

Inter-individual variability in the production of flavan-3-ol colonic metabolites

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

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26

27 **Abstract**

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

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

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

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

49

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

52 **Introduction**

53 Epidemiological and human intervention studies report the **potentially beneficial** effects of
54 (poly)phenolic-rich plant foods against several chronic conditions, including cardiometabolic diseases,
55 neurodegeneration, and certain kinds of cancer [1,2]. Supplementation with (poly)phenolic compounds
56 may represent an effective means of providing potential bioactive compounds to consumers, as part of
57 a strategy to enhance the health benefits attributed to plant-based food products [3]. To exert a
58 systemic biological action, (poly)phenolic compounds must be bioavailable, and this may vary
59 substantially with different plant foods and dietary supplements [4,5]. Following ingestion, most
60 phenolics undergo modifications during passage through the gastrointestinal tract. They are
61 extensively modified by the action of the gut microbiota, being converted to colonic catabolites that
62 are absorbed into the blood stream where they circulate principally as conjugated phase II metabolites
63 [1,2]. Green tea and coffee are, respectively, major sources of bioactive flavan-3-ols, and chlorogenic
64 acids (CGAs) which occur principally as caffeoylquinic acids (CQAs). Both flavan-3-ols and CQAs
65 are bioavailable after acute intake of green tea and coffee [6-8]. However, there is a paucity of
66 information on their bioavailability after combined and prolonged ingestion of green tea and coffee,
67 circumstances which reflect their normal consumption by the general public [9].

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

80 individual variability in the production of metabolites derived from coffee CGAs and green tea flavan-
81 3-ols has been reported [17,8,18,19], no phenotypes in their production have been identified. In this
82 sense, 5-(phenyl)- γ -valerolactones, unique ring-fission microbial products of flavan-3-ol monomers
83 and oligomeric proanthocyanidins, might be selectively produced on the basis of different enterotypes
84 or microbiota profiles.

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

90

91 **Subjects and methods**

92 **Participants**

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

102

103 **Study design and protocol**

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

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

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

124

125 **(Poly)phenol tablets and chemicals**

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

134 3-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-(3'-hydroxyphenyl)propionic acid,
135 hippuric acid, 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, (+)-catechin, and (–)epigallocatechin-

136 3-*O*-gallate were purchased from Sigma-Aldrich (St. Louis, MO, USA), while procyanidin B₂ was
137 supplied by Phytolab (Vestenbergsgreuth, Germany). Ferulic acid-4'-sulfate, isoferulic acid-3'-*O*-
138 glucuronide, dihydroferulic acid-4'-sulfate, caffeic acid-4'-*O*-glucuronide, dihydroisoferulic acid-3'-*O*-
139 glucuronide, and dihydrocaffeic acid-3'-sulfate were obtained from Toronto Research Chemical
140 (Toronto, Canada), while 4'-hydroxyhippuric acid was purchased from Bachem Ltd. (St Helens, UK).
141 5-(4'-hydroxyphenyl)- γ -valerolactone, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-(phenyl)- γ -
142 valerolactone-3'-sulfate, and 5-(phenyl)- γ -valerolactone-3'-*O*-glucuronide were prepared in house
143 using the strategies of Curti et al. [20] for aglycones and Brindani et al. [19] for phase II conjugates.
144 They are catalogued on the standards sharing platform FoodComEx (www.foodcomex.org).
145 Quercetin-3'-sulfate and quercetin-3'-*O*-glucuronide were kindly provided by Denis Barron (Nestle
146 Research Center, Lausanne, Switzerland) and Gary Williamson (School of Food Science and
147 Nutrition, University of Leeds, UK), while feruloylglycine was a gift from Takao Yokota (Teikyo
148 University, Japan). All solvents and reagents were purchased from Sigma-Aldrich, unless otherwise
149 indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout
150 the experiment.

151

152 **Tablets and urine sample preparation for UHPLC-MSⁿ analyses**

153 GTE and GCE tablets were analyzed to determine their (poly)phenolic composition. Three
154 randomly selected tablets of each type were weighted, pulverized and 100 mg aliquots extracted with
155 1 mL of methanol/water/formic acid (74.5:24.5:1, v/v/v). The samples were vortexed, ultrasonicated
156 for 90 min, and centrifuged at 5 °C for 10 min at 4000 g. The supernatants were collected and the
157 pellets re-extracted with 0.5 mL of methanol/water/formic acid (74.5:24.5:1, v/v/v). The samples were
158 vortexed and ultrasonicated for 25 min, and centrifuged at 5 °C for 10 min at 4000 g. The two
159 supernatants were pooled, diluted 1:5 with 0.1% aqueous formic acid prior to ultra-high performance
160 liquid chromatography-mass spectrometry (UHPLC-MSⁿ) analysis for phenolic characterization and
161 quantification.

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

165

166 **Qualitative and quantitative analysis of (poly)phenols in tablets and urine samples by UHPLC-** 167 **MSⁿ**

168 (Poly)phenolic compounds were analyzed using an Accela UHPLC 1250 with a LTQ XL linear
169 ion trap-mass spectrometer fitted with a heated-electrospray ionization (ESI) probe (Thermo Scientific
170 Inc., San Jose, CA, USA). Separation was performed with a XSELECT HSS T3 (50 × 2.1 mm),
171 2.5 µm particle size column (Waters, Milford, MA, USA). The volume injected was 5 µL and the
172 column oven was set to 30°C. Elution was carried out at a flow rate of 0.3 mL/min. The gradient
173 started with 2% acetonitrile in 0.1% aqueous formic acid and, after 0.5 min, an 8.5-min linear gradient
174 of 2% to 45% acetonitrile was applied. From 9 to 9.5 min the acidified acetonitrile increased to 80%,
175 and after 3 min was reduced to 2% acetonitrile to re-equilibrate the column for 3 min at start
176 conditions. The MS was operated in negative ionization mode with a capillary temperature of 275 °C
177 and a source temperature of 250 °C. The sheath gas flow was 40 units and the auxiliary and sweep gas
178 were set to 5 units. The source voltage was 3 kV. The capillary and tube lens voltage were -9 and -
179 53 V, respectively. Analyses were carried out using full scan, data-dependent MS³ scanning from *m/z*
180 100 to 1000, with collision induced dissociation (CID) of 35 (arbitrary units). Helium gas was used for
181 CID. After this first step, further specific MS² and MS³ analyses were carried out to unambiguously
182 identify and quantify the compounds revealed in the first step, by monitoring specific *m/z* transitions.
183 Molecules were fragmented using pure helium (99.99%). CID settings were optimized for each
184 compound in order to produce the highest fragment signals. Identification was performed by
185 comparison with standards, when available, and literature. Data processing was performed using
186 Xcalibur software (Thermo Scientific).

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

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

198

199 **Statistical analysis**

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

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

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

217 SPSS statistics 21.0 software (IBM, Chicago, IL). Then, to better understand the causes behind the
218 observed inter-individual variability in the excretion of flavan-3-ol metabolites, partial least squares
219 discriminant analysis (PLS-DA) was carried out. PLS-DA is a linear projection method, and all
220 metabolites are assumed to be combined in a linear manner to maximize discrimination. Different
221 explorative PLS-DA models were built to define the number of clusters and the subjects in each
222 cluster, by using only the information for GTE flavan-3-ol metabolites. The model that maximized the
223 validation parameters was selected. Model validation was performed by different parameters
224 considering the variation in the metabolite data, $R^2(X)$, variation in the dependent variable (class),
225 $R^2(Y)$ and goodness of fit of the validation (Q^2) of the model [21], and by performing cross validation
226 by random permutation. The selection of the most representative metabolites from the whole set of
227 metabolites (variable selection) was performed by using the Variable Importance in Projection (VIP)
228 scores, estimating the importance of each variable in the projection used in a PLS model [22]: variable
229 with VIP scores greater than 1 were considered important in the given model, while variables with
230 VIP scores less than 1 were less important and good candidates for exclusion from the model. PLS-
231 DA analysis was performed in SIMCA (version 14, Umetrics, Umea, Sweden).

232

233 **Results**

234 **Participant baseline characteristics and treatment adherence**

235 Participants (9 women, 2 men) were aged 28 ± 6 years, had a healthy BMI (21.8 ± 1.6 kg/m²) and
236 normal blood pressure (systolic blood pressure (SBP): 110 ± 6 mm Hg, diastolic blood pressure
237 (DBP): 69 ± 6 mm Hg). On average, 99% of the polyphenol doses were consumed, indicating good
238 adherence to the supplementation for all the participants. Participants also adhered to their usual diets
239 and to the supplement consumption restrictions.

240

241 **Dietary assessment**

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

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

250

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

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

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

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

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

274

275 **Identification of urinary phenolic metabolites**

276 UHPLC-MSⁿ analysis of urine samples allowed the identification and quantification of a total of
277 32 compounds. Retention time and mass spectral data of each metabolite are reported in
278 Supplementary material Table 5. In absence of available standards for some phase II metabolites
279 detected in urine, the criteria of identification were based on previously reported HPLC-MS analyses
280 [18,7]. Phase II metabolites produced by the action of UDP-glucuronosyltransferases and
281 sulfotransferases were identified through the loss of the conjugation group (m/z 176 for glucuronides
282 and m/z 80 for sulfates) to produce the aglycone fragment ion at MS², as previously described [18,7].
283 Where necessary, further MS fragmentation of the aglycone was applied to confirm the identification
284 of the metabolites through their characteristic MS³ fragment ions (Supplementary material Table 5).
285 From the 32 identified compounds, three were glycine conjugates (hippuric acid [metabolite **1**], 4'-
286 hydroxyhippuric acid [**22**], and feruloylglycine [**10**]), two benzoic acid derivatives (3-hydroxy- and
287 3,4-dihydroxybenzoic acid, [**2** and **23**, respectively]), five free and conjugated phenylpropionic acids
288 (dihydroisoferulic acid-3'-*O*-glucuronide [**5**], dihydrocaffeic acid-3'-sulfate [**6**], dihydroferulic acid-4'-
289 sulfate [**8**], 3-(3'-hydroxyphenyl)propionic acid [**9**], 3-(phenyl)propionic acid-sulfate [**13**]), and four
290 hydroxycinnamic acid sulfates and glucuronides (caffeic acid-4'-*O*-glucuronide [**3**], isoferulic
291 acid-3'-*O*-glucuronide [**7**], ferulic acid-4'-sulfate [**11**], and caffeic acid-sulfate [**12**]). However, the
292 large majority of metabolites found in urine were methyl, sulfate, and glucuronide conjugates of
293 (epi)catechins, and phenyl- γ -valerolactones which comprised a total of nine phenyl- γ -valerolactones
294 (three monohydroxyphenyl- γ -valerolactones, 5-(4'-hydroxyphenyl)- γ -valerolactone [**32**], 5-(phenyl)- γ -
295 valerolactone-3'-*O*-glucuronide [**28**], and 5-(phenyl)- γ -valerolactone-3'-sulfate [**31**]; four
296 dihydroxyphenyl- γ -valerolactones, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone [**29**], 5-
297 (hydroxyphenyl)- γ -valerolactone-*O*-glucuronide [**24**], 5-(hydroxyphenyl)- γ -valerolactone-sulfate [**30**],
298 and 5-(phenyl)- γ -valerolactone-sulfate-*O*-glucuronide [**25**]; and two trihydroxyphenyl- γ -

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

305

306 **Effect of tablet supplementation on the urinary excretion of phenolic metabolites**

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

315 Up to six (epi)catechin derivatives increased significantly during the supplementation, as well as
316 all the phenyl- γ -valerolactone derivatives. Some phenolic acids such as
317 3-(3'-hydroxyphenyl)propionic acid [9], phenylpropionic acid-sulfate [13], 3,4-dihydroxybenzoic acid
318 [23], and feruloylglycine [10] were also excreted in significantly higher amounts after the
319 supplementation with GTE and GCE tablets (Fig. 1). Interestingly, not all the 19 compounds increased
320 significantly during the early part of the supplementation period (visit 2, week 2). The excreted
321 amounts of 5-(*O*-methyl-hydroxyphenyl)- γ -valerolactone-sulfate [26], 5-(phenyl)- γ -valerolactone-3'-
322 sulfate [31], and 3-(3'-hydroxyphenyl)propionic acid [9] increased significantly after 4 weeks of
323 treatment (visit 3), while 5-(4'-hydroxyphenyl)- γ -valerolactone [32], 3-(phenyl)propionic acid-sulfate
324 [13], and feruloylglycine [10] increased significantly only after the whole supplementation period of 8

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

327

328 **Lack of good correlation among some metabolites belonging to the same transformation** 329 **pathway**

330 Correlation analysis showed a strong relationship among most of the phenolic metabolites
331 belonging to the same transformation pathway (**Fig. 2**). For instance, all the (dihydro)caffeic acid and
332 (dihydro)ferulic acid derivatives associated with the degradation pathways of GCE and CGA [6] were
333 well and positively correlated ($p < 0.05$, Fig. 2, compounds **3, 5-8, and 10-12**). Similarly, most of the
334 (epi)(gallo)catechin conjugates and 3-(hydroxyphenyl)propionic acids derived from the metabolism of
335 GTE flavan-3-ols [18,7] were also positively correlated (Fig. 2, compounds **4, 9, 13, 15, 16, and 18-**
336 **20**). (Phenyl)- γ -valerolactone derivatives (compounds **24-32**) were positively correlated to
337 (epi)(gallo)catechin conjugates (compounds **4, 15, 16, and 18-20**) but, surprisingly, they did not show
338 good correlations among each other (Fig. 2). Specifically, while a good correlation (r between 0.56
339 and 0.78) was found between dihydroxyphenyl- γ -valerolactones (**24, 25, 29, and 30**) and
340 trihydroxyphenyl- γ -valerolactones (**26 and 27**), monohydroxyphenyl- γ -valerolactones (**28, 31, and 32**)
341 correlated inversely (r between -0.11 and -0.33) with trihydroxyphenyl- γ -valerolactones (**26 and 27**)
342 and positively, but weakly (r between 0.11 and 0.56), with dihydroxyphenyl- γ -valerolactones (**24, 25,**
343 **29, and 30**). It should be also mentioned that only monohydroxyphenyl- γ -valerolactones (**28, 31, and**
344 **32**) correlated positively, but weakly, with 3-(hydroxyphenyl)propionic acids (compounds **9 and**
345 **13**)(Fig. 2).

346

347 **Unsupervised multivariate analysis highlighted inter-individual differences associated with the** 348 **urinary excretion of phenyl- γ -valerolactones and phenolic acids**

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

353 carried out. Three principal components (PCs) explained 64.0% of the total variability (**Fig. 3A and**
354 **3B**). The first PC described the 27.9% of the observed variation and was positively loaded mainly by
355 dihydroxy- and trihydroxy-phenyl- γ -valerolactone derivatives [**24-27, 29, 30**] and
356 methy(epi)(gallo)catechin conjugates [**18-20**]. PC2 explained 18.7% of variability and was positively
357 linked to conjugated dihydrocaffeic, dihydroferulic, and hydroxycinnamic acids [**3, 5-8, 10-12**], while
358 it was inversely correlated to monohydroxyphenyl- γ -valerolactones [**28, 31, 32**]. PC3 (17.3% of total
359 variability) had positive component loadings from hydroxyphenylpropionic acid and hydroxybenzoic
360 acid derivatives [**2, 9, 13, 23**].

361 When the individual scores with respect to each PC were determined for each subject along the
362 different study visits (Fig. 3C and 3D), it was possible to observe how the urinary excretion profiles
363 exerted by GCE and GTE supplementation (visits 2, 3, and 4, positive values for PC1) differed from
364 the profiles at the beginning of the study and after the wash-out period (visits 1 and 5, respectively,
365 negative scores for PC1), which remained well grouped. The inter-individual variability was clear
366 from sample scores for PC2 and PC3. For instance, subjects #11 and #13 showed positive values for
367 PC2 during supplementation (low excretion of monohydroxyphenyl- γ -valerolactones), while subject
368 #8 showed high negative values for this PC (related to a high excretion of monohydroxyphenyl-
369 γ -valerolactones). Similarly, subjects #6 and #15 displayed very high positive scores for PC3 (high
370 excretion of 3-(hydroxyphenyl)propionic acid and hydroxybenzoic acid derivatives), while all the
371 other subjects had low scores for PC3 (limited excretion of 3-(hydroxyphenyl)propionic and
372 hydroxybenzoic acids). These two subjects (#6 and #15) also exhibited low scores for PC1, indicating
373 a limited excretion of dihydroxy- and trihydroxy-phenyl- γ -valerolactone derivatives (Fig. 3C and 3D).
374 Overall, phenolic metabolites associated with the degradation pathways of GTE flavan-3-ols
375 (compounds **2, 9, 13, 18-20, and 23-32**) were the main contributors to the inter-individual variability
376 observed.

377

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

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

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

395 To select the optimal number of metabolites that were important for this PLS-DA model, VIP
396 scores were used. VIP scores >1 were considered as relevant and, hence, nine metabolites were
397 selected (Supplementary material Fig. 3). Trihydroxyphenyl- γ -valerolactone [**26, 27**],
398 dihydroxyphenyl- γ -valerolactone [**24, 25, 29, 30**], and 3-(hydroxyphenyl)propionic acid derivatives
399 [**9, 13**], as well as *O*-methyl-(epi)catechin-*O*-glucuronide [**18**], were the phenolic metabolites
400 facilitating the identification of three clusters characterized by a specific urinary profile of GTE
401 derived flavan-3-ol metabolites.

402

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

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

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

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

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

437 1 and metabotype 2 for the total excretion of dihydroxyphenyl- γ -valerolactone derivatives were not
438 confirmed for individual dihydroxyphenyl- γ -valerolactone derivatives (Supplementary material Fig.
439 4C, 4D, 4E, and 4F), but this did not alter the unique urinary profile of each metabotype.

440

441 Discussion

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

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

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

472 Inter-individual variability is a major factor affecting phenolic bioavailability and, among other
473 factors, can be driven by the activity of gut microbiota [10]. A high inter-individual variability has
474 been reported in the excretion of phenolic metabolites derived from coffee CQAs and green tea flavan-
475 3-ols [17,8,18,19,3], but the underlying causes are not yet fully understood. The results presented here
476 also showed a high inter-individual variation, mainly observed for those metabolites originating from
477 colonic degradation of GTE flavan-3-ols. So far, although individual variation in the production of
478 phenyl- γ -valerolactones had been reported [33,34,27,35], no clear clusters of metabolites have been
479 described. Associations of gut metabolites allowed us to tentatively define three different flavan-3-ol
480 metabotypes among the participants in the present study. The mechanisms behind the selective
481 production of differently-hydroxylated phenyl- γ -valerolactones and 3-(3'-hydroxyphenyl)propionic
482 acid by the gut microbiota may be linked to the capability of specific bacteria to: 1) carry out the C-
483 ring cleavage of the different catechins present in tea [36,37], 2) perform the dehydroxylation of
484 phenyl- γ -valerolactones [38], and 3) convert phenyl- γ -valerolactones into 3-(phenyl)propionic acids,
485 although the species catalyzing this step have yet to be identified [38].

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

493 health effects associated with the consumption of flavan-3-ol sources (i.e. cocoa, tea, wine, apple, etc.)
494 and their main gut microbiota-derived metabolites. In this sense, clustering subjects according to their
495 metabotypes in the production of flavan-3-ol metabolites may provide novel insights in the health
496 benefits attributed to dietary sources of flavan-3-ols on specific groups of population, as it has been
497 described for isoflavones (with equol production) and ellagitannins (with urolithin production) (13, 15,
498 16). In order to simplify future research in the field, some metabolites representative of the three
499 putative metabotypes found in the present study and excreted in high amounts could be used as
500 biomarkers of a specific metabotype after tea consumption: 5-(dihydroxyphenyl)- γ -valerolactone-*O*-
501 glucuronide [27], 5-(hydroxyphenyl)- γ -valerolactone-*O*-glucuronide [24], and 3-(phenyl)propionic
502 acid-sulfate [13] may be good candidates. To fully understand the metabotypes associated with flavan-
503 3-ol microbial metabolites, further studies should target the whole set of metabolites linked to the
504 degradation pathways of different monomeric and oligomeric flavan-3-ols. Studies with larger
505 numbers of subjects and using different sources of flavan-3-ols are needed to confirm the existence of
506 these metabotypes and to ascertain whether there might be more metabotypes than were not apparent
507 in the current preliminary study. The influence of age, sex, (patho)physiological status, **type of diet**,
508 and dosage, among other factors affecting the bioavailability of phenolic compounds [10], should also
509 be investigated. Microbiomics should also be taken into account to unravel the enterotypes, or
510 bacterial species involved in the metabolic transformations yielding flavan-3-ol metabotypes.

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

517

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527
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541
542 **Compliance with ethical standards**

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544

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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

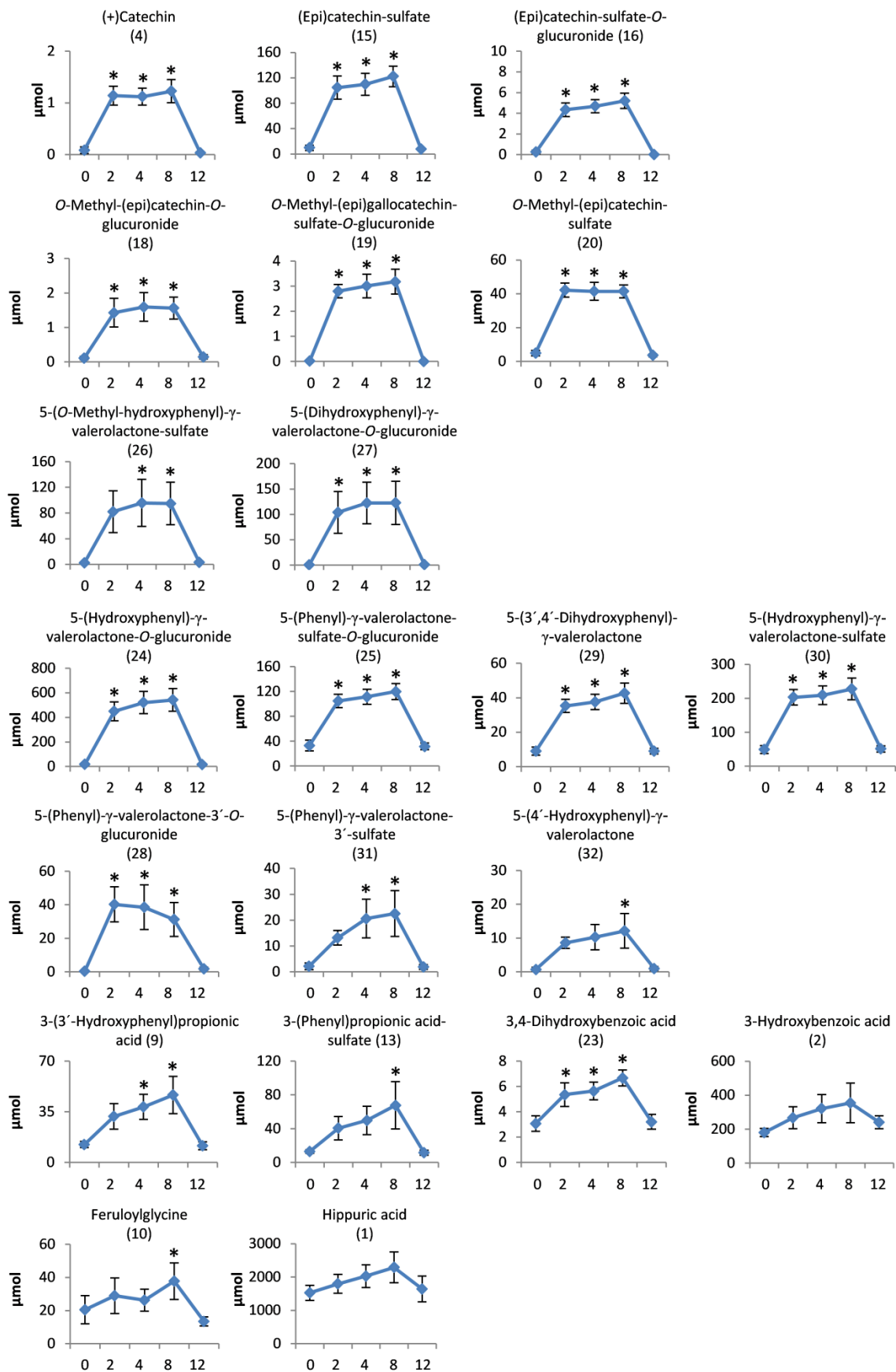


Figure 2

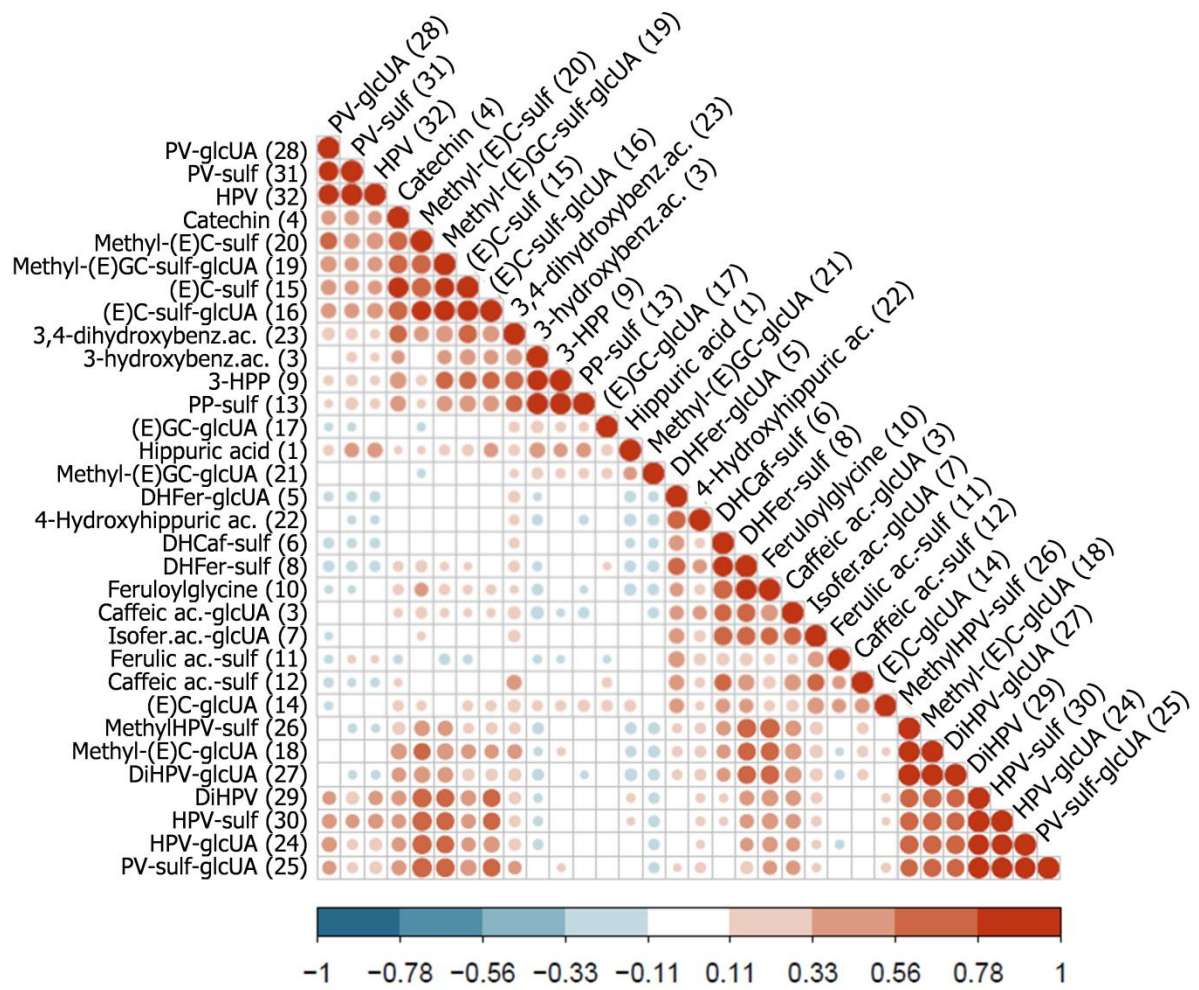


Figure 3

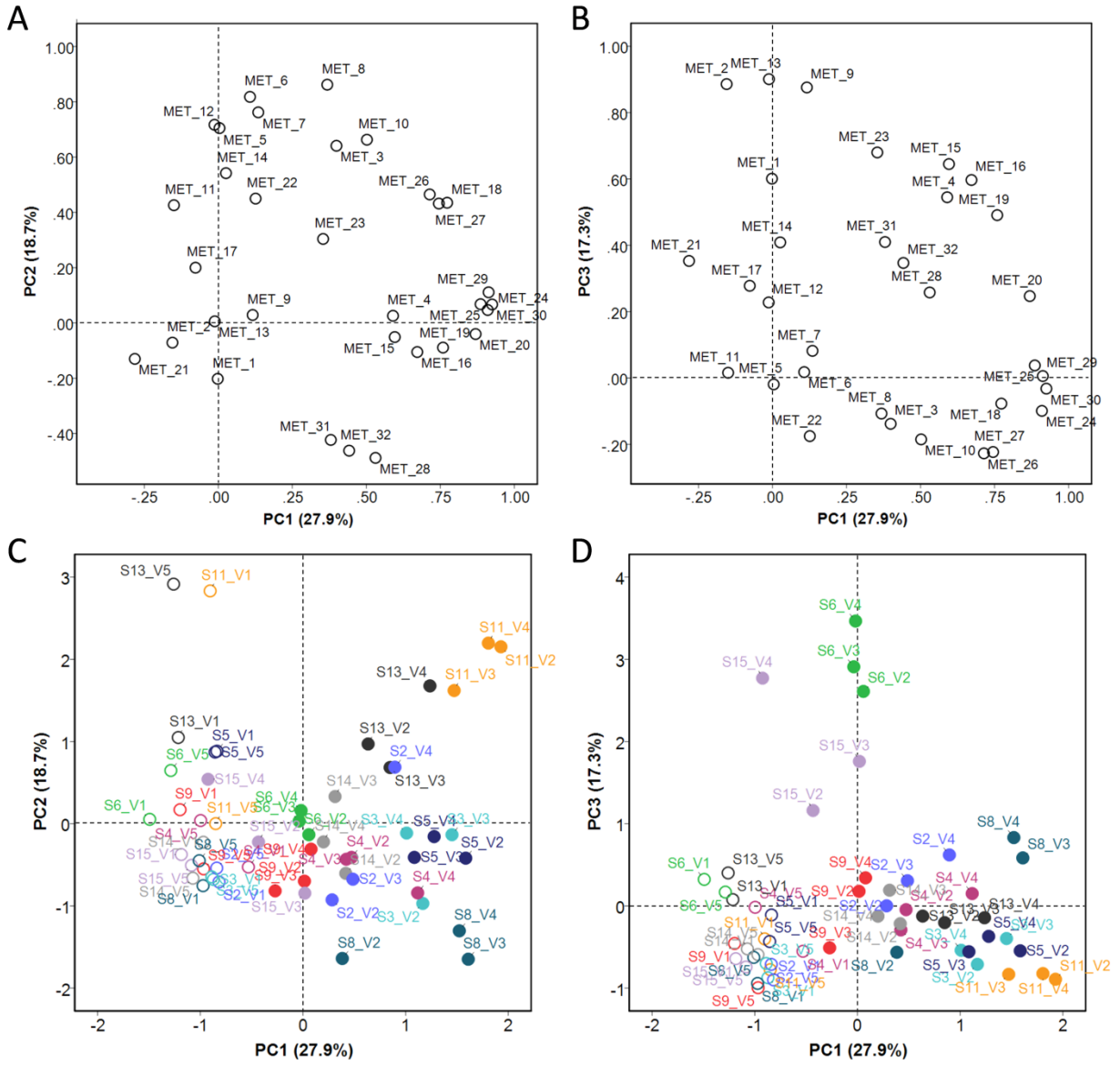


Figure 4

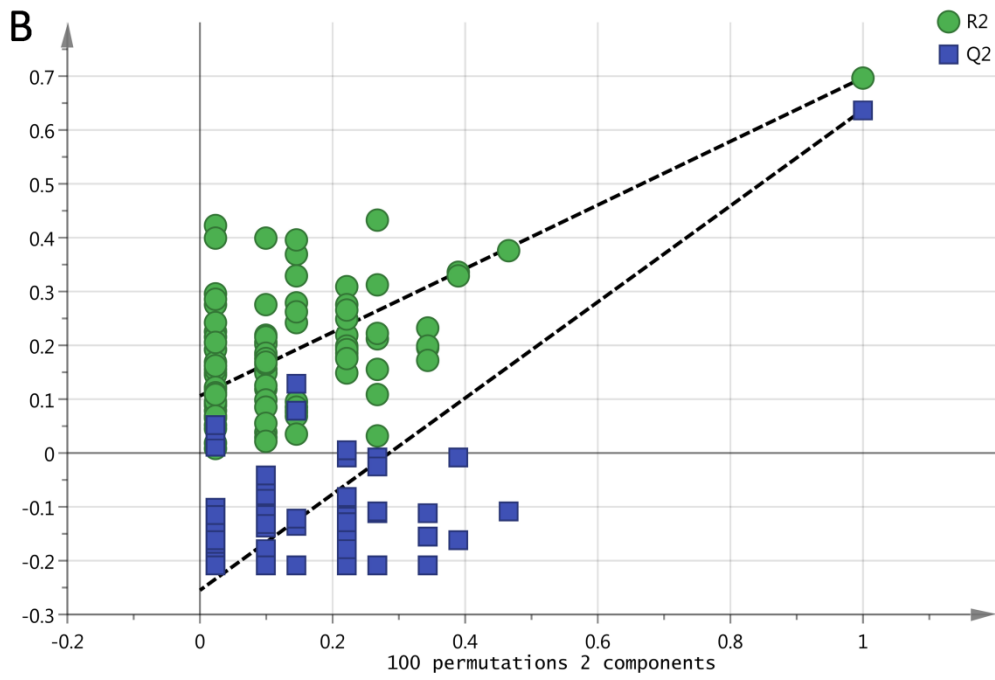
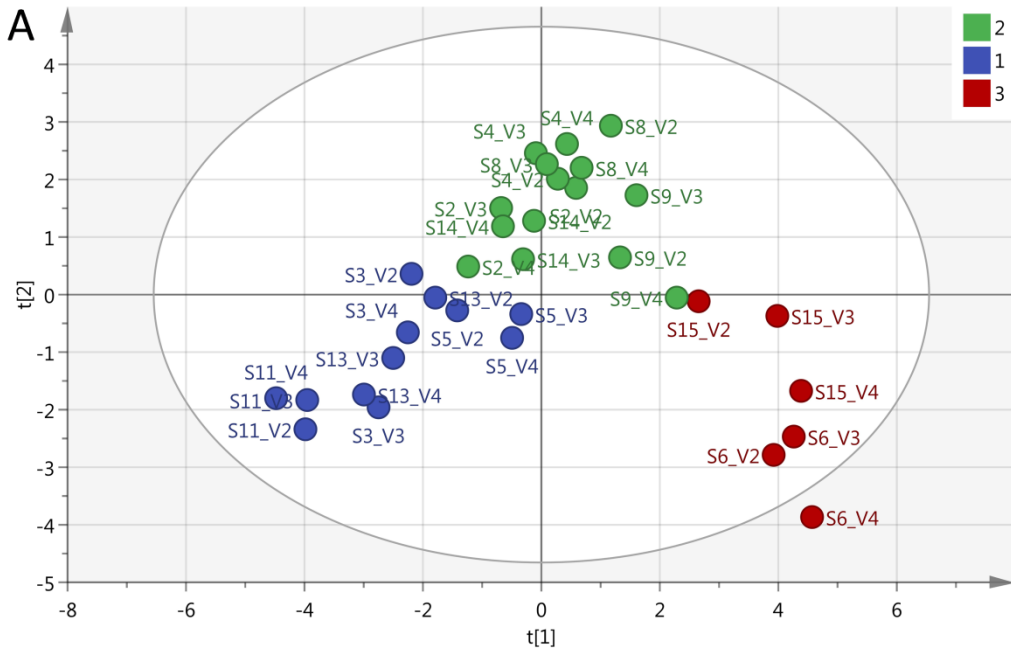


Figure 5

