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Sex Differences in the Absorption, Disposition, Metabolism, and Excretion of Grape Seed Proanthocyanidins in Prepubescent Rats

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Scope: The absorption, disposition, metabolism, and excretion (ADME) of phenolic compounds are key factors in determining their bioactivity. The group demonstrates that the ADME of a Grape Seed Proanthocyanidin Extract (GSPE) depends on sex in adult rats and specifically, methylated metabolites are only quantified in brain male adult rats. The aim of this study is to determine whether these differences exist before puberty.

Methods and results: Prepubescent 4-week-old male and female Wistar rats are administered GSPE at a dose of 1000 mg kg^{-1}. Plasma, liver, mesenteric white adipose tissue (MWAT), brain, and kidneys are extracted excised 2 h after GSPE administration, and the PAs metabolite profile is studied by HPLC-ESI-MS/MS. Moreover, plasma estradiol and brain and liver catechol-*O*-methyltransferase (COMT) protein levels are also studied. Results showed that there are no differences in plasma and brain among sexes and only differences are observed in liver, MWAT, and kidney with individual metabolites. This agrees with the lack of differences in estradiol and COMT levels among sexes. However, the ADME of PAs metabolites is higher in male rats.

Conclusions: The results demonstrate lack of sex-dependence in metabolite profile in prepubescent rats, suggesting that sex differences in the metabolism of GSPE occur due to puberty.

1. Introduction

Flavan-3-ols, also known as flavanols, range from simple monomers, mainly (+)-catechin and (–)-epicatechin, to oligomeric and polymeric proanthocyanidins (PAs). PAs are especially abundant in grape seeds,[1] and they have reported a wide range of health-promoting effects, including decrease of the risk of cardiovascular diseases or type 2 diabetes.[2] PAs present biological functions in children,[3,4] and after a chronic berry consumption, healthy children seem to excrete a similar pattern of (poly)phenol metabolites to adults.[5] However, to our knowledge, the plasma levels of PAs in children is yet to be explored.

Remarkably, the health promoting activities of flavanols have been mainly attributed to their metabolized forms.[6,7] Once ingested, flavanols can be absorbed in the small intestine and are thereafter recognized as xenobiotics,

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undergoing Phase-II enzymatic detoxification both at the small intestine and the liver. Glucuronide-, methyl-, and sulpho-metabolites are formed due to the enzymatic activity of uridine 5'-diphosphate glucuronosyltransferases (UGTs), catechol-*O*-methyltransferase (COMT), and cytosolic sulphotransferases (SULTs), respectively.[8] However, high molecular weight polymeric PAs are not absorbed in the small intestine and reach the colon.[9,10] Gut bacteria are able to hydrolyze flavanols PAs into smaller molecules which can be absorbed in the colon, reach the systemic circulation, and further undergo Phase-II detoxification.[8] Both metabolized and non-metabolized flavanols are known to reach several organs and tissues to finally reach the kidney where they are excreted via the urine.[11–13] An overview of flavonoid absorption and post-absorptive metabolism is described by Cassidy and Minihane.[14]

Our group has previously demonstrated that host's physiology can greatly affect the metabolism, concentration, and body distribution of flavanols and their metabolites[12,15–18] which may affect their bioactivity.[15,19] For example, one of the key differences in PAs absorption, disposition, metabolism, and excretion (ADME) between male and female adult rats was the higher presence of methyl-metabolites in males brain.[12] Also, male young rats presented a sharper, faster plasma (poly)phenolic metabolite kinetic curve than older male rats.[17] Of note, several changes that occur during aging present a sex-related profile.[20] For instances, the expression of several SULT isoforms,[21–23] UGTs isoforms,[23,24] and the COMT enzyme[23,25] change between sexes in adulthood. Moreover, estradiol has been described as a negative regulator of the COMT enzyme,[26–28] while androgens downregulate the expression of several SULT isoforms.[22] Whether these changes are also reported in prepubescent rats, or puberty is their triggering factor is unknown. To shed some light on that matter, in vivo animal-based studies should be firstly performed.

Considering that flavanols are recognized as xenobiotics[8] and that physiological changes occur while rats grow older,[20] we hypothesize that 4-week-old male and female rats will not present a marked difference in the metabolism and body distribution of GSPE flavanols, unlike what we reported for adult rats.[12] Thus, the aim of this study was to elucidate weather GSPE flavanols can be differently absorbed, distributed, and metabolized in a sex-dependent manner at the early age of 4 weeks. To further understand the role of phenolic methyl metabolism, the levels of brain and liver COMT, as well as plasma estradiol, were measured.

2. Experimental Section

2.1. Chemicals and Reagents

Acetone, methanol, acetonitrile (all HPLC analytical grade), and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). (+)-Catechin, (-)-epicatechin, gallic acid, PA B2, epigallocatechin gallate (EGCG), and pyrocatechol (internal standard, IS) were purchased from Fluka/Sigma-Aldrich (Madrid, Spain) and were individually dissolved in methanol at the concentration of 2000 mg L^{-1}. All standard stock solutions were prepared newly every 3 months and stored in amber-glass flasks at −20 °C.

Table 1. Main (poly)phenolic compounds of the Grape Seed Proanthocyanidin Extract (GSPE) used in this study analyzed by HPLC-ESI-MS/MS.

Compound	Concentration [mg g^{-1}]
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
PA dimer B1[a)]	88.80 ± 3.46
PA dimer B3[a)]	46.09 ± 2.07
PA dimer B2	33.24 ± 1.39
Gallic acid	31.07 ± 0.08
ECG	21.24 ± 1.08
Dimer gallate[b)]	8.86 ± 0.14
PA trimer[a)]	4.90 ± 0.47
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
EGC[b)]	0.27 ± 0.03
PA tetramer[a)]	0.05 ± 0.01
EGCG	0.03 ± 0.00

Adapted from Margalef et al.[12] ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; PA, proanthocyanidin. [a)] Quantified using the calibration curve of procyanidin dimer B2. [b)] Quantified using the calibration curve of epigallocatechin gallate.

A mixed standard stock solution at the concentration of 200 mg L^{-1} of each phenolic compound was prepared weekly in methanol and stored in amber-glass flasks at −20 °C. This stock solution was daily diluted to the desired concentration with acetone/Milli-Q water/acetic acid (70/29.5/0.5; v/v/v) solution and stored under the same conditions until chromatographic analysis.

2.2. Grape Seed Procyanidin Extract

A grape seed procyanidin extract (GSPE) was obtained from *Les derives Résiniques et Terpéniques* (Dax, France). GSPE total (poly) phenol content, individual flavanols, and phenolic acids were listed in **Table 1**.

2.3. Experimental Procedure, Plasma, and Tissue Collection

Prepubescent 4-week-old male ($n = 6$) and female ($n = 12$) Wistar rates, weighing 83.0 ± 5.3 g were used in this study. Animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 °C with 12 h light/dark cycle (lights on at 8:00 a.m.). Rats consumed tap water and standard chow diet (Planlab A04, Barcelona, Spain) ad libitum. Female rats were randomly divided into two different groups: the GSPE group ($n = 6$) and the water vehicle (VH) group ($n = 6$). Samples from the VH group were used as controls and blank matrixes for the construction of calibration curves. On the day of the experiment, overnight-fasted animals were individually weighed and GSPE at the dose of 1000 mg kg^{-1} was single administered to the male ($n = 6$) and female ($n = 6$) GSPE groups by oral gavage, while VH female animals were single administered 0.2 mL of tap water. In all groups, oral administration occurred between 8:00 and 9:00 a.m. Two hours after GSPE administration, rats

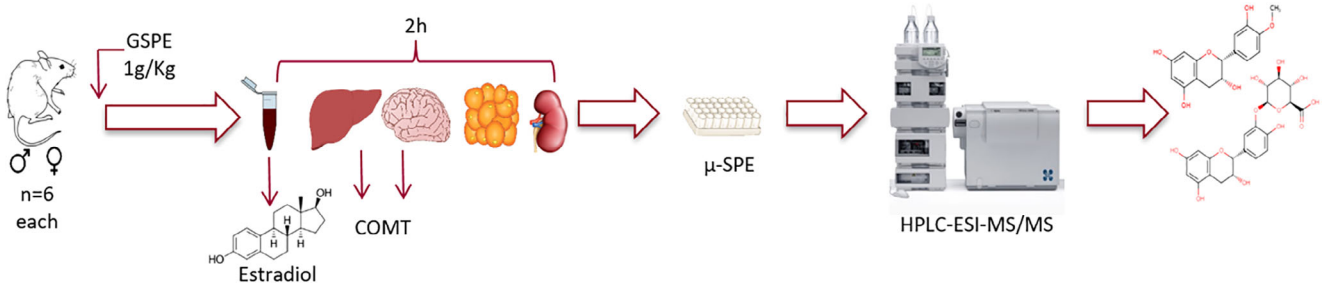

Figure 1. Graphical representation of the experimental design used in this study.

were scarified by decapitation. This time point was selected because it was described that maximum concentrations of flavanols phase-II metabolites in plasma and tissues were reached between 1 and 2 h.[12,17] Brains, livers, mesenteric white adipose tissues (MWAT), and kidneys were excised from all the rats, washed with cold PBS to remove traces of superficial blood and immediately snap-frozen in liquid nitrogen freeze-dried (**Figure 1**). Plasma samples were obtained by centrifuging blood samples (2000 x *g*, 15 min, 4 °C) in Sarstedt heparinized tubes (16 I.U.) (Barcelona, Spain). Freeze-dried tissues and plasma samples were stored at –80 °C until use. This section was conducted following previous experiments of the research group.[11,12] All procedures were performed in accordance with the guidelines for care and use of laboratory animals of the Universitat Rovira i Virgili (Tarragona, Spain, permit number 9495).

2.3.1. Dosage Information/Dosage Regimen

A dose of 1000 mg kg^{-1} of GSPE was single administrated by oral gavage between 8:00 and 9:00 a.m. This dose equated to 8.6 g dose of GSPE for a 70 kg person according to Reagan-Shaw et al.[29] This dose was used in order to compare with a previous study in which differences were observed between sexes in adult rats using this dose.[12] Thus, this dose provided with high enough metabolites' concentrations to stablish clear differences between groups.

2.4. Extraction of Proanthocyanidins and their Metabolites from Plasma

Prior to chromatographic analyses, plasma samples were pretreated using the previously reported methodology based on micro solid-phase extraction (μSPE).[10] Plasma samples were cleaned up by μSPE using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were sequentially conditioned by adding 250 μL of methanol and 250 μL of acetic acid solution (0.2%). Then, 300 μL of phosphoric acid (4%) and 50 μL of IS (2000 μg mL^{-1}) were mixed with 250 μL of plasma aliquots. The mixture was then loaded onto the plate. Loaded plates were sequentially washed with 200 μL of Milli-Q water and 200 μL of acetic acid (0.2%). The retained proanthocyanidins and their metabolites were eluted by adding 50 μL of acetone/Milli-Q water/acetic acid solution (70/29.5/0.5; v/v/v) twice. The eluted solution was directly injected into the HPLC-MS/MS system.

2.5. Extraction of Proanthocyanidins and their Metabolites from Tissues

Prior to chromatographic analyses, proanthocyanidins and their metabolites were extracted from rats tissues by the previously reported methodology based on an off-line solid–liquid extraction (SLE) in tandem with a μSPE.[10,11] Briefly, the LSE procedure involved mixing 60 mg of freeze-dried tissue with 50 μL of ascorbic acid (1%) and 100 μL of phosphoric acid (4%). Tissues were then extracted four times with 400 μL of water/methanol/phosphoric acid (4%) (94.4/4.5/1.5; v/v/v). At every extraction, 400 μL of extraction solution was added and samples were then sonicated in an ice bath for 30 s using a Vibracell Ultrasonic Sonicator (Sonics & Materials, Newtown, CT, USA). Afterwards, samples were centrifuged (17 000 x *g*, 4 °C, 15 min) and supernatants were recollected. Following this, 300 μL of the supernatants form the LSE procedure were processed by the previously described μSPE methodology in Section 2.4.

2.6. Chromatographic Analysis of Proanthocyanidins and their Metabolites

The eluted solutions from the μSPE were directly analyzed using a 6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA) as previously described.[10] Zorbax SE-aq (150 × 2.1 mm id, 3.5 μm particle size, Agilent Technologies) was used as the chromatographic column. The mobile phase consisted of acetic acid 0.2% (solvent A) and acetonitrile (solvent B) in a gradient mode set as follows: initial conditions 5% B; 0–10 min, 5–55% B; 10–12 min, 55–80% B; 12–15 min, 80% B isocratic; and 15–16, 80–85% B. A postrun of 10 min was required for column equilibration. For all runs, 2.5 μL of sample was injected and flow rate was maintained at 0.4 mL min^{-1}. Electrospray ionization (ESI) was conducted at 350 °C and 12 L min^{-1} with 4000 V of capillary voltage and 45 psi of nebulizer gas pressure. The mass spectrometer was operated in the negative mode and MS/MS data were acquired in multiple reaction monitoring (MRM) mode. MRM conditions for the analysis of flavanol and their metabolites using HPLC-ESI-MS/MS had been previously optimized (Table S2, Supporting Information).[10,30]

2.7. Sample Quantification

For sample quantification, plasma and extractions from the LSE procedure from the VH group were spiked with seven different

concentrations of the standard compounds to construct the calibration curves. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). Quality parameters needed to carry out the analyses were listed in Table S1, Supporting Information. Any analyte quantified in the blank plasma or tissue was subtracted from both the calibration curve and the samples. The results were expressed as the mean ± SEM ($n = 6$).

2.8. Western Blot Analysis

COMT enzyme levels were determined in the liver and brain by western blot analyses. Briefly, 20 mg of tissues were homogenated in 500 µL of Radio-Immunoprecipitation Assay lysis buffer containing protease inhibition cocktails by using a TissueLyser LT (Qiagen, Barcelona, Spain). Homogenates were incubated at 4 °C for 30 min and later centrifuge (20 000 × *g*, 15 min, 4 °C). Supernatants were used for total protein quantification and western blot analyses. Total protein was quantified by using the Pierce BCA protein assay kit (Thermo Scientific, Madrid, Spain). A total of 20 µg of protein for liver or 40 µg of protein for brain were solubilized and boiled for 10 min in loading buffer solution that contained Tris–HCl (0.5 M), glycerol, SDS, β-mercaptoethanol, and Bromophenol Blue at pH 6.8. Protein separation of the extracts was achieved by using SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). Proteins were electrotransferred onto polyvinylidene difluoride membranes (Trans-Blot Turbo Mini polyvinylidene difluoride Transfer Packs, Bio-Rad, Barcelona, Spain). After blocking, membranes were incubated overnight at 4 °C and with agitation with specific antibodyes (1:1000) for COMT (Abcam, Cambridge, UK) and actin (Sigma-Aldrich, Madrid, Spain).

For liver tissues, membranes were incubated with goat-anti rabbit secondary antibody (GE Healthcare, Barcelona, Spain) at 1:10 000 for 1 h at room temperature and under coinstant agitation. Protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare, Barcelona, Spain) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Protein band quantification from liver proteins was performed using ImageJ (NIH, MD, USA).

For brain tissues, membranes were incubated with goat anti-mouse secondary fluorescent antibody (LI-COR, USA) at 1:10 000 for 1 h at dark and room temperature and under constant agitation. Protein levels were detected at 800 nm using ODYSSEY CLx (LI-COR, USA) and band quantification was performed using Image Stidio TM 2.0 version (LI-COR, USA) software.

2.9. Plasma Estradiol Assay

Plasma 17β-estradiol levels were analyzed using an ELISA kit (Enzo Life Silences, Barcelona, Spain) according to the manufacturer's instructions.

2.10. Statistical Analysis

The levels of plasma estradiol and GSPE metabolites, and tissue and organ COMT protein and GSPE metabolites levels were compared between male and females ($n = 6$; each) by Student's *t*-test, with statistical significance defined as $p < 0.05$ (GraphPad Prism V10.1.1, La Jolla, CA, USA).

3. Results

The plasma, liver, MWAT, brain, and kidney distribution of GSPE metabolites in male and female prepubescent rats can be found in **Figure** 2, **Tables** 2 and S3, Supporting Information. Plasma estradiol and brain and liver COMT levels were also investigated (**Figures** 3 and 4).

3.1. Sex-Dependent Differences in GSPE Metabolites Distribution in Plasma and Liver

In plasma, male rats reached a total metabolite concentration of 61.50 ± 5.13 µM, which was 1.51-fold higher than the reported for females (40.85 ± 12.10 µM), although not statistically significant. The amounts of individual metabolites and the groups of metabolites were also not different between sexes. In terms of proportions, glucuronidemetabolites accounted for >70% of the total metabolites' concentration in both groups. Although not higher in terms of concentrations, sulpho-, methyl-sulpho-, and methyl-metabolites as well as phenolic acids and free flavanols reached slightly higher proportions in female rats.

In livers, the concentration of total flavanol metabolites was 2.19-fold statistically higher in male (91.06 ± 7.15 nmol g^{-1}) than in female rats (41.63 ± 10.06 nmol g^{-1}). Accordingly, male has higher concentration of most metabolites compared with female rats: epicatechin, 3-*O*-methyl-catechin, 4-*O*-methyl-epicatechin, epicatechin-sulfate, methyl-catechin-sulfate, methyl-epicatechin-sulfate, catechin-glucoronide, epicatechin-glucoronide, methyl-catechin-glucuronide, and methyl-epicatechin-glucuronide. However, these differences were not observed at the proportions of the metabolites groups. Hence, methyl-glucuronide-metabolites accounted for 55.62% and 57.91% of all the metabolites in male and female rats, respectively. Also, glucuronide- and methyl-sulfate-metabolites represented an important proportion of the metabolite forms in a similar proportion in both sexes. Sulpho- and methyl-metabolites as well as phenolic acids and free flavanols were found at very low concentrations in the liver of both sexes.

3.2. Sex-Dependent Differences in GSPE Metabolites Distribution in Mesenteric White Adipose Tissue

In the MWAT, total metabolite concentration reached a 3.67-fold sadistically higher concentration in male rats (105.96 ± 26.85 nmol g^{-1}) than in females (28.84 ± 6.08 nmol g^{-1}). Accordingly, male has higher concentration of those metabolites significantly different from female rats. Specifically, gallic acid, methyl-catechin-sulfate, methyl-epicatechin-sulfate, catechin-glucuronide, epicatechin-glucuronide, methyl-catechin-glucuronide, and methyl-epicatechin-glucuronide. However, in the MWAT the proportions of the metabolite families were also very similar among sexes. Interestingly, phenolic acids and free

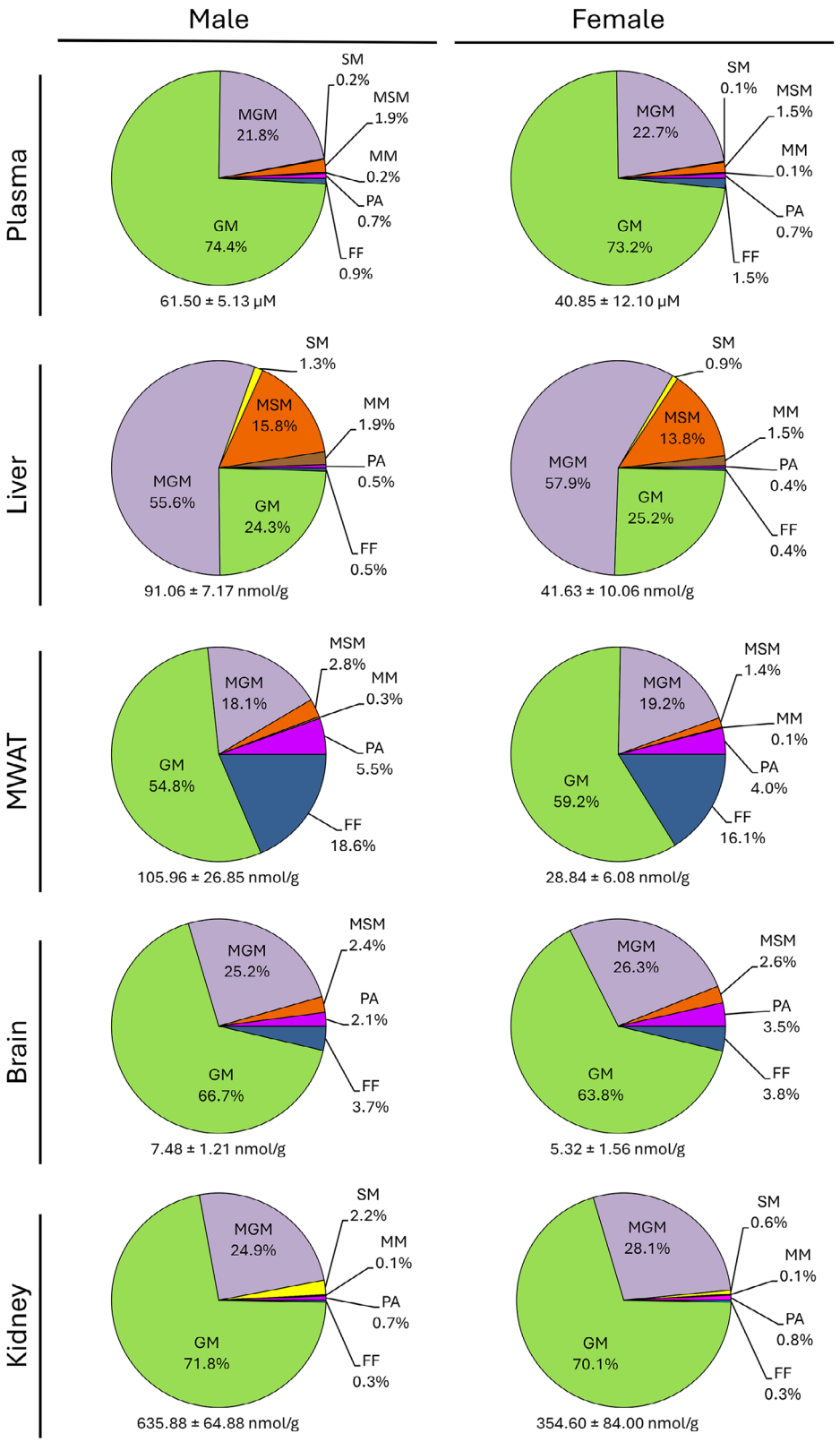

Figure 2. Distribution of metabolized and non-metabolized Proanthocyanidins in mesenteric white adipose tissue (MWAT), liver, plasma, brain, and kidneys of male (left) and female (right) 4-week-old rats 2 h after the ingestion of 1000 mg kg^{-1} of a Grape Seed Proanthocyanidin Extract (GSPE). Data are given as a total mean concentration ± SEM ($n = 6$) and percentage. FF, free flavonols; GM, glucuronide metabolites; MGM, methyl-glucuronide metabolites; MM, methyl metabolites; MSM, methyl-sulfate metabolites; PA, phenolic acids; SM, sulfated metabolites.

Table 2. (Poly)phenols and their phase-II metabolites detected by HPLC-ESI-MS/MS in male and female rat plasma, liver, white mesenteric adipose tissue (MWAT) and brain 2 h after the ingestion of 1000 mg kg^{-1} of Grape Seed Proanthocyanidin Extract (GSPE).

Tissue	Compound	Male	Female	*t*-test
Plasma [µM]	Vanillic acid	0.02 ± 0.00	0.01 ± 0.00	ns
	Gallic acid	0.39 ± 0.05	0.26 ± 0.08	ns
	Catechin	0.08 ± 0.03	0.17 ± 0.10	ns
	Epicatechin	0.13 ± 0.03	0.16 ± 0.07	ns
	3-*O*-methyl-catechin[a)]	0.11 ± 0.02	0.06 ± 0.01	ns
	4-*O*-methyl-epicatechin[b)]	n.q.	n.q.	
	Catechin-sulfate[a)]	0.10 ± 0.02	0.06 ± 0.02	ns
	Epicatechin-sulfate[b)]	0.01 ± 0.00	0.01 ± 0.00	ns
	Methyl-catechin-sulfate[a)]	1.04 ± 0.27	0.57 ± 0.14	ns
	Methyl-epicatechin-sulfate[b)]	0.13 ± 0.02	0.06 ± 0.02	ns
	Epicatechin gallate[c)]	n.q.	n.q.	
	EGCG	n.q.	n.q.	
	Catechin-glucuronide[a)]	22.79 ± 1.98	15.97 ± 4.77	ns
	Epicatechin-glucuronide[b)]	22.94 ± 2.08	13.95 ± 5.03	ns
	Methyl-catechin-glucuronide[a)]	8.02 ± 0.65	5.56 ± 1.27	ns
	Methyl-epicatechin-glucuronide[b)]	5.39 ± 0,50	3.72 ± 0.83	ns
	PA B1[d)]	0.07 ± 0,03	0.08 ± 0.03	ns
	PA B3[d)]	0.08 ± 0,03	0.06 ± 0.02	ns
	PA B2[d)]	0.17 ± 0,06	0.15 ± 0.05	ns
	Epicatechin gallate-glucuronide[c)]	n.d.	n.d.	
	PA dimer-gallate[d)]	n.d.	n.d.	
	PA Trimer[d)]	n.d.	n.d.	
Liver [nmol g^{-1}]	Vanillic acid	0.13 ± 0.03	0.07 ± 0.01	ns
	Gallic acid	0.32 ± 0.16	0.08 ± 0.02	ns
	Catechin	0.07 ± 0.02	0.04 ± 0.01	ns
	Epicatechin	0.17 ± 0.03	0.06 ± 0.02	$p < 0.05$
	3-*O*-methyl-catechin[a)]	0.34 ± 0.03	0.12 ± 0.03	$p < 0.001$
	4-*O*-methyl-epicatechin[b)]	1.41 ± 0.25	0.49 ± 0.11	$p < 0.05$
	Catechin-sulfate[a)]	0.05 ± 0.00	n.q.	
	Epicatechin-sulfate[b)]	1.16 ± 0.23	0.37 ± 0.14	$p < 0.05$
	Methyl-catechin-sulfate[a)]	3.44 ± 0,32	1.11 ± 0.32	$p < 0.001$
	Methyl-epicatechin-sulfate[b)]	10.99 ± 1.19	4.65 ± 0.80	$p < 0.001$
	Epicatechin gallate[c)]	n.d.	n.d.	
	EGCG	0.03 ± 0.01	0.00 ± 0.00	ns
	Catechin-glucuronide[a)]	9.52 ± 1.12	4.69 ± 1.31	$p < 0.05$
	Epicatechin-glucuronide[b)]	12.62 ± 1.22	5.79 ± 1.51	$p < 0.001$
	Methyl-catechin-glucuronide[a)]	33.70 ± 3.39	15.94 ± 3.98	$p < 0.001$
	Methyl-epicatechin-glucuronide[b)]	16.94 ± 1.60	8.16 ± 1.94	$p < 0.001$
	PA B1[d)]	0.03 ± 0.01	0.01 ± 0.01	ns
	PA B3[d)]	0.05 ± 0.02	0.02 ± 0.01	ns
	PA B2[d)]	0.11 ± 0.04	0.03 ± 0.01	ns
	Epicatechin gallate-glucuronide[b)]	n.d.	n.d.	
	PA dimer-gallate[d)]	n.d.	n.d.	
	PA Trimer[d)]	n.d.	n.d.	
Kidney [nmol g^{-1}]	Vanillic acid	0.23 ± 0.01	0.14 ± 0.03	$p < 0.001$
	Gallic acid	3.50 ± 0.76	2.15 ± 0.76	ns
	Catechin	0.39 ± 0.12	0.80 ± 0.52	ns
	Epicatechin	0.97 ± 0.27	1.07 ± 0.50	ns
	3-*O*-methyl-catechin[a)]	0.84 ± 0.16	0.41 ± 0.16	ns
	4-*O*-methyl-epicatechin[b)]	2.85 ± 0.51	1.57 ± 0.42	ns

(*Continued*)

Table 2. (Continued)

Tissue	Compound	Male	Female	*t*-test
Catechin-sulfate[a)]	0.40 ± 0.10	0.37 ± 0.17	ns	
Epicatechin-sulfate[b)]	11.64 ± 7.76	1.62 ± 0.74	ns	
Methyl-catechin-sulfate[a)]	n.q.	n.q.	ns	
Methyl-epicatechin-sulfate[b)]	n.q.	n.q.	ns	
Epicatechin gallate[c)]	n.q.	n.q.	ns	
EGCG	0.03 ± 0.01	0.01 ± 0.00	ns	
Catechin-glucuronide[a)]	187.91 ± 27.27	95.49 ± 27.06	$p < 0.05$	
Epicatechin-glucuronide[b)]	231.89 ± 30.23	116.65 ± 34.56	$p < 0.05$	
Methyl-catechin-glucuronide[a)]	88.25 ± 7.30	51.69 ± 8.98	$p < 0.05$	
Methyl-epicatechin-glucuronide[b)]	62.39 ± 4.80	36.44 ± 6.32	$p < 0.01$	
PA B1[d)]	0.28 ± 0.11	0.21 ± 0.13	ns	
PA B3[d)]	0.77 ± 0.49	0.19 ± 0.11	ns	
PA B2[d)]	0.49 ± 0.19	0.38 ± 0.25	ns	
Epicatechin gallate-glucuronide[c)]	n.d.	n.d.		
PA dimer-gallate[d)]	n.d.	n.d.		
PA Trimer[d)]	n.d.	n.d.		
Brain [nmol g^{-1}]	Vanillic acid	n.d.	n.d.	
	Gallic acid	0.15 ± 0.03	0.22 ± 0.06	ns
	Catechin	0.11 ± 0.03	0.11 ± 0.04	ns
	Epicatechin	0.23 ± 0.05	0.42 ± 0.26	ns
	3-*O*-methyl-catechin[a)]	n.d.	n.d.	
	4-*O*-methyl-epicatechin[b)]	n.d.	n.d.	
	Catechin-sulfate[a)]	n.d.	n.d.	
	Epicatechin-ulphate[b)]	n.d.	n.d.	
	Methyl-catechin-sulfate[a)]	0.04 ± 0.01	0.07 ± 0.03	ns
	Methyl-epicatechin-sulfate[b)]	0.18 ± 0.03	0.16 ± 0.07	ns
	Epicatechin gallate[c)]	n.d.	n.d.	
	EGCG	0.18 ± 0,16	0.04 ± 0.02	ns
	Catechin-glucuronide[a)]	2.16 ± 0.41	1.52 ± 0.49	ns
	Epicatechin-glucuronide[b)]	2.83 ± 0.54	1.88 ± 0.57	ns
	Methyl-catechin-glucuronide[a)]	1.01 ± 0.14	0.77 ± 0.20	ns
	Methyl-epicatechin-glucuronide[b)]	0.88 ± 0.14	0.63 ± 0.14	ns
	PA B1[d)]	0.02 ± 0.00	0.03 ± 0.02	ns
	PA B3[d)]	0.01 ± 0.00	0.02 ± 0.01	ns
	PA B2[d)]	0.05 ± 0.01	0.07 ± 0.03	ns
	Epicatechin gallate-glucuronide[c)]	n.d.	n.d.	
	PA dimer-gallate[d)]	n.d.	n.d.	
	PA Trimer[d)]	n.d.	n.d.	
MWAT [nmol g^{-1}]	Vanillic acid	0.26 ± 0.03	0.26 ± 0.03	ns
	Gallic acid	5.53 ± 1.82	0.88 ± 0.33	$p < 0.05$
	Catechin	7.70 ± 2.78	1.87 ± 0.98	ns
	Epicatechin	5.09 ± 1.88	1.38 ± 0.68	ns
	3-*O*-methyl-catechin[a)]	0.06 ± 0.02	0.01 ± 0.00	ns
	4-*O*-methyl-epicatechin[b)]	0.23 ± 0.08	0.05 ± 0.01	ns
	Catechin-sulfate[a)]	n.d.	n.d.	
	Epicatechin-sulfate[b)]	n.d.	n.d.	
	Methyl-catechin-sulfate[a)]	0.96 ± 0.25	0.05 ± 0.02	$p < 0.01$

(*Continued*)

Table 2. (Continued)

Tissue	Compound	Male	Female	*t*-test
Methyl-epicatechin-sulfate[b)]	2.31 ± 0.86	0.36 ± 0.07	$p < 0.05$	
Epicatechin gallate[c)]	n.d.	n.d.		
EGCG	n.d.	n.d.		
Catechin-glucuronide[a)]	25.20 ± 5.67	7.67 ± 1.66	$p < 0.05$	
Epicatechin-glucuronide[b)]	32.84 ± 7.75	9.41 ± 1.98	$p < 0.05$	
Methyl-catechin-glucuronide[a)]	10.20 ± 2.74	2.45 ± 0.31	$p < 0.05$	
Methyl-epicatechin-glucuronide[b)]	9.01 ± 2.36	3.07 ± 0.42	$p < 0.05$	
PA B1[d)]	1.76 ± 0.64	0.38 ± 0.19	ns	
PA B3[d)]	1.40 ± 0.50	0.27 ± 0.13	ns	
PA B2[d)]	3.59 ± 1.33	0.71 ± 0.36	ns	
Epicatechin gallate-glucuronide[c)]	n.d.	n.d.		
PA dimer-gallate[d)]	n.d.	n.d.		
PA Trimer[d)]	n.d.	n.d.		

Data are given as a total mean concentration ± SEM ($n = 6$). Statistical differences were analyzed by *t*-test. Quantification of conjugated metabolites is tentative and focused on comparing the same metabolite in male versus female rats. EGCG, epigallocatechin gallate; n.d., not detected; n.q., not quantified; ns, not significant, $p > 0.05$, *t*-test; PA, proanthocyanidin. [a)] Quantified using the calibration curve of catechin. [b)] Quantified using the calibration curve of epicatechin. [c)] Quantified using the calibration curve of EGCG. [d)] Quantified using the calibration curve of proanthocyanidin dimer B2.

flavanols accounted for a higher proportion in the MWAT than in other tissues regardless of the sex. Specifically, free flavanols reached 19.68 ± 7.00 and 4.65 ± 1.90 nmol g^{-1} in male and female MWAT, respectively. However, glucuronide-metabolites were the 54.77% and 59.20% in male and female MWAT, respectively. Sulpho-metabolites were not quantified in the MWAT, while methyl-sulpho-metabolites reached very low concentrations.

3.3. Sex-Dependent Differences in GSPE Metabolites Distribution in Brain

The detected metabolites in brain were those present in the cerebral circulation and/or in the brain parenchyma. Brain was the tissue with the lowest GSPE flavanol metabolite's concentration in both male (7.48 ± 1.21 nmol g^{-1}) and female (5.32 ± 1.56 nmol g^{-1}) rats. In addition, there were not differences in total flavanol concentration nor in individual metabolites or their proportions between sexes. Similar to the other tissues studied, glucuronide- and methyl-glucuronide-metabolites accounted for the 91.89% and 89.66% of all the metabolites in male and female rat brains, respectively. Sulpho-metabolites were not quantified and methyl-sulpho-metabolites were quantified at the very low concentrations.

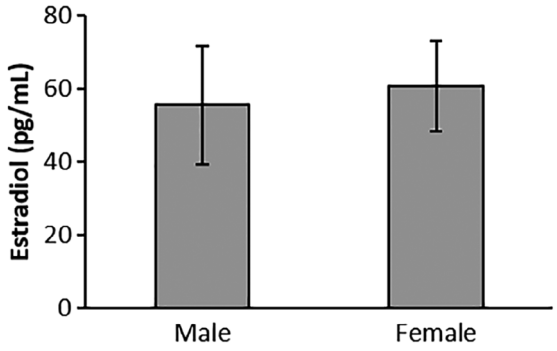

Figure 3. Plasma estradiol levels of male and female 4-week-old rats. Results are expressed as mean ± SEM ($n = 6$).

3.4. Sex-Dependent Differences in GSPE Metabolites Distribution in Kidneys

Kidneys were the organ with the highest concentration of flavanol metabolites in both male (635.88 ± 64.88 nmol g^{-1}) and female (354.60 ± 84.00 nmol g^{-1}) rats. Total metabolites' concentrations in male rats were 1.79-times statistically higher than the reported for females while metabolite proportions remained constant between sexes. Accordingly, vanillic acid, catechin-glucuroronide, epicatechin-glucuronide, methyl-catechin-glucuronide, and methyl-epicatechin-glucuronide were present in higher concentrations in male than in female rats. However, in terms of kidney metabolite propositions there were not differences among sexes. Sulpho- and methyl-metabolites, phenolic acids, and free flavanols represented low proportions in male and female kidneys. Finally, no methyl-sulfated metabolites were reported in the kidney.

3.5. Sex-Related Differences in Plasma Estradiol Levels

Plasma estradiol levels from male (55.51 ± 16.21 pg mL^{-1}) and female (60.75 ± 12.39 pg mL^{-1}) rats showed lack of sex-specificity in 4-week-old rats ($p = 0.7985$, Figure 3).

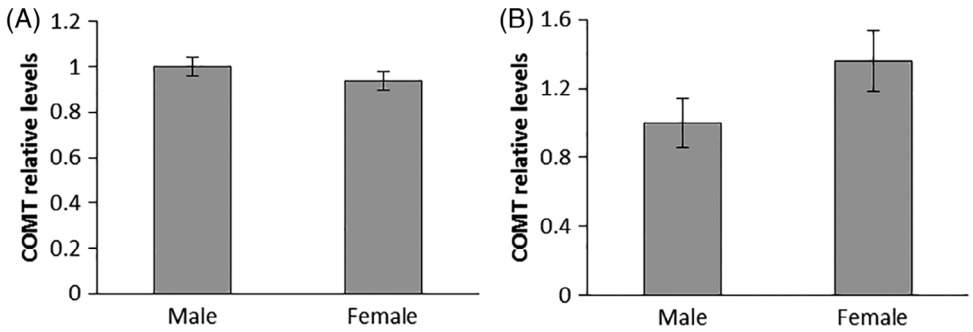

Figure 4. Catechol-*O*-methyltransferase (COMT) protein relative levels in livers (A) and brains (B) of male and female 4-week-old rats. Results are expressed as the mean of relative COMT protein content ± SEM ($n = 6$). Results are expressed relative to male rats.

3.6. Sex-Related Differences in Liver and Brain COMT Protein Levels

The protein relative levels for COMT were also determined in liver (Figure 4A) and brain (Figure 4B). There were not differences among male and female rats ($p = 0.4180$ in liver and $p = 0.0766$ in brain). Thus, no sex-specific differences were found for COMT in these tissues.

4. Discussion

PAs are one of the most predominant classes of dietary (poly)phenols and exert several health-promoting functions,[2,6] and these extend to children.[3,4] However, several physiological factors can greatly affect the metabolism of PAs and, hence, their biological activities.[12,15–17] Specifically, our group has demonstrated that the metabolism of GSPE depend on the sex[12] and age[17] of the model. The differences between sexes may be caused by differences in the levels of sex hormones[22,26–28] or expression of phase-II enzymes.[21–25] Therefore, the aim of this study was to compare the phase-II metabolism of grape seed PAs between sexes before puberty, where poor hormonal differences[31] and sex-specific gene expression[20] exist (i.e., prepubescent physiological conditions). To do so, 4-week-old male and female rats were administered 1000 mg GSPE kg^{-1} and sacrificed 2 h after the administration, which has been reported to be the time point where PA phase-II metabolites reach their maximum concentration.[12,17] Although this high dose can lead to saturation of phase II enzymes in both animals and humans,[11,19,32] it provides with high enough metabolites' concentrations to stablish clear differences between groups. The selected organs and tissues were chosen due to their relevance in phenolic compound ADME, and the reported sex-dependent differences in metabolite profile found in adulthood.[12]

The total amount of metabolites found in plasma was 1.51-fold not significantly higher in male than in female rats. Paradoxically, in our previous study using adult male and female rats, the plasma of females reported a 2.2-fold increase when compared to males'.[12] Thus, these results indicate that the plasma bioavailability of GSPE phenolic compounds depends on both sex and age. In this sense, age has been demonstrated as an important factor determining GSPE ADME in young (8-week-old) and adult (24-week-old) male rats.[17] However, the plasma distribution of GSPE metabolites in prepubescent rats was very similar between male and female rats, which is in disagreement with the results reported in adult male and female rats.[12] For instance, in adult male rats, the percentage of sulfated metabolites in plasma was 9%, while in adult female rats, it was 24%. In their prepubescent counterparts, these metabolites accounted for approximately 2% in both sexes. Thus, it seems that during puberty several physiological changes must occur to explain the increase in phenolic sulfation. In this sense, SULTs reach higher expression rates as rats grow older.[21,33] Similarly, the lack of difference in estradiol plasma levels found in this study is also in line with the lack of difference in the percentage of metabolites with a methyl moiety in the plasma.

The main difference in the ADME of GSPE in the liver of male and female prepubescent rats was the 2.19-fold increase of total metabolites in males. Importantly, GSPE has been reported to produce several effects in the liver.[2] Moreover, changes in the ADME of GSPE has been demonstrated to modulate the biological effects in the liver cell line HepG2.[19] Thus, the differences in the total metabolite amounts between male and female rats could be associated with changes in their biological effects. No sex-specificity in the phenolic metabolites proportions was found in the liver, which disagrees with our previous study in adult rats (i.e., higher proportions of sulfation in female livers).[12] In this sense, such differences could be attributed to the pattern of expression of the phenol SULTs in the liver, which starts to become sex-specific by the 30 days of age both in rats,[21] and greatly varies between sexes by the age of 3 months.[23] The similar percentages of metabolites with a methyl moiety found in our study (≈73%) agrees with the same protein levels of COMT in the liver. These values are similar to adult male rats (72%), and ≈10% lower than in the females' (64%).[12] Of note, this could involve different effects associated with the consumption of GSPE. For example, the methylation of flavan-3-ols has been linked with a higher potential to reduce the lipid accumulation by 3T3-L1 cells.[34]

After being ingested PAs are absorbed in the small intestine and subjected to phase-II detoxification and can subsequently reach the MWAT.[11] Thus, in this study, the results in MWAT provide information for flavanol metabolism in the intestine. Our results suggest enhanced intestinal absorption in male prepubescent rats (3.76-fold increase vs females). Several findings agree with this result, including the fact that the Mrp4 transporter, involved in the transport of flavonoids into the intestinal lumen, is overexpressed in female's colon.[35] In addition, gastric emptying and small-bowel transit has been shown to be faster

in males than females,[36] which would also agree with the increased intestinal absorption of flavanols in males. More importantly, this higher intestinal absorption in male rats would explain the higher metabolite concentrations found in the other organs and tissues in this study. Controversially, in our previous study with 8–10-week-old rats, male and female rats reached similar concentrations in the MWAT,[12] which were very similar to the ones reached in female rats in this study. The percentage of each metabolic family was very similar between male and female prepubescent rats, and the dominating metabolite family was the glucuronide metabolites. The small intestine has been described as a primary source of glucuronidation for the flavanol catechin,[37] and poor statistical differences exist in the expression of the UGT superfamily throughout the small intestine.[24] Remarkably, highest proportion of free flavanols was found in this study in the MWAT. This has been previously reported in different models,[11,12] and could be attributed to the fact that the dose of 1000 mg kg^{-1} can saturate the phase-II enzymes in animals and humans.[11,19,32]

The administration of GSPE is known to promote several health effects in the brain.[38,39] However, the bioavailability of PAs metabolites in the brain is conditioned by their pass throughout the blood brain barrier (BBB).[40] Hence, total PAs metabolites' in brain has the lowest concentration among the studied tissues and similar total metabolite concentration in both sexes. These results could agree with the previous results that indicate that not all (poly)phenolics can cross the BBB.[11,12] Surprisingly, and although previous studies in 8–10 week-old rats showed great differences between sexes, specifically in the level of methylated metabolites,[12] prepubescent rats did not show a sex-specific metabolism of PAs in the brain. The lack of sex-dependent changes in estrogenic, implying the same downregulation of the COMT enzyme,[26–28] and the non-statistically significant changes in the COMT protein level in prepubescent rats' brain, trend also found in the literature, may explain why methyl-metabolites are found at similar proportions and concentrations in male and female prepubescent rats. Shockingly, this was the main difference in PAs' metabolism between adults male and female rats found in the brain.[12] Glucuronide metabolites were also more abundant in adult female brains,[12] pattern that was not found in prepubescent rats. Indeed, this lack of sex-specificity on the glucuronidation pattern in the brain could agree with the lack of changes in the expression of UGT enzymes between sexes in rat cerebral cortex.[24] Therefore, prepubescent rats do not report the changes in PAs brain metabolite profile found in adult rats, which were the most relevant changes throughout our previous experiment.[12] This suggests that puberty might be the triggering factor that will ultimately modulate PAs metabolite profile in adulthood.

The kidneys are the organ that PAs metabolites lastly reach to be excreted via the urine.[11–13] Total metabolite concentration reached the highest concentration in kidneys from both male and female rats, suggesting poor bioavailability and fast pharmacokinetics of bioavailable PA metabolites, which we have previously reported.[11,17] Agreeing with this, cocoa flavanols mostly excreted within the first 4 h after oral administration via the urine.[41] In the same line of liver, total metabolite concentration was 1.79 times higher in male rats than their counterparts. This could be explained by the differences in (poly)phenol absorption at intestinal level. In the kidney, flavanols can be further metabolized by phase-II enzymes. Indeed, kidneys express phenol SULTs,[42] several UGT isoforms,[24] and the COMT enzyme.[28] The proportion of sulfate-metabolites seems to be sex-specific, reaching higher proportions in male rats. Agreeing with this, the phenol SULT1C1 presents a male-dominant expression in several tissues, including the kidney, in mice, and rats.[22]

Viewed as a whole, this data indicate that poor differences exist between male and female prepubescent rats in terms of metabolite proportions, indicating that the sex-related changes in PA metabolite profile found in adult rats[12] are likely to be triggered by puberty. However, this study demonstrates that the bioavailability is different between male and female prepubescent rats. This could involve important changes in the biological effects associated with GSPE consumption at this early stage of life. Overall, this is the first study to evaluate the ADME of PAs in prepubescent rats, providing relevant results as a first connexion between the bioactive effects of PAs in children[3,4] and their ADME. Thus, these results show the need of future studies of PAs bioactivities and ADME in children of both sexes and suggest future applications of PAs in personalized nutrition. However, a limitation of the study is related to the results in brain as these are conducted in brains that were not prefunded and thus, the metabolites detected could be those present in cerebral circulation and/or in brain tissue.

5. Concluding Remarks

Although clear differences in the metabolite's concentrations in plasma and the studied tissues exist between male and female 4-week-old prepubescent rats, in terms of proportion of metabolite families, there does not exist differences. These highlights that most of the reported changes in adult rats are not conserved in young prepubescent rats. Taking into consideration all the data, our results suggest that physiological changes that modulate PA metabolism are triggered during puberty.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, L.I.-C., M.S., A.A.-A., and B.M.; Formal analysis, L.I.-C.; Investigation, L.I.-C. and A.M.-C.; Data curation, L.I.-C.; Writing — original draft, L.I.-C.; Writing — review and editing, L.I.-C., A.A.-A., and B.M.; Supervision, M.S., A.A.-A., and B.M; Funding acquisition, F.I.B., M.S., A.A.-A., and B.M.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bioavailability, phenolic metabolites, polyphenols, procyanidins, sex

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