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Determination of Peroxyl Radical Scavenging Activity of Flavonoids and Plant Extracts Using an Automatic Potentiometric Titrator

MITSUAKI SANO,*,† RISA YOSHIDA,† MASAKUNI DEGAWA,† TOSHIO MIYASE,† AND KYOJI YOSHINO‡

School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan, and Biochemistry, Numazu College of Technology, 3600 Ooka, Numazu-shi, Shizuoka 410-8501, Japan

A novel potentiometric method for evaluation of peroxyl radical scavenging activity of flavonoids and plant extracts was developed. The oxidation of potassium iodide (KI) was performed in acetonitrile-phosphate buffer (1:1) containing antioxidant using 2,2′-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator. The amount of iodine released from KI during a 20-min free radical oxidation was determined quantitatively using an automatic potentiometric titrator with sodium thiosulfate. The radical scavenging activity of the sample was expressed as the inhibition ratio for iodine release of the control group mediated by the radical. The results obtained from some authentic polyphenols correlated well with those of previous reports. This is a simple, time-saving method requiring less than 30 min and is useful in assessing the radical scavenging activity of antioxidants in plant extracts. We describe the radical scavenging activities of various flavonoids including 21 kinds of tea catechins and vegetable extracts by this method.

KEYWORDS: Radical scavenging activity; potentiometric method; tea catechin; flavonoid; antioxidant

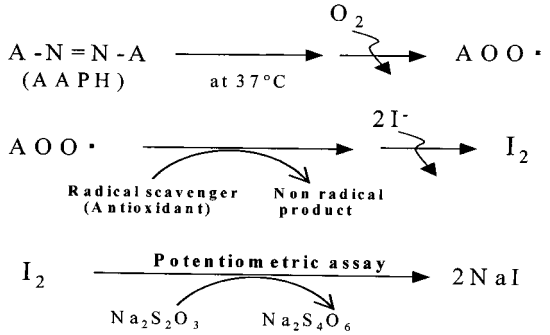
INTRODUCTION

Reactive free radicals, which abstract protons from biological molecules and induce oxidative damage of cellular lipids, nucleic acids and proteins, are thought to be one of the major risk factors for cancer, atherosclerosis, diabetes mellitus, coronary heart disease, and various other degenerative diseases (*1*−*3*). On the contrary, free-radical-scavenging antioxidants derived mainly from dietary sources play an important role in preventing oxidative damage. The flavonoids exist abundantly in vegetables and fruits are good radical scavengers. Many methods to determine the radical scavenging activity of plant extracts have been reported, all of which have different advantages and limitations (*4*−*8*).

In this paper, we report a determination method for peroxyl radical scavenging activity, based upon the principle of the peroxide value assay method. The peroxide value is commonly determined by measuring the amount of iodine liberated from a saturated KI solution by lipid peroxide in fat or oil (*9*).

In our established method, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator was used in place of lipid peroxide. Namely, AAPH decomposes by incubation at 35−40 °C and reacts rapidly with O_2 to give water-soluble peroxyl radicals (*10*). The peroxyl radicals oxidize I^- to I_2. Antioxidants having radical scavenging effects inhibit the peroxyl radical-induced oxidation. The amount of iodine liberated from KI during the oxidation is determined automatically using a potentiometric titrator with sodium thiosulfate (**Scheme 1**).

Scheme 1



This method using an automatic potentiometric titrator was simple and could be successfully applied to the determination of antioxidant ability of various plant extracts and authentic flavonoids. Tea catechins such as epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) are known to be strong antioxidants. Several studies have demonstrated that these catechins exhibit biological and pharmacological properties based on their antioxidant ability (*11*, *12*).

* Corresponding author. Tel/Fax: +81-542-64-5680. E-mail: sano@ys2.u-shizuoka-ken.ac.jp.
† University of Shizuoka.
‡ Numazu College of Technology.

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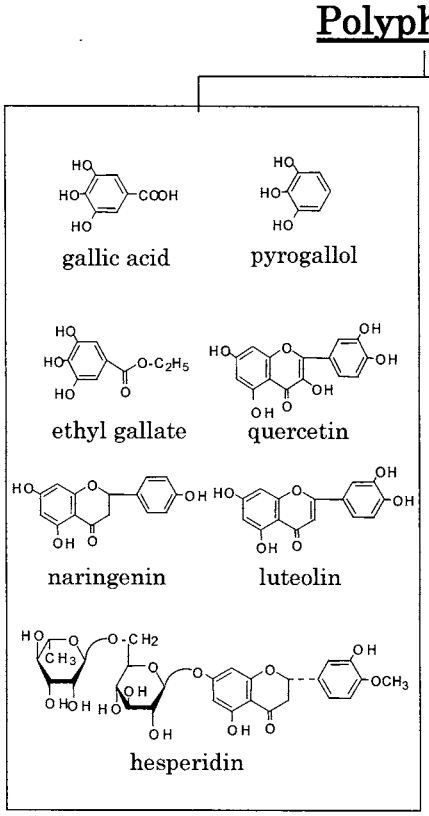

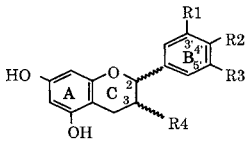

Compounds	2	R1	R2	R3	R4	R5	R6
Epigallocatechin-3-*O*- gallate (EGCG)	*R*	OH	OH	OH	G	OH	OH
Epicatechin-3-*O*- gallate (ECG)	*R*	OH	OH	H	G	OH	OH
Epigallocatechin (EGC)	*R*	OH	OH	OH	OH	-	-
Epicatechin (EC)	*R*	OH	OH	H	OH	-	-
Epigallocatechin-3-*O*- (3-*O*- methyl) gallate (EGCG3"Me)	*R*	OH	OH	OH	G	OCH_3	OH
Epigallocatechin-3-*O*- (4-*O*- methyl) gallate (EGCG4"Me)	*R*	OH	OH	OH	G	OH	OCH_3
4'-*O*-Methyl epigallocatechin-3-*O*- gallate (EGCG4'Me)	*R*	OH	OCH_3	OH	G	OH	OH
4'-*O*-Methyl epigallocatechin-3-*O*- (4-*O*- methyl) gallate (EGCG4', 4"diMe)	*R*	OH	OCH_3	OH	G	OH	OCH_3
Epigallocatechin-3-*O*- (3, 4-*O*- dimethyl) gallate (EGCG3", 4"diMe)	*R*	OH	OH	OH	G	OCH_3	OCH_3
Epicatechin-3-*O*- (3-*O*- methyl) gallate (ECG3"Me)	*R*	OH	OH	H	G	OCH_3	OH
Epicatechin-3-*O*- (4-*O*- methyl) gallate (ECG4"Me)	*R*	OH	OH	H	G	OH	OCH_3
4'-*O*- Methyl epicatechin-3-*O*- gallate (ECG4'Me)	*R*	OH	OCH_3	H	G	OH	OH
3'-*O*- Methyl epicatechin-3-*O*- (4-*O*- methyl)gallate (ECG3'4"diMe)	*R*	OCH_3	OH	H	G	OH	OCH_3
4'-*O*- Methyl epicatechin-3-*O*- (4-*O*- methyl) gallate (ECG4', 4"diMe)	*R*	OH	OCH_3	H	G	OH	OCH_3
Epicatechin-3-*O*- (3, 4-*O*- dimethyl) gallate (ECG3", 4"diMe)	*R*	OH	OH	H	G	OCH_3	OCH_3
Gallocatechin-3-*O*- gallate (GCG)	*S*	OH	OH	OH	G	OH	OH
Catechin-3-*O*- gallate (CG)	*S*	OH	OH	H	G	OH	OH
Gallocatechin (GC)	*S*	OH	OH	OH	OH	-	-
Catechin (C)	*S*	OH	OH	H	OH	-	-
Gallocatechin-3-*O*- (3-*O*- methyl) gallate (GCG3"Me)	*S*	OH	OH	OH	G	OCH_3	OH
Gallocatechin-3-*O*- (4-*O*- methyl) gallate (GCG4"Me)	*S*	OH	OH	OH	G	OH	OCH_3

Figure 1. Chemical structure of plant polyphenols used in this study.

We also describe the radical scavenging activities of 21 kinds of tea catechins including epicatechins and the C-2 epimers and O-methylated catechin derivatives (*13*) (Figure 1).

MATERIALS AND METHODS

Reagents and Samples. Potassium iodide, AAPH, and sodium thiosulfate solution (0.02 mol/L, factor = 1.00 at 20 °C) were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Epicatechins and their epimers except EGCG4′Me and EGCG4′,4″diMe were isolated from green tea leaves according to the method previously reported (*13*). Some catechins (EGCG, EC, ECG, and EGC) purchased from Kurita Co. (Tokyo, Japan) were also used. EGCG4′Me and EGCG4′,4″diMe were synthesized from EGCG using methyl iodide in our laboratory. Naringenin was purchased from Aldrich Chemical Co.(Milwaukee, WI). Other polyphenols and chemicals were purchased from Wako Pure Chemicals. Vegetable samples were obtained from a supermarket in Japan. Water used was purified by the Milli-Q system (Millipore, Bedford, MA).

Apparatus. Titration of sodium thiosulfate solution was performed using an automatic potentiometric titrator equipped with a platinum combination electrode, model AT510 (Kyoto Electronics Manufacturing Co. Ltd., Kyoto, Japan).

Preparation of Vegetable Extracts and Standard Antioxidant. Authentic polyphenol was dissolved in 30 mM phosphate buffer (pH 5.5)-acetonitrile (1:1) and then applied to determine the radical-scavenging activity. Vegetables were homogenized with 5 volumes of water in an ice bath.

The extraction of antioxidants from the vegetable homogenates was done with or without heat treatment. Namely, extract I was obtained by centrifugation of the homogenate at 3000 rpm for 10 min at 4 °C followed by filtration through a filter paper. Extract II was obtained by centrifugation and filtration after heating the homogenate for 30 min in a boiling water bath. Extract III was obtained by heat treatment

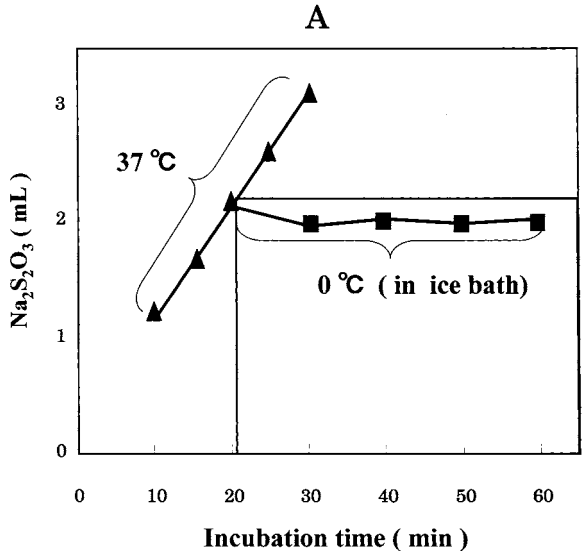

Figure 2. Effect of temperature on AAPH-induced oxidation. The reaction vessel was incubated at 37 °C (▲) and stood in an ice bath (■). Each point was average of duplicate determinations.

of extract I in the same manner as the preparation of extract II. These extracts were used to examine the stability of vegetable antioxidants by extraction with heating.

Potentiometric Method. An aliquot of sample solution was added to 2 mL acetonitrile-phosphate buffer (1:1), followed by addition of 100 μL of saturated KI solution. The total volumes were adjusted to 2.8 mL with water. After preincubation of the mixture at 37 °C for 2 min, radical-induced oxidation was started by the addition of 200 μL of 0.5 M AAPH. After 20 min of incubation at 37 °C in the dark, the

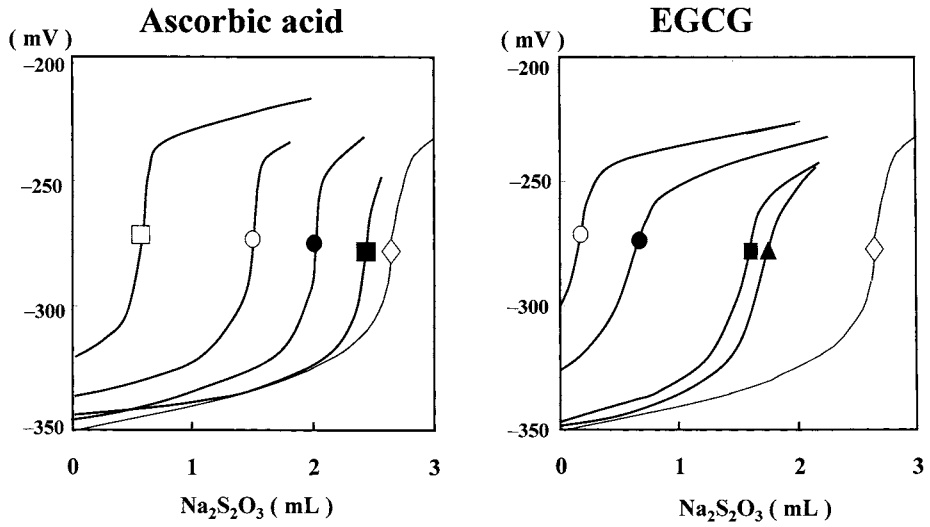

Figure 3. Electric potential changes of ascorbic acid and EGCG by automatic titration with $Na_2S_2O_3$. Antioxidant concentration in reaction mixture: ◇, control; ▲, 1.67 μM; ■, 3.33 μM; ●, 16.67 μM; ○, 33.33 μM; □, 66.67 μM.

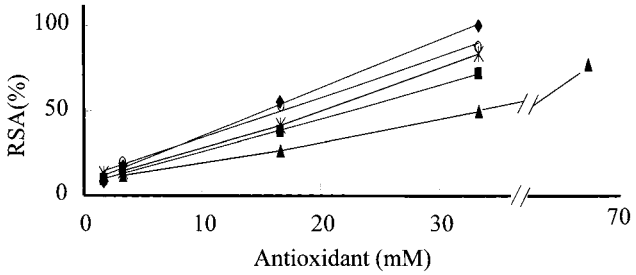

Figure 4. Dose-dependent curves of flavonoids on the determination of radical-scavenging activity: ♦, EGCG; ○, ECG; X, EGC; ▲, EC; ■, quercetin. Correlations between RSA (%) and EGCG, ECG, EGC, EC, and quercetin were 0.9920, 0.9822, 0.9924, 0.9854, 0.9962, respectively.

Table 1. Reproducibility of Radical-Scavenging Activity of Antioxidants by Potentiometric Method[a]

	$Na_2S_2O_3$ (mL)			
statistics	control	ascorbic acid	EGCG	cherry-tomato extract
mean	2.6530	1.1293	1.8738	1.7797
SD	0.0309	0.0474	0.0239	0.0485
CV (%)	1.1648	4.1947	1.2748	4.0891

[a] Data in each group were determined five times. The determination of ascorbic acid and EGCG was done at a concentration of 50 and 3.3 μM, respectively, and that of cherry tomato was done using an extract of 10 g of fresh tomato homogenate/50 mL water.

reaction vessel was chilled immediately in an ice bath to stop the radical production from AAPH and was allowed to stand for 5 min in an ice bath. Subsequently, the volume of the reaction mixture was adjusted to 30 mL with water. The concentration of iodine in the mixture was determined using a potentiometric titrator with 0.25 mM $Na_2S_2O_3$. The minimum titration amount of $Na_2S_2O_3$ was fixed at 0.1 μL and the titration was controlled by the automatic titrator.

Radical Scavenging Activity (RSA). The RSA of plant extracts and standard flavonoids is expressed as the percentage inhibition for iodine release of blank reagent without sample (control). The RSA was calculated from the following equation:

$$\text{RSA (\%)} = \left(1 - \frac{\text{amount of titrant (sample)} - \text{amount of titrant (0 time)}}{\text{amount of titrant (control)} - \text{amount of titrant (0 time)}}\right) \times 100$$

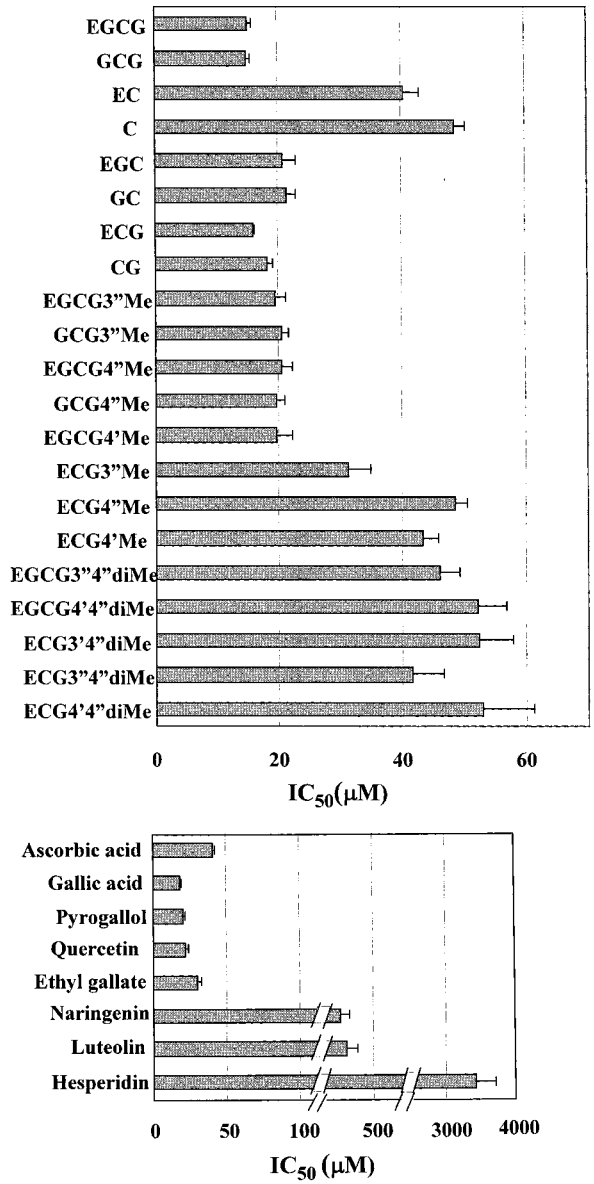

Figure 5. Radical-scavenging activity of plant polyphenols determined by potentiometry. IC_{50} of polyphenol was calculated from the result of RSA (%).

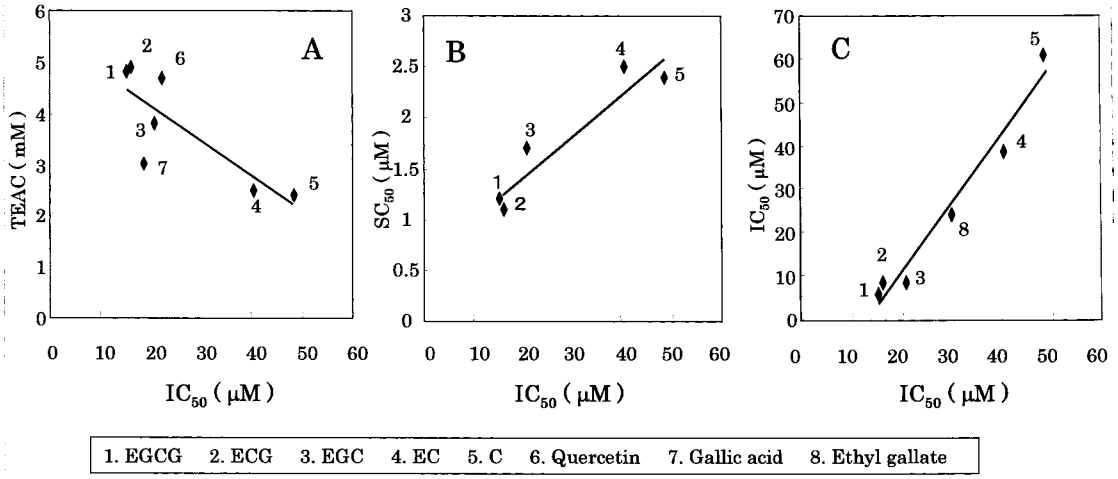

Figure 6. Comparison of the potentiometric method and some other evaluation methods for radical scavenging activity: A, $ABTS^{\cdot+}$ radical scavenging activity; B, DPPH radical scavenging activity; C, alkoxyl radical scavenging activity. Correlations between RSA (%) and method A (ABTS), method B (DPPH), method C (alkoxyl) were 0.6501 ($p < 0.05$), 0.8952 ($p < 0.05$), and 0.9645 ($p < 0.01$), respectively. ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate); DPPH, 1,1-diphenyl-2-picrylhydrazyl; TEAC, trolox equivalent antioxidant capacity; SC_{50}, 50% scavenging concentration.

The 50% inhibitory concentration (IC_{50}) of each polyphenol was calculated from the result of RSA (%) in a dose-dependent manner.

RESULTS AND DISCUSSION

The maximum potential difference was automatically determined as the end point of the titration. The radical-induced oxidation of KI at 37 °C progressed time-dependently and the reaction almost stopped in the ice bath (**Figure 2**). The reproducibility of the method was good under the optimal pH conditions of 4.5−6.0. As shown in **Table 1**, all coefficients of variance were less than 4.2% and mean relative errors were within ±4.85% in the results of control and some samples (ascorbic acid, EGCG, and cherry-tomato extract). **Figure 3** shows the electric potential changes of ascorbic acid and EGCG by the titration of $Na_2S_2O_3$. Ascorbic acid and EGCG in the reaction mixture decreased the titration amount of $Na_2S_2O_3$ in a dose-dependent manner. **Figure 4** shows the doseresponse curves of major tea catechins and quercetin for radical-scavenging activity represented as RSA (%). There was a linear relationship between the concentration of antioxidants and RSA in the range 0.5−35 μM of antioxidant.

Radical-Scavenging Activity of Authentic Polyphenol. Figure 5 shows the 50% inhibitory concentrations (IC_{50}) of various standard polyphenols (see **Figure 1**). Some epimers of epicatechins with the carbon at the C-2 position have been detected in tea infusions extracted with hot water (*14*, *15*). The values represented as IC_{50} showed no significant difference between epicatechins and the C-2 isomers. Radical scavenging activity of the O-dimethylated derivatives on the B ring and/or galloyl moiety was lower than the other catechin and monomethylated catechin derivatives. The results suggest that the trihydroxy moiety on the B ring and the O-dihydroxy moiety on the galloyl group play important roles in the radical-scavenging effect. The major catechins (EGCG, EGC, ECG, EC) in green tea leaves are strong antioxidants (*16*, *17*). These tea catechins were often used as standard samples for the evaluation of radical-scavenging activity. As shown in **Figure 6**, the results of some samples including the major tea catechins correlate well with those of other antioxidant evaluation methods (*4*−*6*).

Radical Scavenging Activity (RSA) of Vegetable Extract. Figure 7 shows RSA of water-soluble extracts of commonly eaten vegetables. Tea extract exhibited a very high RSA value

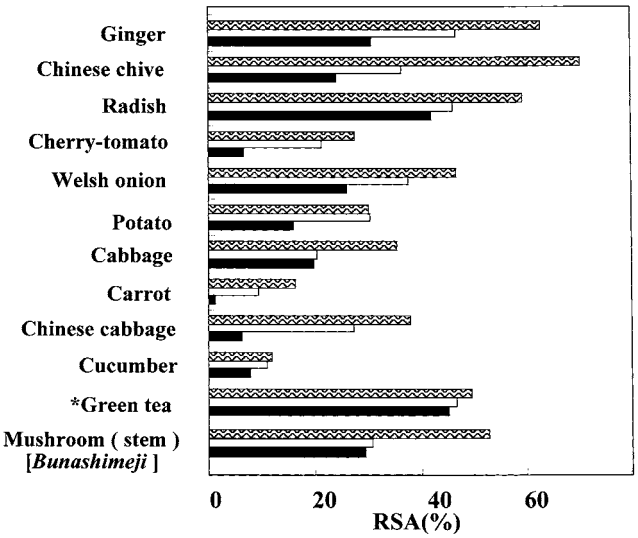

Figure 7. Peroxyl radical scavenging activity of vegetable extracts by potentiometry: (patterned bar), extract I; (white bar), extract II; (black bar), extract III. The preparation of extracts I–III was described in Materials and Methods. *The sample of green tea extract was diluted with 500-fold distilled water.

in comparison with those of other extracts. A marked decrease in RSA of water-soluble extracts, especially that of cherry-tomato, carrot, or Chinese cabbage, was observed by heat treatment (see RSA of extract II and extract III). The results suggest that the peroxyl radical scavengers in these vegetables are unstable in heat extraction. Ascorbic acid, which exists abundantly in fresh vegetables, has a strong peroxyl radical scavenging activity (*18*). However, there was no significant correlation between RSA and ascorbic acid concentration in the vegetables except tea ($r = -0.0984$). Therefore, the RSA of the vegetable extracts was mainly attributed to the combined antioxidant effects of flavonoids, ascorbic acid, and other antioxidants.

This is a simple, time-saving method requiring less than 30 min that uses commonly available, low-cost reagents. The measurement error using the automatic potentiometric titrator is relatively small. This method was suitable as a preliminary screening assay for water-soluble antioxidants in plants and

foods. Since the entire process is performed in one vessel, it is possible to automate all the operations involved in this method.

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