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**Inter-individual variability in the production of flavan-3-ol colonic metabolites:
preliminary elucidation of urinary metabotypes**

Pedro Mena^{1,^}, Iziar A. Ludwig^{1,2,^}, Virginia B. Tomatis^{3,4,^}, Animesh Acharjee^{3,4,5,6}, Luca Calani¹,
Alice Rosi¹, Furio Brighenti¹, Sumantra Ray³, Julian L. Griffin^{3,4}, Les J. Bluck^{3,‡,†} and Daniele Del
Rio^{1,‡}

[^]*Equal contributors*

[‡]*Equal contributors*

¹ Human Nutrition Unit, Department of Food & Drugs, University of Parma, Parma, Italy.

² Food Technology Department, Universitat de Lleida-Agrotecnio Center, Lleida, Spain.

³ UK Medical Research Council Elsie Widdowson Laboratory (formerly MRC Human Nutrition
Research), Cambridge, UK.

⁴ Department of Biochemistry, University of Cambridge, Cambridge, UK.

⁵ Institute of Cancer and Genomic Sciences, Centre for Computational Biology, University of
Birmingham, Birmingham, UK.

⁶ Institute of Translational Medicine, University Hospitals Birmingham NHS Foundation Trust,
Birmingham, UK.

[†] *LB is deceased.*

Address for correspondence: Daniele Del Rio & Pedro Mena, Human Nutrition Unit, Department of
Food & Drugs, University of Parma, Medical School Building C, Via Volturno, 39, 43125 Parma,
Italy. Phone: (+39) 0521-903841. E-mail: daniele.delrio@unipr.it;
pedromiguel.menaparreno@unipr.it.

ORCID: PM 0000-0003-2150-2977; IAL 0000-0001-5506-3293; AA 0000-0003-2735-7010; LC
0000-0002-3516-8976; AR 0000-0001-8533-797X; FB 0000-0001-8441-4611; SR 0000-0002-1411-
0003; JLG 0000-0003-1336-7744; LJB 0000-0001-6776-4281; DDR 0000-0001-5394-1259.

Abstract

Purpose: There is much information on the bioavailability of (poly)phenolic compounds following acute intake of various foods. However, there are only limited data on the effects of repeated and combined exposure to specific (poly)phenol food sources and the inter-individual variability in their bioavailability. This study evaluated the combined urinary excretion of (poly)phenols from green tea and coffee following daily consumption by healthy subjects in free-living conditions. The inter-individual variability in the production of phenolic metabolites was also investigated.

Methods: Eleven participants consumed both tablets of green tea and green coffee bean extracts daily for 8 weeks and 24-h urine was collected on five different occasions. The urinary profile of phenolic metabolites and a set of multivariate statistical tests were used to investigate the putative existence of characteristic metabotypes in the production of flavan-3-ol microbial metabolites.

Results: (Poly)phenolic compounds in the green tea and green coffee bean extracts were absorbed and excreted after simultaneous consumption, with green tea resulting in more inter-individual variability in urinary excretion of phenolic metabolites. Three metabotypes in the production of flavan-3-ol microbial metabolites were tentatively defined, characterized by the excretion of different amounts of trihydroxyphenyl- γ -valerolactones, dihydroxyphenyl- γ -valerolactones, and hydroxyphenylpropionic acids.

Conclusions: The selective production of microbiota-derived metabolites from flavan-3-ols and the putative existence of characteristic metabotypes in their production represent an important development in the study of the bioavailability of plant bioactives. These observations will contribute to better understand the health effects and individual differences associated with consumption of flavan-3-ols, arguably the main class of flavonoids in the human diet.

Keywords: polyphenols; green tea catechins; coffee caffeoylquinic acids; colonic microbiota; urinary phenotype; metabotypes.

Introduction

Epidemiological and human intervention studies report the **potentially beneficial** effects of (poly)phenolic-rich plant foods against several chronic conditions, including cardiometabolic diseases, neurodegeneration, and certain kinds of cancer [1,2]. Supplementation with (poly)phenolic compounds may represent an effective means of providing potential bioactive compounds to consumers, as part of a strategy to enhance the health benefits attributed to plant-based food products [3]. To exert a systemic biological action, (poly)phenolic compounds must be bioavailable, and this may vary substantially with different plant foods and dietary supplements [4,5]. Following ingestion, most phenolics undergo modifications during passage through the gastrointestinal tract. They are extensively modified by the action of the gut microbiota, being converted to colonic catabolites that are absorbed into the blood stream where they circulate principally as conjugated phase II metabolites [1,2]. Green tea and coffee are, respectively, major sources of bioactive flavan-3-ols, and chlorogenic acids (CGAs) which occur principally as caffeoylquinic acids (CQAs). Both flavan-3-ols and CQAs are bioavailable after acute intake of green tea and coffee [6-8]. However, there is a paucity of information on their bioavailability after combined and prolonged ingestion of green tea and coffee, circumstances which reflect their normal consumption by the general public [9].

Inter-individual variability in the bioavailability of (poly)phenolic compounds may have an impact on their putative health effects [10]. Between-subject variability in gut microbial composition can lead to the selective production of specific metabolites. This has been demonstrated with equol and 8-prenylnaringenin [11,12], where metabolism by the colonic microflora potentially determines the benefits associated to the consumption of the parent compounds [13]. Moreover, phenotypical differences in the production and excretion of colonic microbial metabolites are not restricted to a single compound, as they may also involve a set of catabolites originating from the same parent compound, as in the case of urolithins, ellagitannin-derived microbial metabolites. Subjects can be classified into three urolithin phenotypes, or metabotypes, according to the qualitative and quantitative proportions of different urolithins excreted after consumption of ellagic acid or ellagitannins [14]. Interestingly, the benefits associated with the ingestion of pomegranate and walnuts, sources of ellagitannins, may be related to each specific metabotype [15,16,14]. So far, although a high inter-

individual variability in the production of metabolites derived from coffee CGAs and green tea flavan-3-ols has been reported [17,8,18,19], no phenotypes in their production have been identified. In this sense, 5-(phenyl)- γ -valerolactones, unique ring-fission microbial products of flavan-3-ol monomers and oligomeric proanthocyanidins, might be selectively produced on the basis of different enterotypes or microbiota profiles.

The first aim of this study was to evaluate the urinary excretion of the (poly)phenolic compounds of green tea (GTE) and green coffee beans (GCE) following their daily, simultaneous consumption as tablets by subjects in free-living conditions. The inter-individual variability observed in the urinary excretion of phenolic metabolites was assessed by multivariate statistical tests to investigate the existence of metabotypes in the production of flavan-3-ol metabolites.

Subjects and methods

Participants

Thirteen healthy participants, aged 18-45 years, with a body mass index (BMI) of 18.5 – 24.9 kg/m² were enrolled. Exclusion criteria included smoking; chronic/systemic illnesses and/or major surgical operations of renal, hepatic or gastrointestinal origin; significant history or presence of cancer, metabolic, cardiovascular, endocrine and/or inflammatory disease; anemia or other blood disorders; anxiety, depression, psychological problems; eating disorders. Participants were also excluded if they were pregnant, lactating or contemplating pregnancy. A total of 11 participants completed the study. The flow of participants throughout the study is presented in Online Resource Fig. 1 under “Supplementary Material” in the online issue. Sample size was estimated from previous studies on the urinary excretion of phenolic compounds from green tea and coffee (6-8).

Study design and protocol

This was a 12-week, longitudinal experimental study. Healthy participants completed five 24-h urine collections, at weeks 0, 2, 4, 8, and 12 (visits 1, 2, 3, 4, and 5, respectively). After the first urine collection (week 0), participants took six GTE and three GCE tablets daily for a period of 8 weeks. No tablets were taken during the last 4 weeks of the study. The study was approved by the Norfolk

Research Ethics Committee (REC) (Ref: 13/EE/0028) in April 2013. Participants gave their informed written consent before enrollment. A scheme of study protocol is presented in Online Resource Fig. 2.

During the run-in period, participants were asked to maintain their usual dietary habits throughout the study and to avoid consumption of dietary supplements, except for the GTE and GCE tablets provided during the course of the study. Participants kept a daily record of tablet intake. Study visits were conducted upon completion of each 24-h urine collection. Participants returned the collected urine and were assessed for adherence to the urine collection protocol and dietary intake requirements, and adherence to the tablet intake (by using an *ad hoc* questionnaire and by tablet count) during the supplementation period. Blood pressure measurements were carried out at the first visit by automated sphygmomanometer. During visits 1, 3, 4 and 5, participants completed a (poly)phenol questionnaire (PPQ), developed in-house by referring to Phenol-Explorer (Release 3.0) and the United States Department of Agriculture (USDA) database for the Flavonoid Content of Selected Foods (USDA-FD) (Release 3.1), to estimate their intake of flavan-3-ols and CGAs during the course of the study. Participants stopped taking the (poly)phenol tablets after week 8 and completed the last study visit on week 12. The volume of each 24-h urine collection was measured, and five 2 mL aliquots were stored at -80 °C prior to analysis.

(Poly)phenol tablets and chemicals

GTE tablets were purchased from Healthspan (Saint Peter Port, UK) and Verdesse® GCE tablets were purchased from Nature's Best (Kent, UK). Other ingredients included in the GTE tablets as fillers/coating were maltodextrin, cellulose, croscarmellose sodium, stearic acid, silica, magnesium stearate, hypromellose and glycerin. GCE tablets included calcium carbonate, cellulose, silicon dioxide, stearic acid, magnesium stearate, hydroxypropyl methylcellulose, glycerine, and colors (titanium dioxide, curcumin, copper chlorophyllin, and iron oxide). Both supplements were decaffeinated and were produced to pharmaceutical standards under Good Manufacturing Practice (GMP).

3-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-(3'-hydroxyphenyl)propionic acid, hippuric acid, 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, (+)-catechin, and (–)epigallocatechin-

3-*O*-gallate were purchased from Sigma-Aldrich (St. Louis, MO, USA), while procyanidin B₂ was supplied by Phytolab (Vestenbergsgreuth, Germany). Ferulic acid-4'-sulfate, isoferulic acid-3'-*O*-glucuronide, dihydroferulic acid-4'-sulfate, caffeic acid-4'-*O*-glucuronide, dihydroisoferulic acid-3'-*O*-glucuronide, and dihydrocaffeic acid-3'-sulfate were obtained from Toronto Research Chemical (Toronto, Canada), while 4'-hydroxyhippuric acid was purchased from Bachem Ltd. (St Helens, UK). 5-(4'-hydroxyphenyl)- γ -valerolactone, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-(phenyl)- γ -valerolactone-3'-sulfate, and 5-(phenyl)- γ -valerolactone-3'-*O*-glucuronide were prepared in house using the strategies of Curti et al. [20] for aglycones and Brindani et al. [19] for phase II conjugates. They are catalogued on the standards sharing platform FoodComEx (www.foodcomex.org). Quercetin-3'-sulfate and quercetin-3'-*O*-glucuronide were kindly provided by Denis Barron (Nestle Research Center, Lausanne, Switzerland) and Gary Williamson (School of Food Science and Nutrition, University of Leeds, UK), while feruloylglycine was a gift from Takao Yokota (Teikyo University, Japan). All solvents and reagents were purchased from Sigma-Aldrich, unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Tablets and urine sample preparation for UHPLC-MSⁿ analyses

GTE and GCE tablets were analyzed to determine their (poly)phenolic composition. Three randomly selected tablets of each type were weighted, pulverized and 100 mg aliquots extracted with 1 mL of methanol/water/formic acid (74.5:24.5:1, v/v/v). The samples were vortexed, ultrasonicated for 90 min, and centrifuged at 5 °C for 10 min at 4000 g. The supernatants were collected and the pellets re-extracted with 0.5 mL of methanol/water/formic acid (74.5:24.5:1, v/v/v). The samples were vortexed and ultrasonicated for 25 min, and centrifuged at 5 °C for 10 min at 4000 g. The two supernatants were pooled, diluted 1:5 with 0.1% aqueous formic acid prior to ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MSⁿ) analysis for phenolic characterization and quantification.

Urine samples were defrosted, vortexed, centrifuged at 16110 *g* for 10 min at 5 °C, diluted 1:2 with 0.1% aqueous formic, and passed through 0.45 µm nylon filter discs prior to the analysis of 5 µL aliquots by UHPLC-MSⁿ.

Qualitative and quantitative analysis of (poly)phenols in tablets and urine samples by UHPLC-MSⁿ

(Poly)phenolic compounds were analyzed using an Accela UHPLC 1250 with a LTQ XL linear ion trap-mass spectrometer fitted with a heated-electrospray ionization (ESI) probe (Thermo Scientific Inc., San Jose, CA, USA). Separation was performed with a XSELECT HSS T3 (50 × 2.1 mm), 2.5 µm particle size column (Waters, Milford, MA, USA). The volume injected was 5 µL and the column oven was set to 30°C. Elution was carried out at a flow rate of 0.3 mL/min. The gradient started with 2% acetonitrile in 0.1% aqueous formic acid and, after 0.5 min, an 8.5-min linear gradient of 2% to 45% acetonitrile was applied. From 9 to 9.5 min the acidified acetonitrile increased to 80%, and after 3 min was reduced to 2% acetonitrile to re-equilibrate the column for 3 min at start conditions. The MS was operated in negative ionization mode with a capillary temperature of 275 °C and a source temperature of 250 °C. The sheath gas flow was 40 units and the auxiliary and sweep gas were set to 5 units. The source voltage was 3 kV. The capillary and tube lens voltage were -9 and -53 V, respectively. Analyses were carried out using full scan, data-dependent MS³ scanning from *m/z* 100 to 1000, with collision induced dissociation (CID) of 35 (arbitrary units). Helium gas was used for CID. After this first step, further specific MS² and MS³ analyses were carried out to unambiguously identify and quantify the compounds revealed in the first step, by monitoring specific *m/z* transitions. Molecules were fragmented using pure helium (99.99%). CID settings were optimized for each compound in order to produce the highest fragment signals. Identification was performed by comparison with standards, when available, and literature. Data processing was performed using Xcalibur software (Thermo Scientific).

Quantification was performed with calibration curves of pure commercial standards, when available. Caffeic acid-sulfate and phenylpropionic acid-sulfate were quantified as dihydrocaffeic acid-3'-sulfate equivalents. Epicatechin-sulfate, *O*-methyl-(epi)catechin-sulfate, (epi)gallocatechin-

sulfate-*O*-glucuronide, and *O*-methyl-(epi)gallocatechin-sulfate-*O*-glucuronide were quantified as quercetin-3'-sulfate equivalents. (Epi)catechin-*O*-glucuronide, (epi)gallocatechin-*O*-glucuronide, *O*-methyl-(epi)catechin-*O*-glucuronide, and *O*-methyl-(epi)gallocatechin-*O*-glucuronide levels were estimated by reference to a quercetin-3'-*O*-glucuronide standard curve. 5-(*O*-Methyl-hydroxyphenyl)- γ -valerolactone-sulfate and 5-(hydroxyphenyl)- γ -valerolactone-sulfate were expressed as 5-(phenyl)- γ -valerolactone-3'-sulfate equivalents, while 5-(hydroxyphenyl)- γ -valerolactone-*O*-glucuronide, 5-(dihydroxyphenyl)- γ -valerolactone-*O*-glucuronide, and 5-(phenyl)- γ -valerolactone-sulfate-*O*-glucuronide were quantified as 5-(phenyl)- γ -valerolactone-3'-*O*-glucuronide equivalents.

Statistical analysis

Results are presented as mean values \pm standard deviation (SD) for (poly)phenols in GTE and GCE tablets and as mean values \pm standard error of the mean (SEM) for metabolites in urine samples. Log-transformed dietary flavan-3-ol and CGA intake data are presented as geometric mean (95% confidence interval (CI)). Tests for assessing data normality and homoscedastic were performed using the Kolmogorov–Smirnov and Levene's tests, respectively. Logarithmic transformation was used for non-normally distributed flavan-3-ol and CGA dietary intake data. For all the metabolites, one-way repeated measures analysis of variance (ANOVA) was performed to compare mean differences at the five defined time points. Post-hoc analysis was conducted using pairwise comparisons with Bonferroni correction. For the urinary excretion of each metabolite by groups of subjects, one-way ANOVA with post-hoc Dunnett's T3 test was used since variances in groups were not equal. Differences were considered significant at $p \leq 0.05$. All statistical analyses were performed using the STATA v.12.0 software package (Stata Corporation LP, College Station, TX, USA).

Correlation analysis was performed to assess relationships among phenolic metabolites. "R" version 3.3.1 (<https://www.r-project.org/>) was used, and the visualization was achieved using the `corrplot` package.

Multivariate principal component analysis (PCA) with varimax rotation was applied to explore the inter-individual variability observed for the urinary excretion of the phenolic metabolites, by using

SPSS statistics 21.0 software (IBM, Chicago, IL). Then, to better understand the causes behind the observed inter-individual variability in the excretion of flavan-3-ol metabolites, partial least squares discriminant analysis (PLS-DA) was carried out. PLS-DA is a linear projection method, and all metabolites are assumed to be combined in a linear manner to maximize discrimination. Different explorative PLS-DA models were built to define the number of clusters and the subjects in each cluster, by using only the information for GTE flavan-3-ol metabolites. The model that maximized the validation parameters was selected. Model validation was performed by different parameters considering the variation in the metabolite data, $R^2(X)$, variation in the dependent variable (class), $R^2(Y)$ and goodness of fit of the validation (Q^2) of the model [21], and by performing cross validation by random permutation. The selection of the most representative metabolites from the whole set of metabolites (variable selection) was performed by using the Variable Importance in Projection (VIP) scores, estimating the importance of each variable in the projection used in a PLS model [22]: variable with VIP scores greater than 1 were considered important in the given model, while variables with VIP scores less than 1 were less important and good candidates for exclusion from the model. PLS-DA analysis was performed in SIMCA (version 14, Umetrics, Umea, Sweden).

Results

Participant baseline characteristics and treatment adherence

Participants (9 women, 2 men) were aged 28 ± 6 years, had a healthy BMI ($21.8 \pm 1.6 \text{ kg/m}^2$) and normal blood pressure (systolic blood pressure (SBP): $110 \pm 6 \text{ mm Hg}$, diastolic blood pressure (DBP): $69 \pm 6 \text{ mm Hg}$). On average, 99% of the polyphenol doses were consumed, indicating good adherence to the supplementation for all the participants. Participants also adhered to their usual diets and to the supplement consumption restrictions.

Dietary assessment

When the dietary intake of flavan-3-ols and CGAs was assessed, no significant differences in the habitual intakes of CQAs nor (+)-catechin, (–)-epicatechin-3-*O*-gallate, (–)-epigallocatechin, or (–)-epigallocatechin-3-*O*-gallate were observed during the study (Online Resource, Supplementary

material Table 1). Only (–)-epicatechin intake was significantly higher at baseline than at the end of the post-washout period consumption ($p \leq 0.001$). In this sense, no major changes in the intake of phenolics through the diet during the whole intervention were noted. A high inter-individual variability in the intake of these phenolic compounds was observed among participants (Online Resource, Supplementary material, Table 1).

(Poly)phenolic composition of the green coffee extract and green tea extract tablets

The (poly)phenol composition of the GCE and GTE tablets is summarized in **Table 1** while a detailed characterization of the phenolic profile is presented at Supplementary material Table 2. All the compounds were identified according to their retention times and characteristic MS^2 and MS^3 spectra, reported in Supplementary material Tables 3 and 4.

The analysis of GCE tablets led to the identification of 30 compounds, all of them belonging to the phenolic subclass of CGAs. Major components were caffeoylquinic acid, feruloylquinic acid, and dicaffeoylquinic acid isomers, comprising 57.9%, 22.5%, and 10.4% of the total phenolic content, respectively (Table 1). The amount of phenolic compounds per GCE tablet was 311 μmol , which provided a daily supplementation of 933 μmol of CGAs.

In the case of the GTE tablets, a total of 31 polyphenols were identified (Supplementary material Table 4). The most represented class of flavonoids were flavan-3-ols, with 21 compounds, accounting for the 99.5% of the total phenolic content (Table 1). The predominant flavan-3-ols were monomers and, in particular, those presenting three hydroxyl groups in the B-ring (i.e., (epi)gallocatechin derivatives, 93.0% of the GTE phenolic content), followed by those possessing two hydroxyl groups (i.e., (epi)catechin derivatives, 5.8% of the GTE phenolic content). The major individual compounds were epigallocatechin, epigallocatechin-3-*O*-gallate, and gallocatechin, which made up 54.3%, 21.0%, and 17.4% of the total phenolic content, respectively. Six flavonols, representing only 0.5% of the total phenolic content of GTE tablets, were also identified (Table 1). The amount of (poly)phenols provided for each GTE tablet was 1.67 mmol, contributing with up to 10.02 mmol to the daily intake of phenolic compounds. Expressed as milligrams, the daily amounts of CGAs and flavan-3-ols

consumed by participants during the supplementation period were approximately 356 mg and 3469 mg, respectively.

Identification of urinary phenolic metabolites

UHPLC-MSⁿ analysis of urine samples allowed the identification and quantification of a total of 32 compounds. Retention time and mass spectral data of each metabolite are reported in Supplementary material Table 5. In absence of available standards for some phase II metabolites detected in urine, the criteria of identification were based on previously reported HPLC-MS analyses [18,7]. Phase II metabolites produced by the action of UDP-glucuronosyltransferases and sulfotransferases were identified through the loss of the conjugation group (m/z 176 for glucuronides and m/z 80 for sulfates) to produce the aglycone fragment ion at MS², as previously described [18,7]. Where necessary, further MS fragmentation of the aglycone was applied to confirm the identification of the metabolites through their characteristic MS³ fragment ions (Supplementary material Table 5). From the 32 identified compounds, three were glycine conjugates (hippuric acid [metabolite **1**], 4'-hydroxyhippuric acid [**22**], and feruloylglycine [**10**]), two benzoic acid derivatives (3-hydroxy- and 3,4-dihydroxybenzoic acid, [**2** and **23**, respectively]), five free and conjugated phenylpropionic acids (dihydroisoferulic acid-3'-*O*-glucuronide [**5**], dihydrocaffeic acid-3'-sulfate [**6**], dihydroferulic acid-4'-sulfate [**8**], 3-(3'-hydroxyphenyl)propionic acid [**9**], 3-(phenyl)propionic acid-sulfate [**13**]), and four hydroxycinnamic acid sulfates and glucuronides (caffeic acid-4'-*O*-glucuronide [**3**], isoferulic acid-3'-*O*-glucuronide [**7**], ferulic acid-4'-sulfate [**11**], and caffeic acid-sulfate [**12**]). However, the large majority of metabolites found in urine were methyl, sulfate, and glucuronide conjugates of (epi)catechins, and phenyl- γ -valerolactones which comprised a total of nine phenyl- γ -valerolactones (three monohydroxyphenyl- γ -valerolactones, 5-(4'-hydroxyphenyl)- γ -valerolactone [**32**], 5-(phenyl)- γ -valerolactone-3'-*O*-glucuronide [**28**], and 5-(phenyl)- γ -valerolactone-3'-sulfate [**31**]; four dihydroxyphenyl- γ -valerolactones, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone [**29**], 5-(hydroxyphenyl)- γ -valerolactone-*O*-glucuronide [**24**], 5-(hydroxyphenyl)- γ -valerolactone-sulfate [**30**], and 5-(phenyl)- γ -valerolactone-sulfate-*O*-glucuronide [**25**]; and two trihydroxyphenyl- γ -

valerolactones, 5-(dihydroxyphenyl)- γ -valerolactone-*O*-glucuronide [27], and *O*-methyl-(hydroxyphenyl)- γ -valerolactone-sulfate [26]) and nine (epi)catechins (catechin [4], (epi)catechin-*O*-glucuronide [14], (epi)catechin-sulfate [15], (epi)catechin-sulfate-*O*-glucuronide [16], (epi)gallocatechin-*O*-glucuronide [17], *O*-methyl-(epi)catechin-*O*-glucuronide [18], *O*-methyl-(epi)gallocatechin-sulfate-*O*-glucuronide [19], *O*-methyl-(epi)catechin-sulfate [20], and *O*-methyl-(epi)gallocatechin-*O*-glucuronide [21]).

Effect of tablet supplementation on the urinary excretion of phenolic metabolites

From the 32 metabolites detected and quantified in urine, 19 compounds showed statistically significant differences between the visit before the supplementation period (week 0) and during the supplementation (weeks 2, 4, and 8) ($p < 0.05$ for metabolites 4, 9, 10, 13, 15, 16, 18-20 and 23-32). The urinary excretion of compounds 1-3, 5-8, 11, 12, 14, 17, 21, and 22 did not change statistically as a result of the supplementation with the GTE and GCE tablets. The main urinary metabolites and the observed treatment effects are reported in Fig. 1, while numeric data on metabolite excretion at visit 3 are presented in Supplementary material Table 5. A high inter-individual variability was found for most of the metabolites of colonic origin.

Up to six (epi)catechin derivatives increased significantly during the supplementation, as well as all the phenyl- γ -valerolactone derivatives. Some phenolic acids such as 3-(3'-hydroxyphenyl)propionic acid [9], phenylpropionic acid-sulfate [13], 3,4-dihydroxybenzoic acid [23], and feruloylglycine [10] were also excreted in significantly higher amounts after the supplementation with GTE and GCE tablets (Fig. 1). Interestingly, not all the 19 compounds increased significantly during the early part of the supplementation period (visit 2, week 2). The excreted amounts of 5-(*O*-methyl-hydroxyphenyl)- γ -valerolactone-sulfate [26], 5-(phenyl)- γ -valerolactone-3'-sulfate [31], and 3-(3'-hydroxyphenyl)propionic acid [9] increased significantly after 4 weeks of treatment (visit 3), while 5-(4'-hydroxyphenyl)- γ -valerolactone [32], 3-(phenyl)propionic acid-sulfate [13], and feruloylglycine [10] increased significantly only after the whole supplementation period of 8

weeks (visit 4) (Fig. 1). However, after the washout period of 4 weeks (visit 5), all the compounds decreased to the levels observed before the supplementation, at visit 1 (week 0) (Fig. 1).

Lack of good correlation among some metabolites belonging to the same transformation pathway

Correlation analysis showed a strong relationship among most of the phenolic metabolites belonging to the same transformation pathway (**Fig. 2**). For instance, all the (dihydro)caffeic acid and (dihydro)ferulic acid derivatives associated with the degradation pathways of GCE and CGA [6] were well and positively correlated ($p < 0.05$, Fig. 2, compounds **3**, **5-8**, and **10-12**). Similarly, most of the (epi)(gallo)catechin conjugates and 3-(hydroxyphenyl)propionic acids derived from the metabolism of GTE flavan-3-ols [18,7] were also positively correlated (Fig. 2, compounds **4**, **9**, **13**, **15**, **16**, and **18-20**). (Phenyl)- γ -valerolactone derivatives (compounds **24-32**) were positively correlated to (epi)(gallo)catechin conjugates (compounds **4**, **15**, **16**, and **18-20**) but, surprisingly, they did not show good correlations among each other (Fig. 2). Specifically, while a good correlation (r between 0.56 and 0.78) was found between dihydroxyphenyl- γ -valerolactones (**24**, **25**, **29**, and **30**) and trihydroxyphenyl- γ -valerolactones (**26** and **27**), monohydroxyphenyl- γ -valerolactones (**28**, **31**, and **32**) correlated inversely (r between -0.11 and -0.33) with trihydroxyphenyl- γ -valerolactones (**26** and **27**) and positively, but weakly (r between 0.11 and 0.56), with dihydroxyphenyl- γ -valerolactones (**24**, **25**, **29**, and **30**). It should be also mentioned that only monohydroxyphenyl- γ -valerolactones (**28**, **31**, and **32**) correlated positively, but weakly, with 3-(hydroxyphenyl)propionic acids (compounds **9** and **13**)(Fig. 2).

Unsupervised multivariate analysis highlighted inter-individual differences associated with the urinary excretion of phenyl- γ -valerolactones and phenolic acids

The variability registered in the excretion of phenolic metabolites (Fig. 1) and the lack of correlation among some metabolites belonging to the same transformation pathway (Fig. 2) accounted for a high inter-individual difference in the urinary profile of certain metabolites. To better explore this inter-individual variability, and the relationships among metabolites, unsupervised PCA was

carried out. Three principal components (PCs) explained 64.0% of the total variability (**Fig. 3A and 3B**). The first PC described the 27.9% of the observed variation and was positively loaded mainly by dihydroxy- and trihydroxy-phenyl- γ -valerolactone derivatives [24-27, 29, 30] and methy(epi)(gallo)catechin conjugates [18-20]. PC2 explained 18.7% of variability and was positively linked to conjugated dihydrocaffeic, dihydroferulic, and hydroxycinnamic acids [3, 5-8, 10-12], while it was inversely correlated to monohydroxyphenyl- γ -valerolactones [28, 31, 32]. PC3 (17.3% of total variability) had positive component loadings from hydroxyphenylpropionic acid and hydroxybenzoic acid derivatives [2, 9, 13, 23].

When the individual scores with respect to each PC were determined for each subject along the different study visits (Fig. 3C and 3D), it was possible to observe how the urinary excretion profiles exerted by GCE and GTE supplementation (visits 2, 3, and 4, positive values for PC1) differed from the profiles at the beginning of the study and after the wash-out period (visits 1 and 5, respectively, negative scores for PC1), which remained well grouped. The inter-individual variability was clear from sample scores for PC2 and PC3. For instance, subjects #11 and #13 showed positive values for PC2 during supplementation (low excretion of monohydroxyphenyl- γ -valerolactones), while subject #8 showed high negative values for this PC (related to a high excretion of monohydroxyphenyl- γ -valerolactones). Similarly, subjects #6 and #15 displayed very high positive scores for PC3 (high excretion of 3-(hydroxyphenyl)propionic acid and hydroxybenzoic acid derivatives), while all the other subjects had low scores for PC3 (limited excretion of 3-(hydroxyphenyl)propionic and hydroxybenzoic acids). These two subjects (#6 and #15) also exhibited low scores for PC1, indicating a limited excretion of dihydroxy- and trihydroxy-phenyl- γ -valerolactone derivatives (Fig. 3C and 3D). Overall, phenolic metabolites associated with the degradation pathways of GTE flavan-3-ols (compounds 2, 9, 13, 18-20, and 23-32) were the main contributors to the inter-individual variability observed.

Supervised multivariate analysis assisted in subject clustering according to different excretion profiles and indicated the flavan-3-ol metabolites are involved in the definition of inter-individual differences

PLS-DA was applied to predict which metabolite(s) could better explain the inter-individual variation observed from the PCA results and to stratify the individuals into groups sharing a common excretion profile of phenolic metabolites during the supplementation period (visits 2, 3, and 4). By using the preliminary information provided by the PCA, only data from the GTE flavan-3-ol candidate metabolites were used for the PLS-DA.

From the score plot (**Fig. 4A**), groups of volunteers were clearly discriminated according to excreted metabolites. The volunteers were divided into three groups having a different number of subjects (4, 5, and 2 subjects per group; group 1 included subjects #3, #5, #11 and #13; group 2, subjects #2, #4, #8, #9, and #14; and group 3, subjects #6 and #15). The model was quantified based on the variation explained by data. In this situation, $R^2(X)=54\%$, $R^2(Y)=76\%$, and $Q^2=71.2\%$ were found. A Q^2 value higher than 70% accounted for a good model. Additionally, 100 permutation tests (randomly permute classes) were performed to check the difference between the permuted and the original value. It demonstrated the robustness of the model and also proved that R^2 and Q^2 values were not by chance (Fig. 4B).

To select the optimal number of metabolites that were important for this PLS-DA model, VIP scores were used. VIP scores >1 were considered as relevant and, hence, nine metabolites were selected (Supplementary material Fig. 3). Trihydroxyphenyl- γ -valerolactone [26, 27], dihydroxyphenyl- γ -valerolactone [24, 25, 29, 30], and 3-(hydroxyphenyl)propionic acid derivatives [9, 13], as well as *O*-methyl-(epi)catechin-*O*-glucuronide [18], were the phenolic metabolites facilitating the identification of three clusters characterized by a specific urinary profile of GTE derived flavan-3-ol metabolites.

Putative metabotypes in the excretion of flavan-3-ol colonic metabolites

The metabolites contributing to a greater extent the inter-individual variability observed in the urinary excretion of phenolic compounds, and favoring subject clustering during the supplementation period, were identified by PLS-DA. This information was the cornerstone for defining three putative metabotypes in the production of flavan-3-ol metabolites. For that, in order to focus just on metabolites of colonic origin and not on those originating from phase II metabolism of parent

compounds, *O*-methyl-(epi)catechin-*O*-glucuronide was not considered. On the other hand, monohydroxyphenyl- γ -valerolactone derivatives, although not reaching statistical significance in PLS-DA results, were included for the elucidation of the metabotypes. This decision was based on their relevance at PCA level and in order to include a key scaffold in the degradation pathway of dihydroxyphenyl- γ -valerolactone into 3-(hydroxyphenyl)propionic acid. Putative metabotypes were identified by considering the sum of all the derivatives belonging to the same aglycone moiety (for instance, 5-(*O*-methyl-hydroxyphenyl)- γ -valerolactone-sulfate [26] and 5-(dihydroxyphenyl)- γ -valerolactone-*O*-glucuronide [27] were joined together as trihydroxyphenyl- γ -valerolactone). This approach allowed avoiding the confounding factor that individual differences in phase II enzymes might represent, and, at the same time focusing on microbiota-derived differences in the production and excretion of flavan-3-ol catabolites. The urinary excretion of trihydroxyphenyl- γ -valerolactones, dihydroxyphenyl- γ -valerolactones, monohydroxyphenyl- γ -valerolactones and 3-(hydroxyphenyl)propionic acids by clusters of subjects are reported in **Fig. 5**. Except for monohydroxyphenyl- γ -valerolactone derivatives, statistically significant differences were observed in the urinary excretion of all the other flavan-3-ol colonic catabolites among individual clusters ($p < 0.001$). Three putative metabotypes were thus identified among the 11 volunteers:

- metabotype 1, characterized by the presence of high amounts of tri- and di-hydroxyphenyl- γ -valerolactones while a reduced excretion of 3-(hydroxyphenyl)propionic acid
- metabotype 2, associated with a medium excretion of dihydroxyphenyl- γ -valerolactone while a limited excretion of trihydroxyphenyl- γ -valerolactone and 3-(hydroxyphenyl)propionic acid
- metabotype 3, limited in the production of phenyl- γ -valerolactones but producing high amounts of 3-(hydroxyphenyl)propionic acid.

When the excretion of individual compounds was taken into account (Supplementary material Fig. 4), very similar results were achieved. Trihydroxyphenyl- γ -valerolactone, dihydroxyphenyl- γ -valerolactone and 3-(hydroxyphenyl)propionic acid derivatives showed statistically significant differences in their urinary excretion among individual clusters ($p < 0.001$), whereas these differences were not observed for any of the three monohydroxyphenyl- γ -valerolactone derivatives studied ($p > 0.05$, Supplementary material Fig. 4G, 4H, and 4J). The differences observed between metabotype

1 and metabotype 2 for the total excretion of dihydroxyphenyl- γ -valerolactone derivatives were not confirmed for individual dihydroxyphenyl- γ -valerolactone derivatives (Supplementary material Fig. 4C, 4D, 4E, and 4F), but this did not alter the unique urinary profile of each metabotype.

Discussion

Supplementation with (poly)phenolic compounds represents a potentially effective means of providing bioactive compounds to consumers, as part of a strategy to harness the health benefits attributed to plant-based foodstuffs [3]. The bioavailability of phenolic compounds after supplement consumption is attracting increasing attention, and the issue should be tackled in the context of real-life settings. The contribution of GCE tablets to the excretion of phenolic metabolites associated with the degradation pathways of coffee hydroxycinnamates (compounds **3**, **5-8**, and **10-12**), as proposed by Stalmach et al. [6], was very limited and restricted exclusively to feruloylglycine (**10**) at the last visit during the supplementation period (week 12, Fig. 1). The excretion of other metabolites widely linked to coffee consumption [23] was not enhanced as a result of GCE intake. The daily amount of CQAs provided by the GCE supplements corresponded roughly to the intake of 3½ cups of espresso coffee [24]. This intake of CQAs was likely too low to significantly increase the excretion of CQA metabolites in most of the volunteers; they reported a limited consumption of CQAs, with a high inter-individual variation, in accordance with the intake of phenolic acids reported for European populations [25].

The amount of flavan-3-ols provided by the GTE tablets, comparable to up to 23 cups of green tea [26] and far from being achievable in the framework of a regular diet, was enough to increase the urinary excretion of typical flavan-3-ol metabolites during the supplementation period. This effect of supplementation on the urinary profile was observed for both phase II metabolites of flavan-3-ol monomers and metabolites of colonic origin, such as phenyl- γ -valerolactones. Actually, both types of flavan-3-ol metabolites (phase II and colonic) have defined good biomarkers of consumption of foods rich in flavan-3-ols like tea, cocoa, and wine [27-29]. A significantly increased amount of phenyl- γ -valerolactone conjugates has also been reported after consumption of a flavonoid-rich fruits and vegetables diet [30]. On the other hand, the higher excretion of 3-(hydroxyphenyl)propionic and

hydroxybenzoic acids during the supplementation period, although possibly related to coffee CQA metabolism [6,17], was probability also linked to the catabolism of GTE flavan-3-ols, because of the amounts excreted, their lack of correlation with the excretion of other CQA metabolites, and their good correlation with flavan-3-ol monomers (Fig. 2). Moreover, 3-(3'-hydroxyphenyl)propionic and hydroxybenzoic acids, produced from the opening of the phenyl- γ -valerolactone lactone ring and from further β -oxidation of the side chain of phenylvaleric acids [31], have been reported to increase after consumption of flavan-3-ol-rich foodstuffs [32,18,7,27,29].

Inter-individual variability is a major factor affecting phenolic bioavailability and, among other factors, can be driven by the activity of gut microbiota [10]. A high inter-individual variability has been reported in the excretion of phenolic metabolites derived from coffee CQAs and green tea flavan-3-ols [17,8,18,19,3], but the underlying causes are not yet fully understood. The results presented here also showed a high inter-individual variation, mainly observed for those metabolites originating from colonic degradation of GTE flavan-3-ols. So far, although individual variation in the production of phenyl- γ -valerolactones had been reported [33,34,27,35], no clear clusters of metabolites have been described. Associations of gut metabolites allowed us to tentatively define three different flavan-3-ol metabotypes among the participants in the present study. The mechanisms behind the selective production of differently-hydroxylated phenyl- γ -valerolactones and 3-(3'-hydroxyphenyl)propionic acid by the gut microbiota may be linked to the capability of specific bacteria to: 1) carry out the C-ring cleavage of the different catechins present in tea [36,37], 2) perform the dehydroxylation of phenyl- γ -valerolactones [38], and 3) convert phenyl- γ -valerolactones into 3-(phenyl)propionic acids, although the species catalyzing this step have yet to be identified [38].

The existence of metabotypes in the production of phenolic metabolites has been discussed almost exclusively in recent years for equol, 8-prenylnaringenin, and urolithins, compounds derived from the colonic metabolism of the isoflavone daidzein, hop prenylflavonoids, and ellagitannins/ellagic acid, respectively [11,12,14]. The importance of metabotypes relies on the effect that the selective production of microbiota-derived metabolites may have on the health effects of certain foods or specific phenolic compounds [13,16,15]. Taking into account that flavan-3-ols are the main source of flavonoids in Western diets [39,40], these results are key for the further study of the

health effects associated with the consumption of flavan-3-ol sources (i.e. cocoa, tea, wine, apple, etc.) and their main gut microbiota-derived metabolites. In this sense, clustering subjects according to their metabotypes in the production of flavan-3-ol metabolites may provide novel insights in the health benefits attributed to dietary sources of flavan-3-ols on specific groups of population, as it has been described for isoflavones (with equol production) and ellagitannins (with urolithin production) (13, 15, 16). In order to simplify future research in the field, some metabolites representative of the three putative metabotypes found in the present study and excreted in high amounts could be used as biomarkers of a specific metabotype after tea consumption: 5-(dihydroxyphenyl)- γ -valerolactone-*O*-glucuronide [27], 5-(hydroxyphenyl)- γ -valerolactone-*O*-glucuronide [24], and 3-(phenyl)propionic acid-sulfate [13] may be good candidates. To fully understand the metabotypes associated with flavan-3-ol microbial metabolites, further studies should target the whole set of metabolites linked to the degradation pathways of different monomeric and oligomeric flavan-3-ols. Studies with larger numbers of subjects and using different sources of flavan-3-ols are needed to confirm the existence of these metabotypes and to ascertain whether there might be more metabotypes than were not apparent in the current preliminary study. The influence of age, sex, (patho)physiological status, **type of diet**, and dosage, among other factors affecting the bioavailability of phenolic compounds [10], should also be investigated. Microbiomics should also be taken into account to unravel the enterotypes, or bacterial species involved in the metabolic transformations yielding flavan-3-ol metabotypes.

In conclusion, this study demonstrated that (poly)phenolic compounds in GTE and GCE-based dietary supplements are absorbed, metabolized, and excreted following their daily, simultaneous consumption. However, relevant increases in the urinary excretion of some phenolic metabolites may depend on the habitual intake of flavan-3-ol and CQAs. Overall, this study has evidenced the putative existence of specific metabotypes in the production of flavan-3-ol colonic metabolites, for the first time. Based on the relevance of this outcome, further research is guaranteed.

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Compliance with ethical standards

Conflict of Interest: Authors declare no conflict of interest.

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TABLES

Table 1. (Poly)phenolic composition of tablets of green coffee bean extracts (GCE) and green tea (GTE)¹

Compound	μmol/tablet
GCE	
Total caffeoylquinic acids	180 ± 5
Total coumaroylquinic acids	2.9 ± 0.1
Total feruloylquinic acids	76 ± 2
Total dicaffeoylquinic acids	33 ± 2
Total caffeoylferuloylquinic acids	8.7 ± 0.1
Total caffeoylquinic acid lactones	8.2 ± 0.7
Total caffeoyl-dimethoxycinnamoylquinic acids	2.7 ± 0.3
Total phenolic compounds	311 ± 8
GTE	
Total monohydroxy flavan-3-ol monomers	0.5 ± 0.0
Total dihydroxy flavan-3-ol monomers	97 ± 5
Total trihydroxy flavan-3-ol monomers	1553 ± 203
Total dihydroxy flavan-3-ol dimers	7.1 ± 0.2
Total dihydroxy/trihydroxy-flavan-3-ol dimers	3.1 ± 0.1
Total trihydroxy flavan-3-ol dimers	1.3 ± 0.1
Total flavonols	7.5 ± 0.3
Total phenolic compounds	1679 ± 212

¹A detailed characterization of the phenolic composition of the GCE and GTE tablets is provided in Supplementary Table 2. Values presented as mean ± SD (n= 3)

FIGURE LEGENDS

Fig. 1. Urinary excretion (24-h collection) of the 21 main phenolic metabolites during the 5 visits of the supplementation study (in weeks, weeks 0, 2, 4, 8, and 12 correspond to visits 1, 2, 3, 4, and 5 - after washout-, respectively) including the 6 (epi)catechin and (epi)gallocatechin derivatives, 9 phenyl- γ -valerolactone derivatives, and 4 phenolic acid derivatives that increased during the supplementation period. Values, in μmol , are mean \pm SEM ($n=11$). * indicates significant differences ($p<0.05$) compared to visits 1 and 5 according to repeated-measures ANOVA with post-hoc pairwise comparisons.

Fig. 2. Correlation heatmap. Although a strong relationship among most of the phenolic metabolites belonging to the same transformation pathway was found, (phenyl)- γ -valerolactone derivatives did not show good correlations among each other. A scale of correlation ($p<0.05$) is indicated by color. Red indicated positively correlated, whereas dark blue negatively correlated compounds. PV, 5-(phenyl)- γ -valerolactone; HPV, 5-(hydroxyphenyl)- γ -valerolactone; DiHPV, 5-(dihydroxyphenyl)- γ -valerolactone; (E)C, (epi)catechin; (E)GC, (epi)gallocatechin; HPP, 3-(3'-hydroxyphenyl)propionic acid; PP, 3-(phenyl)propionic acid; DHFer, dihydroferulic acid; DHCaf, dihydrocaffeic acid; Isofer. ac., isoferulic acid; glcUA glucuronide; sulf, sulfate. The code assigned to each metabolite, indicated in Supplementary material Table 5, is also reported here in brackets. Taking into account the aglycones, compounds **28**, **31**, and **32** are monohydroxyphenyl- γ -valerolactones; compounds **24**, **25**, **29**, and **30** are dihydroxyphenyl- γ -valerolactones; and compounds **26** and **27** are trihydroxyphenyl- γ -valerolactones.

Fig. 3. Principal component analysis highlighting inter-individual differences associated with the urinary excretion of 5-(phenyl)- γ -valerolactones and phenolic acids. Loading plots of PC1 versus PC2 (A) and PC1 versus PC3 (B); score plots of the excreted phenolic metabolites by each volunteer and visit obtained from PC1 and PC2 (C) and PC1 and PC3 (D). In the loading plots, MET_n indicates the metabolite code, as reported in Fig. 2 and Supplementary material Table 5. In the score plots, empty circles correspond to visits 1 and 5 (no GTE+GCE supplementation), while full circles correspond to

visits 2-4 (GTE + GCE supplementation); codes indicate the subject code and visit number, i.e., S13_V5 means “subject #13, visit 5”.

Fig. 4. Partial least squares-discriminant analysis assisting in subject clusterisation according to different excretion profiles. (A) Score plot shows 3 groups (group 1 -blue-: subjects #3, #5, #11 and #13; group 2 -green-: subject #2, #4, #8, #9, and #14; and group 3 -red-: subjects #6 and #15) for visits 2-4 and by using only GTE flavan-3-ol candidate metabolites with VIP>1 (Supplementary material Fig. 3). (B) Validation plot obtained from 100 permutation tests (randomly permute classes) for the PLS-DA model; R² and Q² mean R² and Q², respectively.

Fig. 5. Urinary excretion of trihydroxyphenyl- γ -valerolactones (A), dihydroxyphenyl- γ -valerolactones (B), monohydroxyphenyl- γ -valerolactones (C), and 3-(3'-hydroxyphenyl)propionic acid (D) by groups of subjects. Boxplots are built using the mean of the urinary excretion of the sum of all the derivatives belonging to the same aglycone moiety, for the three visits under GCE and GTE supplementation (visits 2-4, weeks 2-8): trihydroxyphenyl- γ -valerolactone is calculated of the sum of compounds **26** and **27**; dihydroxyphenyl- γ -valerolactone of compounds **24**, **25**, **29**, and **30**; monohydroxyphenyl- γ -valerolactone of compounds **28**, **31**, and **32**; and (hydroxyphenyl)propionic acid of compounds **9** and **13**. The blue group, defining metabotype 1, is formed by 4 subjects; the green group, metabotype 2, is formed by 5 subjects; and the red group, metabotype 3, is formed by 2 subjects. Different letters indicate statistically significant differences ($p<0.05$) among groups according to ANOVA with post-hoc Dunnett's T3 test. Circles and asterisks indicate outliers and extreme outliers (more than three times the interquartile range), respectively.

Figure 1

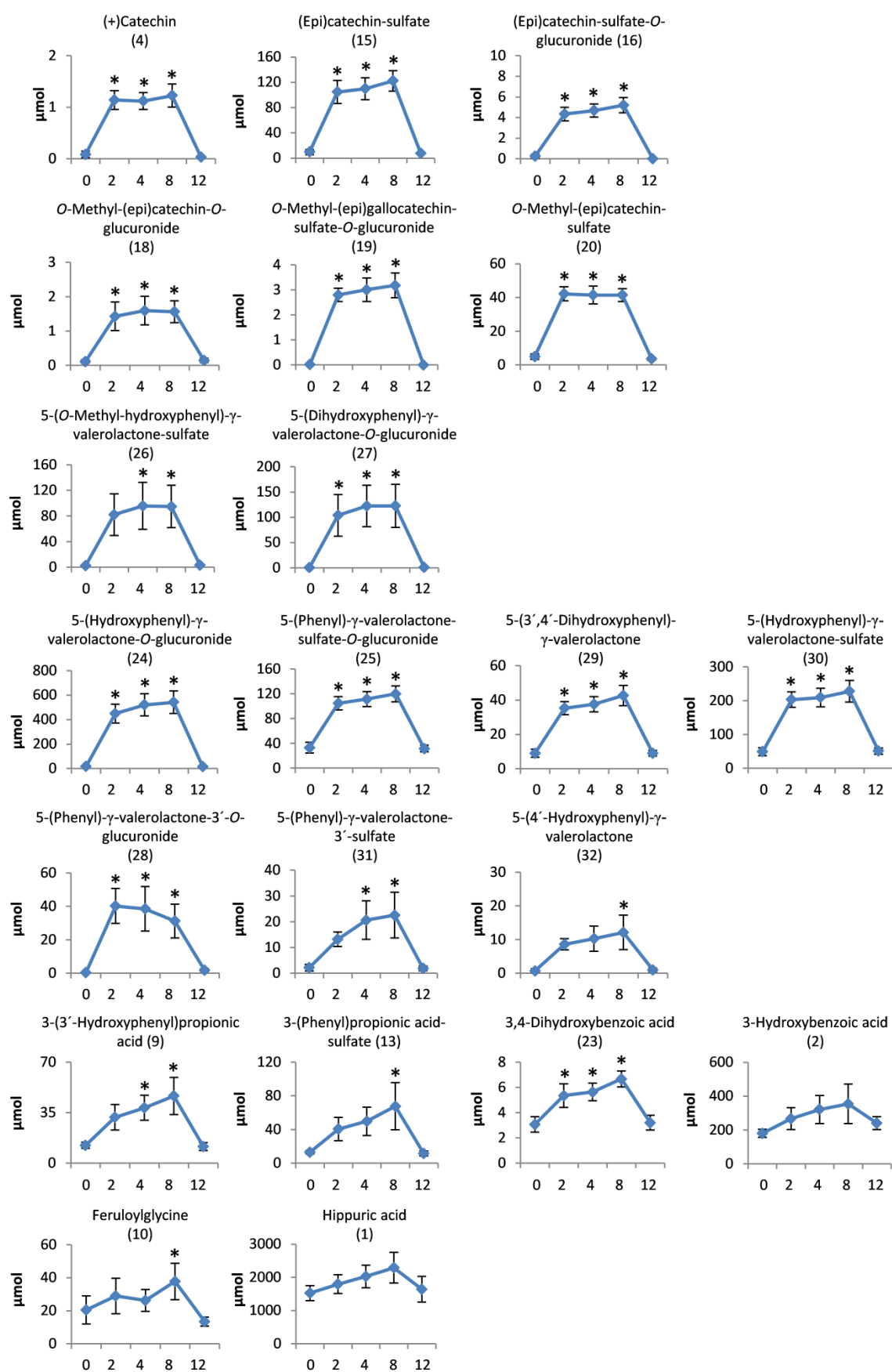


Figure 2

Figure 2

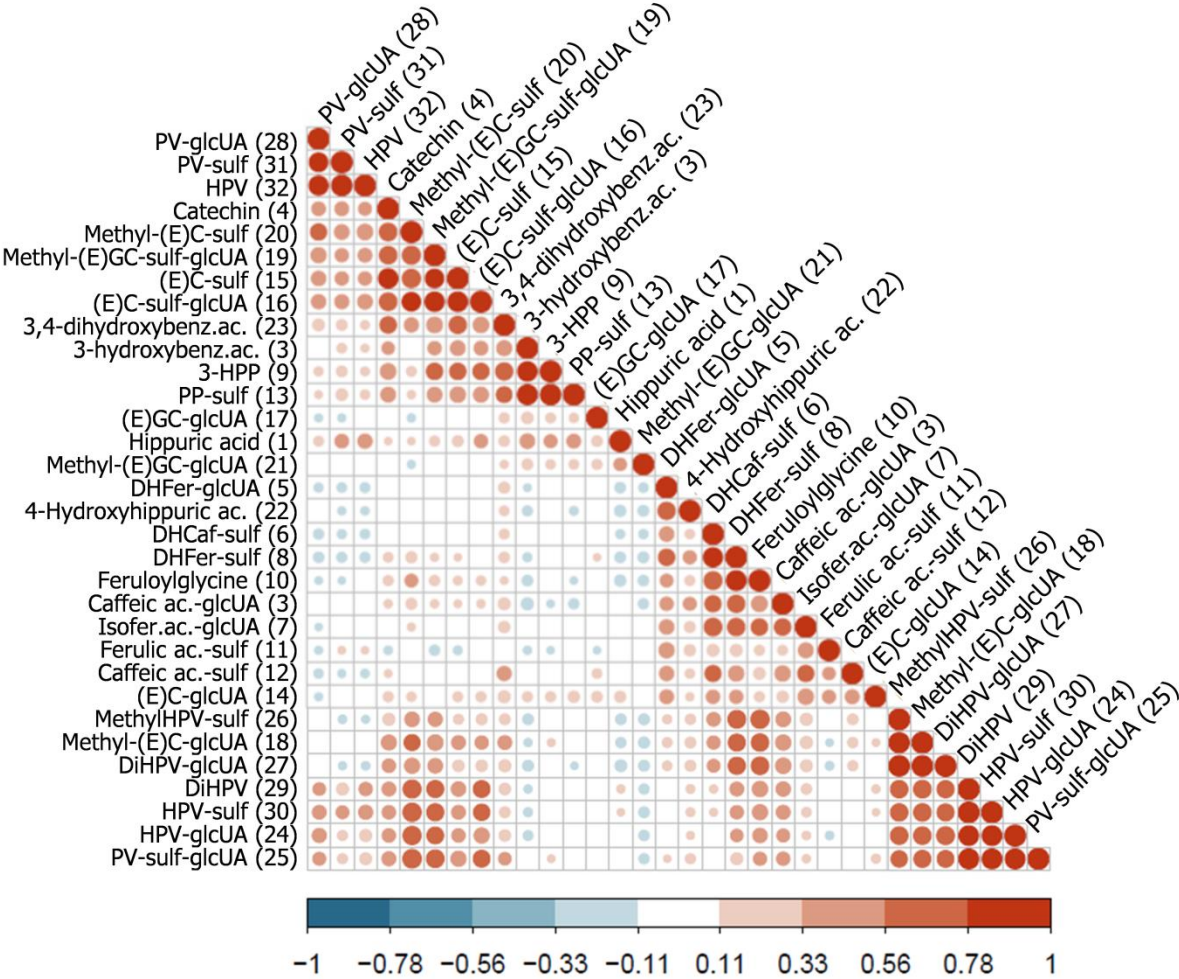


Figure 4

Figure 4

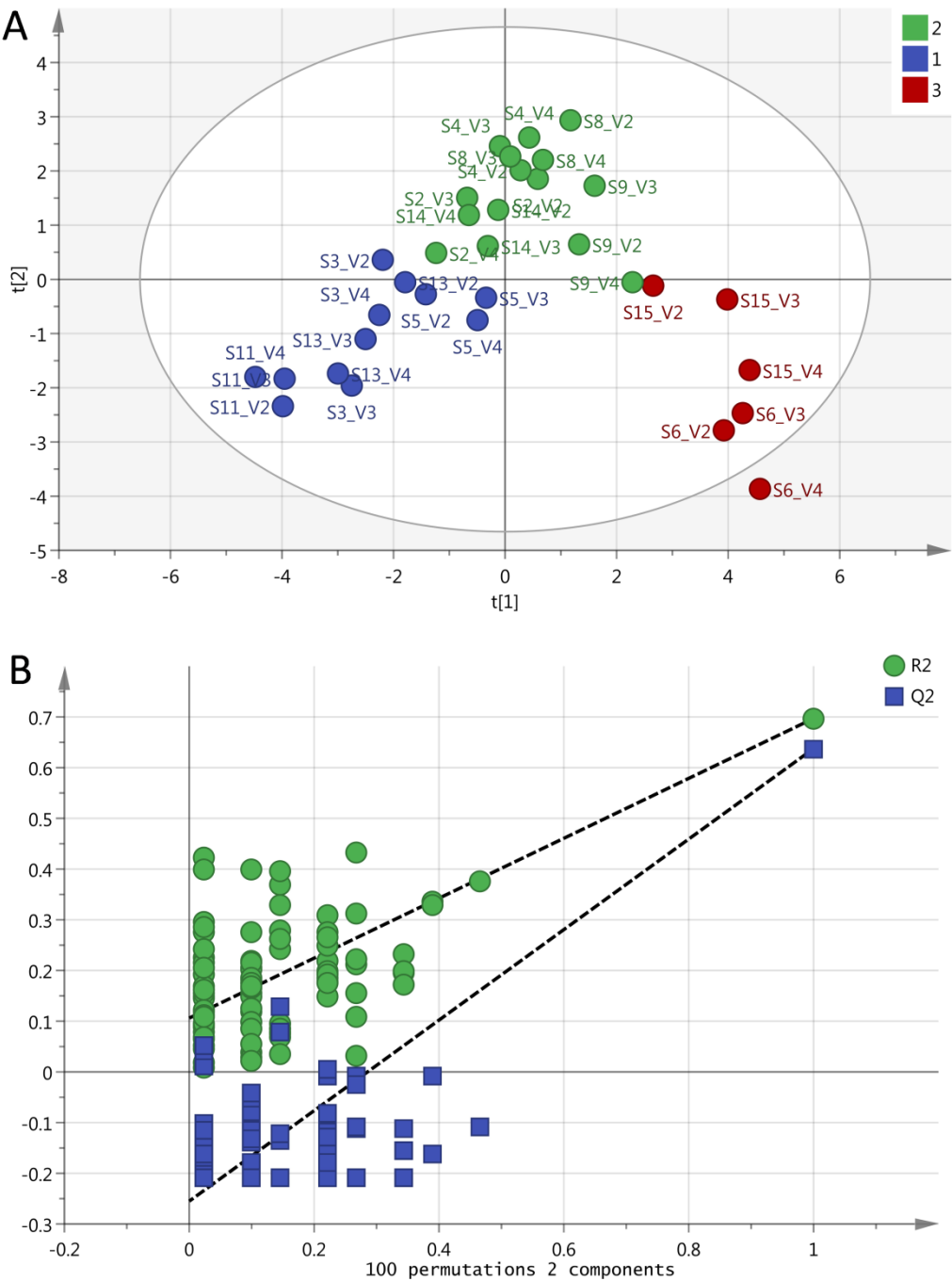


Figure 5

Figure 5

