosolic side of the rough endoplasmic reticulum.

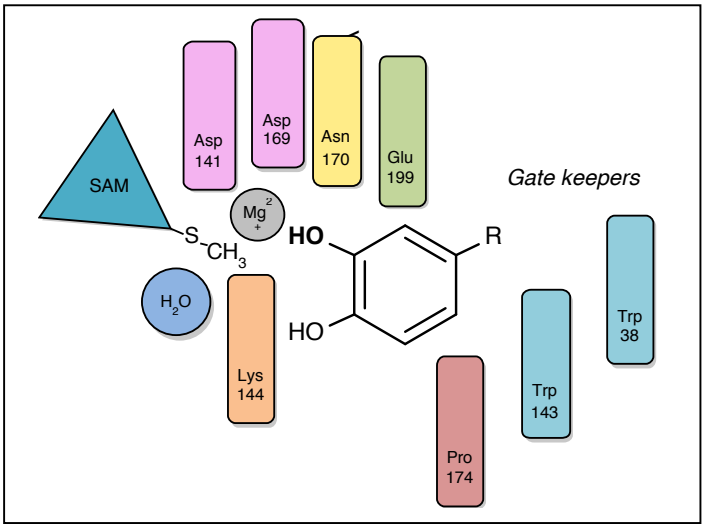

Figure 8. Catechol-*O*-methyltransferase (COMT) catalytic site. Extracted from Männistö et al. 1999 [99]

More recently, research has focused on the description of COMT inhibitors as drugs in the treatment of neurodegenerative diseases like Parkinson [102].

COMT inhibitors have also been found in green tea (i.e. quercetin, and catechins) [103–105].

COMT contributes to individual cognitive differences between animals and humans, as it is directly related to the dopamine concentration in the prefrontal cortex [86]. It has also been hypothesized that COMT activity has been decreased throughout intra-species evolution [101].

2.2. Microbial metabolism

As described above, polyphenols that have not been absorbed at the level of the small intestine, those with a degree of polymerization higher than 2, and the metabolites circulating through the enterohepatic circulation via the bile, reach the colon to be subjected to microbial metabolism. This creates small phenolic structures that can then reach the liver through the portal circulation, undergoing there the phase-II metabolism. These metabolites can also be excreted through the urine or transported to organs and tissues through the systemic circulation [77,6] (Figures 4 and 5).

The specific microbial metabolism of flavanols has been reported for *in vitro* studies, and is based on the ring fission of the main flavonoid structure (Figure 1), or the cleavage of the resulting aliphatic bound [106,107]. The main metabolites related to flavanol catabolism can be divided into three different families named as valerolactones (V), phenolic acids (PhA), and simple phenols (Ph) (Figure 9).

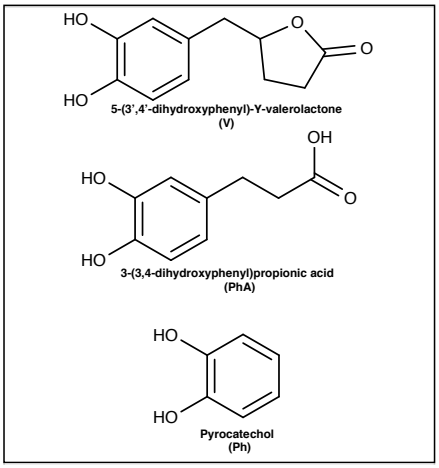

Figure 9. Main structures of flavanol microbial metabolism.

The V family results from the flavanol C-ring fission at the 1-2 and 4-10 positions, by an intermediate of C-ring open catabolites named as propan-2-ols (POLS) [107,108]. By acidic hydrolysis, V can be converted into valeric acids (VA), which by the α- and β- microbial oxidations can be transformed into phenylpropionic and phenylacetic acids, respectively [107,106]. It has been hypothesized that another catalytic route where the PA dimer B2 is converted into V or VA by another VA intermediate (with a double bound in its aliphatic chain) produced by the C-ring opened structure [107]. All of these microbial catabolites, reach the liver where they can also undergo phase-II conjugation being also glucuronidated, sulfated, and/or methylated [77,78] Figure 10 shows the proposed catabolic pathways for the formation of small molecular weight phenolic and phenyl carboxylic acid derivatives from the anaerobic incubation (37ºC) of (–)-epicatechin and PA B2 with human faecal microbiota (5% w/v) in vitro [108].

On the other hand, it has also been described that gallated flavanols undergo microbial catabolism by the cleavage of their galloylated bond, giving by these way the respective flavanol form (monomers or even dimers), and the respective Gallic acid residue [106]. Gallic acid, even being a small phenolic acid, can also undergo microbial metabolism by the microbial cleavage of the carboxylic acid bound, giving simple phenolic forms such as pyrogallol or catechol [25,109,110].

There is some evidence of the bidirectional effects regarding to the relationship between gut bacteria and polyphenols, as it has been shown that polyphenols are widely metabolized by the microbiota [25,78,111,106], but several studies have also reported that changes in gut microbiota can be made by polyphenols, resulting in other collateral health benefits [112].

Individual microbiota differences are widely observed within the same spices [113–116], and between different species [117]. For example, it has been suggested that in humans, Lactobacillus, Chlostridium, Eubacterium, and Bacteroides could have involved in the catabolism of flavonoids [118,119]. Recently, both *Eggerthella lenta* and *Fusobacterium plautii* have been reported as

responsible for the conversion of epicatechin to specific forms of V [120]. Rat microbial cleavage of EGC has also been attributed to *F. plautii* [121], *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, and *Slackia*

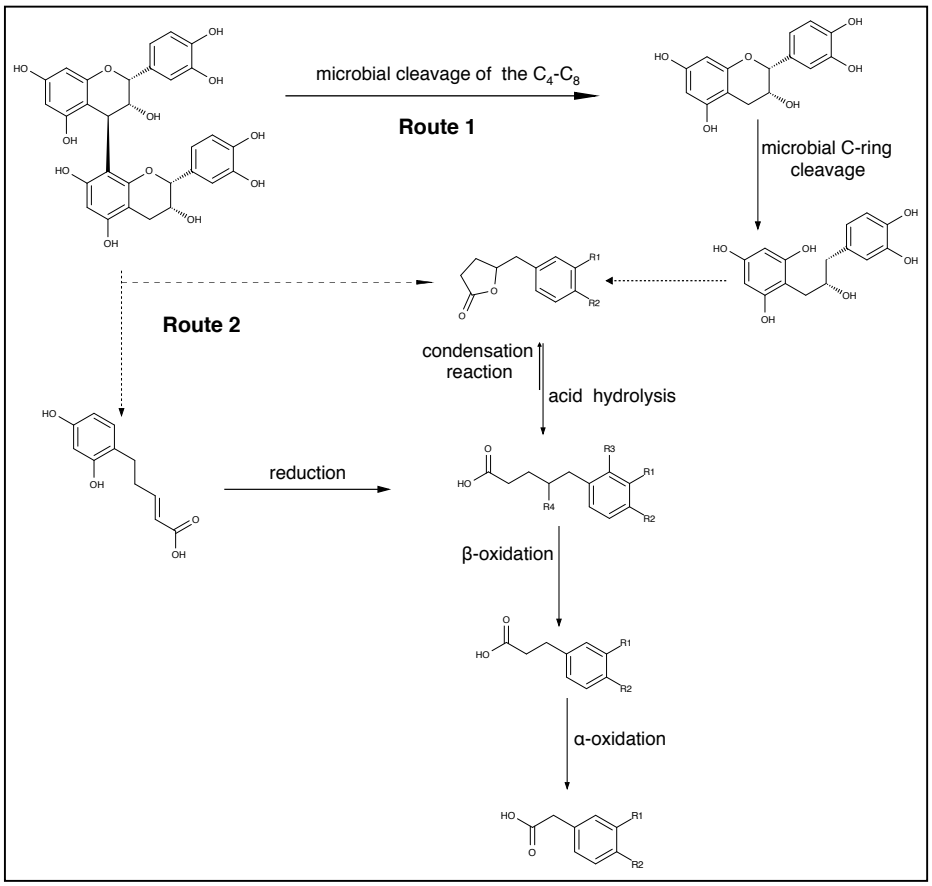

Figure 10. Proposed catabolic pathway for the formation of small phenolic derivatives from (-)-epicatechin and PA B2. Extracted from Stoupi et al. 2010 [108]

equolifacens [122].

3. METHODOLOGIES

A methodology that is able to identify and quantify the largest number of compounds present in the biological samples (fluids and tissues) is crucial to determine how polyphenols, and specially flavanols, are specifically metabolized and distributed though the body, which at the same time is essential to understand their physiological activity.

It is important to have different strategies for the determination of polyphenols in biological samples (Figure 11). They are divided into 2 main steps named as: sample pre-treatment, including the preparation of the sample and the flavanol metabolites extraction, sample quantification, including the separation and detection of each analyte.

The strategic procedure has to be taken into account (Figure 11) because even though polyphenols, and their metabolites, share a common phenolic feature there is significant structural diversity, especially in their metabolites, with different hydroxylation patterns, different kinds of conjugates, and their position in the parent structure. Although there have been recent advances in identification and analysis of polyphenols and their metabolites, there are still some key points to take in account for the quantification of these compounds in biological samples.

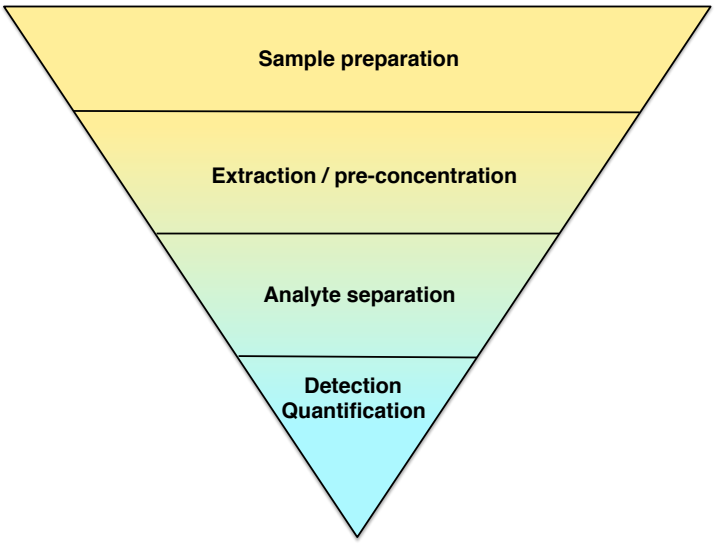

Figure 11. Methodological strategies for polyphenol determination in biological samples. Adapted from Stalikas et al. 2007 [123]

I. **Different products of metabolism.** Products of metabolism of a specific polyphenol, for example flavanols, can differ considerably depending on, for example, the degree of polymerization, leading to phase-II and microbial metabolites. This structural diversity is translated into compounds with different molecular weights, structures and polarities and leading to different behaviours during the analysis. Sometimes all possible metabolites are not commercially available, and their identification and quantification can be hampered.

II. **Complexity of each biological matrix.** The nature of each biological sample and its composition (i.e. water and fat content) differs significantly between matrices, resulting in differences when performing the sample extraction.

III. **Low concentration in the sample.** Flavanols and their metabolites are present at very low concentrations in biological samples as these are poorly absorbed. Thus it is essential to increase their concentration to ensure quantities adequate to preform subsequent analysis.

3.1. Sample pre-treatment

Before flavanols and their metabolites are extracted from the biological matrices, these samples need to be collected, reserved and prepared for extraction. To avoid degradation of the polyphenols, biological samples are always frozen before extraction and quantification [123]. In addition, for the analysis of the tissues, these samples are always lyophilized for two reasons: 1) to avoid the degradation of the analytes during the extraction [124], and 2) to avoid the error produced by the water content in these kind of samples (normalizing all the results by the weight of dried tissue). Temperature, light and oxygen exposure can also affect the polyphenol content [125]. In many cases, an antioxidant agent like ascorbic acid is added to the samples to avoid this oxidation process [123]. When liquid samples such as plasma or serum extraction is required, a pre-centrifugation process may also be

required to avoid interferences produced by collagen aggregates during extraction.

There are several methods available for the extraction of polyphenols from biological samples [126–130]. When the study is carried out on biological tissues, the most important challenge to overcome is obtaining all the analytes included in the study from the solid matrix, hence it is necessary to break down the collagen structure and free the analytes. The most common method of extraction includes a homogenization step in a saline solution [131,132] or in strong acids [133,134], followed by an organic solvent extraction. However, because of new methodologies and equipment with increased sensitivity, these kinds of extractions are not compatible, or more complex than necessary in order to eliminate the salts present in the solution.

In general, liquid-liquid extractions (LLE) or liquid-solid extractions (LSE) have been set as a starting point for these kinds of procedures. The use of organic solvents (methanol, ethanol, acetone, etc.), in this method, allows the liberation and removal of metabolites from the other components of the matrix, and especially during protein denaturalization. Samples can then be analysed directly and quantified, purified and concentrated using solid phase extractions (SPE) with conventional cartridges [135,136] containing a sorbent weight from 60 mg to 6 g [126]. The last procedure is the most frequent method used to remove as much as possible the non-polyphenolic remaining compounds that may interfere in the analysis of the biological sample. The most important disadvantage of traditional SPE is the sample volume, as this kind of technique requires volumes that sometimes are difficult to obtain, especially when the study is done in plasma samples, and more specifically in plasma from small animals like rodents. The latter, is the most important reason why in recent years, the introduction of μ-SPE methodologies have lead to a significant breakthrough in the study of the metabolism and bioavailability of polyphenols, and their use has been rapidly expanded [127,126,137–139]. The μ-SPE methodology allows not only the removal of interferences but also the concentration of the analytes under study, which leads to a decrease in the limits of detection, and the analytes isolation using

ultra-low elution volume, in this way eliminating the post-extraction evaporation and reconstitution steps, that may be time sensitive [126].

3.2. Sample quantification

The analytical technique used to perform the analysis, detection and quantification of polyphenols (in any sample) needs to be sensible, and selective enough to obtain a reliable quantification of the compounds that are found at low concentrations in the sample [140]. There are several techniques that can give the polyphenol content in biological samples, and the literature highlights the strong relationship between the technological evolution of the equipment and the enhanced knowledge of the fate of the polyphenols after ingestion and absorption [124]. The identification and quantification of polyphenols and their metabolites in biological samples are key in understand their metabolism and their possible health promoting effects.

3.2.1. Traditional methods of quantification

Initially, derived metabolites were studied using traditional methods for the identification and quantification of polyphenols such as capillary electrophoresis (CE), nuclear magnetic resonance (NMR) [141] and chromatographic techniques (gas and liquid, GC and LC respectively) [140]. However, some were not extensively used because of the equipment limitations, as is the case for CE where the detection of trace metabolites was hampered. In the case of NMR, limitations appeared because of the complexity of the samples and the difficulties during compound isolation. Nevertheless this technique had a major advantage, as it allowed structural information to be obtained which helped in the elucidation of the structure of multiple compounds generated after the polyphenols ingestion [142].

The main method used for the analysis of polyphenols and their metabolites was chromatography, preferably LC over GC for two main reasons: 1) the introduction of new and better ionization interfaces in the LC [143], 2) LC avoids the sample derivatization step crucial for GC [123]. The chromatographic equipment, where the real work of the separating all the analytes present in

the injected sample occurs, can be coupled to several detectors, such as ultra violet (UV) diode array (DAD), fluorescence (FLD), and/or mass spectrometry (MS) [144,145]. The major drawbacks of UV, DAD, and FLD are their poor specificity, and accuracy. They also are strongly dependent on the separation of the compounds in the LC to avoid interferences for a good and reliable quantification, for this reason significantly extending the time of analysis [146]. This last issue was relieved, in part, improving the chromatographic separations with the appearance of the high performance LC (HPLC) and the ultra high performance LC (UHPLC), which with the introduction of smaller chromatographic columns and adapting the equipment to high pressures, significantly reduced the time of each analysis (critical when the study requires the injection of a large number of samples) [139]. Chromatographic separation is often disregarded when it is in reality essential for a successful analysis [123].

3.2.2. New methodologies

HPLC and UHPLC represent the modern culmination of the development of LC, as they appeared as a result of the improvement in the packaging materials used as stationary phases in the chromatographic columns. Based on the van Deemter equation, which shows that, as particle sizes decrease, there is a significant gain in the efficiency which is not being reduced increasing the flow rates [147,146]. HPLC differs from the traditional LC in its internal pressures (50-350 bar), and the working columns used that are made with smaller sorbent particles (2-50 µm and 1-2 µm for HPLC and UHPLC respectively) giving higher resolutions [148,149]. The emergence of UHPLC led to an enhanced signal-to-noise ration (S/N), shorter times of analysis, and peak resolutions improvement [139,149].

Most of the studies in the 1990s measured total aglycones (total polyphenol content) in plasma and urine after the chemical or enzymatic deconjugation of polyphenol metabolites [150], because the requirement of authentic reference standards which are not still commercial. However, several studies are now able to report the polyphenol conjugate composition in biological samples

after the ingestion of pure polyphenols, polyphenol-rich foods or extracts [130,151,152,138]. This has been made possible because of advances in MS knowledge and technology in combination with high-resolution LC systems [150]. MS detection of bioactive compounds is based on the mass-to-charge ratio (m/z) and abundance measurements of a selected and ionized molecule, leading to the exclusion of other m/s and a higher specificity. Particularly, when the LC is hyphenated with a time-of-flight (TOF) MS the method is permitted to perform untargeted analysis, leading to detect any compound capable to be ionized. This method is usually suitable when new studies have to be carried out, in order to identify any compound in the injected solution when a method of quantification needs to set-up [143].

The use of MS in the tandem mode (MS/MS) has had great impact. This detection technique provides a hard ionization to the studied molecule giving a high degree of fragmentation, which can provide important structural information facilitating the identification of unknown compounds if they are compared to mass spectra libraries always obtained under identical operating conditions [143,153]. The detection and quantification limits are reduced using MS/MS, and the quantification of metabolites at very low concentrations are allowed. All these considerations have led to the scientific community to use different detectors such as triple quadrupole (QqQ), quadrupole coupled to ion trap (Q-TRAP), and quadrupole hyphenated to time of flight (Q-TOF) in the study of these compounds [154], giving the opportunity to perform targeted and/or untargeted metabolomics. Table 2 describes the different applications, advantages, and disadvantages of all available methodologies for the quantification of polyphenols and their metabolites in biological samples.

Table 2. Advantages and disadvantages of the main methodologies used for the identification and quantification of polyphenols

	Technological bases	Main Applications	Advantages	Disadvantages	Ref
HPLC-NMR	Active nuclei (^{13}C, or ^{1}H) absorb electromagnetic radiation	Polyphenol structure characterization	- Structure characterization of unknown compounds - Relatively rapid analysis	- Poor sensibility	142
HPLC-UV/DAD	Light absorption at a specific wave length	Polyphenol semi-quantification	- Do not need expert technicians - Cheap	- Long separation times - Poor selectivity - Poor sensibility	146
HPLC-FLD	Emission of a fluorescent signal	Polyphenol semi-quantification	- Do not need expert technicians - Cheap	- Long separation times - Poor selectivity (needs a strict chromatographic separation) - Poor sensibility - Need to include a fluorescent compound to the sample	146
GC-MS	Sample ionization	Polyphenol quantification	- High selectivity - High sensibility	- Sample derivatization - Not universal - Long separation times	123
HPLC-TOF	Sample ionization	Polyphenol quantification	- High sensibility - Analysis of complex matrices - Short separation times (UHPLC) - Untargeted metabolomics analysis	- Matrix effect - Poor selectivity - Difficulty for transferring methods to other detectors - Expensive technique - Not universal - Need expert technicians - High consumption of mobile phase	139, 143
HPLC-MS/MS	Sample ionization and fragmentation	Polyphenol quantification	- High selectivity - High sensibility - Direct injection of water soluble extracts - Analysis of complex matrices - Short separation times (UHPLC)	- Matrix effect - Absence of complete libraries and databases - Difficulty for transferring methods to other detectors - Expensive technique - Not universal - Need expert technicians - High consumption of mobile phase	139, 143

4. BIOAVAILABILITY AND DISTRIBUTION OF POLYPHENOLS

Several studies support the affirmation that polyphenols, and specifically flavanols, exert a positive modulation in health [155–158]. In addition, the bioactivity of polyphenols has been directly linked with their products of metabolism, the investigation of flavanols metabolism and bioavailability, a key point in understanding the mechanisms involved on their attributed antioxidant and other beneficial properties, it has been strengthened in recent years [151,159–164].

Metabolism, distribution, and bioavailability of polyphenols have been made, using different food sources [165–167], extracts [168–171], amounts (usually very high concentrations) [137,138,169,172], and experimental models (human, animal, or *in vitro*) [151,167,173,174]. In addition, some controversies have been discussed regarding the experimental conditions as in most cases *in vitro* and *in vivo* studies cannot be compared as they are both made with pure compounds or extracts, not paying attention to the products of the metabolism that reach the target tissues [150,175].

In this sense, some flavonoid metabolites (i.e. quercetin, and (-)-epicatechin conjugates) have been reported to be able to retain antioxidant properties and inhibitory oxidase effects *in vitro* and *in vivo* [87,176–178]. All of these facts force the scientific community to reconsider the experimental design when the bioactivity of polyphenols wants to be demonstrated in *in vitro* studies [150].

It has been extensively stated that polyphenol metabolism, plasma and tissue bioavailability are depending on several factors, thus their potential health effects could be consequently different depending on each situation. The metabolism of polyphenols is strongly dependent on two general factors [179]:

- I) External Factors: They can be subdivided into different subcategories such as the polyphenol structure, source, food

processing, quantity of ingested polyphenols, and the length of treatment.

- II) Internal Factors: Characterized by the polyphenol consumer particular biological situation, these factors can be subdivided into different specific situations such as the age, gender, and pathological state.

Unfortunately, until now, there are no specific and comparable studies that allow the comparison all of these factors for a complete study of polyphenol (and specifically for flavanols) metabolism, bioavailability, distribution, and bioactivity.

In addition, the study of the content of polyphenols and their metabolites in the target tissues is a key point in understanding the potential bioactivities that they can exert *in situ*. Many *in vivo* studies have revealed the distribution of polyphenols in different tissues under different conditions and using different polyphenol sources [128,134,138,180] and pure compounds [181,182], revealing that the plasma composition differs significantly with the distribution of polyphenols and their metabolites through the body. As stated in Section 2.1, phase-II enzymes can be located in tissues other than the main metabolic ones (the liver and the intestine) [86]. Moreover, it has been reported that there could be a putative deconjugating enzyme in the endothelial cells which is the responsible to deglucuronidate the epicatechin-glucuronide conjugate to form free epicatechin, which could be responsible for lowering blood pressure an known effect of flavanol-rich fruits [183]. These facts have to be taken into account, as conjugation or deconjugation of metabolites in the target tissues is on of the most important key points for the elucidation of real mechanisms involved into the flavanol health benefits.

Some studies reported techniques for the direct delivery of polyphenols to the target tissues in order to avoid the detoxification metabolism. For example, by the direct injection into the blood stream, infusion in the target organ, by subcutaneous injection, or by three different systems (nanoparticle, liposome, and microemulsion) to the intracellular organelles, the desired polyphenol is

delivered, unchanged, to its target tissue. [184]. However, this may not be the best strategy to study dietary polyphenols, if they are wanted to be used as dietary supplements for their health promoting effects, and their preventive activities.

4.1. External Factors affecting the metabolism and bioavaiablity of polyphenols

There are several external factors that may interfere with the polyphenols metabolism, including environmental factors that can have an important role in the polyphenol content of foods. Polyphenol content may undergo an important quantitative and qualitative variation depending on the harvest season or the environmental conditions under which the fruits and vegetables are subjected during the cultivation [139]. Herein, the external factors that may affect the polyphenols metabolism have been divided into main 5 different categories: structure, source, food processing, quantity of ingested polyphenols, and length of treatment.

4.1.1. Structure

As described above, polyphenols, and especially flavonoids are characterized by their main structural skeleton, and classified depending on their degree of hydroxylation, the presence of the conjugated C-ring by a double bound between C-2 – C-3, and their ketonic functional group in the C-4 position [6,7]. Phase-II metabolism is the responsible of the conjugation of polyphenols in order to enhance their excretion through the urine by the conjugation of specific sites (C-3, C-5, or C-7 for glucuronidation and sulfation); and C-3', or C-4' for methylation) (Figure 1). In addition, the degree of polymerization will govern the involvement of the microbial metabolism [77,78].

Several structure-activity relationship (SAR) studies have revealed the important role of the position and the number of substituents in the polyphenol basic structure and their close connection to their antiproliferative,

cytotoxic, antioxidant, and inhibiting/activating activities [67,104,185–188]. In addition, it is widely known that plasma metabolites composition differs significantly from the compounds present in foods [6,25,34,150]. For this reason, a through study is required, in order to elucidate polyphenols, especially flavanols, and their metabolism, bioavailability, and bioactivity.

4.1.2. Source

Dietary flavonoids are the main polyphenolic source in vegetables and fruits and their distribution is highly variable depending on food type (Table 1). Food matrix effect studies are increasing in the recent years, as there is an important interaction between polyphenols and some food components such as protein, carbohydrates, fat, water, fiber, and alcohol that may affect to their absorption and metabolism [189]. In addition, non-extractable polyphenols (NEP), which are made up of macromolecules, and single phenolic compounds associated with macromolecules, cannot be detected in foods by the usual analytical procedures as there is no solvent that would be suitable for the extraction of all the antioxidants present in foods, especially those associated with complex carbohydrates and proteins as monomers and complex polymers [190]. Monomeric and polymeric forms of polyphenols cannot be quantified using the same chromatographic separation, being the first ones quantified by reversed phase LC, and the last ones by normal phase LC [191]. Therefore, this last group (the polymeric forms) represent a significant fraction of polyphenols that are metabolized by gut microflora and are also reported to increase antioxidant and antiproliferative capacities, reduce the intestinal tumorgenesis, and modify the intestinal gene expression [192].

It has also been reported that lactic matrices [193], sugar [194], fat [195] and carbohydrate content [196] in foods may affect the phenol-metabolic profile, even it has been reported that the absorption of quercetin, catechin, and resveratrol seems to be equivalent in humans using different food sources [197]. More specifically, milk, has been described to exert negative effects on the bioavailability of PAs as they can be linked with lactic proteins, in this way becoming exempt to the gastric breakdown in these kind of matrices [198], or

might hamper the PAs absorption increasing the gastric pH [199]. The absorption of epicatechin has been studied in one crossover study, which concluded that this was inhibited by the interaction between milk proteins and PAs from cocoa [161]. In contrast other studies, demonstrated, that epicatechin concentrations in plasma did not differ between lactic and water-based cocoa beverages under the same nutritional conditions [193,161], and that flavanol rich cocoa milk consumption did not show differences on the absorption of flavanol monomers. In addition, there are descriptions of higher flavanol bioavailability by increased levels of their metabolites in urine and plasma after the ingestion of PAs-rich cocoa powder dissolved in milk [168] .

The high quantity of different polyphenol-rich foods, requires a strategy to study the metabolism and distribution of these compounds and their related bioactivity and how they might be used as extracts and/or pure polyphenols, in order to decrease the interaction of the different components from the food matrix, and to have a more realistic view of the ingested-bioactive amounts of compounds.

4.1.3. Food processing related factors

Several factors affect the content of polyphenols in foods during processing, and consequently their metabolism and bioavailability are both affected [179]. For example, the polyphenol content is significantly influenced by thermal treatments [200,201,168] and other food processing factors such as freeze drying, air-drying and storage [202]. Recently, the effect of cocking on the polyphenol content has also been evaluated in vegetables [203]. Technical processes, such as homogenization [204], and vaporization [205], may also increase the bioavailability of polyphenols.

The high quantity of information about the food processing and the polyphenol content, has allowed the updating of a database on the effects of food processing on polyphenol content which facilitates a more accurately estimation of polyphenol exposure from dietary surveys [206].

4.1.4. Quantity of ingested polyphenols

The hormesis concept is defined as the dose response phenomenon of a toxic or xenobiotic compound, characterized by low dose stimulation and a high dose inhibition [207]. Vitamins and oligoelements are not only beneficial at low quantities, but are also essential for the health [208]. Most drugs are described as hormetic compounds, leading to beneficial health effects at low doses, or giving the opposite desired effect when administered at high doses [209].

Once ingested, polyphenols are recognized as xenobiotics, along with most drugs. Therefore, their metabolism could be comparable in order to provide the most reliable, and realistic amount of ingested polyphenols needed to achieve the maximum bioactivity [210]. The absorption, metabolism and bioavailability of polyphenols depending on their consumption, have been widely studied in recent years [211,172,212,213]. Interestingly, it has been reported that after digestion, the metabolized compounds can lose their original properties or even acquire new activities [214]. In fact, the uptake and metabolism of polyphenols is usually associated with their methylation, sulfation, or glucuronidation by phase-II enzymes [77,109,215]. Moreover, considerable quantities of ingested flavanols are degraded by colonic microbiota upon reaching the large intestine, where they yield other smaller molecules that are also absorbed into the body [6]. Specifically, some studies have demonstrated that after conjugation, flavanols are distributed throughout the body and are found at considerable concentrations in most tissues after an acute intake of a PAs extract [138,169,170]. In addition, it has been shown that, the intake of large amounts of polyphenol-rich products is not directly linked to the concentration of these compounds and their metabolites in blood and tissues [15].

In fact, it has been reported that dietary polyphenols may act hormetically, giving cytoprotective effects at low ingested amounts and the over generation of reactive and damaging intermediates with pathological consequences at high ingested amounts (acting as pro-oxidants) [216]. Interestingly, grape seed

derived PAs have also been reported to exert dose-response activities reducing postprandial lipemia [217], or blood pressure [41] *in vivo*, with insulinomimetic activities insulin-sensitive cell lines [218] *in vitro.* However, bioavailability studies made with grape seed flavanols have been realized mainly using 1000 mg/Kg [129,138,169].

Therefore, realistic bioavailability studies should be made in order to elucidate whether the flavanol metabolism and bioavailability differs between high and the real administrated or bioactive amounts.

4.1.5. Consumption length

There are several pharmacokinetic studies about polyphenol metabolism and distribution in humans [109,196] and animal models [137,152,219]. Most of them reveal that the highest concentrations of polyphenols and their phase-II metabolites in plasma and most tissues are reached between 1 and 4 h after the ingestion of the polyphenol source [137,152]. Acute *in vivo* studies, for example, reported that the most effective effect of flavanols is reached at short times [49,220,221] being reversed on longer time scales [41,196] after their ingestion. Bioavailability studies made after an acute intake of a flavanol extract, reported that these compounds are conjugated to their phase-II metabolites, which are then distributed throughout the body and are found at considerable concentrations in tissues on short time scales [152].

Alternatively, it has been reported that regular consumption of flavonoids in the human diet has a direct association with beneficial health effects for people suffering from several diseases [42,222,223]. However, few studies have evaluated the tissue distribution of flavanols after long-term ingestion [170] or reported the accumulation of polyphenols in tissues after the acute administration of flavonoids, although this accumulation was evidenced on short time scales after ingestion [224,225]. Some compounds, however, such as fat-soluble vitamins or some toxins, accumulate in some organs or tissues after chronic ingestion [226,227].

Therefore, studies assessing the distribution, and accumulation of flavanols after a long-term ingestion need to be done in order to elucidate if flavanols

are accumulated in the tissues over longer time scales, or if the distribution of these compounds differ between acute and chronic studies.

4.2. Internal factors affecting the metabolism and bioavailability of polyphenols

Internal factors, also known as host-related factors, that may affect the polyphenol bioavailability have been divided into intestinal and systemic factors [179]. However, there are bidirectional interactions between these factors, as intestinal factors (enzymatic activity, intestinal transit, and microbiota) are strongly dependent on the systemic conditions and vice versa. For this reason, in this thesis both factors are unified in the common one. Therefore, the host-related factors affecting the metabolism, bioavailability, distribution, and bioactivity of polyphenols have been divided into 3 different categories: age, gender, and health conditions.

4.2.1. Age

There is an increased inter-individual variability directly linked with the age, that may be partially explained by reduced homeostatic ability causing a disruption of some regulatory processes related to the functional integration between cells and organs. Consequently, failures in the maintenance of homeostasis under physiological stress appear [228]. Several of these changes have pharmacokinetic implications [229]. Glomerular filtration rate [230], liver volume and apparent liver blood flow [231] decrease in ageing. There is also a progressive reduction in total body water and lean body mass, resulting in a relative increase in body fat along time [232], and changes in pharmacokinetics and pharmacodynamics with advancing age, including a reduction in renal and hepatic clearance and an increased prolongation of elimination of fat-soluble drugs. In addition the increased sensitivity of specific kinds of drugs such as anticoagulants, cardiovascular and psychotropic drugs are also relevant characteristics in elderly subjects [228]. Lee *et al*. (2008) not only reported that liver expression of xenobiotic metabolizing enzymes depended on the age, but also that several of these enzymatic activities varied also [233].

Although few gastrointestinal functions decline to a significant extent in healthy ageing [234], it leads to physiological changes that affect oral and oesophageal function, gastric pH and emptiness rate and intestinal transit times [235]. In addition, major changes in bacterial population that include metabolically active groups occur [236,237]. This could lead to important changes in the biochemical capacity of the gut. In addition, it should be highlighted that faecal studies demonstrated great variability in bacterial populations in the elderly [238].

Polyphenols have been reported to exert beneficial effects against the most common age-related diseases such as type-II diabetes [47], cardiovascular diseases [42], Alzheimer [239,240], and Parkinson [30,241], among others.

In regards to flavanol metabolism and distribution and their relationship with the age, until now only one human study has been found, where no age-related differences were found in plasma or urine after the ingestion of cocoa flavanols [242].

4.2.2. Gender

Males and females are differenced by several factors such as the fat and the water content as an example of general physiologic features [243], and hormonal activity as the main metabolic feature [244]. Several numerous sex-related differences in both humans and other mammals have been shown in processes such as lipid and glucose metabolism [245], psychiatric disorders [246], coronary artery disease [247], susceptibility to inflammatory [245] and infectious diseases [248]. There are also sex-related differences in the level of protective health effects imparted by the moderate consumption of alcohol [249]. Furthermore, it has been observed that responses to xenobiotics are different between genders. For example, male rats have been observed to metabolize xenobiotics faster, and to have higher phase-II detoxification enzyme activities compared to female rats [250]. Additionally, female rats are known to have less cytochrome P450 (CYP), which facilitates the detoxification and excretion of xenobiotics (phase-I metabolism), than male rats [243].

Interestingly, phase-II enzymes, specially COMT, has been linked directly to the metabolism of catechol-estrogens [251], demonstrating that the are significant differences in the COMT activity between males and females in different tissues such as liver or brain, the female-COMT being more active than the male one [243,246]. Sex-related differences have also been reported regarding to the UGT [252,253], and for SULT enzymatic activities [253,254]. In addition, some studies revealed sex-related factors in the metabolism of quercetin showing greater metabolites in males urine, than in females, with the exception of quercetin sulfate, and quercetin glucuronide sulfate, which were found at higher concentrations in females 24 h urine [255]. Another gender related study demonstrated that females are more efficient at metabolizing resveratrol and pterostilbene than males, showing altered enzymatic specificity of UGT, when male and female human liver microsomes were incubated with these polyphenols for 1 h [256].

Therefore, a complete *in vivo* study is required in order to assess in an holistic way the differences in metabolism, bioavailability, and distribution of polyphenols, specifically flavanols, between males and females.

4.2.3. Health conditions

Physiological conditions such as the body mass index (BMI), the fat and lean body content may affect not only the metabolism of all the ingested foods or drugs [257,258], but also the metabolism of polyphenols. Obesity is one of the most important metabolic disorders that may cause chronic diseases such as type-II diabetes, hypertension, or atherosclerosis, and is also one of the required conditions for the diagnosis of MeS [258–260]. In addition, it has been reported that the phase-II metabolism may also be altered by different metabolic diseases as activities of phase-II enzymes in the intestine, liver, or kidneys have also been shown to be disrupted [86,261].

Gut metabotype, defined as individual bacterial distribution or phenotypic metabolism [262,263], has been studied in the recent years as the study of complex metabolite profiles in biological samples, may provide an approach to understand the global metabolic regulation of the organism in relation to

this peculiar pathology [264], and also for the increased importance of personalized nutrition and the development of functional foods [262,265]. Metabolic profiling has revealed gut microbiome differences depending on the diet [266], the lifestyle [267], even on the pathology of such diseases as type-II diabetes [268], atherosclerosis [269], and hypertension [267]. In addition, gut microbiota profile has also been directly linked to the immune system [270], or colorectal cancer [271], among others.

Polyphenols have been reported as microflora-modulators [272–274]. Their microbial metabolites have also shown health promoting effects as antioxidants [275,276]. As well as polyphenol metabolism is significantly altered depending on the microbiotic profile, and their beneficial health effects might also be disturbed.

Finally, peripheral tissues related pathologies, and specifically non-metabolic related pathologies, are mostly characterized by a mutation on a specific gene [277], or specific polymorphisms [278]. Therefore, it could be possible that the polyphenol metabolism was not altered between healthy and diseased subjects, but the distribution of the products of their metabolism was different in the target and injured tissues because for example an abnormal state of transporters, located inflammation, or disrupted localized deconjugating or conjugating enzymes.

5. REFERENCES

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BOOK CHAPTER:

Analytical Methods for the Identification of Physiologically Bioactive Forms of Food Flavonoids

Anna Arola-Arnal[1*], Manuel Suárez[1], Maria Margalef[1], Gerard Aragonès[1], Lluís Arola[1, 2], Cinta Blade[1], And Begoña Muguerza[1, 2]

[1]Nutrigenomic Research Group Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, Spain. [2] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Catalonia, Spain

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1. INTRODUCTION

Polyphenols are plant secondary metabolites that form one of the major groups of phytochemicals. These compounds are widely dispersed throughout the plant kingdom and are ubiquitous constituents of fruits, vegetables, cereals, olives, dry legumes, chocolate and beverages such as tea and wine. Polyphenols are divided into flavonoids and non-flavonoids. The non-flavonoid group includes several subfamilies depending on the number of phenol rings that they contain and the structural elements that bind these rings to one another. According to the basic structure of their skeletons, they can be differentiated in phenolic acids, acetophenones and phenylacetic acid, hydroxycinnamic acids and coumarins, naphthoquinones, xanthones and stilbenes (reviewed by Crozier *et al*., 2009). Flavonoids (from the Latin word *flavus* for "yellow") are the largest group of phenolic compounds; more than 6,500 flavonoids have been identified.

2. FLAVONOID CLASSIFICATION

The chemical structure of flavonoids is based on a nucleus of three phenolic rings referred to as A, B, and C rings (Fig. 1). Specifically, the flavonoid basic structure is a 2-phenyl benzopyrone in which the three-carbon bridge between the phenyl groups is usually a cyclised oxygen. Flavonoids can be differentiated into 6 subfamilies according to their degree of unsaturation and the oxidation level of the oxygenated heterocycle. Flavanols (essentially, flavan-3-ols) and anthocyanidins have a heterocyclic pyran as their C ring and are the most relevant for the human diet. In contrast, flavonols, flavones, flavanones and isoflavones have a pyrone as their C ring (Aherne and O'Brien, 2002).

In addition, the basic flavonoid skeleton can undergo numerous substitutions, including glycosylation, hydrogenation, hydroxylation, malonylation, methylation, and sulphation. Hydroxyl groups are usually present at the 4-, 5- and 7-positions, sulphate groups and glycosides increase the water solubility of flavonoids. In contrast, methyl and isopentyl groups make flavonoids lipophilic. Flavonoid molecules that are not attached to sugar moieties are referred to as being in the aglycone form, whereas flavonoid molecules attached to sugar moieties are called flavonoid glycosides.

Finally, different studies have revealed the important role that the structure of flavonoid molecules plays in their biological function; the position and number of substitutions in the flavonoid basic structure significantly affect the antiproliferative, cytotoxic, antioxidant, anti-inflammatory and anti-enzymatic activities of such compounds (Amic *et al*., 2007; Guerrero *et al*., 2012).

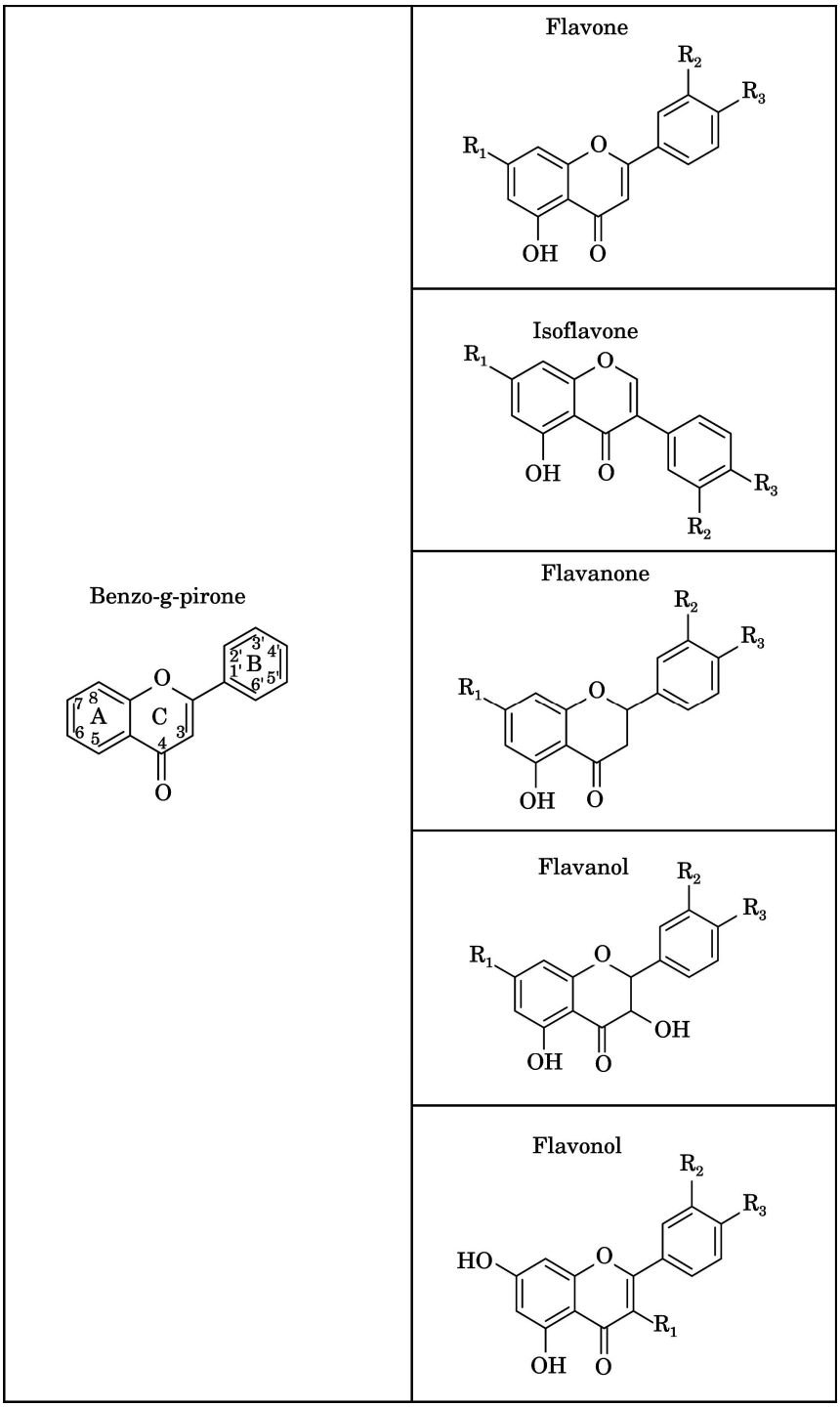

Fig. 1: Structures of the different subfamilies of flavonoids.

berries or cocoa flavonoids lowered the blood pressure (BP), whereas administration of hesperidin or epigallocatechingallate (EGCG) had no effect. In animal studies, a reduction in BP appears to be mechanistically related to improved endothelial function and increased NO bioavailability induced by flavonoids (Galleano *et al*., 2012).

Flavonoid-rich foods and extracts have been shown to reduce the levels of plasma triglycerides (TG), total cholesterol and low-density lipoprotein (LDL)-cholesterol in humans with metabolic syndromes and in rodents (Bladé *et al*., 2010). In addition, a grape seed proanthocyanidin extract (GSPE) reduces plasma TG, apolipoprotein B and LDL-cholesterol concentrations in healthy rats given an acute oral dose (Del Bas *et al*., 2005) and in dyslipidemic rats after chronic administration (Quesada *et al*., 2009). This hypolipidemic effect of GSPE is mainly due to the activation of the nuclear receptor farnesoid-X-receptor (FXR) in the liver, which favours fatty acid oxidation over triglyceride synthesis (Del Bas *et al*., 2009).

Recently, miRNAs have also been described as molecular mechanisms by which flavonoids regulate lipid metabolism. Specifically, proanthocyanidins, EGCG and quercetin repress miR-33a and/or miR-122, two miRNAs that control lipid metabolism. Using Nuclear Magnetic Resonance, it has been evidenced that flavonoids bind directly to these miRNAs and that the binding features depend on the flavonoid's chemical structure (Baselga-Escudero *et al*., 2014).

Epidemiological, animal and *in vitro* studies support the beneficial effects of dietary flavonoids on glucose homeostasis (Wedick *et al*., 2012). Interestingly, some flavonoids have the same potency as the anti-diabetic drugs habitually used in clinics, encouraging the study of the activity of these compounds and their metabolites. Flavonoids exert their anti-diabetic effects by targeting cellular signalling pathways in different organs involved in glucose homeostasis, such as the pancreas, liver, skeletal muscle and white adipose tissue (Babu *et al*., 2013). Specifically, flavan-3-ols, flavanones, anthocyanidins, flavonols, flavones and isoflavones improve glucose homeostasis through several mechanisms; they increase insulin secretion by pancreatic β-cells, lower hyperglycaemia by repressing glucose production in hepatocytes and/or reducing insulin resistance and enhance glucose uptake in muscle and adipose tissue. The inhibition of intestinal starch digestion by specific flavonoids, such as quercetin, may also contribute to the anti-diabetic effect (Li *et al*., 2009). Additional anti-diabetic activities have been described for flavonoids; for example, proanthocyanidins are defined as short-lived insulin mimetics in rats (Pinent *et al*., 2012).

Many studies have focused on flavonoid protection against cancer. A meta-analysis study (Hui *et al*., 2013) shows that the intake of flavonols and flavones, but not flavan-3-ols, flavanones, anthocyanins or total

3. FLAVONOIDS AND THEIR FUNCTIONALITY

Epidemiological studies support that flavonoid-rich diets are associated with a low incidence of chronic diseases, such as cardiovascular diseases (CVD), type II diabetes, neurodegenerative diseases, and cancer. However, as the majority of human studies have examined flavonoids in flavonoid-rich food and fruits and vegetables contain additional nutrients and phytochemicals that could impact the results, it is difficult to conclude whether the beneficial effects can be directly attributed to a specific flavonoid or food.

The best-described functionality of flavonoids is their antioxidant activity by which they suppress the formation of reactive oxygen species (ROS), scavenge ROS and enhance the antioxidant defences. However, it is now recognised that the roles of the majority of flavonoids go beyond their antioxidant properties and involve the targeting of cell signalling pathways and epigenetic factors, such as DNA methylation, histone modifications and mi(cro)RNAs (Bladé *et al*., 2013). Because flavonoids are poorly absorbed, some authors suggest that they provide health benefits by regulating the metabolism of gut microbiota, which in turn, modulates the host metabolism (Lu *et al*., 2013).

Systematic reviews of prospective cohort studies indicate that the intake of anthocyanidins, flavonols, flavones, flavanones, proanthocyanidins and flavan-3-ols significantly reduces the risk (Wang *et al*., 2014) and mortality (McCullough *et al*., 2012) of CVD. However, in young and middle-aged women, only anthocyanin intake, is significantly associated with a reduced risk of myocardial infarction (Cassidy *et al*., 2013). Therefore, it seems that the effectiveness of specific flavonoids in protecting from CVD is dependent on the age of the target population. This cardiovascular protection has been ascribed to the capacity of flavonoids to reduce the risk factors associated with CVD, such as endothelial dysfunction and inflammation, hypertension, dyslipidemia, obesity and diabetes.

Endothelial dysfunction is an important and independent predictor of the future development of CVD and dietary flavonoids can improve this situation. For instance, meta-analysis studies show that the endothelial function, measured as flow-mediated dilation is significantly improved after the ingestion of grape polyphenols (Li *et al*., 2013) or isoflavones (Beavers *et al*. 2012). In this respect, a pleiotropic effect of some flavonoids on endothelial function has been described, which increases the bioavailability of nitric oxide (NO), prevents endothelial cell apoptosis, and reduces inflammation (Dayoub *et al*., 2013).

The effects of flavonoids on hypertension are well documented and their effectiveness is dependent on the molecule's chemical structure. For example, in subjects with metabolic syndrome, supplementation with

flavonoids is associated with a reduced risk of breast cancer, especially among post-menopausal women. In contrast, there is no evidence that dietary flavonoids lower the risk of stomach and colorectal cancer (Jin *et al*., 2012; Woo and Kim, 2013). Several studies note that flavonoids interfere with cancer initiation, promotion and progression by targeting receptors and different components of cellular signal transduction pathways involved in tumour growth and metastasis. Specifically, flavonoids target different protein kinases, such as tyrosine kinases, protein serine-threonine kinases, and cyclin-dependent kinases, inhibiting their activity. Notably, the many cancer-inhibiting activities ascribed to flavonoids have presented them as potential new anti-cancer agents that may replace traditional chemotherapy, and flavonoids are currently investigated in the treatment of ovarian, breast, cervical, pancreatic, and prostate cancers (Ravishankar *et al*., 2013). Additionally, quercetin and genistein have entered late-phase clinical trials for several oncological indications (Lazarevic *et al*., 2011).

Finally, recent epidemiological studies show that increased fruit and vegetable intake is associated with an improvement in cognitive function and reduced risk from age-related neurodegenerative diseases, which are largely attributable to high flavonoid intake (Tangney *et al*., 2011). Although flavonoids are poorly absorbed, they have been found in the brain, indicating that they can cross the hematoencephalic barrier (Arola-Arnal *et al*., 2013). In the brain, flavonoids prevent neurodegeneration, inhibit neuroinflammation and reduce age-related cognitive decline. This occurs through the activation of signalling pathways that are critical for controlling synaptic plasticity and for inducing vascular effects, which promote the growth of new nerve cells in the hippocampus (Rendeiro *et al*., 2012).

4. FLAVONOID METABOLISM AND BIOAVAILABILITY

Flavonoids are known to be poorly absorbed and to be recognised by the body as xenobiotics; in the body, they undergo several modifications, which are most likely intended to increase their water solubility and facilitate their elimination. Hence, to associate flavonoids with their health effects, it is necessary to understand how these compounds are absorbed, metabolised and distributed throughout the body. However, polyphenols, and flavonoids in particular, have a great diversity of chemical structures and molecular weights (degrees of polymerisation), which highly influence their absorption with the monomeric forms having higher bioavailability.

After an acute ingestion, some flavonoids, which are normally glycosidated in plants are hydrolysed and absorbed in the small intestine (Fig. 2). These compounds are thought to be hydrolysed in the limit of the epithelial cells by the catalysis of cytosolic β-glucosidase (CBG) and enter into the cells as aglycones by passive diffusion (Day *et al*., 2000). Another possible mechanism is that conjugated flavonoids are transported into

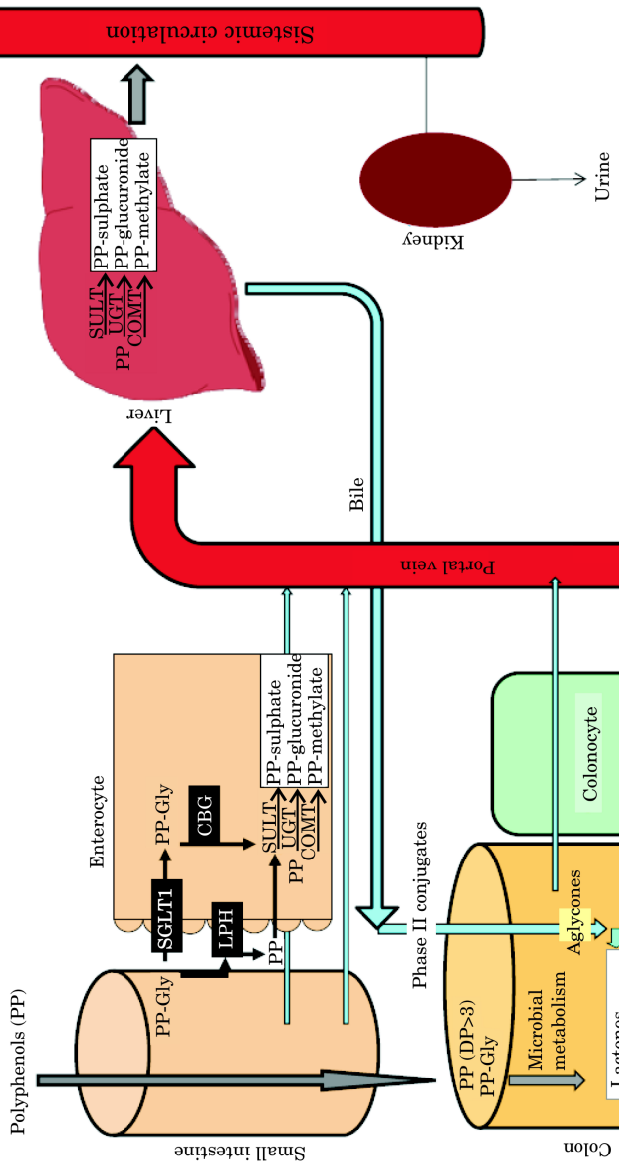

Fig. 2: Schematic representation of the absorption and metabolism of polyphenol compounds in the different organs involved in their bioavailability. CBG, cytosolic β-glucosidase; COMT, catchol-O-methyl transferase; DP, degree of polymerisation; LPH, lactose phloridzin hydrolase; PP, polyphenol aglycone; PP-Gly, polyphenol glycoside; SGLT1, sodium-dependent glucose transporter; SULT, sulphotransferase; UGT, uridine-5'-diphosphate glucuronosyltransferase. Adapted from Monagas *et al.* (2010) and Del Rio *et al.* (2013).

epithelial cells by the sodium-dependent glucose transporter 1 (SGLT1) and hydrolysed within the cells by the action of CBG (Gee *et al*., 2000). Once in the enterocytes, the aglycone flavonoids are rapidly conjugated to form sulphated, glucuronidated and methylated derivatives – by the action of the phase II enzymes sulphotransferases (SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGT) and catechol-*O*-methyltransferases (COMTs), respectively – to reach the portal bloodstream. In the liver, the flavonoids are further subjected to phase II metabolism, excreted via bilis to the intestinal lumen and/or redirected to the systemic circulation to be distributed throughout the body (Del Rio *et al*., 2013).

Those flavonoids forms that cannot be absorbed in the small intestine are known to reach the colon after passing intact throughout the gastrointestinal tract. The forms that are not absorbed in the small intestine are mainly proanthocyanidins (*i.e*., degree of polymerisation greater than 3) and those glucosides that are resistant to the action oflactase-phlorizin hydrolase (LPH) or CBG. In fact, it is estimated that 90-95% of the dietary polyphenols are able to reach the colon, where they are transformed by microbial catabolism (Clifford, 2004). Thus, the colon, is an important organ for the metabolism of flavonoids, and has a great diversity of microbial populations either obligatory or facultative anaerobes (*Bacteriodes*, *Bifidobacterium*, *Enterobacteriaceae* and *Clostridium*), that are responsible of degrading the non-digested food matrix and turning its components in microbial metabolites (Manichanh *et al*., 2010). The metabolites conjugating moieties are cleaved and the oligomers undergo an interflavanic link cleavage to produce monomers. After that, a wide range of enzymes produced by the gut bacteria can hydrolyse, reduce, dehydroxylate, decarboxylate and demethylate several polyphenolic functional groups; these are thus converted into different low-molecular weight metabolites, such as valerolactone-related compounds, valeric acids, phenylpropionic acids, phenylacetic acids, benzoic acids and several conjugated phenolic acids (Dall'Asta *et al*., 2012). Thereafter, the microbial metabolites can reach the portal circulation through the transport of the colonocytes to the liver. In the liver, the metabolites can be further subjected to phase II metabolism before entering the systemic circulation, to finally reach different tissues or be excreted through the urine (Clifford, 2004). However, it is not yet know the complete catabolic pathway of flavonoids – and hence all the potential colonic metabolites – due to several limitations; these include the difference in microbiota composition between subjects, especially in humans and the limited number of identified human gut bacteria able to catabolise flavonoids.

The absorption and phase II metabolism of flavonoids are fast, and they reach the maximal plasma concentrations 1-2 hours post-ingestion (Serra *et al*., 2010). Moreover, although polyphenols and their phase II metabolites are known to be recognised by the body as xenobiotics and thus excreted mainly via renal (Crozier *et al*., 2011), several studies in animals have

demonstrated that at short times post-ingestion, they target most tissues, such as the liver, kidney, adipose tissues, heart and spleen (Juan *et al*., 2010; Serra *et al*., 2013). Interestingly, flavonoids have also been found in the brain (Arola-Arnal *et al*., 2013) and hence are able to cross the blood-brain barrier. However, further studies are needed to understand the details of flavonoid metabolism and bioavaiability. For instance, it is not completely understood how flavonoids and their metabolites travel through the blood; this can be clarified by using pure labelled compounds. It is also largely unknown how these compounds enter into different cells. It has been suggested that the organic anionic transporter polypeptide 2 (OATP2) is involved in the absorption of flavonoids and their conjugated forms into hepatocytes and that the multi-resistant protein 2 (MRP2) is involved in their efflux into the bile (König *et al*., 1999). Therefore, it seems that the tissue uptake of these compounds may depend on specific transporters in the cell membranes. Faria *et al*., (2011) demonstrated a stereoselective process involved in crossing the blood-brain barrier for catechin and epicatechin, suggesting that these compounds have different affinities for their transporters. This can explain at least in part, the observed tissue specificity of the compounds.

After phase II and colonic metabolism, the ingested flavonoid forms that are in the body differ substantially from the original forms present in foods. Hence, the beneficial health effects attributed to flavonoids could be potentially due to the action of their metabolites rather than to the original forms. Moreover, it has also been suggested that as-yet unidentified flavonoid forms present in plasma may contribute to the health effects of flavonoids (Manach *et al*., 2005).

5. *IN VITRO* BIOLOGICAL ACTIVITY

As previously mentioned, flavonoids exert multiple beneficial effects and improve human health. These health effects have been usually tested in animals and in human trials. However, the studies that aimed to test the bioactivity of flavonoids and to identify the molecular mechanisms by which these compound act have mostly utilised cell lines of different tissue origins. As noted above, the postprandial flavonoid forms that circulate in an organism are mainly the products of phase II and colonic microbiota metabolism; yet *in vitro* analyses of the biological activity of flavonoids normally utilise the non-metabolised forms, and most of the mechanisms elucidated *in vitro* have relied on pure flavonoid compounds or plant flavonoid-rich extracts. This is due to the difficulty of obtaining the flavonoid conjugates, especially with plant extracts, which contain more than one type of such compound. Nevertheless, flavonoid metabolites of colonic microbiota can be readily obtained, and some studies have clearly shown the bioactive effects of some gut derivatives on pancreatic beta cells

(Fernández-Millán *et al*., 2014), neuronal PC12 cells (Pavlica and Gebhardt, 2010), Caco-2 cells (Forester *et al*., 2014), human colon cells (Miene *et al*., 2011) and HepG2 cells (Baselga-Escudero *et al*., 2014).

At the moment, little is known about the biological activity and molecular mechanisms involving flavonoid metabolites, particularly phase II metabolites. These conjugated forms are chemically and physically distinct from the aglycone flavonoids and thus also have distinct physiological behaviour. In this regard, several studies have demonstrated that conjugated flavonoids exert different bioactive effects *in vitro* than the non-metabolised forms. Thus, a weaker estrogenic effect was observed for the daidzein and genistein glucuronide conjugates than for their aglycones forms (Zhang *et al*. 1999), and an endothelial property was demonstrated for the (+)-catechin phase II metabolites but not for the aglycone flavonoid (Koga and Meydani, 2001). Moreover, quercetin-3-*O*-glucuronide has been identified as a bioactive compound that reduces β-amyloid peptides in primary neuronal cultures (Ho *et al*., 2013). Hence, several recent authors reported arguments for the use of physiological conjugated flavonoids at appropriate concentrations, rather than the aglycone forms, for testing bioactivity *in vitro*.

A number of *in vitro* analyses have demonstrated that the conjugated moieties of flavonoids can be removed by enzymes inside the cells. As shown by O'Leary *et al*. (2003), β-glucuronidase, which hydrolyses the glucuronide group, deglucuronidates quercetin-glucuronide intracellularly in HepG2 cells to form the quercetin-free aglycone. This aglycone form can be further metabolised to the corresponding glucuronidated, methylated and sulphated derivatives, before they efflux. In addition,Mukai *et al*. (2012) showed that quercetin-glucuronide is deconjugated in Neuro-2a cells. As β-glucuronidase is known to be present in many human tissues, free aglycone flavonoids formed inside the cells could be the bioactive forms responsible of the health properties of flavonoids. Indeed, aglycone quercetin showed a better antioxidant activity than the glucuronide form *in vitro* (Mukai *et al*., 2012).

In summary, it is important to identify the physiological bioactive forms of flavonoids to further advance polyphenol research. Identifying the specific flavonoid metabolites present in blood and tissues is crucial for understanding the biological activities of flavonoids.

6. ANALYTICAL METHODS FOR THE IDENTIFICATION OF BIOACTIVE FLAVONOIDS

6.1. Traditional Analytical Methods

The identification and quantification of flavonoids and their derived metabolites in biological samples (from plasma to faeces, including tissues

such as liver, kidney and adipose tissue) is crucial for determining the fate of these compounds in the organism after ingestion and absorption.

The first point that is necessary to take into account in the analysis of flavonoid metabolites is the analytical techniques used in these procedures. Inspection of the vast literature reveals different methodologies and pieces of equipment that have been used to conduct these analyses and highlights the strong relationship between the technological evolution of the equipment and the enhanced knowledge of these metabolites in the scientific community. Initially, the derived metabolites were studied by traditional procedures for identifying phenolic compounds, namely by the use of capillary electrophoresis (CE), NMR and chromatographic techniques (both gas and liquid, GC and LC, respectively). However, these analyses were difficult to perform because of the great complexity of the biological samples, the high number of possible metabolic forms (glucuronides, sulphates and methylates, their combinations and the microbial-derived compounds) and the extremely low concentration of these metabolites in biological samples.

Among these methodologies, CE was the least used because of the limitations of the equipment used in these procedures, which hindered the detection of trace metabolites. The major advantage NMR is obtaining structural information about the metabolites, which is very important for elucidating the structure of the multiple compounds generated after flavonoid ingestion. However, the results are compromised by the complexity of the samples and the use of NMR is therefore limited to isolated compounds or simple samples.

In general, chromatography has been the method of choice for analysing flavonoid metabolites, with a clear preference for LC over GC due to the introduction of more adequate ionisation interfaces in the LC equipment. Chromatographic equipment can be coupled to a diverse range of detectors, such as ultraviolet (UV), diode array (DAD), fluorescence (FLD) and mass spectrometry (MS). The major drawbacks of UV, DAD and FLD are their lower specificity and accuracy and strong dependence on the correct separation of the compounds to avoid interference, resulting in a long analysis time in most cases. This issue was relieved, in part, by improving the chromatographic separations achieved with the appearance of ultra-performance liquid chromatography (UPLC). The chromatographic separations techniques are sometimes neglected, but these are in fact essential for the success of the analysis. UPLC was developed as a result of an improvement in the packing materials used for the stationary phases of the columns. It is based on the van Deemter equation, which shows that, as the particle sizes decreases to less than 2.5 mm, there is a significant gain in efficiency which does not diminish at increased flow rates (Barceló-Barrachina *et al*., 2006). The emergence of UPLC led to an enhanced signal-to-noise ratio (S/N), a shorter analysis time and improved peak resolution (Motilva *et al*., 2013).

Yet this enhancement of the chromatographic technique was not in itself sufficient to allow accurate and precise identification of flavonoid metabolites. To be able to correctly identify and quantify bioactive compounds in any sample, an authentic reference standard is needed. However, because commercial standards of the conjugated metabolites are usually not available, such standards cannot be used. Consequently, the evolution of the MS detectors and particularly their use in the tandem mode (MS/MS), had a great impact. In mass spectrometric detection, the mass-to-charge ratio (m/z) of a bioactive compound is selected for monitoring, with the exclusion of all the other m/z, thus resulting in high specificity. This property is even more pronounced when working in the tandem mass mode, whereby m/z is selected from both parent and daughter ions, resulting in enhanced specificity and sensitivity. This reduces the detection and quantification limits and allows quantification of metabolites at very low concentrations in the samples. All these considerations have led the scientific community to use detectors such as the triple quadrupole (TQD), quadrupole coupled to ion trap (Q-TRAP) and quadrupole hyphenated to time of flight (Q-TOF) in the study of these compounds (Gray *et al*., 2010).

The second point that has a strong impact on the analysis of flavonoid metabolites is the preparation of the samples. Flavonoid metabolites are distributed in several tissues and therefore, it is necessary to optimise the extraction methodologies to a diverse range of matrices, of both hydrophilic and lipophilic nature. In general, liquid-liquid extraction (LLE) is set as a starting point of the procedures. By this method, the use of organic solvents (methanol, ethanol, acetone, etc.) allows the liberation and removal of metabolites from the other components of the matrix, especially proteins. After that, the samples can be either directly injected into the LC or purified by solid-phase extraction (SPE). This last procedure is usually the best choice for removing from the sample non-flavonoid interferences that are also extracted with the organic solvents. In recent years, the introduction of microSPE methodologies more suitable for biological samples due to the lower amount of sample needed allowed not only the removal of interferences but also concentration of the metabolites, with a consequent decrease in the limits of detection. Therefore, their use has rapidly expanded (Suárez *et al*., 2009; Motilva *et al*., 2013).

The final important point that should be considered is the lack of reference standard compounds for the metabolites. Two different approaches can be followed depending on whether intact metabolites or free flavonoids need to be identified. These basically differ in the use of enzymes as a sample pre-treatment prior to the LLE to hydrolyse the bonds of the flavonoids with the conjugated groups. Thus, sulphates, methylases and glucuronidases can be used to liberate the intact flavonoids. Following these procedures, quantification is performed for the total amount of

flavonoid equivalents distributed in the biological tissue after the ingestion of a certain amount of sample. However, specific information about the different metabolites species remains unknown and thus the particular bioactive forms cannot be identified.

6.2. New Methodologies

The advances in the use of chromatographic analysis have allowed good characterisation of the forms present in the body following flavonoid intake and thus of those metabolites that are physiologically relevant. However, the identification of the bioactive physiological forms of flavonoids is still ambiguous. As stated above, clear identification requires the use of physiologically appropriate conjugates of flavonoids in the *in vitro* analysis of flavonoid bioactivity. Different approaches can be taken to evaluate the effects of metabolites. Some researchers have chosen to chemically synthesise the metabolites and use them both for *in vitro* studies and as reference compounds in LC-MS/MS analysis. For example, Actis-Goretta *et al*. (2012) synthesised standards of epicatechin sulphates, glucuronides and *O*-methylsulphates and used them in LC-MS/MS analysis.

By another approach, the *in vitro* activity of flavonoids was evaluated by treating cells with the sera of rats that had been orally administered a flavonoid-rich extract (Guerrero *et al*., 2013), which would simulate the physiological conditions that occur within the body (Fig. 3). Specifically, this *in vivo* and *in vitro* system was used to establish the bioactivity of flavonoids on *de novo* lipid synthesis and excretion in HepG2 cells. It is important to note that the sera metabolites resulted from the metabolization not only by liver hepatocytes but also by intestinal cells and microbiota. Therefore, the metabolites used contained all the possible bioactive forms of flavonoids that were present in the animal sera 2 hours after the administration of a flavonoid-rich extract. Other researchers have also treated cell cultures with sera of rats that had been previously administered the compounds. Recently, the bioactivity of bezafibrate, which is a known peroxisome proliferator-activated receptor-γ ligand, was demonstrated in HeLa cells using serum and a combined *in vivo* and *in vitro* system (del Bas *et al*., 2012). In these studies, rats were used as a tool to produce flavonoid metabolites. These physiological forms were then utilised to treat cultured cells, allowing the evaluation of the functionality of the bioactive forms.

It is important to note that before its use for the treatment of cells and chromatographic analysis, the serum must be semi-purified and pre-concentrated using microSPE columns. This step is considered crucial for obtaining highly purified bioactive flavonoids and metabolites for both chromatographic analysis and *in vitro* cell treatment.

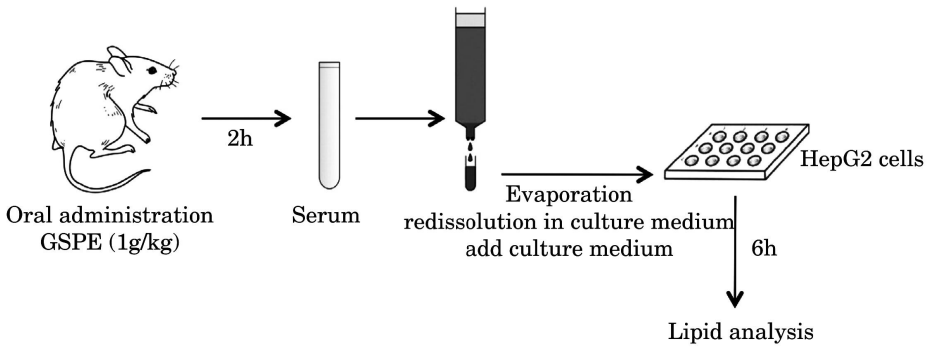

Fig. 3: Graphical representation of the *in vivo* and *in vitro* system used to test the lipid-lowering effect of bioactive polyphenols (from Guerrero *et al*. 2013).

Although the combined *in vivo* and *in vitro* system was used to test the functionality of phase II metabolites and some aglycones with a time-point of 2 hours (Guerrero *et al*., 2013), this methodology can be a particularly useful tool for testing the bioactivity of microbial metabolites by employing serum from rats subjected to a longer treatment with polyphenols. These studies could be especially relevant, considering the fact that microbial metabolites are thought to be the forms responsible for a major part of the health effects of polyphenols.

7. CONCLUSIONS

The impact of the flavonoid forms found in plasma and biological tissues is very different from that of the original flavonoids present in food sources because of the rapid conversion of flavonoids into their phase II and microbial metabolites following absorption. Therefore, *in vitro* experiments utilising food flavonoids to study their functionality or mechanism of action do not necessarily reflect the *in vivo* situation. Hence, the large amount of scientific data that has been generated using non-physiologically relevant forms and/or concentrations of flavonoids may be questionable. In fact, the most important limitation in current flavonoid research is the use of non-physiologically relevant compound forms and/or concentrations for analysing bioactivity. Understanding the bioactivity of flavonoids thus requires advances in analytical methods to allow the detection and quantification of all flavonoid metabolites present in biological samples and the development of *in vitro* models using physiologically appropriate conjugates, forms and concentrations of flavonoids that resemble those observed in tissues after compound intake. This approach is particularly important when extracts and non-purified compounds are used to treat cells because the extracts consist of a complex mixture of different molecules. Moreover, the post-absorption metabolism of the extract mixture, which

yields numerous metabolites, makes it impossible to obtain compounds from other sources that are the same as those in an *in vivo* organism. The use of physiologically appropriate conjugates is therefore essential for the *in vitro* analysis of flavonoid bioactivity.

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II. HYPOTHESIS AND AIMS

Flavanols are considered the most abundant flavonoids in the human diet and their consumption has been associated with health promoting effects. However, their bioactivities are mainly attributed to their metabolic derived products. In fact, many of the *in vitro* studies where polyphenol food extracts were used to treat cells have been recently questioned since bioactive forms of polyphenols are different from those present in food. In this sense, flavonoids are recognized as xenobiotics and undergo phase-II enzymatic detoxification. Moreover, they can also reach the colon to be converted into a wide variety of low molecular weight compounds.

Since there are several conditions that can interfere with xenobiotic -and therefore flavonoid- metabolism, we hypothesized that **external factors like quantity and length of flavanol intake and internal factors such as age, gender and health state of the host may affect the metabolism, bioavailability and tissue distribution of dietary flavanols, influencing the physiological bioactivities of these compounds.**

Therefore, the aim of this thesis was to elucidate whether flavanol metabolism, bioavailability and tissue distribution were affected by different external and internal factors also influencing the bioactivities of these compounds.

The aim of the present research is to elucidate whether metabolism, bioavailability and tissue distribution of flavanol are affected by different external and internal factors and also influence the bioactivities of these compounds.

In order to assess the established assumption, specific objectives were proposed:

1. **To determinate the colonic flavanol microbial biotransformation in rats (Chapter 2)**

While flavanol phase-II metabolism was well described, flavanol colonic microbial metabolism and pathways were studied mostly by *in vitro* studies. Therefore, it was necessary a proper *in vivo* flavanol colonic microbial biotransformation study to completely elucidate the flavanol metabolism. In order to assess this objective two goals were proposed.

a) To develop and validate a method for the quantification of flavanol plasma colonic metabolites **[Manuscript 1].**

b) To determine the colonic biotransformation pathway and the plasma temporal appearance of flavanols colonic metabolites **[Manuscript 2].**

2. **To evaluate external factors affecting flavanol metabolism in rats (Chapter 3).**

In order to assess this objective two goals were proposed.

a) To elucidate whether flavanol metabolism, bioavailability and bioactivity differ depending on the flavanol ingested quantity through an *in vitro-in vivo* model using flavanol physiologically appropriate forms and concentrations **[Manuscript 3]** and **[Manuscript 4]** and if flavanol tissue distribution depends on the ingested amount **[Manuscript 5].**

b) To clarify if flavanols can be metabolized and distributed differently throughout the body depending on the length of flavanol intake by the quantification of flavanols in plasma and tissues after long-term administration **[Manuscript 6].**

3. **To evaluate internal factors affecting flavanol metabolism in rats (Chapter 4)**

In order to assess this objective three goals were proposed.

a) To elucidate whether flavanol metabolism and plasma bioavailability differ along time by the age of the host **[Manuscript 7].**

b) To clarify if gender of the host affects metabolism, plasma bioavailability and tissue distribution of flavanols along time **[Manuscript 8].**

c) To assess whether different health conditions lead to different flavanol metabolism and bioactivity thought the study of flavanol plasma kinetics in a state of disease associated to metabolic syndrome **[Manuscript 9]** and to explore the relationships of flavanol bioactivities with their plasma and aorta levels in a genetically associated pathology **[Manuscript 10].**

To achieve all these objectives, different amounts of grape seed flavanols under different experimental conditions were administered to rats and the plasma and tissue concentrations of flavanols and their metabolites were analyzed by HPLC-MS/MS.

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RESULTS

CHAPTER 1:

Analytical methods for the microbial metabolism

MANUSCRIPT 1:

A rapid method to determine colonic microbial metabolites derived from grape flavanols in rat plasma by liquid- chromatography tandem mass spectrometry

Maria Margalef[†], Zara Pons[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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Article

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A Rapid Method to Determine Colonic Microbial Metabolites Derived from Grape Flavanols in Rat Plasma by Liquid Chromatography−Tandem Mass Spectrometry

Maria Margalef,[†] Zara Pons,[†] Begoña Muguerza,*[,†,‡] and Anna Arola-Arnal[†]

[†]Nutrigenomic Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona 43007, Spain

[‡]Centre Tecnològic de Nutrició i Salut, TECNIO, Campus of International Excellence Southern Catalonia, Reus 43204, Spain

ABSTRACT: This study describes the development and validation of a liquid chromatography−mass spectrometry method for determination of a large number of flavanol colonic derivatives in biological samples. The method was validated with rat plasma after the intake of grape seed flavanols. The minimum plasma volume necessary to maintain good recovery values within the range of 83−110% for all of the standards was determined by micro solid-phase extraction (μ-SPE). In total, 16 commercial standards were used to measure 30 different phenolic compounds present at low concentration levels (micromolar). The chromatographic method enabled reliable quantification of plasma colonic flavanol derivatives with low limits of detection and quantification, achieving values of 0.03 nM and 0.10 nM, respectively. The developed method can be readily applied to determine all of the flavanol metabolites that are most likely responsible for the majority of biological effects of poorly absorbed flavanols.

KEYWORDS: *bioavailability, gut microbial metabolism, colon, polyphenols, proanthocyanidins, flavanols, HPLC-ESI-MS/MS*

INTRODUCTION

Flavanols are one of the most common groups of polyphenols in the human diet and are mainly found in fruit, cocoa, tea, wine, nuts, and beans.[1,2] This group of polyphenols exist in both monomeric (catechin and epicatechin) and oligomeric (proanthocyanidins or condensed tannins, depending on the molecular weight) aglycon forms and esterified by gallic acid.[3] Flavanols improve human health, and grape flavanols, specifically, have been shown to possess several health benefits. In fact, our group has demonstrated that grape seed proanthocyanidin extract (GSPE) exhibits antioxidant capacity,[4] improves lipid metabolism,[5,6] limits adipogenesis,[7] acts as an insulin-mimetic agent,[8] possesses antihypertensive effects,[9] and reduces inflammation.[10]

However, the beneficial health properties of polyphenols are mainly attributed to the compounds derived from their metabolism.[11] Several studies have demonstrated that dietary polyphenols are xenobiotic and, after their absorption in the small intestine, follow the common metabolic pathway of drugs; they undergo phase II enzymatic detoxification with conjugations in the small intestine and/or in the liver to form glucurono, methyl, and sulfo conjugates before entering the bloodstream.[12]

In addition to absorption of polyphenols in the small intestine, it is estimated that 90−95% of dietary polyphenols can reach the colon.[12] In the colon, the wide range of enzymes produced by gut bacteria can hydrolyze several functional groups; reduce, dehydroxylate, decarboxylate, and demethylate the polyphenols; and convert them into different low molecular weight metabolites (valerolactone compounds, valeric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, and several conjugated phenolic acids, consecutively) that are able to be absorbed in situ.[12−14] Subsequently, these colonic metabolites can also be conjugated during metabolism in the small intestine and/or the liver.

As a consequence, multiple metabolites are potentially formed after the ingestion of polyphenols, and therefore, the levels of many of the metabolites in the plasma can be very low. In addition, due to the numerous interferences and, in many cases, the limited available volume of these types of samples, the methodology used to identify these compounds and their metabolites in biological samples is complex. Thus, pretreatment of the sample to reduce contaminants and preconcentrate the compounds is necessary.[15] The most frequently used sample pretreatment for polyphenol determination in the plasma and tissues is off-line microelution solid-phase extraction (μ-SPE) that allows rapid isolation of the compounds to be analyzed by use of a reduced sample volume.[15−19] In addition, the analytical method for compound quantification should guarantee sensitivity, selectivity, and robustness with low limits of detection (LOD) and quantification (LOQ) since polyphenols and their metabolites appear in biological sample at low concentrations and also for the complexity of the biological samples. SPE and μ-SPE, high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) separation, and detection by mass spectrometry (MS) are the most common techniques to identify and quantify polyphenols in biological samples; UHPLC coupled to tandem triple quadrupole MS (UHPLC-MS/MS) detection is the most widely used.[14,16,17,20]

Most human and rat studies have been focused on identifying flavanols and their phase II metabolites (glucuronide, methyl

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and sulfate forms of monomeric flavanols) after the ingestion of proanthocyanidins,[17,21−25] but only a few studies have been focused on colonic microbial metabolites and the use of proanthocyanidins.[13,16,20,21,26,27]

Therefore, the aim of this study was to develop and validate a method that uses improved off-line μ-SPE followed by HPLC-MS/MS detection for quantification of colonic metabolites derived from grape flavanols in the plasma collected from rats.

MATERIALS AND METHODS

Grape Seed Proanthocyanidin Extract. Grape seed proanthocyanidin extract (GSPE) was obtained from Les Dérives Résiniques et Terpéniques (Dax, France). The individual flavanols and phenolic acids contained in the grape seed extract that were used in this study are detailed in Table 1 (adapted from Quiñones et al.[9]).

Table 1. Individual Phenolic Compounds of Grape Seed Proanthocyanidin Extract (Flavanols and Phenolic Acids)[a]

compd	concn[b] (mg/g)
catechin	90.7 ± 7.6
epicatechin	55.0 ± 0.8
procyanidin dimer[c]	144.2 ± 32.2
procyanidin trimer[c]	28.4 ± 2.0
procyanidin tetramer[c]	2.0 ± 0.2
dimer gallate[d]	39.7 ± 7.1
epigallocatechin gallate	0.4 ± 0.1
epicatechin gallate[d]	55.3 ± 1.5
p-coumaric acid	0.1 ± 0.0
gallic acid	17.7 ± 2.0
3,4-dihydroxybenzoic acid	1.0 ± 0.1
vanillic acid	0.1 ± 0.0
quercetin	0.3 ± 0.0
quercetin 3-*O*-gallate	0.2 ± 0.0
naringenin 7-glucoside	0.1 ± 0.0
kaempferol 3-glucoside	0.1 ± 0.0

[a]Determined by reverse-phase HPLC-ESI-MS/TOF. Values are expressed as milligrams of compound per gram of fresh extract and are the means of three samples. [b]Data adapted from Quiñones et al.[9] [c]Quantified by use of the calibration curve of procyanidin B2. [d]Quantified by use of the calibration curve of epigallocatechin gallate.

Chemicals and Reagents. Acetone (HPLC analytical grade), methanol (HPLC analytical grade), and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). (+)-Catechin, (−)-epicatechin, benzoic acid, phloroglucinol, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, and ferulic acid (all from Fluka/Sigma−Aldrich, Madrid, Spain) were individually dissolved in methanol at 4000 mg/L. Procyanidin B2, epigallocatechin gallate (EGCG), and pyrocatechol (internal standard, IS) (all from Fluka/Sigma−Aldrich, Madrid, Spain) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany) were individually dissolved in methanol at 2000 mg/L. All standard stock solutions were prepared every 3 months and stored in dark-glass flasks at −20 °C.

A mixed standard stock solution in methanol of all these compounds [(+)-catechin, (−)-epicatechin, benzoic acid, phloroglucinol, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)-acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, EGCG, and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone] at 200 mg/L and procyanidin B2 at 100 mg/L was prepared weekly and stored in dark-glass flasks at −20 °C. This mixed standard stock solution was diluted daily to the desired concentration by use of an acetone/Milli-Q water/acetic acid (70/29.5/0.5 v/v/v) solution and stored under the same conditions until chromatographic analysis.

Animal and Plasma Collection. Male Wistar rats, weighing between 226 and 260 g, were obtained from Charles River Laboratories (Barcelona, Spain). All of the animals were housed at 22 °C with a light/dark cycle of 12 h (lights on at 9:00 a.m.) and were fed a standard chow diet (AO4, Panlab, Barcelona, Spain) ad libitum during the experiment. The animals were randomly divided into two groups: the control group (n = 6) and the GSPE group (n = 6). On the day of the experiment, the rats were starved for 12 h. To the GSPE group, a dose of 1000 mg of GSPE/kg of body weight (1 mL) was administered by oral gavage. Water (1 mL) was orally administered to the control group. In both groups, oral administration was performed by gastric intubation to the fasted rats between 9:00 and 10:00 a.m. Blood samples were obtained via saphenous vein extraction by use of heparin vials (Starsted, Barcelona, Spain) 7 h after GSPE or water administration. Plasma samples were obtained by centrifugation (2000g, 15 min, 4 °C). The plasma was pooled (n = 6) to obtain sufficient sample volume to perform three replicate chromatographic analyses. The pooled plasma samples were stored at −80 °C until chromatographic analysis was performed. The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of Universitat Rovira i Virgili.

Off-line μ-SPE Extraction. *Optimization of Plasma Volume.* To determine the minimum sample volume with good recovery values, four different spiked blank plasma volumes were tested (350, 250, 200, and 150 μL) at three different levels of concentration (50, 500, and 5000 μg/mL) for all the standards. Briefly, the Oasis HLB μ-Elution Plates 30 μm (Waters, Barcelona, Spain) were conditioned sequentially with 250 μL of methanol and 250 μL of 0.2% acetic acid. To ensure full contact of the compounds with the sorbent column, plasma aliquots were mixed with different volumes of 4% phosphoric acid (300 μL for 350- and 250-μL plasma samples, 350 μL for 200-μL plasma sample, and 400 μL for 150-μL plasma sample) and 50 μL of IS (200 μg/mL). The loaded plates were washed with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid. The retained polyphenols were then 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 HPLC-MS/MS.

Optimization of Cleanup Steps. The cleanup steps were further evaluated following the procedure described under Optimization of Plasma Volume) for a plasma volume of 250 μL. In this case, the loaded plates were washed with 100 μL each or 150 μL each of Milli-Q water and 0.2% acetic acid.

Optimization of Elution Steps. The elution steps were further evaluated following the procedure described under Optimization of Plasma Volume for a plasma volume of 250 μL. In this case, the retained polyphenols were eluted with 2 × 25 μL of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5 v/v/v).

Plasma Flavanol Metabolite Extraction. Prior to extraction, the plasma samples were centrifuged (1500g, 5 min, 4 °C) to remove aggregates. The plasma extraction was performed by off-line μ-SPE as described under Optimization of Plasma Volume for a plasma volume of 250 μL. The eluted solution was directly injected into the HPLC-MS/MS.

Analysis of Plasma Flavanols and Their Metabolites. *Instrumental Conditions.* The HPLC system consisted of a 1200 LC Series (Agilent Technologies, Palo Alto, CA) using Zorbax SB-Aq (150 mm × 2.1 mm i.d., 3.5 μm particle size) chromatographic column, from Agilent Technologies, Palo Alto, CA. The mobile phase was 0.2% acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.4 mL/min. The elution gradient was 5−55% B (0−10 min), 55−80% B (10−12 min), 80% B isocratic (12−15 min), and 80−5% B (15−16 min). A 10 min post run was applied. The sample injection volume used was 2.5 μL.

Table 2. Retention Times, Exact Masses, and Optimized MRM Conditions[a]

		TOF/MS[b]		MS/MS quantification[c]			MS/MS confirmation[c]			
compd	MW	$[M-H]^-$	RT (min)	MRM_1	F (V)	CE (V)	MRM_2	F (V)	CE (V)	ref
catechin	290.27	289.0735	6.6	289 > 203	120	20	289 > 245	120	20	
epicatechin	290.27	289.0735	6.9	289 > 245	130	10	289 > 203	130	20	
procyanidin B1	578.52	577.1360	6.5	577 > 425	130	10	577 > 407	130	30	41
procyanidin B2	578.52	577.1360	6.7	577 > 425	130	10	577 > 407	130	30	
procyanidin B3	578.52	577.1360	6.1	577 > 425	130	10	577 > 407	130	30	41
gallic acid	170.12	169.0147	2.8	169 > 125	90	10	169 > 79	90	40	
vanillic acid	168.15	167.0358	7.09	167 > 152	80	10	167 > 123	80	5	
epigallocatechin gallate	458.37	457.0780	7.3	457 > 169	110	20	457 > 305	110	20	
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	292.28	291.088	7.1	291 > 247	70	20	291 > 96	70	20	27
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	208.21	207.0669	7.8	207 > 85	120	10	207 > 121	120	10	
4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	226.23	225.0779	9.2	225 > 163	70	10	225 > 181	70	10	27
4-hydroxy-5-(3′-hydroxyphenyl)valeric acid	210.23	209.0830	11.9	209 > 147	150	0				27
4-hydroxy-5-phenylvaleric acid	194.23	193.0880	9.8	193 > 175	60	10				27
3-(3,4-dihydroxyphenyl)propionic acid	182.17	181.0512	6.2	181 > 137	60	10				20
3-(3-hydroxyphenyl)propionic acid	166.17	165.0558	6.9	165 > 121	90	10	165 > 59	90	0	20,27
3-(4-hydroxyphenyl)propionic acid	166.17	165.0558	7.2	165 > 121	90	10	165 > 59	90	0	
phenylpropionic acid	150.17	149.0615	9.5	149 > 105	90	10				27
2-(3,4-dihydroxyphenyl)acetic acid	168.15	167.0357	4.6	167 > 123	50	10	167 > 95	50	30	20
2-(3-hydroxyphenyl)acetic acid	152.15	151.0409	6.1	151 > 107	60	5	151 > 93	60	20	27
2-(4-hydroxyphenyl)acetic acid	152.15	151.0409	6.5	151 > 107	60	5	151 > 65	60	30	
phenylacetic acid	136.15	135.0451	9.2	135 > 91	90	5	135 > 100	90	5	20
3,4-dihydroxybenzoic acid	154.12	153.0201	4.1	153 > 109	80	10	153 > 62	80	40	20
3-hydroxybenzoic acid	138.12	137.0243	6.5	137 > 93	70	10				
4-hydroxybenzoic acid	138.12	137.0243	6.8	137 > 93	70	10				20
benzoic acid	122.12	121.0296	7.9	121 > 77	60	5	121 > 59	60	5	
3-*O*-methylgallic acid	184.15	183.0308	5.6	183 > 168	90	10	183 > 124	90	10	20
homovanillic acid	182.17	181.0512	4.5	181 > 163	90	10	181 > 134	90	20	42
homovanillyl alcohol	168.19	168.0715	6.3	167 > 152	150	10	167 > 133	150	10	42
ferulic acid	194.18	193.0510	8.2	193 > 134	60	10	193 > 178	60	10	
hippuric acid	179.17	178.0512	5.5	178 > 134	80	5	178 > 77	80	10	
phloroglucinol	126.11	125.0250	2.27	125 > 57	90	10	125 > 125	90	0	

[a]Abbreviations: MW, molecular weight; RT, retention time; F, fragmentor; CE, collision energy; MRM, multiple reaction monitoring. [b]For compounds studied by HPLC-ESI-TOF/MS. [c]For analysis of phenolic compounds studied by HPLC-ESI-MS/MS.

This HPLC system was coupled to a 6210 time-of-flight (TOF) mass spectrometer (Agilent Technologies, Palo Alto, CA) system or coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA) system. Ionization was done by electrospray (ESI) in the negative mode. When HPLC-ESI-TOF/MS was performed, the ESI conditions were drying gas temperature of 350 °C, flow of 12 L/min, rnebulizer gas pressure of 45 psi, and capillary voltage of 4000 V. Moreover, when HPLC-ESI-MS/MS was performed, the acquisition was done by multiple reaction monitoring (MRM). The ESI working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h; desolvation gas flow rate, 800 L/h; and desolvation temperature, 400 °C. Nitrogen (>99% purity) was used as a nebulizing and collision gas. Cone voltages and collision energies were optimized by infusing a standard solution of 10 mg/L of each standard or treated rat plasma for expected analytes in a mixture of acetone/Milli-Q water/acetic acid (70/29.5/0.5 v/v/v) at a flow rate of 0.4 μL/min. The full-scan mode was first acquired to select the most abundant m/z value, and the fragmentor was optimized. Main $[M-H]^-$ ions were selected as precursor ions. The collision energies were then studied to find the most abundant product ions, and the most sensitive transition was selected for subsequent quantification. The second transition was used for confirmation purposes. All of the HPLC-MS/MS conditions are outlined in Table 2. The dwell time established for each transition was 30 ms. Data were acquired by use of MassHunter qualitative analysis software B.02.00 (Agilent Technologies, Palo Alto, CA). All of the expected product transitions were confirmed in the literature.

Method Validation. To validate the quantitative method, the calibration curves, linearity, extraction recovery, matrix effect, precision, and method detection and quantification limits were studied by analyzing standard solutions and blank plasma samples spiked with the standard polyphenols. Blank plasmas were obtained at 7 h from rats that ingested water (without GSPE); no phenolic acid basal metabolites were present in blank plasmas. The calibration curves were obtained by plotting the analyte/IS peak abundance ratio and the corresponding analyte/IS concentration ratio.

Method precision was determined as the relative standard deviation (% RSD) of the concentration in a triplicate analysis of three different spiked samples (50, 500, and 5000 μg/mL) randomly distributed intraday. Recovery values were calculated by comparing the responses of the abundance ratio analyte/IS spiked in pretreated plasma matrices by adding the standards before and after pretreatment (off-line μ-SPE) at three different concentration levels (50, 500, and 5000 μg/mL). Matrix effects were evaluated by comparing the relative analyte/IS abundance obtained from spiked blank plasma after off-line μ-SPE with those obtained from commercial standard solutions at three different concentration levels (50, 500, and 5000 μg/mL). Sensitivity was evaluated by determining LOD, defined as the concentration corresponding to 3 times the signal/noise ratio, and LOQ_s defined as the concentration corresponding to 10 times the signal/noise ratio. Method detection and quantification limits (MDL and MQL,

Table 3. Recovery Values for Analysis of Phenolic Compounds in Different Volumes of Spiked Plasma Samples by Off-line μ-SPE

	recovery (%)											
	150 μL[a]			200 μL[a]			250 μL[a]			350 μL[b]		
compd	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL
catechin	60	55	60	97	99	91	98	104	107	97	99	101
epicatechin	36	32	38	85	80	88	95	102	100	101	104	106
procyanidin B2	51	48	42	83	78	79	92	102	102	98	107	106
epigallocatechin gallate	54	51	63	82	80	82	93	94	96	90	84	101
gallic acid	54	50	36	65	68	86	85	83	96	97	95	91
vanillic acid	84	77	83	106	96	84	100	103	99	95	98	95
5-(3,4-dihydroxyphenyl)-γ-valerolactone	57	52	70	82	76	75	93	93	96	95	99	109
3-(4-hydroxyphenyl)propionic acid	63	69	65	88	84	81	106	97	92	113	112	94
2-(3,4-dihydroxyphenyl)acetic acid	40	47	49	79	81	76	95	99	98	99	94	97
2-(3-hydroxyphenyl)acetic acid	67	61	57	81	90	100	101	99	97	96	95	88
2-(4-hydroxyphenyl)acetic acid	63	57	78	62	65	79	83	85	100	96	92	110
3-hydroxybenzoic acid	89	76	86	81	78	86	95	98	101	99	102	98
benzoic acid	67	64	78	96	92	90	94	93	90	109	97	101
ferulic acid	17	27	26	75	77	86	102	103	94	108	99	108
hippuric acid	46	51	82	96	92	82	100	99	94	94	92	94
phloroglucinol	25	27	14	86	80	83	100	93	92	96	103	92

[a]Total volume added into μ-SPE sorbent was 600 μL. [b]Total volume added into μ-SPE sorbent was 700 μL.

respectively) were calculated for the analysis of 250-μL plasma sample following the procedure described previously.

RESULTS AND DISCUSSION

Currently, the common approach for quantification of flavanol metabolites is to quantify the phase II metabolites from the small intestine and liver in plasma or other tissues at short times after the intake of proanthocyanidins.[17−19] Additionally, there are a few studies where microbial metabolites derived from flavanols are analyzed in biological samples.[16,21,26] However, recent investigations revealed that the majority of ingested polyphenols reach the colon, where the enzymes of gut bacteria are able to convert polyphenols and their metabolites into a wide variety of low molecular weight compounds, which can then be absorbed in situ.[11,28] Therefore, since colonic metabolites could be responsible for many of the health benefits attributed to flavanols, complete characterization of the colonic metabolism of flavanols will help to elucidate the bioavailability and metabolism of proanthocyanidins and identify the derivatives responsible for the beneficial health effects attributed to proanthocyanidins.[11] Nevertheless, to our knowledge, there are no validated chromatographic methodologies for the analysis of flavanol-derived microbial metabolites in biological samples.[16,20,21,29] Indeed, only a few studies have analyzed colonic microbial metabolites in biological samples after the ingestion of flavanols.[16,21,26] Currently, the most commonly used method for the study of colonic metabolites is based on in vitro models using batch-culture fecal fermentations.[13,27,30] Hence, in this study we set up an improved methodology for analysis of flavanols and their microbial metabolites.

Improved Off-line μ-SPE for Plasma Sample Pretreatment. The off-line μ-SPE method, previously reported for plasma samples treatment prior to analysis of proanthocyanidins, anthocyanins, and their phase II metabolites,[15,17] was here improved to further reduce plasma volume and to enable extraction of colonic metabolites derived from flavanols. The μ-SPE method enabled concentration of the compounds prior to chromatographic analysis, since they are present in plasma samples at very low levels. Furthermore, the plasma samples had a large number of interferences, mainly proteins, which were removed in this methodology by precipitation with-phosphoric acid.[15] In addition, in this study μ-SPE with hydrophilic−lipophilic balanced (HLB) sorbent allowed the extraction of flavanols and their colonic metabolites. Furthermore, previous studies using this sorbent extracted phase II metabolites derived from flavanols (i.e., the glucuronidated, methylated and sulfated forms of catechin and epicatechin).[16,17] However, in this study phase II metabolites were not analyzed, as the methodology was already reported, and phase II metabolites should not be expected to occur in significant amount in plasma 7 h after polyphenol administration.[16,17]

A major limitation for the analysis of biological samples such as plasma or serum is the sample volume.[15] Especially for the study of small subjects such as rats or mice, the sample volume becomes the most critical parameter to consider. Moreover, reducing the plasma volume will enable experimentation without the sacrifice of animals. Hence, in this study the sample volume for μ-SPE was reduced from 350 to 250 μL with preservation of the recovery values (Table 3). All of the recovery values for 350 μL of plasma sample were similar to reported values of Martí et al.[15] When the extraction recovery was tested with 250 μL of plasma sample, its related rates were also suitable and ranged from 83% to 107%. However, when the extraction recovery was tested with 200 or 150 μL, some compounds showed poorer recovery percentages than when a volume of 250 μL was evaluated. Specifically, recoveries showed

Table 4. Recovery Values Obtained after Testing Different Volumes of Clean-up and Elution Solutions[a]

	recovery (%)								
	100 μL clean-up step			150 μLclean-up step			50 μL elution step		
compd	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL
catechin	63.1	9.9	3.3	57.9	20.1	2.6	61.3	86.3	83.8
epicatechin	45.0	60.9	19.0	52.0	58.4	19.6	35.5	89.6	90.1
procyanidin B2	61.4	47.1	19.6	73.0	44.5	20.7	66.8	82.8	97.1
epigallocatechin gallate	5.8	0.8	0.0	9.2	2.9	0.1	50.4	62.5	70.1
gallic acid	0.0	0.0	0.0	0.0	0.0	0.0	43.2	26.8	92.8
vanillic acid	66.1	14.3	2.2	68.6	15.1	2.5	23.5	84.7	79.6
5-(3,4-dihydroxyphenyl)-γ-valerolactone	57.3	4.5	0.1	26.7	7.9	2.4	54.9	82.0	64.5
3-(4-hydroxyphenyl)propionic acid	0.0	0.0	44.5	0.0	0.0	37.5	0.0	99.7	90.9
2-(3,4-dihydroxyphenyl)acetic acid	0.0	0.0	0.0	0.0	0.0	0.0	53.3	73.4	88.5
2-(3-hydroxyphenyl)acetic acid	38.5	59.2	26.7	55.1	77.2	17.8	62.1	52.4	93.6
2-(4-hydroxyphenyl)acetic acid	68.9	65.0	17.7	69.1	69.2	20.6	58.8	15.8	35.2
3-hydroxybenzoic acid	71.7	10.0	7.7	71.7	11.6	1.9	26.5	66.9	75.3
benzoic acid	68.4	73.0	61.4	70.6	78.6	40.6	52.4	96.2	64.9
ferulic acid	20.3	3.4	0.7	14.2	3.1	0.2	15.3	84.9	86.9
hippuric acid	30.1	55.3	94.6	38.0	55.8	95.7	23.5	24.0	103.4
phloroglucinol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.9	19.8

[a]Results are expressed as a percentage after comparing the spiked blank plasma response at three different levels (50, 500, and 5000 μg/mL) before and after off-line μ-SPE.

Table 5. Parameters for Quantification of Phenolic Compounds in Spiked Plasma Samples by HPLC-ESI-MS/MS[a]

					precision (% RSD, n = 3)			% matrix effect						
compd	RT (min)	calibration curve	determ coeff (R^2)	working linearity range (μM)	50 μg/mL	500 μg/mL	5000 μg/mL	50 μg/mL	500 μg/mL	5000 μg/mL	LOD (nM)	LOQ (nM)	MDL[b] (nM)	MQL[b] (nM)
catechin	6.4	y = 0.799x	0.997	0.007−17.225	0.8	3.9	6.6	7.7	3.1	2.7	2.30	7.66	0.66	2.19
epicatechin	6.9	y = 1.674x	0.996	0.007−17.225	1.6	5.6	0.8	−1.0	0.3	1.2	1.66	5.52	0.47	1.58
procyanidin B2	6.7	y = 34.138x	0.991	0.008−4.321	0.6	3.6	5.4	−0.4	1.6	0.0	1.95	6.49	0.56	1.85
epigallocatechin gallate	7.3	y = 8.399x	0.990	0.004−10.908	1.1	4.2	2.0	2.2	1.5	6.0	0.76	2.52	0.22	0.72
gallic acid	2.8	y = 1.035x	0.991	0.059−29.391	1.6	4.6	4.2	0.7	0.8	1.4	11.75	39.22	3.36	11.20
vanillic acid	6.7	y = 0.862x	0.996	0.012−29.762	1.7	6.0	2.7	2.4	−0.2	2.5	4.29	14.29	1.22	4.08
5-(3,4-dihydroxyphenyl)-γ-valerolactone	7.2	y = 0.746x	0.998	0.048−24.038	3.8	3.4	4.9	5.5	4.7	1.8	14.42	48.08	4.12	13.74
3-(4-hydroxyphenyl)propionic acid	7.3	y = 0.063x	0.997	0.060−30.120	3.6	2.6	0.5	2.8	−1.7	0.5	24.10	80.32	6.88	22.95
2-(3,4-dihydroxyphenyl)acetic acid	4,3	y = 0.389x	0.996	0.060−29.762	3.2	4.5	3.4	0.6	0.8	0.3	11.90	39.68	3.40	11.34
2-(3-hydroxyphenyl)acetic acid	5.8	y = 0.775x	0.995	0.013−32.895	2.2	2.2	1.7	5.4	2.9	0.4	3.29	10.96	0.94	3.13
2-(4-hydroxyphenyl)acetic acid	6.1	y = 0.534x	0.994	0.013−32.895	5.0	8.5	2.4	0.1	4.1	−1.5	8.87	29.90	2.56	8.54
3-hydroxybenzoic acid	6.3	y = 0.690x	0.998	0.072−36.232	3.1	1.1	2.8	3.2	2.7	4.2	39.13	130.43	11.28	37.37
benzoic acid	7.8	y = 0.510x	0.995	0.016−40.984	3.4	5.6	3.5	−0.2	1.2	3.2	3.78	12.61	1.08	3.60
ferulic acid	8.1	y = 5.658x	0.995	0.010−25.773	0.5	5.4	2.7	4.9	2.0	7.3	2.13	7.11	0.61	2.03
hippuric acid	5.5	y = 1.981x	0.986	0.011−27.933	2.4	5.0	4.5	−2.7	0.3	−0.1	0.10	0.34	0.03	0.10
phloroglucinol	2.3	y = 0.157x	0.990	0.079−39.683	2.5	6.2	1.7	2.1	1.3	−0.2	23.81	79.37	6.80	22.68

[a]Abbreviations: RT, retention time; LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MQL, method quantification limit. [b]Method detection and quantification limits are given as nanomoles per liter of fresh sample, calculated for analysis of 250-μL plasma sample.

values lower than 80% when the plasma volume was 150 μL. Thus, 250 μL is the lowest plasma sample volume required to achieve acceptable recovery values for all compounds and, hence, was the plasma volume used in this study. On the other hand, decreasing the cleanup solution volume from 200 μL to either 150 or 100 μL did not improve the extraction recovery, as some compounds, like 2-(3,4-dihydroxyphenyl)acetic acid or gallic acid, were not detected by HPLC-ESI-MS/MS. In the same way, decreasing the volume of elution solution from 100 to 50 μL also showed poor recovery values for all compounds tested (Table 4), which indicate that more volume is needed to elute all the compounds. Thus, from all the assessed factors, it

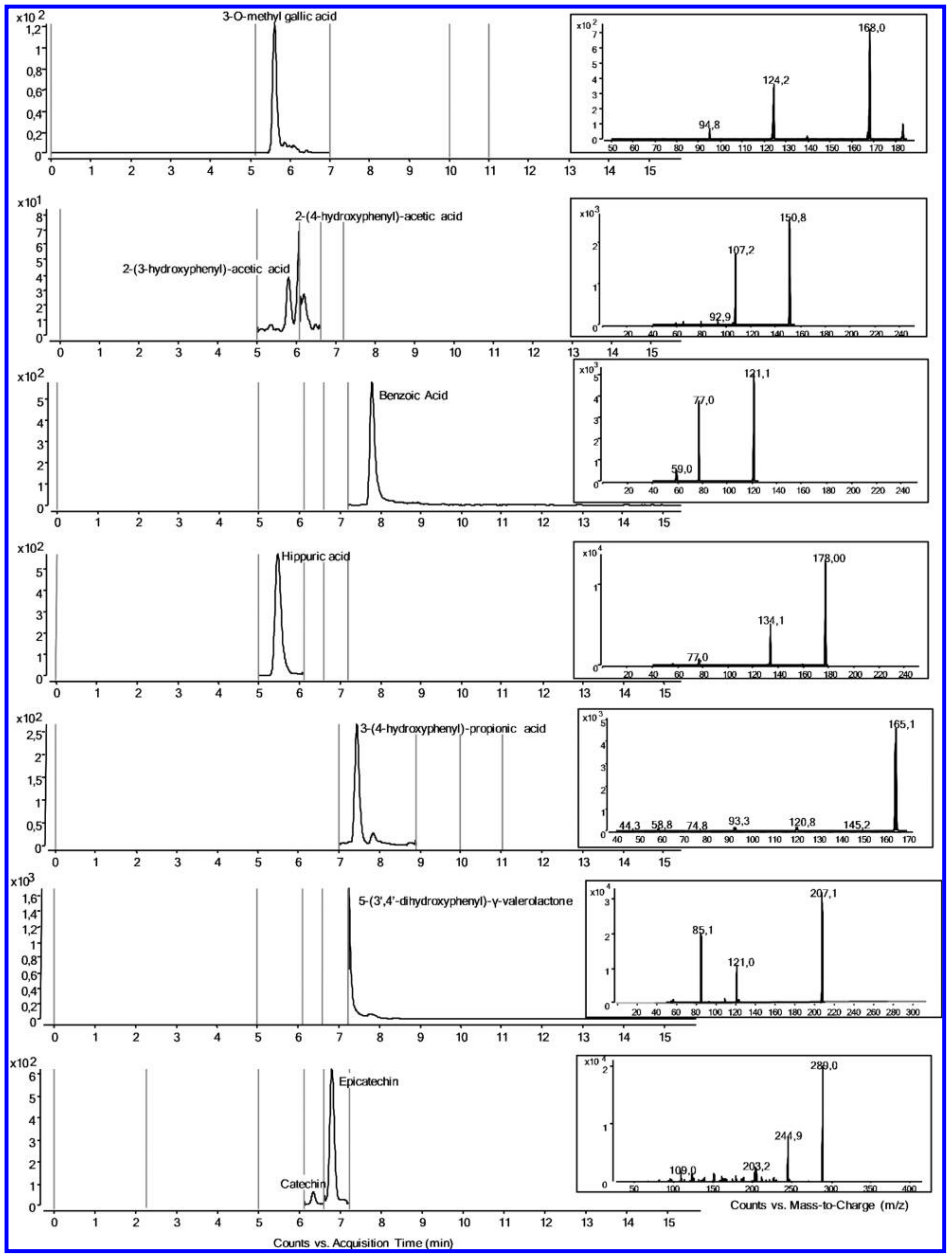

Figure 1. Extracted ion chromatograms of plasma microbial metabolites and their respective mass spectra fragmentation 7 h after ingestion of GSPE (1000 mg/kg).

can be concluded that, for off-line μ-SPE, the plasma volume can be reduced to 250 μL but the cleanup needs to be realized with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid and the elution volume should be 100 μL.

Quality Parameters. Basal plasma spiked with a range of different concentrations of flavanols and phenolic acids was analyzed via off-line μ-SPE coupled to HPLC-ESI-MS/MS to determine the matrix effect, working linearity range, calibration curves, reproducibility, LODs, and LOQs (Table 5). The linearity of flavanols and phenolic acids was between 0.004 and 41 μM. All of the compounds possessed a R^2 value equal or higher than 0.99, even taking into account that the standards present in the spiked blank plasma and the interactions between compounds could have an adverse effect on the working linearity range.[31] The relative standard deviation (% RSD) was calculated at three concentration levels (50, 500, and 5000 μg/mL) for all the standards in three randomly distributed intraday analyses. For the flavanols and phenolic acids studied, RSD values were between 0.51% and 8.5%. The higher percent RSD values are correlated with a higher dispersion; therefore, these RSD percentages indicate a large variance between the compounds. However, all the percent RSD results were considered the correct values because they did not exceed 20%.[32] Furthermore, no matrix effect was observed with rates close to 0% for all the flavanols and phenolic acids evaluated. For all of the phenolic compounds studied, the LODs and LOQs were lower than 40 nM and 131 nM, respectively. The MDL and MQL values were 0.03−11.28 nM and 0.10−37.37 nM, respectively. The use of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone as a standard for quantification of all valerolactone and valeric acids metabolites was an important improvement for the methodology developed in our study, as these metabolites were previously quantified as (−)-epicatechin equivalents.[20]

Identification of Colonic Microbial Metabolites Derived from Grape Seed Proanthocyanidins in Rat Plasma. The strategy applied in this study to determine colonic metabolites derived from GSPE was to identify all of the colonic metabolites, including glucuronidated or sulfated phenolic acids, present in the rat plasma from the exact mass of all potential molecular ions by HPLC-ESI-TOF/MS. For this, we analyzed a pool of rat plasma (n = 6) 7 h after GSPE ingestion. The pooled plasma was necessary to collect enough volume of plasma for three replicate chromatographic analyses without the rat sacrifice (i.e., plasma obtained from saphenous vein). Moreover, pooled plasma increases homogeneity and sensitivity in order to allow the detection of all potential metabolites.[33,34] The 7 h time point was selected to allow the flavanols to reach the colon, to be metabolized by gut bacteria, and to be absorbed.

Of the 43 metabolites described in the literature from human[13,20,28] or rat,[14,16,20] 30 colonic metabolites were detected in the plasma after 7 h. Interestingly, at 7 h post-GSPE administration, no glucuronide or sulfate derivatives of the phenolic acids were detected. The fragmentation pattern and retention behavior were subsequently determined by a literature search for all of the compounds identified (i.e., the exact mass and retention time detected), and the compound parameters were then optimized to be quantified by HPLC-ESI-MS/MS (Table 2). Searching the literature for potential compound fragmentation that could occur is very important because the ionization conditions may vary depending on the equipment used in the analysis. Therefore, all of the compounds quantified that did not have their own commercial standard were confirmed in the literature.[20,29] All of the MRM conditions and quality parameters for quantification by HPLC-ESI-MS/MS are listed in Tables 2 and 5, respectively.

Application to Flavanol Bioavailability. The major breakthrough of the method reported in this study was the ability to analyze more than 20 different rat colonic metabolites and additional nonmetabolized flavanols by the same plasma extraction pretreatment and same chromatographic analysis, with a large number of commercial standards (16) for the most specific and accurate quantification possible.

The method developed was applied to analyze rat plasma obtained from the saphenous vein after administration of 1000 mg/kg GSPE. This dose was selected because it was the previously reported acute dose necessary to reach appropriate levels of metabolized forms in the plasma.[17] However, although the high dose was used in rats, these methodologies are usually further extended to the analysis of human plasma with more physiological concentrations of polyphenols.[26,35−37] HPLC-ESI-MS/MS chromatograms of flavanol colonic products in the plasma from treated rats and the respective mass spectra fragmentations are shown in Figure 1. All of the compound concentrations in rat plasma analyzed by HPLC-ESI-MS/MS are shown in Table 6.

The plasma concentrations of catechin, epicatechin, and procyanidin dimer B2, present in the GSPE, were 0.087, 0.532, and 0.013 μM, respectively. The catechin and epicatechin monomers are rapidly absorbed and appear in plasma between 1 and 2 h after ingestion.[17] In this study, we showed that these nonmetabolized compounds can still be quantified in the plasma 7 h after GSPE ingestion, at even lower concentrations than at the 2 h time point.[16] However, EGCG was not detected, which can be attributed to the low concentration in the GSPE more than the poor solubility of the gallate forms of flavanols.[38] Gallic and vanillic acids were two phenolic acids present in GSPE (Table 1) and in plasma that reached concentrations of 0.745 and 0.555 μM, respectively. This high concentrations at 7 h after GSPE ingestion can be attributed to formation of these phenolic acids as products of microbial metabolism.[12,13,27]

Regarding valerolactone or valeric acid compounds, the results show that the only metabolite found in plasma 7 h after administration of GSPE was 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, although other in vitro experiments performed in human feces with GSPE reported the presence of 5-(3′-hydroxyphenyl)-γ-valerolactone and γ-valerolactone.[13,39] On the other hand, the phenolic acids that presented the highest concentration in rat plasma were 3-(3,4-dihydroxyphenyl)-propionic acid and 3-(4-hydroxyphenyl)propionic acid (9.467 and 7.605 μM, respectively). The rest of the studied phenolic acids were found in concentrations lower than 1 μM. However, it is important to take into account that when microbial metabolites reach the liver, they can be subjected to phase II enzymes and can be conjugated to different final products,[12] as has been observed for 3-*O*-methylgallic acid, the methyl conjugate product from gallic acid[28] whose rat plasma concentration after GSPE intake was 0.217 μM, or ferulic acid, a methyl-conjugated derivative of 3-(3,4-dihydroxyphenyl)propionic acid,[28] which was quantified in the rat plasma at a concentration of 0.241 μM. Unfortunately, no comparable experiments exist in the literature for GSPE colonic metabolites, as they differ in their experimental approach and/or the composition of the extracts. Specifically, when microbial metabolites were analyzed in the plasma, the studies were performed with extracts from cocoa and almonds.[21,26] Almond polyphenols differ considerably in composition from polyphenols found in grapes,[21] and the cocoa-derived polyphenols, which are very similar to grape-

Table 6. Quantified Levels of Flavanols and Their Colonic Metabolites in Rat Plasma (n = 6) at 7 h after an Acute Intake of Grape Seed Proanthocyanidin Extract (1000 mg/kg)[a]

	total concn ± SEM[b] (μM)
Flavanols	
catechin	0.087 ± 0.000
epicatechin	0.532 ± 0.001
procyanidin dimer B1[c]	nq
procyanidin dimer B2	0.013 ± 0.000
procyanidin dimer B3[c]	nq
gallic acid	0.745 ± 0.001
vanillic acid	0.555 ± 0.000
epigallocatechin gallate	nq
Metabolites	
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol[d]	nq
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	1.276 ± 0.005
4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid[d]	nq
4-hydroxy-5-(3′-hydroxyphenyl)valeric acid[d]	nq
4-hydroxy-5-phenylvaleric acid[d]	nq
3-(3,4-dihydroxyphenyl)propionic acid[e]	9.467 ± 0.180
3-(3-hydroxyphenyl)propionic acid[e]	0.135 ± 0.004
3-(4-hydroxyphenyl)propionic acid	7.605 ± 0.084
3-phenylpropionic acid[e]	0.175 ± 0.000
2-(3,4-dihydroxyphenyl)acetic acid	0.637 ± 0.001
2-(3-hydroxyphenyl)acetic acid	0.111 ± 0.000
2-(4-hydroxyphenyl)acetic acid	0.140 ± 0.001
2-phenylacetic acid[f]	0.034 ± 0.000
3,4-dihydroxybenzoic acid[g]	nq
3-hydroxybenzoic acid	0.117 ± 0.000
4-hydroxybenzoic acid[g]	nq
benzoic acid	0.502 ± 0.002
3-O-methylgallic acid[h]	0.217 ± 0.000
homovanillic acid[i]	0.137 ± 0.000
homovanillyl alcohol[i]	nd
ferulic acid	0.241 ± 0.000
hippuric acid	0.669 ± 0.001
phloroglucinol	nd

[a]Data are given as the concentration (micromolar) ± standard error of the mean (SEM) from a triplicate chromatographic analysis. [b]Abbreviations: nd = not detected; nq = not quantified. [c]Quantified as procyanidin B2. [d]Quantified as 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. [e]Quantified as 3-(4-hydroxyphenyl)propionic acid. [f]Quantified as 2-(4-hydroxyphenyl)acetic acid. [g]Quantified as 3-hydroxybenzoic acid. [h]Quantified as gallic acid. [i]Quantified as vanillic acid.

derived polyphenols in composition, were used for chronic treatment in the study.[26] On the other hand, all of the studies performed with grape seed polyphenol extract,[13] its fractions,[40] or a red wine extract[27] were performed in in vitro systems by fecal fermentations.

In conclusion, the present study describes a rapid, simple, and sensitive method for measuring flavanol colonic metabolites in plasma samples by HPLC-ESI-MS/MS. The improved method for off-line μ-SPE plasma pretreatment allowed the flavanols and their colonic metabolites to be determined at low concentrations in a reduced plasma volume, which is an important factor to develop a complete in vivo study. In total, 30 flavanols and their colonic derivatives could be analyzed by a unique pretreatment extraction and chromatographic analysis. Hence, the method developed could be used for completed pharmacokinetics and bioavailability studies in animals.

AUTHOR INFORMATION

Corresponding Author

*Phone +34 977559566; fax +34 977558232; e-mail begona.muguerza@urv.cat.

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Notes

The authors declare no competing financial interest.

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MANUSCRIPT 2:

Plasma kinetics and microbial bioatransformation of grape seed flavanols in rats

Maria Margalef[†], Zara Pons[†], Francisca-Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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Plasma kinetics and microbial biotransformation of grape seed flavanols in rats

Maria Margalef [a], *Zara Pons* [a], *Francisca Isabel Bravo* [a], *Begoña Muguerza* [a,b,*], *Anna Arola-Arnal* [a]

[a] *Nutrigenomic Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona 43007, Spain*

[b] *Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus 43204, Spain*

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ABSTRACT

Flavan-3-ols and their oligomeric forms, proanthocyanidins (PAs), are poorly absorbed in the small intestine and reach the colon where gut bacteria enzymes can hydrolyse them to produce small molecular metabolites, which can reach systemic circulation. However, the microbial metabolism of flavanols has been poorly described *in vivo*. The aim of this study was to determine the colonic biotransformation pathway and the plasma temporal appearance of grape seed flavanols colonic metabolites in rats. Rat plasma colonic metabolites were analysed by HPLC-MS/MS at 2, 7, 24 and 48 h after 1000 mg/kg of a grape seed PA extract (GSPE) administration. Results indicated that non-metabolised flavanols have peak plasma concentrations 2 h after GSPE administration, whereas the colonic metabolites appeared in plasma later, indicating their gradual colonic biotransformation as valerolactone > phenylpropionic acids ≈ phenylacetic acids > benzoic acids. This study shows how flavanols are biotransformed by gut bacteria in rats over time, facilitating potential bioactive compound identification for particular health effects.

1. Introduction

Flavanols are among the most common polyphenols in the human diet and are mainly found in fruit, cocoa, tea, wine, nuts and beans (Aherne & O'Brien, 2002). This polyphenol group exists in both monomeric (catechin and epicatechin) and oligomeric (proanthocyanidins (PAs) or condensed tannins, depending on the molecular weight) aglycone forms and esterified with gallic acid (Aron & Kennedy, 2008). Flavanols improve human health, and our group has demonstrated that grape seed flavanols exhibit antioxidant capacity (Puiggros et al., 2005), improve lipid metabolism (Guerrero et al., 2013), limit adipogenesis (Pinent et al., 2005), act as an insulin-mimetic agent (Pinent et al., 2004), possess antihypertensive effects (Quiñones et al., 2013) and reduce inflammation (Terra et al., 2011).

The beneficial health properties of polyphenols are mainly attributed to the compounds derived from their metabolism (Del Rio et al., 2013). Dietary polyphenols are known to be recognised as xenobiotics and that they undergo phase II enzymatic detoxification at the small intestine and liver to form sulpho-, methyl- or glucorono-conjugates after their

* *Corresponding author.* Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, C/Marcel.lí Domingo s/n, 43007 Tarragona, Spain. Tel.: +34 977559566; fax: +34 977558232.
E-mail address: begona.muguerza@urv.cat (B. Muguerza).

Chemical compounds: Benzoic acid (PubChem CID: 243); 2-(3,4-Dihydroxyphenyl)acetic acid (PubChem CID: 547); (–)-Epicatechin (PubChem CID: 72276); Gallic acid (PubChem CID: 370); Hippuric acid (PubChem CID: 464); Homovanillic acid (PubChem CID 1738); 3-(4-Hydroxyphenyl)propionic acid (PubChem CID: 10394); 3-O-Methylgallic acid (CID: 19829); Procyanidin B2 (PubChem CID: 122738); Vanillic acid (PubChem CID: 8468).

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absorption in the small intestine. These metabolites can be returned to the lumen through the bile by enterohepatic circulation and can reach the systemic circulation to be transported to other tissues or to be excreted by the urine (Monagas et al., 2010). However, the PA absorption in the small intestine is determined by the degree of polymerisation, which has usually been considered to be a limiting factor on the health benefits from flavanol consumption (Del Rio et al., 2013). Whereas monomeric and low molecular-weight forms are mainly absorbed through the small intestine, some monomeric glucosides, oligomers with a degree of polymerisation greater than 2–3 and those forms from enterohepatic circulation cross the gastrointestinal tract and reach the colon, where they are transformed by intestinal microbiota for their absorption or excretion (Aura, 2008; Monagas et al., 2010). It is estimated that 90–95% of dietary polyphenols can reach the colon (Clifford, 2004; Monagas et al., 2010), where they are subjected to microbial catabolism.

The colon has diverse microbial populations composed of either obligatory or facultative anaerobes (including *Bacteriodes*, *Bifidobacterium*, *Enterobacteriaceae* and *Clostridium*) (Manichanh et al., 2010) responsible for degrading undigested food matrix and turn its components in microbial metabolites (Sánchez-Patán et al., 2012a). *In vitro* studies have shown that flavanol oligomers undergo an interflavanic link cleavage and turn into monomers (Monagas et al., 2010). The variety of enzymes produced by the gut bacteria can hydrolyse, reduce, dehydroxylate, decarboxylate and demethylate the polyphenols and convert them into different low molecular weight metabolites (valerolactone compounds, valeric acids, phenylpropionic acids, phenyl acetic acids, benzoic acids and several conjugated phenolic acids, consecutively) (Monagas et al., 2010; Sánchez-Patán et al., 2012a,b). These metabolites may reach the portal circulation through colonocytes being transported to the liver where they can be further metabolised by hepatic phase II enzymes and excreted into the urine or carried to the systemic circulation to reach different tissues (Monagas et al., 2010).

The colon metabolises flavanols and the beneficial health effects accredited to these compounds could be potentially produced not only by phase II but also by the colonic metabolites. Many studies using *in vitro* fermentation systems have evaluated the microbial metabolism of polyphenols (Sánchez-Patán et al., 2012a,b; Serra et al., 2011). However, the study of the microbial catabolism of flavonoids *in vivo* is limited (Serra et al., 2013; Urpi-Sarda et al., 2009a,b). The aim of this study is to evaluate how flavanols are biotransformed by the colon in rats and their colonic metabolites' time of occurrence in plasma and to establish a proposal of the rat flavanol catabolic pathway. Rat plasma colonic metabolites were analysed by high-performance liquid chromatography-tandem triple quadrupole mass spectrometry (HPLC-MS/MS) after different times of grape seed Pas administration.

2. Materials and methods

2.1. *Grape seed proanthocyanidin extract*

A grape seed PA extract (GSPE) was obtained from *Les Dérives Résiniques et Terpéniques* (Dax, France). The total polyphenol content and the individual flavanols and phenolic acids comprising the grape seed extract used in this study are detailed in Table 1 (adapted from Quiñones et al., 2013).

Table 1 – Total polyphenols and individual flavanols and phenolic acids of grape seed proanthocyanidin extract (GSPE).

Compound	Concentration (mg/g)
Total polyphenol content[1]	554.16 ± 13.02
Phenolic compound[2]	
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
Procyanidin dimer[a]	144.2 ± 32.2
Procyanidin trimer[a]	28.4 ± 2.0
Procyanidin tetramer[a]	2.0 ± 0.2
Dimer gallate[a]	39.7 ± 7.1
Epigallocatechin gallate	0.4 ± 0.1
Epicatechin gallate[b]	55.3 ± 1.5
p-coumaric acid	0.1 ± 0.0
Gallic acid	17.7 ± 2.0
3,4-dihydroxybenzoic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Quercetin	0.3 ± 0.0
Quercetin-3-O-gallate	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0

Data adapted from Quiñones et al. 2013.
The results are expressed on a wet basis as the mean ± SD (n = 3).
[1] Measured by Folin–Ciocalteu's method.
[2] HPLC-MS.
[a] Quantified using the calibration curve of proanthocyanidin B2.
[b] Quantified using the calibration curve of epigallocatechin gallate.

2.2. *Chemicals and reagents*

Acetone (HPLC analytical grade), methanol (HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). The following were individually dissolved in methanol at 4000 mg/L: (+)-catechin; (–)-epicatechin; benzoic acid; phloroglucinol; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2-(4-hydroxyphenyl)acetic acid; 2-(3,4-dihydroxyphenyl)acetic acid; 3-(4-hydroxyphenyl)propionic acid; vanillic acid; gallic acid; hippuric acid and ferulic acid (all from Fluka/Sigma-Aldrich, Madrid, Spain). The following were individually dissolved in methanol at 2000 mg/L: procyanidin B2; epigallocatechin gallate (EGCG); and pyrocatechol (internal standard (IS)) (all from Fluka/Sigma-Aldrich) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany). All standard stock solutions were prepared every 3 months and stored in dark-glass flasks at –20 °C.

A mixed standard stock solution in methanol was prepared weekly from the following compounds and stored in dark glass flasks at –20 °C: (+)-catechin; (–)-epicatechin; benzoic acid; phloroglucinol; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2-(4-hydroxyphenyl)acetic acid; 2-(3,4-dihydroxyphenyl)acetic acid; 3-(4-hydroxyphenyl) propionic acid; vanillic acid; gallic acid; hippuric acid; ferulic

acid; EGCG and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone at 200 and 100 mg/L of procyanidin B2, respectively. This mixed standard stock solution was diluted daily to the desired concentration using an acetone/Milli-Q water/acetic acid (70/29.5/0.5, v/v/v) solution and stored in dark glass flasks at –20 °C until chromatographic analysis.

2.3. *Animal and plasma collection*

Male Wistar rats, weighing between 226 and 260 g, were obtained from Charles River Laboratories (Barcelona, Spain). All animals were housed at 22 °C with a light/dark cycle of 12 h (lights on at 09:00 AM) and were fed a standard chow diet (AO4, Panlab, Barcelona, Spain) *ad libitum* during the experiment. The animals were randomly divided into two groups: the control group (n = 6) and the GSPE group (n = 6). A dose of 1000 mg/kg of GSPE (1 mL in water) was administered to the GSPE group by oral gavage. Water (1 mL) was orally administered to the control group. In both groups, oral administration was performed by gastric intubation to fasted rats between 9 and 10 AM. Fasting blood samples were obtained via saphenous vein extraction using heparin vials (Starsted, Barcelona, Spain) at 0, 2, 7, 24 and 48 h after GSPE or water administration, in order to see the kinetic behaviour of microbial metabolites, which are supposed to appear at later times than the flavanol phase-II metabolites. Plasma samples were obtained by centrifugation (2000 × *g*, 15 min, 4 °C) and pooled (n = 6), to have sufficient volume for the analysis and also to remove the biological variability. The pooled plasma samples were stored at –80 °C until chromatographic analysis was performed (Fig. 1). Plasma from the control group (water oral gavage) was used to perform the calibration curves in the chromatography analysis. Any compound present in the plasma control group (0 h time-point) was subtracted from the plasma concentration at all other time-points. The plasma samples were not treated with glucuronidase or sulphatase enzymes. The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of Universitat Rovira i Virgili (permission number 6777).

2.4. *Micro-solid phase plasma polyphenol extraction*

Prior to chromatographic analysis, the pool of rat plasmas (n = 6) for each time-point was pre-treated by off-line micro-Solid Phase Extraction (μ-SPE) as described previously (Margalef, Pons, Muguerza, & Arola-Arnal, 2014) (Fig. 1) using OASIS HLB μ-Elution Plates 30 μm (Waters, Barcelona, Spain). Briefly, the micro-cartridges were conditioned sequentially with 250 μL methanol and 250 μL 0.2% acetic acid. A plasma aliquot (250 μL) was mixed with 300 μL 4% phosphoric acid and 50 μL pyrocatechol (250 ppb), and then this mixture was loaded into the plates. The loaded plates were washed with 200 μL Milli-Q water and 200 μL 0.2% acetic acid. The retained polyphenols were eluted with 2 × 50 μL acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v).

2.5. *Chromatographic analysis*

The eluted solution was directly analysed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, U.S.A.) as previously described (Margalef et al., 2014). Briefly, Zorbax SB-Aq (150 × 2.1 mm i.d., 3.5 μm particle size, Agilent Technologies) was the chromatographic column. The mobile phase was 0.2% acetic acid (solvent A) and acetonitrile (solvent B) with a flow rate of 0.4 mL/min. The elution gradient was 0–10 min, 5–55% B, 10–12 min, 55–80% B, 12–15 min, 80% B isocratic, and 15–16 min 80–5% B. A post run of 10 min was applied and 2.5 μL of sample were injected. Electrospray ionisation (ESI) was conducted at 350 °C and 12 L/min with 45 psi of nebuliser gas pressure, and 4000 V of capillary voltage. The mass spectrometer was operated in negative mode and MS/MS data were acquired in Multiple Reaction Monitoring (MRM) mode. Optimised MRM conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS are presented in Table 2 and all quality parameters required to perform the analysis of these metabolites are shown in Table 3.

3. Results

In this study, we quantified the colonic metabolites that appeared in pooled (n = 6) rat plasma 0, 2, 7, 24 and 48 h after GSPE (1000 mg/kg) ingestion using HPLC-MS/MS. The pooled plasma was necessary to collect enough volume for three replicate chromatographic analyses at the different times without sacrificing the rats. Moreover, pooled plasma increases homogeneity and sensitivity in order to allow the detection of all potential metabolites (Demelbauer, Plematl, Josic, Allmaier, & Rizzi, 2005;

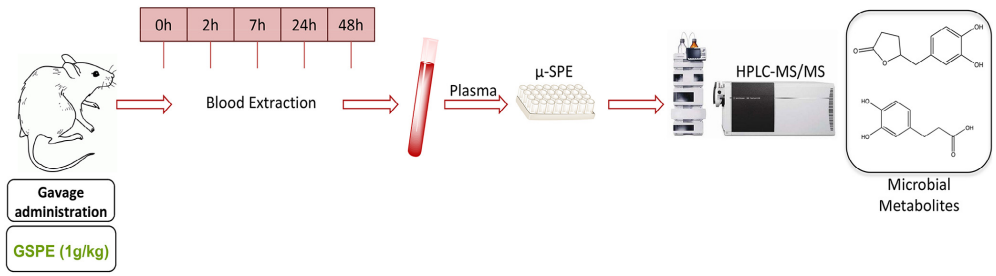

Fig. 1 – Graphical representation of the experimental design used in this study.

Table 2 – Optimized MRM conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS.

Compound	MS/MS Conditions					
	Quantification			Confirmation		
	MRM_1	F (V)	CE (V)	MRM_2	F (V)	CE (V)
Catechin	289 > 203	120	20	289 > 245	120	20
Epicatechin	289 > 245	130	10	289 > 203	130	20
Procyanidin B1	577 > 425	130	10	577 > 407	130	30
Procyanidin B2	577 > 425	130	10	577 > 407	130	30
Procyanidin B3	577 > 425	130	10	577 > 407	130	30
Gallic acid	169 > 125	90	10	169 > 79	90	40
Vanillic acid	167 > 152	80	10	167 > 123	80	5
Epigallocatechin gallate	457 > 169	110	20	457 > 305	110	20
1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	291 > 247	70	20	291 > 96	70	20
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	207 > 85	120	10	207 > 121	120	10
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	225 > 163	70	10	225 > 181	70	10
4-Hydroxy-5-(3′-hydroxyphenyl)-valeric acid	209 > 147	150	0	–	–	–
4-Hydroxy-5-(phenyl)-valeric acid	193 > 175	60	10	–	–	–
3-(3,4-Dihydroxyphenyl)propionic acid	181 > 137	60	10	–	–	–
3-(3-Hydroxyphenyl)propionic acid	165 > 121	90	10	165 > 59	90	0
3-(4-Hydroxyphenyl)propionic acid	165 > 121	90	10	165 > 59	90	0
Phenylpropionic acid	149 > 105	90	10	–	–	–
2-(3,4-Dihydroxyphenyl)acetic acid	167 > 123	50	10	167 > 95	50	30
2-(3-Hydroxyphenyl)acetic acid	151 > 107	60	5	151 > 93	60	20
2-(4-Hydroxyphenyl)acetic acid	151 > 107	60	5	151 > 65	60	30
Phenylacetic acid	135 > 91	90	5	135 > 100	90	5
3,4-Dihydroxybenzoic acid	153 > 109	80	10	153 > 62	80	40
3-Hydroxybenzoic acid	137 > 93	70	10	–	–	–
4-Hydroxybenzoic acid	137 > 93	70	10	–	–	–
Benzoic acid	121 > 77	60	5	121 > 59	60	5
3-O-Methylgallic acid	183 > 168	90	10	183 > 124	90	10
Homovanillic acid	181 > 163	90	10	181 > 134	90	20
Homovanillyl alcohol	167 > 152	150	10	167 > 133	150	10
Ferulic acid	193 > 134	60	10	193 > 178	60	10
Hippuric acid	178 > 134	80	5	178 > 77	80	10
Phloroglucinol	125 > 57	90	10	125 > 125	90	0

Abbreviations: F, Fragmentor; CE, Collision Energy; MRM, Multiple reaction monitoring.

Margalef et al., 2014; McGaw, Phinney, & Lowenthal, 2010). A range of time points was selected to detect all potential colonic metabolites and to study how these metabolites appear in plasma at different times, allowing the different flavanols to reach the colon, to be metabolised by gut bacteria and to be absorbed by rats (Demelbauer et al., 2005; Serra et al., 2011, 2013).

3.1. *Plasma kinetics of non-metabolised PA in rat plasma*

Procyanidin B2 (**1**), catechin (**2**), epicatechin (**3**), gallic acid (**4**) and vanillic acid (**5**) are polyphenols present in GSPE at considerable concentrations (Table 1). These compounds were absorbed, peaked in plasma concentration 2 h after GSPE ingestion and disappeared at 24 h (Fig. 2). Interestingly, the concentration of compound **5** increased again after 24 h but did not reach as high concentrations as at 2 h (Fig. 2C).

3.2. *Plasma kinetics of microbial PA metabolites in rat plasma*

3.2.1. *Valerolactone metabolites*

The colonic metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**6**) that had its highest plasma concentration at 7 h after GSPE administration, was still present in the plasma at 24 h and disappeared after 48 h (Fig. 3). No other valerolactone metabolites were detected in rat plasma (data not shown).

3.2.2. *Phenylpropionic acid metabolites*

The phenylpropionic acids colonic metabolites 3-(4-hydroxyphenyl) propionic acid (**7**), 3-(3,4-dihydroxyphenyl) propionic acid (**8**) and 3-(3-hydroxyphenyl) propionic acid (**9**) peaked in plasma concentrations 24 h post GSPE administration, and at 48 h, there was still a considerable amount of these metabolites in plasma (Fig. 4). Interestingly, **7** and **8** reached very high plasma concentrations, having a concentration of approximately 50 μM and 10 μM at 24 h, respectively (Fig. 4A). Much lower concentrations were found for **9** and for phenylpropionic acid (**10**) (Fig. 4B).

3.2.3. *Phenylacetic acid metabolites*

The phenylacetic acid metabolites had different kinetic profiles depending on the metabolite. Although 2-(4-dihydroxyphenyl) acetic acid (**11**), 2-(3-dihydroxyphenyl) acetic acid (**12**) and phenylacetic acid (**13**) reached their highest concentration in plasma as rapidly as 2 h post-GSPE administration (Fig. 5A), 3,4-dihydroxyphenylacetic acid (**14**) had peak plasma concentration at 24 h post-administration. A higher concentration of **14** was found in plasma (approximately 2 μM

Table 3 – The retention behaviour (RT, min), calibration curve, determination coefficient (R^2), working linearity range, LODs, LOQs, MDLs and MQLs for phenolic compound quantification in spiked plasma samples using HPLC-ESI-MS/MS. (Adapted from Margalef et al. 2014 [19])

Compound	RT (min)	Calibration curve	Determination coefficient (R^2)	Working linearity range (μM)	LOD (nM)	LOQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Catechin	6.4	y = 0.799x	0.997	0.007–17.225	2.30	7.66	0.66	2.19
Epicatechin	6.9	y = 1.674x	0.996	0.007–17.225	1.66	5.52	0.47	1.58
Procyanidin B2	6.7	y = 34.138x	0.991	0.008–4.321	1.95	6.49	0.56	1.85
Epigallocatechin gallate	7.3	y = 8.399x	0.990	0.004–10.908	0.76	2.52	0.22	0.72
Gallic acid	2.8	y = 1.035x	0.991	0.059–29.391	11.75	39.22	3.36	11.20
Vanillic acid	6.7	y = 0.862x	0.996	0.012–29.762	4.29	14.29	1.22	4.08
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	7.2	y = 0.746x	0.998	0.048–24.038	14.42	48.08	4.12	13.74
3-(4-Hydroxyphenyl)propionic acid	7.3	y = 0.063x	0.997	0.060–30.120	24.10	80.32	6.88	22.95
2-(3,4-Dihydroxyphenyl)acetic acid	4,3	y = 0.389x	0.996	0.060–29.762	11.90	39.68	3.40	11.34
2-(3-Hydroxyphenyl)acetic acid	5.8	y = 0.775x	0.995	0.013–32.895	3.29	10.96	0.94	3.13
2-(4-Hydroxyphenyl)acetic acid	6.1	y = 0.534x	0.994	0.013–32.895	8.87	29.90	2.56	8.54
3-Hydroxybenzoic acid	6.3	y = 0.690x	0.998	0.072–36.232	39.13	130.43	11.28	37.37
Benzoic acid	7.8	y = 0.510x	0.995	0.016–40.984	3.78	12.61	1.08	3.60
Ferulic acid	8.1	y = 5.658x	0.995	0.010–25.773	2.13	7.11	0.61	2.03
Hippuric acid	5.5	y = 1.981x	0.986	0.011–27.933	0.10	0.34	0.03	0.10
Phloroglucinol	2.3	y = 0.157x	0.990	0.079–39.683	23.81	79.37	6.80	22.68

Abbreviations: RT (Retention behaviour); LOD (Limit of detection); LOQ (Limit of quantification); MDL (Method detection limit); MQL (Method quantification limit).

[a] Method of detection and quantification limits in nmol/L of fresh sample calculated for the analysis of 250 μL of plasma sample.

at 24 h after the GSPE administration) with respect to the other phenylacetic metabolites (less than 1.2 μM at 2 h) (Fig. 5B).

3.2.4. *Benzoic acid metabolites*

Benzoic acid derivatives can be formed by different flavanol metabolisation pathways (Selma, Espín, & Tomás-Barberán, 2009; Serra et al., 2011; Urpi-Sarda et al., 2009a,b), and a variety of compounds may be formed. However, we were able to quantify only rat plasma 3-hydroxybenzoic acid (**15**) and benzoic acid (**16**), and no other benzoic acid metabolites were detected in plasma (data not shown); **15** and **16** reached considerable plasma concentration levels with comparable kinetic profiles (Fig. 6). These metabolites peak in plasma concentration at 2 h post GSPE administration, and then from 2 to 7 h, the concentration in plasma decreased and was maintained until 48 h.

3.2.5. *Phenolic acids metabolites*

The phenolic acids detected in this study were hippuric acid (**17**), homovanillic acid (**18**), homovanillyl alcohol (**19**) and 3-O-methyl gallic acid (**20**). It is important to note that microbial metabolites can reach the portal circulation through colonocytes being transported to the liver where they can be further subjected to phase II metabolism before entering the circulation (Gonthier et al., 2003). Thus, **18**, **19** and **20** were some of the methylated products detected in rat plasma (Fig. 7), and these final metabolites have different kinetic profiles. Although **18**, **19** and **20** reached the highest concentration in plasma at 2 h post GSPE administration, the concentration of **18** was maintained for 48 h, but the plasma levels of **19** and **20** were decreased within 24 h. Otherwise, **17** had a completely different kinetic profile as it reached peak concentration in plasma at 7 h post GSPE administration, and later levels were decreased down to 24 h and maintained up to 48 h. Moreover, **17** had maximum concentration levels of approximately 0.7 μM, whereas **18**, **19** and **20** did not reach concentrations higher than 0.5 μM. No other phase II conjugated colonic metabolites were detected in rat plasma (data not shown).

4. Discussion

The changes occurring during the first-pass metabolism are an important aspect of flavanols. The molecular forms that reach the peripheral circulation and tissues are different from those that are present in foods (Kroon et al., 2004). Microflora extensively affect flavanol cleavage and hence further contribute to the variation in the molecular forms of the flavanols found in blood and tissues (Dall'Asta et al., 2012; Gonthier et al., 2003). These findings suggest that the bioactive forms of flavanols could be products of their wide metabolism (Del Rio et al., 2013; Guerrero et al., 2013). Although there are several studies in rats and humans evaluating flavanol phase II metabolism (Arola-Arnal et al., 2013; Das & Rosazza, 2006), the microbial colonic catabolism of flavanols has been poorly studied *in vivo*, but it has been evaluated extensively by *in vitro* experiments using a human or rat faecal matrix (Cueva et al., 2013; Sánchez-Patán et al., 2012a,b Serra et al., 2012). As the phase II metabolism of flavanols is already well established, in this study, we focused only on the evaluation of rat microbial biotransformation of flavanols through a kinetic analysis of rat plasma after acute GSPE administration (1000 mg/kg). We propose an *in vivo* microbial metabolic pathway for grape seed flavanols (Fig. 8).

Similar to other studies, we found that the non-metabolised compounds reach their peak plasma concentrations at 2 h post-administration of GSPE (Serra et al., 2013). Although also abundant in GSPE, non-metabolised dimeric procyanidins or those with higher molecular weights showed much less

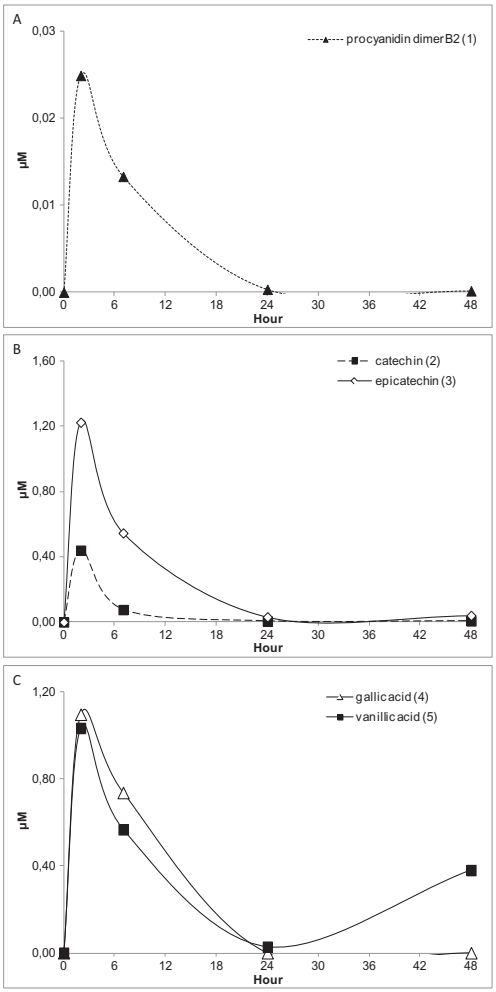

Fig. 2 – Kinetic profiles of rat plasma non-metabolised flavanols from grape seed proanthocyanidin extract (GSPE). Proanthocyanidin dimer B2 (1) (A), Monomeric flavanols (2 and 3) (B) and Phenolic acids (4 and 5) (C). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

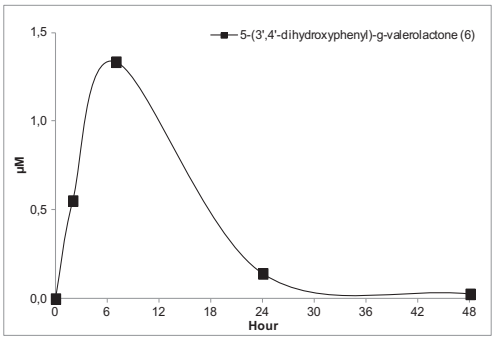

Fig. 3 – Kinetic profiles of rat plasma valerolactone metabolite (6) from grape seed proanthocyanidin extract (GSPE). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

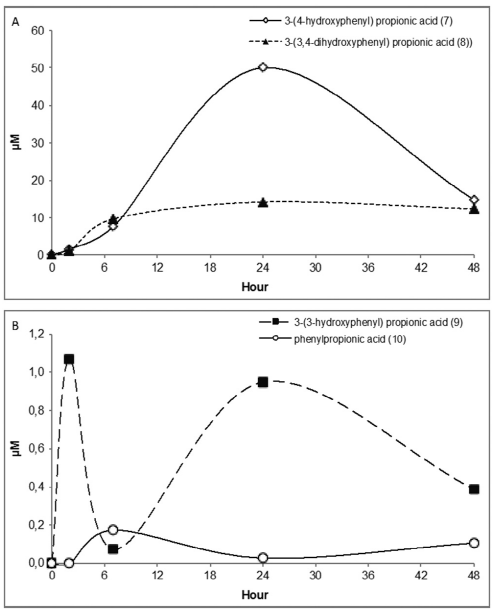

Fig. 4 – Kinetic profiles of rat plasma phenylpropionic acid from grape seed proanthocyanidin extract (GSPE). 3-(4-hydroxyphenyl)propionic acid (7) and 3-(3,4-dihydroxyphenyl)propionic acid (8) (A) and 3-(3-hydroxyphenyl)propionic acid (9) and phenylpropionic acid (10) (B). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

significant plasma levels than non-metabolised flavan-3-ols or did not appear in plasma, respectively. This is attributed to the larger flavanol molecular weight, making small intestine absorption more difficult, and hence the polymeric forms reach the colon to be subjected to microbial metabolism (Del Rio et al., 2013; Monagas et al., 2010). Once in the colon, flavanols can be biotransformed by three different metabolic pathways (Fig. 8). The first pathway is meta-substitution of the flavanol A ring producing 5-(2′,4′-dihydroxy) phenyl-2-ene-valeric acid (21).

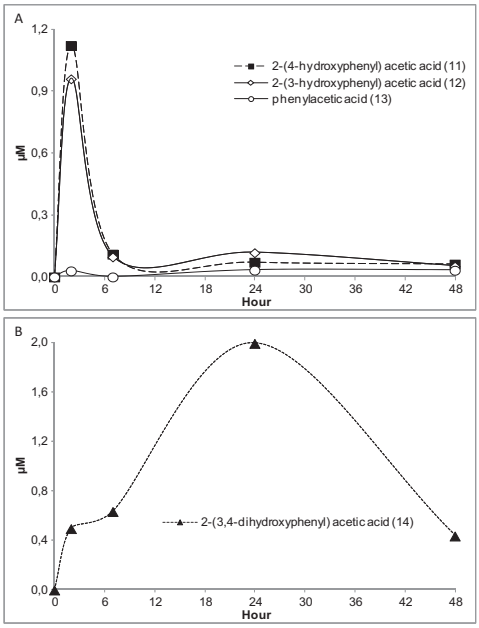

Fig. 5 – Kinetic profiles of rat plasma phenylacetic acids from grape seed proanthocyanidin extract (GSPE). 2-(4-hydroxyphenyil)acetic acid (11), 2-(3-hydroxyphenyil)acetic acid (12) and phenylacetic acid (13) (A) and 2-(3,4-dihydroxyphenyil)acetic acid (14) (B). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

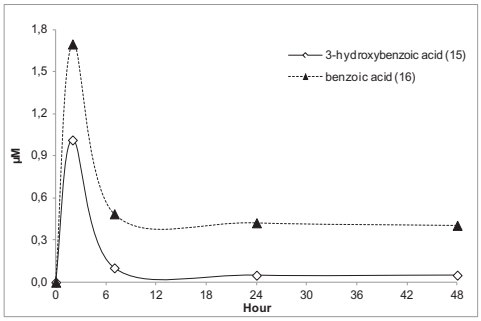

Fig. 6 – Kinetic profiles of rat plasma benzoic acids (15 and 16) from grape seed proanthocyanidin extract (GSPE). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

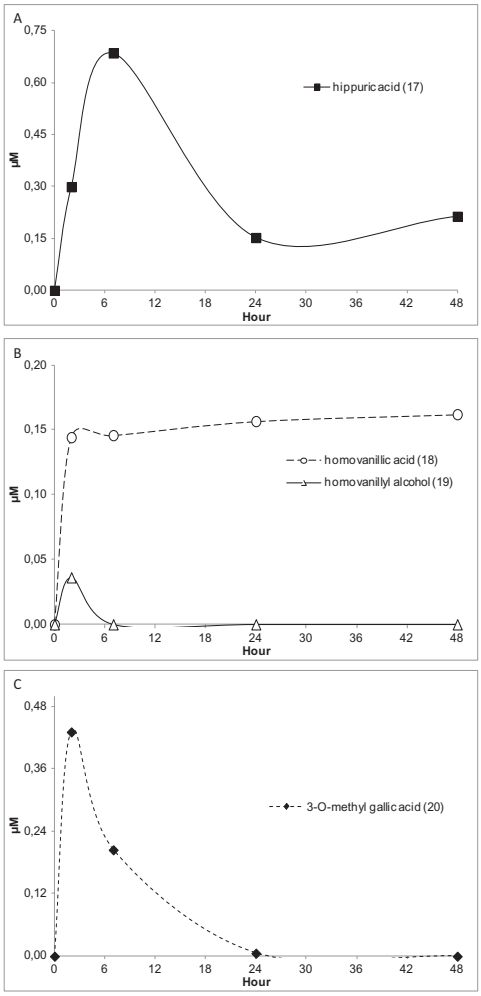

Fig. 7 – Kinetic profiles of rat plasma phenolic acids from grape seed proanthocyanidin extract (GSPE). Hippuric acid (17) (A), homovanillic acid (18) and homovanillyl alcohol (19) (B) and 3-O-methyl gallic acid (20) (C). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

However, this metabolite was not detected in rat plasma (data not shown), suggesting that it could be an exclusively human metabolite because it was detected using human faecal microbiota. Alternatively, it is an intermediate unable to reach the bloodstream as it is known to be further metabolised by reduction reactions (Stoupi, Williamson, Drynan, Barron, &

JOURNAL OF FUNCTIONAL FOODS 12 (2015) 478–488

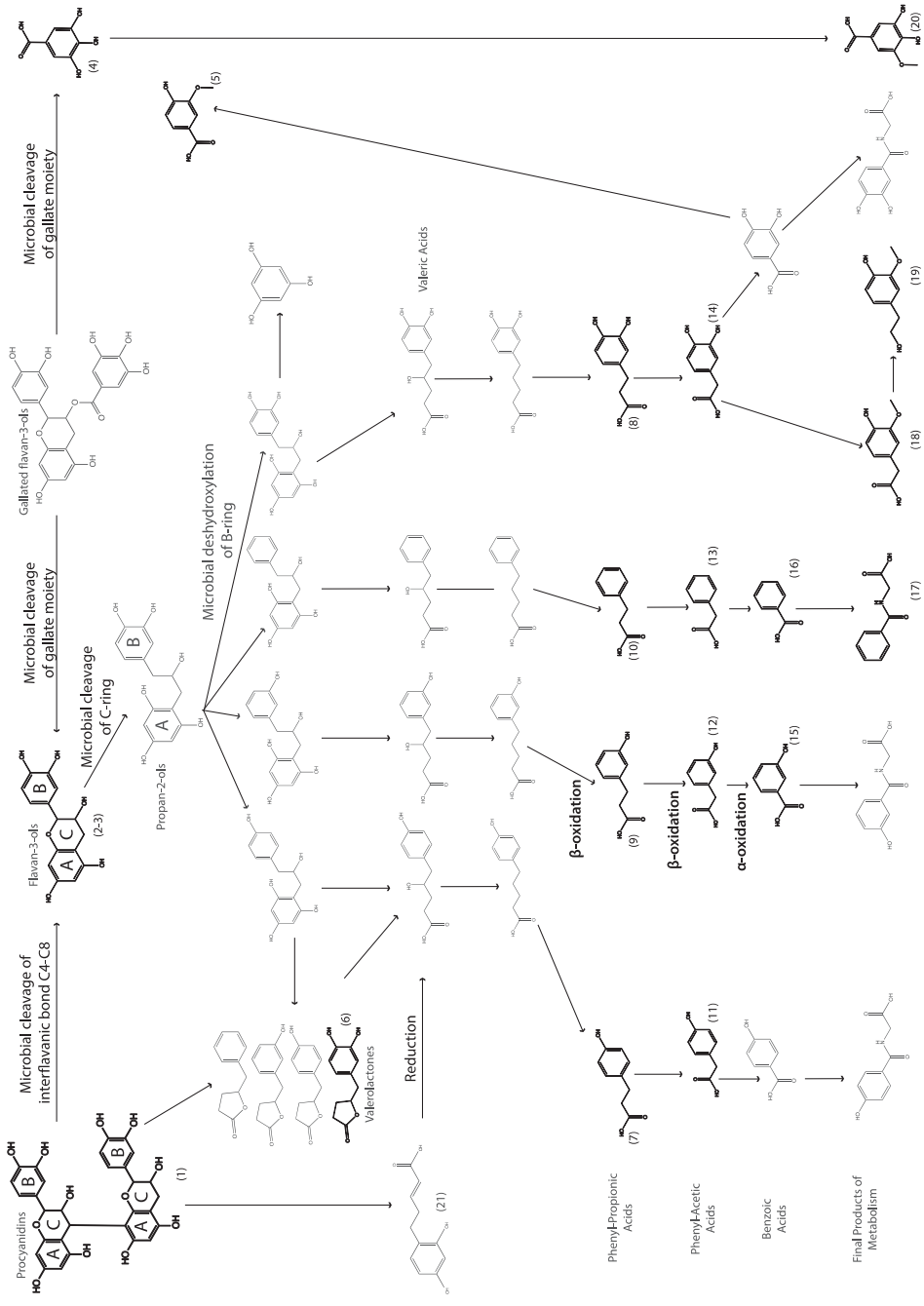

Fig. 8 – The proposed microbial colonic biotransformation pathway of proanthocyanidin in rats according to the detected metabolites in rat plasma after 0 h, 2 h, 7 h, 24 and 48 h of a 1000 mg/kg of a grape seed proanthocyanidin extract (GSPE) administration. The metabolites detected in rat plasma appear in bold, and the metabolites cited in the text are numerated.

Clifford, 2010a). The second pathway is biotransformation to valerolactone compounds, such as **6**, by the microbial cleavage of flavanol C- and A- rings (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009). The final pathway is microbial cleavage of the flavanol interflavanic bond C4–C8 to be transformed in their respective monomeric forms (**2** and **3**) (Appeldoorn et al., 2009). Monomeric aglycones can also get to the colon from conjugated compounds such as epicatechin gallate by microbial cleavage of the gallate moiety (Selma et al., 2009) or from phase II conjugates that reach the colon through bile extraction by enterohepatic recirculation or from non-absorbed monomeric flavanol glucosides that pass intact throughout the gastrointestinal tract. Monomeric flavan-3-ols (**2** and **3**) can undergo a microbial cleavage of the C-ring to produce propan-2-ol metabolites with different degrees of hydroxylation by a microbial dehydroxylation of the B-ring. Propan-2-ol metabolites may become valerolactones (**6**) by microbial A-ring cleavage. Alternatively, it is suggested that these valerolactones could come directly from the PA dimers by the microbial cleavage of the C- and A- rings (Appeldoorn et al., 2009). This metabolic process is in agreement with the kinetic behaviour of **6**, which had a maximum plasma concentration 7 h after the non-metabolised flavanols had sufficient time to be processed. The propan-2-ol metabolites were not detected in rat plasma (data not shown), which supports the metabolic pathway proposed in this study (Fig. 8) as propan-2-ol are intermediates that are further metabolised.

These early metabolites undergo microbial cleavage of the lactone ring by acidic hydrolysis (Dall'Asta et al., 2012; Del Rio et al., 2013) to form valeric acid metabolites, which were also not detected in rat plasma as they are intermediate compounds (data not shown). Although no valeric acid or propan-2-ol metabolites were detected in rat plasma, previous *in vitro* studies with rat (Serra et al., 2011, 2012) or human (Cueva et al., 2013; Sánchez-Patán et al., 2012a,b) faecal microflora detected these compounds. This study found that these intermediates do not reach the systemic circulation at sufficient concentration levels to be detected and quantified by MS, which suggests that they may remain in the colon to be subject to the microbial metabolism.

It has been described that valeric acids may suffer a β-oxidation of the branched chain to form phenyl propionic acids and their derivatives (Selma et al., 2009; Stoupi et al., 2010a), which are the main compounds from the microbial metabolism (Aura, 2008). In this sense, the main grape seed flavanol metabolite product of the microbial biotransformation was **7**, which reached levels of 50 μM in rat plasma at 24 h after GSPE ingestion. Propionic acids appeared in plasma after valerolactone metabolites, which agrees with our proposed metabolic pathway (Fig. 8). Other phenylpropionic acids (such as **8–10**) were quantified in rat plasma. However, **7–10** are further metabolised to form phenylacetic acids (**11–14**) and benzoic acids (**15–17**) by the β- and α-oxidation of their branched chain, respectively (Stoupi, Williamson, Drynan, Barron, & Clifford, 2010b). A different kinetic behaviour was demonstrated between **14** and phenylacetic acids (Fig. 5), which suggests that the hydroxylation pattern could be performed when the compounds reach the systemic circulation by the hydroxylation enzymes, more than by the microbial metabolism (Aura, 2008).

Phenolic acid derivatives (**17–20**) are the final products of the metabolism, which are formed by the enzymatic conjugation of phenolic acids (Gregus, Fekete, Halaszi, & Klaassen, 1996; O'Leary et al., 2001), and hence a heterogeneous kinetic profile was also observed in rat plasma. In this sense, **17**, also known as benzoylglycine, is formed in the liver from benzoic acid in two enzymatic steps with benzoyl-coA synthase and benzoyl-CoA glycine N-transferase (Gregus et al., 1996; Nandi, Lucas, & Webster, 1979); **18** and **19** are methylated derivatives of phenylacetic acids, and **20** is a methylated derivative of gallic acid. The methylation is produced mainly by hepatic catechol-O-methyltransferase (COMT) (O'Leary et al., 2003). As final products of flavanol metabolism, all of these compounds are still present in plasma 48 h post GSPE administration. Particularly, **5** reached maximal concentrations in plasma 2 h post GSPE administration as this compound is also present in GSPE. After 24 h, its concentration in plasma starts to increase again as a product of the flavanol colonic metabolism.

This study shows how grape seed flavanols are biotransformed by rat gut bacteria enzymes and how they appear in rat plasma over time. These results may facilitate identification of flavanol bioactive forms for particular health effects. Further human studies using the described methodology will be interesting due to the different metabolism between species and the intra- and inter-individual colonic bacteria variations (Kleessen, Bezirtzoglou, & Mättö, 2000; Lampe, 2003).

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CHAPTER 2:
External factors affecting the flavanol metabolism

MANUSCRIPT 3:

Serum metabolites of proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells

Ligia Gerrero[†¥1]; Maria Margalef[†1], Zara Pons[†], Mar Quiñones[†], Lluis Arola[†§,] Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [b] Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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Serum metabolites of proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells

Ligia Guerrero[a,b,1], Maria Margalef[a,1], Zara Pons[a], Mar Quiñones[a], Lluis Arola[a,c], Anna Arola-Arnal[a,*], Begoña Muguerza[a,c]

[a]Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona 43007, Spain
[b]Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia
[c]Centre Tecnològic de Nutrició i Salut (CTNS), Reus 43204, Spain

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Abstract

The regular consumption of flavonoids has been associated with reduced mortality and a decreased risk of cardiovascular diseases. The proanthocyanidins found in plasma are very different from the original flavonoids in food sources. The use of physiologically appropriate conjugates of proanthocyanidins is essential for the *in vitro* analysis of flavonoid bioactivity.

In this study, the effect of different proanthocyanidin-rich extracts, which were obtained from cocoa (CCX), French maritime pine bark (Pycnogenol extract, PYC) and grape seed (GSPE), on lipid homeostasis was evaluated. Hepatic human cells (HepG2 cells) were treated with 25 mg/L of CCX, PYC or GSPE. We also performed *in vitro* experiments to assess the effect on lipid synthesis that is induced by the bioactive GSPE proanthocyanidins using the physiological metabolites that are present in the serum of GSPE-administered rats. For this, Wistar rats were administered 1 g/kg of GSPE, and serum was collected after 2 h. The semipurified serum of GSPE-administered rats was fully characterized by liquid chromatography tandem triple quadrupole mass spectrometry (LC–QqQ/MS^2). The lipids studied in the analyses were free cholesterol (FC), cholesterol ester (CE) and triglycerides (TG).

All three proanthocyanidin-rich extracts induced a remarkable decrease in the *de novo* lipid synthesis in HepG2 cells. Moreover, GSPE rat serum metabolites reduced the total percentage of CE, FC and particularly TG; this reduction was significantly higher than that observed in the cells directly treated with GSPE. In conclusion, the bioactivity of the physiological metabolites that are present in the serum of rats after their ingestion of a proanthocyanidin-rich extract was demonstrated in Hep G2 cells.

Keywords: Grape seed proanthocyanidin extract; Serum metabolites; Lipid synthesis; Cell cultures; HepG2 cells

1. Introduction

Cardiovascular disease (CVD) is the main cause of death worldwide. According to the World Health Organization, 17.3 million people died from CVD in 2008; this number represents 30% of the total deaths worldwide. In addition, starting from 2015, it is estimated that approximately 20 million people will die every year from this disease. Among the modifiable risk factors for CVD, dyslipidemia, hypertension, smoking and diabetes mellitus are of particular relevance [1]. Specifically, hyperlipidemia, which is the elevation of cholesterol and/or triglyceride (TG) levels, is a significant risk factor for the development of atherosclerosis and heart disease.

There is a body of evidence that indicates that a diet rich in vegetables and fruits decreases the risk of CVD [2–4]; in addition, this decrease has been attributed to the phenolic compounds that are present in plants. Flavonoids are phenolic compounds that are commonly found in fruits and vegetables at high concentrations, and their regular consumption has been associated with a reduced mortality and a decreased risk of CVD [5–8]. More specifically, grapes, wine, cocoa and pine are known to be significant sources of flavonoids, particularly flavan-3-ols and proanthocyanidins [9,10].

It is well known that proanthocyanidins improve human health by affecting the cellular and physiological processes. Several studies have shown the beneficial effects of proanthocyanidins on lipid metabolism in different experimental models [11,12] and humans [13,14]. Our group has previously shown that the oral administration of grape seed proanthocyanidins significantly reduces the postprandial levels of TG-rich and apolipoprotein-B-containing lipoproteins and improves several atherosclerotic risk indexes in normolipidemic rats [15]. In addition, grape seed proanthocyanidins lead to a reduction in the production and secretion of TG in the human hepatocarcinoma cell line HepG2 [16]. However, the effect of other proanthocyanidin extracts on lipid production and secretion has not been studied in hepatic cells.

* Corresponding author. Dpt. Bioquímica i Biotecnologia, Universitat Rovira i Virgili, C/ Marcel.lí Domingo s/n 43007 Tarragona, Spain. Tel.: +34 977 55 86 30; fax: +34 977 55 82 32.
E-mail address: anna.arola@urv.cat (A. Arola-Arnal).
[1] These authors contributed equally to this work.

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It is generally accepted that polyphenol bioavailability is relatively poor [17], although the monomeric flavan-3-ols (or flavanols) are among the polyphenols that exhibit higher bioavailability [18]. Moreover, the rapid conversion of flavonoids into their metabolites is well known [19]. Therefore, the large amount of scientific data that has been generated using nonphysiologically relevant forms and/or concentrations of flavonoids is questionable [20,21]. In fact, the limited description of flavonoid bioactivity that is based on studies that use nonphysiologically relevant forms and/or concentrations of these compounds is considered the most important limitation in flavonoid research [20]. Thus, the development of *in vitro* models using physiologically appropriate conjugates, forms and concentrations of flavonoids that are more similar to those observed in tissues after the intake of these compounds is an important requirement for the analysis of flavonoid bioactivity [21]. Hence, we hypothesized that this question could be addressed through the treatment of cells with the sera of rats that were orally administered a proanthocyanidin-rich extract. In this study, the cells were incubated directly with the flavonoid metabolites in the rat sera, which would simulate the physiological conditions that occur within the body. The use of the sera of rats that were previously administered the compound under study for the treatment of cell cultures has been recently described. In fact, a previous study confirmed the bioactivity of bezafibrate, which is a known peroxisome proliferator-activated receptor-γ ligand, in HeLa cells using serum [22]. In a similar manner, in this study, rats were used as a tool to produce flavonoid metabolites. These physiological forms were utilized to treat HepG2 cells, allowing the evaluation of the functionality of the bioactive forms.

Therefore, the aim of this study was to first evaluate the effect of different proanthocyanidin-rich extracts on the lipid production and secretion in HepG2 cells. Moreover, the lipid-lowering effect of bioactive proanthocyanidins was also evaluated using an *in vitro* system; in this system, the cells were treated with the physiological metabolites that were present in the serum of rats that ingested a grape seed proanthocyanidin extract (GSPE).

2. Materials and methods

2.1. Chemicals and reagents

Chromatographic analysis: Methanol (Scharlab S.L., Barcelona, Spain), acetone (Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of high-performance liquid chromatography (HPLC) analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). The 1000-mg/L standard stock solutions of (+)-catechin, (−)-epicatechin, gallic acid, epigallocatechin gallate (EGCG), proanthocyanidin B2 and proanthocyanidin B1 (all from Fluka/Sigma-Aldrich, Madrid, Spain) in methanol and the 2000-mg/L standard solution of pyrocatechol in methanol, which was used as an internal standard (Fluka/Sigma-Aldrich), were stored in a dark-glass flask at −20°C.

Standard stock mixtures with a concentration of 200 mg/L of (+)-catechin, (−)-epicatechin, EGCG and gallic acid in methanol and 100 mg/L of proanthocyanidin B2 and proanthocyanidin B1 in methanol were prepared weekly and stored at −20°C. These standard stock solutions were diluted daily to the desired concentration using an acetone/water/acetic acid (70:29.5:0.5, v:v:v) solution.

Cell culture: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Bio Whittaker Europe (Verviers, Belgium). The Bradford protein reagent was obtained from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA). ^{14}C-acetate was purchased from Amersham Biosciences (Buckinghamshire, England).

2.2. Proanthocyanidin-rich extracts

The GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this proanthocyanidin extract contains monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%) and oligomeric (>5) (35.7%) proanthocyanidins.

The cocoa proanthocyanidin extract (CCX) used for this study was provided by Natraceutical (Valencia, Spain). This extract was obtained from a polyphenol-rich cocoa powder that was produced from unfermented, blanch-treated, nonroasted cocoa beans, which preserve the degradation of polyphenols [23]. The data obtained using normal-phase HPLC showed that the proanthocyanidin cocoa extract contains monomeric (23.7%), dimeric (15.8%), trimeric (18.4%), tetrameric (13.9%) and oligomeric proanthocyanidins (>5) (36.2%) [24].

The Pycnogenol extract (PYG) was provided by Shirota Functional Foods (Tarragona, Spain). The PYG contains monomeric (38.0%), dimeric (40.9%) and oligomeric (containing more than three monomeric units, 62.0%) proanthocyanidins. [25].

2.3. Experimental procedure in rats

In this study, 17- to 20-week-old male Wistar rats that weighed 300–350 g were used. The Animal Ethics Committee of our university approved all procedures (reference number 6777 by Generalitat de Catalunya). The animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a 12/12-h light/dark cycle (light from 9:00 a.m. to 21:00 p.m.). The animals consumed tap water and a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum* throughout the experiment. The rats were randomly divided into two groups: control (n=4) and GSPE (n=3). The rats from the experimental group were administered 1 ml of 1 g/kg of body weight of GSPE by oral gavage. The control group was orally administered 1 ml of water. The corresponding treatments were administered between 9 and 10 a.m. after overnight fasting. Two hours after the treatment, the rats were anaesthetized with sodium pentobarbital (80 mg/kg), and their blood was collected by cardiac puncture (Fig. 1). The blood was maintained at room temperature for 30 min. Once the blood coagulated, it was centrifuged at 2000×g and 4°C for 15 min to obtain the serum. The sera were inactivated at 56°C for 30 min to avoid the risk of complement-mediated cell lysis and stored at −80°C until analysis. All of the methods were in accordance with the guidelines for the care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain).

2.4. Extraction of serum proanthocyanidins

Prior to the cell culture and chromatographic analysis, the rat serum samples were pretreated by off-line micro-solid-phase extraction procedure (μSPE) following the methodology that was previously described by Martí et al. (2010) [26] using 30-μm OASIS HLB μElution Plates (186001828BA; Waters, Barcelona, Spain). Briefly, the microcartridges were sequentially conditioned with 250 μl of methanol and 250 μl of 0.2% acetic acid. Prior to extraction, the serum was centrifuged at 2000×g and 4°C for 5 min. Two serum aliquots (each of 350 μl) were mixed, each of them, with 300 μl of 4% phosphoric acid and 50 μl of pyrocatechol (2000 ppb) and then loaded onto two different plates. The two loaded plates were washed with 200 μl of Milli-Q water and 200 μl of 0.2% acetic acid. The retained flavanols on each plate were eluted with 2×50 μl of an acetone/Milli-Q water/acetic acid (70:29.5:0.5, v:v:v) solution. The two elutions were mixed to obtain a final volume of 200 μl. Part of the solution (25 μl) was evaporated to dryness using a SpeedVac Concentrator SPD 2010 SAVANT (Thermo Scientific, USA) at room temperature and redissolved in 25 μl of an acetone/Milli-Q water/acetic acid (70:29.5:0.5, v:v:v) solution. These samples were then directly injected in the liquid chromatography tandem triple quadrupole mass spectrometry (LC–QqQ/MS^2) for chromatographic analysis; the sample volume used was 2.5 μl. The remaining 175 μl of the semipurified serum was also evaporated to dryness at room temperature using the same procedure described above and then stored at −80°C until its use in the cell culture experiments (Fig. 1).

2.5. Chromatographic analysis

The chromatographic analysis was performed using a 1200 LC Series coupled to a 6410 QqQ/MS^2 (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax C18 (100 mm×2.1 mm internal diameter, 1.8-μm particle size) chromatographic column from Agilent Technologies. The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 ml/min. The elution gradient was 0–10 min, 5%–55% B; 10–12 min, 55%–80% B; 12–15 min, 80% B isocratic; and 15–16 min, 80%–5% B. A post run of 10 min was applied. The ESI conditions were the following: drying gas temperature and flow rate of 350°C and 12 L/min, respectively, nebulizer gas pressure of 45 psi and capillary voltage of 4000 V. The QqQ was operated in the negative mode. The QqQ acquisition was performed in the MRM mode for the analysis of the proanthocyanidins and their metabolites.

2.6. Method validation and sample quantification

To validate the quantitative method, the calibration curves, linearity, extraction recovery, precision, sensitivity, and the method detection and quantification limits (MDL and MQL, respectively) were studied through an analysis of the standard solutions and blank serum samples that were spiked with standard flavanols. The calibration curves were obtained by plotting the analyte/IS peak abundance ratios and the corresponding analyte/IS concentration ratios. The extraction recovery was evaluated through a comparison of the responses of the spiked samples with the calibration curves of the standard solutions. The precision of the method was assessed from the relative standard deviation (RSD) in a triplicate analysis of a spiked sample. The sensitivity was evaluated by determining the limit of detection (LoD), which is defined as the concentration that corresponds to three times the signal-to-noise ratio, and the limit of quantification (LoQ), which is defined as the concentration that corresponds to 10 times the signal-to-noise ratio. The MDL and MQL were calculated in

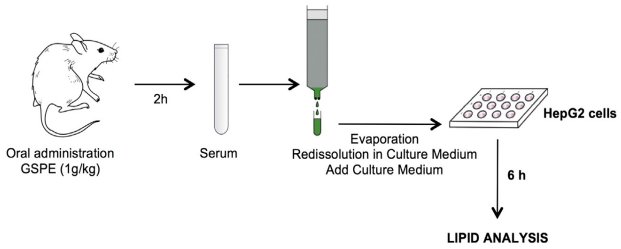

Fig. 1. Graphical representation of the *in vivo* and *in vitro* systems used in this study.

the analysis of 350 μl of a sample. Table 1 shows the values that were obtained for each quality parameter.

To quantify the samples, eight spiked blank samples with different concentration levels were used to obtain the calibration curves. The standard compounds in the samples were then quantified through the interpolation of the analyte/IS peak abundance ratios in these curves. Due to the lack of appropriate standards, the catechin, epicatechin and epicatechin gallate metabolites were tentatively quantified using the calibration curves of the catechin, epicatechin and EGCG standards, respectively. Similarly, the proanthocyanidin B3 dimer and the proanthocyanidin trimer were quantified using the calibration curves of the proanthocyanidin B2 dimer.

2.7. Cell culture

The human hepatocellular carcinoma cell line HepG2 (ATCC code HB-8065, Manassas, VA, USA) was cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a cell culture flask at 37°C in a humidified atmosphere with 5% CO_2. The cells were fed every 2–3 days.

2.8. Lipid analysis

Once they reached 80%–90% confluence, the HepG2 cells were seeded at a cell density of 500×10^3 cell/well in 12-well plates. The growth medium was replaced by supplemented culture medium 12 h before the addition of the treatments. In the first *in vitro* study, the HepG2 cells were treated with 25 mg/L of GSPE, CCX or PYG, which was dissolved in 1% EtOH, or vehicle (1% EtOH), which was used as the negative control. In the second *in vitro* study, the HepG2 cells were treated with the semipurified rat serum. The dried semipurified serum was redissolved in supplemented culture medium and then added to the growth medium on the well (1:10, v/v). GSPE (25 mg/L) and EtOH (1%) were used as positive and negative controls, respectively. In both *in vitro* studies, ^{14}C-acetate (0.6 μCi/ml) was added to the cell culture medium at the same time as the treatment to assess the lipid synthesis.

After 6 h of treatment with the different proanthocyanidin extracts or sera, the media and cells were collected. The lipid fractions were obtained through a hexane/isopropanol (3:2, v:v) extraction and separated through thin layer chromatography (TLC). The lipids analyzed in both studies were cholesterol ester (CE), free cholesterol (FC) and TG. TLC was performed as previously described [27] with an additional separation using a hexane/MTBE/NH_3 (30:20:0.1, v:v:v) solvent to obtain the TG fraction [16]. The obtained lipid fractions were separated, and the radioactivity was measured by scintillation counting. The values were corrected per milligram of protein, which was determined using the Bradford methodology [28].

2.9. Statistical analysis

The results are expressed as the mean±standard error (S.E.M.) of the mean and were analyzed by Student's *t* test and one-way analysis of variance using the SPSS software. The differences between the groups were assessed using the Bonferroni test (to correct for multiple comparisons). The differences between the means were considered significant when $P<.05$.

3. Results

3.1. Proanthocyanidin-rich extracts decrease lipid synthesis and excretion in HepG2 cells

All three tested proanthocyanidin-rich extracts (GSPE, CCX and PYC) induced a 50%–60% decrease in the synthesis of CE in HepG2 cells; no differences were observed between the three extracts (Fig. 2A). Furthermore, GSPE and PYC also induced a 40%–50% decrease in the levels of both FC and TG. However, even though CCX also induced a decrease in the FC and TG levels, this decrease was not statistically significant (Fig. 2B and C).

Moreover, no significant difference was measured in the amount of CE, FC and TG in the medium in response to any of the three proanthocyanidin-rich extracts. Therefore, the observed decrease in the synthesis of FC, CE and TG was due to a significant reduction of the lipids inside the cells (Fig. 2).

3.2. Characterization of the GSPE metabolites in the rat serum

To assess the bioactive compounds in GSPE that affect the *de novo* lipid synthesis and excretion, we characterized the proanthocyanidin metabolites that are present in the sera of GSPE-administered rats by LC–ESI–QqQ/MS. This analysis was performed 2 h after the administration of 1 g/kg GSPE to the rats. Fig. 3 illustrates the extracted ion chromatograms of the analysis of the flavonoids and their metabolites. The results of this analysis are shown in Table 2, which details the amount of each flavonoid and metabolite in the rat serum. Hence, 2 h after GSPE ingestion, the main compounds detected in the rat sera were conjugated forms of the monomeric flavan-3-ols (catechin and epicatechin). Specifically, the flavan-3-ol metabolites (epi)catechin glucuronide, methyl-(epi)catechin-glucuronide, epicatechin-sulfate, methyl-epicatechin and methyl-(epi)catechin-sulfate were detected at concentrations of at least 0.15 μM (Table 2). The glucuronidated forms were present in the sera at a substantially higher concentration than the concentrations of the methylated and sulfated conjugates.

Table 1
Quality parameters of the quantitative method used for the determination of proanthocyanidins by LC-ESI-QqQ/MS^2

Compound	Calibration curve	Determination coefficient (R^2)	Linearity (μM)	Recovery (%)	Precision (%RSD, $n=3$)	LoD (nM)	LoQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Catechin	$y=0.1472x$	0.99	0.19–39	99	0.29	20.70	68.90	5.90	19.70
Epicatechin	$y=0.1127x$	0.99	0.18–36	80	1.06	12.40	41.30	3.50	11.80
B2 dimer	$y=0.2474x$	0.99	0.04–9.0	92	0.27	6.90	23.00	2.00	6.60
B1 dimer	$y=0.3335x$	0.99	0.04–8.6	69	0.85	31.10	103.70	8.90	29.60
EGCG	$y=2.0337x$	0.99	0.20–12	73	8.77	1.20	4.00	0.30	1.10
Gallic acid	$y=0.2719x$	0.98	0.31–61	48	0.42	35.30	117.60	10.10	33.60

[a] Method detection and quantification limits are expressed in μmol/L of fresh sample, which were calculated for the analysis of a 350-μl serum sample.

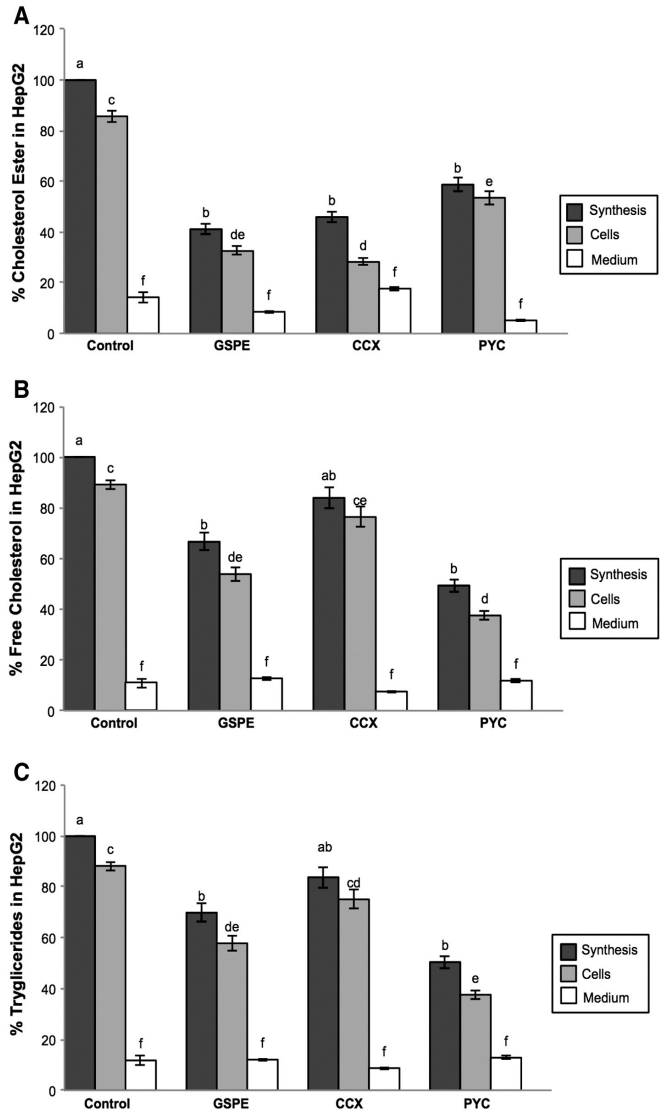

Fig. 2. Effect of proanthocyanidin-rich extracts on lipid synthesis and secretion in HepG2 cells. Changes induced by GSPE, CCX and PYC on the *de novo* synthesis and secretion of CE (A), FC (B) and TG (C). The HepG2 cells were simultaneously incubated with ^{14}C-labeled acetate and 25 mg/L of GSPE, CCX, PYG or vehicle (control). After 6 h of treatment, the radioactivity that was incorporated into the media and cellular lipids was measured. %CE, %TC and %TG were calculated considering the control as the 100%. All of the values are the mean±S.E.M. of triplicates of three independent experiments; the bars with different letters indicate statistically significant differences compared to the control (P<.05).

However, in contrast to the high amount of these compounds in the extract, free forms of unconjugated catechin, epicatechin and dimeric proanthocyanidins were detected in low amounts [29]. Moreover, other compounds that were also abundant in GSPE were not detected in the sera, such as monomeric gallate conjugates and trimeric proanthocyanidins. The retention times of each compound are shown in Fig. 3.

3.3. The metabolites in the serum of GSPE-administered rats decrease lipid synthesis and excretion in HepG2 cells

Two hours after the administration of GSPE, the rat serum was extracted, semipurified and used to treat HepG2 cells as a source of bioactive GSPE metabolites. The results show that the metabolites in the serum extracted from GSPE-administered rats significantly

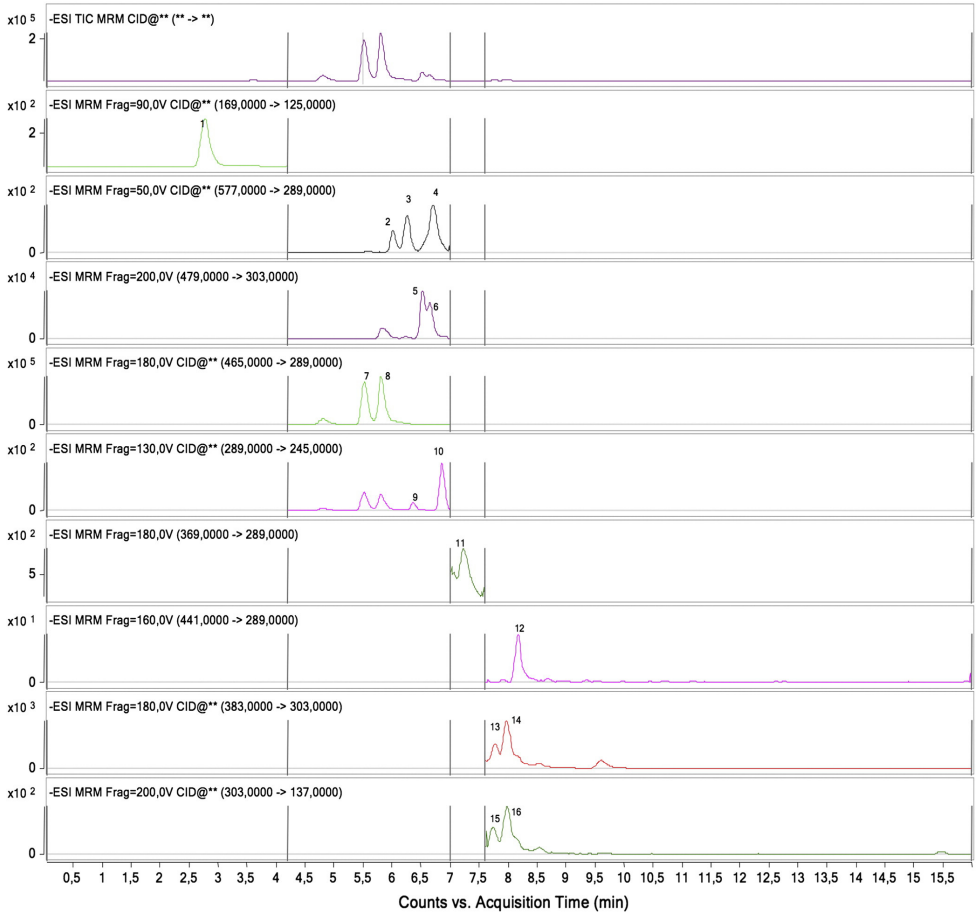

Fig. 3. Extracted ion chromatograms of the compounds in Table 2. These chromatographs were used for the analysis of the serum of GSPE-administered rats. The serum was extracted 2 h after the administration of 1 g/kg of GSPE. The following compounds were analyzed: (1) gallic acid, (2) B1 dimer, (3) B1+B3 dimer, (4) B2 dimer, (5) methyl-catechin-glucuronide, (6) methyl-epicatechin-glucuronide, (7) catechin glucuronide, (8) epicatechin glucuronide, (9) catechin, (10) epicatechin, (11) epicatechin sulfate, (12) epicatechin gallate, (13) methyl-catechin-*O*-sulfate, (14) methyl-epicatechin-*O*-sulfate, (15) 3-methyl-epicatechin and (16) 4-methyl-epicatechin.

reduced the total percentage of CE and FC in both the cells and the culture medium compared with the effect obtained with the sera of rats that were administered water (Fig. 4A and B). The percentage of TG was also reduced inside the cell but not in the medium (Fig. 4C). The GSPE metabolites induced 39%, 52% and 72% reductions in the cellular levels of CE, FC and TG.

4. Discussion

Dietary proantocyanidins are known to have numerous potential health benefits; these compounds present a protective role against different cardiovascular risk factors [30,31], including high serum lipid levels. In fact, our research group has reported a reduction in postprandial TG as a result of the administration of 25 mg/kg of GSPE to normolipidemic rats [16], mice [32] and rats that received a high-fat diet [33]. In addition, the GSPE-induced suppression of some lipogenic enzymes, which are induced by a high-fat diet, has been described [33,34]. Moreover, a previous *in vitro* study reported the reduction of *de novo* TG synthesis and secretion in HepG2 cells that were treated with 50 mg/kg GSPE [16].

In our study, the GSPE-treated HepG2 cells exhibited hypolipidemic effects compared to the control cells. In particular, we found a significant reduction in the accumulation of intracellular ^{14}C-lipids but no significant differences in the lipid contents in the media. This result is likely because the incorporated radioactivity was measured after 6 and not after 12 h of treatment, as previously described [16]. Interestingly, our results show that half of the dose of GSPE (25 mg/L) and half of the treatment duration were sufficient to reduce lipid synthesis and production. Similar results were observed with the other proanthocyanidin-rich extracts (CCX and PYC). However, CCX did not significantly decrease the syntheses and the amount (both the cell and medium contents) of FC and TG (Fig. 4B and C, respectively); this difference is likely because of the different composition of the extracts.

Table 2
Flavonoids and metabolites in the serum of rats that ingested an acute dose of 1 g/kg of GSPE

	Total amount (μM)
Compound	
Catechin	0.08±0.02
Epicatechin	0.52±0.14
Procyanidin B1 dimer	0.10±0.06
Proanthocyanidin B2 dimer	0.19±0.50
Proanthocyanidin B3 dimer [c]	0.10±0.02
Gallic acid	0.89±0.13
Epicatechin gallate [d]	n.d.
Dimer gallate [c]	n.d.
Trimer [c]	n.d.
EGCG	n.d.
Metabolites	
Catechin-glucuronide [a]	>39±14.89
Epicatechin-glucuronide [b]	>36±14.51
Methyl-catechin-glucuronide [a]	14.89±1.96
Methyl-epicatechin-glucuronide [b]	12.35±1.16
Catechin-sulfate [a]	n.d.
Epicatechin-sulfate [b]	0.76±0.16
3-*o*-methyl-epicatechin [b]	0.15±0.03
4-*o*-methyl-epicatechin [b]	0.34±0.05
Methyl-catechin-*o*-sulfate [a]	1.50±0.30
Methyl-epicatechin-*o*-sulfate [b]	3.91±0.63

The quantification was performed 2 h after the administration of GSPE. The data are presented as the mean (μM)±S.E.M. (n=4).
Abbreviations: n.d.=not detected; n.q.=not quantified.
[a] Quantified as catechin.
[b] Quantified as epicatechin.
[c] Quantified as dimer B2.
[d] Quantified as EGCG.

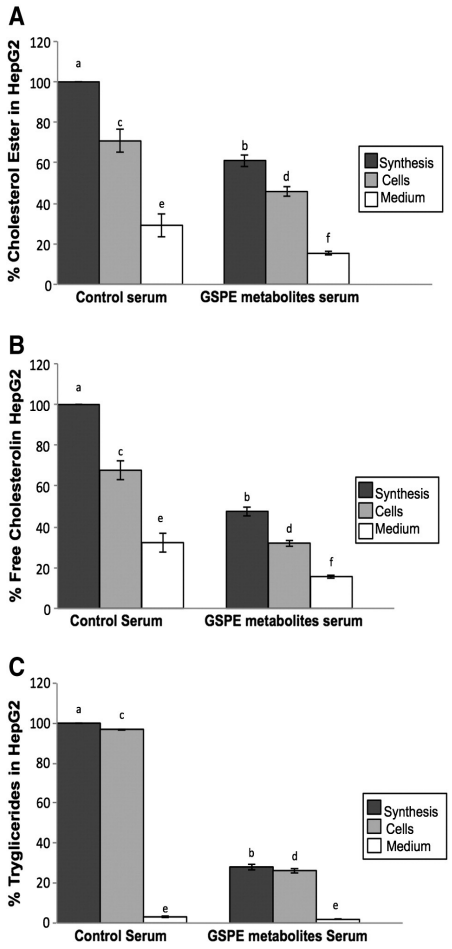

Fig. 4. Effect of semipurified serum from GSPE-administered rats on lipid synthesis and secretion in HepG2 cells. Changes induced by the semipurified serum of GSPE-administered rats on the *de novo* synthesis and secretion of CE (A), FC (B) and TG (C). The serum was extracted 2 h after the administration of water (control group) or 1 g/kg of GSPE. The HepG2 cells were simultaneously incubated with ^{14}C-labeled acetate and semipurified rat serum. After 6 h of treatment, the radioactivity that was incorporated into the media and the cellular lipids was measured. %CE, %TC and %TG were calculated considering the control as the 100%. All of the values are the mean±S.E.M. of triplicates of three independent experiments.

The data obtained using nonbioactive molecules and extracts to treat cultured cells have been questioned [22]. More specifically, the *in vitro* results obtained using nonphysiologically relevant forms and/or concentrations of flavonoids are considered an important limitation of the studies that aim to determine the *in vivo* bioactivity of these compounds [20,21]. An important aspect of polyphenols is the changes that occur to these molecules during first-pass metabolism. Thus, the molecular forms that reach the peripheral circulation and tissues are different from those that are present in foods [20]. In addition, the microflora extensively affects polyphenol hydrolysis and hence further contributes to the variation in the molecular forms of the polyphenols that are found in the blood and tissues [12,35,36]. These findings suggests that the bioactive forms of polyphenols are different from those that are present in food and that *in vitro* experiments with food polyphenols do not necessarily capture the *in vivo* situation [37]. In fact, GSPE is rich in monomers, namely, catechin and epicatechin, which are found both free and conjugated to a gallate moiety, and dimers [29]. However, these free forms are not found in a high concentration in the serum, which is predominantly composed of conjugated metabolites, such as catechin and epicatechin glucuronide. Moreover, only dimeric proanthocyanidins are detected in serum, whereas trimeric proanthocyanidins are not detected despite their abundance in GSPE [29]. The serum metabolites that were detected in this study are similar to those that were previously reported by other authors that analyzed plasma after the administration of the same dose of GSPE [29]. Therefore, the bioactive flavanols in rat serum differ considerably from the compounds that are present in GSPE. Hence, it is important to perform flavan-3-ol functionality studies using the metabolites in the serum of GSPE-administered rats for the treatment of cells. This approach is particularly important when extracts and not pure compounds are used to treat cells since the extracts consist of a complex mixture of different molecules. Moreover, the postabsorption metabolization of the extract mixture, which yields numerous metabolites [29], turns into impossible to obtain the same compounds from other sources rather than from the rat.

In this study, we combined the *in vivo* and *in vitro* system described to establish the bioactivity of proanthocyanidins on *de novo* lipid synthesis and excretion. Thus, HepG2 cells were incubated with the physiological metabolites that are present in the serum of GSPE-administered rats. It is important to point out that the sera metabolites come from the metabolization not only by the liver hepatocytes but also by the intestinal cells and by the microbiota. Therefore, the metabolites used in our study contained all the possible bioactive forms of polyphenols that are present in the sera of the animals 2 h after the administration of an extract rich in proanthocyanidins. The rats were administered with an acute dose of 1 g/kg

body weight of GSPE to obtain a sufficient amount of the proanthocyanidins metabolites to observe a further functionality in HepG2 cells [29]. The rat serum was obtained 2 h after the administration of the proanthocyanidin-rich extract because the highest plasma peak concentrations of flavanols are obtained 2 to 3 h after the ingestion of the extract in a dose-dependent manner [38,39]. Specifically, the maximum peaks were obtained in the plasma of rats that received 1 g/kg of GSPE 1 to 2 h postadministration [29]. Moreover, before its use for the treatment of HepG2 cells and chromatographic analysis, the sera were semipurified using a μSPE, which is a standard method that has been used prior to the chromatographic analysis of the polyphenols in biological samples, such as plasma [40]. Through this method, the numerous interferences in the sera, which are mainly proteins, are reduced. In addition to the pretreatment of the plasma, this method preconcentrates the phenolic compounds because these are present in the plasma and serum in trace amounts [40]. Therefore, we consider this semipurification and preconcentration step crucial to obtain mostly purified bioactive flavonoids and metabolites for both chromatographic analysis and *in vitro* cell treatment.

The results obtained using the metabolites in the sera of GSPE-administered rats, which were compared to the results obtained with the sera of rats that were orally administered water, showed a decrease in the *de novo* lipid synthesis and excretion in HepG2 cells, especially of TG. Similarly, a marked decrease in the TG level was also observed in previous *in vivo* studies that administered GSPE to rats, which showed that GSPE induced a clear hypotriglyceride effect in animals [15]. The lipid-lowering effect of semipurified rat sera is indicative that the physiological forms of the GSPE flavonoids also exhibit hypolipidemic properties. It was recently postulated that orally administered quercetin and likely other flavonoids are conjugated in the intestine and liver prior to their transport to the bloodstream and that the flavonoid released from their glucuronidated metabolite is responsible for its activity [41,42]. Although our study has been performed with other cell types and using other flavonoids that are different from quercetin, as well as with extracts instead of pure compounds, the obtained results also show the bioactivity of the conjugated metabolites.

Finally, although this study was realized with the phase II metabolites and some aglycones (i.e., a time point of 2 h was selected), this methodology can be a particularly useful tool to test the bioactivity of microbial metabolites through the use of serum from rats administered proanthocyadins after long time points. These studies could be especially relevant taking into account that the microbial metabolites are thought to be the responsible forms of a great part of the health effects of proanthocyanidns [43].

In conclusion, this study demonstrates the high hypolipidemic *in vitro* effect of different proanthocyanidin-rich extracts. Furthermore, the bioactivity of the proanthocyanidin rat sera metabolites of GSPE was also demonstrated in HepG2 cells, which exhibited a decrease in lipid synthesis and excretion. All these results demonstrated that the proposed *in vivo–in vitro* combined system is useful to study the functionality of the bioactive forms of flavonoids.

Acknowledgments

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A dose-response study of the bioavailabity of grape seed proanthocyanidins in rat and lipid-lowering effects of generated metabolites in HepG2 cells

Maria Margalef[†1], Ligia Gerrero[†¥1], Zara Pons[†], Mar Quiñones[†], Lluis Arola[†§], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [b] Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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A dose–response study of the bioavailability of grape seed proanthocyanidin in rat and lipid-lowering effects of generated metabolites in HepG2 cells

Maria Margalef [a,1], Ligia Guerrero [a,b,1], Zara Pons [a], Francisca Isabel Bravo [a], Lluís Arola [a,c], Begoña Muguerza [a,c,*], Anna Arola-Arnal [a]

[a] Nutrigenomic Group, Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona 43007, Spain
[b] Department of Research, Nutrition and Innovation, ALPINA S.A., Bogotá, Colombia
[c] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus 43204, Spain

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ABSTRACT

Hyperlipidemia is one of the principal causes of cardiovascular disease and proanthocyanidins (PAs) regulate lipid homeostasis. This study aims to evaluate the concentration of PAs in rat serum after the administration of different doses of PAs and to determine the capacity of these metabolites to reduce *de novo*lipid synthesis in HepG2 cells. Two hours after oral administration of different doses of a grape seed proanthocyanidin extract (GSPE) (1000, 375, 250 and 125 mg/kg), serum was semi-purified and characterised by HPLC–ESI–MS/MS before analysing the synthesis and secretion of lipids in HepG2 cells. Results showed a dose-dependent appearance of metabolised PAs in serum at doses up to 375 mg/kg and saturation at 1000 mg/kg of GSPE. A reduction in cholesterol esters (CE), free cholesterol (FC) and triglycerides (TG) synthesis was observed without dose-dependence when the cells were treated with PAs metabolites. Moreover, a low dose of metabolites (125 mg/kg) was sufficient to reduce FC and TG synthesis. In conclusion, the study demonstrated that PAs metabolise in a dose-dependent manner up to 370 mg/kg but not dose-dependent effect was shown in reducing the *de novo*synthesis of lipids.

1. Introduction

Hyperlipidemia is a metabolic disorder that is characterised by increased blood levels of total cholesterol, low density lipoprotein cholesterol (LDL) and/or triglycerides (TG) all of which are correlated with the development of atherosclerosis, the underlying cause of cardiovascular disease (CVD) and stroke (Yang et al., 2012). On the contrary, high density lipoprotein cholesterol (HDL) prevents atherosclerosis by reverting the stimulatory effect of oxidised-LDL. Several lines of evidence indicate that lipid-lowering treatments can reduce the development of coronary atherosclerosis (Nissen et al., 2004); in fact, a primary goal of clinical treatments for CVD risk reduction is to achieve therapeutic target levels for all lipid parameters (Wang et al., 2011).

Polyphenols are among the most abundant phytochemicals present in the human diet, and increasing evidence points to the important health-promoting effects of select flavonoids (Hertog et al., 1995; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Inverse relationships between plant-derived food intake and coronary heart disease risk have been previously reported (Shivashankara & Acharya, 2010). One of the main contributors to polyphenol intake in humans are the flavanols or proanthocyanidins (PAs), which are found primarily in grapes, beans, nuts, cocoa, tea and wine (Bladé, Arola, & Salvadó, 2010; Borriello, Cucciolla, Della Ragione, & Galletti, 2010). Our group has previously shown that the oral administration of grape seed PAs reduces TG and cholesterol and modulates the hepatic expression of several related genes and microRNAs in fatty acid, TG, and cholesterol metabolism (Baselga-Escudero et al., 2013; Del Bas et al., 2008). However, the intake of large amounts of polyphenol-rich products is not directly linked to the concentration of these compounds and their metabolites in blood and tissues (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). It is generally accepted that the bioavailability of polyphenol is relatively poor, although monomeric flavan-3-ols show higher bioavailability (Tomas-Barberan et al., 2007). It has been proposed that oligomeric and polymeric PAs are degraded into smaller units, especially monomers, by gastric juice (Ottaviani, Kwik-Uribe, Keen, & Schroeter, 2012; Prasain et al., 2009). In addition, after digestion, the metabolised compounds can lose their original properties or even acquire new activities (Gutierrez-Merino et al., 2011). In fact, the uptake and metabolism

* Corresponding author at: Dpt. Bioquímica y Biotecnología, U. Rovira i Virgili, C/ Marcel.li Domingo s/n, 43007 Tarragona, Spain, Tel.: +34 977 55 95 66; fax: +34 977 55 82 32.
E-mail address: begona.muguerza@urv.cat (B. Muguerza).
[1] These authors contributed equally to this work.

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of polyphenols is usually associated with their methylation, sulphation, or glucuronidation. In addition, considerable quantities of ingested flavonoids are degraded by colonic microbiota upon reaching the large intestine, where they yield other, smaller molecules that are also absorbed into the body (Del Rio et al., 2013). Thus, in plasma, polyphenols occur more often in more diverse forms than are present in food (Rice-Evans, 2001). Therefore, the properties of polyphenol compounds or extracts differ depending on whether they are studied using *in vitro*or *in vivo* models (Del Bas, Laos, Caimari, Crescenti, & Arola, 2012; Kroon et al., 2004). Thereby, the development of bioactive *in vitro*models using physiologically appropriate conjugates and concentrations is an important requirement for establishing the flavonoid bioactivity mechanisms (Kay, 2010). Recently, we showed the bioactivity of physiological rat metabolites after the ingestion of grape seed polyphenols using an *in vivo–in vitro*system (Guerrero et al., 2013). This previous study was realised with a dose of 1000 mg/kg of a grape seed proanthocyanidin extract (GSPE) to obtain the metabolites maximum concentrations in serum. Hence, in the present study, we aim to evaluate the absorption and serum bioavailability of PAs at low GSPE doses (i.e. lower than 1000 mg/kg). Moreover, the lipid-lowering effects of rat generated metabolites after the ingestion of different doses of GSPE was evaluated in HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (Scharlab S.L., Barcelona, Spain), acetone (Sigma-Aldrich, Madrid, Spain), and glacial acetic acid (Panreac, Barcelona, Spain) were of high performance liquid chromatography (HPLC) analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Stock standard solutions of 1000 mg/L (+)-catechin, (−)-epicatechin, gallic acid, epigallocatechin gallate (EGCG), PAs B1 and B2 (all from Fluka/Sigma-Aldrich, Madrid, Spain) in methanol and a standard solution of 2000 mg/L of pyrocatechol in methanol as the internal standard (IS) (Fluka/Sigma-Aldrich, Madrid, Spain) were stored in a dark glass flask at −20 °C.

A 200 mg/L stock standard mixture of (+)-catechin, (−)-epicatechin, EGCG, and gallic acid in methanol, and 100 mg/L of PAs B1 and B2 were prepared weekly and stored at −20 °C. The stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.1.1. Cell culture

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Bio Whittaker Europe (Verviers, Belgium). Bradford protein reagent was obtained from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA). ^{14}C-acetate was purchased from Amersham Biosciences (Buckinghamshire, England).

2.2. Grape seed rich-proanthocyanidin extract

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). Table 1 shows the total polyphenol, phenolic compounds (flavan-3-ols and phenolic acids) and the antioxidant capacity of the extract used in this study (adapted from Quiñones et al., 2013).

2.3. Experimental procedure in rats

Seventeen- to twenty-week-old male Wistar rats (n = 15) weighing 300–326 g were used for this study. The animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 °C with 12 h light/dark cycles (light from 9:00 a.m. to 9:00 p.m.). The animals consumed tap water and a standard chow diet (Panlab A04, Barcelona, Spain) ad libitum during the experiment. Rats were randomly divided into five groups, which were administered either 1 mL water (control group) or different doses of GSPE dissolved in 1 mL of water (125, 250, 375, and 1000 mg/kg groups). GSPE doses or water were administered by oral gavage between 9 and 10 a.m. following overnight fasting. Two hours after treatment, rats were anesthetised with sodium pentobarbital (80 mg/kg) and blood was collected by cardiac puncture. To obtain serum samples, blood was left at room temperature for 30 min to coagulate and was then centrifuged (2000 × g, 15 min, 4 °C). Serum were inactivated at 56 °C for 30 min to avoid the risk of complement-mediated cell lysis and stored at −80 °C until analysis. All methods were in accordance with the guidelines for care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain); procedure number 6777.

Table 1
Total polyphenols (mg/g) and main phenolic compounds (flavan-3-ols and phenolic acids) (mg/g) of the grape seed proanthocyanidin extract (GSPE) used in this study.

	Amount
Total polyphenols[a]	516.8 ± 12.1
Phenolic compounds[b]	
Gallic acid	17.7 ± 2.0
Protocatechuic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Proanthocyanidin dimer[1]	144.2 ± 32.2
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
p-Coumaric acid	0.1 ± 0.0
Dimer gallate[1]	39.7 ± 7.1
Epigallocatechin gallate	0.4 ± 0.1
Proanthocyanidin trimer[1]	28.4 ± 2.0
Proanthocyanidin tetramer[1]	2.0 ± 0.2
Epicatechin gallate[2]	55.3 ± 1.5
Quercetin-3-O-galactoside	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0
Quercetin	0.3 ± 0.0
Antioxidant capacity[c]	16,936 ± 651

Adapted from Quiñones et al. (2013).
The results are expressed on a wet basis as the mean ± SD (n = 3).
[a] Spectrophotometric method Folin–Ciocalteu. The results are expressed as mg gallic acid equivalent/g of fresh GSPE.
[b] HPLC–MS. The results are expressed as mg of phenolic compound/g of fresh GSPE.
[c] Hydrophilic ORAC (H-ORAC) assay, expressed as μmol of Trolox equivalents (TE)/g of fresh GSPE.
[1] Quantified using the calibration curve of proanthocyanidin B2.
[2] Quantified using the calibration curve of epigallocatechin gallate.

2.4. Serum proanthocyanidin extraction

Prior to cell culture and chromatographic analysis, rat serum PAs were extracted and semi-purified by off-line micro-solid phase extraction (μSPE) following the previously described methodology (Guerrero et al., 2013), using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of 0.2% acetic acid. Serum was centrifuged prior to extraction (2000 × g, 5 min, 4 °C). Two serum aliquots (350 μL each) were individually mixed with 300 μL of 4% phosphoric acid and 50 μL of pyrocatechol (1000 μg/L) and were then loaded onto two different plates. The two loaded plates were washed with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid. The retained flavanols were eluted with 2 × 50 μL of acetone:Milli-Q water:acetic acid solution (70:29.5:0.5, v:v:v) for each plate. Finally, the two elutions were mixed to obtain a final volume of 200 μL. Part of that solution (25 μL) was evaporated to dryness using a SpeedVac Concentrator SPD 2010 SAVANT (Thermo Scientific, San Jose, CA, USA) at room temperature and redissolved with

Table 2
Flavanols and their metabolites quantified in rat serum over a 2-h period after the ingestion of an acute intake of grape seed proanthocyanidin extract (GSPE) (1000, 375, 250, or 125 mg/kg) by HPLC–ESI–MS/MS.

Compound	Total amount (μM)			
	125 mg/kg	250 mg/kg	375 mg/kg	1000 mg/kg
Catechin	0.04 ± 0.013	0.06 ± 0.023	0.15 ± 0.034	0.12 ± 0.005
Epicatechin	0.24 ± 0.051	0.56 ± 0.188	0.74 ± 0.148	0.48 ± 0.031
Proanthocyaidin dimer B1	n.q.	0.01 ± 0.003[a]	0.03 ± 0.024[ab]	0.09 ± 0.001[ab]
Proanthocyanidin dimer B2	n.q.	0.01 ± 0.003[a]	0.03 ± 0.019[a]	0.27 ± 0.016[ab]
Proanthocyanidin dimer B3[3]	n.q.	0.01 ± 0.002[a]	0.012 ± 0.004[a]	0.07 ± 0.001[ab]
Gallic acid	0.21 ± 0.087	0.46 ± 0.034	0.59 ± 0.238	0.80 ± 0.164
Epicatechin gallate[4]	n.d.	n.d.	n.d.	n.d.
Dimer gallate[3]	n.d.	n.d.	n.d.	n.d.
Trimer[3]	n.d.	n.d.	n.d.	n.d.
EGCG	n.d.	n.d.	n.d.	n.d.
Metabolite				
Catechin-glucuronide[1]	13.09 ± 1.038[a]	29.78 ± 2.134[ab]	40.16 ± 6.389[b]	41.30 ± 5.800[ab]
Epicatechin-glucuronide[2]	11.41 ± 1.058	26.63 ± 0.036	37.56 ± 6.943	37.54 ± 6.750
Methyl-catechin-glucuronide[1]	12.58 ± 1.037	17.71 ± 1.673	22.04 ± 1.678	7.68 ± 0.556
Methyl-epicatechin-glucuronide[2]	9.73 ± 1.064	12.46 ± 0.858	19.01 ± 2.749	36.79 ± 5.889
Catechin-sulphate[1]	0.06 ± 0.010	0.14 ± 0.055	0.53 ± 0.358	0.06 ± 0.010
Epicatechin-sulphate[2]	n.d.	n.d.	n.d.	0.03 ± 0.003
3-o-methyl-epicatechin[2]	0.12 ± 0.010	0.11 ± 0.025	0.33 ± 0.094	0.09 ± 0.006
4-o-methyl-epicatechin[2]	0.15 ± 0.016	0.13 ± 0.019	0.42 ± 0.143	0.10 ± 0.001
Methyl-catechin-o-sulphate[1]	0.62 ± 0.043	0.47 ± 0.122	1.97 ± 0.827	0.19 ± 0.012
Methyl-epicatechin-o-sulphate[2]	1.55 ± 0.308	1.48 ± 0.329	4.49 ± 1.553	0.58 ± 0.045

The data are given as the mean (μM) ± SEM (n = 3); values with different letters indicate statistically significant differences between GSPE doses (One-way ANOVA, $p < 0.05$). Abbreviations: n.d. = not detected. n.q. = not quantified. EGCG = epigallocatechin gallate.
[1] Quantified as catechin.
[2] Quantified as epicatechin.
[3] Quantified as proanthocyanidin dimer B2.
[4] Quantified as EGCG.

25 μL of an acetone:Milli-Q water:acetic acid solution (70:29.5:0.5, v:v:v). These samples were then directly injected in the HPLC tandem triple quadrupole mass spectrometer (HPLC–MS/MS) for chromatographic analysis; the sample volume was 2.5 μL. The remaining 175 μL of the semi-purified serum was also evaporated to dryness using the same procedure described above and was then stored at −80 °C until the cell culture experiment.

2.5. Chromatographic analysis

The chromatographic analysis was performed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150 mm × 2.1 mm i.d., 3.5 μm particle size) as a chromatographic column from Agilent Technologies. The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The elution gradient was 0–10 min, 5–55% B; 10–12 min, 55–80% B; 12–15 min, 80% B isocratic; 15–16 min 80–5% B. A post run of 10 min was applied. The electrospray ionisation (ESI) conditions were 350 °C and 12 L/min of drying gas temperature and flow, respectively, a nebuliser gas pressure of 45 psi, and 4000 V of capillary voltage. MS/MS was operated in negative mode. MS/MS acquisition was performed in multiple reaction monitoring (MRM) mode for PAs and their metabolites. Data acquisition was conducted using the MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

For sample quantification, spiked blank sera at 8 different concentrations were used to obtain calibration curves, and standard compounds in the samples were quantified by interpolating the analyte/IS peak abundance ratio in these curves. Catechin, epicatechin, and EGCG metabolites were tentatively quantified using the standard catechin, epicatechin, and EGCG calibration curves, respectively, due to the lack of standards. In the same way, dimer PA B3 and trimer PAs were quantified using the calibration curve of dimer PA B2 (see Supplementary information SI1).

2.6. Cell culture

The human hepatocellular carcinoma cell line HepG2 (ATCC code HB-8065, Manassas, VA, USA) was cultured in DMEM medium supplemented with 10% (v/v) foetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 2 mM of L-glutamine in a cell culture flask at 37 °C and a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days.

2.7. Lipid analysis

The HepG2 cells were seeded at 500 × 10^3 cells/well in 12-well plates, and they were used upon reaching 80–90% confluence. Growth medium was replaced by supplemented culture media 12 h before the treatments. HepG2 cells were cultivated with the PAs metabolites derived from semi-purified rat serum (Guerrero et al., 2013). Rat serum were obtained for *in vitro* use 2 h after the administration of either water or increasing doses of GSPE (125, 250, or 375 mg/kg). The dried, semi-purified serum was redissolved in supplemented culture medium and was then added to the growth medium in the well (1:10, v/v). GSPE (25 mg/L) and ethanol (1%) were used as a positive and negative control, respectively. ^{14}C-acetate (0.6 μCi/mL) and the appropriate treatment were added simultaneously to the cell culture medium to evaluate lipid synthesis. Six hours after treatment with the purified serum, media and cells were collected and the lipid fraction was obtained via hexane:isopropanol (3:2, v:v) extraction and separated by Thin Layer Chromatography (TLC). In all experiments, the lipids evaluated were cholesterol esters (CE), free cholesterol (FC) and TG. TLC was performed as previously described (Pill, Aufenanger, Stegmeier,

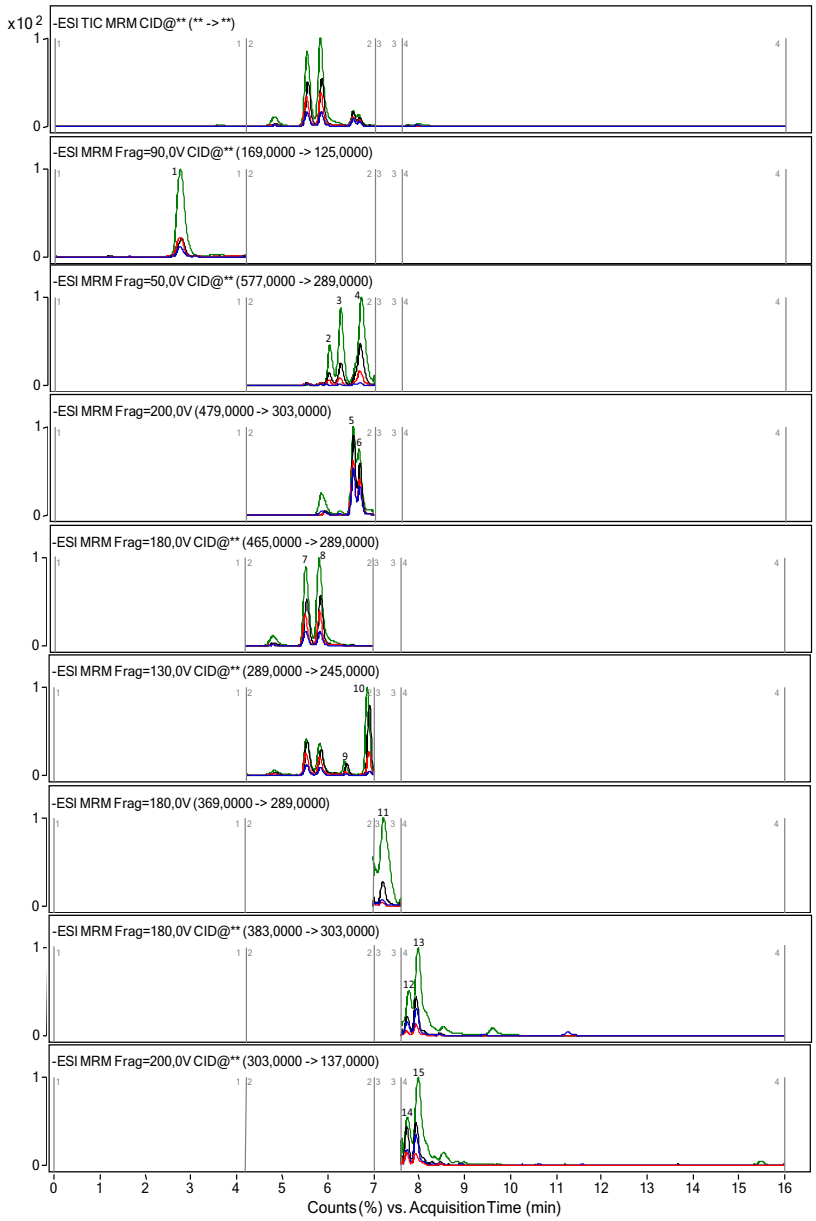

Fig. 1. Extracted ion chromatogram of serum flavonoids 2 h after the administration of 1000 mg GSPE/kg (in green), 375 mg GSPE/kg (in black), 250 mg GSPE/kg (in red), and 125 mg GSPE/kg (in blue). (1) Gallic acid; (2) Dimer B1; (3) Dimer B3; (4) Dimer B2; (5) Methyl-catechin-glucuronide; (6) Methyl-epicatechin-glucuronide; (7) Catechin glucuronide; (8) Epicatechin glucuronide; (9) Catechin; (10) Epicatechin; (11) Catechin sulphate; (12) Methyl-catechin-O-sulphate; (13) Methyl-epicatechin-O-sulphate; (14) 3-methyl-epicatechin; (15) 4-methyl-epicatechin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Schmidt, & Müller, 1987), with an additional separation using a Hexane:MTBE:NH_3 (30:20:0.1, v:v:v) solvent to obtain the TG fraction (Del Bas et al., 2008). The obtained lipid fractions were separated, and radioactivity was measured by scintillation counting. The values were normalised to milligrams of protein, determined using the Bradford methodology (Bradford, 1976).

2.8. Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM) and analysed by Student's t-test and one-way ANOVA using the IBM SPSS Statistics software (Version 20.0.0). Differences between groups were assessed using the Bonferroni test (to correct for multiple comparisons). Differences between means were considered significant when $p < 0.05$.

3. Results

3.1. GSPE serum metabolites determination

The HPLC–ESI–MS/MS analysis of rat serum PAs metabolites collected 2 h after the ingestion of 1000, 375, 250, or 125 mg/kg GSPE is presented in Table 2 and Fig. 1. Free forms of catechin, epicatechin, and dimeric PAs, in addition to phenolic acids such as gallic acid, were detected at low concentrations in serum (up to 0.80 μM), in contrast to the high concentrations of these compounds found in GSPE (Quiñones et al., 2013). Moreover, other compounds abundant in GSPE were not detected in serum, such as monomeric and dimeric gallated conjugates or trimeric PAs (Table 2). However, the primary compounds detected in rat serum were conjugated forms of the monomeric flavan-3-ols (catechin and epicatechin). For all doses tested, the glucuronidated forms of flavanols were present in serum at substantially higher concentrations compared to methylated and sulphated conjugates (Fig. 2). In addition, although the metabolite serum concentration was very compound-specific, the results corresponding to many physiological forms of PAs showed a dose-dependent effect on both metabolised and non-metabolised flavonoids up to a dose of 375 mg/kg, so that at the dose of 1000 mg/kg of GSPE, the metabolism of many compounds was reduced (Figs. 2 and 3).

3.2. GSPE rat serum metabolites decrease lipid synthesis and excretion in HepG2 cells

3.2.1. Effect of different doses of GSPE rat serum metabolites on cholesterol ester synthesis in HepG2 cells

Treating HepG2 cells with semi-purified serum from GSPE-administered rats produced a dose-dependent decrease in CE synthesis relative to the cells treated with serum from water-administered rats (Fig. 4A). However, only serum from the 375 mg/kg GSPE dose significantly reduced the CE synthesis compared to the control animals. The differences in CE synthesis were due to a decrease in the intracellular lipid content (69 ± 4.1%, after setting the CE synthesis of the control group to 100% for the dose of 375 mg/kg GSPE). For the three doses tested, the CE secretion into culture medium was similar to controls.

3.2.2. Effect of different doses of GSPE rat serum metabolites on free cholesterol synthesis in HepG2 cells

The total amount of intracellular FC was reduced when cells were incubated with the metabolites present in serum (Fig. 4B). No differences in the synthesised FC were observed for the three doses studied. The FC secreted by the cells into the culture medium was similar to that of the controls for all three tested doses.

3.2.3. Effect of different doses of GSPE rat metabolites on triglyceride synthesis in HepG2 cells

Although a decrease in TG synthesis and intracellular TG was observed for all doses, surprisingly, only treatment with a low dose of metabolites (GSPE intake of 125 mg/kg) resulted in a statistically significant difference compared to the control (70 ± 4.0%, whereas the control was set to 100%). TG secretion into the cell culture medium was similar to that of the control metabolites for all three doses (Fig. 4C).

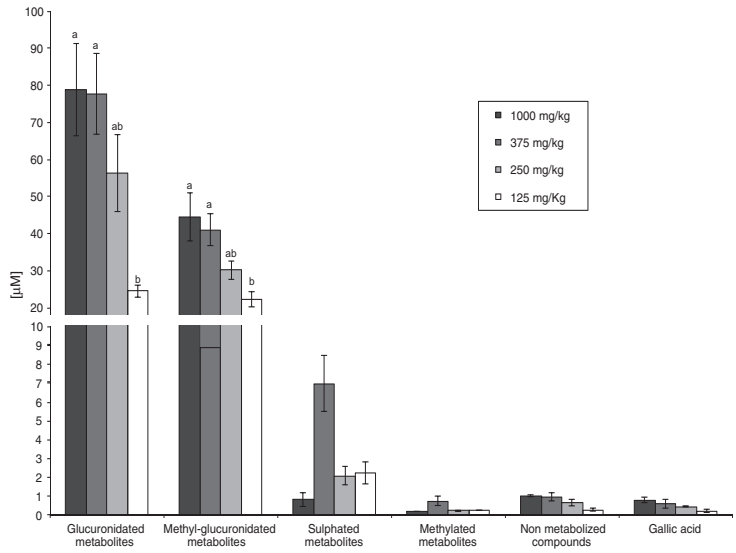

Fig. 2. Rat serum concentrations (μM) of gallic acid and non-metabolised GSPE compounds and their flavan-3-ols glucuronidated, methyl-glucuronidated, sulphated, and methylated metabolites 2 h after the administration of 1000, 375, 250, and 125 mg GSPE/kg. The results are expressed as the mean ± standard error (SEM). Different letters indicate statistically significant differences compared to the control ($p < 0.05$).

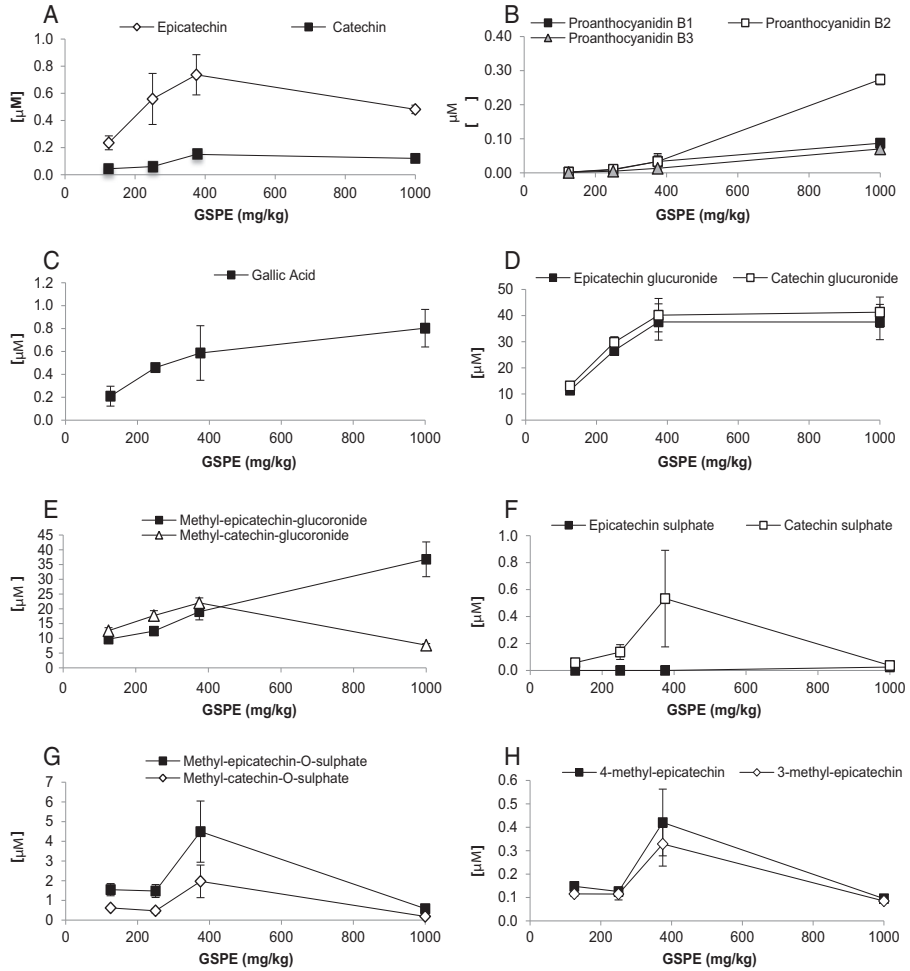

Fig. 3. Rat serum concentrations (μM) of non-metabolised and metabolised GSPE compounds at 2 h after the administration of 1000, 375, 250, and 125 mg GSPE/kg. The results are expressed as the mean ± standard error (SEM). A) catechin and epicatechin, B) dimeric proanthocyanidins, C) gallic acid, D) (epi)catechin-glucuronide, E) methyl-(epi) catechin-glucuronide, F) (epi)catechin-sulphate, G) methyl-(epi)catechin-O-sulphate, H) 3- and 4-methyl-epicatechins.

4. Discussion

The regular consumption of flavonoids in the human diet has been associated with reduced mortality and morbidity of cardiovascular disease (CVD) (Crozier, Jaganath, & Clifford, 2009; Rasmussen et al., 2005). PAs are considered the most abundant flavonoids in the human diet (Bladé et al., 2010) and, similar to other flavonoids, their beneficial effects depend on both the amount consumed and their bioavailability (Manach et al., 2004). It has been shown that low molecular weight forms, especially monomeric flavan-3-ols and dimers, are absorbed in the small intestine and metabolised by the phase-II enzymes, whereas the polymeric forms are metabolised by the colonic microbiota (Aura, 2008; Monagas et al., 2010). It has also been demonstrated that at 2 h after an acute PA administration, the main compounds that reach the systemic circulation and tissues are phase-II metabolites (Serra et al., 2010, 2013). In addition, the bioactive compounds that eventually reach tissues are substantially different from those that are initially present in food (Kroon et al., 2004). In fact, the qualitative and quantitative PAs composition differs substantially between GSPE and the serum of animals administered a 1000 mg/kg dose of this same extract (Guerrero et al., 2013). As a result of these structural changes, many *in vitro* studies with no physiological forms of flavonoids have been questioned because their beneficial effects could be modulated by their metabolic conjugates (Kay, 2010; Kroon et al., 2004). In a previous study, we described a new methodology for evaluating the effects of bioactive forms of PAs on *de novo* lipid synthesis in cultured cells (Guerrero et al., 2013). Hence, the objective of the present work was to determine whether PAs are absorbed or metabolised differently depending on the dose administered to rats and whether the different absorption or amount of metabolites could affect the bioactivity of the PAs in regulation *de novo* lipid synthesis using the previously described methodology (Guerrero et al., 2013).

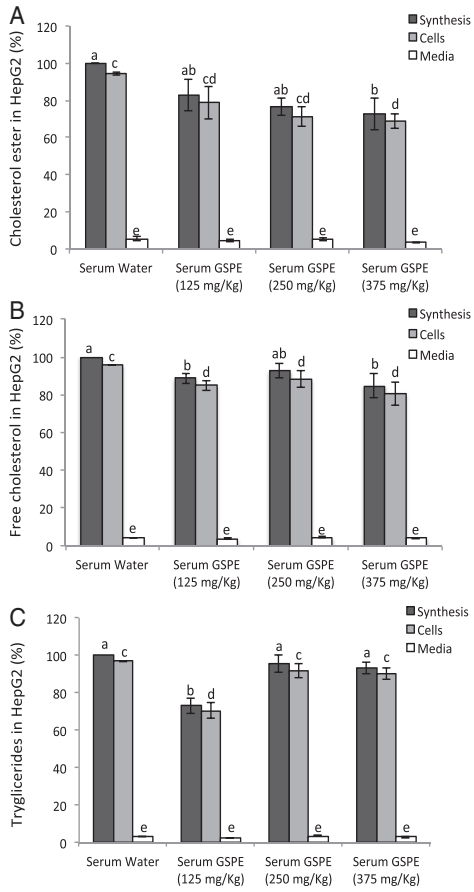

Fig. 4. Effect of rat semi-purified serum obtained 2 h after the administration of GSPE (375, 250, and 125 mg/kg) on HepG2 cells. Cells were simultaneously incubated with ^{14}C-labelled acetate and rat semi-purified serum. Six hours after the treatment, radioactivity incorporated into media (□) and cellular (■) lipids was measured. The total synthesis represents the radioactivity present in the cells and culture medium (■). All values are the mean ± SEM of triplicates of three independent experiments. A. Results related to cholesterol ester synthesis and secretion in HepG2 cells. B. Results related to free cholesterol synthesis and secretion in HepG2 cells. C. Results related to triglycerides synthesis and secretion in HepG2 cells. Different letters indicate statistically significant differences compared to the control ($p < 0.05$).

This study was conducted at 2 h post GSPE administration and focused on flavanol-phase-II metabolites, since these compounds are known to peak serum concentration at 2 h post PAs administration (Serra et al., 2010). In addition, it is known that although smaller phenolic compounds are generated from the action of colonic bacteria after polyphenols intake, these colonic metabolites appear in circulation later in time (Del Rio et al., 2013). Acute PAs bioavailability studies are usually conducted with high, non-physiological doses of PAs extracts, such as 1000 mg/kg of GSPE, to reach a serum or plasma metabolite concentration that is detectable by chromatographic analysis (Arola-Arnal et al., 2013; Guerrero et al., 2013; Serra et al., 2010). However, in this study is demonstrated that following treatment with low physiological doses of GSPE (i.e.; 125 mg/kg GSPE), PAs metabolites can be detected and quantified in serum and that a dose of 1000 mg/kg of GSPE saturates the system. Moreover, a clear dose–response of both metabolised and non-metabolised PAs can be observed in rat serum 2 h following the acute administration of low doses of GSPE (125, 250, and 375 mg/kg). These results indicate that the rat's ability to conjugate many flavonoids could be overwhelmed at high doses. Therefore, the administration of high doses of GSPE (from 375 mg/kg) does not result in a greater presence of serum PAs metabolites. In fact, the concentration of some metabolites at 1000 mg/kg is even decreased respect to lower doses. Only some minority aglycone forms, such as gallic acid and dimeric PAs, had greater serum concentrations at 1000 mg/kg than at 375 mg/kg. Similarly, when a 1000 mg/kg dose is administered to rats, the methyl-epicatechin glucuronide concentration is increased relative to 375 mg/kg. However, there are no differences between methyl glucuronidated metabolites (sum of methyl epicatechin glucuronide and methyl catechin glucuronide) when the doses of 375 and 1000 mg/kg of GSPE are compared. This observation could indicate that the enzyme O-methyl transferase has greater affinity for epicatechin than catechin (Fig. 3).

On the other hand, several studies have shown the beneficial effects of flavonoids in reducing TG levels both *in vitro* (Pal et al., 2003) and *in vivo* (Auger et al., 2002; Vinson, Teufel, & Wu, 2001). Similarly, this is not the first demonstration of GSPE reducing lipid synthesis, especially for TG (Josep Maria Del Bas et al., 2008, 2009; Guerrero et al., 2013; Quesada et al., 2009). Furthermore, we have previously reported that a 1000 mg/kg dose of grape seed PAs metabolites in rat serum reduced *de novo* lipid synthesis in HepG2 cells (Guerrero et al., 2013). In this study, the lipid-lowering effect of metabolites at lower doses of GSPE (125, 250, and 375 mg/kg) on *de novo* lipid synthesis were evaluated because the highest dose (1000 mg/kg) did not yield a higher concentration of serum metabolites than the 375 mg/kg dose (i.e. the system is saturated). The results showed a reduction in *de novo* lipid synthesis at all doses studied. However, although elevating the dose of GSPE to 375 mg/kg increased the metabolite concentrations appearing in serum, a dose-dependent effect was only observed on CE, but not TG or FC. Moreover, the lowest dose of 125 mg/kg showed the strongest effect on TG, indicating that a relatively moderate dose of 125 mg/kg is effective. The lack of dose-dependence effect of PAs has been previously reported by our group, indicating that lower doses of GSPE can be more efficient than higher doses (Quiñones et al., 2013).

Given the high concentrations of conjugate forms present in serum, specifically the glucuronic acid conjugates, these metabolites seem to be involved in reducing the *de novo* synthesis of lipids in hepatic cells. However, these conjugated forms may not act directly at the cellular level. Previous studies have indicated that there is no direct relationship between the plasma concentration and the target tissue concentration of flavonoids, besides varying the distribution between blood and tissues depending on the concerned flavonoid (Hong, Kim, Kwon, Lee, & Chung, 2002; Maubach et al., 2003). In HepG2 cells, O'Leary et al. demonstrated that glucuronidated flavonoids are deconjugated following intact entry into cells by an unidentified transporter (O'Leary et al., 2003). Therefore, the deconjugation of glucuronidated metabolites to their bioactive forms inside HepG2 cells could explain the lack of a direct relationship between the serum concentration of these conjugated compounds and their biological functionality, including any effects on *de novo* lipid synthesis. Additionally, the lack of a relationship between the serum metabolite concentration and the regulation of lipid synthesis could be explained by the presence of other minority compounds in the serum that regulate lipid synthesis and have not been detected.

5. Conclusion

This study showed the dose-dependent appearance of both metabolised and non-metabolised PAs in rat serum 2 h following the acute administration of low doses of GSPE (up to 375 mg/kg) but a saturation of the system when a high dose of GSPE was administered.

Moreover, the study demonstrated that these PAs metabolites exhibit no dose-dependent effects in reducing the *de novo* synthesis of lipids, especially TG, and showed that a relatively moderate dose of 125 mg/kg is effective.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2014.07.019.

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MANUSCRIPT 5:

Tissue distribution of rat flavanol metabolites at different doses

Maria Margalef[†], Zara Pons[†], Francisca Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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Tissue distribution of rat flavanol metabolites at different doses☆

Maria Margalef[a], Zara Pons[a], Francisca Isabel Bravo[a], Begoña Muguerza[a,b,*], Anna Arola-Arnal[a]

[a]Nutrigenomic Group, Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona 43007 Spain
[b]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus 43204, Spain

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Abstract

Flavanols are metabolized in the small intestine and the liver to produce their glucuronidated, sulfated or methylated conjugates that can be body distributed or excreted in the urine. However, the intake of large amounts of flavanols is not directly related to their bioavailability. This study aims to investigate the administered dose dependence of flavanols' conjugation and body distribution. In this study, different doses of a grape seed proanthocyanidin extract (GSPE; 125, 250, 375 and 1000 mg/kg) were orally administered to male Wistar rats. Tissues were collected 2 h after GSPE administration. Flavanols were quantified by HPLC–MS/MS. Results show that the majority of GSPE metabolites are located in the kidney, followed by the liver. Lower concentrations were found in mesenteric white adipose tissue (MWAT) and the brain. Moreover, flavanol metabolites followed a tissue-specific distribution pattern independent of dosage. In the kidney, glucuronidated metabolites were the most abundant; however, in the liver, it was mainly methyl-glucuronidated metabolites. In MWAT, free flavanols were dominant, and methylated metabolites were dominant in the brain. Concentration within a tissue was dependent on the administered dose. In conclusion, flavanol metabolites follow a tissue-specific distribution pattern and only the tissue concentration of flavanol metabolites is dependent on the administered dose.

Keywords: Brain; Grape seed extract; HPLC–MS/MS; Mesenteric white adipose tissue; Proanthocyanidins

1. Introduction

Polyphenols are among the most abundant phytochemicals present in the human diet, and increasing evidence points to the important health-promoting effects of select flavonoids [1,2]. Inverse relationships between plant-derived food intake and coronary heart disease risk have been previously reported [3]. The flavanoin-type flavan-3-ols, or flavanols, are one of the main polyphenols ingested by humans. These phytochemicals are found primarily in grapes, beans, nuts, cocoa, tea and wine [4,5]. They range from the flavanol monomers (+)-catechin and its isomer (−)-epicatechin to more complex structures that include oligomeric and polymeric proanthocyanidins (PAs), which are also known as condensed tannins. Our group has previously shown that the oral administration of grape seed flavanols exerts unique beneficial properties on some metabolic syndrome-related parameters and cardiovascular diseases by acting as antioxidants [6], limiting adipogenesis [7], presenting anti-inflammatory properties [8] and acting either as an insulin-mimetic [9] or as an antihypertensive [10,11] agent. A reduction in the *de novo* synthesis of hepatic lipids, mainly triglycerides, has also been demonstrated [12]. However, the beneficial effects of flavanols are dependent on several factors, such as the model used, the time of treatment or the administered dose of the flavanol extract in both *in vitro* and *in vivo* models [13].

It is generally accepted that the bioavailability of polyphenols is relatively poor, although monomeric flavan-3-ols show higher bioavailability [14]. It has also been proposed that oligomeric and polymeric flavanols, or PAs, are degraded into smaller units, especially monomers, by gastric juices [15,16]. In addition, after digestion, the metabolized compounds can lose their original properties or even acquire new activities [17]. In fact, the uptake and metabolism of polyphenols are usually associated with their methylation, sulfation or glucuronidation by phase II enzymes [18–20]. Considerable quantities of ingested flavanols are degraded by colonic microbiota upon reaching the large intestine, where they yield other smaller molecules that are also absorbed into the body [21]. Some studies have demonstrated that after conjugation, flavanols are distributed throughout the body and are found at considerable concentrations in most tissues after an acute intake of a PA extract [12,13,22–24]. However, the intake of large amounts of polyphenol-rich products is not directly linked to an increase in the concentration of these compounds in the blood and tissues [25]. It has also been demonstrated that different doses of flavanols do not always lead to different concentrations of metabolites in rat sera after an acute administration, and the *in vitro* effects of these metabolites have a dose–response behavior [13]. Therefore, the aim of this study is to elucidate whether flavanols can also be conjugated and distributed differently throughout the body when the intake dosage of a grape seed PAs extract is varied.

☆ Conflict of interest: The authors have declared no conflict of interest.

* Corresponding author at: Department of Biochemistry and Biotechnology, Rovira i Virgili University, C/ Marcel.li Domingo s/n 43007, Tarragona, Spain. Tel.: +34 977 55 95 66; fax: +34 977 55 82 32.
E-mail address: begona.muguerza@urv.cat (B. Muguerza).

RESULTS

2. Materials and methods

2.1. Chemicals and reagents

Methanol (Scharlab S.L., Barcelona, Spain), acetone (Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Individual stock standard solutions of 2000 mg/l in methanol of (+)-catechin, epigallocatechin gallate (EGCG), 3-hydroxybenzoic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, benzoic acid and pyrocatechol as the internal standard (IS) (all from Fluka/Sigma-Aldrich, Madrid, Spain), as well as a standard solution of 1000 mg/l in methanol of procyanidin B_2, (−)-epicatechin (Fluka/Sigma-Aldrich) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany) were prepared and stored in a dark-glass flask at −20°C.

A 20-mg/l stock standard mixture in methanol of (+)-catechin, (−)-epicatechin, 3-hydroxybenzoic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, benzoic acid, procyanidin B_2 and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was prepared weekly and stored at −20°C. This stock standard solution was diluted daily to the desired concentration using an acetone/water/acetic acid (70:29.5:0.5, vol/vol/vol) solution.

2.2. Grape seed polyphenol extract

Grape seed polyphenol extract (GSPE) was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). Table S1 shows the total polyphenol content and the phenolic compound (flavan-3-ols and phenolic acids) concentrations of the extract used in this study (adapted from Quiñones et al. [10]).

2.3. Experimental procedure in rats

Male Wistar rats (17–20 weeks old) weighing 300–326 g were used for this study. The animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with 12-h light/dark cycles (light from 9:00 a.m. to 9:00 p.m.). The animals consumed tap water and a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum* during the experiment. The rats were randomly divided into five groups and administered the following by oral gavage: 1 ml of water (n=3), 125 mg/kg of GSPE (n=3), 250 mg/kg of GSPE (n=3), 375 mg/kg of GSPE (n=3) and 1000 mg/kg of GSPE (n=3). Oral administration in all groups occurred between 9 and 10 a.m. after overnight fasting, and the total oral administered volume was always 1 ml of either water or GSPE–water solution. Rats were anesthetized with sodium pentobarbital (80 mg/kg) and sacrificed by exsanguination 2 h after the GSPE or water ingestion. The liver, kidneys, mesenteric white adipose tissue (MWAT) and brain were excised from the rats and freeze-dried for the extraction of free flavanols and flavanol metabolites. Dried tissues were stored at −80°C. The study was in accordance with the guidelines for care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain).

2.4. Free flavanol and flavanol metabolite extraction from the tissues

Prior to the chromatographic analysis of the free flavanols and their metabolites in rat tissues, the samples were pretreated using previously reported methodology [23,26] based on an offline liquid–solid extraction (LSE) in tandem with a micro-solid-phase extraction (μSPE). Briefly, the LSE procedure involves adding 50 μl of 1% ascorbic acid and 100 μl of 4% phosphoric acid to 60 mg of freeze-dried tissue. All tissue samples were then extracted 4 times with 400 μl of water/methanol/4% phosphoric acid (94.4:4.5:1.5, vol/vol/vol). In each extraction, the 400 μl extraction solution was added, the sample was sonicated for 30 s with the sample in an ice water bath to avoid heating, and then it was centrifuged for 15 min at 17,150×g at room temperature (except for MWAT, which was centrifuged at 4°C to achieve the proper separation between the fat and the aqueous phase). The obtained supernatants from the tissue LSE were cleaned up by μSPE using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). The micro-cartridges were conditioned sequentially with 250 μl of methanol and 250 μl of 0.2% acetic acid. Then, 300 μl of phosphoric acid 4% and 50 μl of the IS (1000 μg/ml) were added to 350 μl of the tissue extract, and the 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 free flavanols and their metabolites were then eluted with 2×50 μl of acetone/Milli-Q water/acetic acid solution (70:29.5:0.5, vol/vol/vol). The eluted solution was directly injected in the HPLC–MS/MS, and the sample volume was 2.5 μl.

2.5. Chromatographic analysis

The chromatographic analysis was performed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150 mm×2.1 mm i.d., 3.5-μm particle size) as the chromatographic column (Agilent Technologies). The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 ml/min. The elution gradient was as follows: 0–10 min, 5%–55% B; 10-12 min, 55%–80% B; 12–15 min, 80% B isocratic; and 15–16 min, 80%–5% B. A post-run of 10 min was applied. Electrospray ionization (ESI) conditions were a drying gas temperature of 350°C and a flow rate of 12 l/min, 45 psi of nebulizer gas pressure, and 4000 V of capillary voltage. The MS/MS was operated in negative mode, and the acquisition was performed in MRM mode for free flavanols and their metabolites. The acquisition method was performed as previously reported for the quantification of phase II and microbial flavanol metabolites [12,13]. Data acquisition was carried out using MassHunter Software (Agilent Technologies).

2.6. Sample quantification

For sample quantification, a pool of blank tissue extracts or sera from rats administered water were spiked with 10 different concentrations to obtain calibration curves, and standard compounds in the samples were quantified by interpolating the analyte/IS peak abundance ratio in the resulting standard curves. Quality parameters, such as calibration curve detection and quantification limits and method detection and quantification limits, were also calculated (Table S2).

2.7. Statistical analysis

Results were expressed as the mean±standard error (S.E.M.) of the mean (n=3) and analyzed by one-way or two-way analysis of variance (ANOVA) using SPSS 21.0 software. One-way ANOVA was applied when the results were compared within the same tissue. Differences between groups were assessed by the Bonferroni test (to correct for multiple comparisons). Two-way ANOVA was applied when the results were compared considering all tissues. Differences between means were considered significant when P<.05.

3. Results

Tables 1 and 2 detail the concentrations of each phase II and colonic flavanol metabolite in the different tissues (i.e., liver, kidney, MWAT and brain) at 2 h after the administration of 125, 250, 375 and 1000 mg/kg of GSPE. These data provide insight into how flavanols are metabolized and distributed throughout the bodies of rats.

3.1. Distribution of free flavanols and their phase II metabolites in rat tissues

When the administered dose of GSPE is varied, free flavanols and their phase II metabolites reach different concentrations in different tissues (Table 1, Fig. S1, and Fig. 1). In fact, it is quite clear that there are large differences in the concentrations of each metabolite depending on the tissue types and the corresponding doses. In all of the tissues evaluated, gallated flavanol could not be detected.

As shown in Fig. 1, in all of the tissues and GSPE doses evaluated, the majority of the flavanol phase II metabolites were found in the kidneys, with total concentrations from 300 to almost 900 nmol/g. These kidney concentrations are approximately 3 times higher than those in the liver. However, in MWAT and brain, the amount of flavanol metabolites targeted to these tissues is lower than that in the kidneys or the liver, with total flavanol metabolite concentrations of lower than 40 nmol/g. Moreover, in the brain, there is a clear dose–response effect up to 1000 mg/kg of GSPE that is not reproduced in the liver, kidneys or MWAT.

Interestingly, the distribution of each specific phase II metabolite was dependent on the tissue and the dose of GSPE (Table 1 and Figs. 2 and 3). In this sense, the main metabolites in liver were the phase II methyl-glucuronidated form for all of the evaluated doses with concentrations of approximately 40–120 nmol/g. In the liver, the concentrations of free flavanols and their glucuronidated, sulfated and methylated derivatives increased as the dose increases. Notably, a strong increase from 375 to 1000 mg/kg for the sulfated and nonconjugated free flavanols (catechin, epicatechin and procyanidin dimers) was observed. Conversely, the amount of gallic acid in the liver was reduced with increasing doses of GSPE (Fig. 2A).

In the kidneys, the main phase II metabolites were the glucuronidated and methyl-glucuronidated forms, with concentrations from 100 to 400 nmol/g, whereas much lower concentrations were found for the sulfated, methylated and nonconjugated free flavanols (Fig. 2B). Furthermore, these concentrations remained similar for nearly all of the doses evaluated, although some variation at the 1000-mg/kg dose was

Table 1
Flavanols and their phase II metabolites detected by HPLC–ESI–MS/MS in different rat tissues at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of a GSPE

Compound	125 mg/kg				250 mg/kg				375 mg/kg				1000 mg/kg			
	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)
Catechin	0.23±0.03	0.41±0.1	0.61±0.25	n.d.	0,16±0.02	1.53±0.15	1.13±0.97	n.d.	0.91±0,16	2.13±0.82	3.09±1.26	n.d.	38.95±7.20	13.85±4.22	5.30±0.72	n.d.
Epicatechin	1.50±0.25	4.63±1.57	2.59±1.82	n.d.	2.20±0.59	6.86±0.33	1.65±0.76	n.d.	19.27±5.54	13.09±3.91	4.32±1.81	n.d.	44.65±5.37	51.75±19.93	7.80±1.27	n.d.
Epicatechin gallate[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EGCG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Procyanidin dimer B_2	n.d.	n.d.	0.49±0.17	n.d.	0.07±0.01	n.d.	0.63±0.42	n.d.	0.12±0.01	n.d.	1.23±0.44	n.d.	13.53±2.40	5.34±2.45	2.27±0.74	n.d.
Procyanidin dimer B_3[b]	n.d.	n.d.	0.17±0.09	n.d.	0.03±0.01	n.d.	0.14±0.05	n.d.	0.29±0.01	n.d.	0.56±0.26	n.d.	7.99±0.84	3.55±1.58	0.82±0.28	n.d.
Procyanidin dimer B_1[b]	n.d.	n.d.	0.28±0.12	n.d.	n.d.	n.d.	0.30±0.12	n.d.	n.d.	n.d.	0.80±0.31	n.d.	7.10±2.30	3.67±1.64	1.11±0.37	n.d.
Gallic acid	82.83±33.04	1.27±0.27	0.20±0.06	n.d.	52.94±12.34	4.15±2.25	0.36±0.22	n.d.	25.74±8.95	15.70±0.69	0.74±0.28	n.d.	3.26±1.36	46.41±12.06	1.27±0.11	n.d.
Catechin glucuronide[c]	3.12±0.53	57.75±13.30	4.50±1.89	0.33±0.07	7.24±1.37	149.72±7.36	2.26±0.78	0.75±0.05	9.41±0.42	231.98±43.15	4.00±0.04	1.26±0.31	13.91±2.34	202.55±53.87	2.06±0.72	2.37±0.18
Epicatechin glucuronide[d]	5.26±1.04	50.55±12.96	3.44±1.24	0.61±0.13	12.47±1.77	114.23±14.06	4.79±1.72	1.59±0.21	18.61±3.88	179.34±64.91	8.18±0.43	2.47±0.28	25.10±5.86	145.68±29.42	4.56±1.43	4.60±0.36
Methyl-catechin glucuronide[c]	4.10±0.09	126.89±16.41	3.05±1.90	0.21±0.01	6.93±0.38	242.21±25.17	0.48±0.18	0.44±0.04	11.07±3.91	257.32±70.24	0.81±0.03	0.68±0.30	12.20±2.27	146.15±22.38	0.43±0.09	0.75±0.10
Methyl-epicatechin glucuronide[d]	45.25±9.81	67.09±11.18	3.16±1.97	0.23±0.05	109.05±8.08	115.40±12.51	0.48±0.14	0.46±0.03	119.30±31.50	121.40±31.41	0.90±0.01	0.85±0.14	114.77±15.13	109.18±14.55	0.56±0.19	1.44±0.11
Catechin-sulfate[c]	0.08±0.02	n.d.	n.d.	n.d.	0.09±0.01	n.d.	n.d.	n.d.	0.25±0.05	0.29±0.02	n.d.	n.d.	8.51±0.17	3.96±1.21	n.d.	n.d.
Epicatechin-sulfate[d]	0.32±0.06	n.d.	n.d.	n.d.	0.33±0.02	n.d.	n.d.	n.d.	1.32±0.41	0.26±0.02	n.d.	n.d.	n.q.	2.93±1.02	n.d.	n.d.
Methyl-catechin-sulfate[c]	1.09±0.15	n.d.	0.13±0.06	n.d.	2.22±0.08	n.d.	0.03	n.d.	6.92±1.73	0.41±0.08	0.04±0.01	n.d.	28.18±6.07	0.91±0.32	0.07±0.02	n.d.
Methyl-epicatechin-sulfate[d]	12.50±2.18	n.d.	0.52±0.22	n.d.	16.83±1.90	n.d.	0.15±0.07	n.d.	29.94±13.24	n.d.	0.27±0.02	n.d.	71.41±12.71	n.d.	0.33±0.09	n.d.
3-*O*-methyl-epicatechin[d]	0.26±0.04	6.07±0.11	n.d.	5.83±0.50	0.28±0.03	6.18±0.50	n.d.	7.05±0.76	0.59±0.14	12.19±1.54	n.d.	7.93±0.95	4.16±0.78	21.83±5.70	n.d.	9.80±0.39
4-*O*-methyl-epicatechin[d]	1.38±0.17	3.45±1.09	n.d.	5.54±0.78	1.88±0.13	3.94±0.69	n.d.	6.19±0.56	3.77±1.43	9.98±0.31	n.d.	7.98±1.27	10.60±2.05	14.26±3.40	n.d.	14.25±0.48

Abbreviations: n.d., not detected; n.q., not quantified.
The results are expressed in nmol/g of dried tissue as the mean±S.D. (n=3).
[a] Quantified using the calibration curve of EGCG.
[b] Quantified using the calibration curve of procyanidin dimer B_2.
[c] Quantified using the calibration curve of catechin.
[d] Quantified using the calibration curve of epicatechin.

Table 2
Microbial colonic flavanol metabolites detected by HPLC–ESI–MS/MS in different rat tissues at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of a GSPE

Compound	125 mg/kg				250 mg/kg				375 mg/kg				1000 mg/kg			
	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)	Liver (nmol/g)	Kidney (nmol/g)	MWAT (nmol/g)	Brain (nmol/g)
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	n.d.	n.d.	0.08±0.003	0.01±0.00	n.d.	n.d.	0.09±0.03	0.02±0.01	n.d.	n.d.	0.05±0.01	0.02±0.01	n.d.	n.d.	0.03±0.01	0.03±0.00
3-(4-hydroxyphenyl) propionic acid	n.d.	1.17±0.32	n.d.	n.d.	n.d.	1.66±0.54	n.d.	n.d.	n.d.	1.99±0.18	n.d.	n.d.	n.d.	2.42±0.36	n.d.	n.d.
Phenylpropionic acid[a]	n.d.	n.d.	0.53±0.20	0.22±0.05	n.d.	n.d.	0.55±0.08	0.28±0.06	n.d.	n.d.	0.46±0.06	n.d.	n.d.	n.d.	0.57±0.09	6.67±0.77
3-Hydroxybenzoic acid	n.d.	n.d.	0.93±0.15	0.61±0.13	n.d.	n.d.	1.29±0.16	0.43±0.18	n.d.	n.d.	1.59±0.52	0.44±0.04	n.d.	n.d.	1.02±0.02	1.06±0.06
Benzoic acid	6.48±2.97	9.08±3.33	2.70±1.30	15.63±3.40	3.23±1.09	10.30±4.85	4.74±2.02	2.53±1.40	n.d.	5.19±1.70	5.05±1.64	18.88±2.96	n.d.	n.d.	n.d.	11.00±3.95
3-*O*-Methylgallic acid[b]	3.08±1.02	162.65±41.26	0.66±0.04	n.d.	5.92±0.94	506.27±96.55	0.37±0.10	n.d.	8.57±4.36	614.54±154.32.	0.77±0.38	n.d.	24.20±5.43	1241.41±260.55	0.87±0.32	n.d.
Homovanillic acid[c]	2.59±1.17	n.d.	n.d.	2.36±0.48	4.23±0.42	n.d.	n.d.	2.11±0.23	2.42±0.10	n.d.	n.d.	1.84±0.44	n.d.	n.d.	n.d.	2.39±0.40
Hippuric acid	1.38±0.17	n.d.	n.d.	0.32±0.10	2.32±0.90	n.d.	n.d.	0.56±0.18	1.41±0.42	n.d.	n.d.	0.42±0.21	n.d.	n.d.	n.d.	0.22±0.01

Abbreviations: n.d., not detected; n.q., not quantified.
Compounds not detected in any tissue: 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid, 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid, 4-hydroxy-5-(phenyl)-valeric acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, phenylacetic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, homovanillyl alcohol and ferulic acid.
The results are expressed in nmol/g of dried tissue as the mean±S.D. ($n=3$).

[a] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid.
[b] Quantified using the calibration curve of gallic acid.
[c] Quantified using the calibration curve of vanillic acid.

observed: the methylated, sulfated and nonconjugated free flavanols increased in concentration, but the glucuronidated and methyl-glucuronidated concentrations decreased (Fig. 3).

In MWAT, the major compounds found were glucuronidated derivatives at low doses of GSPE and nonconjugated free flavanols at the highest dose (1000 mg/kg) (Fig. 2C). Very low concentrations were observed for the other metabolites in the MWAT, and the methylated and sulfated metabolites were not detected in this tissue (Table 1).

In the brain, only a few metabolites were detected (glucuronidated, methyl-glucuronidated and methylated flavanol metabolites) at very low concentrations. The methylated conjugate was the form with the highest concentration at all of the evaluated doses. Moreover, in the brain, the epicatechin metabolites were found in greater concentrations than the catechin conjugates (Table 1). All of these metabolites seem to behave equally at all doses, increasing their concentrations as the dose increases (Fig. 2D).

Overall, in Fig. 3, it can be seen that the metabolite distribution is highly variable between tissues and that this distribution is quite sensitive to different GSPE doses. It can also be observed that at the highest dose (1000 mg/kg), free flavanols (monomers and dimers) increased proportionally while their metabolites decreased.

3.2. Distribution of flavanol colonic metabolites in rat tissues

At 2 h post-GSPE administration (with 125, 250, 375 and 1000 mg/kg of body weight doses), only few microbial metabolites at very low concentrations could be detected in the rat tissues. The majority of these metabolites were not detected by HPLC–ESI–MS/MS. Notably, simple phenols and final products of microbial metabolism (namely, methyl conjugated phenols) were the most abundant compounds identified in all tissues. 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone was only found in MWAT and brain tissue, albeit at low concentration levels. Likewise, 3-(4-hydroxyphenyl)propionic acid was found in the kidneys. Interestingly, 3-*O*-methylgallic acid was the main compound found in kidney and liver tissues, but it was detected at much higher concentrations in the kidneys (160–1240 nmol/g) than in the liver (3-24 nmol/g) (Table 2). However, this compound was not detected in the brain and was found at very low concentrations in MWAT. The other major flavanol colonic metabolite was benzoic acid, which could be found in all tissues at concentrations ranging from approximately 2 to 19 nmol/g of tissue.

4. Discussion

The regular consumption of flavonoids in the human diet has been associated with beneficial health effects for people suffering from several diseases [2,27]. Flavanols are considered the most abundant flavonoids in the human diet [4], and their beneficial effects depend on both the amount consumed and their bioavailability [19]. It has been shown that low molecular weight forms, especially monomeric flavan-3-ols and dimers, are first absorbed and then glucuronidated, methylated and sulfated in the small intestine before they are further metabolized in the liver [18,19,28]. Therefore, the bioactive compounds that eventually reach the tissues are substantially different from those that are initially present in food [29]. In fact, the qualitative and quantitative flavanol composition differs substantially between GSPE and the sera of animals administered a 1000-mg/kg dose of this same extract [12]. Hence, the objective of the present work was to determine whether flavanols are metabolized and distributed differentially throughout the bodies of rats depending on the tissue and on the dose administered.

The present study was realized at 2 h post-GSPE administration since it has been reported between 1 and 2 h the maxim times of appearance of flavanol phase II metabolites in plasma and tissues [14,26,30]. Moreover, at these short times, GSPE has been reported to

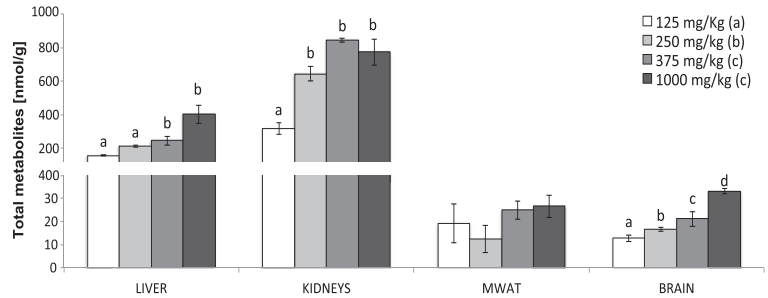


Fig. 1. Total concentrations of flavanol and their phase II metabolites (catechin, epicatechin, procyanidin dimers, gallic acid and their methylated, glucuronidated and sulfated derivatives) quantified by HPLC–ESI–MS/MS in rat tissues at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of a GSPE. Data are given as the means±S.E.M. ($n=3$) and expressed in nmol/g of dried tissue. Different letters indicate statistically significant differences between treatment groups ($P<.05$). *P* was estimated by one-way ANOVA when the treatment groups were compared within the same tissue. *P* was estimated by two-way ANOVA when the treatment groups were compared considering all tissues.

exert some of their health effects in rats as lowering blood pressure [10] or increasing secretion of GLP-1 and insulin and hence a decrease in plasma glucose levels [31]. Furthermore, the doses of GSPE of 125, 250, 375 and 1000 mg/kg were selected as the doses of 250 and 375 mg/kg of GSPE are those acute doses administered to rats for the study of physiological effects of GSPE as in lipid and glucose metabolism [9,32] or hypertension [10,11] among others. On the other hand, bioavailability studies with GSPE have always been realized with a dose of 1000 mg/kg, which we have also used to compare with other bioavailability studies previously realized [12,23,33,34]. Finally, we also selected 125 mg/kg as a lower dose to the normally used in acute studies with GSPE. Moreover, we have realized a previous study in serum with the same doses [13]. After an acute administration of these doses of GSPE, flavanol metabolites were quantified in liver as the main tissue of flavanol phase II metabolization, MWAT as the storage organ, kidney to evaluate one of the excretion ways of flavanols and brain as an important peripheral organ difficult to cross.

The distribution of free flavanols and flavanol metabolites differs considerably in different tissues and at different doses. This result could be attributed to the different solubility proprieties of each flavanol metabolite or to specific transporters in each tissue [13,26,29]. Nevertheless, the functionality of the tissue also needs to be considered. The fact that the majority of the phase II flavanol metabolites were quantified in the kidneys at all of the doses evaluated and shortly after administration (i.e., 2 h post-GSPE administration) may be because these compounds are mainly rapidly excreted in the urine. Thus, the body recognizes them as xenobiotics [21]. In the liver, the quantity of phase II metabolites was also abundant, which is reasonable because the main metabolism of flavanols occurs in this tissue [25] and other studies have demonstrated similar levels of metabolite concentrations at 1000 mg/kg of GSPE in this tissue [22,23,26]. The metabolites present in tissues, mainly in the liver and the kidneys, are equivalent to those found previously in the serum at the same doses [12,13]. Nevertheless, there are important differences in the found amount of the different metabolites. For example, glucuronidated metabolite concentration in kidney is more than 3 times more than in serum. The nonmetabolized flavanols concentrations in serum are mostly lower than those in tissues. For example, aglycone flavanols and gallic acid reached concentrations after 1000 mg/kg of GSPE administration less than 1 μM in serum, but concentrations of 2–112, 5–78 and 5–17 nmol/g were measured in the liver, kidneys and MWAT, respectively. However, these metabolites were not found in the brain.

Finally, the quantity of flavanol metabolites that target the MWAT and brain is fewer than those that target the liver and kidneys but is still significant at 2 h. This observation probably results from the fact that those compounds are the physiological active forms. Not all of the flavanol metabolites are able to cross the blood–brain barrier (BBB), as only a few of these compounds (most notably the methylated forms) were detected in the brain. It has been previously reported that flavanols can cross the BBB, but different results were observed [23]. The study by Arola-Arnal et al. [23] also involved detecting GSPE metabolites at 2 h after the administration of 1000 mg/kg of GSPE, but their findings differ from the results presented herein because no methylated flavanols where detected in the brain, and instead, free flavanols were quantified. These differences could be due to differences in the experimental methods, such as the gender of the rats. Faria et al. [35] suggest that the female hormone progesterone can act as an endogenous factor that modulates P-glycoproteins' abilities to serve as flavanol transporters that could be used to cross the BBB. Moreover, our results showed that the epicatechin forms are more able to cross the BBB than the catechin, suggesting that specific transporters of each polyphenol structure may be involved, as previously suggested by Faria et al. [35] in *in vitro* studies. Furthermore, in general, epicatechin metabolites are the main compounds absorbed, possibly because of a stereospecific mechanism of transport or absorption [35]. Additionally, the MWAT seems to accumulate nonmetabolized flavanols, as previously reported [23,24]. In this tissue, increasing the concentration of GSPE administered to rats decreases the levels of the metabolized forms and increases the levels of those that are not conjugated. The presence of flavanols in adipose tissue is considered important because flavanols have been described to present different beneficial properties relating to metabolic syndrome [11]. The leading cause of metabolic syndrome is excess energy intake. This excess energy is stored in the adipocytes, which suffer from hyperplasia and start releasing proinflammatory cytokines and adipocyte-related hormones. These factors then promote a proinflammatory state and the production of reactive oxygen species. Therefore, the presence of flavanols in adipose tissue could be related to their beneficial effects on this disorder. In fact, it has been demonstrated *in vitro* that grape seed flavanols exhibit beneficial effects in adipose tissue, such as limiting adipogenesis [7]. In summary, our results demonstrated that the metabolites present in the liver and the kidneys are equivalent to those found in the serum or plasma. However, specifically and independently of the GSPE administered, there is an accumulation of the methylated derivatives in the brain and unconjugated free flavanols in the MWAT.

When interpreting the differential flavanol distribution between tissues, it is important to note that different metabolites reach different tissues as the products of metabolism. In addition, the dose–response study is very consistent with previous results in serum using the same doses of GSPE where a saturation of the system was

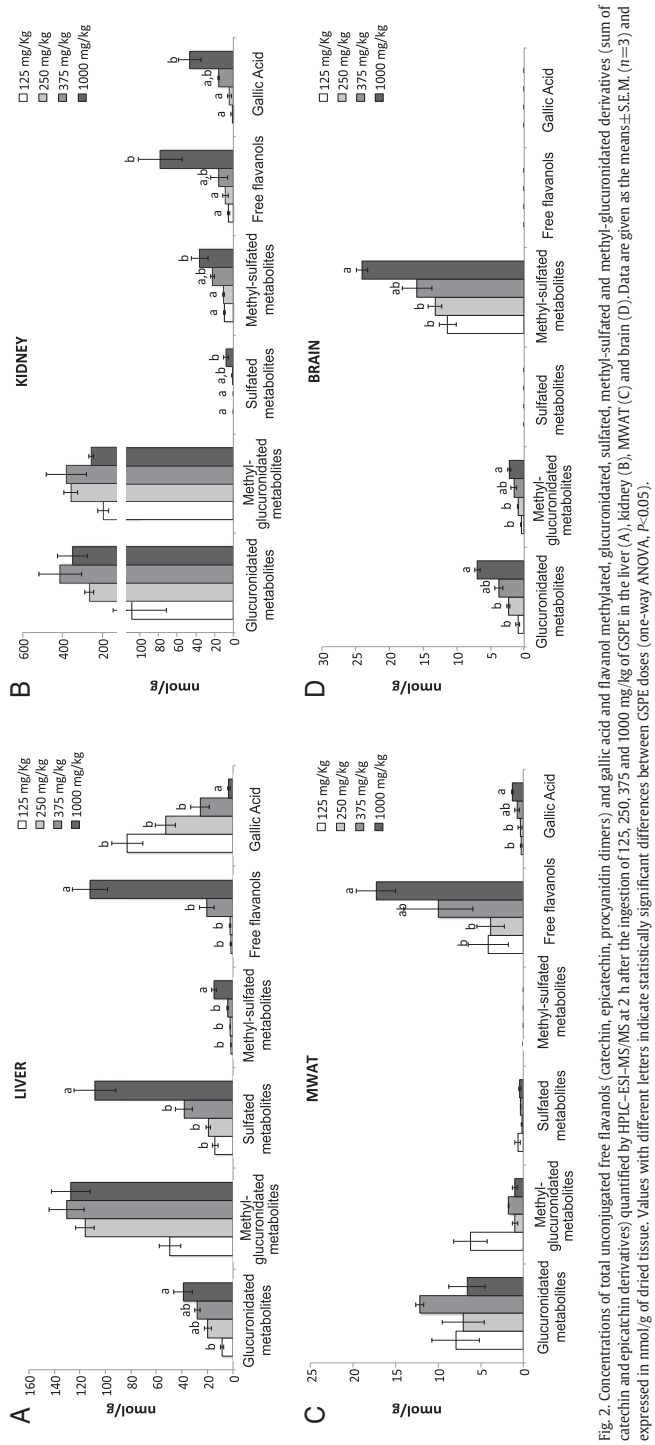
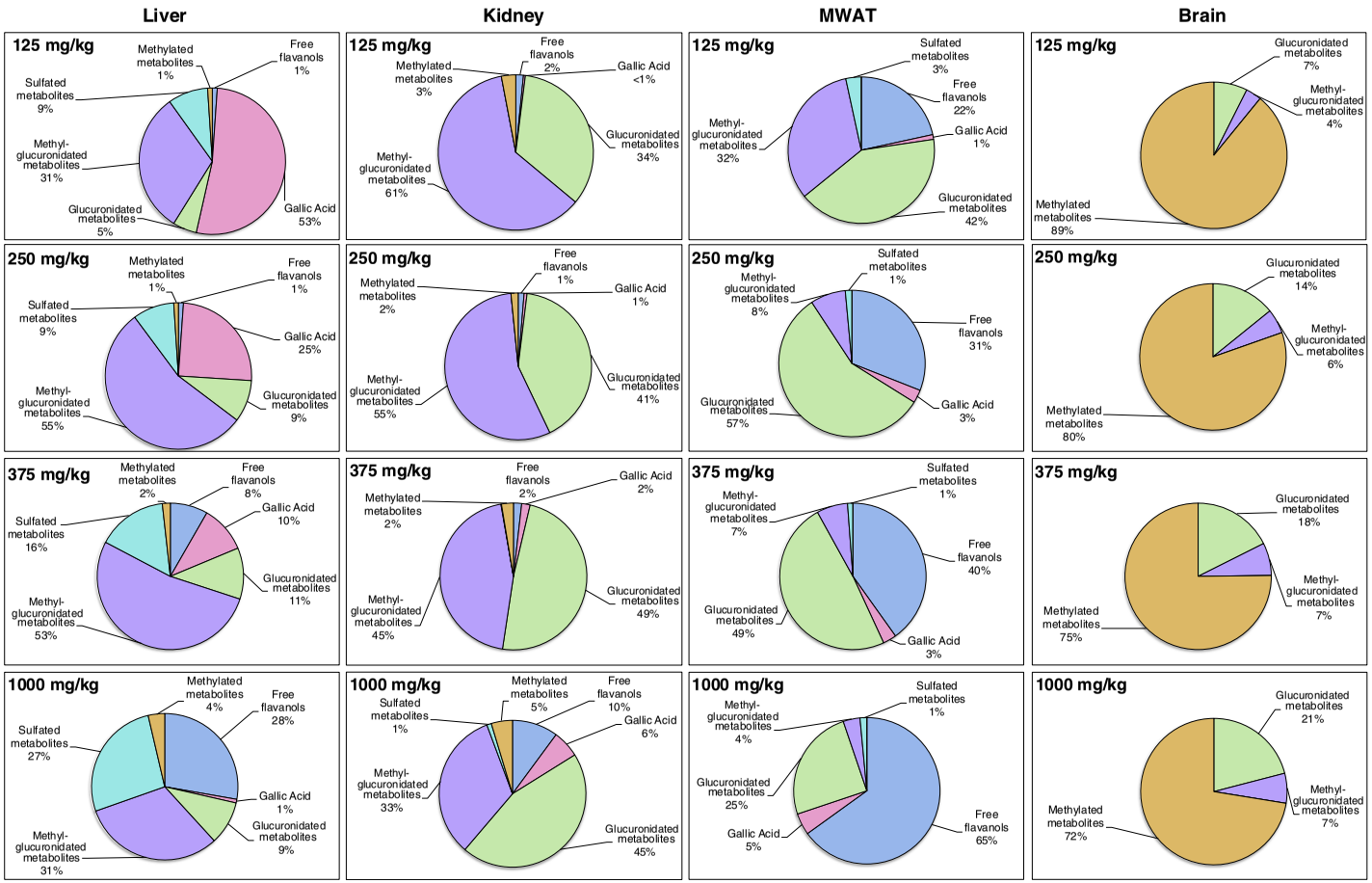


Fig. 2. Concentrations of total unconjugated free flavanols (catechin, epicatechin, procyanidin dimers) and gallic acid and flavanol methylated, glucuronidated, sulfated, methyl-sulfated and methyl-glucuronidated derivatives (sum of catechin and epicatchin derivatives) quantified by HPLC–ESI–MS/MS at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of GSPE in the liver (A), kidney (B), MWAT (C) and brain (D). Data are given as the means ± S.E.M. ($n = 3$) and expressed in nmol/g of dried tissue. Values with different letters indicate statistically significant differences between GSPE doses (one-way ANOVA, $P < 0.05$).



ig. 3. Distributions of free flavanols (catechin, epicatechin and procyanidin dimers), gallic acid and flavanol phase II metabolites quantified by HPLC–ESI–MS/MS in rat tissues (liver, kidney, MWAT and brain) at 2 h after the ingestion of 125, 50, 375 and 1000 mg/kg of a GSPE. Data are given as the means±S.E.M. (n=3) and expressed as percentages.

observed at very high doses of the extract (1000 mg/kg) [13]. In liver and kidney tissue, a dose–response effect up to 375 mg/kg has been observed, but at 1000 mg/kg, the total amount of metabolites did not increase further. Moreover, at this high dose, the quantity of nonconjugated free flavanols increased, but the glucuranidated forms decreased in the MWAT. This result confirms the saturation of the system at 1000 mg/kg of GSPE administration. Therefore, as observed previously in plasma, there is a saturation of the system at 375 mg/kg of GSPE in the liver and kidneys but not in the MWAT or the brain. Otherwise, our results show that, depending on the dose of GSPE administered, flavanol metabolites distribute differently throughout the different tissues. However, further studies will be required to elucidate the characteristic distributions of the flavanol metabolites in tissues such as adipose tissue and brain, as well as to elucidate the biological significance of particular flavanol metabolites in particular tissues.

Finally, once ingested, the large molecular weight flavanols go to the colon, where they are metabolized by the gut microbiota to produce low-molecular-weight phenolic acids [21]. These compounds are products of the catabolism of the gut microbiota and were also analyzed in the various tissues of rats given different doses. However, because the study was conducted 2 h post-GSPE administration, the majority of the colonic flavanol metabolites were not detected as most of them may not appear until later time points. This is because prior to absorption, the compounds need to move to the colon, be metabolized and then be reabsorbed into the circulatory system [21,22]. However, even at only 2 h, some final products of this catabolism, such as 3-*O*-methylgallic acid and benzoic acids, could be detected in the kidneys and liver but not in the brain or the MWAT; at later time points, they might also be target dose tissues.

5. Conclusions

This study demonstrated that the experimental conditions, such as the extract dose administered, influence the metabolism and distribution of flavanols throughout the bodies of rats. This finding may be due to the different functionalities of these compounds in the various tissues because different physiological bioactive forms are generated. In addition, independent of the used doses, a specific distribution of the flavanol derivatives in the various tissues can be observed, with the notable presence of free and methylated flavanols in the MWAT and the brain, respectively. Therefore, that flavanols are conjugated and distributed differently throughout the body when the intake dosage of a grape seed PA extract is varied may involve a difference in their biological effects in the target tissue. These findings point to the clinical research to find the best dose for a specific biological or health effect.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.04.006.

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MANUSCRIPT 6:

Lack of tissue accumulation of grape seed flavanols after daily long-term administration in healthy and cafeteria diet obese rats

Maria Margalef[1], Zara Pons[1], Lisard Iglesias-Carres[1], Francisca Isabel Bravo[1], Begoña Muguerza[1,2*], Anna Arola-Arnal[1]

[1]Nutrigenomic Research Group. Biochemistry and Biotechnology Department. Rovira i Virgili University, Spain, [2]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

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Lack of tissue accumulation of grape seed flavanols after daily long-term administration in healthy and cafeteria diet obese rats

Maria Margalef[1], Zara Pons[1], Lisard Iglesias-Carres[1], Francisca Isabel Bravo[1], Begoña Muguerza[1,2*], Anna Arola-Arnal[1]

[1]Nutrigenomic Research Group. Biochemistry and Biotechnology Department. Rovira i Virgili University, Spain
[2]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

Running title: Long-term non-accumulation of flavanols

*Dra. Begoña Muguerza Marquinez
Biochemistry and Biotechnology Department, Rovira i Virgili University, C/ Marcel·li Domingo s/n 43007 Tarragona, Spain.
Telephone: 34 977 55 9566
Fax: 34 977 55 8232
E-mail: begona.muguerza@urv.cat

Abstract

Flavanols, one of the most abundant dietary polyphenols in human diet and well-known for their health benefits, are absorbed in the small intestine and metabolized by phase-II enzymes and the microflora and are distributed throughout the body depending on several factors. In this study, we aim to evaluate whether flavanols are tissue-accumulated after the long-term administration of a grape seed polyphenol extract (GSPE) in rats and if the compounds that are present in tissues differ in a cafeteria diet obesity state. For that, plasma, liver, mesenteric white adipose tissue (MWAT), brain and aorta flavanol metabolites from standard chow-diet-fed rats (ST) and cafeteria-diet-fed rats (CAF) were analyzed by HPLC-MS/MS 21 h after the last 12-week-daily GSPE (100 mg/kg) dosage. Results showed that the long-term GSPE intake did not trigger a flavanol tissue accumulation, indicating a clearance of products at each daily-dosage. Moreover, the detected compounds differed substantially between ST and CAF-obese rats. Therefore, these results suggest that polyphenol benefits in a disease state would be due to a daily pulsatile effect. Moreover, obesity induced by diet influences the metabolism and bioavailability of flavanols in rats.

Keywords: bioavailability, metabolites, obesity, polyphenol, tissue distribution.

1. Introduction

Flavanols, or flavan-3-ols, are a flavanoid-type polyphenol that is mostly ingested by humans, as these phytochemicals are mainly found in grapes, cocoa, chocolate, red wine, and green tea [1–3]. These flavanols consist of the monomers (+)-catechin and (-)-epicatechin and their polymeric and oligomeric forms, known as proanthocyanidins (PAs) [4,5]. The potential health benefits of flavanols have been widely studied in animal models [6–9] and in humans [10–14], indicating that these compounds exert beneficial health effects on some related cardiovascular [15–18] and metabolic disorders [14,19,20]. Specifically, flavanols from grape seed improve lipid metabolism [21], increase insulin secretion [22], exhibit antioxidant and anti-inflammatory capacities [23], and act as antihypertensive agents [24].

However, the beneficial health properties of flavanols are mainly attributed to the compounds that are derived from their metabolism [4]. In this sense, once ingested, flavanol monomers and dimers are absorbed through the small intestine and recognized as xenobiotics by the body to be subjected to phase-II metabolism by the enterocyte or hepatocyte phase-II enzymes uridin-glucuronil transferases (UGTs), sulfotransferases (SULTs) and/or catechol-*O*-methyl transferases (COMTs) to form their respective glucuronidated, sulfated or methylated metabolites [25]. In addition, flavanols with a degree of polymerization greater than 2 pass intact through the small intestine and reach the colon, where they are subjected to the microbiota metabolism to form small phenolic compounds that could after undergo the phase-II metabolism [4,25]. In fact, after an acute intake of a flavanol extract, these compounds are conjugated to their phase-II metabolites, which are then distributed throughout the body and are found at considerable concentrations in tissues at short times [26]. However, few studies have evaluated the tissue distribution of flavanols after long-term flavanol administration [27] or reported the accumulation of polyphenols in tissues after

the acute administration of flavonoids, although this accumulation was evidenced at short times after ingestion [28,29]. However, some compounds, such as fat-soluble vitamins or some toxins, accumulate in some organs or tissues after chronic ingestion [30,31].

There are several factors that can interfere with the metabolism, the production of a specific metabolite and its body distribution, such as the host-internal factors related to the phase-II enzyme activity, the intestinal transit time, colonic microbiota and host systemic factors, such as the age, gender physiological conditions, genetics, or pathologies/disorders [2]. In particular, we previously reported that the bioavailability and time of appearance of grape seed flavanols in rats after a short-term administration differ between tissues [26], or at varying doses [26,32] and that different metabolites can present distinct bioactivities [21,32]. Otherwise, regarding differences due to pathologies/disorders, obesity changes in the gene expression or in the activity of phase-II enzymes have been reported for rat/mouse obese models [33]. Moreover, changes in the gut microbiome are associated with obesity [34,35]. Thus, the present study aims to evaluate whether flavanols can be accumulated in tissues after 12 weeks of the daily intake of a grape seed polyphenol extract, understanding the term accumulation in the different tissues as a storage process different from those related to the normal flow of the compounds that reach the tissues through the systemic circulation, and whether the compounds that are present in tissues after this long-period differ in a cafeteria diet obesity state.

2. Materials and Methods

2.1. Chemicals and Reagents

Methanol (Scharlab S.L., Barcelona, Spain), acetone, acetonitrile (both from Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Individual stock standard solutions of 2000 mg/L in methanol of (+)-catechin, (-)-epicatechin,

procyanidin B2, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, benzoic acid, and pyrocatechol as the internal standard (IS) (all from Fluka/Sigma-Aldrich, Madrid, Spain), as well as 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany), were prepared and stored in a dark glass flask at -20 ºC.

A 20 mg/L stock standard mixture in methanol of all of the compounds described above was prepared weekly and stored at -20 ºC. This stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.2. Grape Seed Polyphenol Extract (GSPE)

Grape seed polyphenol extract (GSPE) was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). Table 1 shows the phenolic compound (flavan-3-ols and phenolic acids) concentrations of the extract used in this study.

2.3. Experimental Procedure in rats

Six-week-old male Wistar rats Crl:WI (Charles River Laboratories, Barcelona, Spain) were singly housed in animal quarters at 22 ºC with a light/dark period of 12 h. After a quarantine period of 2 weeks, the animals weighed 230-240 g and were divided into two dietary groups. The control group (ST, n=12) was fed the standard chow Panlab A04 (Panlab, Barcelona, Spain) and tap water *ad libitum*. The second group (CAF, n=12) had free access to a fresh cafeteria diet consisting of bacon (10-12 g), sausage (8–12 g), biscuits with *paté* (12–15 g), cheese (10–12 g), *ensaïmada (sweetened pastry)* (4-5 g), carrots (8-10 g), and sweetened milk (20% sucrose (w/v)) daily renewed and tap water in addition to the standard chow diet. The standard chow had a calorie breakdown of 14% protein, 8% fat and 73% carbohydrates, whereas the calorie breakdown of the cafeteria diet was 14% proteins, 35% fat and

51% carbohydrates. All of the animals were fed *ad libitum*, and the diets were maintained for 12 weeks until sacrifice. The ST group was daily administered vehicle (condensed milk and water (1:1 v/v)) (n=6) or with 100 mg/kg GSPE in vehicle (n=6). The CAF group was also daily administered vehicle (n=6) or GSPE (n=6) as described above. All the administrations were daily voluntary licked between 6:00 – 7:00 pm until sacrifice (12 weeks). Rats were weighed and sacrificed by exsanguination after a 6 h fasting period and 21 h after the last GSPE or vehicle administration. Blood, liver, mesenteric white adipose tissue (MWAT), aorta and brain were excised from the rats. Plasma was obtained by blood centrifugation (2000 x *g,* 4 ºC, 15 min), and all of the tissues were freeze-dried. Dried tissues and plasma were stored at –80 ºC until the chromatographic analysis. This study was performed in accordance with the guidelines for the care and use of laboratory animals of the University Rovira i Virgili.

2.4. Flavanol and flavanol metabolite extraction from plasma and tissues

Prior to the chromatographic analysis of the flavanols and their metabolites in rat plasma and tissues, the samples were pre-treated using previously reported methodology based on a micro solid-phase extraction (μSPE) for plasma and an off-line liquid–solid extraction (LSE) in tandem with a μSPE for tissues [26,36].

2.5. Chromatographic analysis

The chromatographic analysis was performed using a 1290 LC Series UHPLC coupled to a 6490 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150 mm × 2.1 mm i.d., 3.5 μm particle size) as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA). The MS system consisted of an Agilent Jet Stream (AJS) ionization source. The mobile phase, electrospray ionization (ESI) conditions and acquisition method were performed as previously reported for the quantification of phase-II and microbial flavanol

metabolites in plasma and tissues [26,36]. Data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

2.6. Sample quantification

For sample quantification, a pool of blank tissue extracts or plasma (n=6) from the rats that were administered the vehicle were spiked with 7 different concentrations of standards to obtain calibration curves for ST and CAF rats. The studied compounds in the samples were quantified by interpolating the analyte/IS peak abundance ratio in the resulting standard curves. Quality parameters, such as calibration curve detection and quantification limits (LOD and LOQ, respectively) and method detection and quantification limits (MDL and MQL, respectively), are shown in Table S1 for ST rats and in Table S2 for CAF rats.

The flavanols and phenolic acids that are present in the blank plasma and tissue (i.e., from the rats administered vehicle) from the diets were quantified by HPLC-MS/MS, and all of the values were subtracted from the final results (i.e., from the rats administered GSPE) in order to quantify only the phenolic compounds from the GSPE.

2.7. Statistical analysis

Animal weights were statistically analyzed by one-way ANOVA using the IBM SPSS Statistics software (Version 20.0.0) and expressed as the mean ± standard error of the mean (SEM). Differences between groups were assessed using the Bonferroni test (to correct for multiple comparisons). Differences between means were considered significant when $p<0.05$.

3. Results

Compared to the control group (mean body weight of 409 ± 19 g), the animals that were fed a cafeteria diet (mean body weight of 523 ± 27 g) had significantly increased body weight. No differences were found in the body weight by the administration of GSPE either in ST rats (406 ± 6 g) or in CAF rats (516 ± 34 g).

Table 2, Table 3 and Figure 1 show the distribution of flavanols, phenolic acids, flavanol phase-II metabolites and microbial flavanol metabolites in rat tissues (i.e., liver, MWAT, aorta and brain) quantified by HPLC-MS/MS after a daily intake of 100 mg/kg GSPE for 12 weeks and 21 h after the last administration of the extract in both the ST and CAF groups. No gallate flavanols or PA trimers were detected in the plasma or in the studied tissues.

3.1. Plasma and liver flavanol and flavanol metabolite concentrations after long-term GSPE administration in ST and CAF rats

The rat plasmas of animals which were daily-administered GSPE for 12 weeks were analyzed by HPLC-MS/MS 21 h after the last administration of GSPE, and no flavanol or flavanol metabolites were quantified (data not shown).

Rat livers were also analyzed (Table 2, Table 3 and Figure 1A) to evaluate whether flavanols accumulate in this tissue after a long-term extract administration (100 mg/kg). Few microbial metabolites and scarcely some flavanol and their phase-II metabolites were quantified in the liver. Otherwise, the results evidenced an effect on the cafeteria diet on these compounds that were quantified in the liver. Although there are no differences in the total amount of flavanols that were detected in this tissue, being 6.96 nmol/g in ST-diet-fed rats and 6.90 nmol/g in CAF-rats, the CAF rats had a greater quantity of non-metabolized flavanols and phenolic acids (14%) than did the ST rats (2%) (Figure 1A). The main phase-II metabolite that was found in this tissue was the methyl-catechin glucuronide, ranging from 0.242 nmol/g in ST rats and 0.063 nmol/g in CAF rats. Furthermore, some compounds were detected specifically in the CAF rat liver but not in the ST rat liver, including the dimer B3 and the sulfated and methyl-sulfated flavanol metabolites (Table 2).

Moreover, the liver of CAF rats also has fewer microbial metabolites (5.81 nmol/g) than the ST rats (6.51 nmol/g) and more non-conjugated microbial metabolites; the benzoic acids (BA) were the major microbial metabolite in

CAF rats, and the 4-hydroxy-5-(3',4'-dihidroxyphenyl)-valeric-acid-specific metabolite was only present in the liver of CAF rats but not in the ST rats (Table 3). In the liver of the ST rats, the main microbial compounds were those of the final products: homovanillic, hippuric and ferulic acids (Figure 1A).

3.2. Mesenteric White Adipose tissue (MWAT) flavanol and flavanol metabolite concentrations after long-term GSPE administration in ST and CAF rats

After 12 weeks of daily GSPE administration (100 mg/kg) and 21 h after the last dose, few flavanols, phenolic acids and microbial metabolites were quantified in MWAT without the detection of any flavanol phase-II metabolite. Both ST and CAF rats have the same total amount of compounds (3.29 nmol/g), with catechin and gallic acid being the predominant polyphenols that were found in this tissue (Table 2, Table 3 and Figure 1B). However, the cafeteria diet influenced those compounds that were present in MWAT; in the MWAT of CAF rats, 64% of the compounds were microbial metabolites and only 36% were non-metabolized compounds, whereas in the MWAT of the ST rats, 61% were non-metabolized compounds and 39% were microbial metabolites (Figure 1B). In addition, phenyl-acetic acids (PAA) and valerolactones (V) seem to be exclusive compounds of the cafeteria diet group.

3.3. Aorta flavanol and flavanol metabolite concentrations after long-term GSPE administration in ST and CAF rats

In the aorta, after 12 weeks of a daily intake of GSPE and 21 h after the last extract dose, only a small amount of compounds were detected in both the ST (1.43 nmol/g) and CAF (1.03 nmol/g) rats. In the aorta of CAF rats, any flavanol or flavanol phase-II metabolite could be quantified, and only microbial metabolites were detected (Table 1, Table 2 and Figure 1C). However, in the aorta of ST rats, 47% of the quantified compounds were the non-metabolized compounds catechin (0.465 nmol/g), epicatechin (0.171

nmol/g) and vanillic acid (0.039 nmol/g) (Table 2). Regarding the microbial metabolites, there are some metabolites that are specific for each group (Table 3). For example, phenylpropionic acid was only found in ST rats, reaching 0.244 nmol/g. For CAF rats, 3-(3,4-dihydroxyphenyl)propionic acid reached a concentration of 0.374 nmol/g and was not found in ST rats. Finally 3,4-dihydroxybenzoic acid (also known as protocatechuic acid) was also a specific compound in the aorta of CAF rats, with a concentration of 0.032 nmol/g.

3.4. Brain flavanol and flavanol metabolite concentrations after long-term GSPE administration in ST and CAF rats

Any flavanol and flavanol phase-II metabolites were quantified in the brain after 12 weeks of daily GSPE administration (100 mg/kg) and 21 h after the last dose, which was not affected by the diet (Table 2). Therefore, in the brain, only few microbial flavanol metabolites were quantified (Table 3). Benzoic acid was the most abundant form that was found in both groups (1.50 nmol/g in ST and 2.43 nmol/g in CAF rats). Benzoic acid hydroxylated on the third position (3-hydroxybenzoic acid) was the second most important microbial metabolite, reaching 0.64 nmol/g in ST and 0.76 nmol/g in CAF rat brain. Homovanillic acid (a methylated form of 2-(3,4-dihydroxyphenyl)acetic acid) was the only final product of the microbial metabolism that could be found in both brain groups, reaching 0.53 nmol/g and 1.74 nmol/g (ST and CAF rats, respectively). However, in the brain of CAF rats, the quantity of microbial metabolites (4.94 nmol/g) is approximately 1.7 times greater than that in the brain of ST rats (2.82 nmol/g) (Figure 1D), and 5-(3',4'-dihidroxyphenyl)-γ-valerolactone seem to be an specific metabolite for ST rats (0.20 nmol/g), as it was not detected in the brain of CAF rats.

4. Discussion

Flavanols are some of most important dietary polyphenols with beneficial health effects [4] described in both *in vitro* [21,22,32] and *in vivo* [16,21,32,37] models. These compounds, which are present in most fruits and vegetables, are

abundant phytochemicals in the human diet and, once ingested, are absorbed in the small intestine and recognized by the body as xenobiotics, which are manly metabolized in the small intestine and liver by phase-II enzymes to their methyl, sulfate and glucuronide forms. Moreover, those forms that are too big to be absorbed in the small intestine pass intact to the colon, where they are metabolized to small phenolic acids by microbiota [36]. Therefore, the health effects of flavanols are mainly attributed to the products of their metabolism [4]. We previously reported that after an acute dose of grape seed flavanols, phase-II and microbial flavanol metabolites are distributed throughout the body and appear in the plasma and tissue short times after their ingestion [21,26,32,36]. However, considering that a Mediterranean diet consists of a daily intake of fruits, nuts and vegetables and hence a daily consumption of flavanols, there are few studies that explore the distribution or accumulation of polyphenols after a long-term intake period [27,38–40]. We have also reported that the metabolism and distribution of these compounds depends on several factors, such as the dosage, and the tissue [26,32]. Therefore, we herein aim to study whether flavanols can accumulate in tissues after a daily long-term administration of grape seed flavanols in rats and whether the compounds that are present in tissues after this long period differ in a cafeteria-diet-obesity state.

We previously reported how flavanols from grape seeds are distributed in rat tissues 2 h after a single acute administration of a low dose of 125 mg/Kg of GSPE [26]. Therefore, in this study, a dose of 100 mg/Kg of GSPE was selected to be compared with our previous acute study. However, in order to evaluate whether these compounds undergo long-term accumulation in tissues, understanding the term of accumulation in the different tissues as a storage process different from those related to the normal flow of the compounds that reach the tissues through the systemic circulation, we administered GSPE to rats daily for 12 weeks together with a standard or cafeteria diet. Most importantly, the last dose of GSPE was administered 21 h before the sacrifice, as it is well known that after an acute dose of GSPE,

the maximal times of appearance of flavanol metabolites in plasma and tissues is 1 to 2 h after ingestion, and after 24 h, there are hardly any of these compounds [36,41,42]. In this sense, the only long-term study with GSPE was performed for 3 weeks and 5 h after the last dose, which does not permit a study of the long-term accumulative effect, as at 5 h, there are still flavanols in the plasma and tissues from the last dosage [27]. Our results clearly indicate that 21 h after GSPE administration, there are no flavanol metabolites in the plasma and that very few of these compounds were detected in the studied tissues (liver, MWAT, aorta and brain) in both ST and CAF rats, indicating that these small amounts of flavanol metabolites are from the last ingested dosage of GSPE and not as a result of a repetitive dosage. Specifically, the concentrations in tissues 21 h after daily GSPE administration for 12 weeks are much lower than the total amounts that were quantified 2 h after a single GSPE ingestion of a similar dose (i.e., in liver, approximately 28 times lower; in MWAT, approximately 7 times lower; and in brain, 3.5 times lower) [26]. Moreover, the majority of the metabolites in tissues at 21 h are products of the colonic microbiota and not phase-II metabolites, as these phenolic compounds require more time to be produced [26,36]. These results indicate that flavanols are not accumulated in tissues after a long-term period independent of the diet or physiological state. According to our study, Bieger et al. [39] showed that quercetin was not accumulated in the studied tissues of pigs (including brain and liver) administered quercetin twice a day for 4 weeks. However, other authors have reported brain accumulation after 4 weeks of oral intake of 213 mg of quercetin per day in rats [38]. Nevertheless, the concentration that was achieved in this tissue is of the pmol/g range, which is significantly lower than the levels reached in the present study. This result suggests that the low concentration of quercetin in the brain could be from the last dose more than from an accumulative effect. In fact, our results after 12 weeks clearly indicate that grape seed flavanols do not accumulate in the liver, brain, MWAT or aorta, which agrees with the fact that flavanols are recognized by the body as xenobiotics and that after ingestion are rapidly

conjugated to increase their solubility and be easily and rapidly excreted [25]. In fact, our results seem to indicate that the tissue-detected compounds do not come from a long-term accumulation but from the 21st acute GSPE administration dosage. Hence, the health effects of flavanols as evidenced in long-term studies with a disease state could interestingly be attributed to a kind of pulsatile and repetitive effect of the bioactive forms of flavanols. This timely strategy would agreement with previous studies that reported that polyphenols exhibited health effects at short times after their acute ingestion, being reversed at longer times [24,37,43,44]. Moreover, rapid reversions of the health benefits of polyphenol-rich products (increasing blood pressure) were observed after halting their long-term administration to hypertensive rats [17,45,46].

Cafeteria diet is a reported robust model for metabolic syndrome that can lead to liver and adipose tissue inflammation [47] and, as shown in this study, increased body weight. In this study, the results indicate that the metabolization and tissue distribution of the scarce concentrations of flavanols 21 h after the last GSPE dosage differed substantiality in an obesity pathological state. This result could be attributed to the fact that diet can rapidly alter the gut microbiome and that, in obesity, phase-II enzymes could also be altered. Obese Zucker [48] or high-fat-diet rats [49] altered the mRNA expression of their main phase-II enzymes (glucuronyltransferases (UGTs), and sulfotransferases (SULTs), respectively). Interestingly, in the liver of CAF rats, there are more non-conjugated free flavanols than phase-II metabolites compared to the liver of ST rats. These results agree with Perez-Viscaino et al. [50], who hypothesized that unconjugated flavanols are responsible for the flavanol health benefits, being unconjugated in the target tissue. Although our results indicate that, in obesity, flavanols are metabolized and distributed differently throughout the body, acute studies with GSPE administration to healthy and obese rats at different time points will be needed to elucidate how different the bioavailability of flavanols is in this pathological state.

This study demonstrates that grape seed flavanols do not accumulate in rat tissues after a long-term daily orally intake of GSPE in ST or obese CAF rats. Moreover, the metabolites that were detected in the tissues after a long-term intake would be the bioactive forms of flavanols from the last dosage, which would indicate that the protective and preventive health effects of flavanols may be not due to an accumulative response of all of the flavanol doses but because of cyclic acute responses. Otherwise, the obesity that is induced by the cafeteria diet influences the metabolization and distribution of phase-II and microbial flavanol metabolites in rats. However, additional studies, which we are currently conducting, are necessary to clarify the differences in flavanol metabolism and distribution in obesity.

Author contributions

M. M., A. A-A. and B. M. analyzed data and wrote the manuscript. M. M., Z. P. F. B. and L. I-C. contributed to researching the data. M. M., Z. P., A. A-A. and B. M. contributed to experimental design, discussion, and review of the manuscript.

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Figure Legends

Figure 1. Distributions of non-metabolized compounds (i.e., the flavanols catechin, epicatechin and proanthocyanidin dimers and the gallic and vanillic acids), the flavanol phase-II metabolites and the microbial metabolites as quantified by HPLC-ESI-MS/MS in rat tissues after 12 weeks of the daily ingestion of 100 mg/kg grape seed polyphenol extract (GSPE) and 21 h after the last extract administration in both Standard (ST, left panels) and Cafeteria (CAF, right panels)-fed rats. (A) Liver. (B) Mesenteric white adipose tissue (MWAT). (C) Aorta. (D) Brain. The final products (FP) are the homovanillic, hippuric and ferulic acids. The data are given as the means (n=6) and expressed as percentages.

Table 1. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1[1]	88.80 ± 3.46
PA dimer B3[1]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[1]	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin[2]	0.27 ± 0.03
PA trimer[1]	4.90 ± 0.47
PA tetramer[1]	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

[1] Quantified using the calibration curve of proanthocyanidin B2.

[2] Quantified using the calibration curve of epigallocatechin gallte.

Table 2. Flavanols and their phase-II metabolites detected by HPLC-ESI-MS/MS in different rat tissues at 21 h after the ingestion of 100 mg/kg of a grape seed proanthocyanidin extract (GSPE) in ST and CAF rats.

Compound	ST				CAF			
	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]
Catechin	n.q.	1.486±0.877	0.465±0.054	n.d.	0.307±0.154	0.573±0.042	n.d.	n.d.
Epicatechin	n.q.	0.109±0.052	0.171±0.015	n.d.	0.092±0.042	0.229±0.031	n.d.	n.d.
Procyanidin dimer B2	n.q.	0.035±0.020	n.d.	n.d.	0.009±0.005	0.029±0.007	n.d.	n.d.
Procyanidin dimer B3[3]	n.d.	0.022±0.007	n.d.	n.d.	0.006±0.003	0.016±0.002	n.d.	n.d.
Procyanidin dimer B1[3]	n.d.	0.019±0.010	n.d.	n.d.	n.d.	0.019±0.002	n.d.	n.d.
Gallic Acid	0.074±0.033	0.304±0.197	n.d.	n.d.	0.435±0.287	0.184±0.027	n.d.	n.d.
Vanillic Acid	0.044±0.006	0.030±0.004	0.039±0.006	n.d.	0.106±0.048	0.130±0.017	n.d.	n.d.
Catechin glucuronide[1]	0.038±0.003	n.d.	n.d.	n.d.	0.307±0.154	n.d.	n.d.	n.d.
Epicatechin glucuronide[2]	0.012±0.003	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methyl-catechin glucuronide[1]	0.242±0.061	n.q.	n.q.	n.q.	0.063±0.034	n.q.	n.q.	n.q.
Methyl-epicatechin glucuronide[2]	0.020±0.005	n.d.	n.d.	n.d.	0.014±0.009	n.d.	n.d.	n.d.
Catechin-sulphate[1]	n.d.	n.d.	n.d.	n.d.	0.012±0.006	n.d.	n.d.	n.d.
Epicatechin-sulphate[2]	n.d.	n.d.	n.d.	n.d.	0.001±0.000	n.d.	n.d.	n.d.
Methyl-catechin-sulphate[1]	n.d.	n.d.	n.d.	n.d.	0.032±0.011	n.d.	n.d.	n.d.
Methyl-epicatechin-sulphate[2]	n.d.	n.q.	n.q.	n.q.	0.009±0.003	n.q.	n.q.	n.q.

Abbreviations: ST (Standard-diet fed rats); CAF (cafeteria-diet fed rats); MWAT (mesenteric white adipose tissue); n.d. (not detected); n.q. (not quantified)

Compounds not detected in any tissue: Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG), 3-*O*-methyl-epicatechin, and 4-*O*-methyl-epicatechin.

The results are expressed in nmol/g of dried tissue as the mean ± SD (n=3).

[1]Quantified using the calibration curve of catechin

[2]Quantified using the calibration curve of epicatechin

[3]Quantified using the calibration curve of procyanidin dimer B2

Table 3. Microbial colonic flavanol metabolites detected by HPLC-ESI-MS/MS in different rat tissues at 21 h after the ingestion of 100 mg/kg of a grape seed proanthocyanidin extract (GSPE) in ST and CAF rats.

Compound	ST				CAF			
	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	0.003±0.001	n.d.	n.d.	0.196±0.021	0.101±0.041	0.080±0.010	n.d.	n.d.
4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid[1]	n.d.	n.d.	0.035±0.001	n.d.	0.301±0.020	n.d.	0.020±0.000	n.d.
3-(3,4-dihydroxyphenyl)propionic acid[2]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.374±0.020	n.d.
3-(3-hydroxyphenyl)propionic acid[2]	n.d.	0.185±0.062		n.d.	n.d.	0.315±0.080	n.d.	n.d.
3-(4-hydroxyphenyl)propionic acid	0.466±0.208	0.128±0.048	n.d.	n.d.	0.688±0.176	0.276±0.070	n.d.	n.d.
Phenylpropionic acid[2]	1.011±0.116	0.475±0.042	0.244±0.168	n.d.	0.981±0.084	0.354±0.081	n.d.	n.d.
2-(3-hydroxyphenyl)acetic acid	0.329±0.050	n.d.	n.d.	n.d.	0.239±0.083	0.182±0.019	n.d.	n.d.
2-(4-hydroxyphenyl)acetic acid	0.263±0.032	n.d.	n.d.	n.d.	0.197±0.075	0.213±0.026	n.d.	n.d.
3,4-dihydroxybenzoic acid[3]	0.017±0.001	n.d.	n.d.	n.d.	0.140±0.061	n.d.	0.032±0.001	n.d.
3-hydroxybenzoic acid	0.506±0.025	0.264±0.017	0.433±0.013	0.635±0.081	1.628±0.107	0.295±0.042	0.465±0.008	0.762±0.072
Benzoic Acid	1.147±0.070	0.096±0.058	n.d.	1.466±0.289	0.989±0.043	0.162±0.037	n.d.	2.432±0.542
Homovanillic acid[4]	0.714±0.097	n.d.	n.d.	0.528±0.072	0.202±0.027	n.d.	n.d.	1.743±0.117
Hippuric acid	1.999±0.157	0.097±0.034	0.020±0.005	n.d.	0.495±0.152	0.131±0.023	0.147±0.014	n.d.
Ferulic Acid	0.060±0.034	0.043±0.016	0.024±0.001	n.d.	0.111±0.033	0.102±0.024	0.002±0.000	n.d.

Abbreviations: ST (Standard-diet fed rats); CAF (cafeteria-diet fed rats); MWAT (mesenteric white adipose tissue); n.d. (not detected); n.q. (not quantified)

Compounds not detected in any tissue: 4-Hydroxy-5-(3'-hydroxyphenyl)-valeric acid, 4-Hydroxy-5-(phenyl)-valeric acid, 2-(3,4-Dihydroxyphenyl)acetic acid, Phenylacetic acid, 4-Hydroxybenzoic acid, Homovanillyl alcohol, and 3-methylgallic acid.

The results are expressed expressed in nmol/g of dried tissue as the mean ± SD (n=3).

[1]Quantified using the calibration curve of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone

[2]Quantified using the calibration curve of 3-(4-hydroxyphenyl)acetic acid

[3] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid

[34]Quantified using the calibration curve of vanillic acid

Figure 1

RESULTS

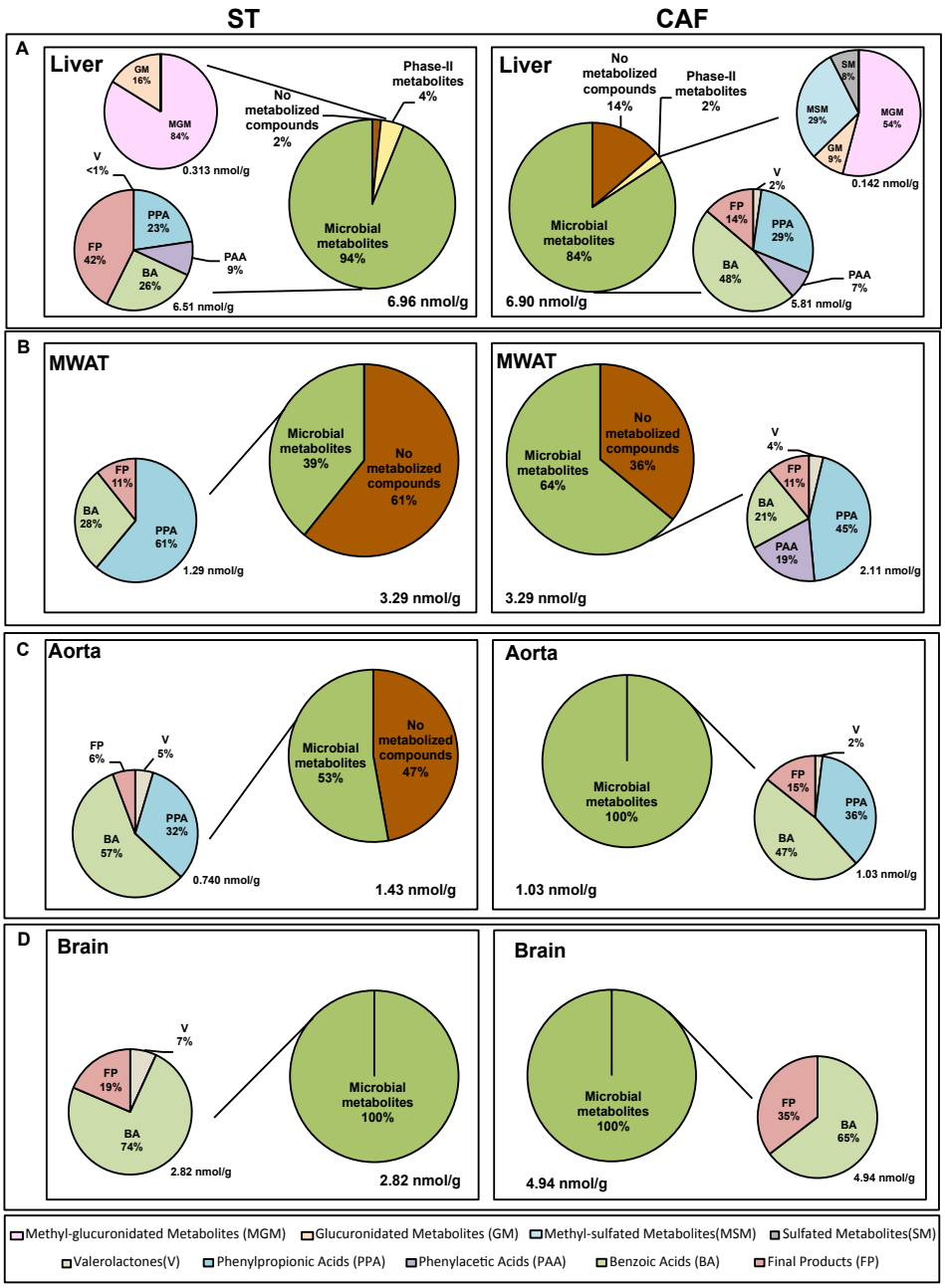

Table S1. Method validation for the determination of flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in the studied tissues from rats fed with standard chow diet assessed by the following parameters: calibration curve, R^2, linearity range, LOD, and LOQ, MDL, and MQL.

Tissue	Compound	Calibration Curve	Determination Coeficient (R^2)	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL* (nmol/g)	MQL* (nmol/g)
Liver	Catechin	y=0.007x	0.998	0.03-17.22	0.86	2.75	0.006	0.020
	Epicatechin	y=0.020x	0.993	0.03-17.22	0.69	2.32	0.005	0.017
	Procyanidin dimer B2	y=0.018x	0.999	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.141x	0.993	0.06-29.39	0.26	0.94	0.002	0.007
	Vanillic Acid	y=0.265x	0.996	0.06-29.76	0.60	2.15	0.004	0.015
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.225x	0.993	0.04-24.04	0.09	0.43	0.001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.027x	0.992	0.06-30.12	0.17	0.60	0.001	0.004
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.014x	0.996	0.06-29.76	0.17	0.60	0.001	0.004
	2-(3-hydroxyphenyl) acetic acid	y=0.054x	0.997	0.06-32.90	0.17	0.60	0.001	0.004
	2-(4-hydroxyphenyl) acetic acid	y=0.035x	0.996	0.06-32.90	0.17	0.60	0.001	0.004
	3,4-dihydroxybenzoic acid	y=0.331x	0.996	0.06-36.23	0.17	0.60	0.001	0.004
	3-hydroxybenzoic acid	y=0.271x	0.995	0.08-36.23	0.26	0.77	0.002	0.006
	Benzoic Acid	y=0.096x	0.997	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=0.106x	0.998	0.08-25.78	0.26	0.77	0.002	0.006
	Hippuric Acid	y=0.310x	0.999	0.05-27.93	0.17	0.52	0.001	0.004
MWAT	Catechin	y=0.037x	0.999	0.03-17.22	0.86	2.75	0.006	0.018
	Epicatechin	y=0.046x	0.993	0.03-17.22	0.69	2.32	0.005	0.015
	Procyanidin dimer B2	y=0.048x	0.999	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.174x	0.999	0.06-29.39	0.26	0.94	0.002	0.006
	Vanillic Acid	y=0.373x	0.995	0.06-29.76	0.60	2.15	0.004	0.014
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.322x	0.992	0.04-24.04	0.09	0.43	0.001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.054x	0.997	0.06-30.12	0.17	0.60	0.001	0.004
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.022x	0.998	0.06-29.76	0.17	0.60	0.001	0.004
	2-(3-hydroxyphenyl) acetic acid	y=0.065x	0.995	0.06-32.90	0.17	0.60	0.001	0.004
	2-(4-hydroxyphenyl) acetic acid	y=0.040x	0.998	0.06-32.90	0.17	0.60	0.001	0.004
	3,4-dihydroxybenzoic acid	y=0.323x	0.991	0.06-36.23	0.17	0.60	0.001	0.004
	3-hydroxybenzoic acid	y=0.182x	0.996	0.08-36.23	0.26	0.77	0.002	0.005
	Benzoic Acid	y=0.295x	0.996	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=0.396x	0.997	0.08-25.78	0.26	0.77	0.002	0.005
	Hippuric Acid	y=0.113x	0.999	0.05-27.93	0.17	0.52	0.001	0.003
Aorta	Catechin	y=0.019x	0.997	0.03-17.22	0.86	2.75	0.012	0.039
	Epicatechin	y=0.058x	0.998	0.03-17.22	0.69	2.32	0.010	0.033
	Procyanidin dimer B2	y=0.049x	0.999	0.02-8.64	0.09	0.26	0.001	0.004
	Gallic Acid	y=0.175x	0.999	0.06-29.39	0.26	0.94	0.004	0.013
	Vanillic Acid	y=0.376x	0.997	0.06-29.76	0.60	2.15	0.009	0.030
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.327x	0.993	0.04-24.04	0.09	0.43	0.001	0.006
	3-(4-hydroxyphenyl) propionic acid	y=0.042x	0.996	0.06-30.12	0.17	0.60	0.002	0.009
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.017x	0.999	0.06-29.76	0.17	0.60	0.002	0.009
	2-(3-hydroxyphenyl) acetic acid	y=0.060x	0.999	0.06-32.90	0.17	0.60	0.002	0.009
	2-(4-hydroxyphenyl) acetic acid	y=0.037x	0.997	0.06-32.90	0.17	0.60	0.002	0.009
	3,4-dihydroxybenzoic acid	y=0.314x	0.999	0.06-36.23	0.17	0.60	0.002	0.009
	3-hydroxybenzoic acid	y=0.328x	0.998	0.08-36.23	0.26	0.77	0.004	0.011
	Benzoic Acid	y=0.240x	0.999	0.07-40.98	0.17	0.69	0.002	0.010
	Ferulic Acid	y=0.542x	0.999	0.08-25.78	0.26	0.77	0.004	0.011
	Hippuric Acid	y=0.114x	0.999	0.05-27.93	0.17	0.52	0.002	0.007
Brain	Catechin	y=0.126x	0.995	0.03-17.22	0.86	2.75	0.010	0.032
	Epicatechin	y=0.029x	0.997	0.03-17.22	0.69	2.32	0.008	0.027
	Procyanidin dimer B2	y=0.027x	0.998	0.02-8.64	0.09	0.26	0.001	0.003
	Gallic Acid	y=0.180x	0.992	0.06-29.39	0.26	0.94	0.003	0.011
	Vanillic Acid	y=0.271x	0.999	0.06-29.76	0.60	2.15	0.007	0.025
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.206x	0.997	0.04-24.04	0.09	0.43	0.001	0.005
	3-(4-hydroxyphenyl) propionic acid	y=0.030x	0.998	0.06-30.12	0.17	0.60	0.002	0.007
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.017x	0.998	0.06-29.76	0.17	0.60	0.002	0.007
	2-(3-hydroxyphenyl) acetic acid	y=0.051x	0.999	0.06-32.90	0.17	0.60	0.002	0.007
	2-(4-hydroxyphenyl) acetic acid	y=0.033x	0.993	0.06-32.90	0.17	0.60	0.002	0.007
	3,4-dihydroxybenzoic acid	y=0.349x	0.993	0.06-36.23	0.17	0.60	0.002	0.007
	3-hydroxybenzoic acid	y=0.170x	0.995	0.08-36.23	0.26	0.77	0.003	0.009
	Benzoic Acid	y=0.152x	0.997	0.07-40.98	0.17	0.69	0.002	0.008
	Ferulic Acid	y=0.411x	0.994	0.08-25.78	0.26	0.77	0.003	0.009
	Hippuric Acid	y=0.117x	0.997	0.05-27.93	0.17	0.52	0.002	0.006

*MDL and MQL for the analysis of 60mg of tissue.

Abreviations: Determination coefficient (R^2); limit of detection (LOD); limit of quantification (LOQ); method detection limit (MDL); method quantification limit (MQL); mesenteric white adipose tissue (MWAT).

Table S2. Method validation for the determination of flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in the studied tissues from rats fed with cafeteria diet assessed by the following parameters: calibration curve, R^2, linearity range, LOD, and LOQ, MDL, and MQL.

Tissue	Compound	Calibration Curve	Determination Coeficient ($R^{2)}$	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL* (nmol/g)	MQL* (nmol/g)
Liver	Catechin	y=0.027x	0.998	0.03-17.22	0.86	2.75	0.007	0.010
	Epicatechin	y=0.077x	0.998	0.03-17.22	0.69	2.32	0.005	0.009
	Procyanidin dimer B2	y=0.099x	0.998	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.145x	0.998	0.06-29.39	0.26	0.94	0.002	0.008
	Vanillic Acid	y=0.637x	0.999	0.06-29.76	0.60	2.15	0.005	0.017
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.438x	0.999	0.04-24.04	0.09	0.43	0.001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.077x	0.998	0.06-30.12	0.17	0.60	0.001	0.005
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.012x	0.998	0.06-29.76	0.17	0.60	0.001	0.005
	2-(3-hydroxyphenyl) acetic acid	y=0.066x	0.999	0.06-32.90	0.17	0.60	0.001	0.005
	2-(4-hydroxyphenyl) acetic acid	y=0.053x	0.999	0.06-32.90	0.17	0.60	0.001	0.005
	3,4-dihydroxybenzoic acid	y=0.328x	0.998	0.06-36.23	0.17	0.60	0.001	0.005
	3-hydroxybenzoic acid	y=0.328x	0.999	0.08-36.23	0.26	0.77	0.002	0.006
	Benzoic Acid	y=0.361x	0.999	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=1.213x	0.998	0.08-25.78	0.26	0.77	0.002	0.006
	Hippuric Acid	y=0.089x	0.998	0.05-27.93	0.17	0.52	0.001	0.004
MWAT	Catechin	y=0.039x	0.998	0.03-17.22	0.86	2.75	0.006	0.020
	Epicatechin	y=0.042x	0.997	0.03-17.22	0.69	2.32	0.005	0.017
	Procyanidin dimer B2	y=0.042x	0.999	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.157x	0.994	0.06-29.39	0.26	0.94	0.002	0.007
	Vanillic Acid	y=0.340x	0.997	0.06-29.76	0.60	2.15	0.004	0.015
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.328x	0.998	0.04-24.04	0.09	0.43	0,001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.046x	0.993	0.06-30.12	0.17	0.60	0.001	0.004
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.019x	0.996	0.06-29.76	0.17	0.60	0.001	0.004
	2-(3-hydroxyphenyl) acetic acid	y=0.060x	0.994	0.06-32.90	0.17	0.60	0.001	0.004
	2-(4-hydroxyphenyl) acetic acid	y=0.037x	0.994	0.06-32.90	0.17	0.60	0.001	0.004
	3,4-dihydroxybenzoic acid	y=0.334x	0.998	0.06-36.23	0.17	0.60	0.001	0.004
	3-hydroxybenzoic acid	y=0.180x	0.995	0.08-36.23	0.26	0.77	0.002	0.006
	Benzoic Acid	y=0.238x	0.996	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=0.366x	0.998	0.08-25.78	0.26	0.77	0.002	0.006
	Hippuric Acid	y=0.107x	0.999	0.05-27.93	0.17	0.52	0.001	0.004
Aorta	Catechin	y=0.017x	0.998	0.03-17.22	0.86	2.75	0.014	0.044
	Epicatechin	y=0.051x	0.995	0.03-17.22	0.69	2.32	0.011	0.038
	Procyanidin dimer B2	y=0.048x	0.999	0.02-8.64	0.09	0.26	0.001	0.004
	Gallic Acid	y=0.168x	0.998	0.06-29.39	0.26	0.94	0.004	0.015
	Vanillic Acid	y=0.378x	0.997	0.06-29.76	0.60	2.15	0.010	0.035
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.305x	0.994	0.04-24.04	0.09	0.43	0.001	0.007
	3-(4-hydroxyphenyl) propionic acid	y=0.041x	0.997	0.06-30.12	0.17	0.60	0.003	0.010
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.017x	0.999	0.06-29.76	0.17	0.60	0.003	0.010
	2-(3-hydroxyphenyl) acetic acid	y=0.063x	0.999	0.06-32.90	0.17	0.60	0.003	0.010
	2-(4-hydroxyphenyl) acetic acid	y=0.038x	0.998	0.06-32.90	0.17	0.60	0.003	0.010
	3,4-dihydroxybenzoic acid	y=0.314x	0.999	0.06-36.23	0.17	0.60	0.003	0.010
	3-hydroxybenzoic acid	y=0.323x	0.999	0.08-36.23	0.26	0.77	0.004	0.013
	Benzoic Acid	y=0.239x	0.997	0.07-40.98	0.17	0.69	0.003	0.011
	Ferulic Acid	y=0.120x	0.995	0.08-25.78	0.26	0.77	0.004	0.013
	Hippuric Acid	y=0.556x	0.999	0.05-27.93	0.17	0.52	0.003	0.008
Brain	Catechin	y=0.012x	0.998	0.03-17.22	0.86	2.75	0.010	0.032
	Epicatechin	y=0.028x	0.993	0.03-17.22	0.69	2.32	0.008	0.027
	Procyanidin dimer B2	y=0.025x	0.994	0.02-8.64	0.09	0.26	0.001	0.003
	Gallic Acid	y=0.183x	0.997	0.06-29.39	0.26	0.94	0.003	0.011
	Vanillic Acid	y=0.264x	0.997	0.06-29.76	0.60	2.15	0.007	0.025
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.213x	0.999	0.04-24.04	0.09	0.43	0.001	0.005
	3-(4-hydroxyphenyl) propionic acid	y=0.032x	0.997	0.06-30.12	0.17	0.60	0.002	0.007
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.018x	0.993	0.06-29.76	0.17	0.60	0.002	0.007
	2-(3-hydroxyphenyl) acetic acid	y=0.052x	0.999	0.06-32.90	0.17	0.60	0.002	0.007
	2-(4-hydroxyphenyl) acetic acid	y=0.033x	0.996	0.06-32.90	0.17	0.60	0.002	0.007
	3,4-dihydroxybenzoic acid	y=0.359x	0.995	0.06-36.23	0.17	0.60	0.002	0.007
	3-hydroxybenzoic acid	y=0167x	0.998	0.08-36.23	0.26	0.77	0.003	0.009
	Benzoic Acid	y=0.167x	0.996	0.07-40.98	0.17	0.69	0.002	0.008
	Ferulic Acid	y=0.455x	0.999	0.08-25.78	0.26	0.77	0.003	0.009
	Hippuric Acid	y=0.117x	0.996	0.05-27.93	0.17	0.52	0.002	0.006

*MDL and MQL for the analysis of 60mg of tissue.

Abreviations: Determination coefficient (R^2); limit of detection (LOD); limit of quantification (LOQ); method detection limit (MDL); method quantification limit (MQL); mesenteric white adipose tissue (MWAT).

CHAPTER 3:

Internal factors affecting the flavanol metabolism

MANUSCRIPT 7:

Age related differences in the plasma kinetics of flavanols in rats

Maria Margalef[1*], Lisard Iglesias-Carres[1*], Zara Pons[1], Francisca Isabel Bravo[1], Begoña Muguerza[1,2*], Anna Arola-Arnal[1]

[1]Nutrigenomic Research Group. Biochemistry and Biotechnology Department. Rovira i Virgili University, Spain, [2]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

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Age related differences in the plasma kinetics of flavanols in rats

Maria Margalef[1*], Lisard Iglesias-Carres[1*], Zara Pons[1], Francisca Isabel Bravo[1], Begoña Muguerza[1,2#], Anna Arola-Arnal[1]

[1]Nutrigenomic Research Group. Biochemistry and Biotechnology Department. Rovira i Virgili University, Spain

[2]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

* These authors contributed equally to this work.

Running title: Age differences in flavanol plasma kinetics

#Dra. Begoña Muguerza Marquinez
Biochemistry and Biotechnology Department, Rovira i Virgili University, C/ Marcel·li Domingo s/n 43007 Tarragona, Spain.
Telephone: 34 977 55 9566
Fax: 34 977 55 8232
E-mail:begona.muguerza@urv.cat

Abstract

Dietary flavanols produce beneficial health effects, and once absorbed, they are recognized as xenobiotics and undergo phase-II enzymatic detoxification. However, flavanols with a degree of polymerisation greater than 2 reach the colon where they are subjected to microbial metabolism and can be further absorbed and suffer phase-II reactions. In this sense, flavanols health-promoting properties are mainly attributed to their metabolic products. Several age-related physiological changes have been evidenced and it is known that flavanols bioavailability is affected by internal factors. Therefore, this study aimed to elucidate whether animals of different age, young and elderly rats, exhibit differences in the flavanol metabolism and plasma bioavailability. To accomplish this aim, an acute dose of a grape seed polyphenol extract was administered to male rats and after 2, 4, 7, 24 and 48 h flavanols and their phase II and microbial metabolites were quantified by HPLC-ESI-MS/MS in plasma. Results indicated important age-related quantitative differences in plasma flavanol metabolites. Interestingly, elderly rats presented a remarkably reduction in flavanol absorption and phase-II flavanol metabolisation. Consequently, microbial-derived flavanol metabolism is triggered by higher flavanol affluence in the colonic tract. Furthermore, young rats presented a faster metabolic profile than elderly rats. Hence, our results indicate that the physiological bioactivities of flavanols may depend on age.

Keywords: Bioavailability, grape seed, metabolites, microbiota, polyphenols.

1. Introduction

Polyphenols are plant secondary metabolites present in the human diet and can be classified in two different groups, the flavonoids and the non-flavonoids. Including flavonols, flavonas, isoflavonas, flavanones, anthocyanidins, flavan-3-ols and dihydrochalcones, flavonoids are the most numerous polyphenols and can be found throughout the plant kingdom. Grapes are a flavonoid-rich food, containing the flavan-3-ols or flavanols (+)-catechin and (-)-epicatechin and their polymeric forms, proanthocyanidins (PA) (1). Flavanols present a wide range of biological activities. Indeed, our group has demonstrated that grape-seed flavanols exert an antioxidant effect (2), restore blood pressure (3), reduce several risk factors of cardiovascular diseases (4), improve lipid profile (5), protect against weigh gain (6), and reduce inflammation (7).

Flavanol-derived metabolites are responsible for most of the health beneficial effects reported (1). In this sense, dietary flavanols after their absorption are recognized as xenobiotics and undergo phase-II enzymatic detoxification at both the small intestine and liver. Methyl-, glucuronido- and sulfo-metabolites are formed due to the enzymatic activity of catechol-*O*-methyltransferase (COMT), uridine 5'-diphosphate glucoronosyltransferases (UGTs) and cytosolyc sulfotransferases (SULTs), respectively (8). It is worth to note that polymers greater than trimers are unlikely to be absorbed in the small intestine and reach the colon where they can undergo microbial biotransformation (8–10). Gut bacteria are able to hydrolyze flavanols into small molecular weight flavanol metabolites which can be also absorbed and reach different tissues (11) and therefore suffer phase-II reactions (8). Metabolised and no metabolised flavanols have been found to reach the kidney (11–13) and to be extracted via urine (14).

Several factors affect xenobiotic metabolism, including gender, physiopathological conditions and age (15). During ageing some of the

regulatory processes providing integration between cells and organs become disrupted. Consequently, failures in the maintenance of homeostasis under physiological stress appear (16). Several of these changes have pharmacokinetic implications (17). In addition, glomerular filtration rate (18), liver volume and apparent liver blood flow (19) decrease in ageing. Lee *et al*. not only reported that liver expression of xenobiotic metabolizing enzymes depended on the age, but also that several of these enzymatic activities did vary as well (20). Although few gastrointestinal functions decline to an important extent in healthy ageing (21), ageing leads to physiological changes that affect oral and esophageal function, gastric pH and intestinal transit times (22). In addition, major changes in bacterial population that include metabolically active groups occur (23). This could lead to important changes in the biochemical capacity of the gut. In addition, it should be highlighted that faecal studies demonstrated great variability in bacterial populations in the elderly (24). Moreover, body composition also changes during ageing. A loss of body weight, body cell mass, body water and a gain in body fat occur. Body weight loss has been strongly associated with lean mass loss (17). It should be highlighted that understanding the metabolism of flavanols in the elderly populations is of key importance as this target population can greatly benefit from the health effects of these compounds. Indeed, health benefits of flavanol consumption have been reported specifically in elderly populations (25,26). Moreover, polyphenols have been reported to exert beneficial functions against most of common age-related diseases such as type-II diabetes, cardiovascular diseases, Alzheimer, Parkinson, among others (1).

Despite we have shown that the flavanol metabolisation and bioavailability differs considerably depending on the experimental conditions like the model used, the time of treatment or the dosage (5,11), there are scare studies comparing different aged populations using polyphenols and more specifically flavanols (14). In addition, as flavanol health-promoting properties are mainly attributed to their metabolic products (1), we hypothesize that

flavanol metabolisation and absorption differ in youth and old age and that these differences could influence the physiological bioactivities of these compounds. Therefore, the aim of this study is to elucidate whether flavanols plasma kinetics are affected by age and if these compounds can be differently absorbed and metabolised in young and elderly rats.

2. Materials and Methods

2.1. Grape seed polyphenol extract

A grape seed polyphenol extract (GSPE) was obtained from *Les Dérives Résiniques et Terpéniques* (Dax, France). The total polyphenol, the individual flavanols and phenolic acids comprising this extract are detailed in Table 1.

2.2. Chemicals and reagents

Acetone (HPLC analytical grade), methanol (HPLC analytical grade), acetonitril (HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Ultrapure water was obtained from Milli-Q Advantage A10 system (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). (+)-catechin, (-)-epicatechin, benzoic acid, phloroglucinol, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, feluric acid, PA B2, epigallocatechin gallate (EGCG), pyrocatechol (internal standard, IS), all purchased from Fluka/Sigma-Aldrich (Madrid, Spain) and 5-(3',4'-dihydroxyphelyl)-γ-valerolactone, purchased from MicroCombiChem e.K. (Wiesbaden, Germany), were individually dissolved in methanol at the concentration of 2000mg/L. All standard stock solutions were prepared every 3 months and stored in dark-glass flasks at -20ºC.

A mixed standard stock solution in methanol at the concentration of 200mg/L of all of these compounds [(+)-catechin, (-)-epicatechin, benzoic acid,

phloroglucinol, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, feluric acid, EGCG and 5-(3',4'-dihydroxyphelyl)-γ-valerolactone] and proanthocyanidin B2 at the concentration of 100mg/L was prepared weekly and stored in dark-glass flasks at -20ºC. This stock solution was daily diluted to the desired concentration with acetone/Milli-Q water/acetic acid (70/29.5/0.5; v/v/v) and stored under the same conditions until chromatographic analysis.

2.3. Animal and Plasma collection

Young 10-week-old (n=6), weighing between 262 g and 288 g, and elderly 24-week-old (n=6) male Wistar rats, weighing between 482 g and 537 g, were obtained from Charles River Laboratories (Barcelona, Spain).. All of the animals were housed at 22ºC with a light/dark cycle of 12 h (lights on at 9:00 a.m.) and consumed tap water and a standard chow diet (AO4, Panlab, Barcelona, Spain) *ab libitum* during the experiment. Each young and elderly animals were randomly divided into 2 different sub-groups: the control group (n=1) and the GSPE group (n=5). A dosage of 1000 mg/Kg of GSPE (1mL in water) was administered to the GSPE group by oral gavage whilst water (1 mL) was orally administered to the control group. Oral administration was performed by gastric intubation to rats between 9 and 11 a.m. Blood samples were obtained via saphenous vein extraction using heparin vials (Starsted, Barcelona, Spain) at 0, 2, 4, 7, 24 and 48 h after GSPE or water administration (Figure 1). Plasma samples were obtained by centrifugation (2000 x g, 15 minutes, 4 ºC) and pooled (n=5) as a way to obtain sufficient volume for the analysis and also remove biological variability. Plasma samples were stored at -80 ºC until chromatographic analysis. The plasma from the control group (water oral gavage) was used to perform the calibration curves in the chromatographic analysis. Any compound present in the plasma GSPE group (0 h time-point) was subtracted from the plasma

concentrations at all other time-points. Plasma samples were not treated with glucuronidase or sulfatase enzymes. The study was performed in accordance with the institutional guidelines for care and use of laboratory animals, and the experimental procedures were approved by the Ethical Cometee for Animal Experimentationof Universitat Rovira i Virgili (reference number 283).

2.4. Micro-solid phase plasma flavanol extraction

Prior to chromatographic analysis, plasma pools (n=5) for each time-point were pre-treated by off-line micro-Solid Phase Extraction (µ-SPE) as described previously (10) using OASIS HLB µ-Elution Plates 30 µm (Waters, Barcelona, Spain). Briefly, the micro-cartridges were sequentially conditioned with 250 µL methanol and 250 µL 0.2 % acetic acid. Plasma aliquots (250 µL) were mixed with 300 µL 4 % phosphoric acid and 50 µL IS (250 ppb) before being loaded into the plates. The loaded plates were washed with 200µL Milli-Q water and 200 µL 0.2 % acetic acid. The retained flavanols and their metabolites were eluted with 2 x 50 µL acetone/Milli-Q water/acetic acid solution (70/29.5/0.5; v/v/v). The eluted solution was directly injected into the HPLC-MS/MS.

2.5. Chromatographic analysis

The eluted solutions were directly analysed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Thechnologies, Palo Alto, U.S.A.) as previously described (10). Briefly, Zorbax SE-aq (150 x 2.1 mm i.d., 3.5 µm particle size, Aglient Technologies) was the used chromatographic column. The mobile phase used for the separation of the flavanols was composed of 0.2 % acetic acid (solvent A) and acetonitrile (solvent B) in a gradient mode set as follows: initial conditions 5 % B; 0-10 min, 5-55 % B; 10-12min, 55-88 % B; 12-15 min, 80 % B isocratic; and 15-16 min, 80-5 % B. A post-run of 10min was applied for column equilibration. 2.5 µL of sample were injected and flow rate

of 0.4 mL/min was fixed for all the runs. Electrospray ionization (ESI) was conducted at 350 ºC and 12 L/min with 45 psi of nebuliser gas pressure, and 4000 V of capillary voltage. The mass spectrometer was operated in the negative mode and MS/MAS data were acquired in Multiple Reaction Monitoring (MRM) mode. Optimised MRM conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS can be found elsewhere (10,11).

2.6. Sample quantification

For sample quantification, plasma from the control group (water) was spiked with 7 different concentrations of the standard compounds to obtain calibration curves. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. All quality parameters required to perform the analysis are presented in Table 2. Data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, U.S.A.).

3. Results

In this study, no metabolised flavanols, phase-II flavanol metabolites and microbial-derived flavanols present in pooled (n=5) rat plasma 0, 2, 4, 7, 24 and 48 h after GSPE (1000 mg/Kg) ingestion were quantified using HPLC-ESI-MS/MS. Pooling the plasma was required to obtain sufficient volume to perform the chromatographic analyses and extract blood from the animals at different times while avoiding their sacrifice. In addition, pooling the plasma increases homogeneity and sensitivity which, in turn, allows the detection of all potential metabolites as we have previously realized (9). A range of time-points was selected in accordance with the literature in order to detect no metabolised flavanols and their phase-II and microbial metabolites (9,11).

Figure 2 shows the plasma kinetic behaviour of total flavanols, no metabolised flavanols, phase-II metabolites and microbial-derived

compounds after an acute dosage of GSPE (1000 mg/Kg) in elderly and young rat and Figure 3 shows their relative abundance in plasma at different times (2, 4, 7, 24 and 48 h). Detailed individual concentrations of these compounds can be found in Table 3 and Table 4. This data provides insights into how flavanols are metabolised depending on the rat age.

3.1. Total flavanols

The highest total flavanol plasma concentration was reached 2 h after GSPE (1000 mg/Kg) administration in young and elderly rats (Figure 2A), and interestingly young rats (218.21 μM) have more than 3 time more concentration than elderly rats (65.09 μM) in plasma at this time-point (Figure 3). Nevertheless, young rats presented a more rapid decrease in total flavanol plasma concentration, being more gradual the one reported in elderly rats (Figure 2A). In this sense, whilst during the first time-points of the kinetic study (i.e. 2, 4 and 7 h) young rats presented a remarkably higher total flavanol concentration, higher concentrations were found in elderly rats at 24 h (42.53 μM) and 48 h (14.35 μM) when compared to young rats (4.92 μM and 4.93 μM respectively) after GSPE administration (Figure 3).

3.2. No metabolised flavanols

Both young and elderly rats presented the highest concentration of no metabolised flavanols in plasma at 2 h after GSPE dosage (Figure 2B). The concentrations reached at this time-point were 4.31 μM for young rats and 12.20 μM for elderly rats, being all compounds but vanillic acid more concentrated in elderly rat than in young (Table 3). Epicatechin and PA dimer B2 were the major compounds detected in young and elderly rats, respectively. Generally speaking, most compounds concentrations decreased during the kinetic study, but catechin, epicatechin and gallic acid increased at 48 h after GSPE dosage in young rats. With regards to their relative abundance, no metabolised compounds represented a small percentage in young rats until 24 h after GSPE administration time when a higher percentage was found in young rat plasma (Figure 3). Interestingly,

their plasma behaviour in elderly rats was different, representing higher amounts at 2 h (19% of 65.09 μM) and 4 h (13% of 65.09 μM) and scarce levels at 7, 24 and 48 h after GSPE administration (Figure 3). Thus, the plasma kinetic profile of no metabolised compounds in young and elderly rats also differs in its reduction rate. However, whilst elderly rats presented a rapid decrease in no metabolised flavanols 2 h after the GSPE dosage, the reduction in young rats was not as sharp.

3.3. Phase-II flavanol metabolites

The highest plasma concentration of phase-II flavanol metabolites was also reached 2 h after the GSPE dosage (1000 mg/Kg), being higher in young rats (209.78 μM) than in elderly rats (32.82 μM) (Figure 2C). Catechin-glucuronide, representing a plasma concentration of 118.528 μM in young rats and 15.358 μM in elderly rats at 2 h, was the compound with the highest plasma concentrations during all the kinetic study in both groups of rats (Table 3). Also, the most representative compounds at all times and in both groups were the glucuronide and methyl-glucuronide forms. Compared with them, few sulfated, methyl-sulfated and methylated forms were detected. Despite that, elderly rats present a wider variety of phase-II flavanol metabolites at 2 and 4 h after GSPE dosage than young rats and at 7 h, only glucuronidated and methyl-glucuronidated metabolites are quantified in elderly rats, whereas a wider range of compounds are detected in young rats at 7 h. Also, phase-II plasma kinetic behaviour is very similar in both cases, presenting a steady decrease 2 h after GSPE administration. It is worth noting, though, that total phase-II flavanol concentration has been found to be lower in young rats only after 24 h after GSPE dosage. With regards to its relative abundance, until 7 h after GSPE dosage phase-II flavanol metabolites represented more than 95 % in young rats and more than 45 % of the quantified compounds in elderly rats and lower percentages are observed 7 h after GSPE dosage in both groups (Figure 3).

3.4. Microbial-derived flavanol metabolites

Total microbial-derived compound were found to be more concentrated at all time-points in elderly rats (Figure 2D) and several differences were evidenced in individual microbial metabolites in plasma between ages (Table 4). Also, the kinetic profile was different between young and elderly rats. In this sense, whilst after 7 h after GSPE dosage microbial-derived flavanols increased their concentration in young rats plasma, their concentration dropped 24 h after in elderly rats plasma. In reference to their relative abundance in young rats, microbial-derived metabolites did not represent a high percentage of the total compounds quantified in plasma until 24 h after GSPE dosage (59 % of 4.92 μM at 24 h and 77 % of 4.93 μM at 48 h) (Figure 3). In contrast, microbial-derived flavanols quantified in elderly rat plasma represent a high percentage of total compounds at all times, reaching their higher percentage at 24 h (76 % of 42.53 μM).

4. Discussion

Flavanols are some of the most abundant dietary polyphenols and exert several biological functions (2–6). Once ingested, these compounds are absorbed in the small intestine and undergo phase-II reactions in the small intestine and liver, generating methyl, sulfate and glucuronide derivates (8). Polymeric forms, unable to be absorbed at this level, reach the colon where they are subjected to microbial biotransformation. The smaller compounds formed can be absorbed via colonocytes and later undergo phase-II reactions (8) to be eliminated by the urine (14). In this sense, the beneficial effects of flavanols are mainly attributed to their metabolized-derived compounds (1) and their metabolism and absorption are known to be affected by multiple factors (5,11). Several are the studies focused on the kinetic behavior of flavanols (9,13,12) but scarce are the ones comparing their behavior within different ages (14), although one of the factors affecting xenobiotic metabolism is ageing (15). Hence, in this study we aim to evaluate whether flavanols can be differently absorbed and metabolized depending on

the age. For this, we compared the pharmacokinetic behavior between young and early rats that were acutely administered grape seed flavanols. Based on previous studies, a dose of 1000 mg/kg was selected for evaluation in acute bioavailability studies following flavanol ingestion in rats (9,27). However, although we have previously reported that this high dose leads to saturation of the system (11,28), it is still a valid dose for comparing differences in young and elderly rats pharmacokinetics since it leads to concentrations of flavanols that are high enough to enable the detection of clear differences between the groups.

Flavanol concentrations were analyzed in plasma before and at 2, 4, 7, 24 and 48 h after an acute administration of GSPE and in both groups of rats the total flavanols, the unconjugated forms and their phase II metabolites have maximum concentrations after 2h indicating no differences in the time point of maximum absorption between ages. Our results are in agreement with many studies (9,12,13,29) that described that these compounds appear in plasma shortly after ingestion, with maximum concentrations of phase-II metabolites being reached between 1 and 2 h, and significant decrease at 4 h after ingestion of flavanols (12,13,30).

Interestingly, the total flavanol concentration in elderly rats was about 3 times lower than young rats at short times (i.e. 2-7 h). Therefore, bearing in mind that oral dosage of GSPE was equal in both groups of rats and that no significant changes are detected in stomach emptiness and small intestine rates depending on age (31), our results seem to indicate intestinal absorption is reduced in elderly animals. Indeed, the motility patterns of the small intestine are maintained during the ageing process (32), whilst paracellular and transcellular transport decreases during ageing (33), facts that also seem to support our results. This low flavanol absorption in old age is in agreement with the fact that elderly rats have a considerable amount of colonic flavanol metabolites at short times after GSPE ingestion (i.e. 2 h).

Regarding to the kinetic profile, the results presented herein clearly evidenced that the phase-II flavanol metabolites in young rats disappeared in plasma much faster than the elderly rats, although the non-metabolised flavanols showed a lighter decrease in plasmas compared with the elderly rats. The differences in decreased concentration rate between ages could be attributed to both decreased renal clearance rate associated with ageing (17) and a higher flavanol income due to reduced small intestine absorption.

In the elderly rats the phase II metabolism is also poorer than young rats as the concentrations of unconjugated flavanols at 2 h is higher than for young rats and at 7 h it seems that elderly rats have all the flavanols metabolised as there are no free flavanols in plasma. A reduction in both phase-II metabolising enzyme expression and activity related to age (20) could potentially be the responsible for this. Moreover, considering that at old age the system is normally more injured, these results also agree with Perez-Viscaino *et al.*, who hypothesized that unconjugated flavanols are responsible for the flavanol health benefits, being unconjugated in the target tissue (34).

Elderly rats showed to have much higher concentrations of microbial metabolites than young rats at all times event at 2 h after GSPE ingestion, indicating a remarkable difference in the microbial metabolism related to age. As indicated before, due to reduced absorption at the small intestine, more compounds are to reach the colon tract, where they are to be metabolised by the microbiota, resulting into higher formation of this compounds at all times of the pharmakokinetic study. In addition, elderly populations have been reported to present a slower colonic transit time (31), lower renal clearance rates for different drugs (17). Moreover, the wider variety of compounds detected in the elderly rats could be attributed to the fact that microflora composition is more variable in elderly subjects (24).

In conclusion, this study demonstrates that elderly rats not only presented a reduced absorption at the small intestine, but also a reduction in phase-II metabolism of flavanols after GSPE ingestion. As a result of decreased absorption, higher amounts of microbial-derived metabolites are found in elderly rats compared to young rats. Furthermore, young rats present a faster metabolic profile than elderly rats. Therefore, the physiological bioactivities of flavanols may depend on the age, and hence this factor should be considered when investigating flavanol compounds *in animal* and clinical studies.

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Figure Legends

Figure 1. Extracted ion chromatograms of flavanols and their phase-II metabolites 2 h after the administration of 1000 mg/kg of a grape seed polyphenol extract (GSPE) in male (continuous line) and female (discontinuous line) rat plasma. (1) Gallic acid, (2) Dimer B1, (3) Dimer B3, (4) Dimer B2, (5) Methyl-catechin-glucuronide, (6) Methyl-epicatechin-glucuronide, (7) Catechin glucuronide, (8) Epicatechin glucuronide, (9) Catechin, (10) Epicatechin, (11) Catechin sulfate, (12) Epicatechin sulfate (13) Methyl-catechin-O-sulfate, (14) Methyl-epicatechin-O-sulfate, (15) 3-Methyl-epicatechin, and (16) 4-Methyl-epicatechin.

Figure 2. Pharmacokinetic profiles of flavanols and their metabolites in rat plasma and tissues after acute ingestion of grape seed polyphenol extract (GSPE) in both male (left panels) and female (right panels) rats. (A) Plasma. (B) Liver. (C) Mesenteric white adipose tissue (MWAT). (D) Brain. Data are displayed as the mean ± standard error of the mean (SEM) (n=6). The results are expressed in μM for plasma samples and in nmol/g for the studied tissue samples.

Figure 3. Distributions of flavanols (catechin, epicatechin and PA dimers) and their phase-II metabolites, as quantified by HPLC-ESI-MS/MS in rat plasma and tissues at 2 h after the ingestion of 1000 mg/kg of grape seed polyphenol extract (GSPE) in both male (left panels) and female (right panels) rats. (A) Plasma. (B) Liver. (C) Mesenteric white adipose tissue (MWAT). (D) Brain. Data are displayed as the mean (n=6) and expressed as percentages.

Table 1. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1[1]	88.80 ± 3.46
PA dimer B3[1]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[1]	8.86 ± 0.14
Epicatechingallate	21.24 ± 1.08
Epigallocatechingallate	0.03 ± 0.00
Epigallocatechin[2]	0.27 ± 0.03
PA trimer[1]	4.90 ± 0.47
PA tetramer[1]	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

[1] Quantified using the calibration curve of proanthocyanidin B2.

[2] Quantified using the calibration curve of epigallocatechingallte.

Table 2. The calibration curve, determination coefficient (R^2), working linearity range, LODs, LOQs, MDLs and MQLs for phenolic compound quantification in spiked plasma samples using HPLC-ESI-MS/MS.

Compound	Calibration Curve	R^2	Linearity (µM)	LOD (nM)	LOQ (nM)	MDL* (nM)	MQL* (nM)
Catechin	y=0.010x	0.991	0.007 - 17.255	6.890	22.967	2.756	9.187
Epicatechin	y=0.030x	0.995	0.007 - 17.255	4.698	15.659	1.879	6.264
PA dimer B2	y=0.022x	0.997	0.003 - 8.643	0.648	2.161	0.259	0.864
Gallic acid	y=0.199x	0.994	0.012 - 29.391	1.102	3.674	0.441	1.470
Vanillic acid	y=0.313x	0.996	0.012 - 29.762	0.116	0.387	0.046	0.155
EGCG	y=0.301x	0.995	0.004 - 10.908	1.608	5.358	0.643	2.143
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.324x	0.990	0.010 - 24.038	0.114	0.381	0.046	0.152
2-(3,4-dihydroxyphenyl)acetic acid	y=0.011x	0.995	0.012 - 29.762	0.116	0.387	0.046	0.155
3-(4-hydroxyphenyl)propionic acid	y=0.055x	0.995	0.012 - 30.120	0.888	2.959	0.355	1.184
2-(3-hydroxyphenyl)acetic acid	y=0.061x	0.990	0.013 - 32.895	0.293	0.976	0.117	0.390
2-(4-hydroxyphenyl)acetic acid	y=0.085x	0.991	0.013 - 32.895	1.715	5.715	0.686	2.286
3,4-dihydroxybenzoic acid	y=0.228x	0.997	0.016 - 40.323	0.916	3.053	0.366	1.221
3-hydroxybenzoic acid	y=0.242x	0.999	0.014 - 36.232	0.539	1.797	0.216	0.719
Benzoic acid	y=0.240x	0.997	0.016 - 40.984	0.277	0.924	0.111	0.370
Ferulic acid	y=0.311x	0.998	0.010 - 25.773	0.336	1.119	0.134	0.448
Hippuric acid	y=0.122x	0.998	0.011 - 27.933	0.018	0.059	0.007	0.024
Phloroglucinol	y=0.011x	0.999	0.016 - 39.683	0.366	1.220	0.146	0.488

* MDL and MQL for the analysis of 250 µL of plasma.

Abbreviations: Determination coefficient (R2); limit of detection (LOD); limit of quantification (LOQ); method detection limit (MDL); method quantification limit (MQL); proanthocyanidin (PA).

Table 3. Flavanols and their phase-II metabolites quantified in pooled rat plasma at 2, 4, 7, 24 and 48 h after the ingestion of an acute dose of a grape seed polyphenol extract (1000 mg/Kg) by HPLC-ESI-MS/MS.

Compound	Total amount (μM)									
	Young					Elderly				
	2h	4h	7h	24h	48h	2h	4h	7h	24h	48h
Catechin	0.799	0.799	0.213	n.d.	0.320	1.623	0.919	n.d.	n.d.	n.d.
Epicatechin	1.357	0.528	0.462	n.d.	0.146	1.566	0.393	n.d.	n.d.	n.d.
PA dimer B1[3]	0.098	0.121	0.088	n.d.	n.d.	2.278	0.523	n.d.	n.d.	n.d.
PA dimer B2	0.672	0.275	0.191	n.d.	n.d.	3.113	1.163	n.d.	n.d.	n.d.
PA dimer B3[3]	0.095	0.136	0.095	n.d.	n.d.	1.343	0.692	n.d.	n.d.	n.d.
Gallic acid	0.002	0.507	0.230	0.013	0.096	1.475	1.661	0.174	0.187	0.129
Vanillic acid	1.283	0.941	0.691	0.471	0.392	0.803	0.465	n.q.	n.q.	n.q.
Metabolite										
Catechin-glucuronide[1]	118.528	87.122	52.897	0.450	0.066	15.358	12.870	11.082	7.522	3.652
Epicatechin-glucuronide[2]	46.484	33.361	19.757	0.202	0.018	4.403	4.589	3.694	2.279	1.008
Methyl-catechin-glucuronide[1]	30.607	28.799	22.247	0.539	0.089	1.277	1.022	0.770	n.d.	n.d.
Methyl-epicatechin-glucuronide[2]	7.068	7.933	6.170	0.262	0.027	3.973	3.102	1.646	0.261	n.d.
Catechin-sulfate[1]	1.527	n.q.	0.196	n.d.	n.d.	1.457	0.722	n.d.	n.d.	n.d.
Epicatechin-sulfate[2]	0.191	n.q.	0.039	n.d.	n.d.	0.225	0.088	n.d.	n.d.	n.d.
Methyl-catechin-*O*-sulfate[1]	2.035	n.q	0.502	n.q.	n.d.	3.072	1.874	n.d.	n.d.	n.d.
Methyl-epicatechin-*O*-sulfate[2]	1.950	1.853	0.785	0.026	n.d.	1.135	0.713	n.d.	n.d.	n.d.
3-*O*-methyl-epicatechin[2]	0.572	n.q.	n.q.	n.q.	n.d.	0.691	0.375	n.d.	n.d.	n.d.
4-*O*-methyl-epicatechin[2]	0.818	n.q.	0.223	0.030	n.d.	1.229	0.780	n.d.	n.d.	n.d.

Abbreviations: proanthocyanidin (PA); not detected (n.d.); not quantified (n.q.); epigallocatechin gallate (EGCG).
Compounds not detected or quantified at any time: epicatechin gallate; dimer gallate; trimer; EGCG
[1] Quantified using the calibration curve of catechin.
[2] Quantified using the calibration curve of epicatechin.
[3] Quantified using the calibration curve of proanthocyanidin dimer B2.

Table 4. Microbial-derived flavanol metabolites quantified in pooled rat plasma at 2, 4, 7, 24 and 48 h after the ingestion of an acute dose of a grape seed polyphenol extract (1000 mg/Kg) by HPLC-ESI-MS/MS.

Compound	Total amount (μM)									
	Young					Elderly				
	2h	4h	7h	24h	48h	2h	4h	7h	24h	48h
1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol[1]	0.001	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-hydroxy-5-phenylvaleric acid[1]	0.002	0.011	n.q.	0.310	0.028	0.049	0.156	0.254	0.054	n.q.
3-(3,4-dihydroxyphenyl)propionic acid[2]	0.022	n.q.	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.
3-(4-hydroxyphenyl)propionic acid	n.q.	n.q.	n.q.	n.q.	n.q.	2.714	3.004	5.488	26.440	2.674
3-(3-hydroxyphenyl)propionic acid[2]	0.021	0.020	0.006	n.q.	n.q	n.q.	n.d.	n.d.	n.d.	n.q.
3-phenylpropionic acid[2]	1.108	0.443	0.282	0.999	0.773	n.q.	0.317	1.642	2.518	1.658
2-(3,4-dihydroxyphenyl)acetic acid[3]	0.016	n.q.	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.
2-(3-hydroxyphenyl)acetic acid	n.q.	n.q.	n.q.	n.q.	n.q.	0.010	0.191	0.441	0.291	n.q
2-(4-hydroxyphenyl)acetic acid	0.015	n.q.	n.q.	0.056	0.082	5.021	0.190	0.203	0.146	n.q
2-phenylacetic acid[3]	n.d.	0.088	0.003	n.q.	0.063	n.d.	n.d.	n.d.	n.d.	n.d.
3,4-dihydroxybenzoic acid	n.q.	n.q.	n.q.	n.q.	n.q.	0.137	0.051	0.046	0.041	0.019
3-hydroxybenzoic acid	n.q.	n.q.	n.q.	n.q.	n.q.	0.312	0.294	0.255	0.080	0.085
Benzoic acid	0.842	0.735	0.586	0.657	0.770	1.898	0.758	0.814	n.q	n.q
3-*O*-methylgallic acid[4]	1.021	1.321	0.680	0.024	0.000	5.115	0.526	0.174	n.d.	n.d.
Homovanillic acid[5]	0.076	0.315	n.q.	n.q.	0.159	0.235	0.228	0.213	0.201	0.168
Homovanillyl alcohol[5]	0.068	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic acid	0.003	0.005	0.013	0.026	0.064	0.044	0.105	0.080	0.032	0.002
Hippuric acid	0.868	n.q.	n.q.	0.860	1.410	4.537	6.623	9.770	2.481	4.955

Abbreviations: n.d. = not detected; n.q. = not quantified.
Compounds not detected or quantified at any time: 5-(3',4'-dihydroxyphenyl)-γ-valerolactone; 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone; 4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid; 4-hydroxybenzoic acid; and phloroglucinol.
[1] Quantified using the calibration curve of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone.
[2] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid.
[3] Quantified using the calibration curve of 2-(4-hydroxyphenyl)acetic acid.
[4] Quantified using the calibration curve of gallic acid.
[5] Quantified using the calibration curve of vanillic acid.

Figure 1.

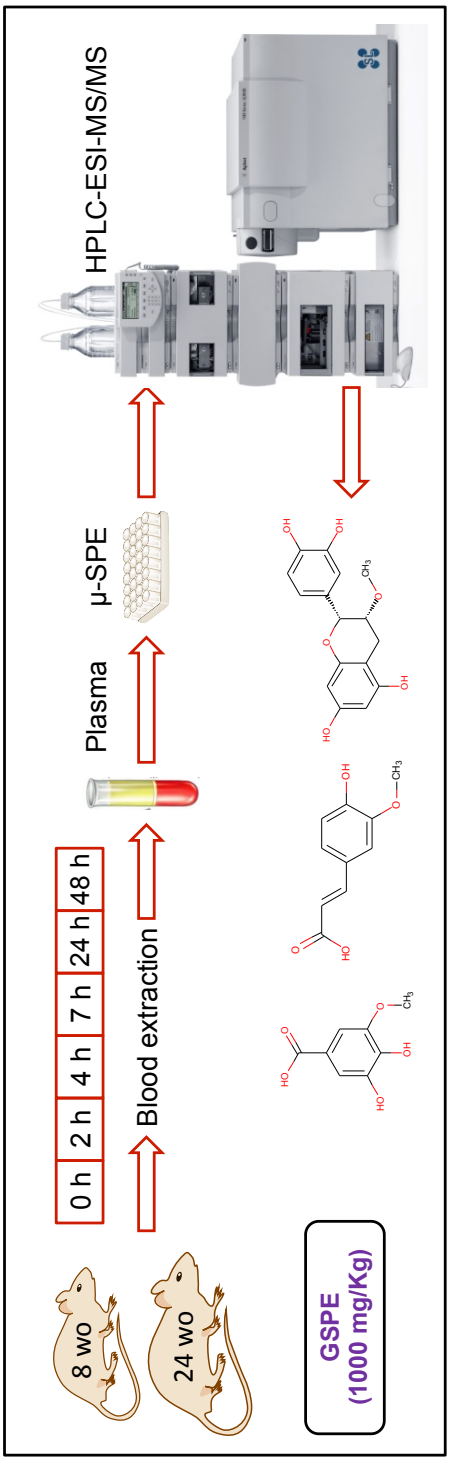

HPLC-ESI-MS/MS
μ-SPE
Plasma
Blood extraction
0 h
2 h
4 h
7 h
24 h
48 h
8 wo
24 wo
GSPE
(1000 mg/Kg)

Figure 2.

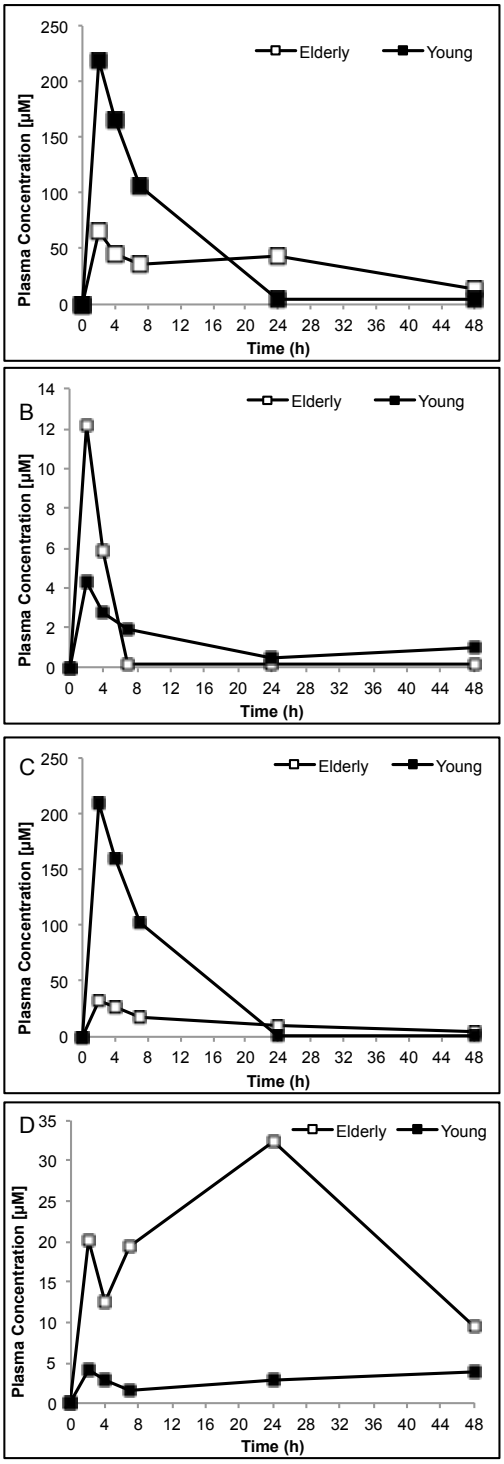
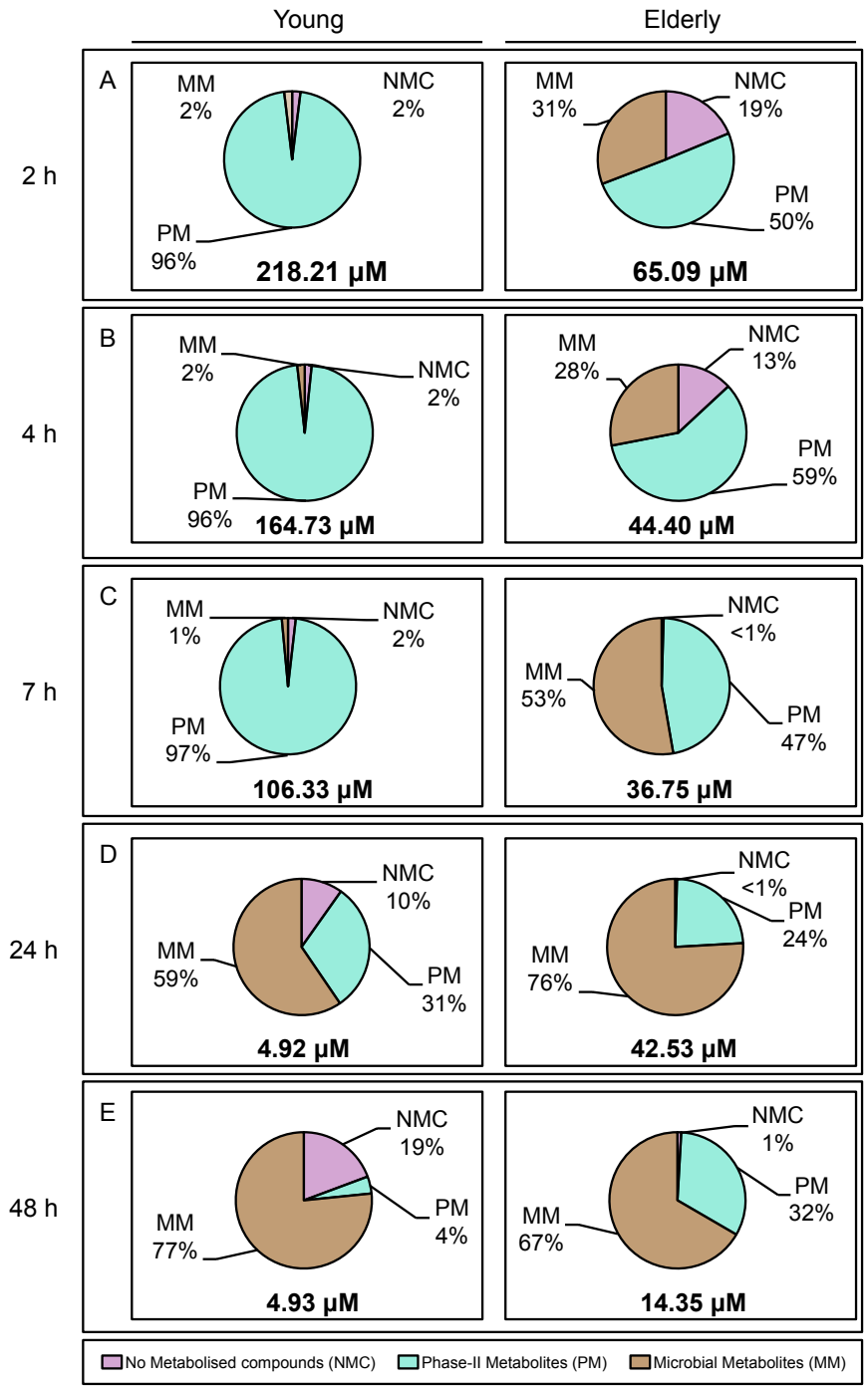


Figure 3.


Young
Elderly
2 h
4 h
7 h
24 h
48 h
A
MM 2%
NMC 2%
PM 96%
218.21 µM
MM 31%
NMC 19%
PM 50%
65.09 µM
B
MM 2%
NMC 2%
PM 96%
164.73 µM
MM 28%
NMC 13%
PM 59%
44.40 µM
C
MM 1%
NMC 2%
PM 97%
106.33 µM
NMC <1%
MM 53%
PM 47%
36.75 µM
D
NMC 10%
MM 59%
PM 31%
4.92 µM
NMC <1%
PM 24%
MM 76%
42.53 µM
E
NMC 19%
MM 77%
PM 4%
4.93 µM
NMC 1%
PM 32%
MM 67%
14.35 µM
No Metabolised compounds (NMC)
Phase-II Metabolites (PM)
Microbial Metabolites (MM)

MANUSCRIPT 8:

Gender related differences in the body distribution of flavanols in rats

Maria Margalef[1*], Lisard Iglesias-Carres[1*], Zara Pons[1], Begoña Muguerza[1,2*], Anna Arola-Arnal[1]

[1]Nutrigenomic Research Group. Biochemistry and Biotechnology Department. Rovira i Virgili University, Spain, [2]Technological Center of Nutrition and Health (CTNS), TECNIO, CEICS, Reus, Spain

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Gender related differences in the body distribution of flavanols in rats

Maria Margalef[a], Zara Pons[a], Lisard Iglesias-Carres[a], Lluís Arola[ab], Begoña Muguerza[ab*], and Anna Arola-Arnal[a].

[a] Nutrigenomic group, Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona, 43007 Spain

[b] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

Running title: Sex differences in flavanol bioavailability

Corresponding author:

*Dra. Begoña Muguerza

Nutrigenomic research group, Department of Biochemistry and Biotechnology, University Rovira i Virgili,

C/ Marcel.li Domingo s/n 43007 Tarragona, Spain

Telephone: +34 977 55 95 66

Fax: +34 977 55 82 32

E-mail: begona.muguerza@urv.cat

Abstract

Dietary flavanols produce beneficial health effects, and once absorbed, they are recognized as xenobiotics and undergo phase-II enzymatic detoxification. Flavanols health-promoting properties are mainly attributed to their metabolic products. This work aimed to elucidate whether rats of the opposite sex exhibited differences in the metabolism and distribution of ingested flavanols. To accomplish this aim, acute doses of grape seed polyphenols were administered to male and female rats. After 1, 2 and 4 h, plasma, liver, mesenteric white adipose tissue (MWAT), brain and hypothalamus flavanol metabolites were quantified by HPLC-MS/MS. Results indicated important sex-related quantitative differences in plasma and in brain. Moreover, remarkable sex-related differences in the distributions and types of flavanol metabolites were also observed between liver and brain. Therefore, this study demonstrated that sex differentially influences the metabolism and distribution of flavanols throughout the bodies of rats, which may affect the physiological bioactivities of flavanols between males and females.

Chemical compounds studied in this article

(+)-Catechin (PubChem CID: 9064); (-)-Epicatechin (PubChem CID: 72276); Epicatechin gallate (PubChem CID: 367141); Gallic acid (PubChem CID: 370); Procyanidin B2 (PubChem CID: 122738); Protocatechuic acid (PubChem CID: 72); Vanillic acid (PubChem CID: 8468)

Keywords: bioavailability; grape seed; metabolites; polyphenol; sex

1. Introduction

Polyphenols are among the most abundant phytochemicals that are present in the human diet, and the flavanoid-type flavan-3-ols, or flavanols, are one of the primary types of polyphenol that are ingested by humans. Flavanols are mainly found in grapes, beans, nuts, cocoa, tea and wine. Flavanols range from the monomers (+)-catechin and (-)-epicatechin to the oligomeric and polymeric proanthocyanidins (PAs) (Bladé, Arola, & Salvadó, 2010). Increasing evidence has indicated the important health-promoting effects of flavonoids (Del Rio et al., 2013). Specifically, grape seed flavanols exhibit antioxidant and anti-inflammatory capacities (Terra et al., 2011), improve lipid metabolism (Guerrero et al., 2013), increase insulin secretion (González-Abuín et al., 2014) and act as antihypertensive agents (Quiñones et al., 2013).

The beneficial health properties of flavanols are mainly attributed to the compounds that are derived from their metabolism (Del Rio et al., 2013). Hence, the absorption and tissue distribution of flavanol metabolites should bear a close relationship to their biological functions and beneficial health effects. These compounds have been shown to be recognized by the body as xenobiotics and to undergo phase-II enzymatic detoxification in the small intestine and liver, leading to the formation of sulfo-, methyl- or glucuronide-conjugates after their absorption. These metabolites can enter systemic circulation to be transported to other tissues or to be excreted by the urine (Monagas et al., 2010). Whereas monomeric and low molecular-weight forms are primarily absorbed through the small intestine, oligomers cross the gastrointestinal tract and reach the colon, where they are transformed by intestinal microbiota to either be absorbed or excreted (Aura, 2008; Monagas et al., 2010).

Recently, we have shown that differences in experimental conditions, such as flavanol dosage, affect the metabolization and bodily distribution of flavanol metabolites (Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015). Moreover, several studies have demonstrated that the beneficial effects of

flavanols that are observed under laboratory conditions are dependent on several experimental factors, such as the model used (*in vitro* or *in vivo* models), the time of treatment or the administered dose of the flavanol extract (Guerrero et al., 2013; Kay, 2010; Kroon et al., 2004; Margalef, Guerrero, et al., 2014; Z Pons et al., 2014). Furthermore, numerous sex-related differences in both humans and other mammals have been shown in processes such as lipid and glucose metabolism (Varlamov, Bethea, & Roberts, 2014), in psychiatric disorders (Harrison & Tunbridge, 2007) and in coronary artery disease (Yahagi, Davis, Arbustini, & Virmani, 2015). There are also sex-related differences in susceptibility to inflammatory and infectious diseases (Liu et al., 2003) and in the level of protective health benefits that are imparted by drinking moderate amounts of alcohol (Taylor et al., 2009). Furthermore, it has been observed that responses to xenobiotics are different between genders. For example, male rats have been observed to more quickly metabolize xenobiotics and to have higher phase-II detoxification enzyme activities compared to female rats (DeBethizy & Hayes, 1994). Additionally, female rats are known to have less cytochrome P450 (CYP), which facilitates the detoxification and excretion of xenobiotics (phase-I metabolism), than male rats (Mugford & Kedderis, 1998).

Therefore, because flavanols are recognized as xenobiotics by the body, we hypothesize that the metabolism and subsequent tissue distribution of flavanols is different between male and female rats and that these properties may also differentially influence the physiological bioactivities of these compounds between males and females. Therefore, the aim of this study was to elucidate whether flavanols can be differentially absorbed, conjugated and distributed throughout the bodies of rats of opposite sexes.

2. Matherials and methods

2.1. Chemicals and reagents

Methanol (Scharlab S.L., Barcelona, Spain), acetone (Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10

system (Madrid, Spain). Phosphoric acid (98%) and ascorbic acid were also purchased from Sigma-Aldrich (Madrid, Spain). Individual, standard stock solutions of 2000 mg/L (+)-catechin, (-)-epicatechin, epigallocatechin gallate (EGCG), gallic acid, vanillic acid, PA B2, and, pyrocatechol as internal standard (all from Fluka/Sigma-Aldrich, Madrid, Spain, except for PA B2, which was from Extrasynthese, Lyon, France) were prepared in methanol and stored in dark-glass flasks at -20 ºC.

Standard 20 mg/L stock mixtures of (+)-catechin, (-)-epicatechin, EGCG, gallic acid, vanillic acid and PA B2 in methanol were prepared weekly and stored at -20 ºC. These solutions were diluted daily to the desired concentrations using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.2. Grape seed polyphenol extract

Grape seed polyphenol extract (GSPE) was obtained from white grape seeds and was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the PA profile of the extract was composed of monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%) of PAs. Chromatographic separation, using a 1290 LC Series, was performed for the quantification of phenolic compounds (flavan-3-ols and phenolic acids). The separations were achieved using a Zorbax SB-Aq (150 mm x 2.1 mm i.d., 3.5 µm particle size) chromatographic column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The elution gradient was as follows: 0-10 min, 5-55% B; 10-12 min, 55-80% B; 12-15 min, 80% B isocratic; 15-16 min 80-5% B. A post run of 10 min was applied. Quantification was performed by coupling the LC system to a 6490 MS/MS (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) conditions included a drying gas temperature of 200 ºC and a flow rate of 14 L/min, 20 psi of nebulizer gas pressure, and 3000 V of capillary voltage. The MS/MS was operated in negative mode, and the acquisition was performed in

Multiple Reaction Monitoring (MRM) mode for all of the phenolic compounds (Table 1). Data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

2.3. Experimental procedures in rats

Male (n=20) and female Wistar rats (n=20) that were 8-10 weeks old and weighed 280-320 g and 190-220 g, respectively, were used for this study. The animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 ºC with 12 h light/dark cycles (light from 9:00 a.m. to 21:00 p.m.). Rats consumed tap water and a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum*. On the day of the experiment, 1000 mg/kg of GSPE was administered to each rat by oral gavage, which was applied to both male (n=18) and female rats (n=18). The rats were divided according to sex into three different groups (n=6) depending on the time of sacrifice (1, 2, or 4 h after GSPE administration). In all groups, oral administration occurred between 9 and 10 am after overnight fasting, and the total orally administered volume per animal was always 1 mL of a GSPE-water solution. Livers, mesenteric white adipose tissues (MWAT), brains and hypothalami were excised from all of the rats and were freeze-dried for later extraction of flavanols and flavanol metabolites. Plasma samples were obtained by centrifuging blood samples (2000 × *g*, 15 min, 4 °C) in Sarstedt heparinized tubes (16 I.U.) (Barcelona, Spain). Dried tissues and plasma samples were stored at -80ºC. Additionally, 1 mL of tap water was administered via oral gavage to each of 2 extra male and 2 extra female rats to obtain blank samples as controls and blank matrix for calibration curves. All procedures were performed in accordance with the guidelines for care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain, permission number 282).

2.4. Extraction of flavanols and flavanol metabolites from plasma

Prior to performing chromatographic analyses of flavanols and flavanol metabolites in rat plasma, the samples were pretreated using previously reported methodology that is based on a micro solid-phase extraction (μSPE)

(Margalef, Pons, Muguerza, & Arola-Arnal, 2014). The plasma samples were cleaned up by µSPE using 30 µm OASIS HLB µ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were sequentially conditioned with 250 µL of methanol and 250 µL of 0.2% acetic acid. Following this, 300 µL of 4% phosphoric acid and 50 µL of IS (2000 µg/mL) were added to 250 µL aliquots of plasma samples, and the mixtures were loaded onto a plate. The loaded plates were washed with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. The retained flavanols and their metabolites were then eluted twice with 50 µL aliquots of an acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solution was directly injected into the HPLC-MS/MS, and the sample volume was 2.5 µL.

2.5. Extraction of flavanols and flavanol metabolites from tissues

Prior to chromatographic analysis of flavanols and their metabolites in rat tissues, the samples were pretreated using previously reported methodology (Margalef et al., 2015; Margalef, Pons, et al., 2014) that was based on an off-line liquid-solid extraction (LSE) in tandem with a micro solid-phase extraction (µSPE). Briefly, the LSE procedure involved adding 50 µL of 1% ascorbic acid and 100 µL of 4% phosphoric acid to 60 mg of freeze-dried tissue. All tissue samples were then extracted 4 times with 400 µL aliquots of water/methanol/4% phosphoric acid (94.4/4.5/1.5, v/v/v). In each extraction, 400 µL of extraction solution was added, after which the sample was sonicated (in an ice water bath to avoid heating) for 30 s using a Vibracell Ultrasonic Sonicator (Sonics & Materials, Newtown, CT, USA). Following this, the sample was centrifuged for 15 min at 17150 x g at room temperature (except for samples of MWAT, which were centrifuged at 4 ºC to achieve proper separation between fat and the aqueous phase). The supernatants that were obtained from the LSE procedure were cleaned up by µSPE following a previously described methodology for plasma but using 350 µL of tissue extract instead of plasma.

2.6. Chromatographic analysis of flavanols and flavanol metabolites

Chromatographic analyses were performed using the same chromatographic system as is described above (Section 2.2). The acquisition method was performed as previously reported for the quantification of phase-II flavanol metabolites (Serra et al., 2009). Data acquisition was conducted using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). The retention times and the ion chromatograms that were extracted from the studied compounds are shown in Figure 1.

2.7. Sample quantification

For sample quantification, either pooled blank plasma or pooled tissue extracts from rats that were administered water were spiked with standard compounds at 7 different concentrations to obtain calibration curves, and standard compounds in the samples were quantified by interpolating the analyte/IS peak abundance ratio in the resulting standard curves. Quality parameters, such as calibration curve detection and quantification limits (LOD and LOQ, respectively) and method detection and quantification limits (MDL and MQL, respectively), are shown in Table 2. Any flavanol concentrations that were quantified in blank plasma and tissues (i.e., from rats administered water) were subtracted from both the calibration curve and from the samples. The results are expressed as the mean ± standard error of the mean (SEM) (n=6).

3. Results

The composition of the extract that was used in this study is described in Table 3. The extract contained most of the representative flavanols of grape seed extract (Quiñones et al., 2013), including gallic acid, monomeric flavan-3-ols (catechin and epicatechin) and their gallate forms (epigallocatechin, epicatechin gallate, and epigallocatechin gallate), and oligomeric PAs (n=2-4). All of the identified compounds in the extract were studied in relation to their bioavailability in male and female rats.

Tables 4 and 5 detail the concentrations of each of the phase-II flavanol metabolites and their free forms in plasma and tissues (i.e., liver, MWAT and brain) at 1, 2 and 4 h after the administration of 1000 mg/kg of GSPE to female and male rats. No gallate flavanols or PA trimers were detected either in plasma or in the studied tissues.

3.1. Sex-related differences in GSPE flavanol distribution in plasma and liver

Following acute administration of GSPE, both male and female rats quickly metabolized epicatechin and catechin into their methylated, glucuronidated and sulfated derivatives, all of which were primarily present in the livers and plasma of both male and female rats alike. Both male and female rats exhibited peak maximum concentrations of unconjugated flavanols and their metabolites in plasma between 1 and 2 h after administration, without indicating evident differences between genders in pharmacokinetic studies (Figure 2A). In liver tissues, the kinetics of the flavanol metabolites were similar between genders, with the maximums occurring between 1 and 4 h; however, for unconjugated flavanols the maximum was at 1 h, and this peak was more prominent for females than for males (Figure 2B and Tables 4 and 5). Moreover, important differences between male and female rats were observed in total polyphenol content and in metabolite distribution. Female rats had twice total metabolites in plasma than male rats (for example, at 2 h the concentration was 65.1 μM in males and 141.4 μM in females; Figure 3A), although the total metabolites in the liver was very similar between genders (for example, at 2 h the concentration was 445 nmol/g in males and 448 nmol/g in females; Figure 3B). More specifically, male and female rats had different proportions of flavanol metabolites in their plasma and livers (Table 4 and 5). For example, when considering the 2 h time point as a maximum for flavanol concentrations, the following observations held true: in plasma, 2 h after the ingestion of GSPE, male rats exhibited a greater proportion of methyl-glucuronidated metabolites (20%) than females (12%) and a reduced proportion of methyl-sulfated metabolites (7%) than females (20%) (Figure 3A). At the 2 h time point, when examining the liver, male rats

had a higher proportion of methyl-glucuronidated metabolites (48%) than females (29%) and reduced proportions of both sulfated (5%) and methyl-sulfated (13%) metabolites than females (15% sulfated and 25% methyl-sulfated) (Figure 3B).

3.2. Sex-related differences in GSPE flavanol distributions in mesenteric white adipose tissue

Aglycone flavanols (catechin and epicatechin) and gallic acid were primarily found in MWAT in both male and female rats, although the quantity of flavanol metabolites was very low and only the glucuronidated forms were detected in both genders. In particular, epicatechin was the major flavanol that was found to be present in this tissue in both females (22.4, 17.4 and 4.5 nmol/g at 1, 2 and 4 h, respectively) and males (10.7, 14.2 and 4.6 nmol/g at 1, 2 and 4 h, respectively) (Table 4 and 5). Unconjugated flavanols reached a maximum in MWAT between 1 and 2 h in male rats, but in female rats they were at a maximum by 1 h, and at 2 h their concentrations in this tissue decreased considerably (Figure 2C). The total amount of metabolites and their distribution patterns were also very similar between male and female rats (Table 4 and 5) in this tissue. For example, at 2 h, the concentrations of total metabolites were 29.7 nmol/g in males and 27.7 nmol/g in females (Figure 3C).

3.3. Sex-related differences in GSPE flavanol distribution in brain tissues

Brain was found to be the tissue type with the lowest concentrations of flavanols, and sulfated metabolites were not detected in either male or female rats. In both genders, the maximum concentrations of flavanols and their metabolized forms in brain were reached at 2 h following GSPE ingestion, and at 4 h these concentrations were dramatically reduced (Figure 2D). However, several sex-related differences were found with respect to flavanol bioavailability in brain tissues following the ingestion of GSPE. Typically, brains from male rats had higher amounts of flavanols and metabolites than brains from female rats. For example, at 2 h, which was the maximum

absorption point in this tissue, the average concentration of total metabolites in brains from male rats was 26 nmol/g, and in brains from female rats it was 15.5 nmol/g. Furthermore, 46, 61 and 51% of flavanols in male rats were methylated epicatechins at 1, 2 and 4 h, respectively, whereas this metabolite was not detected in female rats at any point in time (Table 4 and 5). Moreover, using the 2 h time point as an example, in male rats only 6% of the total metabolites were nonconjugated flavanols, and 24% were glucuronide metabolites (Figure 3D). In contrast, in the brains of female rats, no methylated flavanols were detected, and at 2 h 23-34% of the metabolites were non-conjugated compounds, and the glucuronidated flavanols (46-68%) were the main metabolized compounds quantified in this tissue (Figure 3D, Table 4 and 5). Interestingly, PA dimers were detected in the brains of both sexes, and were more abundant in female rats (0-2.2 nmol/g) than in males (0-0.3 nmol/g) (Table 4 and 5). We also analyzed the hypothalami of rats from both genders, but no metabolites were detected in this tissue in either male or female rats (data not shown).

4. Discussion

Flavanols are abundant phytochemicals in the human diet, and their consumption has been associated with beneficial health effects (Z Pons et al., 2014; Quesada et al., 2009; Terra et al., 2011). Flavanols are recognized by the body as xenobiotics, and in the small intestine and the liver they are subjected to phase-II detoxification enzymes, which convert them into their methylated, sulfated and glucuronidated derivatives. However, their primary health effects, metabolism and bioavailability depend on several factors, such as intestinal enzyme activity, intestinal transit time, colonic microbiota, pathologies, genetics, and physiological conditions, among others (Aherne & O'Brien, 2002; D'Archivio et al., 2007). Furthermore, it has previously been observed that xenobiotic metabolism is different between genders (Mugford & Kedderis, 1998) and that drug-metabolizing enzymes are differentially affected by xenobiotics depending on sex (Finnen & Hassall, 1984; Mugford & Kedderis, 1998). Therefore, in light of these sex-related differences, we analyzed differences in the metabolism and distribution of flavanols between

male and female rats. To accomplish this, we administered acute doses of GSPE to male and female rats and then analyzed the concentrations of flavanols and their metabolites in plasma, liver, MWAT, brain and hypothalamus, which were chosen as representative tissues for elucidating how flavanols are distributed throughout the body. Based on previous studies, a dose of 1000 mg/kg was selected for evaluation in acute bioavailability studies following flavanol ingestion in rats (Margalef et al., 2015; Serra et al., 2009; Shoji et al., 2006). However, although we have previously reported that this high dose leads to saturation of the system (Margalef et al., 2015; Margalef, Guerrero, et al., 2014), it is still a valid dose for comparing differences in male and female tissue distribution and metabolism because it leads to concentrations of flavanols that are high enough to enable the detection of clear differences between the groups. Flavanol concentrations were analyzed in tissues at 1, 2 and 4 h following the acute administration of GSPE, as it has been well described that these compounds appear in plasma and tissues shortly after ingestion, with maximum concentrations of phase-II metabolites being reached between 1 and 2 h, and significant decrease at 4 h after ingestion of flavanols (Serra et al., 2013; Tomas-Barberan et al., 2007). Additionally, GSPE has been reported to exert beneficial health effects at these early time points, such as increasing GLP-1 and insulin secretions (González-Abuín et al., 2014), decreasing plasma glucose levels (Pinent, Cedó, Montagut, Blay, & Ardévol, 2012) and exhibiting antihypertensive effects (Zara Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2015; Quiñones et al., 2013).

It has been reported that male rats metabolize drugs faster than female rats, which has also been proven to be true with other xenobiotics such as polyphenols (Sipes & Gandolfi, 1991). However, in this study, plasma flavanol pharmacokinetics were similar in both genders; although, in the liver, female rats appeared to possess a faster metabolism than male rats, as unconjugated flavanols seemed to disappear from the livers of female rats faster than in males. However, the primary sex-related differences in plasma and liver tissues were in the total amounts and distributions of flavanols and

their metabolites. In this case, female rats were found to have twice the amount of flavanol metabolites in plasma than male rats. Generally, females have less water in their body compositions than males of similar body weights (Harris, Benet, & Schwartz, 1995); in this study, the female rats that were employed weighed an average of 32% less than the male rats, as the rats were age-matched (8-10 weeks old). Therefore, administering equivalent doses of GSPE led to higher concentrations of flavanol metabolites in female versus male rats. Flavanol phase-II metabolism principally occurs in the liver, and therefore the majority of methylated, sulfated and glucuronidated metabolites are found in this tissue in both genders (Del Rio et al., 2013). Previous studies in male (Margalef et al., 2015) and female (Arola-Arnal et al., 2013) rats have demonstrated similar concentrations of metabolites in this tissue following the administration of 1000 mg/Kg of GSPE. Although previous drug and xenobiotic enzymatic detoxification studies have demonstrated that male rats have higher phase-II enzymatic activities than female rats (DeBethizy & Hayes, 1994; Mugford & Kedderis, 1998), in this study, total amounts of flavanols and their metabolites in liver tissues were not affected by gender differences. Rather, the primary sex-related difference in this tissue, which was also true in plasma, was related to the proportions of individual metabolites. Sulfated and methyl-sulfated metabolites were found to be more abundant in the livers of female rats than in males, although male rats exhibited higher quantities of methyl-glucuronide metabolites than female rats at all of the time points that were evaluated. These results suggest that the sulfotransferase (SULT) enzymatic system may be more active in females, whereas the uridine 5'-diphospho-glucuronosyltransferase (UGT) and catechol-*O*-methyltransferase (COMT) enzymatic systems may be more active in males. However, Dellinger et al. (Dellinger, Garcia, & Meyskens, Jr., 2014) demonstrated that women are more efficient at glucuronidating pterostilbene and resveratrol polyphenols than men, indicating that enzymatic system activities could be different for polyphenols other than flavanols. Nevertheless, these results are in agreement with previous studies that have shown that the balance between the sulfation and glucuronidation of

polyphenols is affected by sex (Piskula, 2000) and that there are sex-related differences in how xenobiotics affect metabolic enzymes in the livers of rats (Finnen & Hassall, 1984; Mugford & Kedderis, 1998).

According to other studies that also employed GSPE (Ardévol, Motilva, Serra, Blay, & Pinent, 2013; Arola-Arnal et al., 2013; Margalef et al., 2015), in MWAT, the main compounds that were quantified were non-conjugated flavanols, whereas only very poor concentrations of phase-II metabolites were measured, and only glucuronide and methyl-glucuronide forms were detected. This specific distribution of free flavanols in MWAT is most probably due to the hydrophobic properties of adipose tissue rather than because of differences in enzymatic activities in this tissue. The total concentrations of flavanols and the individual proportions of flavanols and flavanol metabolites in MWAT were not found to be different between male and female rats, indicating that their tissue distributions are similar in both genders. However, in males, the accumulation of free flavanols (i.e., catechin and phenolic acids) in MWAT occurred over a longer period of time than in females, as in females the quantity of these compounds began to decrease at 2 h, which was similar to the results observed for unconjugated metabolites in the liver. These results may be because females have a faster metabolism than males or because of physiological differences (e.g., body weight, height, body surface area, total body water, and quantities of extracellular and intracellular water).

Finally, in brain tissues, the kinetic behaviors of flavanols and their metabolites was the same in both male and female rats: the maximum concentrations of these compounds in brain tissues were reached 2 h after the ingestion of GSPE, and they were later excreted into systemic circulation as opposed to being stored in target tissues. Indeed, only a few of these compounds could be detected in brain tissues, which agrees with previous results that demonstrated that not all flavanols are able to cross the blood-brain barrier (BBB) (Arola-Arnal et al., 2013; Margalef et al., 2015). Furthermore, both the types and quantities of flavanols that targeted the brain were distinct between male and female rats. For example, the quantity of

flavanol metabolites that targeted the brains of male rats was greater than in female rats, and the main metabolites in this tissue in male rats were methyl-epicatechin metabolites, whereas these compounds were not detected in the brains of female rats. However, the brains of female rats were found to have a higher quantity of PA dimers than the brains of male rats. We hypothesize that these gender differences in brain tissues could have arisen for two different reasons: 1) A sex-related specificity was imposed with regard to what types of flavanols were able to cross the BBB. In support of this reasoning, it has been previously reported that estrogens may have an important role in modulating free flavanol uptake by blood brain barrier (BBB) cells *in vitro*, and it has been further suggested that the female hormone progesterone can act as an endogenous factor that modulates the abilities of P-glycoproteins to serve as transporters of flavanols across the BBB (Faria et al., 2011); and 2) Different phase-II enzyme activities exist within the brains of male versus female rats. In support of this reasoning, the majority of the methylated flavanols found within the brains of male rats and the lack of these compounds found within the brains of female rats could be explained because estrogen is an important regulator of COMT activity in the brain (Harrison & Tunbridge, 2007; Mannisto & Kaakkola, 1999). It has further been reported that COMT activity in the prefrontal cortex is 17% higher in men than in women (Harrison & Tunbridge, 2007). These differences are important because polyphenols that target the brain are probably these physiologically active forms. For example, Wang J et al. (Wang et al., 2012) reported that flavanols that were able to target the brain after the ingestion of grape polyphenols increased cognition by improving synaptic plasticity in the brain. Therefore, the fact that different flavanols and flavanol metabolites are targeted to the brains of male versus female rats may be due to different flavanol bioactivities in their brain tissues.

This study demonstrated that the metabolism and tissue distribution of flavanols in rats is influenced by sex. These differences are probably due to inherent physiological differences between the sexes, such as total body water, differences in phase-II enzyme activities in target tissues (i.e., in liver

and in brain), and differences in tissue specificities. It is important to note that these differences in bioavailability may differentially influence the physiological bioactivities of these compounds in males and females. Although further studies will be necessary to elucidate sex-related differences in the physiological bioactivities of flavanols, experimental conditions such as gender should already be taken into consideration when investigating flavanol compounds *in vivo*. Moreover, understanding flavanol metabolism is vital to its interpretation and utility in the clinic and to delineating whether different treatments are necessary for male versus female patients.

Author contributions

M. M., A. A-A. and B. M. analyzed data and wrote the manuscript. M. M., Z. P. and L. I-C. contributed to researching the data. M. M., Z. P., A. A-A. L.A. and B. M. contributed to experimental design, discussion, and review of the manuscript.

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Figure Legends

Figure 1. Extracted ion chromatograms of flavanols and their phase-II metabolites 2 h after the administration of 1000 mg/kg of a grape seed polyphenol extract (GSPE) in male (continuous line) and female (discontinuous line) rat plasma. (1) Gallic acid, (2) Dimer B1, (3) Dimer B3, (4) Dimer B2, (5) Methyl-catechin-glucuronide, (6) Methyl-epicatechin-glucuronide, (7) Catechin glucuronide, (8) Epicatechin glucuronide, (9) Catechin, (10) Epicatechin, (11) Catechin sulfate, (12) Epicatechin sulfate (13) Methyl-catechin-O-sulfate, (14) Methyl-epicatechin-O-sulfate, (15) 3-Methyl-epicatechin, and (16) 4-Methyl-epicatechin.

Figure 2. Pharmacokinetic profiles of flavanols and their metabolites in rat plasma and tissues after acute ingestion of grape seed polyphenol extract (GSPE) in both male (left panels) and female (right panels) rats. (A) Plasma. (B) Liver. (C) Mesenteric white adipose tissue (MWAT). (D) Brain. Data are displayed as the mean ± standard error of the mean (SEM) (n=6). The results are expressed in μM for plasma samples and in nmol/g for the studied tissue samples.

Table 1. Optimized MRM conditions in HPLC-MS/MS for the study of the polyphenols present in the grape seed polyphenol extract (GSPE) and their metabolites studied in plasma and tissues after an acute administration of 1000 mg/kg of GPSE per body weight.

Compound	Quantification		Confirmation			
	MRM	CE (V)	MRM	CE (V)	MRM	CE (V)
Gallic acid	169>125	5	169>169	0	169>79	40
Protocatechuic acid	153>109	10	153>62	40	-	-
Vanillic acid	167>152	10	167>123	5	-	-
PA dimer B2	577>425	10	577>407	20	577>289	20
PA dimer B1	577>425	10	577>407	20	577>289	20
PA dimer B3	577>425	10	577>407	20	577>289	20
Catechin	289>245	5	289>203	10	289>179	5
Epicatechin	289>245	5	289>203	10	289>179	5
Dimer gallate	729>577	20	729>441	30	729>407	50
Epicatechin gallate	441>331	0	441>289	5	-	-
Epigallocatechin gallate	457>169	20	457>305	20	457>457	0
Epigallocatechin	305>125	5	305>179	5	-	-
PA trimer	865>577	20	865>713	20	-	-
PA tetramer	1153>865	40	1153>1153	0	-	-
Catechin glucuronide	465>289	20	465>203	40	-	-
Epicatechin glucuronide	465>289	20	465>203	40	-	-
Methyl-catechin glucuronide	479>303	20	479>289	20	-	-
Methyl-epicatechin glucuronide	479>303	20	479>289	20	-	-
Catechin-sulfate	369>245	20	369>289	20	-	-
Epicatechin-sulfate	369>245	20	369>289	20	-	-
Methyl-catechin-sulfate	383>245	10	383>303	20	-	-
Methyl-epicatechin-sulfate	383>245	10	383>303	20	-	-
3-*O*-methyl-epicatechin	303>137	20	303>285	10	-	-
4-*O*-methyl-epicatechin	303>137	20	303>285	10	-	-

Abbreviations: PA (proanthocyanidin), MRM (Multiple Reaction Monitoring), CE (Collision Energy), V (Volts)

Table 2. Method validation for the determination of flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in the studied tissues.

Tissue	Compound	Male							Female						
		Calibration Curve	Determination Coefficient (R^2)	Linearity Range (μM)	LOD (nM)	LOQ (nM)	MDL* (pmol/g or nM)	MQL* (pmol/g or nM)	Calibration Curve	Determination Coefficient (R^2)	Linearity Range (μM)	LOD (nM)	LOQ (nM)	MDL* (pmol/g or nM)	MQL* (pmol/g or nM)
Plasma	Catechin	y=0.037x	0.997	0.034 - 17.225	0.096	0.320	0.038	0.128	y=0.032x	0.994	0.034 - 17.225	0.172	0.574	0.069	0.230
	Epicatechin	y=0.037x	0.994	0.034 - 17.225	0.159	0.530	0.064	0.212	y=0.027x	0.996	0.034 - 17.225	0.230	0.766	0.092	0.306
	PA B2	y=0.009x	0.999	0.017 - 8.643	0.207	0.691	0.083	0.277	y=0.012x	0.992	0.017 - 8.643	0.691	2.305	0.277	0.922
	Gallic acid	y=0.050x	0.999	0.058 - 29.391	0.261	0.871	0.105	0.348	y=0.089x	0.996	0.058 - 29.391	0.252	0.840	0.101	0.336
	Vanillic Acid	y=0.118x	0.999	0.059 - 29.735	0.193	0.643	0.077	0.257	y=0.092x	0.991	0.059 - 29.735	0.046	0.154	0.019	0.062
Liver	Catechin	y=0.018x	0.998	0.034 - 17.225	1.279	4.262	9.694	32.312	y=0.025x	0.993	0.034 - 17.225	1.494	4.981	11.859	39.530
	Epicatechin	y=0.005x	0.999	0.034 - 17.225	1.074	3.579	8.141	27.136	y=0.008x	0.997	0.034 - 17.225	1.312	4.375	10.416	34.720
	PA B2	y=0.022x	0.993	0.017 - 8.643	0.975	3.250	1.021	3.404	y=0.025x	0.992	0.017 - 8.643	1.062	3.540	2.494	8.314
	Gallic acid	y=0.144x	0.999	0.058 - 29.391	0.452	1.507	3.428	11.427	y=0.173x	0.999	0.058 - 29.391	0.588	1.959	4.665	15.551
	Vanillic Acid	y=0.255x	0.998	0.059 - 29.735	0.135	0.449	7.391	24.638	y=0.318x	0.997	0.059 - 29.735	0.314	1.048	8.428	28.095
MWAT	Catechin	y=0.042x	0.997	0.034 - 17.225	0.101	0.336	0.774	2.579	y=0.040x	0.999	0.034 - 17.225	0.103	0.345	0.840	2.801
	Epicatechin	y=0.037x	0.996	0.034 - 17.225	0.129	0.431	0.991	3.305	y=0.035x	0.997	0.034 - 17.225	0.125	0.418	1.018	3.395
	PA B2	y=0.013x	0.998	0.017 - 8.643	0.189	0.629	1.447	4.824	y=0.015x	0.996	0.017 - 8.643	0.207	0.691	1.686	5.621
	Gallic acid	y=0.051x	0.999	0.058 - 29.391	0.321	1.069	2.461	8.202	y=0.049x	0.999	0.058 - 29.391	0.243	0.811	1.978	6.592
	Vanillic Acid	y=0.112x	0.999	0.059 - 29.735	0.101	0.335	0.771	2.571	y=0.108x	0.998	0.059 - 29.735	0.085	0.283	0.691	2.302
Brain	Catechin	y=0.115x	0.999	0.034 - 17.225	1.590	5.300	12.422	41.407	y=0.163x	0.997	0.034 - 17.225	10.335	34.451	81.508	271.693
	Epicatechin	y=0.113x	0.999	0.034 - 17.225	2.953	9.843	23.070	76.899	y=0.176x	0.999	0.034 - 17.225	3.445	11.484	27.169	90.564
	PA B2	y=0.057x	0.997	0.017 - 8.643	2.593	8.643	20.256	67.521	y=0.046x	0.995	0.017 - 8.643	5.186	17.285	40.896	136.321
	Gallic acid	y=0.037x	0.999	0.058 - 29.391	2.519	8.397	19.681	65.605	y=0.046x	0.998	0.058 - 29.391	3.919	13.063	30.905	103.018
	Vanillic Acid	y=0.064x	0.999	0.059 - 29.735	0.776	2.586	6.060	20.201	y=0.244x	0.997	0.059 - 29.735	2.549	8.496	20.101	67.002

Abbreviations: PA (Proanthocyanidin), LOD (limit of detection), LOQ (limit of quantification), MDL (method detection limit) and MQL (method quantification limit).

*MDL and MQL in pmol/g for the analysis of 60mg of tissue or in nM for the analysis of 250 μL of plasma.

Table 3. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1[1]	88.80 ± 3.46
PA dimer B3[1]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[1]	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin[2]	0.27 ± 0.03
PA trimer[1]	4.90 ± 0.47
PA tetramer[1]	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

[1] Quantified using the calibration curve of proanthocyanidin B2.

[2] Quantified using the calibration curve of epigallocatechin gallte.

Table 4. Flavanols and their phase-II metabolites detected by HPLC-ESI-MS/MS in female rat plasma, liver, white mesenteric adipose tissue (MWAT) and brain at 1, 2, and 4 h after the ingestion of 1000 mg/kg of a grape seed polyphenol extract (GSPE).

Compound	Plasma			Liver			MWAT			Brain		
	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h
Catechin	0.48 ± 0.05	0.33 ± 0.03	0.38 ± 0.06	2.59 ± 0.51	1.24 ± 0.25	0.50 ± 0.21	18.04 ± 3.39	6.83 ± 0.73	2.43 ± 0.33	1.38 ± 0.10	1.87 ± 0.25	0.31 ± 0.05
Epicatechin	1.72 ± 0.34	2.41 ± 0.45	1.57 ± 0.08	18.99 ± 2.81	6.60 ± 1.35	4.05 ± 1.31	22.40 ± 6.54	17.34 ± 7.93	4.47 ± 2.09	0.69 ± 0.08	1.22 ± 0.15	0.36 ± 0.03
Epicatechin gallate[3]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EGCG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PA dimer B2	1.23 ± 0.31	2.09 ± 0.43	1.04 ± 0.13	0.98 ± 0.18	0.62 ± 0.05	0.25 ± 0.10	6.66 ± 0.53	4.04 ± 0.88	1.50 ± 0.57	0.43 ± 0.08	1.58 ± 0.17	n.q.
PA dimer B3[4]	0.28 ± 0.08	0.50 ± 0.09	0.28 ± 0.03	1.03 ± 0.21	0.73 ± 0.27	0.15 ± 0.07	2.88 ± 0.76	2.40 ± 1.04	0.65 ± 0.22	n.d.	n.d.	n.d.
PA dimer B1[4]	0.49 ± 0.08	0.81 ± 0.16	0.52 ± 0.08	0.40 ± 0.19	0.14 ± 0.04	0.11 ± 0.04	3.90 ± 0.42	2.40 ± 0.38	1.03 ± 0.35	0.29 ± 0.03	0.59 ± 0.07	n.q.
PA Trimer[4]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic Acid	2.51 ± 0.26	3.16 ± 0.35	2.17 ± 0.23	0.87 ± 0.30	1.17 ± 0.22	0.91 ± 0.07	15.65 ± 2.03	10.62 ± 1.18	7.36 ± 0.90	n.d.	n.d.	n.d.
Vanillic Acid	0.48 ± 0.04	0.43 ± 0.03	0.31 ± 0.02	1.14 ± 0.16	1.15 ± 0.13	0.45 ± 0.14	1.24 ± 0.21	0.73 ± 0.17	0.38 ± 0.03	n.d.	n.d.	n.d.
Catechin glucuronide[1]	35.08 ± 2.49	31.89 ± 1.53	19.47 ± 1.03	36.44 ± 3.87	40.82 ± 6.76	49.58 ± 2.17	12.56 ± 1.63	12.27 ± 2.55	12.00 ± 0.95	1.23 ± 0.08	2.85 ± 0.22	0.48 ± 0.10
Epicatechin glucuronide[2]	44.08 ± 1.88	46.90 ± 6.64	39.37 ± 1.62	43.11 ± 10.49	40.85 ± 7.50	40.17 ± 3.78	15.51 ± 2.27	15.93 ± 3.51	14.54 ± 1.08	2.86 ± 0.15	4.91 ± 0.35	1.61 ± 0.20
Methyl-catechin glucuronide[1]	6.02 ± 0.17	7.69 ± 0.74	6.82 ± 0.49	34.73 ± 1.12	51.64 ± 5.19	53.96 ± 3.91	2.19 ± 0.34	3.11 ± 0.59	3.13 ± 0.35	0.87 ± 0.03	1.26 ± 0.16	0.14 ± 0.01
Methyl-epicatechin glucuronide[2]	5.00 ± 0.30	9.19 ± 0.88	6.53 ± 0.33	53.01 ± 3.13	77.20 ± 9.77	73.68 ± 4.68	1.79 ± 0.35	2.16 ± 0.37	2.46 ± 0.22	1.11 ± 0.06	1.23 ± 0.12	0.12 ± 0.02
Catechin-sulfate[1]	0.20 ± 0.03	0.27 ± 0.07	0.19 ± 0.02	6.21 ± 0.54	15.39 ± 4.17	8.77 ± 3.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Epicatechin-sulfate[2]	2.26 ± 0.44	5.89 ± 1.27	2.12 ± 0.12	21.03 ± 1.84	52.13 ± 14.11	37.89 ± 10.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methyl-catechin-sulfate[1]	1.75 ± 0.41	2.48 ± 0.58	1.47 ± 0.18	10.71 ± 1.51	16.89 ± 3.57	9.16 ± 1.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methyl-epicatechin-sulfate[2]	18.55 ± 4.01	26.47 ± 4.74	17.72 ± 1.79	87.72 ± 13.54	95.25 ± 11.52	81.94 ± 21.26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-*O*-methyl-epicatechin[2]	0.16 ± 0.02	0.39 ± 0.08	0.35 ± 0.03	13.97 ± 3.09	14.08 ± 2.17	23.43 ± 4.11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-*O*-methyl-epicatechin[2]	0.08 ± 0.02	0.06 ± 0.01	0.04 ± 0.01	24.37 ± 3.70	31.56 ± 4.35	47.64 ± 8.83	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Abbreviations: PA (proanthocyanidin), n.d. (not detected); n.q. (not quantified)

The results are expressed in nmol/g of dried tissue or in μM in plasma as the mean ± SD (n=6).

[1]Quantified using the calibration curve of catechin

[2]Quantified using the calibration curve of epicatechin

[3]Quantified using the calibration curve of EGCG

[4]Quantified using the calibration curve of proanthocyanidin dimer B2

Table 5. Flavanols and their phase-II metabolites detected by HPLC-ESI-MS/MS in male rat plasma, liver, white mesenteric adipose tissue (MWAT) and brain at 1, 2, and 4 h after the ingestion of 1000 mg/kg of a grape seed polyphenol extract (GSPE).

Compound	Plasma			Liver			MWAT			Brain		
	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h
Catechin	0.16 ± 0.01	0.12 ± 0.03	0.09 ± 0.01	0.94 ± 0.26	1.22 ± 0.13	0.38 ± 0.04	11.01 ± 3.39	13.57 ± 1.22	5.00 ± 1.71	0.43 ± 0.09	0.77 ± 0.07	n.q.
Epicatechin	1.52 ± 0.47	0.74 ± 0.17	0.47 ± 0.08	6.08 ± 0.78	3.41 ± 0.51	2.42 ± 0.38	10.67 ± 2.96	14.23 ± 2.65	4.56 ± 1.43	0.68 ± 0.07	0.50 ± 0.03	n.q.
Epicatechin gallate[3]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EGCG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PA dimer B2	1.76 ± 0.44	0.74 ± 0.08	0.39 ± 0.07	0.32 ± 0.07	0.49 ± 0.10	0.15 ± 0.03	6.68 ± 1.94	5.19 ± 1.04	2.86 ± 0.69	0.23 ± 0.03	0.29 ± 0.03	n.q.
PA dimer B3[4]	0.46 ± 0.13	0.27 ± 0.04	0.18 ± 0.03	0.25 ± 0.06	0.42 ± 0.05	0.15 ± 0.03	2.44 ± 0.55	2.06 ± 0.10	1.13 ± 0.31	n.d.	n.d.	n.d.
PA dimer B1[4]	0.80 ± 0.22	0.31 ± 0.02	0.16 ± 0.03	0.31 ± 0.18	0.80 ± 0.11	0.13 ± 0.02	6.29 ± 1.14	2.06 ± 0.73	2.01 ± 0.81	n.d.	n.d.	n.d.
PA Trimer[4]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic Acid	3.38 ± 0.41	2.39 ± 0.57	3.58 ± 0.69	0.97 ± 0.25	0.61 ± 0.15	0.75 ± 0.09	11.75 ± 2.15	6.72 ± 1.35	8.14± 1.09	n.q.	n.q.	n.q.
Vanillic Acid	0.11 ± 0.03	0.07 ± 0.01	0.06 ± 0.00	1.06 ± 0.11	0.62 ± 0.15	0.53 ± 0.07	1.45 ± 0.37	0.34 ± 0.03	0.31 ± 0.06	n.d.	n.d.	n.d.
Catechin glucuronide[1]	23.66 ± 0.37	22.71 ± 1.41	13.00 ± 3.41	26.58 ± 1.73	39.07 ± 9.05	48.91± 1.86	9.13 ± 1.41	8.75 ± 1.58	10.56 ± 1.89	2.10 ± 0.37	2.83 ± 0.37	0.70 ± 0.08
Epicatechin glucuronide[2]	18.65 ± 5.71	19.23 ± 4.28	16.26 ± 2.82	57.50 ± 5.81	59.78 ± 19.13	31.46 ± 5.29	10.44 ± 1.52	10.34 ± 1.70	13.34 ± 2.38	2.84 ± 0.57	3.37 ± 0.44	0.77 ± 0.05
Methyl-catechin glucuronide[1]	5.73 ± 0.18	7.05 ± 0.70	4.09 ± 0.49	34.44 ± 12.96	71.95 ± 9.54	63.85 ± 9.20	2.22 ± 0.29	2.52 ± 0.67	3.14 ± 0.45	0.97 ± 0.08	0.94 ± 0.09	0.14 ± 0.02
Methyl-epicatechin glucuronide[2]	4.01 ± 0.27	5.61 ± 0.31	3.36 ± 0.55	92.28 ± 15.63	132.10 ± 15.63	118.20 ± 19.52	1,41 ± 0.27	1,58 ± 0.32	2,41 ± 0.32	1.17 ± 0.12	1.29 ± 0.13	0.16 ± 0.03
Catechin-sulfate[1]	0.19 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	2.38 ± 0.65	2.96 ± 0.72	4.07 ± 1.71	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
Epicatechin-sulfate[2]	1.26 ± 0.21	0.96 ± 0.28	0.43 ± 0.08	9.07 ± 4.31	17.06 ± 4.14	15.00 ± 3.50	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
Methyl-catechin-sulfate[1]	1.22 ± 0.20	0.94 ± 0.22	0.74 ± 0.13	8.53 ± 1.77	8.42 ± 1.41	7.52 ± 1.57	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
Methyl-epicatechin-sulfate[2]	6.05 ± 0.72	3.79 ± 0.88	2.76 ± 0.44	45.79 ± 13.28	50.65 ± 10.28	35.91 ± 9.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-*O*-methyl-epicatechin[2]	0.18 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	16.67 ± 2.19	16.97 ± 3.60	18.68 ± 4.18	n.d.	n.d.	n.d.	3.87 ± 0.92	9.46 ± 1.64	1.15 ± 0.13
4-*O*-methyl-epicatechin[2]	0.02 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	29.96 ± 5.36	38.46 ± 8.83	42.04 ± 10.15	n.d.	n.d.	n.d.	3.25 ± 0.64	6.54 ± 0.69	0.68 ± 0.10

Abbreviations: PA (Proanthocyanidin), n.d. (not detected); n.q. (not quantified)

The results are expressed in nmol/g of dried tissue or in µM in plasma as the mean ± SD (n=6).

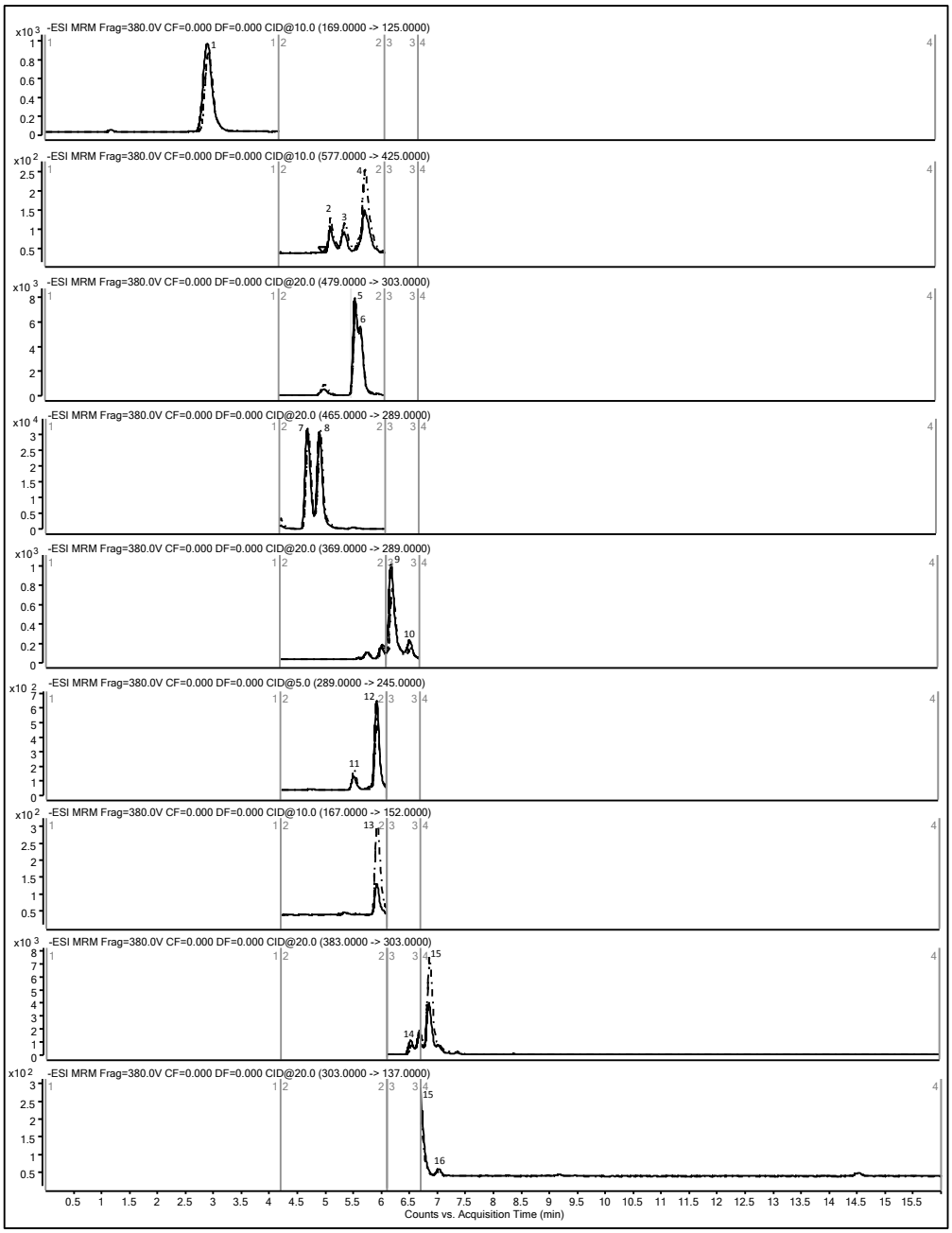
[1]Quantified using the calibration curve of catechin

[2]Quantified using the calibration curve of epicatechin

[3]Quantified using the calibration curve of EGCG

[4]Quantified using the calibration curve of proanthocyanidin dimer B2

Figure 1.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-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@20.0 (479.0000 -> 303.0000)
-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@20.0 (465.0000 -> 289.0000)
-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@20.0 (369.0000 -> 289.0000)
-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@5.0 (289.0000 -> 245.0000)
-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@10.0 (167.0000 -> 152.0000)
-ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@20.0 (383.0000 -> 303.0000)
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Counts vs. Acquisition Time (min)

Figure 2.

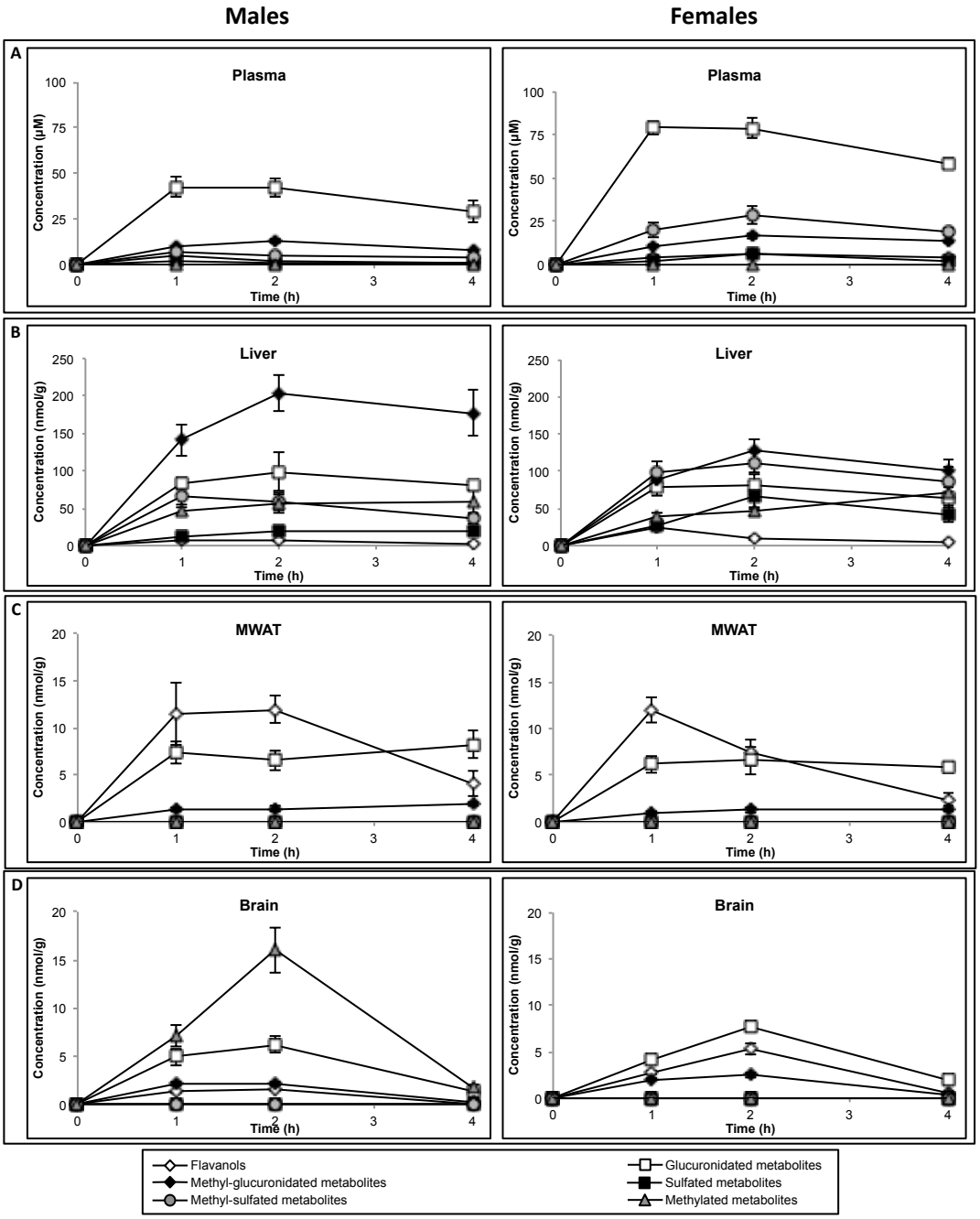
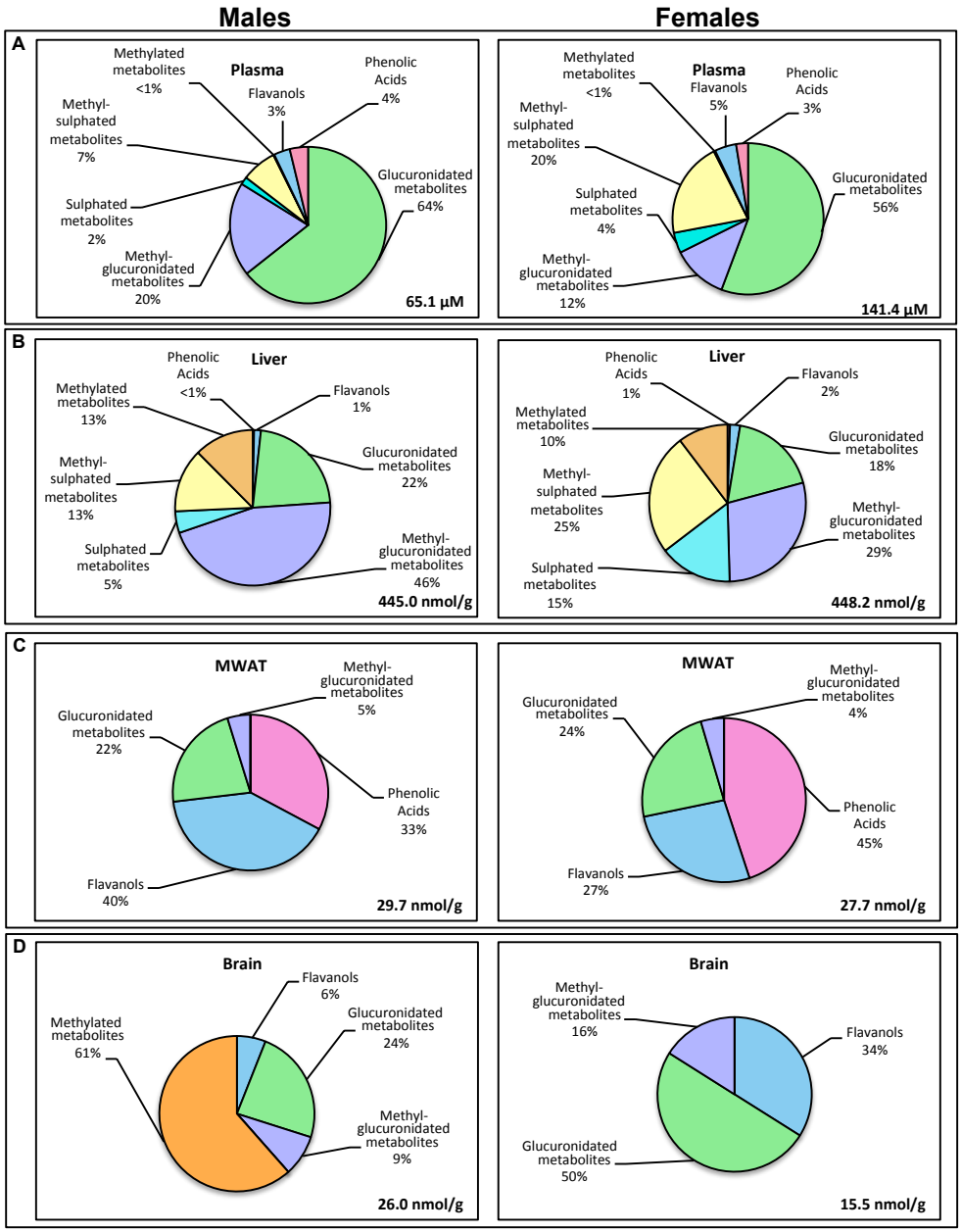

Males
Females
A
Plasma
Concentration (µM)
Time (h)
B
Liver
Concentration (nmol/g)
C
MWAT
D
Brain
Flavanols
Methyl-glucuronidated metabolites
Methyl-sulfated metabolites
Glucuronidated metabolites
Sulfated metabolites
Methylated metabolites

Figure 3.


Males
Females
A
Plasma
Methylated metabolites <1%
Phenolic Acids 4%
Flavanols 3%
Methyl-sulphated metabolites 7%
Glucuronidated metabolites 64%
Sulphated metabolites 2%
Methyl-glucuronidated metabolites 20%
65.1 µM
Plasma
Methylated metabolites <1%
Flavanols 5%
Phenolic Acids 3%
Methyl-sulphated metabolites 20%
Glucuronidated metabolites 56%
Sulphated metabolites 4%
Methyl-glucuronidated metabolites 12%
141.4 µM
B
Liver
Phenolic Acids <1%
Flavanols 1%
Methylated metabolites 13%
Glucuronidated metabolites 22%
Methyl-sulphated metabolites 13%
Methyl-glucuronidated metabolites 46%
Sulphated metabolites 5%
445.0 nmol/g
Liver
Phenolic Acids 1%
Flavanols 2%
Methylated metabolites 10%
Glucuronidated metabolites 18%
Methyl-sulphated metabolites 25%
Methyl-glucuronidated metabolites 29%
Sulphated metabolites 15%
448.2 nmol/g
C
MWAT
Methyl-glucuronidated metabolites 5%
Glucuronidated metabolites 22%
Phenolic Acids 33%
Flavanols 40%
29.7 nmol/g
MWAT
Methyl-glucuronidated metabolites 4%
Glucuronidated metabolites 24%
Phenolic Acids 45%
Flavanols 27%
27.7 nmol/g
D
Brain
Flavanols 6%
Glucuronidated metabolites 24%
Methylated metabolites 61%
Methyl-glucuronidated metabolites 9%
26.0 nmol/g
Brain
Methyl-glucuronidated metabolites 16%
Flavanols 34%
Glucuronidated metabolites 50%
15.5 nmol/g

MANUSCRIPT 9:

Flavanol plasma bioavailability is affected by metabolic syndrome in rats

Maria Margalef[†], Zara Pons[†], Lisard Iglesias-Carres[†], Francisca Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Nutrigenomic Research Group. Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

Submited to Food Chemistry
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Flavanol plasma bioavailability is affected by metabolic syndrome in rats

Maria Margalef[†], Zara Pons[†], Lisard Iglesias-Carres[†], Francisca Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Nutrigenomic Research Group. Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

Running title: Flavanol bioavailability kinetics in rats with metabolic syndrome

***Corresponding author:** Begoña Muguerza
Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili
C/Marcel.lí Domingo s/n, 43007 Tarragona, Spain
Phone: +34 977559566
Fax: +34 977558232
e-mail: begona.muguerza@urv.cat

Abstract

Flavanols, one of the main important dietary polyphenols, once ingested are recognized as xenobiotics, and undergo phase-II colonic and microbial metabolism. As flavanols health benefits have been attributed to their metabolic products, the study of their bioavailability is one of the most important points in understanding their bioactivity. Different factors such as gender, age, or physiological condition may influence the metabolism, bioavailability, and tissue distributions of these compounds. Therefore, the aim of this study was to evaluate whether a pathological state could influence flavanol plasma bioavailability. Standard (ST) and cafeteria diet (CAF) fed rats, a robust model of metabolic syndrome (MeS), were administered 1000 mg/kg of a flavanol enriched grape seed polyphenol extract (GSPE). Flavanols and their metabolites were quantified by HPLC-MS/MS in plasma before and at 2, 4, 7, 24, and 48 h after GSPE ingestion. Increased plasma bioavailability, a 2 h delay in the maximum plasma concentration and lower prevalence of the compounds over time were observed in CAF rats. CAF rats also showed increased plasma concentration of gallic acid and glucuronidated metabolites, lower absorption of flavanol dimers, and reduced flavanol microbial derivative metabolites. This study demonstrates that a pathological state such as MeS modifies flavanol bioavailability, supporting the hypothesis that flavanol metabolism, and therefore flavanol functionality, depend on the organisms' state of health. Hence, many of the polyphenol studies carried out in healthy hosts cannot be extrapolated to health disorder states because many metabolic forms of polyphenols could be different different in healthy and diseased states.

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Key words: bioactivity; cafeteria diet; kinetic profile; metabolism; obesity; polyphenols

1. Introduction

Flavanols comprise one of the most important groups of dietary polyphenols and are mainly found in grapes, cocoa, red wine, and green tea (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). They are also known as flavan-3-ols, and their monomers consist of (+)-catechin, and (-)-epicatechin units. Oligomeric and polymeric forms of flavan-3-ols are commonly named proanthocyanidins (PAs) (Bladé, Arola, & Salvadó, 2010). These phytochemicals have been associated with several health benefits (Del Rio et al., 2013), acting as lipid homeostasis modulators (Guerrero et al., 2013; Margalef, Guerrero, et al., 2014), free radical scavengers (Fraga, Galleano, Verstraeten, & Oteiza, 2010), antihypertensives (Zara Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2015; Quiñones et al., 2013), anti-diabetics (Castell et al., 2009; González-Abuín et al., 2014) and anti-inflammatory agents (Martinez-Micaelo et al., 2015). Nevertheless, as the beneficial health-promoting effects of flavanol are largely attributed to their metabolic products, the study of the metabolism of the ingested compounds, the derived bioavailable products and their distribution through target tissues is essential to elucidating their bioactivities (Bohn et al., 2015).

Once ingested, flavanol monomers and PA dimers are recognized as xenobiotics and undergo a phase-II enzymatic detoxification in the small intestine and liver (Monagas et al., 2010). Uridin-glucuronil transferases (UGTs), sulfotransferases (SULTs) and/or catechol-*O*-methyl transferases (COMTs) are the phase-II enzymes responsible for the detoxification of flavanol parent compounds, producing glucuronidated, sulfated and methylated conjugates, respectively, in order to increase their solubility and enhance their excretion through the urine (Del Rio et al., 2013; Monagas et al., 2010). In addition, PAs with a high degree of polymerization cannot be absorbed at the level of the small intestine and reach the colon to undergo microbial catabolism, leading to the formation of small phenolic compounds able to reach the liver, where they can also be subjected to phase-II conjugation (Aura, 2008; Monagas et al., 2010; Stoupi, Williamson, Drynan, Barron, & Clifford, 2010). Both flavanol phase-II and microbial metabolites

reach the systemic circulation to be distributed to different tissues or reach the kidneys to be excreted through the urine (Monagas et al., 2010).

Flavanol metabolism has been widely studied for years (Slanina & Taborska, 2004; Walle, 2004), but there are still many gaps in our knowledge about the factors affecting the metabolism and bioavailability of these phytochemicals(Bohn et al., 2015). Bioavailability and bioactivity of flavanols depend on two main factors, external and host-related factors (D'Archivio et al., 2007). External factors include those related to ingested flavanol, namely its structure, the food matrix, the food processing, and the ingestion length (Bohn et al., 2015; D'Archivio et al., 2007). The internal factors include those factors related to the internal state of the individual subjects, such as gender, age, physiological condition, and any existing pathology states (D'Archivio et al., 2007). Physiological conditions such as body mass index (BMI), fat, and body lean content may affect the metabolism of all ingested foods or drugs (Isezuo, Badung, & Omotoso, 2003; Tesauro & Cardillo, 2011), including the metabolism of polyphenols. Obesity is one of the most important metabolic disorders that causes chronic diseases such as type-II diabetes, hypertension, or atherosclerosis and is also one of the required conditions for the diagnosis of Metabolic Syndrome (MeS) (Dobrian, Davies, Schriver, Lauterio, & Prewitt, 2001; Sampey et al., 2011; Tesauro & Cardillo, 2011). Cafeteria-diet-fed obese rats are an animal model for the study of MeS and have been used for the study of specific pathologies included in the cluster diseases that define MeS, such as hypertension or obesity (Z Pons et al., 2014; Sampey et al., 2011).

The aim of this study was to evaluate whether flavanol metabolism and plasma bioavailability are affected by the pathological metabolic state induced by a cafeteria diet.

2. Materials and Methods

Methanol (Scharlab S.L., Barcelona, Spain), acetone, acetonitrile (both from Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a

Milli-Q advantage A10 system (Madrid, Spain). Individual stock standard solutions of 2000 mg/L in methanol of (+)-catechin, (-)-epicatechin, procyanidin B2, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, benzoic acid, and pyrocatechol (the internal standard (IS)) (all from Fluka/Sigma-Aldrich, Madrid, Spain), and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (MicroCombiCheme.K., Wiesbaden, Germany), were prepared and stored in a dark glass flask at -20 ºC.

A 20 mg/L stock standard mixture in methanol of all of the compounds described above was prepared weekly and stored at -20 ºC. This stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.2. Grape Seed Polyphenol Extract (GSPE)

A grape seed polyphenol extract (GSPE) rich in flavanols was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). Table 1 shows the phenolic compound (flavan-3-ols and phenolic acids) concentrations of the extract used in this study.

2.3. Experimental Procedure in rats

Six-week-old male Wistar rats Crl:WI (Charles River Laboratories, Barcelona, Spain) were singly housed at 22 ºC with a light/dark period of 12 h. After a quarantine period of 2 weeks, the animals weighed 230-240 g and were divided into two dietary groups. The control group (ST, n=6) was fed standard chow (Panlab A04, Panlab, Barcelona, Spain) and tap water *ad libitum*. The second group (CAF, n=6) had free access to a fresh cafeteria diet consisting of bacon (10-12 g), sausage (8–12 g), biscuits with *paté* (12–15 g), cheese (10–12 g), *ensaïmada* (sweetened pastry) (4-5 g), carrots (8-10 g), and sweetened milk (20% sucrose (w/v)) renewed daily, plus tap water in addition to the standard chow diet. The standard chow diet had a calorie breakdown of 14% protein, 8% fat and 73% carbohydrates, whereas the calorie breakdown of the cafeteria diet was 14% proteins, 35% fat and 51%

carbohydrates. All of the animals were fed *ad libitum*, and the diets were maintained for 12 weeks until the day of the experiment.

The day of the experiment, both animal groups were randomly divided into two different subgroups: the GSPE group (n=5) and the control group (n=1). A dose of 1000 mg/Kg of GSPE (1 mL in water) was administered to the GSPE group by oral gavage. Water (1 mL) was orally administered to the control group. In both groups, oral administration was performed by gastric intubation between 9 and 10 a.m. Blood samples were obtained via saphenous vein extraction using heparin vials (Starsted, Barcelona, Spain) at 0, 2, 4, 7, 24 and 48 h after GSPE or water administration, in order to measure the kinetics of phase-II and microbial metabolites. Plasma samples were obtained by centrifugation (2000 x g, 15 min, 4ºC) and pooled to obtain a sufficient volume for the analysis and also to remove biological variability. The pooled plasma samples were stored at -80ºC until chromatographic analysis was performed. Pooled plasma from the control group (water oral gavage) was used for the calibration curves in the chromatography analysis. Any compound present in the plasma at the 0 h time-point was subtracted from the plasma concentrations at all other time-points in order to remove the compounds endogenous to the diet. This study was performed in accordance with institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili.

2.4. Micro-Solid Phase plasma flavanol extraction

Prior to chromatographic analysis, the pooled rat plasma for each time-point was pre-treated by off-line micro-Solid Phase Extraction (µ-SPE) as described previously (Margalef, Pons, Muguerza, & Arola-Arnal, 2014) using OASIS HLB µ-Elution Plates 30 µm (Waters, Barcelona, Spain). Briefly, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. Plasma aliquots (250 µL) were mixed with 300 µL of 4% phosphoric acid and 50 µL of pyrocatechol (1000 ppb), and this mixture was loaded into the plates. The loaded plates were washed with 200

µL of Milli-Q water and 200 µL 0.2% acetic acid. The retained flavanols were eluted with 2 x 50 µL of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v).

2.5. Chromatographic Analysis

The chromatographic analysis was performed using a 1290 LC Series UHPLC coupled to a 6490 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150 mm × 2.1 mm i.d., 3.5 µm particle size) column as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA). The MS system consisted of an Agilent Jet Stream (AJS) ionization source. The mobile phase, electrospray ionization (ESI) conditions and acquisition method were performed as previously reported for the quantification of phase-II and microbial flavanol metabolites in plasma and tissues (Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015a, 2015b; Margalef, Pons, et al., 2014). Data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

3. Results

Flavanol phase-II and microbial metabolites were quantified in pooled rat plasma using HPLC-MS/MS at different times (0, 2, 4, 7, 24, and 48 h) after the ingestion of GSPE (1000 mg/kg) in both healthy and MeS obese rats. Pooling the plasma was necessary to collect enough volume for three replicate chromatographic analyses at the different times without sacrificing the rats.

Moreover, pooling the plasma increased homogeneity and sensitivity to allow for the detection of all potential metabolites (Demelbauer, Plematl, Josic, Allmaier, & Rizzi, 2005; McGaw, Phinney, & Lowenthal, 2010). A range of time points was selected to detect all potential metabolites and to study how these metabolites appear in plasma at different times. All the flavanols from the extract were able to reach the major absorbing parts of the gastrointestinal tract (small intestine and colon) in the time course, which

assured their metabolization by phase-II enzymes and colonic bacteria (Serra et al., 2011, 2013).

We found increased plasma bioavailability of flavanols and their metabolites and a 2 h delay of the maximum plasma concentration in MeS obese rats compared to healthy rats. Maximum concentration values of total polyphenolic compounds were 170 μM at 4 h for CAF fed rats and 70 μM at 2 h for ST fed rats (Figure 1A). Moreover, the compounds present in ST rat plasma remained in the systemic circulation longer. Twenty-four hours after GSPE consumption, these compounds were present at higher concentrations in ST rats than in their MeS counterparts. Almost all the compounds found in MeS rat plasma were monomers, small phenolic compounds, and phase-II metabolites (Figure 1B). However, a reduced absorption of PA dimers (Figure 1C) and lower microbial metabolism was observed; both were decreased more than 3-fold in MeS rats (Figure 1D).

3.1. Plasma kinetics of non-metabolized flavanols in rat plasma

Figure 2 shows non-metabolized GSPE compounds (i.e. catechin and epicatechin monomers, dimers, and phenolic acids present in the pure extract). The maximum plasma concentration for the non-metabolized compounds was reached at 4 h for CAF rats and at 2 h for ST rats, except for gallic acid, which did not show clear differences in the time of maximum bioavailability between CAF and ST rats. In addition, no metabolized compounds remained in CAF rat plasma longer than in ST rats. Catechin maximum plasma concentrations were decreased in CAF rats compared to ST rats (0.4 and 1.6 μM, respectively), although epicatechin concentrations were equal (Figure 2A). Similarly, a decrease in the bioavailability of PA dimers was observed in CAF rats compared to ST rats. PA B2 maximum concentrations values were 1.2 μM at 4 h in CAF rats and 3.0 μM at 2 h in ST rats (Figure 2B). Gallic acid was the only non-metabolized compound found in plasma that showed an increased bioavailability in the MeS state, reaching plasma maximum concentrations 3 times higher in CAF rats compared to ST rats (5.4 μM and 1.7 μM both at 4 h, respectively) (Figure

2C). However, vanillic acid did not show changes in short-term plasma concentrations, but showed an increased bioavailability in CAF rats 24 h after the ingestion of GSPE.

3.2. Plasma kinetics of phase-II flavanol metabolites in rat plasma

Flavanol phase-II metabolites also showed the highest concentrations in plasma at 2 h after GSPE consumption in the ST group and at 4 h in the CAF group (Figure 3). In addition, all glucuronidated metabolites increased in the plasma of CAF rats compared to ST rats, reaching maximum concentration values of 64 μM in CAF rats and 15 μM in ST rats for catechin glucuronide and of 45 μM in CAF rats and 5 μM in ST rats for epicatechin glucuronide (Figure 3A). Methyl-glucuronidated metabolites also showed an increase in plasma bioavailability in CAF rats compared to the ST dietary group (Figure 3B). Interestingly, an inversion of the major methyl-glucuronidated compound was observed, as a maximum of 5 μM methyl-epicatechin glucuronide was detected in ST rats, increasing to 10 μM in CAF rats. Methyl-catechin glucuronide reached maximum concentrations at 1.3 μM in ST rats and 20 μM in CAF rats, becoming the major methyl-glucuronidated compound in plasma.

Catechin sulfated metabolites showed a reduction in CAF compared to ST rats (Figure 3C), from 1.5 to 0.4 μM, respectively. Epicatechin sulfate was increased 5-fold in CAF rats (1 μM) compared with the ST group (0.2 μM). Methyl-sulfated metabolites (Figure 3D) showed the same crossover kinetics as sulfated metabolites. The methyl-catechin-O-sulfate was the major metabolite in ST rats, whereas the epicatechin homologue was the major metabolite found in CAF obese rats.

Finally, methylated epicatechin metabolites showed an almost two-fold decrease in obese rats compared with ST rats (Figure 3E). 4-methyl-epicatechin was the major metabolite identified in both cases (0.6 μM and 1.2 μM respectively). Metabolites methylated at the third position measured 0.7 μM in ST rats and 0.3 μM in CAF rats.

3.3 Plasma kinetics of microbial flavanol metabolites in rat plasma

The kinetic profiles of all studied microbial flavanol metabolites was significantly different between the two dietary groups (Figure 4). The products of flavanol microbial catabolism showed a very sharp decrease in plasma concentration in the CAF obese rats for almost all compounds (Figure 4).

For valeric acid derivatives (Figure 4A), the main plasma metabolite found in both dietary groups was 4-hydroxy-5-(3',4'-dihydroxyphenyl) valeric acid, which reached maximal concentrations of 0.25 μM in the ST and of 0.10 μM in the CAF group. The microbial metabolite 3-(4-hydroxyphenyl) propionic acid was the main metabolite found in the plasma of healthy rats, with values reaching 25 μM 24 h after the ingestion of GSPE (Figure 4B). However, in the plasma of the CAF group, 3-(4-hydroxyphenyl) propionic acid did not exceed 0.2 μM. 2-(4-hydroxyphenyl)acetic acid was the major phenylacetic compound found in rat plasma, with concentrations of 5 μM in ST rats at 2 h, but only 0.12 μM in the CAF rats (Figure 4C). Benzoic acids are the only non-conjugated catabolic products found at considerable concentrations in CAF rats (Figure 4D). Benzoic acid was the most abundant compound in both groups, reaching maximal concentrations of 1.9 μM in ST rats and 0.7 μM in CAF rats.

The final compounds of microbial metabolism had both quantitative and qualitative differences in ST rats compared with CAF rats (Figure 4E). In ST rats, the major compound was hippuric acid, which reached its maximum concentration 7 h after GSPE consumption (10 μM), but it was not detected in the plasma of CAF rats at any time. The methylated metabolites of gallic acid (3-*O*-methyl gallic acid) and homovanillic acid were the only microbial metabolites that were found at higher concentrations in CAF rats. The microbial derivative 3-*O*-methyl gallic acid reached maximum values of 7 and 5.7 μM in CAF and ST rats, respectively, while homovanillic acid was found only in CAF rats, with maximum values of 2.9 and 3.2 μM at 4 and 24 h after GSPE ingestion, respectively.

4. Discussion

Flavanols are some of most important dietary polyphenols, with beneficial health effects (Del Rio et al., 2013) reported in both *in vitro* (González-Abuín et al., 2014; Guerrero et al., 2013; Margalef, Guerrero, et al., 2014) and *in vivo* (Guerrero et al., 2013; Margalef, Guerrero, et al., 2014; Z Pons et al., 2014; Zara Pons et al., 2015) models. The first pass metabolism is one of the most important aspects of flavanol biotransformation, as the molecular forms that reach the systemic circulation differ significantly from those present in ingested foods (Kroon et al., 2004). Therefore, factors that may affect flavanol metabolism influence its bioactivity. Specifically, the health state is a host-related factor of special relevance, as it may influence both the metabolism and the bioactivity of flavanols and their health-promoting properties. For instance, flavanols exhibit potent antihypertensive effects in hypertensive rats but not in normotensive animals (Cienfuegos-Jovellanos et al., 2009; Zara Pons et al., 2015). Owing to these facts, the aim of this study was to evaluate whether flavanol metabolism and plasma bioavailability kinetics differ in a metabolic pathological state using an animal model of obesity induced by a CAF diet.

Previous studies by our group and others showed that after an acute dose of grape seed flavanols to healthy animals, flavanols and their phase-II metabolites are distributed throughout the body and appear in the plasma and tissues at maximal concentrations between 1 and 2 h after consumption (Guerrero et al., 2013; Margalef et al., 2015a, 2015b; Margalef, Guerrero, et al., 2014). Interestingly, the results obtained in this study clearly showed that when metabolism is disrupted by MeS, maximum concentration values are delayed 2 h, reaching maximal amounts in the plasma 4 h after flavanol ingestion, instead of 2 h as observed in the ST group. These data indicate an alteration of flavanol absorption in obese animals. Our results also showed higher concentrations of total phenolic compounds in CAF rats (non conjugated flavanols, phase-II metabolites and small phenolic compounds), indicating higher absorption at the level of the small intestine of small phenolic compounds and flavanol monomers, which appear in plasma as free

and conjugated monomers. Our combined data together point to the fact that in metabolic disorders, intestinal permeability is altered by the disruption of tight junctions within the small intestine, which affects the passive diffusion of flavanol aglycones through the enterocytes (Manach et al., 2004; Miele et al., 2009). Interestingly, in CAF-fed rats, the plasma levels of PA dimers were significantly lower than in healthy rats. Therefore, PA dimers probably have a specific and undescribed active transport mechanism that is disrupted by the MeS state. Moreover, CAF fed rats showed a faster decrease in total flavanol plasma levels, which could be explained by the over-activation of phase II enzymes increasing the hydrosoluble, easily extractable forms.

This enzymatic alteration is in agreement with the higher plasma levels of phase-II metabolites in CAF rats that would be indicative of over-activation of hepatic phase-II enzymes. Specifically, our data suggest over-activation of UGT activity, while SULT and COMT activities seem to be inhibited by the metabolic disease induced by CAF diet. This fact, our data agree with prior reports of higher expression of UGT genes and lower activity of SULT enzymes in non-alcoholic fatty liver disease obese mice (Merrell & Cherrington, 2011). Interestingly, SULT enzymes showed different steroselectivety for catechin and epicatecnin depending on health status. In fact, catechin-sulfated and methyl-sulfated metabolites showed higher concentrations in ST rats and were decreased in CAF animals. On the contrary, epicatechin metabolites were increased in CAF rats and decreased in ST rats.

Finally, it is known that obesity significantly affects the gut microbiota (DiBaise et al., 2008; Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh et al., 2006). In fact, gut microbiota show daily changes depending on lifestyle (David, Materna, et al., 2014) and diet (David, Maurice, et al., 2014; Graf et al., 2015). In addition, polyphenols have been reported as microbiota modulators (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Duda-Chodak, Tarko, Satora, & Sroka, 2015; Viveros et al., 2011), and their microbial metabolites have health-promoting effects as

antioxidants (Bialonska, Kasimsetty, Khan, & Ferreira, 2009; Ishimoto et al., 2012). Therefore, flavanol microbial metabolites were affected in obese rats. This study clearly shows that obesity leads to a decrease in bioavailable microbial metabolites. The importance of these findings should be studied in greater detail, taking into account that many of the bioactivities of many polyphenols are related to their microbial metabolites (Del Rio et al., 2013; Selma, Espín, & Tomás-Barberán, 2009).

In conclusion, we demonstrated that metabolic disorder induced by the diet significantly influences the metabolism, plasma bioavailability and kinetics of flavanols, leading to qualitative and quantitative differences in circulating flavanol levels, their phase II metabolites and their microbial metabolites over time. These differences could explain why flavanols show health benefits in a pathological state but not in a healthy state. Therefore, flavanol studies carried out in healthy subjects should not be extrapolated to the unhealthy ones.

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Figure Legends

Figure 1. Kinetic profiles of compounds in pooled plasma (n=5), quantified by HPLC-MS/MS in standard (ST) and cafeteria-fed (CAF) rats at 0, 2, 4, 7, 24, and 48 h after the ingestion of a grape seed polyphenol extract (GSPE 1000 mg/kg). Plasma concentrations are given in μM. A) Total plasma polyphenolic compounds; B) Flavanol monomers (catechin and epicatechin), small phenols (gallic and vanillic acids), and flavanol phase-II metabolites; C) PA Dimers; D) Microbial metabolites.

Figure 2. Kinetic behavior of non-metabolized flavanols in pooled plasma (n=6) quantified by HPLC-MS/MS in standard (ST) and cafeteria-fed (CAF) rats at 0, 2, 4, 7, 24, and 48 h after the ingestion of a grape seed polyphenol extract (GSPE 1000 mg/kg). Plasma concentrations are given in μM. A) flavanol monomers; B) PA dimers; C) phenolic acids.

Figure 3. Kinetic behavior of phase-II flavanol metabolites in pooled plasma (n=6) quantified by HPLC-MS/MS in standard (ST) and cafeteria-fed (CAF) rats at 0, 2, 4, 7, 24, and 48 h after the ingestion of a grape seed polyphenol extract (GSPE 1000 mg/kg). Plasma concentrations are given in μM. A) glucuronidated metabolites; B) methyl-glucuronidated metabolites; C) sulfated metabolites; D) methyl-sulfated metabolites; E) methylated metabolites.

Figure 4. Kinetic behavior of microbial flavanol metabolites in pooled plasma (n=6) quantified by HPLC-MS/MS in standard (ST) and cafeteria-fed (CAF) rats at 0, 2, 4, 7, 24, and 48 h after the ingestion of a grape seed polyphenol extract (GSPE 1000 mg/kg). Plasma concentrations are given in μM. A) valeric acids metabolites; B) phenylpropionic acids metabolites; C) phenylacetic acids metabolites; D) benzoic acids metabolites; E) final products of microbial metabolism.

Table 1. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1[1]	88.80 ± 3.46
PA dimer B3[1]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[1]	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin[2]	0.27 ± 0.03
PA trimer[1]	4.90 ± 0.47
PA tetramer[1]	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

1 Quantified using the calibration curve of proanthocyanidin B2.

2 Quantified using the calibration curve of epigallocatechin gallte.

Figure 1

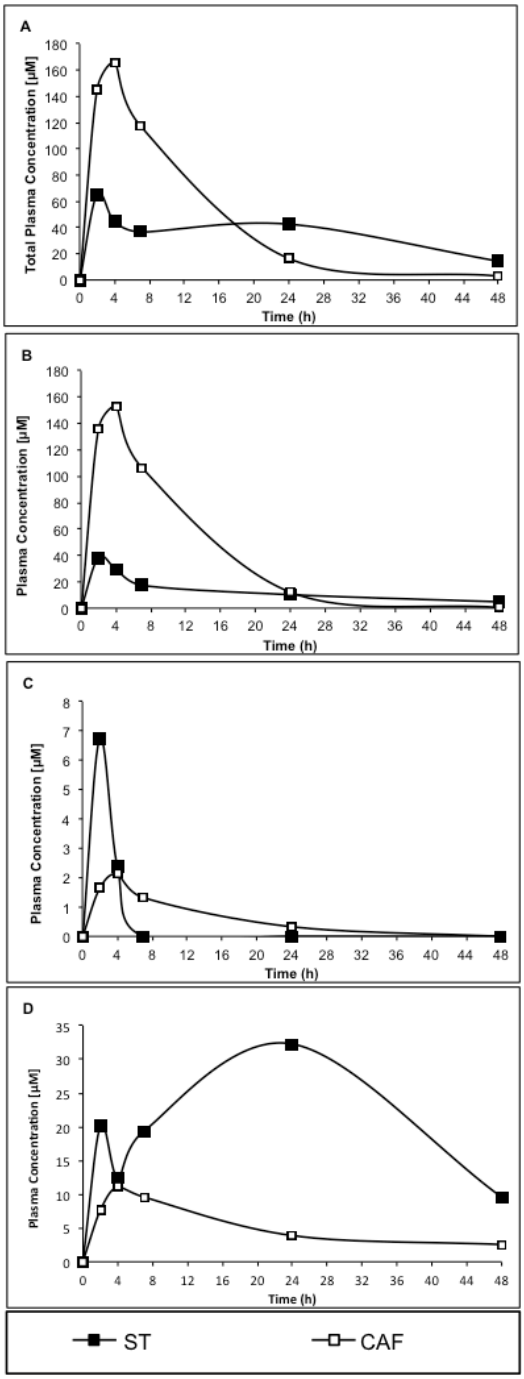
A
Total Plasma Concentration [µM]
Time (h)
B
Plasma Concentration [µM]
Time (h)
C
Plasma Concentration [µM]
Time (h)
D
Plasma Concentration [µM]
Time (h)
ST
CAF

Figure 2.

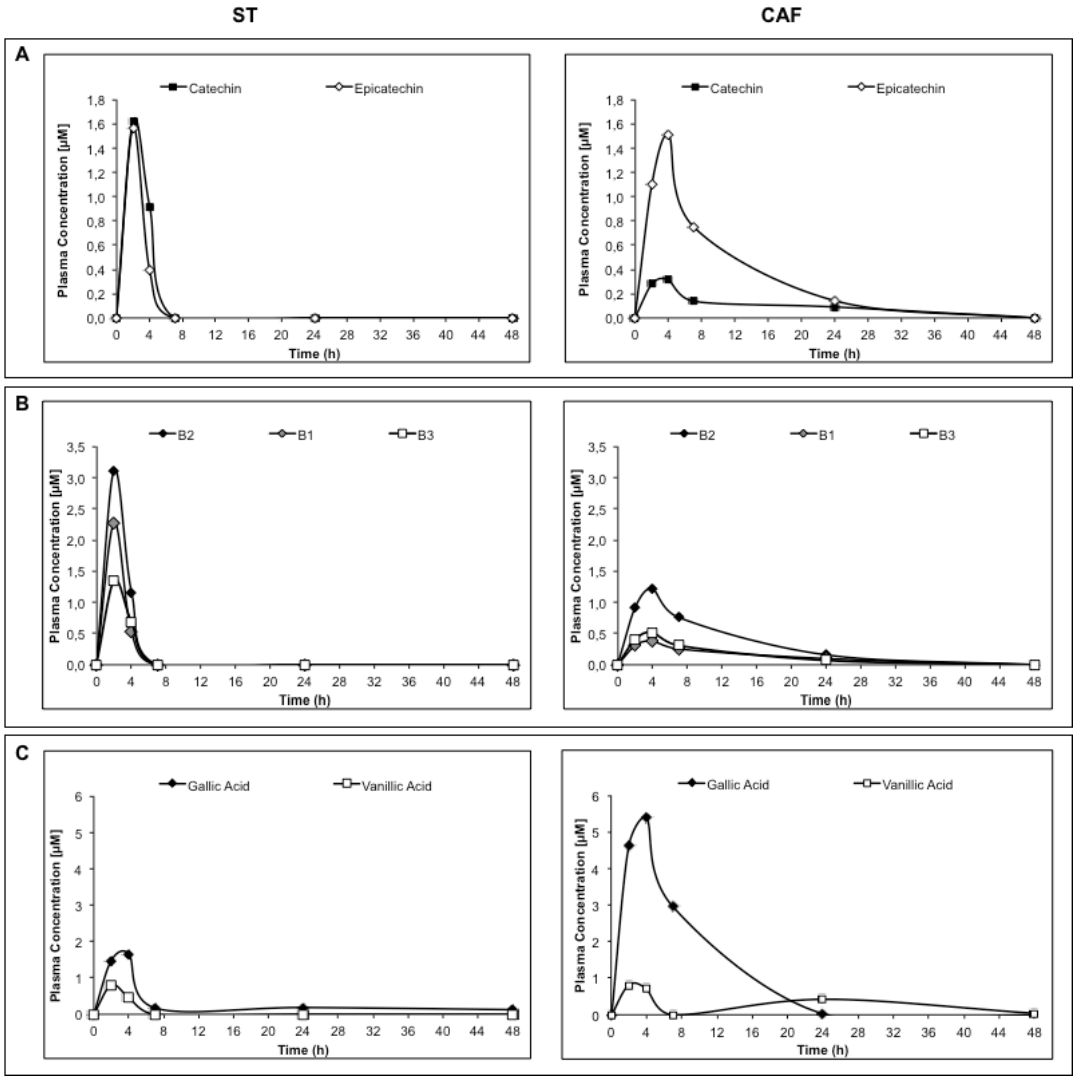
ST
CAF
A
Catechin
Epicatechin
Plasma Concentration [µM]
Time (h)
B
B2
B1
B3
C
Gallic Acid
Vanillic Acid

Figure 3.

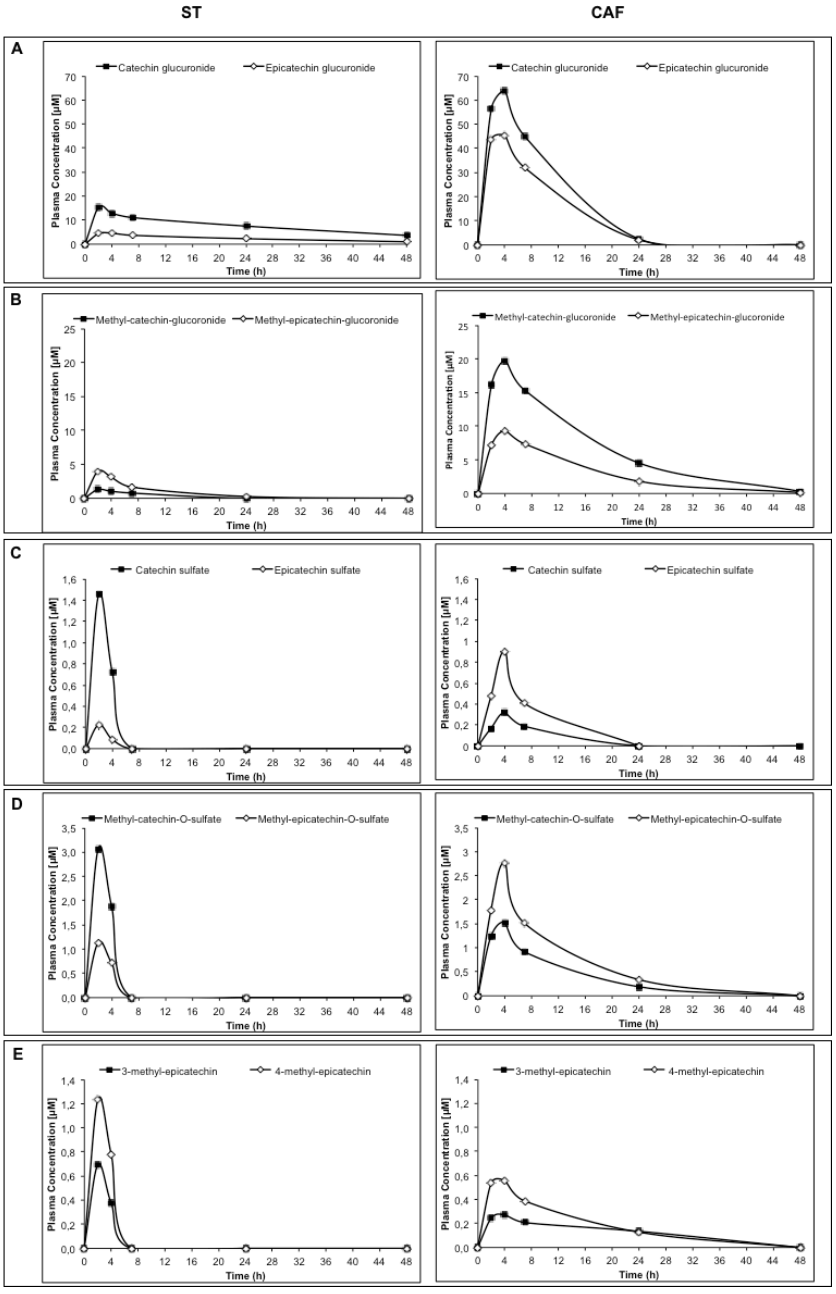
ST
CAF
A
Catechin glucuronide
Epicatechin glucuronide
B
Methyl-catechin-glucoronide
Methyl-epicatechin-glucoronide
C
Catechin sulfate
Epicatechin sulfate
D
Methyl-catechin-O-sulfate
Methyl-epicatechin-O-sulfate
E
3-methyl-epicatechin
4-methyl-epicatechin
Plasma Concentration [μM]
Time (h)

Figure 4.

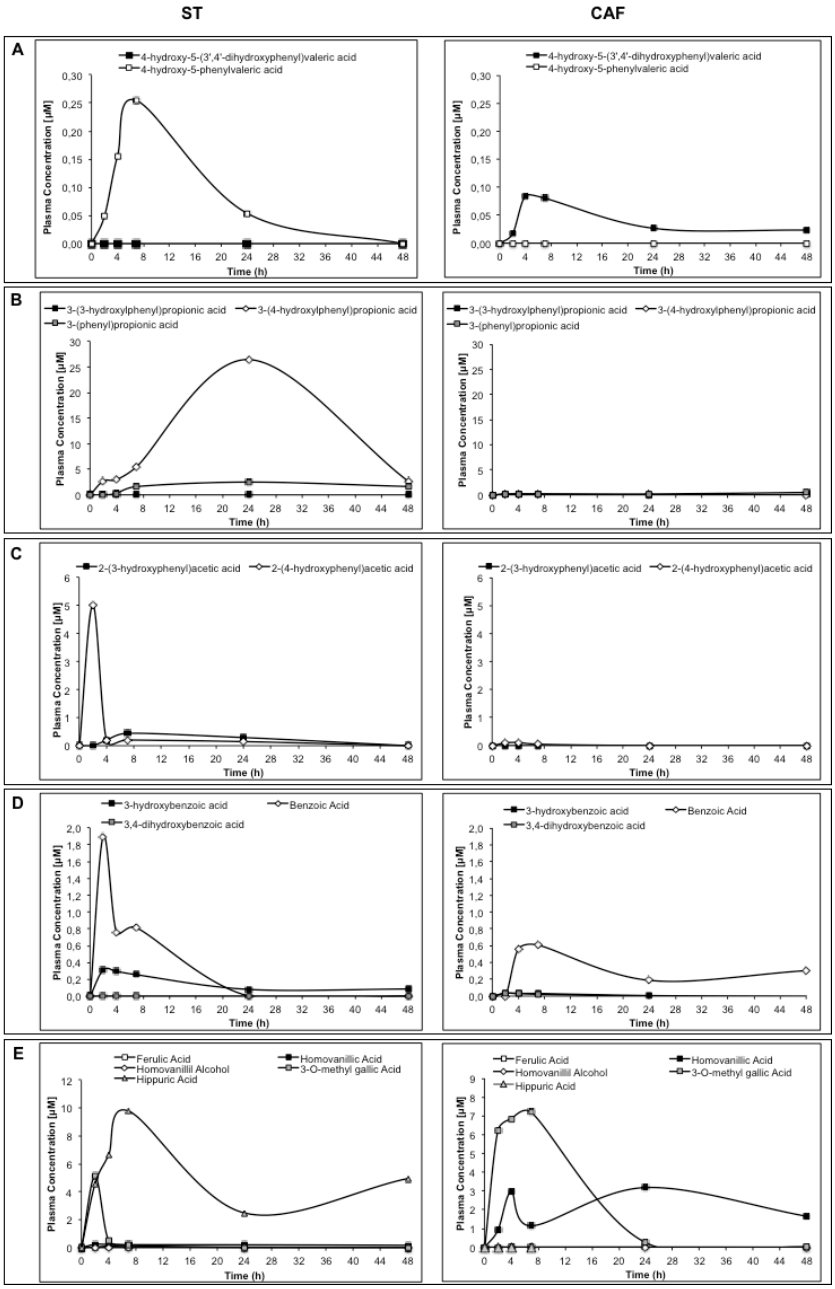
ST
CAF
A
4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid
4-hydroxy-5-phenylvaleric acid
B
3-(3-hydroxylphenyl)propionic acid
3-(4-hydroxylphenyl)propionic acid
3-(phenyl)propionic acid
C
2-(3-hydroxyphenyl)acetic acid
2-(4-hydroxyphenyl)acetic acid
D
3-hydroxybenzoic acid
Benzoic Acid
3,4-dihydroxybenzoic acid
E
Ferulic Acid
Homovanillic Acid
Homovanillil Alcohol
3-O-methyl gallic Acid
Hippuric Acid
Plasma Concentration [μM]
Time (h)

Table S1. Validation of the determination of plasma flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in tissues from both diet groups, assessed by the following parameters: calibration curve, R^2, linearity range, LOD, and LOQ, MDL, and MQL.

Diet Group	Compound	Calibration Curve	Determination Coeficient (R^2)	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL* (nM)	MQL* (nM)
Standard Diet	Catechin	y=0.011x	0.992	0.03-17.22	0.86	2.75	0.006	0.020
	Epicatechin	y=0.031x	0.995	0.03-17.22	0.69	2.32	0.005	0.017
	Procyanidin dimer B2	y=0.022x	0.997	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.120x	0.995	0.06-29.39	0.26	0.94	0.002	0.007
	Vanillic Acid	y=0.313x	0.996	0.06-29.76	0.60	2.15	0.004	0.015
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.324x	0.991	0.04-24.04	0.09	0.43	0.001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.056x	0.997	0.06-30.12	0.17	0.60	0.001	0.004
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.012x	0.996	0.06-29.76	0.17	0.60	0.001	0.004
	2-(3-hydroxyphenyl) acetic acid	y=0.061x	0.991	0.06-32.90	0.17	0.60	0.001	0.004
	2-(4-hydroxyphenyl) acetic acid	y=0.085x	0.992	0.06-32.90	0.17	0.60	0.001	0.004
	3,4-dihydroxybenzoic acid	y=0.228x	0.997	0.06-36.23	0.17	0.60	0.001	0.004
	3-hydroxybenzoic acid	y=0.242x	0.999	0.08-36.23	0.26	0.77	0.002	0.006
	Benzoic Acid	y=0.241x	0.997	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=0.311x	0.999	0.08-25.78	0.26	0.77	0.002	0.006
	Hippuric Acid	y=0.122x	0.999	0.05-27.93	0.17	0.52	0.001	0.004
Cafeteria Diet	Catechin	y=0.018x	0.997	0.03-17.22	0.86	2.75	0.006	0.018
	Epicatechin	y=0.026x	0.994	0.03-17.22	0.69	2.32	0.005	0.015
	Procyanidin dimer B2	y=0.084x	0.997	0.02-8.64	0.09	0.26	0.001	0.002
	Gallic Acid	y=0.093x	0.999	0.06-29.39	0.26	0.94	0.002	0.006
	Vanillic Acid	y=0.045x	0.996	0.06-29.76	0.60	2.15	0.004	0.014
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.374x	0.996	0.04-24.04	0.09	0.43	0.001	0.003
	3-(4-hydroxyphenyl) propionic acid	y=0.045x	0.992	0.06-30.12	0.17	0.60	0.001	0.004
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.012x	0.999	0.06-29.76	0.17	0.60	0.001	0.004
	2-(3-hydroxyphenyl) acetic acid	y=0.064x	0.995	0.06-32.90	0.17	0.60	0.001	0.004
	2-(4-hydroxyphenyl) acetic acid	y=0.045x	0.996	0.06-32.90	0.17	0.60	0.001	0.004
	3,4-dihydroxybenzoic acid	y=0.663x	0.993	0.06-36.23	0.17	0.60	0.001	0.004
	3-hydroxybenzoic acid	y=0.377x	0.995	0.08-36.23	0.26	0.77	0.002	0.005
	Benzoic Acid	y=0.265x	0.996	0.07-40.98	0.17	0.69	0.001	0.005
	Ferulic Acid	y=0.496x	0.997	0.08-25.78	0.26	0.77	0.002	0.005
	Hippuric Acid	y=0.117x	0.991	0.05-27.93	0.17	0.52	0.001	0.003

* MDL and MQL for the analysis of 250 μL of plasma.
Abbreviations: Limit of detection (LOD); limit of quantification (LOQ); method detection limit (MDL); method quantification limit (MQL).

MANUSCRIPT 10:

Rat health status affects target tissue levels and bioactivity of flavanols

Maria Margalef[†], Zara Pons[†], Lisard Iglesias-Carres[†], Francisca Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†] Nutrigenomic Research Group. Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

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Rat health status affects target tissue levels and bioactivity of flavanols

Maria Margalef[†], Zara Pons[†], Lisard Iglesias-Carres[†], Mar Quiñones [†¥], Francisca Isabel Bravo[†], Begoña Muguerza[†§*], Anna Arola-Arnal[†]

[†]Nutrigenomic Research Group. Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, 43007 Spain. [¥] Department of Physiology, CIMUS, University of Santiago de Compostela – Instituto de Investigación Sanitaria, Santiago de Compostela, 15782, Spain. [§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain

Running title: Rat health status affects flavanol bioavailability and bioactivity

*__Corresponding author:__ Begoña Muguerza

Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili

C/Marcel.lí Domingo s/n, 43007 Tarragona, Spain

Phone: +34 977559566

Fax: +34 977558232

E-mail: begona.muguerza@urv.cat

Abstract

The study of the flavanol metabolism is essential to identify the compounds involved in their bioactivity, as beneficial effects of flavanols have been attributed to their metabolic products,. However, host-related factors, such as pathological conditions, may affect flavanol metabolism and in turn, their bioactivity. The aim of this study was to elucidate whether the health status affects the flavanol metabolism, influencing their bioactivity in relation to hypertension. Blood pressure (BP) effect of flavanols was studied in spontaneously hypertensive rats and healthy Wistar rats at 6 h after the administration of 375 mg/kg of a grape seed extract rich in flavanols. Then animals were sacrificed, and plasma bioavailability and aorta distribution of flavanol metabolites were studied by HPLC-MS/MS in both groups. This study demonstrates important differences in bioactivity and target tissue flavanol derivative levels between healthy and diseased rats, indicating those flavanol forms most probably responsible of the antihypertensive effect.

Key words: aorta; bioavailability; colonic metabolites; essential hypertension; grape seed polyphenols.

1. Introduction

Hypertension (HTN) is a major risk factor for the development of cardiovascular disease (Papadogiannis & Protogerou, 2010) and is also the most common disease diagnosed in primary care (Chobanian et al., 2003). The incidence of HTN is estimated to increase over 24% in developed countries and to 80% in developing countries by 2025 (Messerli, Williams, & Ritz, 2007). Spontaneously hypertensive rats (SHR) are one of the most used experimental models of HTN. These animals present high blood pressure (BP) values at approximately 4-6 weeks of life without pharmacological or surgical intervention (Zicha & Kunes, 1999). The importance of this model has been attributed to the similarity of its pathophysiology to essential HTN in humans (Trippodo & Frohlich, 1981).

Increasing evidence suggests that a vegetable and fruit-rich diet, which is abundant in polyphenolic compounds, helps to control BP. In fact, increased fruit and vegetable intake has been included in the guidelines for the management of arterial HTN (Mancia et al., 2007). Grapes are a significant source of polyphenols, particularly flavanols. Flavanols, or flavan-3-ols, are characterized as one of the most important plant dietary polyphenol groups that can be found in grapes, apples, cocoa, red wine, and green tea (Aherne & O'Brien, 2002). Their monomers consist of (+)-catechin and (-)-epicatechin units that can be found in oligomeric and polymeric forms, also known as proanthocyanidins (PAs). Their beneficial health effects have been demonstrated for years in human (Del Rio et al., 2013; Manach et al., 2005; Williamson & Manach, 2005) and animal (Baselga-Escudero et al., 2013; Pinent et al., 2006; Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2015; M. Quiñones et al., 2013) studies, attributing them healthy properties (Fernández-Iglesias et al., 2014; Guerrero et al., 2013; Margalef, Guerrero, et al., 2014; Martinez-Micaelo, González-Abuín, Ardèvol, Pinent, & Blay, 2012; Pinent et al., 2004; Pons et al., 2015; M. Quiñones et al., 2013). Our research group has previously demonstrated an anti-hypertensive effect as a result of grape seed extract rich in flavanols (GSPE) administration in

hypertensive rats, with 375 mg/kg being the most effective dose and 6 h post-administration the time at which the maximum decrease of BP was observed (Pons et al., 2015; M. Quiñones et al., 2013).

However, as the beneficial effects of flavanols have been attributed to their metabolic products, their study is essential to understand the mechanism involved in flavanol bioactivity (Kroon et al., 2004). As non-essential nutrients in the diet, once ingested, flavanols and PA dimers are recognized as xenobiotics (Monagas et al., 2010). Thus, they are absorbed in the small intestine and undergo phase-II enzymatic detoxification *in situ* or in the liver (Del Rio et al., 2013; Monagas et al., 2010). Moreover, non-absorbed flavanols, metabolites from enterohepatic circulation via bilis, and all PAs with a degree of polymerization higher than 2 reach the colon, where they are subjected to microbial metabolism to form smaller phenolic compounds as a result of microbial cleavage of flavanol structures capable of reaching the liver to undergo phase-II metabolism (Aura, 2008; Monagas et al., 2010). Next, all metabolic products and non-metabolized compounds reach the systemic circulation to be distributed throughout the organism or to be excreted through the urine (Del Rio et al., 2013; Monagas et al., 2010). In addition, several external factors (Bohn et al., 2015; D'Archivio et al., 2007), such as the amount of consumed polyphenol (Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015b; Margalef, Guerrero, et al., 2014) or the length of ingestion, and host-related factors, such as the gender, age, or the pathological state of the studied subject (D'Archivio et al., 2007), may affect the metabolism, bioavailability and distribution of polyphenols in general and in particular flavanols. Therefore, the aim of this study was to elucidate whether rat health status affects flavanol metabolism, influencing their bioactivity. To assess this objective, the plasma bioavailability and aorta distribution of flavanols and their metabolites were studied in hypertensive and healthy rats 6 h after the ingestion of 375 mg/kg of GSPE, and the obtained levels of these compounds were related to their BP effects.

2. Material and Methods

Methanol (Scharlab S.L., Barcelona, Spain), acetone, acetonitrile (both from Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Individual stock standard solutions of 2000 mg/L (+)-catechin, (-)-epicatechin, procyanidin B2, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid, gallic acid, hippuric acid, ferulic acid, benzoic acid, and pyrocatechol as the internal standard (IS) (all from Fluka/Sigma-Aldrich, Madrid, Spain), as well as 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany) in methanol were prepared and stored in dark glass flasks at -20 ºC.

A stock standard solution (20 mg/L) of all compounds described above in methanol was prepared weekly and stored at -20 ºC. This stock standard solution was diluted daily to the desired concentrations using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.1. Grape Seed Polyphenol Extract (GSPE)

GSPE was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). Table 1 shows the phenolic compound (flavan-3-ols and phenolic acids) concentrations of the extract used in this study.

2.2. Experimental Procedure in Rats

Male Wistar (Crl:WI, n=12) and SHR (n=12), aged 22 weeks old (Charles River Laboratories, Barcelona, Spain), were singly housed in animal quarters at 22 ºC with a light/dark period of 12 h and fed a standard chow Panlab A04 (Panlab, Barcelona, Spain) diet and tap water *ad libitum* for a quarantine period of 2 weeks. The day of the experiment, animals were weighed (380-400 g for Crl:WI and 307-310 g for SHR) and divided into two different subgroups (water and GSPE, n=6 per group). Rats from the water group

were orally administered tap water (1 mL), while rats from the GSPE group were administered 375 mg/kg of GSPE (1 mL in water). In all groups, oral administration was performed by gastric intubation of fasted rats between 9 and 10 am. BP values were recorded by the tail-cuff method (Buñag, 1973) in both the GSPE and water groups at 6 h after GSPE or water administration. The 6 h time point was selected because it is the time of the maximum drop in BP (M. Quiñones et al., 2013). Next, all animals were sacrificed by exsanguination. Blood and the aorta were excised from the rats. Plasma samples were obtained by centrifugation (2000 x g, 15 min, 4 ºC) and stored at -80 ºC until chromatographic analysis was performed. Aortas were stored under the same plasma conditions. Plasma and aortas from the water group were used to create the calibration curves for the chromatography analysis. The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of Universitat Rovira i Virgili.

2.3. Flavanol and flavanol metabolite extraction from plasma and aorta

Prior to chromatographic analysis of the flavanols and their metabolites in rat plasma and aortas, the samples were pre-treated using the previously reported methodology based on a micro solid-phase extraction (μSPE) for plasma and an off-line liquid–solid extraction (LSE) in tandem with a μSPE for the aorta (Margalef et al., 2015b; Margalef, Pons, Muguerza, & Arola-Arnal, 2014).

2.4. Chromatographic analysis

Chromatographic analysis was performed using a 1290 LC Series UHPLC coupled to a 6490 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150 mm × 2.1 mm i.d., 3.5 μm particle size) as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA). The MS system consisted of an Agilent Jet Stream (AJS) ionization source. The mobile phase, electrospray ionization (ESI) conditions

and acquisition method were performed as previously reported for the quantification of phase-II and microbial flavanol metabolites in plasma and tissues (Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015a; Margalef et al., 2015b). Data acquisition was performed using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

2.5. Sample quantification

For sample quantification, a pool of blank aorta extracts or plasma from the Crl:WI (n=6) and SHR (n=6) rats that were administered water were spiked with 7 different concentrations of standards to obtain calibration curves for Crl:WI and SHR rats. The studied compounds in the samples were quantified by interpolating the analyte/IS peak abundance ratio in the resulting standard curves. Quality parameters, such as calibration curve detection and quantification limits (LOD and LOQ, respectively) and method detection and quantification limits (MDL and MQL, respectively), are shown in Table S1 for Crl:WI and in Table S2 for SHR rats.

The flavanols and phenolic acids present in the blank plasma and tissue (i.e., from the rats administered with water) quantified by HPLC-MS/MS were considered to emerge from the diet. Hence, all quantified values were subtracted from any other concentration found after GSPE ingestion to quantify only the compounds derived from the GSPE.

3. Results

3.1. Effect of GSPE on arterial blood pressure

The administration of GSPE resulted in a significant decrease in BP at 6 h in SHR but not in healthy Wistar rats (Figure 1). Conversely, water administration did not lead to changes in BP (data not shown).

3.2. Plasma flavanol metabolites in healthy and spontaneously hypertensive rats

The total amount of bioavailable plasma flavanols in healthy Wistar rats (135.27 ± 20.63 μM) and SHR rats (162.39 ± 14.69 μM) are very similar

(Figure 2A). However, although the plasma flavanol phase-II metabolites levels in healthy rats (128.91 ± 20.42 μM) and SHR (126.14 ± 12.34 μM) are also very similar, the plasma free flavanol monomer levels in SHR (2.6 ± 0.19 μM) are higher than in healthy rats (1.94 ± 0.55 μM). Nevertheless, important differences are displayed at the level of individual metabolite concentrations in the plasma (Table 2). For instance, plasma gallic acid levels in SHR are approximately 10 times more concentrated than in healthy rats. Moreover, methyl-epicatechin-O-sulfate is more than 4 times more concentrated in SHR than in healthy rat plasma, whereas the sulfated metabolites were not quantified in the plasma of SHR, though their concentrations in Wistar rats ranged from 0.04 ± 0.00 and 0.19 ± 0.04 μM.

Furthermore, significant differences were observed in plasma microbial metabolites, as their concentrations in SHR are much higher (33.65 ± 4.70 μM) than in healthy rats (4.42 ± 0.37 μM). More specifically, this difference is observed for the conjugated microbial flavanol metabolites (Figure 2). In this case, the results showed quantitative differences between both health states and qualitative differences in the type of metabolites found in both groups of plasma (Table 3). Specifically, 3-(4-hydroxyphenyl)propionic acid, 3-*O*-methylgallic acid, and hippuric acid were the metabolites with the highest differences in healthy and hypertensive rats, with all of them exhibiting an increase in SHR plasma.

3.3. Aorta flavanol metabolites in healthy and spontaneously hypertensive rats

Aorta metabolite concentrations were significantly decreased in the tissue of hypertensive rats, with 78.61 ± 23.17 nmol/g in healthy rats and 45.02 ± 5.50 nmol/g in hypertensive rats (Figure 2B). Specifically, regarding the compounds being absorbed at the small intestine, a 2-fold decrease in phase-II metabolites was seen, decreasing from 71.72 ± 21.80 nmol/g to 30.06 ± 1.04 nmol/g in healthy and hypertensive rats, respectively. Moreover, the forms that were directly absorbed and not metabolized also showed a

significant decrease between healthy and hypertensive states, from 5.6 ± 1.35 to almost 0.96 ± 0.14 nmol/g, respectively. These changes arise as a result of decreased concentrations of catechins as unconjugated catechin, catechin-glucuronide and methyl-catechin-glucuronide in SHR aortas (Table 2). Conversely, methyl-epicatechin glucuronidtechin and epicatechin metabolite concentrations in SHR aortas are very similar (16.28 ± 1.33 and 13.78 ± 0.01 nmol/g, respectively), whereas in Wistar rats, catechin concentrations are much higher than epicatequin levels (58.48 ± 18.64 nmol/g and 13.24 ± 3.16 nmol/g, respectively).

The results showed increased microbial metabolite levels in the aortas of hypertensive rats (1.29 ± 0.03 nmol/g in healthy rats and 14.00 ± 4.32 nmol/g in SHR), primarily with increased concentrations of non-conjugated microbial products (Figure 2B). Additionally, individual microbial metabolite concentrations were quantitatively and qualitatively different in both health states, as 3-phenylpropionic acid, 4-hydroxybenzoic acid, benzoic acid and hippuric acid concentrations were increased in aortas of hypertensive rats (Table 3).

When the phase-II conjugated metabolites for both flavanols and microbial acids are compared with the non-conjugated compounds, the conjugated forms in aortas of hypertensive rats (34.71 ± 2.86 nmol/g) are less concentrated than in the aortas of healthy rats (72.31 ± 21.81 nmol/g). On the contrary, the non-conjugated forms in aortas of hypertensive rats (10.31 ± 2.63 nmol/g) are slightly more concentrated than in the aortas of healthy rats (6.30 ± 1.33 nom/g).

4. Discussion

The regular consumption of flavonoids has been associated with health-promoting effects in several diseases (Crozier, Jaganath, & Clifford, 2009; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Nevertheless, beneficial health properties of flavonoids, and in particular flavanols, are mainly attributed to the compounds derived from their

metabolism (Del Rio et al., 2013), as the molecular forms that reach the peripheral circulation and tissues are different from those present in foods due to changes that occur to these molecules during metabolism (Guerrero et al., 2013; Kroon et al., 2004; Margalef et al., 2015b; Margalef, Guerrero, et al., 2014). However, several conditions can interfere with flavanol metabolism (D'Archivio et al., 2007). Overall health status has been described as one host-related factor affecting the metabolism, bioavailability and bioactivity of flavanols (D'Archivio et al., 2007).

Grape seed flavanols showed an anti-hypertensive effect in SHR that was not reproduced in healthy Wistar rats. Thus, flavanols exhibited a selective anti-hypertensive effect specific to the hypertension condition. These results are in agreement with those previously reported by our group that showed an important anti-hypertensive effect of grape seed (M. Quiñones et al., 2013) and cocoa flavanols (Cienfuegos-Jovellanos et al., 2009) in SHR prior to unappreciable BP variations in the normotensive standardized model of SHR, the Wystar Kyoto animal model (WKY). Therefore, the obtained results corroborated that health status affects the bioactivity of flavanols.

Despite the differences found in flavanol bioactivity between both health statuses, the main flavanol metabolites and the total plasma polyphenolic concentrations showed similar values in healthy and hypertensive rats. These results are in agreement with the fact that essential hypertension is a located pathology that can hardly influence the metabolism of flavanols in terms of total plasma bioavailability. However, when results were analyzed individually, significant differences in plasma were shown between both health statues. In fact, unconjugated flavanols showed higher concentrations in SHR than in healthy rats, primarily due to an increase in plasma gallic acid concentrations in hypertensive rats. However, the most important plasma bioavailability difference between healthy and hypertensive rats was in microbial metabolism that seemed to be significantly over-activated in SHR. Nevertheless, these findings agree with the fact that high BP has been recently associated with gut microbiota dysbiosis, both in animal and human

hypertension (Yang et al., 2015). In addition, microbial metabolism over-activation is also in agreement with the high plasma gallic acid levels quantified in SHR rats compared to healthy rats because it has been described that the galloilated moiety is cleaved by the gut microbiota (Margalef et al., 2015a; Selma, Espín, & Tomás-Barberán, 2009). Flavanol monomers and their respective gallic acid residues can be released in the colon and cross the colonocytes to reach the liver and become conjugated by phase-II enzymes producing 3-*O*-methylgallic acid, which interestingly has also been found to be significantly increased in the plasma of hypertensive animals.

The anti-hypertensive effect of grape seed flavanols in SHR is an endothelium-dependent effect mainly mediated by changes in endothelium-derived factors such as nitric oxide and prostacyclin (Mar; Quiñones et al., 2014). Therefore, it would seem plausible that the endothelium of SHR presents higher concentrations of flavanol metabolites than that from healthy animals. However, our results showed lower flavanol endothelial levels in SHR compared to healthy rats. Nevertheless, it is well known that the highest concentrations of flavanols are not always the most effective in decreasing BP (Cienfuegos-Jovellanos et al., 2009; Pons et al., 2015; M Quiñones, Miguel, Muguerza, & Aleixandre, 2011; M. Quiñones et al., 2013). This fact can be explained by the pro-oxidant effects and excessive production of reactive oxygen species instead of the antioxidant properties caused by high doses of flavanols described previously (Azam, Hadi, Khan, & Hadi, 2004; Cotelle, 2001; Lahouel et al., 2007; Procházková, Bousova, & Wilhelmová, 2011). Decreased flavanol levels in aortas of SHR occurs primarily as a result of decreased catechin metabolites, which can be attributed to disrupted active transport driven by endothelial flavonoid transporters, such as bilirubin translocase, which has been revealed as a specific flavonoid transported in the endothelium (Maestro et al., 2010). Methyl-epicatechin glucuronide was the only compound found in the aorta that could be considered an exception to this decreased endothelial absorption, as it showed slightly elevated

concentrations in aortas of hypertensive rats. This result could be due to active transport driven by specific endothelial flavonoid transporters (Maestro et al., 2010) or also to the increased specificity of COMT in the endothelium, which has been reported to exhibit higher activity in aortas of hypertensive rats (Trajkov, Berkowitz, & Spector, 1974). Indeed, although the amount of (–)-epicatechin and its metabolites in aortas of SHR and healthy rats are similar, the proportion of (–)-epicatechin and its metabolites compared to the catechins increases significantly in aortas of hypertensive rats. This result is considered especially relevant because (–)-epicatechin is the monomeric flavanol that possesses better known anti-hypertensive properties (Ellinger, Reusch, Stehle, & Helfrich, 2012; Gómez-Guzmán et al., 2012). Nevertheless, our results showed similar final concentrations of total catechin and epicatechin forms in aortas of SHR and healthy rats. Therefore, based on these results, similar roles of catechin and epicatechin forms toward the anti-hypertensive effect of GSPE should not be ruled out. In fact, the BP lowering effect of catechin, similar to epicatechin, has recently been reported by our group (Quiñones et al., 2015).

Similar to the results reported in plasma, important differences in microbial metabolites between both health statuses were found in the aorta, with higher concentrations of these metabolites in hypertensive rats than in healthy rats. In addition, qualitative differences in compound type detected in this tissue were also observed, with most cases of the microbial metabolite uptake by hypertensive rat aortas being significantly higher. This result can be attributed to over-activation of microbial metabolism previously mentioned. In addition, disruption of tight junctions in the endothelium or a different morphology of the endothelial gap junctions (Hüttner, Costabella, De Chastonay, & Gabbiani, 1982) that allows these small compounds to cross the endothelial barrier in hypertensive rats should not be ruled out. Because the most significant plasma and aorta bioavailability differences between healthy and genetically hypertensive rats were attributed mainly to the microbial metabolism, which seemed to be significantly over-activated in

SHR, it would seem plausible that these metabolites could also be involved in the flavanol anti-hypertensive effect. As far as we know, this is the first time that microbial flavanol metabolites have been related to the compound's anti-hypertensive effect.

This study demonstrates that a health status of essential hypertension extensively affects the levels and bioactivity of flavanols in the aorta as one of the main dysfunctional tissues. The flavanol forms present in the aorta of hypertensive rats are those forms most likely responsible for the anti-hypertensive effects of these compounds, including the flavanol microbial colonic metabolites. Therefore, to assess the real mechanisms involved in flavanols' health benefits, a study of metabolism during this concrete pathological condition is necessary.

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Figure legends

Figure 1. Blood pressure decrements at 6 h after the ingestion of 375 mg/kg of a grape seed polyphenol extract in healthy and spontaneously hypertensive rats (SHR) rats.

Figure 2. Plasma and aorta distribution of total non-conjugated flavanols (i.e., no metabolized compounds and no conjugated microbial metabolites) and conjugated flavanol compounds (i.e., phase-II metabolites and conjugated microbial metabolites), quantified by HPLC-MS/MS in plasma (A) and aorta (B) of healthy rats (n=6) and spontaneously hypertensive rats (SHR) (n=6) at 6 h after the ingestion of 375 mg/kg of a grape seed polyphenol extract (GSPE).

Table 1. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1[1]	88.80 ± 3.46
PA dimer B3[1]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[1]	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin[2]	0.27 ± 0.03
PA trimer[1]	4.90 ± 0.47
PA tetramer[1]	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

[1] Quantified using the calibration curve of proanthocyanidin B2.

[2] Quantified using the calibration curve of epigallocatechin gallte.

Table 2. No metabolised flavanols and their phase-II metabolites from plasma and aorta of healthy rats (n=6) and sponstaneously hypertensive rats (SHR) (n=6) quantified by HPLC-MS/MS, at 6 h after the ingestion of GSPE (375 mg/kg). Data is given as the mean ± standard error of the mean (SEM).

Compound	Healthy rats		SHR	
	Plasma	Aorta	Plasma	Aorta
	µM	nmol/g	µM	nmol/g
Catechin	0.69 ± 0.46	2.56 ± 1.17	0.20 ± 0.05	0.25 ± 0.12
Epicatechin	0.37 ± 0.12	0.73 ± 0.22	0.24 ± 0.04	0.36 ± 0.07
PA dimer B1	0.14 ± 0.03	0.62 ± 0.09	0.03 ± 0.00	0.06 ± 0.02
PA dimer B2	0.25 ± 0.04	0.45 ± 0.05	0.06 ± 0.02	0.12 ± 0.04
PAdimer B3	0.20 ± 0.03	0.37 ± 0.11	0.05 ± 0.01	0.10 ± 0.03
Gallic acid	0.21 ± 0.03	0.75 ± 0.40	1.92 ± 0.20	0.07 ± 0.04
Vanillic	0.07 ±0.02	0.12 ± 0.11	0.10 ± 0.01	n.q
Catechin-glucuronide	58.20 ± 12.84	38.17 ± 15.02	57.73 ± 7.29	10.23 ± 0.64
Epicatechin-glucuronide	20.54 ± 4.33	10.01 ± 2.44	18.03 ± 2.25	8.32 ± 0.09
Methyl-catechin-glucuronide	38.01 ± 4.46	20.31 ± 3.62	29.56 ± 2.38	6.05 ± 0.39
Methyl-epicatechin-glucuronide	8.96 ±0.75	3.22 ± 0.72	14.58 ± 0.84	5.46 ± 0.08
Catechin-sulfate	0.19 ± 0.04	n.d.	n.q.	n.d.
Epicatechin-sulfate	0.04 ± 0.00	n.d.	n.q.	n.d.
Methyl-catechin-*O*-sulfate	1.37 ± 0.24	n.d.	1.38 ± 0.21	n.d.
Methyl-epicatechin-*O*-sulfate	0.95 ±0.21	n.d.	4.15 ± 0.40	n.d.
3-*O*-methyl-epicatechin	0.26 ± 0.04	n.d.	0.27 ± 0.03	n.d.
4-*O*-methyl-epicatechin	0.40 ±0.05	n.d.	0.43 ± 0.04	n.d.

Abbreviations: PA:proanthocyanidin; n.d: not detected; n.q: not quantified

Table 3. Flavanol microbial metabolites from plasma and aorta of healthy rats and spontaneously hypertensive rats (SHR) (n=6) at 6 h after the ingestion of GSPE (375 mg/kg) quantified by HPLC-MS/MS. Data is given as the mean ± standard error of the mean (SEM).

Compound	Healthy rats		SHR	
	Plasma	Aorta	Plasma	Aorta
	μM	nmol/g	μM	nmol/g
1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol	0.07 ± 0.01	n.d.	n.d.	n.d.
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	0.01 ± 0.00	0.23 ± 0.05	n.q.	0.01 ± 0.00
5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone	n.d.	n.d.	n.d.	n.d.
4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid	0.06 ± 0.03	n.d.	0.09 ± 0.05	n.d.
4-hydroxy-5-phenylvaleric acid	0.04 ± 0.02	n.d.	0.01 ± 0.00	n.d.
3-(3,4-dihydroxyphenyl)propionic acid	0.31 ± 0.02	n.d.	n.q.	n.d.
3-(4-hydroxyphenyl)propionic acid	0.65 ± 0.19	n.d.	5.55 ± 1.15	n.d.
3-(3-hydroxyphenyl)propionic acid	0.49 ± 0.16	n.d.	0.63 ± 0.10	n.d.
3-phenylpropionic acid	0.13 ± 0.06	n.d.	1.62 ± 0.52	2.20 ± 0.44
2-(3,4-dihydroxyphenyl)acetic acid	n.d.	n.d.	n.d.	n.d.
2-(4-hydroxyphenyl)acetic acid	0.24 ± 0.02	n.d.	1.03 ± 0.13	n.d.
2-(3-hydroxyphenyl)acetic acid	0.57 ± 0.11	n.d.	0.86 ± 0.12	n.d.
2-phenylacetic acid	n.d.	n.d.	n.d.	n.d.
3,4-dihydroxybenzoic acid	0.02 ± n.q.	n.d.	0.02 ± n.q.	0.08 ± n.q.
4-hydroxybenzoic acid	0.22 ± 0.02	0.47 ± 0.06	0.23 ± 0.03	3.68 ± 1.00
3-hydroxybenzoic acid	n.d.	n.d.	0.12 ± 0.05	n.d.
Benzoic acid	0.67 ± 0.05	n.q.	0.36 ± 0.05	3.37 ± 1.05
3-*O*-methylgallic acid	0.95 ± 0.16	0.28 ±	18.70 ±	0.49 ± 0.09
Homovanillic acid	n.q	n.d.	0.06 ±	0.25 ±0.09
Homovanillyl alcohol	n.d.	n.d.	n.d.	n.d.
Ferulic acid	0.01 ± 0.00	0.08 ± 0.01	0.01 ± 0.00	0.08 ± 0.01
Hippuric acid	n.q.	0.23 ± 0.06	4.35 ± 1.63	3.84 ± 1.63

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

Figure 1.

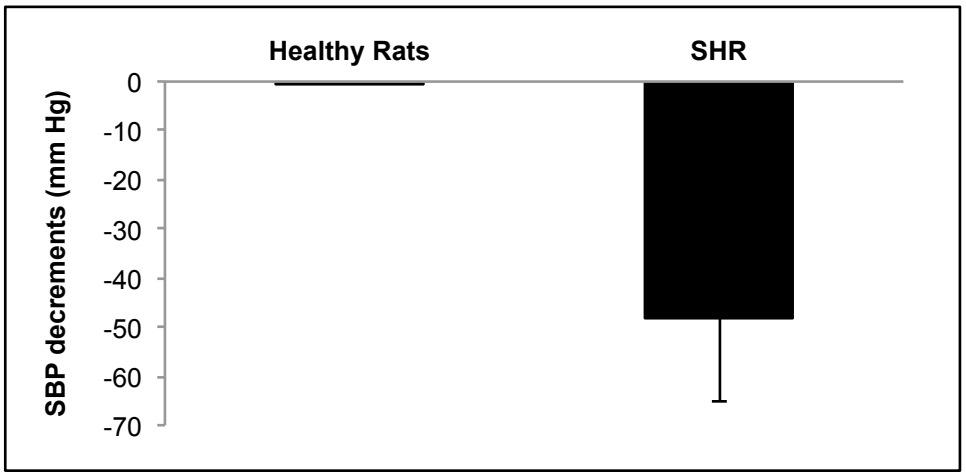

Figure 2.

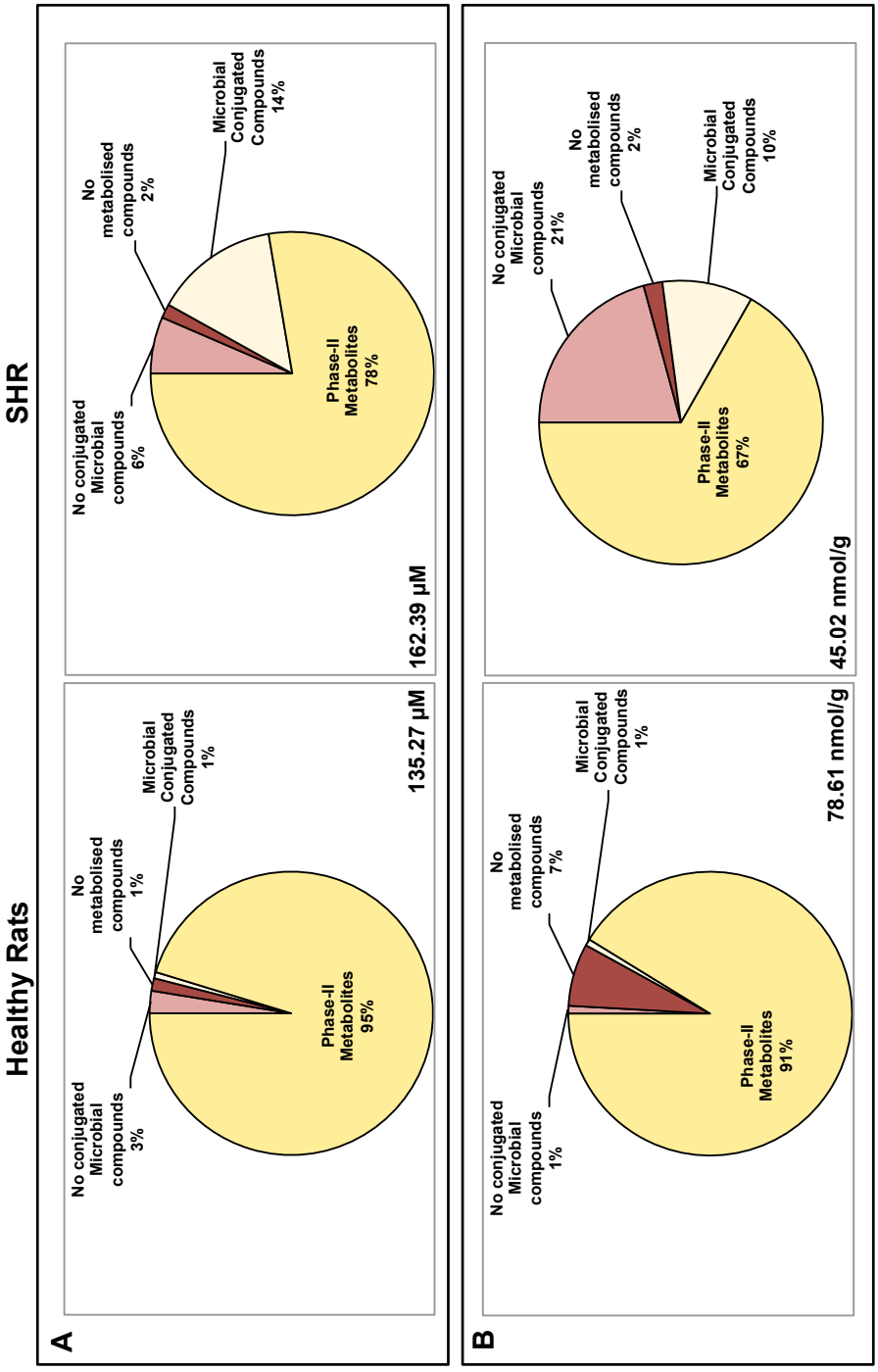
Healthy Rats
SHR
A
No conjugated Microbial compounds 3%
No metabolised compounds 1%
Microbial Conjugated Compounds 1%
Phase-II Metabolites 95%
135.27 µM
No conjugated Microbial compounds 6%
No metabolised compounds 2%
Microbial Conjugated Compounds 14%
Phase-II Metabolites 78%
162.39 µM
B
No conjugated Microbial compounds 1%
No metabolised compounds 7%
Microbial Conjugated Compounds 1%
Phase-II Metabolites 91%
78.61 nmol/g
No conjugated Microbial compounds 21%
No metabolised compounds 2%
Microbial Conjugated Compounds 10%
Phase-II Metabolites 67%
45.02 nmol/g

Table S1. Method validation for the determination of flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in the studied tissues from healthy rats assessed by the following parameters: calibration curve, R^2, linearity range, LOD, and LOQ, MDL, and MQL.

Diet Group	Compound	Calibration Curve	Determination Coeficient (R^2)	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL* (nM or pmol/g)	MQL* (nM or pmol/g)
Plasma	Catechin	y=0.011x	0.992	0.03-17.22	0.86	2.75	0.34	1.10
	Epicatechin	y=0.039x	0.995	0.03-17.22	0.69	2.32	0.28	0.93
	Procyanidin dimer B2	y=0.020x	0.997	0.02-8.64	0.09	0.26	0.04	0.10
	Gallic Acid	y=0.119x	0.994	0.06-29.39	0.26	0.94	0.10	0.38
	Vanillic Acid	y=0.313x	0.996	0.06-29.76	0.60	2.15	0.24	0.86
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.324x	0.991	0.04-24.04	0.09	0.43	0.04	0.17
	3-(4-hydroxyphenyl) propionic acid	y=0.056x	0.997	0.06-30.12	0.17	0.60	0.07	0.24
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.012x	0.996	0.06-29.76	0.17	0.60	0.07	0.24
	2-(3-hydroxyphenyl) acetic acid	y=0.061x	0.991	0.06-32.90	0.17	0.60	0.07	0.24
	2-(4-hydroxyphenyl) acetic acid	y=0.086x	0.992	0.06-32.90	0.17	0.60	0.07	0.24
	3,4-dihydroxybenzoic acid	y=0.228x	0.997	0.06-36.23	0.17	0.60	0.07	0.24
	3-hydroxybenzoic acid	y=0.242x	0.999	0.08-36.23	0.26	0.77	0.10	0.31
	Benzoic Acid	y=0.241x	0.997	0.07-40.98	0.17	0.69	0.07	0.28
	Ferulic Acid	y=0.311x	0.999	0.08-25.78	0.26	0.77	0.10	0.31
	Hippuric Acid	y=0.122x	0.998	0.05-27.93	0.17	0.52	0.07	0.21
Aorta	Catechin	y=0.024x	0.999	0.03-17.22	0.86	2.75	10.05	32.13
	Epicatechin	y=0.081x	0.999	0.03-17.22	0.69	2.32	8.06	27.10
	Procyanidin dimer B2	y=0.072x	0.999	0.02-8.64	0.09	0.26	1.05	3.04
	Gallic Acid	y=0.254x	0.999	0.06-29.39	0.26	0.94	3.04	10.98
	Vanillic Acid	y=0.629x	0.999	0.06-29.76	0.60	2.15	7.01	25.12
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.875x	0.999	0.04-24.04	0.09	0.43	1.05	5.02
	3-(4-hydroxyphenyl) propionic acid	y=0.067x	0.998	0.06-30.12	0.17	0.60	1.99	7.01
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.025x	0.999	0.06-29.76	0.17	0.60	1.99	7.01
	2-(3-hydroxyphenyl) acetic acid	y=0.057x	0.999	0.06-32.90	0.17	0.60	1.99	7.01
	2-(4-hydroxyphenyl) acetic acid	y=0.096x	0.999	0.06-32.90	0.17	0.60	1.99	7.01
	3,4-dihydroxybenzoic acid	y=0.511x	0.999	0.06-36.23	0.17	0.60	1.99	7.01
	3-hydroxybenzoic acid	y=0.517x	0.999	0.08-36.23	0.26	0.77	3.04	9.00
	Benzoic Acid	y=0.388x	0.998	0.07-40.98	0.17	0.69	1.99	8.06
	Ferulic Acid	y=0.882x	0.999	0.08-25.78	0.26	0.77	3.04	9.00
	Hippuric Acid	y=0.163x	0.999	0.05-27.93	0.17	0.52	1.99	6.07

Abbreviations: LOD: limit od detection; LOQ: limit of quantification; MDL: method detection limit; MQL: method quantification limit
*MDL and MQL in pmol/g for the analysis of 60mg of aorta or in nM for the analysis of 250 μL of plasma.

Table S2. Method validation for the determination of flavanols and phenolic acids by off-line μSPE-HPLC-MS/MS in the studied tissues from hypertensive rats assessed by the following parameters: calibration curve, R^2, linearity range, LOD, and LOQ, MDL, and MQL.

Diet Group	Compound	Calibration Curve	Determination Coeficient (R^2)	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL* (nM or pmol/g)	MQL* (nM or pmol/g)
Plasma	Catechin	y=0.011x	0.999	0.03-17.22	0.86	2.75	0.34	1.10
	Epicatechin	y=0.034x	0.999	0.03-17.22	0.69	2.32	0.28	0.93
	Procyanidin dimer B2	y=0.024x	0.998	0.02-8.64	0.09	0.26	0.04	0.10
	Gallic Acid	y=0.009x	0.999	0.06-29.39	0.26	0.94	0.10	0.38
	Vanillic Acid	y=0.309x	0.999	0.06-29.76	0.60	2.15	0.24	0.86
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.571x	0.998	0.04-24.04	0.09	0.43	0.04	0.17
	3-(4-hydroxyphenyl) propionic acid	y=0.031x	0.997	0.06-30.12	0.17	0.60	0.07	0.24
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.010x	0.999	0.06-29.76	0.17	0.60	0.07	0.24
	2-(3-hydroxyphenyl) acetic acid	y=0.030x	0.992	0.06-32.90	0.17	0.60	0.07	0.24
	2-(4-hydroxyphenyl) acetic acid	y=0.044x	0.999	0.06-32.90	0.17	0.60	0.07	0.24
	3,4-dihydroxybenzoic acid	y=0.245x	0.999	0.06-36.23	0.17	0.60	0.07	0.24
	3-hydroxybenzoic acid	y=0.243x	0.999	0.08-36.23	0.26	0.77	0.10	0.31
	Benzoic Acid	y=0.234x	0.999	0.07-40.98	0.17	0.69	0.07	0.28
	Ferulic Acid	y=0.359x	0.999	0.08-25.78	0.26	0.77	0.10	0.31
	Hippuric Acid	y=0.127x	0.994	0.05-27.93	0.17	0.52	0.07	0.21
Aorta	Catechin	y=0.053x	0.998	0.03-17.22	0.86	2.75	9.01	28.83
	Epicatechin	y=0.085x	0.993	0.03-17.22	0.69	2.32	7.23	24.32
	Procyanidin dimer B2	y=0.075x	0.999	0.02-8.64	0.09	0.26	0.94	2.73
	Gallic Acid	y=0.254x	0.999	0.06-29.39	0.26	0.94	2.73	9.85
	Vanillic Acid	y=0.646x	0.999	0.06-29.76	0.60	2.15	6.29	22.54
	5-(3',4'-dihydroxyphenyl)-γ-valerolactone	y=0.705x	0.997	0.04-24.04	0.09	0.43	0.94	4.51
	3-(4-hydroxyphenyl) propionic acid	y=0.070x	0.996	0.06-30.12	0.17	0.60	1.78	6.29
	2-(3,4-dihydroxyphenyl) acetic acid	y=0.025x	0.998	0.06-29.76	0.17	0.60	1.78	6.29
	2-(3-hydroxyphenyl) acetic acid	y=0.065x	0.999	0.06-32.90	0.17	0.60	1.78	6.29
	2-(4-hydroxyphenyl) acetic acid	y=0.105x	0.999	0.06-32.90	0.17	0.60	1.78	6.29
	3,4-dihydroxybenzoic acid	y=0.538x	0.999	0.06-36.23	0.17	0.60	1.78	6.29
	3-hydroxybenzoic acid	y=0.549x	0.999	0.08-36.23	0.26	0.77	2.73	8.07
	Benzoic Acid	y=0.428x	0.999	0.07-40.98	0.17	0.69	1.78	7.23
	Ferulic Acid	y=0.906x	0.999	0.08-25.78	0.26	0.77	2.73	8.07
	Hippuric Acid	y=0.165x	0.999	0.05-27.93	0.17	0.52	1.78	5.45

Abbreviations: LOD: limit od detection; LOQ: limit of quantification; MDL: method detection limit; MQL: method quantification limit
*MDL and MQL in pmol/g for the analysis of 60mg of aorta or in nM for the analysis of 250 μL of plasma

IV. GENERAL DISCUSSION

Polyphenols are recognized by the body as xenobiotics, and in the small intestine and the liver they are subjected to phase-II detoxification enzymes, which convert them into their methylated, sulfated and glucuronidated derivatives [1,2]. Polymeric forms, unable to be absorbed at this level, reach the colon where they are subjected to microbial biotransformations [3,4]. The changes occurring during metabolism make the circulating forms of flavanols that reach the peripheral tissues, and hence the bioactive forms of flavanols, to be different from those present in foods [5]. On the other hand, metabolism of xenobiotics depends on several factors, such as intestinal enzyme activity, intestinal transit time, colonic microbiota, pathologies, genetics, and physiological conditions, among others [6]. In addition, other factors, such as ingested flavanol quantity or the duration of the polyphenol intake may also influence the metabolism of these compounds. Therefore, in light of these differences the objective of this thesis was to elucidate whether flavanols metabolism, bioavailability and tissue distribution were affected by different external and internal factors also influencing the bioactivities of these compounds. For this, different quantities of grape seed flavanols were administered during different times to rats under different conditions and the flavanol metabolites in plasma and tissues were quantified by HPLC-MS/MS.

Flavanol compounds are found primarily in grapes, beans, nuts, cocoa, tea and wine [7,8]. Grape seeds are a by-product of the grape/wine industry, but they are one of the richest sources of flavanols [9]. Flavanols from grape seed were selected because this source contains important amounts of all the types of flavanols, including gallated forms [10]. In addition, health beneficial effects of the grape seed flavanol rich extract used in this thesis have been extensively investigated [11–17]. In fact, our research group has demonstrated that GSPE exhibits antioxidant capacity [18], improves lipid metabolism [19],

limits adipogenesis [20], exhibits antihypertensive properties [10], acts as an insulin-mimetic agent [21], and reduces inflammation [16].

Although the phase-II metabolism of flavanols was very well described [2,22–24], there was an important need of information of how flavanols are metabolized in rat colon by the gut microbiota, as only *in vitro* studies were performed to elucidate the specific metabolic pathway occurring in there [25–28]. In order to completely understand the metabolism of flavanols we firstly needed to determine the colonic microbial metabolic pathway of flavanols in rats. However, there were no validated chromatographic methodologies for the analysis of flavanol-derived microbial metabolites in biological samples. Hence, a methodology was set up to develop and validate a rapid, simple and highly sensitive analytical method using HPLC-ESI-MS/MS for the determination of a large number of compounds derived from colonic metabolism of flavanols in plasma **[Manuscript 1]**. A total of 16 commercial standards were used to measure 30 different phenolic compounds present at low concentration levels (μM). In addition, a 30% reduction of plasma volume, which is an important limitation for the analysis of biological samples, especially for small study subjects such as rats or mice, was achieved. As a result, the developed method can be readily applied to determine the flavanol metabolites, including the colonic metabolites that are most likely responsible for many biological effects of poorly absorbed flavanols, and it could also be easily adjusted to be used in other hosts.

Then, the flavanol colonic metabolites quantification along time was performed in rat plasma after grape seed flavanol ingestion. In addition, a potential colonic flavanol biotransformation pathway in rats was also proposed. The results indicated that non-metabolised flavanols have peak plasma concentrations 2 h after GSPE administration, whereas the colonic metabolites appeared in plasma at later times, indicating their gradual colonic biotransformation as valerolactone > phenylpropionic acids ≈ phenylacetic acids > benzoic acids **[Manuscript 2]**. Hence, this study showed how flavanols were biotransformed by gut bacteria in rats over time, which would

facilitate the identification of potential bioactive compounds for particular health effects.

Once both phase-II and microbial flavanol metabolites were able to be analysed in rat plasma, external factors as the quantity and duration of the flavanol intake were studied to assess their influence on the dietary flavanol metabolism, as well as on their bioactivities. Regarding to the quantity of flavanols ingested, initially an *in vitro-in vivo* model to use flavanol physiologically appropriate forms was set out **[Manuscript 3].** In this study, the *in vitro* lipid homeostatic effect of flavanol metabolites was assessed using flavanol-administered rat semi-purified sera. Results showed a decrease in lipid synthesis and excretion in HepG2 cells, placing the use of semi-purified serum rich in flavanol metabolites as *in vivo* bioactive compounds suitable to be used in *in vitro* studies. As soon as the semi-purified sera compounds were confirmed as a good model to study the bioactivity of physiological forms of flavanols, the effect of appropriate amounts of flavanols intake on flavanol metabolism, bioavailability, tissue distribution and bioactivity was started to be performed. Firstly a dose-response study using this *in vivo* - *in vitro* model was carried out to clarify whether different concentrations of ingested flavanol were differently absorbed and metabolized in rats, and if the obtained semi-purified metabolites could differently modulate the lipid homeostasis in HepG2 cells **[Manuscript 4].** Results showed that up to a dose of 375 mg/Kg of GSPE flavanols, were absorbed and metabolized in a dose-response manner, but at 1000 mg/Kg, the system becomes saturated. Interestingly, the lipid lowering effect of the flavanol metabolites present in the semi-purified sera was not dependent on the metabolite concentration, showing effectiveness at very low amounts of ingested flavanols. Finally, the distribution of flavanols throughout the body depending on the quantity of ingested flavanols was also evaluated through an *in vivo* study where phase-II and microbial flavanol metabolites were quantified in rat tissues after an acute intake of different amounts of flavanols **[Manuscript 5].** It could be stated that the tissue distribution of flavanols was also affected by the quantity of flavanols

ingested, showing also a clear metabolic saturation as in the previous studies (up 375 mg/Kg). Also, a specific distribution of the metabolic products in the studied tissues was evidenced, highlighting the presence of free and methylated flavanols in the white adipose tissue and the brain, respectively. Therefore, the fact that flavanols are conjugated and distributed differently throughout the body when the intake of flavanols is varied, may involve a difference in their biological effects in the target tissue because different physiological bioactive forms are generated. On the other hand, the ingestion length as an external factor affecting the flavanol metabolism, bioavailability, tissue distribution and accumulation was also studied **[Manuscript 6].** This study showed that flavanols did not accumulate in rat tissues after a long-term daily orally intake in rats. Paradoxically, the detected metabolites in tissues after long-term intake would be the bioavailable forms from the last intake, which would indicate that the protective and preventive health effects of flavanols may be not due to an accumulative response of all of the flavanol ingestions but because of cyclic acute responses.

The influences of age, gender and health conditions of the host as internal factors able to modify the metabolism, bioavailability, tissue distribution and bioactivity of flavanols were evaluated. Results indicated that ageing resulted in a slower metabolic profile, a reduced small-intestine flavanol absorption, and phase-II metabolism of flavanols after the flavanol intake **[Manuscript 7]**. In addition, according to the observed decreased absorption, and metabolism at the level of the small intestine, an increased microbial metabolism activity was also stated in ageing. Therefore, it was demonstrated that ageing influence differently in metabolism, and plasma bioavailability of flavanols. Age-related changes in renal clearance, phase-II enzymatic activities and gut microbial profile were pointed out as potential responsible of the different flavanol metabolism and plasma bioavailability over the time in ageing**.** To study the gender as an individual internal factor affecting the metabolism, bioavailability and distribution of flavanols, another study was set out, comparing the flavanol metabolites content in plasma and tissues of male and female rats at different times after the flavanols intake

[Manuscript 8]. In this study, results also demonstrated that the metabolism and distribution of flavanols throughout the bodies of rats is influenced by sex. Specifically, results showed that flavanols and their metabolites were widely distributed in tissues at 1, 2 and 4 h after ingestion of grape seed flavanols in both genders, but important quantitative gender differences in plasma (female had more flavanol metabolites than male rats) and in brain (male have more flavanol metabolites than female rats) were observed. Moreover, gender differences in the distribution and type of flavanols metabolites in liver and in brain were also observed between rats of different genders. Specifically, male rats present brain specificity for methylated metabolites and female rats for dimeric PA. These differences were most probably due to inherent physiological differences between genders, such as total body water, differences in phase-II enzyme activities in target tissues (i.e., in liver and in brain), and differences in tissue specificities. However, in white adipose tissue, there were no differences in either the quantity or the quality of the flavanols metabolites between male and female rats. Therefore, the observed differences in the flavanol metabolism, bioavailability and tissue distribution may differentially influence the physiological bioactivities of these compounds depending on the internal factors as the age or gender in healthy animals.

Finally, the health status as the last internal factor affecting the metabolism, bioavailability, tissue distribution, and hence the bioactivity of dietary flavanols was studied. For this, non-healthy animals were used in the different studies. Firstly, a kinetic study of plasma flavanol metabolism and bioavailability under a pathological status of MeS induced by the diet was assessed **[Manuscript 9]**. Results showed that MeS pathology significantly influenced the metabolism, and plasma bioavailability of flavanols leading to a 2 h delay of the time of plasma *maximum* bioavailability. Moreover, a higher phase-II glucuronidated bioavailability was observed, indicating a clear over-activation of these specific phase-II enzymes in a MeS disease. In addition, the results also showed a higher absorption of monomeric flavanols and small phenolic acids at the level of the small intestine, indicating that

enterocyte permeability could be compromised in obesity. On the contrary, a lower absorption of flavanol dimers was showed, suggesting different transport mechanisms for these compounds. Interestingly, a significant reduction of the microbial metabolism was also observed in MeS rats respect to healthy rats. These facts would show that animals with MeS presented a strong disruption in all over the gastrointestinal tract, including the microbiota. Secondly, a last study was performed in order to evaluate the relationships of flavanol bioactivities with their plasma and aorta levels in a genetically associated pathology. Specifically a model of SHR was used **[Manuscript 10]**. The obtained results showed flavanol antihypertensive bioactivity in SHR but not in healthy rats. Demonstrating that, as our group previously reported, dietary flavanols decreased blood pressure in a hypertensive state [10]. The most important plasma and aorta flavanol differences between healthy and genetically hypertensive rats were referred to the microbial metabolism, which seemed to be significantly over-activated in the SHR, and that makes plausible the fact that theses metabolites would be involved in this effect. Nevertheless, plasma flavanol compounds were not decreased in SHR respect to Wistar animals, indicating that, differently as in the study related to the MeS **[Manuscript 9],** in this pathological state a disruption in the gastrointestinal tract cannot be observed. But, when results were analysed individually, significant differences in plasma were evidenced between both health statues. The most important plasma bioavailability difference between healthy and hypertensive rats was referred to the microbial metabolism that seemed to be significantly over-activated in SHR. Nevertheless, these finding agree with the fact that high blood pressure has been recently associated with gut microbiota dysbiosis, both in animal and human hypertension [29].

However, as aorta flavanols were reduced in SHR comparing to those healthy counterparts, a disruption of the active transport driven by some endothelial flavonoid transporters [30] could be related. The higher aorta levels of some phenolic acids in SHR comparing to healthy rats could be due to a potential disruption of tight junctions in the endothelium, or the different morphology of the endothelial gap junctions that has been demonstrated in

essential hypertension [31], leading these small phenolic compounds to cross the endothelial barrier in hypertensive rats. In addition, similarly to the results reported in plasma, important differences in microbial metabolites between both health statuses were found in aorta, showing higher concentrations of these metabolites in hypertensive rats than in healthy rats. And thus, a health status of hypertension, as a located disease, affects the levels and bioactivity of flavanols in the dysfunctional related tissues, like aorta. The flavanols forms present in aorta of hypertensive rats are those flavanols forms most probably responsible of the antihypertensive effects of these compounds including flavanol microbial colonic metabolites since these forms are those which present the major aorta qualitative and quantitative differences between healthy and hypertensive rats.

Therefore, these studies demonstrated how a pathological state influences significantly the metabolism and plasma bioavailability of flavanols, leading to differences in flavanols bioactivity. In summary, in order to assess the real mechanisms involved into the flavanol health benefits, the study of the flavanol metabolism at the concrete physiopathological condition should be started to be established in order to assess the plasma bioavailable metabolites that will reach the target tissues, and exert the bioactive function.

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V. CONCLUSIONS

1. **Flavanols are biotransformed to small phenolic acids by rat gut bacteria enzymes indicating in plasma their gradual colonic biotransformation as valerolactone, following phenylpropionic and phenylacetic acids and finally benzoic acids.**
2. **Flavanol metabolites exert *in vitro* bioactivity by modulating lipid homeostasis in HepG2 cells**
3. **Different flavanol ingested amounts present different metabolism, bioavailability, tissue distribution and bioactivity in rats**
    - Flavanol metabolism is saturated up to 375 mg/kg
    - Increased amounts of flavanols are not necessary related with effectiveness
    - Flavanol metabolites distribution is tissue specific, being free flavanols mostly found in adipose tissue and methylated flavanols in brain
4. **Flavanols do not accumulate in rat after a long-term daily orally intake**
5. **Ageing influence differently the flavanols metabolism, and plasma bioavailability**
    - Elderly rats present a slower flavanol metabolic profile, reduced first-pass flavanol absorption and phase-II metabolism and an over-activated microbial flavanol catabolism.
6. **Gender significantly influences the metabolism, plasma bioavailability and tissue distribution of flavanols in rats**
    - Female rats have increased plasma bioavailability of flavanol metabolites along time, decreased liver bioavailability of flavanol metabolites and faster flavanol clearance in adipose tissue.
    - Female rats present brain specificity for dimeric PAs and male rats

for methylated metabolites.

7. Health status significantly influence flavanol metabolism, bioavailability, tissue distribution, and bioactivity

- Rats affected by metabolic syndrome present a delay in the flavanol maximum bioavailability, increased levels of Gallic acid and flavanol monomeric forms but decreased concentrations of flavanol dimeric forms in plasma.
- Flavanol microbial metabolism is altered by metabolic syndrome induced by cafeteria diet and essential hypertension. While it is disrupted in rats affected by metabolic syndrome, this is over-activated in essential hypertension.
- Essential hypertension affects extensively aorta flavanol levels and their bioactivity, highlighting microbial metabolites as those forms which present the major aorta qualitative and quantitative differences between healthy and hypertensive rats.
- In order to assess the flavanol health benefits, the study of the metabolism at the concrete pathological condition is needed.

The ingested amount of flavanols and the age, gender and health status of the host affect the metabolism, bioavailability, tissue distribution and therefore the bioactivity of these compounds. Paradoxically the consumption length does not contribute to the flavanol tissue accumulation.

ANNEX

List of Publications

FULL PAPERS

Maria Margalef; Zara Pons; Lisard Iglesias-Carres; Francisca I Bravo; Begoña Muguerza; Anna Arola-Arnal. Lack of tissue accumulation of grape seed flavanols after daily long-term administration in healthy and cafeteria diet obese rats. (*Submitted).*

Maria Margalef; Lisard Iglesias-Carres; Zara Pons; Francisca I Bravo: Begoña Muguerza; Anna Arola-Arnal. Age related differences in the plasma kinetics of flavanols in rats. *(Submitted).*

Maria Margalef; Zara Pons; Lisard Iglesias-Carres; Lluis Arola; Begoña Muguerza; Anna Arola-Arnal. Gender related differences in the body distribution of flavanols in rats. *(Submitted).*

Maria Margalef; Zara Pons; Lisard Iglesias-Carres; Francisca I Bravo; Begoña Muguerza; Anna Arola-Arnal. Flavanol plasma bioavailability is affected by metabolic syndrome in rats. *(Submitted).*

Maria Margalef; Zara Pons; Lisard Iglesias-Carres; Mar Quiñones; Francisca I Bravo; Begoña Muguerza; Anna Arola-Arnal. Rat health status affects target tissue levels and bioactivity of flavanols. *(Submitted).*

Mar Quiñones; **Maria Margalef**; Anna Arola-Arnal; Begoña Muguerza; Marta Miguel; Amaya Aleixandre. Blood pressure effect and related plasma levels of flavan-3-ols in spontaneously hypertensive rats.
Food & Function (2015), *in press.* DOI: <10.1039/C5FO00547G>.

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Maria Margalef; Zara Pons; Francisca I Bravo; Begoña Muguerza; Anna Arola-Arnal Plasma kinetics and microbial biotransformation of grape seed flavanols in rats.
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Maria Margalef; Ligia Guerrero; Zara Pons; Francisca Isabel Bravo; Lluis Arola; Begoña Muguerza; Anna Arola-Arnal. A dose-response study of the bioavailability of grape seed proanthocyanidin in rat and lipid-lowering effects of generated metabolites in HepG2 cells.
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Zara Pons; Ligia Guerrero; **Maria Margalef**; Lluis Arola; Anna Arola-Arnal; Begoña Muguerza. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. Journal of Physiology and Biochemistry (2014), 70(2), pp. 629-637

Leire Martínez-Fernández; Zara Pons; **Maria Margalef**; Anna Arola-Arnal; Begoña Muguerza. Regulation of Vascular Endothelial Genes by Dietary Flavonoids: Structure-Expression Relationship Studies and the Role of the Transcription Factor KLF-2.
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Noemí González-Abuín; Neus Martínez-Micaelo; **Maria Margalef**; Maite Blay; Anna Arola-Arnal; Begoña Muguerza; Anna Ardévol; Montserrat Pinent. A grape seed extract increases active glucagon-like peptide-1 levels after an oral glucose load in rats.
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Francisca I Bravo; Zara Pons, **Maria Margalef**; Anna Arola-Arnal; Begoña Muguerza. Grape seed flavanols block the renin-angiotensin aldosteron system in obese-hypertensive rats. SEBBM (2015).

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BOOK CHAPTERS

Anna Arola-Arnal; Manuel Suárez; **Maria Margalef**; Gerard Aragonès; Lluís Arola; Cinta Bladé; Begoña Muguerza. Analytical methods for the identification of physiologically bioactive forms of food flavonoids. Recent progress in medical plants. 40. Favonoids and Antioxidants. *In press.*