Determination of Free and Total Phenolic Acids in Plant-Derived Foods by HPLC with Diode-Array Detection

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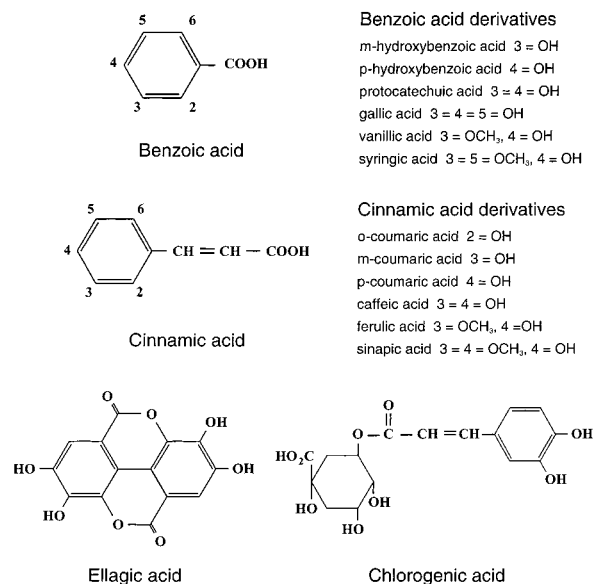MTT Agrifood Research Finland, Food Research, ET-talo, 31600 Jokioinen, Finland

A high-performance liquid chromatographic (HPLC) method with diode-array detection (DAD) was used to identify and quantify free and total phenolic acids (*m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, syringic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, and ellagic acid) in plant foods. Free phenolic acids were extracted with a mixture of methanol and 10% acetic acid. Bound phenolic acids were liberated using first alkaline and then acid hydrolysis followed by extraction with diethyl ether/ethyl acetate (1:1). All fractions were quantified separately by HPLC. After HPLC quantification, results of alkali and acid hydrolysates were calculated to represent total phenolic acids. Ellagic acid was quantified separately after long (20 h) acid hydrolysis. The methods developed were effective for the determination of phenolic acids in plant foods. DAD response was linear for all phenolic acids within the ranges evaluated, with correlation coefficients exceeding 0.999. Coefficients of variation for 4−8 sample replicates were consistently below 10%. Recovery tests of phenolic acids were performed for every hydrolysis condition using several samples. Recoveries were generally good (mean >90%) with the exceptions of gallic acid and, in some cases, caffeic acid samples.

KEYWORDS: Phenolic acids; HPLC; diode-array detection; foods; vegetables; fruits; berries

INTRODUCTION

Phenolic acids have received considerable attention as potentially protective factors against cancer and heart diseases in part because of their potent antioxidative properties and their ubiquity in a wide range of commonly consumed foods of plant origin (*1*, *2*). Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids (**Figure 1**). The most common hydroxycinnamic acid derivatives are *p*-coumaric, caffeic, and ferulic acids which frequently occur in foods as simple esters with quinic acid or glucose. Likely the most familiar of these is chlorogenic acid. Unlike hydroxycinnamates, hydroxybenzoic acid derivatives are mainly present in the form of glucosides in foods. The most common forms are *p*-hydroxybenzoic, vanillic, and protocatechuic acids (*3*). Ellagic acid is a dilactone of hexahydroxydiphenic acid, which in turn is a dimeric condensation product of gallic acid (*4*).

Different chemical natures and varying sensitivities to the conditions of extraction and hydrolysis complicate the determination of phenolic acids. Numerous extraction procedures have been described in the literature. A common feature of these extraction methods is their suitability to only one plant material type for certain phenolic acid(s) or certain form(s) of phenolic acid(s). Soluble phenolic acids have been extracted using mixtures of methanol, acetone, and/or water (*5−7*), cold or hot methanol (*8−10*), or mixtures of methanol, water, and acetic

Benzoic acid derivatives
m-hydroxybenzoic acid 3 = OH
p-hydroxybenzoic acid 4 = OH
protocatechuic acid 3 = 4 = OH
gallic acid 3 = 4 = 5 = OH
vanillic acid 3 = OCH₃, 4 = OH
syringic acid 3 = 5 = OCH₃, 4 = OH

Cinnamic acid derivatives
o-coumaric acid 2 = OH
m-coumaric acid 3 = OH
p-coumaric acid 4 = OH
caffeic acid 3 = 4 = OH
ferulic acid 3 = OCH₃, 4 =OH
sinapic acid 3 = 4 = OCH₃, 4 = OH

Figure 1. Chemical structures of analyzed phenolic acids.

acid (*11*, *12*). Bound phenolic acids have been liberated using acid hydrolysis (*9*, *13*) or alkaline hydrolysis (*5*, *14−17*), or both (*18*, *19*).

A number of analytical techniques have been presented for the quantification of phenolic acids, including thin-layer chromatography (*19*), gas−liquid chromatography (*5*, *13*, *20*), gas chromatography−mass spectrometry (*5*, *21*), or capillary elec-

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trophoretic methods (*22*). However, high-performance liquid chromatography (HPLC) is presently the most widely used quantification method (*8*, *9*, *15*, *16*, *23*).

Despite the numerous methods for phenolic acid determination described in the literature, an optimized and validated method for the determination of a variety of phenolic acids in different kinds of plant foods is lacking. Hence, this study aimed to develop an optimized method for the determination of phenolic acids (*m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, syringic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, and ellagic acid) in different types of plant foods.

MATERIALS AND METHODS

Standards. Standards of phenolic acids (**Figure 1**) were obtained from two manufacturers. The following standards were obtained from Sigma Chemical Co. (St. Louis, MO): protocatechuic acid (P-5630), *p*-hydroxybenzoic acid (H-5376), vanillic acid (V-2250), gallic acid (G-7384), syringic acid (S-6881), *p*-coumaric acid (C-9008), *m*-coumaric acid (C-5017), *o*-coumaric acid (C-4400), tr-cinnamic acid (C-6004), chlorogenic acid (5-caffeoylquinic acid; C-3878), caffeic acid (C-0625), ferulic acid (F-3500), and ellagic acid (E-2250). Sinapic acid was purchased from Fluka (Buchs, Switzerland). All standards were prepared as stock solutions at 2 mg/mL in methanol, except ellagic acid (1 mg/mL in dimethyl sulfoxide (DMSO)/methanol, 1:1, v/v). Stock solutions of the standards were stored in darkness at −18 °C. Standard solutions remained stable over three months.

Samples. Samples of potato (Nicola, 1 kg), apple (Granny Smith, 4 apples), apple juice (1 L), tomato (1 kg), carrot (0.5 kg), red raspberry (frozen, 0.5 kg), strawberry (frozen, 0.5 kg), ground coffee (1 kg), and rye crispbread (7 subsamples, 300−500 g) were obtained from local food outlets. Rosé wine (Mateus, Portugal, 0.75 L) was purchased from a local wine store (Oy ALKO Ab). Apple juice and rosé wine samples were analyzed as such, strawberry sample was analyzed after thawing and homogenization, unpeeled apple samples were analyzed after coring, and crispbread samples were analyzed after pooling and homogenization. Chopped tomato, carrot, and red raspberry samples were freeze-dried, homogenized, and stored at −18 °C before analysis. Potato samples were cooked in boiling water for 20 min, then peeled, homogenized, and stored at −18 °C before analysis. Coffee sample was brewed with a conventional coffee maker using 38 g of coffee powder and 700 mL of water. The coffee sample was analyzed immediately after preparation.

In-House Reference Sample. To prepare an in-house reference sample containing a variety of different phenolic acids, 0.25 kg of orange, 0.5 kg of lemon, 0.25 kg of cranberry, 0.38 L of red wine, 0.38 L of infused green tea, 0.5 kg of black currant, and 0.5 kg of spinach were mixed, homogenized, and lyophilized. The reference material was stored in airtight plastic containers in darkness at −20 °C and found to be very stable for flavonoids (*24*).

Hydrolysis and Extraction of Phenolic Acids. A homogenized dried (0.2−0.5 g) or fresh (2 g) sample (except apple) was weighed into a 50-mL graduated plastic test tube and homogenized in 7 mL of the mixture of methanol (containing 2 g/L of 2,(3)-*tert*-butyl-4-hydroxyanisole) and 10% acetic acid (85:15) using a Heidolph Diax 600 homogenizer (Heidolph, Germany). Apple samples were cut into 4 identical pieces and one piece of each apple was collected for analysis. The pieces were then weighed and the same weight as the apple weight of extraction solution (see above) was added. The mixture was then homogenized using a Pamix homogenizer. A 4-g sample of the homogenized mixture was then weighed into a 50-mL test tube and 5 mL of the extraction solution was added. Homogenization was performed as described above. After homogenization all sample extracts were ultrasonicated for 30 min, made up to 10 mL with distilled water, and mixed, and 1 mL was filtered through a membrane filter (0.45 μm, 25 mm; Pall Gelman Laboratory) for the HPLC analysis of free phenolic acids.

After taking the sample for analyzing free phenolic acids, 12 mL of distilled water and 5 mL of 10 M NaOH were added into the test tube, and its contents were then bubbled with nitrogen, sealed, and stirred overnight at room temperature (about 16 h) using a magnetic stirrer. The solution was then adjusted to a pH of 2, and liberated phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA, 1:1) by manually shaking and centrifuging. DE/EA layers were combined, evaporated to dryness, and dissolved into 1.5 mL of methanol. After samples were filtered through a membrane filter (see above), the HPLC run was performed.

After the above alkaline hydrolysis was completed, an acid hydrolysis was then performed by adding 2.5 mL of concentrated HCl into the test tube and incubating the tube in a water bath (85 °C) for 30 min. After acid hydrolysis, the sample was allowed to cool, and the pH was adjusted to 2. The DE/EA extraction performed was similar to that for alkaline hydrolysis. Evaporated extract was then dissolved into 1.5 mL of methanol, filtered through a membrane filter (see above), and analyzed by HPLC. After HPLC quantification, the results from alkali and acid hydrolysates were calculated to represent total phenolic acids.

Hydrolysis of Ellagic Acid. The extraction method employed for ellagic acid determinations in berries was based on the method of Häkkinen et al. (*25*) with the following modifications: a homogenized dried red raspberry (0.5 g) or fresh strawberry (5 g) sample was weighed into a rotavapor flask and 10 mL (dried red raspberry) or 5 mL (fresh strawberry) of water, 20 mL of methanol, and 5 mL of concentrated HCl were added. The mixture was refluxed for 20 h at 85 °C. The extract was allowed to cool, made up to 50 mL with methanol, and part of it was filtered with a membrane filter (see above) for the HPLC analysis.

Quantification of Phenolic Acids. The analytical HPLC system employed comprised an Agilent 1100 Series high-performance liquid chromatograph equipped with an Agilent 1100 Series diode-array detector (Agilent Technologies, Germany). The HPLC pumps, autosampler, column oven, and diode-array system were monitored and controlled using the HP Chem Station computer program (Agilent). Wavelengths used for the identification of phenolic acids with the diode-array detector were 254 nm for protocatechuic acid, *p*-hydroxybenzoic acid, and vanillic acid; 270 nm for gallic acid; 280 nm for syringic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, and tr-cinnamic acid; 329 nm for chlorogenic acid, caffeic acid, and ferulic + sinapic acid. Ferulic and sinapic acids were quantified together as ferulic acid.

Phenolic acid separation was done by an Inertsil ODS-3 (4.0 × 150 mm, 3 μm; GL Sciences, Inc., Japan) column with a C-18 guard column. Temperature of the column oven was set at 35 °C. Gradient elution was employed with a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) as follows: isocratic elution 95% A/5% B, 0−5 min; linear gradient from 95% A/5% B to 50% A/ 50% B, 5−55 min; isocratic elution 50% A/50% B, 55−65 min; linear gradient from 50% A/50% B to 95% A/5% B, 65−67 min; post-time 6 min before next injection. Ellagic acid was quantified separately using shortened gradient elution: isocratic elution 95% A/5% B, 0−5 min; linear gradient from 95% A/5% B to 50% A/ 50% B, 5−35 min; isocratic elution 50% A/50% B, 35−40 min; linear gradient from 50% A/50% B to 95% A/5% B, 40−43 min; post-time 6 min before the next injection.

Flow rate of the mobile phase was 0.7 mL/min, and the injection volumes were 10 μL of the standards and sample extracts. All phenolic acids were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were made by diluting stock standards in methanol to yield 30−300 (ellagic acid) or 10−80 μg/mL (other phenolic acids).

Method Validation. Linearity of the detector responses and the detection limits were tested for the phenolic acid standards. Recovery tests were performed for every hydrolysis condition with several food samples (**Table 1**). Repeatability of the method was tested by analyzing all samples 4−8 times and analyzing the reference sample 7 times. Identity and purity of the phenolic acid peaks was monitored (in addition to monitoring retention times and the symmetry of the peak), comparing the spectral data of the sample peaks with that obtained for the phenolic acid standards.

Table 1. Recoveries of Phenolic Acids (%) after Different Hydrolysis Conditions ($n = 1$–5)

compound/ procedure[a,b]	potato	apple	tomato	carrot	crisp-bread	red raspberry	strawberry	apple juice	rose wine	coffee
5-CQA/										
1	80, 101	102–110[c]								
2	0									
caf/										
1	95, 113	111–115						120	115	120
2								99	75	1
p-coum/										
1	93	109–119		120, 113	125					
2	107	89–106	95, 101	87, 105	117	101, 96	110			
3			65, 67	70, 88	83	74, 63	62			
fer/										
1	87, 111	116, 117		109, 119	110					
2	94–105	89, 103	99	85, 104	119					
3				70, 82	110					
p-OH-b/										
1		116–119		113, 119	122		107			
2		100, 106		103, 103	107	106, 82	111			
3				98, 95	105	101, 86	90			
van/										
1	95			111, 119	107					
2	94, 113			108, 108	105	110, 94				
3				76	111	89, 87				
syr/										
1					125					
2					118					
3					104					
proto/										
1	94	108–118		90, 99						
2	89	86–103	107, 93	91, 86		101, 73	103			
3				94, 109	104	95, 75	102			
gal/										
1						121, 122	119			
2						15, 0	19			
3						61, 50	68			

[a] Compounds: 3-CQA, 3-caffeoylquinic acid; 5-CQA, 5-caffeoylquinic acid (chlorogenic acid); 4-CQA, 4-caffeoylquinic acid; caf, caffeic acid; p-coum, p-coumaric acid; fer, ferulic acid; p-OH-b, p-hydroxybenzoic acid; van, vanillic acid; syr, syringic acid; proto, protocatechuic acid; gal, gallic acid; tr-cin, tr-cinnamic acid. [b] Procedures: 1, methanol-acetic acid extraction; 2, alkaline hydrolysis; 3, acid hydrolysis. [c] Recoveries are presented as ranges if more than two recovery tests were performed.

RESULTS AND DISCUSSION

The difficulty of phenolic acid determination arises from the extraction of these compounds from food matrixes. Previously, very complicated extraction methods have been employed to determine free, esterified, and glycolysated phenolic acids in plant material (*5*, *7*, *26*). The present study has resulted in a simplified method for a variety of food samples. This method enables the determination of free and total phenolic acids using three different hydrolysis procedures in the same test tube.

Free phenolic acids were first extracted using methanol/acetic acid as the extraction solution. Ultrasonication times of 30 min were adequate because, according to our studies, 1 h of sonication or 30 min of sonication plus 30 min of shaking did not result in better extraction values.

Different hydrolysis time (2, 4, and 16 h) and alkaline strength (2.5, 5, and 7 mL of 10 M NaOH) combinations were tested to optimize the alkaline hydrolysis procedure (results not shown). The most effective combination for most phenolic acids was 16 h and 5 mL of 10 M NaOH. During these conditions the largest results were obtained, and the recoveries of added phenolic acids were good in most cases (**Table 1**). However, an exception was gallic acid which was unstable during alkaline conditions. Hence, the results obtained for this phenolic acid after alkaline hydrolysis were too low and are not shown in **Table 3**. Under alkaline conditions, chlorogenic and other caffeoylquinic acids are hydrolyzed rapidly to caffeic acid (*27*). In some samples (e.g., coffee), caffeic acid further decomposed as a result of the alkali treatment (**Table 1**). In plants, however,

most of the caffeic acid occurs as caffeoylquinic acids (*3*) and these could be quantified after the methanol/acetic acid extraction. Generally, alkaline hydrolysis liberated most of the bound phenolic acids. Acid hydrolysis was unnecessary in the cases of apple, apple juice, and potato because the hydrolysis liberated only very low contents of phenolic acids after the alkali treatment.

Following alkaline hydrolysis, acid hydrolysis was performed to liberate the rest of the bound phenolic acids. Different hydrolysis times (15 min, 30 min, 1 h, and 16 h), acid strengths (2.5, 3, 5, and 10 mL of concentrated HCl and 5 mL of 6 M HCl) and temperature (35 °C and 85 °C) combinations were tested to optimize the acid hydrolysis procedure (results not shown). The compromise combination turned out to be 2.5 mL of concentrated HCl, 30 min, and 85 °C. These conditions were sufficiently effective with minimal losses. However, the recovery of p-coumaric acid was quite variable (**Table 1**).

When developing the method, extractability of phenolic acids into the DE/EA solution was examined by recovery tests. These recovery tests showed that, generally, free phenolic acids extracted well into DE/EA solution with recoveries ranging from 87 to 112%. In addition to free phenolic acids, also some forms of bound phenolic acids extracted to some degree into the DE/ EA solution. For example, recovery of chlorogenic acid (esterified phenolic acid) was 54−63% and that of ellagic acid was 42%.

The above extraction methods were not suitable for ellagic acid as this phenolic acid was only partly soluble in the mixture

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Table 2. Contents of Free Phenolic Acids in Tested Foods, mg/kg Fresh Weight, Mean ± SD (key for phenolic acids is given in footnote *a* of Table 1)

	potato Nicola (*n* = 8)	apple Granny Smith (*n* = 5)	apple juice (*n* = 4)	tomato (*n* = 7)	carrot (*n* = 4)	rosé wine (*n* = 4)	coffee drink (*n* = 4)	crisp-bread rye (*n* = 4)	red raspberry (*n* = 8)	straw-berry (*n* = 4)
3-CQA	14.5 ± 0.23	nd[a]	nd	nd	nd	40.8 ± 0.47	400 ± 10	nd	nd	nd
5-CQA	91.2 ± 0.29	32 ± 1.3	58 ± 1.5	24 ± 1.2	120 ± 9.4	nd	960 ± 29	nd	15 ± 1.0	29 ± 2.5
4-CQA	23.0 ± 0.20	7.6 ± 0.17	2.75 ± 0.058	11.7 ± 0.59	1.19 ± 0.095	nd	530 ± 16	nd	nd	nd
caf	1.01 ± 0.021	1.4 ± 0.12	1.92 ± 0.081	1.5 ± 0.16	0.37 ± 0.062	2.93 ± 0.023	1.3 ± 0.13	7.7 ± 0.22	nd	nd
p-coum	nd	nd	0.62 ± 0.035	nd	nd	1.08 ± 0.069	nd	nd	nd	nd
fer	1.16 ± 0.015	nd	nd	nd	nd	1.8 ± 0.14	nd	39 ± 1.1	nd	nd
p-OH-B	nd	nd	nd	nd	0.59 ± 0.063	0.6 ± 0.10	nd	nd	nd	nd
van	nd	nd	nd	nd	nd	nd	nd	5.7 ± 0.49	nd	nd
syr	nd	nd	nd	nd	nd	1.45 ± 0.064	nd	nd	nd	nd
proto	nd	nd	nd	nd	nd	2.6 ± 0.23	nd	nd	nd	nd
tr-cin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
gal	nd	nd	nd	nd	nd	9.4 ± 0.79	nd	nd	nd	nd

a nd, not detected.

Table 3. Contents of Total Phenolic Acids in Foods (mg/kg fresh weight) Obtained in the Present Study (mean ± SD) and Other Studies (key for phenolic acids is given in footnote *a* of Table 1)

	potato, Nicola (*n* = 8)[d]	apple, Granny Smith (*n* = 5)	apple juice (*n* = 4)	tomato (*n* = 7)	carrot (*n* = 4)	rosé wine (*n* = 4)	coffee, drink (*n* = 4)	crisp-bread, rye (*n* = 4)	red raspberry (*n* = 8)	strawberry (*n* = 4)
5-CQA	91.2 ± 0.29 164 (*32*) 100–190 (*33*) 30–900 (*12*)	32 ± 1.3	58 ± 1.5 16.6 (*23*) 1.41–214 (*34*)	24 ± 1.2 42 (*32*) 12–71 (*35*)	120 ± 9.4 83 (*32*)	nd[a]	960 ± 29 1340–3653 (*37*)	nd	15 ± 1.0	29 ± 2.5
caf[b]	36 ± 8.6 28.3 (*31*) 3–33 (*39*)	14.0 ± 0.71 57.5 (*31*) 52–191 (*36*)	24 ± 2.8 22.6 (*31*)	24 ± 1.4 23.8 (*31*)	140 ± 14 45.6 (*31*)	17.7 ± 0.53	960[c] 631.3 (*31*)	13 ± 0.36	5.1 ± 0.10 3.0 (*31*) 6–10 (*38*)	1.86 ± 0.040 3.0 (*31*)
p-coum	1.00 ± 0.031 4–40 (*12*) 1.0 (*31*) <0.5–4 (*39*)	5.0 ± 0.67 8.7 (*6*) 15–22 (*36*)	9.9 ± 0.43 11.7 (*31*)	9.5 ± 0.67 11.0 (*31*)	1.47 ± 0.064 1.4 (*31*)	7.6 ± 0.39	13.7 ± 0.60 nd (*31*)	32 ± 1.5	16 ± 1.0 4.8 (*31*) 7–20 (*38*)	26 ± 1.2 nd (*31*)
fer	5.1 ± 0.27 nd (*31*) 3–6 (*39*)	1.3 ± 0.19 2.2 (*31*) 4–8 (*36*)	2.1 ± 0.16 nd (*23*) 0.9 (*31*)	3.0 ± 0.16 6.4 (*31*)	14.2 ± 0.72 2.0 (*31*)	2.3 ± 0.24	90 ± 3.0 nd (*31*)	1099 ± 54	nd 2.9 (*31*) 3–17 (*38*)	nd 0.5 (*31*)
p-OH-B	<0.1	nd	0.66 ± 0.061 nd (*31*)	0.51 ± 0.076 nd (*31*)	55 ± 2.5 nd (*31*)	1.24 ± 0.069	1.5± 0.11 nd (*31*)	8.5 ± 0.73	26 ± 1.5 20 (*31*) 15–27 (*38*)	75 ± 1.3 12.6 (*31*)
van	nd 5–40 (*12*) nd (*39*)	nd	nd	0.23 ± 0.015 0.5 (*31*)	8.9 ± 0.37 4.4 (*31*)	4.0 ± 0.18	0.7 ± 0.14 nd (*31*)	25 ± 1.3	5.2 ± 0.34 nd (*31*)	1.0 ± 0.10 1.3 (*31*)
syr	nd	nd 22.5 (*31*)	nd	nd	0.18± 0.025 nd (*31*)	5.8± 0.43	nd	19 ± 1.6	nd	nd
proto	nd 50–200 (*12*) nd (*39*)	4,9 ± 0.25 nd (*31*)	2.4 ± 0.13 0.5 (*31*)	nd	1.3 ± 0.073 nd (*31*)	3.4 ± 0.37	nd	10 ± 1.4	13 ± 1.0 31 (*31*) 25–37 (*38*)	4.0 ± 0.41 12.8 (*31*)
tr-cin	0.18 ± 0.015	nd	nd	nd	nd	nd	0.13 ± 0.010	nd	1.01 ± 0.046 nd (*31*)	9.0 ± 0.38 nd (*31*)
ella									1600 ± 55 499–708 (*25*) 650.1 (*31*)	310 ± 26 249–403 (*25*) 226.6 (*31*)

a nd, not detected. *b* Including caffeic acid decomposed from caffeoyl quinic acids. *c* Decomposed during alkaline hydrolysis; the value is calculated from caffeoyl quinic acid results. *d* In references (*12*), (*32*), and (*33*) uncooked potato was used.

of DE/EA (1:1). For ellagic acid, a modification of the method by Häkkinen et al. (*25*) was used. Ellagic acid has often been extracted using trifluoroacetic acid solutions (*28*, *29*). However, Häkkinen and co-workers (*25*) compared trifluoroacetic acid and HCl extractions and found the latter to be far more efficient. In addition to testing hydrolysis conditions by Häkkinen et al. (*25*), 5-h and 20-h hydrolyses using twice as much HCl were tested in the present study. The conditions optimized by Häkkinen et al. (*25*) were the most efficient and these conditions

were therefore chosen. The HPLC run was shortened for ellagic acid quantification to speed up the analysis time.

All other phenolic acids separated well from the matrix and each other except ellagic, ferulic, and sinapic acids, which eluted at the same retention time (**Figures 2−4**). Because sinapic acid is quite rare as compared with ferulic acid, and the spectral differences between ferulic and sinapic acids at 329 nm are small, the quantification of sinapic acid was performed together with ferulic acid as ferulic acid. Instead, quantification of ferulic
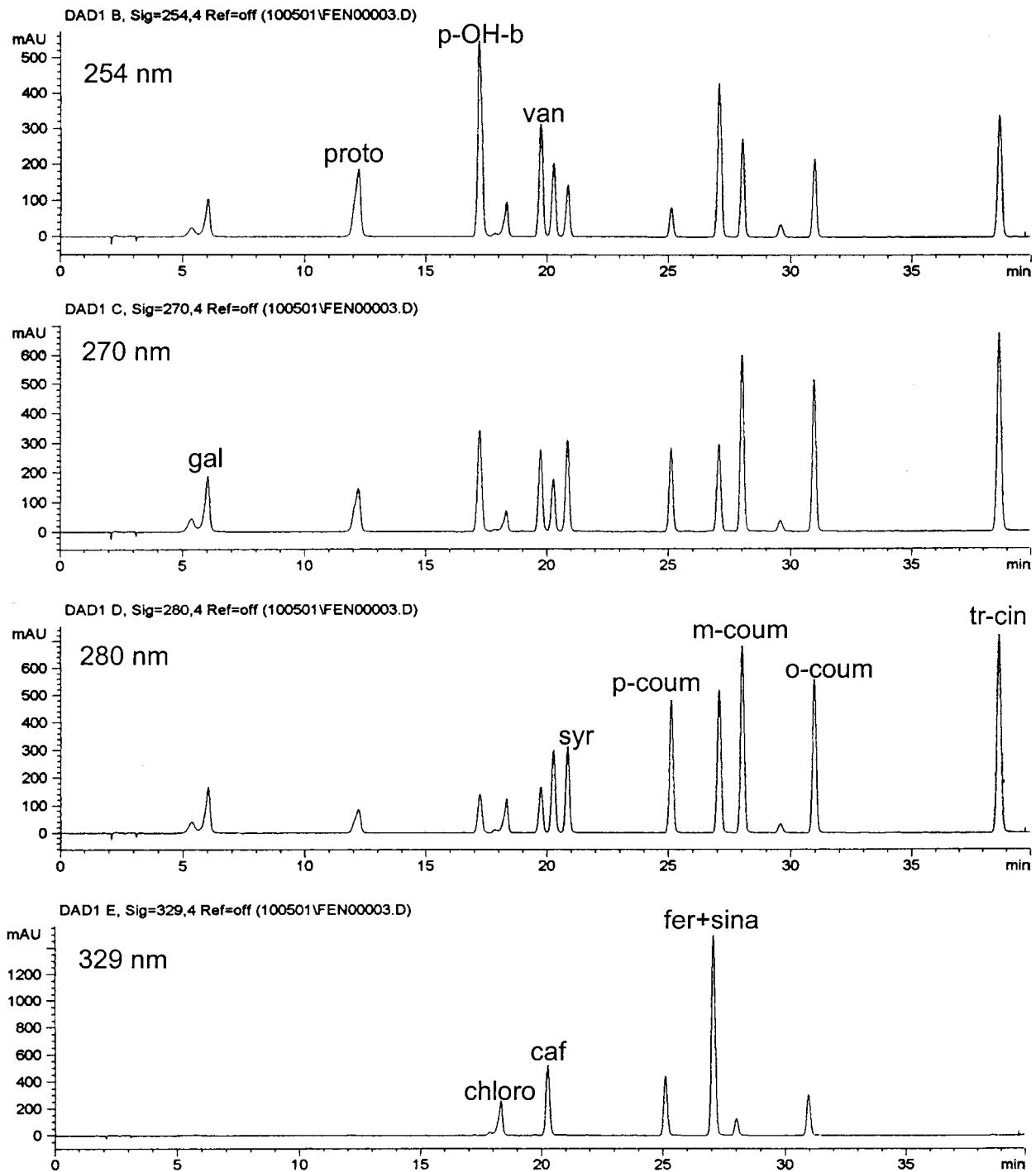
Figure 2. HPLC chromatograms of the phenolic acid standard mixture recorded at 254, 270, 280, and 329 nm. Column: Inertsil ODS-3(4.0 × 150 mm, 3 μm; GL Sciences, Inc, Japan). Mobile phase: gradient elution consisting of 50 mM H_3PO_4, pH 2.5, and acetonitrile. abbreviations p-OH-b, p-hydroxybenzoic acid; van, vanillic acid; gal, gallic acid; syr, syringic acid; p-coum, p-coumaric acid; m-coum, m-coumaric acid; o-coum, o-coumaric acid; tr-cin, tr-cinnamic acid; chloro, 5-caffeoylquinic acid (chlorogenic acid); caf, caffeic acid; fer, ferulic acid; sina, sinapic acid.

and sinapic acids is impossible if the sample contains large amounts of ellagic acid. Thus, in that situation, ferulic and sinapic acids are obviously less important from a nutritional point of view. It turned out to be possible to combine the DE/EA extracts obtained after alkaline and acid hydrolysis before the HPLC analysis, although separation of phenolic acids from the matrix was not as good as that by analyzing the extracts separately.

Comparing the spectral data of the sample peaks with those obtained for the phenolic acid standards confirmed the reliability of quantification (matches were generally 90–100%). Reliability

of the method was further shown by recovery and repeatability tests. Recovery tests were performed for every hydrolysis condition using several samples. Generally the recoveries were good (mean >90%, **Table 1**). However, it appeared that phenolic acids were more stable during alkaline than acid hydrolysis. The method proved to be repeatable: coefficient of variation for the reference sample varied from 4.0% (p-coumaric acid) to 14.1% (p-hydroxybenzoic acid). For other samples, coefficients of variation were mostly under 10% (**Tables 1** and **2**). Detection limits for phenolic acids, defined as the signal three times the height of the noise level, were estimated at 0.02–
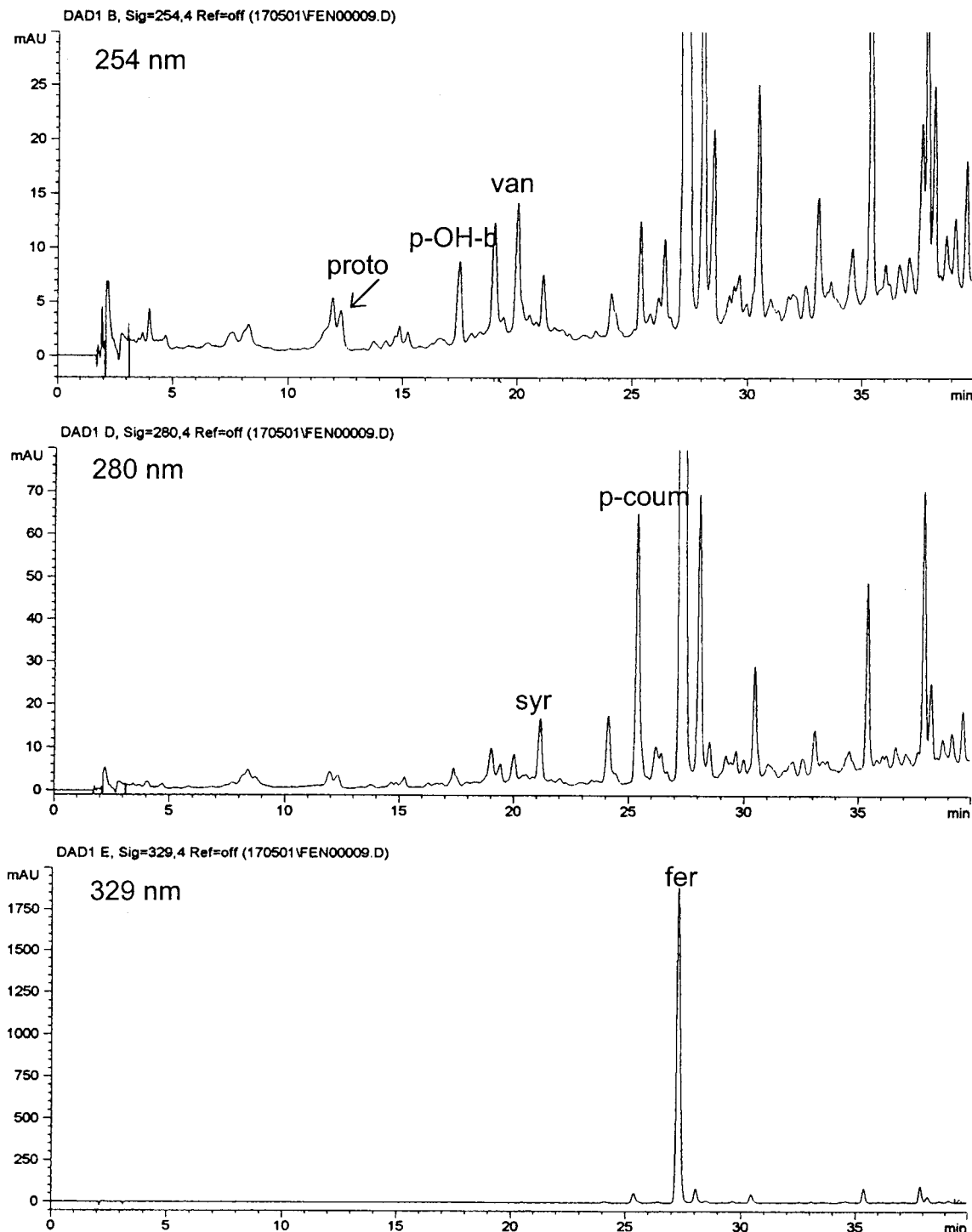
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Figure 3. HPLC chromatograms of crispbread sample after alkaline hydrolysis. Key for phenolic acids is same as for **Figure 2**.

0.07 μg/mL. Response of the detector was linear within the tested ranges. Coefficients of correlation were >0.999 for all phenolic acids.

Suitability of the method was tested by analyzing several food samples. Results of the quantification of phenolic acids (average values of all replicated analyses) are presented in **Tables 2** and **3**. Total phenolic acids (**Table 3**) represent the calculated results obtained after alkaline and acid hydrolyses. The main phenolic acid in the majority of the samples analyzed (potato, apple, apple juice, tomato, carrot, and coffee) was chlorogenic acid (5-caffeoylquinic acid). In addition to chlorogenic acid, other forms of caffeoylquinic acids were also found, namely 3-caffeoylquinic acid and/or 4-caffeoylquinic acid. We identified 3- and 4-caf-

feoylquinic acids using spectral data and the data produced by Brandl and Herrmann (*30*) and quantified as 5-caffeoylquinic acid.

The best phenolic acid sources of the samples analyzed were red raspberry, coffee, and crispbread. Caffeoylquinic acids were abundant in coffee, and ferulic acid was abundant in crispbread. Red raspberry had a high content of ellagic acid: 1.6 ± 0.055 g/kg fw. Ellagic acid content in strawberry was 0.31 ± 0.026 g/kg fw. In addition to ellagic acid, some unidentified compounds with absorbance spectra very similar to that of ellagic acid were detected in berries. The unidentified compounds were not taken into account when calculating ellagic acid results. Moderately good phenolic acid sources were cooked potato with
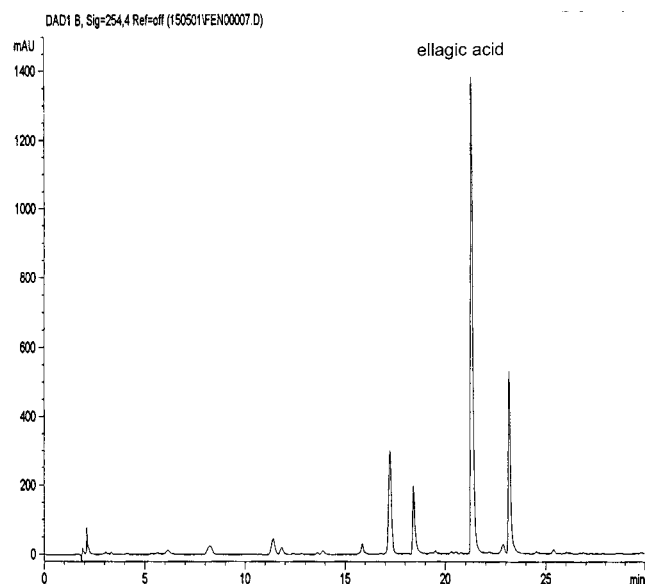
Figure 4. HPLC chromatogram of the analysis of ellagic acid in red raspberry sample.

high caffeoylquinic acid content and carrot with abundant caffeoylquinic and *p*-hydroxy benzoic acid contents.

Sporadic reports on the content of phenolic acids in food items are available in the literature. Results of the present study are compared with previous publications, in particular, with those by Radtke et al. (*31*; **Table 3**). When considering that our test samples were not representative, and the likely high variation of phenolic acid contents in plant foods, the data of the present study were in moderate agreement with earlier reported values.

CONCLUSION

The HPLC methods described here were well-suited for determining most free and total phenolic acids in various plant foods. The exception was gallic acid, and in some cases caffeic acid (in coffee), which decomposed during alkaline hydrolysis. The sensitivity, accuracy, and repeatability of the methods were acceptable.

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