A comparative study on the bioavailability of phenolic compounds from organic and nonorganic red grapes.

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Abstract:

The health-promoting functions of fruit phenolic compounds are mainly attributed to their metabolites. The organic cultivation of fruits is becoming increasingly popular. Thus, this study evaluates whether the differences in red Grenache grapes derived from organic culture conditions influence the bioavailability and metabolism of phenolic compounds in rats. Organic and nonorganic (conventional) red Grenache grapes (OG and CG, respectively) were characterized and administered to Wistar rats (65 mg gallic acid equivalents/kg bw). Serum was recollected at different time points, and the phenolic metabolites were quantified by HPLC-ESI-MS/MS. The results showed that organic cultivation increased the oligomeric proanthocyanidin and anthocyanidin contents and decreased the content of free flavanols and dietary fiber. The serum profile of OG-administered rats showed higher metabolite concentrations at 2 h and reduced metabolite concentration at 24 h compared with the CG-administered rats. Thus, this particular serum kinetic behavior might influence the bioactivity of their phenolic compounds.

Keywords: Anthocyanins; Flavonoids; Phase-II metabolism; Phenolic compounds; Microbial metabolism.

Abbreviations: Body weight (bw); nonorganic conventional grapes (CG); conventional grape-administered rats (CGR); organic grapes (OG); organic grape-administered rats (OGR).

Chemical compounds: Benzoic acid (PubChem CID: 243), Catechin (PubChem CID: 9064), Cyanidin-3-*O*-rutinoside (PubChem CID: 441674), Epicatechin (PubChem CID: 72276), Epigallocatechin gallate (PubChem CID: 65064), Hippuric acid (PubChem CID: 464), Homovanillic acid (PubChem CID: 1738), Malvidin-3-*O*-glucoside (PubChem CID: 443652), *p*-Coumaric acid (PubChem CID: 637542), and Phenylpropionic acid (PubChem CID: 107).

1. Introduction

Fruit consumption is known to be healthy, and this is mainly attributed to their phenolic constituents (Liu, 2003). In this sense, red grapes are rich dietary sources of phenolic compounds (Del Rio et al., 2013). The main types of phenolic families found in red grapes are flavan-3-ols, anthocyanins, flavonols, hydroxycinnamic acids and stilbenes (Iacopini, Baldi, Storchi, & Sebastiani, 2008; Iglesias-Carres et al., 2018; Mulero, Pardo, & Zafrilla, 2010). Flavan-3-ols in grapes occur in monomeric as well as oligomeric and polymeric forms. Several anthocyanidins, including cyanidin, petunidin, malvidin and delphinidin glycosides, can also occur in grapes (Dani et al., 2007; Mulero et al., 2010). Flavonols occur in grapes mainly as quercetin and kaempferol glycosyl derivates, and resveratrol is mainly found in grape skins (Iacopini et al., 2008; Mulero et al., 2010). The synthesis of phenolic compounds in plants can be influenced by environmental conditions such as water availability and soil mineral content. In particular, the phenolic profile of fruits is known to vary depending on culture practices (Heimler, Romani, & Ieri, 2017). Currently, organic grape cultivation systems are increasing, and their production is subjected to strict rules that regulate the type and amount of chemicals used in their production. In fact, organic production techniques are defined as all crop growing systems that promote and enhance biodiversity, biological cycles and soil biological activity. In organic production systems, the use of off-farm inputs is minimized, and the use of synthetic pesticides, growth hormones, antibiotics, modern engineering techniques, chemical fertilizers and sewage sludge are not allowed. However, organic materials from organic farms, natural substances, materials obtained naturally and mineral fertilizers with low solubility are allowed ("EUR-Lex-32007R0834-EN-EUR-Lex," n.d.; Winter & Davis, 2006). Additionally, organic (organic grapes, OG) and nonorganic (conventional grapes, CG) grapes have been shown to have different health effects in animal models of obesity and epilepsy (Cardozo et al., 2013; Rodrigues et al., 2012). Moreover, the *in vitro* antioxidant

activities of grape juices produced from OG are usually higher than those reported from juices produced from CG (Granato, de Magalhães Carrapeiro, Fogliano, & van Ruth, 2016).

The beneficial health effects of phenolic compounds have been attributed to their metabolic products rather than the naturally occurring forms (Margalef et al., 2014). In this sense, phenolic compounds must be bioavailable to exert their systemic function, and bioavailability requires their digestion, absorption and metabolism (Sasot et al., 2017). Buccal, pancreatic and intestinal enzymes can cleave the glycosyl moiety of phenolic compounds, generating aglycone phenolics (Sasot et al., 2017; Stalmach, Edwards, Wightman, & Crozier, 2012). Nevertheless, glycosylated phenolic compounds can be absorbed by the host and found in the plasma (Miyazawa & Nakagawa, 1999). Importantly, anthocyanidins can be absorbed by the stomach (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003) and reach the systemic circulation as glycosides (Miyazawa & Nakagawa, 1999). Phenolic compounds, including anthocyanins, can be absorbed by the small intestine (Monagas et al., 2010; Passamonti et al., 2003). There, these compounds undergo phase-II detoxification, which includes glucuronidation and methylation by uridine 5´-diphosphate glucuronosyltransferases (UGTs) and catechol-*O*-methyltransferase (COMT), respectively. Before reaching the plasma, these compounds can undergo further phase-II metabolism in the liver, which also includes sulfuration by cytosolic sulfotransferases (SULTs) (Andres-Lacueva et al., 2012; Monagas et al., 2010; Motilva et al., 2016). Non-absorbed phenolic compounds are known to reach the colon where they can undergo microbial metabolism (Monagas et al., 2010). For example, high-molecular-weight proanthocyanidins are unlikely to be absorbed in the small intestine and instead reach the colon (Margalef, Pons, Bravo, Muguerza, & Arola-Arnal, 2015; Monagas et al., 2010). When these non-absorbed phenolics reach the gut, microbiota can hydrolyze them (A. Aura, 2008; Margalef et al., 2015). In fact, *in vitro* studies show

that colon polyphenol bioavailability is affected by gastrointestinal digestion (Annunziata et al., 2018). Metabolic products are known to reach the systemic circulatory system, reach different organs and tissues (Andres-Lacueva et al., 2012), and finally reach the kidneys, where they are eliminated via the urine (Motilva et al., 2016; Sasot et al., 2017).

Importantly, different factors are known to modulate the bioavailability and metabolism of phenolic compounds. Some of these factors include the administration dose, content of dietary components in the food matrix (i.e., dietary fiber and fat) and metabolic state of the host (Margalef et al., 2014, 2016, 2017). Therefore, this study aimed to evaluate whether the bioavailability and metabolism of the phenolic compounds in OG-administered rats are distinct from those of CG.

2. Materials and methods

2.1. Chemicals and reagents

Acetone, acetonitrile, methanol (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). Folin-Ciocalteu reagent was purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Eriodyctiol-7-*O*-glucoside, quercetin-3-*O*-galactoside (hyperoside), isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside were purchased from Exrtasynthese (Lyon, France). Benzoic acid, caffeic acid, (+)-catechin, epigallocatechin gallate (EGCG), *p*-coumaric acid, (-)-epicatechin, ferulic acid, gallic acid, hippuric acid, 3-hydroxybenzoic acid, 3-(4-hydroxy)phenylpropionic acid, phloroglucinol, proanthocyanidin dimer B2, protocatechuic acid, pyrocatechol (internal standard, IS), quercetin and vanillic acid were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Cyanidin-3-*O*-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were purchased from PhytoLab

(Vestenbergsgreuth, Germany). Resveratrol was purchased from Quimivita (Barcelona, Spain), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone was purchased from MicroCombiChem e.K. (Wiesbaden, Germany), and rutin was kindly provided by Nutrafur (Murcia, Spain).

Standard compounds were individually dissolved in methanol (MeOH) at 2000 mg/L, with the exceptions of isorhamnetin-3-*O*-glucoside, which was dissolved at 1000 mg/L, and hyperoside, which was dissolved at 500 mg/L. Additionally, cyanidin-3-*O*-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were individually dissolved in MeOH (0.01 % HCl) at 500 mg/L. All standard stock solutions were freshly prepared every 3 months and stored in amber-glass flasks at -20 °C. Mixed standard stock solutions of the standard compounds were prepared in acetone/water/acetic acid (70/29.5/0.5; v/v/v) or MeOH to construct the calibration curves for the phenolic metabolites or quantification of the grape phenolics, respectively.

2.2. Plant fruit material

OG and CG from the red Grenache variety were harvested at maturity on the same day from contiguous vineyards in the same geographical region of Rasquera (Tarragona, Spain). According to the farmers, the antifungals copper(II) sulfate and conazole and the pesticide chlorpyrifos were used during the conventional cultivation of the red Grenache grapes.

Grape pedicles were manually removed, and grapes, including the seeds, skins and pulp, were frozen in liquid nitrogen. The frozen grapes were grounded to homogeneity. The homogenates were then freeze-dried for one week at -85 °C using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain). Lyophilized grapes were further grounded to obtain a fine powder, which was kept at room temperature and protected from light and humidity until further use.

148 *2.3. Characterization of the dietary components of grapes*

149 The dietary components of the OG and CG were characterized according to the official
150 AOAC methods (AOAC, 1990). Briefly, the water content was determined from the
151 difference in the weight of the samples before and after dehydration (98 °C, 24 h). The
152 ash content was determined as the difference in the weight of the samples before and
153 after complete incineration of the organic matter (500 °C, 24 h). The protein was
154 quantified by the Kjeldahl method (conversion factor 6.25), and the lipid content was
155 determined by continuous extraction with n-hexane in a Soxhlet extractor. The total
156 dietary fiber (TDF) content was determined by treatment of the grapes with heat-stable
157 α-amylase, protease from *Bacillus licheniformis* and amyloglucosidase from *Aspargilus*
158 *niger* (Sigma-Aldrich, Madrid, Spain) and sequential weights of the dry residue. The
159 carbohydrate content was calculated by subtracting the water, ash, lipid, protein and
160 TDF contents from the OG and CG. All methodologies were applied to freeze-dried OG
161 and CG in triplicate.

162 *2.4. Extraction, detection and quantification of the phenolic compounds from grapes*

163 The phenolic compounds in OG and CG were extracted according to Iglesias-Carres *et*
164 *al*. (Iglesias-Carres et al., 2018). First, the total phenolic contents in the OG and CG
165 extracts were analyzed by the Folin-Ciocalteu method as described by Iglesias-Carres
166 *et al*. (Iglesias-Carres et al., 2018). Then, the extracts were directly analyzed using a
167 1200 LC series instrument coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto,
168 CA, USA). Two different HPLC-ESI-MS/MS systems were used to separate, detect and
169 quantify the non-anthocyanin and anthocyanin phenolic compounds. A ZORBAX
170 Eclipse XDB-C18 column (150 mm x 2.1 mm i.d., 5 µm particle size) equipped with a
171 narrow-bore guard column (2.1 mm x 12.5 mm, 5 µm particle size) (Agilent
172 Technologies, Palo Alto, CA, USA) was used for the separation of non-anthocyanin
173 compounds. The mobile phase consisted of (A) water:acetic acid (95:5, v:v) and (B)

174 acetonitrile:acetic acid (95:5, v:v). The gradient mode was as follows: initial conditions,
175 0 % B; 0-0.5 min, 0 % B; 0.5-2 min, 0-10 % B; 2-12 min, 10-30 % B; 12-16 min, 30-60
176 % B; 16-17 min, 60-100 % B; 17-20 min, 100 % B; and 20-21 min, 100-0 % B. A
177 postrun of 6 min was required for column re-equilibration. The flow rate was set at 0.4
178 mL/min, and the injection volume was 2.5 µL for all runs. Electrospray ionization (ESI)
179 was conducted at 200 °C, the flow rate was 14 L/min with a nebulizer gas pressure of
180 20 psi, and the capillary voltage was 3000 V. The mass spectrometer was operated in
181 negative mode, and the MS/MS data were acquired in dynamic mode. The
182 anthocyanins were separated on an Acquity BHE C18 column (50 mm x 2.1 mm, 1.7 5
183 µm particle size) (Waters, Milford, MA, USA). The mobile phases consisted of
184 water:formic acid (9:1, v:v) (A) and acetonitrile (B). The gradient mode was as follows:
185 initial conditions, 0 % B; 0-1 min, 0 % B; 1-5 min, 0-9 % B; 5-10 min, 9-15 % B; 10-15
186 min, 15-45 % B; 15-16 min, 45-100 % B; 16-17 min, 100 % B; and 17-18 min, 100-0 %
187 B. A postrun of 6 min was required for column re-equilibration. The flow rate was set at
188 0.4 mL/min, and the injection volume was 2.5 µL for all runs. ESI was conducted as
189 previously described. The mass spectrometer was operated in positive mode, and the
190 MS/MS data were acquired in dynamic mode. The method quality parameters can be
191 found in Table S1.

192 *2.5. Experimental procedure for rat serum collection*

193 Male Wistar rats (640 ± 43 g, 30 weeks of age) were housed at 22 °C with a light/dark
194 cycle of 12 h (lights on at 9:00 am), and the animals were provided tap water and a
195 standard chow diet (AO4, Panlab, Barcelona, Spain) *ab libitum* during the experiment.
196 The rats were randomly distributed into two groups (n=6, each): the OG-administered
197 and the CG-administered rat (OGR and CGR, respectively) groups. In both groups, rats
198 were orally administered a dose of 65 mg gallic acid equivalent (GAE)/kg body weight
199 (bw), as determined by the Folin-Ciocalteu method (Iglesias-Carres et al., 2018), which
200 corresponds to 2.45 g dw OG/kg bw and 2.71 g dw CG/kg bw. The rats were fasted for

8 h prior to oral administration by intragastric intubation between 9 and 10 am. Blood samples were obtained from the saphenous vein using nonheparinized vials (Sarstedt, Barcelona, Spain) before (0 h) and 2, 4, 7, 24 and 48 h after grape administration (Figure 1). The blood samples were maintained at room temperature for 1 h and then centrifuged (2000 x g, 15 min, 4 °C) to obtain the serum. The serum samples were stored at – 80 °C until use. The samples were pooled (n=6) to obtain sufficient volumes to perform the chromatographic analyses while avoiding sacrificing the animals. Additionally, pooling biological samples increases homogeneity and sensitivity, and consequently, this allows the detection of all potential metabolites as previously described by Margalef. *et al*. (Margalef et al., 2015). This study was performed in accordance with institutional guidelines for the care and use of laboratory animals. Additionally, the experimental procedure was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249).

2.6. Extraction of phenolic metabolites from the serum

Prior to quantification of the phenolic metabolites in the serum, the samples were pretreated following the previously developed methodology based on micro solid-phase extraction (μ-SPE). The serum samples were cleaned and concentrated by μ-SPE using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain) as previously described by Margalef *et al.* (Margalef et al., 2016).

2.7. Serum phenolic metabolite quantification

Grape serum metabolites were analyzed with two different chromatographic systems depending on their structure. Chromatographic separation of the non-anthocyanin metabolites was achieved with a Kinetex EVO C18 column (2.6 μm, 150 x 2.1 mm). Mobile phase consisted of water/acetic acid (99.8/0.2; v/v) (mobile phase A) and acetonitrile (mobile phase B) with the following gradient: initial conditions, 0 % B; 0 – 0.5 min, 0 % B; 0.5 – 15 min, 0 – 40 % B; 15 – 15.5 min, 40 – 100 % B; 15.5 – 19 min,

100 % B; 19 – 20 min, 100 – 0 % B. A post run of 3 min was required for column re-equilibration. The flow rate was set at 0.4 mL/min. Quantification was achieved by coupling the above system with a 6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was conducted at 200 °C, the flow rate was 14 L/min, the nebulizer gas pressure was 20 psi, and the capillary voltage was 3000 V. The mass spectrometer was operated in negative mode, and the data were acquired using dynamic mode. Optimized fragmentation conditions for the analysis of the phenolic metabolites are summarized in Supplementary Table S2. The analysis of the anthocyanin metabolites was conducted as described by Nagy *et al.* (Nagy, Redeuil, Bertholet, Steiling, & Kussmann, 2009). ESI was conducted as previously described, and the mass spectrometer was operated in the positive mode. For the quantification of phenolic metabolites, the serum obtained from the 0 h time-point was spiked with standards at 8 different concentrations to construct calibration curves. Any compound present at the 0 h time-point was subtracted from the serum concentration at all other time-points. Samples were quantified by interpolating the analyte/IS peak abundance ratio using the standard curves. Data acquisition was performed using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). The method quality parameters can be found in Table S3.

2.8. Statistics

Student's t-test (SPSS, SPSS Inc., Chicago, IL, USA) was used to estimate any differences in the phenolic and nonphenolic composition of OG and CG.

3. Results

This study aimed to elucidate whether the organic cultivation system influences the bioavailability and metabolism of red Grenache grape polyphenols. Thus, the characterization of the food matrix, which included the phenolic and nonphenolic dietary constituents of both grape varieties, was required. OG and CG were harvested

from contiguous vineyards and on the same day to assure that the only agronomic difference between them was the cultivation system. Serum samples were obtained before and 2, 4, 7, 24 and 48 h after the administration of 65 mg GAE/kg bw of OC or CG to study the bioavailability and metabolism of OG and CG polyphenols.

3.1. Nonphenolic constituents of organic and conventional red Grenache grapes

The water, ash, protein, lipid, fiber and carbohydrate contents of freeze-dried OG and CG were determined (Table 1). The quantities of food constituents in the two cultivars were very similar, although total dietary fiber, was 1.7 times higher in conventional grapes. Specifically, OG and CG were dominated by carbohydrates (proportions higher than 70 %). Low water contents remained after freeze-drying, and the content in OG (12.44 ± 0.07) was statistically higher than in CG (11.51 ± 0.04). The lipids, protein and ash contents in the grapes represented a small proportion of the grape dietary components.

3.2. Phenolic profile of organic and conventional red Grenache grapes

OG and CG showed different phenolic profiles (Table 2). OG had higher quantities of anthocyanidins than CG. In this sense, OG showed content of malvidin-based anthocyanidins 1.6-times higher than that in CG. Indeed, the most abundant anthocyanidin, malvidin *O*-coumaroylglucoside d3, was significantly more abundant in OG than in CG. However, other members of this family, such as delphinidin *O*-glucoside and petunidin *O*-glucoside, were significantly more abundant in CG. Regarding flavan-3-ols, OG had higher concentrations of proanthocyanidin dimers and trimers and of the monomeric glycosylated and gallate flavan-3-ols, and some of these differences in individual compounds were statistically significant. Although free catechin and epicatechin were more abundant in CG, this was only statistically significant for catechin. Moreover, CG presented significantly higher quantities of the predominant flavonol quercetin-3-*O*-glucoside and of kaempferol *O*-galactose, kaempferol-3-*O*-

279 glucose, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside. Additionally,
280 caffeoyltartaric acid, the second most abundant phenolic acid in both grape varieties,
281 was significantly more abundant in CG, although protocatechuic acid *O*-glucoside, was
282 significantly more abundant in OG. Regarding stilbene derivatives, CG had significantly
283 higher concentrations of these compounds than OG.

284 *3.3. Serum kinetic behavior of organic and conventional red Grenache grapes*
285 *metabolites*

286 After oral administration of whole OG and CG at the same phenolic dose (65 mg
287 GAE/kg bw) to rats, which corresponded to doses of 2.45 g dw OG/kg bw and 2.71 g
288 dw CG/kg bw, the kinetic profiles of the sum of all detected grape phenolic metabolites
289 in rat serum from the groups presented similar behaviors (Figure 2 A and Table 3), and
290 this included two serum peaks, one at 2 h and the other at 24 h, while very few
291 metabolites were detected at 7 and 48 h. However, important differences were
292 observed. In this sense, OGR presented total metabolite concentrations of 4289.75 nM
293 2 h after grape administration, while CGR presented a 1.8-fold lower total concentration
294 (2390.11 nM). In contrast, the total metabolite concentration at 24 h in CGR was
295 2495.41 nM, which represents a 1.4-fold increase in the total metabolite concentration
296 relative to OGR (1822.21 nM).

297 3.3.1. Flavonoid metabolites

298 Flavonoid glycosides (i.e., rutin) and nonconjugated flavan-3-ols (i.e., catechin and
299 epicatechin) were not detected in the serum of these animals. However, flavonoid
300 phase-II metabolites were detected, but those were only flavan-3-ol phase-II
301 metabolites. The kinetic plasma behavior of this group of metabolites was similar to
302 that of the total phenolic metabolite concentration and showed two serum peaks, one at
303 2 h and the other at 24 h with concentrations that ranged between 1500 and 50 nM
304 (Figure 2B and Table 3). Specifically, OGR presented higher concentrations of total

flavan-3-ol phase-II metabolites than CGR at all the time points studied. The maximum concentrations of these compounds were found 2 h after grape administration, with the exception of methylcatechin, which reached its maximum at 7 h. Methylcatechin glucuronide was the flavonoid phase II metabolite that reached the highest serum concentration in both OGR and CGR.

3.3.2. Phenolic acid metabolites

The serum kinetic profile of phenolic acids also presented two serum peaks, one at 2 and one at 24 h, and at 2 h, total concentrations were higher in OGR (2031.82 nM) than in CGR (791.69 nM) (Figure 2C and Table 3). In this sense, methylgallic, homovanillic, hippuric and phenylpropionic acids were the most abundant compounds at 2 h in OGR, but the last two were found at significantly lower concentrations in CGR at the same time point. While the highest concentrations were found in the 2 h serum sample from OGR, the contents in the 24 h sample were higher than in the 2 h serum sample from CGR. In this sense, at 24 h, CGR reported a higher total phenolic acid concentration (2110.21 nM) than OGR (1191.27 nM). Indeed, the higher total concentrations at 24 h in CGR were mainly due to the much greater increase in the hippuric acid concentration in CGR (1395.63 nM in CGR and 570.86 nM in OGR).

3.3.3. Cinnamic acid metabolites

The cinnamic acids detected in this study were *p*-coumaric, caffeic and ferulic acids. The total serum kinetic profile of cinnamic acid derivatives was very similar following administration of OG or CG to rats, and like the other types of metabolites, both showed two serum peaks, one at 2 and the other at 24 h after grape administration as well as a lack of detection of these metabolites at 7 and 48 h (Figure 2D and Table 3). However, the kinetic profile of this metabolic family in serum was mainly due to the *p*-coumaric acid concentration. In this sense, *p*-coumaric acid was present at very similar concentrations in serum after OG or CG administration.

331 3.3.4. Valerolactone and valeric acid metabolites

332 In CGR, 4-hydroxy-(3',4'-dihydroxyphenyl)valeric acid first appeared in serum at 4 h
333 (5.99 nM), was more abundant at 7 h (17.13 nM), and remained constant in serum until
334 48 h. The behavior of this compound in serum was different in ORG. In this sense, it
335 first appeared at 2 h (12.79 nM) and reached its maximum by 7 h (19.59 nM). The
336 concentrations between 24 and 48 h, although lower than that at 7 h, were maintained
337 (11.87 and 11.48 nM, respectively). For 5-(3,4-dihydroxyphenyl)-γ-valerolactone
338 glucuronide, both rat groups reported maximum concentrations 24 h after grape
339 administration (Table 3), and the content was higher in OGR (76.98 nM) than in CGR
340 (13.65 nM). However, a less intense serum peak also appeared at 4 h for both OGR
341 (19.92 nM) and CGR (7.75 nM).

342 **4. Discussion**

343 The consumption of fruits is known to produce health effects, and these effects are
344 partially attributed to their phenolic content (Liu, 2003). However, recent studies
345 suggest metabolic products after phenolic ingestion are the real effectors of those
346 biological functions (Margalef et al., 2014). Thus, the study of the factors that modulate
347 polyphenol bioavailability and metabolism is essential to understanding their bioactivity.
348 In this sense, many factors including dose and food matrix can modulate polyphenol
349 bioavailability and metabolism (Bohn, 2014; Margalef et al., 2014). Currently, the
350 production of grapes grown under organic cultivation systems is increasing (European
351 Comission, 2016), and growing conditions are known to modulate the phenolic profile
352 of fruits and their juices (Granato et al., 2016; Granato, Koot, Schnitzler, & van Ruth,
353 2015; Heimler et al., 2017). Thus, this change in the production of grapes could alter
354 the bioavailability and metabolism of grape polyphenols. Therefore, this study aimed to
355 elucidate whether organic cultivation of grapes can alter grape polyphenol
356 bioavailability and metabolism in rats. To ensure that the only independent variable

was the cultivation system, OG and CG were harvested at maturity on the same day from contiguous vineyards.

To provide relevant information about the food matrixes administered to rats, the phenolic profiles of OG and CG were determined by HPLC-ESI-MS/MS, and the contents of their relevant dietary components were also determined. The profiles of nonphenolic constituents in the cultivars were very similar, but OG had a 1.7-fold lower dietary fiber content than CG. Similarly, Dani *et al.* reported a reduced fiber content in Niagara grape juices produced organically when compared to their conventional counterparts (Dani et al., 2007). The phenolic profiles of both varieties are consistent with the major phenolic families in several grape varieties (Iacopini et al., 2008; Iglesias-Carres et al., 2018; Mulero et al., 2010). OG presented a higher content of phenolic compounds, which agrees with the fact that phenolic compounds are synthetized under stressful conditions (Del Rio et al., 2013; Winkel-Shirley, 2002). Thus, the limited amount of chemical treatment allowed in organic cultivation systems might have resulted in higher stress growth conditions, altering the phenolic profile of the grapes. Accordingly, OG presented a higher anthocyanin content than CG, and this flavonoid family is a hallmark of plant stress (Winkel-Shirley, 2002). Moreover, organic cultivation systems have previously been demonstrated to modulate anthocyanin (Dani et al., 2007; Rodrigues et al., 2012), oligomeric flavan-3-ol (Dani et al., 2007) and flavonol (Mulero et al., 2010) contents in grapes and grape-derived products following the same trends observed in this study. Indeed, anthocyanins have been reported at higher concentrations in grape juices produced from OG than from CG (Granato et al., 2016). Paradoxically, stilbenes, which are considered phytoalexins produced by plants under stress, injury or disease (Del Rio et al., 2013), were found in greater amounts in CG. Similarly, catechin was found at significantly higher concentrations in CG. However, higher concentrations of catechin, as well as epicatechin, were reported as

hallmarks of organic grape juice production in the study of Granato *et al*. (Granato et al., 2015).

The bioavailability of grape phenolics from OG and CG was studied in rat serum at different times after acute administration. Polyphenol dose administration is a well-known factor affecting the bioavailability and metabolism of phenolic compounds (Margalef et al., 2014). To avoid any differences in phenolic bioavailability that could arise from differences in phenolic compound dose, rats were administered a total of 65 mg GAE/kg bw, which in the case of OGR corresponded to 2.45 g OG/kg bw and 2.71 g CG/kg bw in CGR. This total polyphenol dose was selected considering the total polyphenol content in a dose of 125 mg/kg bw of a grape seed flavan-3-ol extract in which the flavanol metabolites were correctly detected in plasma (Margalef et al., 2014). The time points for blood collection were based on the fact that the early extraction times (i.e., 2 – 4 h) give information about the small intestinal absorption of the phenolic compounds, while later time points (i.e., 7 – 48 h) give information about colonic metabolism of the phenolic compounds (Margalef et al., 2016). For instance, a recent study by Annunziata *et al*. in humans determined that the maximum content of resveratrol in serum appears 1 h after the administration of a nutraceutical based on a grape pomace polyphenol extract rich in resveratrol (Annunziata et al., 2019). Remarkably, different studies have evaluated the bioavailability and metabolism of grape polyphenols in rats (Margalef et al., 2014, 2016, 2015, 2017; Rodriguez Lanzi et al., 2018) and humans (Castello et al., 2018; Sasot et al., 2017; Stalmach et al., 2012), but only a few have monitored the concentrations for more than 7 h and evaluated the contributions of microbial-derived metabolites in the serum and/or plasma metabolic profile (Castello et al., 2018; Margalef et al., 2015, 2017). Importantly, the study of the microbial metabolism of polyphenols is relevant because these compounds also hold the key to the bioactivities associated with polyphenol consumption (Del Rio et al., 2013; ~~Krga et al., 2018;~~ Margalef et al., 2015).

The only flavonoid phase-II metabolites detected in this study were flavan-3-ol metabolites, and those were mainly glucuronide metabolites; this is consistent with trends observed for grape and cocoa flavan-3-ols (Margalef et al., 2017; Serra et al., 2013). Although some authors have quantified the phase-II metabolites of flavonols and stilbenes in serum, plasma or other tissues after the consumption of foods or extracts rich in those compounds, the administration doses were much higher (Andres-Lacueva et al., 2012; Miyazawa & Nakagawa, 1999; Motilva et al., 2016; Passamonti et al., 2003; Rodriguez Lanzi et al., 2018). For example, in rats administered a grape pomace polyphenol extract, Rodriguez *et al.* found quercetin methyl-glucuronide in plasma when quercetin was administered at a dose of 0.84 mg/kg bw but not when it was administered at a dose of 0.42 mg/kg bw (Rodriguez Lanzi et al., 2018). In the case of anthocyanin phase-II metabolites, the kinetics of these compounds are fast, as they can appear as early as 6 min after oral administration (Passamonti et al., 2003) and can be almost absent from plasma by the 4th hour after administration (Miyazawa & Nakagawa, 1999). Additionally, their bioavailability seems to be low, as reported by Kuntz *et al.* (Kuntz et al., 2015). In this sense, in humans, the administration of 0.33 L of a smoothie rich in anthocyanins (total anthocyanin concentration of 983 ± 38 mg/L) resulted in a maximum plasma concentration of glycosylated compounds of 2.79 nM (maximum concentration approximately 55 – 60 min after smoothie intake) and a maximum plasma concentration of glucuronides of 2.8 nM (maximum concentration approximately 110 min after smoothie intake) (Kamiloglu et al., 2016). In addition, anthocyanidins, especially acylated anthocyanidins, are very unstable under intestinal conditions (Kamiloglu et al., 2016; Stalmach et al., 2012). In this sense, Kuntz *et al.* attributed the low anthocyanin bioavailability in human plasma due to their degradation under physiological pH. Another group of metabolites that were generally not detected in OGR and CGR were phenylacetic acid derivates (Kuntz et al., 2015). In our study, homovanillic acid was the only phenylacetic representative observed. Similarly,

Castello *et al.* did not report phenylacetic acid derivatives after administration of a grape pomace extract to humans (Castello et al., 2018).

Important differences were found in the phenolic bioavailability and metabolism of OG and CG in rats in this study. Based on the first serum peak (2 h) the total phase-II flavan-3-ol metabolite concentration was higher in OGR than in CGR. However, CG presented a higher content of monomeric flavan-3-ols than OG, which are more bioavailable than their oligomeric counterparts (Margalef et al., 2016). Therefore, the food matrix seems to influence the bioavailability and metabolism of grape phenolic compounds (Bohn, 2014). Indeed, the plasma kinetics of different phenolic compounds that are present in wine depend on their administration (wine, grape juice or vegetable juice) in humans according to the study of Goldberg *et al.* (Goldberg, Yan, & Soleas, 2003). Specifically, the dietary components of the fruit matrix, such as the fiber or lipid contents, can modulate the bioavailability and metabolism of the phenolic compounds (Bohn, 2014). In this sense, CG presented a higher content in dietary fiber, which is known to bind phenolic compounds under gastrointestinal conditions, impeding their absorption in the small intestine and promoting the increased passage of these compounds to the colon (Bohn, 2014). This would also be in agreement with the higher total phenolic acid concentration at 24 h and the short side-chain phenolic acids, such as benzoic acid, at 48 h in CGR relative to OGR. The content of flavan-3-ols phase-II metabolites peaked a second time in serum 24 h after grape administration in both treatment groups, and OGR reached higher concentrations than CGR. The higher contents of dimeric and trimeric flavan-3-ols in OG could contribute to the higher concentration of flavan-3-ol phase-II metabolites 24 h after grape administration. In this sense, high-molecular-weight flavan-3-ols are known to be hydrolyzed by gut microbiota to form monomeric flavan-3-ols, and these structures can be absorbed *in situ* and undergo phase-II metabolism (Margalef et al., 2015; Monagas et al., 2010). Thus, the phenolic profile of the red grapes used in this study does not appear to be

464 the only factor modulating the bioavailability of flavan-3-ols in rats, but rather other fruit-
465 related factors, such as dietary fiber, seem to significantly contribute to the differences
466 between the serum metabolite profiles of OGR and CGR. Grapes are known to contain
467 nonextractable proanthocyanidins (NEPAs), which are linked to food matrix
468 constituents, and cannot be extracted by either digestive enzymes or acidic methanol-
469 water solvents (Saura-Calixto et al., 2010). Thus, these proanthocyanidins, which
470 cannot be extracted and quantified by our grape extraction method, could also
471 contribute to the differences observed in OGR and CGR in the formation of the 24-h
472 flavan-3-ol phase-II metabolite peak since these compounds are not absorbed in the
473 small intestine (Saura-Calixto et al., 2010). The higher content of oligomeric flavan-3-
474 ols in OG also seemed to have an impact on the profile of valerolactones, which are
475 mainly formed by the microbial metabolism of dimeric flavan-3-ols (Margalef et al.,
476 2015), and were found at higher concentrations at 24 h in ORG than in CGR.

477 At 2 h, the serum of both treatment groups also showed relevant concentrations of
478 phenolic acids, which, in the case of OGR, were even higher than the ones observed at
479 24 h. Phenolic acids, such as hippuric or homovanillic acids, are abundant microbial-
480 derived metabolites from flavan-3-ols, flavonols and anthocyanins (A. Aura, 2008; A. M.
481 Aura et al., 2002; Margalef et al., 2015). In our study, the main phenolic acids found at
482 2 h in both administration groups were hippuric, homovanillic, phenylpropionic and
483 methylgallic acids. Notably, in grape-seed flavan-3-ol bioavailability studies involving
484 rats, phenolic acids such as phenylpropionic and homovanillic acid also peaked at 2 h
485 after administration, and their concentrations were also higher at 2 h than at 24 h
486 (Margalef et al., 2015). Moreover, when whole grapes are consumed, under small
487 intestinal conditions, anthocyanins can be degraded to phenolic acids (Yang, Yuan,
488 Wang, Han, & Liu, 2018), and thus, the higher anthocyanin content in OG could also
489 explain the higher serum concentration of phenolic acids at 2 h in OGR. The phenolic
490 acids found at 24 h were generated by the microbial metabolism of flavan-3-ols,

491 flavonols and anthocyanins (A. Aura, 2008; A. M. Aura et al., 2002; Margalef et al.,
492 2015). For example, hippuric acid was formed by the microbial metabolism of flavan-3-
493 ols (Margalef et al., 2015).

494 The highest serum concentration of cinnamic acid derivatives was found at 2 h after
495 grape administration, and this was similar for both OGR and CGR. In both cases, *p*-
496 coumaric acid accounted for > 95 % of the cinnamic acids found in serum at 2 h
497 despite their low concentration in both OG and CG. However, anthocyanins acetylated
498 with *p*-coumaric acid were abundant in both grape cultivars. These compounds are
499 unstable under gastrointestinal conditions and can be hydrolyzed, releasing acylated
500 compounds such as *p*-coumaric acid that can then be absorbed (Kamiloglu et al., 2016;
501 Stalmach et al., 2012). However, despite the higher content of acylated anthocyanins
502 with *p*-coumaric acid in OG, OGR did not show higher concentrations of *p*-coumaric
503 acid than CGR. Thus, these results suggest another metabolic route for the formation
504 of *p*-coumaric acid in CGR at 2 h. The only cinnamic acid derivative present at 24 h
505 was *p*-coumaric acid. In this sense, caffeic acid derivatives such as caffeoyltartaric
506 acid, which was found at higher concentrations in CG than in OG, could undergo
507 hydrolysis and dehydroxylation to form *p*-coumaric acid, and this would agree with the
508 higher *p*-coumaric concentration at 24 h in CGR than OGR. Similarly, gallic acid
509 dehydroxylation has been proposed as a possible metabolic route for the generation of
510 protocatechuic acid in humans (Motilva et al., 2016).

511 Importantly, the bioavailability and metabolism of phenolic compounds are the main
512 limiting factors for their bioactivity (Bohn, 2014). Thus, the differences reported in this
513 study could be associated with important changes in the biological effects associated
514 with the consumption of the OG and CG used in this study. In agreement with this,
515 Cardozo *et al.* reported that chronic intake of organic grape juice for 12 weeks
516 normalized the nitric oxide levels in the cerebral cortex and hippocampus of rats fed a
517 high fat diet, which was not found when rats consumed conventional grape juice

(Cardozo et al., 2013). Similarly, Rodrigues *et al.* observed different effects on the activity of antioxidant enzymes in the cerebellum of rats that consumed organic or conventional juices in a chemical rat model of epilepsy (Rodrigues et al., 2012). However, functional studies should be performed to elucidate whether the differences in the bioavailability and metabolism triggered by OG cultivation affect the bioactivities associated with red Grenache grape consumption.

This study demonstrates that the differences in the phenolic and nonphenolic composition of red Grenache grapes grown with an organic cultivation system relative to those grown conventionally resulted in different grape phenolic serum metabolite kinetic profiles in rats. The serum metabolite concentration in OGR suggested higher metabolism of OG polyphenols in the small intestine. In contrast, OGR suggested lower metabolism of OG phenolic compounds by the gut microbiota. As a result of this difference in bioavailability and metabolism of the phenolic compounds from the OG used in this study, the potential health effects associated with their consumption could differ from the studied CG. However, more studies should be carried out with different organic and conventional grapes to confirm these results.

Declarations of interest: None

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702 **Figure legends:**

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

704 **Figure 2.** Kinetic profile of organic (organic grapes, OG) and non-organic
705 (conventional grapes, CG) red Grenache grapes in rat serum: (a) total serum
706 metabolites; (b) total flavan-3-ol metabolites; (c) total phenolic acid metabolites; (d)
707 total cinnamic acid metabolites; (e) total other metabolites. Concentrations (nM)
708 were quantified using the HPLC-ESI-MS/MS method in rat pooled serum (n=6,
709 each) 2, 4, 7, 24 and 28 h after the ingestion of red Grenache grapes at a dose of
710 65 mg GAE/kg bw, corresponding to 2.45 g dw OG/kg bw and 2.71 g dw CG/kg
711 bw.

712

713 **Table 1.** Nonphenolic constituents in freeze-dried powders organic (organic
714 grapes, OG) and nonorganic (conventional grapes, CG) red Grenache grapes. The
715 results are expressed as g/100 g dw ± SD (n=3).

	OG	**CG**
Water	12.44 ± 0.07	11.51 ± 0.04*
Ashes	1.46 ± 0.04	1.88 ± 0.09
Protein	2.29 ± 0.07	3.44 ± 0.03
Lipids	2.02 ± 0.16	1.91 ± 0.05
Total fiber	4.51 ± 0.31	7.71 ± 0.04
Carbohydrates	77.29 ± 0.64	73.55 ± 0.25

716 * Indicates a significant difference (p<0.05) between OG and CG by the Student-t test.

717 **Table 2.** Phenolic compounds in organic (organic grapes, OG) and nonorganic

718 (conventional grapes, CG) red Grenache grapes quantified by HPLC-ESI-MS/MS.

719 The results are expressed as mg/kg dw ± SD (n=3).

Compound	OG	CG
Flavanols		
Catechin	609.62 ± 155.92	1365 ± 44
Epicatechin	280.81 ± 75.60	429.92 ± 4.28
Gallocatechin [a]	n.d.	0.06 ± 0.01
Epigallocatechin [a]	0.11 ± 0.01	n.q.
Catechin gallate [a]	85.25 ± 24.64	43.69 ± 3.85*
(Epi)catechin *O*-glucoside d1 [b]	12.29 ± 2.74	11.14 ± 1.07
(Epi)catechin *O*-glucoside d2 [b]	6.40 ± 1.74	5.95 ± 1.29
(Epi)catechin *O*-glucoside d3 [b]	6.94 ± 1.49	6.43 ± 1.33
(Epi)catechin *O*-glucoside d4 [b]	46.52 ± 8.24	35.35 ± 3.95
Gallocatechin gallate [a]	0.23 ± 0.04	0.11 ± 0.02*
Epigallocatechin gallate	0.86 ± 0.08	0.78 ± 0.07
Procyanidin dimer d1 [c]	339.90 ± 91.28	239.90 ± 12.42
Procyanidin dimer d2 [c]	115.20 ± 28.92	103.03 ± 3.97
Procyanidin dimer B2	319.50 ± 70.84	189.61 ± 12.28
Procyanidin dimer d3 [c]	9.98 ± 2.51	7.84 ± 0.83
Procyanidin dimer d4 [c]	34.40 ± 4.81	25.30 ± 2.09*
Procyanidin dimer d5 [c]	11.20 ± 2.42	6.42 ± 0.12
Procyanidin trimer d1 [c]	3.95 ± 0.80	3.27 ± 0.20
Procyanidin trimer d2 [c]	2.98 ± 0.72	1.94 ± 0.05
Anthocyanins		
Pelargonidin *O*-glucoside [d]	1.80 ± 0.15	5.30 ± 0.11*
Cyanidin *O*-glucoside [d]	48.44 ± 6.39	31.72 ± 1.57*
Delphinidin *O*-glucoside [d]	106.59 ± 10.26	199.42 ± 26.66*
Petunidin *O*-glucoside [d]	122.59 ± 16.59	311.93 ± 21.00*
Malvidin-3-*O*-glucoside	564.55 ± 24.67	854.56 ± 50.43*
Malvidin *O*-acetylglucoside [e]	7.41 ± 1.27	7.93 ± 0.29
Peonidin *O*-coumaroylglucoside d1 [f]	35.11 ± 5.41	22.14 ± 1.83
Peonidin *O*-coumaroylglucoside d2 [f]	2.04 ± 0.26	1.44 ± 0.26
Peonidin 3-*O*-rutinoside	n.d.	n.d.
Delphinidin *O*-coumaroylglucoside d1 [d]	n.q.	15.29 ± 2.70
Delphinidin *O*-coumaroylglucoside d2 [d]	3.13 ± 0.30	6.27 ± 0.60
Delphinidin *O*-coumaroylglucoside d3 [d]	2.02 ± 0.34	1.17 ± 0.03
Petunidin *O*-acetylglucoside d1 [d]	1.08 ± 0.15	1.37 ± 0.08
Petunidin *O*-acetylglucoside d2 [d]	1.91 ± 0.33	n.q.
Petunidin *O*-acetylglucoside d3 [d]	1.71 ± 0.16	3.46 ± 0.23
Petunidin *O*-acetylglucoside d4 [d]	32.67 ± 4.05	57.43 ± 3.35
Malvidin *O*-coumaroylglucoside d1 [e]	13.61 ± 1.47	1.27 ± 0.10*
Malvidin *O*-coumaroylglucoside d2 [e]	50.60 ± 6.19	5.50 ± 0.23*
Malvidin *O*-coumaroylglucoside d3 [e]	830.52 ± 111.56	82.99 ± 9.16*
Flavonols		
Quercetin	1.39 ± 0.19	1.46 ± 0.10
Kaempferol *O*-galactose [g]	8.61 ± 2.00	14.08 ± 1.19*
Kaempferol-3-*O*-glucose	33.45 ± 7.33	56.81 ± 4.09*
Hyperoside	n.d.	n.d.
Quercetin-3-*O*-glucoside [h]	187.43 ± 60.16	470.51 ± 96.61*
Isorhamnetin-3-*O*-glucoside	23.40 ± 2.77	75.62 ± 3.26*

Kaempferol-3-*O*-rutinoside	1.84	±	0.40	2.72	± 0.31*
Rutin	20.46	±	3.47	24.82	± 1.86
Stilbenes					
Resveratrol	4.46	±	1.59	7.78	± 0.39*
Resveratrol *O*-glucoside d1 [i]	7.49	±	1.12	60.96	± 7.38*
Resveratrol *O*-glucoside d2 [i]	17.47	±	6.72	151.04	± 17.37*
Phenolic acids					
Benzoic Acid	9.55	±	0.79	7.46	± 0.41*
3-Hydroxybenzoic acid [j]	n.q.			n.q.	
Dihydroxybenzoic acid d1 [k]	4.78	±	0.72	2.06	± 0.16*
Protocatechuic acid	15.76	±	5.42	11.08	± 1.50
p-Coumaric acid	0.90	±	0.14	0.44	± 0.05*
Gallic acid	3.51	±	1.25	2.54	± 0.39
Caffeic acid	n.q.			0.63	± 0.08
Ferulic acid	n.q.			n.q.	
Caffeoyltartaric acid [l]	3434	±	276	5864	± 362*
Protocatechuic acid *O*-glucoside [k]	4382	±	361	3657	± 41*
Coumaric acid *O*-glucoside [m]	5.32	±	1.44	1.23	± 0.02*
Gallic acid *O*-glucoside d1 [n]	0.70	±	0.17	0.79	± 0.07
Gallic acid *O*-glucoside d2 [n]	13.54	±	4.56	35.23	± 0.34*
Caffeic acid *O*-glucoside d1 [l]	n.d.			13.46	± 1.02
Caffeic acid *O*-glucoside d2 [l]	n.d.			7.74	± 0.44
Caffeic acid *O*-glucoside d3 [l]	65.57	±	2.12	67.68	± 3.92
Flavanones					
Eriodictyol	n.q.			n.q.	
Eriodictyol-7-*O*-gucoside	0.44	±	0.04	0.40	± 0.00

* Indicates a significant difference (p<0.05) between OG and CG by Student's t test. Different isomers are indicated by d1, d2, d3, d4 and d5. Abbreviations: n.d., not detected; n.q., not quantified. [a] Quantified using the calibration curve of epigallocatechin gallate. [b] Quantified using the calibration curve of catechin. [c] Quantified using the calibration curve of procyanidin dimer B2. [d] Quantified using the calibration curve of cyanidin-3-*O*-rutinoside. [e] Quantified using the calibration curve of malvidin-3-*O*-glucoside. [f] Quantified using the calibration curve of peonidin-3-*O*-rutinoside. [g] Quantified using the calibration curve of kaempferol-3-*O*-glucoside. [h] Quantified using the calibration curve of hyperoside. [i] Quantified using the calibration curve of resveratrol. [j] Quantified using the calibration curve of benzoic acid. [k] Quantified using the calibration curve of protocatechuic acid. [l] Quantified using the calibration curve of caffeic acid. [m] Quantified using the calibration curve of *p*-coumaric acid. [n] Quantified using the calibration curve of gallic acid.

Table 3. Grape phenolic metabolites quantified in pooled (n=6, each) rat serum at 2, 4, 7, 24 and 48 h after the administration of organic (organic grapes, OG) or nonorganic (conventional grapes, CG) red Grenache grapes at a dose of 65 mg GAE/kg bw, corresponding to doses of 2.45 g dw OG/kg bw and 2.71 g dw CG/kg bw.

	Metabolite	Serum concentration (nM)									
		OG					CG				
		2 h	4 h	7 h	24 h	48 h	2 h	4 h	7 h	24 h	48 h
FM	Catechin glucuronide [a]	210.92	124.64	n.d.	31.46	n.d.	226.05	108.46	n.d.	31.14	n.d.
	Epicatechin glucuronide [b]	110.07	70.83	n.d.	30.72	n.d.	80.48	44.18	n.d.	15.06	n.d.
	Methylcatechin glucuronide [a]	1431	912.64	3.80	358.25	35.55	998.24	566.59	0.00	201.36	25.99
	Methylepicatechin glucuronide [b]	373.68	279.15	2.60	93.26	3.54	199.44	126.00	2.54	47.21	15.00
	Methylcatechin [a]	n.d.	14.11	52.30	11.30	19.83	n.d.	n.d.	47.67	39.56	34.59
PAM	3-(4-Hydroxyphenyl)propionic acid	n.q.	23.65	5.79	31.71	4.17	n.d.	15.66	n.d.	48.82	n.d.
	3-(3-Hydroxyphenyl)propionic acid [c]	11.55	11.04	n.d.	2.15	n.d.	3.02	6.46	n.d.	7.81	n.d.
	3-(3,4-Dihydroxyphenyl)propionic acid [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Phenylpropionic acid [c]	407.96	333.39	n.d.	464.55	n.d.	8.25	136.10	n.d.	352.11	n.d.
	Homovanillic [d]	495.95	152.58	n.d.	28.27	n.d.	430.47	112.50	88.00	133.17	3.48
	Hippuric acid	570.86	599.20	n.d.	610.93	n.d.	n.d.	178.12	n.d.	1396	n.d.
	Methylgallic acid [e]	384.04	193.99	n.d.	37.77	2.08	288.94	103.64	n.d.	25.57	1.55
	Dihydrocaffeic acid glucuronide [f]	8.61	n.q.	n.d.	n.d.	n.d.	4.44	3.98	1.86	n.q.	n.q.
	Dihydroferulic acid glucuronide [g]	14.64	n.d.	n.d.	n.d.	n.d.	12.52	n.d.	n.d.	n.d.	n.d.
	Vanillic Acid	18.56	n.d.	n.d.	5.88	n.d.	6.22	n.q.	7.03	9.39	7.17
	Benzoic acid	92.13	12.50	n.d.	10.02	n.d.	37.83	21.45	27.93	132.59	83.70
	3-Hydroxybenzoic	27.54	n.d.	n.d.	n.q.	n.d.	n.q.	n.d.	2.05	5.13	14.43
CAM	*p*-Coumaric acid	110.26	31.19	n.d.	17.09	n.d.	90.68	29.44	n.d.	25.15	1.50
	Caffeic	n.q.	15.85	n.d	n.d	n.d	n.q.	5.71	n.d	n.d.	n.d.
	Ferulic acid	5.16	n.d.	n.d.	n.q.	n.d.	1.68	n.q.	n.d.	n.d.	n.d.
OM	4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid [h]	12.79	8.79	19.56	11.87	11.48	n.d.	5.99	17.13	12.07	19.36
	5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide [h]	3.81	19.92	n.d.	76.98	n.d.	1.85	7.75	n.d.	13.65	n.d.
Total metabolites		**4290**	**2803**	**84.05**	**1822**	**76.65**	**2390**	**1472**	**194.21**	**2495**	**206.78**

737 Abbreviations: FM, flavan-3-ol phase-II metabolites; PAM, phenolic acid metabolites; CAD, cinnamic acid metabolites; OM, other
738 metabolites. [a] Quantified using the calibration curve of catechin. [b] Quantified using the calibration curve of epicatechin. [c] Quantified using
739 the calibration curve of 3-(4-hydroxyphenyl)propionic acid. [d] Quantified using the calibration curve of vanillic acid. [e] Quantified using the
740 calibration curve of gallic acid. [f] Quantified using the calibration curve of caffeic acid. [g] Quantified using the calibration curve of ferulic acid. [h]
741 Quantified using the calibration curve of 5-(3,4-dihydroxyphenyl)-*γ*-valerolactone glucuronide.