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Administration Time Significantly Affects Plasma Bioavailability of Grape Seed Proanthocyanidins Extract in Healthy and Obese Fischer 344 Rats

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Scope: Phenolic compounds are bioactive molecules that are associated with several health benefits. Metabolization and absorption are the main determinants of their bioavailability and bioactivity. Thus, the study of the factors that modulate these processes, such as sex or diet is essential. Recently, it has been shown that biological rhythms may also play a key role. Hence, the aim of this study is to evaluate if the bioavailability of a grape proanthocyanidin extract (GSPE) is affected by the administration time in an animal model of metabolic syndrome (MetS).

Methods and Results: Female and male Fischer 344 rats are fed either a standard or a cafeteria diet (CAF) for 9 weeks, and an oral dose of GSPE (25 mg kg^{-1}) is daily administered either at 8:00 am (zeitgeber time (ZT)-0) or at 8:00 pm (ZT-12) during the last 4 weeks. Plasma phenolic compounds are then quantified by liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Phase-II and gut microbiota-derived phenolic metabolites are affected by ZT in all conditions or only in obese rats, respectively. CAF feeding affected the bioavailability of phenolic acids and free flavan-3-ols. Differences due to sex are also observed.

Conclusion: These findings demonstrate that ZT, diet, and sex are key factors influencing phenolic compounds bioavailability.

1. Introduction

Phenolic compounds, known as polyphenols when including more than one aromatic ring, are a group of bioactive molecules produced by plants in response to stress.[1] These compounds can be classified into four main groups: phenolic acids, flavonoids, stilbenes, and lignans. This family of compounds is highly distributed in plants, being flavonoids the most consumed. Flavonoids comprise flavonols, flavones, flavan-3-ols, isoflavones, flavanones, and anthocyanidins.[2] Consumption of phenolic compounds has been associated with several health benefits in cardiovascular and metabolic disorders and in some cancers.[1]

It has been estimated that only a small part of the ingested polyphenols is absorbed by the small intestine (5–10%). The rest (90–95%) reach the colon, where they are subjected to extensive microbial metabolism. For example, high-molecular-weight proanthocyanidins are transformed into small phenolic acid microbial derived metabolites.[3] Once absorbed, phenolic compounds and their metabolites are transported through the systemic circulation to the different tissues and organs where they are recognized as xenobiotics and undergo extensive phase II reactions including glucuronidation, sulfation, and/or methylation, all of them catalyzed by the action of uridine 5'-diphospho-glucuronyltransferase (UGTs), sulphotransferases (SULTs), and catechol-*O*-methyltransferase (COMT), respectively.[4] In addition, these metabolites may be transferred back into the intestine through the enterohepatic cycle together. Finally, the metabolites transported via systemic circulation reach the kidneys and are excreted through the urine. Non-absorbed polyphenols are excreted through feces.[4]

Several factors affect the metabolism and bioavailability of these bioactive compounds.[5] Actually, it has been shown that external and internal factors such as the environment,[6] polyphenol structure,[6] age,[7] sex,[8] gut microbiota,[3] and animal strain[9] determine the bioavailability of phenolic compounds.

In addition to these factors, circadian rhythm has emerged as potential key modulator of bioactivity due to its effect on metabolism, this could be caused by changes in bioavailability. Circadian clocks have evolved in each organism so that they can adapt their behavior and physiology to the most appropriate time of day. The central system of the circadian clock depends on a

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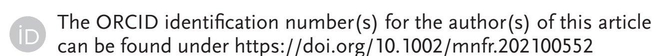
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central clock located in the hypothalamus and secondary clocks placed in peripheral tissues.[10] The intrinsic circadian clock is based on the oscillation of the transcription and translation of the clock genes. In this regard, it is known that there are certain external signals such as light[11] and food[12] that regulate metabolism by modulating circadian clocks. The term "zeitgeber time" (ZT), which in German means "timer," is used to describe a unit of time based on the period of a zeitgeber, such as the 12:12 light:dark cycle.[13]

Several studies have demonstrated that circadian rhythms can influence the bioactivity of phenolic compounds and, at the same time, the intake of these compounds can modulate biological rhythms.[14] For example grape seed proanthocyanidins extract (GSPE) has been shown to exert properties by modulating the level of melatonin and the expression of Clock genes in the hypothalamus depending on the administration time.[15] This extract is rich in flavonoids including flavan-3-ols such as catechin, epicatechin and their polymeric forms, proanthocyanidins. Furthermore, GSPE contain a wide variety of phenolic acids.[7] In this regard, GSPE has been shown to exert several beneficial effects including, protection against weight gain,[16] decrease in inflammation,[17] restoration of blood pressure, improvement of the diversity of gut microbiota and regulation of circadian rhythms.[18]

Considering all this evidence, we hypothesize that metabolism and absorption of phenolic compounds may be influenced by circadian rhythms. Therefore, the aim of this study was to evaluate if the plasma bioavailability of phenolic compounds from GSPE is affected by the administration time in an animal model of metabolic syndrome (MetS) induced by cafeteria diet feeding.

2. Experimental Section

2.1. Grape Seed Polyphenol Extract (GSPE)

GSPE was obtained from white grape seed and provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). The main phenolic compounds (flavan-3-ols and phenolic acids) present in the extract used for this study were shown in **Table 1**. Nomenclature according to Kay et al.[19]

Table 1. Main phenolic compounds (flavan-3-ols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analyzed by HPLC-MS / MS.

Compound	Concentration [mg g^{-g}]
3,4,5-trihydroxybenzoic acid	31.07 ± 0.08
3,4-dihydroxybenzoic acid	1.34 ± 0.02
4-hydroxy-3-methoxybenzoic acid	0.77 ± 0.04
PC dimer B2	33.24 ±1.39
PC dimer B1[a)]	88.80 ± 3.46
PC dimer B3[a)]	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate[a)]	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin[b)]	0.27 ± 0.03
PC trimer[a)]	4.90 ± 0.47
PC tetramer [a)]	0.05 ± 0.01

Adapted from Margalef, Pons, Iglesias-Carres et al. (2017). The results are expressed on a wet basis as the means ± SD ($n = 3$) in mg of phenolic compound per g of GSPE. PC, proanthocyanidin. [a)] Quantified using the calibration curve of proanthocyanidin B2. [b)] Quantified using the calibration curve of epigallocatechin gallate.

2.2. Chemicals and Reagents

Acetone, acetonitrile and phosphoric acid (Sigma-Aldrich, Madrid, Spain), glacial acetic acid (Panreac, Barcelona, Spain), and methanol (Scharlab S.L., Barcelona, Spain) were all of HPLC analytical grade. Ultrapure water was obtained from a MilliQ Advantage A10 system (Millipore, Madrid, Spain).

Individual standard stock solutions of 2000 mg L^{-1} (+) -catechin, (−) epicatechin, epigallocatechin gallate (EGCG), 3,4,5-trihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3-hydroxybenzoic acid, 3″-hydroxyphenylacetic acid, 4″-hydroxyphenylacetic acid, 3″,4″-dihydroxyphenylacetic acid, 3-(4′-hydroxyphenyl)propanoic acid, benzoic acid, hippuric acid, 4'-hydroxy-3'-methoxycinnamic acid and benzene-1,2-diol (internal standard; IS) (all purchased from Fluka/Sigma-Aldrich, Madrid, Spain), and proanthocyanidin B2 (Extrasynthese Lyon, France), were prepared in methanol and stored in dark glass flasks at −20 °C. In addition, a stock solution containing all individual compounds was prepared weekly at a concentration of 20 mg L^{-1} in methanol. This standard solution was diluted to the desired concentration using a solution of acetone: water: acetic acid (70:29.5:0.5, v:v:v) and 40 µL IS at 20 ppm, in order to obtain straight standards at different concentrations of the pure metabolites. This solution was stored in dark glass containers at −20 °C until chromatographic analysis.

2.3. Experimental Procedure in Rats

Sixty-four 8-week-old male ($n = 32$) and female ($n = 32$) Fischer 344 rats (Charles River Laboratories, Barcelona, Spain), weighing 236.36 ± 30.55 and 181.94 ± 19.14 g, respectively, were housed in pairs at 22 °C with ad libitum access to tap water and food under a light/dark cycle of 12 h (light from 8:00 a.m. to 8:00 p.m.). The animals were randomly divided into eight groups according to time of treatment (ZT), sex, and diet (standard and cafeteria) (**Figure 1**).

Standard chow diet (ST) (72% carbohydrates (CH) (being 4% sugars), 8% lipid, and 19% protein; Safe-A04c, Germany) or cafeteria diet (CAF) (55% CH (being 35% sugars), 34% lipid, and 11% protein) were administered for 9 weeks. CAF was a hypercaloric diet, highly palatable, that induced hyperphagia and obesity.[20] This length of CAF administration had been shown to be adequate for a proper development of metabolic syndrome.[21] CAF diet was freshly prepared every day and included the following regular human food products (g per rat): bacon (12–15 g), biscuits with pâté (12 g), biscuits with cheese (14 g), sweet roll (8–10 g), carrots (8–10 g) and sweetened milk (20% sucrose (w/v)), and standard chow diet (10–12 g).

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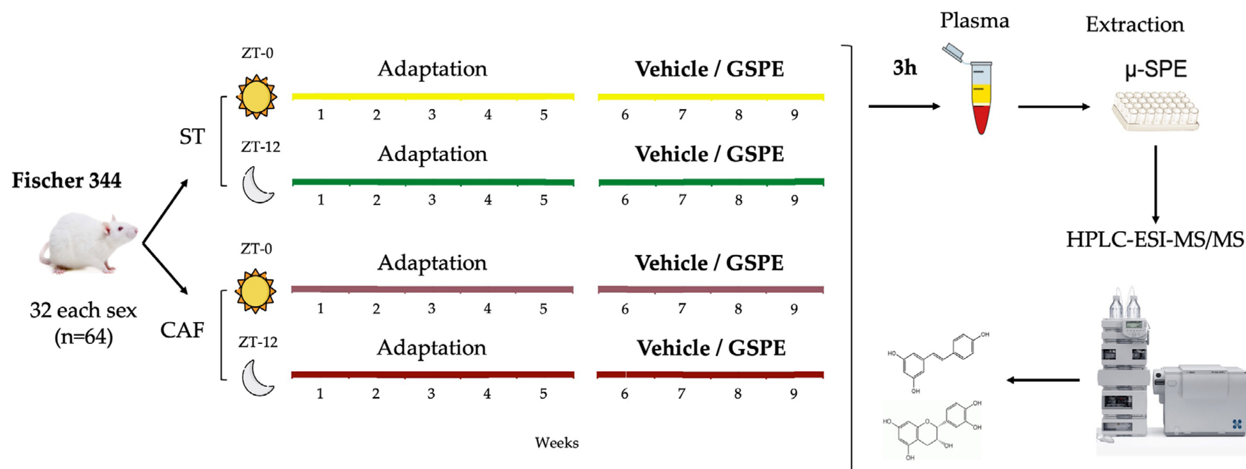

Figure 1. Graphical representation of the experimental design used in this study. CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract; ST, standard chow diet; ZT-0, GSPE administration time at 8 a.m.; ZT-12, GSPE administration time at 8 p.m. Copyright white laboratory rat: Rosa Jay/Shutterstock.com.

2.3.1. Dosage Information / Dosage Regimen

During the last 4 weeks of the experiment, a daily dose of GSPE equivalent to dietary intake levels (25 mg kg^{-1} of body weight) was orally administered in condensed milk diluted in water (1:4 v:v) by allowing rats to drink it from the tip of a syringe. This dose has been widely used by the group and had been shown to be the lowest most effective dose in modulating many central metabolic pathways in healthy rats.[22] In addition, taking into account a translation of animal to human doses and estimating the daily intake for a 70 kg human,[23] the phenol extract dose of 25 mg kg^{-1} per day corresponds to an intake of approximately 370 mg of phenols per day. This amount of phenolic compounds can be easily achieved in humans with a polyphenol-rich diet. Animals receiving only vehicle were included as controls. Vehicle (condensed milk diluted in water (1:4 v:v)) and GSPE treatments were administrated either at ZT-0 (8 a.m.; light or resting phase) or at ZT-12 (8 p.m.; dark or active phase). The sacrifice of the rats was 3 h after the last dose.

2.3.2. Sacrifice and Plasma Collection

After sacrifice by decapitation, plasma samples were obtained by centrifugation (2000 x *g*, 15 min, 4 °C) in heparinized tubes (Started, Barcelona, Spain) and stored at –80 °C until chromatographic analysis was performed.

All procedures were performed in accordance with the guidelines for the care and use of laboratory animals, and the experimental procedure was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 9495 by Generalitat de Catalunya) in accordance with the EU Directive 2010/63/EU.

2.4. Micro-Solid Phase Plasma Phenolic Metabolites Extraction

In order to analyze the content of polyphenols and its derivatives of the plasma samples it was necessary to make an extraction to eliminate possible interferences. For this purpose, the previous developed methodology based on micro solid-phase extraction (μSPE) was used using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain).[24]

Briefly, the micro-cartridges were sequentially conditioned with 250 μL of methanol and 250 μL of 0.2% acetic acid. Plasma samples (250 μL) were mixed with 300 μL 4% H_3PO_4 and 50 μL IS at 20 ppm, and this mixture was loaded into the plates. Subsequently, a washing process was carried out to remove interferences that may have been retained in the plates with 200 μL Milli-Q water and then 200 μL acetic 0.2%. Finally, the samples were eluted twice with 50 μL of acetone: water: acetic acid (70:29.5:0.5, v:v:v). The eluted solutions were directly injected into the chromatography equipment.

2.5. Chromatographic Analysis (HPLC-ESI-MS/MS)

Chromatographic separation of phenolic compounds in μ-SPE eluted solutions was performed using an Agilent 1290 LC Series and Zorbax SB-Aq chromatographic column (150 mm x 21 mm i.d., 3.5 μm particle size, Agilent Technologies Palo Alto, CA, USA). The mobile phase consisted of 0.2% acetic acid in water (solvent A) and 100% acetonitrile (solvent B) with the following elution gradient: initial conditions started at 5% of eluent B and was linearly increased to 55% after 10 min, further increased to 80% B in 2 min. Then, it was kept isocratic for 3 min and back to initial conditions for 1 min. A post run of 10 min was applied for column equilibration. The flow rate was set at 0.4 mL min^{-1} and the injection volume at 2.5 μL. Quantification was performed by coupling the LC system to a 6490 (MS/MS) tandem mass spectrometer (Agilent Technologies) following the method described by Margalef et al.[24] Electrospray ionization (ESI) was performed at 350 °C and 12 L min^{-1} with 45 psi nebulizer gas pressure and 4000 V capillary voltage. The mass spectrometer operated in negative mode and MS/MS data was acquired in "Multiple Reaction Monitoring" (MRM) mode. Optimized MRM conditions for the analysis were performed as previously reported for the quantifica-

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tion of phase-II and microbial flavan-3-ols metabolites in plasma and tissues.[24]

2.6. Sample Quantification

For the quantification of the samples, control group blank plasma was spiked with standard compounds at nine different concentrations to obtain calibration curves in both ZT-0 and ZT-12 for each group (CAF and ST). Compounds present at the 0 ppb were subtracted from the plasma concentration at all other concentration-points. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standards curves. All quality parameters are presented in Tables S1 and S2 (Supporting Information). Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

2.7. Statistics

Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, IL, USA). The differences among groups were assessed using one- and three-way ANOVA. First, three-way ANOVA was carried out to evaluate the effects of diet, ZT, sex, and their interactions. The results were reported in tables and figures with italic capital letters indicating significant effects of diet (*D*), GSPE administration time (*ZT*), sex (*S*), or their interaction (*DxZT, DxS, ZTxS, DxZTxS*). When one or more main effects were statistically significant, one-way ANOVA was used to determine the differences between the means. When only the interactions were statistically significant according to the three-way ANOVA model, a one-way ANOVA was performed followed by multiple comparisons. The assumption of normality was determined using the Shapiro-Wilk test, and the homoscedasticity between groups was determined using Levene's test. LSD *post hoc* contrast was used when variances between groups were similar, and Tamhane's T2 test was used if this assumption was not fulfilled. Non-normally distributed data were analyzed by non-parametric multiple comparisons Kruskal-Wallis test. All results represent the mean ± SD from N independent experiments (see figure and table legends for statistical details for each case). Principal component analysis (PCA) was performed to evaluate, under a multivariate approach, the influence of different factors on the metabolization of phenolic compounds using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

3. Results

The effects of sex and time of administration on phenolic compounds plasma bioavailability were investigated under healthy and obesogenic conditions induced by CAF feeding. To this aim, circulating levels of phenolic compounds were analyzed in plasma samples obtained from ST- and CAF-fed rats of both sexes after chronic administration at different daytime of a proanthocyanidins extract. A three-way analysis of variance was conducted to analyze the main effect of diet (*D*), administration time (*ZT*), sex (*S*) and their interaction on the plasma bioavailability of the main phenolic compounds metabolites (flavan-3-ols and phenolic acids, phase-II and microbial colonic metabolites).

3.1. Flavan-3-ols and Phenolic Acids

Figure 2a and **Table 2** show the quantified phenolic compounds belonging to the group of flavan-3-ols and phenolic acids. Remarkably, although CAF-fed animals presented decreased plasma total levels, a wider variability of this group of compounds was observed. Indeed, all the members of this group of phenolic metabolites were significantly ($p < 0.001$) influenced by diet except for 4-hydroxy-3-methoxybenzoic acid. Catechin, epicatechin, and procyanidin dimers levels were detected only in CAF-fed animals. 3,4,5-trihydroxybenzoic acid was the most abundant phenolic acid, and its concentration was significantly higher in ST-fed animals ($p < 0.001$).

Overall, from a multivariate approach, diet was the main factor affecting the flavan-3-ols and phenolic acids overall profile (F (1, 51) = 1358.307, $p < 0.001$) (Table 2). Indeed, groups clustered differently accordingly to diet when analyzed by PCA (Figure 2b). No significant differences due to sex were observed for any compound in this large group.

3.2. Phase-II Flavan-3-ols Metabolites

Figure 2c and Table 2 show the different metabolites identified and quantified from phase II metabolism. The majority of the compounds of this group were methyl-epicatechin glucuronide, epicatechin glucuronide and catechin glucuronide. A significant overall impact of GSPE administration time (F (1, 51) = 33.326, $p < 0.001$) was observed. Indeed, this factor significantly impacted on the plasma bioavailability of all the analyzed metabolites in this group except for the sulfate forms. Thus, metabolites levels were higher when GSPE was administered at ZT-0 ($p < 0.001$). In addition, diet did also affect the plasma levels of most of these phase II metabolites. Methyl-epicatechin glucuronide ($p < 0.01$) showed higher values in rats fed a cafeteria diet, while catechin glucuronide, methyl-catechin glucuronide, catechin sulphate, methyl-catechin sulphate, and methyl-epicatechin sulphate ($p < 0.001$) were found at higher levels in the plasma from rats fed a standard diet. Epicatechin sulphate levels were not altered by any of the studied factors. When analyzed by PCA, groups clustered together accordingly to administration time and type of diet (Figure 2d). No significant differences due to sex were observed.

3.3. Microbial Colonic Metabolites

Microbiota metabolites were the second largest group of compounds detected in the samples. Figure 2e and Table 2 show all the quantified phenolic compounds belonging to this group. An overall significant effect of GSPE administration time (F (1, 51) = 11.180, $p < 0.01$) as well as a significant interaction effect of diet and administration time (F (1, 51) = 6.133, $p < 0.01$) were observed when analyzing plasma levels of these metabolites. Thus, significant higher overall levels of microbial-derived metabolites were found when GSPE was administered at ZT-12 in CAF-fed rats. This ZT effect was not observed in ST-fed rats. In addition, diet significantly altered the overall levels of these metabolites only when GSPE was administered at ZT-0 (see Figure 2e). Moreover, this group was the only one showing a significant overall

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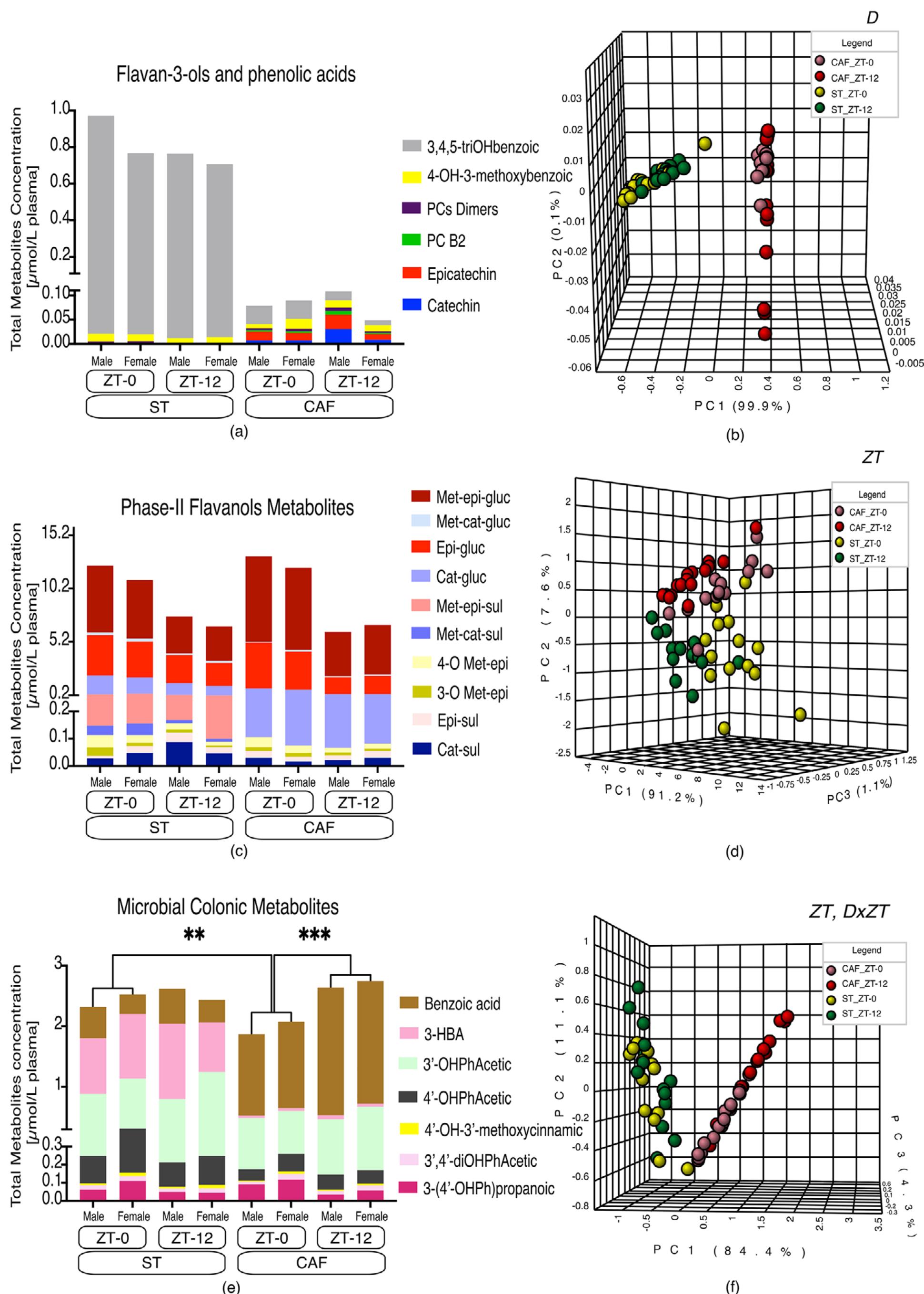

Figure 2. Distribution of phenolic compounds and their derivatives quantified in plasma 3 h after the last dose (4 weeks) of GSPE (25 mg kg^{-1}) by HPLC-ESI-MS/MS in Flavan-3-ols and phenolic acids a), Phase II metabolites c) and microbiota-derived metabolites e). Rats were divided into eight groups, depending on diet (ST and CAF), sex (male and female), and time of administration of GSPE (ZT-0 and ZT-12). Data are presented as means ($n = 8$). Principal component analysis (PCA) b, d, f) graphs show the statistical effect of diet (*D*), GSPE administration time (*ZT*), sex (*S*) or their interaction (*DxZT, DxS, ZTxS, DxZTxS*). The entire statistical procedure is described in the Section 2.7 Statistics. *Indicates significant effects (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). 3-HBA, 3-hydroxybenzoic acid; cat, catechin; epi, epicatechin; gluc, glucuronide; met, methyl; OH, hydroxyl; OHPh, hydroxyphenyl; PC, Procyanidin; sul, sulfate.

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Table 2. Phenolic compounds and their derivatives quantified in plasma (μM) 3 h after the last acute dose for 4 weeks of GSPE (25 mg kg^{-1}) by HPLC-ESI-MS/MS.

Compound	Effect	Standard diet				Cafeteria diet			
		ZT-0		ZT-12		ZT-0		ZT-12	
		Male	Female	Male	Female	Male	Female	Male	Female
Σ Flavan-3-ols and phenolic acids	*D**	0.973 ± 0.033	0.768 ± 0.152	0.764 ± 0.113	0.707 ± 0.040	0.079 ± 0.030	0.089 ± 0.024	0.108 ± 0.051	0.049 ± 0.024
Catechin	*D**	n.d.	n.d.	n.d.	n.d.	0.007 ± 0.001	0.007 ± 0.004	0.031 ± 0.021	0.009 ± 0.006
Epicatechin	*D**	n.d.	n.d.	n.d.	n.d.	0.018 ± 0.008	0.016 ± 0.004	0.029 ± 0.013	0.012 ± 0.007
Procyanidin B2	*D**	0.002 ± 0.001	0.002 ± 0.001	n.q.	n.q.	0.003 ± 0.002	0.003 ± 0.001	0.008 ± 0.005	0.003 ± 0.001
Procyanidin dimers[a)]	*D**	0.004 ± 0.001	0.004 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.004 ± 0.001	0.005 ± 0.002	0.007 ± 0.003	0.003 ± 0.001
3,4,5-trihydroxybenzoic acid	*D**	0.952 ± 0.032	0.747 ± 0.153	0.752 ± 0.110	0.693 ± 0.040	0.038 ± 0.022	0.038 ± 0.022	0.019 ± 0.010	0.010 ± 0.005
4-hydroxy-3-methoxybenzoic acid		0.016 ± 0.006	0.015 ± 0.004	0.011 ± 0.005	0.012 ± 0.005	0.009 ± 0.001	0.020 ± 0.010	0.015 ± 0.004	0.013 ± 0.004
Σ Phase-II flavan-3-ols	***ZT*** *	**12.339 ± 5.626**	**11.005 ± 2.383**	**7.599 ± 1.780**	**6.647 ± 3.504**	**13.217 ± 4.262**	**12.134 ± 3.074**	**6.136 ± 2.378**	**6.796 ± 4.378**
Catechin gluc[b)]	*ZT**, *D**	1.784± 0.774	1.530 ± 0.0.466	1.119 ± 0.241	0.885 ± 0.572	0.757 ± 0.252	0.660 ± 0.176	0.266 ± 0.120	0.263 ± 0.163
Epicatechin gluc[c)]	*ZT**	3.751 ± 2.139	3.336 ± 1.033	2.626 ± 0.763	2.128 ± 1.166	4.254 ± 1.600	3.569 ± 1.304	1.591 ± 0.935	1.690 ± 1.294
Methyl-catechin gluc[b)]	*ZT**, *D**	0.256 ± 0.139	0.289 ± 0.049	0.138 ± 0.049	0.194 ± 0.066	0.103 ± 0.037	0.144 ± 0.032	0.065 ± 0.025	0.112 ± 0.044
Methyl-epicatechin gluc[c)]	*ZT**, *D**	6.249 ± 2.506	5.483 ± 1.038	3.479 ± 0.702	3.231 ± 1.665	7.999 ± 2.485	7.686 ± 1.652	4.149 ± 1.284	4.649 ± 2.894
Catechin sulphate[b)]	*D**	0.027 ± 0.021	0.048 ± 0.013	0.087 ± 0.049	0.046 ± 0.026	0.030 ± 0.013	0.016 ± 0.009	0.022 ± 0.009	0.030 ± 0.023
Epicatechin sulphate[c)]		0.009 ± 0.008	0.024 ± 0.006	0.035 ± 0.021	0.022 ± 0.013	0.024 ± 0.010	0.016 ± 0.009	0.016 ± 0.007	0.025 ± 0.017
3-O Methyl epicatechin[c)]	*ZT**	0.031 ± 0.017	0.013 ± 0.010	0.011 ± 0.005	0.007 ± 0.003	0.014 ± 0.007	0.015 ± 0.004	0.012 ± 0.006	0.009 ± 0.004
4-O Methyl epicatechin[c)]	*ZT**	0.045 ± 0.028	0.028 ± 0.012	0.023 ± 0.011	0.014 ± 0.009	0.036 ± 0.009	0.027 ± 0.006	0.017 ± 0.005	0.018 ± 0.010
Methyl-catechin sulphate[b)]	*D**	0.034 ± 0.024	0.043 ± 0.029	0.011 ± 0.006	0.009 ± 0.004	n.d.	n.d.	n.d.	n.d.
Methyl-epicatechin sulphate[c)]	*D**	0.153 ± 0.097	0.210 ± 0.107	0.070 ± 0.032	0.111 ± 0.088	n.d.	n.d.	n.d.	n.d.
Σ Microbial metabolism	***ZT*** * ***DxZT****	**2.318 ± 0.397**#	**2.521 ± 0.561**#	**2.621 ± 0.547**	**2.433 ± 0.482**	**1.870 ± 0.262**$	**2.075 ± 0.453**$	**2.637 ± 0.382**	**2.747 ± 0.592**
3-(4'-hydroxyphenyl) propanoic acid	*ZT**	0.063 ± 0.020	0.111 ± 0.051	0.051 ± 0.018	0.045 ± 0.019	0.092 ± 0.041	0.118 ± 0.019	0.034 ± 0.009	0.059 ± 0.020
3',4'-dihydroxyphenylacetic acid	*S**, *SxD**	0.026 ± 0.012	0.027 ± 0.007	0.019 ± 0.008	0.025 ± 0.008	0.014 ± 0.003†	0.032 ± 0.011	0.020 ± 0.005†	0.026 ± 0.004
3′-hydroxyphenylacetic acid	*S**, *D**	0.633 ± 0.137	0.825 ± 0.196	0.582 ± 0.197	0.994 ± 0.224	0.311 ± 0.066	0.343 ± 0.164	0.324 ± 0.094	0.500 ± 0.187
4′-hydroxyphenylacetic acid	*D**	0.152 ± 0.013	0.155 ± 0.047	0.134 ± 0.034	0.160 ± 0.020	0.062 ± 0.031	0.099 ± 0.035	0.082 ± 0.019	0.075 ± 0.019
3-hydroxybenzoic acid	*D**	0.918 ± 0.439	1.067 ± 0.354	1.244 ± 0.370	0.817 ± 0.277	0.045 ± 0.018	0.051 ± 0.016	0.066 ± 0.012	0.055 ± 0.019
Benzoic acid	*D**, *DxZT**	0.518 ± 0.083	0.318 ± 0.054	0.581 ± 0.090	0.373 ± 0.070	1.339 ± 0.229$	1.420 ± 0.409$	2.101 ± 0.346	2.022 ± 0.675
4'-hydroxy-3'-methoxycinnamic acid	*S**	0.009 ± 0.004	0.019 ± 0.006	0.009 ± 0.006	0.020 ± 0.014	0.007 ± 0.002	0.013 ± 0.004	0.008 ± 0.002	0.010 ± 0.007

gluc, glucuronide; n.d., not detected; n.q., not quantified; ZT-0, GSPE administration time at 8 a.m.; ZT-12, GSPE administration time at 8 p.m. [a)] Quantified using the calibration curve of procyanidin dimer B2. [b)] Quantified using the calibration curve of catechin. [c)] Quantified using the calibration curve of epicatechin. The "effect" column represents the statistical results of diet (*D*), GSPE administration time (*ZT*), sex (*S*) or their interaction (*DxZT, DxS, ZTxS, DxZTxS*). #, $ and † indicate D, ZT and S effects respectively by one- and three-way ANOVA followed by LSD or Tamhane's T2 post hoc test, $p < 0.05$. Further details are described in the Section 2.7 Statistics. Results are expressed as μM ± SD ($n = 8$). *Indicates significant effects (*$p < 0.01$, **$p < 0.001$).

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impact of sex for some of the analyzed metabolites. When analyzing by PCA, groups clustered together according to diet and, in the case of metabolites from CAF-fed rats, according to ZT (Figure 2f). Therefore, ZT seems to have an effect on microbial-derived metabolites plasma levels only in CAF-fed rats but not in ST-fed rats.

Plasma levels of benzoic acid were significantly increased ($p < 0.001$) by cafeteria diet. In addition, benzoic acid levels were also affected by *ZT* ($p < 0.001$), being higher in ZT-12 compared to ZT-0 in animals fed with cafeteria diet. On the other hand, 3-hydroxybenzoic acid was significantly increased ($p < 0.001$) in rats fed a standard diet. 3′-hydroxyphenylacetic acid was also significantly reduced ($p < 0.001$) in CAF-fed rats compared to ST and was increased in females compared to males ($p < 0.001$). The remaining metabolites described in Table 2 represent a small part of the microbiota-derived metabolite group and are mainly influenced by diet and sex (see Table 2).

4. Discussion

Consumption of phenolic compounds has been associated with several health benefits.[1] However, their bioactivity is dependent on bioavailability, including both metabolization and absorption. Thus, the study of the factors that modulate the bioavailability and metabolism of phenolic compounds, such as the gut microbiota, sex, or diet, is essential.[3,8] In addition to these factors, it has recently been shown that biological rhythms may also play a key role on phenolic compounds bioavailability.[14] However, little is known about the relationships between flavonoids and circadian rhythms. Therefore, the aim of this study was to evaluate if the plasma bioavailability of phenolic compounds from GSPE is affected by the administration time in healthy rats and in a model of metabolic syndrome (MetS) induced by cafeteria diet feeding. Fischer 344 rats were selected because they are characterized by a high degree of sensitivity to circadian rhythms.[9] The administered dose of GSPE (25 mg kg^{-1} BW per day) is equivalent to a human dose of 370 mg per day.[23] Considering that it is estimated that daily intake of total polyphenols in humans can be up to 1 g per day and doses up to 1 g per day GSPE are not associated with detectable adverse effects, this dose of 370 mg per day is appropriate for long-term administrations in humans.[25]

In this chronic study we investigated the main GSPE-derived metabolites, including flavan-3-ols and phenolic acids, phase II metabolites and gut microbiota-derived metabolites at 3 h after the last GSPE administration. Phase II-metabolites were the most abundant while microbiota-derived metabolites were detected at lower levels, corresponding to the remaining in the blood from the chronic administration of GSPE in the previous days. This is in accordance with previous studies that have shown that flavan-3-ols and their phase II metabolites reach their maximum concentration in tissue and plasma during the first hours after consumption,[4,26,27] while colonic microbial metabolites reach the maximum concentration from 7 to 24 h after consumption, in some cases remaining up to 48 h in the blood.[28]

Regarding flavan-3-ols and phenolic acids metabolites, they were detected at higher levels in ST-fed rats compared to CAF-fed rats, especially in the case of 3,4,5-trihydroxybenzoic acid. This may be due to the fact that 3,4,5-trihydroxybenzoic acid may be mainly produced as a result of microbial metabolism since its intestinal absorption is very low.[3] CAF diet is known to alter gut microbiota composition[29] and consequently this can determine a lack of enzymes involved in polyphenols metabolism such as microbial esterases. These enzymes are responsible for the rapid cleavage of the 3,4,5-trihydroxybenzoic acid ester from the galloylated monomeric flavan-3-ols,[30] thus explaining the lower values observed in the animals fed CAF diet. Besides, free sugar rich-foods such as white bread and grape fruit juice has been shown to significantly increase monomeric flavonols bioavailability, specifically epicatechin and catechin.[31] This is in accordance with our results, as we found increased absorption of these phenolic compounds while reduced absorption of galloylated monomeric flavan-3-ols (EGC, ECG, EGCG) was observed. This may translate into a reduction of 3,4,5-trihydroxybenzoic acid in animals with metabolic syndrome.[32] Also, the higher presence of flavan-3-ols in CAF but not in ST may be caused by increased intestinal permeability due to metabolic syndrome.[27] On the other hand, CAF diet is rich in fat which can also have an impact on polyphenols bioavailability. Thus, it has been shown that dietary fats may alter the transit of polyphenols through gastrointestinal tract impacting the absorption of more hydrophobic polyphenols.[33] However, our study is mainly focused on hydrophilic polyphenols, so the fat content has a lower impact on these. This study shows a strong influence of circadian rhythm on the plasma bioavailability of phase-II metabolites. Many biological processes involved in metabolism and absorption of functional food factors follow circadian rhythm.[34] According to the time of the day, metabolization and absorption processes change, being most optimal during the active phase of the organism[35] and reaching the highest levels of most intermediate metabolites. In the case of the rodents, the maximum level of these metabolites is observed at night. In addition, Arola-Arnal et al.[14] described the “timing” of obtaining food as being closely related to metabolism. Examples of this are the rhythmic fluctuations of metabolites such as butyrate in response to circadian rhythms,[36] variation in the expression of hexose carriers SGLT1, GLUT2, and GLUT5[37] or the gastric emptying time of anthocyanins, which was significantly faster when administered in the dark period (active phase) than in the light period (inactive phase).[34] Our results show that the group treated during its resting phase (ZT-0) showed a higher average concentration of total phenolic compounds compared to those treated during the active phase (ZT-12). In the same line Zhang et al[38] described in C57BL/6 mice a higher expression of carriers and phase II enzymes during the light phase, or what is the same in rodents, their resting phase. In this line, Zmrzljak and Rozman observed that mRNA levels of UGTs were maximal during the light time of the day and SULTs during light to dark transition.[39] Similarly, in our study the levels of phase 2-derived metabolites are higher during the day phase. In addition, a higher concentration of glucuronide metabolites is observed compared to sulfated and methylated metabolites, which could be attributed to a higher expression of UGTs at early light hours compared to SULTs and COMTs. Furthermore, these results could indicate that expression of SULTs is not as sensitive to zeitgeber time as in other enzymes. An increase in epicatechin:catechin (EPI:CAT) ratio was

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observed for both free and glucuronide forms. In the same way, the absorption rate of four different types of monomeric proanthocyanidins in human at 2–4 h has been shown to have a higher EPI:CAT ratio.[32] This increase in the EPI:CAT could be the consequence of the depolymerization of GSPE into monomeric forms, which is mainly composed of epicatechin units.[26]

On the other hand, it is known that gut microbiota is altered in obesity and related disorders, and that diet is one of the main factors shaping the gut microbiota composition.[29] Moreover, gut bacteria directly affect the metabolism of phenolic compounds and as a result their bioavailability and composition.[3] The results obtained from microbiota-derived metabolites show a variation in the concentration of metabolites such as benzoic acid, 3-hydroxybenzoic acid, and 3′-hydroxyphenylacetic acid according to the type of diet consumed. This may be caused by changes in the relative abundance of different strains in charge of the bacterial β-oxidation to produce hydroxybenzoic acids.[30] Furthermore, we observed an influence of ZT in animals treated with a cafeteria diet. Unpublished results from our group observed differences due to ZT in the ratio of Bacteroidetes/Firmicutes only in animals that have consumed a cafeteria diet. This could explain the difference by ZT found only in CAF group.

Finally, sex effects were only observed for some microbiota-derived metabolites depending on the diet. In addition, no significant intra-group differences in the bioavailability of phenolic compounds were observed in female rats. Therefore, we assume that if there were differences in the estrous cycle this did not impact on polyphenols bioavailability. Previous studies have shown increased phase-II enzymatic activity in male rats compared to female rats. Margalef et al.[8] demonstrated differences in phase-II enzymatic activity at the level of tissues such as the brain and liver, however the total amount of flavan-3-ols and their metabolites in liver tissues were not affected by gender differences. However, those results were obtained in adult rats from a different strain (Wistar) and diet. Several studies such as those published by Margalef et al.[7] and Lee et al.[40] concluded that the age factor plays a key role in sex differences, being the hormonal development one of the main responsible for differences in metabolism between males and females. The age of the animals in this study (8 weeks) may indicate that they are still growing and therefore attenuate the difference between sexes.

This study clearly shows a strong influence of administration time on the plasma bioavailability of phase-II metabolites and gut microbiota-derived phenolic metabolites treated with cafeteria diet. Levels of phase 2-derived metabolites showed a higher average concentration of total polyphenols during the day phase (ZT-0) compared to the active phase (ZT-12). In addition, a higher concentration of glucuronide metabolites was observed compared to sulfated and methylated metabolites. Furthermore, we observed an influence of ZT in an animal model of metabolic syndrome (MetS) induced by cafeteria diet feeding. This could be due to the alteration in the rhythms of both gut microbiota and circadian clock hosts induced by cafeteria diet feeding. Further studies about gut microbiota changes mediated by diet and circadian rhythms are needed in order to elucidate which bacterial taxa are linked to specific polyphenols metabolites. Diet-induced obesity also affected the plasma bioavailability of phenolic acids and free flavan-3-ols. Finally, the levels of some microbiota-derived metabolites were affected by sex.

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

I.E.-M.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft. V.A.-G.: Formal analysis; Investigation. B.M.: Conceptualization; Funding acquisition; Supervision. A.A.-A.: Conceptualization; Funding acquisition; Supervision. F.I.B.: Conceptualization; Funding acquisition; Supervision. C.T.-F.: Conceptualization; Funding acquisition; Supervision; Investigation; Methodology; Writing – Reviewing and Editing. M.S.: Conceptualization; Funding acquisition; Supervision; Investigation; Methodology; Writing – Reviewing and Editing.

Data Availability Statement

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

Keywords

bioavailability, circadian rhythms, GSPE, metabolic syndrome, phenolic compounds

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