Distribution of grape seed flavanols and their metabolites in pregnant rats and their foetuses

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Abbreviations: detection limit (LoD), grape seed proanthocyanidin extract (GSPE), internal standard (IS), liquid-solid extraction (LSE), mesenteric white adipose tissue (MWAT), method detection limit (MDL), method quantification limit (MQL), micro solid-phase extraction (μSPE), quantification limit (LoQ), relative standard deviation (% RSD).

Key words: amniotic fluid, bioavailability, placenta, polyphenols, proanthocyanidins.
Abstract

Scope: Polyphenols have been demonstrated to provide health benefits affecting cellular and physiological processes. This study aims to evaluate the bioavailability and distribution of grape seed flavanol compounds during pregnancy and whether foetuses could be exposed to these compounds.

Methods and results: The distribution of flavanols and their metabolites in rat plasma, liver, white adipose tissue, brain, amniotic fluid, placenta and foetuses after 1 and 2 h of an acute intake of a grape seed proanthocyanidin extract (GSPE) was examined by LC-ESI-TOF/MS. Flavanols and their metabolites were widely distributed in both pregnant and non-pregnant rat plasma and tissues. In liver, the conjugated forms of flavanols were less available in pregnant than non-pregnant rats. Flavanol metabolites were abundant in maternal placenta but detected at low levels in foetuses and amniotic fluid.

Conclusion: Flavanol metabolisation appears to be less active in the liver during pregnancy. Moreover, data indicated that transport across the placenta is not efficient and for flavanols and their metabolites the placenta seems to act as a barrier. However, these compounds target the foetus and are excreted in the amniotic fluid.
1 Introduction

Maternal over-nutrition, overweight and obesity during pregnancy have been associated with increased obesity and with several health complications of the offspring, such as type 2 diabetes. These complications have been attributed to maternal nutrition, as increasing evidence suggests that maternal nutrition may induce epigenetic modifications of the foetal genome perspective [1-2]. The so-called epigenetic changes are heritable changes that influence gene expression without altering the DNA sequence (i.e., DNA methylation, chromatin remodelling or mi(cro)RNA expression) [3]. Hence, preventive treatments during pregnancy could be beneficial to decrease the health complications of the offspring. These complications appear to be affected by maternal diet control and nutritional epigenetic influences. Hence, a prevention treatment based on bioactive compounds with epigenetic capacities appears to be a good approach for preventing health complications of the offspring. Interestingly, bioactive dietary polyphenol compounds have been demonstrated to induce epigenetic modifications [4-5].

The most abundant polyphenols in the human diet are flavanols, which consist of the monomeric flavan-3-ol and the proanthocyanidin tannins. Flavanols are primarily provided by fruits, cocoa, tea, wine, nuts and beans [6-7]. Flavanols provide health benefits that affect cellular and physiological processes. Specifically, flavanols improve human health, with cardioprotectant [8], antioxidant [8], anti-inflammatory [9], antigenotoxic [10] and anticarcinogenic [11] activities. Moreover, flavanols have been widely studied for their positive health effects, such as improving dyslipidemia [12] and insulin resistance [13]. Different flavanol extracts have been studied to determine their health and biological effects [14-15]. Among them, a grape seed proanthocyanidin extract (GSPE) has been used extensively and has demonstrated
many beneficial health properties in different animal models [12, 13, 16-20]. GSPE flavanols can range in molecular weight from monomers (i.e., catechin, epicatechin and their gallate forms) to long-chain polymers and is primarily composed of dimeric and trimeric procyanidins [21]. Several in vivo studies have demonstrated that after administration, flavanols are absorbed and further metabolised, primarily forming glucuronide, sulphate and methylate conjugates [22]. Flavanols and their metabolites are distributed to different tissues and have been observed in target tissues such as liver, brain or kidney in rats [22-24]. Hence, the absorption and tissue distribution of the flavanol metabolites should bear a close relationship with their biological function and beneficial health effects. In these studies, the distribution of procyanidins and their metabolites in rat plasma and tissues after an acute intake of a polyphenolic extract has been reported in male Wistar rats. As far as we know, pharmacokinetic studies in rat maternal plasma and foetuses have only been performed with green tea catechins [25]. Nevertheless, the conjugated forms of green tea catechins were not determined. Although maternal nutrition during pregnancy is important, the distribution of flavanol metabolites in pregnant rats and their foetuses has never been reported. Therefore, the present work aims to evaluate the bioavailability and distribution of grape seed flavanols and their metabolites in plasma and tissues of pregnant rats and to evaluate whether foetuses might be exposed to these compounds.

2 Materials and methods

2.1 Grape seed extract
The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The content in individual flavanols and phenolic acids of the grape seed extract used in this study are detailed in Table 1 (adapted from Quiñones et al [26]).

2.2 Chemicals and reagents

Acetone (HPLC grade), L-ascorbic acid (>99%) and orthophosphoric acid (>85%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was provided by Scharlab S.L. (Barcelona, Spain), and glacial acetic acid (>98%) was provided by Panreac Química S.L.U. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Oasis HLB 96-well μElution Plates, 2 mg of sorbent and 30 μm particle size, were purchased from Waters Corp. (Milford, USA).

Stock standard solutions of 2000 mg/L in methanol of (+)-catechin, (-)-epicatechin and pyrocatechol (Fluka/Sigma-Aldrich, St. Louis, MO, USA) and a standard solution of 1000 mg/L in methanol of procyanidin B2 (Fluka/Sigma-Aldrich, St. Louis, MO, USA) were stored in dark-glass flasks at -20°C. A 100 mg/L stock standard mixture in methanol of (+)-catechin, (-)-epicatechin and procyanidin B2 were 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, v:v:v) solution.

2.3 Animals

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures. The animals used were 12-week-old female virgin Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 210-220 g. All
the animals were housed at 22°C with a light/dark cycle of 12 h (lights on at 09:00) and fed with a standard chow diet (AO4, Panlab, Barcelona, Spain).

Twelve virgin rats were mated with males until impregnation (confirmed by the presence of sperm in vaginal smears). The day of impregnation was designated as day 0 of pregnancy. The day before the treatment (day 18 of pregnancy), the animals were deprived of food overnight (for 14 h). On day 19 of pregnancy, the rats (n=6 animals per group) were intragastrically gavaged at 09:00 a.m. with GSPE (1 g per kg of body weight) diluted in distilled water and sacrificed 1 or 2 h after the treatment under anaesthesia (pentobarbital sodium, 80 mg/kg body weight). The blood was collected by cardiac puncture, and the plasma was obtained by centrifugation (2000 x g, 15 min at 4°C). Amniotic fluid samples were collected from the foetal sac using a 1-ml syringe with a 21-guage needle. The liver, brain, placenta, foetus and mesenteric white adipose tissue depots (MWAT) were rapidly removed after death, rinsed with Milli-Q water and frozen in liquid nitrogen. Plasma, amniotic fluid, foetuses and the different tissues were stored at -80°C for procyanidin extraction and chromatographic analysis. The tissues were freeze-dried prior to extraction and stored at -80°C.

Twelve virgin rats were used as control non-pregnant groups and treated with an identical dose of GSPE (1 g/kg body weight). The animals were sacrificed 1 or 2 h after the treatment, and the blood and the different tissues were collected, processed and stored in conditions identical to those in the pregnancy experiment.

Plasma and tissues free of procyanidins were obtained from non-pregnant rats that were not treated with GSPE. These sample matrices were used as blanks to validate the procedure.
2.4 Extraction of flavanols from plasma, amniotic fluid and tissues

Tissue samples (liver, brain, placenta, foetus tissue homogenate and MWAT) were pretreated prior to micro solid phase extraction (μSPE) by the off-line liquid-solid extraction (LSE) method following the methodology previously described by Serra et al. [27]. Briefly, to 60 mg of freeze-dried tissue, 50 μl of 1% L-ascorbic acid and 100 μl of 4% orthophosphoric acid were added. The tissue sample was extracted four times with 400 μl of water/methanol/4% orthophosphoric acid (94/4.5/1.5, v/v/v). In each extraction, 400 μl of extraction solution was added; the sample was sonicated for 30 s and then centrifuged for 15 min at 17,150 x g at 20ºC. The supernatants were collected, and then the extracts were purified by μSPE to determine the presence or the amount of procyanidins.

Prior to chromatographic analysis, the rat plasma, amniotic fluid samples and the tissue extractions were pretreated by off-line μSPE following the methodology previously described by Martí et al. [28] using Oasis HLB μElution Plates, 30 μm (Waters Corp., Milford, USA). Briefly, the micro-cartridges were conditioned sequentially with 250 μl of methanol and 250 μl of 0.2% acetic acid. A volume of 350 μl of plasma or amniotic fluid was mixed with 300 μl of 4% phosphoric acid and 50 μl of pyrocatechol (2000 ppb), and this mixture was then loaded onto the plate. The loaded plates were washed with 200 μl of Milli-Q water and 200 μl of 0.2% acetic acid. The retained procyanidins were 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 into the LC-TOF/MS or in the LC-QqQ-MS².

2.5 Analysis of flavanols from plasma, amniotic fluid and tissues

2.5.1 Instrumental conditions
A 1200 Series RRLC Liquid Chromatograph coupled to a 6210 TOF/MS was used for the determination of flavanols and conjugated metabolites. To confirm the identity of the metabolites, a 1200 Series LC coupled to a 6410 QqQ-MS/MS (Agilent Technologies, Palo Alto, U.S.A.) was used. In both cases, an identical chromatographic method was performed using a Zorbax C18 (100 mm length x 2.1 mm i.d., 1.8 μm particle size) chromatographic column from Agilent Technologies. Mobile phases were 0.2% acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was 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 performed.

ESI conditions for both TOF and QqQ analysis were 350°C and 12 l/min of drying gas temperature and flow, respectively, 45 psi of nebuliser gas pressure, and 4000 V of capillary voltage. TOF and QqQ were operated in negative mode. TOF conditions were 135 V of fragmentor and a scan range between 40 and 1200 m/z. Internal calibration of the m/z axis was performed during the entire run with the reference masses 112.9855 and 1033.9881. QqQ acquisition was performed in product ion mode.

2.5.2 Identification of flavanol metabolites and method validation.

Five microlitres of extracted samples was directly injected into the LC-TOF/MS for the detection of flavonols and their metabolites.

A volume of 2.5 μl of a pool of samples was injected into the LC-QqQ-MS² in product ion mode to confirm the identity of the detected metabolites. We compared the obtained MS² transitions with those described in the literature. The metabolites
whose identities were confirmed are shown in Table 2 together with the observed MS$^2$ transitions and references.

The quantitative method validation, calibration curves, extraction recovery, precision, sensitivity, method detection and quantification limits were studied by the analysis of standard solutions and samples in which procyanidin standards were included. Calibration curves were obtained by plotting the analyte/internal standard (IS) peak abundance ratio and the corresponding analyte/IS concentration ratio. Extraction recovery was evaluated by comparison of the response of samples containing procyanidin standards with the calibration curve of standard solutions. Method precision was determined from the relative standard deviation (% RSD) in a triplicate analysis of a sample containing standards. Sensitivity was evaluated by determining the limit of detection (LoD), defined as the concentration corresponding to three times the signal/noise ratio, and the limit of quantification (LoQ), defined as the concentration corresponding to 10 times the signal/noise ratio. Method detection and quantification limits (MDL and MQL, respectively) were calculated for the analysis of 350 $\mu$l of sample for placenta and amniotic fluid and 60 mg of dried sample for the rest of the tissues following the procedure described previously.

2.5.3 Sample quantification

For the quantification of samples, blank samples containing standards at 5 different levels of concentration 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. Because of the lack of standards, catechin and epicatechin metabolites were tentatively quantified using the standard catechin and
epicatechin calibration curves, respectively. Similarly, dimeric procyanidin B1 was quantified using the calibration curve of dimeric procyanidin B2.

2.6 Statistical analysis
The data for the polyphenol metabolite levels are presented as mean values±standard error (n=4). Data were analysed by a multifactor ANOVA to detect differences in polyphenol metabolite levels among different pregnant rat samples between post-ingestion time points (1 and 2 h) and to compare these differences with those of non-pregnant rats. For each treatment condition, data were also analysed by a one-way ANOVA to detect differences among polyphenol metabolite levels. A significant difference was considered at a level of $p \leq 0.05$ using Statgraphics Plus v. 5.1 software (Manugistics, Inc., Rockville, MA, USA). When effects were statistically significant, one-way ANOVA followed by a Duncan multiple range test was applied to determine differences among means, with a significance level of $p \leq 0.05$. Tukey’s test was used to determine treatment differences between means.

3 Results
3.1 Validation of the analytical procedure
The analysis of the extracted samples by LC-TOF/MS allowed the detection of different conjugated metabolites of both catechin and epicatechin by the exact mass, detecting in all cases the molecular ion [M-H$^-$]. In most cases, the error between the detected and calculated mass was below 5 ppm. Moreover, the identified metabolites were confirmed by LC-QqQ-MS$^2$. Detailed data regarding flavanols and metabolites in LC-ESI-TOF/MS and LC-ESI-QqQ/MS$^2$ analysis are given in Table 2.
The analytical characteristics such as the determination coefficient (R²), linearity, LoD and LoQ, MDL and MQL, extraction recovery and method precision were determined by including standards in blank plasma, tissues and maternal samples (i.e., placental homogenate, foetal homogenate and amniotic fluid) with different standard concentrations of catechin, epicatechin and dimeric procyanidin B2 by μSPE-LC-TOF/MS. The results are shown in Table 3.

The linearity was evaluated following the procedure developed in the range from 0.03 to 34.60 μM for catechin and epicatechin and from 0.05 to 17.33 μM for dimeric procyanidin B2. All the compounds demonstrated determination coefficients (R²) higher than 0.996. The intra-day precision (within-day precision, n = 3) of the methods was expressed as the % RSD of the concentration (Table 3). These values were lower than 15.1% in plasma, 17.7% in the liver, 2.7% in the MWAT, 24.3% in the foetal homogenate, 14.3% in the placental homogenate and 11.7% in the amniotic fluid. The lowest intra-day precision of flavanols (catechin, epicatechin and dimeric procyanidin B2) was obtained in the MWAT, with values between 0.6% and 2.7%.

The extraction recoveries were determined for the analysis of plasma, liver, MWAT, brain, foetal homogenate, placental homogenate and amniotic fluid (Table 3). The results indicated that more than 85% of flavanols were recovered in plasma and amniotic fluid. In liver, adipose tissue brain, placenta and foetal homogenates, recoveries ranging from 50 to 100% were obtained depending of the flavanol and the tissue analysed (Table 3). The MDLs and MQLs were lower than 7 and 25 nM, respectively, for plasma and amniotic fluid and lower than 0.3 and 1 μmol/kg, respectively, in most of the tissues. The obtained results have demonstrated the analytical method to be suitable for the determination of selected flavanols in the studied samples.
3.2 Flavanol distribution in maternal rat plasma and tissues during pregnancy

The composition of the GSPE used in this study is described in the Material and Methods (section 2.1.). The extract contained the most representative flavanols of grape seed extract [21], including monomeric flavan-3-ols (catechin and epicatechin) and their gallate forms (epigallocatechin, epicatechin gallate and epigallocatechin gallate) and polymeric proanthocyanidins (n=2-6). These compounds were studied in relation to their bioavailability in the present work. The major compounds detected in the plasma of both pregnant and non-pregnant rats 1h and 2 h after GSPE ingestion were conjugated forms of the monomeric catechin and epicatechin. The flavan-3-ol metabolites catechin glucuronide, epicatechin glucuronide, methyl-epicatechin-glucuronide and methyl-epicatechin-sulphate were detected at concentrations ranging from 23.21 to 5.45 µM (Table 4). The glucuronidated forms were present in plasma in a substantially higher concentration than other methylated and sulphated conjugates. Moreover, the free forms of catechin and epicatechin were detected in plasma at low concentrations (3.43 - 0.03 µM). Despite the high percentage of dimeric and trimeric procyanidins in the GSPE, procyanidins were detected as unconjugated forms in low amounts in plasma (0.04 - 1.57 µM). The physiological state of pregnancy did not affect to a major extent the bioavailability of flavanols and their metabolites in plasma at either 1 or 2 h after ingestion of the GSPE. However, 2 h after GSPE ingestion, the amount of catechin glucuronide appeared to be higher in pregnant than non-pregnant rats. In contrast, concentrations of methyl-epicatechin-glucuronide 1 h after GSPE ingestion significantly decreased in pregnant than non-pregnant plasma (Table 4).
Flavanols and their metabolites were detected in liver, MWAT and brain, demonstrating a wide distribution range throughout the body (Table 5). Nevertheless, the concentration of flavanols and their metabolites were greatly altered among the different tissues studied. Hence, MWAT was found to be the organ with the greatest presence of the unconjugated flavanols (i.e., catechin, epicatechin, procyanidin dimers B1 and B2), ranging from 15.89 to 118.79 nmol/g tissue. These compounds were found in much lower concentrations in the liver (2.03-5.17 nmol/g tissue) and in the brain, where catechin, epicatechin and dimeric procyanidin B2 were detected at low levels (1.27-2.39 nmol/g tissue). Catechin and epicatechin were substantially conjugated in liver rather than in MWAT and brain (Table 5). In the latter tissue, some glucuronidated forms of catechin and epicatechin were observed, and catechin glucuronide, epicatechin glucuronide and methyl-epicatechin glucuronide were quantified at 1.15-2.20 nmol/g tissue. Similar amounts of these metabolites were found in the MWAT (0.40-1.75 nmol/g tissue). However, in contrast to observations in the plasma and in the liver, the sulphated metabolites of flavanols were not detected or were present at very low levels in the MWAT and in the brain. Interestingly, during pregnancy the content of the most conjugated flavon-3-ols in the liver was lower than in non-pregnant rats. However, pregnancy appears to not affect the accumulation of the unconjugated flavanols in MWAT and brain (Table 5). Additionally, differences in the metabolite concentrations between the 1 h and 2 h time points after ingestion of the GSPE were not significant in liver, MWAT and brain (data not shown).

3.3 Flavanol distribution in placenta, amniotic fluid and foetuses
The content of flavanols and their metabolites in placental homogenate, foetal homogenate and amniotic fluid at the 1 h or 2 h time points after ingestion of the GSPE is shown in Table 6. Homogenates of all placentas and all foetuses for each pregnant rat were used as pools to avoid large standard deviations in metabolite concentrations due to biological variations between individual dams. As it was expected, flavanols and their metabolites were quantified in placental and in foetal homogenate 1h post-ingestion, whereas these compounds were quantify in amniotic fluid after 2h of GSPE ingestion. The major flavanols quantified in the GSPE used in this study (i.e., catechin, epicatechin, dimeric procyanidins (B1 and B2)) were quantified in placental homogenate with concentrations ranging from 3.06 to 13.29 nmol/g tissue. However, in foetal homogenates, these compounds were present at low concentrations or they were not detected. In amniotic fluid, the free forms of catechin and epicatechin were detected, but the amounts were so low that they could not be quantified. For instance, concentrations of unconjugated epicatechin in placental homogenate reached 13.29 nmol/g tissue 2 h after ingestion of the GSPE. However, unconjugated epicatechin was present in very low concentrations in foetal homogenate (0.43 nmol/g tissue), and it was present in trace levels in amniotic fluid 2 h after ingestion. Dimeric procyanidins reached concentrations of 3.06-4.45 nmol/g tissue in placental homogenate, but they were present in low concentrations or not detected in foetal homogenate and amniotic fluid. The placenta was the organ with the greatest presence of metabolites compared with the foetal homogenate and amniotic fluid. Regarding flavanol metabolites, the highest levels were also found in the placenta in which the glucuronidated and/or methylated forms of catechin and epicatechin were present in a higher amount than the sulphated forms. Moreover, in this tissue, the concentrations of these conjugated forms were significantly higher
than the free forms of dimeric procyanidins. Regarding the sulphated metabolites, epicatechin sulphate could be detected in placenta in very low amounts, but it was not found in foetuses. Catechin sulphate, which was present in maternal plasma, was not detected in placenta, foetal homogenate or amniotic fluid (Table 6).

Although the free forms of flavanols in amniotic fluid were not detected or not quantified, trace levels of some flavanol metabolites (i.e., catechin and epicatechin glucuronide, methyl-catechin glucuronide, methyl-epicatechin glucuronide and methyl-epicatechin-O-sulphate) could be quantified 2 h after GSPE ingestion (Table 6).

4 Discussion

The aim of our study was to evaluate how flavanols from a GSPE are distributed during pregnancy in maternal plasma and tissues and to compare the results to those in non-pregnant rats. This is of special interest due to the potential beneficial health effects of flavanols during this physiological state for both the mother and the offspring. In both physiological states in rats (i.e., pregnant and non-pregnant rats), a rapid absorption of the GSPE was observed with phenolic compounds detected in plasma 1 h after ingestion, although maximal concentrations for some compounds were observed after 2 h. These results are similar to those obtained by others after administration of an identical extract used in this study (i.e., GSPE) who reported maximal concentrations in plasma between 1 h and 2 h after administration of the extract to male Wistar rats [21].

The concentrations of the non-metabolised flavan-3-ols and their metabolised forms in non-pregnant rats after an acute intake of the GSPE were in the range of those previously reported by Serra et al. [22] in the plasma of male Wistar rats. In this
study, the GSPE was also used as the proanthocyanidin extract at an identical dose with identical kinetics. Our results indicated that in both pregnant and non-pregnant physiological states, free forms of catechin and epicatechin were found in plasma at low concentrations. In contrast, most of the metabolites identified in plasma appeared in their conjugated form, primarily glucuronidated likely by liver, and this agrees with the studies performed with an identical GSPE by Serra et al. [21].

Pregnancy could involve several physiological changes, such as in plasma protein composition or in renal plasma flow [31], which could alter the absorption, distribution and elimination of flavanols. To our knowledge, although there are no studies of GSPE bioavailability during pregnancy, there are several studies regarding drug kinetics during pregnancy. In these studies, it has been demonstrated that various pregnancy-related haemodynamic changes such as an increase in cardiac output, blood volume, the volume of distribution, renal perfusion and glomerular filtration may affect drug disposition and elimination and cause an increase or decrease in the terminal elimination half-life of drugs [32]. For many drugs, absorption is decreased and elimination increased, thus tending to reduce plasma concentrations [33]. Hence, in our study, it appears that pregnancy did not affect to a major extent the absorption of GSPE flavanols. However, the absorption of some glucuronidated flavanol metabolites was disturbed during pregnancy.

Once administered, monomeric flavan-3-ol are absorbed in the small intestine and metabolised into glucuronide, sulphate and/or methylated conjugates. Further conjugation occurs in the liver. Once the conjugation is complete, these metabolites enter the bloodstream, where they are transported to the different tissues or are eliminated. Furthermore, flavanols can also enter the colon where are fragmented by microbial catabolism and reabsorbed in the liver, where additional conjugated can
take place, and further enter the circulation or eliminated in the bile [34-35].

However, in this study, not only were the conjugated forms of catechin and epicatechin able to reach the bloodstream, but proanthocyanidin dimers were also detected in plasma 1 h and 2 h after the acute intake of the GSPE, as was also reported by others [36-37]. The enterohepatic recirculation of these compounds and their different affinities with proteins also affect their concentration in plasma [38]. Catechin and epicatechin monomers and their methyl, sulphate and glucuronidate conjugates are rapidly absorbed and appear in plasma within short time periods between 1-2 h after ingestion [21]. The low plasma levels of the gallate forms of proanthocyanidins in pregnant and non-pregnant rats can be attributed to the poor solubility of these compounds when compared with catechins [25]. The distribution of flavanols in tissues of male Wistar rats has been reported using natural extracts other than GSPE [23-24]. Our results indicated that flavanol metabolites were uniformly distributed in both pregnant and non-pregnant rats in all the analysed tissues with a high number of metabolites at high concentrations in the liver and placenta. No significant differences between 1 h and 2 h after ingestion of the GSPE were observed in the studied tissues (data not shown). The high presence of metabolites in the liver indicated active metabolism by this organ in which catechin and epicatechin are conjugated to their methylated, sulphated and glucuronidated forms. Interestingly, liver metabolism appears to be less active during pregnancy in which the content of conjugated flavon-3-ols in the liver was lower than that in non-pregnant rats. This conjugation is a common metabolic detoxification process for most xenobiotics to assist urinary elimination of phenolics [39]. In this sense, the placenta acts as a barrier between maternal and foetal circulation and prevents xenobiotics from entering into the foetus. Furthermore, hydrophilic drugs are able to
cross the placenta by means of transporters [31, 40], which would explain the relatively high concentration of metabolites compared with the non-conjugated forms. Moreover, a high accumulation of glucuronidated, methylated and sulphated forms in the liver and placenta could indicate major excretion of these phenolic conjugates [41,42].

In contrast, the non-metabolised forms of catechin and epicatechin are accumulated in the MWAT in both physiological states, and a low amount of the conjugated compounds at low concentrations were present in this tissue. Fewer data are available in the literature regarding the determination of flavanols and their metabolites in adipose tissue. The free form of dimeric procyanidin B2 was previously found in adipose tissue [27], but these authors did not identify catechin, epicatechin and their metabolites in this tissue. White adipose tissues store excess fat and fat-soluble nutrients. The extremely high dose of GSPE administered to the rats (i.e., 1 g/kg body weight) is sufficiently high to allow flavanols to penetrate into the foetus. Of special interest is the presence of flavanols and their metabolites in the brain. Previously, Urpi-Sarda et al. [43] demonstrated that catechins, epicatechins and their metabolites can cross the blood-brain barrier and target the brain after three weeks of a cocoa diet. Our results confirm that monomeric flavan-3-ol and its glucuronidated forms target the brain. These conjugated metabolites were also found at 4 h after the administration of 1 g of cocoa extract/kg rat by Serra et al. [27], although they did not indentify the monomeric catechin and epicatechin but the free form of dimeric procyanidin B2. Initial in vitro studies have indicated that less polar polyphenols or metabolites better penetrate the blood-brain barrier [44]. Indeed, our results indicated that less polar non-conjugated flavanols penetrated the brain. However, we also demonstrated that polar metabolites (i.e., glucuronidated) are taken up by the brain.
Interestingly, in our study, phenolic compounds were found in the brain at short time periods of 1 and 2 h after ingestion of the GSPE, although monomeric and dimeric flavanols were present in small amounts. In fact, some studies suggested that polyphenols typically accumulate in the brain at levels below 1 nmol/g tissue [45].

The second aim of this study was to investigate whether flavanols and their metabolites could cross the placenta barrier and enter the foetuses of pregnant rats. As previously mentioned, a high concentration of flavanols and primarily their conjugated forms are located in the placenta of pregnant rats because this tissue acts as a barrier for xenobiotics entering the foetuses. Therefore, data indicated that transport across the placenta is not efficient and for these compounds the placenta seems to act as a barrier. However, our results indicated that flavanols and their metabolites are able to cross the placental barrier to target the foetuses and be excreted to the amniotic fluid. The presence of these compounds in foetuses suggests that they could be able to exert a biological effect on the offspring. However, it is difficult to know if the concentrations achieved in the foetuses are comparable to the concentrations at which flavanols have been observed to provide beneficial health effects on adults. Moreover, the mechanism by which these compounds can exert their biological effects remains unknown, although increasing evidence indicates an induction of epigenetic modifications [4-5]. Hence, further studies will be necessary to evaluate the potential protective and/or epigenetic role of the intake of flavanols during pregnancy acting on the mothers and their offspring.

In conclusion, in this study, we demonstrated that flavanols and their metabolites were widely distributed in maternal plasma and tissues 1 and 2 h after ingestion of a GSPE. A pregnant physiological state of rats appeared not to alter to a major extent the absorption and distribution of grape seed flavanols, even though the metabolism
of flavanols in the liver appeared to be less active during pregnancy. Placenta seems
to act as a barrier for flavanols and their metabolites and that transport across the
placenta is not efficient. However, trace amounts of these compounds were detected
in both foetuses and amniotic fluid.
Acknowledgments

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The authors have declared no conflict of interest.
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1 [31] Morgan, J.D., Drug disposition in mother and foetus. *Clinical and Experimental


Table 1. Amount of the individual flavanols and phenolic acids of the grape seed proanthocyanidin extract (GSPE) used in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (mg compound/g extract)</th>
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<tr>
<td>Gallic acid</td>
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<tr>
<td>Protocatechuic acid</td>
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<td>Vanillic acid</td>
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<td>Procyanidin dimer(^a)</td>
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<tr>
<td>p-coumaric acid</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Dimer gallata(^a)</td>
<td>39.7 ± 7.1</td>
</tr>
<tr>
<td>Epigallocatechinch gallate</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Procyanidin trimer(^a)</td>
<td>28.4 ± 2.0</td>
</tr>
<tr>
<td>Procyanidin tetramer(^a)</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Epicatechin gallate(^a)</td>
<td>55.3 ± 1.5</td>
</tr>
<tr>
<td>Quercetin-3-O-galactoside</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Naringenin-7-glucoside</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Kaempferol-3-glucoside</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.3 ± 0.0</td>
</tr>
</tbody>
</table>

(Adapted from Quiñones et al. [26])

Phenolic components were determined by reverse-phase HPLC-MS. The results are expressed as mean ± SD (n=3).  
\(^a\) Quantified using the calibration curve of procyanidin B2.  
\(^b\) Quantified using the calibration curve of epigallocatechin gallate.
Table 2. Detailed data for the studied procyanidins and their metabolites by LC-ESI-TOF/MS in negative mode, and transitions obtained in analysis by LC-ESI-QqQ/MS² in product ion mode used to confirm the metabolite identity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula</th>
<th>Exact MW (g/mol)</th>
<th>tR TOF (min)</th>
<th>Detected ion [M-H(^+)]</th>
<th>tR QqQ (min)</th>
<th>Observed transitions in MS²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standards</strong></td>
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</tr>
<tr>
<td>Pyrocatechol</td>
<td>C₆H₆O₂</td>
<td>110.036</td>
<td>2.90</td>
<td>109.030</td>
<td>2.76</td>
<td>109&gt;109</td>
<td>[27]</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>C₁₅H₁₄O₆</td>
<td>290.079</td>
<td>7.11</td>
<td>289.072</td>
<td>6.84</td>
<td>289&gt;245 289&gt;203 289&gt;179</td>
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</tr>
<tr>
<td><strong>Identified metabolites</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Procyanidin B1, B3 dimer</td>
<td>C₆₀H₅₀O₁₂</td>
<td>578.142</td>
<td>6.26, 6.49</td>
<td>577.136, 577.135</td>
<td>6.00, 6.26</td>
<td>577&gt;425 577&gt;407 577&gt;289</td>
<td></td>
</tr>
<tr>
<td>Catechin glucuronide</td>
<td>C₂₁H₂₂O₁₂</td>
<td>466.111</td>
<td>5.76</td>
<td>465.105</td>
<td>5.50</td>
<td>465&gt;289 465&gt;203</td>
<td></td>
</tr>
<tr>
<td>Epicatechin glucuronide</td>
<td>C₂₁H₂₂O₁₂</td>
<td>466.111</td>
<td>6.04</td>
<td>465.105</td>
<td>5.80</td>
<td>465&gt;289 465&gt;203</td>
<td>[27], [29], [30]</td>
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<tr>
<td>4-O-methyl-Epicatechin</td>
<td>C₂₁H₂₂O₁₂</td>
<td>304.097</td>
<td>7.64</td>
<td>303.088</td>
<td>7.74</td>
<td>303&gt;137 303&gt;285</td>
<td>[30]</td>
</tr>
<tr>
<td>Methyl-catechin-glucuronide</td>
<td>C₂₂H₂₄O₁₂</td>
<td>480.126</td>
<td>6.77</td>
<td>479.121</td>
<td>6.52</td>
<td>479&gt;303 479&gt;289</td>
<td>[27]</td>
</tr>
<tr>
<td>Methyl-epicatechin-glucuronide</td>
<td>C₂₂H₂₄O₁₂</td>
<td>480.126</td>
<td>6.90</td>
<td>479.121</td>
<td>6.65</td>
<td>479&gt;303 479&gt;289</td>
<td>[27]</td>
</tr>
<tr>
<td>Catechin sulphate</td>
<td>C₁₅H₁₄O₉S</td>
<td>370.035</td>
<td>7.34</td>
<td>369.029</td>
<td>7.20</td>
<td>369&gt;289 369&gt;245</td>
<td>[27]</td>
</tr>
<tr>
<td>Epicatechin sulphate</td>
<td>C₁₅H₁₄O₉S</td>
<td>370.035</td>
<td>7.52</td>
<td>369.029</td>
<td>7.24</td>
<td>369&gt;289 369&gt;245</td>
<td>[27], [29], [30]</td>
</tr>
<tr>
<td>Methyl-catechin-O-sulphate</td>
<td>C₁₅H₁₄O₉S</td>
<td>384.0515</td>
<td>8.04</td>
<td>383.045</td>
<td>7.93</td>
<td>383&gt;303 383&gt;245</td>
<td>[27]</td>
</tr>
</tbody>
</table>
Table 3. Method validation for the determination of flavanols by off-line μSPE-LC-TOF/MS in the studied tissues assessed by the following parameters: determination coefficient ($R^2$), linearity, detection and quantification limits (LoD and LoQ), method detection and quantification limits (MDL and MQL), extraction recovery and intra-day method precision.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Procyanidin</th>
<th>Det. Coeff. ($R^2$)</th>
<th>Linearity (μM)</th>
<th>LoD (μM)</th>
<th>LoQ (μM)</th>
<th>MDL (μM)</th>
<th>MQL (μM)</th>
<th>Recovery (%)</th>
<th>Precision (RSD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
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</tr>
<tr>
<td>Catechin</td>
<td>0.9991</td>
<td>0.04-34.60</td>
<td>0.01</td>
<td>0.04</td>
<td>0.003</td>
<td>0.011</td>
<td>88</td>
<td>11.1</td>
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<tr>
<td>Epicatechin</td>
<td>0.9990</td>
<td>0.03-34.60</td>
<td>0.01</td>
<td>0.03</td>
<td>0.002</td>
<td>0.008</td>
<td>100</td>
<td>7.4</td>
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<tr>
<td>Procyanidin B2</td>
<td>0.9999</td>
<td>0.05-17.33</td>
<td>0.02</td>
<td>0.05</td>
<td>0.004</td>
<td>0.015</td>
<td>100</td>
<td>15.1</td>
<td></td>
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<tr>
<td><strong>Liver</strong></td>
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<tr>
<td>Catechin</td>
<td>0.9991</td>
<td>0.19-34.60</td>
<td>0.06</td>
<td>0.19</td>
<td>0.480</td>
<td>1.602</td>
<td>61</td>
<td>13.3</td>
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<tr>
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<td>0.17-34.60</td>
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<td>0.17</td>
<td>0.432</td>
<td>1.441</td>
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<td>14.0</td>
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<td>0.34</td>
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<td>2.825</td>
<td>9.417</td>
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<td><strong>Adipose tissue</strong></td>
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<td>Catechin</td>
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<td>0.04-34.60</td>
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<td>0.04</td>
<td>0.102</td>
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<td>0.03-34.60</td>
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<td>0.079</td>
<td>0.262</td>
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<td>0.09-17.33</td>
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<td>0.09</td>
<td>0.217</td>
<td>0.722</td>
<td>68</td>
<td>0.6</td>
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<tr>
<td><strong>Brain</strong></td>
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<tr>
<td>Catechin</td>
<td>0.9999</td>
<td>0.09-34.60</td>
<td>0.03</td>
<td>0.09</td>
<td>0.231</td>
<td>0.769</td>
<td>70</td>
<td>-</td>
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<tr>
<td>Epicatechin</td>
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<td>0.12-34.60</td>
<td>0.03</td>
<td>0.12</td>
<td>0.288</td>
<td>0.961</td>
<td>53</td>
<td>-</td>
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<tr>
<td>Procyanidin B2</td>
<td>0.9999</td>
<td>0.09-17.33</td>
<td>0.03</td>
<td>0.09</td>
<td>0.217</td>
<td>0.722</td>
<td>78</td>
<td>-</td>
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<tr>
<td><strong>Foetal homogenate</strong></td>
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<tr>
<td>Catechin</td>
<td>0.9999</td>
<td>0.06-34.60</td>
<td>0.02</td>
<td>0.06</td>
<td>0.154</td>
<td>0.515</td>
<td>80</td>
<td>20.5</td>
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<tr>
<td>Epicatechin</td>
<td>0.9997</td>
<td>0.04-34.60</td>
<td>0.01</td>
<td>0.04</td>
<td>0.090</td>
<td>0.300</td>
<td>99</td>
<td>24.3</td>
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<tr>
<td>Procyanidin B2</td>
<td>1.0000</td>
<td>0.07-17.33</td>
<td>0.02</td>
<td>0.07</td>
<td>0.180</td>
<td>0.602</td>
<td>100</td>
<td>16.2</td>
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<tr>
<td><strong>Placental homogenate</strong></td>
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<tr>
<td>Catechin</td>
<td>0.9993</td>
<td>0.09-34.60</td>
<td>0.03</td>
<td>0.09</td>
<td>0.231</td>
<td>0.769</td>
<td>50</td>
<td>3.3</td>
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<tr>
<td>Epicatechin</td>
<td>0.9998</td>
<td>0.04-34.60</td>
<td>0.01</td>
<td>0.04</td>
<td>0.102</td>
<td>0.339</td>
<td>68</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>0.9999</td>
<td>0.10-17.33</td>
<td>0.03</td>
<td>0.10</td>
<td>0.241</td>
<td>0.802</td>
<td>68</td>
<td>14.3</td>
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<td><strong>Amniotic fluid</strong></td>
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<tr>
<td>Catechin</td>
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<td>0.03</td>
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<td>0.008</td>
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<td>11.7</td>
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<tr>
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<td>0.03</td>
<td>0.003</td>
<td>0.010</td>
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<td>10.7</td>
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<tr>
<td>Procyanidin B2</td>
<td>0.9984</td>
<td>0.09-17.33</td>
<td>0.03</td>
<td>0.09</td>
<td>0.007</td>
<td>0.025</td>
<td>100</td>
<td>10.4</td>
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</tr>
</tbody>
</table>

* Method detection and quantification limits for the analysis of 350 μl of plasma and amniotic fluid, in μmol/litre, and 60 mg of the rest of the tissues, in μmol/kilogram of dried tissue.
Table 4. Flavanols and their metabolites detected in rat plasma (µM) 1 h and 2 h after ingestion of 1 g/kg of body weight of GSPE.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>PREGNANT 1h</th>
<th>PREGNANT 2h</th>
<th>NON-PREGNANT 1h</th>
<th>NON-PREGNANT 2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>0.06±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.19±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.03±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1.37±0.39&lt;sup&gt;Abd&lt;/sup&gt;</td>
<td>0.98±0.19&lt;sup&gt;Re&lt;/sup&gt;</td>
<td>3.43±1.41&lt;sup&gt;Ac&lt;/sup&gt;</td>
<td>0.67±0.18&lt;sup&gt;Bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>n.q.</td>
<td>n.q.</td>
<td>0.04±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>n.q.</td>
</tr>
<tr>
<td>Procyanidin dimer B2</td>
<td>1.38±0.22&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.19±0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.11±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.87±0.27&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Procyanidin dimer B1</td>
<td>1.57±0.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.02±1.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.02±0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.89±0.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catechin glucuronide</td>
<td>8.77±0.95&lt;sup&gt;Abh&lt;/sup&gt;</td>
<td>11.04±0.25&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>10.63±0.72&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.18±0.55&lt;sup&gt;Bb&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epicatechin glucuronide</td>
<td>14.10±1.59&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>12.31±3.12&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>23.21±3.23&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>12.91±0.91&lt;sup&gt;Ba&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-O-methyl-epicatechin</td>
<td>0.40±0.08&lt;sup&gt;Abd&lt;/sup&gt;</td>
<td>0.32±0.08&lt;sup&gt;Be&lt;/sup&gt;</td>
<td>0.63±0.17&lt;sup&gt;Ad&lt;/sup&gt;</td>
<td>0.25±0.05&lt;sup&gt;Abd&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-O-methyl-epicatechin</td>
<td>0.15±0.03&lt;sup&gt;Abd&lt;/sup&gt;</td>
<td>0.09±0.02&lt;sup&gt;Bc&lt;/sup&gt;</td>
<td>0.28±0.08&lt;sup&gt;Ad&lt;/sup&gt;</td>
<td>0.09±0.03&lt;sup&gt;Abd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methyl-epicatechin-glucuronide</td>
<td>0.48±0.09&lt;sup&gt;dc&lt;/sup&gt;</td>
<td>0.61±0.11&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>1.34±0.51&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.34±0.07&lt;sup&gt;dc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methyl-epicatechin-sulphate</td>
<td>5.39±0.89&lt;sup&gt;Ce&lt;/sup&gt;</td>
<td>8.61±0.75&lt;sup&gt;Abb&lt;/sup&gt;</td>
<td>8.59±1.33&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>5.45±0.29&lt;sup&gt;Bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catechin sulphate</td>
<td>0.07±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.08±0.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.19±0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.02±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epicatechin sulphate</td>
<td>1.06±0.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.83±0.41&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.18±0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.56±0.17&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methyl-catechin-O-sulphate</td>
<td>4.78±1.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.58±1.77&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.61±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.75±1.47&lt;sup&gt;ce&lt;/sup&gt;</td>
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<tr>
<td>Methyl-epicatechin-O-sulphate</td>
<td>7.88±1.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.55±2.91&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>7.59±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.83±2.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data are given as the means±s.e.m. (n=4-6). Abbreviations: n.q.= not quantified; n.d.= not detected.

The last column shows the significant effects for pregnancy and treatment time: P, a significant effect between pregnant or non-pregnant rats; T, a significant effect between 1 and 2h after ingestion of 1 g/kg of body weight of a grape seed proanthocyanidin extract (GSPE); P x T, a significant effect between the interaction of the physiological state (pregnant and non-pregnant rats) of rats and time after GSPE ingestion (two-way ANOVA, p<0.05).

For each metabolite, different letters within an identical row indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, p<0.05).

For an identical physiological state of rats and an identical time after ingestion, different letters within an identical column indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, p<0.05).
Table 5. Flavanols and their metabolites detected in rats 2 h after ingestion of 1 g/kg of body weight of a GSPE extract.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LIVER (nmol/g tissue)</th>
<th>MWAT (nmol/g tissue)</th>
<th>BRAIN (nmol/g tissue)</th>
<th>Primary and interaction effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-pregnant</td>
<td>Pregnant</td>
<td>Non-pregnant</td>
<td>Pregnant</td>
</tr>
<tr>
<td>Catechin</td>
<td>4.75±1.46</td>
<td>2.03±0.46</td>
<td>118.79±33.83</td>
<td>104.61±19.81</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>5.17±0.72</td>
<td>2.21±0.27</td>
<td>94.84±27.36</td>
<td>81.64±15.02</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6.09±2.53</td>
<td>5.17±1.67</td>
</tr>
<tr>
<td>Procyanidin dimer B2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>44.94±15.58</td>
<td>60.34±14.59</td>
</tr>
<tr>
<td>Catechin glucuronide</td>
<td>5.81±0.58</td>
<td>2.79±0.60</td>
<td>1.75±0.43</td>
<td>0.77±0.07</td>
</tr>
<tr>
<td>Epicatechin glucuronide</td>
<td>3.23±0.38</td>
<td>n.q.</td>
<td>1.71±0.42</td>
<td>0.93±0.17</td>
</tr>
<tr>
<td>3-O-methyl-epicatechin</td>
<td>7.64±0.52</td>
<td>9.57±1.35</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>4-O-methyl-epicatechin</td>
<td>4.15±0.72</td>
<td>6.25±0.99</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methyl-catechin-glucuronide</td>
<td>3.34±0.47</td>
<td>1.88±0.76</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methyl-epicatechin-glucuronide</td>
<td>13.74±0.46</td>
<td>11.17±1.37</td>
<td>0.74±0.20</td>
<td>0.40±0.10</td>
</tr>
<tr>
<td>Epicatechin sulphate</td>
<td>3.67±1.20</td>
<td>n.q.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methyl-catechin-O-sulphate</td>
<td>9.15±1.27</td>
<td>9.13±2.26</td>
<td>n.d.</td>
<td>0.41±0.18</td>
</tr>
<tr>
<td>Methyl-epicatechin-O-sulphate</td>
<td>24.62±2.65</td>
<td>6.67±0.78</td>
<td>0.27±0.09</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The data are given as the means±s.e.m. (n=4-6). Abbreviations: MWAT, mesenteric white adipose tissue; n.q.= not quantified; n.d.= not detected.

Last column shows the significant effects for pregnancy and tissue type: P, a significant effect between pregnant or non-pregnant rats; T, a significant effect between flavanol amount in different tissues; P × T, a significant effect between the interaction of the physiological state (pregnant and non-pregnant rats) of rats and the flavanol amount in different tissues (two-way ANOVA, \( p \leq 0.05 \)).

abcd For each metabolite, different letters within an identical row indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, \( p \leq 0.05 \)).

abcdef For each tissue and an identical physiological state of rats, different letters within an identical column indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, \( p \leq 0.05 \)).
Table 6. Flavanols and their metabolites detected in pregnant rats 1 h and 2 h after ingestion of a grape seed procyanidin extract.

<table>
<thead>
<tr>
<th></th>
<th>PLACENTAL HOMOGENATE (nmol/g tissue)</th>
<th>FOETAL HOMOGENATE (nmol/g tissue)</th>
<th>AMNIOTIC FLUID (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
<td>2h</td>
<td>1h</td>
</tr>
<tr>
<td>Catechin</td>
<td>5.24±0.75 te</td>
<td>7.88±1.91 ade</td>
<td>n.d.</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>11.80±2.57 Ad</td>
<td>13.29±1.57 Aed</td>
<td>0.31±0.08 Ac</td>
</tr>
<tr>
<td>Procyanidin dimer B2</td>
<td>3.06±0.75 aef</td>
<td>4.45±0.47 Ac</td>
<td>1.27±0.16 Ab</td>
</tr>
<tr>
<td>Procyanidin dimer B1</td>
<td>3.34±0.77 aef</td>
<td>3.36±0.49 Ac</td>
<td>n.d.</td>
</tr>
<tr>
<td>Catechin glucuronide</td>
<td>59.79±3.82 Aa</td>
<td>81.29±11.29 Aa</td>
<td>0.88±0.11 Acd</td>
</tr>
<tr>
<td>Epicatechin glucuronide</td>
<td>17.91±1.04 bc</td>
<td>28.00±3.64 Ab</td>
<td>0.44±0.06 Ae</td>
</tr>
<tr>
<td>3-O-methyl-epicatechin</td>
<td>17.12±1.77 Ac</td>
<td>15.35±1.24 Aed</td>
<td>1.15±0.23 Acd</td>
</tr>
<tr>
<td>4-O-methyl-epicatechin</td>
<td>23.88±2.72 Ab</td>
<td>20.02±2.40 Ac</td>
<td>0.93±0.09 Acd</td>
</tr>
<tr>
<td>Methyl-catechin-glucuronide</td>
<td>4.71±1.10 Ae</td>
<td>4.23±0.39 Ae</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methyl-epicatechin-glucuronide</td>
<td>15.18±1.76 Acd</td>
<td>18.16±1.14 Ac</td>
<td>0.75±0.14 Ad</td>
</tr>
<tr>
<td>Catechin sulphate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Epicatechin sulphate</td>
<td>1.72±0.81 erf</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methyl-catechin-O-sulphate</td>
<td>n.d.</td>
<td>1.94±1.02 e</td>
<td>n.q.</td>
</tr>
<tr>
<td>Methyl-epicatechin-O-sulphate</td>
<td>1.50±0.15 def</td>
<td>3.21±0.62 Ae</td>
<td>2.53±0.09 Aa</td>
</tr>
</tbody>
</table>

The data are given as the means±s.e.m. (n=4-6). Abbreviations: n.q.= not quantified; n.d.= not detected.

TIS, the effect of the tissue type; T, the effect of the period after ingestion of 1 g/kg of body weight of a grape seed procyanidin extract (GSPE); P × T, the interaction of the physiological state of rats and time after GSPE ingestion (two-way ANOVA, p≤ 0.05).

For each metabolite, different letters within an identical row indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, p≤ 0.05).

For an identical physiological state of rats and an identical time after ingestion, different letters within an identical column indicate significant differences among mean values (one-way ANOVA and Duncan’s multiple-range test, p≤ 0.05).