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Analysis of Flavonoids and Other Phenolic Compounds Using High-Performance Liquid Chromatography with Coulometric Array Detection: Relationship to Antioxidant Activity

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High-performance liquid chromatography coupled with a coulometric array detector was used to characterize the electrochemical behavior of 17 flavonoids and three cinnamic acid derivatives. The antioxidant activity of these phenolic compounds was evaluated by the ferric reducing activity power (FRAP), the oxygen radical absorbance capacity (ORAC), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assays. All flavonoids, except kaempferol-3-rutinoside, malvidin-3-glucoside, and peonidin-3-glucoside, had two oxidation potentials (100−300 and 700−800 mV). Quercetin and myricetin had an additional oxidation wave at 400 mV. The electrochemical responses at a relatively low oxidation potential (300 mV) and the cumulative responses at medium oxidation potentials (400 and 500 mV) had the highest correlations with antioxidant activities. The highest correlations between electrochemical characteristics and antioxidant activities were found between electrochemical responses and antioxidant activities obtained in the FRAP assay and in the DPPH assay after short reaction periods. Lower correlations were revealed between electrochemical responses and antioxidant activities obtained in the ORAC assay.

KEYWORDS: HPLC; electrochemical detection; coulometric array; antioxidant capacity; ORAC; FRAP; DPPH; ARP; flavonoids; anthocyanins; phenolic compounds

INTRODUCTION

In recent years, there has been an increased interest in flavonoids due to the possible health benefits of eating fruits and vegetables containing these compounds (*1*−*3*). The anticarcinogenic, antimutagenic, and cardioprotective effects reported are generally associated with the flavonoids' antioxidant properties. Flavonoids may act as reducing agents and donors of hydrogen and thereby function as free radical scavengers, or they may function as antioxidants by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating pro-oxidant trace metals (*3*, *4*).

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6−C3−C6), labeled A, B, and C (**Figure 1**). The various classes of flavonoids differ in the level of oxidation and saturation of ring C, while individual compounds within a class differ in the pattern of the substitution of rings A and B. Among the flavonoids are flavonols, flavanols, anthocyanidins, flavones, and flavanones. Differences in structure and substitution will influence the phenoxyl radical stability and thereby the antioxidant properties of the flavonoids (*4*−*6*).

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A number of methods have been developed and applied to measure the antioxidant activities of pure compounds, plant extracts, and human plasma (*7*−*11*). The principles of the methods differ; for example, in the FRAP method, the antioxidants' ability to reduce a ferric complex to the ferrous form is measured (*11*). FRAP was developed to analyze the reducing ability of plasma (*11*) but has recently been applied to other samples such as tea (*12*), *Rubus* species (*13*), other small fruits (*14*), and edible plants (*15*). The antioxidant activity of dietary polyphenols has been determined by a modified FRAP assay (*16*). In the TEAC (*10*), TRAP (*9*, *17*), and ORAC (*8*) assays, scavenging of free radicals generated in the reaction mixture is measured. The ORAC assay has been used for the analysis of a range of antioxidant systems, from pure compounds to plant extracts and biological samples (*14*, *18*−*21*). Other methods measure the antioxidant scavenging ability toward stable radical species such as *N*,*N*-dimethyl-*p*-phenylenediamine (*22*) and DPPH• (*7*, *23*).

Flavonoids are UV absorptive and have traditionally been analyzed by HPLC with UV/visible detection. Electrochemical detection is sensitive, selective, and gives useful information about polyphenolic compounds in addition to spectra obtained by photodiode array detectors (*24*). Differences in electrochemically active substituents on analogous structures can lead to characteristic differences in their voltammetric behavior (*25*).

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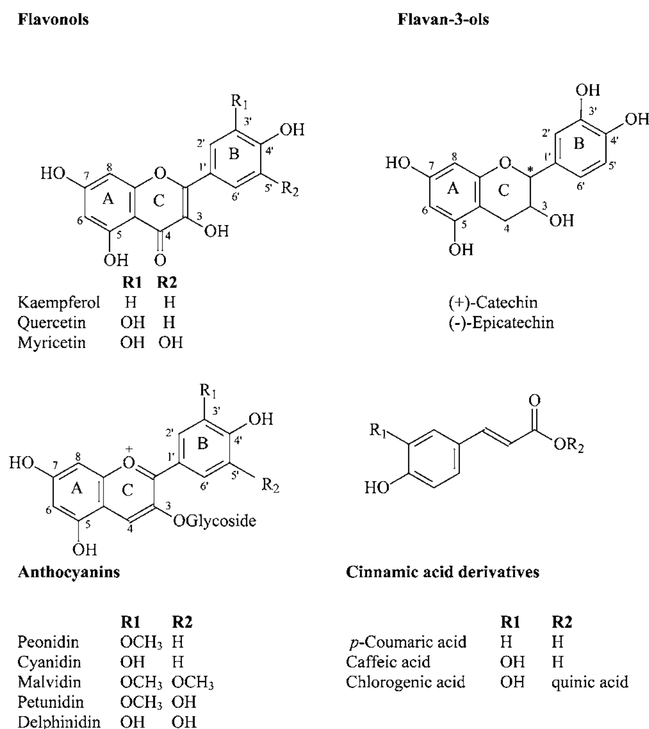

Figure 1. Structural formulas of the analyzed compounds.

Because the response profile across several cell potentials is representative for the voltammetric properties of a compound, useful qualitative information can be obtained using electrochemical detection.

A positive, linear correlation between the antioxidant activity of fruits and vegetables measured as ORAC values and the total electrochemical responses, obtained by HPLC coupled to a coulometric array detector, has been found (*26*). In another study, the electrochemical potential corresponding to the MDRP was found to be inversely proportional to the antioxidant efficiency of phenolic acids (*27*). However, no correlation was established for flavonoids.

In the present study, the electrochemical behaviors and antioxidant activities of 20 flavonoids and cinnamic acid derivatives, predominant in berries, were investigated. Electrochemical characteristics were obtained by HPLC with a coulometric array detector. The antioxidant activity was evaluated by three common in vitro methods with different working principles, the FRAP, the DPPH, and the ORAC assays. The purpose of the work was to determine if analysis of phenolic compounds by HPLC with a coulometric array detector could be utilized to predict antioxidant activity assessed by the three methods. Furthermore, the study was aimed at determining which parameters from the coulometric analysis would best describe the antioxidant activities achieved by the FRAP, the DPPH, and the ORAC assays.

MATERIALS AND METHODS

Chemicals. Quercetin, (+)-catechin, (−)-epicatechin, quercetin-3-rhamnoside (quercitrin), quercetin-3-rutinoside (rutin), *p*-coumaric acid, *β*-PE, and DPPH• were purchased from Sigma Chemical Co. (St. Louis, MO). Myricetin, kaempferol, chlorogenic acid, caffeic acid, 2,4,6-tripyridyl-*s*-triazine, and Trolox were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Quercetin-3-galactoside (hyperoside) and quercetin-3-glucoside (isoquercitrin) were obtained from Carl Roth GmbH (Karlsruhe, Germany). Kaempferol-3-rutinoside and kaempferol-7-neohesperidoside were obtained from Indofine Chemical Co., Inc. (Somerville, NJ). Cyanidin-3-glucoside, cyanidin-3-galactoside, peonidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside were purchased from Polyphenols AS (Sandnes, Norway). AAPH was obtained from Polysciences Inc. (Warrington, PA). Acetic acid, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, sodium acetate, HCl, acetonitrile, phosphoric acid, and methanol, all analytical or HPLC grade, were obtained from Merck KGAa (Darmstadt, Germany).

Standard Preparation. The chemical structures of the phenolic compounds analyzed are shown in **Figure 1**. Stock standard solutions of these compounds were prepared by dissolving the phenolic compounds in methanol at a concentration of 0.5 mg/mL. The anthocyanins were in addition dissolved in acidic methanol (0.01% HCl). The stock solutions were stored at −75 °C before further dilution by methanol prior to analysis. For HPLC analysis, two seven-component working standard solutions of polyphenolics were prepared and diluted by methanol to concentrations within the linear range of the detector, about 5−5000 ng/mL. The six anthocyanins were diluted in acidic (0.01% HCl) methanol.

HPLC with Coulometric Array Detection. HPLC analyses were performed using an HP 1050 series HPLC (Hewlett-Packard GmbH, Waldbronn, Germany) interfaced to an ESA coulometric array detector (ESA Inc., Chelmsford, MA) with eight porous graphite working electrodes with associated palladium reference electrodes. The detector array was set from 100 to 800 mV in increments of 100 mV. The ESA CoulArray operating software (ESA Inc.) was used to collect voltammetric data. Raw data were processed using Microsoft Excel. The results were presented as peak areas at the electrodes, expressed as microcoulombs per nanomole of antioxidant (μC/nmol), and as cumulative peak areas (μC/nmol). The cumulative peak area was the response across several electrodes; for example, the cumulative response at 300 mV (C300 mV) was the sum of the peak areas at 100, 200, and 300 mV. The cumulative response as a function of oxidation potential for a compound was plotted as HDV (**Figure 2**). As a compound moves through the coulometric array, it is oxidized in a stepwise fashion until complete electrochemical conversion at a current plateau. When the analyzed compound contains more than one oxidizable moiety, more than one current plateau occurs, and a characteristic multiwave HDV is generated. In the present study, DP, defined as the voltage (mV) with the maximum signal, was determined for each of these oxidation waves.

Chromatographic separation was performed on a Luna C_{18} column (250 mm × 2.0 mm i.d., 5 μm particle size) equipped with a 5 μm C_{18} (ODS) guard column (4.0 mm × 3.0 mm i.d.) both from Phenomenex (Torrance, CA).

The mobile phase for separation of polyphenols other than anthocyanins consisted of acetonitrile (A), methanol (B), and 70 mmol/L (mM) KH_2PO_4 adjusted to pH 2.4 with phosphoric acid (85%) (C) (*28*). The initial mobile phase composition was 10% B and 90% C, followed by a linear gradient to 22% B and 78% C in 10 min; a linear gradient with A from 0 to 25%, B constant, and C from 78 to 53% in 25 min; and finally a linear gradient with A from 25 to 45%, B constant, and C from 53 to 33% in 10 min.

For the separation of anthocyanins, the mobile phase consisted of acetonitrile (A) and 1% phosphoric acid, 10% acetic acid, and 5% acetonitrile (v/v/v) in water with 50 mM NaCl (B) (*28*). The program followed a linear gradient from 2 to 12% A in 15 min, from 12 to 22% A in 5 min, and isocratic conditions with 22% A for 5 min.

In both chromatographic systems, the solvent flow rate was 1 mL/min and the column was allowed to equilibrate for 10 min before each sample was injected. The samples were filtered through a Millex HA 0.45 μm filter (Millipore, Molsheim, France) before injection (50 or 20 μL).

FRAP Assay. The FRAP assay was carried out according to the procedure described by Benzie and Strain (*11*) with some modifications. Briefly, 2.4 mL of freshly prepared FRAP reagent containing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 300 mM acetate buffer, pH 3.6, in the ratio of 1:1:10 was mixed with 80 μL of antioxidant solution (250 μM). The absorbance at 593 nm was recorded every 6 s using an UV/vis scanning spectrophotometer (UV-2101PC, Shimadzu Corp., Kyoto, Japan). The reaction was monitored at room temperature (22 °C) for at least 60 min. Aqueous solutions of Fe(II) ($FeSO_4 \cdot 6H_2O$)

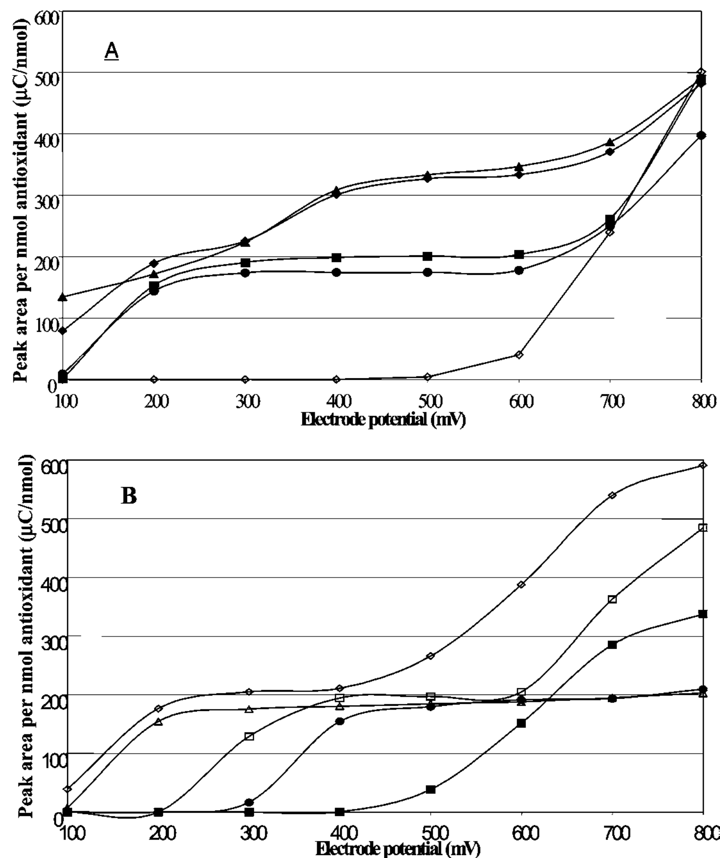

Figure 2. HDVs of representative phenolic compounds. Responses (peak areas) are corrected to 1 nmol for all compounds. (**A**) Myricetin (▲), quercetin (◆), quercetin-3-glucoside (■), kaempferol (●), and kaempferol-3-rutinoside (◇). (**B**) (+)-Catechin (◇), caffeic acid (△), cyanidin-3-glucoside (□), malvidin-3-glucoside (●), and *p*-coumaric acid (■).

in the concentration range of 125−1000 μmol/L were used for calibration of the FRAP assay. FRAP values, derived from triplicate analysis, were expressed as μmol TE per μmol of antioxidant (μmol TE/μmol). FRAP values obtained at different reaction times were designated $FRAP_{time}$; for example, $FRAP_{10}$ was the FRAP value obtained after 10 min of reaction time.

DPPH Assay. The scavenging effects of the phenolic compounds toward the stable free radical DPPH$^•$ were measured according to the procedure by Brand-Williams et al. (*7*). Briefly, the antioxidant in methanol (0.1 mL) was added to 2.4 mL of methanolic DPPH$^•$ solution (25 mg/L). The reaction mixture was covered and left in the dark at room temperature (22 °C). The absorbance at 515 nm was recorded spectrophotometrically (HP 8452A, Hewlett-Packard GmbH) after 0.5, 1, 3, 15, 30, and 60 min and thereafter every hour until the steady state. Each antioxidant was prepared in duplicates for each of at least three concentrations. The amount of sample required to decrease the initial DPPH$^•$ concentration by 50% (EC_{50}) was calculated by linear regression of remaining DPPH$^•$ (%) vs sample concentration. The antioxidant activity was given as the reciprocal of EC_{50}, the ARP in units of μmol TE per μmol of antioxidant (μmol TE/μmol). ARP values obtained at different reaction times were designated ARP_{time}; for example, ARP_{10} was the ARP value obtained after 10 min of reaction time.

ORAC Assay. The ORAC assay was performed as described by Cao et al. (*8*) with some modifications. The measurements were carried out on a Wallac 1420 Victor^2 96 well plate reader (EG & Wallac, Turku, Finland) with a fluorescence filter (excitation 540 nm/8 nm, emission 570 nm/7 nm). β-PE (16.7 nM) was the fluorescence probe and target molecule for free radical attack from AAPH (4 nM) as the peroxyl radical generator. The reaction was conducted at 37 °C at pH 7.0 with Trolox (1 μM) as the control standard and phosphate buffer as the blank. All standards dissolved in methanol (0.5 mg/mL) were diluted with buffer (1:125−500, v/v) prior to analysis. The same concentration of methanol (0.8%) was used in all samples, blank, and standard. The β-PE fluorescence was recorded every 3 min after the addition of AAPH. All measurements were expressed relative to the initial reading. The final results were calculated using the differences of areas under the β-PE decay curves between the blank and a sample and were expressed in μmol of TE per μmol of antioxidant (μmol TE/μmol).

Statistical Analysis. Regression analysis to determine ORAC and ARP values was performed by Microsoft Excel. Univariate correlation analysis (linear regression) between electrochemical responses and antioxidant activities was performed by Minitab Statistical Software (Release 13.30, Minitab Inc., State College, PA). PLS regression was performed using The Unscrambler (v7.6 SR-1, CAMO ASA, Oslo, Norway) software program. The data were analyzed by PLS regression using electrochemical responses (peak areas) as X variables and antioxidant activities (FRAP, ARP, and ORAC values) as Y variables. All variables were weighed by 1/standard deviation before analysis. Full cross-validation was used to validate the PLS model.

RESULTS

Electrochemical Characteristics. Electrochemical profiles, the HDVs, of representative phenolic compounds are shown in **Figure 2**. When more than one electrochemical active functional group was present, more than one oxidation potential occurred and a characteristic multiwave HDV was generated, as illustrated in the HDV of quercetin and myricetin (**Figure 2A**). The first oxidation wave for the polyphenols occurred from 100 to 300 mV, the next from 400 to 500 mV, and the last from 700 to 800 mV. Cumulative responses at the end of these oxidation waves (at 300, 500, and 800 mV) and DPs for the phenolic compounds are shown in **Table 1**. All of the phenolic compounds analyzed, except kaempferol-3-rutinoside, malvidin-3-glucoside, peonidin-3-glucoside, and *p*-coumaric acid, had their first oxidation wave at a low potential (100−300 mV). The flavonoids, except kaempferol-7-neohesperidoside and

Table 1. DPs (mV) and Cumulative Responses (μC/nmol) at Three Oxidation Potentials for 20 Flavonoids and Cinnamic Acid Derivatives Analyzed by HPLC with Coulometric Array Detection

phenolic compound	DPs[a] (mV)	cumulative peak area per nmol compound (μC/nmol)[b] at 300 mV	500 mV	800 mV	literature values DPs (mV) (ref)
		flavonols			
quercetin	200, 400, 800	225	327	482	120 (*34*), 770 (*26*), 300 (*27*), 900 (*27*)
quercetin-3-rutinoside	200, 800	174	183	348	180 (*34*), 780 (*34*), 700 (*26*), 300 (*27*), 900 (*27*)
quercetin-3-glucoside	200, 800	190	201	489	250 (*27*), 800 (*27*)
quercetin-3-galactoside	200, 800	191	201	563	
quercetin-3-rhamnoside	200, 800	151	163	423	180 (*34*), 780 (*34*)
myricetin	100, 400, 800	223	333	489	60 (*34*), 100 (*27*), 700 (*27*)
kaempferol	200, 800	174	175	398	180 (*34*), 700–770 (*26*)
kaempferol-7-neohesperidoside	200, 800	173	177	294	
kaempferol-3-rutinoside	800	0	4	501	
		flavan-3-ols			
(+)-catechin	200, 700	205	266	591	480 (*34*), 120 (*25*), 490 (*26*), 100 (*27*), 600 (*27*)
(–)-epicatechin	200, 700	209	256	547	490 (*26*), 100 (*27*), 600 (*27*)
anthocyanins					
cyanidin-3-glucoside	300, 700	129	197	485	
cyanidin-3-galactoside	300, 700	125	194	480	
petunidin-3-glucoside	300, 700	151	205	551	
delphinidin-3-glucoside	200, 800	213	250	455	
malvidin-3-glucoside	400	17	180	209	
peonidin-3-glucoside	500	0	154	395	
		cinnamic acid derivatives			
caffeic acid	200	175	185	203	120 (*34*), 60 (*25*), 140 (*26*), 150 (*27*)
chlorogenic acid	200	164	168	186	120 (*34*), 120 (*25*), 150 (*27*)
p-coumaric acid	700	0	39	338	540 (*34*), 540 (*25*), 490 (*26*), 750 (*27*)

[a] DPs were defined as the voltages (mV) with a maximum signal for each oxidation wave. [b] Cumulative peak area, the response across several potentials, expressed as μC per nmol of antioxidant. The average standard deviation was 8% ($n = 3$).

malvidin-3-glucoside, had comparable cumulative responses, about 400−550 μC/nmol compound, at the end of the coulometric array (at 800 mV) (**Table 1**).

All flavonoids, except kaempferol-3-rutinoside, malvidin-3-glucoside, and peonidin-3-glucoside, had more than one oxidation potential. Quercetin had three oxidation waves at 200, 400, and 800 mV. Myricetin had a similar HDV as quercetin, but the first DP was at a lower potential (100 mV). All of the quercetin-3-glycosides had the same oxidation profile and DP at 200 and 800 mV, as illustrated by the HDV of quercetin-3-glucoside (**Figure 2A**). Kaempferol and kaempferol-7-neohesperidoside had similar HDV as the quercetin-3-glycosides, while kaempferol-3-rutinoside required an electrode potential of at least 600 mV to be oxidized (**Figure 2A**). The flavan-3-ols, (+)-catechin and (−)-epicatechin, had identical electrochemical responses with oxidation waves at 200 and 700 mV (**Table 1** and **Figure 2B**). Delphinidin-3-glucoside had the lowest first oxidation potential of the anthocyanins, followed by cyanidin-3-glucoside, cyanidin-3-galactoside, and petunidin-3-glucoside, which were oxidized at 300 mV (**Table 1**). All of these compounds had a second oxidation wave at 700−800 mV. The first DP of malvidin-3-glucoside was at 400 mV, with no considerable further oxidation at higher potentials. Peonidin-3-glucoside started to oxidize at 400 mV and continued to oxidize throughout the coulometric array. Caffeic and chlorogenic acid had only one DP, at 200 mV, and low total responses at the end of the coulometric array.

Antioxidant Activities. The FRAP values of the phenolic compounds after 60 min of reaction time are given in **Table 2**. The ferric reducing ability of representative phenolic compounds as a function of time is presented in **Figure 3**. The reaction with the ferric−TPTZ complex was not finished after 60 min for most of the compounds but continued to increase for several hours. However, after the fast initial reaction, the reaction rate declined, and after 60 min, the order of antioxidant activity between the compounds was constant. Besides, the correlations between FRAP values obtained at different reaction times, from 1 to 120 min, were high ($r > 0.93$, $p < 0.001$). The FRAP values for the flavan-3-ols, the quercetin-3-glycosides, and the

Table 2. Antioxidant Activities of 20 Flavonoids and Cinnamic Acid Derivatives Analyzed in the FRAP, the DPPH• (ARP Values), and the ORAC Assay

phenolic compound	μmol TE/μmol FRAP$_{60}$[a]	ARP$_{120}$[b]	ORAC[c]
	flavonols		
quercetin	4.0 ± 0.0	3.8 ± 0.1	2.7 ± 0.1
quercetin-3-rutinoside	2.4 ± 0.0	3.3 ± 0.1	3.6 ± 0.3
quercetin-3-glucoside	2.3 ± 0.0	3.1 ± 0.0	3.2 ± 0.2
quercetin-3-galactoside	2.0 ± 0.1	3.1 ± 0.4	3.2 ± 0.1
quercetin-3-rhamnoside	2.3 ± 0.0	3.4 ± 0.1	3.7 ± 0.2
myricetin	2.9 ± 0.1	3.0 ± 0.1	2.6 ± 0.2
kaempferol	1.8 ± 0.0	1.2 ± 0.0	2.1 ± 0.1
kaempferol-7-neohesperidoside	1.8 ± 0.1	1.3 ± 0.1	2.1 ± 0.3
kaempferol-3-rutinoside	0.1 ± 0.0	ND[d]	2.2 ± 0.2
	flavan-3-ols		
(+)-catechin	1.8 ± 0.0	3.5 ± 0.2	3.9 ± 0.2
(–)-epicatechin	1.7 ± 0.1	3.4 ± 0.1	4.0 ± 0.2
	anthocyanins		
cyanidin-3-glucoside	3.9 ± 0.0	2.8 ± 0.2	2.8 ± 0.3
cyanidin-3-galactoside	3.7 ± 0.2	2.9 ± 0.1	2.7 ± 0.2
petunidin-3-glucoside	3.5 ± 0.1	2.5 ± 0.2	2.6 ± 0.1
delphinidin-3-glucoside	3.1 ± 0.0	1.9 ± 0.1	2.4 ± 0.3
malvidin-3-glucoside	2.4 ± 0.0	1.6 ± 0.1	2.3 ± 0.2
peonidin-3-glucoside	1.8 ± 0.0	1.6 ± 0.0	2.3 ± 0.3
	cinnamic acid derivatives		
caffeic acid	1.5 ± 0.0	1.5 ± 0.0	2.1 ± 0.1
chlorogenic acid	1.5 ± 0.0	1.9 ± 0.1	2.0 ± 0.1
p-coumaric acid	0.3 ± 0.0	0.01 ± 0.00	1.6 ± 0.2

[a] FRAP of antioxidants (250 μM) after 60 min of reaction time, expressed as μmol TE per μmol of antioxidant ± SE ($n = 3$). [b] ARP as μmol of DPPH• reduced after 120 min by the amount (μmol) of antioxidant necessary for 50% reduction of DPPH•, expressed as μmol TE per μmol of antioxidant ± SE ($n = 6$). [c] ORAC expressed as μmol of TE per μmol of antioxidant ± SE ($n = 6$). [d] No detectable reaction with DPPH•.

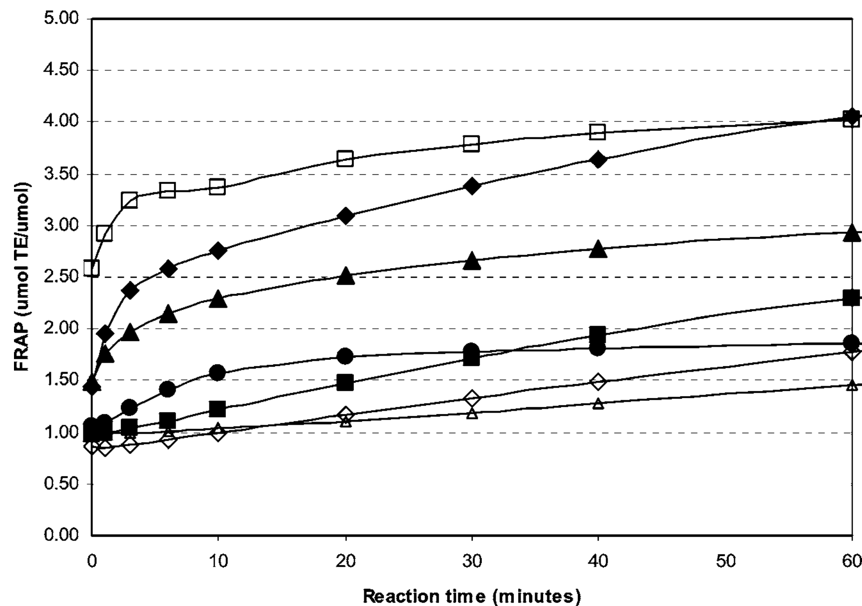

Figure 3. FRAP reaction kinetics of methanolic solutions of 250 μM quercetin (◆), quercetin-3-glucoside (■), myricetin (▲), kaempferol (●), (+)-catechin (◇), cyanidin-3-glucoside (in acidic methanol) (□), and chlorogenic acid (△).

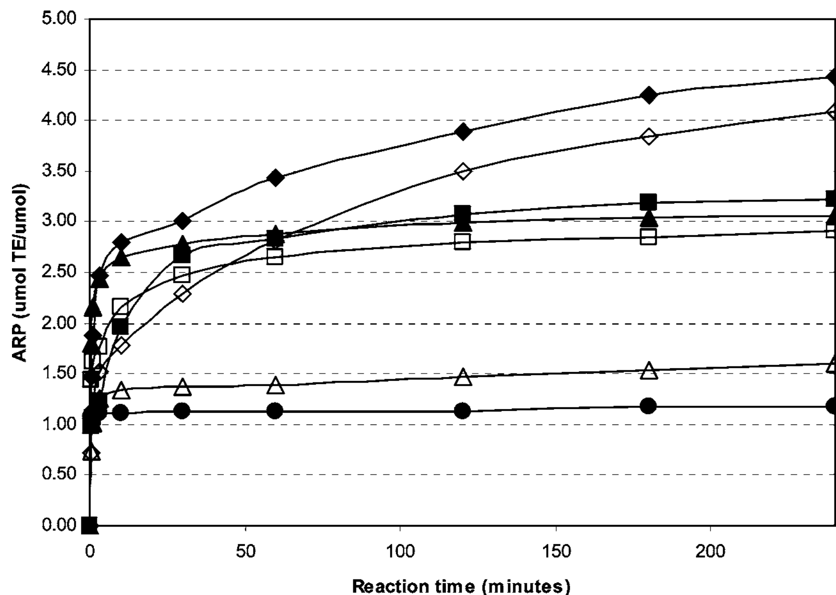

Figure 4. DPPH• reaction kinetics of methanolic solutions of quercetin (◆), quercetin-3-glucoside (■), myricetin (▲), kaempferol (●), (+)-catechin (◇), cyanidin-3-glucoside (□), and caffeic acid (△).

cinnamic acid derivative showed a linear increase with time (**Figure 3**), while the flavonol aglycons, the anthocyanins, and kaempferol-7-neohesperidoside had a nonlinear increase in FRAP values with time. All of the quercetin-3-glycosides had similar FRAP values with initial values of 1.0 μmol TE/μmol and linear reaction profiles with slopes about 0.22 FRAP units per 10 min. The anthocyanins all had the same reaction profile as cyanidin-3-glucoside (**Figure 3**) but varied in $FRAP_{60}$ values from 1.8 to 3.9 μmol TE/μmol. Kaempferol-3-rutinoside hardly reduced Fe(III), while kaempferol-7-neohesperidoside showed the same reaction kinetic as kaempferol.

Kinetics of the DPPH radical scavenging reaction of representative polyphenols are shown in **Figure 4**. The ARP values given in **Table 2** were achieved after 120 min of reaction time. The reaction between DPPH• and the flavonoids had by that time reached the steady state, except for quercetin and the flavan-3-ols where the ARP values continued to increase about 10% per hour after 2 h of reaction time. The correlations between ARP values obtained at reaction times longer than 10 min were high ($r > 0.92$, $p < 0.001$). Shorter reaction times caused a different antioxidant activity ranking order for the components. The quercetin-3-glycosides had comparable ARP_{120} values (**Table 2**) and the same, quite slow reaction with DPPH• (**Figure 4**). After 3 min of reaction time, about 42% of the DPPH• was reduced (as compared to after 120 min), and after 30 min, 85% reduction had taken place. Kaempferol-3-rutinoside had no detectable radical scavenging effect on DPPH•. Kaempferol-7-neohesperidoside, however, had about the same scavenging effect on DPPH• as kaempferol and the same fast reaction rate, i.e., 100% reaction within 3 min. All anthocyanins had similar reaction kinetics, illustrated by the reaction of cyanidin-3-glucoside (**Figure 4**).

In the ORAC assay, the loss of fluorescence of β-PE when exposed to the free radical generator AAPH is an index of oxidation. The inhibition of the reaction by an antioxidant is measured as the area under the curve and takes into account

both inhibition time and degree of inhibition. Trolox, the standard, acted fast and displayed an initial 100% inhibition of the free radical action, thus producing a lag phase. The cinnamic acid derivatives had a similar antioxidant action, while the flavonoids reacted slower and showed no lag phase in inhibiting peroxyl radical-initiated oxidation of β-PE. The ORAC values, given in **Table 2**, varied less between different phenolic compounds than the FRAP and ARP values.

Correlations between Electrochemical Characteristics and Antioxidant Activity. Multivariate regression analysis was performed to explain the relations between electrochemical responses (X), given as peak areas at different potentials (100−800 mV) and cumulative peak areas at different potentials (C100−C800 mV), and antioxidant activities (Y) as FRAP, ARP, and ORAC values. The covariance between X and Y was modeled by PLS regression. In PLS, many collinear variables are reduced to a few noncorrelated (orthogonal) PCs (*29*). The first component (PC1) captures most of the data variance, while each successive PC describes less of the information in the original data. Here, the first three PCs explained 74% of the variance in the Y data and 67% of the variance in the X data. A plot of the samples in the PC space is called a score plot. From the score plot, the samples' relations between one another are viewed. Samples close to each other in the diagram have similar attributes (response variables), while samples farther apart are more different. The loading plot is a map of variables (X and Y) and shows how the PCs are related to the original variables. The corresponding score and loading plots are complementary and give valuable information about samples and variables when studied simultaneously. Score and loading plots of the first two PCs in the present study are shown in **Figure 5**. Flavonoids with a 1,2-dihydroxy group (catechol) or a 1,2,3-trihydroxy group (pyrogallol) were located to the right in the score plot (**Figure 5A**). High antioxidant activities, high peak areas at 300 and 400 mV, and high cumulative peak areas at 300−800 mV characterized these compounds (**Figure 5A**,**B**) Compounds with no ortho hydroxy group were located to the left in the diagram and were characterized by relatively high electrochemical responses at 600 mV. Along PC2, the flavan-3-ols were separated from the anthocyanins. The latter were characterized by high FRAP values and peak areas at 300 and 400 mV, while (+)-catechin and (−)-epicatechin had high ORAC values and electrochemical responses at 200 mV. The relation between antioxidant activities and electrochemical responses is illustrated in the loadings plot (**Figure 5B**). Variables close in the diagram had the highest correlations; that is, $FRAP_{60}$ correlated best with peak areas at 300 mV, ORAC correlated best with cumulative peak areas at 800 mV, and ARP_{120} correlated best with cumulative peak areas at 400 and 500 mV. ARP values obtained at short reaction times had the highest correlation with FRAP values, especially those at long reaction times. Only weak connections were revealed between FRAP and ORAC activities.

The plots from the PLS regression analysis (**Figure 5**) illustrate the connection between samples and variables. Numeric values were obtained for the PLS model, as well. These results are not shown, since the same conclusions were drawn from univariate regression analysis, and for clarity, we chose to discuss the latter results. Univariate regression analysis for all electrochemical responses and antioxidant activities was performed. The electrochemical responses that had the highest correlations with FRAP and ARP values were peak areas at 300 mV and cumulative peak areas at 400 mV (C400 mV), while cumulative responses at the end of the coulometric array,

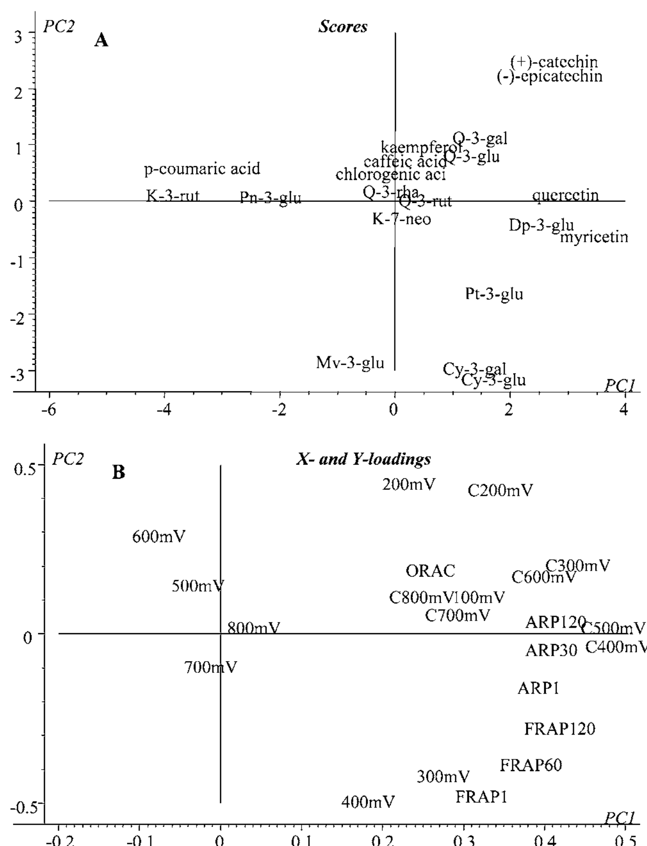

Figure 5. Scores and loadings plots for factors 1 (PC1) and 2 (PC2) from the PLS regression analysis of electrochemical responses as X data and antioxidant activities as Y data. (K, kaempferol; Q, quercetin; Cy, cyanidin; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pt, petunidin; gal, galactoside; glu, glucoside; rha, rhamnoside; and rut, rutinoside.)

at 800 mV, had the highest correlations with ORAC values. Regression coefficients (r) for the correlations between these responses and antioxidant activities are shown in **Table 3**. When all phenolic compounds were included in the regression analysis, FRAP values had the highest correlations with peak areas at 300 mV ($0.71 < r < 0.82$). There were only minor differences for FRAP values obtained at different reaction times. ARP values were best predicted by cumulative responses at 400 mV ($0.76 < r > 0.93$). The highest correlation between coulometric array responses and ARP values was obtained after a short reaction time (3 min). Correlations decreased with increasing reaction times. There were low correlations between electrochemical data and ORAC values when all compounds were included in the regression analysis. The highest correlation was found between cumulative responses at the end of the coulometric array and ORAC values ($r = 0.57$, $p = 0.008$). The correlations between electrochemical responses and antioxidant activities increased when anthocyanins ($n = 6$) and other polyphenols ($n = 14$) were treated separately. **Table 3** shows that antioxidant activities of anthocyanins were best predicted by electrochemical responses at 300 mV ($0.88 < r < 0.99$), while for the other polyphenolics the highest correlations with antioxidant activities, excluding ORAC values, were obtained with cumulative coulometric array responses at 400 mV ($0.80 < r < 0.97$). ORAC values were less predictable from electrochemical responses, the highest correlation coefficient being $r = 0.63$ ($p = 0.03$) for cumulative coulometric array responses at 800 mV.

Table 3. Univariate Correlation[a] between Coularray Response[b] and Antioxidant Activity of 20 Flavonoids and Cinnamic Acid Derivatives

	all compounds (n = 20) (mV)			anthocyanins (n = 6) (mV)			polyphenolics[c] (n = 14) (mV)		
	300	C400	C800	300	C400	C800	300	C400	C800
ORAC	0.20	0.40	0.57**	0.94**	0.51	0.65	0.45	0.41	0.63*
ARP_3	0.54*	0.93***	0.28	0.99***	0.71	0.76	0.69**	0.97***	0.18
ARP_{10}	0.53*	0.87***	0.36	0.96**	0.58	0.73	0.76**	0.93***	0.30
ARP_{30}	0.40	0.79***	0.42	0.95**	0.52	0.71	0.74**	0.84***	0.38
ARP_{120}	0.41	0.76***	0.50*	0.93**	0.48	0.68	0.63*	0.81**	0.49
ARP_{240}	0.38	0.77***	0.53*	0.88	0.15	0.75	0.57	0.80**	0.52
$FRAP_1$	0.82***	0.62**	0.16	0.92*	0.76	0.53	0.68*	0.96***	0.08
$FRAP_{10}$	0.82***	0.62**	0.19	0.98**	0.82	0.69	0.69**	0.89***	0.07
$FRAP_{60}$	0.78***	0.73***	0.29	0.98***	0.77	0.75	0.70**	0.90***	0.20
$FRAP_{120}$	0.71***	0.79***	0.35	0.98**	0.78	0.76	0.64*	0.91***	0.29

[a] Correlation coefficient, *r*. Significance: *, $p \leq 0.05$; **, $0.05 < p \leq 0.01$; and ***, $0.01 < p \leq 0.001$. [b] Area (μC/nmol) at 300 mV and cumulative area (μC/nmol) at 400 (C400) and 800 mV (C800). [c] Phenolic compounds other than anthocyanins.

DISCUSSION

Antioxidant Activity. Many of the 20 flavonoids and cinnamic acid derivatives studied are prevalent components in fruits and berries and have thus previously been examined for their antioxidant properties. In the present study, ARP values obtained at the steady state and FRAP values after 60 min reaction times at room temperature are tabulated (**Table 2**). These results were in agreement with earlier findings for most of the compounds. However, in the FRAP and DPPH assays, where reaction rates are functions of both reaction time and temperature, the results will differ depending on the conditions used in the assays.

FRAP values for quercetin, quercetin-3-rutinoside, catechin, and caffeic acid reported earlier (*16*) were slightly higher than the present results, probably due to a higher reaction temperature (37 vs 23 °C). ARP values were similar to previously reported values for the same compounds (*7*, *23*, *30*). ARP values measured after 360 min (*30*) were higher than our results obtained after 120 min for slow reacting compounds but similar for fast reacting compounds. Compounds that react fast reduce a number of DPPH• molecules corresponding to the number of free hydroxyl groups on the compound (*7*). However, for slower reacting compounds, as were the majority of the compounds tested, the reaction mechanisms are more complex due to potential side reactions in the DPPH• assay, as dimerization between two phenoxyl radicals and complexation between phenoxyl radicals and DPPH• (*7*, *31*). ORAC values of the flavonoids were in the same magnitude range, 2.1−4.0 μmol TE/μmol, as previously reported (*26*, *32*, *33*), although some deviation in the ranking order occurred.

Electrochemical Characteristics. DPs determined by HPLC with a coulometric array detector reported in the literature are given together with our results in **Table 1**. The differences between the results for the flavonoids are probably due to how DP is defined. DP has been defined either as the potential where the maximum signal occurs (*25*, *26*, *34*) or as the potential corresponding to MDRP for each current plateau (*27*). The latter results in higher values for DP. For compounds with one oxidation wave, as the cinnamic acid derivatives, comparable DPs are reported in all studies (*25*−*27*, *34*). However, for compounds with more than one oxidation wave, information about further oxidation potentials is lost when DP is ascribed to the potential where the maximum signal occurs. Guo et al. (*26*) determined DP in that way and stated that flavonoids had high oxidation potentials (700−770 mV). However, other studies confirm the presence of two oxidation waves for the flavonoids (*25*, *27*). The first oxidation wave, from 100 to 300 mV, for the phenolic compounds is most likely attributed to oxidation of a catechol or a pyrogallol group. The presence of *o*-hydroxyl is important due to stabilization of the phenoxyl radical by hydrogen binding between the adjacent hydroxyls and the possible regeneration of another diphenol (*7*, *35*, *36*). Oxidation of flavonoids is known to occur in ring B, and substitution of this ring is of major importance for electron donating ability and thus antioxidant activity (*4*−*6*, *36*). In accordance with this, a decrease in the flavonoids' first oxidation potential was found to be a function of the number of hydroxyl groups on ring B (**Table 1** and **Figure 2**). When a flavonoid has a monohydroxyl on ring B, the structure of the remaining part of the molecule becomes more important (*37*). Conjugation with rings C and A can lead to a lower oxidation potential, as shown for kaempferol (**Table 1**). The electroactive groups responsible for the later oxidation waves (400−500 and 700−800 mV) are not as obvious. All flavonoids investigated had a 5,7-dihydroxyl on ring A; therefore, oxidation of this meta dihydroxyl group should not contribute to differences in electrochemical properties or antioxidant activities between the flavonoids. The 3-OH moiety of the C ring plays an important role for the antioxidant activity of flavonols via its interaction with ring B. Through a hydrogen bond, the B ring is held in the same plane as rings A and C, thus resulting in a completely planar molecule structure (*36*). Removal or substitution of the 3-OH induces a torsion angel in ring B and loss of conjugation. An increased oxidation potential and a lowered antioxidant activity of quercetin and kaempferol when glycosylated at 3-OH can be due to this reduced electron delocalization ability across the molecule. Moreover, the oxidizable (acidic) hydroxyl in C-3 is lost when glycosylated. Glycosylation of 7-OH in kaempferol did not affect electrochemical behavior or antioxidant activity of the molecule. No effect of different sugar moieties in the 3-OH position of quercetin or cyanidin was revealed in the electrochemical response, ORAC values, or ARP values measured at the steady state. However, quercetin and cyanidin glucosides had slightly higher FRAP activities and ARP values after a short reaction time (≤3 min) than the corresponding galactosides. Similar results have been reported for cyanidin, peonidin, and malvidin glycosides in a DPPH assay with a short reaction time (*38*). No explanation to these effects of sugar types has so far been given.

Electrochemical profiles and antioxidant activities of (+)-catechin and (−)-epicatechin were equal, meaning that diastereomization does not affect electrochemical properties. (+)-Catechin and (−)-epicatechin have, as quercetin, a catechol group in ring B and an OH in C-3 but lack the 2,3-double bond conjugated with the 4-carbonyl group in ring C, and therefore contain no oxidizable (acidic) hydroxyl in C-3 (**Figure 1**). This

results in a different electrochemical profile and antioxidant activity of flavan-3-ol as compared with the corresponding flavon-3-ol.

Dihydroxylation of a mono-hydroxycinnamic acid, i.e., *p*-coumaric acid vs caffeic acid, had a pronounced effect on the electrochemical response and radical scavenging ability, most likely due to the stabilization of the phenoxyl radical by the adjacent hydroxyl group. Esterification of the carboxylate group of caffeic acid had no influence on HDV or antioxidant activity. In contrast to when flavonoids are glycosylated, no electron delocalization can be destroyed in these one-ringed structures.

The accuracy at which DPs can be determined depends on the number of working electrodes and thereby voltage increments. Oxidation intervals of 60 mV have been suggested to be sufficient to allow for voltammetric resolution of compounds based on ease of oxidation (*25*). In the present study, increments of 60 mV were tested (results not shown). DPs achieved under these conditions were, as expected, more accurate and lower than the values presented in **Table 1**. As an example, the first DP of the quercetin glycosides was at 180 mV, while the first DPs for quercetin, the flavan-3-ols, and caffeic acid were at 120 mV as compared with DPs at 200 mV obtained for all of these compounds with increments of 100 mV. Different chromatographic conditions will most likely influence the electrochemical responses, as well (*39*). In the present study, the anthocyanins were analyzed in a separate chromatographic system. Therefore, a comparison between anthocyanins and other phenolic compounds should be made with care.

HDV depends on the concentration of the analyte. When the concentration exceeds the linear range for a single cell, the response will be shifted toward higher potentials. At low concentrations, however, the response may be too low to obtain a complete HDV. The linear concentration range for the coulometric array detector was high when the total response was considered, whereas the concentration range was considerably narrower for individual cells.

The importance of how electrochemical responses are achieved and defined has to be emphasized. For selectivity and identification of compounds, it is important to be aware of the linear concentration range for the individual cells in the coulometric array, to utilize shorter oxidation intervals for a more accurate determination of DP and to report all oxidation waves (DPs) obtained for a compound.

Electrochemical Profile—Prediction of Antioxidant Activity. Polyphenolic compounds are reducing agents, and their electrochemical responses are due to the donation of electrons. By evaluating electrochemical profiles and DPs of individual polyphenolics, it should be possible to estimate their antioxidant activity. The lower the oxidation potential, the higher is the ability to act as an antioxidant, since the value provides an estimate of the energy required to donate an electron.

The DPPH method is based on the reduction of a stable free radical ($DPPH^{\bullet}$) by an antioxidant. The initial fast electron and proton transfer is analogous to what happens in the coulometric detector, while several side reactions have been shown to occur in the DPPH assay when slow reacting compounds are involved (*7*, *31*). In the FRAP assay, the ability of the antioxidant to reduce a ferric complex to the ferrous form is measured. The redox potential for Fe(III)/Fe(II) is about 0.77 V. Thus, high oxidation potentials, close to 700 mV, for compounds such as kaempferol-3-rutinoside and *p*-coumaric acid, appeared to account for the poor antioxidant activity measured in the FRAP and probably the DPPH assay. The reducing ability measured in the FRAP assay did, not surprisingly, correlate well with electrochemical responses. A high correlation was obtained with ARP values determined at short reaction times, as well. The highest correlation between electrochemical response and antioxidant activity, as FRAP and ARP values, occurred at relatively low oxidation potentials (areas at 300 mV and cumulative areas at 400 mV). This is reasonable since many compounds with high oxidation potentials, which responded poorly in the FRAP and DPPH assays, were oxidized to the same extent as compounds with a lower oxidizing potential at the end of the coulometric array (at 800 mV). ORAC values were best predicted by a cumulative response at the end of the coulometric array. This could be explained since the radical scavenging reaction is taken to completion in the ORAC assay. A positive, linear correlation between ORAC activity and total peak height or area has previously been reported (*26*). MDRP for phenolic acids was inversely proportional to antioxidant efficiency as determined in a lipidic model or by the DPPH assay (determined by others) (*27*). However, no correlation was established between antioxidant activity and MDRP of flavonoids in that study. Even if MDRP or DP provides useful information, more knowledge about electrochemical behavior and thereby antioxidant activity would be obtained if responses and cumulative responses at different potentials were included in the analysis. Multivariate regression analysis can be utilized to find correlations between several X variables, as electrochemical responses, and one or more Y variables, as antioxidant activities. The relationships between samples and variables were illustrated in an informative way in the scores and loadings plots (**Figure 5**). These relationships were further confirmed by univariate regression analysis.

A screening method for radical scavenging components using postcolumn derivatization with DDPH has been developed (*40*). HPLC coupled with a coulometric array detector may be an easier alternative for the screening of antioxidant activities of unknown components. In addition to information about electrochemical behavior and antioxidant activity, results from a coulometric array detector may contribute to valuable knowledge about molecular structures due to the close relationship to the electrochemical profile.

ABBREVATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; ARP, antiradical power; β-PE, β-phycoerythrin; DP, dominant potential; DPPH, 2,2-diphenyl-1-picrylhydrazyl; C, cumulative; FRAP, ferric reducing activity power; HDV, hydrodynamic voltammogram; HPLC, high-performance liquid chromatography; MDRP, maximum detector response potential; ORAC, oxygen radical absorbance capacity; PC, (principal) component; PLS, partial least squares; SE, standard error; TEAC, Trolox equivalent antioxidant capacity; TRAP, total radical-trapping antioxidant parameter; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; TE, Trolox equivalents.

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