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Plasma kinetics and microbial biotransformation of grape seed flavanols in rats

Maria Margalef [a], *Zara Pons* [a], *Francisca Isabel Bravo* [a], *Begoña Muguerza* [a,b,*], *Anna Arola-Arnal* [a]

[a] *Nutrigenomic Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona 43007, Spain*

[b] *Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus 43204, Spain*

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ABSTRACT

Flavan-3-ols and their oligomeric forms, proanthocyanidins (PAs), are poorly absorbed in the small intestine and reach the colon where gut bacteria enzymes can hydrolyse them to produce small molecular metabolites, which can reach systemic circulation. However, the microbial metabolism of flavanols has been poorly described *in vivo*. The aim of this study was to determine the colonic biotransformation pathway and the plasma temporal appearance of grape seed flavanols colonic metabolites in rats. Rat plasma colonic metabolites were analysed by HPLC-MS/MS at 2, 7, 24 and 48 h after 1000 mg/kg of a grape seed PA extract (GSPE) administration. Results indicated that non-metabolised flavanols have peak plasma concentrations 2 h after GSPE administration, whereas the colonic metabolites appeared in plasma later, indicating their gradual colonic biotransformation as valerolactone > phenylpropionic acids ≈ phenylacetic acids > benzoic acids. This study shows how flavanols are biotransformed by gut bacteria in rats over time, facilitating potential bioactive compound identification for particular health effects.

1. Introduction

Flavanols are among the most common polyphenols in the human diet and are mainly found in fruit, cocoa, tea, wine, nuts and beans (Aherne & O'Brien, 2002). This polyphenol group exists in both monomeric (catechin and epicatechin) and oligomeric (proanthocyanidins (PAs) or condensed tannins, depending on the molecular weight) aglycone forms and esterified with gallic acid (Aron & Kennedy, 2008). Flavanols improve human health, and our group has demonstrated that grape seed flavanols exhibit antioxidant capacity (Puiggros et al., 2005), improve lipid metabolism (Guerrero et al., 2013), limit adipogenesis (Pinent et al., 2005), act as an insulin-mimetic agent (Pinent et al., 2004), possess antihypertensive effects (Quiñones et al., 2013) and reduce inflammation (Terra et al., 2011).

The beneficial health properties of polyphenols are mainly attributed to the compounds derived from their metabolism (Del Rio et al., 2013). Dietary polyphenols are known to be recognised as xenobiotics and that they undergo phase II enzymatic detoxification at the small intestine and liver to form sulpho-, methyl- or glucorono-conjugates after their

* *Corresponding author.* Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, C/Marcel.lí Domingo s/n, 43007 Tarragona, Spain. Tel.: +34 977559566; fax: +34 977558232.
E-mail address: begona.muguerza@urv.cat (B. Muguerza).

Chemical compounds: Benzoic acid (PubChem CID: 243); 2-(3,4-Dihydroxyphenyl)acetic acid (PubChem CID: 547); (–)-Epicatechin (PubChem CID: 72276); Gallic acid (PubChem CID: 370); Hippuric acid (PubChem CID: 464); Homovanillic acid (PubChem CID 1738); 3-(4-Hydroxyphenyl)propionic acid (PubChem CID: 10394); 3-O-Methylgallic acid (CID: 19829); Procyanidin B2 (PubChem CID: 122738); Vanillic acid (PubChem CID: 8468).

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absorption in the small intestine. These metabolites can be returned to the lumen through the bile by enterohepatic circulation and can reach the systemic circulation to be transported to other tissues or to be excreted by the urine (Monagas et al., 2010). However, the PA absorption in the small intestine is determined by the degree of polymerisation, which has usually been considered to be a limiting factor on the health benefits from flavanol consumption (Del Rio et al., 2013). Whereas monomeric and low molecular-weight forms are mainly absorbed through the small intestine, some monomeric glucosides, oligomers with a degree of polymerisation greater than 2–3 and those forms from enterohepatic circulation cross the gastrointestinal tract and reach the colon, where they are transformed by intestinal microbiota for their absorption or excretion (Aura, 2008; Monagas et al., 2010). It is estimated that 90–95% of dietary polyphenols can reach the colon (Clifford, 2004; Monagas et al., 2010), where they are subjected to microbial catabolism.

The colon has diverse microbial populations composed of either obligatory or facultative anaerobes (including *Bacteriodes*, *Bifidobacterium*, *Enterobacteriaceae* and *Clostridium*) (Manichanh et al., 2010) responsible for degrading undigested food matrix and turn its components in microbial metabolites (Sánchez-Patán et al., 2012a). *In vitro* studies have shown that flavanol oligomers undergo an interflavanic link cleavage and turn into monomers (Monagas et al., 2010). The variety of enzymes produced by the gut bacteria can hydrolyse, reduce, dehydroxylate, decarboxylate and demethylate the polyphenols and convert them into different low molecular weight metabolites (valerolactone compounds, valeric acids, phenylpropionic acids, phenyl acetic acids, benzoic acids and several conjugated phenolic acids, consecutively) (Monagas et al., 2010; Sánchez-Patán et al., 2012a,b). These metabolites may reach the portal circulation through colonocytes being transported to the liver where they can be further metabolised by hepatic phase II enzymes and excreted into the urine or carried to the systemic circulation to reach different tissues (Monagas et al., 2010).

The colon metabolises flavanols and the beneficial health effects accredited to these compounds could be potentially produced not only by phase II but also by the colonic metabolites. Many studies using *in vitro* fermentation systems have evaluated the microbial metabolism of polyphenols (Sánchez-Patán et al., 2012a,b; Serra et al., 2011). However, the study of the microbial catabolism of flavonoids *in vivo* is limited (Serra et al., 2013; Urpi-Sarda et al., 2009a,b). The aim of this study is to evaluate how flavanols are biotransformed by the colon in rats and their colonic metabolites' time of occurrence in plasma and to establish a proposal of the rat flavanol catabolic pathway. Rat plasma colonic metabolites were analysed by high-performance liquid chromatography-tandem triple quadrupole mass spectrometry (HPLC-MS/MS) after different times of grape seed Pas administration.

2. Materials and methods

2.1. Grape seed proanthocyanidin extract

A grape seed PA extract (GSPE) was obtained from *Les Dérives Résiniques et Terpéniques* (Dax, France). The total polyphenol content and the individual flavanols and phenolic acids comprising the grape seed extract used in this study are detailed in Table 1 (adapted from Quiñones et al., 2013).

Table 1 – Total polyphenols and individual flavanols and phenolic acids of grape seed proanthocyanidin extract (GSPE).

Compound	Concentration (mg/g)
Total polyphenol content[1]	554.16 ± 13.02
Phenolic compound[2]	
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
Procyanidin dimer[a]	144.2 ± 32.2
Procyanidin trimer[a]	28.4 ± 2.0
Procyanidin tetramer[a]	2.0 ± 0.2
Dimer gallate[a]	39.7 ± 7.1
Epigallocatechin gallate	0.4 ± 0.1
Epicatechin gallate[b]	55.3 ± 1.5
p-coumaric acid	0.1 ± 0.0
Gallic acid	17.7 ± 2.0
3,4-dihydroxybenzoic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Quercetin	0.3 ± 0.0
Quercetin-3-O-gallate	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0

Data adapted from Quiñones et al. 2013.
The results are expressed on a wet basis as the mean ± SD (n = 3).
[1] Measured by Folin–Ciocalteu's method.
[2] HPLC-MS.
[a] Quantified using the calibration curve of proanthocyanidin B2.
[b] Quantified using the calibration curve of epigallocatechin gallate.

2.2. Chemicals and reagents

Acetone (HPLC analytical grade), methanol (HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). The following were individually dissolved in methanol at 4000 mg/L: (+)-catechin; (–)-epicatechin; benzoic acid; phloroglucinol; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2-(4-hydroxyphenyl)acetic acid; 2-(3,4-dihydroxyphenyl)acetic acid; 3-(4-hydroxyphenyl)propionic acid; vanillic acid; gallic acid; hippuric acid and ferulic acid (all from Fluka/Sigma-Aldrich, Madrid, Spain). The following were individually dissolved in methanol at 2000 mg/L: procyanidin B2; epigallocatechin gallate (EGCG); and pyrocatechol (internal standard (IS)) (all from Fluka/Sigma-Aldrich) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (MicroCombiChem e.K., Wiesbaden, Germany). All standard stock solutions were prepared every 3 months and stored in dark-glass flasks at –20 °C.

A mixed standard stock solution in methanol was prepared weekly from the following compounds and stored in dark glass flasks at –20 °C: (+)-catechin; (–)-epicatechin; benzoic acid; phloroglucinol; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2-(4-hydroxyphenyl)acetic acid; 2-(3,4-dihydroxyphenyl)acetic acid; 3-(4-hydroxyphenyl) propionic acid; vanillic acid; gallic acid; hippuric acid; ferulic

acid; EGCG and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone at 200 and 100 mg/L of procyanidin B2, respectively. This mixed standard stock solution was diluted daily to the desired concentration using an acetone/Milli-Q water/acetic acid (70/29.5/0.5, v/v/v) solution and stored in dark glass flasks at –20 °C until chromatographic analysis.

2.3. *Animal and plasma collection*

Male Wistar rats, weighing between 226 and 260 g, were obtained from Charles River Laboratories (Barcelona, Spain). All animals were housed at 22 °C with a light/dark cycle of 12 h (lights on at 09:00 AM) and were fed a standard chow diet (AO4, Panlab, Barcelona, Spain) *ad libitum* during the experiment. The animals were randomly divided into two groups: the control group (n = 6) and the GSPE group (n = 6). A dose of 1000 mg/kg of GSPE (1 mL in water) was administered to the GSPE group by oral gavage. Water (1 mL) was orally administered to the control group. In both groups, oral administration was performed by gastric intubation to fasted rats between 9 and 10 AM. Fasting blood samples were obtained via saphenous vein extraction using heparin vials (Starsted, Barcelona, Spain) at 0, 2, 7, 24 and 48 h after GSPE or water administration, in order to see the kinetic behaviour of microbial metabolites, which are supposed to appear at later times than the flavanol phase-II metabolites. Plasma samples were obtained by centrifugation (2000 × *g*, 15 min, 4 °C) and pooled (n = 6), to have sufficient volume for the analysis and also to remove the biological variability. The pooled plasma samples were stored at –80 °C until chromatographic analysis was performed (Fig. 1). Plasma from the control group (water oral gavage) was used to perform the calibration curves in the chromatography analysis. Any compound present in the plasma control group (0 h time-point) was subtracted from the plasma concentration at all other time-points. The plasma samples were not treated with glucuronidase or sulphatase enzymes. The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals, and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of Universitat Rovira i Virgili (permission number 6777).

2.4. *Micro-solid phase plasma polyphenol extraction*

Prior to chromatographic analysis, the pool of rat plasmas (n = 6) for each time-point was pre-treated by off-line micro-Solid Phase Extraction (μ-SPE) as described previously (Margalef, Pons, Muguerza, & Arola-Arnal, 2014) (Fig. 1) using OASIS HLB μ-Elution Plates 30 μm (Waters, Barcelona, Spain). Briefly, the micro-cartridges were conditioned sequentially with 250 μL methanol and 250 μL 0.2% acetic acid. A plasma aliquot (250 μL) was mixed with 300 μL 4% phosphoric acid and 50 μL pyrocatechol (250 ppb), and then this mixture was loaded into the plates. The loaded plates were washed with 200 μL Milli-Q water and 200 μL 0.2% acetic acid. The retained polyphenols were eluted with 2 × 50 μL acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v).

2.5. *Chromatographic analysis*

The eluted solution was directly analysed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, U.S.A.) as previously described (Margalef et al., 2014). Briefly, Zorbax SB-Aq (150 × 2.1 mm i.d., 3.5 μm particle size, Agilent Technologies) was the chromatographic column. The mobile phase was 0.2% acetic acid (solvent A) and acetonitrile (solvent B) with a flow rate of 0.4 mL/min. The elution gradient was 0–10 min, 5–55% B, 10–12 min, 55–80% B, 12–15 min, 80% B isocratic, and 15–16 min 80–5% B. A post run of 10 min was applied and 2.5 μL of sample were injected. Electrospray ionisation (ESI) was conducted at 350 °C and 12 L/min with 45 psi of nebuliser gas pressure, and 4000 V of capillary voltage. The mass spectrometer was operated in negative mode and MS/MS data were acquired in Multiple Reaction Monitoring (MRM) mode. Optimised MRM conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS are presented in Table 2 and all quality parameters required to perform the analysis of these metabolites are shown in Table 3.

3. Results

In this study, we quantified the colonic metabolites that appeared in pooled (n = 6) rat plasma 0, 2, 7, 24 and 48 h after GSPE (1000 mg/kg) ingestion using HPLC-MS/MS. The pooled plasma was necessary to collect enough volume for three replicate chromatographic analyses at the different times without sacrificing the rats. Moreover, pooled plasma increases homogeneity and sensitivity in order to allow the detection of all potential metabolites (Demelbauer, Plematl, Josic, Allmaier, & Rizzi, 2005;

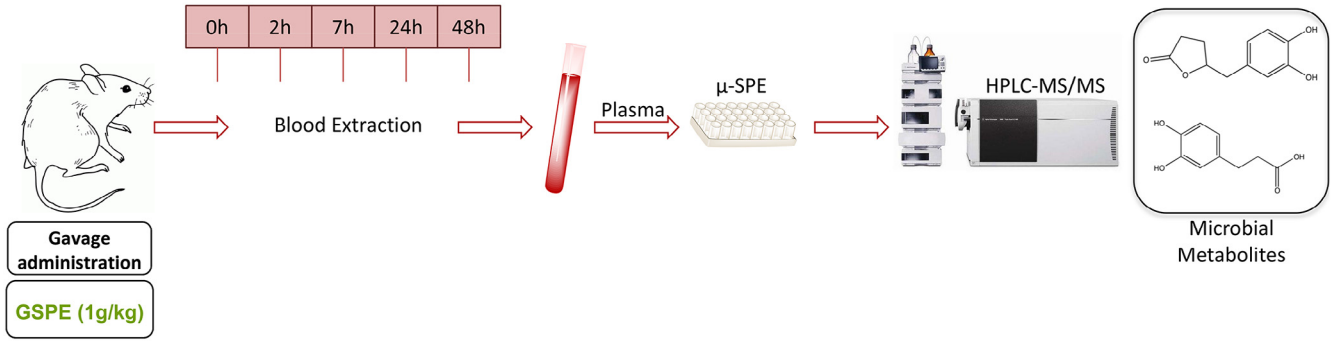

Fig. 1 – Graphical representation of the experimental design used in this study.

Table 2 – Optimized MRM conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS.

Compound	MS/MS Conditions					
	Quantification			Confirmation		
	MRM_1	F (V)	CE (V)	MRM_2	F (V)	CE (V)
Catechin	289 > 203	120	20	289 > 245	120	20
Epicatechin	289 > 245	130	10	289 > 203	130	20
Procyanidin B1	577 > 425	130	10	577 > 407	130	30
Procyanidin B2	577 > 425	130	10	577 > 407	130	30
Procyanidin B3	577 > 425	130	10	577 > 407	130	30
Gallic acid	169 > 125	90	10	169 > 79	90	40
Vanillic acid	167 > 152	80	10	167 > 123	80	5
Epigallocatechin gallate	457 > 169	110	20	457 > 305	110	20
1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	291 > 247	70	20	291 > 96	70	20
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	207 > 85	120	10	207 > 121	120	10
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	225 > 163	70	10	225 > 181	70	10
4-Hydroxy-5-(3′-hydroxyphenyl)-valeric acid	209 > 147	150	0	–	–	–
4-Hydroxy-5-(phenyl)-valeric acid	193 > 175	60	10	–	–	–
3-(3,4-Dihydroxyphenyl)propionic acid	181 > 137	60	10	–	–	–
3-(3-Hydroxyphenyl)propionic acid	165 > 121	90	10	165 > 59	90	0
3-(4-Hydroxyphenyl)propionic acid	165 > 121	90	10	165 > 59	90	0
Phenylpropionic acid	149 > 105	90	10	–	–	–
2-(3,4-Dihydroxyphenyl)acetic acid	167 > 123	50	10	167 > 95	50	30
2-(3-Hydroxyphenyl)acetic acid	151 > 107	60	5	151 > 93	60	20
2-(4-Hydroxyphenyl)acetic acid	151 > 107	60	5	151 > 65	60	30
Phenylacetic acid	135 > 91	90	5	135 > 100	90	5
3,4-Dihydroxybenzoic acid	153 > 109	80	10	153 > 62	80	40
3-Hydroxybenzoic acid	137 > 93	70	10	–	–	–
4-Hydroxybenzoic acid	137 > 93	70	10	–	–	–
Benzoic acid	121 > 77	60	5	121 > 59	60	5
3-O-Methylgallic acid	183 > 168	90	10	183 > 124	90	10
Homovanillic acid	181 > 163	90	10	181 > 134	90	20
Homovanillyl alcohol	167 > 152	150	10	167 > 133	150	10
Ferulic acid	193 > 134	60	10	193 > 178	60	10
Hippuric acid	178 > 134	80	5	178 > 77	80	10
Phloroglucinol	125 > 57	90	10	125 > 125	90	0

Abbreviations: F, Fragmentor; CE, Collision Energy; MRM, Multiple reaction monitoring.

Margalef et al., 2014; McGaw, Phinney, & Lowenthal, 2010). A range of time points was selected to detect all potential colonic metabolites and to study how these metabolites appear in plasma at different times, allowing the different flavanols to reach the colon, to be metabolised by gut bacteria and to be absorbed by rats (Demelbauer et al., 2005; Serra et al., 2011, 2013).

3.1. *Plasma kinetics of non-metabolised PA in rat plasma*

Procyanidin B2 (**1**), catechin (**2**), epicatechin (**3**), gallic acid (**4**) and vanillic acid (**5**) are polyphenols present in GSPE at considerable concentrations (Table 1). These compounds were absorbed, peaked in plasma concentration 2 h after GSPE ingestion and disappeared at 24 h (Fig. 2). Interestingly, the concentration of compound **5** increased again after 24 h but did not reach as high concentrations as at 2 h (Fig. 2C).

3.2. *Plasma kinetics of microbial PA metabolites in rat plasma*

3.2.1. *Valerolactone metabolites*

The colonic metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**6**) that had its highest plasma concentration at 7 h after GSPE administration, was still present in the plasma at 24 h and disappeared after 48 h (Fig. 3). No other valerolactone metabolites were detected in rat plasma (data not shown).

3.2.2. *Phenylpropionic acid metabolites*

The phenylpropionic acids colonic metabolites 3-(4-hydroxyphenyl) propionic acid (**7**), 3-(3,4-dihydroxyphenyl) propionic acid (**8**) and 3-(3-hydroxyphenyl) propionic acid (**9**) peaked in plasma concentrations 24 h post GSPE administration, and at 48 h, there was still a considerable amount of these metabolites in plasma (Fig. 4). Interestingly, **7** and **8** reached very high plasma concentrations, having a concentration of approximately 50 μM and 10 μM at 24 h, respectively (Fig. 4A). Much lower concentrations were found for **9** and for phenylpropionic acid (**10**) (Fig. 4B).

3.2.3. *Phenylacetic acid metabolites*

The phenylacetic acid metabolites had different kinetic profiles depending on the metabolite. Although 2-(4-dihydroxyphenyl) acetic acid (**11**), 2-(3-dihydroxyphenyl) acetic acid (**12**) and phenylacetic acid (**13**) reached their highest concentration in plasma as rapidly as 2 h post-GSPE administration (Fig. 5A), 3,4-dihydroxyphenylacetic acid (**14**) had peak plasma concentration at 24 h post-administration. A higher concentration of **14** was found in plasma (approximately 2 μM

Table 3 – The retention behaviour (RT, min), calibration curve, determination coefficient (R^2), working linearity range, LODs, LOQs, MDLs and MQLs for phenolic compound quantification in spiked plasma samples using HPLC-ESI-MS/MS. (Adapted from Margalef et al. 2014 [19])

Compound	RT (min)	Calibration curve	Determination coefficient (R^2)	Working linearity range (μM)	LOD (nM)	LOQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Catechin	6.4	y = 0.799x	0.997	0.007–17.225	2.30	7.66	0.66	2.19
Epicatechin	6.9	y = 1.674x	0.996	0.007–17.225	1.66	5.52	0.47	1.58
Procyanidin B2	6.7	y = 34.138x	0.991	0.008–4.321	1.95	6.49	0.56	1.85
Epigallocatechin gallate	7.3	y = 8.399x	0.990	0.004–10.908	0.76	2.52	0.22	0.72
Gallic acid	2.8	y = 1.035x	0.991	0.059–29.391	11.75	39.22	3.36	11.20
Vanillic acid	6.7	y = 0.862x	0.996	0.012–29.762	4.29	14.29	1.22	4.08
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	7.2	y = 0.746x	0.998	0.048–24.038	14.42	48.08	4.12	13.74
3-(4-Hydroxyphenyl)propionic acid	7.3	y = 0.063x	0.997	0.060–30.120	24.10	80.32	6.88	22.95
2-(3,4-Dihydroxyphenyl)acetic acid	4,3	y = 0.389x	0.996	0.060–29.762	11.90	39.68	3.40	11.34
2-(3-Hydroxyphenyl)acetic acid	5.8	y = 0.775x	0.995	0.013–32.895	3.29	10.96	0.94	3.13
2-(4-Hydroxyphenyl)acetic acid	6.1	y = 0.534x	0.994	0.013–32.895	8.87	29.90	2.56	8.54
3-Hydroxybenzoic acid	6.3	y = 0.690x	0.998	0.072–36.232	39.13	130.43	11.28	37.37
Benzoic acid	7.8	y = 0.510x	0.995	0.016–40.984	3.78	12.61	1.08	3.60
Ferulic acid	8.1	y = 5.658x	0.995	0.010–25.773	2.13	7.11	0.61	2.03
Hippuric acid	5.5	y = 1.981x	0.986	0.011–27.933	0.10	0.34	0.03	0.10
Phloroglucinol	2.3	y = 0.157x	0.990	0.079–39.683	23.81	79.37	6.80	22.68

Abbreviations: RT (Retention behaviour); LOD (Limit of detection); LOQ (Limit of quantification); MDL (Method detection limit); MQL (Method quantification limit).
[a] Method of detection and quantification limits in nmol/L of fresh sample calculated for the analysis of 250 μL of plasma sample.

at 24 h after the GSPE administration) with respect to the other phenylacetic metabolites (less than 1.2 μM at 2 h) (Fig. 5B).

3.2.4. *Benzoic acid metabolites*

Benzoic acid derivatives can be formed by different flavanol metabolisation pathways (Selma, Espín, & Tomás-Barberán, 2009; Serra et al., 2011; Urpi-Sarda et al., 2009a,b), and a variety of compounds may be formed. However, we were able to quantify only rat plasma 3-hydroxybenzoic acid (**15**) and benzoic acid (**16**), and no other benzoic acid metabolites were detected in plasma (data not shown); **15** and **16** reached considerable plasma concentration levels with comparable kinetic profiles (Fig. 6). These metabolites peak in plasma concentration at 2 h post GSPE administration, and then from 2 to 7 h, the concentration in plasma decreased and was maintained until 48 h.

3.2.5. *Phenolic acids metabolites*

The phenolic acids detected in this study were hippuric acid (**17**), homovanillic acid (**18**), homovanillyl alcohol (**19**) and 3-*O*-methyl gallic acid (**20**). It is important to note that microbial metabolites can reach the portal circulation through colonocytes being transported to the liver where they can be further subjected to phase II metabolism before entering the circulation (Gonthier et al., 2003). Thus, **18**, **19** and **20** were some of the methylated products detected in rat plasma (Fig. 7), and these final metabolites have different kinetic profiles. Although **18**, **19** and **20** reached the highest concentration in plasma at 2 h post GSPE administration, the concentration of **18** was maintained for 48 h, but the plasma levels of **19** and **20** were decreased within 24 h. Otherwise, **17** had a completely different kinetic profile as it reached peak concentration in plasma at 7 h post GSPE administration, and later levels were decreased down to 24 h and maintained up to 48 h. Moreover, **17** had maximum concentration levels of approximately 0.7 μM, whereas **18**, **19** and **20** did not reach concentrations higher than 0.5 μM. No other phase II conjugated colonic metabolites were detected in rat plasma (data not shown).

4. Discussion

The changes occurring during the first-pass metabolism are an important aspect of flavanols. The molecular forms that reach the peripheral circulation and tissues are different from those that are present in foods (Kroon et al., 2004). Microflora extensively affect flavanol cleavage and hence further contribute to the variation in the molecular forms of the flavanols found in blood and tissues (Dall'Asta et al., 2012; Gonthier et al., 2003). These findings suggest that the bioactive forms of flavanols could be products of their wide metabolism (Del Rio et al., 2013; Guerrero et al., 2013). Although there are several studies in rats and humans evaluating flavanol phase II metabolism (Arola-Arnal et al., 2013; Das & Rosazza, 2006), the microbial colonic catabolism of flavanols has been poorly studied *in vivo*, but it has been evaluated extensively by *in vitro* experiments using a human or rat faecal matrix (Cueva et al., 2013; Sánchez-Patán et al., 2012a,b Serra et al., 2012). As the phase II metabolism of flavanols is already well established, in this study, we focused only on the evaluation of rat microbial biotransformation of flavanols through a kinetic analysis of rat plasma after acute GSPE administration (1000 mg/kg). We propose an *in vivo* microbial metabolic pathway for grape seed flavanols (Fig. 8).

Similar to other studies, we found that the non-metabolised compounds reach their peak plasma concentrations at 2 h post-administration of GSPE (Serra et al., 2013). Although also abundant in GSPE, non-metabolised dimeric procyanidins or those with higher molecular weights showed much less

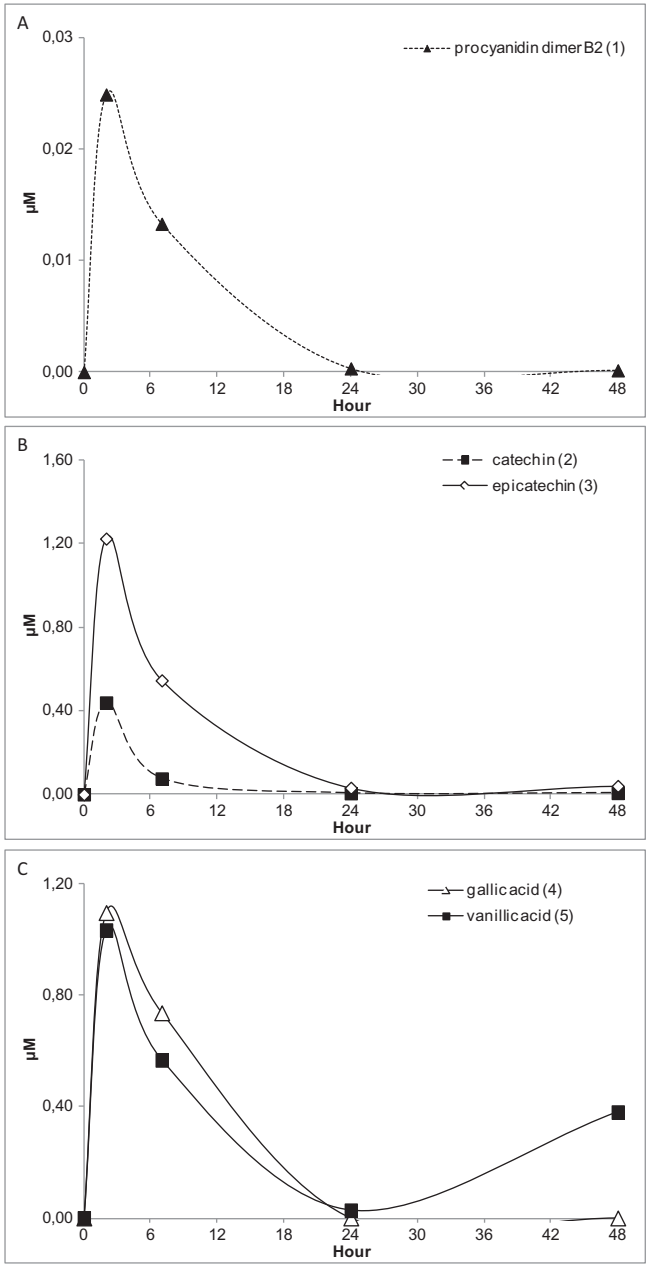

Fig. 2 – Kinetic profiles of rat plasma non-metabolised flavanols from grape seed proanthocyanidin extract (GSPE). Proanthocyanidin dimer B2 (1) (A), Monomeric flavanols (2 and 3) (B) and Phenolic acids (4 and 5) (C). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

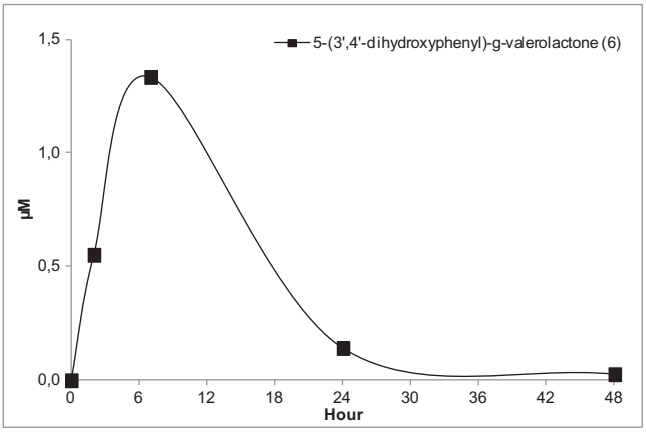

Fig. 3 – Kinetic profiles of rat plasma valerolactone metabolite (6) from grape seed proanthocyanidin extract (GSPE). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

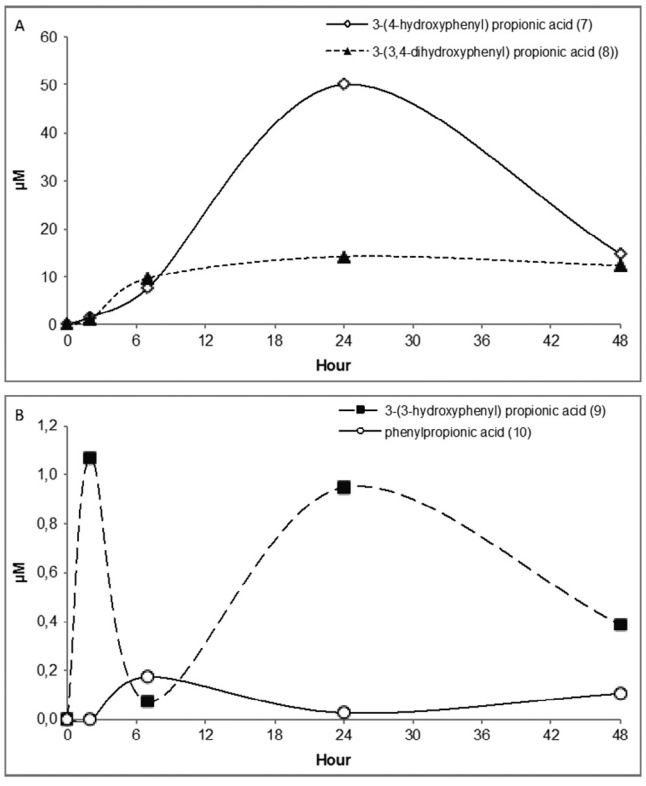

Fig. 4 – Kinetic profiles of rat plasma phenylpropionic acid from grape seed proanthocyanidin extract (GSPE). 3-(4-hydroxyphenyl)propionic acid (7) and 3-(3,4-dihydroxyphenyl)propionic acid (8) (A) and 3-(3-hydroxyphenyl)propionic acid (9) and phenylpropionic acid (10) (B). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode in the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

significant plasma levels than non-metabolised flavan-3-ols or did not appear in plasma, respectively. This is attributed to the larger flavanol molecular weight, making small intestine absorption more difficult, and hence the polymeric forms reach the colon to be subjected to microbial metabolism (Del Rio et al., 2013; Monagas et al., 2010). Once in the colon, flavanols can be biotransformed by three different metabolic pathways (Fig. 8). The first pathway is meta-substitution of the flavanol A ring producing 5-(2′,4′-dihydroxy) phenyl-2-ene-valeric acid (21).

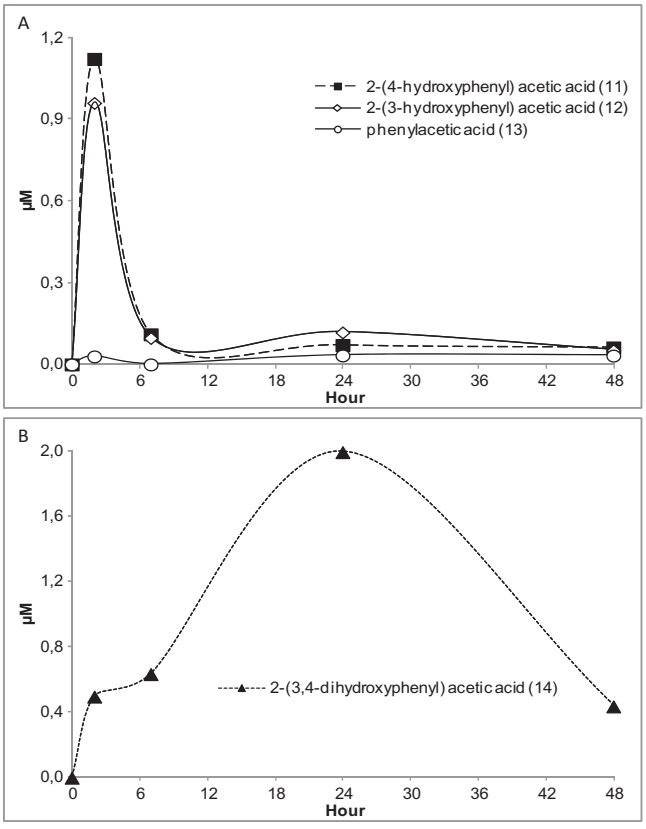

Fig. 5 – Kinetic profiles of rat plasma phenylacetic acids from grape seed proanthocyanidin extract (GSPE). 2-(4-hydroxyphenyil)acetic acid (11), 2-(3-hydroxyphenyil)acetic acid (12) and phenylacetic acid (13) (A) and 2-(3,4-dihydroxyphenyil)acetic acid (14) (B). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

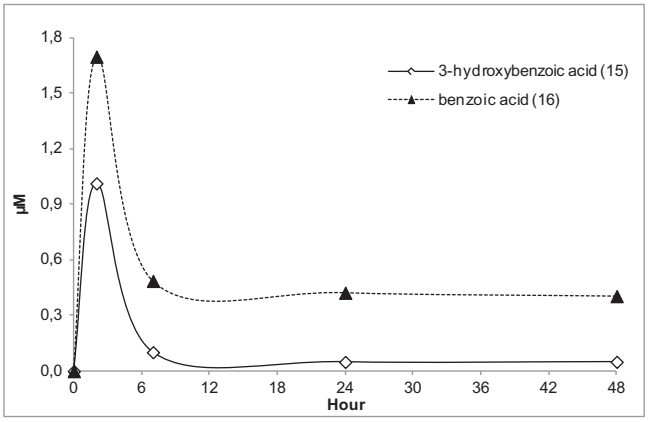

Fig. 6 – Kinetic profiles of rat plasma benzoic acids (15 and 16) from grape seed proanthocyanidin extract (GSPE). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

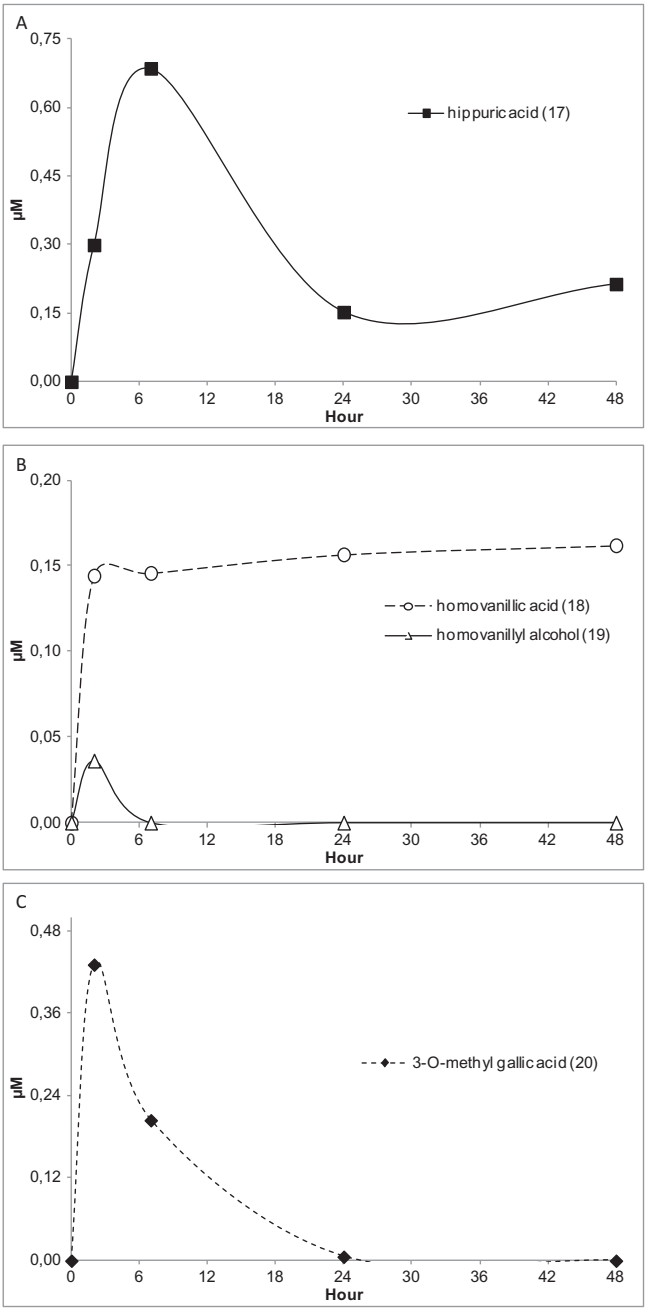

Fig. 7 – Kinetic profiles of rat plasma phenolic acids from grape seed proanthocyanidin extract (GSPE). Hippuric acid (17) (A), homovanillic acid (18) and homovanillyl alcohol (19) (B) and 3-O-methyl gallic acid (20) (C). Concentrations (µM) were quantified using HPLC-MS/MS in negative mode on the plasma of rats (n = 6) treated with GSPE (1000 mg/kg) for 0 h, 2 h, 7 h, 24 h and 48 h.

However, this metabolite was not detected in rat plasma (data not shown), suggesting that it could be an exclusively human metabolite because it was detected using human faecal microbiota. Alternatively, it is an intermediate unable to reach the bloodstream as it is known to be further metabolised by reduction reactions (Stoupi, Williamson, Drynan, Barron, &

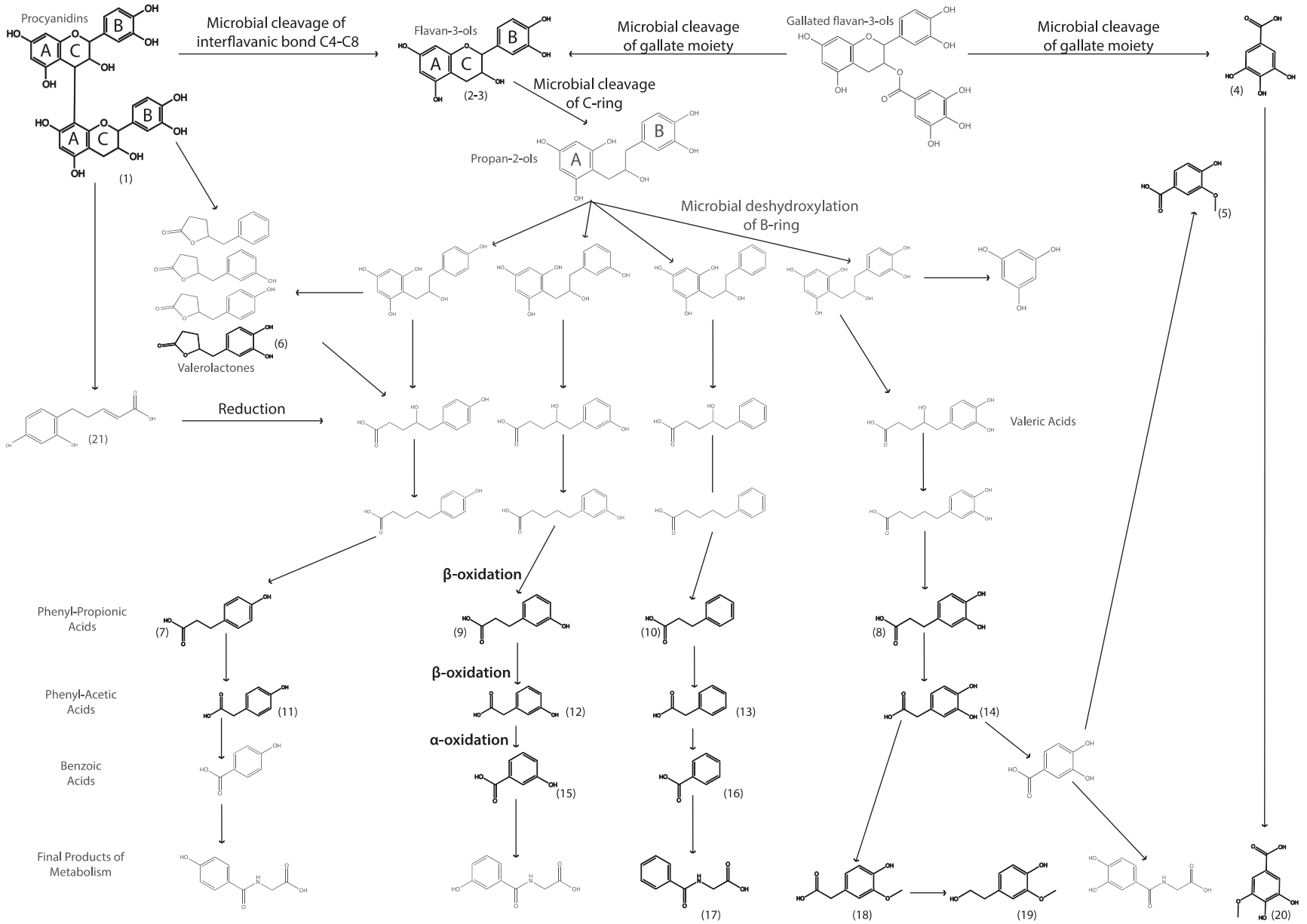

Fig. 8 – The proposed microbial colonic biotransformation pathway of proanthocyanidin in rats according to the detected metabolites in rat plasma after 0 h, 2 h, 7 h, 24 and 48 h of a 1000 mg/kg of a grape seed proanthocyanidin extract (GSPE) administration. The metabolites detected in rat plasma appear in bold, and the metabolites cited in the text are numerated.

Clifford, 2010a). The second pathway is biotransformation to valerolactone compounds, such as **6**, by the microbial cleavage of flavanol C- and A- rings (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009). The final pathway is microbial cleavage of the flavanol interflavanic bond C4–C8 to be transformed in their respective monomeric forms (**2** and **3**) (Appeldoorn et al., 2009). Monomeric aglycones can also get to the colon from conjugated compounds such as epicatechin gallate by microbial cleavage of the gallate moiety (Selma et al., 2009) or from phase II conjugates that reach the colon through bile extraction by enterohepatic recirculation or from non-absorbed monomeric flavanol glucosides that pass intact throughout the gastrointestinal tract. Monomeric flavan-3-ols (**2** and **3**) can undergo a microbial cleavage of the C-ring to produce propan-2-ol metabolites with different degrees of hydroxylation by a microbial dehydroxylation of the B-ring. Propan-2-ol metabolites may become valerolactones (**6**) by microbial A-ring cleavage. Alternatively, it is suggested that these valerolactones could come directly from the PA dimers by the microbial cleavage of the C- and A- rings (Appeldoorn et al., 2009). This metabolic process is in agreement with the kinetic behaviour of **6**, which had a maximum plasma concentration 7 h after the non-metabolised flavanols had sufficient time to be processed. The propan-2-ol metabolites were not detected in rat plasma (data not shown), which supports the metabolic pathway proposed in this study (Fig. 8) as propan-2-ol are intermediates that are further metabolised.

These early metabolites undergo microbial cleavage of the lactone ring by acidic hydrolysis (Dall'Asta et al., 2012; Del Rio et al., 2013) to form valeric acid metabolites, which were also not detected in rat plasma as they are intermediate compounds (data not shown). Although no valeric acid or propan-2-ol metabolites were detected in rat plasma, previous *in vitro* studies with rat (Serra et al., 2011, 2012) or human (Cueva et al., 2013; Sánchez-Patán et al., 2012a,b) faecal microflora detected these compounds. This study found that these intermediates do not reach the systemic circulation at sufficient concentration levels to be detected and quantified by MS, which suggests that they may remain in the colon to be subject to the microbial metabolism.

It has been described that valeric acids may suffer a β-oxidation of the branched chain to form phenyl propionic acids and their derivatives (Selma et al., 2009; Stoupi et al., 2010a), which are the main compounds from the microbial metabolism (Aura, 2008). In this sense, the main grape seed flavanol metabolite product of the microbial biotransformation was **7**, which reached levels of 50 μM in rat plasma at 24 h after GSPE ingestion. Propionic acids appeared in plasma after valerolactone metabolites, which agrees with our proposed metabolic pathway (Fig. 8). Other phenylpropionic acids (such as **8–10**) were quantified in rat plasma. However, **7–10** are further metabolised to form phenylacetic acids (**11–14**) and benzoic acids (**15–17**) by the β- and α-oxidation of their branched chain, respectively (Stoupi, Williamson, Drynan, Barron, & Clifford, 2010b). A different kinetic behaviour was demonstrated between **14** and phenylacetic acids (Fig. 5), which suggests that the hydroxylation pattern could be performed when the compounds reach the systemic circulation by the hydroxylation enzymes, more than by the microbial metabolism (Aura, 2008).

Phenolic acid derivatives (**17–20**) are the final products of the metabolism, which are formed by the enzymatic conjugation of phenolic acids (Gregus, Fekete, Halaszi, & Klaassen, 1996; O'Leary et al., 2001), and hence a heterogeneous kinetic profile was also observed in rat plasma. In this sense, **17**, also known as benzoylglycine, is formed in the liver from benzoic acid in two enzymatic steps with benzoyl-coA synthase and benzoyl-CoA glycine N-transferase (Gregus et al., 1996; Nandi, Lucas, & Webster, 1979); **18** and **19** are methylated derivatives of phenylacetic acids, and **20** is a methylated derivative of gallic acid. The methylation is produced mainly by hepatic catechol-O-methyltransferase (COMT) (O'Leary et al., 2003). As final products of flavanol metabolism, all of these compounds are still present in plasma 48 h post GSPE administration. Particularly, **5** reached maximal concentrations in plasma 2 h post GSPE administration as this compound is also present in GSPE. After 24 h, its concentration in plasma starts to increase again as a product of the flavanol colonic metabolism.

This study shows how grape seed flavanols are biotransformed by rat gut bacteria enzymes and how they appear in rat plasma over time. These results may facilitate identification of flavanol bioactive forms for particular health effects. Further human studies using the described methodology will be interesting due to the different metabolism between species and the intra- and inter-individual colonic bacteria variations (Kleessen, Bezirtzoglou, & Mättö, 2000; Lampe, 2003).

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REFERENCES

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: Chemistry, food content, and metabolism. *Nutrition (Burbank, Los Angeles County, Calif.)*, 18(1), 75–81.

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)-gamma-valerolactone as the major metabolites. *Journal of Agricultural and Food Chemistry*, 57(3), 1084–1092.

Arola-Arnal, A., Oms-Oliu, G., Crescenti, A., Del Bas, J. M., Ras, M. R., Arola, L., & Caimari, A. (2013). Distribution of grape seed flavanols and their metabolites in pregnant rats and their fetuses. *Molecular Nutrition & Food Research*, 57(10), 1741–1752.

Aron, P. M., & Kennedy, J. A. (2008). Flavan-3-ols: Nature, occurrence and biological activity. *Molecular Nutrition & Food Research*, 52(1), 79–104.

Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews*, 7(3), 407–429.

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica*, 70(12), 1103–1114.

Cueva, C., Sánchez-Patán, F., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., Bartolomé, B., & Moreno-Arribas, M. V. (2013). In vitro fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: Changes in microbial groups and phenolic metabolites. *FEMS Microbiology Ecology*, *83*(3), 792–805.

Dall'Asta, M., Calani, L., Tedeschi, M., Jechiu, L., Brighenti, F., & Del Rio, D. (2012). Identification of microbial metabolites derived from in vitro fecal fermentation of different polyphenolic food sources. *Nutrition (Burbank, Los Angeles County, Calif.)*, *28*(2), 197–203.

Das, S., & Rosazza, J. P. N. (2006). Microbial and enzymatic transformations of flavonoids. *Journal of Natural Products*, *69*(3), 499–508.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*, *18*(14), 1818–1892.

Demelbauer, U. M., Plematl, A., Josic, D., Allmaier, G., & Rizzi, A. (2005). On the variation of glycosylation in human plasma derived antithrombin. *Journal of Chromatography. A*, *1080*(1), 15–21.

Gonthier, M. P., Donovan, J. L., Texier, O., Felgines, C., Remesy, C., & Scalbert, A. (2003). Metabolism of dietary procyanidins in rats. *Free Radical Biology and Medicine*, *35*(8), 837–844.

Gregus, Z., Fekete, T., Halaszi, E., & Klaassen, C. (1996). Lipoic acid impairs glycine conjugation of benzoic acid and renal excretion of benzoylglycine. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *24*(6), 682–688.

Guerrero, L., Margalef, M., Pons, Z., Quiñones, M., Arola, L., Arola-Arnal, A., & Muguerza, B. (2013). Serum metabolites of proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells. *The Journal of Nutritional Biochemistry*, *24*(12), 2092–2099.

Kleessen, B., Bezirtzoglou, B., & Mättö, J. (2000). Culture-based knowledge on biodiversity, development and stability of human gastrointestinal microflora. *Microbial Ecology in Health and Disease*, *12*(2), 53–63.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., & Williamson, G. (2004). How should we assess the effects of exposure to dietary polyphenols in vitro? *The American Journal of Clinical Nutrition*, *80*(1), 15–21.

Lampe, J. W. (2003). Isoflavonoid and lignan phytoestrogens as dietary biomarkers. *The Journal of Nutrition*, *133*(3), 956S–964.

Manichanh, C., Reeder, J., Gibert, P., Varela, E., Llopis, M., Antolin, M., Guigo, R., Knight, R., & Guarner, F. (2010). Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome Research*, *20*(10), 1411–1419.

Margalef, M., Pons, Z., Muguerza, B., & Arola-Arnal, A. (2014). A rapid method to determine colonic microbial metabolites derived from grape flavanols in rat plasma by liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, *62*, 7698–7706.

McGaw, E. A., Phinney, K. W., & Lowenthal, M. S. (2010). Comparison of orthogonal liquid and gas chromatography–mass spectrometry platforms for the determination of amino acid concentrations in human plasma. *Journal of Chromatography. A*, *1217*(37), 5822–5831.

Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., Andres-Lacueva, C., & Bartolomé, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food & Function*, *1*(3), 233–253.

Nandi, D. L., Lucas, S. V., & Webster, L. T. J. (1979). Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. *The Journal of Biological Chemistry*, *254*(15), 7230–7237.

O'Leary, K. A., Day, A. J., Needs, P. W., Mellon, F. A., O'Brien, N. M., & Williamson, G. (2003). Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: The role of human β-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. *Biochemical Pharmacology*, *65*(3), 479–491.

O'Leary, K. A., Day, A. J., Needs, P. W., Sly, W. S., O'Brien, N. M., & Williamson, G. (2001). Flavonoid glucuronides are substrates for human liver β-glucuronidase. *FEBS Letters*, *503*(1), 103–106.

Pinent, M., Bladé, M. C., Salvadó, M. J., Arola, L., Hackl, H., Quackenbush, J., Trajanoski, Z., & Ardévol, A. (2005). Grape-seed derived procyanidins interfere with adipogenesis of 3T3-L1 cells at the onset of differentiation. *International Journal of Obesity (2005)*, *29*(8), 934–941.

Pinent, M., Blay, M., Bladé, M. C., Salvadó, M. J., Arola, L., & Ardévol, A. (2004). Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology*, *145*(11), 4985–4990.

Puiggros, F., Llópiz, N., Ardévol, A., Bladé, C., Arola, L., & Salvadó, M. J. (2005). Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems. *Journal of Agricultural and Food Chemistry*, *53*(15), 6080–6086. doi:10.1021/jf050343m.

Quiñones, M., Guerrero, L., Suarez, M., Pons, Z., Aleixandre, A., Arola, L., & Muguerza, B. (2013). Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Research International*, *51*(2), 587–595.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R. M., Quintanilla-López, J. E., Lebrón-Aguilar, R., Martín-Álvarez, P. J., Moreno-Arribas, M. V., & Bartolomé, B. (2012a). In vitro fermentation of a red wine extract by human gut microbiota: Changes in microbial groups and formation of phenolic metabolites. *Journal of Agricultural and Food Chemistry*, *60*(9), 2136–2147. doi:10.1021/jf2040115.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., Moreno-Arribas, M. V., & Bartolomé, B. (2012b). Gut microbial catabolism of grape seed flavan-3-ols by human faecal microbiota. Targetted analysis of precursor compounds, intermediate metabolites and end-products. *Food Chemistry*, *131*(1), 337–347.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: Role in human health. *Journal of Agricultural and Food Chemistry*, *57*(15), 6485–6501.

Serra, A., Macià, A., Romero, M. P., Anglés, N., Morelló, J. R., & Motilva, M. J. (2011). Metabolic pathways of the colonic metabolism of procyanidins (monomers and dimers) and alkaloids. *Food Chemistry*, *126*(3), 1127–1137.

Serra, A., Macià, A., Romero, M. P., Reguant, J., Ortega, N., & Motilva, M. J. (2012). Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids. *Food Chemistry*, *130*(2), 383–393.

Serra, A., Macià, A., Rubió, L., Anglès, N., Ortega, N., Morelló, J. R., Romero, M. P., & Motilva, M. J. (2013). Distribution of procyanidins and their metabolites in rat plasma and tissues in relation to ingestion of procyanidin-enriched or procyanidin-rich cocoa creams. *European Journal of Nutrition*, *52*(3), 1029–1038. doi:10.1007/s00394-012-0409-2.

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

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010b). Procyanidin B2 catabolism by human fecal

microflora: Partial characterization of "dimeric" intermediates. *Archives of Biochemistry and Biophysics*, 501(1), 73–78.

Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J., Arola, L., & Blay, M. (2011). Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of Nutritional Biochemistry*, *22*(4), 380–387.

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

Urpi-Sarda, M., Monagas, M., Khan, N., Llorach, R., Lamuela-Raventós, R. M., Jáuregui, O., Estruch, R., Izquierdo-Pulido, M., & Andrés-Lacueva, C. (2009b). Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography. A*, *1216*(43), 7258–7267.