Determination of Flavonoids in Plant Material by HPLC with Diode-Array and Electro-Array Detections

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A high-performance liquid chromatographic (HPLC) method with in-line connected diode-array (DAD) and electro-array (EC) detection to identify and quantify 17 flavonoids in plant-derived foods is described. Catechins were extracted from the samples using ethyl acetate, and quantification of these compounds was performed with the EC detector. Other flavonoids were quantified with DAD after acid hydrolysis. The methods developed were effective for the determination of catechins and other flavonoids in plant-derived foods. Responses of the detection systems were linear within the range evaluated, 20−200 ng/injection (DAD) and 20−100 ng/injection (EC), with correlation coefficients exceeding 0.999. Coefficient of variation was under 10.5%, and recoveries of flavonoids ranged from 70 to 124%. Purity of the flavonoid peaks was confirmed by combining the spectral and voltammetric data.

Keywords: *Flavonoids; HPLC; diode array; electro array; foods; vegetables; fruits; berries*

INTRODUCTION

Flavonoids comprise flavonols, flavones, flavanones, anthocyanidins, catechins, and biflavans. Many of these compounds exist as sugar conjugates in foods. Interest in flavonoids has increased among food chemists and nutritionists as flavonoids are ubiquitous in vegetables, berries, and fruits, and several of these compounds act as antioxidants (Heinonen et al., 1998; Igile et al., 1994; Meyer et al., 1998; Teissedre et al., 1996; Wanasundra et al., 1997), and may have a protective role in carcinogenesis, atherosclerosis, and thrombosis (Hertog, 1994; Hollman et al., 1996; Knekt et al., 1996; Verma et al., 1988; Wattenburg, 1990; Wei et al., 1990).

A traditional method for flavonoid determination in food material is UV−Vis absorption spectroscopy. These colorimetric procedures rely on the reaction of flavonoids with one of a number of reagents of varying selectivity. Flavonoids have been recorded unspecifically, as “total phenolics”, or the compounds have been isolated by preparative chromatography (for example, open-column and TLC) before spectrophotometric measurement (Harborne et al., 1975; Markham, 1982; Robards and Antolovich, 1997).

If a study seeks to separately determine individual flavonoid compounds, then a high-resolution chromatographic technique is the method of choice. HPLC methods have become especially common because HPLC combines the advantages of simultaneous separation and quantification of the flavonoid compounds under study without needing a preliminary derivatization (Hertog, 1994; Robarts and Antolovich, 1997). Because of the lack of standard compounds for many flavonoid glycosides and their great number, it has become an accepted practice to hydrolyze the glycosides into aglycones with HCl before HPLC analysis.

Detection of individual flavonoid compounds by HPLC methods has mostly been based on UV−Vis absorption. Use of diode array detection (which enables the collection of on-line spectra and simultaneous quantification by several wavelengths) has become especially common (Chilla et al., 1996; Hertog et al., 1992a,b, 1993; Justesen et al., 1998; Martos et al., 1997; Nogata et al., 1994; Rhodes and Price, 1996; Simonetti et al., 1997). Recently, coulometric electrode array detection has been shown to be a promising technique for flavonoid analysis. Coulometric array detection enables increased selectivity and sensitivity for the HPLC analysis of phenolic and flavonoid compounds based on differences of their voltammetric properties (Achilli et al., 1993; Gamache et al., 1993; Guo et al., 1997). Recently, HPLC−MS and capillary electrophoresis methods have also been employed to determine phenolic compounds in foods (Fernandez de Simon et al., 1995; He et al., 1997; Justesen et al., 1998; Poon, 1998; Tomas-Barberan and Garcia-Viguera, 1997).

Diode array and electrochemical detections produce different types of data which can be used in identifying the peaks. This paper will describe methods for 17 flavonoids (Figure 1): an HPLC−EC method for catechins and an HPLC−DAD method for other flavonoids. DAD and electro array detection were performed simultaneously in this system, thus aiding the identification of individual flavonoids. Combining the spectral and voltammetric information obtained by the two detection techniques resulted in specific, selective, and precise flavonoid analysis.

MATERIALS AND METHODS

Standards. Standards of flavonoid aglycones (Figure 1) were obtained from different manufacturers. Standards, purity grade, and manufacturers varied by aglycone: (+)-catechin (≥98%), (−)-epicatechin gallate (≥98%), (−)-epigallocatechin (≥98%), (−)-epigallocatechin gallate (≥98%), myricetin (≥95%), eriodictyol (≥95%), quercetin (≥98.5%), naringenin (≥96%), luteolin (≥95%), hesperetin (≥95%), isorhamnetin (≥95%), apigenin (≥95%), rhamnetin (≥95%), galangin (≥95%), tangeretin (≥95%) (Roth); (−)-epicatechin (>90%) (Aldrich); and

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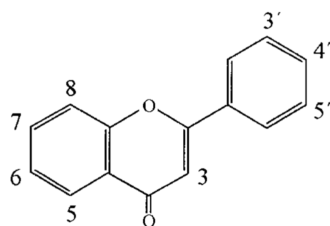
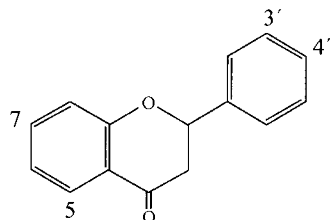


Flavones

apigenin	4′,5,7 = OH
luteolin	3′,4′,5,7 = OH
tangeretin	4′,5,6,7,8 = OCH_3

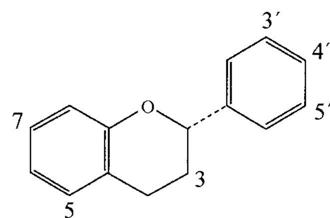
Flavonols

galangin	3,5,7 = OH
kaempferol	3,4′,5,7 = OH
quercetin	3,3′,4′,5,7 = OH
myricetin	3,3′,4′,5′,5,7 = OH
rhamnetin	3,3′,4′,5 = OH; 7 = OCH_3
isorhamnetin	3,4′,5,7 = OH; 3′ = OCH_3



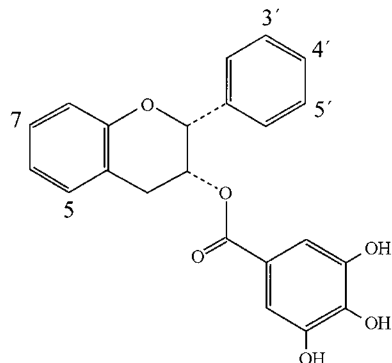
Flavanones

naringenin	3′,5,7 = OH
eriodictyol	3′,4′,5,7 = OH
hesperetin	3′,5,7 = OH; 4′ = OCH_3



Catechins (Flavanols)

(+)-catechin	3 = ◄OH; 3′,4′,5,7 = OH
(-)-epicatechin	3 = ···OH; 3′,4′,5,7 = OH
(-)-epigallocatechin	3,3′,4′,5′,5,7 = OH



Gallic acid esters of catechins

(-)-epicatechin gallate	3′,4′,5,7 = OH
(-)-epigallocatechin gallate	3′,4′,5′,5,7 = OH

Figure 1. Chemical structures of analyzed flavonoid aglycones.

kaempferol (>95%) (Fluka). All standards were prepared as stock solutions at 5 mg/50 mL in MeOH, except for luteolin and apigenin (5 mg/50 mL in DMF/MeOH, 1:6, v/v), and isorhamnetin and rhamnetin (5 mg/50 mL in DMF/MeOH, 1:10, v/v).

Working standards were made by diluting stock solutions in methanol to yield 2−4 μg/mL. Stock/working solutions of the standards were stored in darkness at −18 °C. Standard solutions remained stable over three months.

Samples. Samples of lingonberry, cranberry, red onion, yellow onion, broccoli, green tea, black tea, red wine, apple, lemon, orange, and dried parsley were obtained from Finnish wholesalers and supermarkets. Edible parts of subsamples, excluding teas and red wine, were pooled and homogenized in a blender. The pooled fresh samples were then frozen and freeze-dried and stored frozen at −18 °C before analysis. Tea samples were infused according to the label instructions (one 2-g tea bag was steeped at 90 °C for 3 min in 200 mL of water) and immediately analyzed.

In-house Reference Sample. To prepare an in-house reference sample containing a variety of different flavonoids, 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 homogenized and lyophilized.

Extraction and Hydrolysis. The extraction method employed for dried samples was the method of Hertog et al. (1992a) modified as described in the following. A dried sample (0.5 g) was weighed into a 100-mL Erlenmeyer flask then dispersed in 40 mL of 62.5% aqueous methanol containing 2 g/L of 2,(3)-*tert*-butyl-4-hydroxyanisole (BHA). The mixture was then ultrasonicated for 5 min. To this extract 10 mL of 6

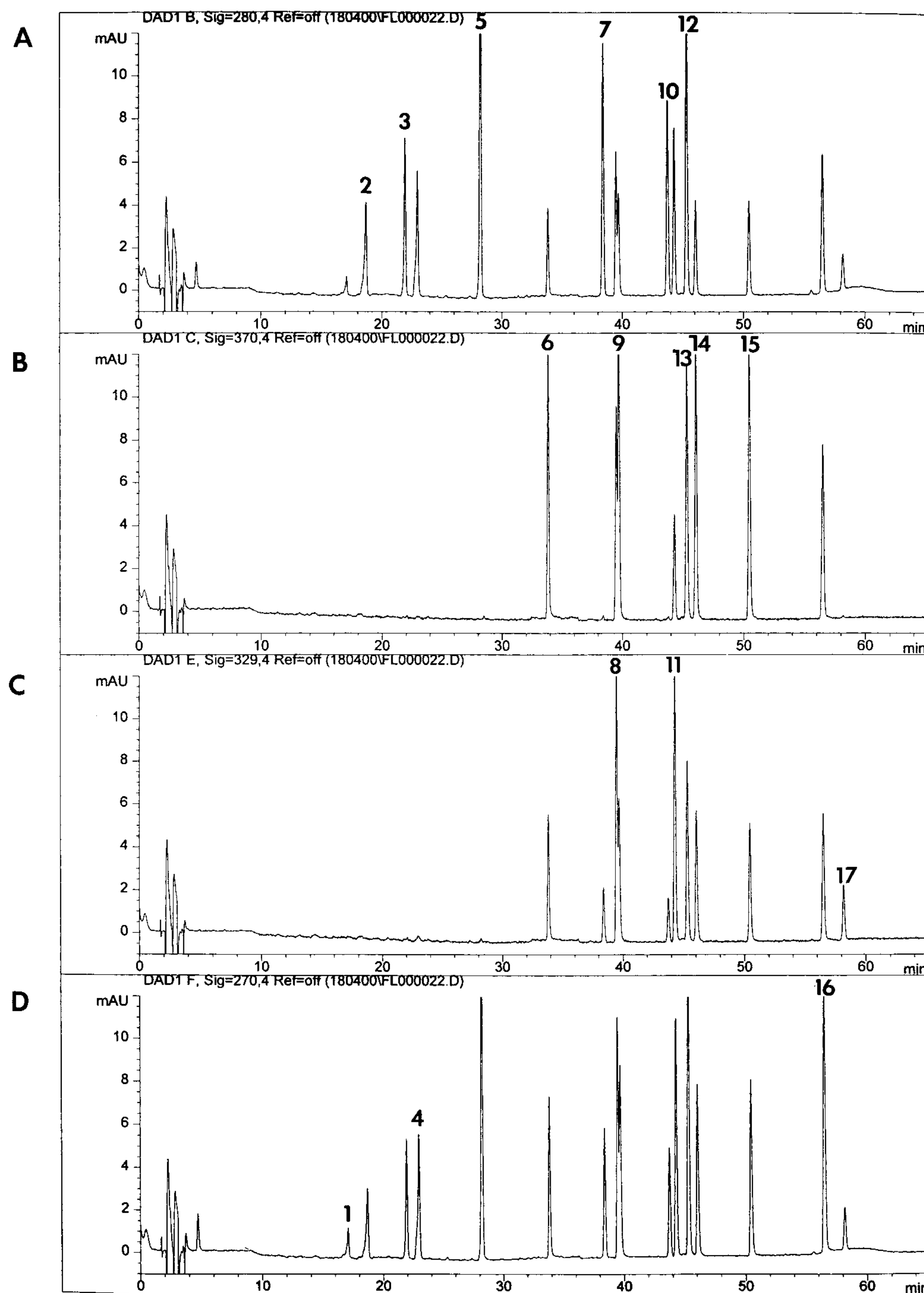


Figure 2. HPLC−DAD chromatograms of the flavonoid standard mixture recorded at 280.4 (A), 370.4 (B), 329.4 (C), and 270.4 (D) nm. 1, (−)-epigallocatechin; 2, (+)-catechin; 3, (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-epicatechin gallate; 6, myricetin; 7, eriodictyol; 8, luteolin; 9, quercetin; 10, naringenin; 11, apigenin; 12, hesperetin; 13, kaempferol; 14, isorhamnetin; 15, rhamnetin; 16, galangin; 17, tangeretin.

M HCl was added. The sample was bubbled with nitrogen for ca. 40−60 s after which the flask was sealed tightly. Hydrolysis was carried out in a shaking waterbath at 90 °C for 2 h. After hydrolysis the sample was allowed to cool, then it was filtered, made up to 100 mL with methanol, and ultrasonicated for 5 min. Before quantification by HPLC the sample was filtered through a 0.2-μm membrane filter.

Red wine and tea samples were prepared by adding 25 mL of 62.5% aqueous methanol (containing BHA as above) to a 15-mL sample, followed by ultrasonication for 5 min and addition of 10 mL of 6 M HCl. The rest of the sample handling was the same as that for the dried samples, except that the hydrolysis time was 4 h at 90 °C.

Catechins in tea and red wine samples were extracted by adding 3 × 3 mL of ethyl acetate into a 5-mL (black tea or red wine) or 0.5-mL (green tea) sample. The extraction was performed in a test tube fitted with a corkscrew cap by manually shaking the sample for 1 min. The ethyl acetate layer was separated from the water layer and evaporated to dryness with nitrogen stream. The residue obtained was solubilized into methanol and diluted to 20 mL with methanol, resulting in a sample ready for the HPLC analysis. Approximately 2 mL of the final sample extract was filtered through a 0.2-μm filter before the HPLC analysis.

Quantification of Flavonoids. The analytical HPLC system employed consisted of an HP 1090M Series II high-

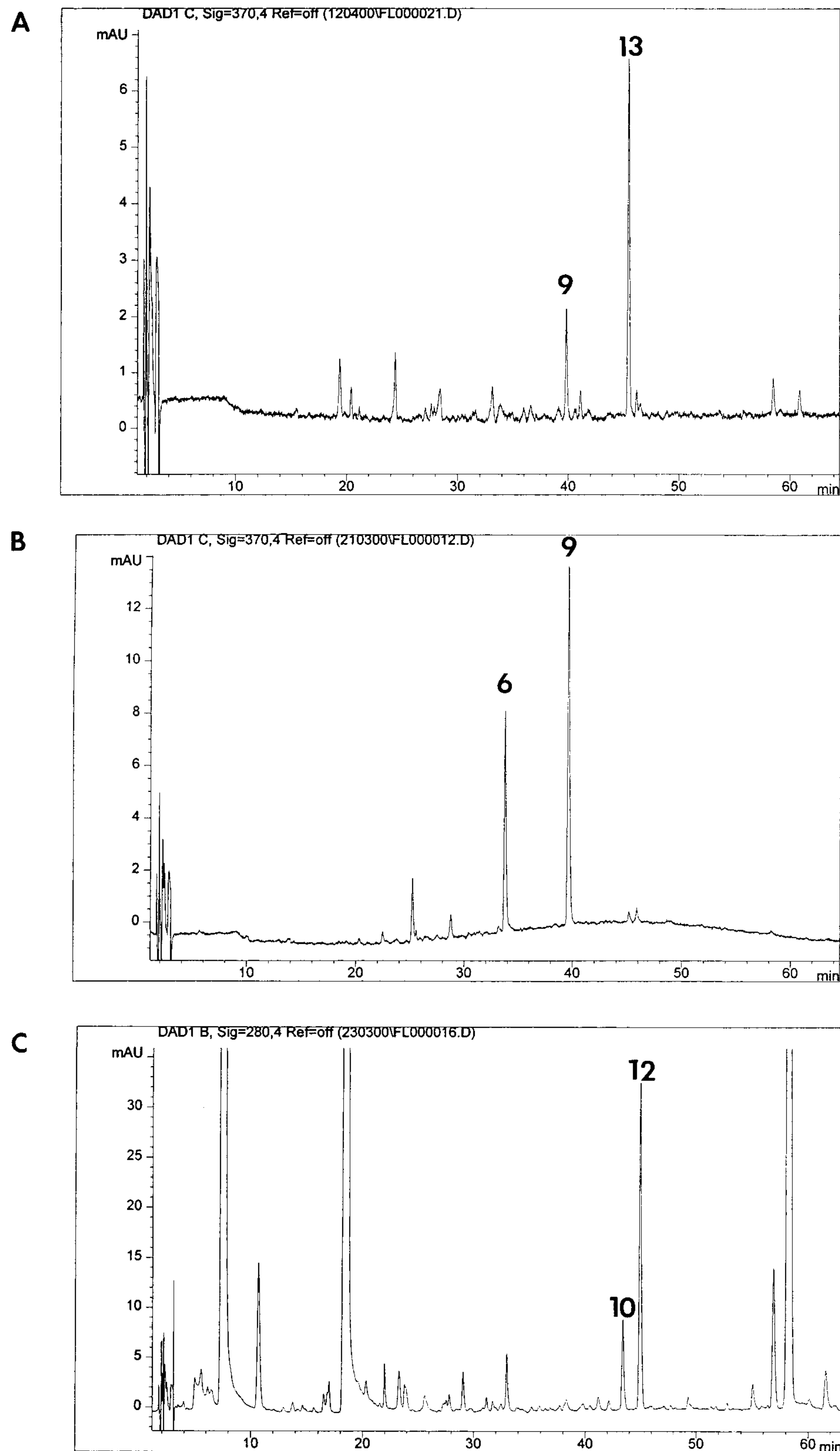


Figure 3. HPLC–DAD chromatograms of broccoli (A), cranberry (B), and orange (C). Key for flavonoid peaks is same as for Figure 2.

performance liquid chromatograph equipped with an HP1090 Series II diode array and an eight-channel electrochemical coulometric array detector (EC; Esa Inc., USA). The HPLC pumps, autosampler, column oven, and diode array system were monitored and controlled and the analytical data were evaluated using the HP 3D Chem Station computer program. Wavelengths used for identification of catechins, and identification and quantification of other flavonoids, with the diode-array detector were 270.4 nm for galangin, (−)-epigallocatechin, and (−)-epigallocatechin gallate; 280.4 nm for eriodictyol, naringenin, (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, and hesperetin; 329.4 nm for luteolin, apigenin, and tangeretin; and 370.4 nm for myricetin, kaempferol, rhamnetin, quercetin, and isorhamnetin.

The electro-array detector (EC) was controlled using ESA CoulArray for Windows Data Processing Module Version 1.00

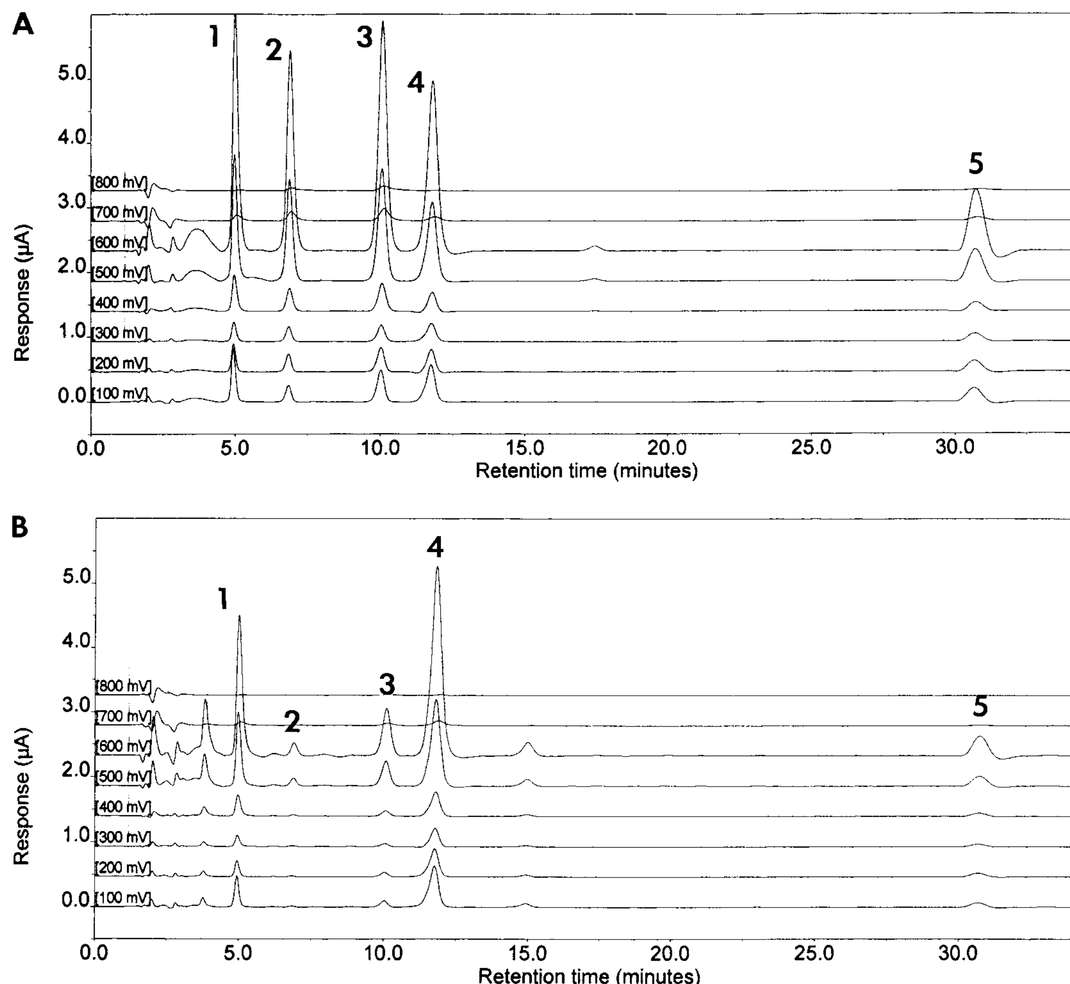


Figure 4. HPLC−EC chromatograms of a standard mixture of catechins (A) and green tea (B). Key for flavonoid peaks is same as for Figure 1.

computer program. The EC detection system consisted of two cell packs in series, both containing four electrochemical detector cells with porous graphite working electrodes and associated palladium reference electrodes. The EC was operated using 100−800 mV potentials (100 mV intervals). The detector array was housed in a temperature-regulated compartment at 35 °C.

Flavonoid separation was done by an Inertsil (GL Sciences, Inc., Japan) ODS-3 (4.0 × 150 mm, 3 μm) column with a C-18 guard column. Temperature of the column oven was set at 35 °C. Gradient elution was employed for flavonoids other than catechins 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. Catechins were quantified using isocratic elution: 86% solution A and 14% solution B.

The 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 flavonoids were quantified using the external standard method. Quantification was based on peak area (DAD) or peak height (EC). Calibration curves of the standards were made by diluting stock standards in methanol to yield 2−10 μg/mL (catechins) and 2−20 μg/mL (other flavonoids). The samples were prepared and analyzed in duplicate.

Method Validation. Linearity of the detector responses and the detection and determination limits were tested for the flavonoids. Orange, apple, cranberry, green tea, yellow onion, parsley, and the reference sample were selected for recovery tests. Day-to-day repeatabilities of the methods were tested by analyzing the reference sample 5 (catechins) or 10 (other flavonoids) times. Identity and purity of the flavonoid peaks was monitored (in addition to monitoring retention times and the symmetry of the peak) with a DAD and an EC system using the spectral and voltammetric data, respectively. By EC, the purity match of the sample peaks was obtained by comparing channel height ratios (dominant vs post- and predominant channel heights) with flavonoid standard peaks. In the DAD system, purity analysis was made by comparing the spectral data of the sample peaks with those obtained for the flavonoid standards.

RESULTS AND DISCUSSION

Optimization of acidic conditions for the hydrolysis of flavonoid glycosides in a range of fruits, vegetables, and beverages has been described by Hertog et al. (1992b). Several acid concentrations and hydrolysis times were also tested in the present study (results not shown), and the resulting data were in agreement with the results of Hertog et al. (1992b). Moreover, no glycosides were found in the sample extracts after sample preparation. Bronner and Beecher (1998) and Dalluge et al. (1998) used minimal sample preparation to analyze catechins from infused tea samples. After infusion, they only added internal standard and filtered the samples prior to HPLC analysis. In the present study, extraction of catechins from infused tea using ethyl acetate enabled better separation of the compounds from the matrix, thus improving quantification.

Table 1. Flavonoid Contents in Foods

food	compound	contents (mg/100 g fw[a]) this study	recovery (%)	contents (mg/100 g fw or 100 mL) other studies[c]
reference sample	eriodictyol	33.8	82	
	hesperetin	80.4	93	
	myricetin	24	107	
	quercetin	45.4	93	
	epigallocatechin	77.4	105	
	(+)-catechin	27.1	90	
	(−)-epicatechin	18.1	120	
	epigallocatechin gallate	56.6	70	
	(−)-epicatechin gallate	19.3	124	
lingonberry	quercetin	10		7.4, 14.6[5], 16.9[12]
cranberry	myricetin	6.9	108	23[1], 7.4, 14.2[5], 7.7[7], 0.4−2.7[9]
	quercetin	10.4	107	16[1], 8.3, 12.1[5], 17.2[7], 7.3−25[9]
red onion	quercetin	30.7		45 ± 21[1], 20.1[4]
yellow onion	quercetin	19.2	105	34 ± 7[1], 18.5−63.4 [4], 28.4−48.6[6]
broccoli	quercetin	1.5	103	3.7 ± 0.1[1], 3.0[6], 2.1[11]
	kaempferol	6.0	85	6.0 ± 3.4[1], 7.2[6], 4.12[11]
green tea	epigallocatechin	3.9	95	2[2], 1.5[14], 32.2, 32.5[15]
	(+)-catechin	0.8	85	-[2],0.6 [14], 0.7, 0.8[15]
	(−)-epicatechin	2.7	92	2[2], 3.8[14], 6.5, 7.9[15]
	epigallocatechin gallate	28.6	115	5[2], 21.7[14], 45.9, 57.4[15]
	(−)-epicatechin gallate	6.9	94	3[2], 3.2[14], 8.4, 8.8[15]
	quercetin	2.3	82	1.4−2.3[3]
	kaempferol	1.3	70	0.91−1.5[3]
	myricetin	0.8	75	0.52−1.2[3]
black tea	epigallocatechin	0.5		6[2], 7.3−31.1[15]
	(+)-catechin	0.4		-[2], 0.6−4.8[15]
	(−)-epicatechin	0.7		4[2], 1.0−5.2[15]
	epigallocatechin gallate	2.9		12[2], 9.3−28.7[15]
	(−)-epicatechin gallate	2.0		11[2], 2.9−11.6[15]
	quercetin	1.7		1.4 ± 0.5[1], 1.0−2.5[3]
	kaempferol	1.4		1.6 ± 0.5[1], 0.63−1.7[3]
	myricetin	0.3		0.4 ± 0.2[1], 0.17−0.52[3]
red wine	quercetin	0.7	104	0.8 ± 0.5[1], 0.41−1.6[3], 0.4[13]
	myricetin	0.6	101	1.0 ± 0.6[1], 0.69−0.93[3], 0.3[13]
	(+)-catechin	6.3	71	2.2−20.8[8], 3.8[13]
	(−)-epicatechin	3.2	107	1.5−8.8[8], 6.0[13]
apple	quercetin	4.8	79	2.0 ± 0.4[1], 2.1−7.2[6]
	kaempferol	0.5		<0.2[6]
lemon	naringenin	0.6		0.5[1]
	hesperetin	17.2		17[1]
	luteolin	1.5		- [1]
	eriodictyol	17.6		- [1]
	quercetin	1.1		- [1]
orange	naringenin	11.9	85	11 ± 2[1]
	hesperetin	41.4	93	31 ± 2[1]
parsley[b]	luteolin	21.7	106	1.1 ± 1.1[1], -[10]
	apigenin	1484,2	82	185 ± 5[1], 105−366[10]
	isorhamnetin	36,4	110	-[1], − [10]
	kaempferol	-		1.1[1], 0−2.1[10]

[a] fw, fresh weight. [b] Results obtained in this study are given as μg/100 g dry weight. [c] References: (1), Justesen et al. (1998); (2), Bronner & Beecher (1998); (3), Hertog et al. (1993); (4), Crozier et al. (1997); (5), Häkkinen et al. (1999a); (6), Hertog et al. (1992a); (7), Hertog (1994); (8), Goldberg et al. (1998); (9), Bilyk & Sapers (1986); (10), Knuthsen and Justesen (1999); (11), Lugasi et al. (1999); (12), Häkkinen et al. (1999b); (13), Pellegrini et al. (2000); (14), Dalluge et al. (1998); (15), Kuhr & Engelhardt (1991).

Our preliminary studies indicated that a solvent comprising a mixture of acetonitrile and buffer provided a more efficient HPLC separation of flavonoids than a methanol-buffer system. The acetonitrile-based mobile phase also resulted in a lower pressure in the pump and column as compared with the methanol-buffer system. According to Crozier et al. (1997) wide variations exist in the effectiveness of different reversed-phase HPLC columns and mobile phases when used to analyze free and conjugated flavonoids. They tested several columns and found C_{18} Nova-Pak (150 × 3.9 mm, 4 μm; Waters) to be the most efficient for flavonoid analysis. We tested C_{18} Nova-Pak and Inertsil ODS-3 and obtained better efficiency by using the latter. Using the HPLC conditions described above, flavonoids eluted as sharp symmetrical peaks and generally separated from each other and from the different matrixes well, although the number of individual flavonoids in the standard mixture was as high as 17 (Figures 2−4). Luteolin and quercetin had slightly different retention times, but baseline separation was not achieved. Moreover, kaempferol and hesperetin eluted at exactly the same retention time. However, the incomplete separation was not problematic because these coeluting flavonoids rarely coexist in food samples. In addition, the spectral and voltammetric properties of the flavonoids in question differ, thus enabling their identification.

Monitoring the spectral and voltammetric data produced by the in-line connected DAD and EC, respectively, confirmed the identity and purity of the flavonoid peaks. When purity of the sample peaks was monitored by comparing their spectra or channel-height ratios with those obtained for flavonoid standards, a good match (90−100%) was found in both cases. Quantification of catechins was performed using EC because the responses of these flavonoids were, depending on the compound, 7.5−100 times higher by EC than DAD. In

the case of other flavonoids DAD was sufficiently sensitive for quantification.

Reliability of the methods was further shown by recovery and repeatability tests. Recoveries of added flavonoids were generally good (Table 1), indicating the accuracy of the methods. Repeatability tests were conducted by analyzing an in-house reference sample over 5 (catechins) or 10 (other flavonoids) days and monitoring the contents of (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, (−)-epicatechin gallate (EC) and eriodictyol, hesperetin, myricetin, and quercetin (DAD). The methods proved to be highly repeatable; coefficients of variation (CV%) varied from 2.9 ((−)-epicatechin) to 10.4 ((−)-epicatechin gallate).

Detection limits for catechins and other flavonoids, defined as the signal three times the height of the noise level, were estimated at 0.004−0.04 and 0.08−0.25 μg/mL, respectively. Responses of the detectors were linear within the tested ranges. Coefficients of correlation were >0.999 for all flavonoids.

Suitabilities of the methods were tested by analyzing several food samples. Results from the quantification of flavonoids (average values of duplicate analyses) are presented in Table 1, as well as the data derived from earlier studies. Contents of different flavonoids may vary considerably in plant-derived foods, according to pigmentation, cultivar, regional and other cultivation differences, part of the plant, ripeness, vinification technique, and length of storage time (Bilyk and Sapers, 1986; Crozier et al., 1997; Goldberg et al., 1998; Gomez-Plaza et al., 2000; Herrmann, 1976; Häkkinen and Törrönen, 2000; Justesen et al., 1999). Considering that the variation in flavonoid contents could be high, the data of the present study were in agreement with earlier reported values (Table 1). As expected, quercetin was the major flavonoid present in nearly all analyzed samples. Naringenin and hesperetin were typical flavonoids of citrus fruits. On the other hand, apigenin was found only in parsley.

The present methods were effective for the determination of catechins and other flavonoids in various plant-derived foods. The diode-array and electro-array systems chosen for peak identity and purity analysis, as well as the recovery tests, showed the quantification of flavonoids to be reliable and accurate. In addition, repeatability of the method was good.

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