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

Muñoz-González, C., Moreno-Arribas, M.V, Rodríguez-Bencomo, J.J., Cueva, C.,
Martín Álvarez, P.J., Bartolomé, B., Pozo-Bayón, M.A*

Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM).

C/ Nicolás Cabrera, 9, Campus de Cantoblanco, 28049 Madrid, Spain.

* corresponding author: mdelpozo@ifi.csic.es

Abstract

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

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

Introduction

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

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

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

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

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

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

different phenolic acid groups that might come from the degradation of dietary polyphenols by gut microbiota, and to apply it for the characterisation of the phenolic metabolites resulting from the incubation, in anaerobic conditions, of a commercial grape seed extract and their corresponding flavan-3-ol monomeric and oligomeric fractions with faecal samples from three healthy volunteers.

2. Materials and methods

2.1. Chemicals, reagents and standard solutions

For this study, 43 phenolic compounds which belong to seven different chemical structure families that have been described in the literature as phenolic microbial-derived metabolites were selected (**Table 1**). Phenolic standards were provided by Sigma-Aldrich Chemical Co (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) and Extrasynthèse (Genay, France) and had purity greater than 98 %. A standard solution formed with the 43 compounds at a concentration of 250 mg L⁻¹ was prepared in ethanol (Panreac, Spain). In order to determine the performance characteristics of the method, working solutions were prepared by diluting different amounts of the standard solution in ethanol. The concentration of the working solutions ranged between 0.1-25 mg L⁻¹.

2.2 Liquid-liquid extraction (LLE)

The liquid-liquid extraction procedure was basically performed as described by Grün et al (2008). To 500 µL of sample (aqueous solution of 43 phenolic compounds or faecal samples spiked with the same compounds), 65 µL of 1M hydrochloric acid (Prolabo, France)) were added for acidifying the samples (pH 2-3) prior to the extraction. After the addition of the

internal standard (50 μL of 100 $\mu\text{g mL}^{-1}$ *trans*-cinnamic acid-d7 (Sigma-Aldrich, Switzerland) in 1:1 methanol/water (v/v)), the samples were kept at 4 °C for 10 min. Two mL of ethyl acetate were added, the samples were vortexed for 30 s, and centrifuged at 3000 g for 10 min. The supernatants were transferred and the extraction was repeated twice. The resulting extracts were combined and concentrated under nitrogen stream. Samples were further dried by the subsequent addition and evaporation of 1 mL of dichloromethane. To the samples, 100 μL of BSTA/TMCS (N,O-Bis(trimethylsilyl)trifluoroacetamide with 10 % trimethylchlorosilane) (Sigma-Aldrich, Switzerland) was added for the formation of the corresponding silyl-derived compounds. The samples were incubated at 90 °C for 30 min. After cooling down to room temperature, 100 μL of hexane were added to 100 μL of the derivatized samples and the mixtures were vortex-mixed. Then, GC-MS analysis was carried out. Five levels of concentrations of each phenolic acid (0.1, 1, 5, 10, 25 mg L^{-1}) were tested in duplicate.

2.3 Gas chromatography-Mass spectrometry analysis

An Agilent 6890N GC system (Agilent, Palo Alto, CA) with a split/splitless injector and interfaced with an Agilent 5973N mass spectrometer was used for sample analysis. The injector was set at 280 °C. An Agilent MSD ChemStation Software (E.02.00.493 version) was used to control the system. For separation, an HP-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 μm) Quadrex Co. (Woodbridge, CT) was used. Helium was the carrier gas (1 mL min^{-1}). One μL of the sample was injected in split mode at a split ratio of 20:1. The GC conditions were as follows: initial temperature of 45 °C for 1 min to 100 °C (held for 5.5 min) at 10 °C/min. Then, the temperature was increased up to 300 °C at 7.5 °C/min and held for 2.5 min giving a total run time of 41 min.

For the MS system, the temperatures of the transfer line, quadrupole and ionization source were 270, 150 and 230 °C respectively; electron impact mass spectra were recorded at

70eV ionization voltages and the ionization current was 10 μ A. The acquisitions were performed in scan mode (from 35 to 450 amu). Peak identification was carried out by analogy of mass spectra with those of the mass library (Wiley 6.0 and Nist 2.0) and comparing the calculated retention times and the mass spectra with those corresponding to commercial standards. Quantitative data were obtained by calculating the relative areas (area of the quantification ion of the phenolic compound / area of the quantification ion of the internal standard (*trans*-cinnamic acid-d7)). Calibration curves representing relative areas of quantification ions vs. concentration were built for quantification purposes.

2.4 Fermentation of phenolic extracts by faecal microorganisms

Faecal samples were collected from three separate individuals (females). All volunteers were in good health, they were nonsmokers, aged from 25 to 30 years, had not ingested antibiotics for at least 6 months before the study, and had no history of any gastrointestinal disease. Samples were collected, on site, on the day of the experiment and were used immediately. The *in vitro* batch culture fermentations were basically performed as described by Tzounis and collaborators (Tzounis et al., 2008). The samples were diluted 1:10 (w/v) with anaerobic phosphate buffer (1 M; pH 7.4) and homogenized in a stomacher for 2 min. Resulting faecal slurries from each individual (i.e. faecal samples were not pooled) were used to inoculate the batch-culture vessels.

Three commercial extracts kindly supplied by Dr. Piriou (Les Dérives Resiniques & Terpéniques, S.A., France) were used in this study: a grape seed extract (Vitaflavan®) (GSE) and two rich-monomeric (GSE-M) and rich-oligomeric (GSE-O) fractions obtained from Vitaflavan®. According to the producer, the monomeric fraction contained around 58 % monomers and 42 % procyanidins whereas the oligomeric fraction contained around 7 % monomers and 93 % procyanidins. More detailed composition of the grape seed extract and their

fractions is reported in Monagas, Hernández-Ledesma, Garrido, Martín-Álvarez, Gómez-Cordovés & Bartolomé, (2005).

Batch-culture fermentation vessels (300 mL volume; one vessel per treatment group) were sterilised and filled with 135 mL basal nutrient medium (peptone water (2 g L⁻¹), yeast extract (2 g L⁻¹), NaCl (0.1 g L⁻¹), K₂HPO₄ (0.04 g L⁻¹), KH₂PO₄ (0.04 g L⁻¹), NaHCO₃ (2 g L⁻¹), MgSO₄·7H₂O (0.01 g L⁻¹), CaCl₂·6H₂O (0.01 g L⁻¹), Tween 80 (2 mL L⁻¹), haemin (50 mg L⁻¹), 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 distilled water. The pH of the basal medium was adjusted to 7 and autoclaved before dispensing it into the vessels. Medium was then gassed overnight with O₂-free N₂. Before the addition of faecal slurry samples, the temperature of the basal nutrient medium was set at 37 °C by using a circulating water-bath and the pH was maintained at 6.8, using an Electrolab pH controller, in order to mimic conditions located in the distal region of the human large intestine (anaerobic; 37 °C; pH about 6.8). Vessels inoculated with 15 mL of faecal slurry (1:10, w/v) and with the three extracts were tested. As a control, incubations with human faecal slurries were carried out without the addition of any extract. Samples were collected at different times (30 minutes and 10 hours). Prior to analysis, the samples were centrifuged at 13000 rpm for 10 min at room temperature and the supernatant was passed through 0.22 µm PVDF filters (OlimPeak, Tecknokroma). The supernatant was stored at -80 °C until the extraction.

2.6 Statistical analysis

The statistical methods used for the data analysis were: Linear Regression to establish the calibration curves of each phenolic compound and the Lack of Fit test to judge the adequacy of the models; Minimal Differences Tests (LSD) to check differences in mean and Cluster Analysis to search how the samples are grouped. Statgraphics Centurion XV, version 15.2

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

3. Results and discussion

3.1 Linear regressions

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

Most of the phenolic compounds belonging to the phenylpropionic, phenylacetic, cinnamic, mandelic and benzoic acids showed a good linearity, as it can be seen by checking the determination coefficients (R²), which in general showed values > 99 % (**Table 2**). However,

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

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

3.2.1 Precision

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

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

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

3.2.3 Recovery

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

3.3. Application of LLE-GC-EI-MS method to faecal samples fermented with three commercial grape seed phenolic extracts

The LLE-GC-EI-MS method was applied to know the phenolic metabolites produced from the degradation of three types of phenolic grape seed extracts by faecal microbiota from three individuals. The extracts essayed were the whole grape extract (GSE) and its corresponding purified fractions: rich in monomers (GSE-M) and in oligomers (GSE-O). Moreover, control samples (C-S), consisting in the faeces of each individual without the addition of phenolic extracts were also analyzed. The essays were performed in batch culture systems, and the samples for phenolic metabolites analysis were collected from the *in vitro* fermentation systems after 30 minutes and 10 hours of incubation.

Figure 1 shows an example of the chromatograms corresponding to the reference phenolic compounds (Figure 1a) and to the phenolic compounds identified in a faecal sample incubated with the whole grape extract (GSE) and analyzed after 30 minutes of incubation (Figure 1b). From the 43 phenolic compounds essayed, 27 were identified in the faecal samples but only 17 compounds were above the calculated limit of quantification (**Table 4**). The highest concentration values corresponded to the phenylpropionic acids (1.65 mg L⁻¹ in average), phenylacetic acids (1.31 mg L⁻¹) and gallic acid (1.49 mg L⁻¹). Benzoic acids (except gallic acid), showed the lowest concentration values (0.18 mg L⁻¹). This is in agreement with Gross and collaborators (Gross et al., 2010), who found phenylpropionic and phenylacetic acids and their corresponding hydroxylated derivatives as the major metabolites produced from wine/grape polyphenols by faecal microbiota while benzoic acids were found in lower amounts. On the other hand, although some mandelic and cinnamic acids (mandelic, 4-hydroxymandelic, ferulic acid, among others) were identified in the samples, they could not be quantifying because they gave very low responses, bellow the calculated LOQ. Some mandelic acids are known urinary metabolites resulting from different dietary components. For example, 4-

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

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

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

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

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

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

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

4. Conclusions

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

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

Acknowledgments

Authors are grateful to the volunteers that provided the samples and to Dr. Glen Gibson (Department of Food Biosciences, University of Reading, UK) and his group for their supervision and assistance with the fermentation experiments carried out in this laboratory during the placement of CC. CMG and JJRB acknowledge CSIC for their respective research grants. CC thanks MICINN for her research grant. This work has been funded by the Spanish Ministry for Science and Innovation (AGL2009-13361-C02-01 and CSD2007-00063 Consolider Ingenio 2010 FUN-C-FOOD Projects), and the Comunidad de Madrid (ALIBIRD P2009/AGR-1469 Project).

References

- Appeldoorn, M. M., Vincken, J. P., Aura, A. M., Hollman, P. C. H., & Gruppen, H. (2009). Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)- γ -valerolactone as the major metabolites. *Journal of Agricultural and Food Chemistry*, 57(3), 1084-1092.
- Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews*, 7(3), 407-429.
- Aura, A. M., Martin-Lopez, P., O'Leary, K. A., Williamson, G., Oksman-Caldentey, K. M., Poutanen, K., & Santos-Buelga, C. (2005). In vitro metabolism of anthocyanins by human gut microflora. *European Journal of Nutrition*, 44(3), 133-142.
- Braune, A., Engst, W., & Blaut, M. (2005). Degradation of neohesperidin dihydrochalcone by human intestinal bacteria. *Journal of Agricultural and Food Chemistry*, 53(5), 1782-1790.

- Cerdá, B., Tomás-Barberán, F. A., & Espín, J. C. (2005). Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: Identification of biomarkers and individual variability. *Journal of Agricultural and Food Chemistry*, 53(2), 227-235.
- Cueva, C., Moreno-Arribas, M. V., Martín-Álvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., Rivas, C. L., Requena, T., Rodríguez, J. M., & Bartolomé, B. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology*, 161(5), 372-382.
- Das, N. P., & Griffiths, L. A. (1969). Studies on flavonoid metabolism. Metabolism of (+)-[14C] catechin in the rat and guinea pig. *Biochemical Journal*, 115(4), 831-836.
- Deprez, S., Brezillon, C., Rabot, S., Philippe, C., Mila, I., Lapiere, C., & Scalbert, A. (2000). Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *Journal of Nutrition*, 130(11), 2733-2738.
- Donovan, J. L., Manach, C., Rios, L., Morand, C., Scalbert, A., & Rémésy, C. (2002). Procyanidins are not bioavailable in rats fed a single meal containing a grapeseed extract or the procyanidin dimer B₃. *British Journal of Nutrition*, 87(4), 299-306.
- Gill, C. I. R., McDougall, G. J., Glidewell, S., Stewart, D., Shen, Q., Tuohy, K., Dobbin, A., Boyd, A., Brown, E., Haldar, S., & Rowland, I. R. (2010). Profiling of phenols in human fecal water after raspberry supplementation. *Journal of Agricultural and Food Chemistry*, 58(19), 10389-10395.
- Gonthier, M. P., Cheynier, V., Donovan, J. L., Manach, C., Morand, C., Mila, I., Lapiere, C., Rémésy, C., & Scalbert, A. (2003a). Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *Journal of Nutrition*, 133(2), 461-467.
- Gonthier, M. P., Rémésy, C., Scalbert, A., Cheynier, V., Souquet, J. M., Poutanen, K., & Aura, A. M. (2006). Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomedicine and Pharmacotherapy*, 60(9), 536-540.
- Gonthier, M. P., Rios, L. Y., Verny, M. A., Rémésy, C., & Scalbert, A. (2003b). Novel liquid chromatography-electrospray ionization mass spectrometry method for the quantification in human urine of microbial aromatic acid metabolites derived from dietary polyphenols. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 789(2), 247-255.
- Groenewoud, G., & Hundt, H. K. L. (1986). The microbial metabolism of condensed (+)-catechins by rat-caecal microflora. *Xenobiotica*, 16(2), 99-107.
- Gross, G., Jacobs, D. M., Peters, S., Possemiers, S., Van Duynhoven, J., Vaughan, E. E., & Van De Wiele, T. (2010). In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *Journal of Agricultural and Food Chemistry*, 58(18), 10236-10246.
- Grün, C. H., van Dorsten, F. A., Jacobs, D. M., Le Belleguic, M., van Velzen, E. J. J., Bingham, M. O., Janssen, H. G., & van Duynhoven, J. P. M. (2008). GC-MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and in vitro

intervention studies. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 871(2), 212-219.

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S., & Prior, R. L. (2004). Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. *Journal of Nutrition*, 134(3), 613-617.

Jenner, A. M., Rafter, J., & Halliwell, B. (2005). Human fecal water content of phenolics: The extent of colonic exposure to aromatic compounds. *Free Radical Biology and Medicine*, 38(6), 763-772.

Massart, D. L., Vandeginste, B. G. M., Deming, S. N., Michotte, Y. & Kaufman, L., ed (1990). *Chemometrics: A Textbook*. Amsterdam: Elsevier.

Monagas, M., Hernández-Ledesma, B., Garrido, I., Martín-Álvarez, P. J., Gómez-Cordovés, C., & Bartolomé, B. (2005). Quality assessment of commercial dietary antioxidant products from *Vitis vinifera* L. grape seeds. *Nutrition and Cancer*, 53(2), 244-254.

Peters, S., Janssen, H. G., & Vivo-Truyols, G. (2010). Trend analysis of time-series data: A novel method for untargeted metabolite discovery. *Analytica Chimica Acta*, 663(1), 98-104.

Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., Moore, K. P., & Rice-Evans, C. A. (2004). Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. *Free Radical Biology and Medicine*, 36(2), 212-225.

Ríos, L. Y., Gonthier, M. P., Rémésy, C., Mila, I., Lapiere, C., Lazarus, S. A., Williamson, G., & Scalbert, A. (2003). Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *The American journal of clinical nutrition*, 77(4), 912-918.

Roowi, S., Stalmach, A., Mullen, W., Lean, M. E. J., Edwards And, C. A., & Crozier, A. (2010). Green tea flavan-3-ols: Colonic degradation and urinary excretion of catabolites by humans. *Journal of Agricultural and Food Chemistry*, 58(2), 1296-1304.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., & Jiménez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition*, 45(4), 287-306.

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010). A comparison of the in vitro biotransformation of (-)-epicatechin and procyanidin B2 by human faecal microbiota. *Molecular Nutrition and Food Research*, 54(6), 747-759.

Tzounis, X., Vulevic, J., Kuhnle, G. G. C., George, T., Leonczak, J., Gibson, G. R., Kwik-Urbe, C., & Spencer, J. P. E. (2008). Flavanol monomer-induced changes to the human faecal microflora. *British Journal of Nutrition*, 99(4), 782-792.

Urpí-Sarda, M., Monagas, M., Khan, N., Lamuela-Raventos, R. M., Santos-Buelga, C., Sacanella, E., Castell, M., Permanyer, J., & Andres-Lacueva, C. (2009). Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Analytical and Bioanalytical Chemistry*, 394(6), 1545-1556.

522 VanT Slot, G., Mattern, W., Rzeppa, S., Grewe, D., & Humpf, H. U. (2010). Complex
523 flavonoids in cocoa: Synthesis and degradation by intestinal microbiota. *Journal of Agricultural*
524 *and Food Chemistry*, 58(15), 8879-8886.

525
526 van Dorsten, F. A., Grün, C. H., van Velzen, E. J. J., Jacobs, D. M., Draijer, R., & van
527 Duynhoven, J. P. M. (2010). The metabolic fate of red wine and grape juice polyphenols in
528 humans assessed by metabolomics. *Molecular Nutrition and Food Research*, 54(7), 897-908.

529
530 Williams, R. E., Eyton-Jones, H. W., Farnworth, M. J., Gallagher, R., & Provan, W. M. (2002).
531 Effect of intestinal microflora on the urinary metabolic profile of rats: A ¹H-nuclear magnetic
532 resonance spectroscopy study. *Xenobiotica*, 32(9), 783-794.

533
534
535
536

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-M**: 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.