Research Article

Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: A study on the Spanish diet

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Received: December 1, 2009
Revised: March 22, 2010
Accepted: April 26, 2010

Scope: Dietary polyphenols (PP) can be divided into two groups: extractable polyphenols (EPP) or compounds solubilized by aqueous organic solvents, and nonextractable polyphenols (NEPP) or compounds that remain in their corresponding extraction residues. Most studies on food polyphenols and dietary intakes address exclusively EPP. The objective of this work was to determine the actual amount of PP, including NEPP, in food and in a whole diet.
Methods and results: HPLC-MS analyses were performed to identify EPP in methanol–acetone extracts and NEPP in the acidic hydrolyzates of their extraction residues in cereals, fruits, vegetables, nuts, and legumes. NEPP contents, estimated as hydrolyzable PP plus nonextractable proanthocyanidins (PA), ranged from 880 mg/100 g dry weight in fruits to 210 mg/100 g in cereals and were substantially higher than the contents of EPP. NEPP intake (day/person) in the Spanish diet (942 mg) is higher than EPP intake (258 mg) fruits and vegetables (746 mg) are the major contributors to the total PP intake (1201 mg).
Conclusion: Non extractable polyphenols are the major part of dietary polyphenols. The knowledge of intakes and physiological properties of NEPP may be useful for a better understanding of the potential health effects of dietary PP.

Keywords:
Dietary polyphenols / Extractable polyphenols / Nonextractable polyphenols / Polyphenol intake / Spanish diet

1 Introduction

Polyphenols (PP) are the most abundant phytochemicals in the human diet and their biological properties, bioavailability, metabolism, and effects on the prevention of chronic diseases risk are nowadays the focus of a wide and intense research [1–4].

Dietary polyphenols can be divided into two groups on the basis of their solubility properties: extractable polyphenols (EPP) or compounds solubilized by aqueous organic solvents, and nonextractable polyphenols (NEPP) or compounds that remain in their corresponding extraction residues. EPP are a complex mixture of low-molecular-weight compounds (monomers to decamers), that include extractable proanthocyanidins (PA) and hydrolyzable tannins along with other flavonoids and phenolic acids. NEPP are polymeric polyphenols, including nonextractable PA and hydrolyzable PP or low-molecular-weight PP linked to cell wall constituents (polysaccharides and protein) or trapped within the food matrix. PA and hydrolyzable tannins can be distributed in both EPP and NEPP fractions.

EPP are analyzed in food extracts while the analysis of NEPP requires acidic hydrolysis of their corresponding residues to release the polyphenolic compounds from the food matrix. The presence of NEPP has been reported in plant materials and plant-derived foods [5–7].

There is a wealth of articles in the literature dealing with physiological properties and potential health effects of EPP,

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Abbreviations: EPP, extractable polyphenols; **NEPP,** non extractable polyphenols; **PA,** proanthocyanidins; **PP,** polyphenols but NEPP are usually ignored although they are also bioactive compounds with potential health properties. Similarly, studies of PP intake in diets are based on the EPP contents in food, without taking NEPP into account. As a result, the available data in the literature on food PP and dietary intakes are partial or incomplete; thus, clinical and nutritional studies on dietary PP have considered only a part of the total ingest.

A major proportion of EPP can be expected to be dissolved or bioaccessible in small intestine fluids where they pass at least partially the intestinal barrier (mucosa), followed by metabolism and systemic effects. On the other hand, NEPP cross the gastrointestinal tract associated with the insoluble food matrix (indigestible and cell wall compounds of plant foods) and reach the colon unaltered, where they become a fermentable substrate for bacterial microflora, causing the original structure to break down, either into absorbable metabolites (phenylacetic, phenylpropionic, urolithine, *etc.*) or nonabsorbable metabolites which remain in the colonic lumen where they may counteract the effects of dietary prooxidants [8, 9].

Knowledge of the actual amount of PP, including NEPP, in diets may be useful for a better understanding of their potential health effects. The objective of this work was to determine the EPP and NEPP in a whole diet, the Spanish diet. To that end, EPP in methanol–acetone extracts and NEPP in the acidic hydrolyzates of their extraction residues were analyszd by HPLC-MS in different plant-derived foods (cereals, fruits, vegetables, nuts, and legumes).

2 Materials and methods

2.1 Reagents

All solvents used for HPLC analysis were liquid chromatography grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) and the water used was ultra pure. All other chemicals (Iron III-chloride-6-hydrate, acetone, methanol, butanol, hydrochloric acid 37%, and sulfuric acid) were obtained from Panreac (Castellar del Vallés, Barcelona, Spain). Standards of hydroxybenzoic acids (gallic acid, vanillic acid, syringic acid, tannic acid, ellagic acid, and protocatechuic acid), hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid, and cinnamic acid), flavan-3-ols (catechin, epicatechin, gallocatechin, epicatechin gallate, epigallocatechin gallate, gallocatechin gallate, procyanidin dimers B1 and B2), flavanones (naringin, hesperetin, hesperedin, and phloridzin), flavonols (quercetin-3-glucoside, quercetin-galactoside, quercetin-xyloside, quercetin-rahmnoside, quercetin-arabinoside, rutin, and quercetin), and anthocyanins (cyanidin-3-glucoside, malvidin-3-glucoside, peonidin-3-glucoside, and pelargonidin-3-glucoside) were purchased from Sigma-Aldrich or Extrasynthése (Genay, France). A proanthocyanidin concentrate from Mediterranean carob pod (*Ceratonia siliqua* L.) were supplied by Nestlé S.A.

2.2 Equipment

An Agilent series 1100 HPLC was used coupled with DAD and quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) and Agilent ChemStation software (Agilent, v. 08.03). Mass spectra were acquired in scan mode (negative for EPP and positive for hydrolyzable PP) with an ESI source.

Perkin Elmer Lambda 12 (MA, USA) UV–VIS spectrophotometer was used at 555 nm for nonextractable PA determination.

2.3 Samples

Fruits (26), vegetables (18), cereal foods (6), nuts (5), and legumes (3) of the kinds most commonly consumed in the Spanish diet were purchased at local supermarkets at the optimal stage of maturity. The amount of the edible part of each food consumed daily *per* capita reported in the National Dietary Survey [10] was weighed out and mixed into five samples, one *per* group (fruits, vegetables, cereals, nuts, and legumes) (Table 1). Legumes, some vegetables, and some cereals were previously boiled as they are usually consumed. These samples were freeze dried and milled to a particle size of 0.5 mm in a Rest ZM 200 centrifugal mill (Haan, Germany). Nuts were then defatted at room temperature with petroleum ether.

These five groups of foods represent 97% of total daily *per* capita intake of plant-derived foods in the Spanish diet and were the five test samples used in this work for HPLC analysis of EPP and NEPP.

2.4 Analysis of EPP and NEPP

Analysis of EPP in methanol/acetone extracts and NEPP in acidic hydrolyzates of extraction residues from samples was performed by RP HPLC-MS following the procedures recently described [11].

EPP solutions were obtained by extraction in acidic methanol/water (50:50 v/v, pH = 2) followed by acetone/water extraction (70:30, v/v) performed in triplicate as is described [12]. HPLC analysis was performed in a reverse-phase C18 column (Nucleosil, 150 × 4.6 mm, 5 μm) (Agilent) following the methodology described before [13] with some modifications. Chromatograms were acquired on DAD at 280, 320, 360, and 520 nm. The MS was fitted to an atmospheric pressure, ESI source operated in negative ion mode. The electrospray capillary voltage was set to 3000 V, with a nebulizing gas flow rate of 12 L/h and a drying gas temperature of 300°C. Mass spectra were acquired

Table 1. Samples of plant-derived foods[a]

Samples	Variety	Intake in Spanish diet	
		g fresh matter/day	g edible portion/day
Sample 1: Fruits			
Yellow apple with skin	Golden delicious	15.4	12.8
Red apple with skin	Red delicious	15.4	12.8
Apricot with skin	Moniquí	2.6	2.4
Avocado	Hass	0.93	0.35
Banana	Cavendish	24.6	16.3
Cherry	Navalinda	3.7	3.2
Grape	Moscatel	6.4	5.8
Kiwi fruit	Hayward	8.3	7.1
Lemon	Primofiori	5.2	1.9
Mandarin	Hernandina	17.8	12.8
Melon	Piel de sapo	22.3	13.4
Green table olive	Carbonell	3.4	2.7
Black table olive	Carbonell	3.4	2.7
Orange	Navel-Late	52.6	38.4
Peach with skin	Royal	14.03	12.3
Pear	Blanquilla	19.1	16.8
Pineapple	Sativus	3.6	2.0
Green plum	Metley	2.1	1.8
Red plum	Metley	2.1	1.8
Strawberry	Camarrosa	5.7	5.4
Watermelon	Piel negra	17.4	9.0
Blackberry	Rubus glaucus	2.41	2.4
Chirimoya	Fino de Jete	2.41	1.5
Fig	Cuello dama	2.41	2.0
Mango	Keitt	2.41	1.6
Pomegranate	Grano Elche	2.41	1.5
Total		**258.1**	**190.7**
Sample 2: Vegetables			
Green bean[b]	Helda	7.4	6.8
Chard[b]	White lyon	5.5	1.7
Lettuce	Romana	20.7	12.4
Onion	White	25.9	22.1
Potato[b]	Oria	94.08	84.7
Red pepper	Lamuyo	15.3	12.4
Tomato	Red for salad	45.5	42.8
Garlic	Red	5.91	4.5
Cucumber	Alexandex	5.91	4.6
Mushroom	White	5.91	4.7
Asparagus[b]	Green	5.91	3.5
Carrot	Nantesa	5.91	4.9
Auvergine[b]	Belleza negra	5.91	5.0
Artichoque[b]	White	5.91	2.1
Celery[b]	Afrodita	5.91	4.1
Leek[b]	Buljina	5.91	5.0
Pumpkin[b]	Carruecano	5.91	4.1
Cabbage[b]	Oliver	5.91	4.7
Total		**279.4**	**230.1**
Sample 3: Cereals			
Rice[b]	White and round	6.7	6.7
White bread	White (wheat)	96.9	96.9
Spaghetti[b]	Wheat	9.1	9.1
Biscuits	María Fontaneda	12.5	12.5
Croissant	El Corte Ingés	10.7	10.7
Breakfast cereals	Frosties	3.8	3.8
Total		**139.7**	**139.7**

Table 1. Continued

Samples	Variety	Intake in Spanish diet	
		g fresh matter/day	g edible portion/day
Sample 4: Nuts			
Walnut	Jumbo California	1.9	0.75
Almond	Marcona	1.2	1.2
Peanut	With shell	1.2	0.3
Hazelnut	With peel	1.3	1.3
Pistacho	With shell	1.3	0.6
Total		**6.9**	**4.2**
Sample 5: Legumes			
Chickpeas[b)]	White	4.4	4.4
Red beans[b)]	Red	1.9	1.9
White beans[b)]	White	1.9	1.9
Lentils[b)]	Pardina	3.6	3.6
Total		**11.8**	**11.8**

a) From Ministry of Agriculture, Fisheries and Food, 2006.
b) Boiled.

in the scan mode (mass range *m*/*z* 100–1000). Quantification was done by comparison with known standards when possible. When pure standards were not available, concentration of PP in extracts were calculated using the calibration curves of the standards closest in chemical structure.

NEPP were determined as hydrolyzable PP plus nonextractable PA after subjecting the residue of these extractions to two different hydrolysis treatments: methanol/H_2SO_4 90:10 v/v, 85°C, 20 h, to obtain hydrolyzable PP, including hydrolyzable tannins, and butanol/HCl 97.5:2.5 v/v, 100°C, 60 min to obtain cyanidins/delphinidins solutions derived from nonextractable PA. For hydrolyzable PP, a HPLC reverse-phase C18 column (Gemini, 250 × 4.6 mm, 5 μm) appropriate for acid samples was used applying the method previously reported [14]. Chromatograms were recorded on DAD at 280, 320, 360, and 520 nm. The MS conditions were the same as those for EPP analysis except that the MS operated in positive mode. Quantification was achieved by comparison with known standards when possible.

Nonextractable PA were quantified by measuring the anthocyanidin concentration in butanol/HCl/$FeCl_3$ hydrolyzates at 555 nm and referenced to a calibration curve of a carob pod proanthocyanidin concentrate [5, 15].

Determinations were performed in triplicate in extracts and hydrolyzates. All results were reported as mean ± SD on a dry matter basis.

2.5 Food consumption data

Consumption data were obtained from the National Survey [10], which is compiled from over six thousand questionnaires in households, hotels and restaurants, schools, and numerous institutions (confidence level 95%; error range 2% in amount of food).

3 Results and discussion

The five samples analyzed are representative of the plant-derived foods consumed daily *per* capita in the Spanish Mediterranean diet (Table 1). High consumption of a large variety of fresh fruits and vegetables is a characteristic feature of the Mediterranean diet and the intake of legumes and nuts is also appreciable, higher than in non-Mediterranean areas. The consumption of cereals is also important, but generally lower than in Northern and Central European countries.

Determination of the actual PP content in food requires the analysis of EPP followed by analysis of NEPP. The available HPLC methodologies for determining different classes of EPP in aqueous organic extracts are well known and quite efficient. In this work, we paid special attention to NEPP, a largely unknown food fraction. NEPP are determined in acidic hydrolyzates of extraction residues, as the sum of hydrolyzable PP and nonextractable PA. Sulfuric acid hydrolysis releases hydrolyzable tannins and other PP, and HCl/BuOH treatment of nonextractable PA yields cyanidin/delphinidin solutions.

3.1 NEPP in plant-derived foods

Table 2 shows NEPP contents, estimated as the sum of hydrolyzable PP and nonextractable PA. The highest concentrations of NEPP are in dietary fruits (880 mg/100 g dry weight), followed by legumes (568 mg/100 g), vegetables (326 mg/100 g), nuts (333 mg/100 g), and cereals (210 mg/100 g).

Table 2. NEPP in plant-derived foods

Sample	Hydrolyzable PP[a)]			Nonextractable PA[b)]	Total NEPP[c)]
	[M-H]$^+$	Structure	mg/100 g dry weight	mg/100 g dry weight	mg/100 g dry weight
Sample 1: Fruits					
	195	Ferulic acid	224.8 ± 17.0		
	139	*p*-Hydroxybenzoic acid	69.6 ± 4.9		
	291	Catechin	43.7 ± 5.9		
	307	Gallocatechin	11.1 ± 2.1		
	171	Gallic acid	10.8 ± 0.9		
	181	Caffeic acid	4.0 ± 1.8		
		Other	3.1 ± 1.0		
Total			**366.5 ± 18.8**	**514.1 ± 11.9**	**880.5 ± 22.3**
Sample 2: Vegetables					
	155	Protocatechuic acid	150.5 ± 4.1		
	139	*p*-Hydroxybenzoic acid	116.3 ± 16.5		
	291	(−)-Epicatechin	58.9 ± 3.7		
	181	Caffeic acid	Trace		
	165	*p*-Coumaric acid	Trace		
	195	Ferulic acid	Trace		
Total			**325.7 ± 17.4**	**n.d.**	**325.7 ± 17.4**
Sample 3: Cereals					
	181	Caffeic acid	77.9 ± 4.1		
	291	Catechin	70.1 ± 7.0		
	171	Gallic acid derivative	48.4 ± 2.7		
	139	*p*-Hydroxybenzoic acid	13.5 ± 0.7		
Total			**209.9 ± 5.4**	**n.d.**	**209.9 ± 5.4**
Sample 4: Nuts					
	307	Gallocatechin	27.5 ± 1.7		
	443	Epicatechin gallate	25.7 ± 1.5		
	181	Caffeic acid	17.4 ± 0.9		
	259, 634	Hexahydroxydiphenic acid	15.9 ± 1.0		
	141, 171	Gallate	12.5 ± 1.2		
	303	Ellagic acid	11.7 ± 1.0		
	291	(−)-Epicatechin	8.7 ± 1.9		
	171	Gallic acid	8.7 ± 0.5		
	367	Malvidin	8.1 ± 0.1		
Total			**136.2 ± 3.1**	**198.8 ± 8.0**	**332.7 ± 8.6**
Sample 5: Legumes					
	290	Catechin	139.3 ± 9.1		
	181	Caffeic acid	60.5 ± 4.3		
	291	(−)-Epicatechin	36.1 ± 1.9		
	141, 171	Gallic acid derivative	12.2 ± 1.4		
	171	Gallic acid	7.4 ± 0.5		
Total			**255.7 ± 10.1**	**312.5 ± 53.4**	**568.2 ± 54.3**

n.d., not detected.

a) Determined in hydrolysates by HPLC-MS and spectrophotometrical assay. Triplicate determinations (mean ± SD.

b) Determined in hydrolysates by spectrophotometrical assay. Triplicate determinations (mean ± SD).

c) NEPP content (mg/100 g fresh weight): Fruits: 168.8 ± 4.4; vegetables: 53.4 ± 2.8; cereals: 171.1 ± 4.4; nuts: 314.4 ± 8.3; legumes: 497.7 ± 47.5. Moisture: fruits: 80.9%, vegetables: 83.6%, cereals: 18.5%, nuts: 2.6% and legumes: 12.4%.

PA are oligomers and polymers of flavan-3-ol and flavan-3,4-diols that are distributed in both the EPP and NEPP fractions. Oligomeric PA or extractable PA are soluble in aqueous organic solvents, while most polymeric PA remain in the extraction residues (nonextractable PA). The absence of nonextractable PA in vegetables and cereals, and the appreciable amount of these compounds in fruits (514 mg/100 g), legumes (312 mg/100 g), and nuts (199 mg/100 g) determine the differences in total NEPP contents.

Similarly, hydrolyzable PP may appear in both EPP and NEPP fractions. Fruits, especially strawberries, grapes, pomegranates, peaches, cherries, plums, and mangos, have been reported to be rich sources of hydrolyzable tannins, which are analyzed in aqueous organic solvents [16–19]. Our results indicate that fruits are also an important source of hydrolyzable PP much of which remains in the residue from these extractions. Ferulic acid, *p*-hydroxybenzoic acid, catechin, gallocatechin, gallic acid, and caffeic acid were identified as the main nonextractable hydrolyzable PP in fruits. Protocatechuic acid, *p*-hydroxybenzoic acid, and epicatechin were the major compounds identified in vegetables.

Cereals are a good source of NEPP associated with dietary fiber [20]. Caffeic acid, catechin, gallic acid derivative, and *p*-hydroxybenzoic acid were the main hydrolyzable PP identified in dietary cereals.

Legumes are a good source of NEPP containing high concentrations of both hydrolyzable PP (256 mg/100 g) and nonextractable PA (312 mg/100 g). Catechin, caffeic acid, and epicatechin were the main compounds identified. Finally, the main hydrolyzable PP identified in nuts were gallocatechin, epicatechin gallate, caffeic acid, hexahydroxydiphenic acid, gallate, and ellagic acid.

There is little information regarding the identification of NEPP. In the case of nonextractable PA, acid-catalyzed depolymerization in the presence of suitable nucleophilic reagents, such as benzylmercaptan, toluene-α-thiol (thiolysis) and phloroglucinol, or pectinolytic and cellulolytic enzymes to release PP from the food matrix, has been used as a preliminary step to HPLC analysis [21–27]. However, the PA contents reported in these studies were very low compared with the values reported after butanol/HCl/Fe extractions [28].

For their part, hydrolyzable tannins have been identified in aqueous–organic extracts of fruits, cereals and nuts but not in residues of these extractions [29, 19]. Recent research has provided new data about NEPP, including hydrolyzable PP, in apple, nectarine, and peach [11].

3.2 EPP in plant-derived foods

Fruits also present the highest amount of EPP (dry matter basis) followed by vegetables and nuts. Lower amount were found in cereals and legumes. Regarding the chromatographic profile, the compounds identified as major constituents are logically provided by the highly consumed food. Thus, in the fruit samples, hesperidin, naringin, and epicatechin mainly derived from citrus, apples, and grapes intake, were the main identified compounds. In vegetables, catechin, sinapic acid, quercetin-3-glucoside, and caffeoylquinic acid may come from onion, lettuce, and tomatoes. Epicatechin and ellagic acid were the major constituents in cereals. Epicatechin, gentisic acid genistin, and protocatechuic acid were the main compounds identified in nuts, while in legumes the main compounds were *p*-hydroxybenzoic acid, and protocatechuic acid. Other identified compounds are listed in Table 3.

PA can be present in methanol–acetone extracts as extractable PA (monomers to decamers) and they can be determined by HPLC-DAD jointly with other EPP. For instance, epicatechin and cyanidins in EPP of fruits (Table 3) may come partially from extractable PA. The amount of extractable PA analyzed in our work may differ from literature data because of differences in extraction and HPLC methods. The usual methodology determines extractable PA in acetone:acetic acid:water by HPLC coupled with fluorescence detector (http://www.nal.usda.gov/fnic/foodcomp/Data/PA/PA.html) [23, 30].

There are hundreds of articles in the literature addressing EPP in plant foods. Our results on concentration and composition of this PP fraction are in the same range as the literature data (http://www.nal.usda.gov) [31–35].

3.3 NEPP *versus* EPP

NEPP and EPP were determined by analyzing the dry powder samples, and therefore the results (Tables 2 and 3) are expressed as dry matter. The total PP contents in the different plant-derived foods, expressed also in fresh matter, as the foods are consumed are also indicated in Tables 2 and 3. Typical chromatograms of EPP, hydrolyzable PP, and UV–VIS spectrum of nonextractable PA representative of fruits are shown in Figs. 1 and 2 for visual evidence of the important presence of NEPP.

All plant-derived foods contain more NEPP than EPP in both dry and fresh matter basis.

Low moisture foods (legumes, nuts, and cereals) present the highest content of NEPP when results are expressed in fresh weight, while fruits show the highest amount of both EPP and NEPP on dry weight basis.

Regarding intakes in the Spanish diet, NEPP are the major fraction of total PP where fruits and cereals are the major contributors (Fig. 3).

3.4 Dietary intake of PP

As mentioned above, most studies on intake of dietary PP are limited to extractable compounds and there are no literature data on intake of NEPP.

Ovaskainen *et al.* [36] reported the total PP intake in the Finnish diet (817–919 mg/day/person), with phenolic acids and PA as the main compounds identified. In the Vietnamese, diet intake was 595 mg/day/person, with leafy vegetables as the main contributors [37]. In Spain, EPP intake was estimated around 1100 mg/day/person, where beverages (juice, coffee, and wine) are the main source of EPP [38].

The dietary intake of specific groups of PP have been previously reported: anthocyanins, 82 mg/day; flavonols, especially quercetin, 20–35 mg/day; catechins

Table 3. EPP in plant-derived foods

Sample	EPP[a]		
	$[M-H]^-$	Structure	mg/100 g dry weight[b]
Sample 1: Fruits			
	609	Hesperidin	147.5±6.9
	579	Naringin	52.8±10.9
	289	(−)-Epicatechin	33.2±12.8
	457	Gallocatechin gallate	15.5±8.5
	353, 707	Chlorogenic acid	12.2±3.6
	431	Pelargonidin-3-glucoside	11.5±2.1
	223	Sinapic acid	8.4±1.0
	577	Procyanidin B2	4.8±2.0
	193	Ferulic acid	5.2±1.0
	447	Cyanidin-3-glucoside	4.8±0.1
		Others	10±2.1
Total			**305.9±20.9**
Sample 2: Vegetables			
	289	Catechin	97.9±10.2
	223	Sinapic acid	40.7±1.5
	463	Quercetin-3-glucoside	24.2±2.8
	515	Caffeoylquinic acid	15.6±0.2
	625, 179	Caffeic acid derivative	7.2±0.3
	559	Caffeoylsinapylquinic acid	4.7±0.1
	179	Caffeic acid	5.4±0.2
Total			**195.8±10.7**
Sample 3: Cereals			
	289	(−)-Epicatechin	29.6±2.9
	301	Ellagic acid	18.5±1.4
	137	*p*-Hydroxybenzoic acid	7.0±3.2
	515	Caffeoylquinic acid	1.5±0.1
		Others	0.5±0.1
Total			**57.2±4.6**
Sample 4: Nuts			
	289	(−)-Epicatechin	31.9±5.1
	153	Gentisic acid	23.2±2.3
	431	Genistin	18.1±0.9
	153	Protocatechuic acid	16.9±1.4
	167	Vanillic acid	9.1±1.2
	577	Procyanidin B2	8.4±1.6
	457	Epigallocatechin gallate	3.8±1.5
	353	Chlorogenic acid derivative	4.1±0.9
	301	Ellagic acid	2.4±0.3
		Others	5.2±1.1
Total			**129.7±6.1**
Sample 5: Legumes			
	137	*p*-Hydroxybenzoic acid	35.6±0.6
	153	Protocatechuic acid	14.1±1.1
	193	Ferulic acid	5.8±0.2
	289	(−)-Epicatechin	2.7±0.1
Total			**58.2±1.4**

a) Determined in methanol/acetone extracts by HPLC-MS. Triplicate determinations (mean±SD).

b) EPP content (mg/100 g fresh weight): fruits: 58.4±3.8; vegetables: 32.1±1.7; cereals: 43.8±3.2; nuts: 125.6±5.8; legumes: 51.7±1.2. Moisture: fruits: 80.9%, vegetables: 83.6%, cereals: 18.5%, nuts: 2.6%, and legumes: 12.4%

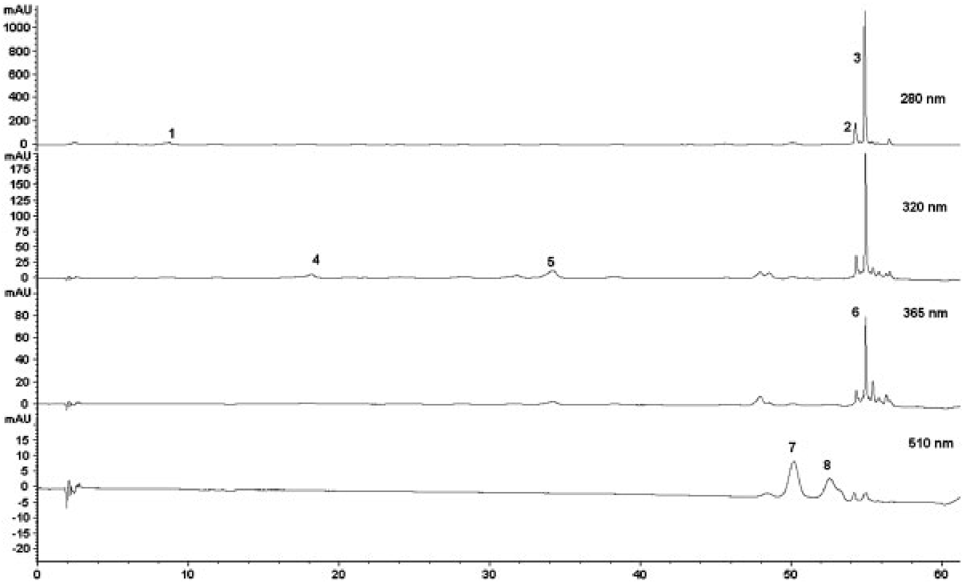

Figure 1. HPLC-DAD chromatogram of EPP of fruits. **1**, sinapic acid; **2**, naringin; **3**, hesperidin; **4**, chlorogenic acid; **5**, ferulic acid; **6**, quercetin-3-glucoside; **7**, malvidin-3-glucoside; **8**, cyanidin-3-glucoside.

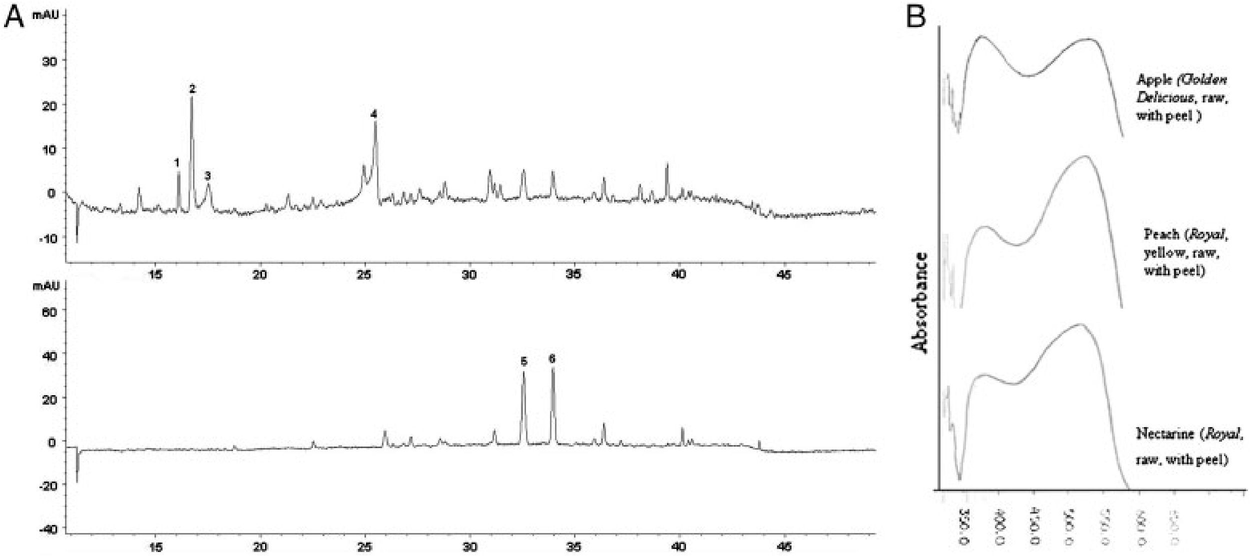

Figure 2. HPLC-DAD chromatograms of hydrolyzable PP of fruits (**A**) and UV-VIS spectrum of nonextractable PA from fruits (**B**). (**A**) **1**, *p*-hydroxybenzoic acid; **2**, catechin; **3**, gallocatechin; **4**, gallic acid derivative; **5**, ferulic acid; **6**, caffeic acid.

and proanthocyanidin dimers and trimers, 18–50 mg/day; polymeric PA, 128 mg/day; chlorogenic, up to 800 mg/day; and isoflavone intake, less than 50 mg/day/person [39, 40].

Table 4 shows the estimated intake of EPP and NEPP in the Spanish diet. Figure 3 summarizes the contribution of each group of plant-derived foods to the total intake of different classes of PP.

Note that NEPP are the major fraction of dietary plant-derived foods PP. NEPP intake (942 mg/day/person) is four times higher than EPP intake (258 mg/day/person). Fruits (550 mg/day/person) and cereals (353 mg/day) are the largest contributors to the total PP intake in the diet (EPP plus NEPP), followed by vegetables (196 mg/day).

In the case of NEPP, hydrolyzable PP intake (633 mg/day/person) is greater than nonextractable PA intake (309 mg/day). Cereals and fruits are the largest contributors to hydrolyzable PP and nonextractable PA intake, respectively. In the EPP fraction, fruits are the largest contributor, with an intake of 110 mg/day (45% of total EPP) followed by vegetables with 74 mg/day (30% of total EPP).

Fruits and vegetables are addressed jointly in nutritional and epidemiological studies, and their health benefits in the diet are well known and supported by strong scientific

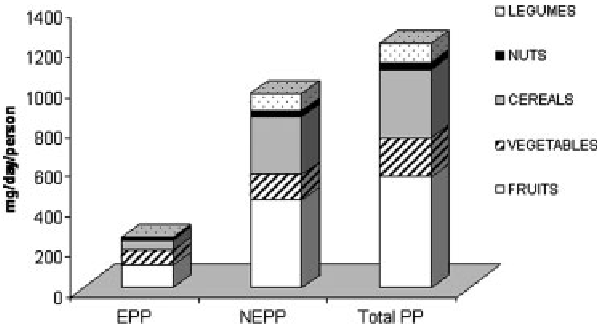

Figure 3. Intake of PP from plant-derived foods in the Spanish diet (EPP; NEPP including hydrolyzable PP and nonextractable PA and total PP).

evidence [41, 42]. A daily consumption of at least 400 g of fruits and vegetables is recommended by the national and international health institutions. The consumption of these foods (537 g/day/person) in the Spanish diet provides approximately a total PP intake of 750 mg (184 mg of EPP plus 562 mg of NEPP), which is equivalent to 60% of the total PP intake from plant-derived foods.

Ferulic acid, caffeic acid, hesperidin, naringin, catechin, epicatechin, ellagic acid, gallic acid derivatives, protocatechuic, and *p*-hydroxybenzoic acid are the major individual PP in the diet. The intake of specific individual compounds lies outside the main objective of this work, but these data (Table 4) can be calculated from the values listed in Tables 2 and 3.

In this work, we show the PP dietary intake from plant-derived foods in the Spanish diet. However, it should be noted that beverages are also an important source of EPP and therefore estimations of the actual total PP intake should include the contribution of PP-rich beverages and vegetable oils common in the diet, especially coffee, wine, beer, tea, fruit juices, and olive oil. Fortunately, we have comprehensive information about PP contents and composition in beverages and oils [1, 43–47], so that it is possible to calculate their contribution to PP intake in the whole diet.

In case of the Spanish diet, we already have an estimation of the EPP intake from beverages and oils (622 mg/day/person) [12]. If we add the figures to the results reported here, we find that the total PP intake in the Spanish diet is around 1822 mg/day/person, of which 942 mg are NEPP and 880 mg EPP, *i.e.* 48% higher than the values cited in the literature for common diets (around 1 g), because of the important contribution of NEPP.

To our knowledge, this is the first report on dietary intakes of PP including NEPP. The HPLC-MS analysis of NEPP performed in acidic hydrolyzates provides data on content and composition of polyphenols in the main groups of plant-derived foods (samples prepared as a pool of individual food). The application of this methodology to individual foods may allow the identification of novel polyphenolic compounds.

3.5 Nutritional implications

The presence of considerable amounts of NEPP could change the current approach to the nutrition and health properties of dietary PP. In fact, most studies in this field almost exclusively address the biological properties, bioavailability, metabolism, and systemic effects of EPP or PP-rich foods and beverages. Our results suggest that around 1 g of NEPP may reach the human colon daily and they may be expected to have a significant physiological impact within the large intestine, affecting microflora development and intestinal antioxidant status by producing metabolites that can be absorbed through the mucosa [12, 48].

Most of the ingested NEPP reach the colon intact. We believe that NEPP may exert their biological effects mainly as a fermentable substrate for bacterial microflora and also in the form of an unabsorbable complex structure with binding properties that may produce local effects in the gastrointestinal tract. The abundant microflora in the colon plays a critical role in the metabolism of NEPP [49, 50]. After microbial enzyme metabolism of any NEPP that reaches the colon, there are two possible routes available: breakdown of the original tannin structure into absorbable metabolites that may produce systemic effects in various organs, or breakdown into nonabsorbable metabolites (probably mid-molecular-weight tannins) which remain in the colonic lumen, where they may counteract the effects of dietary prooxidants in the colon produced during colonic bacterial metabolism.

Several authors have reported that PA are metabolized to a large extent by gut microflora, the main metabolites produced being phenylacetic, phenylpropionic, and phenylbutyric acids [51, 52]. We recently found that the colonic fermentation of nonextractable PA associated with dietary fiber released hydroxyphenylacetic acid, hydroxyphenylvaleric acid and two isomers of hydroxyphenylpropionic acid, and that human plasma contained the same metabolites as after *in vitro* colonic fermentation of nonextractable PA [9].

For their part, hydrolyzable tannins are subject to the action of certain lactobacilli with distinct tannase activity, which hydrolyze hexahydroxydiphenoyl groups in ellagitannins and galloyl groups in gallotannins [53]. A pathway has been proposed for degradation of ellagitannin by human microbiota *via* hydrolysis to ellagic acid and its microbial transformation to urolithin A and urolithin B, which are detected in plasma as glucuronides after absorption [51, 54].

Several studies report that tannins, the major structure of NEPP, may produce chemopreventive effects through various different mechanisms such as induction of apoptosis, inhibition of the inflammation signaling pathway or generation of an antioxidant environment in the colon [55].

3.6 Limitations

This work clearly shows that the NEPP content in different types of plant-derived foods (fruits, vegetable, cereals,

Table 4. Total polyphenol intakes from plant-derived foods in the Spanish diet (mg/day/person)

Sample (intake)[a]	EPP		NEPP[b]		Total
	Structure	Intake	Structure	Intake	
Fruits (192.8)	Hesperidin	54.1±2.5	Ferulic acid	112.0±8.4	
	Naringin	19.3±3.9	Cinnamic acid	34.7±2.3	
	(−)-Epicatechin	12.1±4.6	Catechin	21.7±2.9	
	Gallocatechingallate	5.6±3.0	Gallic acid	5.4±0.4	
	Chlorogenic acid	4.4±1.3	Gallocatechin	5.4±0.9	
	Pelargonidin-3-glucoside	4.2±0.9	Caffeic acid	1.9±0.7	
	Sinapic acid	3.0±0.6	Protocatechuic acid	1.3±0.6	
	Other	7.7±1.1	Vanillic acid	0.2±0.1	
			Nonextractable PA	**256.4±5.8**	
Total		**110.4±7.6**		**439.0±8.1**	**549.5±5.9**
Vegetables (230.1)	Catechin	36.9±3.8	Protocatechuic acid	56.8±1.5	
	Sinapic acid	15.3±0.5	*p*-Hydroxybenzoic acid	43.8±6.2	
	Quercein-3-glucoside	9.1±1.0	(−)-Epicatechin	22.2±1.4	
	Caffeoylquinic acid	5.8±0.1	**Nonextractable PA**	**n.d.**	
	Caffeic acid derivative	2.7±0.1			
	Caffeoylsinapylquinic acid	1.7±0.0			
	Caffeic acid	2.0±0.1			
Total		**73.6±4.0**		**122.9±6.5**	**196.5±7.6**
Cereals (139.8)	(−)-Epicatechin	32.7±4.0	Caffeic acid	108.2±5.7	
	Ellagic acid	21.1±1.5	Catechin	97.9±9.1	
	p-Hydroxybenzoic acid	5.1±1.2	Gallate	67.2±3.4	
	Caffeoylquinic acid	1.7±0.1	*p*-Hydroxybenzoic acid	18.2±0.9	
	Other	0.5±0.1	**Nonextractable PA**	**n.d.**	
Total		**61.1±1.9**		**291.7±11.3**	**352.8±11.4**
Nuts (4.3)	(−)-Epicatechin	1.9±0.0	Gallocatechin	1.8±0.1	
	Protocatechuic	1.2±0.1	Epicatechin gallate	1.7±0.1	
	Gentisic acid	1.6±0.2	Caffeic acid	1.2±0.1	
	Vanillic acid	0.6±0.1	Hexahydroxydiphenic acid	1.1±0.1	
	Procyanidin B2	0.6±0.1	(−)-Epicatechin	0.6±0.1	
	ECG	0.5±0.0	Ellagic acid	0.8±0.1	
	Other	0.4±0.1	Gallic acid	0.6±0.0	
			Malvidin	0.5±0.0	
			Nonextractable PA	**20.2±0.5**	
Total		**6.8±1.1**		**28.8±1.2**	**35.3±2.3**
Legumes (12.1)	Hydroxybenzoic acid	3.8±0.1	Catechin	14.7±0.9	
	Protocatechuic	1.5±0.1	Caffeic acid	6.4±0.4	
	Ferulic acid	0.6±0.1	(−)-Epicatechin	3.8±0.2	
	(−)-Epicatechin	0.3±0.0	Gallic acid derivative	1.3±0.1	
			Protocatechuic acid	0.8±0.1	
			Nonextractable PA	**33.1±5.6**	
Total		**6.2±0.8**		**60.1±5.7**	**66.3±5.7**
Total intake		**258.1±8.9**		**942.5±16.4**	**1200.6±18.5**

n.d., not detected.

a) Gram fresh edible portion/day/person.

b) NEPP: hydrolysable PP plus nonextractable PA.

nuts, and legumes) and in the diet is higher than the EPP content.

However, this work presents some limitations. First, the analysis of NEPP requires strong acidic treatment that may degrade some phenolic compounds, especially flavonoids. Therefore, our results even may underestimate the actual NEPP content.

Second, different analytical procedures used to determine PP in extracts and acidic hydrolyzates (HPLC with different columns and detectors and spectrophotometry) make difficult a direct comparison of quantitative results.

It is reasonable to consider that in some cases, especially in nonextractable PA analysis, we may be closer to an estimation than to an accurate determination. Anyway, there is evidence of the appreciable presence of NEPP in plant-derived foods.

Novel analytical methods that avoid or minimize degradation of NEPP and allow quantitative comparison are needed.

On the other hand, the polyphenolic compounds that are released by chemical hydrolysis may differ from those released in the human gut. Preliminary results suggest that digestive enzymes (proteases, amylases, and lipases) trigger the release of polyphenols from the food matrix more efficiently than aqueous-organic solvents and that NEPP undergo colonic fermentation releasing absorbable metabolites [9, 12]. The relative contribution of EPP and NEPP to the level of phenolic compounds and metabolites in human plasma reported in clinical studies remain to be elucidated.

4 Concluding remarks

The HPLC-MS analysis of NEPP in acidic hydrolyzates of the main groups of plant foods provide data on content and composition of dietary polyphenols and may allow the identification of novel polyphenolic compounds.

Dietary plant-derived foods contain substantially more NEPP than EPP. Fruits and vegetables supply a major proportion of the NEPP in the diet (over 60% in the Spanish diet), followed by cereals. Further studies are needed to determine NEPP concentrations, composition, and physiological properties in specific foods and diets. Over the last three decades, research on polyphenols has addressed almost exclusively extractable compounds. At this time, a wider approach including NEPP may be useful to gain a better understanding of the potential health effects of dietary PP.

This work is supported by the Spanish Ministry of Science (project AGL2008-02541-ALI). Sara Arranz also thanks the Spanish Ministry for granting her an FPI scholarship.

The authors have declared no conflict of interest.

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