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Estimation of the Antioxidant Activities of Flavonoids from Their Oxidation Potentials

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A simple electrochemical method for estimating the antioxidant activity (AA) of flavonoids has been developed. The proposed method is based on a measurement of the half-wave potential ($E_{1/2}$) of the first oxidation wave of flavonoids by using flow-through column electrolysis. At the same time, the lipid peroxidation (LPO) inhibiting effects of these flavonoids were determined. A quantitative structure–activity relationship was obtained to describe the AA of flavonoids: $IC_{50}(\mu M) = 30.36 + 151.50E_{1/2}$ (V) – 12.63log P (r = 0.852), where IC_{50} represents the concentration for 50% inhibition of LPO, and P represents the octanol/water partition coefficient. This method is expected to be useful for the quick screening of flavonoid antioxidants, and evaluating the AA of flavonoid-containing foods and medicinal plants.

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Introduction

In addition to various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices, and different medicinal plants as well in beverages, such as wine, tea, and beer. They are frequently components of the human diet, and intake may reach 800 mg/day.[1] Over the past decade, evidence has been accumulated that flavonoids are an important class of antioxidants. They effectively suppress lipid peroxidation (LPO) in biological tissues and subcellular fractions, such as mitochondria, microsomes, liposomes, low-density lipoprotein (LDL), and erythrocyte membrane.[2-5]

Many evaluation methods of the antioxidant activity (AA) of flavonoids have been developed, such as active oxygen species (for example, superoxide anion, peroxyl radical, and hydroxyl radical) scavenging capability determination,[6-10] radical (not a natural free radical found in the body) scavenging activity determination, including 1,1-diphenyl-2-picrylhydrasyl (DPPH) radical[11] and 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonate radical cation ($ABTS^{\cdot+}$),[12] and enzymatic or nonenzymatic measurement of LPO-inhibiting effects.[13,14]

In order to predict the AA of flavonoids, flavonoid-containing foods, and medicinal plants, as well as for separating antioxidative ingredients, a simple method is needed. In this work, the AA of a series of flavonoids (Fig. 1) belonging to several subclasses was measured using flow-through column electrolysis, and a quantitative structure–activity relationship was proposed linking the LPO inhibitory effects of flavonoids to their half-wave potentials ($E_{1/2}$) and lipophilicity.

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Experimental

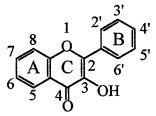
Chemicals

Quercetin dihydrate, baicalein, baicalin (>99.0%), daidzein (>97%), galangin, rutin, kaempferol (95%), daidzin (>99%), wogonin (>98%), fisetin, luteolin (>90%), naringenin, and puerarin (98%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (+)-Epicatechin (>98%), myricetin, silibinin, and morin were purchased from Sigma Chemical Co. (St. Louis, MO). Fustin, apigenin, and hyperoside were from Funakoshi Co. (Tokyo, Japan). (–)-Epicatechin (>98%), (–)-gallocatechin (>98%), (–)-gallocatechin gallate (>98%), (–)-catechin gallate (>98%), (–)-catechin (>98%), (–)-epigallocatechin (>98%), (–)-epicatechin gallate (>98%), (–)-epigallocatechin gallate (>98%), and (+)-catechin were obtained from Kurita Industrial Co. (Tokyo, Japan). All of these chemicals were used as received. Water was purified through a Nanopure II purification train (Sybron Branstead, USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), adenosine 5′-diphosphate monosodium salt (ADP), $FeCl_3$, and other reagents were from Wako Pure Chemical Industries, Ltd.

Flow-through column electrolysis

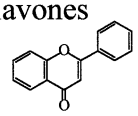
The half-wave potentials ($E_{1/2}$) of the first oxidation waves of flavonoids and the number of electrons (n) transferred in the first oxidation process were determined using flow-through column electrolysis. The system consisted of a PU-980 pump (Jasco, Tokyo, Japan), a DG-908-50 Degasser (Jasco, Tokyo, Japan), an 8125 injector fitted with a 5-μl injection loop (Reodyne, Cotati, USA), and a Potentiostat/Galvanostat HAB-151 (Hokuto Denko Ltd., Tokyo, Japan). The working electrode was carbon fiber threads (Nihon Carbon GF-20-P7 carbon cloth) packed in a Vycor glass cylinder (4 mm inner diameter and 10 mm length). The reference electrode was an

Flavonols



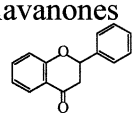
1) Galangin (3,5,7-trihydroxyflavone)
2) Fisetin (3,7,3',4'-tetrahydroxyflavone)
3) Kaempferol (3,5,7,4'-tetrahydroxyflavone)
4) Morin (3,5,7,2',4'-pentahydroxyflavone)
5) Quercetin (3,5,7,3',4'-pentahydroxyflavone)
6) Myricetin (3,5,7,3',4',5'-hexahydroxyflavone)
7) Rutin (5,7,3',4'-tetrahydroxyflavone-3-rutinoside)
8) Hyperoside (5,7,4',5'-tetrahydroxyflavone-3-glycoside)

Flavones



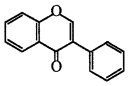
1) Baicalein (5,6,7-trihydroxyflavone)
2) Baicalin (5,6-dihydroxyflavone-7-glycoside)
3) Apigenin (5,7,4'-trihydroxyflavone)
4) Wogonin (5,7-dihydroxy-8-methoxyflavone)
5) Luteolin (5,7,3',4'-tetrahydroxyflavone)

Flavanones



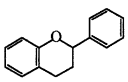
1) Fustin (3,7,3',4'-tetrahydroxyflavanone)
2) Naringenin (5,7,4'-trihydroxyflavanone)

Isoflavones



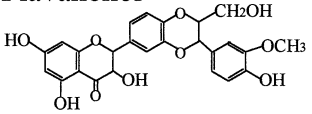
1) Daidzein (7,4'-dihydroxyisoflavone)
2) Daidzin (4'-hydroxyisoflavone-7-glycoside)
3) Puerarin (7,4'-dihydroxyisoflavone-8-C-glycoside)

Flavans



1) (-)-Catechin (3,5,7,4',5'-pentahydroxyflavan)
2) (+)-Catechin (3,5,7,4',5'-pentahydroxyflavan)
3) (-)-Epicatechin (3,5,7,4',5'-pentahydroxyflavan)
4) (+)-Epicatechin (3,5,7,4'-5'-pentahydroxyflavan)
5) (-)-Epicatechin gallate (5,7,4',5'-tetrahydroxyflavan-3-gallate)
6) (-)-Catechin gallate (5,7,4',5'-tetrahydroxyflavan-3-gallate)
7) (-)-Epigallocatechin (3,5,7,3',4',5'-hexahydroxyflavan)
8) (-)-Gallocatechin (3,5,7,3',4',5'-hexahydroxyflavan)
9) (-)-Epigallocatechin gallate (5,7,3',4',5'-pentahydroxyflavan-3-gallate)
10) (-)-Gallocatechin gallate (5,7,3',4',5'-pentahydroxyflavan-3-gallate)

Flavanonol



Silibinin {2,3-Dihydro-3-[4-hydroxy-3-methoxyphenyl]-2-[hydroxymethl]-6-[3,5,7-trihydroxy-4-oxobenzopyran-2-yl] benzodioxin}

Fig. 1 Structures of flavonoids.

Ag/AgCl electrode. The counter electrode was a platinum wire. The carrier solution was methanol:0.1 M phosphate buffer (pH 7.5) (1:1, v/v).

Microsomal preparations

Wistar rats (♂, *ca*. 300 g, 13 weeks) were decapitated. Their livers were excised immediately after being washed with 1.15% KCl *via* the portal vein, and stored at –80˚C. They were thawed in ice-cold water and homogenized in an ice-cold phosphate buffer [1.15% KCl, 10 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 3 ml/g tissue]. The homogenate was centrifuged at 10000*g* (20 min at 4˚C). Subsequently, the supernatant was centrifuged at 105000*g* for 60 min and again for 20 min. The microsomal pellet was resuspended in a phosphate buffer (50 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.4, 0.1 mM EDTA), and stored at –80˚C. The protein concentration was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, U.S.A.).

LPO assay

LPO was monitored by detecting the formation of malondialdehyde (MDA) using the thiobarbituric acid (TBA) assay.[15] Briefly, liver microsomes (1 ml) (*ca*. 500 μg of protein/ml) were incubated at 37˚C for 30 min in a water bath with ADP-$FeCl_3$, NADPH, and a series of concentrations of flavonoids. All substances were added on ice. The reaction was stopped by adding 50 μl of an aqueous solution of

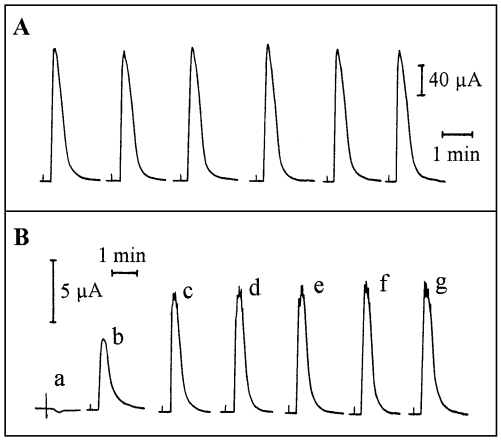


Fig. 2 Current-time curve of baicalein obtained from flow-through column electrolysis. Panel A was obtained at the applied potential of 0.05 V, when the concentration is 1.12 mM. The peak area is used to calculate the electrolytic charge and further the *n* value. Panel B was recorded at the different potentials of a) –0.10; b) –0.05; c) 0.00; d) 0.05; e) 0.10; f) 0.20 and g) 0.30 V, when the concentration is 0.26 mM.

trichloracetic acid (TCA) (100 w/v%). Upon cooling and subsequent centrifugation (10000 rpm, 10 min), the supernatant solution was mixed with TBA solution. The reaction mixture was heated in a water bath at 80˚C for 60 min. After cooling, the absorbance at 532 nm was read by a V-550 UV/Vis spectrophotometer (Jasco, Tokyo, Japan). The AA was calculated as percent inhibition relative to control using the following equation

$$\mathrm{AA}(\%) = \frac{(A_{c(30)} - A_{c(0)}) - (A_{s(30)} - A_{s(0)})}{(A_{c(30)} - A_{c(0)})} \times 100, \quad (1)$$

where $A_{c(30)}$ and $A_{c(0)}$ are the absorbance of the control at t = 30 and 0 min, respectively and $A_{s(30)}$ and $A_{s(0)}$ are the absorbance of the sample at t = 30 and 0 min, respectively. The IC_{50} values (concentrations needed to inhibit the reaction by 50%) were determined by interpolating the 50% inhibition point on a straight line fitted through concentrations which resulted in 10 to 90% inhibition.

The octanol/water partition coefficients of flavonoids were calculated using a log *P* calculation software (Pallas, CompuDrug Chemistry Ltd.). A multiparameter regression analysis was performed using NLRAna software (version 4.1f).

Results

Figure 2 shows typical current-time curves obtained by column electrolysis. The *n* value was calculated from the electrolytic charge (*Q*), determined from the peak area under the current-time curve at a potential of *ca*. 100 mV more positive than $E_{1/2}$, and the Faraday equation, written as

$$Q = Fcvn, \quad (2)$$

where *c* is the concentration (M) of flavonoids, *v* is the solution volume (l), and *F* is the Faraday constant (96500 coulomb/equivalent). The rate of the electrolysis was fast, and the *n* value was obtained with high reproducibility (Table 1). For most flavonoids tested, the *n* value was 2, which suggests

Table 1 The concentration of flavonoids for 50% inhibition of lipid peroxidation (IC_{50}), the half-wave potential ($E_{1/2}$) of the first oxidation wave, the number of electrons (n) transferred in the first oxidation, and their octanol/water partition coefficients (P)

Flavonoid	Substituent	IC_{50}/μM	$E_{1/2}$[a]/V	log P	$n \pm$ SD[b]
Flavonols					
Myricetin	3,5,7,3′,4′,5′-OH	10.5	–0.030	0.61	2.19 ± 0.03
Quercetin	3,5,7,3′,4′-OH	8.5	0.020	1.15	1.87 ± 0.05
Fisetin	3,7,3′,4′-OH	20.8	0.030	1.60	1.74 ± 0.03
Kaempferol	3,5,7,4′-OH	19.0	0.080	1.69	1.70 ± 0.01
Morin	3,5,7,2′,4′-OH	23.0	0.105	1.25	1.16 ± 0.01
Galangin	3,5,7-OH	13.8	0.280	2.11	1.25 ± 0.02
Rutin	5,7,3′,4′-OH-3-rutinose	93.5	0.180	–1.87	2.39 ± 0.04
Hyperoside	5,7,4′,5′-OH-3-glucose	59.5	0.185	–1.08	2.44 ± 0.02
Flavones					
Baicalein	5,6,7-OH	6.3	–0.060	2.14	1.83 ± 0.04
Baicalin	5,6-OH-7-glucose	20.1	0.080	–0.04	1.76 ± 0.04
Luteolin	5,7,3′,4′-OH	26.2	0.180	1.82	2.66 ± 0.03
Wogonin	5,7-OH-8-OCH_3	—[d]	0.360	2.65	1.67 ± 0.05
Apigenin	5,7,4′-OH	—[d]	>0.500[c]	2.36	
Flavanones					
Fustin	3,7,3′,4′-OH	66.0	0.132	0.95	2.08 ± 0.07
Naringenin	5,7,4’-OH	—[d]	0.590	1.85	2.46 ± 0.36
Isoflavones					
Daidzein	7,4′-OH	—[d]	0.500	2.69	1.76 ± 0.18
Daidzin	4′-OH-7-glucose	—[d]	0.538	0.86	0.96 ± 0.12
Puerarin	7,4′-OH-8-C-glucose	—[d]	0.540	–0.88	1.25 ± 0.10
Flavans					
(–)-(2*S*,3*R*)-Gallocatechin	3,5,7,3′,4′,5′-OH	29.3	–0.030	0.43	1.69 ± 0.02
(–)-(2*R*,3*R*)-Epigallocatechin	3,5,7,3′,4′,5′-OH	16.0	–0.035	0.43	1.95 ± 0.03
(–)-(2*R*,3*R*)-Epigallocatechin gallate	5,7,3′,4′,5′-OH-3-gallate	11.0	–0.020	1.67	1.43 ± 0.06
(–)-(2*S*,3*R*)-Gallocatechin gallate	5,7,3′,4′,5′-OH-3-gallate	13.0	–0.010	1.67	1.20 ± 0.04
(–)-(2*S*,3*R*)-Catechin gallate	5,7,3′,4′-OH-3-gallate	13.0	0.040	2.10	3.36 ± 0.16
(–)-(2*R*,3*R*)-Epicatechin gallate	5,7,3′,4′-OH-3-gallate	10.0	0.080	2.10	3.54 ± 0.20
(+)-(2*S*,3*S*)-Epicatechin	3,5,7,3′,4′-OH	25.0	0.082	0.86	2.32 ± 0.07
(–)-(2*R*,3*R*)-Epicatechin	3,5,7,3′,4′-OH	30.0	0.082	0.86	2.29 ± 0.05
(–)-(2*S*,3*R*)-Catechin	3,5,7,3′,4′-OH	38.0	0.092	0.86	2.06 ± 0.07
(+)-(2*R*,3*S*)-Catechin	3,5,7,3′,4′-OH	51.0	0.102	0.86	1.81 ± 0.06
Flavanonol					
Silibinin		98.5	0.450	1.70	1.90 ± 0.31

a. $E_{1/2}$ values were measured using column electrolysis. WE, carbon fibers; RE, Ag/AgCl; CE, Pt; carrier solution, methanol:0.1 M phosphate buffer (pH 7.5) (1:1, v/v). b. Standard deviation obtained using the data repeated 6 times. c. No obvious plateau was observed. Apigenin was oxidized at potentials higher than 0.500 V. d. IC_{50} > 100 μM.

quinones as a possible oxidation intermediate. A hydrodynamic voltammogram was generated by repeatedly injecting a sample into a flow-through system with the applied potential set to a new value for each injection. The resulting peak current of the current–time curve (Fig. 2 panel B) was measured and plotted against the applied potential. Figure 3 displays the hydrodynamic voltammograms of flavonoids, from which $E_{1/2}$ was measured. Most flavonoids showed an obvious oxidation peak. The potential range of the flavonols was from – 0.05 V to 0.40 V. The oxidation potentials of five flavones tested spanned a wide potential range of more than 0.6 V. Although only three flavonoids were tested, it is obvious that the flavanones exhibit a wide potential range. It was characteristic for the three isoflavones that they displayed the first oxidation peak at *ca*. 0.5 V. For the flavans, there is not much difference in the oxidation potential in this group. There was an appreciable difference in the $E_{1/2}$ value of the flavonoids; the range was about 0.60 V. The $E_{1/2}$ values were found to depend greatly on the structures, as shown in Table 1. Pyrogallol-containing flavonoids [except for (–)-epicatechin gallate and (–)-catechin gallate] had rather low $E_{1/2}$ values, less than –0.01 V. Baicalein, having an *o*-trihydroxyl group on the A ring, showed the lowest $E_{1/2}$ value among the flavonoids tested. The $E_{1/2}$ values of (–)-epicatechin gallate and (–)-catechin gallate were on the same order as the catechol-containing flavonoids and catechol-free flavonols. The range was from 0.02 V to 0.28 V. Among these flavonoids, quercetin and fisetin [which contain both an *o*-dihydroxyl group on the B ring and a flavonol basic structure (a 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group)] showed lower $E_{1/2}$ values than those flavonoids having either a catechol structure or the flavonol basic structure. Isoflavones and other catechol-free and pyrogallol-free flavonoids all possessed high $E_{1/2}$ values, more positive than 0.36 V. The structural principles governing the $E_{1/2}$ values of the flavonoids were found to be: 1) the pyrogallol group; 2) the catechol group; 3) the coexistence of the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group; and 4) additional resonance-effective substituents.

The values of IC_{50} in Table 1 show that 23 of the 29 flavonoids investigated exhibited obvious AA in LPO. Good linear relationships between the concentration and the activity were found for these flavonoids. All of the flavonols (free 3-

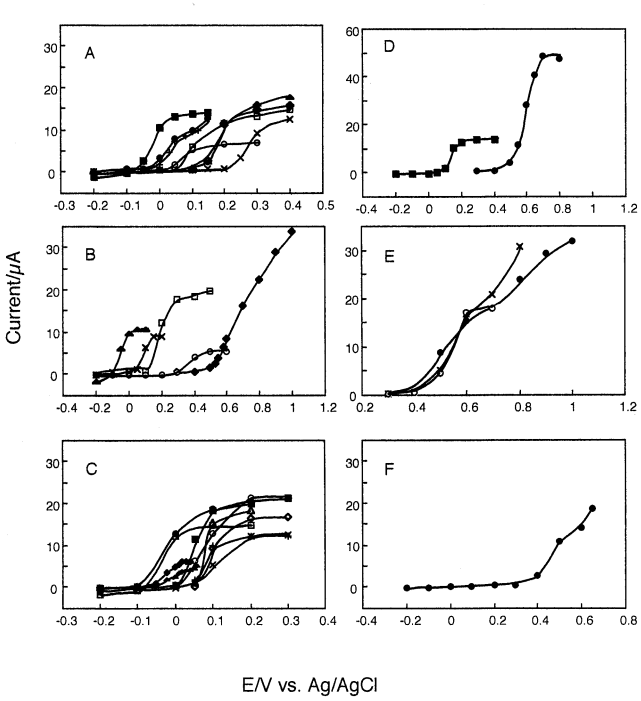


Fig. 3 Hydrodynamic voltammograms of flavonoids obtained in methanol:0.1 M phosphate buffer (pH 7.5) (1:1) carrier solution with a flow rate of 0.5 ml/min. A: flavonols, myricetin (0.27 mM, ■), quercetin (0.21 mM, ●), fisetin (0.24 mM, +), kaempferol (0.15 mM, ○), morin (0.38 mM, □), galangin (0.32 mM, ×), rutin (0.24 mM, ◆) and hyperoside (0.25 mM, ▲); B: flavones, baicalein (0.26 mM, ▲), baicalin (0.18 mM, ×), luteolin (0.25 mM, □), wogonin (0.11 mM, ○) and apigenin (0.14 mM, ◆); C: flavans, (–)-gallocatechin (0.31 mM, □), (–)-epigallocatechin (0.33 mM, ●), (–)-catechin gallate (0.14 mM, ■), (–)-epicatechin gallate (0.11 mM, ○), (–)-epicatechin (0.16 mM, △), (+)-epicatechin (0.13 mM, +), (–)-catechin (0.18 mM, ◇), (+)-catechin (0.17 mM, ×), (–)-epigallocatechin gallate (0.14 mM, ◆) and (–)-gallocatechin gallate (0.10 mM, ▲); D: flavanones, fustin (0.18 mM, ■), naringenin (0.33 mM, ●); E: isoflavones, daidzein (0.21 mM, ●), daidzin (0.26 mM, ○) and puerarin (0.32 mM, ×); F: flavanonol, silibinin (0.18 mM, ●).

hydroxyl group in the C ring) showed a high AA, with little difference. Among these, quercetin (3,5,7,3′,4′-pentahydroxyflavone) was the most active antioxidant with an IC_{50} of 8.5 μM, in the range of the most powerful flavonoid antioxidant. Blocking the 3-hydroxyl group of quercetin with a glycoside as in rutin and hyperoside greatly increased the IC_{50} to a value of 93.5 μM and 59.5 μM, respectively. Within the flavone subclass, baicalein (a pyrogallol-containing flavonoid) was the most powerful antioxidant among the flavonoids investigated. Its corresponding glycoside baicalin showed a large increase in the IC_{50} value of 20.1 μM, on the same order as luteolin. As for the flavanones, fustin (a catechol-containing flavanonol) had an IC_{50} of 66 μM. In the flavan series, nongalloylated catechins were moderately active antioxidants, while the galloylated catechins were highly active antioxidants. Among the flavonoids tested, pyrogallol-containing flavonoids showed a more powerful AA. Flavonoids having an $E_{1/2}$ value higher than 0.45 V, including all of the isoflavones investigated, were inactive. A quantitative structure–activity relationship (QSAR) was obtained to describe the AA of 23 flavonoid antioxidants, shown by a multiparameter equation (Fig. 4)

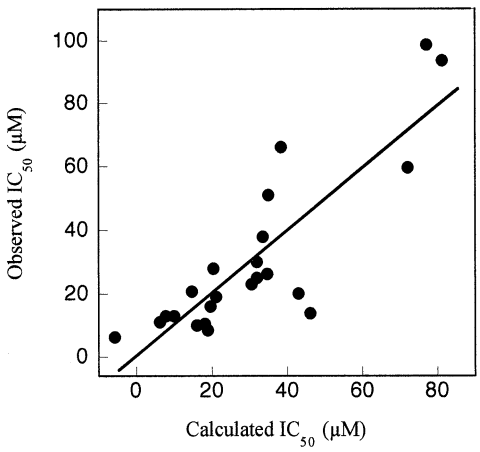


Fig. 4 Plot of observed IC_{50} values against calculated IC_{50} values using Eq. (3) (r = 0.852).

$$IC_{50}(\mu M) = 30.36 + 151.50\,E_{1/2}(V) - 12.63 \log P \quad (r = 0.852), \qquad (3)$$

where IC_{50} represents the concentration for 50% inhibition of LPO, $E_{1/2}$ represents the half-wave potential of the first oxidation wave measured by flow-through column electrolysis, and P represents the octanol/water partition coefficient calculated by software. One should note that when the value of $E_{1/2}$ exceeds 0.45 V, this equation is not applicable, because flavonoids having higher $E_{1/2}$ values are inactive in this assay.

Discussion

Determination of the antioxidant activity

Many methods have been established to study the AA of flavonoids. Accumulated data demonstrate that the AA of individual flavonoids is easily affected by the experimental environment; *i.e.*, the AA data reflect only the specific ability in each corresponding system. For example, data obtained in a trolox (a water-soluble vitamin E analog) equivalent antioxidant capacity (TEAC) assay indicated the ability of flavonoids to scavenge $ABTS^{\cdot+}$.[12] In the oxygen radical absorbance capacity (ORAC) assay,[16] the AA data stood for the capacity inhibiting β-phycoerythrin (β-PE) peroxidation initiated by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). In order to screen the antioxidant flavonoids, a simple method reflecting the essential AA is needed. In general, the AA of flavonoids is regarded to be related to 1) scavenging free radicals; due to their lower redox potentials, flavonoids are thermodynamically able to reduce highly oxidizing free radicals, such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals;[17-19] 2) chelating transition-metals involved in free-radical production;[8,20] and 3) inhibiting the enzymes participating in free-radical generation, such as xanthine oxidase:[21] this effect may be partially due to flavonoids' undergoing redox cycling of enzymes. Among these three factors, the iron-chelating effect is confusing. Some investigators recognized that the antioxidative and lipid peroxidation-inhibiting potential of flavonoids predominantly resided in the radical-scavenging capacity rather than the chelation of metals.[22,23] Other reports demonstrated that the presence of transition metals caused flavonoids to act as prooxidants instead of antioxidants.[16] Thus, irrespective of the iron-chelating parameter, the chemical characteristics in terms of ease of oxidation for flavonoids could be used as an index of

their AA, at least for the essential AA. The present work confirmed this assumption from two aspects. First, the AA of flavonoids is inversely proportional to their $E_{1/2}$ values, *i.e*., the lower the $E_{1/2}$ of flavonoids is, the higher the AA is. This was also supported by van Acker *et al*.[14,23] Second, the structural principles governing the $E_{1/2}$ values of flavonoids (pyrogallol group, catechol group, and the coexistence of the 2,3-double bond in conjugation with 4-oxo and 3-hydroxyl groups) are also the determinants for radical scavenging and/or antioxidative potential suggested by Bors *et al*.:[24] 1) the *o*-dihydroxy structure of the B ring; 2) the C2–C3 double bond in conjugation with a 4-oxo function; and 3) the additional presence of both 3- and 5-hydroxyl groups.

In van Acker's investigations,[14,23] the midpoint potentials of the flavonoids were determined by cyclic voltammetry (CV). When using CV, some compounds were irreversibly oxidized along with adsorption or a kinetic reaction, resulting in a modification of the cyclic voltammograms. In the case of flow-through column electrolysis, the large surface area of the working electrode and the thin layer of the test solution made high efficiency and rapid electrolysis possible,[25,26] and thus less interference reaction was expected to affect the electrode process, compared to CV. Consequently, accurate data can be obtained. At the same time, the number of electrons transferred in the oxidation process can be simultaneously measured with a higher reproducibility compared to that obtained from a carbon packed-bed bulk electrolysis flow cell.[27]

The continuous-flow column electrolytic method, in which the sample solution flows continuously through the column electrode, can usually produce efficient and rapid electrolysis.[25] Comparatively, our method proposed herein has some advantage features, such as a requirement of smaller amounts of samples, easier operation and a shorter assay time. These advantages made the assay more efficient when scanning antioxidant flavonoids in plants or estimating the AA of natural compounds.

Using HPLC coupled with coulometric array detection, Peyrat-Maillard *et al*. confirmed a similar relationship between the AA of phenolic acids and their potential corresponding to maximal detector response (MDRP).[28] Unfortunately, no relationship could be established for flavonoids. In our understanding, this might be attributed to the evidently overestimated MDRP values. For example, the same MDRP data of quercetin and naringenin are in marked contrast to their structures, and quite different from our own and other investigators' data: the oxidation potential of quercetin is much lower than that of naringenin.[23,29,30]

Effect of lipophilicity

The ability of the flavonoids to interact with the lipid bilayers, including their incorporation rate into cells and their orientation, was suggested to be an important factor of their AA in biological systems.[31] The present work showed that a flavonoid lipophilicity too high or too low hampers the AA in LPO system. For example, luteolin, rutin, and hyperoside all have the same $E_{1/2}$ value; the additional sugar moiety, in the case of rutin and hyperoside, greatly decreased the lipophilicity, resulting in the corresponding great increase in the IC_{50} value. In the case of wogonin, although its $E_{1/2}$ value was 0.09 V lower than that of silibinin (IC_{50} = 98.5 μM), it was inactive possibly due to its too high lipophilicity. It should be noted that the effect of a biomembrane might be complicated, not simply increasing or decreasing linearly with increasing lipophilicity. This was supported by the findings of Beyeler *et al*.,[32] who reported that the effects of cianidanols on rat hepatic monooxygenase increased with lipophilicity, reached a plateau, decreased and leveled off for the most lipophilic compounds. Using our $E_{1/2}$ data, log *P* values, and the antioxidant data in references, linear relationships were established with good *r* (0.81 – 0.91);[13,14,16,33] the $E_{1/2}$ term always has a negative coefficient, implying an inverse proportion, while the coefficient of the log *P* term changed in each assay. This also implied the complex effect of membranes.

Relationship between AA and structure

Many investigators have focused on establishing the relationship between flavonoid structure and AA.[34-36] The SAR obtained from the present work showed that structures facilitating electron delocalization across the molecule of parent flavonoids and contributing to an increase in the stability of the aroxyl radicals[24,37] are important determinants of the AA of flavonoids, such as *o*-trihydroxyl groups, *o*-dihydroxyl groups, and the coexistence of the 2,3-double bond in conjugation with 4-oxo and 3-hydroxyl groups. The last two structures were consistent with what Bors suggested, and the 3-hydroxyl group was suggested to determine the fate of the flavonoid aroxyl radical.[38,39] Lacking any one of these structures would influence the delocalization, resulting in a decrease of LPO inhibition. More specifically, quercetin, a 3,5,7,3′,4′-pentahydroxyflavone, was the second strongest antioxidant. Glycosylation of the 3-hydroxyl group, as in rutin and hyperoside, or removal of the 3-hydroxyl group, as in luteloin, greatly reduced the activity. The saturated heterocyclic ring and no 4-oxo function of (+/–)-(E)C lead to a decrease of the IC_{50} values. Lacking the 2,3 double bond, fustin (3,7,3′,4′-tetrahydroxyflavanone) showed a lower inhibitory effect than that of fisetin (3,7,3′,4′-tetrahydroxyflavone). Catechol-containing flavonoids are highly active compared with the corresponding catechol-free flavonoids.

Completing what Bors suggested, we proposed that the pyrogallol group is an important component of the AA of flavonoids, its location also affecting the AA. Compared with baicalein (with an *o*-trihydroxyl group on the A ring), those having an *o*-trihydroxyl group on the B ring [as in myricetin and (–)-(epi)gallocatechin] and/or galloylation of the 3-hydroxyl group [as for (–)-(epi)catechin gallate and (–)-(epi)gallocatechin gallate] are less active. Similarly, baicalin was more potent than the corresponding flavonoids [such as luteolin, rutin, hyperoside, fustin and (+/–)-(epi)catechin] because of the presence of the *o*-dihydroxyl group on the A ring.

Conclusion

Flavonoids are important components in the human diet. The level of intake of flavonoids from the diet is quite high compared to those of vitamin C (70 mg/day), vitamin E (7 – 10 mg/day), and carotenoids (*β*-carotene, 2 – 3 mg/day). The intake of flavonoids can range between 50 and 800 mg/day, depending on the consumption of vegetables and fruits, and of specific beverages, such as red wine, tea, and unfiltered beer.[1] The dietary intake of flavonoid-containing foods was suggested to be of benefit in lowering the risk of certain pathophysiologies that have been associated with free-radical-mediated events, including coronary heart disease and ischemia-reperfusion injury.[36] In our work, the tested flavonoids commonly found in plants covered six important subclasses: flavonols, flavones, flavanones, flavanonols, flavans, and isoflavones. The evidence presented herein and previously suggests that the electrochemical characteristics of flavonoids may play a crucial

role in their AA, and thus the method established herein is expected to be a simple method for screening flavonoid antioxidants and estimating the AA of flavonoid-containing foods and medicinal plants.

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