RESEARCH ARTICLE

Proanthocyanidin metabolites associated with dietary fibre from *in vitro* colonic fermentation and proanthocyanidin metabolites in human plasma

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Proanthocyanidins (PAs) or condensed tannins, a major group of dietary polyphenols, are oligomers and polymers of flavan-3-ol and flavan-3, 4-diols widely distributed in plant foods. Most literature data on PAs' metabolic fate deal with PAs that can be extracted from the food matrix by aqueous-organic solvents (extractable proanthocyanidins). However, there are no data on colonic fermentation of non-extractable proanthocyanidins (NEPAs), which arrive almost intact to the colon, mostly associated to dietary fibre (DF). The aim of the present work was to examine colonic fermentation of NEPAs associated with DF, using a model of *in vitro* small intestine digestion and colonic fermentation. Two NEPA-rich materials obtained from carob pod (*Ceratonia siliqua* L. proanthocyanidin) and red grapes (grape antioxidant dietary fibre) were used as test samples. The colonic fermentation of these two products released hydroxyphenylacetic acid, hydroxyphenylvaleric acid and two isomers of hydroxyphenylpropionic acid, detected by HPLC-ESI-MS/MS. Differences between the two products indicate that DF may enhance the yield of metabolites. In addition, the main NEPA metabolite in human plasma was 3,4-dihydroxyphenyl acetic acid. The presence in human plasma of the same metabolites as were detected after *in vitro* colonic fermentation of NEPAs suggests that dietary NEPAs would undergo colonic fermentation releasing absorbable metabolites with potential healthy effects.

Keywords:
Colonic fermentation / Dietary fibre / Human plasma / Metabolites / Proanthocyanidins

1 Introduction

Proanthocyanidins (PAs) or condensed tannins, a major group of dietary polyphenols, are oligomers and polymers of flavanols that have shown health-related biological properties, particularly with regard to the prevention of chronic diseases and gastrointestinal disorders [1–3].

PAs can be divided in two groups in regards to the extraction technique: those that can be extracted from the food matrix by aqueous-organic solvents (''extractable proanthocyanidins'' or EPAs) and those that remain in the residues of these extractions, called ''non-extractable

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Abbreviations: a.u., arbitrary unit; **CSPA,** *Ceratonia siliqua* L. proanthocyanidin; **DF,** dietary fibre; **GADF,** grape antioxidant dietary fibre; **EPAs,** extractable proanthocyanidins; **IF,** indigestible fraction; **IF-GADF,** indigestible fraction of grape antioxidant dietary fibre; **MRM,** multiple reaction monitoring; **NEPAs,** non-extractable proanthocyanidins; **PAs,** proanthocyanidins; **SCFA,** short-chain fatty acid

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proanthocyanidins'' (NEPAs). EPAs include low- or intermediate-molecular weight PAs (dimers to decamers), while NEPAs are mainly structures linked to the protein and polysaccharides of the dietary fibre (DF) matrix, which cannot be extracted either by the action of digestive enzymes or by the usual aqueous acidic methanol-acetone solvents [4–6].

Dietary EPAs are partially hydrolysed to epicatechin and absorbed in the small intestine [7–10], after which they are extensively conjugated in the liver [11, 12] and another fraction reaches the colon – along with DF and indigestible dietary substrates – where they are metabolized to a large extent by gut microbiota, producing metabolites such as phenylacetic, phenylpropionic and phenylbutyric acids [7, 13].

On the other hand, absorption in the small intestine is much lower or entirely absent in the case of NEPAs, including higher oligomers, polymers and PAs associated with DF within the food matrix [7, 13]. These NEPAs will therefore reach the colon nearly intact.

Articles dealing with fermentation of PAs mainly focus on EPAs, whereas there are very few articles dealing with NEPAs. Moreover, most studies use isolated standards or concentrated extracts of PAs as test samples [9–10, 13–16], whereas it is less common to study PAs as part of the food matrix [17, 18].

Some studies have addressed the interaction between extractable phenolic compounds and DF [17, 19]. However, we are unaware of any studies dealing with NEPAs or PAs associated with DF. However, an appreciable part of dietary PAs reach the human colon along with DF, and both non-digestible carbohydrates and PAs may be expected to interact with the bacterial enzymes, affecting the extent of fermentation.

In this context, the present work examines colonic fermentation (extent and end-products) of NEPAs associated with DF, using a model of *in vitro* small intestine digestion and colonic fermentation. Two NEPA-rich materials obtained from carob pod and red grapes were used as test samples.

The identification of phenolic metabolites after *in vitro* fermentation of PAs, mainly EPAs, has been reported, but there have been few studies on the bioavailability of PAs in humans. With regard to dietary polyphenols, it is known that gallic acid and isoflavones are the best absorbed in the human small intestine, while EPAs are partially absorbed and NEPAs are not absorbed at all. The production of fermentation metabolites from unabsorbed PAs may be quantitatively and biologically important. However, to the best of our knowledge it has yet to be determined whether these potentially bioavailable metabolites are present in human plasma in detectable amounts. Another aim of this work was therefore to determine PAs fermentation metabolites in plasma of healthy subjects.

2 Materials and methods

2.1 Reagents

Pancreatin, lipase, bile extract porcine and α-amylase were from Sigma-Aldrich Química, S.A. (Madrid, Spain). Amyloglucosidase was from Roche Diagnostics, Mannheim, Germany.

Standards of (−)-epicatechin (≥97%), (+)-catechin (≥98%), (−)-epicatechin gallate (≥98%), 4-hydroxybenzoic acid (≥97%), 2-hydroxybenzoic acid (≥97%), 3-hydroxybenzoic acid (≥97%), 2-hydroxyphenylacetic acid 3,4-dihydroxyphenylacetic acid (≥97%), 3,4-dihydroxyphenylpropionic acid (>98%) , 4-hydroxyphenylpropionic acid (phloretic acid) (≥98%), syringic acid (≥95%), 3,4-dihydroxybenzoic acid (≥97%), *p*-coumaric acid (≥98%), *o*-coumaric acid (≥97%), hippuric acid (≥97%), caffeic acid (≥98%), ferulic acid, phenylacetic acid (>99%) and chlorogenic acid (≥95%) were from Sigma Chemical (Saint Louis, MO, USA).

Reagents (analytical grade) for the extraction and fractionation of metabolites and degradation products were: methanol, ethyl acetate and 37% hydrochloric acid from Panreac (Montcada i Reixac, Spain); acetone and diethyl ether from Carlo Erba (Milano, Italy); ACN and formic acid from Merck (Darmstadt, Germany). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA) to a resistivity of 18.2 MΩcm.

2.2 Samples

PAs concentrate from Mediterranean carob pod, *Ceratonia siliqua* L., (CSPA, *Ceratonia siliqua* L. proanthocyanidin) was supplied by Nestlé S.A. (Vevey, Switzerland). This concentrate is a polymerised PA made up of flavan-3-ol units with an MW around 32 000 D.

Grape antioxidant dietary fibre (GADF) is a natural product obtained from red grapes (Cencibel variety, vintage year 2005, La Mancha region, Spain). GADF combines large amounts of DF and phenolic antioxidants such as phenolic acids, anthocyanidins, PAs, catechins and other flavonoids [20]. The proximate composition of GADF and CSPA is presented in Table 1. Methods used to determine DF as indigestible fraction, PAs, non-starch polysaccharides, protein and others have been previously described [21–23].

2.3 Methods

2.3.1 *In vitro* colonic fermentation

This procedure has two main steps: (i) isolation of the indigestible fraction and (ii) colonic fermentation of the indigestible fraction (Fig. 1).

Table 1. Composition of GADF and CSPA (g/100 g dry matter)

	GADF	CSPA
Total dietary fibre[a)]	73.48±0.79	15±0.3
Soluble dietary fibre	15.53±0.11[b)]	0
Insoluble dietary fibre	57.95±0.78[c)]	15±0.3[b)]
PAs	14.81±0.19	78.30±1.83
Fat	7.69±0.49	0
Protein	11.08±0.46	4±0.1
Ash	5.25±0.19	3±0.1

a) Determined as indigestible fraction, ref 21.
b) Non-starch polysaccharides.
c) Major constituents: non-starch polysaccharides (23.9±0.6); PAs (13.6±0.2); resistant protein (7.2±0.3); lignin and others – by difference – (28.78).

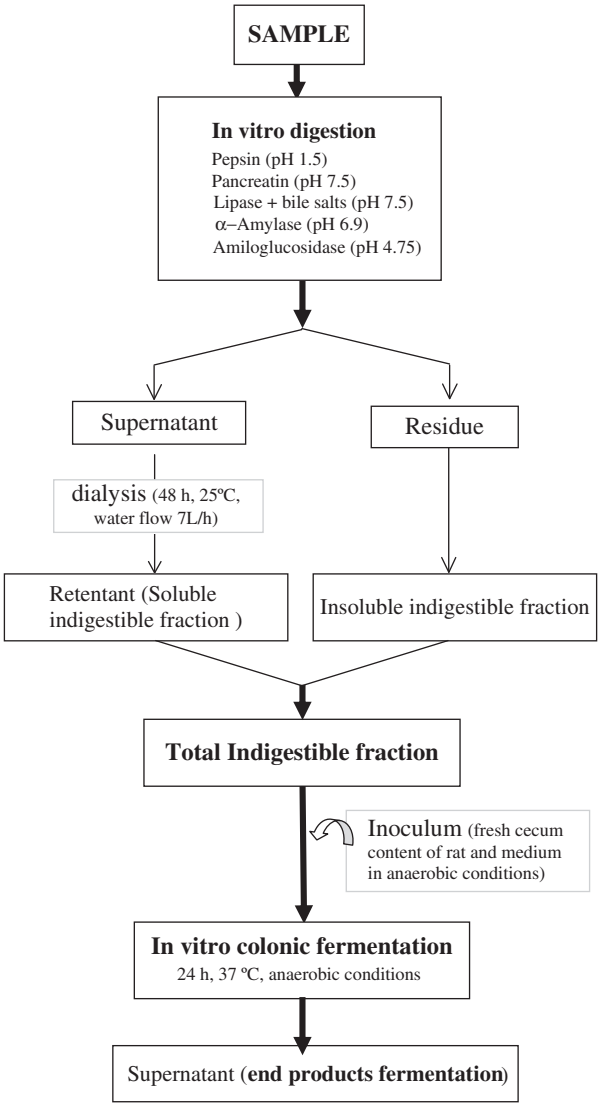

Figure 1. Schematic of *in vitro* colonic fermentation procedure.

2.3.2 Isolation of the indigestible fraction

The procedure described by Saura-Calixto *et al.* [21] was applied to GADF. Samples were successively incubated with digestive enzymes to simulate digestion in the small intestine. Briefly, 300 mg of sample was incubated with pepsin (0.2 mL of a 300 mg/mL solution in HCl-KCl 0.2 M buffer, pH 1.5, 40°C, 1 h, Merck 7190), pancreatin (1 mL of a 5 mg/mL solution in phosphate buffer 0.1 M; pH 7.5, 37°C, 6 h), and α-amylase (1 mL of a 120 mg/mL solution in tris-maleate buffer 0.1 M; pH 6.9, 37°C, 16 h). Then samples were centrifuged (15 min, 25°C, 3000 × *g*) and supernatants removed. Residues were washed twice with 5 mL of distilled water and all supernatants combined. Residues are the insoluble indigestible fraction. Each supernatant was incubated with 100 μL of amyloglucosidase for 45 min at 60°C. They were transferred into dialysis tubes (12 000–14 000 MWCO; Dialysis Tubing Visking, Medicell International, London, UK), and dialyzed against water for 48 h at 25°C (water flow 7 L/h). The dialyzed supernatants are the soluble indigestible fraction. Total indigestible fraction (soluble indigestible fraction+insoluble indigestible fraction) was the substrate to *in vitro* colonic fermentation. This indigestible fraction (IF-GADF) contains non-starch polysaccharides, PAs, resistant protein and others (see Table 1). Isolation of the indigestible fraction was not necessary for CSPA because the physicochemical procedure to obtain this material removed all digestible compounds.

2.3.3 *In vitro* colonic fermentation [4, 24]

Samples (epicatechin, IF-GADF and CSPA) were fermented in a batch culture system under strict anaerobic conditions for 24 h. Fresh cecal contents from fed controlled rats were used as inoculum. Anaerobic conditions were maintained using oxygen-free carbon dioxide. Male Wistar rats (body weight of 200±5 g) fed with standard maintenance diets adjusted to rat dietary requirements were supplied by the breeding Center at the Faculty of Pharmacy (University Complutense of Madrid, Spain). After sacrifice, ceca contents from five rats were diluted (100 g/L) with anaerobic medium, mixed (10 min) in a Stomacher 80 Lab Blender (Seward Medical, London, UK) and filtered (1 mm mesh) to obtain the inoculum.

Triplicate substrates (100 mg of IF-GADF, epicatechin and CSPA, control without substrate and control containing lactulose) were hydrated with the fermentation medium (8 mL).Two millilitres of inoculum were added and the headspace rinsed with carbon dioxide (1 min) in order to keep anaerobic conditions. Tubes were placed in a shaking water bath (80 strokes/min, 37°C, 24 h). Controls without substrate (blank) and controls containing lactulose were included in the experiment as non-fermentable and completely fermentable substrate, respectively. After incubation time (24 h), pH was measured. A manometer was

adapted to each fermentation tube to measure gas pressure. Samples were centrifuged ($2500 \times g$, 10 min) and the supernatants in duplicate were taken for analytical determinations (short-chain fatty acids (SCFAs) and phenolic metabolites).

SCFAs were measured by the method of Spiller *et al* [25], slightly modified. Supernatant (400 μL), internal standard (4-mehtyl valeric acid, 2 mM) (500 μL) and 50 μL of formic acid 12% were made up to 1 mL with Milli-Q water and centrifuged (4°C, 7300 *g*, 15 min). An aliquot of 1 μL of supernatant was injected into a 5890 Hewlett Packard gas chromatograph equipped with a flame ionization detector and a fused silica column (Carbowax 20 M, 10 m × 0.53 mm id). Nitrogen was the carrier gas at a pressure of 17 kPa. Injector and detector temperature was 200°C and column temperature 120°C (isothermal). SCFAs were identified and quantified by comparison with SCFA standards. The results were corrected with blanks of fermentation.

SCFA produced were expressed as mmol *per* gram of dry substrate and as molar proportions acetate:propionate:butyrate. The percentage of fermentability was calculated as the relative percentage of total SCFA produced by the samples with respect to the total SCFA produced by the lactulose at 24 h.

2.4 Design of the study in humans

Twelve non-smoker healthy subjects (eight women and four men with an average age of 25.5 ± 2.8 years) were recruited within the university community. Three 24 h dietary recalls were performed during the weeks previous to the assay and were analyzed by the DIAL System (Departamento de Nutrición y Bromatología, Universidad Complutense de Madrid, Spain). Subjects were instructed to follow a low-polyphenol diet the day before the assay (excluding wine, coffee, tea, chocolate, fruits, vegetables and whole-grain cereals). After an overnight fast, venous blood (12 mL) was collected in EDTA tubes and plasma was obtained by centrifugation. Subjects gave their informed consent to participate in the intervention, and the study protocols were approved by the Ethics Committee of the Hospital Carlos III (Madrid, Spain).

2.5 Sample preparation for analysis by HPLC-ESI-MS/MS

2.5.1 Metabolites from *in vitro* fermentation

Fermentation samples from epicatechin, FI-GADF, CSPA and blanks were processed in duplicate (10 mg each). Then, 10 ppm of salicylic acid was added in each sample as internal standard. An Oasis HLB (3 cc, 60 mg) cartridge from Waters (Mildford, MA, USA) was used for SPE. The cartridge was activated with 1 mL of methanol and 2 mL of water. Then samples were loaded onto the cartridge. To remove interfering components, the sample was washed with 9 mL of water with 5% of methanol and 2% formic acid. The phenolic compounds were then eluted with 1 mL of methanol. The eluates were evaporated under nitrogen until dryness and reconstituted with 200 μL of 0.1% aqueous formic acid (initial HPLC mobile phase). The temperature in the evaporation step was controlled (< 30°C) so as to avoid the deterioration of the phenolic compounds. The samples were filtered through a PTFE 0.45 μ membrane filter from Waters into amber vials for HPLC-MS/MS analysis and stored at −20°C.

2.5.2 Plasma metabolites

To precipitate interfering proteins, 700 μL of plasma were treated with CH_3CN (1.5 mL). Then, after adding internal standard (taxifolin, 10 ppm, 40 μL) the mixtures were centrifuged ($3000 \times g$, 10 min); the supernatant evaporated by nitrogen stream and the pellet taken up in water (1.5 mL) and homogenized on a vortex. The samples were submitted to SPE and work-up as described in Section 2.5.1.

2.6 Analysis by HPLC-ESI-MS/MS

An Agilent series 1100 HPLC instrument (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and an autosampler was used. The loads were of 5 μL of filtered sample. The separations were conducted using a Phenomenex Luna C18(2) (Torrance, CA, USA) 3.5 μm particle size column (50×2.1 mm id) equipped with a Phenomenex Securityguard C18 column (4×3 mm id). The mobile phase consisted of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN. The gradient elution was 8–60% [B] in 12 min followed by washing and re-equilibration step, at flow rate of 400 μL/min.

An API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ont., Canada) equipped with a TurboIon spray source was used to obtain MS/MS data. The settings were: capillary voltage −3500 V (negative mode), nebulizer gas (N_2) 10 arbitrary units (a.u.), curtain gas (N_2) 12 a.u., collision gas (N_2) 10 a.u., declustering potential 50 V, focusing potential −200 V, entrance potential 10 V and collision energy (CE) −30 V. The drying gas (N_2) was heated to 400°C and introduced at a flow rate of 8000 cm^3/min.

2.6.1 Metabolite characterization

The characterization and quantification of the metabolites in plasma and in colonic fermentation supernatants was based on their ion fragmentation in the multiple reaction monitoring (MRM) mode. The following transitions were monitored: benzoic acid m/z 121 → 77; dihydroxybenzoic acid m/z

153→109; phenylacetic acid *m/z* 135→91; hydroxyphenylacetic acid *m/z* 151→107; dihydroxyphenylacetic acid *m/z* 167→123; hippuric acid *m/z* 178→154; hydroxyhippuric acid *m/z* 194→150; caffeic acid *m/z* 179→135; hydroxyphenylpropionic acid *m/z* 165→121; dihydroxyphenylpropionic acid and homovanillic acid *m/z* 181→137; 2-propenoic acid (ferulic acid) *m/z* 193→134; hydroxyphenylvaleric acid *m/z* 193→175; coumaric acid *m/z* 163→119; syringic acid *m/z* 181→163; chlorogenic acid *m/z* 353→191; enterodiol *m/z* 297→253; enterolactone *m/z* 209→137. Metabolites were quantified using calibration curves of hydroxyhippuric acid; hydroxybenzoic acid, dihydroxyphenylacetic acid; and dihydroxyphenylpropionic acid, under same experimental conditions. The use of these phenol standards is justified by the similarity of their chemical properties to those of the metabolites studied. The samples were processed using Analyst 1.4.2 software. The metabolites described here as coming from IF-GADF and CSPA where those giving after 24 h of fermentation signals clearly stronger than those in the controls (fermentation medium without adding the samples at 24 h).

3 Results and discussion

3.1 *In vitro* fermentation

A part of the dietary polyphenols is solubilized in the intestinal fluid during digestion by pH- and temperature-driven physicochemical modifications and/or by digestion of the food matrix by the action of digestive enzymes.

Polyphenols available in soluble form in the small intestine (flavonols, flavones, catechins, phenolic acids, *etc.*), including low molecular weight PAs (dimers and trimers), may be partially absorbed in the small intestine and metabolized. However, a major fraction of dietary PAs reaches the colon, where they become a substrate for fermentation by the action of bacterial enzymes. The potentially fermentable fraction of dietary PAs is made up of: (i) EPAs solubilized in intestinal fluids but unabsorbed because they do not pass the intestinal mucose or because they form less digestible complexes with protein, starch and digestive enzymes [26], plus (ii) a substantial amount of NEPAs associated with DF that are neither solubilized nor degraded by digestive enzymes. The present work addresses the fermentation of two materials containing DF and NEPAs, in different proportions (Table 1) GADF is made up of grape seeds and peels and contains a large amount of DF, a high percentage of which is soluble, while CSPA contains only insoluble DF. CSPA is made up of NEPAs with a basic flavan-3-ol structure and molecular weight over 30 000 [27] plus an appreciable amount of non-digestible polysaccharides (DF). Isolation of polimeric PAs from carob pods has been reported [28], which consisted of subunits of flavan-3-ols and their gallate esters among others, and that has been proven to be associated with insoluble DF [29–32].

Samples were treated under physiological conditions to simulate digestion (Fig. 1). The samples were treated with digestive enzymes to remove the digestible constituents (protein, fat, carbohydrates, *etc.*) and were then inoculated with rat cecal content for colonic fermentation (Fig. 1). A European Interlaboratory Study presents comparable results for DF fermentation end products using inoculum either from rat ceca or from human faeces [24].

EPAs and nutrients (sugars, minerals, amino acids, lipids, *etc.*) were removed by dialysis (48 h). The remaining residues of the enzymatic treatments – containing the insoluble DF – and the retentate or non-dialysed solubilized fraction – containing the soluble DF – were combined and inoculated with rat cecal content for *in vitro* colonic fermentation following the procedure described above. This insoluble and soluble DF from GADF is composed of indigestible complexes containing non-digestible protein and NEPAs in addition to polysaccharides and lignin.

The SCFA production from the *in vitro* fermentation is shown in Table 2. The extent of fermentation or fermentability is a parameter calculated on the basis of the total amount of SCFA produced after 24 h of colonic fermentation in comparison with lactulose standard (100% fermentability). IF-GADF presented greater fermentability (39%) than the CSPA (23%). This was presumably because the GADF contained more DF than the CSPA (73 *versus* 15%), and in particular it contained an appreciable amount of soluble DF (15.5% *versus* 0). It is well known that insoluble DF presents low colonic fermentability while soluble DF is usually highly fermentable, the main end products being SCFA and gas [33]. The molar proportion of SCFA (acetic:propionic:butyric) was comparable in the two samples, which is consistent with the typical SCFA profile of DFs.

SCFAs are the end-fermentation products of DF or non-digestible polysaccharides-carbohydrates, and it was assumed that fermentation of PAs does not yield SCFA. This was corroborated by a control fermentation test performed with monomeric epicatechin, the basic structure of NEPAs. No SCFA were detected in the fermentation supernatant, while significant amounts of the typical end products of PAs (hydroxyphenyl acetic acid or other acids) were found. Studies on fermentation of PAs occasionally

Table 2. Fermentability of NEPA-rich materials

	IF-GADF	CSPA
Fermentability	49.9	23.1
Molar proportion (acetate:propionate:butyrate)	50:39:10	59:29:12
ΔpH[a)]	0.71	0.46
Δpress (psi)[b)]	5.0	2.7

Calculated from $(SCFA_{(sample)}/SCFA_{(lactulose)}) \times 100$.
a) pH value at time zero – pH value at 24 h.
b) Gas pressure at 24 h – gas pressure at time zero.

report SCFA in the supernatants, but this may be because it is not unusual for samples of PA concentrates or extracts to contain appreciable amounts of DF. One example of this is Bazzoco *et al.* [18], who reported that the colonic fermentation of PAs from apple (with a mean degree of polymerization of 71 after enzymatic digestion) caused the release of phenolic acids and production of SCFA.

SCFA and phenolic metabolites reduced the pH of the fermentation medium, which was naturally higher in IF-GADF (0.71) than in the assay with CSPA (0.46). Similarly, the increase of pressure due to gas production (CO_2, H_2, CH_4, *etc.*) was greater during the fermentation of IF-GADF.

The PA-derived polyphenolic metabolites identified after 24 h of colonic fermentation are listed in Table 3. The main metabolite detected from IF-GADF and CSPA samples was hydroxyphenylacetic acid with concentrations of 8.7 and 8.3 ng/mg of dry sample, respectively. This metabolite may be the result of dehydroxylation of dihydroxyphenylacetic acid, which is typically formed during *in vitro* colonic fermentation of flavonols [15]. Hydroxyphenylvaleric acid was the second main metabolite detected in the samples (2.7 ng/mg for IF-GADF and 2.1 ng/mg for CSPA). Again, this metabolite comes from the proathocyanidins included in both IF-GADF and CSPA [10]. Two isomers of hydroxyphenylpropionic acid (3-hydroxyphenylpropionic acid and 4-hydroxyphenylpropionic acid) were also detected by monitoring of the MRM transition 165 → 121. 4-hydroxyphenylpropionic acid was identified by the use of the standard. Both were presumably formed by hydroxylation of 3,4-dihydroxyphenylpropionic acid, which was in turn formed by ring-fission of epicatechin [33].

The total production of metabolites (ng of metabolites *per* g of dry sample) was similar in both IF-GADF and CSPA fermented samples (13 *versus* 11.5 ng). However, if results are expressed as ng of metabolites *per* g of NEPAs in the original samples, the yield is considerably higher in IF-GADF than in CSPA (87.8 *versus* 14.7); in other words, the fact that concentrations of both soluble and insoluble DF were higher in IF-GADF may have enhanced the fermentability of NEPAs, probably due to an increase of bacterial enzymatic activity. Similarly, Aprikian *et al.* [17] reported higher fermentability of apple polyphenols when fermentation was performed in the presence of pectins – soluble DF – and, interestingly, that the combined fermentation of pectins and polyphenols led to a more pronounced improvement in plasma lipid profile than supplementation with either of the two separately. On the other hand, Bazzoco *et al.* [18] reported that the presence of PAs suppressed SCFA formation from carbohydrates, suggesting that PAs inhibit enzymes from the colon microbiota, possibly the same type of enzymes that degrade PAs into small metabolites. There are various possible explanations for the differences between this work and our results. Firstly, the inhibitory effect on microbial activity may depend on the type of PA (molecular size, links with other compounds) and also on the microbiota composition. Bazzoco *et al.* used EPAs and not NEPAs and the effect of these two types of PA may be different. Second, both total concentrations of NEPAs and DF, as well as the NEPAs/DF ratio may be a key factor determining whether bacterial activity is inhibited or enhanced. Finally, DF and EPAs/NEPAs may not be degraded by the same enzymes. In any case, more research is needed to confirm both a possible synergistic action between NEPAs and DF and different effects of EPAs and NEPAs on colonic microbiota.

3.2 PA metabolites in human plasma

The intake of DF and indigestible food constituents in the whole diet in Western countries is around 45–60 g/person/day, while the intake of total PAs, including EPAs and NEPAs, has been estimated at from 0.1 to 1 g/person day depending on the methodology and the population chosen [4, 34]. NEPAs represent a significant part of the total dietary PA. Therefore, there is a high ratio DF/PAs in the human colon, which may favour DF-associated fermentation of this type of polyphenols.

To our knowledge, the possible presence of NEPA fermentation metabolites in human plasma has not been reported before. Our results show that the main PA metabolite in human plasma was 3,4-dihydroxyphenyl acetic acid (also detected in its methylated and sulphated forms), followed by hydroxyphenylvaleric and hydroxyphenylpropinic acids. Figure 2 shows the HPLC-ESI-MS/MS profile corresponding to the detection of hydroxyphenylpropionic acid in the MRM mode (165121 transition) detected in the plasma.

Hydroxyphenylacetic acid was also the main metabolite from *in vitro* fermentation of IF-GADF, which is particularly rich in NEPAs. Polymeric epicatechin, the main phenolic component of NEPAs, ultimately yields absorbable phenolic

Table 3. Main polyphenolic metabolites from *in vitro* fermentation of IF-GADF and CSPA

Phenolic metabolites detected	MRM transition	Metabolite name	ng metabolite/mg of IF-GADF	ng metabolite/mg of dry CSPA
1	151 → 107	Hydroxyphenylacetic acid	8.7	8.3
2	193 → 175	Hydroxyphenylvaleric acid	2.7	2.1
3	165 → 121	3- or 4-Hydroxyphenylpropionic acid	1.2	0.9
4	165 → 121	3- or 4-Hydroxyphenylpropionic acid	0.4	0.2

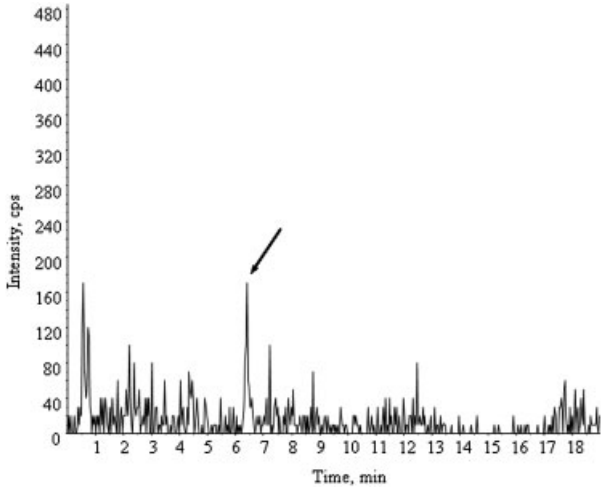

Figure 2. HPLC-ESI-MS/MS profile corresponding to the detection of hydroxyphenylpropionic acid in human plasma. Separation conditions were as stated in the text.

acids after colonic fermentation. Moreover, the presence of methylated and sulphated forms would indicate partial conjugation in the liver. The metabolic pathway that leads to the formation of hydroxyphenylacetic acid and other aromatic acids has been partially elucidated: catechin is degraded into progressively shorter acids starting with 3,4-dihydroxyphenylvaleric acid and 3-hydroxyphenylvaleric acids [13, 15].

Interestingly, supplementation with a grape seed polyphenol extract containing 20.5% polymeric PAs (mean degree of polymerization of 2.7) to hypertensive subjects resulted in increased urinary excretion of 3-hydroxyphenylpropionic acid and 3-hydroxyphenylacetic acid [35]. Regarding hydroxyphenylacetic acid, other authors have reported its excretion after the intake of other foods and beverages rich in EPAs such as beer [36] or cranberry juice [37], so it might be thought that the detection of phenolic acids in plasma in the present study would be due to the intake of other polyphenols and not only NEPAs. However, the measurements in these studies were taken between 60 and 270 min after intake of the corresponding product. Since in the present study subjects had taken a low polyphenol diet the day before and at the time the blood was taken they had been fasting for 12 h, it would seem more likely that the phenolic acids found in plasma came from colonic fermentation of polyphenols, mainly NEPAs, since these are the ones that most reach the colon intact (Fig. 2).

4 Concluding remarks

NEPAs associated with DF, which are not extractable by aqueous-organic solvents or by digestive enzymes, undergo fermentation in the colon, where the extent of fermentation and the yield of metabolites may be enhanced by the presence of DF. Since NEPAs constitute an important fraction of PAs daily intake, the potential significance of this process for gastrointestinal health and other systemic effects remains to be elucidated. The presence in human plasma of the same metabolites as were detected after *in vitro* colonic fermentation of NEPAs means that NEPAs included in a regular diet would undergo colonic fermentation, releasing absorbable metabolites with potential healthy effects.

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The authors have declared no conflict of interest.

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