High-Performance Liquid Chromatography Coupled with Coulometric Array Detection of Electroactive Components in Fruits and Vegetables: Relationship to Oxygen Radical Absorbance Capacity

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An HPLC procedure utilizing reversed-phase chromatography coupled with a coulometric array detection system was developed for the characterization of overall antioxidant status in fruits and vegetables. The method was reliable and sensitive (20 pg to 1 ng detection limit) and can also be used to identify and simultaneously quantify multi-antioxidants including vitamin C, glutathione, phenolic acids, and flavonoids in fruits and vegetables. Each fruit and vegetable aqueous extract showed an unique distribution of chromatographic peaks that could serve as a "fingerprint" for the fruit or vegetable. A significant positive linear correlation was demonstrated in fruit and vegetable aqueous extracts between the total antioxidant activities determined by using oxygen radical absorbance capacity ($ORAC_{ROO\bullet}$) assay and the electrochemical data generated from the coulometric array detectors. From these data, the $ORAC_{ROO\bullet}$ assay appears to be a valid and useful procedure for measuring total antioxidant activity in extracts of fruits and vegetables.

Keywords: *Fruit; vegetable; antioxidant; phenolic acid; flavonoid; anthocyanin*

INTRODUCTION

Accumulated evidence indicates that fruits and vegetables can provide protection against various diseases, including cancer and cardio- and cerebrovascular diseases (Acheson and Williams, 1983; Ames et al., 1993; Armstrong et al., 1975; Verlangieri et al., 1985; Willet, 1994a). This protection is generally attributed to classic antioxidants including vitamins C and E contained in these fruits and vegetables (Ames, 1983; Gey, 1990; Gey et al., 1991; Stähelin et al., 1991a,b; Steinberg et al., 1989; Steinberg, 1991; Willett, 1994b). However, there is a large variety of fruits and vegetables in nature; the overall antioxidant status of fruits and vegetables and the protection provided by fruits and vegetables against diseases may vary considerably from one kind of fruit or vegetable to another. Our laboratory has reported previously that the total antioxidant activity (based upon the fresh weight), determined using the automated oxygen radical absorbance capacity ($ORAC_{ROO\bullet}$) assay with a peroxyl radical ($ROO^{\bullet}$) generator (Cao et al., 1995), of strawberry was seven times the activity measured in apple and banana and sixteen times the activity measured in honeydew melon (Wang et al., 1996). The total antioxidant activity against peroxyl radicals measured in kale was similar to that measured in strawberry but eight to nine times the activity measured in carrots and string beans and 29−35 times the activity measured in celery and cucumber (Cao et al., 1996).

From our previous studies we concluded that the vitamin C content in fruits and vegetables was not directly related to their total antioxidant activities (Cao et al., 1996; Wang et al., 1996). Therefore, other antioxidants or components of fruits and vegetables may play an important role in the prevention of diseases. Among them, the flavonoids have been the subjects of intensive studies because of their antioxidant, anticancer, antihemorrhagic, antiallergic, and anti-inflammatory properties (Cao et al., 1997; Das, 1994). Recently, Hertog et al. (1993) found a significant inverse relationship between flavonoid intake and mortality from coronary heart disease and a borderline significance with the incidence of a first fatal or nonfatal myocardial infarction. Therefore, it is becoming increasingly important to characterize fruits and vegetables according to their total antioxidant activity and to identify those compounds that are responsible for the strong antioxidant activity found in fruits and vegetables.

With the recent advances in electrochemical detection, the multielectrode array detector is becoming a powerful tool for detecting phenolic acids and flavonoids in a wide range of samples (Achilli et al., 1993, 1995; Chiavari et al., 1988; Gamache et al., 1993; Joerg and Sontag, 1993; Lee et al., 1995; Wakui et al., 1992), although the diversity of compounds found in fruits and vegetables is still a major obstacle to the identification and quantitation of all antioxidant constituents in fruits and vegetables. However, the multichannel coulometric detection system may serve as a highly sensitive tool for the overall characterization of antioxidants in fruits and vegetables after separation by HPLC, since all antioxidants are electroactive and the coulometric efficiency of each element of the array allows a complete voltammetric resolution of analytes as a function of their reaction potential. Some peaks may be resolved by the

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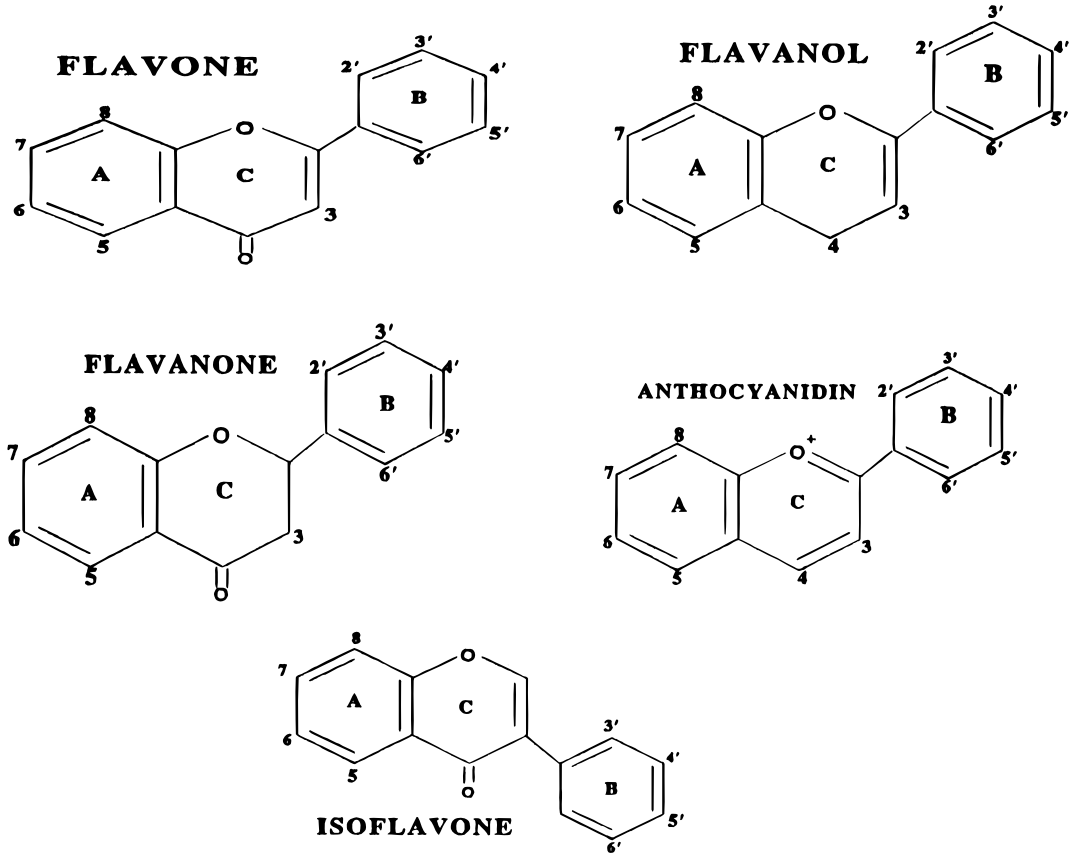

Figure 1. Chemical structures of basic compounds used in standard mixture. See Table 1 for structure configurations for hydroxybenzoic, hydroxyphenylacetic, and hydroxycinnamic acids. See Table 2 for the structure configurations for flavones, flavanones, flavanols, isoflavones, and anthocyanidins.

detector even if they are unresolved when they leave the chromatographic column.

The purpose of this study was to (1) develop a HPLC procedure utilizing reversed-phase chromatography coupled with a coulometric array detection system for characterization of fruits and vegetables and identification of their antioxidant constituents and (2) correlate the electrochemical data generated from the fruits and vegetables with their antioxidant activities determined by using the $ORAC_{ROO\cdot}$ assay.

MATERIALS AND METHODS

Chemicals. *R*-Phycoerythrin (*R*-PE), ascorbic acid, glutathione (GSH), 3,4-hydroxybenzylamine, tyrosine, gallic acid, protocatechuic acid, 4-hydroxyphenylacetic acid, vanillic acid, catechin, epicatechin, caffeic acid, *p*-coumaric acid, gallocatechin gallate, 4-hydroxycoumarin, and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO). Fustin, callistephin, taxifolin, pelargonidin, rutin, naringin, hesperidin, eriodictyol, quercetin, genistein, and kaempferol were purchased from Indofine Chemical Co., Inc. (Somerville, NJ). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), gentisic acid, and ferulic acid were obtained from Aldrich (Milwaukee, WI). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA Inc. (Richmond, VA). Methanol (HPLC grade) was from Fisher Scientific (Boston, MA). HPLC grade water was obtained from J. T. Baker Inc. (Phillipsburg, NJ).

Apparatus. A Dionex HPLC gradient pump (Dionex Corp., Marlton, NJ) was coupled with an ESA coulometric detection system (ESA Inc., Chelmsford, MA), which consisted of three cell packs in series, each pack containing four porous graphite working electrodes with associated palladium reference electrodes and platinum counter electrodes. Chromatographic separation was performed on an ODS (octadecylsiloxane) Hypersil column (C_{18}; 150 mm × 4.6 mm i.d.; particle size, 5 μm; pore size, 120 Å) from Keystone Scientific Inc. (Bellefonte, PA). The column and detector array were housed in a thermostatic chamber maintained at 35 °C.

Chromatographic Methods. The mobile phases used in the gradient runs were prepared with HPLC grade water. A stock solution of 0.2 M NaH_2PO_4, which was adjusted to pH 3.0 with phosphoric acid (85%) and then filtered through a 0.2 μM membrane filter, was used for preparing the mobile phases. Mobile phase A was 50 mM sodium phosphate buffer diluted from the stock solution (0.2 M), while mobile phase B was composed of 50 mM sodium phosphate, methanol, and acetonitrile (30/20/50, v/v/v). Both mobile phases were degassed for 10 min prior to use. The following elution profile was used (% solvent B): 0% (initial, 0−6 min), 2% (6−8 min), 5% (8−10 min), 6% (10−12 min), 7% (12−14 min), 8% (14−32 min), 10% (32−38 min), 18% (38−55 min), 22% (55−65 min), 38% (65−80 min), 100% (80−91 min). The solvent flow rate was 0.4 mL/min initially (0−8 min); 0.6 mL/min (8−10 min), 0.7 mL/min (10−12 min), 0.8 mL/min (12−14 min), 0.9 mL/min (14−87 min). Then, the flow rate was decreased gradually to 0.4 mL/min again within 5 min. The twelve electrode detector potentials were from 0 to 770 mV in increments of 70 mV. The column was allowed to equilibrate for 10 min before each sample was injected.

Standard and Sample Preparation. The stock standard solutions were prepared by dissolving approximately 1.0 mg of the standard flavonoid components in the appropriate volume of 50% aqueous methanol to produce a final concentration of 1 mg/mL. A 0.01 N NaOH solution was used to dissolve hesperidin. The stock standard solutions were stored at −75 °C. A 31-component working standard solution was prepared by combining and diluting each of the stock standard solutions to the desired concentrations in the range of 1−2000 ng/mL. The structures of the standards are shown in Figure 1 and Tables 1 and 2.

The fruits and vegetables were purchased from a local supermarket. The edible portion was weighed and homogenized in deionized water (1:2, w/v) using a commercial blender. The homogenate was centrifuged at 16000*g* for 20

Table 1. Chemical Structures and $ORAC_{ROO\cdot}$ and Dominant Peak Potential (DP) of Monophenolic Compounds Included in Standard Mixture for HPLC

compound	peak no.[a]	position on benzene ring 1	2	3	4	5	$ORAC_{ROO\cdot}$[b]	DP[c]
hydroxybenzoic acids								
4-hydroxybenzoic acid	8 (26)	COOH			-OH		0.17 ± 0.01	700
protocatechuic acid	6 (19)	COOH		-OH	-OH		2.06 ± 0.21	210
gallic acid	5 (14)	COOH		-OH	-OH	-OH	1.74 ± 0.08	140
2,5-dihydroxybenzoic acid	7 (24)	COOH	OH			-OH	1.20 ± 0.07	0−70
vanillic acid	10 (36)	COOH		OCH_3	-OH		1.11 ± 0.04	420
syringic acid	13 (43)	COOH		OCH_3	-OH	OCH_3	1.27 ± 0.02	350
hydroxyphenylacetic acids								
4-OH-phenylacetic acid	9 (31)	CH_2—COOH			-OH		0.41 ± 0.04	560
hydroxycinnamic acids								
caffeic acid	12 (40)	CH=CHCOOH		-OH	-OH		2.23 ± 0.25	140
p-coumaric acid	15 (50)	CH=CHCOOH			-OH		1.09 ± 0.06	490
ferulic acid	18 (56)	CH=CHCOOH		OCH_3	-OH		1.33 ± 0.06	350
sinapinic acid	20 (60)	CH=CHCOOH		OCH_3	-OH	OCH_3	1.66 ± 0.07	210

[a] Peak no., peak number corresponding to Table 3 and Figure 2. Retention time (min) in parentheses. [b] Data are expressed as mean ± SE (n = 4−6). Units are presented as μmol of Trolox equiv per μmol of compound. [c] DP, dominant potential (mV).

Table 2. Chemical Structures and $ORAC_{ROO\cdot}$ and Dominant Peak Potential (DP) of Flavonoid Compounds Included in Standard Mixture for HPLC

compound	peak no.[a]	-OH substitution	substitutions[b]	$ORAC_{ROO\cdot}$[c]	DP[d] (mV)
flavanol					
catechin	11 (37)	3,5,7,3′,4′		2.49 ± 0.07	490
epicatechin	14 (48)	3,5,7,3′,4′		2.36 ± 0.13	490
gallocatechin gallate	17 (54)	-,5,7,3′,4′	3-gallate	2.43 ± 0.13	490
flavanone					
fustin	16 (53)	3,-,7,3′,4′		3.91 ± 0.10	770
taxifolin	21 (60)	3,5,7,3′,4′		3.59 ± 0.07	700
naringin	25 (70)	-,5,-,-,-,4′	7-rha	0.37 ± 0.02	700
hesperidin	26 (71)	3,5,-, 3′,-	4′-OMe; 7-rut	0.04 ± 0.01	440
eriodictyol	28 (73)	-, 5,7,3′4′		3.41 ± 0.28	770
flavone					
rutin	23 (67)	-,5,7,3′,4′	3-rut	0.56 ± 0.07	700
quercetin	29 (75)	3,5,7,3′,4′		3.29 ± 0.12	770
kaempferol	31 (77)	3,5,7,-, 4′		2.67 ± 0.13	700−770
isoflavone					
genistein	30 (76)	-,5,7,-,4′		2.38 ± 0.18	700
anthocyanidin					
pelargonidin chloride	22 (63)	3,5,7,-,4′		0.96 ± 0.02	420
	27 (71)				700
callistephin	19 (58)	-,5,7,-,4′	3-glu	1.09 ± 0.06	700

[a] Peak no., peak number corresponding to Table 3 and Figure 2. Retention time (min) in parentheses. [b] OMe, O-methylation; Rut, rutinoside; Rha, rhamnoglucoside; Glu, glucoside. [c] $ORAC_{ROO\cdot}$ activities (mean ± SE, n = 4−6) of flavones, flavanones, and isoflavones were cited from Cao et al. (1997). [d] DP, dominant potential (mV).

min, and the collected supernatant was stored at −75 °C until analyzed. Before being loaded onto the column, the supernatant was diluted 20 times with 50 mM sodium phosphate buffer, 3,4-dihydroxybenzylamine was added to the diluted samples as an internal standard at a final concentration of 0.5 ng/μL, and 20 μL was injected.

Automated $ORAC_{ROO\cdot}$ Assay. The automated $ORAC_{ROO\cdot}$ assay of the standards and aqueous extracts of fruits and vegetables (Cao et al., 1996; Wang et al., 1995) was based on a previous report of Cao and co-workers (1993), as modified for the COBAS FARA II spectrofluorometric centrifugal analyzer (Cao et al., 1995). Briefly, in the final assay mixture (0.4 mL total volume), *R*-PE (16.7 nM) was used as a target of free radical attack with 4 mM of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical ($ROO^{\bullet}$) generator. Trolox was used as a control standard. The analyzer was programmed to record the fluorescence of *R*-PE every 2 min after AAPH was added. Final results were calculated using the differences of areas under the *R*-PE decay curves between the blank and a sample and were expressed in Trolox equiv.

Data Analysis. The ESA CoulArray operating software (ESA Inc., Chelmsford, MA) was used to collect, store, and analyze voltammetric data. Chromatographic peaks were identified by retention times as well as by the hydrodynamic voltammograms (HDV). In most cases analytes responded, as determined by either peak height or peak area, over three adjacent sensors, termed the lower (preceding), dominant, and upper (following) sensors. The software compares the voltammetric response of an unknown compound to that of an external standard and assigns two "ratio accuracies." The response of an analyte across these three channels is a characteristic of that analyte and is independent of the analyte's concentration. The analyte's response ratio on the lower to dominant channels is compared to the unknown's response ratio for the same channels to produce the first ratio accuracy. The second ratio accuracy is obtained by comparing the response ratio for the upper and dominant channels. When expressed as a percentage, this gives a numerical indicator as to the authenticity and purity of the unknown peak in the sample. This ratio as described below should not be more than 100%.

ratio accuracy (%) =

[ratio of the adjacent (lower or upper) channel to the dominant channel in extract or standard]/ [ratio of the adjacent (lower or upper) channel to the dominant one in the standard or extract] × 100

The identities of some of the peaks were further assessed by spiking samples of interest with the relevant standards and comparing the height or area ratios of the adjacent peaks to the dominant one between a standard and the actual sample. The quantification of identified compounds in the samples was

Table 3. Regression Coefficients of Peak Area (μC) and Concentration (ng/mL) for Known Compounds Utilized in HPLC Analyses

peak no.	name	t_R[a]	DP[b]	slope ± SE[c] ($\times 10^{-3}$)	intercept ± SE[b]	R_{xy}[d]
1	ascorbic acid	6.4	140	4.88 ± 0.033	−0.0014 ± 0.024	1.000
2	glutathione	8.0	700	9.33 ± 0.240	−0.2209 ± 0.175	0.995
3	3,4-dihydroxybenzylamine	9.2	210	5.30 ± 0.151	−0.2069 ± 0.110	0.994
4	tyrosine	11.5	560	10.3 ± 0.499	−0.0172 ± 0.364	0.981
5	gallic acid	13.8	140	7.08 ± 0.269	0.0944 ± 0.196	0.989
6	protocatechuic acid	18.7	210	12.6 ± 0.262	−0.2144 ± 0.191	0.997
7	gentisic acid	24.3	70	9.04 ± 0.092	0.0070 ± 0.067	0.999
8	4-hydroxybenzoic acid	26.1	700	29.9 ± 0.287	0.3258 ± 0.210	0.999
9	4-hydroxyphenylacetic acid	31.1	560	29.6 ± 0.713	−0.6782 ± 0.521	0.995
10	vanillic acid	35.6	420	14.2 ± 0.228	−0.1304 ± 0.166	0.998
11	catechin	37.2	490	10.3 ± 0.098	−0.0580 ± 0.072	0.999
12	caffeic acid	39.5	140	7.92 ± 0.057	−0.0201 ± 0.041	1.000
13	syringic acid	42.7	350	9.92 ± 0.112	−0.1173 ± 0.082	0.999
14	epicatechin	47.5	490	10.3 ± 0.359	−0.2620 ± 0.262	0.990
15	*p*-coumaric acid	49.5	490	22.8 ± 0.347	−0.0151 ± 0.253	0.998
16	fustin	52.8	770	11.8 ± 0.355	−0.0104 ± 0.259	0.993
17	gallocatechin gallate	54.2	490	3.63 ± 0.125	−0.0399 ± 0.048	0.992
18	ferulic acid	56.4	350	10.7 ± 0.116	0.0325 ± 0.084	0.999
19	callistephin	57.7	700	1.92 ± 0.0367	−0.0423 ± 0.027	0.997
20	sinapinic acid	60.0	210	11.8 ± 0.105	−0.0479 ± 0.077	0.999
21	taxifolin	60.2	700	17.8 ± 0.318	−0.1075 ± 0.232	0.997
22	pelargonidin #1	62.7	420	3.06 ± 0.142	−0.0742 ± 0.103	0.983
23	rutin	66.5	700	7.80 ± 0.147	−0.0461 ± 0.107	0.997
24	4-hydroxycoumarin	67.8	770	2.38 ± 0.174	−0.1226 ± 0.067	1.000
25	naringin	69.6	700	3.53 ± 0.039	0.0106 ± 0.028	0.999
26	hesperidin	70.5	490	2.19 ± 0.015	0.0097 ± 0.011	1.000
27	pelargonidin #2	71.3	700	1.69 ± 0.018	0.0058 ± 0.013	0.999
28	eriodictyol	73.4	770	7.38 ± 0.207	−0.1235 ± 0.151	0.994
29	quercetin	74.7	770	2.92 ± 0.249	−0.2465 ± 0.128	0.979
30	genistein	76.3	700	6.01 ± .0121	0.3459 ± 0.089	0.997
31	kaempferol	76.9	700−770	12.6 ± 0.327	−0.3775 ± 0.239	0.995

[a] t_R, retention time (min). [b] DP, dominant potential. [c] Regression equations obtained from responses to injections of (20 μL) of 1, 2, 10, 50, 100, 250, 500, 1000, and 2000 ng/mL standard mixtures except quercetin, 4-hydroxycoumarin, and gallocatechin gallate (see Results). SE, standard error. [d] Correlation coefficients.

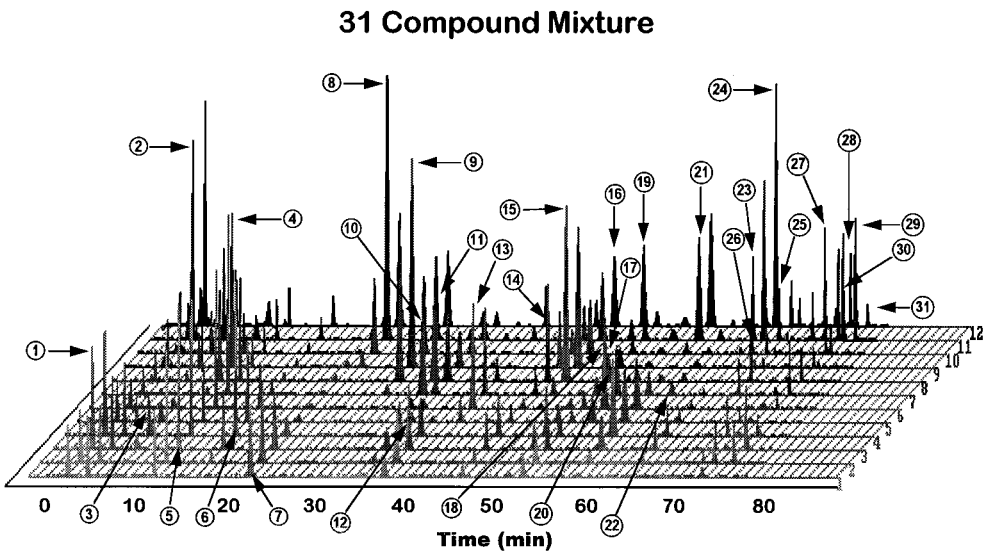

Figure 2. Twelve-channel chromatogram of 31-component standard (20 μL of a solution containing 500 ng/mL of each component). Sensor potentials were 0 mV (channel 1) to 770 mV (channel 12) with 70 mV increments. All channels are displayed at 1.5 μA full scale. See Methods for further details on analytical conditions and Table 3 for compound listing, retention times, and dominant oxidation potential.

performed by fitting the peak area of the dominant channel to the standard calibration curve. Linear correlations were calculated between ORAC activity (Y) and voltammetric data (X) using Slide Write (Advanced Graphics Software, Inc., Carlsbad, CA).

RESULTS

Standard Retention Time, Reproducibility, Sensitivity, and Voltammetric Behavior. The retention time of 31 standard compounds including flavonoids and hydroxybenzoic and cinnamic acids are present in Table 3. Figure 2 shows a chromatogram of these standard compounds resulting from the injection of 20 μL at the concentration of 500 ng/mL. Except for pelargonidin, all standards had only one peak. The pelargonidin produced two peaks with different dominant oxidation potentials. They were identified as pelargonidins #1 and #2. No attempt was made to identify the structures corresponding to these two peaks. However, peak 27, labeled as pelargonidin #2, may be the true compound because its dominant potential is 700 mV, which is the same as callistephin, an anthocyanidin similar to pelargonidin, except for a 3-glucosidic substitution.

Table 4. Voltammetric Response of Components in the Extracts of Fruits and Vegetables

item	no. of peaks[a]	peak area (μC/μL)[b]		peak height (μA/μL)[b]		$ORAC_{ROO\cdot}$[c] (nmol of Trolox equiv/μL)
		total	dominant	total	dominant	
kale	117 ± 4	317.5 ± 7.5	141.0 ± 6.4	19.5 ± 0.6	8.4 ± 0.5	2.70
strawberry	75 ± 4	379.6 ± 9.8	131.0 ± 0.2	27.0 ± 0.4	9.0 ± 0.2	2.68
brussel sprouts	74 ± 2	225.3 ± 0.9	109.9 ± 2.2	12.5 ± 0.3	5.7 ± 0.1	1.73
spinach	67 ± 3	839.4 ± 10.7	367.5 ± 4.2	49.8 ± 1.2	21.8 ± 0.04	1.94
orange	65 ± 3	429.2 ± 23.4	162.6 ± 5.9	28.7 ± 0.1	10.9 ± 0.2	1.97
onion	46 ± 3	271.2 ± 14.2	144.6 ± 5.3	15.4 ± 1.5	7.8 ± 0.7	1.27
garlic	45 ± 3	1108.8 ± 14.7	562.6 ± 7.9	46.9 ± 1.8	24.1 ± 0.8	5.15
alfalfa sprouts	45 ± 4	260.9 ± 16.5	138.5 ± 7.0	16.9 ± 1.8	8.7 ± 0.7	2.15
cabbage	33 ± 4	73.7 ± 8.3	36.5 ± 2.7	3.8 ± 0.3	1.8 ± 0.2	0.49
red grape	31 ± 3	92.5 ± 5.7	45.5 ± 3.4	4.3 ± 0.3	2.1 ± 0.1	1.24
cauliflower	29 ± 4	104.8 ± 2.7	61.6 ± 6.4	5.1 ± 0.2	2.8 ± 0.2	0.79
red pepper	28 ± 2	562.5 ± 0.4	172.6 ± 2.5	38.5 ± 5.8	11.8 ± 1.6	2.39
kiwifruit	20 ± 8	201.1 ± 11.0	71.6 ± 5.1	13.8 ± 0.8	4.5 ± 0.4	1.08
apple	20 ± 3	17.6 ± 5.9	9.7 ± 2.8	0.8 ± 0.3	0.4 ± 0.2	0.49
green pepper	20 ± 1	400.4 ± 17.5	122.5 ± 6.4	24.2 ± 3.8	7.4 ± 1.1	1.79
potato	19 ± 1	89.8 ± 1.6	106.7 ± 9.1	12.5 ± 0.1	6.1 ± 0.4	1.08
banana	15 ± 2	40.9 ± 6.6	23.1 ± 1.3	2.2 ± 0.2	1.2 ± 0.03	0.46
tomato	15 ± 4	100.0 ± 10.3	41.9 ± 7.3	6.5 ± 0.4	2.6 ± 0.3	0.45
pear	12 ± 1	27.5 ± 3.0	11.0 ± 0.7	1.5 ± 0.1	0.5 ± 0.02	0.46
eggplant	8 ± 1	70.9 ± 0.7	35.0 ± 3.1	3.6 ± 0.1	1.6 ± 0.1	0.90
honeydew melon	7 ± 3	24.3 ± 4.1	21.1 ± 1.7	0.9 ± 0.2	0.9 ± 0.1	0.20
leaf lettuce	6 ± 2	15.7 ± 2.4	6.8 ± 0.9	0.9 ± 0.2	0.2 ± 0.09	0.40
cucumber	3 ± 4	26.9 ± 1.1	15.6 ± 1.4	1.3 ± 0.02	0.7 ± 0.07	0.17
carrot	3 ± 1	13.9 ± 3.1	7.8 ± 0.6	0.5 ± 0.2	0.2 ± 0.05	0.34

[a] Peak heights greater than 10.0 nanoamperes. [b] All data are from the diluted aqueous extracts of fruit and vegetable and expressed as mean ± SD (from triplicate determinations). The diluted extracts were prepared by homogenization (1:2, w/v) and centrifugation. μC, microcoulomb; μA, microampere. [c] Data are from the diluted aqueous extracts of fresh fruit and vegetable (nmol of Trolox equiv per μL).

The reproducibility of the method was tested by repeated injections of a standard solution containing the standards reported in Table 3. The within-day retention time variation (8 replicates injected on a single day) of each individual standard ranged from 0.09% to 1.38% (coefficient of variation, CV). The between-day retention time variation (a single injection was made on each of 10 days) for the standard solution had a CV that ranged from 0.11% to 2.10%. The within-day and between-day voltammetric response (peak height) variabilities were tested by repeated injections of the standard mixture (500 ng/mL). The CV of within-day responses ranged from 3.08% to 13.46% (8 replicates injected on a single day) while the CV of between-day responses for most standards in Table 3 was from 4.38% to 17.74% (a single injection was made on each of 10 days). However, the between-day voltammetric response variabilities for genistien, quercetin, and pelargonidin #2 were higher and ranged from 23.40% to 38.06% which may be at least partially because of their low solubility. The detection limit (signal-to-noise ratio > 3) for all compounds except quercetin was 20 pg. The detection limit for quercetin was 1 μg.

The regression coefficients for peak area (microcoulombs, μC) vs concentration (ng/mL) calculated for the 31 standards are presented in Table 3. All compounds except quercetin, 4-hydroxycoumarin, and gallocatechin gallate gave a linear response at concentrations from 1 to 2000 ng/mL. 4-Hydroxycoumarin and gallocatechin gallate both gave a linear response at concentrations from 1 to 1000 ng/mL. Quercetin produced a linear response at concentrations from 50 to 1000 ng/mL.

Different compounds behaved differently in terms of their chromatographic and voltammetric behaviors. In general, for compounds of the same basic structure, hydroxyl groups (-OH) increase mobility (shorter retention time) while methoxyl groups (-OCH_3) decrease mobility (Table 1). For the phenolic acids (Table 1), benzoic acid derivatives eluted earlier than the cinnamic acid derivatives; while for the flavonoids (Table 2), flavanols eluted first, followed by flavanones, anthocyanidins, flavones, and isoflavones. For the flavonoids (Table 2), sugar substitution also increases their mobility (i.e., rutin vs quercetin). In regard to voltammetric behavior, the phenolic acids usually responded dominantly at low potentials (0−560 mV) (Table 1), while the flavonoids responded at high potentials (700−770 mV), except for the flavanols, which had relatively low reaction potentials (490 mV). Ascorbic acid, tyrosine, and glutathione had dominant potentials at 140, 560, and 700 mV, respectively. In addition, a compound with more hydroxyl groups tended to respond on several channels.

$ORAC_{ROO\cdot}$ Activities of the Standards. The $ORAC_{ROO\cdot}$ activities of the phenolic acids and flavonoids used as standards in the characterization of fruits and vegetables by HPLC coupled with coulometric array detection are shown in Tables 1 and 2. It can be seen that the phenolic acids (Table 1), in general, had lower antioxidant activities against peroxyl radicals ($ORAC_{ROO\cdot}$) than flavonoids (Table 2) that contain multiple free hydroxyl groups. However, the glycosidic flavonoids (including rutin, naringin, hesperidin, and callistephin) (Table 2) usually had low $ORAC_{ROO\cdot}$ activities.

Voltammetric Characterization of Electroactive Components in Fruit and Vegetable Aqueous Extracts and Correlations with $ORAC_{ROO\cdot}$ Activity. The peak number, total peak area, and height summed both from dominant channels and across all channels of the 24 fruit and vegetable aqueous extracts that were analyzed are shown in Table 4. Among these fruits and vegetables, kale, strawberry, Brussels sprouts, spinach, and orange eluted more than 65 peaks (peak height > 10 nA) while eggplant, honeydew melon, leaf lettuce, cucumber, and carrot had fewer than 10 peaks. Garlic had the largest total peak area and height summed from dominant channels and the largest total peak area across all channels. However, spinach had the largest total peak height across all channels. The chromatograms of strawberry, kale, spinach, cucumber, and carrot are presented in Figures 3−7 as examples. It was shown that each fruit and vegetable had an

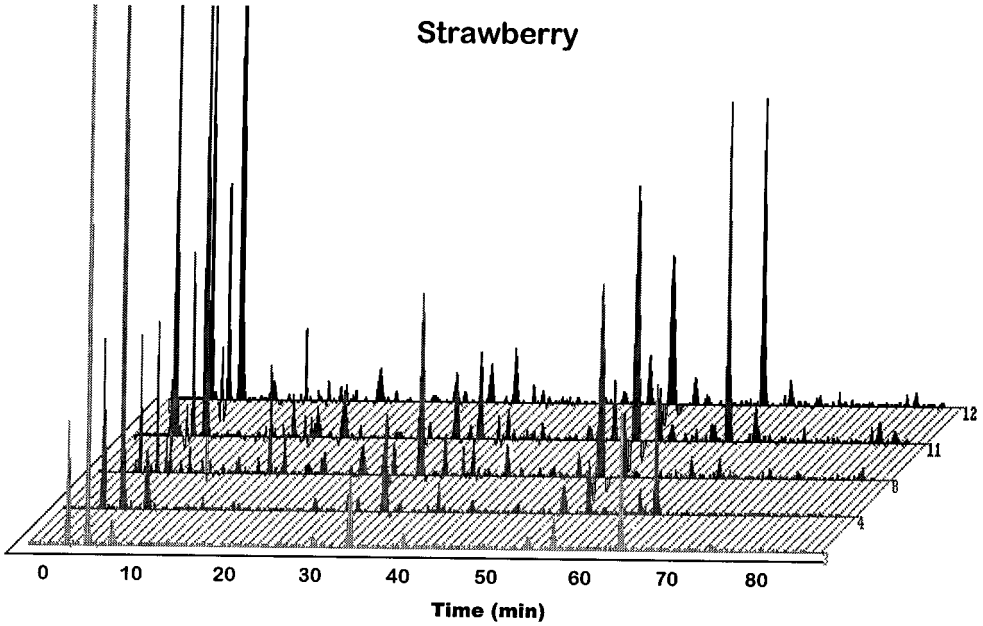

Figure 3. Five-channel chromatogram of strawberry extract (20 μL of the supernatant from a homogenate of 1 g plus 2 mL of water diluted 20-fold before injection). Sensor potentials were 0 mV (channel 1) to 770 mV (channel 12) with 70 mV increments. Only channels 3, 4, 8, 11, and 12 are displayed, corresponding to 140, 210, 490, 700, and 770 mV potentials, respectively. All channels are displayed at 500 nA full scale. See Methods for further details on analytical conditions.

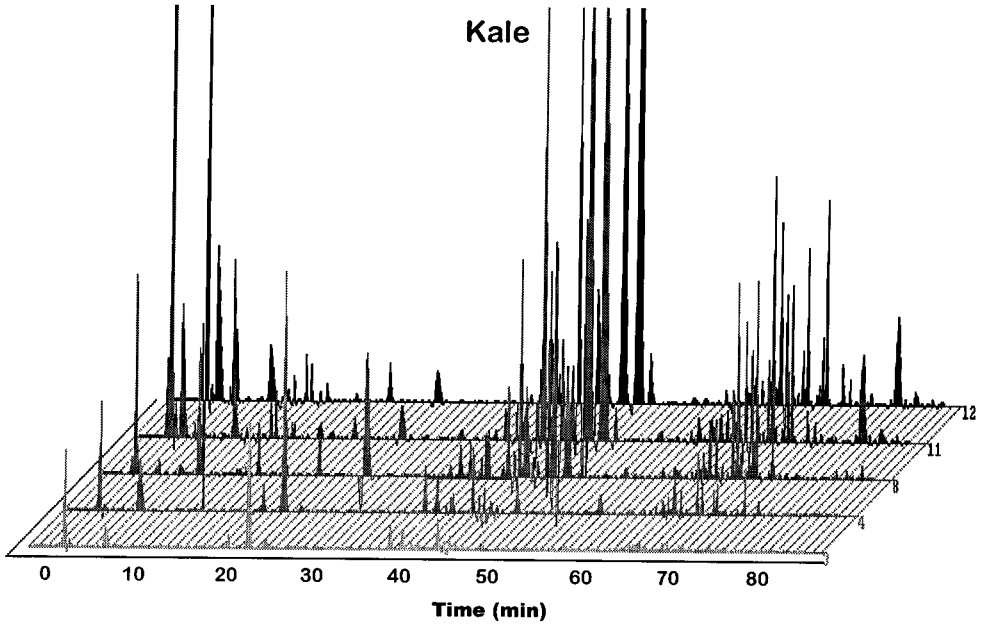

Figure 4. Five-channel chromatogram of kale extract. See Figure 3 for additional details about chromatogram.

unique chromatographic peak distribution. For fruits and vegetables that had only a few peaks, such as cucumber and carrot, most of their constituents usually eluted before 35 min, while for fruits and vegetables that had more peaks, such as strawberry, kale, and spinach, more of the chromatographic peaks eluted after 35 min. Interestingly, most of the constituents (54.8% peaks) in spinach eluted after 65 min.

Figure 8 shows the significant positive linear correlation found in aqueous extracts of various fruits and vegetables between their $ORAC_{ROO\cdot}$ activities and their electrochemical data obtained with the HPLC coupled with coulometric array detection, which included the total peak area and height summed both from dominant channels and across all channels. However, as is evident in Figure 8, spinach was an outlier in the correlation analysis.

Quantification of Some Identified Compounds in Fruits and Vegetables. The concentrations of some electroactive compounds identified in this study in the aqueous extracts of strawberry, spinach, kale, carrot, and cucumber are presented in Table 5. Catechin, rutin, naringin, vitamin C, and glutathione were all detected in strawberry. Ferulic acid was detected in both spinach and kale. Tyrosine and 4-hydroxyphenylacetic (4-HPA) acid were found in all of these fruits and vegetables.

Positive detection of compounds in the fruit and vegetable aqueous extracts was done by matching retention times and peak purity. Peak purity was determined using peak area ratio accuracy (%) calculations as described in the Methods section. Ratio accuracy (%) values in Table 6 greater than 70% were considered to be good matches, while values less than 70% probably indicated a different compound that coeluted with the compound of interest. The 70% ratio accuracy point was the default setting recommended by the ESA CoulArray software.

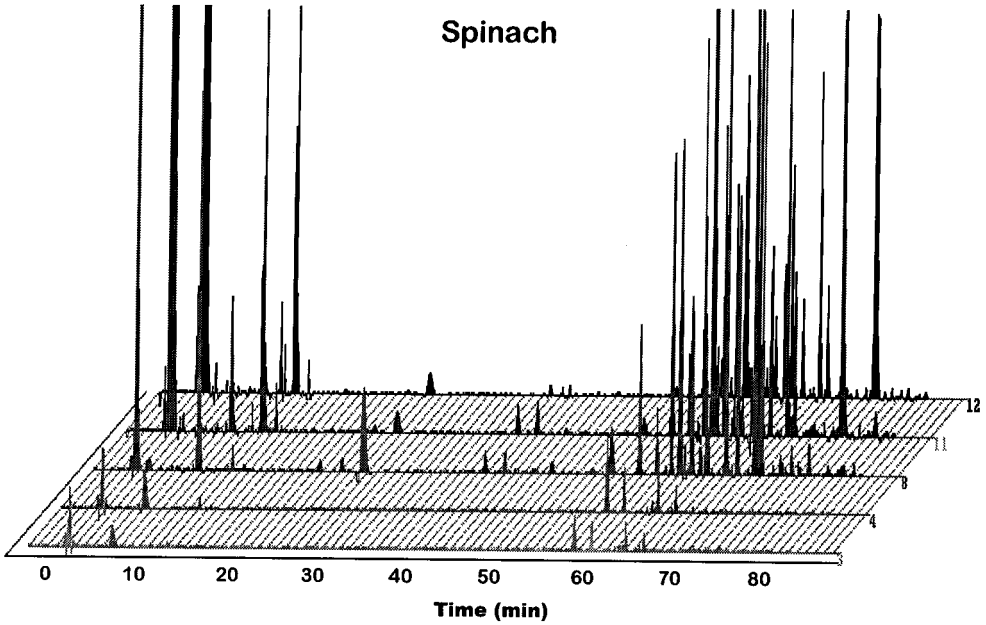

Figure 5. Five-channel chromatogram of spinach extract. See Figure 3 for additional details about chromatogram.

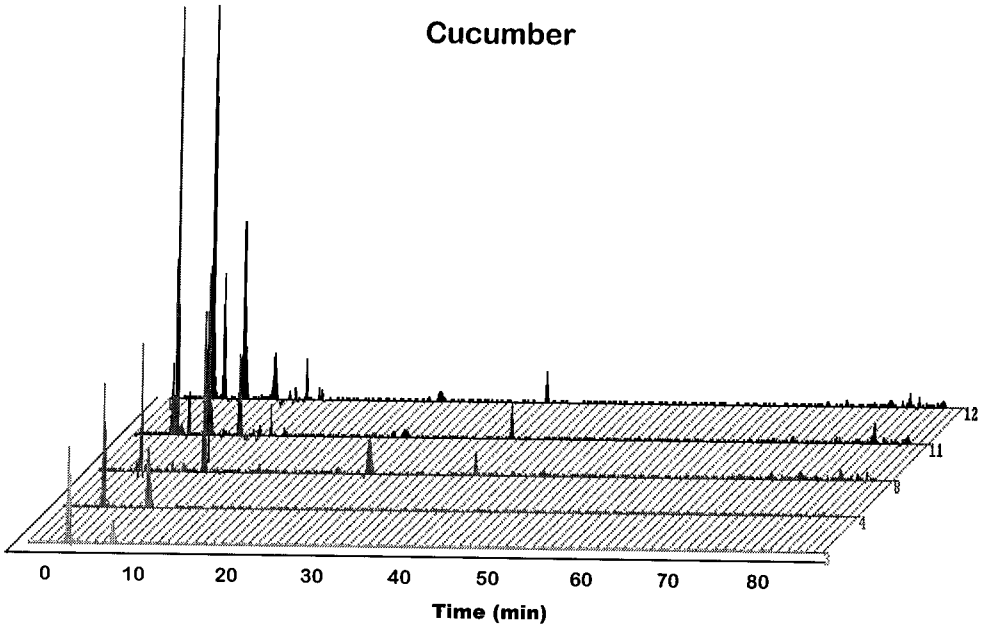

Figure 6. Five-channel chromatogram of cucumber extract. See Figure 3 for additional details about chromatogram.

DISCUSSION

Reversed-phase HPLC with ultraviolet and visible absorbance detection is a common technique for the analysis of various antioxidants in fruits and vegetables, especially phenolic acids and flavonoids (Bakker et al., 1994; Bronner and Beecher, 1995; Elujoba et al., 1991; Hardin and Stutte, 1980; Hertog et al., 1992; Li et al., 1993; Pietta et al., 1995). A sample preparation procedure including extraction and hydrolysis is usually included in this technique because of the great complexity of fruit and vegetable samples. Thus, it is a technique for the selective analysis of certain antioxidants in fruits and vegetables. In this study we describe a nonselective method that characterized overall antioxidant status in fruits and vegetables and separated thirtyone electroactive compounds, including vitamin C, glutathione, and a group of phenolic acids and flavonoids. This method, using the recently developed coulometric array detection technique, showed good reproducibility and sensitivity for the simultaneous detection of these external standards. The limits of detection using a 20-μL injection volume were typically in the low ng/mL range ($\sim$20 pg) with a linear response range of at least 2−3 orders of magnitude. Other investigators using UV detection require amounts in the ng range (Wang et al., 1993).

The procedure described in this paper measured from 3 to 117 electroactive compounds (peaks) in the chromatograms of different fruit and vegetable extracts (Table 4). There are many compounds unidentified but well separated in fruit and vegetable extracts. These chromatograms of fruit and vegetable extracts represent basically a "fingerprint" that characterizes the overall antioxidant status of these fruits and vegetables because (i) these samples were directly used in the assay without any pretreatment; (ii) each fruit and vegetable had a unique chromatographic peak distribution; and (iii) there was a positive linear correlation between their $ORAC_{ROO\cdot}$ activities and their electrochemical data including the total peak area and/or height summed across all peaks from the dominant channels or across all channels.

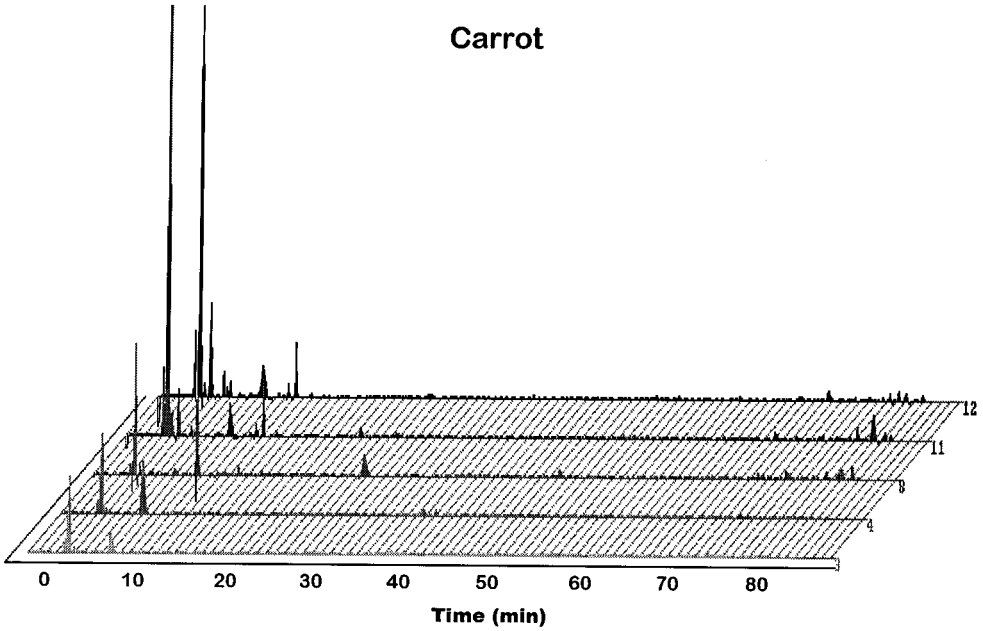

Figure 7. Five-channel chromatogram of carrot extract. See Figure 3 for additional details about chromatogram.

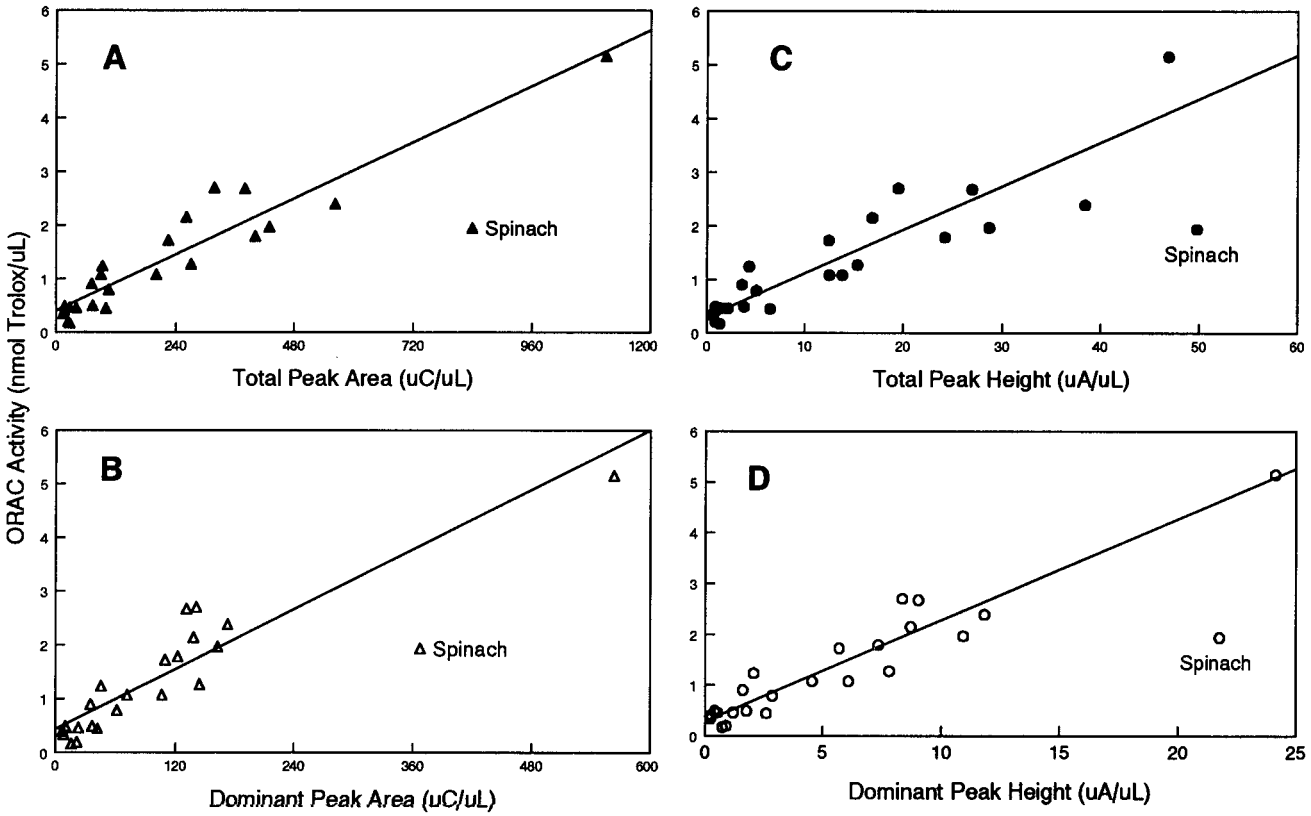

Figure 8. Oxygen radical absorbing capacity ($ORAC_{ROO\cdot}$) (nmol of Trolox equiv/μL) (Y) versus (A) total peak area summed across all channels (μC/μL) ($Y = 0.0044 + 0.3991X$; $r_{XY} = 0.947$, $P < 0.01$); (B) total peak area summed from dominant channels (μC/μL) ($Y = 0.0093 + 0.4408X$; $r_{XY} = 0.932$, $P < 0.01$); (C) total peak height summed across all channels (μA/μL) ($Y = 0.0812 + 0.3078X$; $r_{XY} = 0.914$, $P < 0.01$); and (D) total peak height summed from dominant channels (μA/μL) ($Y = 0.1995 + 0.2837X$; $r_{XY} = 0.956$, $P < 0.01$). Each data point represents the mean of triplicate determinations of one fruit or vegetable.

However, spinach was an outlier in the correlation analysis between the $ORAC_{ROO\cdot}$ activities and electrochemical data of fruits and vegetables. It may be related to the finding that most of the constituents (55% of the peaks) of spinach eluted after 65 min. These components may not contribute much to ORAC activity even though they are likely to be flavonoid type compounds as the dominant responses were at high potentials (see Figure 5); however, they may be glycosylated and acylated by different sugars and acids which would decrease the $ORAC_{ROO\cdot}$ activity (see Table 2).

The linear correlation found in fruit and vegetable extracts between the $ORAC_{ROO\cdot}$ activities and electrochemical data were not surprising since all antioxidants are electroactive chemicals and can be theoretically detected by using the HPLC coupled with coulometric array detectors (however, it is possible that not all electrochemical responses from sample extracts were from antioxidants only). The $ORAC_{ROO\cdot}$ activities presented in Table 4 were determined in an aqueous extract of the fruits and vegetables which was the same extract used for injection into the HPLC. The estimated

Table 5. Concentrations of Some Electroactive Components Identified in Fruits and Vegetables[a]

compound	strawberry	kale	spinach	cucumber	carrot
vitamin C	583 ± 8.1	nd[b]	nd	nd	nd
glutathione	83 ± 1.5	1.2 ± 0.1	nd	nd	nd
tyrosine	4.1 ± 0.1	46 ± 0.5	98 ± 1.7	52.4 ± 0.6	20.6 ± 0.4
4-HPA acid	4.0 ± 0.3	24 ± 1.2	12 ± 0.7	6.8 ± 0.6	2.9 ± 0.1
ferulic acid	nd	2.0 ± 0.1	2.1 ± 0.1	nd	nd
catechin	39 ± 5.6	nd	nd	nd	nd
rutin	66 ± 3.9	nd	nd	nd	nd
naringin	10.8 ± 0.7	nd	11.2 ± 0.1	nd	nd

[a] All data are expressed as mean ± SE (from triplicate determination) of μg per g fresh weight of the fruit or vegetable. [b] nd, not identified or detectable.

Table 6. Analyte Peak Area Ratio Accuracy (%) between Extract and Standard Solutions[a]

compound	strawberry	kale	spinach	cucumber	carrot
vitamin C					
lower/dominant	86 ± 1.3	nd	nd	nd	nd
upper/dominant	84 ± 2.2	nd	nd	nd	nd
glutathione					
lower/dominant	59 ± 0.5	31 ± 27	nd	nd	nd
upper/dominant	73 ± 0.2	52 ± 4.9	nd	nd	nd
tyrosine					
lower/dominant	34 ± 1.2	36 ± 3.2	45 ± 0.9	39 ± 4.8	43 ± 3.9
upper/dominant	61 ± 2.5	62 ± 1.8	61 ± 1.0	64 ± 1.6	66 ± 0.1
4-HPA acid					
lower/dominant	58 ± 2.1	50 ± 1.2	62 ± 3.4	51 ± 1.7	63 ± 0.3
upper/dominant	80 ± 16	80 ± 1.5	83 ± 5.5	69 ± 9.9	53 ± 2.7
ferulic acid					
lower/dominant	nd	63 ± 3.5	77 ± 1.7	nd	nd
upper/dominant	nd	85 ± 3.4	81 ± 3.0	nd	nd
catechin					
lower/dominant	92 ± 3.6	nd	nd	nd	nd
upper/dominant	64 ± 3.8	nd	nd	nd	nd
rutin					
lower/dominant	68 ± 3.0	nd	nd	nd	nd
upper/dominant	97 ± 2.2	nd	nd	nd	nd
naringin					
lower/dominant	83 ± 3.0	nd	67 ± 3.1	nd	nd
upper/dominant	93 ± 8.8	nd	69 ± 2.2	nd	nd

[a] Analyte peak area ratio (lower/dominant, upper/dominant) accuracy (%) between extract and standard solutions are expressed as mean ± SE (from triplicate determinations).

$ORAC_{ROO\cdot}$ per g fresh weight (data not presented) in these extracts is lower than that reported by Wang et al. (1996), which is to be expected since these values represent only the aqueous extract and not the acetone extract which was included in the totals reported by Wang et al. (1996) and Cao et al. (1996). Since the contribution of the acetone extract to the total ORAC of a fruit or vegetable is usually less than 10% (Wang et al., 1996), the electrochemical data expressed as total peak height or area should be useful for roughly evaluating the overall antioxidant status of a fruit or vegetable sample even when some electroactive constituents of this sample cannot be identified or quantitated precisely against known standards. The response of the electrochemical detector was linear over a wide range of concentrations using peak area (Table 3) and peak height (data not presented). Thus, summing the peak areas across all peaks in a chromatogram should provide a reasonable estimate of the total electroactive compounds in the extract. The uniqueness of the ORAC assay system (Cao et al., 1993, 1995) for the total antioxidant capacity determination was demonstrated in the present study.

Most of the external standards used in this study were phenolic acids and flavonoids that commonly occur in fruits and vegetables. These phenolic acids, except 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid, were found to have antioxidant activities (1.09−2.23 Trolox equiv) at least as strong as Trolox, a water soluble vitamin E analogue. However, flavonoids including flavanols, flavones, and isoflavones, in general, had stronger antioxidant activities than these phenolic acids, including benzoic and cinnamic acid derivatives (Cao et al., 1997). We reported previously that some fruits and vegetables, such as strawberry, kale, and spinach, had much higher antioxidant activities than some other fruits and vegetables, such as honeydew melon, carrot, and cucumber (Cao et al., 1996; Wang et al., 1996). One explanation for this great difference in antioxidant activity found among different fruits and vegetables was related to the different flavonoid concentrations (Wang et al., 1996). The results from the present study support this assumption. It can be clearly seen in this study that carrot and cucumber had only a few peaks and most of these peaks eluted before 35 min; however, strawberry, kale, and spinach had a large number of peaks and/or large quantities of individual components and most of these peaks eluted after 35 min. All tested flavonoids, including flavanols (catechin, epicatechin, and gallocatechin gallate), flavones (quercetin, kaempferol, rutin), isoflavones (genistein), flavanones (fustin, taxifolin, naringin, hesperidin, and eriodictyol), and anthocyanidins (pelargonidin and callistephin), also were eluted after 35 min.

Efforts were made in this study to identify some antioxidants, especially phenolic acids and flavonoids in some fruits and vegetables. However, not many of the phenolic acids and flavonoids were found or identified in the fruits and vegetables tested. This is not surprising since the external standards used in this study were very limited, especially considering that phenolic acids and flavonoids encompass such a large family of compounds; there are over 4000 flavonoids found in plants and plant foods (Das, 1994). In addition, most of these external standards were in free form (aglycone), but many phenolic acids and flavonoids exist as their esters or glycosides in fruits and vegetables (Elujoba et al., 1991; Hardin and Stutte, 1980; Li et al., 1993). With more external standards and/or with the help of other techniques like mass spectrometry, we believe it should be possible to identify more, if not all, antioxidant constituents in the fruits and vegetables that have high antioxidant activities.

In conclusion, an HPLC coupled with coulometric array detection procedure was developed for the characterization of overall antioxidant status in fruits and vegetables. The technique was sensitive, with linear responses from flavonoid type compounds in the 20 pg to 40 ng range. The HPLC procedure can be used to identify and quantify simultaneously multi-antioxidants including vitamin C, glutathione, and a group of phenolic acids and flavonoids in fruits and vegetables. A significant linear correlation was demonstrated in fruits and vegetables between the total antioxidant activity determined by using $ORAC_{ROO\cdot}$ assay and the electrochemical data generated from the coulometric array detector. The combination of these two techniques may allow for the identification of specific fruits and vegetables and/or their chemical components that may be important in the prevention of some of the age-related diseases.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; $ORAC_{ROO\bullet}$, oxygen radical absorbance capacity; *R*-PE, *R*-phycoerythrin; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; ODS, octadecylsiloxane; CV, coefficient of variation; 4-HPA acid, 4-hydroxyphenylacetic acid; μC, microcoulomb; μA, microampere.

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