

## Novel Fluorometric Assay for Hydroxyl Radical Prevention Capacity Using Fluorescein as the Probe

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A novel fluorometric method has been developed to evaluate hydroxyl radical prevention capacity using fluorescein (FL) as the probe. The hydroxyl radical is generated by a Co(II)-mediated Fenton-like reaction, and the hydroxyl radical formation under the experimental condition is indirectly confirmed by the hydroxylation of *p*-hydroxybenzoic acid. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidant, the area under the fluorescence decay curve (AUC) is integrated, and the net AUC, which is an index of the hydroxyl radical prevention capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant. Gallic acid is chosen as a reference standard, and the activity of sample is expressed as gallic acid equivalents. The method is rigorously validated through linearity, precision, accuracy, and ruggedness. A wide range of phenolic antioxidants is analyzed, and the hydroxyl radical prevention capacity is mainly due to the metal-chelating capability of the compounds.

**KEYWORDS:** Assay; polyphenolics; hydroxyl radicals; preventive antioxidant; Fenton reaction

### INTRODUCTION

Accumulated evidence indicates that reactive oxygen species (ROS), such as peroxy radicals (ROO<sup>•</sup>), hydroxyl radicals (HO<sup>•</sup>), superoxide ion (O<sub>2</sub><sup>•-</sup>), and singlet oxygen (<sup>1</sup>O<sub>2</sub>), are involved in the pathophysiology of aging and a multitude of diseases, such as cancer, Alzheimer's disease, and Parkinson's disease (1, 2). To counteract the damages of the ROS on living cells, a defense system is designed biologically to neutralize the ROS or to prevent the ROS from being generated in the first place. Depending on the reaction mechanisms, antioxidants are often classified into two major categories: radical chain-breaking antioxidants and preventive antioxidants (3). Chain-breaking antioxidants convert reactive free radicals (e.g., HO<sup>•</sup>) to stable and thus nonaggressive molecules through hydrogen atom transfer reactions between HO<sup>•</sup> and the antioxidants. As a result, the autoxidation chain reactions between the free radicals and the cellular molecules are terminated. Preventive antioxidants inhibit the oxidation reaction from occurring by either converting the precursors of the ROS to unreactive species or inhibiting the oxidation reaction. On the basis of their molecular nature, the preventive antioxidants include enzymatic and nonenzymatic antioxidants. Well-known enzymatic antioxidants are superoxide dismutase (SOD), catalase, and glutathione peroxidase. Nonenzymatic antioxidants include oxidative

enzyme inhibitors and metal chelators (L). Examples of the former include anti-inflammatory medicines such as ibuprofen, which inhibits the activity of cyclooxygenase. Metal chelators deactivate transition metals from reaction with hydrogen peroxide to form reactive oxygen species believed to be hydroxyl radicals. It is evident that the antioxidant defense "team" in living cells contains individual antioxidants that function in very different tasks in the battles against oxidative stress and ROS. Therefore, it is imperative that to comprehensively evaluate the antioxidant activity of food nutrients in vitro, we need a broad range of assays that can cover all aspects of antioxidant activity. It is impossible to have a one-fits-for-all assay. Although there is a validated assay for peroxy radical absorbance capacity (ORAC) (4, 5), no such assay has been reported for hydroxyl radicals. This paper will address that issue.

One condition for hydroxyl radical generation in vitro and in vivo involves two essential components: oxidizable metal ions and hydrogen peroxide. The fact that the mixture of hydrogen peroxide and transition metals such as Fe(II) and Co(II) is a strongly oxidizing and hydroxylating reagent prompted the suggestion of hydroxyl radical involvement during the reaction. The exact mechanisms of Fenton-like reaction are extremely complex, and there is no conclusive answer for or against the involvement of hydroxyl radicals (6). The plain fact all have agreed upon is that the mixture of Fe(II)/Co(II) and H<sub>2</sub>O<sub>2</sub> is an extremely powerful oxidant that is fatal to living cells, which have an antioxidant defense system to eliminate the possibility of reaction between H<sub>2</sub>O<sub>2</sub> and metal ions (7).

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Removal of either of the two reactants will accomplish such a task. Catalase converts  $\text{H}_2\text{O}_2$  to  $\text{O}_2$  and  $\text{H}_2\text{O}$ , and metal chelators bind metal ions so that they become inert toward  $\text{H}_2\text{O}_2$ . Dietary nutrients contain metal chelators that will act as preventive antioxidants against the formation of hydroxyl radicals. Quantifying the activity of the phytochemicals in preventing hydroxyl formation in vitro will be a valuable guide to antioxidant clinical research. Herein we present a fluorometrically based assay for the metal-chelating capacity of polyphenolic compounds and a food matrix. For simplification purposes, we termed this new assay HORAC, standing for hydroxyl (HO) radical (R) averting (A) capacity (C).

## MATERIALS AND METHODS

**Chemicals and Apparatus.** All flavonoid compounds and 30% hydrogen peroxide were purchased from Sigma (St. Louis, MO). Iron(II) fluoride, cobalt(II) fluoride tetrahydrate, picolinic acid (PA), gallic acid, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, and fluorescein disodium were obtained from Aldrich (Milwaukee, WI). Various fruit extracts were obtained in house. All analyses were performed on a COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ; excitation wavelength = 493 nm and emission filter = 515 nm). Identification of the reaction product of *p*-hydroxybenzoic acid and  $\text{CoF}_2/\text{PA}-\text{H}_2\text{O}_2$  was performed by an HP 1100 high-performance liquid chromatography system (Hewlett-Packard, Palo Alto, CA) coupled with a Finnigan MAT LCQ ion trap mass spectroscopic detector (ThermoFinnigan, San Jose, CA): column, Zorbax (Hewlett-Packard) C18 (2.1  $\times$  150 mm, 3  $\mu\text{m}$ ); mobile phase, 70% methanol; flow rate, 0.3 mL/min; UV detector at 280 nm.

**Reagent Preparation.** Fluorescein solution was prepared as follows: 0.0225 g of fluorescein disodium (70%) was dissolved in 50 mL of phosphate buffer and vortexed to homogeneity. The solution, denoted concentrated stock solution, was kept in a  $-80^\circ\text{C}$  freezer for future use. Fifty microliters of concentrated stock solution was diluted to 10 mL with phosphate buffer to yield the stock solution (4.19  $\mu\text{M}$ ); 0.24 mL of stock solution was further diluted to 15 mL with phosphate to give a working solution with a fluorescein concentration of  $6.7 \times 10^{-2}$   $\mu\text{M}$ . A 1.1 M hydrogen peroxide solution was prepared by diluting 30%  $\text{H}_2\text{O}_2$  solution (8.8 M) with distilled water. A cobalt solution was prepared by dissolving 15.7 mg of cobalt(II) fluoride tetrahydrate and 20 mg of picolinic acid in 10 mL of distilled water.

**Sample Preparation.** Gallic acid, flavonoids, and phenolic acids (see Table 4 for individual compounds) were dissolved in methanol and diluted with pH 7.4 phosphate buffer (75 mM) for analysis. The fruit samples were initially ground in a mechanical mill to produce a fine powder; then 0.5 g was accurately weighed, and 20 mL of acetone/water (50:50, v/v) was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with phosphate buffer solution. Fruit extracts (~0.2 g) were dissolved into 50% acetone and water for analysis. For liquid samples, a 20 mL aliquot of sample was centrifuged for 15 min, and the supernatant was ready for analysis after further dilution with phosphate buffer.

**Hydroxyl (HO $\cdot$ ) Radical Averting Capacity Assay.** The COBAS FARA II was programmed to use a two-reagent system (reaction mode 3, P-I-SR1-I-SR2-A). The reaction mode pipetted and transferred the sample (20  $\mu\text{L}$ ) and main reagent (360  $\mu\text{L}$  of FL,  $8.61 \times 10^{-8}$  M) into the main reagent wells of their respective cuvette rotor positions. The rotor spins and the reagents are mixed and incubated for 30 s. After the rotor stops spinning, a start reagent (SR1), 10  $\mu\text{L}$  of hydrogen peroxide (1.1 M), is pipetted into the appropriate start reagent well in the cuvette rotor. Next, the analyzer starts spinning, mixing the sample/FL with  $\text{H}_2\text{O}_2$  for 30 s. The initial reading of fluorescence was taken. Finally, 10  $\mu\text{L}$  of cobalt(II) fluoride solution ( $[\text{Co}] = 9.2$  mM) was pipetted into the appropriate start reagent well in the cuvette rotor. The analyzer then spins to mix the solutions, and the oxidative reaction starts. Hence, the sample makes up 5% of the reaction volume, and the final concentrations of FL, Co(II), and  $\text{H}_2\text{O}_2$  are 0.062  $\mu\text{M}$ , 230

$\mu\text{M}$ , and  $2.75 \times 10^{-2}$  M, respectively. Between transfers, both sample and reagent transfer pipets are washed with cleaning solution to eliminate sample cross-contamination. Fluorescence readings are taken at 0.5 s and then every minute thereafter for 35 min ( $f_1, f_2, f_3, \dots$ ). If the fluorescence of the final reading has not declined by >95% from the first reading, the diluted sample is reanalyzed until a satisfactory fluorescent reading is achieved. FL was prepared in a 75 mM phosphate buffer. FL working solution was routinely incubated in a water bath at  $37^\circ\text{C}$  for 15 min before loading into the COBAS reagent rack. Phosphate buffer was used as a blank, and gallic acid concentrations of 800, 600, 400, 200, and 100  $\mu\text{M}$  were used as standards. Catechin concentrations of 300, 200, and 100  $\mu\text{M}$  were used as QC samples, and caffeic acid (125  $\mu\text{M}$ ) was used as the ruggedness test. The final values are calculated by using a regression equation between the gallic acid concentration and the net area under the FL decay curve. The area under curve (AUC) is calculated as

$$\text{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \dots + f_{34}/f_0 + 0.5(f_{35}/f_0) \quad (1)$$

where  $f_0$  = the initial fluorescence reading at 0 min and  $f_i$  = the fluorescence reading at time  $i$ . The data were analyzed by applying eq 1 in a Microsoft Excel (Microsoft, Roselle, IL) spreadsheet to calculate the AUC. The net AUC is obtained by subtracting the AUC of the blank from that of the sample. The relative HORAC value (gallic acid equivalents, GAE) is calculated as for pure compounds (5):

$$\text{relative HORAC value} = \frac{[(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{gallic acid}} - \text{AUC}_{\text{blank}})] \times (\text{molarity of gallic acid/molarity of sample})}{1} \quad (2)$$

For mixtures, such as food samples, eq 3 is used instead:

$$\text{relative HORAC value} = \frac{[(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{gallic acid}} - \text{AUC}_{\text{blank}})] \times (\text{molarity of gallic acid/concentration of sample})}{1} \quad (3)$$

The HORAC unit is expressed as micromoles of GAE per gram for solid samples and as micromoles of GAE per liter for liquid samples.

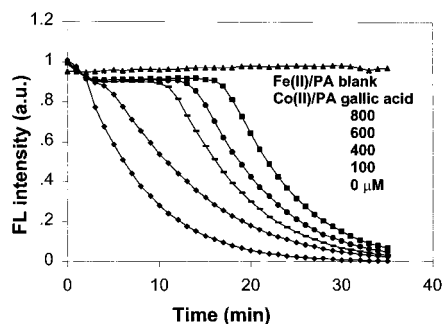
**Hydroxylation of *p*-Hydroxybenzoic Acid.** *p*-Hydroxybenzoic acid (1000 mM, in water, 1.0 mL) was incubated with  $\text{H}_2\text{O}_2$  (1.1 M, 0.5 mL) and  $\text{CoF}_2\text{-PA}$  (0.0162 M) for 30 min. The reaction mixture was analyzed by HPLC-MS. A Zorbax (Hewlett-Packard) C18 column (2.1  $\times$  150 mm, 3  $\mu\text{m}$ ) was used. The mobile phase was 70% methanol with a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized by using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an ESI source. The ionization mode was negative, and the auxiliary gas and sheath gas were set to 72 and 14 units, respectively. An ionization reagent of 1.5 mM ammonium hydroxide was added at a rate of 0.05 mL/min through a tee device by using a secondary HPLC pump before the API chamber. *p*-Hydroxybenzoic acid was used as a standard for calibrating the system.

## RESULTS

Metal ion induced hydroxyl radical generation reaction can be conveniently monitored by the fluorescence decay of fluorescein (FL) due to reaction 4. In the presence of preventive

$$\text{FL} + \text{M(II)} + \text{H}_2\text{O}_2 \rightarrow \text{oxidized FL (loss of fluorescence)} \quad (4)$$


antioxidant, the formation of hydroxyl radical can be inhibited because the metal is deactivated due to coordination with antioxidant, L (eq 5). The product, M(II)L, is not reactive to  $\text{H}_2\text{O}_2$  for generating hydroxyl radicals. As such, the degree of inhibition of reaction 4 is the index of preventive antioxidant capacity, which can be quantified from the fluorescence decay curves.



**Figure 1.** Fluorescent decay curve of fluorescein in the presence of gallic acid.  $[\text{FeF}_2] = 230 \mu\text{M}$ ,  $[\text{PA}] = 460 \mu\text{M}$ ,  $[\text{CoF}_2] = 230 \mu\text{M}$ ,  $[\text{H}_2\text{O}_2] = 0.055 \text{ M}$ , and  $[\text{FL}] = 6.20 \times 10^{-8} \text{ M}$ .  $T = 310 \text{ K}$  ( $37^\circ\text{C}$ ). Gallic acid concentration is given in the figure.

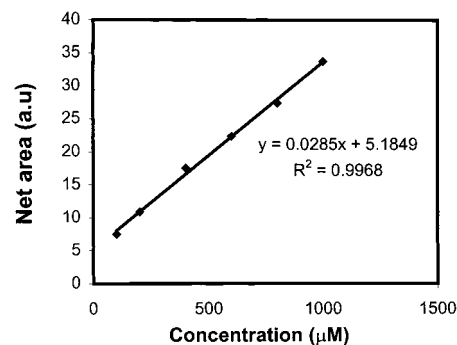
**Selection of Metals for Fenton-like Reaction.** We have tested three different metal salts for Fenton reactions, Cu(I), Fe(II), and Co(II), and found that Co(II) is the most suitable metal for analytical application. Cu(I) salts are only sparingly soluble, and the Fe(II) salts, albeit the most often used for Fenton reaction under acidic conditions, are too air sensitive and somehow do not cause fluorescence intensity to decay under neutral pH conditions (Figure 1). Cobalt(II) salts such as cobalt chloride and fluoride are not soluble in water either, but their complexes with 2 equiv of picolinic acid (PA) are readily soluble. Figure 1 shows the fluorescence decay curve in the presence of  $\text{CoF}_2/\text{PA}$  and  $\text{FeF}_2/\text{PA}$ . Apparently, under the physiological pH conditions, the  $\text{CoF}_2/\text{PA}$  system gives convenient fluorescence decay curves. The net area under the curve is sensitive to the concentration of antioxidants. Co(II) and  $\text{H}_2\text{O}_2$  concentrations are selected so that the reaction finishes within 35 min, the same as the ORAC assays. Therefore, this assay can be conveniently integrated to the same instrument settings as the ORAC assay.

**Evidence of Hydroxyl Radical Involvement.** The involvement of  $\text{HO}^\bullet$  in the  $\text{CoF}_2/\text{PA}-\text{H}_2\text{O}_2$  system is indirectly confirmed by using *p*-hydroxybenzoic acid as an  $\text{HO}^\bullet$  trap (8). The HPLC chromatogram for the reaction between *p*-hydroxybenzoic acid and  $\text{CoF}_2/\text{PA}-\text{H}_2\text{O}_2$  is shown in Figure 5, in which the major oxidized product has been identified as 3,4-dihydroxybenzoic acid. The hydroxylation of *p*-hydroxybenzoic acid clearly indicates that  $\text{HO}^\bullet$  is involved in the mixture of *p*-hydroxybenzoic acid and  $\text{CoF}_2/\text{PA}-\text{H}_2\text{O}_2$ .

**Method Validation.** After the assay protocol was established, we then fully examined the validity of the assay by testing the following validity parameters.

(a) **Linearity and Range.** The linear relationship between the net area and the antioxidant concentration was evaluated using gallic acid at different concentrations. By integrating the areas under the fluorescent decay curve, we are able to quantify the hydroxyl radical inhibition capacity of antioxidants. The inhibition capacity is expressed as gallic acid equivalent (GAE) or HORAC value, which is quantified by the integration of the area under the curve (AUC), similar to that of ORAC assay (9). Figure 2 shows the nearly perfect linear relationship ( $R^2 > 0.99$ ) between the gallic acid concentration and the AUC.

Table 1 summarizes the correlation coefficient, slope, and intercept of the gallic acid standard curve. The linearity range of the assay is between 100 and 800  $\mu\text{M}$ . The slopes of the linear curve fall in the range of  $0.0317 \pm 0.002$ . The intercept has a much larger variability from one run to the other. Therefore, for each HORAC assay, a standard curve should be obtained within the run and used for computation of HORAC values of samples within the same run in order to eliminate the



**Figure 2.** Linear curves of net area under the fluorescence decay curve and the gallic acid concentrations.

**Table 1.** Summary of Gallic Acid Calibration Curve [ $Y(\text{Net Area}) = a + bX (\mu\text{M})$ ] Concentrations Used: 800, 400, 200, and 100  $\mu\text{M}$

run	$R^2$	slope ( $b$ )	intercept ( $a$ )
1	0.9885	0.0281	4.8571
2	0.9991	0.0306	4.3244
3	0.9865	0.0317	4.4690
4	0.9922	0.0320	4.7747
5	0.9990	0.0335	3.1196
6	0.9925	0.0347	3.6113
7	0.9861	0.0324	2.1751
8	0.9946	0.0310	5.550
9	0.9951	0.0330	1.2938
10	0.9971	0.0296	5.201
av	$0.9931 \pm 0.0048$	$0.0317 \pm 0.002$	$3.94 \pm 1.37$

**Table 2.** Net Area under the Curve Corresponding to Different Compounds and Food Matrix

compound	concn ( $\mu\text{M}$ )	net AUC	HORAC (GAE)	$R^2$
rutin	100	21.1	3.82	0.9955
	80	15.91	3.61	
	60	12.68	3.83	
	40	8.31	3.77	
caffeic acid	500	26.40	1.43	0.9859
	250	16.54	1.20	
	187.5	12.46	1.30	
	125	10.40	1.51	
ferulic acid	500	23.02	1.31	0.9941
	250	12.91	1.37	
	187.5	9.40	1.33	
	125	6.85	1.46	
quercetin	250	18.38	1.59	0.9876
	125	9.90	1.72	
	100	8.50	1.84	
	75	6.28	1.81	
wild blueberry	1.3 <sup>a</sup>	24.39	284 <sup>b</sup>	0.9967
	0.65	10.16	237	
	0.325	4.60	215	
cranberry juice	0.2 <sup>c</sup>	30.45	3043 <sup>d</sup>	0.9976
	0.1	14.39	2876	
	0.05	6.584	2632	
	0.025	3.24	3236	
green tea leaf	0.66 <sup>a</sup>	20.64	476 <sup>b</sup>	1
	0.26	10.52	606	
	0.13	5.42	624	

<sup>a</sup> g/L. <sup>b</sup>  $\mu\text{mol}$  of GAE/g. <sup>c</sup> v/v. <sup>d</sup>  $\mu\text{mol}$  of GAE /L.

uncertainty of the intercepts. Table 2 shows the net areas corresponding to the different concentrations of representative phenolic antioxidants and food samples. All analyzed samples demonstrate a good linear relationship between the net area and certain concentration ranges. Therefore, this assay is readily applicable to food antioxidant activity measurements.

(b) **Ruggedness.** Day to day reproducibility was evaluated using gallic acid. HORAC values of 125  $\mu\text{M}$  caffeic acid using

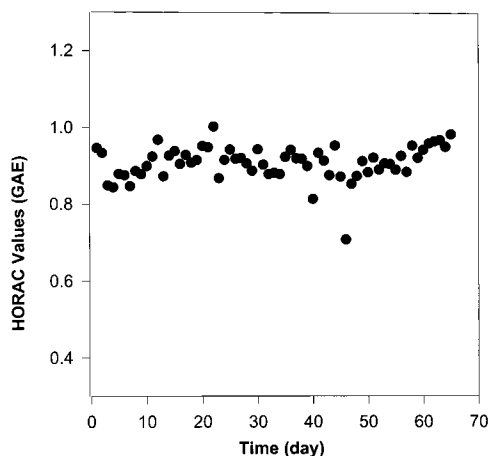


Figure 3. HORAC values of caffeic acid (125  $\mu\text{M}$ ) over 65 days.

Table 3. Precision and Accuracy of Quality Control (QC) Samples

catechin	QC1	QC2	QC3
normal concn ( $\mu\text{M}$ )	300	200	100
run 1			
intramean ( $\mu\text{M}$ )	289.23	217.05	93.70
SD	18.14	11.79	2.69
%RSD	6.27	5.43	2.87
%REC	96.41	108.52	93.70
n	4	4	4
run 2			
intramean ( $\mu\text{M}$ )	292.05	213.22	94.71
SD	6.41	3.50	3.22
%RSD	2.19	1.64	3.40
%REC	97.3	106.61	94.71
n	4	4	4
run 3			
intramean ( $\mu\text{M}$ )	270.74	225.25	87.19
SD	9.96	4.70	6.84
%RSD	3.68	2.09	7.85
%REC	90.20	112.61	87.23
n	4	4	4
pooled runs			
intermean ( $\mu\text{M}$ )	284.01	218.51	91.87
SD	11.58	76.15	4.08
%RSD	7.82	2.81	4.44
%REC	94.67	109.25	91.87
n	12	12	12

two COBAS FARA II analyzers for 65 days are shown in Figure 3. Within the time of testing, the average HORAC value of caffeic acid is 0.90, and the percent relative standard deviation (%RSD) is 4.9%. Therefore, the assay is stable from day to day.

(c) *Accuracy and Precision.* Table 3 summarizes the precision and accuracy. The precision, which is expressed as the relative standard deviation (%RSD) for all QC concentrations, was within  $\pm 15\%$ . The accuracy of the method varies from 87 to 112% within individual batches and from 92 to 109% among all of the batches. The limit of quantitation (LOQ) is 100  $\mu\text{M}$ , and the limit of detection (LOD) is 50  $\mu\text{M}$  GAE.

**HORAC Values of Antioxidant Samples.** To obtain structure and HORAC activity relationships of common phenolic compounds, we selectively examined representative phenolic compounds, and the results are shown in Table 4. For application purposes, we measured the HORAC values of common fruits and extracts as shown in Figure 4.

## DISCUSSION

**Conditions of Metal-Mediated Fenton Reaction.** The name "Fenton reagent" refers to a mixture of hydrogen peroxide and

Table 4. HORAC Values of Common Natural Antioxidants

compound	HORAC (GAE) <sup>a</sup>	ORAC (TE) <sup>b</sup>
protocatechuic acid	1.04 $\pm$ 0.27	5.21 $\pm$ 0.10
gallic acid	1.00	0.90
caffeic acid	1.51 $\pm$ 0.06	4.37 $\pm$ 0.24
chlorogenic acid	1.30 $\pm$ 0.07	3.14 $\pm$ 0.19
ferulic acid	1.36 $\pm$ 0.05	3.77 $\pm$ 0.10
ECG	2.28 $\pm$ 0.19	3.6 $\pm$ 0.07
EGC	1.97 $\pm$ 0.07	2.5
catechin	2.15 $\pm$ 0.16	6.40 $\pm$ 0.23
EGCG	3.10 $\pm$ 0.41	4.94 $\pm$ 0.21
isoquercitrin	5.10 $\pm$ 0.58	4.50 $\pm$ 0.20
rutin	4.5 $\pm$ 0.2	4.28 $\pm$ 0.25
quercetin	1.79 $\pm$ 0.05	4.38 $\pm$ 0.22
kaempferol	1.71 $\pm$ 0.03	5.22 $\pm$ 0.41
genistein	2.48 $\pm$ 0.37	5.93 $\pm$ 0.45
Trolox	$\sim$ 0	1.0
vitamin C	$\sim$ 0	0.95

<sup>a</sup> GAE, gallic acid equivalent. <sup>b</sup> TE, Trolox equivalent.

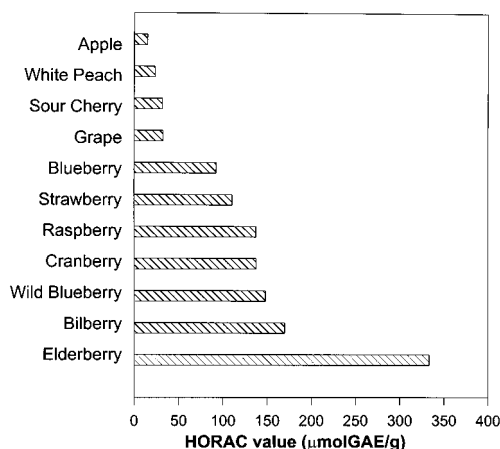
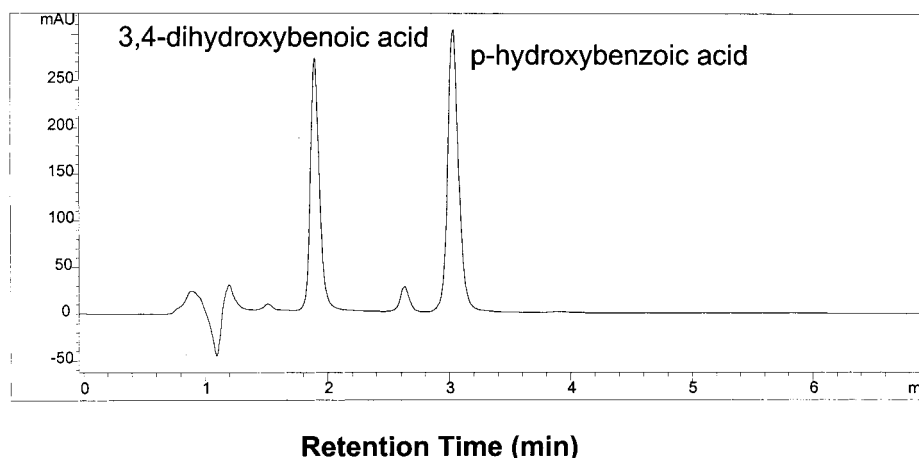


Figure 4. HORAC rankings of fruit extracts ( $n = 3$ ).

ferrous salts, which is an effective oxidant of a large variety of organic substrates. In 1894, Fenton discovered that in the presence of a low concentration of ferrous salts and  $\text{H}_2\text{O}_2$ , tartaric acid is oxidized to dihydroxymaleic acid (10). In a later paper by Haber and Weiss, the authors suggested that in the decomposition of  $\text{H}_2\text{O}_2$  catalyzed by iron salts,  $\text{HO}^\bullet$  is formed as an active intermediate. The involvement of hydroxyl radicals as a reactive intermediate in the Fenton reagent was suggested by the fact that it is an efficient hydroxylating agent (11). It was found later that numerous metal ions and their complexes in their lower oxidation states [e.g., Cu(I), Ti(III), Cr(II), and Co(II)] react with  $\text{H}_2\text{O}_2$  in a similar pattern as Fe(II), and the mixtures of these metals with  $\text{H}_2\text{O}_2$  were thus christened "Fenton-like" reagents (12, 13). Studies have demonstrated that the electron transfer reaction between a transition metal and  $\text{H}_2\text{O}_2$  does not follow an outer-sphere electron transfer mechanism. Instead, the reaction occurs through an inner-sphere electron transfer process, in which  $\text{H}_2\text{O}_2$  forms a complex with transition metals before electron transfer takes place (14). Therefore, if the transition metal is coordinatively saturated, it does not react with  $\text{H}_2\text{O}_2$  to give reactive oxygen species. Indeed, Halliwell et al. have shown that iron chelators inhibit the Fe(II)-mediated Fenton reaction and therefore prevent the oxidative damage caused by Fe(II)/ $\text{H}_2\text{O}_2$  (3). However, quantitative measurement of hydroxyl radical scavenging capacity has been a challenging task because of a lack of a controllable hydroxyl radical source. Cao et al. reported that the hydroxyl radical was generated by the mixture of  $\text{H}_2\text{O}_2$  and Cu(II), and its scavenging capacity was quantified by the ORAC assay using



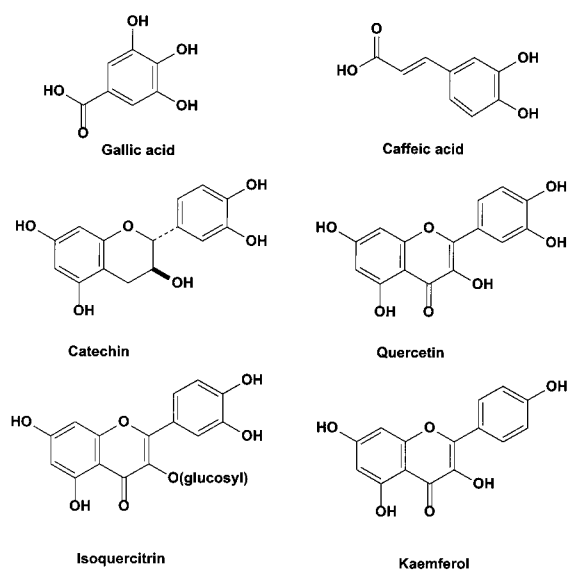
**Figure 5.** HPLC chromatogram of a reaction mixture of *p*-hydroxybenzoic acid and  $\text{CoF}_2/\text{PA}-\text{H}_2\text{O}_2$ . A mixture of *p*-hydroxybenzoic acid (1000  $\mu\text{M}$ , in water, 1.0 mL)  $\text{H}_2\text{O}_2$  (1.1 M, 0.5 mL), and  $\text{CoF}_2\text{-PA}$  [0.0162 M  $\text{Co(II)}$ , 0.5 mL] was incubated at 37  $^\circ\text{C}$  for 30 min. HPLC conditions: column, Zorbax (Hewlett-Packard) C18 (2.1  $\times$  150 mm, 3  $\mu\text{m}$ ); mobile phase, 70% methanol; flow rate, 0.3 mL/min; UV detector at 280 nm.

B-phycoerythrin (B-PE) as the fluorescent probe (15). We were unable to replicate Cao's work when fluorescein was used in place of B-PE. Although the standard redox potential of  $\text{H}_2\text{O}_2$  (1.77 V, NHE) is much larger than that of  $\text{Cu(III)/Cu(II)}$  (0.45–1.02 V, NHE) (16), kinetically, the reaction may be too slow for any practical significance.

Our study also found that  $\text{Fe(II)}$  and  $\text{Cu(I)}$  compounds are prone to be oxidized in the air under neutral conditions (pH 7.4), and thus the mixture of  $\text{Fe(II)}$  or  $\text{Cu(I)}$  with  $\text{H}_2\text{O}_2$  is not a stable source of hydroxyl radicals for quantitation. Therefore, the key elements of our study involve the selection of a metal ion stable at pH 7.4 and a means to control the oxidative reaction rate so that the decay of FL fluorescence can be completely monitored in a certain period of reaction time. After testing several metal ions, we found that  $\text{Co(II)}$  salts are air stable under physiological conditions and that the rate of hydroxyl radical formation can be controlled by the addition of PA as a chelating ligand of  $\text{Co(II)}$ . At present the exact nature of the reaction between  $\text{Co(II)/PA}$  and  $\text{H}_2\text{O}_2$  is not known and demands further investigation.

**Hydroxyl Radical Prevention Capacity and Molecular Structure Relationship.** After surveying the HORAC values of various phenolic compounds, we observed that the compounds that can chelate metals show significant HORAC values. Those without chelating ability have negligible HORAC values. For example, vitamin C and Trolox, the well-known radical chain-breaking antioxidants, show no hydroxyl radical prevention capability under current experimental conditions. This seems to be very puzzling at this moment. Although the lack of metal-chelating ability of vitamin C and Trolox certainly does not slow the reaction between  $\text{Co(II)}$  and  $\text{H}_2\text{O}_2$ , we still expect some protective effect due to their radical absorption capacity as reflected by their ORAC values. We suggest that they act as pro-oxidants by reduction of  $\text{Co(III)}$  to  $\text{Co(II)}$ , thus initiating a catalytic cycle of hydroxyl radical generations. By doing so, they are destroyed quickly under the assay conditions, which include a large excess of hydrogen peroxide. Therefore, overall they do not show protective effects on fluorescence decay, although they may consume equal molar hydrogen peroxide. In fact, vitamin C has been used as a component of  $\text{HO}^\bullet$  radical source mediated by  $\text{Fe(II)}$ .

**Figure 6** represents the chemical structures of three common types of phenolics studied herein. The first group, including gallic acid, caffeic acid, ferulic acid, protocatechuic acid, and chlorogenic acid, are phenolic acids with an *ortho* diphenol



**Figure 6.** Chemical structures of some phenolics studied in this paper.

group. Their HORAC values fall in a narrow range of 1.0–1.51. The second group belongs to flavanol compounds including catechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. Besides the *ortho* dihydroxyl groups on the B ring, they also have hydroxyl groups on the A and C rings. These compounds have higher HORAC values than the phenolic acid group (2.15–3.1 GAE). The third type is flavones with a ketone group on the 4-position of the C ring, including quercetin, genistein, kaempferol, isoquercitrin, and rutin. The HORAC values of these vary largely from 1.71 (kaempferol) to 5.10 (isoquercitrin). The glucose and rutinose groups have significantly positive influences on the HORAC values as the hydroxyl groups may increase the coordination ability. We have suggested that the phenolics act as metal chelators by coordination to  $\text{Co(II)}$  and therefore block the reaction sites for  $\text{H}_2\text{O}_2$ . This coordination reduces the concentration of cobalt species that are active toward  $\text{H}_2\text{O}_2$  and thus effectively averts hydroxyl radical formation. The HORAC values primarily reflect metal coordination ability of the phenolics. The HORAC values as shown in **Table 4**, however, do not correlate with the number of hydroxyl groups in the phenolics and the number of potential chelating sites. This is understandable because the factor that governs the HORAC values are the stability of the complex formed between  $\text{Co(II)}$  and the phenolics (eq 6;  $\text{AO} =$

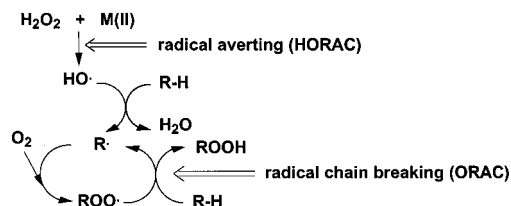
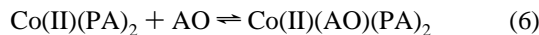


Figure 7. Relationship of HORAC and ORAC.

antioxidants). The higher equilibrium constant of eq 6 should, in principle, contribute to higher HORAC values. Further study is needed to obtain equilibrium constants of all the phenolics with Co(II) and to qualitatively establish a structure–activity relationship.



The preventive antioxidant capacity of some flavones has been well documented; for instance, metal chelation has been proposed as a mechanism for the antioxidant activities of rutin, quercetin (17), and butein (18). In addition, coordination of iron with phenolic compounds has also been employed by Yoshino and co-workers to characterize antioxidant actions of flavonoids and phenolics (19). Compared with the previous studies that lack a quantitation method of antioxidant capacity, the assay described herein is the first to quantitatively measure the metal-chelating antioxidant capacity of food and natural products.

**Comparison of HORAC and ORAC.** As shown in Table 4, there is no correlation between HORAC and ORAC values. For example, genistein, quercetin, and kaempferol have very high ORAC values but their HORAC values are modest, whereas EGCG and rutin have high values for both ORAC and HORAC. In sharp contrast, the most common antioxidants, such as Trolox and vitamin C, do not show significant HORAC values, and that of melatonin is only half that of gallic acid. This phenomenon is in agreement with the fact that the HORAC and ORAC assays measure two different but equally important aspects of antioxidant properties—radical chain breaking and radical prevention. The HORAC primarily reflects metal-chelating radical prevention ability, and the ORAC reflects peroxy radical absorption capacity. It is, therefore, expected that the samples with high HORAC values do not necessarily have high ORAC values and vice versa. Figure 7 illustrates chemically the different aspects of HORAC and ORAC assays in measuring antioxidant activity.

**HORAC Values of Common Fruit Extracts.** Some common fruit extracts were analyzed using the HORAC assay. The results are shown in Figure 4. Obviously, HORAC values are very sensitive to the type of fruits, and the HORAC values range broadly from 15 (apple powder) to 333 (elderberry). These values reflect the nutritional values for different fruits in hydroxyl radical prevention activity *in vitro* only. It would be interesting to see how the HORAC values correlate with the oxidative stress relief capacity of a biological system by these fruits *in vivo*.

In summary, for the first time, a novel method specific for the preventive antioxidant capacity against hydroxyl radical formation was developed and validated. As a complement to the ORAC assay that provides peroxy radical chain-breaking capacity, the HORAC assay will be an important quantitation tool for the study of the preventive antioxidant capacity of food.

#### ABBREVIATIONS USED

ORAC, oxygen radical absorbance capacity; FL, fluorescein; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; HORAC,

hydroxyl (HO·) radical averting capacity; GAE, gallic acid equivalent; AUC, area under the curve; AO, antioxidants; PA, picolinic acid.

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