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Tissue distribution of rat flavanol metabolites at different doses.

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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 1000mg/kg) were orally administered to male Wistar rats. Tissues were collected 2h 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 were 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 (CVD) 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 (TG), 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 is 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 PAs 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.

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 B2; (-)-epicatechin (Fluka/Sigma-Aldrich, Madrid, Spain) and 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 (+)-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 B2 and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone 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

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., 2013 [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 ºC with 12 h light/dark cycles (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* 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-GSPE (n=3), 250

mg/Kg-GSPE (n=3), 375 mg/Kg-GSPE (n=3), and 1000 mg/kg-GSPE (n=3). Oral administration in all groups occurred between 9 and 10 am 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 −80ºC. 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 off-line 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, v/v/v). 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 x g at room temperature (except for MWAT, which was centrifuged at 4 ºC 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 x 50 µL of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solution was directly injected in the 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 x 2.1 mm i.d., 3.5 µm particle size) as the 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. Electrospray ionization (ESI) conditions were a drying gas temperature of 350 ºC 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, Palo Alto, CA, USA).

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 (LOD and LOQ, respectively) and method detection and quantification limits (MDL and MQL, respectively), were also calculated (Table S2).

2.7. Statistical analysis

Results were expressed as the mean ± standard error (SEM) of the mean (n=3) and analyzed by one-way or two-way 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 $p < 0.05$.

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, Figure S1, Figure 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 Figure 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, Figure 2 and Figure 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 non-conjugated 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 (Figure 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 non-conjugated free flavanols (Figure 2B). Furthermore, these concentrations remained similar for nearly all of the doses

evaluated, although some variation at the 1000 mg/kg dose was observed: the methylated, sulfated and non-conjugated free flavanols increased in concentration, but the glucuronidated and methyl-glucuronidated concentrations decreased (Figure 3).

In MWAT, the major compounds found were glucuronidated derivatives at low doses of GSPE and non-conjugated free flavanols at the highest dose (1000 mg/Kg) (Figure 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 (Figure 2D).

Overall, in Figure 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 although 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 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 metabolisation, 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. Respect to the non-metabolized flavanols concentrations, in serum is mostly lower than these 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 nmol/g, 5-78 nmol/g and 5-17 nmol/g were measured in liver, kidneys, and MWAT, respectively. However, these metabolites were not found in 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] suggests 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 in *in vitro* studies [35]. 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 non-metabolized 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 hyperplasia and start releasing pro-inflammatory cytokines and adipocyte-related hormones. These factors then promote a pro-inflammatory 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 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 non-conjugated 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 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 PAs 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.

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Figure Legends

Figure 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 grape seed proanthocyanidin extract (GSPE). Data are given as the means ± SEM (n=3) and expressed in nmol/g of dried tissue. Different letters indicate statistically significant differences between treatment groups (p < 0.05). 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.

Figure 2. Concentrations of total unconjugated free flavanols (catechin, epicatechin, procyanidin dimers and gallic acid) and their methylated, glucuronidated, sulfated, methyl-sulfated and methyl-glucuronidated derivatives (sum of catechin and epicatchin derivatives) quantified by HPLC-ESI-MS/MS at 2h after the ingestion of 125, 250, 375 and 1000 mg/Kg of grape seed proanthocyanidin extract (GSPE) in liver (A), kidney (B), mesenteric white adipose tissue (MWAT) (C) and brain (D). Data are given as the means ± SEM (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).

Figure 3. Distributions of free flavanols (catechin, epicatechin and procyanidin dimers) and their phase-II metabolites quantified by HPLC-ESI-MS/MS in rat tissues (liver, kidney,

mesenteric white adipose tissue (MWAT) and brain) at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of a grape seed proanthocyanidin extract (GSPE). Data are given as the means ± SEM (n=3) and expressed as percentages.

Table 1

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 grape seed proanthocyanidin extract (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[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.	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 B2	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 B3[4]	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 B1[4]	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[1]	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[2]	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[1]	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[2]	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-sulphate[1]	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-sulphate[2]	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-sulphate[1]	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-sulphate[2]	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[2]	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[2]	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: MWAT (mesenteric white adipose tissue); EGCG (epigallocatechingallate); n.d. (not detected); n.q. (not quantified)
The results are expressed in nmol/g of dried tissueas the mean ± SD (n=3).
[1]Quantified using the calibration curve ofcatechin
[2]Quantified using the calibration curve ofepicatechin
[3]Quantified using the calibration curve of EGCG
[4]Quantified using the calibration curve ofprocyanidin dimer B2

Table 2

Table 2. Microbial colonic flavanol metabolites detected by HPLC-ESI-MS/MSin different rat tissues at 2 h after the ingestion of 125, 250, 375 and 1000 mg/kg of a grape seed proanthocyanidin extract (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±0.03	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[1]	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[2]	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[3]	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: MWAT (mesenteric white adipose tissue); 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 expressed in nmol/g of dried tissueas the mean ± SD (n=3).

[1] Quantified using the calibration curve of3-(4-hydroxyphenyl)propionic acid

[2] Quantified using the calibration curve ofgallic acid

[3] Quantified using the calibration curve ofvanillic acid

Figure 1
Figure 1

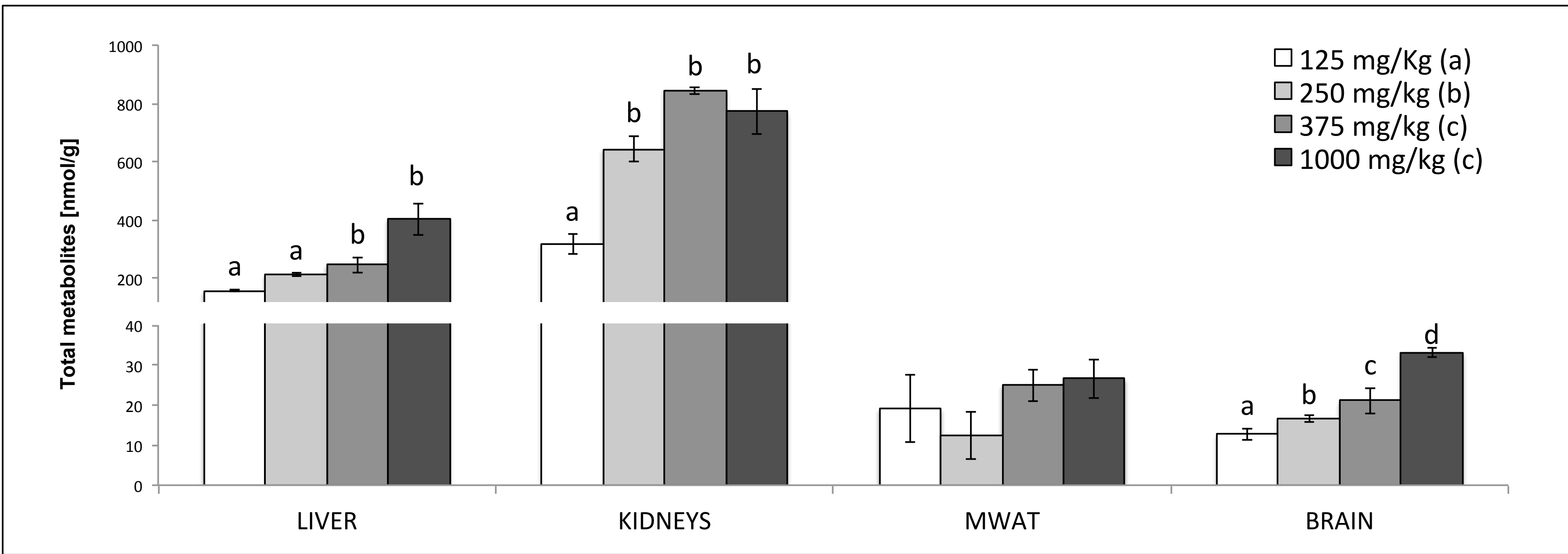
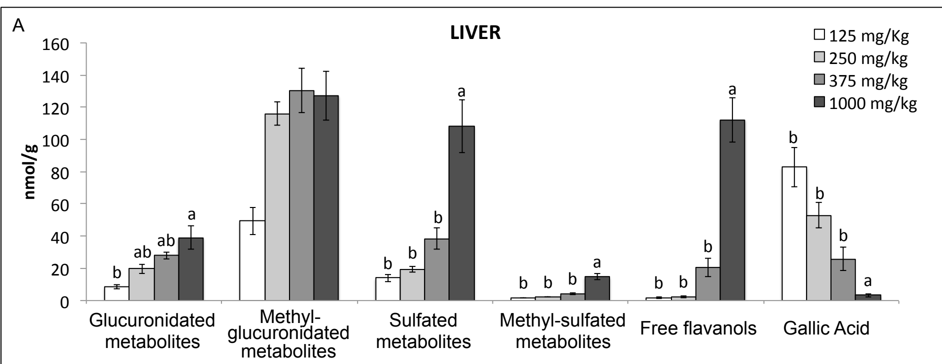
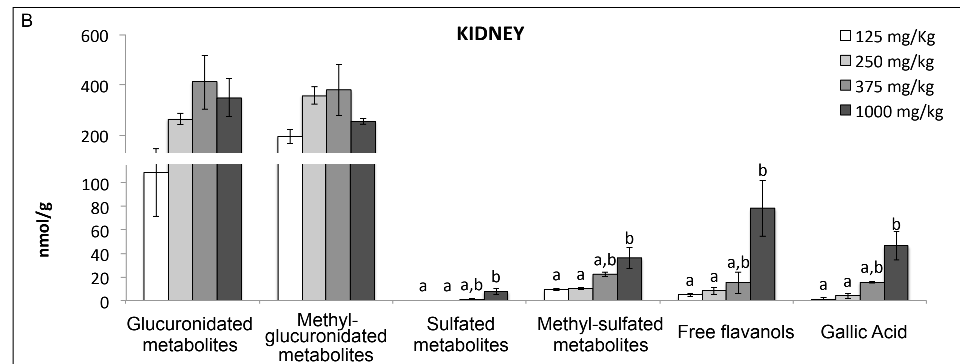
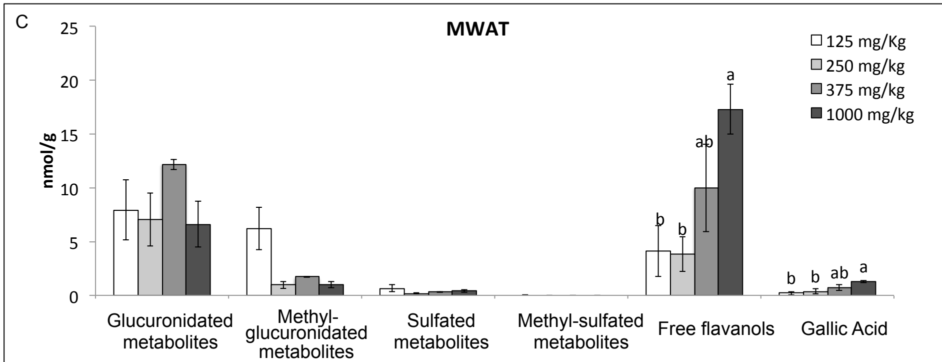


Figure 2 Figure 2







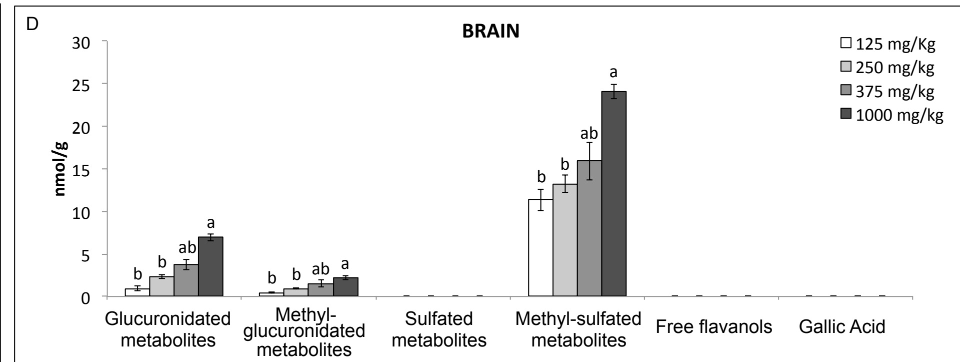
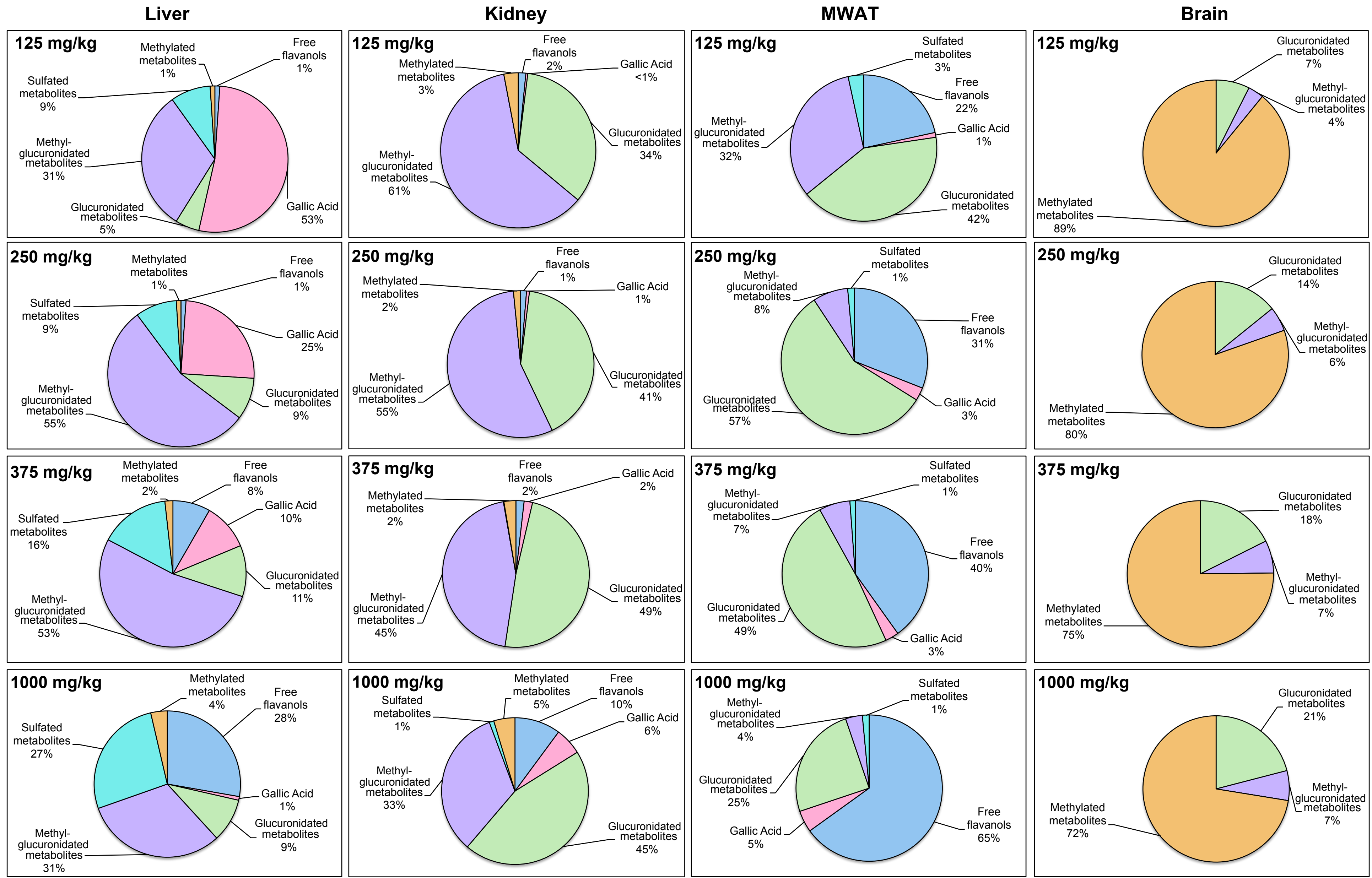


Figure 3Figure 3



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