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Evaluation of the potential of total proanthocyanidin content in feces as an intake biomarker

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ABSTRACT

Due to the health benefits associated with proanthocyanidins (PAs), it is useful to identify dietary PA biomarkers that can be determined by simple methods. Since increased levels of circulating PA metabolites are associated with increased fecal PA content, this study explores the spectrophotometric measurement of fecal PA content and its use as a biomarker of PA intake. To this end, fecal PA content was measured using an adaptation of Porter's spectrophotometric method in samples from a preclinical study and an observational study. In the former, excretion of 250–400 mg PA polymer equivalents/100 g feces was observed during supplementation and the day after, together with a significant association ($p < 0.05$) between PA intake and the excretion of both intact PAs and some PA metabolites, i.e., (+)-catechin, (−)-epicatechin and syringic acid. No relationship between intake and excretion was found in the observational study, either for the entire group (mean excretion of 240 ± 226 mg PA polymer equivalents/100 g feces) or after stratification into tertiles of consumption. In conclusion, the spectrophotometric determination of total PA content in feces proved to be a valid compliance marker in a preclinical study, but it was not associated with PA intake in free-living subjects. The potential of total PA excretion in observational studies, determined in fecal samples collected the day before dietary recall or in several fecal samples from the same subject, remains to be elucidated, as does a complete validation of the method proposed here.

1. Introduction

Proanthocyanidins (PAs) are a class of dietary polyphenols that form part of the family of flavonoids. They include from dimers to high-molecular-weight polymers, consisting of a small number of flavan-3-ols, and can be divided into subclasses such as procyanidins, which are composed exclusively of (epi)catechin units, or prodelphinidins, which include among their constituents at least one unit of (epi)gallocatechin. A schematic overview of PAs is shown in Figure S1. Depending on the linkages between their constituent monomers, PAs may be classified as type B (the most common ones, including only a covalent bond between C4 of the upper monomer and C6 or C8 of the lower monomer) or as type A (which have an additional ether bond between C2 and the hydroxyl group of the C5 or C7 of the lower monomer). While PA oligomers and some polymers may be extracted from food by aqueous–organic solvents and are thus called extractable proanthocyanidins (EPAs), other polymeric PAs remain in the residues from such extractions, showing different associations with the food matrix, and are therefore called non-extractable proanthocyanidins (NEPAs). PAs, including EPAs and NEPAs, are present in many common foods, such as

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certain fruits, legumes, nuts or cocoa (Santos-Buelga and Scalbert, 2000).

Cumulative results, based on mechanistic, preclinical and clinical trials, as well as observational studies, demonstrate the capacity of PAs to modulate several factors related to cardiovascular diseases, such as the lipid profile, blood pressure or flow-mediated dilation (Kruger, Davies, Myburgh and Lecour, 2014; Nunes, Pimentel, Costa, Alves and Oliveira, 2016). Moreover, evidence increasingly shows their potential to modulate type 2 diabetes (Burton-Freeman, Brzezinski, Park, & Sandhu, 2019; González-Abuín, Pinent, Casanova-Martín, Arola, Blay and Ardevol, 2015). Furthermore, observational studies have reported an inverse association between PA intake and colorectal cancer, which is greater the higher the degree of polymerization of the PAs (Rossi, Bosetti, Negri, Lagiou and La Vecchia, 2010). Finally, several *in vitro* and animal studies have reported mechanisms of action by which PAs help prevent colorectal cancer (Ravindranathan, Pasham, Bajaji, Cardenas, Gu, Toden and Goel, 2018), although these results remain to be confirmed in clinical trials.

Once ingested, PAs are known to be extensively metabolized (Rodríguez-Mateos, Vauzour, Krueger, Shanmuganayagam, Reed, Calani, Mena, del Rio and Crozier, 2014). Briefly, we know that only dimers, and rarely trimers, are absorbed in the small intestine; in contrast to monomeric flavanols, known to conjugate with methyl, sulphate or glucuronic acid substituents (Mateos-Martín, Pérez-Jiménez, Fuget and Torres, 2012), PA dimers or trimers have only been detected *in vivo*, as far as we know, in their intact forms (Tsang, Auger, Mullen, Bornet, Rouanet, Crozier and Teissedre, 2005; Pereira-Caro et al., 2018). All PAs that are not absorbed in the small intestine reach the colon, where they are transformed by colonic microbiota, leading to a wide variety of derived phenolic acids. These metabolites may then be absorbed or conjugated in the liver and transferred to the bloodstream, with final urinary excretion. Alternatively, they may be excreted with non-transformed PAs in feces. A fraction of absorbed PA metabolites may return to the small intestine via enterohepatic circulation. Interestingly, a fraction of PAs that is not commonly included in food analysis due to analytical limitations, the high-molecular-weight NEPAs, has also been shown to be at least partially transformed by the action of colonic microbiota (Mateos-Martín et al., 2012). Overall, the metabolic fate of PAs has been explored in both animal and human studies; notably, studies in pigs have shown a profile of PA metabolites similar to that reported in human studies (Choy et al., 2014), which makes this model suitable for work in this field.

Currently, interest is growing in discovering and validating food component intake biomarkers: compounds present in biological samples that when determined allow us to estimate recent or average intakes of specific food groups, foods or food components (Gao et al., 2017). Intake biomarkers may help to overcome well-known limitations of dietary questionnaires, e.g., inaccurate or mistaken intake estimation in observational studies as well as problems evaluating compliance in clinical trials. Such biomarkers may be measured in different fluids. Urine has been suggested as the most appropriate for evaluating polyphenol intake (Pérez-Jiménez, Hubert, Hopper, Cassidy, Manach, Williamson and Scalbert, 2009), although some bioavailability studies have also evaluated polyphenols in feces, since these correlate with certain circulating metabolites (Stoupi, Williamson, Viton, Barron, King, Brown and Clifford, 2010). Also, fecal metabolite values are associated with microbiota profiles (Choy et al., 2014). Moreover, since increased oxidative stress is present in several colorectal pathologies (Wang, Li, Cao and Tian, 2016), the presence of polyphenol metabolites in the colon, contributing to a more antioxidant local environment, could have potential benefits in such cases. However, to the best of our knowledge, the determination of PAs in feces as a biomarker of PA intake has not previously been explored.

With regards to PAs, the use of dietary questionnaires has even more limitations than for other dietary constituents. In particular, most food composition tables only include EPAs, due to the analytical limitations in NEPA determination alluded to earlier. Nevertheless, NEPAs also contribute to the total PA content of common foods (Pérez-Jiménez and Saura-Calixto, 2015) and, as described above, to the PA metabolites in circulation (Mateos-Martín, Pérez-Jiménez, Fuguet and Torres, 2012; Rodríguez-Mateos et al., 2014). Meanwhile, evaluation of the whole variety of PA metabolites or of intact PAs in biological samples is a complex matter that requires specific techniques such as HPLC-MS (high-performance liquid chromatography-mass spectrometry) or HPLC with a fluorescence detector. In contrast, some spectrophotometric methods have been developed to determine the PA content of foods. Such is the case of the DMAC (4-dimethylaminocinnamaldehyde) method in which, under strongly acidic conditions, DMAC gives rise to an electrophilic carbocation which reacts with the C8 of the terminal A-ring in the PA structure, leading to a green compound that can be determined spectrophotometrically (Li, Tanner and Larkin, 1996). An alternative colorimetric test is Porter's method (Porter, Hrstich and Chan, 1986). It consists of treatment with butanol in an acid medium, using iron as a catalyst, in order to breakdown the PAs, releasing anthocyanidins. This method has been used in food samples to analyze both EPAs and NEPAs (Pérez-Jiménez, Arranz and Saura-Calixto, 2009), although it has not previously been applied to fecal samples.

Therefore, the aim of this study is to assess the potential use of total fecal PA content, as determined using Porter's method, as a biomarker of total PA intake. To this end, samples from two different studies were evaluated: a) a preclinical study in which pigs were supplemented with grape seed extract (GSE), in order to evaluate the validity of this determination as a compliance marker in intervention studies; b) an observational study of healthy subjects, in order to explore the potential of total fecal PAs as an intake biomarker in free-living subjects.

2. Experimental section

2.1. Materials

Iron (III) chloride, procyanidin B1 and hydrochloric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butanol and hexane were purchased from Panreac Química S.L.U. (Castellar del Vallès, Barcelona, Spain). MegaNatural® Gold GSE was kindly donated by Polyphenolics Inc. (Madera, CA, USA); it contains 91.9% phenolics (w/w), in terms of gallic acid equivalents, as determined by the Folin-Ciocalteu assay.

2.2. Animal study

2.2.1. Animals and study design

Animal protocols were conducted in accordance with the ILAR Guide for the Care and Use of Laboratory Animals, with approval from the University of California Davis Institutional Animal Care and Use Committee (Protocol #:17257). As previously reported (Choy et al., 2014), six crossbred female pigs weighing 130–150 kg were kept in their standard shared pen throughout the experiment. The animals were fed a standard diet (2 kg per day) composed mostly of corn and soy, neither of which contains PAs, along with nutritional supplements (Ref. 320354 M, Associated Feed & Supply Co., Turlock, CA, USA); diet details are provided as Supplementary **Table S1**. The treatment diet was the control diet supplemented with 1% w/w GSE. The pigs were fed (always at 8:00–8:30 am) with the standard diet for 3 days, then they were fed with the treatment diet for 6 days and finally there was a 3-day post-supplementation period, when they received the control diet again. Fecal samples were collected daily after feeding, put on ice and stored at −80 °C within one hour of collection. The data for total PA excretion obtained from these samples were correlated with data previously reported (including information on analytical conditions and the standards used) for individual metabolites measured by HPLC-MS as well as for PAs according to the degree of polymerization, in all cases using aliquots of the samples (Choy et al., 2014).

2.2.2. Dosage information

Since the pigs consumed about 2 kg of the treatment diet containing 1% of GSE daily and they weighed about 140 kg, the daily dose was 143 mg GSE/kg body weight. This amount may be transformed to the human equivalent dose (HED), i.e., the dose in humans anticipated to have the same degree of effect as that observed in the animals. To perform this calculation, we need to know the Km value: a correction factor for transforming a mg/kg dose to mg/m^2, in order to be able to come back to mg/kg for a different species. Since the Km value has not been reported for pigs, it is recommended to take the mean human body weight (with a standard value of 60 kg) and that of the animal, and apply the formula HED = animal dose in mg/kg × (animal weight in kg/human weight in kg) (FDA, 2005). In the present study, this led to an HED of 190 mg GSE/kg.

2.3. Observational study

The subjects formed part of an observational study that aimed to evaluate the association between diet and macular pigment optical density; full details are provided elsewhere (Olmedilla-Alonso, Rodríguez-Rodríguez, Beltrán-de-Miguel, Estévez-Santiago and Sánchez-Prieto, 2021). Briefly, the inclusion criteria were: age, 45–65; total cholesterol, <240 mg/dL; BMI, ≤30 kg/m^2; adherence to a varied diet; no medication for obesity or cholesterol; no use of dietary supplements; and no diagnosis of cardiovascular diseases, diabetes or inflammatory diseases. The study was approved by the Drug Research Ethics Committee of the Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain (acta n° 03.17, dated 13 February 2017) as well as by the CSIC Ethics Committee. All the subjects signed written informed consent to participate in the study. Of a total of 101 subjects, a sample of 85 (62 women and 23 men) were used for the estimation of total PA excretion and intake, due to the availability of both fecal samples and dietary recall reports.

Recent dietary intake was evaluated using three 24 h recalls, one of which corresponded to the weekend or a holiday, carried out over a period of 7 to 10 days. The participants recalled their first report face to face with a specialized interviewer and then performed the other two by telephone. Additionally, the subjects provided a fecal sample from a representative day within a range of two days before or after the first recall, which was stored frozen until analysis; this approach was chosen because the aim of the study was to evaluate the potential association between habitual (and not specific) food intake and PA excretion. The criteria for a representative day were: 1) a day where the subject followed their usual diet, i.e., did not test a new food or consume some food item eaten only rarely; and 2) exclusion of gastrointestinal disorders (the specific instruction was: "Avoid collecting the sample on an unusual day, with a possible gastrointestinal disorder. If this has not been possible, please inform us." No subject informed of any such situation).

2.4. Study design

2.4.1. Evaluation of PA intake

The first step to evaluate PA intake was the processing of the 24 h recall reports in order to obtain the daily consumption of food containing PAs. When needed, recipes or serving sizes were taken from the DIAL System (Universidad Complutense de Madrid, Spain; Ortega, López-Sobaler, Andrés, Requejo, Aparicio and Molinero, 2008). Since the 24 h recall reports corresponded to three different days, an average value for the three was obtained, considered as the principal daily consumption for each subject. Then, an in-house database of the PA content of foods was created, combining data for EPAs from existing databases, such as that of the United States Department of Agriculture and Phenol-Explorer (USDA, 2015; Neveu et al., 2010), with data for NEPAs from selected publications (Pérez-Jiménez and Saura-Calixto, 2015; Arranz, Silván and Saura-Calixto, 2010). In the case of EPAs, the databases used express the results based on the degree of polymerization, determined by HPLC; while for NEPAs, the chosen publications measured the content by the Porter method. This information is provided as Supporting Information (Table S1). Finally, this database was combined with the daily food consumption reported by the subjects to yield their daily PA intake.

2.5. Assessment of total proanthocyanidins in feces

Total PAs were evaluated in the fecal samples from both the preclinical and the observational study. The feces were freeze-dried and the extraction procedure was performed on the dried samples. A defatting step was applied before performing PA extraction, in order to remove possible confounding compounds (false positives were detected in initial trials without this step, which was introduced when the extraction protocol was optimized). 8 mL of hexane was added to 0.1 g of sample and shaking was applied for 20 min. After centrifugation (10 min, 1814*g*, Selecta Macrotronic, Abrera, Barcelona, Spain), the supernatant was discarded and PA extraction was performed on the residue, applying a spectrophotometric procedure developed for foods (Porter et al., 1986; Pérez-Jiménez et al., 2009). Briefly, 10 mL of butanol/HCl 37% (97.5:2.5) 0.7% $FeCl_3$ was added, and incubation at 100 °C was applied for one hour (GFL 1083, Lauda, Lauda-Königshofen, Germany). Centrifugation was applied (10 min, 1814*g*) and the supernatant was collected. Since after this procedure some released NEPAs were still present in the residue, it was subjected to two additional washings with the same solvent mixture: 5 mL was added in each case with centrifugation, and the supernatant was collected. All the supernatants were combined and taken up to a final volume of 25 mL.

Absorbance was measured in the extracts at 555 nm in a Synergy MX well-plate reader (BioTek, Winooski, Vermont, USA). The results were interpolated into a procyanidin dimer B1 standard curve. Since there are no commercial standards for polymeric PAs, but this is the common form of PAs in feces, the proportion between the absorbance of dimers and polymers previously reported (Porter et al., 1986) was used to transform the dimer B1 curve into a curve of polymeric PA equivalents. The PA extractions were performed in duplicate for each sample, followed by a duplicate absorbance measurement. Intra-day and inter-day precision were determined for a selection of three samples from the observational study, and showed variation coefficients of below 10% in both cases (**Table S2**); no further validation parameters were obtained.

2.6. Statistical analysis

PA excretion data from the preclinical study were processed by one-way ANOVA followed by Tukey's post-hoc HSD test, once the normal distribution of the data and the homogeneity of the variances were verified. Correlation between these data and those for individual PA metabolites previously reported in aliquots from exactly the same fecal samples (Choy et al., 2014) were assessed using Spearman's rank correlation coefficient, since most of the data did not follow a normal distribution.

The PA excretion data from the observational study did not follow a normal distribution, so non-parametric tests were used for their evaluation. The Mann–Whitney *U* test was used to compare PA excretion depending on age and sex. Spearman's rank correlation coefficient was used to assess correlation between PA intake and excretion in this population. Additionally, the subjects were stratified based on tertiles of PA intake; these tertiles were again compared using the Mann–Whitney *U* test.

In both studies, differences with $p < 0.05$ were considered significant. Statistical analysis was performed with SPSS 25 (SPSS Inc., Chicago, USA).

3. Results

3.1. Evaluation of total fecal PAs after PA supplementation: Preclinical study

Mean values for total fecal content of PAs, as measured by the adaptation of Porter's method, are shown in Fig. 1. No content was detected on days 0–2, when the animals did not receive the GSE supplement, or on day 3: the first day of supplementation (when the feces still corresponded to food from the day before). In contrast, PAs were detected on all the days of the supplementation and the first post-supplementation day (day 9 of the experiment). Significant differences were only found between day 5 and day 9 of the experiment; this seems logical, since day 9 was the first one without receiving the supplementation and it agrees with the fact that one day later (day 10 of the experiment) PAs were not detected in the feces. These null values on days without supplementation indicate the absence of a constant background response in the samples, thus demonstrating the specificity of the method even in such complex matrices as feces.

Individual values for total fecal PAs were used to establish correlations with data previously reported in this study for individual PA metabolites as well as for PAs by degree of polymerization (Choy et al., 2014). As shown in Table 1, several significant associations ($p < 0.05$) were found for some individual PA metabolites: (+)-catechin, (-)-epicatechin, 3-hydroxyphenylvaleric acid and syringic acid. An association close to significance ($p = 0.056$) was found for 4-hydroxyhippuric acid. In contrast, no significant association ($p > 0.05$) was found between total fecal PAs and the other 12 individual PA metabolites evaluated. Additionally, significant associations ($p < 0.05$) were found between total fecal PAs and fecal PA dimers, trimers, tetramers and pentamers.

3.2. Evaluation of total fecal PAs in a habitual diet: Observational study

Total fecal PAs were evaluated, using the Porter method, in a total sample of 85 subjects, due to the availability of both fecal samples and dietary recall reports. Of these subjects, 77% were younger than 60 and 73% were female. The excretion values for the whole group, as well as values according to sex and age, are shown in Table 2. The mean excretion value for the whole group was 241 ± 226 mg PA/100 g feces, ranging from 0 to 877 mg/100 g feces and with an interquartile range of 378 mg/100 g feces. A null value was obtained in 29% of the subjects, showing that, as in the animal study, there was no constant background response in the samples. No significant differences were found between the sexes or by age.

In order to evaluate possible associations between the intake and the total excretion of PAs in this population, first their PA intake was estimated (**Table S3**). The intake of EPAs ranged from 17 ± 20 mg/day for trimers to 65 ± 77 mg/day for polymers; while NEPA intake was 330 ± 333 mg/day. The main individual foods contributing to PA intake were banana, apple, dark chocolate and lentils (data not shown).

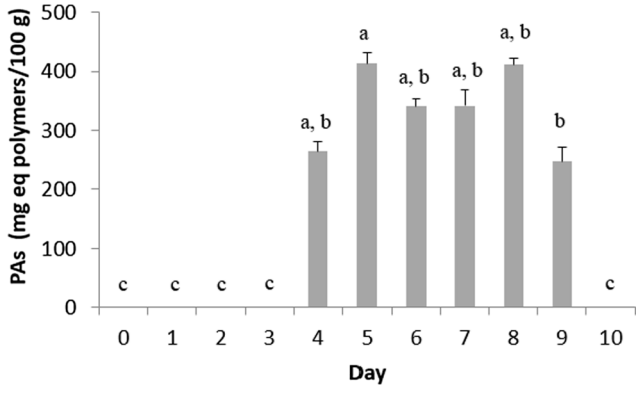

Fig. 1. Total fecal concentration (mg/100 g) of proanthocyanidins detected in pigs following intake of 1% (w/w) grape seed extract (GSE). Days 0–2, non-supplemented; days 3–8, GSE; days 9–10, post-feeding. Results are expressed as mean ± SD. Different superscript letters indicate significant differences ($p < 0.05$, resulting from one-way ANOVA followed by Tukey's *post hoc* test).

Table 1
Correlation analysis (R and *p*-values) between total fecal proanthocyanidins (measured by spectrophotometry analysis) and flavanols or derived metabolites (measured by HPLC-MS analysis) detected in pig feces following intake of 1% (w/w) GSE.

	Correlation coefficient	*p*-value (two-tailed)
Individual metabolites		
(+)-Catechin	0.598	**0.002**
(-)-Epicatechin	0.607	**0.002**
4-Hydroxyphenylacetic acid	−0.290	0.150
3-Hydroxyphenylacetic acid	−0.158	0.460
3,4-Dihydroxyphenylpropionic acid	−0.224	0.291
3-Hydroxyphenylvaleric acid	0.626	0.001
5-Phenylvaleric acid	0.168	0.430
m-Coumaric acid	−0.140	0.513
4-Hydroxybenzoic acid	0.153	0.475
3-Hydroxybenzoic acid	0.321	0.126
Hippuric acid	−0.052	0.808
4-Hydroxyhippuric acid	−0.396	0.056
Protocatechuic acid	0.238	0.261
Homovanillic acid	0.073	0.733
Vanillic acid	0.025	0.907
Syringic acid	−0.407	**0.048**
Caffeic acid	−0.034	0.875
Proanthocyanidins by class		
Dimers	0.599	**0.004**
Trimers	0.599	**0.004**
Tetramers	0.706	**0.001**
Pentamers	0.712	**0.001**

GSE, grape seed extract. HPLC-MS data determined in the same samples provided in Choy et al., 2014 and not from the present study. Values in bold print indicate significant differences ($p < 0.05$, resulting from Spearman's rank correlation coefficient).

No significant associations were found between total fecal PAs and PA intake, or when divided into the different EPA classes, or for total EPAs and NEPAs (Table 3). Partial correlations were also tested for, adjusting for age, sex, BMI and caloric intake, separately and together; and again we found no significant associations (data not shown). Nevertheless, correlations were found between the different PA categories, including between EPAs and NEPAs ($p = 0.001$), where food composition data came from non-related sources. Additionally, the subjects were classified into intake tertiles and we evaluated total fecal PAs in them (Fig. 2); again, no significant differences were found between the three intake tertiles.

4. Discussion

Current interest in biomarkers of the intake of bioactive food components is motivated by two different potential applications: their use as compliance indicators, in order to increase the robustness of clinical trials; and their potential capacity to classify the diet of free-living subjects, in the search for associations with health outcomes. Based on these possibilities, in this study we focused on the potential application of a spectrophotometric method for the determination of total PA content in feces, selecting samples from two different studies: a preclinical study where acute modifications were measured, and an observational study seeking associations with habitual PA intake. Validated methods for the determination of either intact PAs in feces or their derived metabolites do exist (Choy et al., 2014; Pereira-Caro et al., 2018), but they have analytical requirements and demand technical knowledge that may not be available in all laboratories interested in nutrition, despite the

Table 2
Fecal proanthocyanidin excretion (mg/100 g) from healthy subjects in an observational study.

Group	n	Mean ± SD	Median	IQR	Minimum	Maximum	*p*-value for median
Total	85	241.3 ± 225.6	219.3	378.3	0	876.6	
Sex							
Male	23	266.5 ± 195.5	247.4	181.1	0	744.8	0.216
Female	62	232.5 ± 236.0	186.3	402.5	0	876.5	
Age							
<60	65	225.2 ± 224.7	186.3	376.2	0	876.6	0.314
≥60	20	293.7 ± 226.2	273.2	241.0	0	760.6	

IQR, interquartile range. No significant differences ($p < 0.05$) were found, resulting from the Mann-Whitney *U* test.

Table 3
Correlation analysis (R and *p*-values) between proanthocyanidin intake and total fecal proanthocyanidins from healthy subjects in an observational study.

	Correlation coefficient	*p*-value (two-tailed)
Extractable proanthocyanidins		
Dimers	0.20	0.07
Trimers	0.13	0.26
Tetramers to hexamers	0.13	0.25
Heptamers to decamers	0.85	0.19
Polymers	0.84	0.22
Total	0.88	0.15
Non-extractable proanthocyanidins	0.27	0.58

No significant differences ($p < 0.05$) were found, resulting from Spearman's rank correlation coefficient.

increasing importance of polyphenols as key bioactive dietary components. Therefore, a spectrophotometric method such as the one proposed here could be a useful approach to the evaluation of total PA intake, even though it provides less detailed information than other existing techniques. It is becoming more common to collect fecal samples in nutritional studies for microbiota assessment, although the best biological fluid for evaluating polyphenol availability is urine (Pérez-Jiménez et al., 2009). Moreover, there is increasing evidence of the relevance of feces in the search for markers of polyphenol-rich foods (Jiménez-Girón, Ibáñez, Cifuentes, Simó, Muñoz-González, Martín-Álvarez, Bartolomé, Moreno-Arribas, 2015). Additionally, the presence of some metabolites of phenolic compounds in feces has recently been associated with a decrease in fecal water cytotoxicity (Zorraquín-Peña, González de Llano, Tamargo, Moreno-Arribas, Bartolomé, 2020), showing the biological relevance of the determination of phenolic metabolites in this fluid.

In the case of the acute supplementation with PAs via GSE, no traces were detected in fecal samples corresponding to the days before supplementation or the two days after, which demonstrates that the adaptation of Porter's method proposed here does not lead to false positives. At the same time, the alterations in total fecal PA content were coherent with the days of supplementation (with a mean daily dose of 143 mg GSE/kg body weight, see the heading Dosage information). Moreover, total fecal PA content showed significant correlations ($p < 0.05$) with values for PAs according to the degree of polymerization previously obtained in the same biological samples (Choy et al., 2014). Although this correlation may be expected when total PA content is measured in both cases, it should be highlighted that here, we used a less sensitive method than for the previous data obtained by HPLC with a fluorescence detector; thus, this also indicates the validity of the new approach.

While the correlation of total fecal PA content measured here by spectrophotometry with total fecal PA content determined by HPLC allows us to validate the new method, correlation with individual metabolites allows us to determine whether there is some association between non-transformed PAs and metabolized PAs. Thus, it is relevant that, in the preclinical supplementation study, the total PA content detected in the samples correlated significantly ($p < 0.05$) with several PA metabolites -monomeric flavanols and syringic acid- detected in the feces by HPLC-MS analysis (Choy et al., 2014), which have also been reported to be increased after supplementation of rats with a PA concentrate (Wu et al., 2014). However, for other metabolites (hydroxyphenylpropionic and hydroxyphenylacetic acid, among others), no correlation was found; this would seem to call for further research into the metabolic transformation of PAs. It has been reported that high levels of polyphenol metabolites are associated with a healthy dietary pattern, such as a Mediterranean diet (Gutiérrez-Díaz et al., 2017), and with beneficial health outcomes, such as a decrease in the severity of metabolic syndrome (Mora-Cubillos, Tulipani, García-Aloy, Bulló, Tinahones and Andrés-Lacueva, 2015). Although the present study did not focus on the health-related properties of PA metabolites, the associations found here show that PA intake leads to the generation

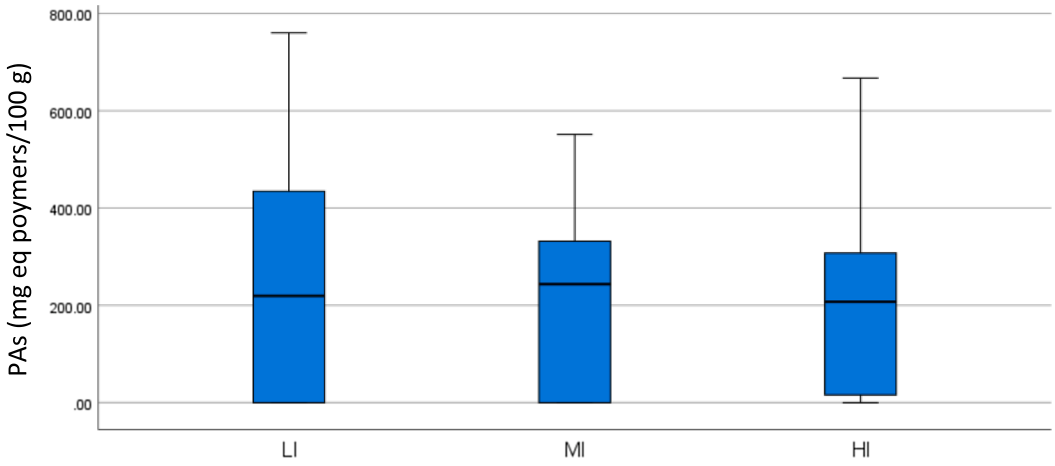

Fig. 2. Total fecal concentration (mg/100 g) of proanthocyanidins in healthy subjects from an observational study after low (LI), medium (MI) or high (HI) proanthocyanidin intake. No significant differences ($p < 0.05$) were found, resulting from the Mann-Whitney *U* test.

of beneficial PA metabolites together with excretion of PAs, since they are not completely transformed. Therefore, the determination of total PA content in feces by a simple method after specific supplementations would indirectly provide an estimation of the levels of PA metabolites. Since the determinations are performed in feces, and the PA metabolites would therefore have previously been in contact with the colonic epithelium, these results are particularly relevant for potential local effects. Moreover, it should be highlighted that an association between fecal PA metabolites and circulating PA metabolites has also been reported (Stoupi et al., 2010). So, the present study provides additional information on the effects of GSE supplementation of pigs, showing that the increase in intact PA families as measured by HPLC may also be determined by a less sophisticated spectrophotometric method.

Regarding the observational study, total PA intake (including EPAs and NEPAs) was estimated as a previous step, to assess the potential association between PA intake and excretion. The results for PA intake were in the same range as those reported in previous studies focused on EPAs or NEPAs (Pérez-Jiménez et al., 2015; Tresserra-Rimbau et al., 2013; Arranz et al., 2010). It should be borne in mind that the latter, which have been studied much less than EPAs, were major contributors to total PA intake in our population (although the methods used to determine the content of these two PA classes are not directly comparable). Indeed, a high contribution of banana to total PA intake was observed, due to the high NEPA content of this fruit (Pérez-Jiménez et al., 2009). Since the database we used for PA content was compiled from different sources for EPA and NEPA content, the correlations found between the intake of the two categories (data not shown) represent indirect evidence of its consistent design because, in general terms, foods containing PAs have both classes (Pérez-Jiménez et al., 2009).

We determined total fecal PAs in the observational study, providing the first results by the method proposed here in a free-living population. No significant differences were found according to either age or sex. There was a wide dispersion of the excretion data, principally as a result of two characteristics: the broad variation in PA intake, as also reported in other cohorts (Tresserra-Rimbau et al., 2013; Zamora-Ros et al., 2018); and the combination of interindividual aspects that led to considerable variability in polyphenol excretion, similarly as reported in other studies, even when using chromatography techniques (Garro-Aguilar et al., 2020). This interindividual variability is derived from several aspects, including physiological state, with recent studies suggesting a different polyphenol metabolic fate in obese and lean subjects (Barnes et al., 2019), and differences in basal microbiota, which is key for the fecal metabolome (Zierer et al., 2018).

In contrast to the results of the supplementation study, no significant associations were found between PA intake (total or by classes) and PA excretion in this population. Although it is evident that an observational study implies much broader differences than those in a preclinical study, at the same time some biomarkers have been reported for polyphenol intake in this kind of study (Zamora-Ros et al., 2018), so the range of characteristics may not be the cause of this discrepancy. Similarly, although limitations associated with food intake estimation based on 24 h recall are well known, they would be expected to be similar here to those in other studies using the same approach (Zamora-Ros et al., 2018). Additionally, we explored stratification of the cohort subjects by consumption level, but found that the tertiles of consumption were not associated with differences in PA excretion either (Fig. 2); despite this kind of stratification having previously been reported as effective in revealing biomarkers that are sensitive to consumption for a wide range of foods (Pujos-Guillot et al., 2013).

One approach for detecting a biomarker of a particular class of polyphenol intake is to analyze a biological sample obtained the same day the food items of interest were consumed (Lacalle-Bergeron et al., 2020). However, in this study, we decided to use fecal samples that did not correspond to the day before the dietary recall since, when significant associations are found in these cases, the proposed biomarkers are particularly useful in large-scale studies when samples may not correspond to the day before the dietary information was obtained. Indeed, the approach of performing measurements on biological samples that are representative of habitual food intake, without collecting dietary consumption information for that specific day, has been successful in other studies in the field of intake biomarkers, e.g., for citrus fruit or coffee consumption (Pujos-Guillot et al., 2013; Rothwell et al., 2014) and for total polyphenol intake (Medina-Remón et al., 2009). The difference between the results in our study and those others may stem from the fact that, in contrast to coffee or citrus fruit, where subjects may be clearly classified as consumers and non-consumers, or to total polyphenols that are present in all plant foods, PAs are found at very different concentrations but only in some specific foods. This means that the amount consumed by a subject may be quite different from one day to another. Moreover, while it has been possible to find metabotypes associated, for instance, with ellagitannin consumption (González-Sarrías et al., 2017), it is not clear that this is the case for PAs (Cortés-Martín et al., 2019; Mena et al., 2019), possibly due precisely to the differences in daily consumption for the same subject. Thus, a better approach for this study, besides the possibility of collecting fecal samples the day before the dietary recall, might have been to pool different fecal samples from each subject, which may then have been more representative of habitual PA intake. Indeed, in a recent study focused on dietary biomarkers of flavanone intake, where some compounds were found to be representative of acute but not of chronic intake, the authors suggested the need to collect several 24 h urine samples (Tahiri et al., 2020). An alternative approach to explore applications of the colorimetric determination of total fecal PAs would be to collect fecal samples corresponding to a recent intake and determine whether the intact compounds may be associated with the consumption of some specific PA-rich foods, and not with all foods containing PAs in general.

It may also be the case that the method we used here is sensitive to interference from the feces themselves. Thus, it is possible that there are other substances in feces that absorb at the same wavelength as we used. Although the fact that a null baseline value was obtained before supplementation in pigs and also in 29% of the human sample means that there is no constant background in all the samples, it is evident that colorimetric methods always exhibit limitations and further improvement may be necessary. Clearly, complete analytical validation of the method remains to be performed before it can be put to wider use.

In conclusion, the present study proposes an adaptation to fecal samples of a spectrophotometric method, in order to determine total PA content. We have shown that the method provides a valid compliance marker, as the results were significantly associated with PA supplementation, as well as with fecal metabolites measured by more sophisticated methods, in a preclinical study. Nevertheless, no associations were found between habitual PA intake and PA excretion measured by this method in an observational study of free-living subjects. The potential application of total PA excretion in observational studies, performing the determination in fecal samples collected the day before dietary recall reports or in several fecal samples from the same subject, remains to be elucidated, as does a complete validation of the method proposed here.

Author contributions

R. M. L.-R., B. O.-A. and J. P.-J. designed the study. C. M.-T., P. Q.-R., E. R.-R. and R. E.-S. performed the experimental assays. C. M.-T. and J. P.-J. carried out the data processing and statistical analysis. J. P.-J. wrote the first draft of the manuscript. All the authors approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no relationships that are in any way relevant to the contents of this paper to disclose.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110390.

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