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

2   **preliminary elucidation of urinary metabotypes**

3   Pedro Mena[1,^], Iziar A. Ludwig[1,2,^], Virginia B. Tomatis[3,4,^], Animesh Acharjee[3,4,5,6], Luca Calani[1],

4   Alice Rosi[1], Furio Brighenti[1], Sumantra Ray[3], Julian L. Griffin[3,4], Les J. Bluck[3,‡,†] and Daniele Del

5   Rio[1,‡]

6   *^Equal contributors*

7   *‡Equal contributors*

8   [1] Human Nutrition Unit, Department of Food & Drugs, University of Parma, Parma, Italy.

9   [2] Food Technology Department, Universitat de Lleida-Agrotecnio Center, Lleida, Spain.

10   [3] UK Medical Research Council Elsie Widdowson Laboratory (formerly MRC Human Nutrition

11   Research), Cambridge, UK.

12   [4] Department of Biochemistry, University of Cambridge, Cambridge, UK.

13   [5] Institute of Cancer and Genomic Sciences, Centre for Computational Biology, University of

14   Birmingham, Birmingham, UK.

15   [6] Institute of Translational Medicine, University Hospitals Birmingham NHS Foundation Trust,

16   Birmingham, UK.

17   [†] *LB is deceased.*

18   **Address for correspondence**: Daniele Del Rio & Pedro Mena, Human Nutrition Unit, Department of

19   Food & Drugs, University of Parma, Medical School Building C, Via Volturno, 39, 43125 Parma,

20   Italy. Phone: (+39) 0521-903841. E-mail: daniele.delrio@unipr.it;

21   pedromiguel.menaparreno@unipr.it.

22

23   **ORCID:** PM 0000-0003-2150-2977; IAL 0000-0001-5506-3293; AA 0000-0003-2735-7010; LC

24   0000-0002-3516-8976; AR 0000-0001-8533-797X; FB 0000-0001-8441-4611; SR 0000-0002-1411-

25   0003; JLG 0000-0003-1336-7744; LJB 0000-0001-6776-4281; DDR 0000-0001-5394-1259.

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.

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-

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/m^2 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 B_2 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-MS^n 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-MS^n) 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-MS^n.

165

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

167   **MS^n**

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 MS^3 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 MS^2 and MS^3 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 ± standard deviation (SD) for (poly)phenols in GTE and

201   GCE tablets and as mean values ± 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/m^2) 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

Identification of urinary phenolic metabolites

276   UHPLC-MS^n 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 MS^2, 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 MS^3 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 cofounding 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 probability 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

541

542   **Compliance with ethical standards**

543   **Conflict of Interest:** Authors declare no conflict of interest.

544

References

1. Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A (2013) Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 18 (14):1818-1892. doi: 10.1089/ars.2012.4581

2. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, Mena P, Del Rio D, Crozier A (2014) Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Arch Toxicol 88 (10):1803-1853. doi: 10.1007/s00204-014-1330-7

3. Bresciani L, Martini D, Mena P, Tassotti M, Calani L, Brigati G, Brighenti F, Holasek S, Malliga D-E, Lamprecht M, Del Rio D (2017) Absorption Profile of (Poly)Phenolic Compounds after Consumption of Three Food Supplements Containing 36 Different Fruits, Vegetables, and Berries. Nutrients 9 (3):194 doi: 10.3390/nu9030194

4. Shi Y, Williamson G (2015) Comparison of the urinary excretion of quercetin glycosides from red onion and aglycone from dietary supplements in healthy subjects: a randomized, single-blinded, cross-over study. Food Funct 6:1443-8. doi:10.1039/c5fo00155b

5. Borel P, Desmarchelier C, Nowicki M, Bott R, Morange S, Lesavre N (2014) Interindividual variability of lutein bioavailability in healthy men: characterization, genetic variants involved, and relation with fasting plasma lutein concentration. Am J Clin Nutr 100 (1):168-175. doi:10.3945/ajcn.114.085720

6. Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C, Steiling H, Williamson G, Crozier A (2009) Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. Drug Metab Dispos 37 (8):1749-1758. doi: 10.1124/dmd.109.028019

7. Stalmach A, Mullen W, Steiling H, Williamson G, Lean MEJ, Crozier A (2010) Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. Mol Nutr Food Res 54 (3):323-334. doi: 10.1002/mnfr.200900194

8. Stalmach A, Williamson G, Crozier A (2014) Impact of dose on the bioavailability of coffee chlorogenic acids in humans. Food Funct 5 (8):1727-1737. doi:10.1039/c4fo00316k

9. Williamson G, Clifford MN Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. Biochem Pharmacol 39:24-39. doi: 10.1016/j.bcp.2017.03.012

10. Manach C, Milenkovic D, Van de Wiele T, Rodriguez-Mateos A, de Roos B, Garcia-Conesa MT, Landberg R, Gibney ER, Heinonen M, Tomas-Barberan F, Morand C (2016) Addressing the inter-individual variation in response to consumption of plant food bioactives - towards a better understanding of their role in healthy ageing and cardiometabolic risk reduction. Mol Nutr Food Res 61 (6). doi:10.1002/mnfr.201600557

11. Setchell KD, Clerici C (2010) Equol: history, chemistry, and formation. J Nutr 140 (7):1355S-1362S. doi:10.3945/jn.109.119776

12. Bolca S, Possemiers S, Maervoet V, Huybrechts I, Heyerick A, Vervarcke S, Depypere H, De Keukeleire D, Bracke M, De Henauw S, Verstraete W, Van de Wiele T (2007) Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: A dietary intervention trial with fifty healthy post-menopausal Caucasian women. Br J Nutr 98 (5):950-959. doi:10.1017/s0007114507749243

13. Hazim S, Curtis PJ, Schar MY, Ostertag LM, Kay CD, Minihane AM, Cassidy A (2016) Acute benefits of the microbial-derived isoflavone metabolite equol on arterial stiffness in men prospectively recruited according to equol producer phenotype: a double-blind randomized controlled trial. Am J Clin Nutr 103 (3):694-702. doi:10.3945/ajcn.115.125690

14. Tomás-Barberán FA, González-Sarrías A, García-Villalba R, Núñez-Sánchez MA, Selma MV, García-Conesa MT, Espín JC (2017) Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. Mol Nutr Food Res 61 (1). doi:10.1002/mnfr.201500901

15. Gonzalez-Sarrias A, Garcia-Villalba R, Romo-Vaquero M, Alasalvar C, Orem A, Zafrilla P, Tomas-Barberan FA, Selma MV, Espin JC (2017) Clustering according to urolithin metabotype explains the interindividual variability in the improvement of cardiovascular risk biomarkers in overweight-obese individuals consuming pomegranate: A randomized clinical trial. Mol Nutr Food Res 61 (5). doi:10.1002/mnfr.201600830

16. Selma MV, Gonzalez-Sarrias A, Salas-Salvado J, Andres-Lacueva C, Alasalvar C, Orem A, Tomas-Barberan FA, Espin JC (2017) The gut microbiota metabolism of pomegranate or walnut ellagitannins yields two urolithin-metabotypes that correlate with cardiometabolic risk biomarkers: Comparison between normoweight, overweight-obesity and metabolic syndrome. Clin Nutr S0261-5614 (17): 30103-6. doi:10.1016/j.clnu.2017.03.012

17. Tomas-Barberan F, García-Villalba R, Quartieri A, Raimondi S, Amaretti A, Leonardi A, Rossi M (2013) In vitro transformation of chlorogenic acid by human gut microbiota. Mol Nutr Food Res 58(5):1122-1131. doi:10.1002/mnfr.201300441

18. Del Rio D, Calani L, Cordero C, Salvatore S, Pellegrini N, Brighenti F (2010) Bioavailability and catabolism of green tea flavan-3-ols in humans. Nutrition 26 (11-12):1110-1116. doi: 10.1016/j.nut.2009.09.021

19. Brindani N, Mena P, Calani L, Benzie I, Choi SW, Brighenti F, Zanardi F, Curti C, Del Rio D (2017) Synthetic and analytical strategies for the quantification of phenyl-gamma-valerolactone conjugated metabolites in human urine. Mol Nutr Food Res 61 (9). doi:10.1002/mnfr.201700077

20. Curti C, Brindani N, Battistini L, Sartori A, Pelosi G, Mena P, Brighenti F, Zanardi F, Del Rio D (2015) Catalytic, Enantioselective Vinylogous Mukaiyama Aldol Reaction of Furan-Based Dienoxy Silanes: A Chemodivergent Approach to γ-Valerolactone Flavan-3-ol Metabolites and δ-Lactone Analogues. Adv Synth Catal 357 (18):4082-4092. doi:10.1002/adsc.201500705

21. Westerhuis JA, van Velzen EJJ, Hoefsloot HCJ, Smilde AK (2008) Discriminant Q2 (DQ2) for improved discrimination in PLSDA models. Metabolomics 4 (4):293-296. doi:10.1007/s11306-008-0126-2

22. Acharjee A, Finkers R, Visser RG, Maliepaard C (2013) Comparison of regularized regression methods for omics data. Metabolomics 3:126-134. doi:10.4172/2153-0769.1000126

23. Edmands WMB, Ferrari P, Rothwell JA, Rinaldi S, Slimani N, Barupal DK, Biessy C, Jenab M, Clavel-Chapelon F, Fagherazzi G, Boutron-Ruault MC, Katzke VA, Kühn T, Boeing H, Trichopoulou A, Lagiou P, Trichopoulos D, Palli D, Grioni S, Tumino R, Vineis P, Mattiello A, Romieu I, Scalbert A (2015) Polyphenol metabolome in human urine and its association with intake of polyphenol-rich foods across European countries. Am J Clin Nutr 102 (4):905-913. doi:10.3945/ajcn.114.101881

24. Ludwig IA, Mena P, Calani L, Cid C, Del Rio D, Lean MEJ, Crozier A (2014) Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? Food Funct 5 (8):1718-1726. doi:10.1039/c4fo00290c

25. Zamora-Ros R, Rothwell JA, Scalbert A, Knaze V, Romieu I, Slimani N, Fagherazzi G, Perquier F, Touillaud M, Molina-Montes E, Huerta JM, Barricarte A, Amiano P, Menéndez V, Tumino R, de Magistris MS, Palli D, Ricceri F, Sieri S, Crowe FL, Khaw KT, Wareham NJ, Grote V, Li K, Boeing H, Förster J, Trichopoulou A, Benetou V, Tsiotas K, Bueno-de-Mesquita HB, Ros M, Peeters PH, Tjønneland A, Halkjær J, Overvad K, Ericson U, Wallström P, Johansson I, Landberg R, Weiderpass E, Engeset D, Skeie G, Wark P, Riboli E, González CA (2013) Dietary intakes and food sources of phenolic acids in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Br J Nutr 110 (8):1500-1511. doi: 10.1017/S0007114513000688

26. Roowi S, Stalmach A, Mullen W, Lean ME, Edwards CA, Crozier A (2010) Green tea flavan-3-ols: Colonic degradation and urinary excretion of catabolites by humans. J Agric Food Chem 58 (2):1296-1304. doi: 10.1021/jf9032975

27. van der Hooft JJ, de Vos RC, Mihaleva V, Bino RJ, Ridder L, de Roo N, Jacobs DM, van Duynhoven JP, Vervoort J (2012) Structural elucidation and quantification of phenolic conjugates present in human urine after tea intake. Anal Chem 84 (16):7263-7271. doi:10.1021/ac3017339

28. Urpi-Sarda M, Monagas M, Khan N, Llorach R, Lamuela-Raventós RM, Jáuregui O, Estruch R, Izquierdo-Pulido M, Andrés-Lacueva C (2009) Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. J Chromatogr A 1216 (43):7258-7267. doi: 10.1016/j.chroma.2009.07.058

29. Urpi-Sarda M, Boto-Ordóñez M, Queipo-Ortuño MI, Tulipani S, Corella D, Estruch R, Tinahones FJ, Andres-Lacueva C (2015) Phenolic and microbial-targeted metabolomics to discovering and evaluating wine intake biomarkers in human urine and plasma. Electrophoresis 36 (18):2259-2268. doi:10.1002/elps.201400506

30. Ulaszewska MM, Trost K, Stanstrup J, Tuohy KM, Franceschi P, Chong MFF, George T, Minihane AM, Lovegrove JA, Mattivi F (2016) Urinary metabolomic profiling to identify biomarkers

of a flavonoid-rich and flavonoid-poor fruits and vegetables diet in adults: the FLAVURS trial. Metabolomics 12 (2):1-22. doi:10.1007/s11306-015-0935-z

31. Meselhy MR, Nakamura N, Hattori M (1997) Biotransformation of (-)-epicatechin 3-O-gallate by human intestinal bacteria. Chem Pharm Bull 45 (5):888-893. doi:

32. Ward NC, Croft KD, Puddey IB, Hodgson JM (2004) Supplementation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. J Agric Food Chem 52 (17):5545-5549. doi:10.1021/jf049404r

33. van Velzen EJJ, Westerhuis JA, Grün CH, Jacobs DM, Eilers PHC, Mulder TP, Foltz M, Garczarek U, Kemperman R, Vaughan EE, van Duynhoven JPM, Smilde AK (2014) Population-based nutrikinetic modeling of polyphenol exposure. Metabolomics: 10:1059-73. doi: 10.1007/s11306-014-0645-y

34. Cueva C, Sánchez-Patán F, Monagas M, Walton GE, Gibson GR, Martín-Álvarez PJ, Bartolomé B, Moreno-Arribas MV (2013) In vitro fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: Changes in microbial groups and phenolic metabolites. FEMS Microbiol Ecol 83 (3):792-805. doi:10.1111/1574-6941.12037

35. Muñoz-González I, Jiménez-Girón A, Martín-Álvarez PJ, Bartolomé B, Moreno-Arribas MV (2013) Profiling of microbial-derived phenolic metabolites in human feces after moderate red wine intake. J Agric Food Chem 61 (39):9470-9479. doi:10.1021/jf4025135

36. Takagaki A, Nanjo F (2015) Bioconversion of (-)-epicatechin, (+)-epicatechin, (-)-catechin, and (+)-catechin by (-)-epigallocatechin-metabolizing bacteria. Biol Pharm Bull 38 (5):789-794.doi: 10.1248/bpb.b14-00813

37. Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C, Scalbert A (2003) Metabolism of dietary procyanidins in rats. Free Radic Biol Med 35 (8):837-844. doi:10.1016/s0891-5849(03)00394-0

38. Kutschera M, Engst W, Blaut M, Braune A (2011) Isolation of catechin-converting human intestinal bacteria. J Appl Microbiol 111 (1):165-175. doi:10.1111/j.1365-2672.2011.05025.x

39. Zamora-Ros R, Knaze V, Rothwell JA, Hémon B, Moskal A, Overvad K, Tjønneland A, Kyrø C, Fagherazzi G, Boutron-Ruault M-C, Touillaud M, Katzke V, Kühn T, Boeing H, Förster J, Trichopoulou A, Valanou E, Peppa E, Palli D, Agnoli C, Ricceri F, Tumino R, de Magistris MS, Peeters PHM, Bueno-de-Mesquita HB, Engeset D, Skeie G, Hjartåker A, Menéndez V, Agudo A, Molina-Montes E, Huerta JM, Barricarte A, Amiano P, Sonestedt E, Nilsson LM, Landberg R, Key TJ, Khaw K-T, Wareham NJ, Lu Y, Slimani N, Romieu I, Riboli E, Scalbert A (2016) Dietary polyphenol intake in Europe: the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Eur J Nutr 55 (4):1359-1375. doi:10.1007/s00394-015-0950-x

40. Bai W, Wang C, Ren C (2014) Intakes of total and individual flavonoids by US adults. Int J Food Sci Nutr 65 (1):9-20. doi: 10.3109/09637486.2013.832170

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

[1]A 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 ± 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 R^2 and Q^2, 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.
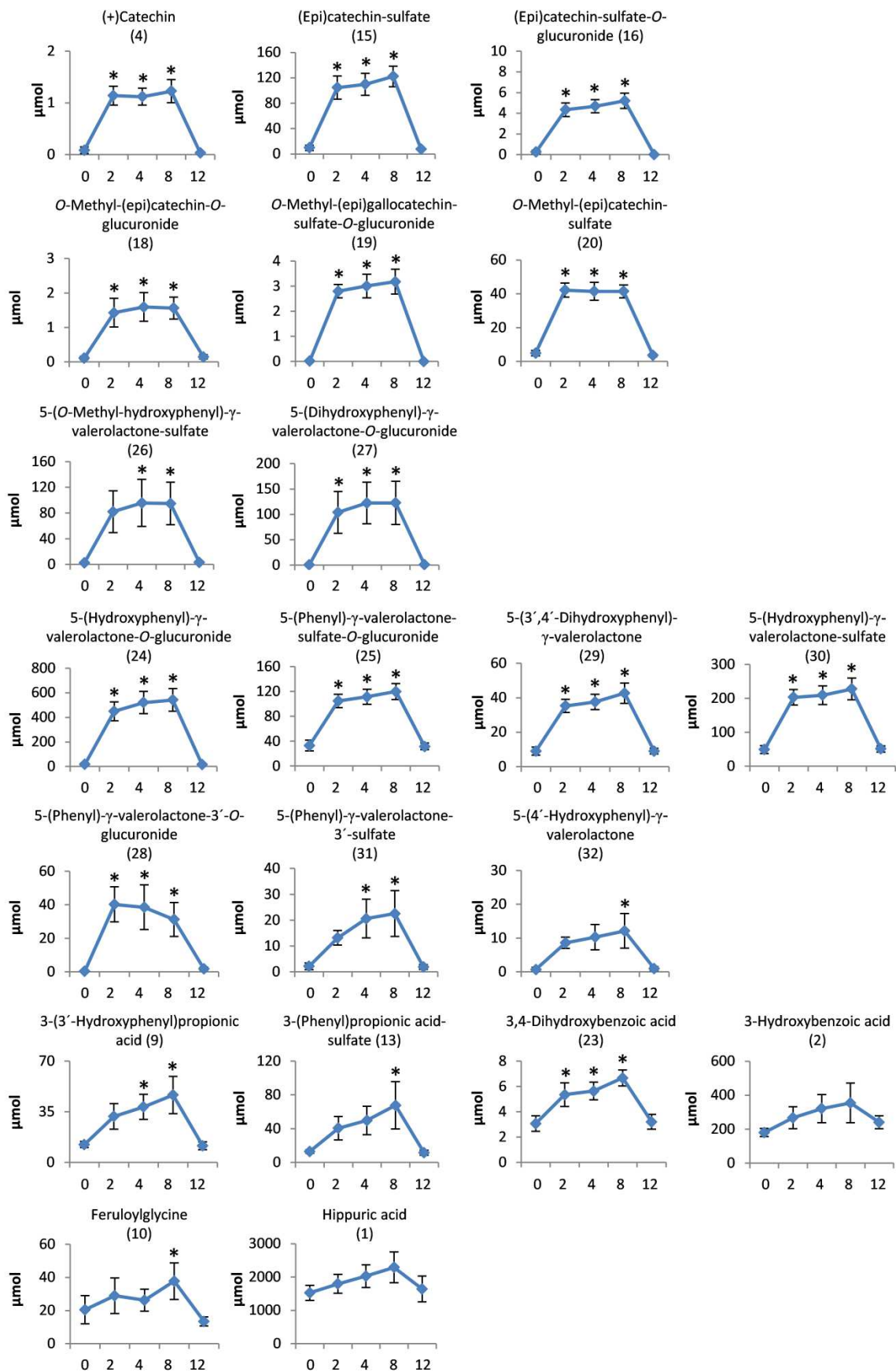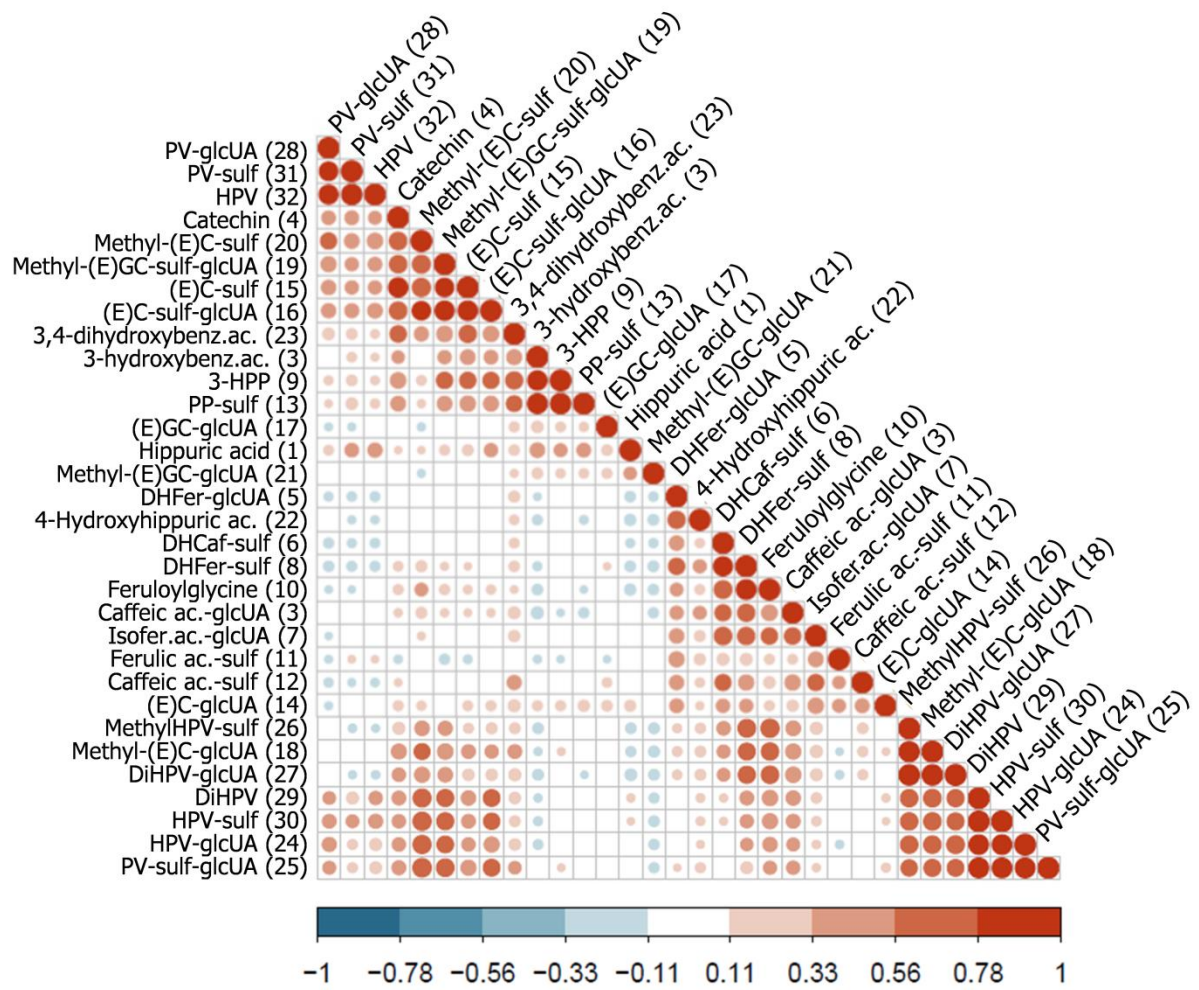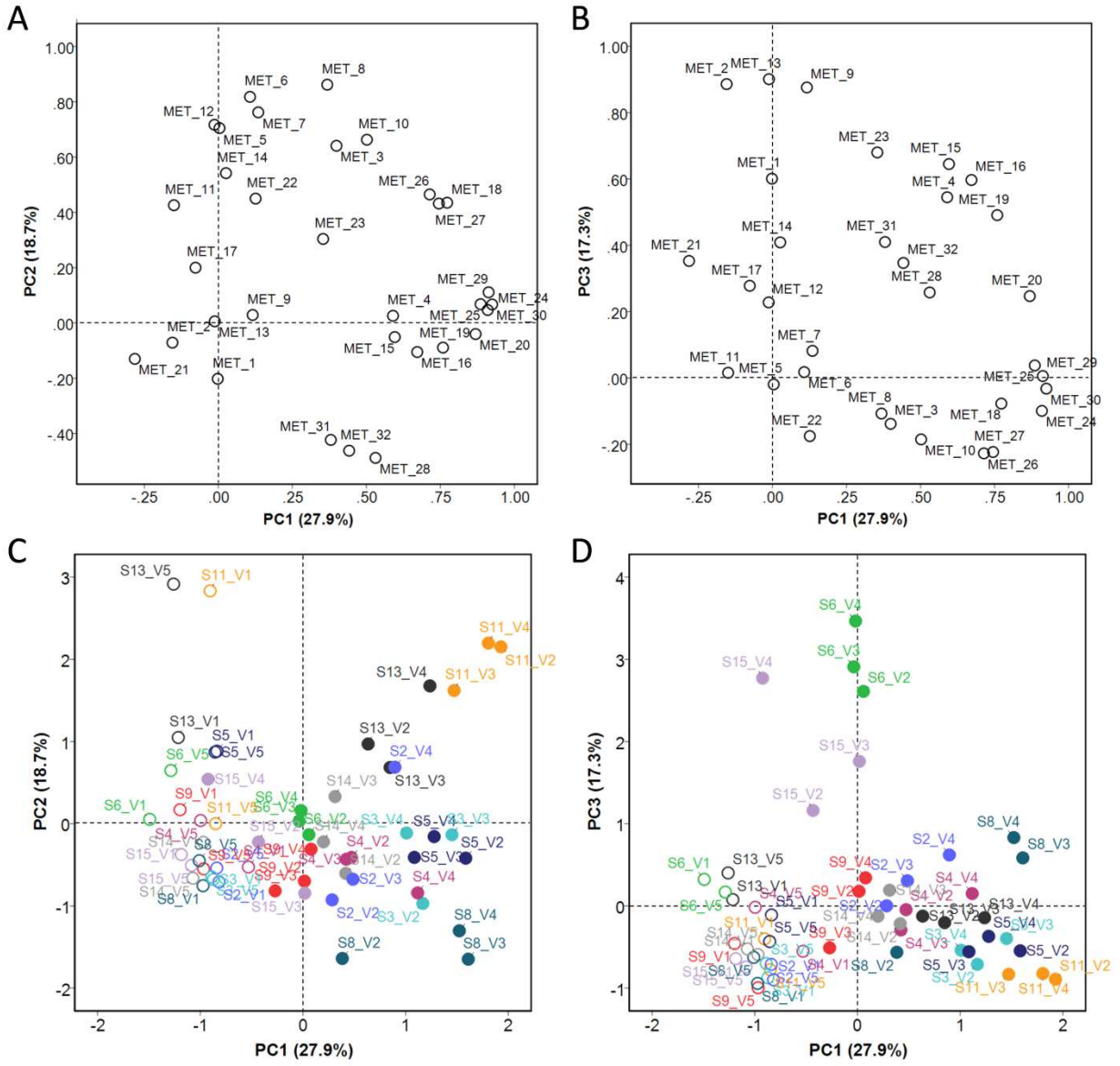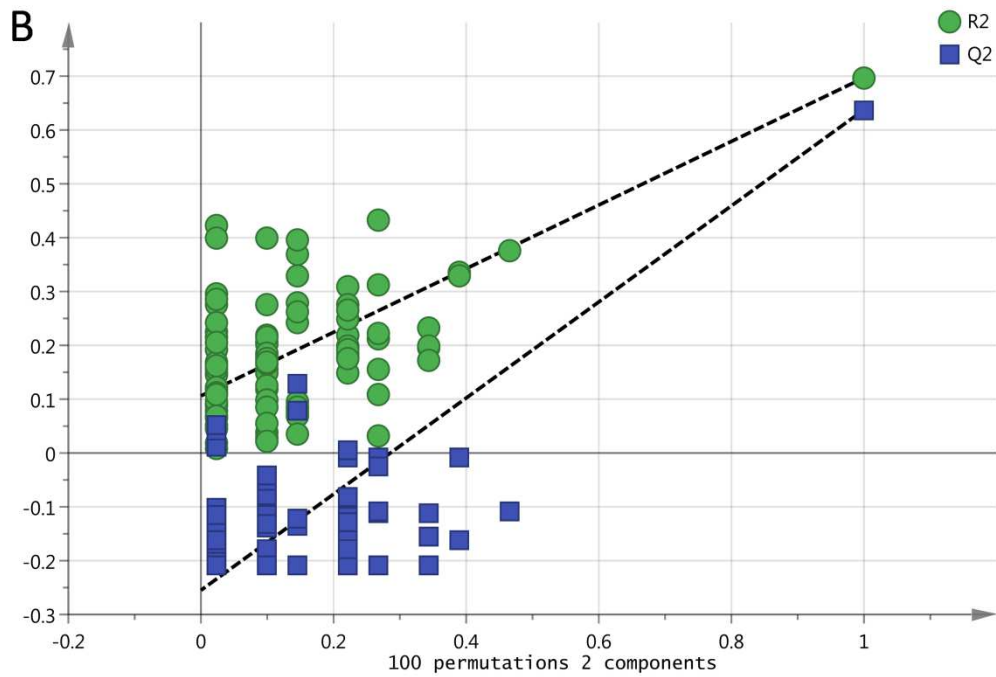
Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

A Trihydroxyphenyl-γ-valerolactone

B Dihydroxyphenyl-γ-valerolactone

C Monohydroxyphenyl-γ-valerolactone

D Hydroxyphenyl propionic acid