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3 FEASIBILITY AND APPLICATION OF LIQUID-LIQUID EXTRACTION 4 COMBINED WITH GAS CHROMATOGRAPHY-MASS 5 SPECTROMETRY FOR THE ANALYSIS OF PHENOLIC ACIDS FROM 6 GRAPE POLYPHENOLS DEGRADED BY HUMAN FAECAL 7 MICROBIOTA

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19 **Abstract**

20 In this study the feasibility of a LLE-GC-EI-MS method for the analysis of 43 phenolic acids
21 belonging to different chemical structure families which have been described in the literature as
22 microbial-derived metabolites after consumption of dietary polyphenols was proved. In addition,
23 the method was applied for the characterisation of phenolic metabolites resulting from the
24 incubation, in anaerobic conditions, of a commercial grape seed extract (GSE) and their
25 corresponding flavan-3-ol monomeric (GSE-M) and oligomeric (GSE-O) fractions with human
26 faeces from healthy volunteers (n=3). The method showed average values of repeatability and
27 reproducibility of 5.0 % and 6.3 %, respectively, adequate and low detection (1.8-30.8 μg L^{-1})
28 and quantification limits (6.0-102.8 μg L^{-1}) and good recovery values (95 %, as average value).
29 A total of 27 phenolic acids were identified in the faecal solutions after incubation with the
30 grape seed extracts. In general, faecal samples incubated with GSE and GSE-M (monomeric
31 fraction) yield a higher formation of phenolic acids compared to the samples incubated with the
32 oligomer fraction (GSE-O).

33

34

35 **Key words:** LLE-GC-EI-MS, phenolic metabolites, gut microbiota, grape polyphenols.

36 **Introduction**

37

38 Flavanols or flavan-3-ols are a unique class of flavonoids that are present as monomers, 39 oligomers and polymers in the human diet. Oligomers and polymers are called 40 proanthocyanidins or condensed tannins, and they are some of the most abundant phenolic 41 compounds in some foods such as cacao, tea and wine (Gu et al., 2004). They are mainly made 42 up of (epi)catechin or (epi)gallocatechin units that are known as procyanidins or 43 prodelphinidins, respectively (Donovan, Manach, Rios, Morand, Scalbert & Rémésy, 2002).

44

45 The most common subclass of proanthocyanidins is the procyanidins. Their monomeric 46 units [(+)-catechin and (-)-epicatechin)] are linked through a C4-C8 or C4-C6 bond (B-Type), 47 which can coexist with an additional C2-O-C7 bond (A-Type). Absorption of procyanidins after 48 food intake has been reported in several earlier studies (Donovan et al., 2002; Groenewoud & 49 Hundt, 1986; Van'T Slot, Mattern, Rzeppa, Grewe & Humpf, 2010) and it is known that only 50 procyanidin dimers are believed to be absorbed intact, although with lower efficiency than the 51 monomeric units (Appeldoorn, Vincken, Aura, Hollman & Gruppen, 2009). Due to the low 52 absorption of intact procyanidins, gut microbial metabolism might play an important role. In 53 fact, monomeric flavan-3-ols are known to be degraded in the gut by colonic microbiota into 54 several phenolic acids with varying hydroxylation patterns, namely, phenylvaleric, 55 phenylpropionic, phenylacetics, benzoic and hippuric acids (Ríos et al., 2003), and other non-56 phenolic catabolites (Das & Griffiths, 1969). These metabolites may selectively influence the 57 growth of intestinal bacteria (both beneficial and pathogenic bacteria) (Cueva et al., 2010), thus 58 the consumption of foods rich in procyanidins, like wine, might have a direct impact in human 59 gut health (Stoupi, Williamson, Drynan, Barron & Clifford, 2010).

60

61 In trying to know more in deep about the absorption and metabolism of these 62 procyanidins, *in vitro* fermentations can be a useful tool to carry out these studies because there

63 are easier to perform than *in vivo* studies. They have the additional advantage that relatively
64 high concentrations of metabolites, not yet affected by absorption and metabolism, are present,
65 which facilitates detection (Appeldoorn et al., 2009). In addition, it has been indicated, that
66 studies performed with animals may result in substantially different bioconversions compared to
67 humans, as has been shown for (+)-catechin and (-)-epicatechin (Scalbert, Manach, Morand,
68 Rémésy & Jiménez, 2005; Williams, Eyton-Jones, Farnworth, Gallagher & Provan, 2002).
69

70 Therefore, the identification and quantification of phenolic metabolites in physiological
71 samples is becoming an outstanding task in different fields, such as food science, nutrition and
72 clinic. The availability of sensitive and roughness analytical tools is an aspect of great
73 importance for their characterization. Although there are many studies based on the use of
74 Liquid Cromatography (LC) coupled to mass spectrometry techniques (MS) for the analysis of
75 these compounds (Appeldoorn et al., 2009; Aura et al., 2005; Gonthier et al., 2006; Gonthier,
76 Rios, Verny, Rémésy & Scalbert, 2003b; Urpí-Sarda et al., 2009), Gas Chromatography-MS
77 (GC-MS) is a complementary analytical technique that can be also adequate for their analysis
78 (Gonthier et al., 2003a; Rechner et al., 2004; Ríos et al., 2003). Recently, Grün and collaborators
79 (Grün et al., 2008) have published a method for the analysis of phenolic acids in physiological
80 samples based on the use of liquid-liquid extraction (LLE). Since then, the use of this isolation
81 technique combined with GC-MS analysis is increasing (Gross et al., 2010; Peters, Janssen &
82 Vivo-Truyols, 2010; van Dorsten, Grün, van Velzen, Jacobs, Draijer & van Duynhoven, 2010).
83 However, as far as we know, none study in the scientific literature has been devoted to show the
84 analytical performance of this method and its feasibility for a large group of phenolic acids
85 belonging to different chemical structure families which could be present in physiological
86 samples.
87

88 Therefore, the aim of the present work has been to show the analytical performance of
89 the LLE-GC-EI-MS for the quantification of a wide range of phenolic metabolites belonging to

90 different phenolic acid groups that might come from the degradation of dietary polyphenols by
91 gut microbiota, and to apply it for the characterisation of the phenolic metabolites resulting from
92 the incubation, in anaerobic conditions, of a commercial grape seed extract and their
93 corresponding flavan-3-ol monomeric and oligomeric fractions with faecal samples from three
94 healthy volunteers.
95
96
97 **2. Materials and methods**
98
99 2.1. Chemicals, reagents and standard solutions
100
101 For this study, 43 phenolic compounds which belong to seven different chemical
102 structure families that have been described in the literature as phenolic microbial-derived
103 metabolites were selected (**Table 1**). Phenolic standards were provided by Sigma-Aldrich
104 Chemical Co (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) and Extrasynthèse
105 (Genay, France) and had purity greater than 98 %. A standard solution formed with the 43
106 compounds at a concentration of 250 mg L^{-1} was prepared in ethanol (Panreac, Spain). In order
107 to determine the performance characteristics of the method, working solutions were prepared by
108 diluting different amounts of the standard solution in ethanol. The concentration of the working
109 solutions ranged between 0.1-25 mg L^{-1}.
110
111 2.2 Liquid-liquid extraction (LLE)
112
113 The liquid-liquid extraction procedure was basically performed as described by Grün et
114 al (2008). To 500 µL of sample (aqueous solution of 43 phenolic compounds or faecal samples
115 spiked with the same compounds), 65 µL of 1*M* hydrochloric acid (Prolabo, France)) were
116 added for acidifying the samples (pH 2-3) prior to the extraction. After the addition of the

117 internal standard (50 µL of 100 µg mL^{-1} *trans*-cinnamic acid-d7 (Sigma-Aldrich, Switzerland) in
118 1:1 methanol/water (v/v)), the samples were kept at 4 ºC for 10 min. Two mL of ethyl acetate
119 were added, the samples were vortexed for 30 s, and centrifuged at 3000 g for 10 min. The
120 supernatants were transferred and the extraction was repeated twice. The resulting extracts were
121 combined and concentrated under nitrogen stream. Samples were further dried by the
122 subsequent addition and evaporation of 1 mL of dichloromethane. To the samples, 100 µL of
123 BSTA/TMCS (N,O-Bis(trimethylsilyl)trifluoroacetamide with 10 % trimethylchlorosilane)
124 (Sigma-Aldrich, Switzerland) was added for the formation of the corresponding silyl-derived
125 compounds. The samples were incubated at 90 ºC for 30 min. After cooling down to room
126 temperature, 100 µL of hexane were added to 100 µL of the derivatized samples and the
127 mixtures were vortex-mixed. Then, GC-MS analysis was carried out. Five levels of
128 concentrations of each phenolic acid (0.1, 1, 5, 10, 25 mg L^{-1}) were tested in duplicate.
129
130 2.3 Gas chromatography-Mass spectrometry analysis
131
132 An Agilent 6890N GC system (Agilent, Palo Alto, CA) with a split/splitless injector and
133 interfaced with an Agilent 5973N mass spectrometer was used for sample analysis. The injector
134 was set at 280 ºC. An Agilent MSD ChemStation Software (E.02.00.493 version) was used to
135 control the system. For separation, an HP-5MS fused silica capillary column (30 m x 0.25 mm x
136 0.25 µm) Quadrex Co. (Woodbridge, CT) was used. Helium was the carrier gas (1 mL min^{-1}).
137 One µL of the sample was injected in split mode at a split ratio of 20:1. The GC conditions were
138 as follows: initial temperature of 45 ºC for 1 min to 100 ºC (held for 5.5 min) at 10 ºC/min.
139 Then, the temperature was increased up to 300 ºC at 7.5 ºC/min and held for 2.5 min giving a
140 total run time of 41 min.
141
142 For the MS system, the temperatures of the transfer line, quadrupole and ionization
143 source were 270, 150 and 230 ºC respectively; electron impact mass spectra were recorded at

144 70eV ionization voltages and the ionization current was 10 μA. The acquisitions were performed
145 in scan mode (from 35 to 450 amu). Peak identification was carried out by analogy of mass
146 spectra with those of the mass library (Wiley 6.0 and Nist 2.0) and comparing the calculated
147 retention times and the mass spectra with those corresponding to commercial standards.
148 Quantitative data were obtained by calculating the relative areas (area of the quantification ion
149 of the phenolic compound / area of the quantification ion of the internal standard (*trans*-
150 cinnamic acid-d7). Calibration curves representing relative areas of quantification ions vs.
151 concentration were built for quantification purposes.
152
153 2.4 Fermentation of phenolic extracts by faecal microorganisms
154
155 Faecal samples were collected from three separate individuals (females). All volunteers
156 were in good health, they were nonsmokers, aged from 25 to 30 years, had not ingested
157 antibiotics for at least 6 months before the study, and had no history of any gastrointestinal
158 disease. Samples were collected, on site, on the day of the experiment and were used
159 immediately. The *in vitro* batch culture fermentations were basically performed as described by
160 Tzounis and collaborators (Tzounis et al., 2008). The samples were diluted 1:10 (w/v) with
161 anaerobic phosphate buffer (1 M; pH 7.4) and homogenized in a stomacher for 2 min. Resulting
162 faecal slurries from each individual (i.e. faecal samples were not pooled) were used to inoculate
163 the batch-culture vessels.
164
165 Three commercial extracts kindly supplied by Dr. Piriou (Les Dérives Resiniques &
166 Terpéniques, S.A., France) were used in this study: a grape seed extract (Vitaflavan®) (GSE)
167 and two rich-monomeric (GSE-M) and rich-oligomeric (GSE-O) fractions obtained from
168 Vitaflavan®. According to the producer, the monomeric fraction contained around 58 %
169 monomers and 42 % procyanidins whereas the oligomeric fraction contained around 7 %
170 monomers and 93 % procyanidins. More detailed composition of the grape seed extract and their

171 fractions is reported in Monagas, Hernández-Ledesma, Garrido, Martín-Álvarez, Gómez-
172 Cordovés & Bartolomé, (2005).
173
174 Batch-culture fermentation vessels (300 mL volume; one vessel per treatment group)
175 were sterilised and filled with 135 mL basal nutrient medium (peptone water (2 g L^{-1}), yeast
176 extract (2 g L^{-1}), NaCl (0.1 g L^{-1}), K_2HPO_4 (0.04 g L^{-1}), KH_2PO_4 (0.04 g L^{-1}), $NaHCO_3$ (2 g L^{-1}),
177 $MgSO_4.7H_2O$ (0.01 g L^{-1}), $CaCl_2.6H_2O$ (0.01 g L^{-1}), Tween 80 (2 mL L^{-1}), haemin (50 mg L^{-1}),
178 vitamin K1 (10 mL L^{-1}), L-cysteine (0.5 g L^{-1}), bile salts (0.5 g L^{-1}), resazurin (1 mg L^{-1}) and
179 distilled water. The pH of the basal medium was adjusted to 7 and autoclaved before dispensing
180 it into the vessels. Medium was then gassed overnight with O_2-free N_2. Before the addition of
181 faecal slurry samples, the temperature of the basal nutrient medium was set at 37 ºC by using a
182 circulating water-bath and the pH was maintained at 6.8, using an Electrolab pH controller, in
183 order to mimic conditions located in the distal region of the human large intestine (anaerobic; 37
184 ºC; pH about 6.8). Vessels inoculated with 15 mL of faecal slurry (1:10, w/v) and with the three
185 extracts were tested. As a control, incubations with human faecal slurries were carried out
186 without the addition of any extract. Samples were collected at different times (30 minutes and
187 10 hours). Prior to analysis, the samples were centrifuged at 13000 rpm for 10 min at room
188 temperature and the supernatant was passed through 0.22 μm PVDF filters (OlimPeak,
189 Tecknokroma). The supernatant was stored at -80 ºC until the extraction.
190
191
192 2.6 Statistical analysis
193
194 The statistical methods used for the data analysis were: Linear Regression to establish
195 the calibration curves of each phenolic compound and the Lack of Fit test to judge the adequacy
196 of the models; Minimal Differences Tests (LSD) to check differences in mean and Cluster
197 Analysis to search how the samples are grouped. Statgraphics Centurion XV, version 15.2

198 (2006, Statistical Graphics Corporation, Manugistics, Inc., MD, www.statgraphics.com) and
199 Statistica of Windows, version 7.1 (StatSoft Inc., Tulsa, Ok 74104, USA, www.statsoft.com9)
200 were used for data processing.

201

202 **3. Results and discussion**

203

204 3.1 Linear regressions

205

206 Data about retention times, molecular masses and characteristics ions used for
207 quantitative analysis of the 43 phenolic compounds and the internal standard (IS) are reported in
208 **Table 1**. For each compound, a linear regression of the relative area (area of the quantification
209 ion of the phenolic compound / area of the quantification ion of the internal standard) versus
210 concentration was calculated to determine the linearity of the analytical method, using two
211 replicates of the aqueous solutions composed by the mix of 43 phenolic compounds at five
212 levels of concentration (0.1, 1, 5, 10 and 25 mg L^{-1}). These concentration levels covered the
213 concentration ranges expected for the phenolic compounds found in physiological samples
214 (Grün et al., 2008). To judge the adequacy of the linear models, the F-ratio for lack of fit was
215 calculated (Massart, 1990). The regression results and the linear ranges are shown in **Table 2**.
216 As can be seen, the linear ranges were in general quite wide for most of the compounds. For
217 most of the compounds they were between 0.1-25 mg L^{-1} except for *p*-coumaric, 4-
218 hydroxymandelic and 4-hydroxy-3-methoxy mandelic acids that showed a narrow range
219 between 0.1-10 mg L^{-1}, and for the acids, phenylacetic, 4-methoxyphenylacetic and 4-
220 hydroxyhippuric acids, which were between 1-25 mg L^{-1}.

221

222 Most of the phenolic compounds belonging to the phenylpropionic, phenylacetic,
223 cinnamic, mandelic and benzoic acids showed a good linearity, as it can be seen by checking the
224 determination coefficients (R^2), which in general showed values > 99 % (**Table 2**). However,

225 other compounds, such as 4-hydroxyhippuric acid, catechol/pyrocatechol, phloroglucinol and
226 pyrogallol showed lower R^2 values, which could be related to their lower molecular weight and
227 therefore their losses during the derivatization step. In addition, the residual standard deviation
228 expressed as a percentage of the mean value (CV %) was lower than 15 % for most of the
229 phenolic compounds studied, which showed the adequacy of the calculated regressions models
230 (data not shown).

231

232 3.2. Analytical quality of the LLE-GC-EI-MS method

233

234 3.2.1 Precision

235

236 The intra-day method repeatability was determined by analyzing in triplicate and in the
237 same day the aqueous solution containing the 43 phenolic acids and the internal standard.
238 Results were expressed as relative standard deviation (RSD) and they are shown in **Table 3**.
239 Relative standard deviations (RSD) ranged between 0.5 to 14.9 %, except for 4-
240 hydroxymandelic acid, which showed an RSD of 16.0 %. In fact, mandelic acids showed the
241 highest RSD values, while phenylpropionic and phenylacetic acids exhibited the lowest. The
242 average value for all compounds was 5.0 %. The inter-day method reproducibility was
243 determined in the same way but analyses were performed in three different days. The RSD for
244 all the phenolic compounds ranged between 2.1 % for gallic acid and 14.2 % for 4-
245 hydroxymandelic acid. The average value for all compounds was above 6.3 %, except for *p*-
246 coumaric acid, which exhibited RSD of 29.2 %.

247

248

249 3.2.2 Limits of detection (LOD) and quantification (LOQ)

250

251 The LOD and LOQ were calculated as the lowest concentration yielding a signal-to-
252 noise ratio of at least 3:1 and 10:1, respectively, and were determined for the 43 phenolic acid
253 standards in a faecal sample spiked with the phenolic mixture at low concentration (1 mg L^{-1}).
254 The LOD values ranged between 1.81 μg L^{-1} for 4-hydroxy-3-methoxy-mandelic acid to 30.84
255 μg L^{-1} for trimethoxycinnamic acid (**Table 3**). The lowest values corresponded to mandelic acids
256 and the highest to phenylacetic acids. In addition, the LOQ ranged from 6.02 to 102.81 μg L^{-1}
257 for 4-hydroxy-3-methoxy-mandelic and trimethoxycinnamic acids, respectively. Mandelic acids
258 showed the lowest values (6.02 to 28.34 μg L^{-1}) and phenylacetic acids showed the highest
259 values (30.38 to 93.44 μg L^{-1}). These values were low enough to use the LLE-GC-EI-MS
260 method for the quantification of all the studied analytes in faecal samples (Gill et al., 2010;
261 Jenner, Rafter & Halliwell, 2005).

262

263 3.2.3 Recovery

264

265 Extraction recovery was calculated after spiking faecal samples from the same individual
266 at two levels of concentration (1 and 10 mg L^{-1}) with the mixture of 43 phenolic acids plus the
267 internal standard. All analyses were carried out in duplicate. Recovery average obtained for all
268 the phenolic compounds was 95 % (**Table 3**), which showed a low effect of the matrix sample
269 on the phenolic acid analysis by using the proposed methodology. Differences in the recovery
270 between the different phenolic acid families were observed. Phenylpropionic, phenylacetic and
271 benzoic acids showed higher recovery values compared to cinnamic and mandelic acids.
272 Exceptionally, some compounds such as phloroglucinol and pyrogallol showed very low or very
273 high recovery values respectively. The low recovery of phloroglucinol could be associated to the
274 strong interaction of this compound with the matrix sample, because of their low affinity by the
275 extraction solvent.

276

277

278 3.3. Application of LLE-GC-EI-MS method to faecal samples fermented with three commercial
279 grape seed phenolic extracts
280
281 The LLE-GC-EI-MS method was applied to know the phenolic metabolites produced
282 from the degradation of three types of phenolic grape seed extracts by faecal microbiota from
283 three individuals. The extracts essayed were the whole grape extract (GSE) and its
284 corresponding purified fractions: rich in monomers (GSE-M) and in oligomers (GSE-O).
285 Moreover, control samples (C-S), consisting in the faeces of each individual without the
286 addition of phenolic extracts were also analyzed. The essays were performed in batch culture
287 systems, and the samples for phenolic metabolites analysis were collected from the *in vitro*
288 fermentation systems after 30 minutes and 10 hours of incubation.
289
290 **Figure 1** shows an example of the chromatograms corresponding to the reference phenolic
291 compounds (Figure 1a) and to the phenolic compounds identified in a faecal sample incubated
292 with the whole grape extract (GSE) and analyzed after 30 minutes of incubation (Figure 1b).
293 From the 43 phenolic compounds essayed, 27 were identified in the faecal samples but only 17
294 compounds were above the calculated limit of quantification (**Table 4**). The highest
295 concentration values corresponded to the phenylpropionic acids (1.65 mg L^{-1} in average),
296 phenylacetic acids (1.31 mg L^{-1}) and gallic acid (1.49 mg L^{-1}). Benzoic acids (except gallic
297 acid), showed the lowest concentration values (0.18 mg L^{-1}). This is in agreement with Gross
298 and collaborators (Gross et al., 2010), who found phenylpropionic and phenylacetic acids and
299 their corresponding hydroxylated derivatives as the major metabolites produced from
300 wine/grape polyphenols by faecal microbiota while benzoic acids were found in lower amounts.
301 On the other hand, although some mandelic and cinnamic acids (mandelic, 4-hydroxymandelic,
302 ferulic acid, among others) were identified in the samples, they could not be quantifying because
303 they gave very low responses, bellow the calculated LOQ. Some mandelic acids are known
304 urinary metabolites resulting from different dietary components. For example, 4-

305 hydroxymandelic acid is a natural occurring metabolite from the degradation of tyramine and/or
306 other similar biogenic amines (van Dorsten et al., 2010). But recently, and using a metabolomic
307 approach, this acid has been found as a strong urinary marker for the intake of polyphenols from
308 wine and grape juice (van Dorsten et al., 2010). In the present work, the detection of 4-
309 hydroxymandelic acid in the fermented samples with grape polyphenols extracts confirms its
310 formation by gut-microbial degradation.

311

312 The influence of the type of polyphenol extract in the formation of phenolic metabolites
313 can be evidenced when performing a cluster analysis. **Figure 2** shows the dendrogram obtained
314 after applying this treatment to the data. Herein, is possible clearly distinguishing between two
315 groups of samples. The first one included all the control samples (C-S) and most of the faecal
316 samples incubated with the polyphenol fraction rich in oligomeric procyanidins (GSE-O). The
317 second group contained the faecal samples supplemented with the whole polyphenol fraction
318 (GSE) and those incubated with the fraction with higher monomer content (GSE-M). These
319 results seemed to indicate a similar behaviour induced by the whole grape extract and the
320 purified monomeric fraction compared to the control and the oligomeric fraction.

321

322 The statistical test of least significant difference (LSD test) was also applied to compare
323 the means corresponding to the concentration of phenolic metabolites detected in the four types
324 of faecal systems (controls and faecal samples fermented with the three types of polyphenol
325 extracts) (**Table 4**). As can be seen, six metabolites showed differences depending on the type of
326 polyphenol extract essayed. Some of them, (e.g. 4-hydroxybenzoic acid, phloroglucinol, vanillic
327 acid) showed lower values in the control samples compared to the samples incubated with the
328 polyphenolic extracts, which shows the ability of human faecal microbiota to degrade grape
329 seeds polyphenols. In addition, others compounds such as catechol were detected in the samples
330 supplemented with the polyphenol extracts but absent in the control sample.

331

332 Nonetheless, for some compounds, the concentrations calculated in the samples
333 supplemented with the polyphenol grape extracts did not increase, or they were even higher in
334 the control samples. Compounds such as benzoic acid, phenyl acetic acid, phenylpropionic acid,
335 4-hydroxyphenyl acetic and 4-hydroxyphenylpropionic were already present in the faecal
336 samples without extract addition (C-S) likely because of the bioconversion of other polyphenol
337 sources from the diet of each individual. The lower amount of these metabolites detected in the
338 samples supplemented with the polyphenol extracts could be due to the inhibition produced by
339 the own extracts in the metabolic transformation of these compounds by the gut microbiota. The
340 inhibition ability of some diet polyphenols on specific gut microorganisms has been recently
341 shown (Cueva et al., 2010).

342 In addition, **Table 4** shows that faecal samples incubated with the whole extract (GSE),
343 and with the monomeric fraction (GSE-M) yield, in general, a higher formation of some
344 phenolic acids compared to the samples incubated with the oligomeric rich extract (GSE-O).
345 These compounds were phloroglucinol, vanillic acid, protocatechuic and gallic acids, among
346 others. These results seem to indicate that the monomers are likely more accessible to gut
347 microbiota than the polymeric procyanidins. The bioconversion of procyanidin monomers
348 (catechin and epicatechin) in *in vitro* systems to some phenolic metabolites such as
349 hydroxyphenylpropionic or hydroxyphenylvaleric acids derivatives has been already shown
350 (Aura, 2008). Our results are indeed in agreement with other published works in which a
351 decrease in the yield of formation of phenolic metabolites from procyanidins by gut microbiota
352 with an increase in the degree of polymerization has been observed (Deprez et al., 2000;
353 Gonthier et al., 2003b; Jenner et al., 2005; Ríos et al., 2003).
354

355 It was also observed some compounds exhibiting significant differences in their
356 concentration according to the type of extract added. While some of them increased in their
357 concentration, other decreased over time. For example, the concentration of protocatechuic
358 decreased with an increase in the incubation time. However, the concentration of other

359 compounds such as phloroglucinol, increased after 600 minutes (10 hours) of incubation. It is
360 known that this metabolite comes from quercetin (Aura, 2008) but also gallic acid can be
361 degraded to pyrogallol and it can be further degraded via phloroglucinol to butyrate and acetate
362 (Gross et al., 2010). Therefore, an increase in the incubation time might promote its degradation
363 by gut bacteria (Braune, Engst & Blaut, 2005). Nonetheless, it is important to underline, that the
364 incubation times selected for this study could not be optimal for monitoring the formation of
365 phenolic metabolites and more samplings points at different incubation times would be
366 necessary to see the influence of this factor. Moreover, differences in the composition of the
367 extract, or interindividual differences in microbiota performance may also affect the
368 bioconversion ratio, therefore, microbial bioconversion of dietary polyphenols cannot be
369 generalized. In fact, it is important to underline the great dispersion observed in the
370 concentration of some phenolic metabolites **(Table 4)** that was likely due to the large
371 interindividual variability in human microbiota, which could condition the phenolic metabolite
372 production. Multiple factors such as age, diet, intestinal health, etc, might influence the
373 composition of human microbiota (Cerdá, Tomás-Barberán & Espín, 2005; Roowi, Stalmach,
374 Mullen, Lean, Edwards And & Crozier, 2010). **Figure 3** illustrates this fact. Herein, a
375 representative example corresponding to the interindividual differences in the production of two
376 hydroxyphenyl acetic acids by gut microbiota can be seen. Therefore, different kinetics of the
377 polyphenol catabolism may contribute to the interindividual variability in the metabolite profiles
378 observed at specific sampling times. However, a large number of samples might be necessary to
379 evidence greater differences between the essayed extracts.

380

381 **4. Conclusions**

382

383 LLE-GC-EI-MS has been proved to be useful for the characterization and quantification of
384 phenolic metabolites from degradation of dietary polyphenols by faecal microbiota, in particular
385 of grape seed flavan-3-ols. In addition, we have found that gut microbiota have preference for

386 flavan-3-ol monomers than for procyanidin oligomers. However, large interindividual
387 differences in the bioconversion rate have been also noticed. Future works will be carried out
388 applying this LLE-GC-EI-MS methodology in intervention studies involving wine consumption
389 by a large and representative number of individuals.

390

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392

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401

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FIGURE CAPTIONS

Figure 1. Example showing the chromatograms corresponding to the mixture of silylated phenolic standards (1a) and to the phenolic metabolites identified in a faecal sample incubated with the whole grape extract (GSE) and collected after 30 minutes of incubation (1b). Peaks identification as in Table 1.

Figure 2. Dendrogram resulting from applying cluster analysis to the data corresponding to the concentration of phenolic metabolites analyzed in the faecal samples after the incubation with the different polyphenolic extracts. Samples are identified by a code formed by the type of extract essayed (**GSE:** commercial extract of grape seed, **GSE-O:** oligomeric fraction of procyanidins, **GSE-O:** monomeric fraction of procyanidin, **C-S:** control sample), the incubation times 30 and 600 minutes (10 hours) (00 and 10 in the diagram) and the different individuals (1, 2 and 3)

Figure 3. Example showing the interindividual differences in the formation of 3-hydroxyphenylacetic acid (on the left) and 4-hydroxyphenylacetic acid (on the right) in faecal samples supplemented with the monomeric fraction of the commercial polyphenolic grape seed extract after 10 hours of incubation.