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ARTICLES

Free-Radical Scavenging Capacity Using the Fenton Reaction with Rhodamine B as the Spectrophotometric Indicator

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A spectrophotometric method was developed to measure antioxidant free-radical scavenging capacity. Rhodamine B (RhB) was oxidized by hydroxyl radical generated via the Fenton reaction to yield a photoinactive RhB product. RhB absorption at 550 nm was restored when antioxidant agents scavenged hydroxyl radical to protect RhB from oxidation. On the basis of the dose response of antioxidant recovery capacity, a model was developed to calculate the free-radical scavenging ability. This method was sensitive to a wide range of antioxidant activity with ascorbic acid reference set as one; the antioxidant recovery capacity of quercetin was 635 compared to 2 for benzoic acid.

KEYWORDS: Hydroxyl radical; rhodamine B; antioxidant

INTRODUCTION

Reactive oxygen species (ROS) have been both positive and negative agents in many living physiological processes. In some cases, ROS were produced specifically to serve essential biological functions, whereas in other cases, ROS were normal metabolic byproducts (*1*). Physiologically, ROS has played a very important role in cellular responses to noxia, defense against infectious agents, and cell signaling. At high concentrations, ROS has been an important mediator of cell structure damage to lipids, proteins, and nucleic acids (*2, 3*); thus, ROS has been associated with aging (*4*), atherosclerosis (*5*), cancer (*6*), smoking (*7*), and respiratory disease (*8*). Host antioxidant defenses control the level of reactive free radicals, but cellular damage occurs when free radicals have overwhelmed this defense (*9*).

Hydroxyl radical (·OH), one of the major ROS in human body, has been generated through a number of mechanisms, including ionizing radiation to decompose water and photolytic decomposition of alkylhydroperoxides. The majority of the hydroxyl radical have come from the metal-catalyzed decomposition of hydrogen peroxide, via the Fenton reaction (*10, 11*) M^{n+} ($= Cu^{+}$, Fe^{2+}, Ti^{3+}, or Co^{2+}) + $H_2O_2 \rightarrow M^{(n+1)+}$ ($= Cu^{2+}$, Fe^{2+}, Ti^{4+}, or Co^{3+}) + ·OH.

It has been suggested that iron regulation prevented free intracellular iron; however, at *in vivo* stress conditions an excess of superoxide released "free iron" from iron-containing molecules (*12*). The released Fe^{II} participated in the Fenton reaction generating highly reactive hydroxyl radicals close to its site of formation (*13*). ROS levels could be reduced by ingesting antioxidant containing food and dietary supplements. Several methods have been developed for measuring the antioxidant capacity of food and dietary supplements. Examples include ORAC assay (*14, 15*), TEAC assay (*16*), TRAP assay (*17*), and FRAP assay (*18*).

The Fenton reaction was selected as the hydroxyl radical source to measure the antioxidant capacity in a manner similar to the *in vivo* generation of the hydroxyl radical. Hydroxyl radical detection methods have included electron-spin resonance (ESR), chemiluminescence, fluorescence, and spectrophotometric methods. ESR detected hydroxyl radical species with unpaired electrons, usually with a spin-trap reagent to form a more stable hydroxyl radical adduct (*19, 20*). Fluorescence has been a sensitive technique for radical quantification that has used one of two mechanisms (*1*): free-radical oxidation of nonphotoactive substrate to produce an enhanced photoactive fluorescent species and signal (*2*) and oxidation of photoactive substrate by free radicals to produce nonphotoactive species to quench fluorescence (*21*). With chemiluminescence, an enhanced luminescence was obtained with chemiluminogenic probes, such as luminol or lucigenin, that reacted with free radicals to produce luminescence (*22*). Other methods for free-radical detection have focused on oxidation or antioxidation, e.g., atomic force microscopy (AFM) to image DNA molecules exposed to hydroxyl radical (*23*). Mello et al. designed an electrochemical biosensor for hydroxyl radical, which consisted of an immobilized dsDNA on a screen-printed electrode surface (*24*). The listed methods have been either time-consuming or

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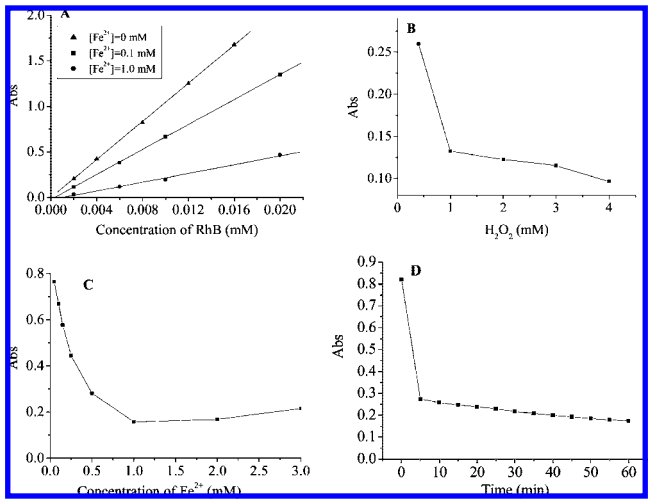

Figure 1. Effect of experimental conditions on the Fenton−RhB system: (**A**) RhB concentration with 2.0 mM [H_2O_2], (**B**) H_2O_2 with 1.0 mM [Fe^{2+}] and 0.010 mM [RhB], (**C**) Fe^{2+} with 0.010 mM [RhB] and 2.0 mM [H_2O_2], and (**D**) reaction time with 1.0 mM [Fe^{2+}], 0.010 mM [RhB], and 2.0 mM [H_2O_2].

expensive; thus, a cost-effective, simpler method for free-radical determination and antioxidant assessment was sought.

Spectrophotometry can be used for hydroxyl radical detection much like fluorescence has been used to measure quenching and enhanced fluorescence. Spectrophotometry may not be the most sensitive method, but it is simple, reproducible, and cost-effective.

Fenton reaction with rhodamine B (RhB) as an indicator has been used to determine the ability of the Fenton reaction to degrade organic dyes, which have been persistent organic pollutants (*25–27*). Limited papers on antioxidant detection indicated that problems remained for the assessment of free radical scavenging capacity by this system (*28, 29*).

The majority of antioxidants, such as polyphenols, are insoluble or slightly soluble in the acidic pH test media necessary to avoid iron hydroxo complex formation. Organic solvents and surfactants could increase antioxidant solubility, but the reaction of such additives with the hydroxyl radicals generated by the Fenton reaction had to be determined.

The purpose of this work was to develop a spectrophotometric method for the evaluation of the free-radical scavenging capacity of antioxidants using RhB and the Fenton reaction.

MATERIALS AND METHODS

Materials. L-Ascorbic acid, rutin, benzoic acid, and benzoic acid derivatives were purchased from Beijing Chemical Reagent Company (Beijing, China); trolox and quercetin were purchased from Aldrich-Sigma (St. Louis, MO); catechin was purchased from TCI (Tokyo, Japan); and 3-methoxybenzoic acid was purchased from Fluka A. G. (Buchs, Switzerland). All other reagents used in this study were of analytical grade. Doubly deionized water was used throughout the study.

Instrumentation. Hitachi U-3010 spectrophotometer (Hitachi, Japan) was used for measurements. The RhB maximum at 550 nm could be measured by a number of instruments sensitive to change in the 550 nm range.

General Procedure. The stock solutions for Fenton reaction were 0.2 mM RhB, 10 mM $FeSO_4$ in 2 mM H_2SO_4, and 20 mM H_2O_2, respectively. Antioxidants were added to media containing 20 mM acetic acid at pH 2.8, 0.010 mM RhB, and 1.0 mM $FeSO_4$. H_2O_2 (2.0 mM) was added last. The test solution was then diluted to 10 mL with deionized water. The RhB absorption at 550 nm was measured 10 min after the hydrogen peroxide was added.

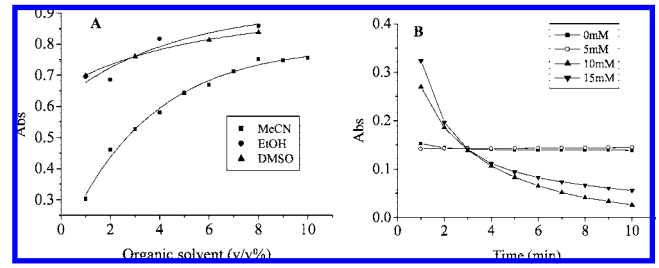

Figure 2. Effect of coexisting substances with 1.0 mM [Fe^{2+}], 0.010 mM [RhB], and 2.0 mM [H_2O_2]: (**A**) organic solvent and (**B**) CTAB.

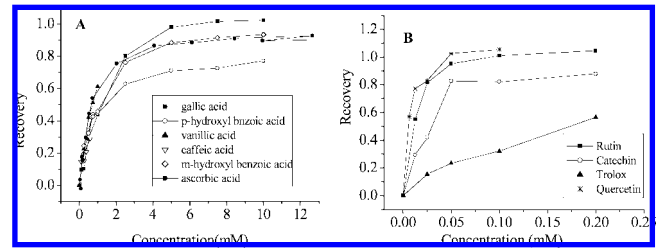

Figure 3. Recovery versus antioxidant concentration: (**A**) ascorbic acid, benzoic acid, and derivatives and (**B**) flavonoids and trolox with 5 mM CTAB.

Table 1. Hydroxyl Radical Scavenging Capacity of Antioxidant Agents and Products[a]

	antioxidant	fitting results			scavenging capacity
		R_{max}	k_2/K	R^2	
antioxidant agents	quercetin	1.10	171.68	0.9761	635.8
	rutin	1.16	63.49	0.8688	235.1
	catechin	1.15	27.08	0.9606	100.3
	trolox	0.66	11.8	0.9751	43.7
	2,4-dihydroxybenzoic acid	0.99	1.96	0.9834	7.3
	m-methoxybenzoic acid	0.96	1.56	0.9952	5.8
	m-hydroxybenzoic acid	0.90	1.36	0.9596	5.0
	p-hydroxybenzoic acid	0.85	1.21	0.9903	4.5
	3,4-dihydroxybenzoic acid	0.85	0.79	0.9782	2.9
	gallic acid	1.11	0.83	0.9980	2.8
	vanillic acid	1.49	0.73	0.9948	2.7
	o-hydroxybenzoic acid	1.03	0.64	0.9838	2.4
	benzoic acid	1.33	0.60	0.9933	2.2
	L-ascorbic acid	1.27	0.27	0.9785	1.0
product samples	wine A	0.90	247.7	0.9472	0.7
	wine B	1.13	90.3	0.8906	0.3
	kiwi juice	0.89	286.4	0.9878	0.8
	L-ascorbic acid	1.27	339.5	0.9785	1.0
	tea A	0.64	2.20	0.9374	0.9
	tea B	0.72	1.62	0.9433	0.7
	tea C	0.76	2.60	0.9947	1.1
	L-ascorbic acid	1.27	2.46	0.9785	1.0

[a] Note: (1) for wine and kiwi juice, the concentration of sample is denoted as v/v. Accordingly, L-ascorbic acid was defined as v/v from a 1 mol/L stock solution; (2) for tea samples, the concentration is denoted as g/L. Accordingly, the concentration of L-ascorbic acid was defined as g/L for tea sample calculations.

Preparation of Standard Curve. Water soluble antioxidants were dissolved in deionized water. Antioxidants with less aqueous solubility were dissolved in cetyl trimethyl ammonium bromide (CTAB), with the concentration of this surfactant kept at 5 mM for all final solutions.

Sample Test. To obtain a dose response curve, 0.02, 0.04, 0.1, 0.2, 0.3, 0.4, or 0.5 mL aliquots or 1 mL of CTAB-solubilized sample solution was added to Fenton reagents. Red wines were added directly. Kiwi juice from the fruit was centrifuged at 12 000 rpm for 20 min and stored at 4 °C, until used. For tea samples, 1 g of tea leaves were boiled with 50 mL of deionized water by gentle stirring. After 10 min, the tea was centrifuged and tested immediately (*30*) by the addition of

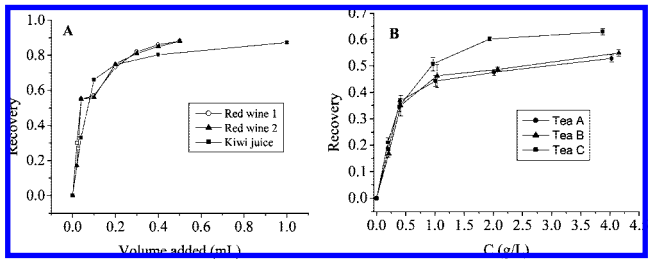

Figure 4. Recovery curves by red wine, kiwi juice, and tea: (**A**) red wine and kiwi juice and (**B**) tea.

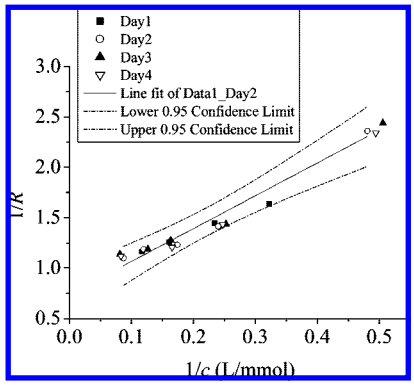

Figure 5. 1/*R* versus 1/*c* plots for L-ascorbic acid data from 4 day test.

0.1, 0.2, 0.5, 1, or 2 mL aliquots to Fenton reagents according to the general procedure.

RESULT AND DISCUSSION

Optimization of Experimental Conditions. The useful concentration range for each of the primary agents of the proposed Fenton-based method, i.e., RhB, H_2O_2, and Fe^{2+}, and the influence of these entities on absorption and sensitivity were determined. RhB absorption increased linearly as RhB was added to the Fenton reaction system containing 2.0 mM H_2O_2 with Fe^{2+} at 0, 0.1, and 1.0 mM concentrations (**Figure 1A**). RhB was not oxidized by H_2O_2 in the absence of Fe^{II}, but there was slight decrease in Fe^{II} absorption in the absence of H_2O_2.

In the presence of the Fenton reaction system, RhB absorption was reduced as RhB was oxidized by hydroxyl radical generated via the Fenton reaction to its photoinactive form with decreased absorption at 550 nm. The degree of RhB oxidation increased as the H_2O_2 concentration increased up to 1.0 mM (**Figure 1B**). Oxidation slowed and approached plateau with H_2O_2 greater than 1.0 mM; thus, 2.0 mM H_2O_2 was chosen for the following experiments.

Fe^{2+} played an important role in the Fenton reaction. The RhB oxidation increased as the Fe^{2+} concentration increased from 0 to 1.0 mM (**Figure 1C**) with 2.0 mM H_2O_2. At 1.0 mM Fe^{2+}, absorption reached a plateau. The hydroxyl radical probably reacted with the extra Fe^{2+} but not H_2O_2. The reaction between ·OH and Fe^{2+} (rate constant $k = 3.2 \times 10^8\ M^{-1}\ s^{-1}$) was 10 times faster than that between ·OH and H_2O_2 (rate constant $k = 3.3 \times 10^7\ M^{-1}\ s^{-1}$) (*31, 32*); thus, 1.0 mM Fe^{2+} was selected for the following measurements.

The hydroxyl radical generation and RhB oxidative reaction occurred rapidly (**Figure 1D**); thus, 10 min was selected for the following measurements.

Effects of Coexisting Substances and Surfactants. Water-miscible organic solvents, acetonitrile, ethanol, and dimethyl sulfoxide (DMSO) (**Figure 2A**), as well as other organic solvents, such as *N*,*N*-dimethyl formamide (DMF) and dioxane, were used to improve the solubility of antioxidants. Ethanol and DMSO showed significantly hydroxyl radical scavenging effects at all tested concentrations, thus interfering with this method. DMSO captured ·OH to form the $\cdot CH_3$ free radical (*33*); the reactive H atom of ethanol reacted with hydroxyl radical. At <3%, acetonitrile exhibited slight interference, but there was limited improvement of antioxidant solubility at this concentration.

Surfactants were also tested to increase the solubility of antioxidants. Triton X-100 and Tween 40 had a scavenging effect at test level concentrations with a trend similar to acetonitrile, thus limiting their use to improve antioxidant solubility. When concentration was greater than 13 mM Triton X-100 and 30 mM Tween 40, RhB absorption was covered by surfactant, not allowing a reasonable detection range for antioxidant recovery. The OH group containing surfactants competed with RhB for reaction with hydroxyl radical.

In contrast, CTAB at 5 mM did not affect the Fenton reaction and RhB oxidation (**Figure 2B**). At higher concentration, CTAB enhanced RhB oxidation at a slow rate to expand the range of reduced absorbance. At 10 min, the absorption reached a stable value. If necessary, it was possible to use CTAB at a concentration greater than 5 mM to increase antioxidant solubility.

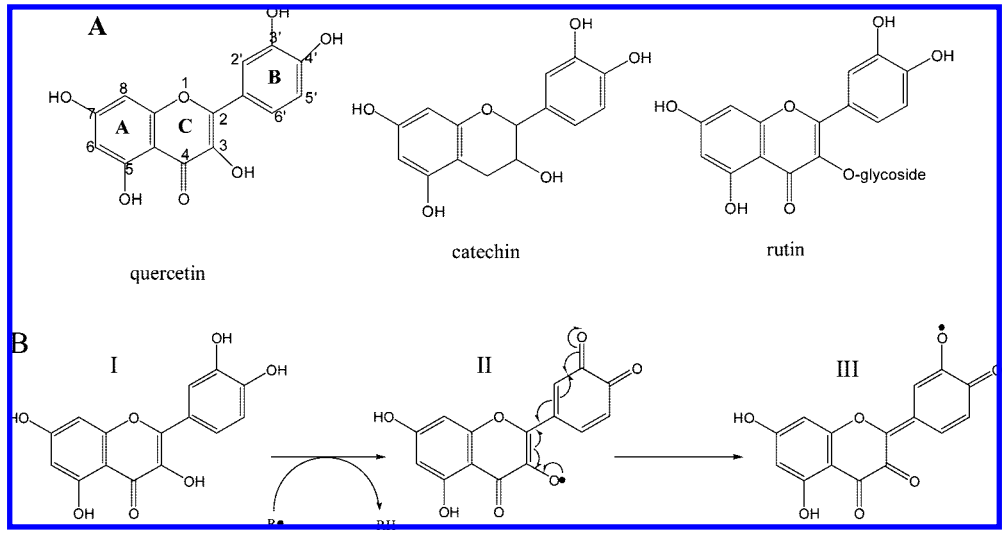

Figure 6. Molecular structure of flavonoids tested (**A**) and resonance structure of quercetin upon oxidation by hydroxyl radical (**B**).

In addition to Fe^{II}, transition-metal ions, such as Co^{I}, Co^{II}, and Mn^{II}, catalyzed the decomposition of H_2O_2. The catalytic ability of Co^{2+} was compared to Fe^{2+} using test method conditions. The Co^{2+} catalytic ability on the Fenton reaction was limited. At a Co^{2+} concentration 10 times Fe^{2+}, the decrease in RhB absorption was just 12% of that with Fe^{2+} (data not shown).

Development of Free-Radical Scavenging Capacity Model. The antioxidant ability at individual concentration levels was represented by recovery (R) of the absorbance of RhB. $R = (A_{AO} - A_b)/(A_0 - A_b)$, where A_0 denoted the absorption of RhB with Fe^{2+} and buffer only, A_b denoted the absorption of RhB in the presence of the Fenton reagent, and A_{AO} represented the absorption with the addition of antioxidant.

The water-soluble antioxidants, e.g., L-ascorbic acid, benzoic acid, and its derivatives, were dissolved in water. Less water-soluble antioxidants, e.g., trolox and flavonoids, including quercetin, rutin, and catechin, were dissolved in 50 mM CTAB aqueous solution. The antioxidant recovery (R) was plotted versus concentration has been shown in **Figure 3**.

The dose response curves were not linear; therefore, a mathematical model was necessary to calculate the scavenging ability of the antioxidants based on the method proposed in this study. The model was based on the assumption that the rate for RhB oxidation was constant during the short reaction time and nearly all ·OH was produced instantly at the beginning of the reaction. Therefore, in the absence of antioxidant, rate for RhB oxidation was denoted by eq 1

$$\mathrm{RhB} + n\cdot\mathrm{OH} \xrightarrow{k_1} \mathrm{oxRhB} \tag{1}$$

where RhB and oxRhB denote rhodamine B and its oxidized form, respectively. k_1 was the rate constant.

The apparent oxidation rate within time t was expressed as

$$v_1 = \frac{([\mathrm{RhB}]_0 - [\mathrm{RhB}]_b)}{t} = \frac{(A_0 - A_b)}{t} = k_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n \tag{2}$$

where $[RhB]_0$ denoted initial concentration of RhB and$[RhB]_b$ denoted concentration of RhB at time t.

When antioxidant was added, the hydroxyl radical was partially scavenged by the antioxidant as shown in eq 3

$$\mathrm{AO} + m\cdot\mathrm{OH} \xrightarrow{k_2} \mathrm{oxAO} \tag{3}$$

where AO and oxAO denoted antioxidant agent and its oxidized form, respectively. k_2 was the rate constant.

In the presence of an antioxidant, eq 4 represented the apparent total reaction rate of ·OH and eq 5 represented the apparent rate of RhB oxidation. Because consumption of ·OH contributed from eqs 1 and 3 depend upon the rate constant of the two reactions, eq 5 was modified to give eq 6.

$$v_{OH} = nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n + mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m \tag{4}$$

$$v_2 = \frac{[\mathrm{RhB}]_0 - [\mathrm{RhB}]_{AO}}{t} = \frac{A_0 - A_{AO}}{t} \tag{5}$$

$$v_2 = k_1[\mathrm{RhB}]_0 \frac{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n}{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n + mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m}[\cdot\mathrm{OH}]^n \tag{6}$$

As defined previously, recovery $R = (A_{AO} - A_b)/(A_0 - A_b)$. Here, R was introduced to connect v_1 and v_2 through deduction of eq 7 to eq 9. Ultimately, the reciprocal of R was obtained from reactions involved in the test system as denoted in eq 10.

$$\frac{v_2}{v_1} = \frac{A_0 - A_{AO}}{A_0 - A_b} = \frac{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n}{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n + mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m} \tag{7}$$

Because

$$\frac{A_0 - A_{AO}}{A_0 - A_b} = 1 - R \tag{8}$$

Therefore,

$$R = 1 - \frac{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n}{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n + mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m} = \frac{mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m}{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n + mk_2[\mathrm{AO}][\cdot\mathrm{OH}]^m} \tag{9}$$

Hence,

$$\frac{1}{R} = \frac{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^n}{mk_2[\cdot\mathrm{OH}]^m[\mathrm{AO}]} + 1 = \frac{nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^{n-m}}{mk_2[\mathrm{AO}]} + 1 \tag{10}$$

In eq 10, $(nk_1[\mathrm{RhB}]_0[\cdot\mathrm{OH}]^{n-m})/m$ was a constant, with the assumption for the model and was denoted as K. Therefore, eq 10 was transformed to eq 11.

$$\frac{1}{R} = \frac{K}{k_2}\frac{1}{[\mathrm{AO}]} + 1 \tag{11}$$

Theoretically, when [AO] was sufficiently large, $1/R$ approached 1, the theoretical maximum value (R_{max}). However, for different antioxidants, the R_{max} varied slightly. It was probable that R_{max} was governed by the reactivity of the radical produced from antioxidant oxidation by the hydroxyl radical because this radical could have participated in the RhB oxidation process; thus, eq 11 was modified to eq 12.

$$\frac{1}{R} = \frac{K}{R_{max} \times k_2}\frac{1}{[\mathrm{AO}]} + \frac{1}{R_{max}} \tag{12}$$

The value of k_2/K was obtained by linear regression of $1/R$ versus 1/[AO] (**Table 1**). Because K was constant, the scavenging capacity of antioxidants was evaluated by comparing k_2/K. The higher the k_2/K value, the more potent the antioxidant.

Analytical Results. The hydroxyl radical scavenging capacity was calculated using L-ascorbic acid as the reference antioxidant set as 1.0 (**Table 1**).

At the test method concentrations of 0.02−0.2 mM, the recovery of trolox was in the 60% range because of limited solubility under test conditions.

A few products were purchased locally to illustrate the method use. These products were analyzed as such, e.g., wine, or prepared in the usual manner, e.g., tea. A response curve to the Fenton reagents was obtained, and the calculations were made to obtain the antioxidant scavenging capacity (**Figure 4** and **Table 1**).

The reproducibility of the method was evaluated by comparing L-abscorbic acid standard curves prepared on each of four consecutive days (**Figure 5**). Using day 2 as an example, linear

fitting and confidence limits (95) were given. All of the data from the other 3 days were within the confidence limits of day 2, indicating reasonable method reproducibility.

The tested antioxidants were divided into two groups based on their hydroxyl radical scavenging ability by the proposed method. The effective concentration of flavonoids was at least 1 order of magnitude lower than L-ascorbic acid, confirming that these flavonoids were very high potent antioxidants. Quercetin was the most potent antioxidant of the three flavonoids tested.

The oxidation of the 3-OH of quercetin produced an −O• through H atom subtraction, and the two hydroxyl groups on ring B was also oxidized to form a quinone, shown in II of **Figure 6B**. The 3-hydroxyl group in ring C of rutin was a glycoside, which blocked a resonance structure and reduced the antioxidant potential. The 2,3 double bond was still conjugated with ring B, thus maintaining the antioxidant capability at a level higher than catechin without 2,3 double bond or 4-oxo group feature. Catechin and quercetin have an identical number of hydroxyl groups in the same ring positions. The 3-hydroxy group in ring C did not contribute to free-radical scavenging activity because there was no conjugate structure without 2,3 double bond and 4-oxo group in ring C. Therefore, other than 3′,4′-hydroxyl groups in ring B, the 2,3 double bond, 3-hydroxyl, and 4-oxo group in ring C were key to free-radical scavenging activity (*34*).

The benzoic acid derivatives in olive oil have been suggested as effective antioxidants because of their tendency to hydroxylation and high reactivity with hydroxyl radical. The scavenging capacity of benzoic acid derivatives depends upon benzene ring substituents and their ring positions. The *meta*-hydroxyl benzoic acid was more effective than the *ortho*- and *para*-hydroxyl derivatives, because the *ortho* and *para* derivatives were affected by the electron-withdrawing potential of the carboxyl functional group (*35, 36*). However, multihydroxyl derivatives did not exhibit a synergistic antioxidant effect.

Conclusion. Hydroxyl radical produced during mitochondrial electron transfer is a source of many other secondary free radicals; thus, it is important to evaluate hydroxyl radical scavenging capability. Antioxidants may show different scavenging capability toward different free radicals. The spectrophotometric method was developed for assessment of hydroxyl radical scavenging capacity of antioxidants with the Fenton reaction as the hydroxyl radical generation system and RhB as a spectrophotometric indicator. The hydroxyl radical oxidation RhB decreased the absorption at 550 nm. Antioxidants restored this 550 nm absorption. A mathematical model for the calculation of free-radical scavenging ability was developed on the basis of the dose response of antioxidant recovery capacity. L-Ascorbic acid was used as a reference with its radical scavenging capacity set at 1.0. Flavonoids had scavenging hydroxyl radical capability 10−300 times greater than benzoic acid and derivatives. The 2,3 double bond, 3-hydroxyl, and 4-oxo groups in ring C of flavonoids played important roles in hydroxyl radical scavenging activity. For benzoic acid and derivatives, the benzene ring substituents enhanced the hydroxyl radical scavenging capability. This method was able to measure the hydroxyl radical scavenging capability of individual antioxidants with a wide dynamic activity range, i.e., 635−2. This simple, sensitive, and cost-effective method may be valuable in antioxidant studies.

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