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ARTICLE

High Contents of Nonextractable Polyphenols in Fruits Suggest That Polyphenol Contents of Plant Foods Have Been Underestimated

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The content of polyphenols in fruits reported in the literature normally refers to extractable polyphenols (EPP) analyzed in aqueous−organic extracts. However, significant amounts of bioactive compounds that are usually not considered in nutritional studies remain in the residue from extraction as nonextractable polyphenols (NEPP). The main objective of this work was to analyze both EPP and NEPP (hydrolyzable polyphenols and proanthocyanidins). EPP were analyzed in methanol/acetone/water extracts, and NEPP were determined in acidic hydrolysates of extraction residue from apple, peach, and nectarine using HPLC-MS and spectrophotometry. Results showed that the NEPP content (112−126 mg/100 g of fresh fruit) was higher than the EPP content (18.8−28 mg/100 g of fresh fruit). Further analyses of NEPP in other fruits and plant foods consumed in diets are needed to compile a complete database of use for nutritional and biological studies.

KEYWORDS: Extractable polyphenols; nonextractable polyphenols; proanthocyanidins; liquid chromatography; mass spectrometry

INTRODUCTION

Dietary polyphenols may have a significant role in the prevention of chronic disease (*1*). Comprehensive knowledge of the total contents of these bioactive compounds in foods and diets is essential for biological, epidemiological, and clinical studies addressing their potential health effects. For polyphenols, these data are becoming more complete and are being assembled in databases (*2*, *3*). The first epidemiology study with polyphenols was concerned only with flavonols and flavones (*4*); more recent studies have been facilitated by more recent data being available for anthocyanins and other flavonoids (*5*). As yet there are no complete works relating proanthocyanidins (PA) intake with disease risk because the proanthocyanidin food composition data are referred only to a fraction of PA. Most literature data on PA content come from HPLC analysis of aqueous−organic extracts of foods, that is, extractable proanthocyanidins (EPA), assuming that all or most of the PA are extracted by aqueous−organic solvents (*6*). However, an important fraction of oligomeric PA remains as nonextractables PA (NEPA) that may escape analysis and are usually not taken into account in chemical and nutritional studies.

The literature data may be of limited use for these studies because most reports on concentrations and compositions of food polyphenols deal with extractable polyphenols (EPP) analyzed in aqueous organic extracts, whereas significant amounts of bioactive polyphenols that remain in the residues from extraction, nonextractable polyphenols (NEPP), are not taken into account (*7*, *8*).

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NEPP are mainly hydrolyzable tannins and proanthocyanidins associated with dietary fiber and protein. Determination of NEPP requires chemical or enzymatic treatment of the aqueous organic residues to release polyphenols from the food matrix before chromatographic or spectrophotometric analysis of the corresponding solutions or hydrolysates (*9*, *10*).

Nonextractable polyphenols, which are not usually considered in nutritional studies, may exert antioxidant activity through a surface reaction in the small intestine (*11*) and reach the colon in association with vegetable cell walls. There they are fermented by bacterial microflora, yielding different metabolites that may counteract the effects of dietary pro-oxidants. Generation of an antioxidant environment in the colon may have important effects on gastrointestinal health, including a chemopreventive effect for colorectal cancer (*12*, *13*).

Fruits are one of the main sources of polyphenols in the diet. There is abundant literature data on EPP in fruits including hydroxybenzoic and hydroxycinnamic acids, flavanols, proanthocyanidins, anthocyanidins, flavones, flavonols, flavanones, and chalcones and extractable hydrolyzable tannins (*14*). Although there is evidence that NEPP remain in the residues of extraction, there are very few or no data on NEPP analysis.

The main objective of this work was to extract and quantify NEPP from fruits and to determine their concentration compared to EPP. Accordingly, HPLC-ESI-MS methodology was applied to determine EPP and hydrolyzable NEPP in three fruits (apple, peach, and nectarine) that are widely consumed as part of the Spanish Mediterranean diet. A complementary quantification of extractable proanthocyanidins (EPA) and nonextractable proanthocyanidins (NEPA) was also performed.

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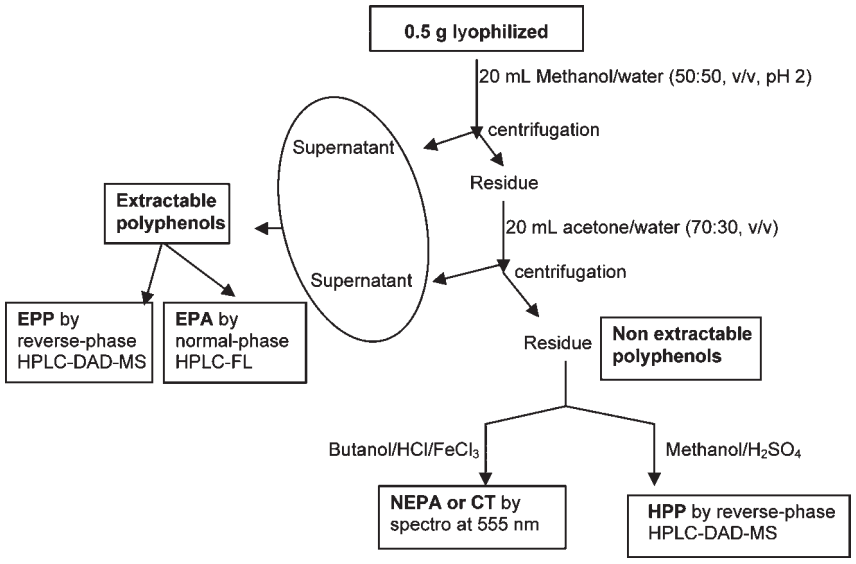

Figure 1. Diagram of determinations performed for analysis of extractable (EPP) and nonextractable polyphenols (NEPP). EPA, extractable proanthocyanidins; NEPA, nonextractable proanthocyanidins; HPP, hydrolyzable polyphenols.

MATERIALS AND METHODS

Reagents. All solvents used for HPLC analysis were of liquid chromatography grade and were obtained from Sigma-Aldrich, and all water was ultrapure. All other chemicals (acetone, methanol, and sulfuric acid) were obtained from Panreac. Standards of hydroxybenzoic acids (gallic acid, vanillic acid, syringic acid, tannic acid, ellagic acid, protocatechuic acid), hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, sinapinic acid, cinnamic acid), flavan-3-ols (catechin, epicatechin, gallocatechin, epicatechin gallate (ECG), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), procyanidin dimers B1 and B2), flavanones (naringin, hesperetin, hesperedin, phloridzin), flavonols (quercetin-3-glucoside, quercetin-galactoside, quercetin-xyloside, quercetin-rhamnoside, quercetin-arabinoside, rutin, quercetin), and anthocyanins (cyanidin-3-glucoside, malvidin-3-glucoside, peonidin-3-glucoside, pelargonidin-3-glucoside) were purchased from Sigma Chemical Co. (St. Louis, MO) or Extrasynthése (Genay, France).

A condensed tannin concentrate from the Mediterranean carob pod (*Ceratonia siliqua* L.) was supplied by Nestlé S.A. Iron(III) chloride-6-hydrate, hydrochloric acid 37%, butanol, acetone, and methanol were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). All solvents used were of analytical grade.

Preparation of Fruit Samples. Apples (var. Golden Delicious; raw, with peel, grown in France), peaches (var. Royal; yellow, raw, with peel, grown in Sevilla), and nectarines (var. Royal; raw, with peel, grown in Sevilla) were purchased in local supermarkets in Madrid, Spain.

All fruits were purchased at the optimal stage of maturity to be consumed. Several pieces of each fruit were selected, and their edible parts were freeze-dried and milled to a particle size of < 0.5 mm in a centrifugal mill Retsch ZM 200 (Haan Germany); the homogenate was stored for not more than 1 week at -20 °C until analysis.

Analytical Methods. Extractions were performed in three different replicates of each fruit homogenate. Determinations were performed per triplicate in each extract and are reported on a fresh matter basis. Results are expressed as mean value ± standard deviation. A diagram summarizing the general procedure is shown in **Figure 1**.

Procedure for Obtaining EPP Solutions and NEPP Hydrolysates. Half a gram of freeze-dried fruit was placed in a capped centrifuge tube; 20 mL of methanol/water (50:50, v/v; pH 2) acidified with 2 N HCl was added, and the tube was thoroughly shaken at room temperature for 1 h. The sample was centrifuged at 3500 rpm for 10 min and the supernatant recovered. Twenty milliliters of acetone/water (70:30, v/v) was added to the residue, and the shaking and centrifugation steps were repeated (*12*). The methanol and acetone extracts were combined and used to quantify and identify EPP by reverse-phase HPLC-MS and EPA by normal-phase HPLC.

Residues obtained after methanol/acetone extraction were used to determine HPP and NEPA following two different procedures.

(*1*) *Hydrolyzable polyphenols* (*HPP*) were released from the food matrix by methanol/H_2SO_4 90:10 (v/v) hydrolysis of 200 mg of the residues at 85 °C for 20 h (*15*). The hydrolysate was analyzed for hydrolyzable polyphenols by reverse-phase HPLC-MS.

(*2*) *Nonextractable Proanthocyanidins* (*NEPA*). Two hundred milligram samples of residue were treated with 10 mL of butanol/HCl (97.5:2.5 v/v) and 0.7 g of $FeCl_3$ to increase the yield of anthocyanidin at 100 °C for 60 min. The tube was centrifuged at 2500*g* for 10 min, and the supernatant containing the resulting anthocyanidin solution was collected. After two washings with 5 mL of butanol, supernatants were combined (*7*, *16*).

Analysis of Extractable Polyphenols and Phenolic Acids. Analyses of EPP and EPA in methanol/acetone extracts of apple, peach, and nectarine were performed in an Agilent series 1100 HPLC (Agilent Technologies, Waldbronn, Germany) coupled with Agilent ChemStation software (Agilent, v. 08.03).

For ***EPP***, 20 μL of filtered extract (0.45 μm Teknokroma filter) was injected into a HPLC reverse-phase C18 Nucleosil column (150 × 4.6 mm i.d.; particle size = 5 μm) (Agilent) with a guard column containing the same stationary phase. The method applied was based on that of Tomás-Barberan et al. (*17*) with some modifications. Chromatograms were acquired at 280, 320, 360, and 520 nm on the DAD.

The MS was fitted to an atmospheric pressure electrospray ionization (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 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, concentrations of polyphenols in extracts were calculated using the calibration curves of the standards closest in chemical structure.

For ***EPAs***, 20 μL of aqueous−organic extracts was injected onto a Phenomenex (Torrance, CA) 5 μm Luna silica (2), 100 A column (25 × 4.6 mm) at 37 °C HPLC column coupled to an Agilent 1100 HPLC system with fluorescence detector and analyzed according to the method described by Gu et al. (*18*) with the additional relative fluorescence response data published by Prior and Gu (*19*). Samples of grape seed procyanidin extract, pure standards of catechin and epicatechin, and procyanidin dimers B1 and B2 were run under the same normal-phase HPLC conditions.

Analysis of NEPP. ***HPP*** were determined in methanol/H_2SO_4 hydrolysates using a method similar to that described elsewhere (*20*). Samples (20 μL) of filtered hydrolysates (0.45 μm Teknokroma filter) were injected into a HPLC reverse-phase C18 Gemini column (250 × 4.6 mm i.d.; particle size = 5 um) with a guard column containing the

Table 1. Determination of Extractable Polyphenols (EPP) determined in Methanol/Acetone Extracts of Apple, Peach, and Nectarine by Using Spectral Characteristics in HPLC (Retention Times, λ_{max}) and Mass Ions in LC-MS[a]

proposed structure	$[M-H]^-$	extractable polyphenols (mg/100 g of fw)
apple		
gallic acid	169	0.53 ± 0.2
chlorogenic acid	353, 707[b]	6.22 ± 0.66
procyanidin B1	577	0.34 ± 0.32
epicatechin gallate	441	0.46 ± 0.09
epicatechin	289	0.2 ± 0.1
phloretin-2′-xyloglucoside	569	0.74 ± 0.02
phloretin-2′-glucoside (phloridzin)	435	0.87 ± 0.14
quercetin-3-glucoside	463	2.80 ± 0.64
quercetin-3-xyloside	433	0.46 ± 0.43
quercetin-3-rhamnoside	447	2.26 ± 0.09
		14.9 ± 2.3
peach		
catechin	289	5.66 ± 0.52
epicatechin gallate	441	1.06 ± 0.2
chlorogenic acid	353, 707[b]	10.24 ± 0.34
caffeoylquinic acid	353	4.10 ± 0.12
procyanidin B1	577	4.69 ± 0.3
quercetin-3-glucoside	463	0.17 ± 0.03
		25.91 ± 1.06
nectarine		
catechin	289	0.7 ± 0.35
epicatechin	289	1.72 ± 0.03
epicatechin gallate	441	0.96 ± 0.15
chlorogenic acid	353, 707[b]	2.5 ± 0.5
caffeoylquinic acid	353	1.06 ± 0.2
quercetin-3-glucoside	463	0.97 ± 0.04
cyanidin-3-glucoside	447	2.32 ± 0.03
		10.27 ± 1.27

[a] Extractions were performed in three different replicates for each product. Determinations were performed per triplicate in each extract and are reported on a fresh matter basis. Results are expressed as mean value ± standard deviation. [b] Dimeric adduct.

same stationary phase. This column is suitable for a wide range of pH values and is appropiate for acid samples. Chromatograms were recorded at 280, 320, 360, and 520 nm on the DAD. 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.

NEPA were quantified by measuring the anthocyanidin concentration in butanol/HCl/$FeCl_3$ hydrolysates at 555 nm and reference to a calibration curve of a carob pod tannin concentrate (*7*, *16*).

RESULTS AND DISCUSSION

Analysis of Extractable Polyphenols. To elucidate the relationship between structure and biological properties of PP and relationships between dietary intakes and health outcomes, it is essential to have accurate data describing the content and composition of these compounds in foods. Because existing composition data are almost exclusively concerned only with EPP, we investigated the content and composition of both the EPP and the polyphenols not extracted (NEPP) by classic solvent extraction methods.

EPP and EPA were quantified in methanol/acetone extracts of apple, peach, and nectarine by HPLC-DAD-MS and normal phase-HPLC−fluorescence detector, respectively, as described earlier. The HPLC-DAD-MS technique allows separation of flavonoids including anthocyanins, benzoic acids, and hydroxycinnamic acids, whereas the HPLC−fluorescence technique is used for separating monomers and oligomers up to polymers of flavan-3-ols.

Results of EPP for apple, nectarine, and peach, expressed as milligrams per 100 g of fresh edible weight, are shown in Table **1**.

HPLC-MS analysis indicated a single major peak with a UV−visible spectrum and $[M-H]^- = 353, 707$ (dimer) parent ion masses that are consistent with the presence of 5-caffeoylquinic acid (chlorogenic acid) in apple sample. Peach and nectarine samples contained 5-caffeoylquinic acid and an additional peak with a different retention time but with the same MS spectrum, and this is likely to be 4-caffeoylquinic acid, the identification of which has been reported previously (*17*). In the 280 nm chromatogram of apple a peak with *m*/*z* 169 was identified as gallic acid. Another two peaks presented similar UV spectrum and retention time as the phloridzin standard and MS ions at *m*/*z* 569 and 437 that may correspond with molecular weights of phloretin-xyloglucoide and phloretin-glucoside identified previously by Alonso-Salces et al. (*21*). A number of other peaks with UV spectra (maximum at 280 nm) characteristic of flavan-3-ols were identified in apple, peach, and nectarine by comparison with standards. Peach was the fruit with the highest concentration of total flavan-3-ols (6.72 mg/100 g of fresh weight (fw)) mainly present as (+)-catechin and (−)-epicatechin gallate (ECG). The sum of catechin, epicatechin (EC), and ECG in nectarine was 3.3 mg/100 g of fresh weight, whereas EC and ECG in apple was 1.4 mg/100 g of fw, amounts that are in accordance with previously reported values (*22*).

The procyanidin dimer B1 was also identified in apple (0.34 mg/100 g of fw) and peach (4.68 mg/100 g of fw) by comparison with standard and mass spectrum, which indicated the characteristic fragment at *m*/*z* 577. Other minor compounds were identified in the 360 nm chromatogram with a characteristic spectrum of flavonols. In apple, quercetin-3-glucoside with mass ion at *m*/*z* 463 and two more compounds coincided with quercetin-3-xyloside with *m*/*z* 433 and quercetin-3-rhamnoside with *m*/*z* 447. In nectarine and peach only one flavonol was identified with *m*/*z* 463, which could correspond to quercetin-3-glucoside. The chromatograms at 510 nm, characteristic of anthocyanins, revealed that only nectarine contains an anthocyanin pigment, in this case, cyanidin-3-glucoside.

Overall, the EPP values presented here are similar to those reported previously in the literature (*23*, *24*), and the small differences are likely due to differences in variety, maturity of fruits, extraction procedures, or HPLC methods. Napolitano et al. (*25*) reported EPP values for apple (Golden Delicious) in the range of 20−24 mg/100 g of fw and reported that chlorogenic acid and flavan-3-ols (catechin and epicatechin) were the major extractable compounds (7.8 and 11.1 mg/100 g of fw, respectively). Quercetin-glucoside, -xyloside, and -rhamnoside have been previously described by other authors with similar amounts (*17*, *26*) reported, and in an extensive study, similar EPP values for nectarine and peach depending on the part of fruit. Hydroxycinnamic acids (chlorogenic and neochlorogenic acids), flavan-3-ols (catechin, epicatechin, and procyanidin B1), flavonols (quercetin-3-glucoside, galactoside, and rutinoside), and anthocyanins (cyanidin-3-glucoside and rutinoside) were the polyphenols identified in the three fruits. EPA content in fruits determined by normal-phase HPLC is presented in **Table 2**. These data show that methanol/acetone extracts contain the flavan-3-ol monomers ((+)-catechin and (−)-epicatechin)) and flavan-3-ol oligomers with degrees of polymerization (DP) from 2 to 10 and polymers that are quantified as a single peak at the end of the chromatogram (*18*). Monomers of catechin and epicatechin and dimers (B1 and B2) were also quantified by reverse-phase HPLC. The EPA contents of the three fruits reported here are similar to values reported elsewhere (*27*, *28*). Monomers and oligomers with DP between 4 and 6 are the major procyanidins in all three fruits. Polymers represent just 10.3−16.6% of the total EPA fraction.

Table 2. Extractable Proanthocyanidins (EPA) Present in Methanol/Acetone Extracts Determined by Normal-Phase HPLC (Milligrams per 100 g of Fresh Weight)[a]

	apple	nectarine	yellow peach
monomers	1.42 ± 0.31	1.05 ± 0.01	3.3 ± 0.46
dimers	0.89 ± 0.11	0.26 ± 0.04	1.31 ± 0.12
trimers	0.7 ± 0.2	0.16 ± 0.02	0.92 ± 0.02
4−6-mers	1.6 ± 1	0.38 ± 0.1	2.04 ± 0.96
7−10-mers	tr[b]	nd[b]	tr
polymers	0.64 ± 0.1	0.37 ± 0.05	0.89 ± 0.12
total EPA	**5.3 ± 1.8**	**2.22 ± 0.6**	**8.56 ± 1.83**

[a] Extractions were performed in three different replicates for each product. Determinations were performed per triplicate in each extract and are reported on a fresh matter basis. Results are expressed as mean value ± standard deviation. [b] tr, trace; nd, not detected.

Analysis of Nonextractable Polyphenols. *Hydrolyzable Polyphenols* (*HPP*). Hydrolyzable tannins are polyesters of a sugar moiety and organic acids. These compounds undergo hydrolytic cleavage to the respective sugar and acid moiety upon treatment with diluted acids. They can be divided into gallotannins and ellagitannins depending on whether the acid component is gallic acid or hexahydroxydiphenic acid.

Different HPLC methods have been reported for the analysis of HT in fruits, but all of them refer to HT present in aqueous−organic extracts conjugated with other molecules (*29*), whereas hydrolyzable tannins, hydroxycinnamic acids, and phenolic acids present in the corresponding residues usually escape analysis (*30*). Moreover, most results are qualitative, mainly due to the lack of chromatographic standards.

To our knowledge, this is the first time that methanolysis has been performed with sulfuric acid in residues to analyze nonextractable HPP in apple, peach, and nectarine.

There are different methods to release these polyphenols from residues of aqueous−organic extraction. The most common procedures are based on acid and alkali hydrolysis. The main objective of our acidic hydrolysis of the residues is to release the NEPP from the cell wall matrix (dietary fiber, protein). This acidic hydrolysis is necessary to release the major part of NEPP trapped in cores or bound to cell wall constituents, although some losses of hydroxycinammic acids may occur (*31*, *32*).

The HPLC-DAD/MS procedure reported by Bennett et al. (*33*) was applied using different conditions to prevent the formation of a precipitate in the ionization source due to the formation of salts (appropriate column for acid pH, different mobile phases, and positive ionization mode in MS detector). Results are shown in **Table 3**.

Chromatograms show that hydroxybenzoic and hydroxycinnamic acids are the major compounds produced by hydrolysis of residue. In three fruits, the compound detected in the 320 nm chromatogram with m/z 195 $[M - H]^+$ was the most abundant, which indicates the presence of ferulic acid. As some authors have reported, acid hydrolysis can break arabinoxylan bonds with ferulic acid in cell wall plants, releasing free ferulic acid (*34*).

Four more compounds were identified as benzoic acids when their UV and MS spectra were compared with standards. The most abundant among them was the m/z 139, identified as *p*-hydroxybenzoic acid; m/z 171 corresponded to gallic acid, probably derived from galloyl derivates; m/z 155 was identified as protocatechuic acid; and m/z 169 was identified as vanillic acid.

Another two peaks with MS ions at m/z 307 and 291 were identified as gallocatechin and catechin, respectively. It is possible that these monomers of flavan-3-ol originated from some proanthocyanidin present in the residue and were hydrolyzed to yield monomers. However, these values are believed to be insignificant compared with NEPA content as commented below.

Table 3. Characterization of Hydrolyzable Polyphenols (Milligrams per 100 g of Fresh Weight) of Apple, Peach, and Nectarine by Using Their Spectral Characteristics in LC-DAD (Retention Time, λ_{max}) and Positive Ions in LC-MS[a]

proposed structure	$[M - H]^+$	apple	peach	nectarine
gallic acid	171	2.9 ± 0.8	0.97 ± 0.2	1.93 ± 0.05
p-hydroxybenzoic acid	139	19.3 ± 3.2	13.5 ± 1.3	14.4 ± 0.8
protocatechuic acid	155	1.3 ± 0.3	1.24 ± 0.4	1.4 ± 0.16
ellagic acid	303	0.18 ± 0.3	tr	0.13 ± 0.03
catechin	291	5.4 ± 2.7	2.5 ± 0.4	4.9 ± 0.2
gallocatechin	307	2.8 ± 1.5	0.6 ± 0.4	1.6 ± 0.4
epicatechin	291	tr	0.14 ± 0.03	0.11 ± 0.04
vanillic acid	169	0.28 ± 0.3	0.14 ± 0.03	tr
ferulic acid	195	48.9 ± 4.5	33.8 ± 3.5	40.8 ± 1.9
cinnamic acid	149	tr	tr	tr
caffeic acid	181	tr	tr	0.9 ± 0.1
sinapinic acid	225	tr	tr	tr
		80.3 ± 9.4	**52.8 ± 2.9**	**65.8 ± 1**

[a] Extractions were performed in three different replicates for each product. Determinations were performed per triplicate in each extract and are reported on a fresh matter basis. Results are expressed as mean value ± standard deviation. tr, trace.

The MS spectrum presented one strong peak with m/z 303, which may correspond to ellagic acid (by comparison with standard) and/or to some hexahydroxydiphenoyl (HHDP) derivative with similar UV spectra.

Other peaks were tentatively identified as cinnamic, caffeic, and sinapic acids by comparison with m/z ions 149, 181, and 225, respectively, and their UV spectra, but they were beneath the detection limit and therefore quantification was not possible. These compounds were considered as traces.

Some of the compounds identified in this work, such as gallic acid, ellagic acid, catechin, and gallocatechin, have also been identified in aqueous−organic extracts of muscadine grapes, raspberry, pomegranate, strawberry, and mango (*35*−*37*).

Regarding the quantitative data, we note the large amount of NEPP as compared with extractable compounds (see **Tables 1** and **2**). NEPP contents are up to 5 times higher than EPP contents (sum of EPPs and EPAs).

Although there is no information on HPLC quantification of NEPA in the literature, our values are consistent with other reported data from spectrophotometric analyses (*7*, *15*).

Nonextractable Proanthocyanidins (*NEPA*). Data on proanthocyanidin contents of selected foods in some databases (*2*) report EPA with degrees of polymerization up to decamers and polymeric procyanidins with DP > 10 eluting as a single peak at the end of the run (*18*, *38*). However, an appreciable amount of high molecular weight PA complexed with polysaccharides and protein may remain unextractable (*39*).

Some attempts to determine NEPA by HPLC-ESI-MS following derivatization, thiolysis, or enzymatic treatments of residues manage to analyze only a small fraction of the total NEPA content (*9*, *40*). The most common procedure for estimating the actual amount of NEPAs is therefore one based on spectrophotometric analysis of anthocyanidin solutions obtained by butanol/HCl/Fe treatment of the residues (*16*). Acid-catalyzed oxidative depolymerization of the interflavan bonds in the proanthocyanidins yield red anthocyanidin solutions, which are measured spectrophotometrically at 555 nm. Carob pod tannin concentrates were selected as representative of a natural sample rich in high molecular weight proanthocyanidins as standards of this analysis. The isolation of carob pod tannin concentrate is based on an extraction of sugars and soluble compounds of carob pod, posterior treatment at high pressure and temperature, and

purification to obtain carob pod tannins concentrate (Nestlé, S.A. Research Centre, Vevey, Switzerland).

Results for apple, peach, and nectarine were 45.77 ± 0.3, 59.1 ± 0.3, and 45.8 ± 1.2 mg/100 g of fresh matter, respectively. If we compare these NEPA values with results obtained for EPA, it seems clear that there is an appreciable underestimation of PA if NEPA are not considered.

Apart from the analytical data, our results may have biological significance.

Absorption, bioavailability, and metabolism of EPP have been extensively studied in both animals and humans, but little is known about the bioavailability of polymeric tannins (*41*).

Recent studies have demonstrated that HT present in fruits are not absorbed in humans but hydrolyzed to yield ellagic acid, or another simple structure may be further metabolized by the human colonic microflora to yield bioavailable derivatives that are able to induce apoptosis in tumor cells (*42*, *43*).

In the case of NEPA, most of the capabilities of procyanidins, including free radical scavenging, largely depend on their structure, particularly their degree of polymerization. Antimicrobial properties, antimutagenic activity, and cardioprotective effects have all been attributed to proanthocyanidins (*44*, *45*).

In conclusion, a determination of total polyphenols in fruits requires specific analysis of NEPP in acid hydrolysates of the corresponding extraction residues in addition to the usual analysis of EPP in aqueous−organic extracts. With that in mind, the quantification of polyphenols in some fruits performed in this work shows that NEPP concentrations may be higher than EPP concentrations. Further analysis of NEPPs in other fruits and plant foods consumed in the diet are needed to build a comprehensive database of use for nutritional and biological studies.

ABBREVIATIONS USED

EPP, extractable polyphenols; NEPP, nonextractable polyphenols; PA, proanthocyanidins; EPA, extractable proanthocyanidins; NEPA, nonextractable proanthocyanidins; HT, hydrolyzable tannins; HPP, hydrolyzable polyphenols; HHDP, hexahydroxydiphenoyl.

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