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Bioavailability and pharmacokinetic profile of grape pomace phenolic compounds in humans

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ABSTRACT

Grape pomace, the major byproduct of the wine and juice industry, is a relevant source of bioactive phenolic compounds. However, polyphenol bioavailability in humans is not well understood, and the inter-individual variability in the production of phenolic metabolites has not been comprehensively assessed to date. The pharmacokinetic and excretive profiles of phenolic metabolites after the acute administration of a drink made from red grape pomace was here investigated in ten volunteers. A total of 35 and 28 phenolic metabolites were quantified in urine and plasma, respectively. The main circulating metabolites included phenyl-γ-valerolactones, hydroxybenzoic acids, simple phenols, hydroxyphenylpropionic acids, hydroxycinnamates, and (epi)catechin phase II conjugates. A high inter-individual variability was shown both in urine and plasma samples, and different patterns of circulating metabolites were unravelled by applying unsupervised multivariate analysis. Besides the huge variability in the production of microbial metabolites of colonic origin, an important variability was observed due to phase II conjugates. These results are of interest to further understand the potential health benefits of phenolic metabolites on individual basis.

1. Introduction

The study of foods containing components with putative beneficial effects on human health has become a topic of increasing importance during the last decades. Epidemiological evidence has widely associated phenolic-rich foods with the prevention of several chronic pathologies, including cardiovascular diseases, diabetes, and neurodegenerative diseases, as well as some types of cancer [1–3]. In this framework, the demand by consumers of foods exerting beneficial effects on human health leads to the introduction into the global market of new products rich in phenolic compounds [4,5]. Grape pomace, the major by-product of wine and grape juice industry, is a rich source of phenolic compounds, including flavonoids (anthocyanins, flavan-3-ols, flavonols, and flavanones) and non-flavonoids (phenolic acids and their derivatives, stilbenes, and lignans) [6]. The phenolic profile of grape pomace extracts has been related to antioxidant and anti-inflammatory effects, the prevention of cardiovascular and intestinal diseases, anti-aging and anti-diabetic properties [7–9].

It should be highlighted that the capability of phenolic compounds to exert beneficial actions is strictly related to their bioavailability and the products of their metabolism [1,2,10]. Therefore, the study of the chemical transformations undergone by phenolic compounds during their passage through the human organism is a tipping point to properly understand their biological effects [10,11]. In this sense, several studies have reported that certain phenolic compounds are highly available, whereas most of them are scarcely or not absorbed in the small intestine [12–14]. The vast majority of dietary phenolic compounds reach therefore the large intestine, where they are further metabolized into various phenol derivatives by the local gut microbiota [15,16]. The main phenolic catabolites are formed by the cleavage of ester and

Abbreviations: PC, principal component; PCA, principal component analysis; RGPD, red grape pomace drink; SRM, selective reaction monitoring; SPE, solid phase extraction

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glycoside bonds, ring-fission, and chemical modifications of their more complex precursors. Once absorbed at colonic level, these newly formed metabolites may undergo phase I/II transformations exerted by both gut epithelial cells and hepatocytes. In addition, some phenolic metabolites may undergo an intense enterohepatic recirculation [5,15–18]. Despite the increase in the number of research studies on this subject, the metabolic fate of phenolic compounds after ingestion in humans still remains poorly understood. One of the main reasons behind this lack of knowledge is related to the high inter-individual variability existing in the bioavailability of these compounds, this being even more relevant when it comes to colonic catabolites [19]. The absence of appropriate reference standards is another factor hampering the adequate identification and quantification of phenolic metabolites in biological fluids [20].

In the light of the above, we aimed to expand the knowledge on the metabolic fate of the phenolic compounds contained in a phenolic-rich drink made from grape pomace. Specifically, this study was focused on the analysis, by UHPLC-ESI-MS/MS techniques, of human and/or microbial phenolic metabolites present in plasma and urine samples of 10 volunteers after the acute administration of a drink prepared with a red grape pomace extract.

2. Material and methods

2.1. Materials

All chemicals and solvents used in this study were of analytical grade. Gallic acid, (−)-epicatechin, (+)-catechin, protocatechuic acid, 3-hydroxyphenylpropionic acid, hippuric acid, rutin, and cyanidin-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydrocaffeic acid-3′-sulphate, ferulic acid-4′-sulphate, ferulic acid-4′-glucuronide, and dihydroferulic acid-4′-sulphate were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(phenyl)-γ-valerolactone-3′-sulphate, 5-(phenyl)-γ-valerolactone-3′-glucuronide, 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-sulphate, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulphate, and 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide were synthesized in house [20,21]. Most of these molecules are catalogued on the standards sharing platform FoodComEx (www.foodcomex.org). Feruloylglycine and 4-hydroxyhippuric acid were kindly supplied by Prof. Alan Crozier (University of California Davis), while hydroxybenzoic acid conjugates were provided by Dr. Colin Kay (N.C. State University's Plants for Human Health Institute). All solvents and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

2.2. Clinical design

Ten men, age 26 ± 2 years, BMI 27 ± 3 kg/m^2 (mean ± SD) were recruited through advertising at the Department of Clinical Medicine and Surgery of the Federico II University, Naples (Table 1).

Table 1
Characteristics of participants in the study. Data are expressed as means ± SD.

Age (years)	26 ± 2
BMI (kg/m^2)	27 ± 3
Systolic blood pressure (mm Hg)	122 ± 13
Diastolic blood pressure (mm Hg)	78 ± 8
Fasting plasma glucose (mg/dL)	102 ± 6
Fasting plasma insulin (μU/mL)	10 ± 5
Fasting plasma triglycerides (mg/dL)	85 ± 38
Fasting plasma total cholesterol (mg/dL)	154 ± 31
HDL-cholesterol (mg/dL)	38 ± 5

Inclusion criteria were as follows: age 20–40 years, BMI 20–30 kg/m^2, fasting plasma glucose < 110 mg/dL, fasting plasma triglycerides ≤200 mg/dL and fasting plasma cholesterol ≤250 mg/dL. Exclusion criteria were: known chronic illness, diabetes, regular intensive physical activity (defined as more than five training units/week), renal failure (serum creatinine > 1.7 mg/dL), liver disease, anaemia, any other chronic diseases, use of drugs able to influence glucose and lipid metabolism. All participants provided written informed consent, and the study was approved by the Ethics Committee of the "Federico II" Naples University. This trial was registered at clinicaltrials.gov as NCT02865278.

2.3. Study design

Participants were asked to consume a low-polyphenol diet during the experimental period (3 days before and 2 days after the test day). To facilitate participant adherence to the dietary restrictions, a list of permitted and forbidden foods was supplied to the volunteers. On the day of the test, participants were admitted at the Clinical Research Center, after 12 h overnight fast for the baseline blood drawing; thereafter, they consumed 250 mL of an aqueous extract drink of red grape pomace (RGPD; 9.8 g/100 mL of soluble carbohydrates and 625 mg/100 mL of total polyphenols). Three hours after RGPD consumption, participants consumed a standard low-polyphenol meal, consisting in white bread (150 g), fatless ham (70 g), spreadable cheese (80 g) and plumcake (33 g) (903 kcal, 18% protein, 30% fat, 52% carbohydrates). Plasma samples were collected every hour over 8 h after drink intake. The next day, a fasting plasma sample was also collected, 24 h after the test drink. In addition, urine samples were collected at fasting and after the RGPD consumption at: 0–3, 3–6, 6–10, 10–24, 24–36, and 36–48 h. The urine volumes were recorded and two aliquots of 2 mL each were stored at −80 °C until analysis.

2.4. Plasma and urine processing

Plasma samples of all volunteers were extracted using a solid phase extraction (SPE) method previously reported by Feliciano and colleagues [22]. Briefly, 350 μL of plasma samples were diluted (1:1) with *o*-phosphoric acid 4% (v/v). After plate activation, 600 μL of the diluted plasma samples were loaded on a 96 well μ-SPE HLB plate (Oasis® HLB μElution Plate 30 μm, Waters, Milford, Massachusetts, MA, USA). Samples were then washed with 200 μL of water and with 200 μL of 0.2% (v/v) acetic acid. Finally, samples were eluted with 60 μL of methanol for UHPLC-ESI-MS/MS analysis.

Urine samples were prepared as previously reported by Brindani and colleagues [20]. Briefly, urine samples were defrosted, vortexed, diluted in 0.1% formic acid in water (1:4, v/v), and centrifuged at 12000 rpm for 5 min. Finally, urine samples were filtered (0.45 μm nylon filter) prior to the UHPLC-ESI-MS/MS analysis.

2.5. UHPLC-ESI-QqQ-MS/MS

Plasma and urine samples were analysed by UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (QqQ-MS/MS, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Chromatographic and ionization parameters for the analysis of the samples were set as previously described [20]. Metabolite identification was carried out by comparing the retention time with authentic standards and/or MS/MS fragmentation patterns. Up to 160 compounds related mainly to the metabolism of anthocyanins, flavan-3-ols, flavonols and some phenolic acids, were monitored in selective reaction monitoring (SRM) mode. Quantification was performed with calibration curves of standards, when available. When not available, the conjugated metabolites were quantified with the most structurally similar compound.

2.6. Data analysis and statistics

All data were expressed as mean values ± SEM. PKsolver add-on program was used to perform pharmacokinetic data analysis in Microsoft Excel [23]. Multivariate principal component analysis (PCA) with varimax rotation was applied to explore the inter-individual variability observed for the plasma profile of phenolic metabolites, by using SPSS statistics 24.0 software (IBM, Chicago, IL).

3. Results

3.1. Phenolic composition of grape pomace drink

A total of 25 phenolic compounds were identified and quantified in the RGPD. The retention times and spectrometric characteristics of the compounds detected are reported in Table S1 of the Supplementary Information. The 250 mL of RGPD used in this study contained 3.7 mmol of total phenolic compounds. Anthocyanins were the most abundant class of phenolic compounds (70%), followed by flavan-3-ol monomers (23%) and procyanidins (4%). Small amounts of flavonols, galloyl glucose, and gallic acid were also present in the drink.

3.2. Excretion of phenolic metabolites in urine

Up to 35 phenolic compounds were identified in urine over 48 h after ingestion of the RGPD. The UHPLC-ESI-MS/MS parameters applied to the characterization of these metabolites in urine are reported in Table S2 of the Supplementary Information. Several classes of phenolic compounds were detected, including simple phenols (catechol derivatives), hydroxybenzoic acids, hydroxyphenylpropionic acids, hydroxycinnamic acids, phenyl-valeric acids, phenyl-γ-valerolactones, and (epi)catechin conjugates (Table 2). A few anthocyanin derivatives were tentatively detected at trace levels in the 0–3 h urine samples of only some among the volunteers and, hence, they were not taken into account. Although a high excretion of hippuric acid and 4′-hydroxyhippuric acid was observed, these were not included in the calculation of the total amount of phenolic compounds since they also originate from other dietary and endogenous sources [10,16].

Phenyl-γ-valerolactones were the main compounds excreted in urine, followed by simple phenols and hydroxybenzoic acids, hydroxyphenylpropionic and hydroxycinnamic acids and (epi)catechins (Fig. 1). Basal amounts of phenyl-γ-valerolactones in urine were negligible as a consequence of the 3-day low-polyphenol diet (Table 2). A measurable urinary excretion of phenyl-γ-valerolactones was already observed after 3 h from the consumption of the RGPD, accounting for the 7.2% of the total excretion of these colonic metabolites. The maximum excretion of phenyl-γ-valerolactones was found in the 3–6 h urine samples (32.5% of the total excretion), followed by a continuous excretion until the 24–36 h urine sample (Table 2). In all volunteers, the major contribution to their excretion was from the phase II conjugates of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (92.2%), mainly the sulphate (compound **30**, 56.2%) and glucuronide forms (**17** and **19**, 33.5%). Instead, 5-hydroxyphenyl-γ-valerolactone derivatives contributed to the 6.7% of the total excretion of phenyl-γ-valerolactones (compounds **25** and **35**) and, contrary to dihydroxyphenyl-γ-valerolactones, their excretion peaked in the 10–24 urine sample (Table 2).

The 9 simple phenols and hydroxybenzoic acids detected in urine samples had a total 0–48 h excretion of 202.0 ± 13.6 μmol (Table 2). The maximum excretion of these metabolites was reached after 6 h from the ingestion of RGPD, like for phenyl-γ-valerolactones. Simple phenols and hydroxybenzoic acids also showed an additional peak after 36 h following RGPD consumption (Fig. 1). Methylpyrogallol-sulphate (**7**), benzoic acid-4-sulphate (**10**), and the glucuronide and sulphate forms of vanillic acid (**2** and **13**, respectively) were the main simple phenols and hydroxybenzoic acids excreted in urine (Table 2).

A total of 7 hydroxyphenylpropionic acids and hydroxycinnamic acids were identified in urine (Table 2). The total 0–48 h excretion of these metabolites was 38.7 ± 1.8 μmol, much lower than for phenyl-γ-valerolactones and simple phenols and hydroxybenzoic acids. The urinary excretion of hydroxyphenylpropionic and hydroxycinnamic acids was almost constant along the 48 h, with a small excretion peak recorded for the 3–6 h urine samples.

Finally, a total of 6 phase II (epi)catechin metabolites were detected in urine (Table 2), with a total excretion equal to 9.6 ± 1.7 μmol. In contrast to the other phenolic classes, these flavan-3-ol metabolites were excreted more rapidly, reaching a maximum excretion rate in the first 3 h (0–3 h urine samples). The excreted amount decreased drastically after 6 h from the intake of the RGPD (Table 2).

3.3. Plasma pharmacokinetics

Up to 28 compounds were identified in the plasma samples collected from the volunteers following RGPD consumption (details on their identification are presented in Table S2, Supplementary Information). Maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), area under the plasma concentration-time curve ($AUC_{0\text{-}24}$) and half-life of elimination ($t_{1/2}$) were calculated for each compound (Table 3). The pharmacokinetic profiles of the main groups of phenolic metabolites identified in plasma are illustrated in Fig. 2. Circulating anthocyanins were not detected. The relative contribution of all the classes of phenolic metabolites detected in plasma to the pool of circulating metabolites is presented in Fig. 3.

Phenyl-γ-valerolactones were the most abundant class of phenolic metabolites in circulation. As in the case of the urinary samples, the glucuronide- and sulphate-conjugated isomers of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**17**, **19**, and **30**) represented the most abundant compounds, with mean C_{max} ranging from 268 to 1171 nM. The rest of the phenyl-γ-valerolactones showed a C_{max} that never exceeded 100 nM. Phenyl-γ-valerolactones were not detectable during the first 2 h after RGPD intake, but started to peak after 4 h (Fig. 2). T_{max} for dihydroxyphenyl-γ-valerolactone derivatives was about 5–7 h, while it was 9–11 h for the monohydroxyphenyl-γ-valerolactone conjugates (Table 3). Their amount decreased progressively 8 h after RGPD consumption, reaching concentration values < 100 nM for all the phenyl-γ-valerolactones after 24 h from the drink intake. Nevertheless, a high inter-individual variability was observed and a subject was characterised by values close to 1 μM for the 5-(hydroxyphenyl)-γ-valerolactone-sulphate isomers (**30**) at 24 h.

A total of 10 hydroxybenzoic acids and simple phenols were detected in plasma, being methylpyrogallol-sulphate (**7**) and protocatechuic acid-3-sulphate (**9**) the most representative compounds. These two compounds were detected at high concentrations in plasma, reaching high nM levels (C_{max} values of 512.4 ± 117.5 and 408.5 ± 68.7 nM, respectively). Hydroxybenzoic acids identified included protocatechuic acid-3-glucuronide (**3**), which was not detected in urine samples. The maximum C_{max} of hydroxybenzoic acids and simple phenols varied significantly among metabolites (Table 3). For instance, C_{max} peak for gallic acid (**1**), protocatechuic acid derivatives (**3**, **4**, and **9**), benzoic acid-sulphate (**10**), and methylcatechol-sulphate (**8**) were observed during the first few hours after RGPD intake, with t_{max} ranging from 2 to 3 h. On the contrary, vanillic acid derivatives (**2** and **13**) and methylpyrogallol-sulphate (**7**) had t_{max} between 4 and 6 h, with catechol-sulphate (**6**) peaking at 11.2 h (Table 3). Moreover, some compounds peaking during the first few hours (**1**, **3**, **4**, **9**, and **10**) showed a second concentration peak around 6–8 h (data not shown). The clearance of these compounds was relatively slow, since their levels remained constant in plasma within the first 8 h. Then, for all volunteers, plasma concentrations went below 100 nM for all the metabolites at time 24 h after RGPD intake.

Regarding hydroxyphenylpropionic acids and hydroxycinnamic acids, up to 5 compounds were identified in plasma (Table 3). In contrast to urine samples, sinapic acid-sulphate (**33**) and

Table 2
Phenolic metabolites detected in urine. Data are expressed in μmol (mean ± SEM). N = 10 except for the last time points where n = 9 (subject#3 was excluded from these calculations because of a lack of compliance with the study protocol during the period 24–48 h).

Id.#	Phenolic compounds	Urine collection periods (hours)								
		0	0–3	3–6	6–10	10–24	24–36	36–48	Total (0–48 h)	CV (%)
		n = 10	n = 10	n = 10	n = 10	n = 10	n = 9	n = 9	n = 9	n = 9
1	Gallic acid	traces	0.41 ± 0.07	0.29 ± 0.04	0.12 ± 0.05	0.12 ± 0.05	traces	traces	0.93 ± 0.06	19.9
2	Vanillic acid-4-glucuronide	0.12 ± 0.07	0.26 ± 0.04	2.17 ± 0.35	2.37 ± 0.56	1.93 ± 0.40	10.84 ± 3.44	1.40 ± 0.35	19.17 ± 4.17	68.7
4	Protocatechuic acid	traces	0.08 ± 0.02	0.08 ± 0.01	0.05 ± 0.02	0.07 ± 0.01	0.14 ± 0.02	0.09 ± 0.03	0.55 ± 0.06	32.8
9	Protocatechuic acid-3-sulphate	0.32 ± 0.11	5.81 ± 0.85	2.43 ± 0.49	0.48 ± 0.08	1.18 ± 0.25	1.24 ± 0.15	2.52 ± 0.84	13.68 ± 1.11	25.6
10	Benzoic acid-4-sulphate	2.68 ± 0.79	2.38 ± 0.94	3.49 ± 1.00	3.25 ± 1.16	3.73 ± 0.94	5.05 ± 0.73	6.50 ± 1.36	26.02 ± 4.27	51.8
13	Vanillic acid-4-sulphate	0.30 ± 0.11	1.16 ± 0.28	9.59 ± 1.69	6.37 ± 1.90	2.54 ± 0.43	21.23 ± 3.35	2.53 ± 0.47	42.12 ± 5.12	38.4
6	Catechol-sulphate	0.12 ± 0.11	0.12 ± 0.10	0.24 ± 0.14	0.21 ± 0.09	0.25 ± 0.10	0.31 ± 0.15	0.08 ± 0.03	1.25 ± 0.66	166.8
7	Methylpyrogallol-sulphate	0.15 ± 0.09	7.79 ± 6.38	33.52 ± 7.59	30.13 ± 6.32	15.47 ± 3.67	4.91 ± 1.94	1.05 ± 0.16	93.61 ± 10.83	36.6
8	Methylcatechol-sulphate	0.10 ± 0.08	1.88 ± 0.29	1.78 ± 0.24	0.59 ± 0.09	0.34 ± 0.08	0.10 ± 0.02	0.12 ± 0.03	4.72 ± 0.37	24.5
	Total simple phenols and hydroxybenzoic acids	**3.84 ± 1.10**	**19.88 ± 7.53**	**53.60 ± 8.16**	**43.59 ± 9.02**	**25.57 ± 3.76**	**43.95 ± 7.22**	**14.32 ± 2.07**	**202.05 ± 13.59**	**21.3**
12	Ferulic acid-4-glucuronide	0.19 ± 0.09	0.18 ± 0.05	0.56 ± 0.08	0.58 ± 0.14	0.99 ± 0.17	1.02 ± 0.31	1.52 ± 0.48	5.09 ± 0.73	45.3
24	Feruloylglycine	0.98 ± 0.36	0.91 ± 0.21	0.80 ± 0.22	0.68 ± 0.21	2.44 ± 0.28	3.98 ± 0.45	2.72 ± 0.36	12.43 ± 1.12	28.5
23	Dihydrocaffeic acid-sulphate	0.06 ± 0.03	0.07 ± 0.01	0.27 ± 0.05	0.23 ± 0.08	0.27 ± 0.06	0.31 ± 0.09	0.20 ± 0.07	1.39 ± 0.30	67.3
26	Dihydroferulic acid-sulphate	0.12 ± 0.04	0.18 ± 0.04	0.15 ± 0.03	0.13 ± 0.03	0.36 ± 0.06	0.46 ± 0.11	0.34 ± 0.08	1.69 ± 0.22	42.0
28	Ferulic acid-4-sulphate	0.11 ± 0.03	0.14 ± 0.04	0.43 ± 0.07	0.56 ± 0.12	0.57 ± 0.13	0.97 ± 0.17	0.97 ± 0.24	3.69 ± 0.53	45.7
33	Sinapic acid-sulphate	traces	traces	0.40 ± 0.10	0.32 ± 0.07	0.44 ± 0.08	0.27 ± 0.08	0.07 ± 0.02	1.50 ± 0.22	45.7
14	Hydroxyphenylpropionic acid-sulphate	0.81 ± 0.19	0.60 ± 0.18	1.09 ± 0.22	1.51 ± 0.36	3.38 ± 0.81	2.82 ± 0.43	2.84 ± 0.53	12.94 ± 1.75	42.7
	Total hydroxyphenylpropionic and hydroxycinnamic acids	**2.28 ± 0.60**	**2.12 ± 0.36**	**3.71 ± 0.58**	**4.01 ± 0.72**	**8.46 ± 0.93**	**9.83 ± 1.16**	**8.66 ± 1.13**	**38.73 ± 1.77**	**14.5**
11	(Epi)catechin glucuronide-sulphate	traces	0.06 ± 0.02	0.18 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	traces	traces	0.37 ± 0.08	66.4
20	(Epi)catechin-glucuronide	traces	0.94 ± 0.13	0.54 ± 0.07	0.16 ± 0.03	0.06 ± 0.01	traces	traces	1.74 ± 0.15	28.0
27	(Epi)catechin-sulphate, isomer 1	traces	1.13 ± 0.60	1.07 ± 0.27	0.29 ± 0.17	traces	traces	traces	2.63 ± 0.73	88.0
29	(Epi)catechin-sulphate, isomer 2	traces	0.98 ± 0.37	0.97 ± 0.20	0.33 ± 0.11	0.06 ± 0.03	traces	traces	2.44 ± 0.49	62.9
32	Methyl(epi)catechin-sulphate, isomer 1	traces	0.15 ± 0.06	0.13 ± 0.02	0.06 ± 0.02	traces	traces	traces	0.38 ± 0.07	59.1
34	Methyl(epi)catechin-sulphate, isomer 2	traces	0.78 ± 0.24	0.89 ± 0.13	0.21 ± 0.06	0.06 ± 0.02	traces	traces	1.98 ± 0.32	51.2
	Total (epi)catechin derivatives	**traces**	**4.03 ± 1.32**	**3.78 ± 0.63**	**1.12 ± 0.36**	**0.28 ± 0.09**	**0.08 ± 0.03**	**traces**	**9.55 ± 1.67**	**55.4**
18	5-(phenyl)-γ-valerolactone-sulphate-glucuronide	traces	0.28 ± 0.11	3.09 ± 0.72	2.12 ± 0.43	1.52 ± 0.43	0.78 ± 0.28	0.18 ± 0.07	7.52 ± 1.13	47.5
31	5-Phenyl-valeric acid-sulphate-glucuronide	traces	traces	traces	traces	0.22 ± 0.03	0.07 ± 0.01	traces	0.39 ± 0.05	43.4
36	5-(phenyl)-γ-valerolactone-sulphate-methoxy	traces	traces	0.08 ± 0.03	traces	0.05 ± 0.01	traces	traces	0.24 ± 0.03	44.1
17	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide	traces	1.15 ± 0.66	12.33 ± 2.93	5.24 ± 1.08	4.36 ± 1.73	0.76 ± 0.31	traces	22.91 ± 3.51	48.4
19	5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	traces	5.18 ± 2.70	53.57 ± 12.84	25.71 ± 5.10	16.89 ± 5.40	3.25 ± 1.22	0.18 ± 0.13	99.47 ± 15.35	48.8
22	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulphate	traces	traces	0.34 ± 0.16	0.62 ± 0.21	0.87 ± 0.24	1.51 ± 0.64	0.40 ± 0.16	3.62 ± 1.03	90.2
15	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	traces	traces	traces	0.15 ± 0.05	traces	0.06 ± 0.05	traces	0.29 ± 0.06	70.0
30	5-(Hydroxyphenyl)-γ-valerolactone-sulphate isomers	0.09 ± 0.04	19.37 ± 7.59	44.24 ± 17.47	46.68 ± 8.81	56.19 ± 13.93	30.39 ± 9.83	5.27 ± 2.16	205.05 ± 35.34	54.5
25	5-Phenyl-γ-valerolactone-3′-glucuronide	traces	0.09 ± 0.05	2.78 ± 1.84	2.31 ± 0.81	5.61 ± 2.30	2.56 ± 0.85	0.71 ± 0.43	14.28 ± 3.52	77.9
35	5-Phenyl-γ-valerolactone-3′-sulphate	traces	0.08 ± 0.03	1.78 ± 0.86	1.14 ± 0.30	3.26 ± 0.88	3.02 ± 1.18	1.22 ± 0.72	10.37 ± 2.20	67.1
21	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	traces	traces	0.26 ± 0.08	0.11 ± 0.02	0.15 ± 0.04	0.07 ± 0.03	traces	0.66 ± 0.09	41.5
	Total phenyl-γ-valerolactones and phenyl-valeric acids	**0.12 ± 0.05**	**26.23 ± 10.85**	**118.50 ± 28.56**	**84.14 ± 15.18**	**89.14 ± 22.09**	**42.50 ± 13.90**	**8.08 ± 3.59**	**364.80 ± 48.93**	**42.4**
	Total phenolic metabolites	**6.25 ± 1.66**	**52.27 ± 15.11**	**179.58 ± 31.75**	**132.86 ± 19.84**	**123.45 ± 25.68**	**96.37 ± 16.03**	**31.10 ± 5.41**	**615.13 ± 51.87**	**26.7**
16	Hippuric acid	56.16 ± 16.81	66.05 ± 16.86	94.70 ± 18.94	109.22 ± 17.77	198.28 ± 50.80	234.60 ± 31.34	191.28 ± 26.68	953.65 ± 143.22	47.5
5	4-Hydroxyhippuric acid	4.73 ± 1.71	7.96 ± 0.88	11.63 ± 1.54	9.66 ± 2.50	14.94 ± 0.93	18.65 ± 1.91	20.46 ± 3.03	88.38 ± 5.49	19.7

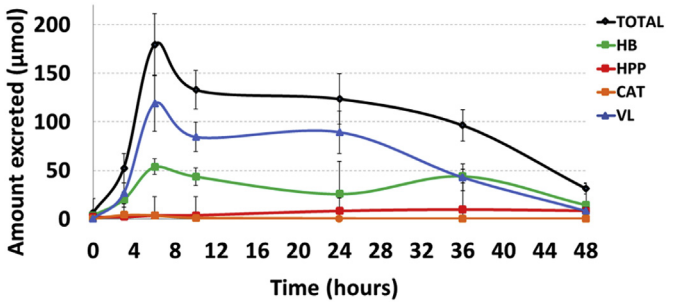

Fig. 1. Total 0–48 h excretion of phenolic metabolites after RGPD consumption: phenyl-γ-valerolactones (VL, including the phenyl-valeric acid-sulphate-glucuronide, dark cyan line), simple phenols and hydroxybenzoic acids (HB, green line), hydroxyphenylpropionic acids and hydroxycinnamic acids (HPP, red line), and (epi)catechins (CAT, orange line). The black line corresponds to the total phenolic metabolites (hippuric acid derivatives were not included). N = 10 except for the last 2 time points where n = 9 (subject #3 was excluded from these calculations because of a lack of compliance with the study protocol during the period 24–48 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

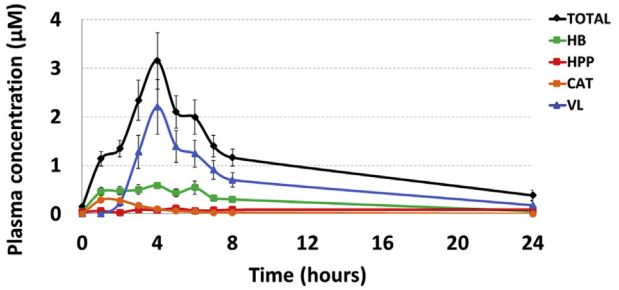

Fig. 2. Metabolites detected in plasma: phenyl-γ-valerolactones (VL, including the phenyl-valeric acid-sulphate-glucuronide, dark cyan line), simple phenols and hydroxybenzoic acids (HB, green line), hydroxyphenylpropionic acids and hydroxycinnamic acids (HPP, red line), and (epi)catechins (CAT, orange line). Black line corresponds to the total phenolic metabolites excluding 4-hydroxyhippuric acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hydroxyphenylpropionic acid-sulphate (**14**) were not detected in plasma. Ferulic acid-4-glucuronide (**12**) was the most abundant compound, followed by another phase II conjugate of ferulic acid, feruloylglycine (**24**). Most of these compounds had a t_{max} at 5–9 h (Table 3), although both ferulic acid-4-glucuronide (**12**) and ferulic acid-4-sulphate (**29**) presented a first peak at 1 h.

A total of 5 (epi)catechin derivatives were detected in plasma (Table 3). One of the two methyl(epi)catechin-sulphate isomers detected in urine was not detected in plasma. The C_{max} of (epi)catechin conjugates in plasma were around 100 nM, peaking during the first hours after RGPD intake.

Last, 4′-hydroxyhippuric acid (**5**) was found in all samples from all

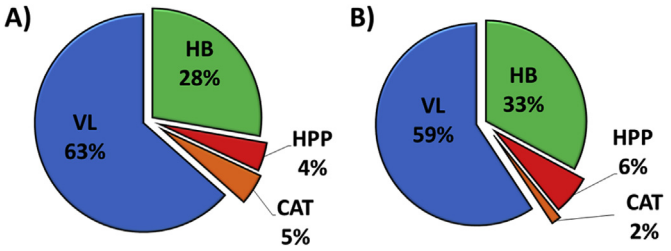

Fig. 3. A) Relative plasma AUC_{0-24} calculated for phenolic metabolites by class after RGPD consumption. B) 48 h-excretion in urine of phenolic metabolites. Phenyl-γ-valerolactones (VL, including phenyl-valeric acid-sulphate-glucuronide), simple phenols and hydroxybenzoic acids (HB), hydroxyphenylpropionic acids and hydroxycinnamic acids (HPP), and (epi)catechins (CAT). Hippuric acid derivatives were not included.

Table 3
Pharmacokinetic parameters of phenolic metabolites detected in plasma (mean ± SEM, n = 10).

Id.#	Phenolic compounds	Cmax (nM)	Tmax (h)	$t_{1/2}$ (h)	AUC_{0-24} (nmol h L^{-1})
Simple phenols and hydroxybenzoic acids					
1	Gallic acid	124.3 ± 31.9	3.8 ± 0.7	20.5 ± 9.4	607.2 ± 211.3
2	Vanillic acid-4-glucuronide	61.3 ± 9.0	5.7 ± 0.4	6.2 ± 1.4	410.9 ± 82.2
4	Protocatechuic acid	5.9 ± 1.2	4.1 ± 0.8	40.4 ± 13.7	40.9 ± 7.5
3	Protocatechuic acid-3-glucuronide	3.1 ± 0.6	3.0 ± 0.8	6.7 ± 2.3	20.5 ± 3.8
9	Protocatechuic acid-3-sulphate	408.5 ± 68.7	2.1 ± 0.3	3.8 ± 0.4	1088.6 ± 126.3
10	Benzoic acid-4-sulphate	56.7 ± 9.4	3.0 ± 0.7	37.2 ± 14.6	521.3 ± 76.3
13	Vanillic acid-4-sulphate	117.0 ± 30.2	4.0 ± 0.5	4.8 ± 0.6	381.9 ± 121.7
6	Catechol-sulphate	2.9 ± 1.0	11.2 ± 2.9	6.3 ± 1.2	20.8 ± 7.5
7	Methylpyrogallol-sulphate	512.4 ± 117.5	5.9 ± 0.5	3.6 ± 0.5	2724.2 ± 584.2
8	Methylcatechol-sulphate	47.1 ± 7.6	2.4 ± 0.5	3.3 ± 0.6	184.6 ± 23.6
5	4-Hydroxyhippuric acid	98.7 ± 15.1	4.6 ± 0.5	14.8 ± 4.2	874.0 ± 173.4
Hydroxyphenylpropionic and hydroxycinnamic acids					
12	Ferulic acid 4-glucuronide	72.8 ± 19.9	7.0 ± 2.0	5.7 ± 1.4	567.5 ± 208.1
24	Feruloylglycine	26.0 ± 3.9	9.3 ± 3.3	26.3 ± 4.9	175.1 ± 28.0
23	Dihydrocaffeic acid-sulphate	8.3 ± 1.5	7.0 ± 2.0	40.1 ± 17.0	50.8 ± 19.9
26	Dihydroferulic acid-sulphate	7.7 ± 1.3	8.5 ± 2.7	17.5 ± 6.8	56.4 ± 16.9
28	Ferulic acid-4-sulphate	10.0 ± 1.8	5.2 ± 0.6	17.9 ± 7.0	63.4 ± 16.2
(Epi)catechin derivatives					
11	(Epi)catechin-glucuronide-sulphate	6.8 ± 1.5	4.8 ± 0.6	0.9 ± 0.3	20.0 ± 4.5
20	(Epi)catechin-glucuronide	135.5 ± 14.2	1.7 ± 0.3	2.3 ± 0.4	459.9 ± 44.5
27	(Epi)catechin-sulphate, isomer 1	87.0 ± 22.8	1.6 ± 0.2	1.9 ± 0.4	166.2 ± 31.1
29	(Epi)catechin-sulphate, isomer 2	94.9 ± 22.8	2.5 ± 0.5	2.9 ± 0.5	290.2 ± 50.1
32	Methyl(epi)catechin-sulphate, isomer 1	12.6 ± 1.9	2.7 ± 0.8	8.1 ± 1.3	53.1 ± 10.2
Phenyl-γ-valerolactones and phenyl-valeric acids					
31	5-Phenyl-valeric acid-sulphate-glucuronide	11.0 ± 1.1	7.9 ± 2.0	36.0 ± 14.1	106.2 ± 29.1
17	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide	268.4 ± 68.2	5.3 ± 0.6	2.1 ± 0.5	1098.2 ± 219.2
19	5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	1171.2 ± 242.7	5.2 ± 0.6	12.4 ± 8.3	6224.8 ± 1175.4
30	5-(Hydroxyphenyl)-γ-valerolactone-sulphate isomers	893.7 ± 201.3	6.3 ± 2.0	4.8 ± 1.1	5196.3 ± 1457.9
25	5-Phenyl-γ-valerolactone-3′-glucuronide	88.4 ± 44.7	9.1 ± 2.5	11.0 ± 2.7	617.0 ± 178.2
35	5-Phenyl-γ-valerolactone-3′-sulphate	69.2 ± 25.2	11.0 ± 2.9	8.1 ± 1.9	441.0 ± 135.7
21	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	14.3 ± 3.2	7.0 ± 2.0	39.5 ± 26.4	66.1 ± 16.5

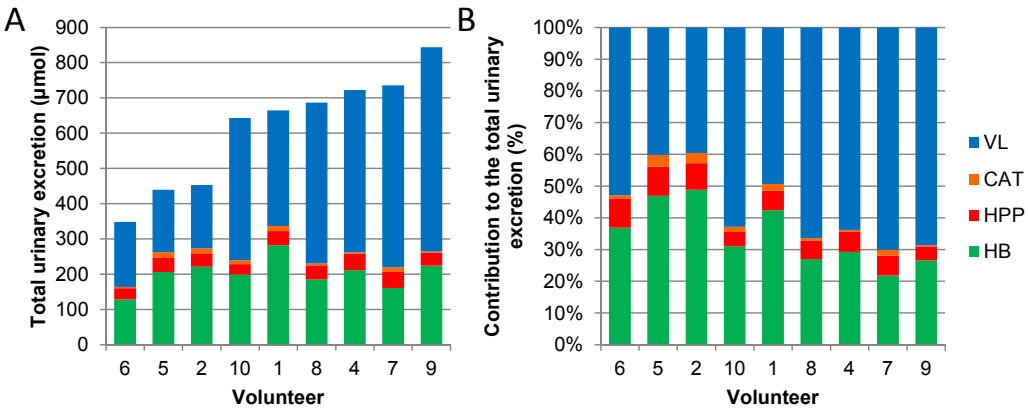

Fig. 4. A) Cumulative urinary excretion of phenolic metabolites at 48 h. B) Relative contribution of each class of phenolic compounds to the total urinary excretion at 48 h. Phenyl-γ-valerolactones (VL, including phenyl-valeric acid-sulphate-glucuronide), simple phenols and hydroxybenzoic acids (HB), hydroxyphenylpropionic acids and hydroxycinnamic acids (HPP), and (epi)catechins (CAT). Hippuric acid derivatives were not included. Subject #3 was excluded from these calculations because of a lack of compliance with the study protocol during the period 24–48 h.

volunteers (Table 3). Although some subjects showed a clear pharmacokinetic profile, other subjects did not shown a trend associated with the intake and metabolism of phenolic compounds.

The relative contribution of the individual classes of phenolic metabolites detected in plasma to the pool of circulating metabolites is shown in Fig. 3A and it was quite similar to that registered for urine (Fig. 3B).

3.4. Inter-individual variability in the production and excretion of phenolic metabolites

A high inter-individual variability was observed in the urinary excretion of phenolic metabolites, as indicated by the high coefficients of variation (CV) registered (Table 2). The CV% for each metabolite ranged from 20 to 167%, with a mean CV of 52%. Total phenolic compounds urinary excretion varied between 348 and 844 µmol (Fig. 4A), the CV being 27% (Table 2). This inter-individual variability in the urinary excretion was also observed among classes of phenolic compounds (Fig. 4A), and entailed notable differences in the qualitative urinary profile of each volunteer, mainly linked to variations in phenyl-γ-valerolactones, hydroxybenzoic acids and simple phenols. (Fig. 4B).

The profile of plasma circulating metabolites after RGPD intake also showed an important inter-subject variability. An unsupervised multivariate analysis (PCA) of the plasmatic concentrations for each metabolite and individual sample revealed the existence of different patterns among volunteers (Fig. 5). Three principal components (PCs) explained up to 44.3% of the total variance (Fig. 5A and B). The first PC (PC1) accounted for the 20.1% of the total variability and it was positively loaded mainly by metabolites peaking in plasma at 4–7 h, such as phenyl-γ-valerolactones (**17**, **19**, **21**, **25**, **30**, and **35**) and vanillic acid derivatives (**2** and **13**). PC2, representing the 14.9% of the variance, was positively linked to compounds achieving maximum plasma concentrations during the first hours after RGPD intake, like (epi)catechin conjugates (**20**, **27**, **29**, and **32**), protocatechuic acid-3-sulphate (**9**), and methylcatechol-sulphate (**8**). PC3 (9.3% of total variance) had positive loadings for 4-hydroxyhippuric acid (**5**), dihydrocaffeic acid-sulphate (**23**) and dihydroferulic acid-sulphate (**26**), and was correlated negatively for catechol-sulphate (**6**).

Plasma sample scores for each PC confirmed the pharmacokinetic profiles previously described, with the samples at collection times 1 and 2 h presenting positive PC2 values and the samples at time points 3–7 h having positive PC1 scores (Fig. 5C). This accounted for the increases in the production of epicatechin derivatives during the first hours after RGPD intake and the subsequent production of phenyl-γ-valerolactones and vanillic acid derivatives. Interestingly, the shift of the different time points for each subject on the plot, and the area covered by the surface delimited by these points, was quite different among subjects. For instance, while volunteer #05 showed PC1 scores close to 0 and maximum PC2 scores about 1, volunteer #10 showed PC1 scores close to 6 and PC2 scores by 4 (Fig. 5C and Supplementary Fig. 1A). This fact demonstrates, through a comprehensive approach, the inter-individual variability existing on the pharmacokinetic profile of phenolic metabolites from RGPD. Lastly, scores for PC3 (Fig. 5D) also indicated inter-subject differences in the pharmacokinetic profiles. In this sense, volunteers #04, #05, and #10 had negative scores for PC3 at times 1–8 h, while #06 and #09 showed positive scores (Fig. 5D and Supplementary Fig. 1B). This may account for individual differences in the production of catechol-sulphate and dihydrocaffeic/dihydroferulic acid sulphates.

4. Discussion

Grape pomace, the major byproduct of the wine and juice industry, is a relevant source of bioactive phenolic compounds [7,24–27]. However, the human bioavailability of its phenolic compounds is relatively unknown, precluding an adequate understanding of the phenolic metabolites behind the putative protective effects of grape pomace against several chronic diseases [7,24–28]. Although the work conducted by Sasot et al. [28] provided great insights in the metabolism of grape pomace phenolics, the quantification of the identified phenolic metabolites and the assessment of the inter-individual variability in their production were not carried out. In the present work, most of the phenolic metabolites formed *in vivo* after the consumption of a phenolic-rich RGPD were quantified using reliable analytical standards, a key step to further carry out proper *in vitro* studies on grape pomace bioactives [20]. This is of particular interest for the unambiguous identification and absolute quantification of phenyl-γ-valerolactones, for which reference standards have only recently become available [20,21].

Data showed that phenolic metabolites increased in plasma up to three different times, at ~2 h, 4 h and 6 h after RGPD intake, with different classes of phenolic metabolites peaking at different time points (Fig. 2, Table 3). In urine, maximum phenolic concentration was found at the collection interval 3–6 h (Table 1), which was in line with the appearance of metabolites in plasma samples. Moreover, most of the phenolic compounds underwent an intense phase II metabolism, likely at the intestinal epithelium level and/or in the liver [18,20,29,30], being the sulphate-conjugates predominant over the glucuronides. These results are in agreement with previous reports assessing the bioavailability of different classes of phenolic compounds consumed simultaneously, like it happens for grape products [5,28,31,32].

Peak plasma concentrations of several hydroxybenzoic acid derivatives occurred approximately at 2- and 4 h after the RGPD supplementation, suggesting absorption in the first part of the gastrointestinal tract and in the small intestine, respectively. Only catechol sulphate peaked later, which may suggest that it was formed in the large intestine after an intense microbial metabolism of other phenolic scaffolds [1,2]. The biphasic pharmacokinetic profile of some compounds such as gallic and protocatechuic acid derivatives may occur as result of the

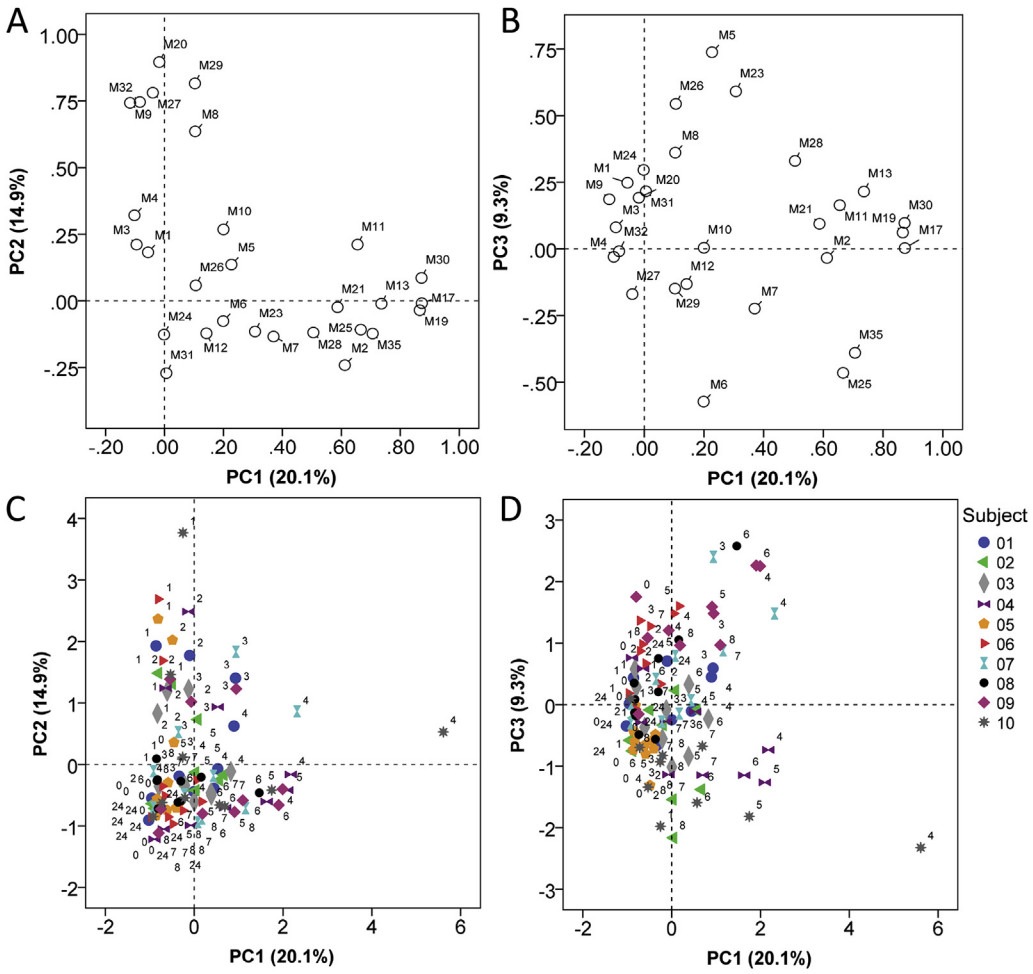

Fig. 5. Principal component analysis highlighting inter-individual differences associated with the pharmacokinetic profile of phenolic metabolites. A) Loading plots of PC1 versus PC2 and B) PC1 versus PC3; C) score plots of the plasma circulating phenolic metabolites for all the volunteers at all the time points (0–24 h) obtained from PC1 and PC2, and D) from PC1 and PC3. Mn indicates the metabolite number, as reported in Table 3. In the score plots, the number accounts for the collection time (0, 1, 2, 3, 4, 5, 6, 7, 8, and 24). Details on individual trends are highlighted in Supplementary Fig. 1.

metabolism of different phenolic structures at multiple tissue level, including the upper and lower parts of the gastrointestinal tract [1,2]. A recent study on the human bioavailability and metabolism of phenolic extracts prepared from grape pomace has underlined that both gallic and protocatechuic acids can be generated from the cleavage of various anthocyanins [30], from the metabolism of syringic acid [33], and also from the gallic acid already present in the fed matrix [34,35]. Similarly, the presence in plasma of ferulic acid, dihydroferulic acid, and dihydrocaffeic acid conjugates followed kinetics in line with what previously reported [5,36].

Grape pomace has been reported to be an important source of bioavailable flavan-3-ol monomers [24]. In this study, the absorption profile of (epi)catechin conjugates showed maximum plasma concentrations around 1–2 h, with $t_{1/2}$ of ~1–3 h, in accordance with previous reports [5,32,36]. With the exception of a methyl(epi)catechin sulphate isomer, (epi)catechin derivatives were rapidly cleared from plasma, just a few hours after RGPD intake, being eliminated in urine during the first 6 h. Moreover, these metabolites only accounted for the 2% of the total phenolic compounds excreted in urine. On the contrary, their main ring fission metabolites of microbial origin, phenyl-γ-valerolactones and phenyl-valeric acids, had longer $t_{1/2}$ and resulted to be the most representative compounds among the phenolic metabolites identified in plasma and urine samples. Phenyl-γ-valerolactones peaked after 4–10 h from the RGPD intake, in good agreement with a recent study, by Bresciani et al., where three phenyl-γ-valerolactones [5-(phenyl)-valerolactone-3′-sulphate, 5-(4′-hydroxyphenyl)-valerolactone-3′-sulphate and 5-(4′-hydroxyphenyl)-valerolactone-3′-glucuronide] were shown peaking between 5 and 10 h [5]. Moreover, a previous work on the metabolism of green tea flavan-3-ols in humans reported high concentrations of phenyl-γ-valerolactones in plasma 12 h after tea ingestion [29,37]. This late appearance of phenyl-γ-valerolactones as a result of flavan-3-ol degradation by the gut microbiota has also been observed in other studies on different food matrices [38–41] or after the intake of radiolabelled epicatechin [18]. Regarding the urinary excretion of phenyl-γ-valerolactones, this went on up to 36 h, in line with a sustained production and absorption of these metabolites (and/or enterohepatic recirculation) before their complete elimination in urine [1,42]. Thus, opposite to the other phenolic metabolites discussed above, phenyl-γ-valerolactones exhibit high concentrations for prolonged periods of time after consumption of plant foods rich in flavan-3-ols. This aspect, also highlighted previously [14,18], points at these ring fission colonic metabolites as good candidates to further explore the health benefits attributed to the consumption of catechin and proanthocyanidin food sources [3,43].

The high inter-individual variability in the absorption and excretion of phenolic derivatives observed in plasma and urine samples is a key point to be considered, as highlighted in other studies on phenolic compounds bioavailability [11,19,35,44]. It is now more than clear that the gut microbiota plays an important role in the inter-individual variability existing in the biochemical transformations of several phenolic compounds. For instance, a recent study on the *in vitro*

gastrointestinal digestion of grape pomace extracts has demonstrated that certain phenolic extracts can promote the growth of specific components of the intestinal microbiota and shift bacteria proportions [45]. Nevertheless, variability due to phase II metabolism, therefore linked to human enzyme expression and activity, should not be ruled out, since a part of the variability observed in the present study was associated with plasma levels of (epi)catechin derivatives.

5. Conclusions

This work on the absorption, metabolism and excretion of red grape pomace phenolic compounds in humans highlighted a very high number of phenolic metabolites appearing in circulation and following different pharmacokinetic profiles after the intake of RGPD. Some of these compounds were excreted in urine up to 48 h after intake. Phenyl-γ-valerolactones were the most relevant and abundant circulating metabolites, followed by some hydroxybenzoic acids and by simple phenols, (epi)catechin conjugates and hydroxyphenylpropionic acids. A high inter-individual variability was registered, and different profiles of plasma circulating metabolites were unravelled. These results highlighted differences not only in the production of gut microbial-derived metabolites, but involved also the phase II metabolic step, which is purely mammalian. Regardless of the individual differences, it can easily be concluded that the intake of a drink prepared from red grape pomace can deliver significant amounts of different phenolic metabolites to the human system. Future investigations should focus both on the causes behind the observed inter-individual variability and how these differences may impact on the putative health properties of this drink.

Author contributions

Study conception and design: RG, DN, DL, and DDR.

Screening of subjects and implementation of the clinical intervention: PC, MV, and CV.

Acquisition of data: GC, LB, MT, and PM.

Analysis and interpretation of data: FC and PM.

Drafting of manuscript: FC and PM.

Critical revision: LB, GG, RG, FB, and DDR.

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Conflicts of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2018.03.021.

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