JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Analysis of the Antioxidant Capacities of Flavonoids under Different Spectrophotometric Assays Using Cyclic Voltammetry and Density Functional Theory

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ABSTRACT: Flavonoids often show inconsistent antioxidant activities (AAs) depending on the assay used. The electrochemical properties of 14 flavonoid standards in cyclic voltammetry [area under anodic wave (Q) and oxidant peak potentials (E_{pa})] and the structural parameters [bond dissociation enthalpy (BDE) and ionization potential (IP)] were investigated. They were compared with the results of four spectrophotometric assays, namely, diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent (FCR), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), to analyze the chemical reasons for the varying AAs of flavonoids under different assays. Using the cyclic voltammetry method, the AAs of the flavonoids in the DPPH, FCR, and FRAP assays were mainly determined by the ease of charge transferring in the first oxidation step. Meanwhile, the results of TEAC assays were primarily influenced by the amount of charge transfer in the multiple oxidation steps (MOS) of flavonoids. In the theoretical calculation, the BDE values of the selected flavonoids had considerably higher correlations with the results of the DPPH assay ($r^2 = 0.89$) compared with the other three assays, which indicates that the oxidant-scavenging reaction of the tested flavonoids in the DPPH assay is closer to a hydrogen atom transfer mechanism. Neither the IP values nor BDE values had satisfactory correlation with the AAs of the flavonoids in the TEAC assay ($r^2 = 0.57$, $r^2 = 0.54$, respectively), Therefore, complex reaction mechanisms underlie this method and appropriate structural descriptors for reflecting the AAs of flavonoids based on MOS (e.g., TEAC values) need further investigation.

KEYWORDS: DPPH, FCR, FRAP, TEAC, area under anodic wave, oxidation peak potential, multiple oxidation steps, BDE, IP

INTRODUCTION

Flavonoids are a class of widely distributed phytochemicals with broad pharmacological activities, including antitumor, antiviral, and anti-inflammatory actions.[1] Interest has been focused to the antioxidant activities (AAs) of flavonoids such as their ability to inhibit free radical formation and scavenge free radicals.[2] Many chemical methods have been developed for assessing their antioxidant capacity. The majority of prevailing methods are based on the scavenging capacity assays against specific oxidants such as the diphenyl-1-picrylhydrazyl (DPPH) radical, Fe(III), and so on.[3] However, different oxidants may oxidize flavonoids to different degrees, depending on the thermodynamic properties of the oxidants and the reactivity of the flavonoid hydroxyl groups.[4] These factors provide flavonoids with different antioxidant capacities.[5] Therefore, assessing their antioxidant capacity is difficult. Meanwhile, the use of only one method is unsuitable for evaluating antioxidant capacity, because multiple oxidants are present in biological systems and food materials. On the other hand, the results obtained using different methods in the various reaction systems are inconsistent and are often not comparable. This contradiction hinders the understanding of the antioxidant capacity of flavonoids.

The aforementioned problem caused by the diversity of oxidants can be resolved using electrochemical oxidation. It can be used as a model for oxidant-scavenging reactions because both reactions involve the cleavage of the same O−H bond and the donation of e^- and H^+.[6] In addition, it can realize the electrooxidation at the desired oxidation strength through setting certain potentials. Hence, different oxidant-scavenging assays, especially for methods containing electron-transferring process, can be simulated in the same experimental system.

Cyclic voltammetry (CV) is a widely used electrochemical technique. Its three parameters are (1) the anodic peak current (I_a), (2) the peak oxidation potential (E_{pa}), and (3) the area under anodic current wave (Q). These parameters have been applied to analyze the antioxidant capacity of samples and they have different chemical meanings. The E_{pa} value reflects the redox properties of antioxidants, and the I_a and Q values are related to the amount of charge transferred. The area under the anodic wave (Q) has been proposed as a better parameter for reflecting the antioxidant capacity of samples rather than I_a.[7] However, the relationships between the CV parameters (E_{pa}, Q) and the results of the antioxidant capacity assessment were unclear. Specifically, the correlations between the AAs of samples differed with the applied methods.[8,9] Similarly, the selectivity of reverse potential has great influence on the correlations between the Q values and the results of chemical assessment assays,[10−12]

Received: May 5, 2011
Accepted: August 8, 2011
Revised: July 9, 2011
Published: August 08, 2011

but the reverse potentials adapted to each antioxidant capacity evaluation assay are still undetermined. Moreover, most previous studies compared the electrochemical index of mixtures (plasma, drinks, plant extracts, and so on) with their antioxidant capacity in some spectrophotometric methods.[13] With these results, elaborating the chemical connections between the electrochemical assay and common spectrophotometric assays was difficult because of the complex compositions of mixtures. Recently, He et al.[4] clarified the assay-dependent antioxidant capacity of only two flavonol standards through voltammetric study, but it could not clarify the regularity of flavonoids on antioxidant activity variation in different assays.

In the current study, the E_{pa} values of 14 flavonoid standards and their Q values, which corresponds to the different reverse potentials, were investigated and fully compared with the results of four spectrophotometric assays. This will be helpful in finding possible an explanation for the antioxidant capacity of flavonoids in different spectrophotometric assays. The selected flavonoids, namely, flavonol, flavones, flavanones, flavanol, and isoflavones, represent the different basic structural units. The ferric-reducing ability of plasma (FRAP), DPPH, Trolox equivalent antioxidant capacity (TEAC), and Folin−Ciocalteu reagent (FCR) assays, the most widely used antioxidant capacity assessment methods, were chosen as tested spectrophotometric methods. In addition, the structural parameters of flavonoids [bond dissociation enthalpy (BDE) and ionization potential (IP)] were also introduced to discuss further the AAs of flavonoids in terms of reaction mechanisms.

MATERIALS AND METHODS

Chemical Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri-(2-pyridyl)-*s*-triazine (TPTZ),Trolox, apigenin, (+)-catechin, daidzein, (−)-epicatechin, genistein, hesperetin, isorhamnetin, kaempferol, luteolin, myricetin, quercetin, quercitrin, naringenin, and taxifolin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

DPPH Assay. All the spectrophotometric assays in this study were conducted using a microplate spectrophotometer system (Spectra Max M2^e, Molecular Devices). The radical-scavenging activity of 2,2-diphenyl-1-picryhydrazyl (DPPH) was assayed according to the method described by Tadolini et al.,[14] which was modified for use in a 96-well microplate. Briefly, a 20 μL sample (flavonoid standards or Trolox) and 180 μL of 0.06 mM DPPH solution were mixed in each microplate. The absorbance of samples at 517 nm was determined after 1 h. For each sample, a blank with 180 μL methanol instead of the DPPH was included to correct for any sample absorbance at 517 nm. The standard curve was linear between 0.1 and 0.5 mM Trolox.

TEAC Assay. The procedure for the TEAC assay followed that by Re et al.[15] with some modifications. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and kept in the dark for 12−16 h before being used. The $ABTS^{\bullet+}$ solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 at 734 nm. In the assay, 10 μL of the sample (flavonoid standards or Trolox) and 190 μL of $ABTS^{\bullet+}$ solution were mixed in microplate wells. The absorbance at 734 nm was determined after 6 min. For each sample, a blank with 190 μL of PBS instead of the ABTS reagent was included to correct any sample absorbance at 734 nm. The standard curve was linear between 0.1 and 1.0 mM Trolox.

FRAP Assay. The FRAP reagent was prepared according to the report by Benzie and Strain.[16] The reagents included 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$. The working FRAP reagent was prepared as required by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. Then, 150 μL of freshly prepared FRAP reagent was heated to 37 °C and 5 μL of the sample was then added into the wells, along with 15 μL of H_2O. After 30 min, absorbance readings were recorded at 593 nm.

FCR Assay. The FCR assay was conducted according to the method described by Singleton and Rossi[17] with some modifications. Appropriately diluted flavonoids (20 μL) were mixed with 150 μL of Folin−Ciocalteu reagent, and after 5 min, 30 μL of sodium carbonate (20% w/v) was added. The mixture was maintained at 37 °C for 30 min. The absorbance was measured at 750 nm. Chlorogenic acid (CA) (0.25−2.0 mM) was used as the standard. The results are expressed as CA equivalent (CAE) per millimole of standard.

Cyclic Voltammograms of Flavonoids. Electrochemical measurements were conducted on a Model CHI 620 C electrochemical analyzer (CHENHUA, Shanghai, China). Cyclic voltammetric measurements were done using a three-electrode system. The working electrode is a glassy carbon electrode with a Ag/AgCl electrode as reference. A platinum foil served as the auxiliary electrode. The glassy carbon working electrode was polished successively with 1, 0.3, and 0.05 μm alumina powder before each scanning. The supporting electrolytes were the 0.2 M Britton−Robinson buffered solutions (BRS) at pH 7.0 with 0.3 M KCl. Flavonoid standards were prepared with methanol as the solvent and diluted to the same 150 μM concentration using a mixing solvents of BRS:methanol (80:20, v:v). Prior to each run, the dissolved oxygen in flavonoid solutions was removed by bubbling with N_2 for about 15 min. Voltammetric scans were carried out from −0.4 V versus Ag/AgCl to different reverse potentials at a scan rate of 400 mV s^{-1} at room temperature (about 25 °C). The range of reverse potential was from 0.2 to 1.2 V. The area under the anodic current wave (Q) and oxidation peaks (E_{pa}) were obtained with EC Application Software.

Quantum Chemical Calculations. The geometries of all molecules studied were fully optimized with the density functional theory (DFT), using the B3LYP functional (UB3LYP for the corresponding radicals), and a 6-311++G(d,p) basis set as implemented in the Gaussian 03 computational programs suite (Gaussian Inc., Pittsburgh, PA).[18] All structures were true minima on the calculated potential energy surface (PES), verified by final frequency calculations that provided energy minima with certainty. The bond dissociation enthalpies (BDE) for the O−H bond in the parent flavonoid molecules were calculated as follows:

$$\mathrm{BDE} = H(\mathrm{ArO}^{\bullet}) + H(\mathrm{H}^{\bullet}) - H(\mathrm{ArOH})$$

where ArOH represents the parent molecule and $ArO^{\bullet}$ is the corresponding radical. The $H^{\bullet}$ enthalpy value was calculated to be −0.499 897 hartree at 6-311G++(d,p). The ionization potential (IP) is expressed as the energy level of highest occupied molecular orbital (E_{HOMO}) according to Koopmans' theory.[19]

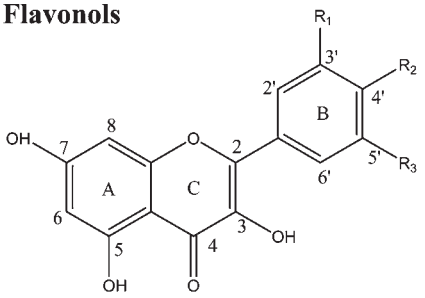
Statistical Analysis. All samples were prepared in triplicate. Each sample analysis was performed in triplicate. All results presented are means (±SD) of at least three independent experiments. Statistical analysis (ANOVA with a statistical significance level set at $P < 0.05$ and linear regression) was carried out with SPSS 13.0 (SPSS, Inc., Chicago, IL).

RESULTS

Antioxidant Activities (AAs) of Selected Flavonoids with Different Spectrophotometric Assays. The antioxidant capacity of flavonols, flavones, flavanones, flavanols, and isoflavones (Figure 1) were evaluated using DPPH, TEAC, FRAP, and FCR assays. The results are shown in Table 1.

In the DPPH assay, the AA of quercetin was over twice higher than that of Trolox. Following this, (+)-catechin, myricetin, isorhamnetin, luteolin, (−)-epicatechin, taxifolin, and quercitrin

Flavonols



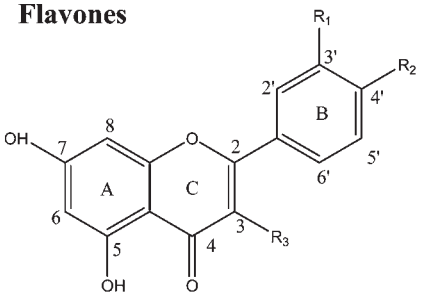
Quercetin: R_1= OH R_2 = OH R_3 = H

Myricetin: R_1 = OH R_2 = OH R_3 = OH

Isorhamnetin: R_1 = OCH_3 R_2 = OH R_3 = H

Kaempferol: R_1 = H R_2 = OH R_3 = H

Flavones

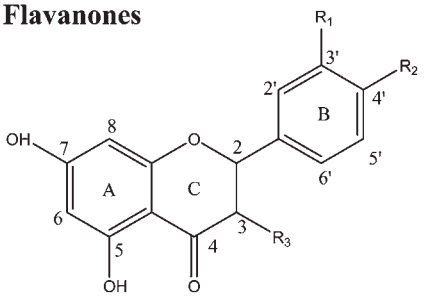


Luteolin: R_1= OH R_2 = OH R_3 = H

Apigenin: R_1= OH R_2 = OH R_3 = H

Quercitrin: R_1= OH R_2 = OH R_3 = O-glu

Flavanones

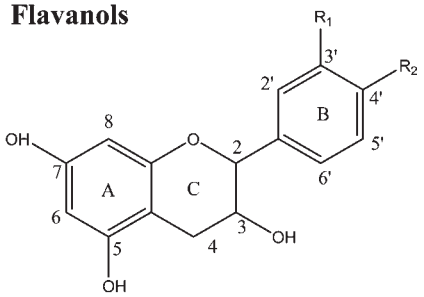


Taxifolin: R_1= OH R_2 = OH R_3 = OH

Hesperetin: R_1= OH R_2 = OCH_3 R_3 = H

Naringenin R_1= OH R_2 = OH R_3 = H

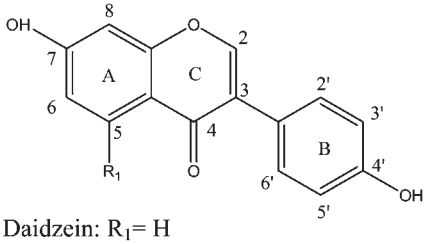
Flavanols



Catechin: R_1= OH R_2 = OH

Epicatechin: R_1= OH R_2 = OH

Isoflavones



Daidzein: R_1= H

Genistein: R_1= OH

Figure 1. Basic structures of flavonoids considered in the present study.

were more efficacious than Trolox, whereas kaempferol, naringenin, and hesperetin presented lower activity than Trolox. Daidzein, genistein, and apigenin had no quantifiable scavenging activity against DPPH radical. The aforementioned ranking order is similar to the results reported by Cai et al.[20]

All the selected flavonoids exhibited greater radical scavenging capacity than Trolox in the TEAC assay. Quercetin and isorhamnetin were the best radical scavengers. Their Trolox equivalent (TE) values were 5.72 and 5.06 mM, respectively, followed by myricetin, taxifolin, and kaempferol, which had similar TE values ($P > 0.05$). The AAs of the remaining flavonoids in the $ABTS^{\bullet+}$ radical scavenging reaction were (−)-epicatechin ≈ (+)-catechin, and genistein ≈ quercitrin ≈ luteolin ≈ apigenin > daidzein > hesperetin > naringenin. The AAs of taxifolin and kaempferol in the TEAC assay were different from the result reported by Rice-Evans et al.,[21] but the antioxidant activity ranking of taxifolin in the TEAC assay was similar with the result reported by Ishige et al.[22]

The total reducing activities of the selected flavonoids were measured with a FRAP assay in which quercetin had the highest reducing capacity among the selected flavonoids. The reducing activity was 5 times higher than that of Trolox. Myricetin had the second highest reducing activity. On the other hand, hesperetin, naringenin, daidzein, apigenin, and genistein showed weak reducing activities, which were similar to the results by Firuzi.[7]

Several selected flavonoids, such as quercitrin, (+)-catechin, hesperetin, naringenin, daidzein, apigenin, and genistein, had

Table 1. AAs of Flavonoids in Different Spectrophotometric Assays and Their Structural Parameters

	spectrophotometric assays						
	TEAC[a]	FCR[b]	FRAP	DPPH	n_{OH}	IP[d]	BDE
			Flavonols				
quercetin	5.72 ± 0.16 a[c]	1.24 ± 0.09 a	5.57 ± 0.50 a	2.25 ± 0.09 a	5	553.826	299.284
myricetin	4.54 ± 0.32 c	1.02 ± 0.10 b	4.15 ± 0.38 b	1.89 ± 0.05 b	6	555.755	308.016
isorhamnetin	5.06 ± 0.15 b	1.04 ± 0.06 b	3.81 ± 0.29 bc	1.40 ± 0.08 d	4	554.790	310.112
kaempferol	4.19 ± 0.21 cd	1.08 ± 0.03 b	3.15 ± 0.34 de	0.83 ± 0.03 e	4	557.685	334.362
			Flavones				
luteolin	3.03 ± 0.19 gh	0.91 ± 0.02 cd	2.42 ± 0.06 f	1.65 ± 0.17 c	4	592.420	307.981
apigenin	2.83 ± 0.09 h	0.39 ± 0.01 g	0 h	0 h	3	603.033	342.181
quercitrin	3.28 ± 0.20 fg	0.81 ± 0.08 de	2.92 ± 0.18 ef	1.65 ± 0.22 c	4	579.854	309.100
			Flavanones				
taxifolin	4.23 ± 0.50 c	0.99 ± 0.12 bc	3.54 ± 0.40 cd	1.35 ± 0.01 d	5	606.893	313.569
hesperetin	2.01 ± 0.04 j	0.53 ± 0.03 f	0.83 ± 0.39 g	0.48 ± 0.04 f	3	586.631	348.245
naringenin	1.29 ± 0.09 k	0.55 ± 0.03 f	0.08 ± 0.03 h	0.27 ± 0.03 g	3	612.682	346.865
			Isoflavones				
daidzein	2.43 ± 0.16 i	0.40 ± 0.02 g	0.20 ± 0.02 h	0 h	2	586.631	339.025
genistein	3.50 ± 0.32 ef	0.33 ± 0.06 g	0.27 ± 0.03 h	0 h	3	576.017	337.779
			Flavanols				
catechin	3.75 ± 0.06 e	0.77 ± 0.06 e	3.12 ± 0.54 de	1.78 ± 0.14 bc	5	575.052	306.477
epicatechin	3.84 ± 0.11 de	1.05 ± 0.07 b	3.38 ± 0.45 cde	1.64 ± 0.14 c	5	575.052	303.126

[a] The results of TEAC, FRAP, and DPPH were expressed as Trolox equivalent (TE) per mmol standard. [b] The results of FCR assay was expressed as chlorogenic acid equivalent (CAE) per mmol standard. [c] Values with no letter in common are significantly different ($P < 0.05$). [d] The BDE and IP values were expressed as kJ mol^{-1}. BDE value refers to the bond dissociation enthalpy of the most easily dehydrogenable OH group (according to calculations).

obviously lower reducing capacity than chlorogenic acid in the FCR assay. Kaempferol, myricetin, (−)-epicatechin, and isorhamnetin showed activities similar to that of chlorogenic acid, and quercetin showed the highest reducing activity.

The DPPH and TEAC assays are two of the most important chemical methods for evaluating the radical-scavenging capacity of antioxidants. Both of the assays used electron-deficient radicals. However, the correlations between their results were not high ($r^2 = 0.47$), which is similar to the result reported by Tabart et al.[5] In the DPPH assay, no antioxidant capacity was observed in daidzein, genistein, and apigenin, whereas all of these flavonoids exhibited greater activities than Trolox in TEAC assay.

The FRAP and FCR assays measure reducing capacity based on the reduction of Fe(III) and Mo(VI), respectively. The results showed that the Fe(III)-reducing activities of selected flavonoids are well-correlated with their Mo(VI)-reducing capacity ($r^2 = 0.90$). This is identical to the results of a previous report in which the data of the two assays are strongly correlated.[23] Moreover, the results of the FCR assays were moderately correlated with those of the TEAC and DPPH assays ($r^2 = 0.76$ and 0.75, respectively). The AAs of the flavonoids in the FRAP assay were highly correlated with the results of the DPPH assay ($r^2 = 0.86$) but were less correlated with antioxidant values in the TEAC assay ($r^2 = 0.75$).

Cyclic Voltammograms Behavior of Flavonoids. The electrode reactions that characterize the electrochemical oxidation of selected flavonoids at the glassy carbon electrode (GCE) were studied using cyclic voltammetry method. All the flavonoids had at least two oxidation peaks in the anodic wave. The peaks correspond to the sequent oxidation of the hydroxyl groups at different positions.[24] The voltammetric behaviors of some flavonoids are depicted in Figure 2. In part A, the flavonols group, including quercetin and isorhamnetin, showed three characteristic peaks in the anodic wave. The middle peak (peak 2) is related to the oxidation of the hydroxyl group in the C ring.[25] In the flavones group (Figure 2B), the luteolin had two anodic peaks; the first peak was remarkable, whereas the second peak was obscure. The two peaks of apigenin overlapped. They were irreversible peaks, which suggest the slow reaction rate and low reactivity of hydroxyl substituents. Flavanones, flavanol, and isoflavones are shown in parts C, D, and E of Figure 2, respectively. Catechin and epicatechin had similar anodic waves, with the first oxidation peak potential of epicatechin lower than that of catechin. In Figure 2C, hesperetin showed two distinct anodic peaks, whereas the two irreversible oxidation peaks of naringenin were closer. The difference between the two flavanones is an extra 4′-methoxy substituent in hesperetin, which can stabilize the semiquinone radical through an intramolecular hydrogen bond.[26] In the isoflavones group, the difference between genistein and daidzein in the cyclic voltammograms is that the area underthe anodic wave (Q) of genistein was higher than that of daidzein within the reverse potential range of 0.6−1.4 V. It resulted from an additional oxidation of the 5-hydroxyl group in genistein.

From the anodic wave of each flavonoid, oxidation peak potentials (E_{pa1}, E_{pa2}) of the tested flavonoids were obtained, and they are shown in Table 2. The flavonol group had the lower E_{pa1} than the other flavonoids. Isorhamnetin and myricetin had the lowest E_{pa1} (0.116 and 0.119 V versus Ag/AgCl), followed by kaempferol and quercetin (0.194 and 0.202 V, respectively). The ranking order of the first oxidation peak potentials of the

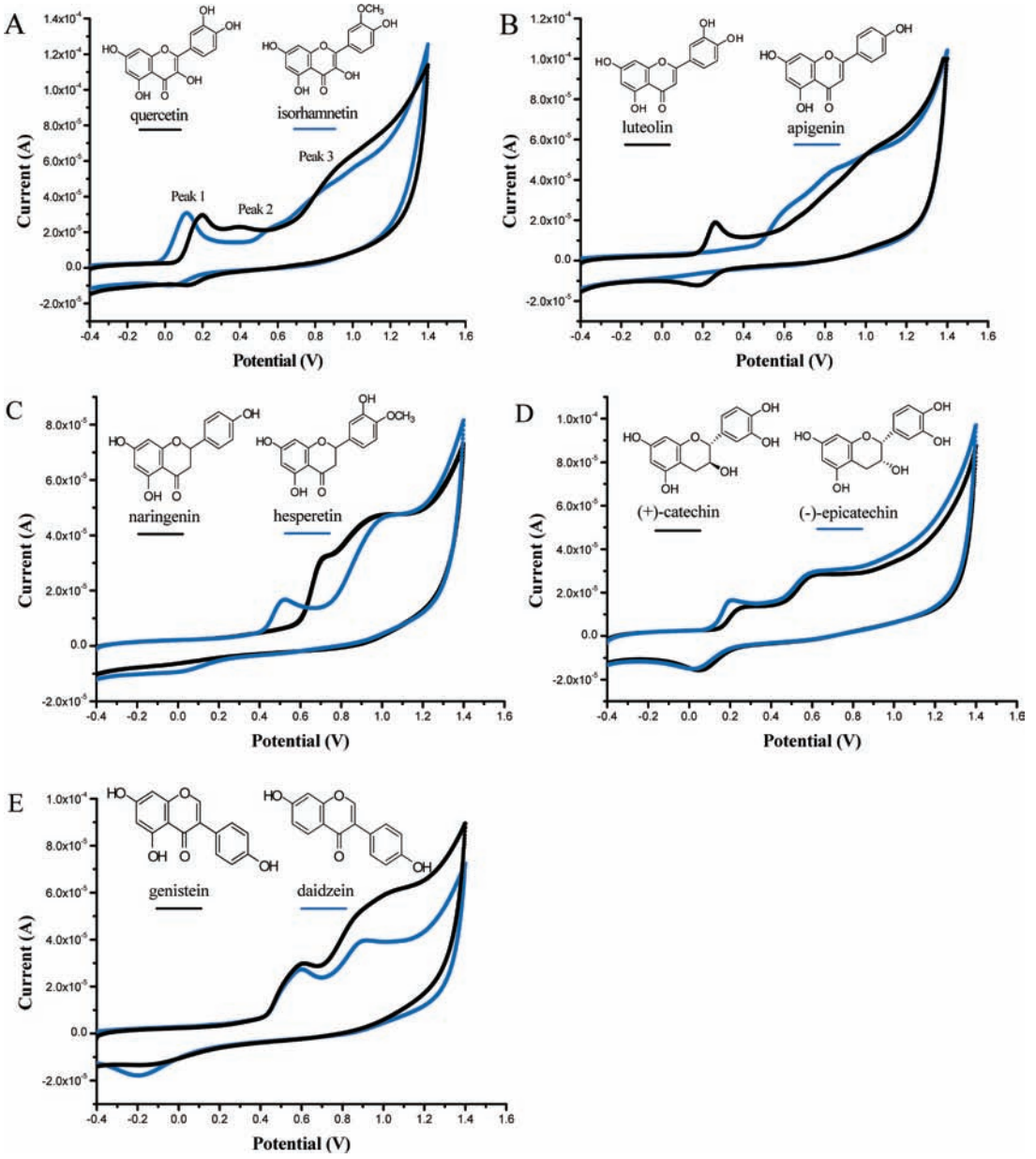


Figure 2. Cyclic voltammograms of 150 μM standard solutions of flavonols (A), flavones (B), flavanones (C), flavanols (D), and isoflavones (E) in the BRS with pH 7.0; scan rate, 400 mV s^{-1}.

remaining flavonoids was as follows: (−)-epicatechin < taxifolin < luteolin < (+)-catechin < quercitrin < hesperetin < daidzein < genisten < apigenin < naringenin. Furthermore, the E_{pa2} values also greatly varied among flavonoid groups. All the flavonols had E_{pa2} values lower than 0.6 V, whereas the second peak potentials of the other flavonoids were higher than 0.6 V, which were contributed by the oxidation of hydroxyl groups in the A ring.[27]

DISCUSSION

As shown in Table 1, the results of the four methods (DPPH, TEAC, FRAP, and FCR) are inconsistent. The chemical reasons for differences in the results are explained in the following discussion.

Comparison of the Electrochemical Indices of the Flavonoids with Their AAs in the Spectrophotometric Assays. Electrochemistry is the conceptual base of the selected antioxidant capacity assays because the transference of charge involved in these assays is based on redox reactions. By comparing the values of electrochemical parameters obtained in the CV assay with the antioxidant results of the selected assays, the variable AAs of the flavonoids in the different spectrophotometric assays can be interpreted.

Relationships among the AAs of Flavonoids in Spectrophotometric Assays and Their Q Values. The area under the anodic wave (Q) represents the charge transfer of flavonoids at certain reverse potentials (Figure 3). The oxidant degree of flavonoids changes when different reverse potentials are used. As shown in Figure 4, the correlations between the Q values of flavonoids and the results of the three spectrophotometric assays (DPPH, FRAP, and FCR) increased from the reverse potential of 0.2 V. The Q values attained maximal correlations with the results of the FCR, FRAP, and DPPH assays at the reverse potentials of

Table 2. *Q* Values of Flavonoids at Different Reverse Potentials and Their Oxidant Peak Potentials

	area under anodic wave (Q/μC)						peak potentials (V)	
	Q_{200}	Q_{400}	Q_{600}	Q_{800}	Q_{1000}	Q_{1200}	E_{pa1}	E_{pa2}
				Flavonols				
quercetin	4.15 ± 0.18	12.07 ± 0.40	18.75 ± 0.57	27.40 ± 0.84	44.27 ± 1.40	64.48 ± 1.68	0.202	0.395
myricetin	6.02 ± 0.76	12.15 ± 0.81	21.32 ± 0.82	26.13 ± 1.89	42.38 ± 1.15	58.59 ± 1.20	0.119	0.598
isorhamnetin	7.62 ± 0.30	12.38 ± 0.45	17.93 ± 0.80	27.87 ± 1.38	43.01 ± 2.08	60.24 ± 2.93	0.116	0.564
kaempferol	3.10 ± 0.18	8.49 ± 0.32	11.87 ± 0.36	19.92 ± 0.69	35.14 ± 1.38	48.40 ± 2.03	0.194	0.384
				Flavones				
luteolin	0.21 ± 0.01	4.05 ± 0.30	7.32 ± 0.56	14.12 ± 1.09	26.61 ± 2.04	41.47 ± 3.23	0.261	1.021
apigenin	0.57 ± 0.02	1.30 ± 0.04	4.59 ± 0.07	14.66 ± 0.34	28.86 ± 0.79	42.59 ± 1.35	0.623	0.821
quercitrin	0.06 ± 0.04	2.57 ± 0.09	5.61 ± 0.14	10.94 ± 0.92	22.52 ± 0.74	35.36 ± 1.42	0.326	0.960
				Flavanones				
taxifolin	1.00 ± 0.10	6.36 ± 0.09	10.86 ± 0.24	19.55 ± 0.52	36.43 ± 0.95	54.19 ± 1.75	0.238	0.966
hesperetin	0.04 ± 0.03	0.12 ± 0.02	3.35 ± 0.40	6.91 ± 0.69	17.57 ± 1.48	28.70 ± 1.78	0.524	1.002
naringenin	0.03 ± 0.01	0.11 ± 0.06	0.77 ± 0.21	8.68 ± 1.12	21.74 ± 2.02	32.69 ± 2.65	0.710	0.955
				Isoflavones				
daidzein	0.37 ± 0.04	1.07 ± 0.10	6.73 ± 0.61	14.47 ± 0.86	25.45 ± 1.12	33.63 ± 1.52	0.601	0.922
genistein	0.15 ± 0.00	0.66 ± 0.03	5.92 ± 0.41	15.67 ± 0.64	31.96 ± 1.02	48.31 ± 1.40	0.614	0.903
				Flavanols				
catechin	0.58 ± 0.16	4.43 ± 0.36	10.39 ± 0.59	19.07 ± 0.83	27.21 ± 1.25	34.72 ± 1.95	0.266	0.609
epicatechin	1.37 ± 0.06	5.87 ± 0.35	12.42 ± 0.56	21.39 ± 0.92	30.23 ± 1.39	39.00 ± 2.03	0.208	0.595

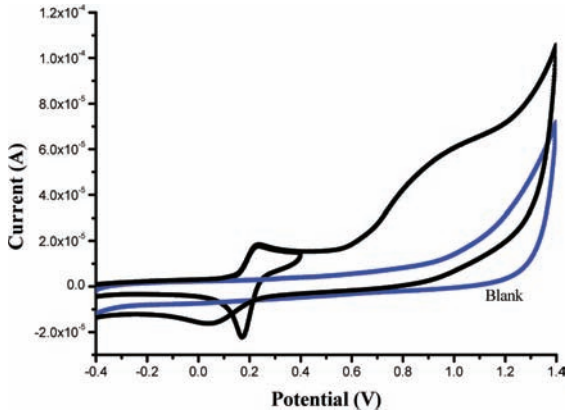


Figure 3. Cyclic voltammograms of flavonoid standard solution (e.g., taxifolin, 150 μM) at different reverse potentials (0.4 and 1.4 V). The Q values of flavonoids were obtained by the integral area under anodic wave subtracting the blank buffer solution at corresponding potentials.

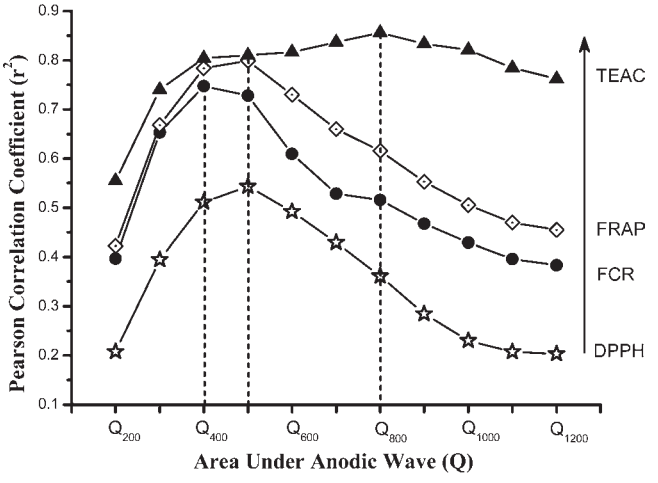


Figure 4. Correlations among the results of four spectrophotometric assays and the *Q* values of the selected flavonoids at the reverse potentials ranging from 0.2 to 1.2 V.

0.4, 0.5, and 0.5 V, respectively. At reverse potentials exceeding 0.5 V, the correlation coefficients fell sharply. At high reverse potentials (>1.0 V), low correlations were observed between the AA values of the tested flavonoids in these assays and the Q values. The flavonoids were oxidized to a similar extent. Figure 2 shows that most of the tested flavonoids cannot reach the second oxidation step at reverse potentials lower than 0.5 V. Thus, the results of DPPH, FRAP, and FCR assays are primarily based on the first oxidation step of flavonoids. This means that the AAs of the flavonoids in the three assays account for the oxidation of most of the active hydroxyl groups (in the B ring).

The TEAC assay, however, showed quite different trends compared with those of the three other assays. It did not reach the maximal correlation with the Q_{500} value. With the reverse potentials increased from 0.5 V, correlation coefficients were still increased (Figure 4). It exhibited the highest correlation between the Q_{800} value of selected flavonoids and their corresponding AAs in the TEAC assay (r^2 = 0.86). After that, the correlations dropped slightly as the reverse potential exceeded 0.8 V. The r^2 value was 0.76 at 1.2 V. Therefore, the oxidation of the flavonoids at high reverse potentials (>0.8 V) does not remarkably change the ranking order of antioxidant activity of these flavonoids in the TEAC assay.

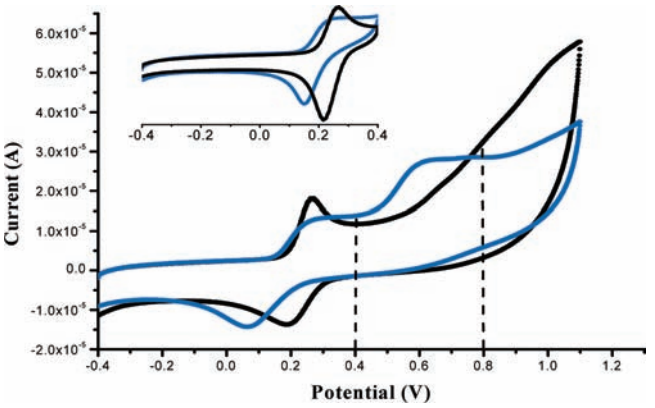


Figure 5. Comparison of the anodic wave areas of catechin and luteolin at two potentials (0.4 and 0.8 V). The blue lines represent catechin, the black lines represent luteolin.

On the other hand, the flavonoids with high peak potentials (e.g., genistein and apigenin) had similar TE values as those of flavonoids with low peak potentials in the TEAC assay (e.g., luteolin, quercitrin) (Table 2). Accordingly, in the CV assay, the charge transfer of genistein and apigenin began to increase at 0.6 V. They all had no detectable current when the oxidant potential was lower than 0.5 V. At 0.8 V, the Q values of apigenin and genistein increased to 14.66 and 15.67 μC, respectively, which exceeded the transfer charge of luteolin and quercitrin (14.12 and 10.94 μC, respectively) (Table 2). Therefore, the oxidation degree of flavonoids, as indicated by the $ABTS^{\bullet+}$ cation radical in the TEAC assay, is deeper than the other three oxidants in DPPH, FRAP, and FCR assays. This explains why the correlation between the results of TEAC assay and the Q values of flavonoids reached their maximal r^2 values at high reverse potentials. Moreover, the flavonols and flavanols had their second oxidation step within the potential range 0.5−0.8 V. This process can contribute to the AAs of flavonoids in the TEAC assay, as well as the first oxidation step. For instance, catechin and luteolin showed similar net area under the anodic wave at 0.4 V (Figure 5), whereas the Q value of catechin was significantly higher than that of luteolin at potential 0.8 V because the second anodic peak of catechin appeared. Correspondingly, the TE value of catechin was significantly higher than that of luteolin in the TEAC assay. The AAs of flavonoids in the TEAC assay may represent the sum of activities of different functional regions (the B and C ring or the A ring).

Relationships between the AA of Flavonoids in Spectrophotometric Assays and Their Oxidation Peak Potentials. The oxidant peak potentials reflect electron-donating capacities, which are determined by the reactivity of the hydroxyl groups. Recently, some researchers have already reported a direct correlation between antioxidant activity of flavonoids and their first oxidation peak potentials.[7,28] It was considered that flavonoids with lower peak potentials would have higher radical-scavenging activity. In this study, the first peak potentials of selected flavonoids were moderately correlated with their TE values in the DPPH assay ($r^2 = 0.75$). The first peak potentials of daidzein, genistein, apigenin, and hesperetin were higher than 0.5 V. From 0.2 to 0.4 V, there is weak charge transfer between the electrode and these flavonoids (Table 2, Figure 2). Furthermore, the redox potential of the DPPH radical is 0.34 V versus Ag/AgCl;[29] therefore, the four flavonoids cannot reduce the DPPH radical. Correspondingly, they showed unquantifiable activity in the DPPH radical scavenging reaction.

The correlations between TEAC values and the first peak potentials (E_{pa1}) were not high ($r^2 = 0.67$), being much lower than those of the FCR ($r^2 = 0.85$) and FRAP assay ($r^2 = 0.87$). When the second peak potentials (E_{pa2}) were taken into account, the correlation ($r^2 = 0.76$) between TE values in TEAC assay and peak potentials (E_{pa1}, E_{pa2}) was improved (eq 1). In addition, when the Q values were introduced, obvious improvement was obtained in following eq 2. The peak potentials alone likely could not fully represent the results of the TEAC assay. The Q value combined with peak potentials would be more suitable for predicting the AAs of the flavonoids in the TEAC assay.

$$\mathrm{TEAC} = 6.267 - 3.295E_{pa1} - 2.016E_{pa2} \qquad r^2 = 0.76 \tag{1}$$

$$\mathrm{TEAC} = 1.635 - 1.513E_{pa1} + 0.073E_{pa2} + 0.137Q_{800} \qquad r^2 = 0.90 \tag{2}$$

Adversely, as shown in the following equations (eqs 3−8), the E_{pa2} and Q values exhibited slight effects on the changes of correlations in multiple linear regressions.

$$\mathrm{FCR} = 1.356 - 1.205E_{pa1} - 0.173E_{pa2} \qquad r^2 = 0.86 \tag{3}$$

$$\mathrm{FCR} = 1.130 - 1.010E_{pa1} - 0.063E_{pa2} + 0.014Q_{400} \qquad r^2 = 0.87 \tag{4}$$

$$\mathrm{FRAP} = 5.818 - 7.219E_{pa1} - 1.113E_{pa2} \qquad r^2 = 0.88 \tag{5}$$

$$\mathrm{FRAP} = 3.769 - 5.480E_{pa1} - 0.190E_{pa2} + 0.103Q_{500} \qquad r^2 = 0.89 \tag{6}$$

$$\mathrm{DPPH} = 2.049 - 3.580E_{pa1} + 0.413E_{pa2} \qquad r^2 = 0.76 \tag{7}$$

$$\mathrm{DPPH} = 1.881 - 3.437\mathrm{E}_{pa1} + 0.488E_{pa2} + 0.008Q_{500} \qquad r^2 = 0.76 \tag{8}$$

The AAs of flavonoids in FCR, FRAP, and DPPH assays are strongly influenced by the ease of charge transfer (reducing reactivity) in the first oxidation step, and the Q value and E_{pa2} are the secondary factors.

In general, the degrees of oxidation of the flavonoids, which are the main chemical differences among these methods, determine their AAs in each assay. The results of the TEAC method more appropriately reflect the overall effect of the different reactive hydroxyl groups in the flavonoids compared with the other three methods.

The Results of FCR and FRAP Assays Cannot Be Defined as "Total Antioxidant Power". The redox potential of the Fe(III) salt in FRAP assay is about 0.7 V, which is identical to that of $ABTS^{\bullet+}$ and much higher than that of the DPPH radical (0.34 V). Conversely, the AAs of the flavonoids in FRAP differed from that of the TEAC assay. The acidic solvent condition can decrease the oxidizing strength of the FRAP assay because the high proton concentration (H^+) inhibits the dissociation of phenolic

hydroxyl. This shifts the redox potentials of the flavonoids to high potentials, inducing the Fe(III) and the DPPH radical to exhibit similar oxidant strength in each assay. Therefore, the results of FRAP do not accurately reflect the "total reducing capacity".

The FCR assay, traditionally called "total phenols assay", has been used to quantify the total reducing capacity of antioxidants. Nevertheless, the results of the FCR assay in the current study could not represent the term of total reducing capacity because the multiple oxidation steps of flavonoids do not show in this assay. The reaction temperature was modified to 37 °C, which was much lower than the regularly applied temperature in FCR assays (90 °C). The oxidation degree of flavonoids is moderate under this physiological condition; thus, FCR assays have the similar thermodynamic significance as the DPPH and FRAP assays when applied to assess the AAs of flavonoids.

To sum up, the results of FCR and FRAP assay cannot be defined as total antioxidant power because the multiple oxidation steps of flavonoids are not reflected.

Effect of Reaction Mechanisms on the AAs of Flavonoids in Different Assays. As primary antioxidants, flavonoids inactivate oxidants according to the hydrogen atom transfer (HAT) and the single electron transfer (SET) mechanisms. Theoretically derived bond dissociation enthalpy (BDE) and ionization potential (IP) have been reported as supportive means to investigate the potential of a compound to act as oxidant scavenger.[30] The BDE value characterizes the hydrogen-atom-donating ability of flavonoids, which corresponds to the HAT reaction mechanism and is determined by the stability of the phenoxyl radical.[26] In the SET mechanism, the IP value is the most significant parameter for the scavenging activity evaluation: the lower the IP value, the easier the electron abstraction and the reaction with free radicals.[31]

The BDE values of the selected flavonoids greatly correlated with the results of DPPH (r^2 = 0.89), whereas the IP values weakly correlated with the DPPH radical-scavenging capacity of the tested flavonoids (r^2 = 0.26, P > 0.05). This trend is similar to that of the scavenging activities of aurones and simple phenols against DPPH radicals.[32] The BDE value seems a more proper parameter for characterizing the antiradical properties of the studied compounds in the DPPH assay. The DPPH assay is the only method that uses organic solvent among the four spectrophotometric assays. Although methanol is a polar solvent, its dielectric constant is lower than that of water.[33] This may cause the transformation of radical-scavenging mechanisms. This indicates that the SET and HAT mechanisms may coexist in this assay,[34] which makes the process of DPPH radical scavenging of phenols very different from the SET mechanism, although the DPPH is customarily classified as an ET-based method. For example, kaempferol had much lower TE values in the DPPH assay than those of flavonoids with a 3′,4′-*o*-dihydroxy group in the B ring (luteolin, taxifolin, and so on) (Table 1), which is the same as the results reported by Hotta et al.[9] Similarly, the BDE value of kaempferol is higher than those of luteolin and taxifolin, among others. However, the charge transfer value (Q) of kaempferol was higher than those of luteolin, taxifolin, catechin, and epicatechin from 0.2 to 1.2 V (Table 2). Consequently, the results of DPPH assay showed the lowest correlation with charge under anodic wave (Q) among the selected assays. (The maximal correlation coefficient square was below 0.6). Therefore, the solvent effect greatly influenced the AAs of the flavonoids in the DPPH assay and the correlations with those of other assays.

In addition, the AAs of flavonoids in the FRAP assay were closely related to the BDE values (r^2 = 0.76) and lowly correlated with IP values (r^2 = 0.44). Meanwhile, the reducing activities of the flavonoids in the FCR assay were not highly correlated with either their BDE (r^2 = 0.62) or IP values (r^2 = 0.33). This may be account for the proton dissociation of the hydroxyl group before reacting with the oxidants under alkaline basic conditions (pH 10), which causes the oxidant-scavenging process of the flavonoids to differ from simple HAT or SET mechanisms.

On the other hand, both the BDE and the IP values had low correlations (r^2 = 0.54 and r^2 = 0.57, respectively) with the $ABTS^{\bullet+}$ radical scavenging capacity of the tested flavonoids. This point contrasts with the results of a previous report.[35] In fact, only the BDE or IP values of flavonoids do not reflect the complex characteristics of antioxidant processes. The multiple oxidation process of flavonoids in oxidant-scavenging reactions could not be supported by the respective BDE or IP values, because the two descriptors represent the reactivity of most active hydroxyl group rather than stoichiometric factors. Furthermore, the number of hydroxyl groups (n_{OH}) has been cited as the biggest determinant of AAs of flavonoids.[36] However, the experimental data did not support that idea, and the correlation between the n_{OH} and the TEAC values was low (r^2 = 0.50). Moreover, the degree of hydroxylation does not reflect the amount of reactive hydroxyl groups. The reactivity of hydroxyl groups is mainly influenced by their positions in the basic structure of the flavonoids. Hence, there is a lack of appropriate structural descriptors or calculation criteria for reflecting the stoichiometry of the oxidant-scavenging reaction. We are exploring a theoretical approach to predict the degree of multiple oxidation of flavonoids, which will be more useful for the quantitative structure−activity relationships studies between the structural properties of the flavonoids and their scavenging capacity against active species with high oxidizing potentials like $ABTS^{\bullet+}$.

Above all, the oxidant-scavenging reaction of the tested flavonoids in the DPPH assay is closer to the HAT mechanism than the other three assays. Complex reaction mechanisms underlie the TEAC method, and appropriate structural descriptors for reflecting the AAs of flavonoids based on multiple oxidation steps need further investigation.

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Funding Sources

This work was supported financially by funds from the National Natural Science Foundation of China (No. 31171647).

ACKNOWLEDGMENT

The authors are grateful for Prof. Wang Zhixiang's help in the theoretical calculations.

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