Gender related similarities and differences in the body distribution of grape seed 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, and are the only flavonoid type structures that can be found in nature in their aglycone forms. They range from the monomers (+)-catechin and (-)-epicatechin to the oligomeric and polymeric proanthocyanidins (PAs) [1]. Increasing evidence has indicated the important health-promoting effects of flavonoids [2]. Specifically, grape seed flavanols exhibit antioxidant and anti-inflammatory capacities [3], improve lipid metabolism [4], increase insulin secretion [5] and act as antihypertensive agents [6].

The beneficial health properties of flavanols are mainly attributed to the compounds that are derived from their metabolism [2]. 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 [7]. 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 [7,8].

Recently, we have shown that differences in experimental conditions, such as flavanol dosage, affect the metabolization and body distribution of flavanol metabolites [9]. 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 [4,10–13]. Furthermore, numerous sex-related differences in both humans and other mammals have been shown in processes such as lipid and glucose metabolism [14], in psychiatric disorders [15] and in coronary artery disease [16]. There are also sex-related differences in susceptibility to inflammatory and infectious diseases [17] and in the level of protective health benefits that are imparted by drinking moderate amounts of alcohol [18]. 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 [19]. 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 [20].

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. The selection of these specific tissues was also encouraged by the well-reported bioactivities of grape seed flavanols in these tissues, such as the lipid homeostasis in the liver [4,10], the anti-inflammatory effects in the adipose tissue [3], and the hormonal regulation in the brain [21].

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). 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, animals were individually weighed and the GSPE dose of 1000 mg/kg was calculated and administrated to both male (n=18) and female rats (n=18) by oral gavage. 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, permit 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) [22]. 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 water with 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 [9,22] 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 [23]. 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 S1. 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 2. The extract contained most of the representative flavanols of grape seed extract [6], 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 3 and 4). 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 3 and 4). 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 3 and 4) 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 3 and 4). 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 3 and 4). 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 3 and 4). 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 [3,11,24]. 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 [25,26]. Furthermore, it has previously been observed that xenobiotic metabolism is different between genders [20] and that drug-metabolizing enzymes are differentially affected by xenobiotics depending on sex [20,27]. 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, different hours after the acute administration of GSPE (1000 mg/kg of body weight), flavanols and their phase-II metabolites were quantified in plasma as the systemic circulation representation, liver as the main phase-II metabolic tissue for flavanols, MWAT as the storage location, and

brain and hypothalamus as important peripheral organs likely to present gender related differences in order to elucidate how flavanols are distributed throughout the body. In addition, the selection of these specific tissues was also encouraged by the well-reported bioactivities of grape seed flavanols in these tissues, such as the lipid homeostasis in the liver [4,10], the anti-inflammatory effects in the adipose tissue [3], and the hormonal regulation in the brain [21]. Based on previous studies, a dose of 1000 mg/kg was selected for evaluation in acute bioavailability studies following flavanol ingestion in rats [9,23,28]. However, although we have previously reported that this high dose leads to saturation of the system [9,10], 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 [29,30]. Additionally, GSPE has been reported to exert beneficial health effects at these early time points, such as increasing GLP-1 and insulin secretions [5], decreasing plasma glucose levels [31] and exhibiting antihypertensive effects [6,32].

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 [33]. 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 [34]; 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 [2]. Previous studies in male [9] and female [35] 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 [19,20], 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. [36] 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 [37] and that there are sex-related differences in how xenobiotics affect metabolic enzymes in the livers of rats [20,27].

According to other studies that also employed GSPE [9,35,38], 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) [9,35]. 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 [39]; 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 [15,40]. It has further been reported that COMT activity in the prefrontal cortex is 17% higher in men than in women [15]. These differences are important because polyphenols that target the brain are probably these physiologically active forms. For example, Wang J et al. [41] 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 distribution of grape seed flavanols in the rat studied tissues 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 and to determine the gender influence on the microbiota and consequently on the microbial flavanol metabolism, 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. All authors have read and approved the final manuscript.

Acknowledgments

This study was supported by the Ministerio de Educación cultura y Deporte of the Spanish Government, grant number AGL2013-40707-R. M.M is recipient of a fellowship from Universitat Rovira i Virgili. Grant Number: DL003693. Z.P is recipient of a predoctoral fellowship from Universitat Rovira i Virgili. Grant Number: 2011BRDI-06-28. L.I-C is recipient of a predoctoral fellowship from Universitat Rovira i Virgili - Martí Franquès. Grant Number: 2015PMF-PIPF-50.

We gratefully acknowledge Niurka Llópiz and Yaiza Tobajas for their technical assistance.

Competing financial interests

The authors have declared no conflict of interest.

References

[1] Bladé, C., Arola, L., Salvadó, M.J., Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol. Nutr. Food Res.* 2010, *54*, 37–59.

[2] Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P.E., Tognolini, M., Borges, G., Crozier, A., Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* 2013, *18*, 1818–1892.

[3] Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., et al., Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *J. Nutr. Biochem.* 2011, *22*, 380–387.

[4] Guerrero, L., Margalef, M., Pons, Z., Quiñones, M., et al., Serum metabolites of proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells. *J. Nutr. Biochem.* 2013, *24*, 2092–2099.

[5] González-Abuín, N., Martínez-Micaelo, N., Margalef, M., Blay, M., et al., A grape seed extract increases active glucagon-like peptide-1 levels after an oral glucose load in rats. *Food Funct.* 2014, *5*, 2357–2364.

[6] Quiñones, M., Guerrero, L., Suarez, M., Pons, Z., et al., Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Res. Int.* 2013, *51*, 587–595.

[7] Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., et al., Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* 2010, *1*, 233–253.

[8] Aura, A.M., Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* 2008, *7*, 407–429.

[9] Margalef, M., Pons, Z., Bravo, F.I., Muguerza, B., Arola-Arnal, A., Tissue distribution of rat flavanol metabolites at different doses. *J. Nutr. Biochem.* 2015, *26*, 987–995.

[10] Margalef, M., Guerrero, L., Pons, Z., Bravo, F.I., et al., A dose–response study of the bioavailability of grape seed proanthocyanidin in rat and lipid-lowering effects of generated metabolites in HepG2 cells. *Food Res. Int.* 2014, *64*, 500–507.

[11] Pons, Z., Guerrero, L., Margalef, M., Arola, L., Arola-Arnal, A., Muguerza, B., Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *J. Physiol. Biochem.* 2014, *70*, 629–637.

[12] Kay, C.D., The future of flavonoid research. *Br. J. Nutr.* 2010, *104*, s91–s95.

[13] Kroon, P.A., Clifford, M.N., Crozier, A., Day, A.J., et al., How should we assess the effects of exposure to dietary polyphenols in vitro? *Am J Clin Nutr*. 2004, *80*, 15–21.

[14] Varlamov, O., Bethea, C.L., Roberts, C.T., Sex-specific differences in lipid and glucose metabolism. *Front. Endocrinol. (Lausanne).* 2014, *5*, 241.

[15] Harrison, P.J., Tunbridge, E.M., Catechol-O-Methyltransferase (COMT): A Gene Contributing to Sex Differences in Brain Function, and to Sexual Dimorphism in the Predisposition to Psychiatric Disorders. *Neuropsychopharmacology*. 2007, *33*, 3037–3045.

[16] Yahagi, K., Davis, H.R., Arbustini, E., Virmani, R., Sex differences in coronary artery disease: Pathological observations. *Atherosclerosis*. 2015, *239*, 260–267.

[17] Liu, H.B., Loo, K.K., Palaszynski, K., Ashouri, J., Lubahn, D.B., Voskuhl, R.R., Estrogen Receptor Mediates Estrogen's Immune Protection in Autoimmune Disease. *J. Immunol.* 2003, *171*, 6936–6940.

[18] Taylor, B., Irving, H.M., Baliunas, D., Roerecke, M., et al., Alcohol and hypertension: gender differences in dose-response relationships determined through systematic review and meta-analysis. *Addiction*. 2009, *104*, 1981–1990.

[19] DeBethizy, J.D., Hayes, J.R., Metabolism: a determinant of toxicity, in: W.A. Hayes (Ed.), Princ. Methods Toxicol., 3rd ed., Raven Press, New York, NY, 1994: pp. 59–100.

[20] Mugford, C.A., Kedderis, G.L., Sex-dependent metabolism of xenobiotics. *Drug Metab. Rev.* 1998, *30*, 441–498.

[21] Ardévol, A., Bladé, C., Salvadó, M.J., Arola, L., Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. *Int. J. Obes. Relat. Metab. Disord.* 2000, *24*, 319–24.

[22] Margalef, M., Pons, Z., Muguerza, B., Arola-Arnal, A., A Rapid Method to Determine Colonic Microbial Metabolites Derived from Grape Flavanols in Rat Plasma by Liquid Chromatography-Tandem Mass Spectrometry. *J. Agric. Food Chem.* 2014, *62*, 7698–7706.
[23] Serra, A., Macià, A., Romero, M.P., Salvadó, M.J., et al., Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B*. 2009, *877*, 1169–1176.
[24] Quesada, H., del Bas, J.M., Pajuelo, D., Díaz, S., et al., Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obes. (Lond).* 2009, *33*, 1007–1012.
[25] D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanita*. 2007, *43*, 348–361.
[26] Aherne, S.A., O'Brien, N.M., Dietary flavonols: chemistry, food content, and metabolism. *Nutrition*. 2002, *18*, 75–81.
[27] Finnen, M., Hassall, K., Effects of hypophysectomy on sex differences in the induction and depression of hepatic drug-metabolizing enzymes in the rat. *J. Pharmacol. Exp. Ther.* 1984, *229*, 250–254.
[28] Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., et al., Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* 2006, *54*, 884–892.
[29] Serra, A., Macià, A., Rubió, L., Anglès, N., et al., Distribution of procyanidins and their metabolites in rat plasma and tissues in relation to ingestion of procyanidin-enriched or procyanidin-rich cocoa creams. *Eur. J. Nutr.* 2013, *52*, 1029–1038.
[30] Tomas-Barberan, F.A., Cienfuegos-Jovellanos, E., Marín, A., Muguerza, B., et al., A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J. Agric. Food Chem.* 2007, *55*, 3926–3935.
[31] Pinent, M., Cedó, L., Montagut, G., Blay, M., Ardévol, A., Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. *Crit. Rev. Food Sci. Nutr.* 2012, *52*, 569–584.
[32] Pons, Z., Margalef, M., Bravo, F.I., Arola-Arnal, A., Muguerza, B., Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. *Eur. J. Nutr.* 2015, *In press*, .
[33] Sipes, I.G., Gandolfi, A.J., Biotransformation of Toxicants, in: M.. Amdur, J. Doull, C.D. Klasses (Eds.), Casarett Doull's, Toxicol. - Basic Sci. Poisons, 4th ed., Pergamon Press., New York, NY, 1991: pp. 64–98.
[34] Harris, R.Z., Benet, L.Z., Schwartz, J.B., Gender Effects in Pharmacokinetics and Pharmacodynamics. *Drugs*. 1995, *50*, 222–239.
[35] Arola-Arnal, A., Oms-Oliu, G., Crescenti, A., Del Bas, J.M., et al., Distribution of grape seed flavanols and their metabolites in pregnant rats and their fetuses. *Mol. Nutr. Food Res.* 2013, *57*, 1741–1752.
[36] Dellinger, R.W., Garcia, A.M.G., Meyskens, Jr., F.L., Differences in the Glucuronidation of Resveratrol and Pterostilbene: Altered Enzyme Specificity and Potential Gender Differences. *Drug Metab. Pharmacokinet.* 2014, *29*, 112–119.
[37] Piskula, M.K., Soy Isoflavone Conjugation Differs in Fed and Food-Deprived Rats. *J. Nutr.* 2000, *130*, 1766–1771.
[38] Ardévol, A., Motilva, M.J., Serra, A., Blay, M., Pinent, M., Procyanidins target mesenteric adipose tissue in Wistar lean rats and subcutaneous adipose tissue in Zucker obese rat. *Food Chem.* 2013, *141*, 160–166.
[39] Faria, A., Pestana, D., Teixeira, D., Couraud, P.-O., et al., Insights into the putative catechin and epicatechin transport across blood-brain barrier. *Food Funct.* 2011, *2*, 39–44.
[40] Mannisto, P.T., Kaakkola, S., Catechol-O-methyltransferase (COMT): Biochemistry, Molecular Biology, Pharmacology, and Clinical Efficacy of the New Selective COMT Inhibitors. *Pharmacol. Rev.* 1999, *51*, 593–628.
[41] Wang, J., Ferruzzi, M.G., Ho, L., Blount, J., et al., Brain-targeted proanthocyanidin metabolites for

445 Alzheimer's disease treatment. *J. Neurosci.* 2012, *32*, 5144–5150.
446
447

448 **Figure Legends**

449 **Figure 1.** Extracted ion chromatograms of flavanols and their phase-II metabolites 2 h after the administration of
450 1000 mg/kg of a grape seed polyphenol extract (GSPE) in male (continuous line) and female (discontinuous line)
451 rat plasma. (1) Gallic acid, (2) Dimer B1, (3) Dimer B3, (4) Dimer B2, (5) Methyl-catechin-glucuronide, (6)
452 Methyl-epicatechin-glucuronide, (7) Catechin glucuronide, (8) Epicatechin glucuronide, (9) Catechin, (10)
453 Epicatechin, (11) Catechin sulfate, (12) Epicatechin sulfate (13) Methyl-catechin-O-sulfate, (14) Methyl-
454 epicatechin-O-sulfate, (15) 3-Methyl-epicatechin, and (16) 4-Methyl-epicatechin.

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

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

465

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)

472 **Table 2.** Main phenolic compounds (flavanols and phenolic acids)
473 of the grape seed polyphenol extract (GSPE) used in this study,
474 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

475 Abbreviations: PA (proanthocyanidin)
476 The results are expressed on a wet basis as the mean ± SD (n=3).
477 The results are expressed as mg of phenolic compound/g of GSPE
478 **[1]** Quantified using the calibration curve of proanthocyanidin B2.
479 **[2]** Quantified using the calibration curve of epigallocatechin gallte.

Table 3. 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 4. 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